

" A CYTOCHEMICAL AND BIOCHEMICAL STUDY OF ACID HYDROLASES
IN DEVELOPING PLANT CELLS, WITH PARTICULAR REFERENCE
TO NON-SPECIFIC ESTERASES "

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TABLE OF CONTENTS

	Page
TITLE	i
ACKNOWLEDGEMENTS	ii
TABLE OF CONTENTS	iii
ABSTRACT	vi
GENERAL INTRODUCTION	1
CHAPTER I: A cytochemical and statistical investigation of β -glycerophosphatase and naphthol AS BI phosphatase during cell division in root tips of <u>Vicia faba</u>	
I.1. Introduction	15
I.2. Materials and Methods	18
I.2.1 Plant material	18
I.2.2 Enzyme localisation procedures for acid phosphatases	18
I.2.3 Counterstaining procedure	21
I.2.4 Defining the tissues of the root tip	21
I.2.5 Counting procedure	22
I.2.6 Presentation of data	24
I.2.7 Statistical methods for the evaluation of significance	24
I.2.8 The effect of counterstaining on particle number	25
I.3. Results	27
I.3.1 Cytology	27
I.3.2 Statistics	28
I.4. Discussion	34
CHAPTER II: A biochemical study of non-specific esterases from the lateral root tips of <u>Vicia faba</u>	
II.1. Introduction	41
II.2. Materials and Methods	44
II.2.1 Chemicals	44
II.2.2 Enzyme extraction and purification	44
II.2.3 Enzyme assay procedure	46
II.2.4 Protein estimation	50
II.3. Results	51
II.3.1. Post-coupling. Factors affecting colour reaction and development	51
II.3.2 Characterisation studies	53
II.4. Discussion	63

CHAPTER III:	A cytochemical and biochemical study of non-specific esterases in the differentiating root cells of <u>Vicia faba</u>	
III:1.	Introduction	67
III.2.	Materials and Methods	70
III.2.1	Preparation of tissues for cytochemical and biochemical studies	70
III.2.2	A note on the accuracy of the microtome setting for section thickness	72
III.2.3	Homogenisation of tissue sections	73
III.2.4	Enzyme assay	74
III.2.5	Protein estimation	75
III.2.6	Photomicrography	75
III.2.7	Cytochemical studies	75
III.3.	Results	77
III.3.1	Cytochemical studies	77
III.3.2	Definition of tissues	78
III.3.3	The pattern of esterase localisation in the terminal 3.4mm of the root	79
III.3.4	Biochemical studies	85
III.3.5	A note on enzyme activity expressed on a per cell basis	89
III.4.	Discussion	90
CHAPTER IV:	A cytochemical and biochemical study of non-specific esterases during soft fruit ripening	
IV.1.	Introduction	97
IV.2	Materials and Methods - Cytochemistry	103
IV.2.1	Growth and selection of fruits	103
IV.2.2	Measurements of cell size in strawberry receptacles	106
IV.2.3	Tissue preparations for the cytochemical localisation of non-specific esterases	106
IV.2.4	Enzyme localisation procedure for non-specific esterases	108
IV.2.5	Photomicrography	108
IV.3.	Results - Cytochemistry	109
IV.3.1	Strawberry	109
IV.3.2	Esterase activity in developing achenes	115
IV.3.3	Raspberry	115
IV.3.4	Blackberry	117
IV.4.	Materials and Methods - Biochemistry	118
IV.4.1	Selection of fruit	118
IV.4.2	Enzyme extraction procedures	119
IV.4.3	Preparation of a crude enzyme extract from achenes removed from the 3 stages of receptacle development C, D and E	124
IV.4.4	The affinity of pectin methyl esterase for naphthol AS D acetate	125

IV.4.5	Biochemical assay	126
IV.4.6	Protein estimation	127
IV.5.	Results - Biochemistry	128
IV.5.1	Changes in esterolytic activity during the ripening of strawberry receptacles	128
IV.5.2	Changes in esterolytic activity of crude extracts from developing achenes of receptacles at stages C, D and E	132
IV.5.3	The affinity of pectin methyl esterase for naphthol AS D acetate	135
IV.5.4	Biochemical characterisation of non-specific esterase from the 3 stages of ripening	136
IV.6	Discussion	141
CHAPTER	V: The development of methods for the ultrastructural localisation of non-specific esterases in plant cells	
V.1	Introduction	151
V.2.	Materials and Methods	154
V.2.1	Durcupan vs. Ethanol dehydration	154
V.2.2	Esterase localisation in strawberry fruits	156
V.2.3	Tissue preparation and the localisation of esterase activity employing diazotised LPED as coupling salt	157
V.2.4	Preparation of ultrathin sections for electron microscopy	157
V.3.	Results	159
V.3.1	Durcupan vs. Ethanol dehydration	159
V.3.2	Esterase localisation in strawberry fruits	159
V.3.3	Esterase localisation in root tips using LPED as coupling salt	163
V.4.	Discussion	166
	GENERAL DISCUSSION	171
	PUBLICATIONS	186
	BIBLIOGRAPHY	187

ABSTRACT

Investigations have been carried out into the occurrence and functions of acid hydrolases during plant cell division, differentiation and senescence using histochemical and biochemical procedures.

Statistical analyses of histochemical data have shown that particles staining for naphthol AS BI phosphatase in dividing unfixed root cells of Vicia faba do not act as a 'trigger' in the way suggested for animal cells, since there were significantly greater numbers of particles in mitotic cells as opposed to interphase cells in all 3 root tissues, apical initials, procortex and central cylinder. Increasing the length of incubation time markedly increased the number of particles in all 3 tissues.

The procedure for determining β -glycerophosphatase activity in glutaraldehyde-fixed root tips was found to be unsatisfactory since cells in sections of tissues incubated without substrate contained lead-staining particles and in both test and control sections diffuse cytoplasmic and nuclear staining were apparent.

Using the histochemical reagents, naphthol AS D acetate and the diazonium salt, fast red violet LB salt, a biochemical study of non-specific esterases extracted from broad bean root tips, has shown that these enzymes have an estimated K_m of 0.07 mM, a pH optimum of 5.5 and that they lose substantial activity when dialysed against distilled water as opposed to 5mM magnesium chloride and 10mM phosphate buffer. However, adding magnesium and/or chloride ions to the assay system reduced enzyme activity.

A combined cytochemical and biochemical analysis of non-specific esterases in differentiating root tissues of the broad bean showed that maximal enzyme activity, expressed on a per segment, per unit protein or per unit area of average-sized section basis, occurred within the zone of cell expansion, elongation and differentiation of procambial tissues.

Cytochemical studies showed that in glutaraldehyde-fixed roots activity was only slightly inhibited when compared with unfixed material, whereas biochemical studies revealed that only about $\frac{1}{8}$ of the activity of enzyme extracted from unfixed roots was present in homogenates of fixed roots.

In a study of non-specific esterases in soft fruit ripening and senescence, cytochemical studies demonstrated that enzyme activity was associated with subcellular structures in the pulp cells of the ripening strawberry. The fleshy mesocarp tissues of raspberry and blackberry, however contained no enzyme end-product at any of the stages of ripening examined.

In older strawberry receptacles, also examined biochemically, enzyme end-product was associated with large spherical lipid-droplet-like bodies absent in younger receptacles.

Assays of non-specific esterases in immature, maturing and mature pulp tissues showed that these enzymes increased several fold during ripening when expressed on a per g fresh weight or per berry basis. Although esterase activity changed little during the transition from 'maturing' to 'mature' when expressed on either basis, the specific activity of the enzymes fell markedly.

The K_m values of enzyme extracts from the 3 stages of berry development decreased as ripening proceeded denoting an

increased affinity of these enzymes for their substrates possibly due to the removal of an inhibitor or a change in iso-enzyme pattern. Esterases from the three ripening stages exhibited broad pH optima (pH 5.5 - 6.5) although limitations of the assay procedure prevented the detection of possible alkaline optima.

Ultrastructural studies showed that changes in the subcellular organelles of ripening strawberry pulp cells were similar to that reported for other senescing plant material, vesiculation of many organelles and loss of ribosomal material being the most characteristic. Attempts to localise esterases in fruit cells at the ultrastructural level were unsuccessful since electron dense end-product of diazotised lead phthalocyanin bound to a variety of structures even in the absence of the enzymes' substrate. In contrast the use of the diazotate of LPED in Vicia faba root cells was more promising in that in test tissue end-product was found in small vacuoles and rough endoplasmic reticulum, whilst control cells incubated in the absence of substrate lacked enzyme end-product.

The water-soluble epoxy resin, Durcupan, was assessed for its effect on plant cell organelle morphology by comparing similar tissue conventionally dehydrated in alcohol. No gross changes in ultrastructure were observed.

GENERAL INTRODUCTION

Interest in acid hydrolases has escalated since de Duve and his collaborators (1955) first showed that in rat liver cells, these enzymes were mainly, although not exclusively, concentrated in a distinct cytoplasmic organelle, 'the lysosome' (de Duve 1959). In their early biochemical cell fractionation studies five separate acid hydrolases - acid phosphatase, acid deoxyribonuclease, (DNAase), acid ribonuclease, (RNAase), cathepsin and β -glucuronidase - were found to be present within the same particle. The list is now more extensive and up to 12 or more hydrolases have been reported from such particles derived from rat lymphoid tissue, (Bowers and de Duve, 1967a, 1967b).

As the name 'lysosome' suggests, these bodies with their hydrolytic enzymes were initially believed to be the foci of controlled intracellular lysis and digestion of metabolically labile cell macromolecules. This process was given the name autophagy or heterophagy depending upon whether the material undergoing digestion was of endogenous or exogenous origin. This whole intracellular digestive system in animal cells was given the name "vacuolar apparatus" (de Duve and Wattiaux, 1966).

This early work, which saw the emergence of a 'new' and 'distinct' group of subcellular particles with a specialised cell role, has since led to voluminous literature on the subject of lysosomes and their functions in animal cell biochemistry, physiology and pathology, which is summarised in a recent two volume treatise on 'Lysosomes in biology and pathology', (Dingle and Fell, 1969).

As a result of their work, de Duve and his associates were able to establish a number of biochemical criteria whereby lysosomes could be characterised and recognised. The hydrolases exhibited

'structure-linked latency' since the enzymes were separated from other cytoplasmic constituents by a lipoprotein membrane that restricted their accessibility to external substrates. Moreover, this membrane could be disrupted by a number of physical and chemical treatments. Homogenisation in a Waring blender, repeated freezing and thawing, ultrasonic vibration, osmotic shock, detergent action or certain enzyme action (protease, lecithinase) all rendered the acid hydrolases freely accessible and soluble (de Duve 1963). It followed too, that if the enzymes were particle-bound then a percentage of the enzyme activity should be sedimentable in homogenates of the tissue. In addition, each of the membrane-bound hydrolases should exhibit acid pH optima, when tested against their respective substrates.

When recounting the history of the discovery of the lysosome de Duve (1969) makes reference to the advances made early on in their studies to the application of cytochemical staining methods using one of the acid hydrolases, acid phosphatase, as a marker enzyme for the intracellular localisation of lysosomal particles (Novikoff, 1961). These staining methods could be used at both the light and electron microscope levels on thin sections of tissue which could also be studied biochemically in whole homogenates. This emphasis on combined biochemical and cytochemical investigations of certain acid hydrolases has been the cornerstone of much subsequent research with animal tissues.

In plant cells, the cytochemical localisation of acid phosphatase, in connection with both cytological and physiological studies, had been attempted by a number of workers (e.g. McGregor and Street, 1953; Wilson and Cutter, 1955; Jensen, 1956; Avers, 1961 and Avers and King, 1960) before widespread interest in this

enzyme had been aroused by de Duve's discovery of the lysosome in animal tissues. Jensen (1956) had proposed that acid phosphatase-staining particles in root tip cells were mitochondrial in nature on account of their size and shape, but later work by Avers and King (1960) and Avers (1961) showed that mitochondrial populations of root meristem cells were heterogeneous with respect to staining for acid phosphatase and respiratory enzymes, and hence the possibility of distinct acid phosphatase-rich particles in plant cells had to be considered. Interest in these particles in plant cells quickened in the train of de Duve's publications on lysosomes (1959, 1963). A search for the existence of corresponding lysosomal particles in plant cells began during the mid-1960's when both cytochemical and biochemical methods were being used to define the source of acid hydrolase activity in a variety of plant tissues. Unfortunately the two approaches were seldom used together. Most cytochemical evidence at both the light and electron microscope levels for the existence of plant organelles with a lysosome-like function came from the use of the Gomori acid phosphatase test (Gomori, 1952). β -glycerophosphate, in the presence of lead nitrate, is the substrate used (e.g. Poux, 1963; Gahan, 1965; Gahan and Maple, 1966; Figier, 1968; Hall, 1969; Sexton and Sutcliffe, 1969) and the precipitated lead phosphate is converted to the sulphide. The ultrastructural evidence suggested that the enzyme activity resided particularly in the vacuoles of a variety of plant tissues (Figier, 1968; Gahan and McLean 1969; Halperin, 1969; Poux, 1970; Sexton et al, 1971).

It was perhaps understandable that cytochemical methods for acid hydrolase localisation in tissue sections should be largely limited to acid phosphatase in view of both Gomori's

(1948, 1952) and Holt's (1958, 1959) work and the original pronouncement of de Duve et al., (1955) that this enzyme was lysosomal. Alternative cytochemical methods to that of Gomori for acid phosphatase detection were available based either on simultaneous or post-coupling azo-dye techniques. These used either the phosphate esters of naphthol AS derivatives (Burstone, 1958a), or α -naphthol phosphate (Grogg and Pearse, 1952; Barka and Anderson, 1962) as substrates, but they were never as widely used. However, with the ever-increasing interest in lysosomal particles, their application to animal cells in conjunction with Gomori's method, or in their own right became widespread (see review by Gahan, 1967). It is only recently that the use of the naphthol AS series for acid phosphatase localisation in plant cells at the light and electron microscope levels has been reported to any extent (Gahan and McLean, 1969; Ashford and McCully, 1970; McLean and Gahan, 1970; Hall and Davie, 1971).

Methods for the histochemical demonstration of acid hydrolases other than acid phosphatase, e.g. DNAase, RNAase, β -glucuronidase, β -galactosidase, β -glucosidase, arylsulphatases, and non-specific esterases have been attempted and of these the most successful have been those for esterases, arylsulphatases, and β -glucuronidase (e.g. Gahan, 1967). The substrates for these 3 groups of enzymes are all derivatives of the naphthol AS series (Burstone, 1958a, 1962); that for esterases often being naphthol AS D acetate, the substrate widely used in this work.

After enzymic hydrolysis the freed substituted naphthol is captured by a suitable diazonium salt and the extremely insoluble azo-dye so formed is deposited at the site of enzyme action. Diffusion artefacts are minimised and localisation has often been referred to as 'excellent', (Pearse, 1960; Burstone, 1962).

Besides the use of naphthol AS derivatives as substrates for non-specific esterases, the two other widely used methods for detection of these enzymes should be mentioned. Wachstein and Meisel (1960) used thiolacetic acid as the esterase substrate. Upon hydrolysis this releases hydrogen sulphide which converts any lead nitrate present to lead sulphide, which can be seen at both the light and electron microscope levels. Perhaps even more widely used is Holt's indigogenic method (1958). Indoxyl acetate or its various substituted derivatives are hydrolysed by esterases to free indoxyl which is then oxidised via its leuco derivative to the corresponding indigoid dye.

Once these histochemical procedures for enzyme localisation became established in studies with animal cells it was not long before the same methods were applied to plant cells. Besides the studies on plant acid phosphatase previously mentioned, Walek-Czernecka (1962, 1963, 1965) localised a number of acid hydrolases using naphtholic or naphthyl substrates in onion bulb-scale epidermis, and suggested that the particles so stained were 'spherosomes', plant cell organelles first identified by Dangeard many years previously (1922) as small, spherical and highly refractile particles in the epidermal cells of Iris leaves. Other workers also identified the acid hydrolase-containing organelles as spherosomes in a variety of plant tissues: Gorska-Brylass (1965) in pollen tubes, Olszewska and Gabara (1964) in the phragmoplast of dividing root cells of Zea mays and Holcomb et al., (1967), in normal and tumour cells cultured from sunflower stems and crown galls.

In addition to vacuoles and spherosomes as possible sites of acid hydrolase activity in plant cells, Dauwalder et al., (1969),

Zee (1969), and Poux (1970), identified the Golgi bodies as staining positively for acid phosphatase.

Cytochemical evidence alone does not provide unequivocal evidence for the existence of a plant cell organelle having a comparable structure and function to the animal lysosome. It became quite clear that to identify such particles, if they existed, it would be necessary to show that isolated organelles from tissue homogenates had the biochemical characteristics that matched the biochemical definitions and criteria, at least, of animal lysosomes i.e. the enzyme would show latency, be sedimentable, and several acid hydrolases with acid pH optima would exist within the same particle.

The sedimentability of acid hydrolases from a variety of plant tissues has been demonstrated by several investigators. Harrington and Altschul (1963) first showed latent, sedimentable acid phosphatase activity in germinating onion seed extracts. Matile et al., (1965) later presented evidence for the sedimentability of 3 other enzymes, non-specific esterases, protease, and RNAase, in cell-free extracts from tobacco and Zea mays seedlings. These observations also showed that only a percentage of such activity was sedimentable and that after homogenisation and high speed centrifugation a fairly high proportion of the enzyme's activity was recovered in the supernatant fraction.

The degrees of sedimentability, varying from negligible (Corbett and Price, 1967) to considerable (Balz, 1966; Matile, 1968b; Semadeni, 1967) have been attributed to inappropriate or inadequate extraction techniques used in the isolation of the acid hydrolase-bearing particles (Matile, 1969).

Recently Gibson and Paleg (1972), illustrated the structural latency of α -amylase in wheat aleurone cells by the use of the surface active agent, Triton X-100. This detergent, along with deoxycholate, crude snake venom and a variety of purified phospholipases was used by Pitt and Galpin (1973) in their study of acid phosphatase latency in cell-free extracts of potato shoots. They found that whilst the phospholipases were ineffective in extracting the enzyme, the other 3 reagents released the enzyme from its particle-bound state.

The demonstrations of sedimentability and latency by the above named workers were often carried out simultaneously with biochemical investigations of the source of acid hydrolase activity in the various cell fractions, separated on appropriate differential density gradients. This information was then related to the evidence obtained from the ultrastructural study of the separated fractions and the subcellular localisation of the respective enzymes confirmed (Matile, 1968b; Matile and Moor 1968; Pitt and Galpin, 1973).

In this way Matile (1968b) showed that the mitochondrial fraction of a cell-free extract prepared from root tips of Zea mays seedlings could be resolved into 'heavy' and 'light' lysosome fractions, there being a total of nine identifiable acid hydrolases, including acid phosphatases and non-specific esterases. Ultrastructural freeze-etch studies showed that the 'light' lysosomal fraction was identifiable with meristematic vacuoles with diameters of 0.5-2 μ m or more. 'Heavy' lysosomes on the other hand were smaller (0.1-0.3 μ m diameter) but had the same fine structure. Similar biochemical and ultrastructural evidence supplied by Pitt and Galpin (1973) suggests that heterogeneous populations of

vesicles and vacuoles contain most of the acid hydrolase activity in cell fractions taken from potato shoots.

Therefore biochemical, ultrastructural and some cytochemical studies suggest that plant vacuoles of varying sizes and development contain appreciable acid hydrolase activity. However Matile and Spichiger (1967) and Semadeni (1967) showed that whilst acid phosphatase and non-specific esterase were concentrated in spherosomes, the nucleases (RNAase and DNAase) and α -amylase were always absent. It must therefore be concluded that the histochemical enzyme staining pattern for spherosomes, mentioned previously, could equally well have represented the distribution of small vacuoles, particularly since the recent finding of Yatsu et al., (1971) shows that in a highly purified preparation of spherosomes from onions and cabbages acid phosphatase activity could not be detected in biochemical assays.

On biochemical and cytological grounds, therefore it seems that plant cells possess organelles that are equivalent to the animal lysosome but it is much more difficult to establish whether they have similar physiological functions. Obviously the heterotrophic nature of animal cells leads to large differences between animal and plant cell metabolism and any functional lysosomal differences are probably a reflection of this.

In animal cells lytic processes, causing certain intracellular organelles or structures to disappear are known to be involved in many phenomena of cellular differentiation and metamorphosis. Such processes have been observed in erythrocyte maturation (Tooze and Davies, 1965), skin keratinization (Farquhar and Palade, 1965), follicular atresia (Guraya and Greenwalt, 1964)

and mammary involution (Slater et al., 1963). Lysosomal enzymes have also been implicated in more dramatic developmental changes such as tissue regression in chick embryo (Scheib, 1965) amphibian tadpoles (Weber 1963; Eeckhout, 1965) and metamorphosing insects (Lockshin and Williams, 1964, 1965) where whole tissues are lost by widespread cell death.

Besides their involvement in cell differentiation and cell death Allison and Mallucci (1964) have claimed that the intracellular release of lysosomal enzymes acts as the 'trigger' for mitosis.

The roles of lysosomes in the pathogenicity of animal cells are known to be numerous and have been the subject of much recent medical literature. This aspect of lysosomal activity is dealt with extensively in the previously mentioned treatise, 'Lysosomes in Biology and Pathology' (Dingle and Fell, 1969). Since it is only recently that lysosome-like organelles in plant cells have been characterised biochemically, studies on their physiological roles have only just begun. Already, however, Matile and Winkenbach (1971) have shown that lysosomal enzymes are involved in ageing and senescence of corolla segments of Ipomea, whilst Heftmann (1971) has shown changes in various acid hydrolases during the ripening of tomato fruits. In addition Gibson and Paleg (1972) have produced evidence for the hormonal induction of lysosomal enzyme synthesis in wheat aleurone cells, where acid hydrolysis of the starchy endosperm is one of the initial events of germination.

It was in an attempt to add to the knowledge of the roles of plant acid hydrolases in the processes of cell division, differentiation and senescence in plant development that the present work was undertaken. A combined cytochemical and biochemical study of selected acid hydrolases was to be undertaken to answer two

specific questions in relation to the physiological and biochemical significance of acid hydrolases in plant cells.

1) Can acid phosphatases act as a 'trigger' for mitosis in plant cells in the same way that has been claimed for animal cells? By using the young root tips of Vicia faba for the cytochemical localisation of acid phosphatases, employing both the lead salt precipitation and azo-dye methods, particles staining for the enzymes could be counted in a large number of dividing and non-dividing cells (Chapter I). Statistical methods could then be used to analyse the significance of the numbers of particles at the different phases of cell division and in each of the different tissues.

Attempts were not made to investigate any possible triggering capacity of non-specific esterases in plant cell division since McLean (1970) has already shown that in broad bean root tips the number of esterase sites represented by azo-dye particles is dependent on the type of cell, not on the stage of cell division reached.

2) How does the activity of nonspecific esterases vary with increasing cell differentiation, maturation and senescence?

The growing root tip serves as an ideal model for the study of cell growth and differentiation (Esau, 1943; Jensen, 1955;

Holmes et al., 1955; Heyes and Brown, 1956). A longitudinal section of the root reveals a region behind the root cap, the apical meristem, where cells are constantly dividing and cutting off cells both to the cap and to the main body of the root.

By examining those cells cut off towards the base of the root a linear sequence is obtained of increasing cell differentiation and maturation with increasing distance from the tip. Consequently by taking serial segments of tissue several workers have investigated changes in cellular morphology, cytology and biochemistry during the differentiation process. Jensen (1958), Holmes et al., (1955) and Sunderland and McLeish (1961) measured changes in nucleic acids and protein whilst studies have also been made of changing enzyme levels in a variety of roots (Bottelier et al., 1943; Robinson and Brown; 1952, 1954; Brown and Robinson, 1955; Van Fleet, 1959; Czernik and Avers, 1964; Sutcliffe and Sexton, 1969). The rationale for such work is that cell growth in intact organs is often accompanied by large increases in protein content (Blank and Frey-Wyssling, 1941; Kopp, 1948; Brown and Broadbent, 1951) and other metabolic events such as respiration (Kopp, 1948).

However, only Sutcliffe and Sexton (1969) have used a combined cytochemical and biochemical approach to studies of this nature, despite the advantages of being able to correlate the cell and tissue localization of the enzyme(s) with their biochemical activity at any given region of the differentiating root.

Non-specific esterases were the enzymes chosen for the present study since there are established and reliable methods for their cytochemical localisation in both plant and animal cells. (Burstone, 1958a, 1962; McLean and Gahan, 1970). Furthermore, the lateral roots of Vicia faba have frequently been used in the histochemical study of non-specific esterases in cell differentiation (Benes, 1962; Sahulka and Benes, 1968; McLean, 1970; Benes, 1971) but no quantitative biochemical data were provided. Sahulka and Benes (1968) did, however, examine this group of enzymes semi-

quantitatively by subjecting enzyme extracts of the roots from the dividing, enlarging and maturing zones, to acrylamide gel electrophoresis and comparing the zymograms produced.

Since methods for the biochemical assay of non-specific esterases using cytochemical reagents had not previously been devised it was necessary first to define the optimal conditions for enzyme assay and then to examine critically and to assess the possible parameters that affect the intensity of colour development of the azo-dye in solution (Chapter II).

In Chapter III a procedure is described for the preparation of extracts and assay of non-specific esterases using homogenates from very small (approx. 0.5 mm) segments of young lateral roots of broad bean, from 0 - 3 mm, a zone covering the events of cell vacuolation, expansion and differentiation. At the same time the opportunity was taken to assess the effect of the universally-used electron microscope chemical fixative, glutaraldehyde, on enzyme activity detected both cytochemically and biochemically, since fixatives generally are known to reduce or alter enzyme activity (Burstone, 1958b; Hopwood, 1972).

The ripening of fruits is generally regarded as a process leading to the senescence of a variety of cells and tissues. Marked metabolic changes involving both the controlled synthesis and breakdown of cell constituents are known to occur during fruit maturation, (Bain and Mercer, 1964; Sacher, 1967). It was proposed that acid hydrolases might be important in certain of the breakdown processes; possibly being involved in the intracellular lysis of cell organelles and macromolecules as senescence proceeded (Barton, 1966).

It was planned to monitor the changes in non-specific esterase activity at the cytochemical level in 3 different soft fruits during ripening; blackberry, raspberry and strawberry. Additional, paralleled cytological and biochemical studies were to be undertaken of one of these fruits, the strawberry, in order to relate changes in enzyme activity to cellular and subcellular changes occurring as the receptacles ripened, (Chapter IV.).

To obtain quantitative data on the changes in esterase activity during ripening the assay method used in the root differentiation studies was applied to enzyme extracts prepared at different stages of berry maturation.

Mention has previously been made of the value of combining biochemical methods with ultrastructural studies when investigating the possible existence of plant lysosomes. It was therefore proposed to carry out some preliminary electron microscope cytochemistry on both differentiating root cells and developing strawberry receptacles to ascertain the subcellular sites of esterase activity and record morphological changes in cell structure with differentiation and ripening.

Shnitka and Seligman (1971) have reviewed some of the present-day limitations of the methodology for the ultrastructural localisation of enzymes. McLean (1970) was aware of these limitations when using lead phthalocyanin as an electron dense coupling salt for the localisation of naphthol AS D esterase activity and naphthol AS BI phosphatase activity in Vicia faba root tips. In an extension of her findings the same coupling salt has here been used for the subcellular localisation of esterase activity at the various stages of strawberry development.

In addition a newly synthesized and improved lead compound triphenyl-p-phenethyl lead (LPED), (Livingston et al., 1969) was used to localise these enzymes in differentiating root cells.

In view of the known solubility of the lead end-product in conventional dehydrating reagents, such as acetone or alcohol (Livingston et al., 1969; Cahan and McLean, 1969), the effect of incorporating a water soluble epoxy resin, Durcupan (Fluka Ltd.,) into the processing of tissues for electron microscopy needed to be assessed. It was hoped that these electron microscope studies would throw further light on the role of esterases in cell differentiation and cell senescence by enabling suitable comparisons to be made with esterase localisation studies performed at the light microscope level.

To sum up, the purpose of this investigation was to examine the involvement of two separate groups of acid hydrolases in the developmental processes of plant cell division (acid phosphatases), differentiation, maturation and senescence (non-specific esterases). This was to be achieved by obtaining quantitative data on esterase activity at the different stages of cell growth and development and combining it with qualitative data from both cytochemical and cytological techniques. In this way it was hoped that a picture could be built up of the ontogeny of the enzymes throughout plant cell growth, maturation and senescence, thereby allowing better comparisons to be made between the known functions of acid hydrolases in animal cell physiology (de Duve and Wattiaux, 1966) and the suggested ones for plant cells (Matile, 1969).

CHAPTER I

A CYTOCHEMICAL AND STATISTICAL INVESTIGATION OF
 β -GLYCEROPHOSPHATASE AND NAPHTHOL AS BI PHOSPHATASE
DURING CELL DIVISION IN ROOT TIPS OF VICIA FABA

I.1.

INTRODUCTION

A number of workers, Flaxman and Mulnard (1961), Allison and Mallucci (1964), Robbins and Gonatas (1964) and Maggi (1965), have shown that in animal cells, acid phosphatase-staining particles or lysosomes undergo apparent changes in number and configuration prior to mitosis. The number of such particles is reduced in dividing cells and their position in the cells changes from a predominantly polar one at interphase to a circum-nuclear one at prophase.

These observations prompted Allison and Mallucci (1964), to suggest that prior to cell division there is a release of lysosomal enzymes into the cytoplasm which acts as the 'trigger' for mitosis in those cells that are 'primed' for the event. They tested this idea by stimulating normally non-dividing tissue, in this case peripheral human blood lymphocytes, to divide by means of the plant mitogen, phytohaemagglutinin (PHA), and observing the ensuing changes in the number of lysosomal particles by acridine orange staining and fluorescence microscopy. In lymphocytes so treated there was an enlargement of the cells, accompanied by an increase in the number of β -glycerophosphatase-rich particles, and a subsequent reduction in number when the cells were dividing. Furthermore, substances such as chloroquinone or prednisolone, that are known to stabilize lysosomal membranes, inhibit this mitogenic effect, (Hirschhorn and Hirschhorn, 1965). Hirschhorn and coworkers (1968) provided added biochemical evidence for this hypothesis when they found a qualitative change in the distribution of β -glucuronidase and acid phosphatase in subcellular fractions isolated from PHA-stimulated human blood lymphocytes. The amounts of these acid hydrolases in the granular fraction derived by

centrifugation at 20,000 x g for 20 min decreased, but the activity in the corresponding supernatant increased, suggesting an increased fragility of the lysosome fraction after treatment. However, there was no increase in the total acid hydrolase activity per cell, and treatment with other membrane-disrupting agents such as Triton X-100 failed to release acid hydrolases from these particles.

In plant cells McLean (1970) reported that she could find no difference in number or pattern of distribution of acid hydrolase-containing particles in the dividing and non-dividing cells of the root and shoot meristems of Vicia faba L. No quantitative evidence was provided to support these preliminary findings, however, and it was in an attempt to extend these findings that a quantitative and statistical study of the distribution during mitosis of acid-phosphatase-staining particles was undertaken.

β -glycerophosphatase and naphthol AS BI phosphatase have been shown to be histochemically and biochemically distinct in both animal tissues (Neil and Horner, 1962; Maggi et al., 1966; Nelson, 1966; and Bowen, 1968) and plant tissues (Gahan and McLean, 1967). For this reason both the metal salt precipitation method of Gomori (1950) for β -glycerophosphatase and the azo-dye method of Burstone (1958a) for naphthol AS BI phosphatase were used in a study of the 'triggering' capacity of these enzymes in both fixed and unfixed root tips of Vicia faba L.

When using the Gomori procedure, fixation is usually recommended, since it has been maintained that undenatured protein produces false positive results (see for instance Gomori, 1950; 1952). Fresh, frozen sections will therefore adsorb nonspecific lead very strongly and not all will be removed in the 'acid rinse' (see Holt,

1959, for a critical assessment of the Gomori procedure). Also, in plant cells Sexton et al.,(1971) found that a large amount of β -glycerophosphatase leaked into the incubation medium when unfixed, frozen sections of root tips of maize and pea were used. Cold, glutaraldehyde-fixed material, on the other hand, gave both good cellular preservation and little enzyme inactivation. For these reasons root tips were first fixed in glutaraldehyde before preparing sections for the investigation of β -glycerophosphatase activity.

I.2.

MATERIALS AND METHODS

I.2.1: Plant material.

The procedure for growing plants of the broad bean, Vicia faba var. 'Aquadulce' was as follows: Seeds were soaked in running tap water for 16-24h, and then grown in tap water culture for 10-14 days under continuous aeration at 20°C. A light regime of 16h light and 8h darkness was maintained throughout the growth period. Lateral roots were washed in a jet of tap water and rinsed in distilled water before removal of the terminal 2 or 3 cm portions with a clean razor blade. All root material was immediately frozen for enzyme studies when unfixed material was required, or treated as in I.2.2.1.1. if fixed sections were needed.

I.2.2. Enzyme localisation procedures for acid phosphatases.

I.2.2.1. The lead salt precipitation method for β -glycerophosphatase (Gomori, 1950).

I.2.2.1.1. Fixation.

Whole roots were first fixed in 2.5% glutaraldehyde solution in 0.1M cacodylate buffer, pH 7.2, at +2°C for 3h with continuous shaking. The tissue was then frozen and sectioned according to the method of Gahan et al., (1967). Sections 10-20 μ m thick were cut on a Bright's cryostat at temperatures between -20°C and -25°C.

I.2.2.1.2. Incubation and staining procedure.

A modification of the Gomori procedure (Holt, 1959) was used. 0.316 g of sodium β -glycerophosphate (Sigma Chem. Co. Ltd.) and 0.12 g lead nitrate (B.D.H. Ltd.) were each separately

dissolved in 5 ml aliquots of distilled water; the two solutions were mixed, and added to 90 ml of 0.05M acetate buffer, pH 5.0. This solution was then left for 8-12h at 37°C before filtering and was used immediately afterwards.

Fixed, frozen sections were removed from the cryostat and allowed to dry in air for about 1 min, after which they were placed in Coplin jars containing the incubation medium. Incubation was allowed to proceed for 1h at 37°C before removing the slides and washing them in distilled water for 1 or 2 min. This was followed by a rinse in 1% (v/v) aqueous glacial acetic acid for 15 sec. The sections were washed once more in distilled water and finally placed in a solution of hydrogen sulphide to convert precipitated lead phosphate to black lead sulphide. The reactions are summarised below in Fig. I.1.

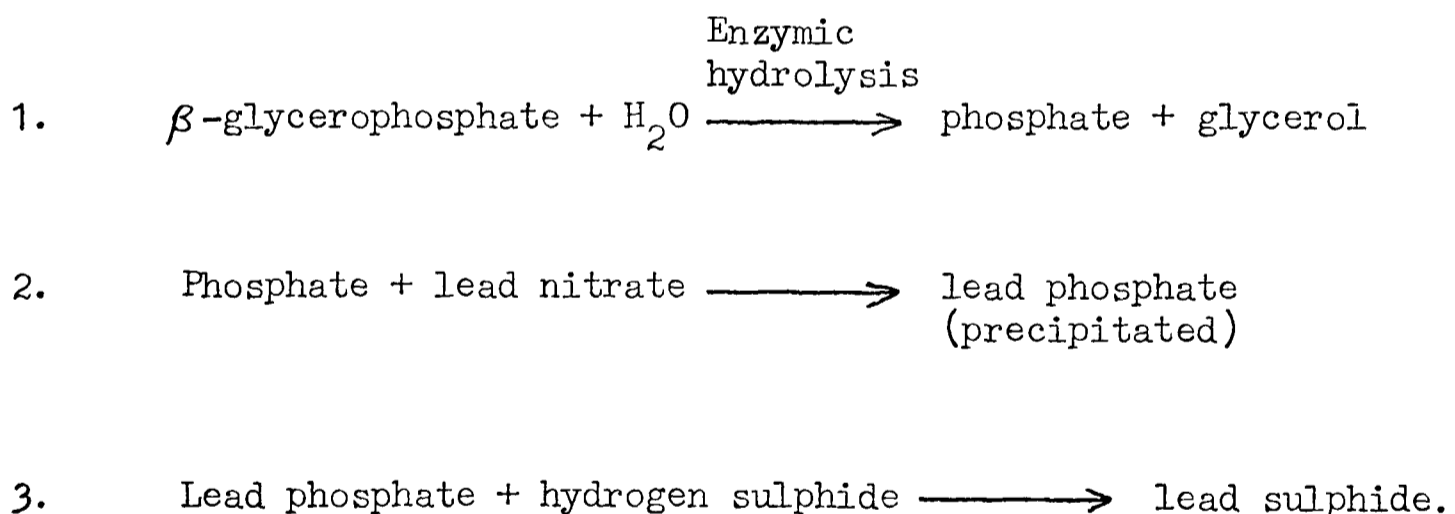


Fig. I.1: The reactions involved in the production of insoluble lead sulphide at acid pH, (Gomori, 1950).

The sections were finally washed again in distilled water before counterstaining, as detailed in Section I.2.3.

I.2.2.1.3. Controls.

i) No-substrate controls.

These were prepared by omitting the substrate, β -glycerophosphate, from the incubation medium. Treatment was otherwise identical with the procedure detailed in Section I.2.2.1.2.

ii) Heat-treated deactivated sections.

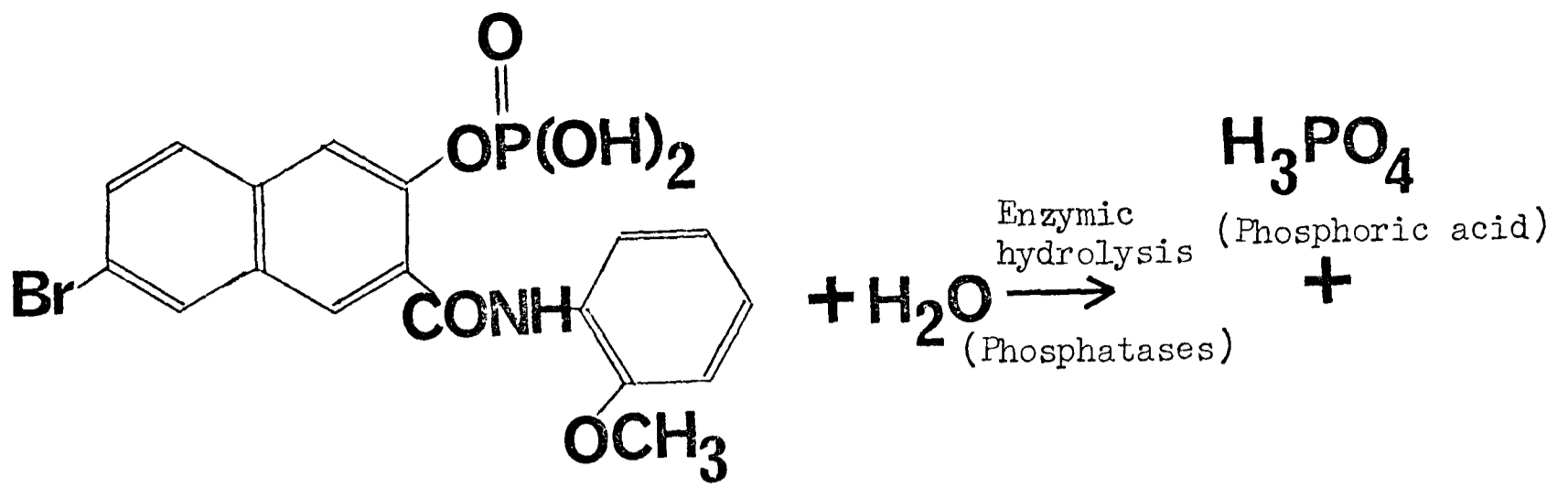
Cryostat-cut sections were placed directly into an incubator at 120°C for 1h and then incubated as detailed in I.2.2.1.2.

I.2.2.2. The azo-dye coupling method for naphthol AS-BI phosphatase (Burstone, 1958a).

A simultaneous coupling procedure was used as follows:

16 mg of naphthol AS-BI phosphate (Sigma Chem. Co. Ltd.) were dissolved in 0.5 ml of dimethylformamide (D.M.F., - B.D.H. Ltd., Dorset). To this solution were added 100 ml of 0.1M acetate buffer, pH 5.0, and 60 mg of fast red violet LB salt (Sigma Chem. Co. Ltd.). The mixture was shaken vigorously and filtered into a Coplin jar. Longitudinal sections 10-20 μ m thick were prepared as described in Section I.2.2.1.1. except that glutaraldehyde fixation was omitted. Sections were incubated for 30, 40 and 50 min at 37°C.

The chemistry of the phosphatase reactions and subsequent coupling is shown below in Fig. I.2.



Naphthol AS BI phosphate

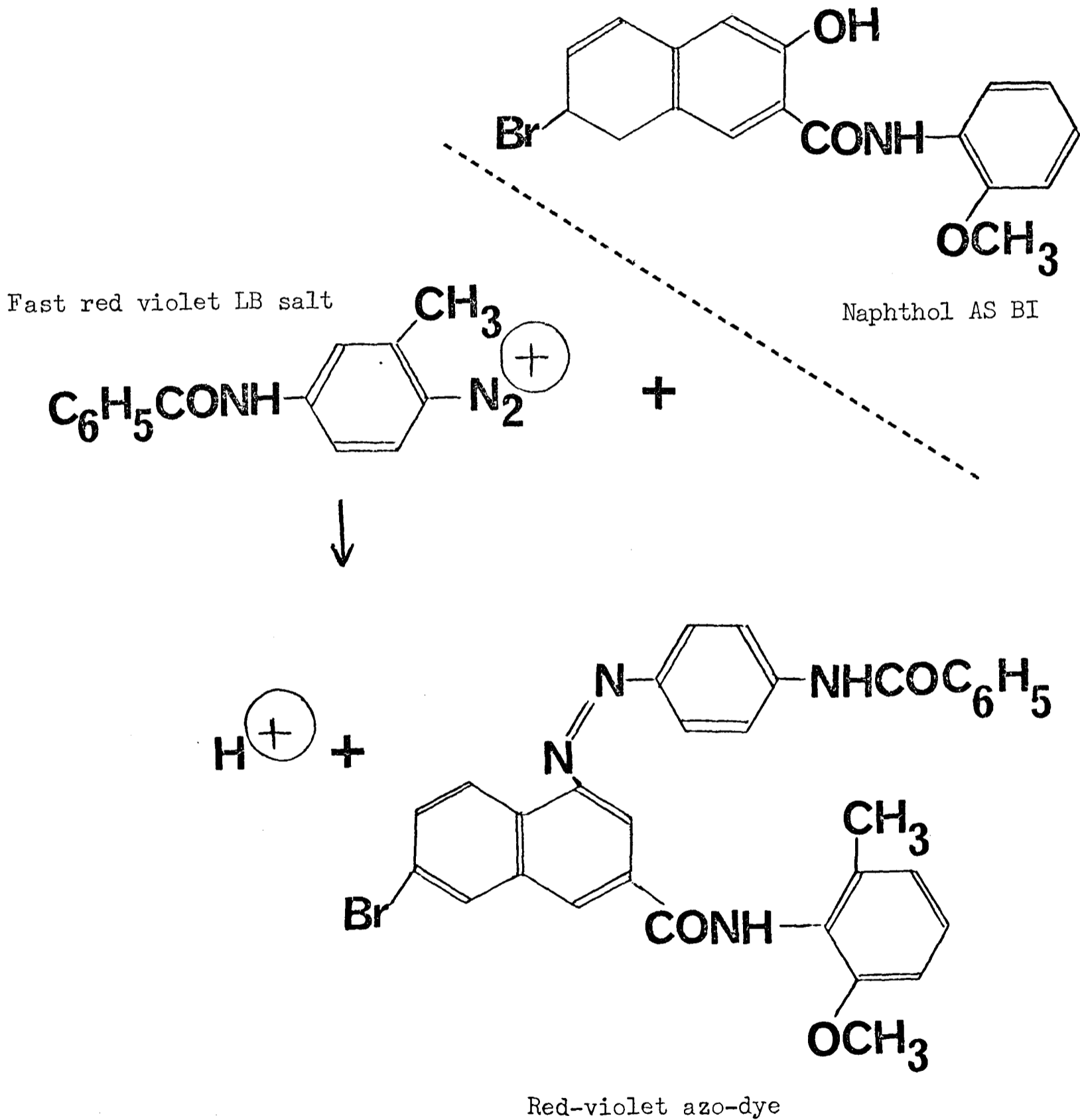


Fig. 1.2. The chemistry of the azo-dye coupling procedure for the demonstration of naphthol AS BI phosphatase. (Burstone, 1958a)

I.2.2.2.1. Controls.

The substrate, naphthol AS BI phosphate, was omitted from the incubation medium when control sections were required.

I.2.3. Counterstaining procedure.

To visualize mitotic figures Kurnick's methyl green (Kurnick, 1950) was used as a counterstain. (The methyl green used was of 'Revector' grade, obtained from Hopkin and Williams Ltd. Essex.) The procedure was as follows:

After enzyme incubation and distilled water wash, slides were immersed in 0.2M acetate buffer, pH 4.2, and then stained with aqueous methyl green (1% w/v) for 15 min at room temperature. The sections were then washed in 2 changes of 0.05M acetate buffer pH 4.2 for 10 min each wash. Slides were finally blotted dry, dehydrated in absolute ethanol for 1 min and mounted in 'Euparal'.

A number of slides were not counterstained after incubation for either β -glycerophosphatase or naphthol AS-BI phosphatase to ensure that the counterstaining procedure had no effect on the number and distribution of particles in mitotic and interphase cells.

I.2.4. Defining the tissues of the root tip.

Although Jensen (1955) has reported that in the primary broad bean root tip, mitotic figures can be found up to a distance of 3 mm from the root tip, in lateral roots it is rare to see mitotic figures more than 2 mm from the apex. This study was therefore confined to the first 2 mm, and within this zone 3 tissues were distinguished by microscopical examination; the apical initials, the procortex and the central cylinder. Using

a graticule in the eye-piece of the microscope these tissues were arbitrarily defined by a standard set of dimensions (given in Fig. I.3.) derived by microscopical measurements made on 4 longitudinal sections of the root tip. The figures are the averages for each of the three tissues.

I.2.5. Counting procedure.

Sites of acid phosphatase activity detected by either procedure were counted using a hand tally counter. Azo-dye particles were of more or less uniform size ca. 0.5 - 1.0 μ m diameter (Fig. I.4a and 4b), whereas lead sulphide deposits varied in size from 0.5-1.5 μ m diameter (Fig. I.5). A green filter in the microscope provided more contrast for viewing the green-stained chromosomes and aided identification of the stages of division, particularly in unfixed material.

For the azo-dye stained sections, ten cells from each stage of mitosis were counted for each incubation time and each tissue type, making a total of 360 mitotic cells. The same number of counts were made on interphase cells.

However, since only a small number of root tip cells are undergoing mitosis at any one time (Gray and Scholes, 1951) many sections had to be examined to fill the quota of mitotic cell counts. Interphase cells were counted from the same number of sections as mitotic cells to reduce sampling error. Since very large numbers of interphase cells are available for selection and scoring a system was devised to reduce bias.

A rectangular area of the slide encompassing the whole root section was defined by measurements of the length and breadth of the root with an eye piece graticule at low power magnification

Fig.I.3: The dimensions of the tissues in a longitudinal section of the lateral root of Vicia faba. (CX = cortex and procortical cells.

Fig.I.4a: Section of procortical tissue incubated for naphthol AS BI phosphatase for 40min. Azo-dye particles are clearly visible and are found in both interphase and mitotic cells. Magnification: x 500.

Fig.I.4b: Tissue incubated for naphthol AS BI phosphatase for 40min. Methyl green staining of mitotic figures is evident and a cell in telophase can be seen in the centre of the photomicrograph. Most of the procortical cells (pc) are without azo-dye particles at this magnification although they can be observed in root cap cells (RC). Magnification: x 500.

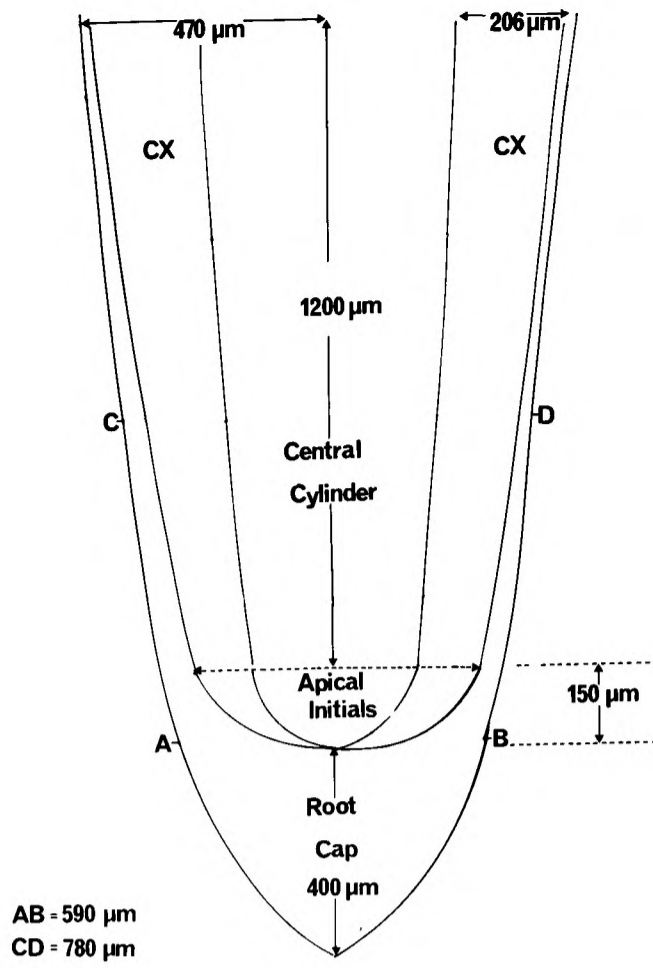


Fig.I.3

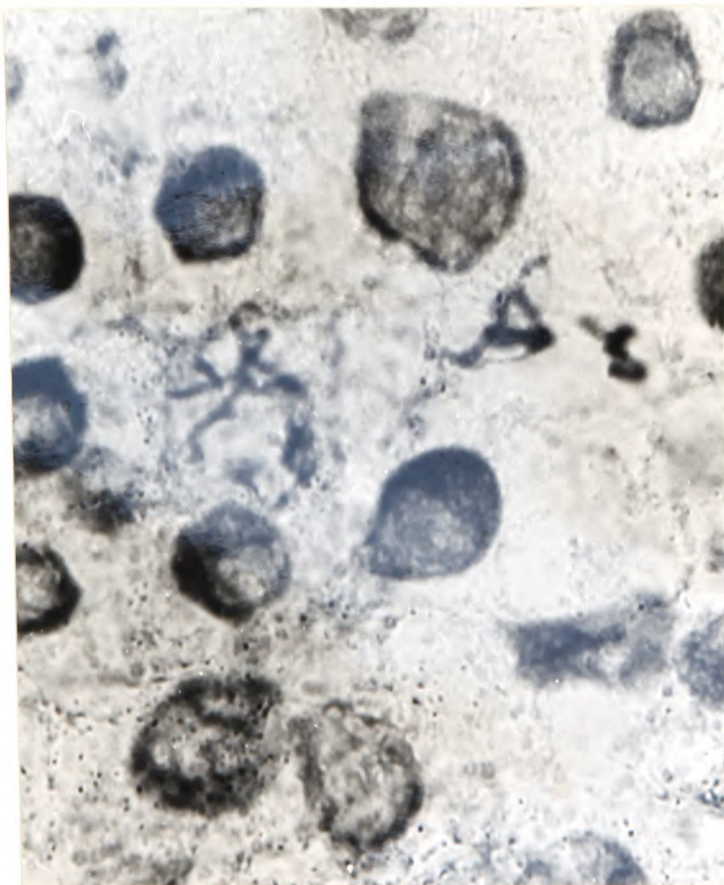


Fig.I.4a)

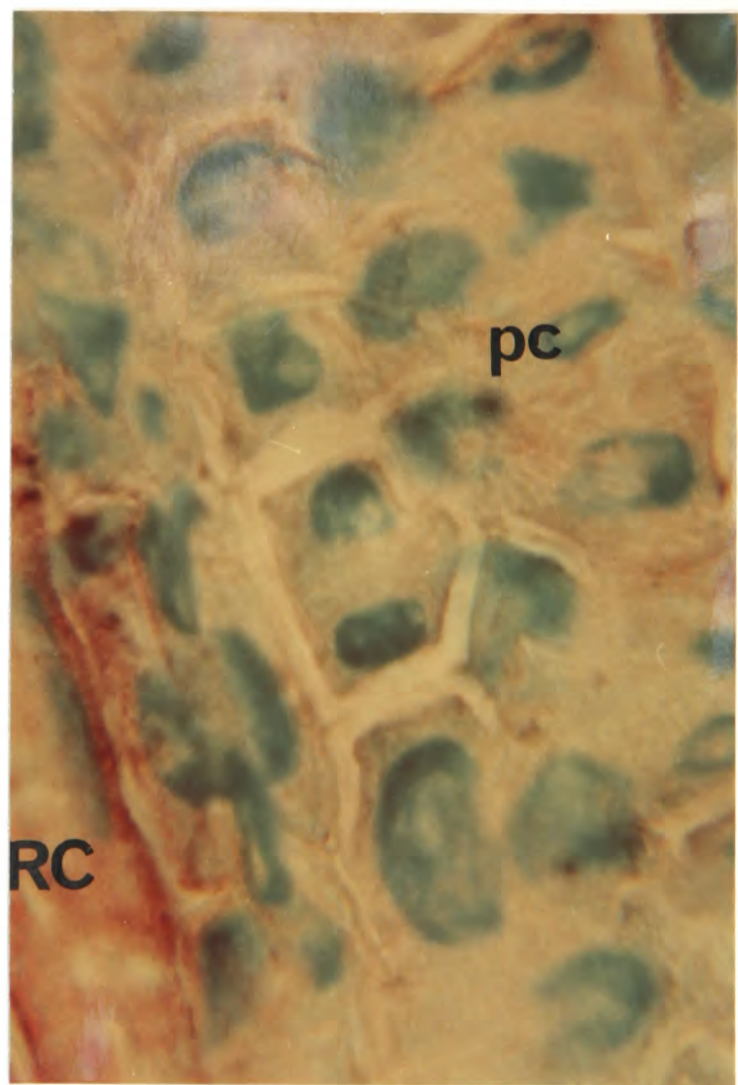


Fig.I.4b)

(approx. x 160). The 'eye piece units' defining the dimensions of the rectangle (approx. 500 units long by 300 units at the widest part of the root, at a magnification of x 160) were then divided by 10 to give a suitable range of numbers for selection from a table of random numbers (Fisher and Yates, 1963). Numbers were chosen in pairs, one for each axis to define any given point on the root surface. When the point was located 10 interphase cells were counted in a clockwise direction from the point source at high power magnification (x 1000). This process was repeated for each incubation time and tissue type until the quota of counts was filled.

When counting non-counterstained cells it was often necessary to locate the selected point using phase-contrast optics since cell outlines were often not visible under bright-field and then to revert to 'bright-field' at high power magnification for counting.

The method of cell selection was not entirely without bias however, since the non-rectangular root shape meant that points selected which fell outside the root section had to be discarded. When selecting numbers sampling was without replacement.

The above method was used for scoring interphase cells from both β -glycerophosphatase-stained and naphthol AS BI phosphatase-stained tissues, except that for the observation of β -glycerophosphatase activity 20 cells instead of 10 from each stage of mitosis and from each tissue type for both test and control (no-substrate) sections were counted, making a total of 480 mitotic cells, half from tests and half from control sections. The same number of counts were made for interphase cells.

Boiled control sections showed that no lead was deposited in the cells of any tissues except those of the root cap. Thus, no counts were made on these sections.

Many cells, both in mitosis and interphase, showed nuclear or DNA staining with lead, (Fig.I.6), but particle counts were confined to the cytoplasm, (see Danielli, 1953 and Holt, 1959, for a discussion of artefactual staining of DNA with the Gomori procedure).

I.2.6. Presentation of data.

Since the range in particle number per cell was large in relation to sample size, individual particle counts for each cell were grouped into class intervals of 5 counts viz., 1-5, 6-10 etc. with a separate class for a 'zero score'.

I.2.7. Statistical methods for the evaluation of
significance.

I.2.7.1. β -glycerophosphatase.

Total particle counts for no-substrate controls were 20-80% of those for test sections, depending on the type of tissue (see Table I.3.). It was therefore considered that a statistical treatment of the data for this enzyme was precluded, although it was considered worthwhile to compare counts for mitotic and interphase cells from both test and no-substrate controls by means of diagrams, (Fig. I.10, I.11, and I.12). For comparative purposes the data from Table I.1. for test counts are presented again in Table I.2. after subtracting counts for no-substrate controls.

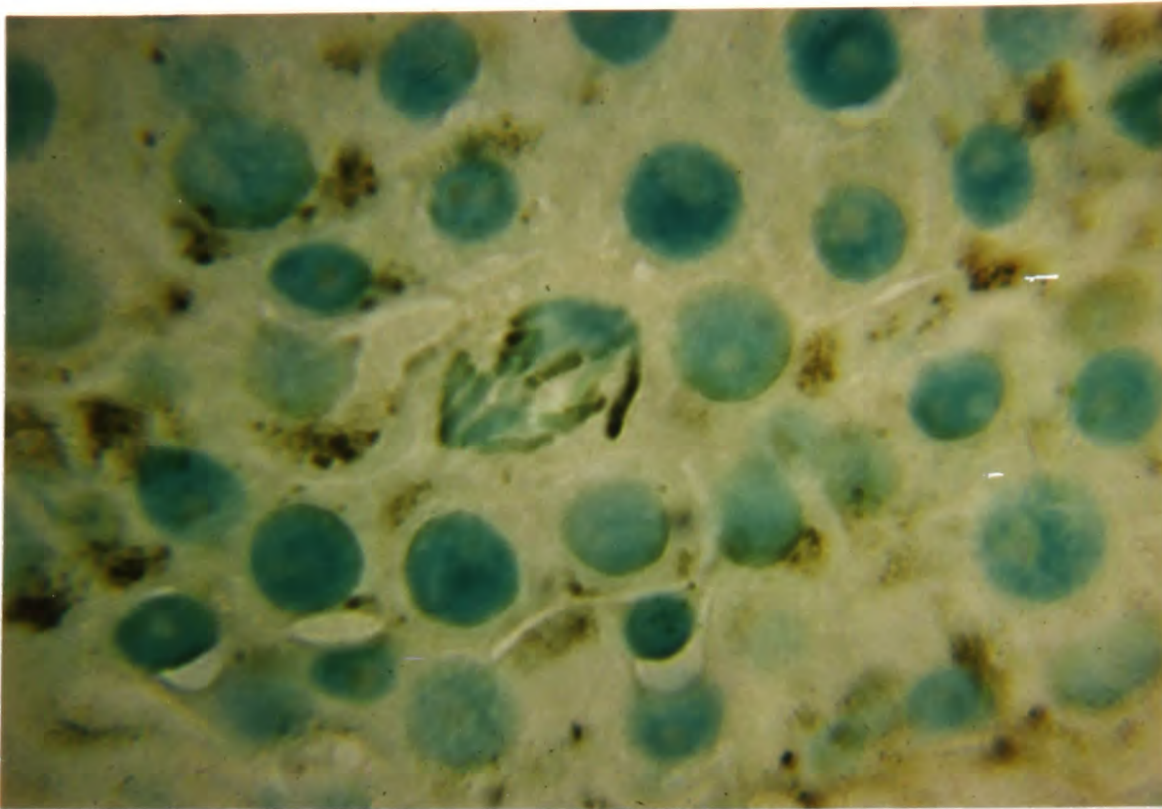


Fig.I.5: Apical Initials stained for B-glycerophosphatase activity and counterstained with methyl green. Note the grouping of the lead-stained particles within the cytoplasm and the staining of anaphase chromosomes. (x 500).

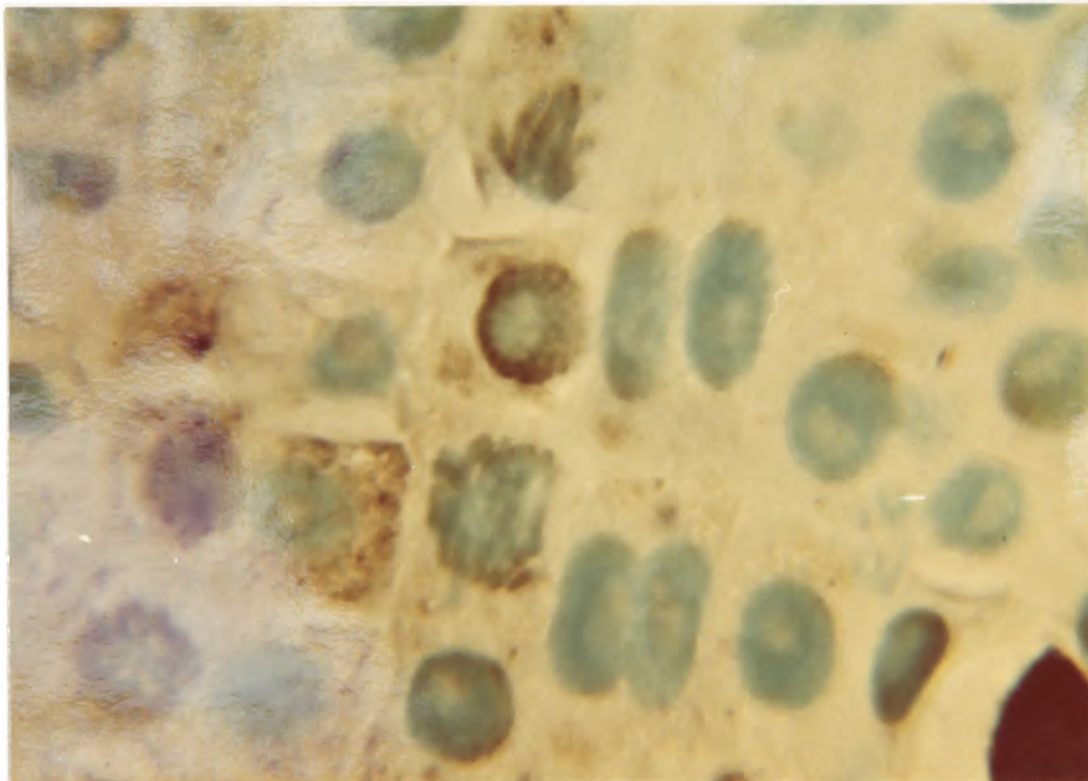


Fig.I.6: Cells from the central cylinder. Varying degrees of nuclear staining for B-glycerophosphatase are evident. (x 500).

I.2.7.2. Naphthol AS BI phosphatase.

Since no-substrate controls were always negative it was appropriate to use particle counts from test sections to calculate the level of significance between scores for mitotic and interphase cells from all 3 tissue types and incubation times.

The statistical procedure used was that for a comparison of means of two large samples of sizes $n_1 > 30$ and $n_2 > 30$. The ratio 'd' is calculated from the formula;

$$d = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}} \quad (\text{see for example, Bailey, 1959}).$$

where the two groups of observations, mitotic and interphase cells, are labelled '1' and '2' respectively and \bar{x}_1 and \bar{x}_2 are the means of n_1 and n_2 observations ($n = 40$ for each of the incubation times for mitotic and interphase cells) and S_1^2 and S_2^2 the estimated variances of the two populations. Data were also plotted in the form of histograms (Figs. I.13. and I.14.) to show the effect of incubation time and the stage of cell division reached on particle counts for mitotic and interphase cells.

I.2.8. The effect of counterstaining on particle number.

Since mitotic figures cannot readily be seen without counterstaining it was necessary to pool data from mitotic and interphase cells from counterstained sections in order to make a valid comparison with data from non-counterstained cells. The procedure for each enzyme is described below.

I.2.8.1. β -glycerophosphatase.

For each tissue type from both test and no-substrate controls, 40 cell counts from a possible 80 were selected at random (Fisher and Yates, 1963) from the data of counterstained material. 80 cells were then counted from non-counterstained material (for both test and no-substrate controls) using the method previously described for selecting interphase cells (I.2.5.). 40 counts from these data were again chosen at random and the statistic 'd' calculated for the difference in means of counterstained and non-counterstained populations.

I.2.8.2. Naphthol AS-BI phosphatase.

Sample sizes and selection of cells for counting were as for β -glycerophosphatase except that 80 cells had to be counted from each incubation time for each tissue type from both counterstained and non-counterstained cells. No-substrate control cells showed no staining and were therefore not counted.

I.3.

RESULTS

I.3.1. Cytology.

I.3.1.1. β -glycerophosphatase.

A mosaic of the terminal two millimetres of a lateral root tip was made from photomicrographs of a section incubated for this enzyme for 60 min, (Fig. I.7.). A similar mosaic was also made (Fig. I.8.) from no-substrate controls to provide a direct visual contrast.

Most obvious in the test roots are the intense reactions of the root cap and certain files of cells of pro-vascular tissues with obvious, clear areas in some cells that probably correspond to large vacuoles. Even at low magnifications mitotic and interphase cells may be distinguished and both particulate and diffuse cytoplasmic stain may be observed. Although in some cells the β -glycerophosphatase particles take up a polarized position as in animal cells (Robbins and Gonatas, 1964), this is by no means always the case in root tip tissue and a number of different configurations of these particles are seen in both mitotic and interphase cells; in some cells particles are completely absent (Fig. I.5 and I.6).

I.3.1.2. Naphthol AS-BI phosphatase.

In contrast to the fixed cells mentioned above, the visualization of mitotic figures in unfixed material was not as clear-cut. (These cells would, however, have been fixed to a certain extent during the counterstaining procedure).

A similar mosaic to that presented in Fig. I.7. is shown in Fig. I.9. and permits a comparison of the overall pattern of



Fig.I.7: Mosaic of a longitudinal section of a root tip fixed in glutaraldehyde and stained for B-glycerophosphatase (1h). Intense activity is obvious in the cells of the central cylinder and root cap. (x 50).

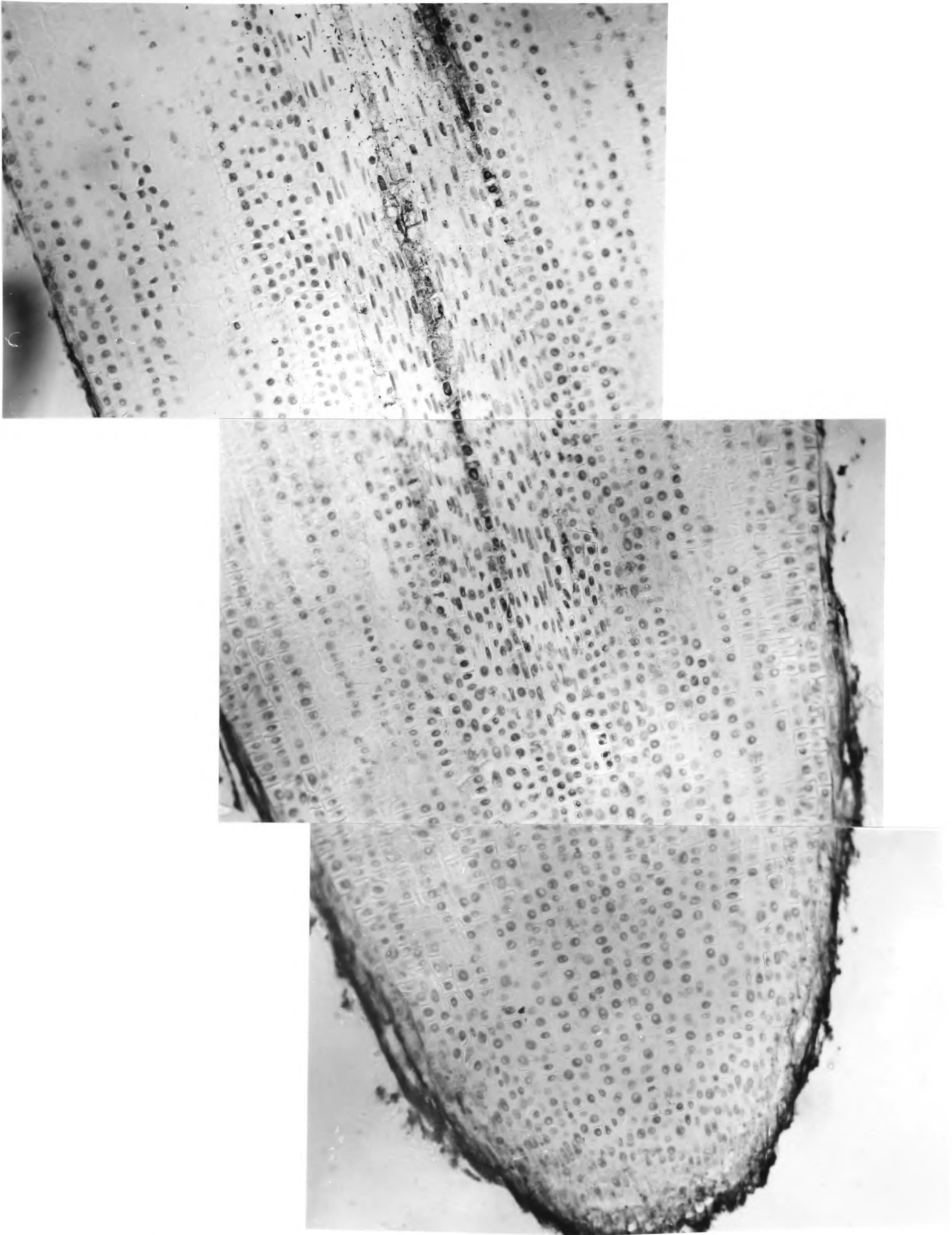


Fig.I.8: As Fig.I.7 but sections incubated in the absence of substrate.

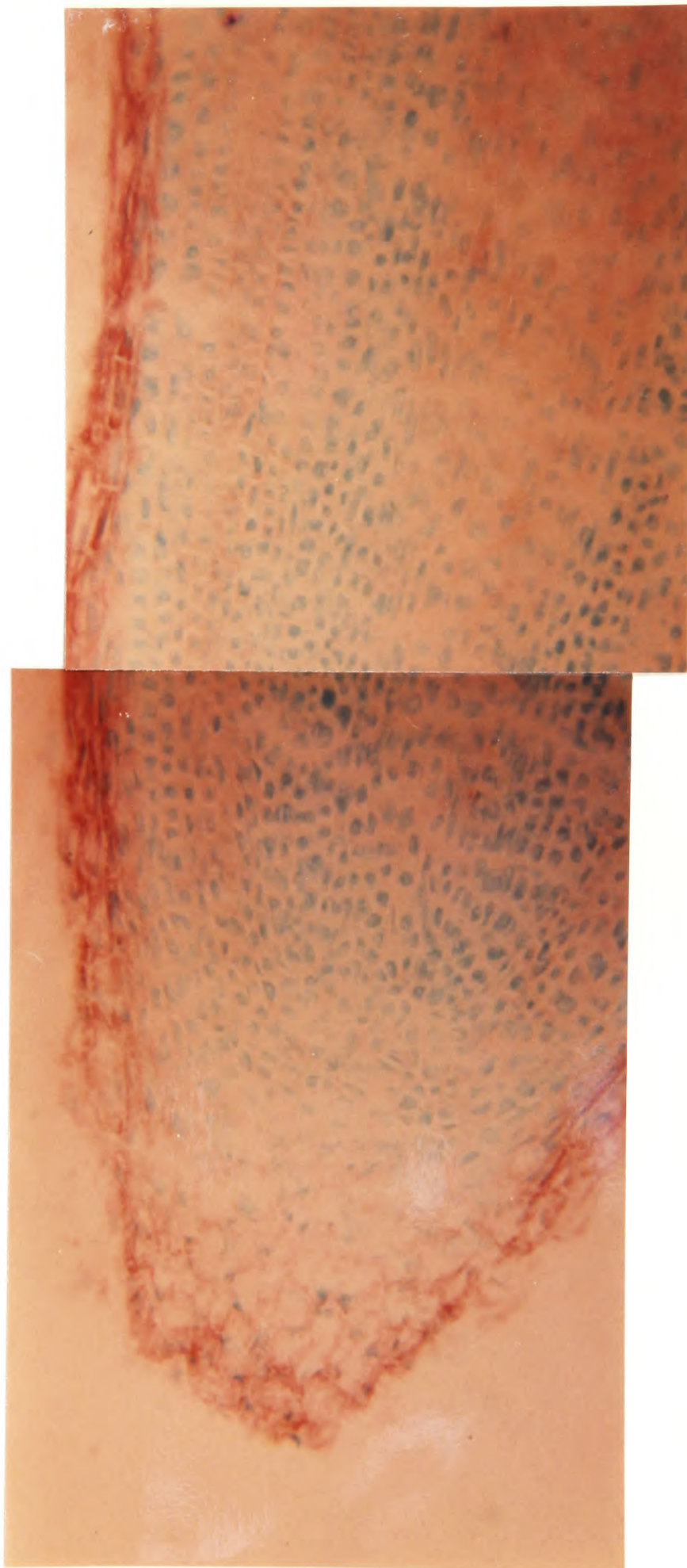


Fig.I.9: Mosaic of unfixed root tip sections stained for naphthol AS BI phosphatase (50min). Red azo-dye is most noticeable in the central cylinder, tracts of cells in the procortex and in the root cap cells. (x 50).

staining intensity. Fig. I.9. shows that, when assessed visually, most of the dye (with the exception of the root cap cells) appeared in the central cylinder and outer cortical layers.

High power photomicrographs (Fig. I.4a) show that the cells lack any diffuse staining (again with the exception of root cap cells) and only a particulate stain is visible. This was true even of sections incubated for 50 minutes.

Particles never appeared grouped or located in particular areas of the cell, but seemed to be spread generally in the cytoplasm. Unlike sections stained for β -glycerophosphatase, no nuclear staining was evident.

I.3.1.3. Effect of counterstaining on cytology.

For both β -glycerophosphatase and naphthol AS-BI phosphatase, sections not counterstained showed no apparent difference in form or distribution of stained particles when compared with counterstained material.

Nuclear staining was evident in non-counterstained material incubated for β -glycerophosphatase, a finding in keeping with other observations on glutaraldehyde-fixed root sections (Sexton et al., 1971).

I.3.2. Statistics.

I.3.2.1. Histograms.

I.3.2.1.1. β -glycerophosphatase.

The frequency distribution curves (Fig. I.10.) in test sections indicate that, during division, there was a progressive decline in the total number of particles up to anaphase. However,

- Fig.I.10: Frequency distribution curves of particle counts of β -glycerophosphatase activity in both test and 'no-substrate' control cells at the different stages of mitosis.
- Fig.I.11: Data from dividing cells (test and controls) grouped and compared with histograms from interphase cells.
- Fig.I.12: Breakdown of control tissue data from Fig.I.11 into tissue types

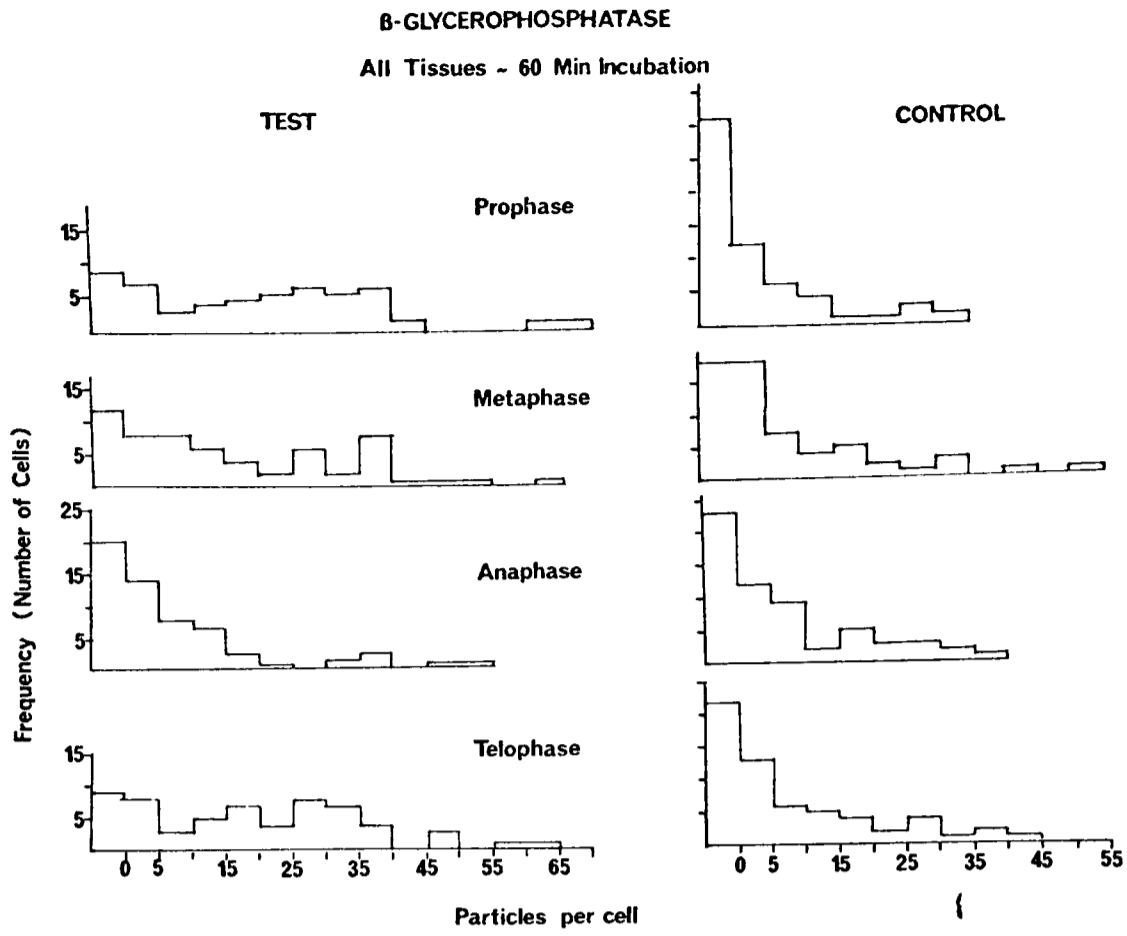


Fig.I.10.

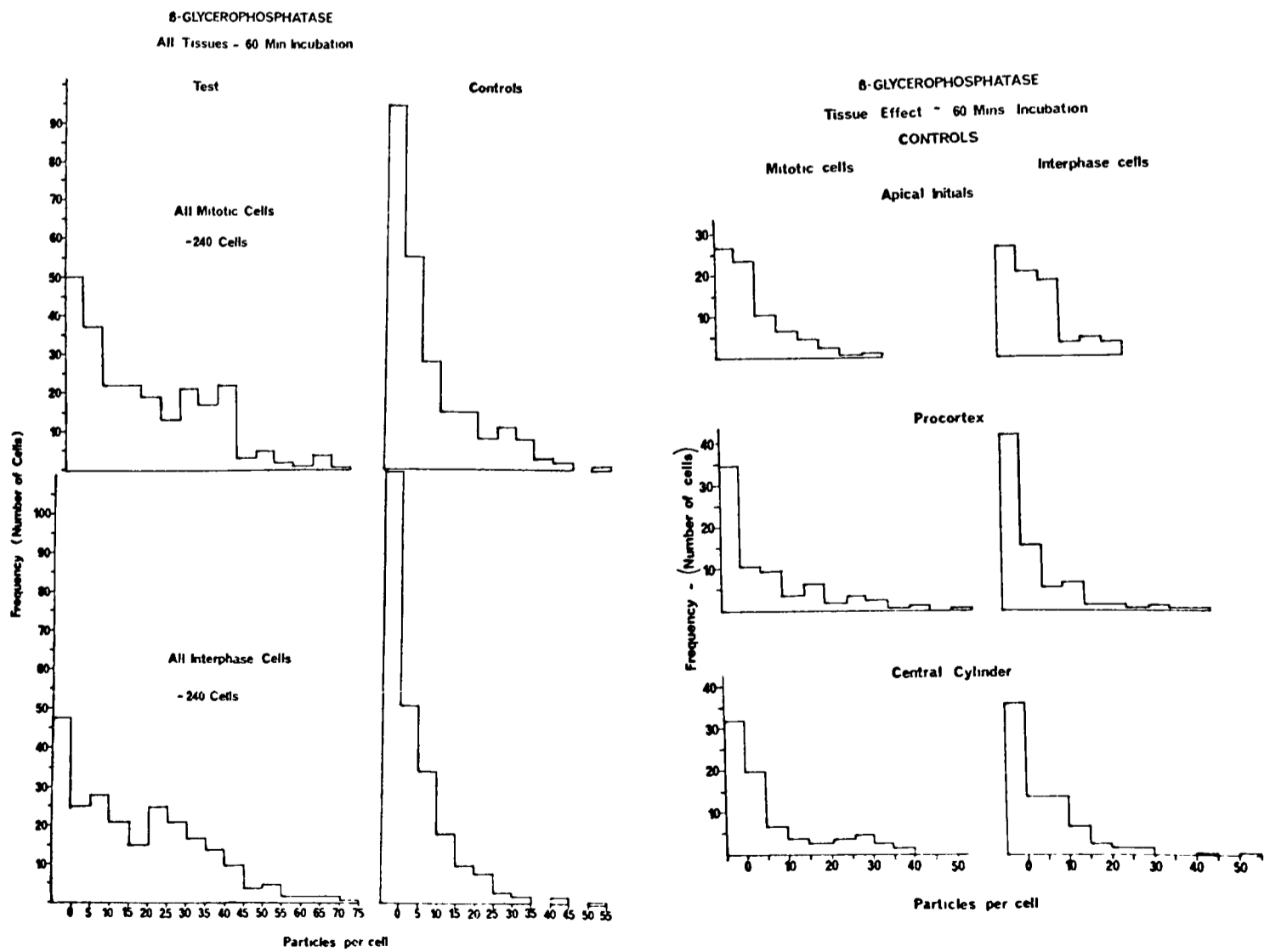


Fig.I.11.

Fig.I.12.

by telophase, particle numbers had increased almost to prophase numbers. In contrast, counts from control sections at all stages of karyokinesis exhibited the same 'J' shaped curve. These results are reflected by Table I.2., where no-substrate control counts have been subtracted from test scores (Table I.1.). All tissues showed, to varying degrees, a cycle of decrease in particle number from prophase to anaphase and a subsequent rise to telophase.

Furthermore, when all the data are grouped for mitotic cells and interphase cells from both test and control sections (Fig. I.11.) the visual similarity is very marked between the curves for mitotic and interphase test scores on the one hand and no-substrate control mitotic and interphase scores on the other hand. It will be noted that, the number of cells lacking any particle staining is twice as great in control sections as in test sections in both dividing and non-dividing cells.

The frequency distribution curves of β -glycerophosphatase-staining particles in test sections is possibly bimodal for both dividing and non-dividing cells, in contrast to the pronounced 'J' shape of curves for control cells. A breakdown of the data from control sections (Fig. I.12.) into tissue types shows no large departures in any of the 6 curves from the 'J' shape. However, the data from Table I.3. show that in dividing procortical cells about 50% of the total lead-staining particles formed on test sections were also formed in the absence of substrate. Figures from the other two tissues were lower but still make significant contributions to the staining of test sections.

In all 3 tissues the percentage of cells staining with lead in the absence of substrate was higher in mitotic cells than

Table I.1. Total counts of β -glycerophosphatase particles from equal numbers (480) of dividing and non-dividing cells.

Phase of Cell Division	Apical Initials	Procortex	Central Cylinder	Totals	n
Prophase	540 (110)	421 (88)	391 (128)	1352 (326)	60
Metaphase	395 (162)	295 (231)	351 (113)	1041 (506)	60
Anaphase	252 (69)	120 (92)	203 (168)	575 (329)	60
Telophase	364 (154)	362 (200)	478 (153)	1204 (507)	60
Totals	1551 (495)	1198 (611)	1388 (562)	4172 (1668)	240
Interphase	1595 (382)	1414 (400)	1423 (484)	4432 (1266)	240

Control values (no substrate) are in brackets.

n = No. of cells counted.

Table I.2. No-substrate control values subtracted from test sections for β -glycerophosphatase in Table I.1.

Phase of Cell Division	Apical Initials	Procortex	Central Cylinder	Totals
Prophase	430	333	263	1026
Metaphase	233	64	238	535
Anaphase	183	38	35	256
Telophase	210	162	325	697
Totals	1056	592	861	2514
Interphase	1213	1014	939	3166

Table I.3. No-substrate control counts as a percentage of test counts for β -glycerophosphatase.

Phase of Cell Division	Apical Initials	Procortex	Central Cylinder	Totals
Prophase	20.4	20.9	31.1	24.1
Metaphase	41.0	78.3	32.2	48.6
Anaphase	27.4	76.7	82.7	65.0
Telophase	23.1	55.2	32.0	42.1
Totals (Mitotic)	31.9	51.0	40.5	40.0
Interphase	23.9	28.3	34.0	28.6

All percentage values are calculated from Table I.1.

interphase cells. It is probable too, that the high no-substrate control counts for cells at anaphase (65% of test sections) is a reflection of the decrease of particle number at this stage of cell division.

I.3.2.1.2. Naphthol AS-BI phosphatase.

Frequency distribution curves for this enzyme at the different incubation times showed that there was a definite shift in the population medians between incubation times 40 and 50 minutes in both mitotic and interphase cells, although between 30 and 40 min there was no perceptible change (Fig. I.13.).

By 50 minutes of incubation the curves for both mitotic and interphase cells were approximately normal, whereas the curves for 30 and 40 minutes were skewed. This means then that at low incubation times (30 and 40 minutes) a large number of cells contained relatively few particles per cell but by 50 minutes many more cells contained greater numbers of particles. For instance, in interphase cells incubated for 30 min there were 30 cells containing between 5-10 particles per cell, by 40 min only 14 cells did so and by 50 min the figure had fallen to only 2 cells. By comparison, 25-30 particles per cell were found in only 7 cells after 30 min incubation, in 12 cells after 40 min and the figure had risen to 20 cells with 50 min incubation.

A statistical treatment of these data is deferred to Section I.3.2.2.1. and the significance of incubation time in determining particle numbers per cell is discussed in I.4.

Since the results of the azo-dye staining were qualitatively the same at all 3 incubation times and other data (Fig. I.15.)

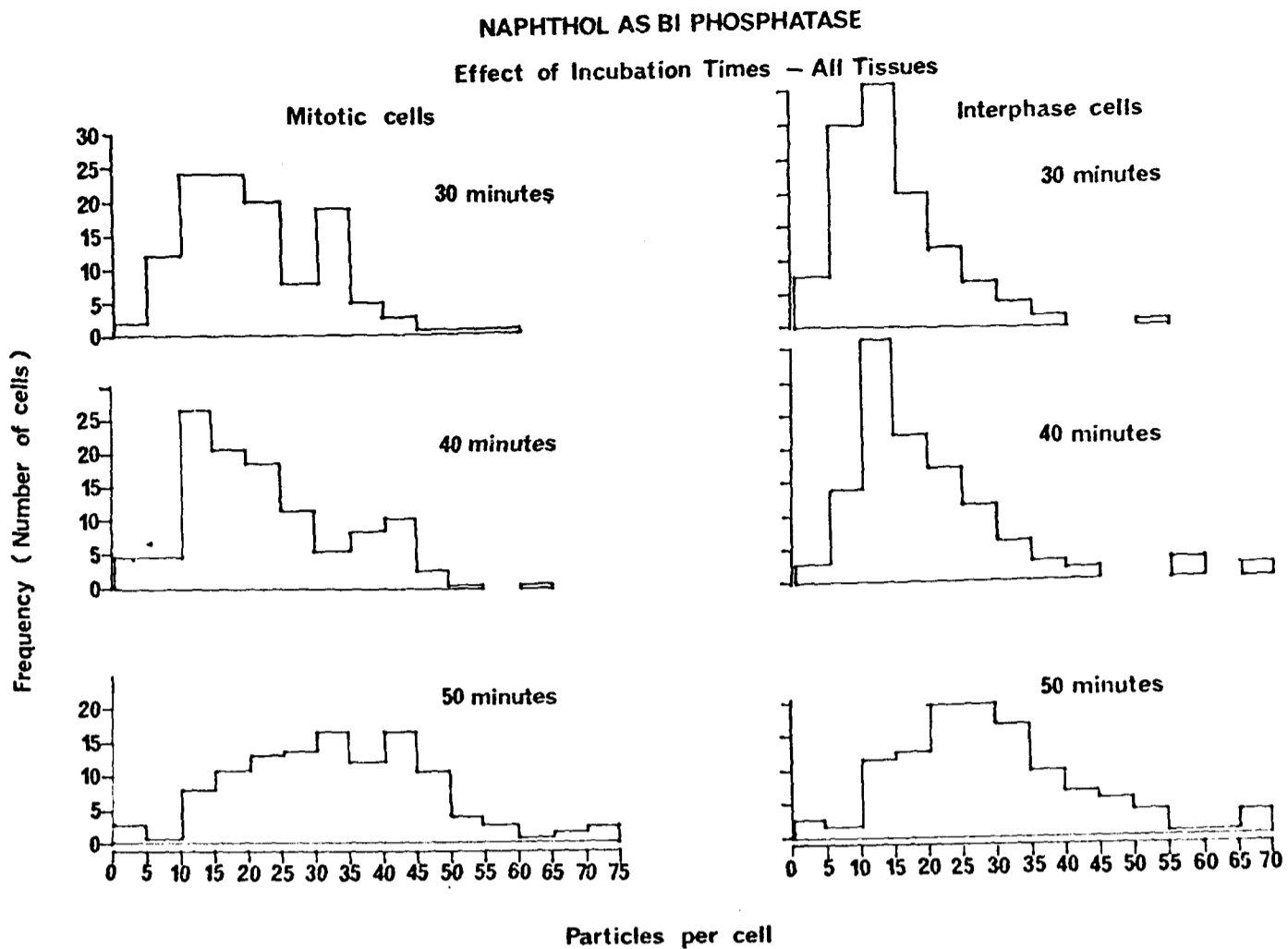


Fig.I.13: The effect of incubation time on the frequency distribution curves for azo-dye particles in both mitotic and interphase cells.

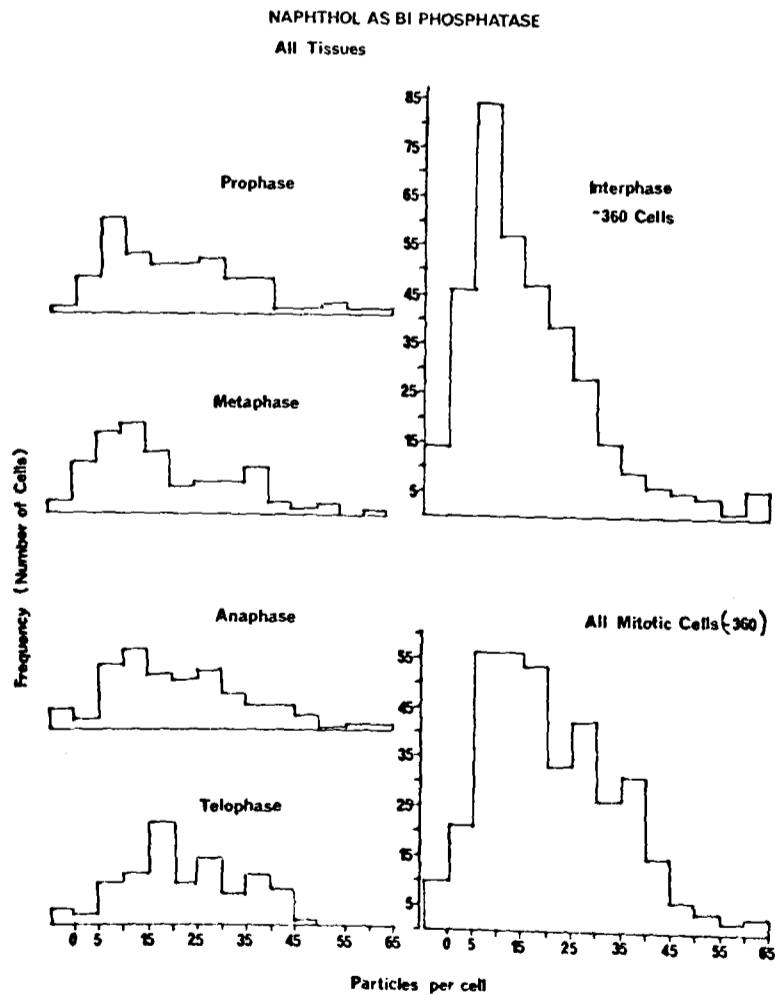


Fig.I.14: Data from Fig.I.13 grouped to show frequency distribution curves for all mitotic and all interphase cells. Mitotic data is also broken down into the respective phases of cell division.

Fig.I.15: The effect of incubation time on the mean numbers of azo-dye particles per cell in mitotic (●—●) and interphase cells (○—○) in the three tissues of Vicia faba root tips.

NAPHTHOL AS BI PHOSPHATASE

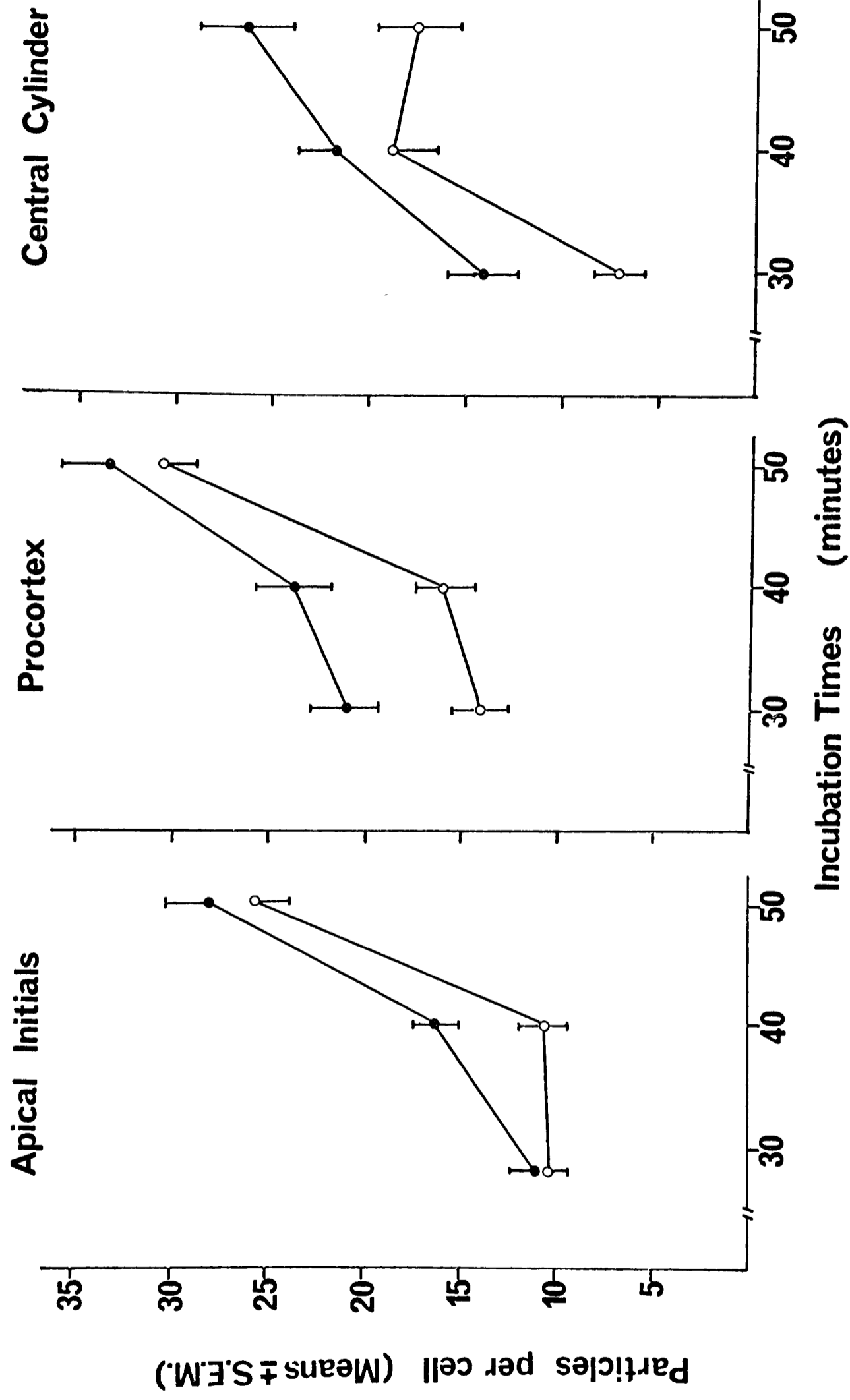


Fig. I.15

showed that curves for mean particle number per cell were similar in all 3 tissues it was decided to group the data from the 3 incubation times (Fig. I.14).

Treating the data in this way obscured the trend from skewness to normality for both mitotic and interphase cells. The raw data from which these histograms were prepared is given in Table I.4; the most noticeable feature of which is the greater number of particles present in mitotic as opposed to interphase cells at all incubation times and in all 3 tissues. It is also evident from this table (and Fig. I.14.) that particle numbers during the different stages of cell division did not show the cyclic decrease to anaphase and increase during telophase observed with β -glycerophosphatase-staining particles (I.3.2.1.1.).

I.3.2.2. Statistical analysis.

I.3.2.2.1. Naphthol AS BI phosphatase.

The means and standard errors of the means were calculated for mitotic and interphase cells at each incubation time and for each tissue type. The results (Fig. I.15.) show a similar pattern for all 3 tissues. As would be expected from Table I.4., the means for mitotic cells were higher than their interphase counterparts at all incubation times and in all 3 tissues.

The statistic 'd' was calculated for the difference in means between mitotic and interphase cells at all incubation times and in all 3 tissues, and the corresponding level of significance determined (Appendix I, Bailey, 1959). The results (Table I.5.) are largely as expected from Fig. I.15.: apical initials and the

Table I.4. Total counts of naphthol AS BI phosphatase particles in equal numbers (720) of dividing and non-dividing cells.

Phase of Cell Division	Apical Initials			Procortex			Central Cylinder			Totals
	30	40	50	30	40	50	30	40	50	
Prophase	108	153	370	231	262	302	84	164	223	1897
Metaphase	113	141	178	215	215	397	96	239	231	1825
Anaphase	118	172	251	209	220	312	190	225	310	2007
Telophase	103	184	326	188	257	327	199	245	300	2129
Totals	442	650	1125	843	954	1338	569	873	1064	7858
Interphase	414	426	1035	561	641	1229	287	761	705	6059

No particles were detected in control sections.

Table I.5. Calculation of the 'd' statistic for a comparison of means between mitotic and interphase cells in each of the 3 tissues of the root tip.

Tissue	Time of Incubation (min)	Mitotic Cells	Interphase Cells	Observed 'd'	Significance Level
Apical Initials	30	11.0 ± 1.36	10.3 ± 1.02	0.41	n.s.
	40	16.2 ± 1.22	10.6 ± 1.25	3.21	**
	50	28.1 ± 2.26	25.9 ± 2.02	0.70	n.s.
Procortex	30	21.1 ± 1.71	14.0 ± 1.45	3.16	**
	40	23.8 ± 1.95	16.0 ± 1.63	3.06	**
	50	33.4 ± 2.47	30.7 ± 1.83	0.88	n.s.
Central Cylinder	30	14.2 ± 1.85	7.2 ± 1.12	3.28	**
	40	21.8 ± 2.08	19.0 ± 2.37	0.88	n.s.
	50	26.6 ± 2.59	17.6 ± 2.18	2.66	**

n.s. = not significant

** = 0.001 < p < 0.01

* = 0.01 < p < 0.05

*** = p < 0.001

∅ Figures are mean number of particles per cell, ± standard error of the mean.

procortex show similar patterns. Taken as a whole mitotic means were significantly greater ($0.001 < p < 0.01$) than interphase means in 5 out of the possible 9 comparisons, with 2 of these at 30 minutes (procortex and central cylinder) 2 at 40 minutes (apical initials and procortex) and one at 50 minutes (central cylinder).

Since incubation time is of obvious importance in determining mean particle number per cell, 'd' was also calculated for a difference in means between 30 and 40 minutes incubation time and 40 and 50 minutes, for each tissue and for both groups of cells (Table I.6.). Comparisons were only made amongst populations of interphase cells and amongst populations of mitotic cells and no attempt was made, for example, to calculate 'd' for a mitotic mean at 30 minutes' incubation and the interphase mean at 40 minutes, in any given tissue.

Data expressed in this way show there are differences between apical initials and procortex on the one hand and the central cylinder on the other, with the levels of significance being $p < 0.01$ in 3 comparisons out of 7 and $p < 0.001$ in the other 4. In practical terms this means that for the apical initials and the procortical cells the increase in numbers of azo-dye particles is greatest between 40 and 50 min of incubation whilst in the central cylinder greatest increases occur between 30 and 40 min.

It is interesting that, in this respect, there are no marked differences between mitotic and interphase cells.

I.3.2.2.2. The effect of counterstaining.

Table I.6. Calculation of significance levels between incubation times for naphthol AS BI phosphatase-staining particles.

Tissue	Comparison made. Incubation time (min)	Interphase Cells		Mitotic Cells	
		Observed 'd'	Significance Level	Observed 'd'	Significance Level
Apical Initials	30/40	0.186	n.s.	2.84	**
	40/50	6.45	***	4.63	***
Procortex	30/40	0.92	n.s.	1.04	n.s.
	40/50	5.97	***	3.04	**
Central Cylinder	30/40	4.50	**	2.76	***
	40/50	0.43	n.s.	1.45	n.s.

Symbols and abbreviations used are as for Table I.5.

I.3.2.2.2.1. β -glycerophosphatase.

A comparison of means between counterstained and non-counterstained sections entailed the calculation of 12 'd' values for this enzyme, 6 for the comparison, (test, counterstained vs. test, non-counterstained) and likewise 6 for the equivalent 'no-substrate control' sections. Each 'd' value was calculated in respect to tissue type (3) and cell type (mitotic or interphase) - (2) giving, $3 \times 2, = 6$ comparisons of means.

For test sections the statistic 'd' ranged from 0.28 to 0.91 and for no-substrate controls from 0.32 to 1.23. Thus at the 5% level of significance counterstaining has no effect on the number of β -glycerophosphatase particles stained.

I.3.2.2.2.2. Naphthol AB BI phosphatase.

Although there were no control sections to count for this enzyme, the use of 3 incubation times meant the calculation of 18 'd' values i.e. 3 (incubation times) x 2 (types of cell), x 3 (tissue types). None of the 'd' values was significant at the 5% level: the range varied from 0.29 to 1.44.

I.4.

DISCUSSION

The high particle counts for sections of meristematic root tissue incubated for β -glycerophosphatase in the absence of substrate has precluded a statistical study of the capacity of this enzyme to 'trigger' mitosis. This is unfortunate since this was the marker enzyme chosen by Allison and Mallucci (1964) in their original studies of lysosomal enzymes in cell division.

The above problem, however, does not apply to naphthol AS-BI phosphatase. The results of statistical analysis for this enzyme indicate that, since there were significantly greater numbers of azo-dye particles in dividing cells than interphase cells in all 3 meristematic tissues investigated, then it must be concluded that naphthol AS BI phosphatase in plant cells, at least, cannot be implicated in the triggering process for cell division, as suggested by the work of Allison and Mallucci (1964). The results are in fact a reverse of what would be expected if the enzymes were triggering mitosis by being released into the cytoplasm just before the commencement of prophase. This finding, however, does not rule out the possibility that acid hydrolases are involved in mitosis since Mazia (1961) has suggested that if the mechanism for the disruption of the nuclear membrane is enzymic, lysosomal enzymes may be involved.

Indeed, since the breakdown of the nuclear membrane commonly marks the end of prophase it may explain the high prophase counts for β -glycerophosphatase. It is noteworthy, however, that prophase counts for naphthol AS BI phosphatase were of the same order as counts for the other stages of mitosis.

In the present work, the effect of increasing the length of incubation time on the number of particles staining for naphthol AS-BI phosphatase is important and warrants further discussion. It is best considered in the light of some closely related work on animal cells. Maggi (1966), working with the same enzyme(s) and investigating its possible triggering effect in the mitosis of HeLa cells, found that the length of incubation time for the enzyme markedly influenced the number of azo-dye particles deposited. During the first 10 min of incubation of these cultured cells there were more phosphatase-staining particles per cell in mitotic cells (approximately 3x) than in interphase cells. After 45 min of incubation, however, the number of particles per cell in the two groups of cells was almost the same, although a heavy diffuse deposition of stain in mitotic cells led her to postulate that prolonged incubation in the acetate buffer had caused this 'artefact'. With the data presented from this work on plant cells there is some support for her findings, since one could likewise postulate that the large increases in mean particle counts per cell between 40 and 50 min for apical initials and procortex indicates the release of the enzyme's latency by destruction of the membrane that previously separated enzyme from substrate (Gahan, 1965). There would thus be an increased availability of enzyme sites and a corresponding increase in the number of azo-dye particles. However, it must not be forgotten that in these plant studies both mitotic and interphase cells reacted in fundamentally the same way to increased incubation times and there was no appearance of diffuse staining in cells (of the tissues investigated) after 50 min as in studies with HeLa cells.

Of the incubation times tested in the present work, 50 min may be considered optimal, since the shapes of the curves for mean particle number per cell against time suggest that the rate of formation of dye particles may be exponential. Low incubation times, i.e. 30 min and less, may therefore represent the lag phase where dye deposition is restricted by the small number of available enzyme sites. With increasing incubation time continued contact between acetate buffer and the membranes inclosing the enzyme, may release more enzyme sites (logarithmic phase) until the diminishing number of latent and potentially available enzyme sites slow down the rate of azo-dye formation (stationary phase). If this is the case then at 50 min in apical initials and procortex tissues dye deposition is in its logarithmic phase whilst at this time central cylinder cells appear to be approaching their stationary phase.

The accurate cytochemical localisation of β -glycerophosphatase in plant cells, fixed in glutaraldehyde, is still fraught with problems. Other investigators working with plant cells (Sexton et al., 1971), and animal cells (Beadle and Dawson, personal communication), obtain apparent staining for β -glycerophosphatase in no-substrate controls when glutaraldehyde is used as the tissue fixative, although as in the present study the levels are much reduced, when compared with test material. The quantitative evidence provided here, however, shows that some cell types stain more heavily with lead, in the absence of β -glycerophosphate than others e.g. procortical cells and mitotic cells generally.

When Holt (1959) was investigating the validity of the Gomori procedure in sections of rat liver and kidney, substrate-omitted control sections were always negative, but the fixative used in his work was formol-calcium. It is an unexplored

possibility that glutaraldehyde may in some way make endogenous substrate more freely available to the lead-containing medium, than other fixatives.

Other major problems involved with the Gomori procedure in fixed tissues are the appearance of diffuse end-product in the cytoplasm and the staining of nuclear structures.

In the present study both diffuse end-product and particulate staining were visible in some cells. Initially it was thought that the incubation time used (1h) may have been responsible for this phenomenon but Sexton and co-workers (1971) have observed diffuse staining in plant tissues incubated for as little as 8 min in maize roots and 12 min in pea roots. In addition recent studies by Brunk and Ericsson (1973) on cultured human glial cells have shown that if buffered fixatives of the correct osmolarity were not used β -glycerophosphatase could leak from ultrastructurally 'intact' lysosomes due to hypo-osmotic damage, even within short incubation times.

This point assumes particular relevance here since it could mean that so called 'triggering' events due to lysosomal leakage as assessed by changes in particle size, number and configuration in animal cells may partly be explained by incorrect fixation procedures. Such leakage may also account for nuclear staining in both this and other studies employing β -glycerophosphate as substrate.

Set against these arguments, however, is the fact that even if diffuse staining were the result of improper fixation and buffering the regular cyclic nature of decrease and increase during mitosis, observed both in the present work with plant cells and the work previously noted with animal cells, would be difficult to explain since a more random distribution of counts throughout mitosis would otherwise be expected.

Nuclear staining has been widely reported in both plant cells (Cahan, 1965; De Jong et al., 1967, and Sexton et al., 1971) and animal cells (Danielli, 1953; Holt, 1959, and Deane, 1963). The phenomenon has been attributed to diffusion and absorption of cytoplasmic enzyme by the nucleus, but the controls carried out by Holt (1959) with liver and rat tissues and by Sexton and coworkers (1971) make this appear unlikely. The latter group of workers suggest therefore that the 'activation' of (a) nuclear acid phosphatase(s) by glutaraldehyde, as originally suggested by De Jong et al., (1967), cannot be ruled out. This idea, however, would seem to conflict with Holt's finding that nuclear staining is an artefact, since no such staining was evident in his studies using frozen sections of gum-sucrose-treated, formol-calcium-fixed animal tissues incubated in fresh Gomori medium. Nuclear staining was detected however if the same medium was stored for any length of time prior to tissue incubation.

Since, in both the studies of Sexton and his associates and those undertaken here on broad bean root tips, fresh incubation media were always used the problems associated with glutaraldehyde-fixation and β -glycerophosphatase staining need to be comprehensively investigated and the causes of nuclear staining made known.

From the foregoing, it would appear that shortening the length of incubation time from 1 h in the present studies may not have eliminated the problems of non-particulate and nuclear staining. However, the possibility needs to be examined that a range of lower incubation times may give quantitatively different results.

The distinctness of the two enzymes, β -glycerophosphatase and naphthol AS-BI phosphatase, examined here should now be obvious. Clearly the reliability of the azo-dye method exceeds the lead salt precipitation procedure for the detection of acid phosphatase activity (at least as far as the meristematic tissues of Vicia faba roots are concerned).

The possibility that naphthol AS-BI phosphate undergoes spontaneous hydrolysis with longer incubation times is extremely unlikely since Burstone (1962) has already shown that the esters of substituted naphthols are extremely stable at acid pH in aqueous media, a fact reflected by the range of incubation times suggested by various authors for naphthol AS BI phosphatase detection (i.e. $\frac{1}{2}$ to 8h) in a variety of tissues, (See e.g. Pearse 1960).

It should be emphasised here that the present study using azo-dye techniques was made exclusively with unfixed cells; the effect of fixation on particle distribution during the mitosis of root tip cells remains unknown.

By the very nature of this study, it has not been possible to follow any one cell through its mitotic cycle, whereas the process is very much a continuous and dynamic one, occupying in Vicia a relatively short period of time. Evans and Savage, (1959) calculated the time of mitosis to be 3h at 19°C, whilst the cells remained in interphase for 26h, a stage in the cell cycle covering events of G₁, S, and G₂ (see for instance Howard and Pelc, 1953). Using the methods described here it is not possible to distinguish between these different stages of the cell cycle and only total counts for all stages of interphase have been made. It may well be that particulate staining varies considerably with the physiological and biochemical status of the interphase cell.

These methods can tell us nothing of the configurational rearrangements of these particles, and their association with other cell structures such as the mitotic spindle, during the phases of division.

What may be required is a dual approach to this problem employing, on the one hand, a process such as time-lapse cinematography to follow the movement of vitally-stained particles so that a qualitative study of their origin and fate may be determined, combined on the other hand with a statistical, quantitative procedure as used here. With regard to the latter it would be advisable to employ microdensitometer tracings as a faster, labour-saving method of particle-counting, as suggested by Altmann, (1971).

An alternative, perhaps simpler and more effective way of testing the 'triggering' capacity of lysosomal enzymes is to use synchronously dividing plant cell cultures such as those used by Yeoman et al., (1968). A full consideration of this possibility is given in the General Discussion (p. 171).

CHAPTER II

A BIOCHEMICAL STUDY OF NON-SPECIFIC ESTERASES
FROM THE LATERAL ROOT TIPS OF VICIA FABA

II.1.

INTRODUCTION

For the studies of acid hydrolases in cell differentiation (Chapter III) and senescence (Chapter IV) attention was switched from acid phosphatases (Chapter I) to non-specific esterases since the latter group of enzymes have been extensively studied cytochemically in relation to the cellular differentiation of the lateral roots of the broad bean (Benes, 1962, 1971; Gahan and McLean 1969; McLean and Gahan 1970; McLean 1970; and Sahulka and Benes, 1969), but no quantitative biochemical data were ever provided.

Before proceeding to a combined cytochemical and biochemical study of the involvement of non-specific esterases in cell differentiation and senescence it was therefore necessary i) to devise a method of assaying quantitatively the esterolytic activity of cell-free extracts, using the reagents normally employed in cytochemical tests and ii) to characterise more closely the kinetic parameters that govern the activity of these enzymes in order that the optimal conditions for assay could be defined.

For the quantitative determination of non-specific esterases, recourse is usually made to the method of Seligman and Nachlas (1950), in which α -naphthol, hydrolysed from the acetate ester, is coupled with a diazotised amine and the resultant azo-dye is estimated colorimetrically. Alternatively, use is made of the p-nitrophenyl esters as substrates in which hydrolysis is measured by the release of acid or of p-nitrophenol (Huggins and Lapidés, 1947). These substrates are usually chosen because of their relatively high solubility compared with naphthol AS esters

and their derivatives, often used in the cytochemical localisation of esterases (Burstone, 1957, 1962). There seems little justification for using α -naphthyl acetate or p-nitrophenyl esters in a quantitative assay of esterase activity after using the highly insoluble naphthol AS esters for comparative cytochemical studies. Indeed, Sahulka and Benes (1969), and Sahulka (1970), have already shown that different electrophoretic staining patterns are obtained on polyacrylamide gels when α -naphthyl acetate and naphthol AS acetate are used as substrates for non-specific esterases extracted from different growth zones of broad bean roots. In addition if p-nitrophenyl esters are used as substrates for the colorimetric determination of esterase activity, there is a strong possibility that proteolytic enzymes such as trypsin and chymotrypsin, if present, may also cleave the ester linkage (Hartley and Kilby, 1952, 1954).

These problems can be overcome to a certain extent if the same reagents are used in both cytochemical and biochemical tests on the enzymes in question. It is then probable that whatever their degree of specificity the enzymes measured quantitatively are the same as those assessed visually in intact cells.

Whilst this rationale has been used extensively for other enzymes such as acid phosphatase (Hall and Butt, 1968), and alkaline phosphatase (Hopsu and McMillan, 1964) only Gomori, (1953) has previously reported on the use of naphthol AS esters for the combined biochemical and cytochemical estimation of non-specific esterases and it is his method that has been used in a slightly modified form for the current work. Gomori overcame the problems of substrate insolubility by incorporating large amounts of propanediol into his assay procedure and adding sodium dodecyl

sulphate at the end of the incubation period to arrest enzyme action and further enhance the solubility of the azo-dye formed upon post-coupling with the diazonium salt.

It was the initial intention of the present work to use this procedure but some modification proved necessary since using these reagents alone failed to maintain the azo-dye in solution. The alternative methods used are described in the following sections.

II.2. MATERIALS AND METHODS

II.2.1. Chemicals.

Tris-hydroxymethylaminomethane, citric acid, magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), disodium hydrogen phosphate (Na_2HPO_4 , anhydrous), sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) were all of 'Analar' grade and were purchased from BDH. Ltd., Poole, Dorset, England. Ethanediol and sodium dodecyl sulphate (SDS), 'specially pure', and dimethylformamide (DMF; laboratory reagent grade) were obtained from the same company.

Fast red violet LB salt, naphthol ASD and its acetate ester, sodium L-ascorbic acid and maleic anhydride were all of the highest purity obtainable from Sigma Chemical Company Ltd., London.

McIlvaine's (citrate-phosphate) buffer, sodium phosphate buffer and Tris-maleate buffer were all prepared and used according to the methods specified by Gomori (1955).

Insoluble polyvinyl pyrrolidone (PVP), brand name 'Polyclar AT', was obtained from General Aniline and Film Corporation, Manchester, England.

Bovine Serum Albumen, Fraction V, (BSA) was obtained from Armour Pharmaceuticals Ltd., Eastbourne, Sussex, England.

II.2.2. Enzyme extraction and purification.

All tissue homogenates were obtained from the whole lateral roots, 2 - 3 cm in length, of the broad bean Vicia faba L. var. Aquadulce. Growing conditions for the plants were those given in Section I.2.1. Quantities of 10 - 40 g fresh weight of roots

were routinely taken for extraction. Before removal the roots were washed for 20-30 min with a pressurised jet of tap water to remove surface contaminants, and finally rinsed in double-distilled water. Roots were first ground, using a pestle and mortar, with their own weight of acid-washed sand for 10-15 min at 4°C. The homogenate was then slowly frozen to -20°C for at least 24h before thawing to approximately 4°C. The homogenate was then added to a thin PVP paste (Loomis and Battaile, 1966), made up 24h in advance with the following constituents:

- a) Insoluble PVP 1g/g fresh weight of roots.
- b) 0.1M sodium phosphate buffer, pH 6.5.
1ml/g fresh wt.
- c) 0.05M Mg Cl₂ 1ml/10g fresh wt.
- d) L-ascorbic acid (sodium salt) to a final concentration of 0.1M.

If necessary the pH of the paste was adjusted to 6.5. with NNaOH, before adding it to the homogenate. If PVP and sodium L-ascorbate were not added the root extract quickly discoloured to produce blue-black solutions containing the oxidation products of the polyphenols. The problems of plant enzyme denaturation by polyphenols and other agents during extraction is comprehensively reviewed by Anderson (1968).

The combined paste and homogenate, at pH 6.5, was then expressed as a slurry through four layers of cheesecloth and the retained solids washed twice with two volumes of chilled double-distilled water. The crude extract was then centrifuged at

3,000xg_{av} at 4°C for 20 min and the supernatant dialysed against 2 litres of 10mM phosphate buffer containing 5mM MgCl₂, pH 6.5 overnight at 4°C (Mendoza et al., 1969). Centrifugation and dialysis were repeated once. Sucrose was then added to the extract, to a concentration of 0.5M, and the mixture centrifuged at 20,000xg_{av} at 4°C for 30 min. The supernatant was further dialysed against 2 litres of the buffer - MgCl₂ mixture as before. The extract was finally centrifuged at 20,000xg_{av}; the supernatant proteins were concentrated by pervaporation at 4°C and stored until needed for a period not exceeding 24 h.

II.2.2.1. Bacterial plate counts of the crude enzyme extract.

In one experiment, the supernatant liquid from the 3,000xg_{av} centrifugation was subjected to bacterial plate counting.

Washing the roots before homogenisation gave plate counts of 1.1×10^4 cells/ml of extract. This figure corresponds to 8.3×10^{-6} μg total dry weight of the cells (Lambert, personal communication) and in turn is equivalent to 6.45×10^{-6} μg protein/ml of enzyme extract according to the data of Herbert et al., (1971).

II.2.3. Enzyme assay procedure.

II.2.3.1. Preliminary considerations.

As previously explained (II.1.) the chief obstacle to the use of naphtholic substrates for colorimetric assay is their insolubility in water. Gomori (1953) circumvented this difficulty by incorporating sodium dodecyl sulphate and propanediol into the incubation mixtures. In the present investigation the incorporation of DMF (The solvent used for naphthol AS D acetate

in cytochemical tests), ethanediol (in place of propanediol) and SDS, proved inadequate in maintaining an appropriate level of solubility; use was therefore made of the ability of proteins to bind azo-dyes and maintain them in colloidal solution (Rutenberg and Seligman, 1955). Preliminary experiments demonstrated that addition of BSA to a final concentration of 0.67 mg/ml prevented, indefinitely, the precipitation of dye crystals from solution, within the concentration ranges of the reactants employed i.e. 0 - 0.53 μ mole/ml naphthol AS D. Analysis showed that the spectrum of the azo-dye solution (Fig. II.2.) in SDS-DMF-ethanediol-water was identical with that of the same solvent containing protein, thereby suggesting that no phase change had occurred upon association.

II.2.3.2. Fixed incubation time assays.

To a solution containing 1 ml of a 10mg/ml solution of BSA and 2 ml of 0.1M sodium phosphate buffer, pH 6.5, were added 3 ml of naphthol AS D acetate solution prepared in the following manner. Weighed amounts of substrate were dissolved in 10 ml of DMF, to which were added 30 ml of ethanediol and a further 60 ml of chilled 0.1M Tris-maleate buffer, pH 5.5. The final pH of the combined solutions was 6.5. It was necessary to maintain pH on the acid side of neutrality to avoid non-enzymic hydrolysis of the substrate (see Section II.3.2.5. and Fig. II.10.). The extent of non-enzymic hydrolysis was measured by use of the appropriate control solutions containing buffer in place of extract.

Both the substrate solution and the enzyme extract were brought independently to incubation temperature before mixing the two, by placing the solutions in a water bath at 32°C for 10 min.

Unless otherwise stated all reactions were performed at pH 6.5 and 32°C for 1h. The choice of pH 6.5. for enzyme assays was made after examining the effect of pH on both esterase activity (Fig. II.11.) and non-enzymic hydrolysis (Fig. II.10). A detailed justification for adopting pH 6.5. for all future assays is given in Section II.3.2.5. The enzyme reaction was stopped by adding 1ml of a 5% (w/v) solution of sodium dodecyl sulphate (Gomori, 1953). Previously it had been shown (Hopsu and McMillan, 1964) that when histochemical enzyme methods are modified for biochemical assay, the stabilized diazonium salts partially inhibit as well as alter the kinetic characteristics of the enzyme reaction. Consequently a post-coupling rather than a simultaneous azo-dye coupling technique was adopted in the present work. Cytochemically, McLean (1970) has shown that when using either coupling technique for the demonstration of nonspecific esterases in root tips of Vicia faba the same staining pattern is obtained, and it was therefore felt that the results obtained employing the biochemical assay system developed in the present study may be justifiably compared with the results of the histochemical studies made in Chapters III and IV, for which a simultaneous coupling procedure was used. Post-coupling was achieved by adding 5ml of a 30mg-% (w/v) solution of fast red violet LB salt in McIlvaine's buffer, pH 5.4. The reactions believed to account for the hydrolysis of the substrate and post-coupling with the diazonium salt are shown in Fig. II.1.

After mixing the solutions by inversion a violet colour resulted. All solutions were returned to the incubation bath and colour development was allowed to proceed for 2h at 32°C (Fig. II.3.). The absorption spectrum of the coloured azo-dye was initially scanned

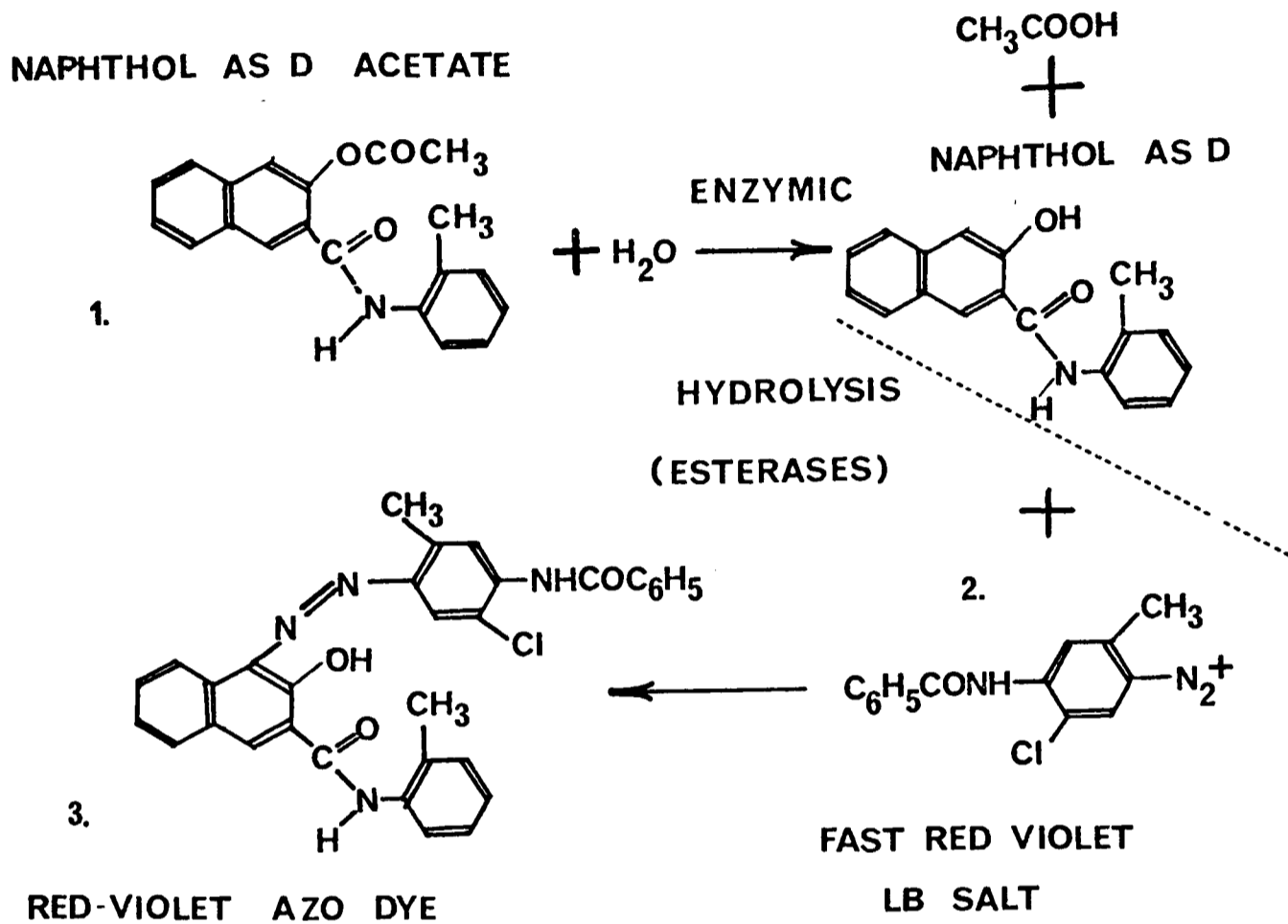


Fig.II.1: The chemistry of the reactions used in the cytochemical and biochemical demonstration of non-specific esterases. Step 1: The hydrolysis of the substrate, naphthol AS D acetate, produces free naphthol AS D and acetic acid. In cytochemical studies the free naphthol AS D is simultaneously coupled with an excess of fast red violet LB salt - diazotised 5-benzamido 4-chloro-*o*-toluidine, (Step 2). In biochemical studies the coupling reagent is added as a separate stage (post-coupling). In either case the product is a red-violet azo-dye (Step 3). (From Burstone, 1962).

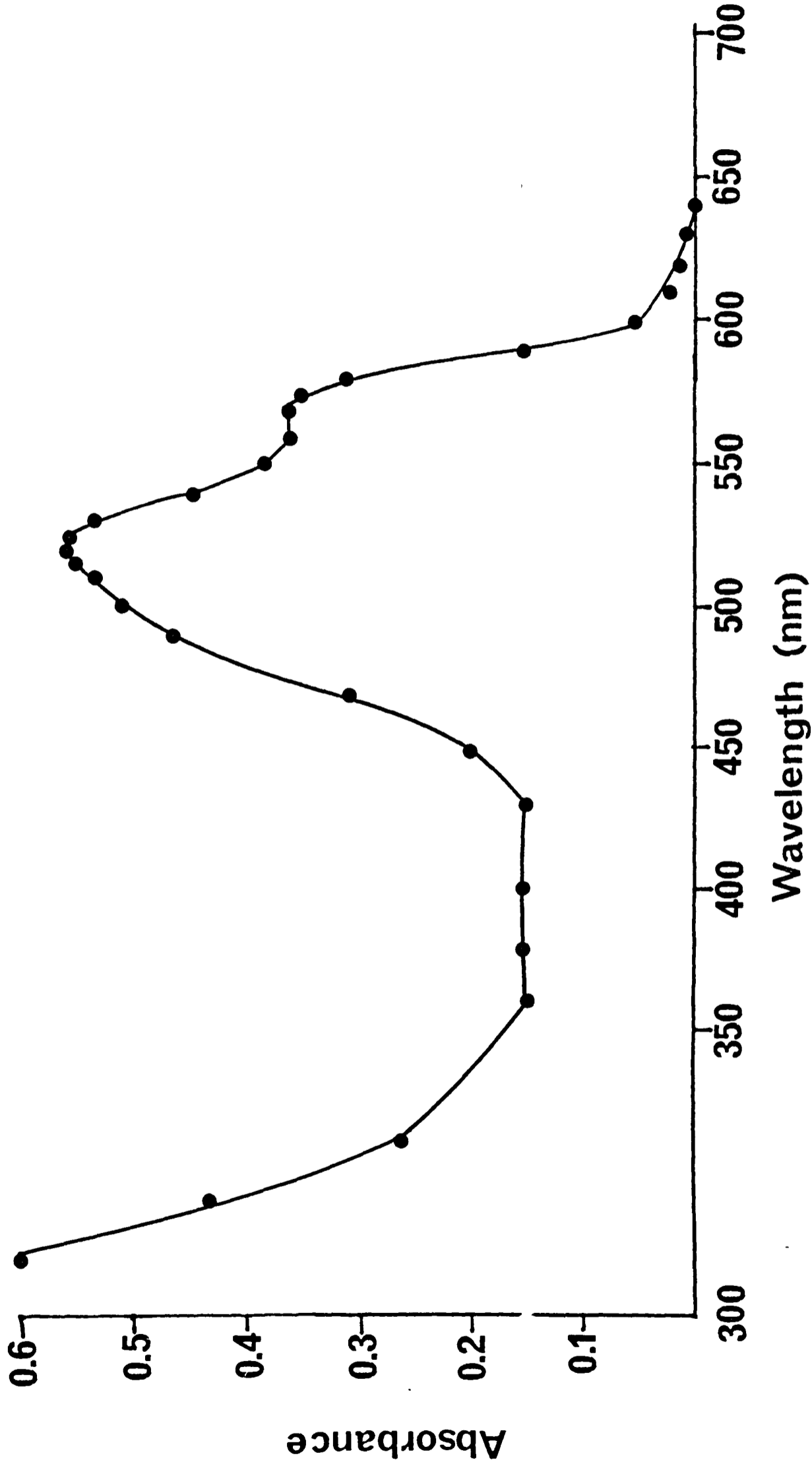


Fig. II.2: Absorption spectrum of the coloured azo-dye product formed by coupling 0.375 μ mole of naphthol AS D in 0.1M tris-maleate buffer, pH 6.5 (8ml), with 5 ml of a 30mg%(w/v) solution of fast red violet LB salt in McIlvaine's buffer, pH 5.4. Also added were 1ml of a 5% solution of sodium dodecyl sulphate (w/v) and 1ml of a 10mg/ml solution of bovine serum albumen (BSA).

on a Model 402 Ultraviolet-Visible Spectrophotometer, (Perkin-Elmer, Beaconsfield, England), to determine λ max. This peak of absorbance was recorded as 520 nm by measuring absorbance over a range of wavelengths (Fig. II.2.), on a Uvispek H700 spectrophotometer (Hilger and Watts, London). The same instrument was used for individual assays of enzyme activity at 520 nm, employing 1 cm silica cuvettes.

II.2.3.3. Controls.

'No enzyme' controls contained all the constituents mentioned above, except for the enzyme solution. This was always added at the end of the incubation time and before post-coupling with fast red violet LB salt. Both test and control solutions were always read against glass double-distilled water. The difference between the two readings was taken to represent enzymic hydrolysis.

In earlier experiments boiled enzyme controls were used. These were prepared by exposing the enzyme extract in a test tube to a temperature of 100°C, in a beaker of boiling water, for 50 min. During the cooling period a flocculent precipitate appeared which was removed by centrifugation. The supernatant was then included in the substrate solution and incubated together with test solutions as 'boiled controls'. However, absorbance readings for 'no enzyme' and 'boiled extract' controls were almost the same, maximum differences being of the order ± 0.006 absorbance units.

For convenience therefore 'no enzyme' controls were used thereafter throughout this work.

II.2.3.4. Calibration.

A calibration curve was obtained by submitting quantities of between 0.1 and 0.8 μ mole naphthol AS D to an exactly

Fig.II.3: Development of the coloured azo-dye product in solution as a function of time, at 32°C. 0.25µmole of free naphthol AS D was post-coupled with a standard excess of diazonium salt.

Fig.II.4: Calibration curve showing the relationship between chromogenesis and absorbance at 520nm. Varying amounts of free naphthol AS D were post-coupled with 5ml of a 30mg% (w/v) solution of fast red violet LB salt in 0.1M McIlvaine's buffer, pH 5.4.

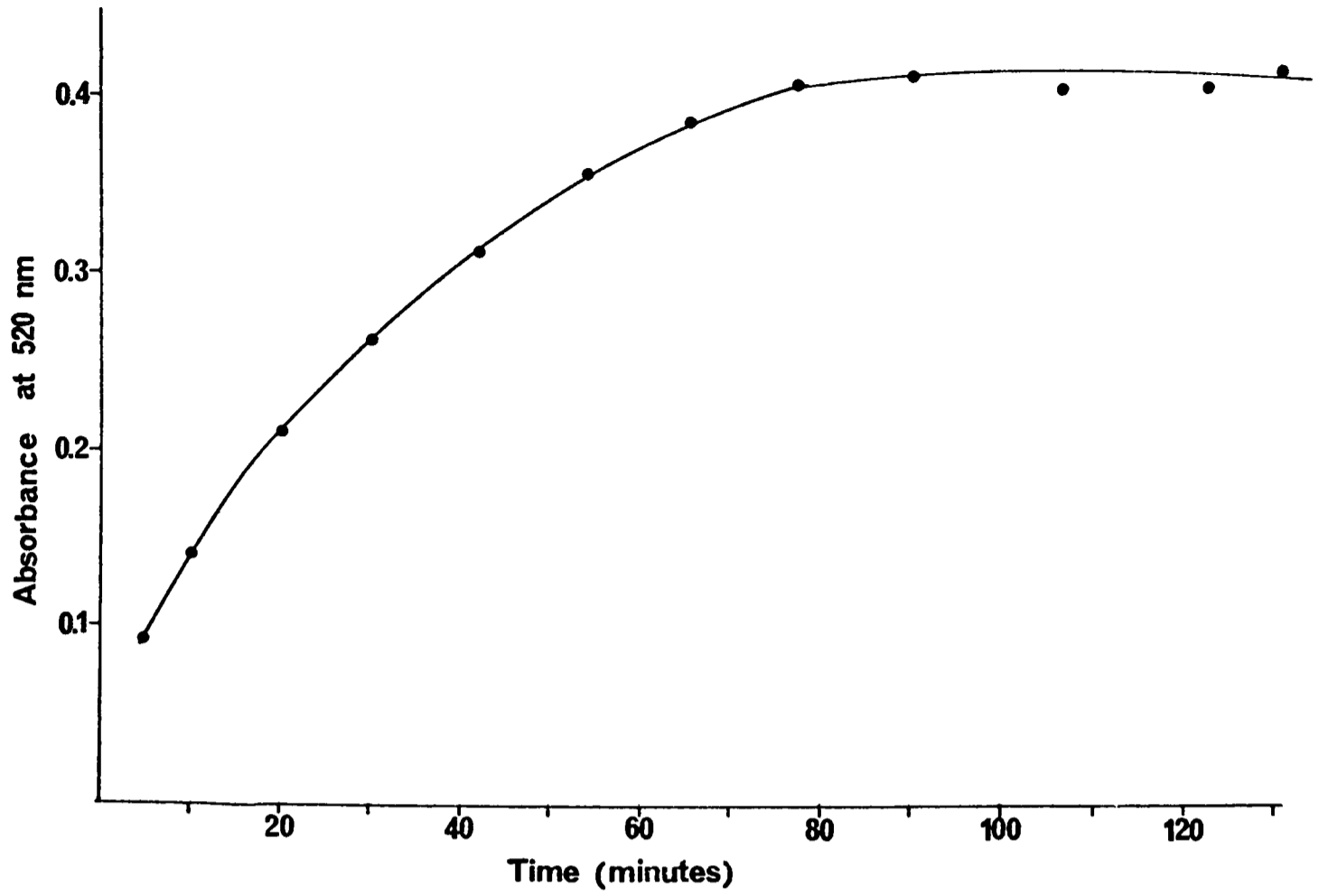


Fig. II.3.

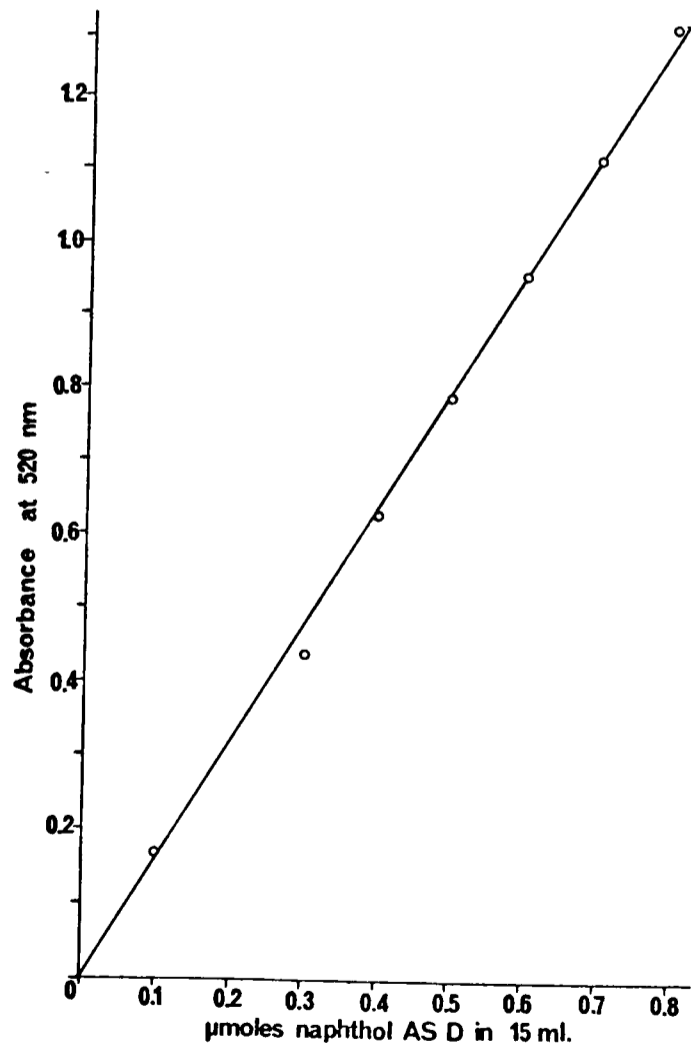


Fig. II.4.

analogous treatment as that adopted in Section II.2.3.2., but in the absence of enzyme extract. A linear relationship was observed. (Fig. II.4.).

II.2.4. Protein estimation.

Soluble proteins from purified and dialyzed extracts were estimated using the colorimetric method of Lowry et al.,(1951).

II.3.

RESULTS

II.3.1. POST-COUPLING:

Factors affecting colour reaction and development.

The factors which might possibly affect the coupling of naphthol AS D and the diazonium salt and the subsequent development of the azo-dye were investigated, using the standard estimation procedure given in 'Materials and Methods' section (II.2.3.2.).

II.3.1.1. pH of the solution containing free naphthol AS D.

A series of solutions was prepared in which 0.1 μ mole/ml of naphthol AS D was maintained in solution at pH values ranging from 3.1 to 7.8, using 0.1M McIlvaine's buffer. Fast red violet (30mg-%) was then added and the solutions were allowed to post-couple for 2h at 32°C. The results are shown in Table II.1. Each optical density value is the mean of 3 readings. The pH of each solution is expressed to the first place of decimals.

After 2h post-coupling, colour development of the dye product only decreased when the pH of the free naphthol AS D solution was 'pH 7.8'. This may reflect the known instability of 'diazonium salts at alkaline pH (Burstone, 1962).

II.3.1.2. Length of the post-coupling period.

A total of 0.3 μ mole of naphthol AS D was post-coupled with a 30mg-% solution of fast red violet at pH 6.5 and 32°C from 5 to 130 min. Absorbance increased for a period of about 90 min from the time of post-coupling, after which little further change occurred. (Fig. II.3.).

Table II.1. The effect of pH of the free naphthol AS D solutions on colour development.

pH	Absorbance at 520 nm	Equivalent concentration of naphthol AS D (μmole)
3.1	0.54	0.34
5.5	0.56	0.35
6.5	0.57	0.36
7.1	0.57	0.36
7.8	0.49	0.31

Table II.2. The effect of coupling salt concentration on colour development.

Fast red violet LB salt (mg-%) (w/v)	Absorbance at 520 nm	Equivalent concentration of naphthol AS D (μmole)
10	0.53	0.33
20	0.53	0.33
30	0.55	0.34
40	0.54	0.34
50	0.54	0.34
70	0.55	0.34
80	0.56	0.35
90	0.55	0.34

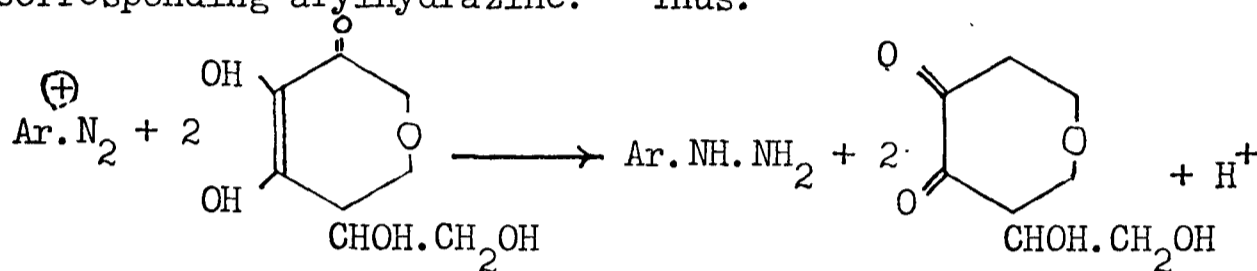
II.3.1.3. Concentration of coupling salt.

Varying amounts of coupling salt, ranging from 10-90 mg% (w/v) were added to a solution of naphthol AS D (0.1 μ mole/ml) maintained at pH 6.5 and at 32°C, (Table II.2.). The increase in concentration of diazonium salt caused no significant change in colour yield, after the standard 2h post-coupling period. Stoichiometrically, it can be shown that a 30mg% solution of fast red violet LB salt contains an excess of the salt. Since a maximum of 0.3 μ mole of naphthol AS D is present in the incubation mixtures a 30mg% (approx. 1 μ mole/ml) solution of the diazonium salt would be in excess in the ratio of approximately 1:8. The figure for the concentration of fast red violet is based on the assumption that the coupling salt is no more than 50% pure (personal communication; Sigma Chemical Co. Ltd.).

II.3.1.4. Reducing agents.

The effect of sodium L-ascorbic acid on the reaction has already been published (James and Smith, 1972); an outline of the procedure is given here. In the early stages of the work, buffer or distilled water extracts made from acetone-dried powders of root tissues were prepared in which sodium L-ascorbate was added to a final concentration of 100mM to prevent tissue discolouration (see II.2.2.). Lower colour intensities were obtained in the enzyme assay when ascorbate was added than in control solutions, prepared for the measurement of non-enzymic hydrolysis, which lacked sodium L-ascorbate. Upon further examination it was shown that at pH 6.5 and 32°C as little as 0.1mM L-ascorbic acid prevented the coupling of more than 60% of the naphthol AS D supplied in a 30mg% solution of the diazonium salt. An examination of the U.V

absorption spectra of mixtures of naphthol AS D, the diazonium salt and different concentrations of ascorbate showed that absorbancy at 343nm due to fast red violet diminished rapidly with increasing concentrations of the reducing agent. At the same time the U.V. spectrum of ascorbate also underwent modification. It was suggested that the diazonium salt is removed by its reduction to its corresponding arylhydrazine. Thus:



As a result of these findings all enzyme extracts from plant tissues were tested for the presence of ascorbate by coupling 0.3 μmole of naphthol AS D with a standard excess of the diazonium salt in the presence of the enzyme solution and comparing absorbance of the preparations with those for control solutions lacking enzyme but containing an equal volume of phosphate buffer.

II.3.2. CHARACTERISATION STUDIES.

II.3.2.1. Choice of working substrate concentration.

The extreme insolubility of the naphthol AS compounds and their derivatives in water has made them particularly useful in enzyme histochemistry since diffusion artifacts are reduced to a minimum and enzyme localisations have been reported to be excellent (Burstone, 1962). However, in order to study the kinetics of enzyme-catalysed hydrolysis, higher concentrations of the substrate, naphthol AS D acetate, are required than those

normally employed in histochemistry. (Standard histochemical procedures using naphthol compounds do not permit accurate recording of substrate concentration since only small amounts of organic solvent - normally about 1% of the final volume - are used to bring the substrate into solution initially, and filtration of the turbid solutions, resulting from the subsequent addition of buffer is a prerequisite for the histochemical localisation of enzymes). In biochemical studies it is essential to maintain the product of the enzyme action (i.e. the free naphthol AS D) in solution long enough to ensure efficient, stoichiometric post-coupling with the diazonium salt, and the dye product must remain in stable solution for spectrophotometric assay.

It has already been shown (Section II.2.3.1.) that the addition of ethanediol -DMF-SDS-BSA mixtures enhanced the stability of solutions of the free naphthol AS D up to a value of at least 0.53mM (0.8 μ mole/15ml, Fig. II.4.) at 32°C. The linear correlation between the amount of naphthol AS D added and the intensity of absorbance in subsequent spectrophotometric assay has already been noted (Section II.2.3.4.). The acetate ester was, however, notably different in its solubility characteristics. Solutions of naphthol AS D acetate in ethanediol-buffer mixtures (pH 6.5), left to equilibrate for at least 60 min at 32°C remained in stable solution up to concentrations of about 0.20mM only (Fig. II.5.) beyond which spontaneous recrystallisation of the substrate occurred. For practical purposes it was assumed that concentrations greater than 0.20mM might be supersaturated and therefore being unstable could have led to errors in assays for hydrolysis. Therefore 0.167mM, or half this value, (0.0835mM), was adopted as the working concentration of the substrate in subsequent assays.

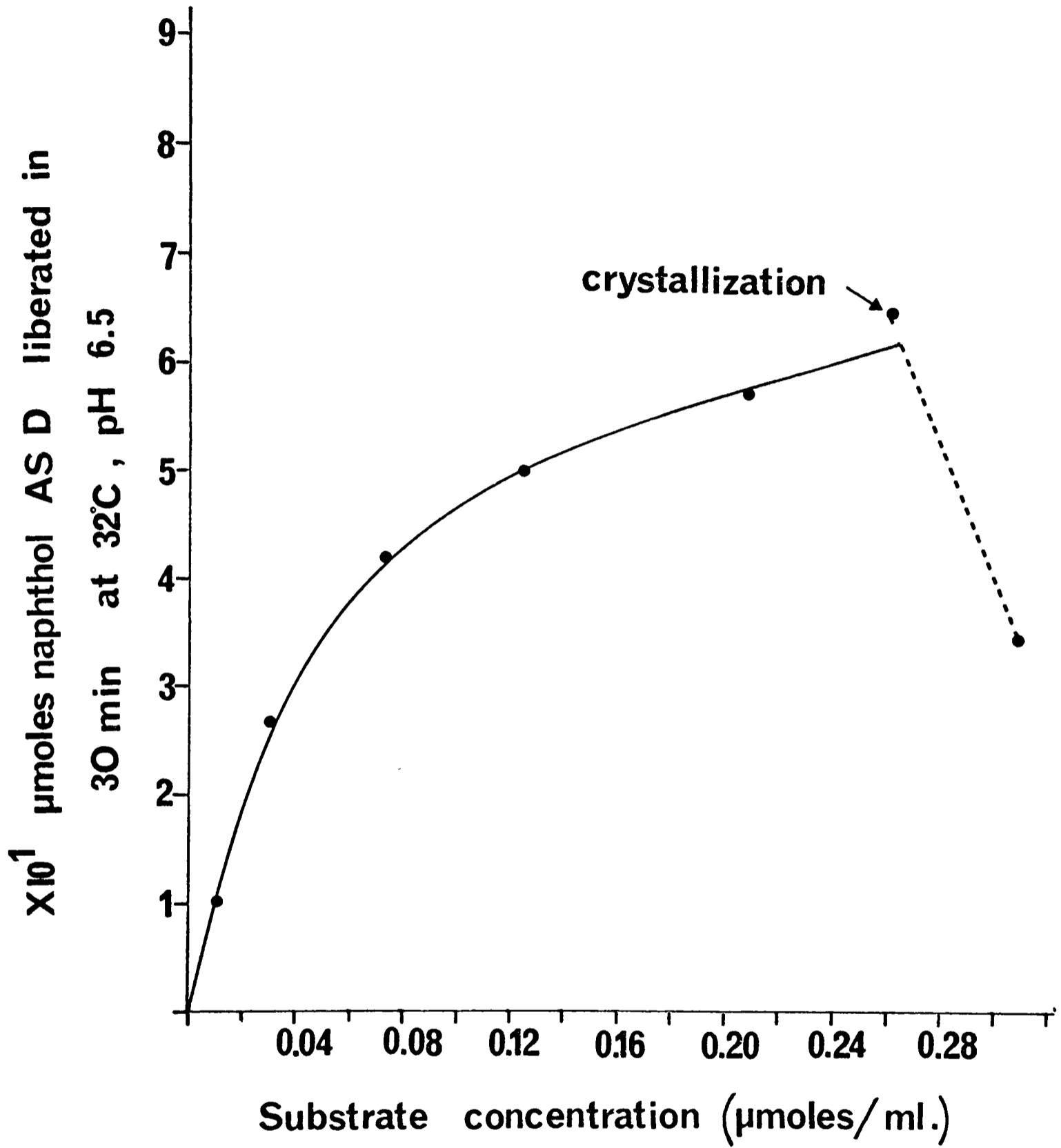


Fig.II.5: The velocity of esterase hydrolysis as a function of substrate concentration. Crystallization of the substrate occurred at about 0.25mM.

I.3.2.2. Enzyme concentration and velocity of hydrolysis.

Incubation over a 60 min period of increasing amounts of enzyme extract with 0.167mM substrate resulted in an apparently proportionate, linear increase in the amount of naphthol AS D released over a range encompassing a ten-fold increase in the amount of enzyme present (Fig.II.6.). The slope of the graph was extrapolated to zero after adjusting for non-enzymic hydrolysis at each enzyme concentration.

II.3.2.3. Time course study of enzyme-catalysed hydrolysis.

The progress of enzyme-catalysed hydrolysis with respect to time was followed by stopping substrate hydrolysis at intervals and assaying spectrophotometrically for product in replicate incubation mixtures. In one experiment, at an initial substrate concentration of 0.083mM, and a protein concentration of 178µg/ml, enzyme catalysed hydrolysis proceeded linearly for 95 min when approximately 37% of the substrate initially supplied had undergone hydrolysis (Fig. II.7.). This result suggests the operation of zero order kinetics for the first 95 min of incubation. However, when the same data were plotted according to the equation describing 1st order kinetics (i.e. $\log \frac{a}{a-x}$ versus 't' where 'a' is the initial substrate concentration and 'x' is the amount of substrate consumed at time 't') a straight line was also obtained for the first 90 min (Fig. II.8.). Since the same data yield straight line plots in both graphs it may be assumed that mixed kinetics are in operation. A further complicating factor that warrants consideration is that water may not be present in excess. The actual concentration of water in the system is not, as is usually assumed for dilute aqueous solutions, 55M but is nearer 30M

Fig.II.6: The relationship between the velocity of esterase activity and enzyme concentration. The initial substrate concentration was 0.167mM.

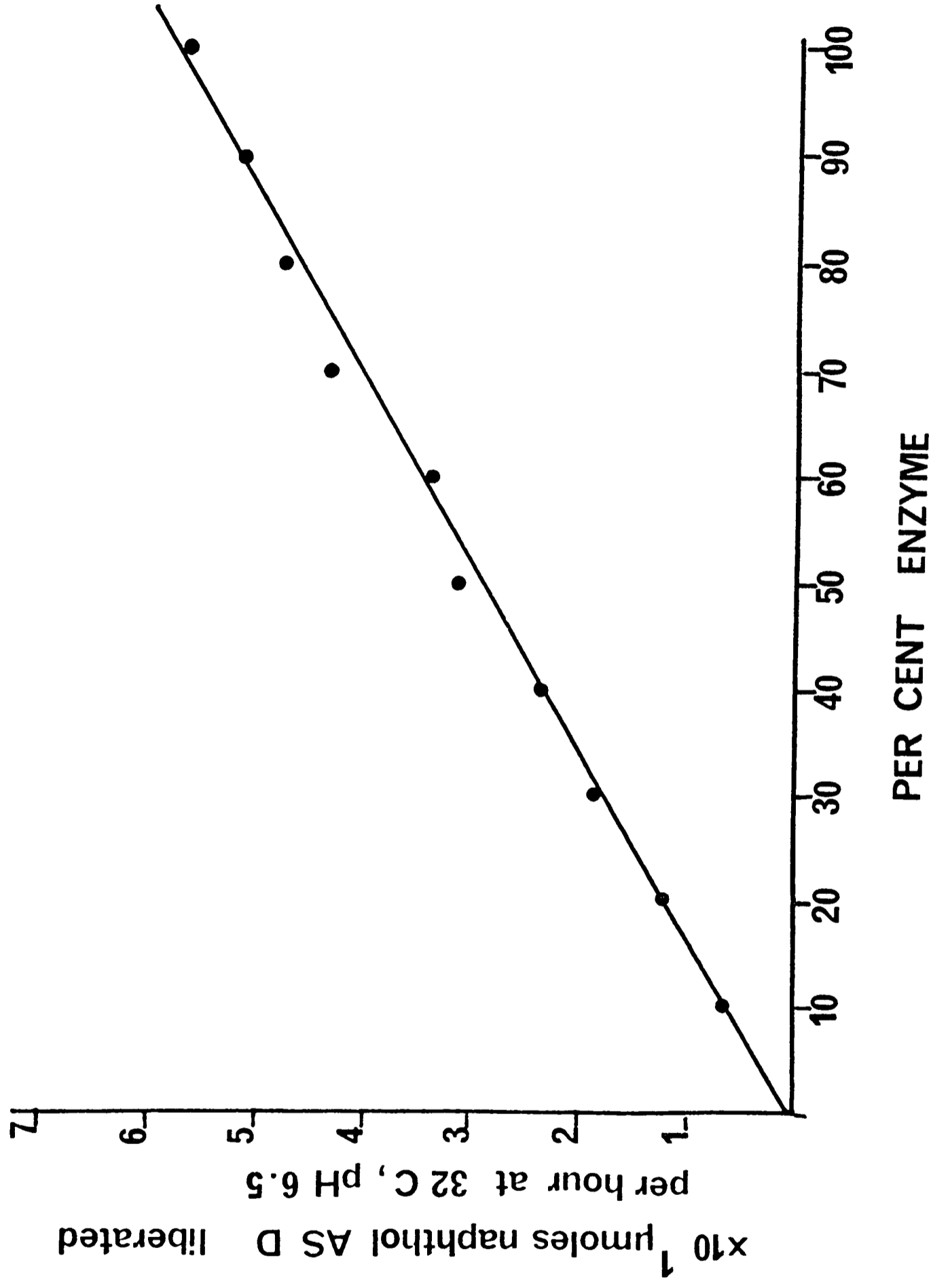


Fig. II.6

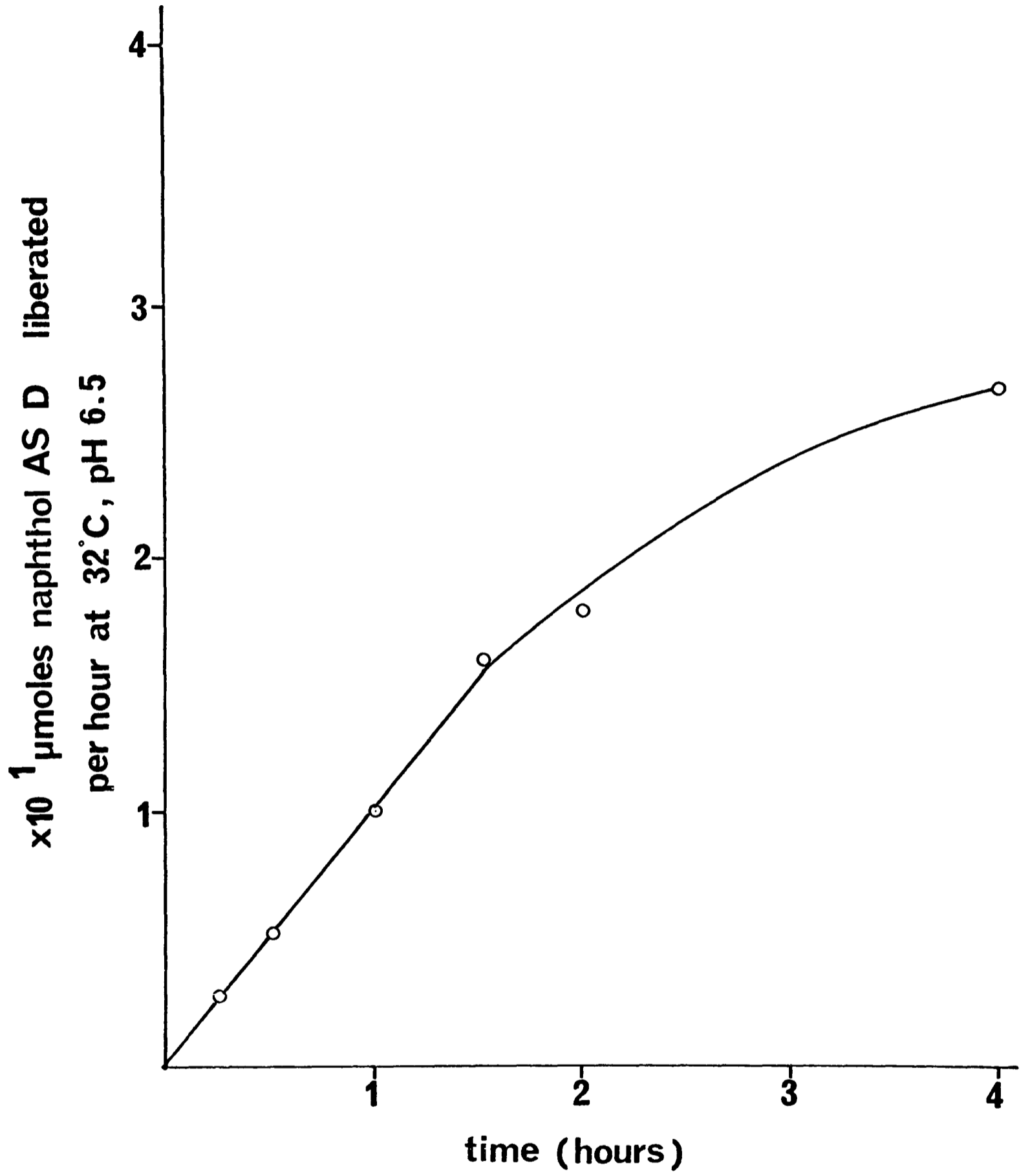


Fig.II.7: The rate of formation of enzyme product as a function of time, with an initial substrate concentration of 0.0835mM.

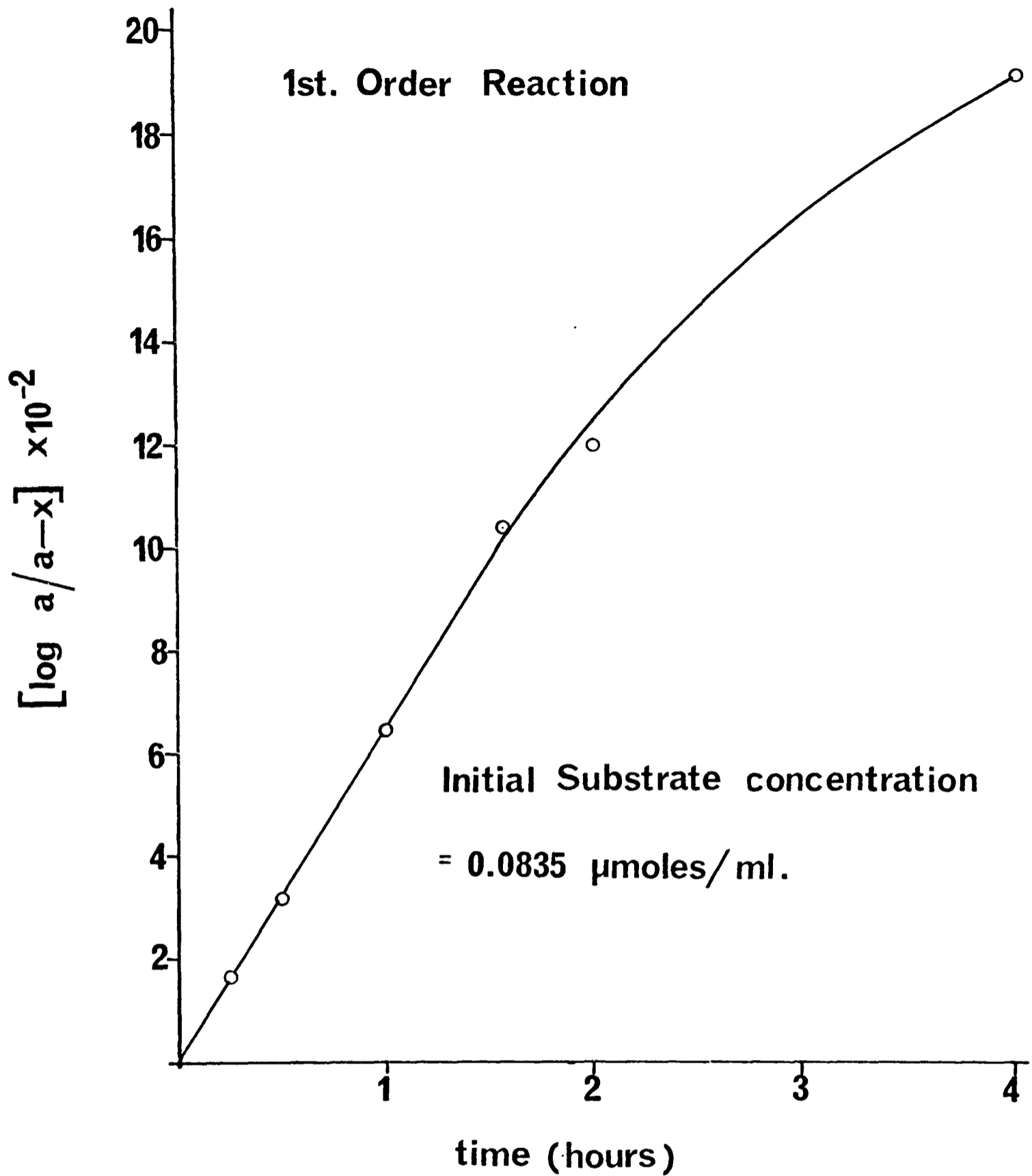


Fig.II.8: The data from Fig.II.7 were plotted according to the equation describing 1st. order reaction kinetics with an initial substrate concentration of 0.0835mM.

and the activity may well be even lower as some water will almost certainly be associated with organic molecules in the form of hydration shells.

II.3.2.4. Estimation of the Michaelis constant.

The Michaelis constant (K_m ; Michaelis and Menten, 1913) of the Vicia faba root tip enzyme extract was derived using the graphical method of Lineweaver and Burke (1934) and a value of 0.07mM was found (Fig. II.9.). The data employed for the determination were obtained by measurement of the extent of hydrolysis after 30 min of incubation at varying concentrations of substrate.

Unfortunately this procedure will not have estimated accurately the initial rates of hydrolysis at the two lowest substrate concentrations, where more than 20% of the substrate had been consumed after 30 min. At an initial substrate concentration of 0.0835mM it has already been shown that linearity in the rate of reaction was observed over 95 min (Fig. II.7.).

More precise determination of K_m was not possible since the range of substrate concentrations available for test was somewhat restricted. At the upper limits of solubility, solutions with substrate concentration greater than approx. 0.20mM recrystallised (Fig. II.5.), and at the lower limit, with substrate concentration less than 0.05mM it was difficult to achieve a significant degree of colour development.

In theory V_{max} is achieved only when the substrate concentration approaches $10 \times K_m$ (i.e. approx. 0.7mM in this system), whereas in this case the upper limit of substrate concentration that could be used was little more than $2 \times K_m$.

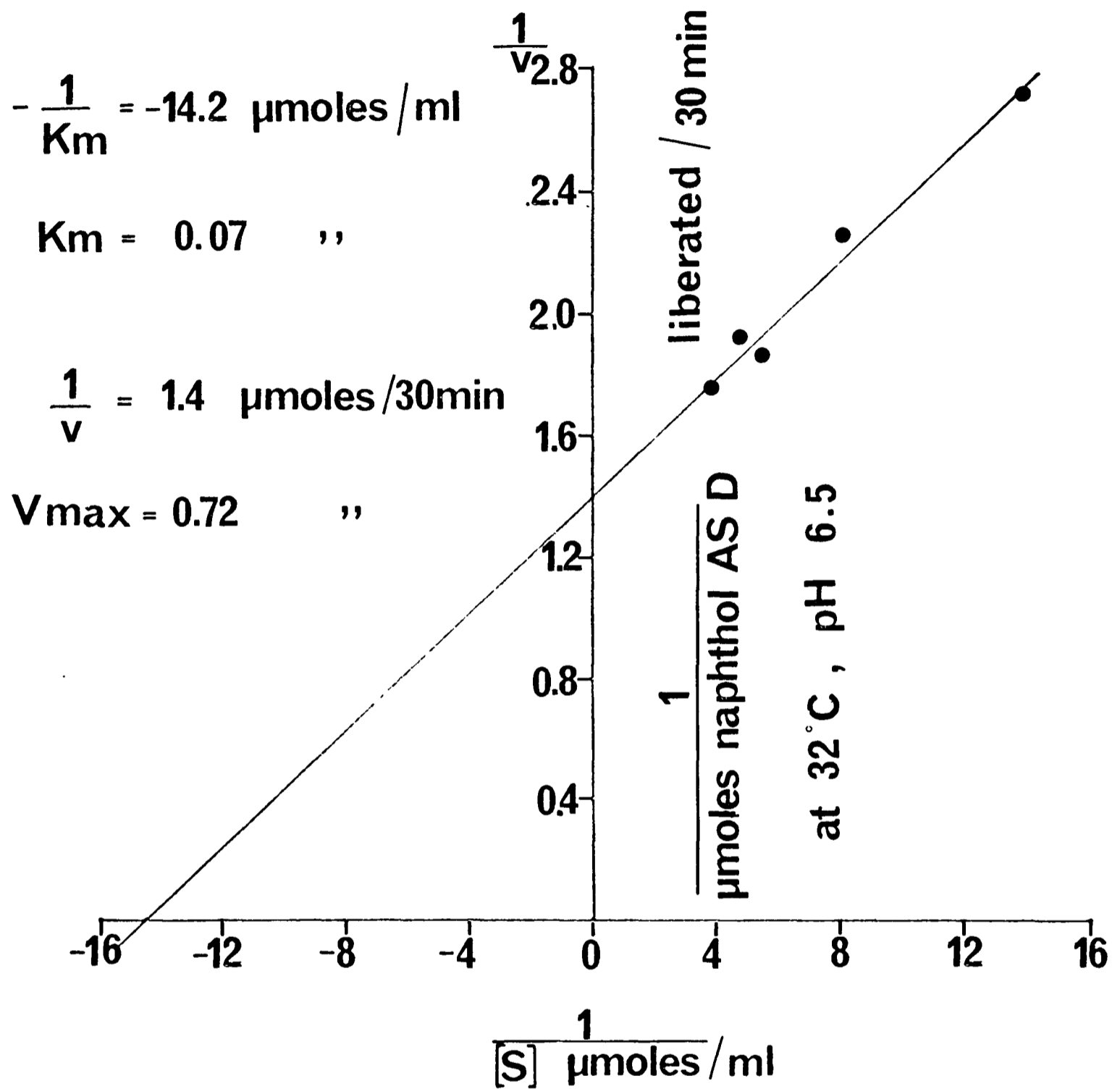


Fig.II.9: The estimation of the Michaelis constant for non-specific esterases from *Vicia faba* root tips, using the double reciprocal plot method ($1/v$ against $1/S$).

It is possible that the calculated K_m value of 0.07mM is higher than the true value, since it does not accord with the character of the plot obtained of enzyme concentration against velocity of hydrolysis. Thus, at the highest enzyme concentration at the end of the hour, the concentration of available substrate had fallen to 0.117mM and assuming $K_m = 0.07mM$ and absolute adherence to the Michaelis model for catalysed hydrolysis, the situation should have changed from one in which the velocity of the reaction approximated to 70% of V_{max} (at 0.167mM) to the point where only 60% of V_{max} obtained. With one-tenth this enzyme concentration the diminution in substrate concentration (due to total hydrolysis) to a value of 0.144mM still allowed a rate of reaction equal to 64% of V_{max} . Therefore the plot should have assumed the form of a convex curve, unless the K_m value was somewhat lower; in which case the instantaneous rate of reaction would have remained closer to a constant value and the plot would have tended towards the straight line observed.

II.3.2.5. The effect of pH on esterase activity.

There are practical difficulties involved in measuring the effect of pH on root tip esterases due to inherent limitations of this particular assay procedure. One problem is that naphthol AS D acetate is very unstable at alkaline pH (Burstone, 1962; Pearse, 1955, 1960) and it was therefore not possible to test for esterase activity at pH values above 7.2 since non-enzymic hydrolysis then became unacceptably rapid. The effect of pH upon the rate of non-enzymic hydrolysis in the test system employed for the present work is shown in Fig. II.10.

Fig.II.10: The relative stability of the naphthol ester under varying pH conditions. Non-enzymic hydrolysis of naphthol AS D acetate is plotted as a function of pH. The initial concentration of naphthol AS D acetate was 0.167 $\mu\text{mole/ml}$.

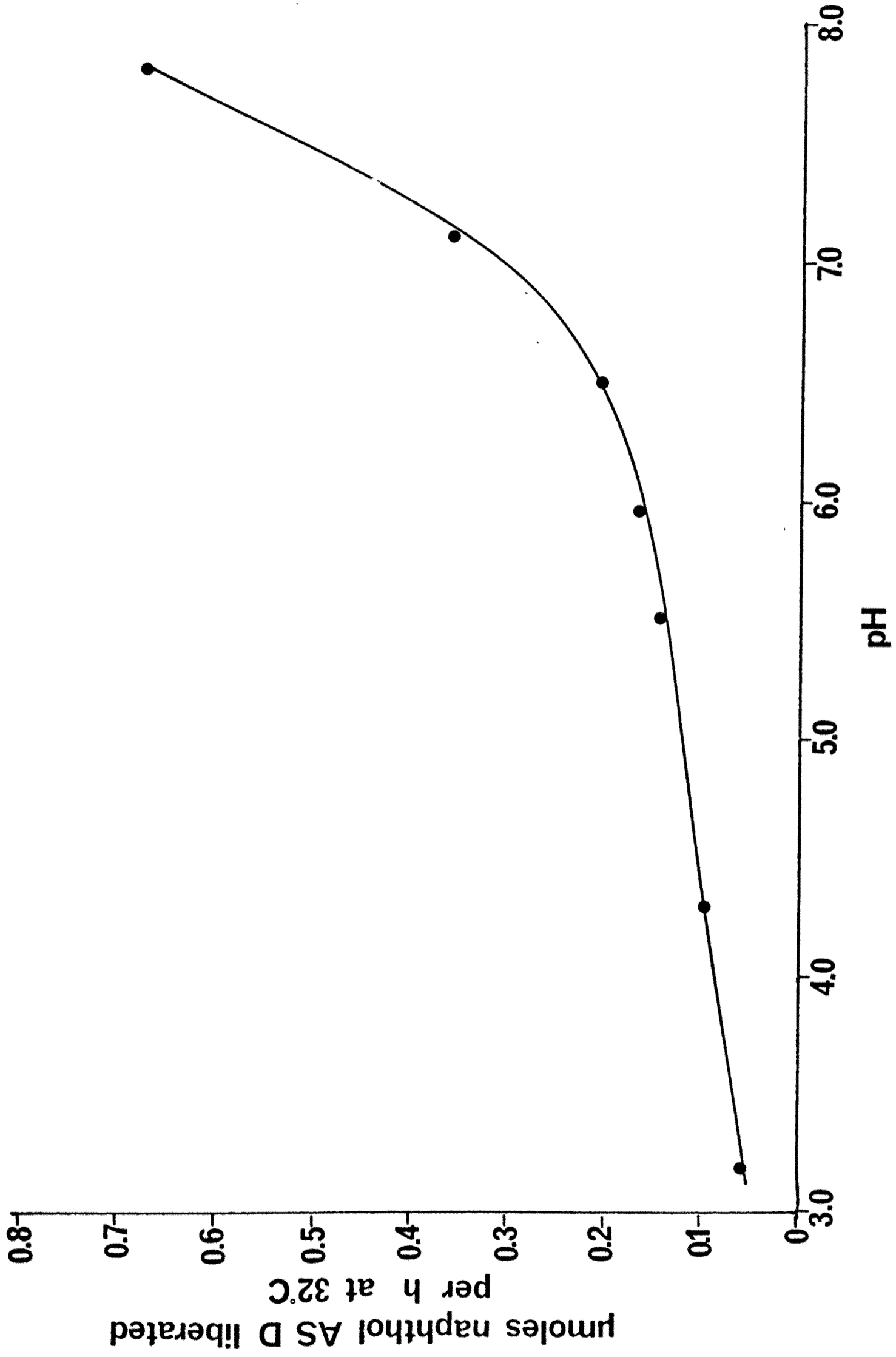


Fig. II. 10

Table II.3. Changes in the pH of buffers as a result of adding DMF to the substrate solutions.

Buffer	pH of buffer before adding to *DMF	pH** of buffer after adding to *DMF
Citrate phosphate	2.6	3.2
"	3.8	4.3
"	4.8	5.6
Tris-maleate	5.0	5.8
"	5.4	6.3
"	5.5	6.5
"	6.4	7.3
"	7.6	8.2

* Final concentration of DMF in the substrate solution was 10% (v/v).

See Section II.2.3.2. for details.

**These values were used in the determination of the effect of pH on esterase activity, (Fig.II.11).

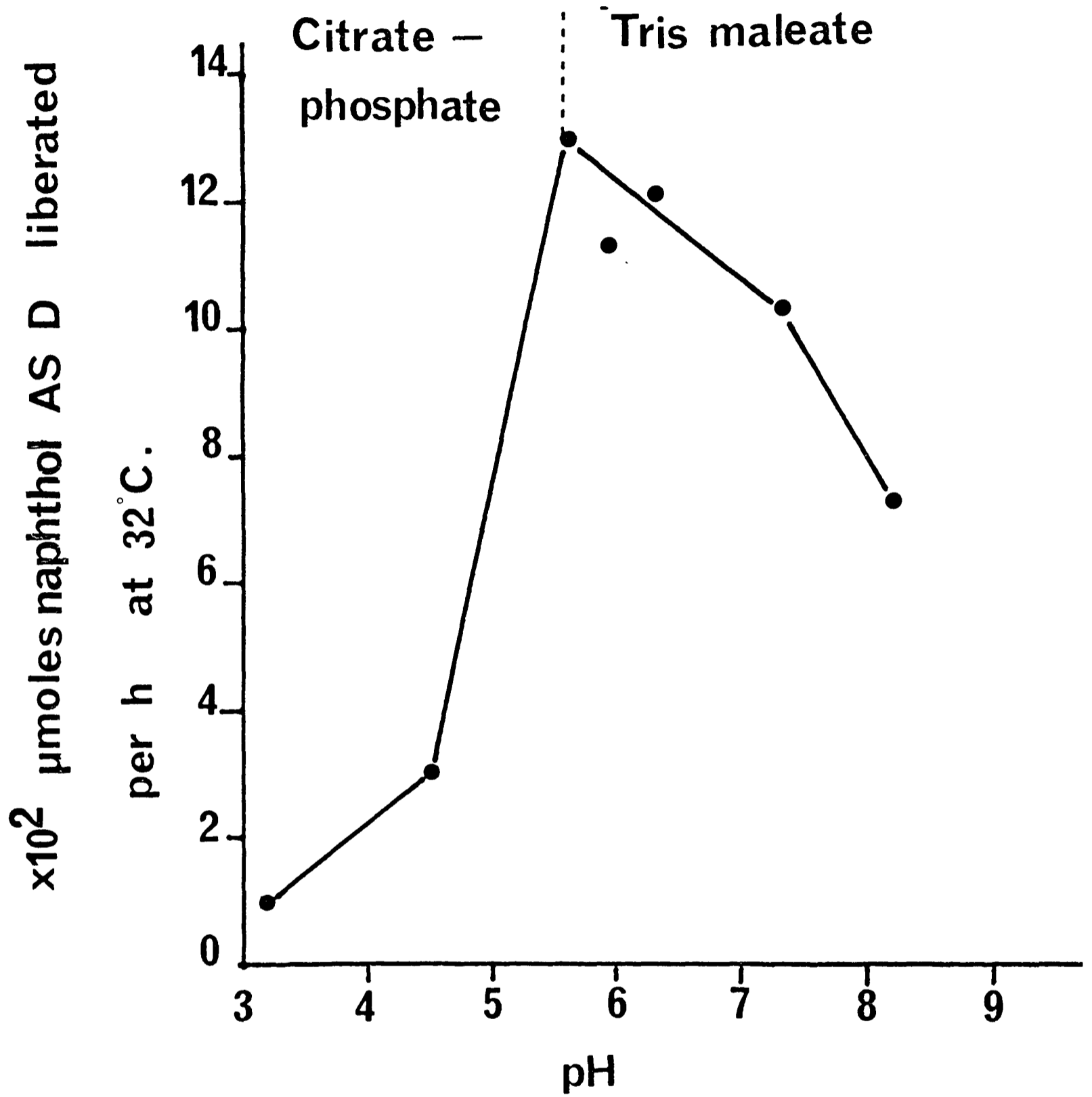


Fig.II.11: The effect of pH on esterase activity. The values plotted represent the differences between the rates of hydrolysis of naphthol AS D acetate in the presence of enzyme and in its absence, at each pH value.

Instead, therefore, it was only possible to define the pH value at which a maximum difference between test and control systems was obtained. In addition, the DMF employed as the substrate solvent gave an alkaline reaction and the large amounts required (10% v/v) in the substrate solution created problems in adjusting pH values since the two buffers used, namely, 0.1M McIlvaine's citrate phosphate and 0.1M Tris-maleate displayed altered buffering capacities in the presence of this reagent. Thus, it was only possible to record final pH values of the substrate solutions, before addition of enzyme. These final pH values were therefore different from the original pH values chosen for the buffers because of the effect of DMF. They are given in Table II.3. and were the values chosen for the determination of the effect of pH on esterase activity (Fig. II.11.).

The enzyme solution was prepared by diluting several fold in double-distilled water a pervaporated concentrate of the enzymes and adding magnesium chloride to a final concentration of 5mM. The pH values of the substrate solutions, accurate to within ± 0.02 pH units, were checked before and after assay, and before post-coupling with the diazonium salt. Replicate control solutions were prepared for each pH value.

A series of buffers was prepared giving a pH range extending from 3.2 to 8.2, using McIlvaine's citrate-phosphate between values 3.2 and 5.6 and 0.1M Tris-maleate between 5.8 and 8.2. (The overlap in pH originally sought between the two buffer systems to take account of possible salination effects could not be achieved because of the effect of DMF.). Fig. II.11. shows a maximum difference between test and control systems at about pH 5.5. The steep fall in esterase activity at pH values beyond 5.5 must

partly be due to the increasing rate of non-enzymic hydrolysis with increasing pH (Fig. II.10.) besides a possible decrease in esterase activity.

It is also evident from Fig. II.11. that in Tris-maleate buffer the greatest esterase activity was recorded at pH 6.3, although there is a distinct possibility that higher activity may have been recorded between 6.3 and 7.3, and the graph could have been drawn to show two pH optima for non-specific esterases, one in citrate phosphate buffer at pH 5.5 or beyond and the other in Tris-maleate buffer between 6.3 and 7.3. It would appear likely therefore that the pH optimum for esterase activity is dependent upon the buffer in which the reaction is taking place.

The recorded optimum of pH 5.5 was, however, not used in further cytochemical and biochemical work for the following reasons.

Histochemical methods for the detection of non-specific esterases are conventionally carried out at pH 7.1 and it was considered that, by using pH 5.5 for future estimations of esterolytic activity different populations of esterase isoenzymes may be detected (e.g. Markert and Moller, 1959).

Moreover the choice of 7.1 as a working pH for cytochemical work using Tris-HCl buffer is a poor one, firstly because Tris-HCl does not buffer effectively at pH 7.1 (Gomori, 1955) and secondly because the substrate is markedly unstable at about this pH (Fig. II.10.).

After taking account of all these factors a 'compromise' value of pH 6.5 was chosen as the standard pH for all subsequent biochemical and cytochemical work since it served the purpose of

i) avoiding large amounts of non-enzymic hydrolysis ii) being near enough to the pH value of 7.1 used in standard cytochemical tests (Burstone, 1962) to permit comparisons with other work and iii) approximately 90% of the esterase activity at pH 5.5 was still recorded at 6.5.

II.3.2.6. The effect of temperature on esterase activity.

A substrate concentration of 0.167mM was used for the determination of an optimum temperature and incubation was allowed to proceed for 1 h in 0.1M Tris-maleate buffer at pH 6.5. Reaction mixtures were incubated in an aluminium block polythermostat (Cross, 1968), with a temperature gradient ranging from 10° to 54°C. Substrate and enzyme solutions were incubated at the desired temperature for 10 min before mixing. This time interval was shown to be sufficient to allow all solutions to reach their desired temperatures.

Experiments showed that the rate of non-enzymic hydrolysis increased markedly beyond 35°C whilst the values for total hydrolysis (enzymic and non-enzymic) increased steadily over the whole range of temperatures used. By subtracting values for non-enzymic hydrolysis from total hydrolysis at each of the selected temperatures the curve in Fig. II.12. was obtained. The highest difference between test and control tubes was recorded at 38°C but at this temperature about 12% of the substrate supplied had been hydrolysed non-enzymically compared to 7% at 32°C.

Moreover it is well known that the optimum temperature is determined by the balance between the effect of temperature on the rate of the enzyme reaction and its effect on the rate of destruction of the enzyme and therefore the actual values of the optimum

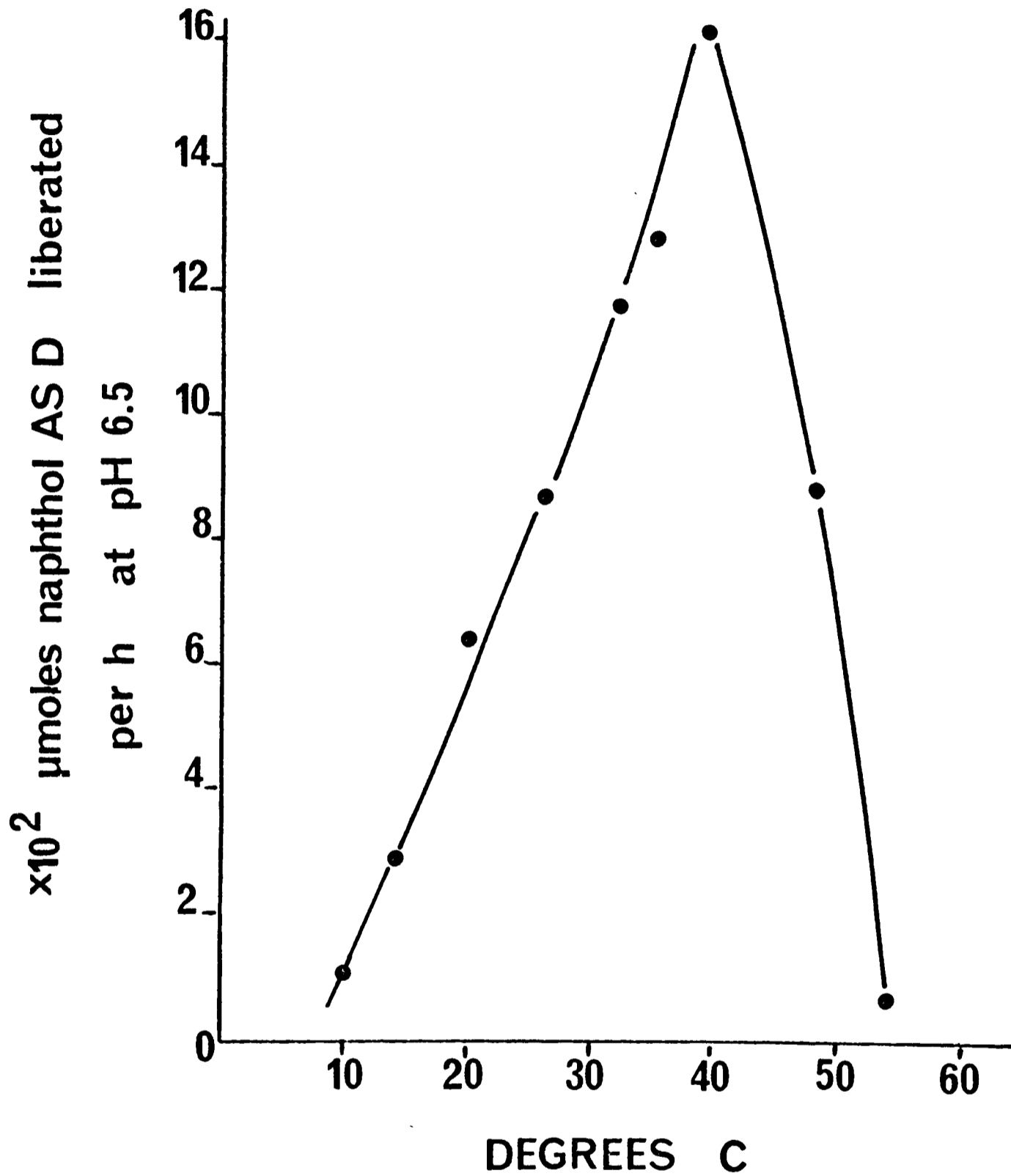


Fig.II.12: Effect of temperature on the activity of root tip esterases at pH 6.5. The results shown are the difference between total hydrolysis and non-enzymic hydrolysis at each temperature value.

temperatures have no special significance (e.g. Dixon and Webb, 1958). To avoid high levels of non-enzymic hydrolysis and to obtain a significant degree of colour development, 32°C was chosen as the standard temperature for incubation for all subsequent assays of esterolytic activity.

II.3.2.7. Ion effects and esterase activity.

Mendoza et al., (1969) have shown that magnesium ions have a marked stimulatory effect on pea and bean esterase activity. Norgaarde and Montgomery, (1968), however, showed that these ions were inhibitory to pea esterases at concentrations in excess of 10mM when phenyl propionate was used as the esterase substrate. Results presented here suggest that these ions may be important in a different way. A crude extract of the root tip esterases from the first 3,000xg_{av} centrifugation (see Section II.2.2.) was divided into three equal aliquots. Each aliquot was then dialysed against 8 litres of one of the following three solutions:

- a) 10mM sodium phosphate buffer, containing 5mM MgCl₂, or
- b) 10mM sodium phosphate buffer only, or
- c) double-distilled water.

The final pH of solutions 'a' and 'b' was 6.5, as was that of the enzyme extracts when removed from their dialysis bags. The pH of the double-distilled water was 5.4, no doubt because of dissolved CO₂. At the end of the treatment volume changes were found to be negligible. The protein content of each preparation was estimated using Lowrys' method and then esterase activity was assayed using the standard system containing supplements of MgCl₂ ranging in concentration from 0 to 50mM. In treatment 'a' the final concentration of MgCl₂ was marginally higher than in treatments 'b' or 'c' due to the presence of MgCl₂ from the dialysing medium.

When the absolute values for esterase activity in each preparation were plotted (Fig. II.13.) it could be seen that the addition of increasing quantities of magnesium chloride to the preparations from treatments 'a' and 'b' caused increasing inhibition of esterase activity whereas very little effect was observed upon the preparation dialysed against double-distilled water. However when the data are expressed on a specific activity basis, i.e. μ moles naphthol AS D liberated per h per mg of protein, it can be seen that the omission of magnesium chloride during enzyme extraction did not alter the catalytic activity of these plant esterases, although omitting all ions reduced the specific activity fourfold (Table II.4.). In this case 'ion effect' and 'buffer effect' cannot be separated, and it is very likely that both are important.

From Table II.4. it may be concluded that magnesium and/or chloride ions exert a non-specific effect by maintaining all or some of the proteins in the 'native' state during dialysis, although since the specific activity of the esterases is unaltered in the presence or absence of 5mM $MgCl_2$ it can be stated that at this concentration the ions are not effective in altering the catalytic properties of these enzymes. The role of phosphate and/or sodium ions is more difficult to assess. Evidence from Fig. II.11. suggests that phosphate ions may be important for enzyme activity, since the changeover point in buffers from citrate-phosphate to Tris-maleate apparently coincides with a decrease in enzyme activity. To determine the role of phosphate ions it would be necessary to substitute for phosphate a different sodium salt with similar buffering capacities, or simply to add phosphate to the tris-maleate buffer.

Fig.II.13: The effect of adding magnesium and chloride ions to the assay system for esterase activity. During enzyme preparation extracts were dialysed against a) 10mM phosphate buffer containing 5mM $MgCl_2$ (●—●); b) 10mM phosphate buffer only (○—○); and c) double distilled water (▲—▲). The pH of all reaction mixtures was adjusted to 6.5.

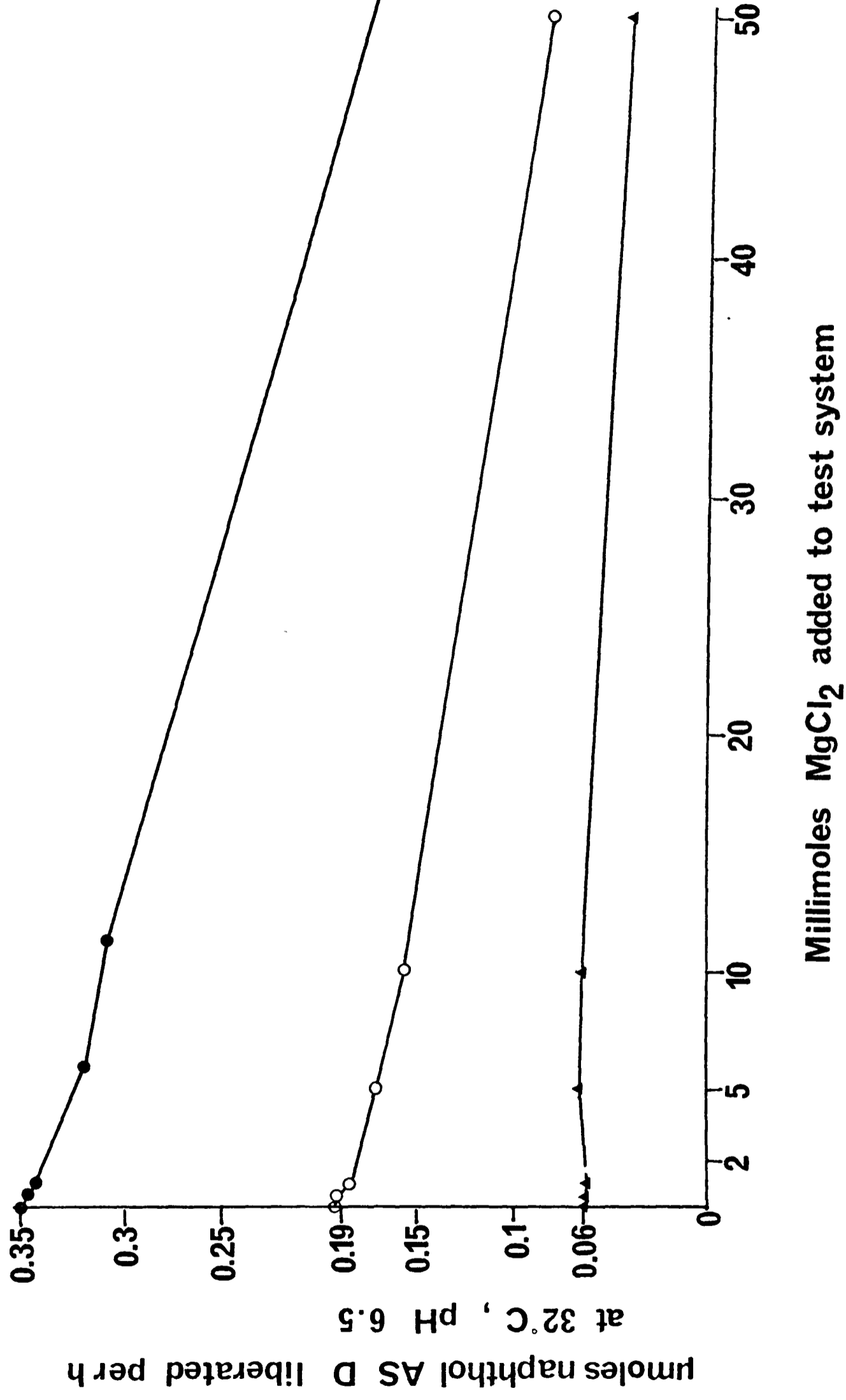


Fig. II.13

Table II.4. Activities of the respective extracts dialysed against
a) 10mM sodium phosphate buffer and 5mM MgCl₂,
b) 10mM sodium phosphate buffer only, and
c) double-distilled water.

Extract	μ moles naphthol AS D** liberated per h.	Protein (μ g/ml)	Specific* Activity	Increase of a) and b) over c). (c) taken as 100).
a)	0.35	350	1.01	419
b)	0.19	172	1.11	422
c)	0.06	264	0.24	100

* Specific activity defined as ' μ moles naphthol AS D liberated per mg protein per h' at pH 6.5, 32^oC without additional Mg⁺⁺ supplement.

** These figures are taken from the '0' addition of Mg⁺⁺ ions in Fig. II.13.

II.4.

DISCUSSION

The use of enzyme histochemical reagents in the biochemical assay and characterisation of enzymes is potentially of great value to their study at the cellular level, although often in practice difficulties are met with in making strict comparisons between cytochemical and biochemical enzyme systems. Cytochemically, parameters such as the penetration of substrate into intact cells and its concentration both inside and outside tissue sections, are of critical importance in achieving valid staining patterns (Holt and O'Sullivan, 1959). Obviously in a biochemical study of these same enzymes these particular problems do not exist, although, as in the present study, there are problems that are peculiar only to the biochemical system; for instance, maintaining the azo-dye in stable solution. In the present work this could only be achieved by adding the catalytically inactive BSA to the assay system. Previously, Gomori (1953), had noted that azo-dyes formed from the coupling of commercially available diazonium salts and substrates derived from the naphthol AS series were too insoluble for spectrophotometric assay even though sodium dodecyl sulphate and, propanediol were added to enhance solubility. For this reason he used the diazonium salt 2-diazonaphthalene-6, 8 disulphonic acid instead of the more commonly used salts which are commercially available. Clearly then the addition of BSA is essential to maintain the product of the reaction between naphthol AS D and the coupling salt (the azo-dye) in stable, clear colloidal solution.

Technical problems aside, however, the correlation of cytochemical and biochemical results are invaluable in that they permit a comparison of the behaviour of the same group of enzymes both

in intact cells and those solubilised from tissue homogenates. This is particularly useful in the study of non-specific esterases, since in animal and plant cells these enzymes can be localised cytochemically in particles identical with those bearing acid phosphatase activity (Holt, 1956; Gahan, 1968), whereas biochemical assays of various subcellular fractions from tissue homogenates of liver cells apparently show that esterase activity is associated almost exclusively with the microsomal (rather than the lysosomal) fraction (Novikoff et al., 1953; Underhay et al., 1956; Novikoff, 1961). This discrepancy may be partly explained by possible 'contamination' i.e. by transference of enzyme from lysosomal particles to microsomes during homogenisation (Holt, 1963; Bowers and de Duve, 1967a). In some investigations however, identical substrates for esterases were not used in cytochemical and biochemical tests and therefore the possibility that the same group of esterases were not being examined must be accepted.

Furthermore, increasing evidence from studies of animal tissues, both cytochemical and biochemical, points to two separate populations of non-specific esterases; a lysosomal one with a pH optimum around 5, and the other microsomal, with a pH optimum of approximately 8.5 (Shibko and Tappel, 1964). In addition, Holt (1963) has pointed out that esterases from liver tissue homogenates display two distinct pH optima; one for indoxyl esters just below pH 4, and another for naphthyl esters at about pH 8.4. Evidence from ultrastructural enzyme localisation studies of non-specific esterases from root cells of Vicia faba (Gahan and McLean, 1969, and Chapter V) suggests that a similar situation may exist in plant cells. These enzymes were localised both on rough endoplasmic

reticulum (a component of the microsomal fraction) and in a variety of different sized vacuoles (probably the lysosome fraction).

In the present investigation, the pH optimum of 5.5 found for broad bean esterases is similar to that recorded by Semadeni (1967) for cell-free extracts of maize root tip non-specific esterase, the optimal activity of which was observed at pH 5.2. Furthermore, Jansen et al., (1947) reported a pH optimum range of 5.5 to 6.5 for citrus acetyl esterase. This evidence taken together with the known instability of naphthol AS D acetate at alkaline pH and the weak buffering capacity of Tris-HCl at pH 7.1, make the choice of pH 7.1 for the cytochemical detection of non-specific esterases (Burstone, 1962) a rather surprising one.

The 'compromise' value of pH 6.5 seems more appropriate in the light of evidence from the present work (see Section II.3.2.5.).

K_m values have only rarely been recorded for esterases extracted from higher plants, but that calculated here for Vicia faba lateral roots, 0.07mM, is considerably lower than that for citrus acetyl esterase recorded by Jansen and coworkers (1947).

They reported K_m values of 370 mM when glycerol monoacetate was used as substrate; 80mM for glycerol diacetate and 30mM for glycerol triacetate. In contrast K_m values of non-specific esterases extracted from a wide range of animal tissues have frequently been published. Bosmann (1972) found the K_m value for a highly purified aryl esterase from pig cerebral cortex to be 0.43 mM. The substrate used was p-nitrophenyl acetate, the same as that used by Huggins and Lapidus (1947) several years earlier in their study of human sera arylesterase. The K_m value for this enzyme was 4.15mM. For those esterases hydrolysing esters of higher fatty acids, Mahadevan

and Tappel (1968) found the K_m for liver lysosomal esterases acting on *p*-nitrophenyl myristate to be 1mM.

It might be argued that when different substrates are employed in the calculation of K_m values for the same group of enzymes, a subsequent comparison of such values is not justified. It is such difficulties that have led to the adoption of arbitrary criteria, as for example the relative rates of substrate hydrolysis and sensitivity to various activators and inhibitors, as the bases of non-specific esterase classification. The shortcomings of dividing non-specific esterases into 3 major groups, *viz*; carboxylesterases (E.C.3.1.1.1.), arylesterases (3.1.1.2.), and acetylersterases (3.1.1.6.) have recently been pointed out by Choudhury (1972). He has produced electrophoretic and biochemical evidence in favour of the view that non-specific esterases belong to a single enzyme system, with each esterase species built on a subunit structure. Thus, he maintains that it is the sharing of common subunits that produces the overlapping specificities of esterases so often observed in biochemical and electrophoretic studies.

At the present time there is conflicting evidence for the role various ions play in esterase activity and function. Bosmann (1972), for instance, has shown that purified arylesterase from guinea pig cerebral cortex was unaffected by magnesium and cadmium ions but required manganese. On the other hand, Mendoza *et al.*, (1969) and Norgaarde and Montgomery (1968) showed that manganese inhibited the esterases of certain leguminous plants. Evidence presented here favours the view that magnesium ions are important in retaining proteins in their native state during the extraction procedure.

CHAPTER III

A CYTOCHEMICAL AND BIOCHEMICAL STUDY OF NON-
SPECIFIC ESTERASES IN THE DIFFERENTIATING ROOT
CELLS OF VICIA FABA

III.1.

INTRODUCTION

Enlargement, elongation, vacuolation, and maturation occur during the differentiation of root cells, processes which require a high degree of controlled lytic and synthetic activity. Differentiating cells undergo gross changes in structure and function and a variety of enzymes must play an important role in the underlying changes.

Recently biochemical and cytochemical investigations have shown that acid hydrolases, including non-specific esterases, may be associated with plant cell structures such as vacuoles and spherosomes (Matile and Wiemkin, 1967; Matile and Spichiger, 1967; Matile, 1968, 1969; Wardrop, 1968; Berjak, 1972; Pitt and Galpin, 1973). If the vacuolar system of plant cells is regarded as the physiological and biochemical counterpart of the lysosomal system in animal cells (de Duve, 1959, 1963; Novikoff et al., 1964) then the elaboration of such a system is a characteristic feature of the process of cell differentiation in roots where all degrees of vacuolation may be observed. In this context, Gahan and Maple (1966) showed that acid phosphatase was associated with the developing protoxylem tissue in the lateral root tips of Vicia faba. The loss of all cytoplasmic constituents during the differentiation of this tissue prompted these authors to suggest that intracellular digestion based on the model of de Duve (1963) for animal cells may be responsible for this phenomenon.

McLean (1970) carried out cytochemical investigations of non-specific esterases in the differentiation of lateral roots of Vicia faba and showed that when visual assessment of staining

intensity was the criterion used, naphthol AS D esterase activity increased with increasing distance from the root tip. Biochemical measurements of changes in enzyme activity along the length of a variety of roots have been made by a number of workers in the past. The basic procedure common to all these studies has been to take serial transverse sections or segments of root tissue and to subject them to chemical and biochemical analysis. These data can then be correlated with cytological and morphological changes occurring in the root. In this way Bottelier et al., (1943) examined dipeptidase activity in barley roots, and Robinson and Brown (1952) measured dipeptidase, glycine oxidase, acid phosphatase and invertase activities in the roots of Vicia faba. More recently Sutcliffe and Sexton (1969) made a combined histochemical and biochemical study of β -glycerophosphatase and adenosine triphosphatase in pea roots, so giving a qualitative and a quantitative picture of the involvement of both enzymes in differentiation.

The aim of the present investigation was to use this approach for the study of non-specific esterases in the terminal 3 mm of the lateral roots of Vicia faba. Previous investigations of these enzymes in root cell differentiation (Benes, 1962, 1971; Hadacova and Sahulka, 1967; Sahulka and Benes, 1969 and McLean, 1970) have been histochemical in approach and so no attempt has been made to compare histochemical and biochemical changes at known distances along the roots. In the present work, therefore, emphasis was laid on delineating and measuring enzyme activity as accurately as possible in serial, frozen root tissue sections of known thickness. The enzyme activity from homogenised tissue

sections could then be expressed on a 'per segment', 'per unit protein' or 'per unit area of average-sized root section' basis and this data related to the histochemical staining pattern for esterases in both transverse and longitudinal sections of root tissue.

The use of aldehyde fixation in enzyme histochemistry, both at the light and electron microscope levels is now widespread (see Hopwood, 1972, for review). Glutaraldehyde and formaldehyde have been found to give the best results for cytological preservation and retention of enzyme activity (Sabitini et al., 1963). Since glutaraldehyde is now a commonly used fixative for electron microscope cytochemistry, a comparison has been made at the light microscope level between the esterase-staining patterns in unfixed and glutaraldehyde-fixed frozen cryostat sections of differentiating roots.

Parallel biochemical studies were also performed on root segments of fixed and unfixed material to determine the extent to which glutaraldehyde affected esterase activity.

III.2.

MATERIALS AND METHODS

III.2.1.

Preparation of tissues for cytochemical and biochemical studies.

Approximately 100 lateral roots of the broad bean Vicia faba were removed with a new razor blade from the parent plants, grown under the conditions previously reported (Section I.2.1.). Roots up to 3 cm in length, and between 10 and 14 days old were selected and used either fresh or fixed in glutaraldehyde by the methods given in Section I.2.2.1.1.

Ten roots at a time were placed in a hollow open glass tube, with an internal diameter of 4 mm. The traces of buffer remaining on the roots, served to hold them in place whilst other roots were being placed in position beside them. When all roots were in place the extreme tips were pushed through the open end with a dissecting needle and blotted dry. Blotting enabled the tips to be marked with a small dot of Indian ink for the identification of alignment in subsequent cryostat sectioning (Jensen, 1962). By gently tapping the glass tube downwards several times on the bench surface the root tips could be brought exactly into line (Fig. III.1.). While this was being done the vessel containing the freezing mixture and block holder (Fig. III.1.f.) was being cooled to -60°C , on a large glass plate. This provided a large cooled surface for subsequent preparation of the root, since the heat was quickly conducted from the glass to the freezing mixture nearby.

Holding the glass tube upright and with the root tips in contact with the cooled surface, tempered (40°C), 3.5% (w/v) Ionagar No.2 (Oxoid Ltd., London) was then delivered into the open end by

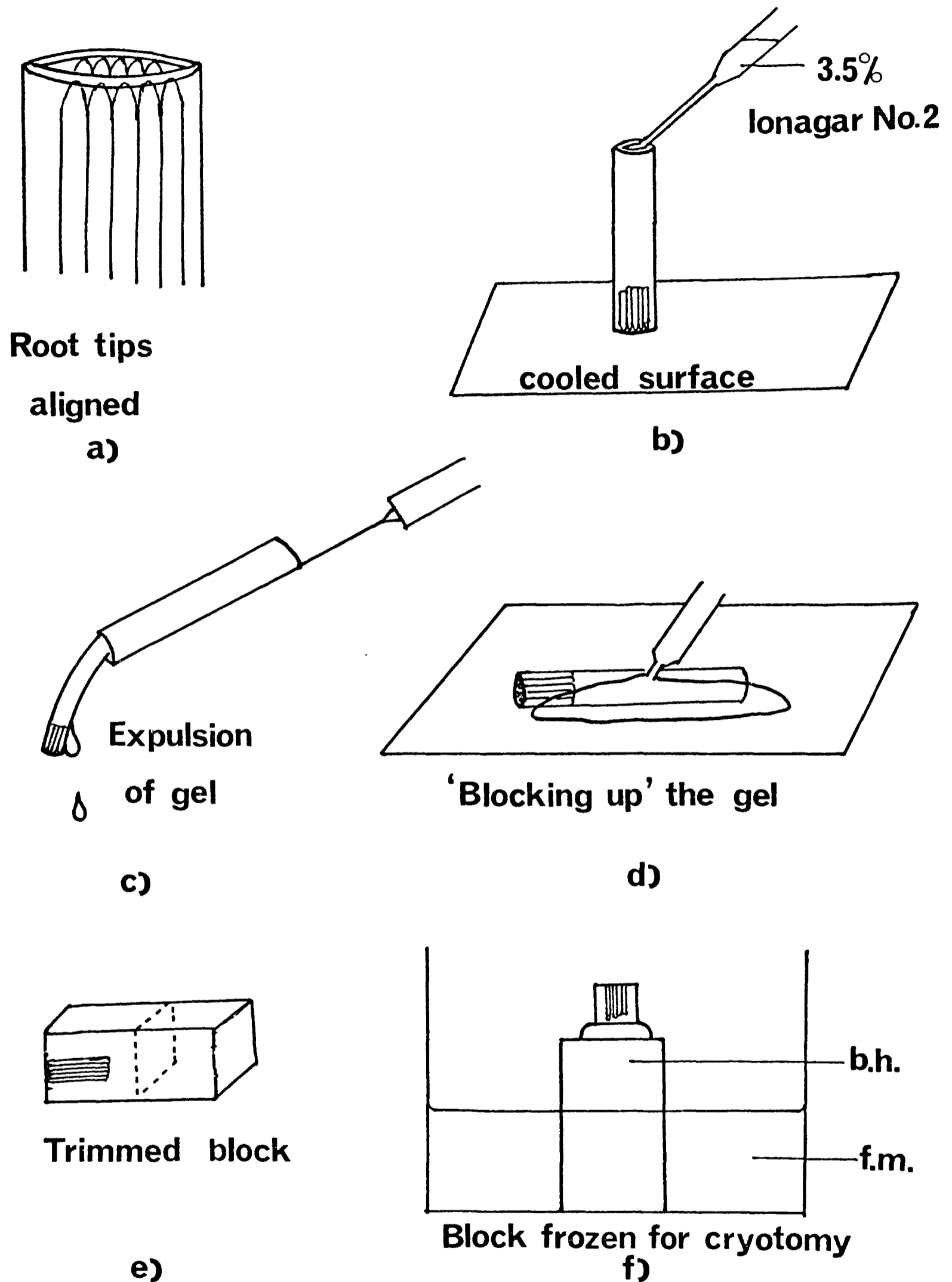


Fig.III.1a) to f): Preparation of fixed and unfixed root tips for cryotomy. Each block of agar contained 10 roots. Abbreviations: b.h., block holder; f.m., freezing mixture, consisting of absolute ethanol cooled with solid carbon dioxide.

means of a Pasteur pipette. In this way all the roots quickly became encased in a cylinder of molten agar, which quickly cooled and gelled. The cylinder of agar-covered roots was then rapidly expelled from its glass container by injecting distilled water between the gel and the sides of the glass tube, and then laid lengthwise on the cooled surface. Additional molten agar was added around the cylinder of gel to make a more manageable shape. It was then trimmed so that about 5 mm of the root tips were retained. The agar block was quickly transferred to a freezing bath of solid carbon dioxide and absolute ethanol mounted on the block holder, and completely frozen (Gahan et al., 1967).

After freezing, the block holder and the frozen roots were transferred to a cryostat either for immediate sectioning or for storage at -25°C . If the blocks were stored for any length of time it was important to prevent desiccation of the frozen agar since this changed its consistency and seriously hindered subsequent section cutting. Desiccation of the agar was reduced by placing polythene hoods over the tissue blocks and securing them to the block holder with elastic bands.

Continuous serial transverse sections were cut at a thickness of $20\mu\text{m}$ for biochemical assay or $10\mu\text{m}$ for cytochemical investigations and at a temperature of -20° to -25°C , using a Bright's cryostat. Each section thus contained a ring of 10 roots. 12 such sections were accommodated on one side of a large coverslip and this represented the first $240\mu\text{m}$ of the root tip. Two coverslips would therefore contain the first $480\mu\text{m}$ of the root tip cut into $20\mu\text{m}$ sections. $480\mu\text{m}$ thereby served as an arbitrary segment of root tip upon which biochemical assay could be performed. By continuing serial sectioning to $3360\mu\text{m}$ 7 such segments were delineated

as shown in Table III.1.

This process needed to be repeated 5 times (i.e. 50 roots were sectioned in all) to give sufficient tissue for enzyme and protein assay.

Table III.1. The segments of the root with their arbitrarily defined limits.

Segment	Distance of root from apex (μm)
I	0 - 480
II	481 - 960
II	961 - 1440
IV	1441 - 1920
V	1921 - 2400
VI	2401 - 2880
VII	2881 - 3360

III.2.2. A note on the accuracy of the microtome setting for section thickness.

It is clearly imperative that desired section thickness (in this case $20\mu\text{m}$) corresponds accurately to the microtome setting in the cryostat cabinet, in order that the segments I - VII spatially correspond each time a whole root is sectioned from the apex ($0\mu\text{m}$) to $3360\mu\text{m}$. In this connexion Butcher (1971) has used a chemical method for accurately determining section thickness based on measurements of the nucleic acid content of liver sections. Using a Brights cryostat at a setting of $20\mu\text{m}$

Butcher calculated the section thickness of liver tissue to be $20.8 \pm 0.21 \mu\text{m}$.

In the present investigation 24 sections were cut, each $20 \mu\text{m}$ thick, per root segment. As there are seven segments, a total of $24 \times 7 = 168$, sections of $20 \mu\text{m}$ thickness was cut for each root 0 to $3360 \mu\text{m}$ in length. Using Butcher's estimation, on the assumption that a similar error existed in the cryostat used here, it appears that a total length of $3494 \pm 35.3 \mu\text{m}$ from the root tip, (i.e., there was an error of $3.8 \pm 1\%$) was taken. Over the 3.4 mm of root examined the error is therefore negligible.

Perhaps of greater significance was Butcher's finding that 'too rapid' or 'too slow' rotations of the cryostat handle caused large variations in section thickness. In the present study it was found through experience that intact sections were only obtained if sectioning, once started, was not interrupted, and was performed at an even pace until the requisite length of root had been cut.

III.2.3. Homogenisation of tissue sections.

Enzyme extracts were prepared by ultrasonication of tissue sections at 21 Kc/s using an MSE 100 watt ultrasonic disintegrator.

In a preliminary experiment 10 unfixed roots were sectioned from the tip to $3360 \mu\text{m}$ by the method described (Section III.2.1.) and all the sections sonicated for 15 sec. This was repeated with another 3 batches of 10 roots using further times of 45, 75 and 120 sec respectively. This established that for roots sections as a whole, 2 min was the time required to release the maximum available quantity of enzyme (Fig. III.22.).

It was impracticable to determine the most effective sonication time for each of the 7 segments since this would have required 200 roots, (50 roots x 4 - number of sonication times = 200). In turn this would have meant cutting 20 section blocks, so that the time between cutting the first section to the time of the first biochemical assay would have been inordinately long and could have resulted in enzyme inactivation of long-stored tissue sections.

10 coverslips, i.e. 2 for each 480 μ m segment repeated 5 times and each containing 120 root sections, from each of the appropriate segments, were placed in a double-walled glass thimble and gently broken up in the presence of 8 ml of chilled 0.1M phosphate buffer pH 6.5 and then submitted to sonication for 2 min. Heating was minimised by the circulation of coolant (-70°C) between the 2 walls of the thimble. Following sonication extracts were dialysed for 24h against 10mM phosphate buffer, pH 6.5 containing 5mM MgCl₂ at 4°C, and then centrifuged for 30 min at 20,000 x g_{av} at 4°C.

The supernatant was used as the source of the enzyme, 1 ml aliquots of this being used for assays of esterolytic activity. 1 ml aliquots of the same solutions were also used for protein determinations.

III.2.4. Enzyme assay.

The method of measuring esterase activity was as given in Chapter II, Section II.2.3., except that only one third of all volumes of each of the constituents of the assay system were used. This was necessary to avoid dilution of the very small

amounts of enzyme and protein extracted during sonication. The final volume of the reaction mixtures after post-coupling therefore was 5 ml. Specific esterase activity is expressed as μ moles naphthol AS D liberated per h per mg protein at 32°C, pH 6.5.

III.2.5. Protein estimation.

The method of Lowry et al., (1951) using bovine serum albumen, Fraction V (Armour Pharmaceuticals Co. Ltd.) as standard, was adopted for the measurements of soluble protein.

III.2.6. Photomicrography.

Colour photographs were taken on Agfachrome 50L or Kodachrome II film and black and white prints made from the transparencies where necessary.

III.2.7. Cytochemical studies.

Non-specific esterase localisation was carried out on unfixed and fixed cryostat sections by the simultaneous azo-dye coupling method of Burstone (1962), employing naphthol AS D acetate as substrate and fast red violet LB salt as coupling reagent. The procedure was as follows:-

To 5.0 mg of naphthol AS D acetate was added 0.5 ml DMF, followed by 25 ml of distilled water, 25 ml of Tris-maleate buffer (pH 6.5) and 30 mg of fast red violet LB salt. The mixture was shaken to dissolve the substrate and filtered through Whatman No.1 filter paper into a Coplin jar. Both fixed and unfixed sections were incubated for 15 min at 37°C.

Between 8 and 30 min of incubation the staining pattern in unfixed tissues was essentially the same but incubation times

longer than 30 min produced staining of an intensity that obscured cytological detail. The same was true of glutaraldehyde-fixed tissues.

After incubation the sections were briefly rinsed in distilled water and counterstained when needed in 1% (w/v) methyl green in 0.05M acetate buffer, pH 4.2 (see Section I.2.3. for details).

Control sections were incubated without substrate; inhibitors were not used because McLean (1970) has already shown that a variety of known esterase inhibitors had a negligible effect on the cytochemical localisation of non-specific esterases in Vicia faba lateral roots.

Tissue sections were examined by light microscopy using bright field illumination and phase contrast optics.

III.3.

RESULTS

III.3.1. Cytochemical studies.

The distribution of the coloured azo-dye end-product was examined in all the differentiating tissues of the terminal 3 mm of the lateral root and compared with the results of the biochemical studies.

Cytochemically, diffuse staining tended to occur in cells in which particulate deposition of end-product was densest, as previously observed by McLean and Gahan (1970). Particular emphasis was, however, made in the present studies in noting the relative intensity of the stain in tissues at different stages of development and comparing such qualitative, or at best semi-quantitative observations with the measurements of esterolytic activity made in the assay procedure on the various tissue segments.

In order to assess visually the amount of end-product deposited in the various tissues a modified and simplified version of the table devised by McLean (1970) was used (Table III.2.)

Each tissue was then examined in turn, its distance from the root tip noted and its staining intensity was classified according to the scheme described in Table III.2.

Colour transparencies of representative tissue sections were made at a magnification of x60. They were then looked at in a hand viewer (x2) for assessment and corresponding tissue sections were then examined under the microscope. Fixed and unfixed tissue sections were examined in the same way, and the results obtained are presented in Tables III.3. and III.4.

Table III.2. Arbitrary visual assessment of the esterase-staining intensity in tissue sections.

Symbol	Appearance of cells after enzyme incubation. Microscopical examination at x160 to x800.	Corresponding appearance of tissue viewed at magnification of x120.
++	Cytoplasm contains numerous close packed particles and some diffuse staining.	Heavy staining.
+	Cytoplasm contains many particles no diffuse staining.	Light staining.
0	Cytoplasm contains few particles; no diffuse staining.	No staining.
0-	Cytoplasm contains very few particles; no diffuse staining.	No staining.
-	Cytoplasm contains no particles; no diffuse staining	No staining.
A	Tissue absent	

Table III.3. Staining intensities for non-specific esterases in the various tissues of differentiating unfixed roots. (For key to symbols see Table III.2.)

Photographic Reference	Root Cap	Epidermal Tissues	Cortical Tissues	Endodermoid Cells	Pericycle	Xylem	Phloem	Apical Meristem	Procambium	Distance from apex (μ m)
V	++	A	A	A	A	A	A	A	A	0-250
VII	++	A	O	A	A	A	A	O	A	250-450
IX & X	++	+	O	A	A	A	A	O	++	450-600
XI	++	+	O	O	O	A	A	A	++	600-650
XII	++	++	O	+	O	A	A	A	++	700-800
XV	+	O	O	++	+	++	++	A	A	1000-1100
XVI	+	O	O	++	+	++	++	A	A	1100-1200
XIX	A	O-	O	++	+	++	++	A	A	1400-1450
XX	A	O-	O	+	+	++	++	A	A	1750-1850
XXIII	A	O-	O	+	+	++	++	A	A	2150-2250
XXVIII	A	O-	O	O	O	++	++	A	A	2250-2350
XXIX	A	O-	O	O	O	++	++	A	A	2350-2450
XXX to XXXIV	A	O-	O	O	O	+	+	A	A	2450-3000

Table III.4. Staining intensities for non-specific esterases in the various tissues of differentiating, fixed roots. (For key to symbols see Table III.2.)

Photographic Reference	Root Cap	Epidermal Tissues	Cortical Tissues	Endodermoid Cells	Pericycle	Xylem	Phloem	Apical Meristem	Procambium	Distance from apex (μ m)
I-IV	++	A	A	A	A	A	A	A	A	0-250
VI	++	A	A	A	A	A	A	O	A	250-400
VII	++	+	O	A	A	A	A	O	++	400-500
IX	++	+	O	A	A	A	A	O	++	500-600
XI	++	+	O	O-	O-	A	A	A	++	600-650
XII	++	++	O	+	O-	A	A	A	++	650-800
XIII	++	+	O	++	O	A	O	A	++	800-900
XIV	+	+	O	++	O	++	+	A	A	900-1000
XV	+	+	O	++	O-	++	+	A	A	1000-1100
XVI	+	+	O-	++	-	++	++	A	A	1100-1200
XVII	+	O	O-	++	-	++	++	A	A	1200-1300
XX	A	O-	O-	++	-	+	+	A	A	1450-1550
XXIII	A	O-	O-	++	-	+	+	A	A	1750-1850
XXIV	O-	O-	O-	++	-	+	+	A	A	1850-1950
XXV	-	O-	O-	+	-	+	+	A	A	1950-2050
XXVI	-	O-	O-	O	-	+	+	A	A	2050-2150
XXVII to XXXV	-	-	O	-	-	O	O	A	A	2150-3000

III.3.2. Definition of tissues.

The dimensions which enable calculation to be made of the areas and volumes of tissue described as root cap and epidermis are easily measured, and to obtain approximate values for them is relatively simple. However, since we are here dealing with the phenomenon of differentiation, there are several instances where it is difficult, if not impossible, to determine the point at which one tissue begins and another one ends. This is particularly true of the procambial tissue, that later gives rise to primary xylem, phloem and endodermoid cells. However, at about 7-800 μ m from the root apex the four arms of the protoxylem are distinguishable, and at this distance the term 'procambium' may be discarded. Throughout the length of the root examined it is difficult to distinguish a single layer of cells that may be distinctly referred to as the 'endodermis', since even high power examination of 1 μ m thick araldite-embedded sections (Chapter V Section V.2.4.) failed to reveal the presence of the Casparian Strip on the radial walls of cells at the stele-cortex boundary. It is quite likely that in the length of root examined (0-3360 μ m) the endodermis, complete with Casparian strip, had not yet differentiated. However, the 2 or sometimes 3, innermost layers of cortical cells were more rectangular than those surrounding them, tended to vacuolate later, and stained more heavily for non-specific esterase. Esau (1953) has suggested the name 'endodermoid cells' be used when an endodermis cannot be properly distinguished, and this is the name given to this tissue in the present work. The pericycle cells are distinguishable by their more rounded appearance when compared to adjacent endodermoid cells, (Fig. III.19.).

Between the epidermis and the endodermoid cells there are approximately seven layers of rapidly vacuolating cortical cells. These isodiametric cells have their own enzyme staining pattern and vacuolate early.

III.3.3. The pattern of esterase localisation in the terminal 3.4mm of the root. A comparison between fixed and unfixed tissue.

The detailed cellular and tissue distribution of non-specific esterase activity in unfixed lateral roots of Vicia faba has previously been recorded by McLean (1970). The following descriptions concentrate on i) recording changing patterns of activity with cell and tissue differentiation and ii) noting the effects of glutaraldehyde fixation on esterase activity by making suitable comparisons with corresponding unfixed tissues.

Figs. III.2. and III.3. with their designated symbols show the gross differences existing between the esterase-staining patterns of fixed and unfixed roots. The vertical distances between the symbols represent relative intensities in the enzyme-staining pattern. A distance of 0.5 cm or less corresponds to the symbol '+' in Table III.2., and a distance of 1-0.5cm corresponds to '+'. Non-visible staining at a magnification of x120 is represented by blank areas. The broken lines shown at 2240 μ m from the apex and beyond in fixed roots (Fig. III.3.) indicate a lessening in the stain intensity and correspond to the symbol '0'.

General Comparison.

A comparison of Fig. III.2. and III.3. shows that the pattern of enzyme localisation in fixed and unfixed tissue is not

UNFIXED, FRESH FROZEN ROOT TIPS

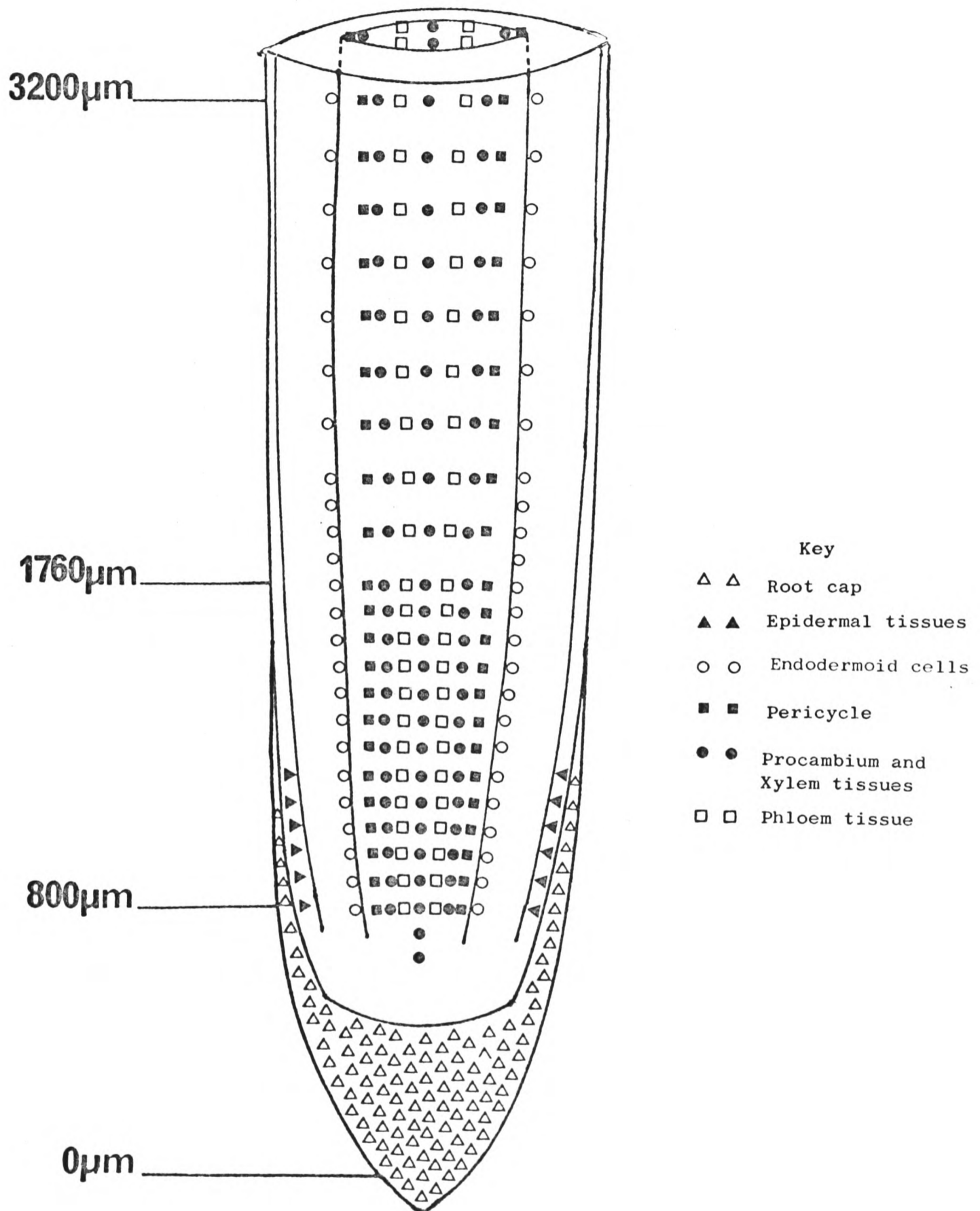


Fig.III.2: Diagrammatic representation of the esterase staining pattern in unfixed lateral roots of *Vicia faba*. The intensity of stain is related to the closeness of the symbols. A vertical distance of 0.5cm or less corresponds to the symbol '+' in Table III.2 and a distance of 1cm-0.5cm to the symbol '++'. Blank areas correspond to non-visible staining at a magnification of x120.

GLUTARALDEHYDE FIXED ROOT TIPS

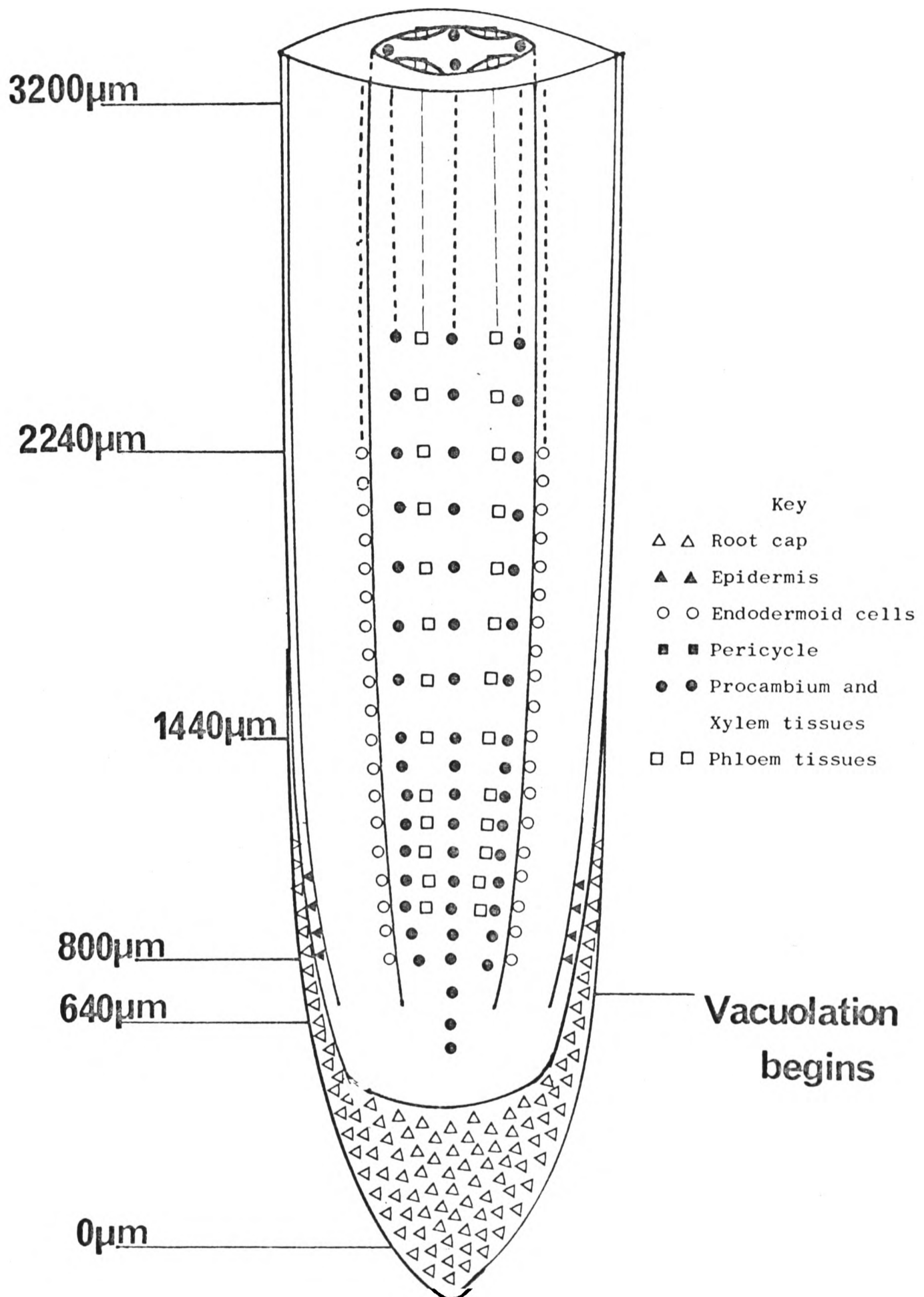


Fig.III.3: Diagrammatic representation of the esterase staining pattern in fixed lateral roots of *Vicia faba*. The method of depicting stain intensity is as explained in Fig.III.2. The broken lines correspond to the symbol '0' of Table III.2.

the same, although there is general agreement in the overall pattern of end-product staining. The esterase end-product in fixed roots was not entirely lost from tissue sections more than 2.6mm from the root tip, and in longitudinal sections particulate end-product could still be seen several millimetres behind the root tip. This applied also to unfixed sections of root tissue in which staining was noticeably heavier between 2.6 and 3.2 mm.

Root Cap.

In the root cap, which extended back approximately 1.5 mm from the root apex, the pattern of end-product deposition was essentially similar in both fixed and unfixed tissues, with characteristically dense and diffuse end-product present in and between the cell walls. Particulate end-product was also observed in the peripheral cytoplasm where cells had vacuolated extensively.

Apical Initials.

Cells from both fixed and unfixed initials showed the presence of several particles, these being generally scattered in the cytoplasm and lacking the longitudinal orientation so characteristic of those in vascular and provascular tissues. The nuclei did not show any evidence of esterase activity in either fixed or unfixed initials. (Figs. III.15. and III.17.).

Epidermal Tissues.

Epidermal cells only became distinguishable at about 800 μ m from the apex, at about the same level as that at which protophloem poles became distinguishable.

FIGS. III.4. - III.14: Comparisons of esterase-staining patterns in differentiating tissues of unfixed (series 'a') and fixed (series 'b') root tips of Vicia faba. Roman numerals in brackets at the end of each legend refer to Tables. III.3. and III.4.

- Fig. III.4: Transverse section of roots approximately 300-400 μm from the apex. Intense activity of the root cap cells is to be contrasted with the very low esterase activity of the meristem. Magnifications: Fig. III.4a) x150, III.4b) x135. (VI).
- Fig. III.5: Sections approximately 400-500 μm from the root tip. Esterase activity in the 2-3 layers of the root cap cells is still evident. Magnifications: Fig. III.5a) x150; III.5b) x135. (VIII).
- Fig. III.6: Sections approximately 500-600 μm from the apex. The root cap esterase activity is still evident. Procambial tissue (in the centre of the root) and the epidermis have acquired end-product staining. Meristematic tissue and procortex do not show staining at this magnification. Magnifications: Fig. III.6a) x150; Fig. III.6b) x100. (IX).

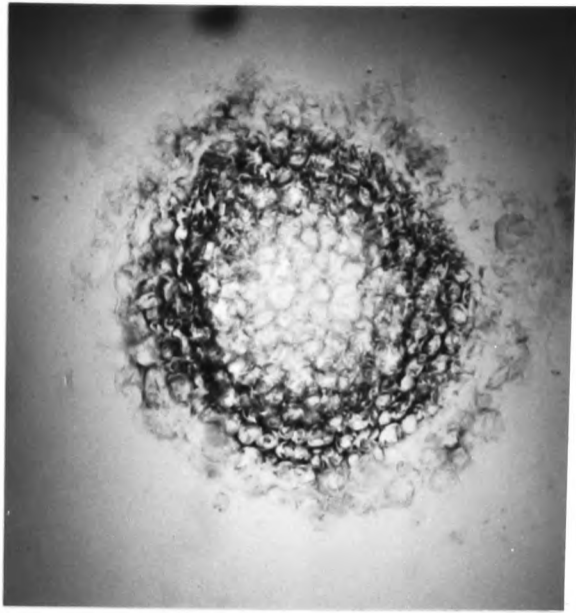


Fig.III.4a)

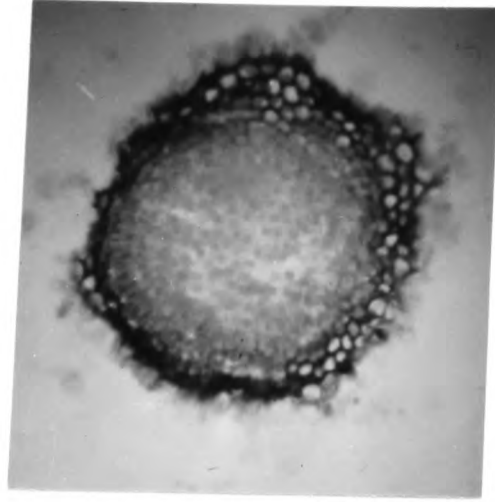


Fig.III.4b)

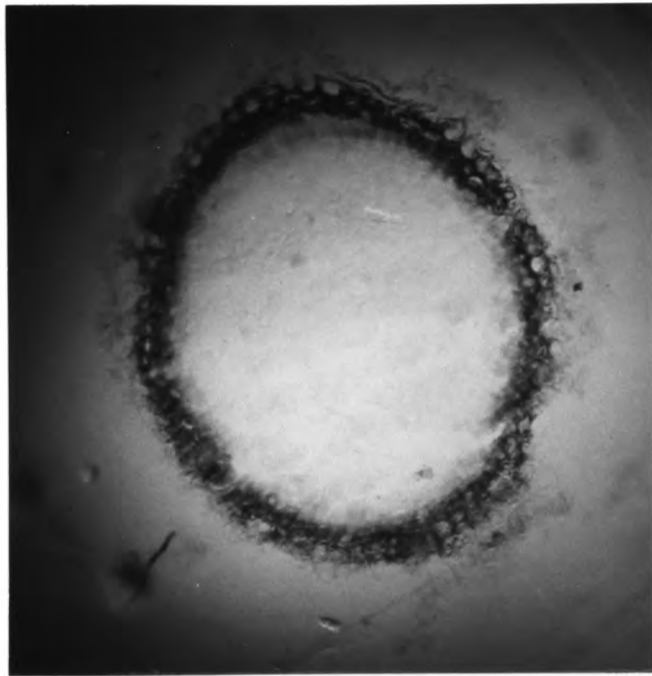


Fig.III.5a)

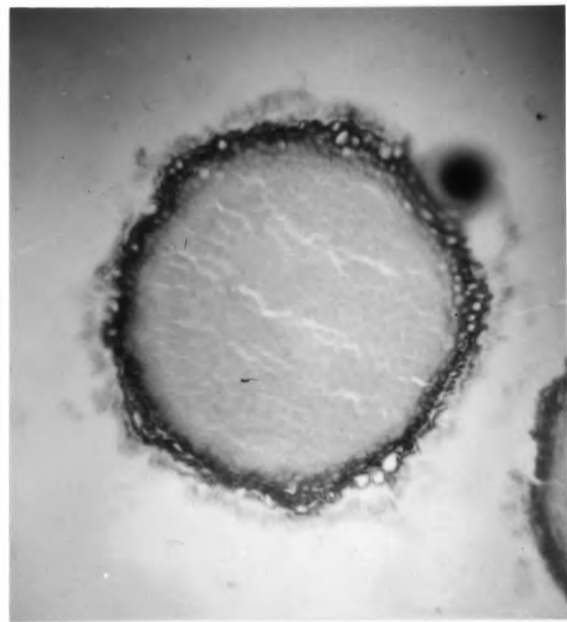


Fig.III.5b)

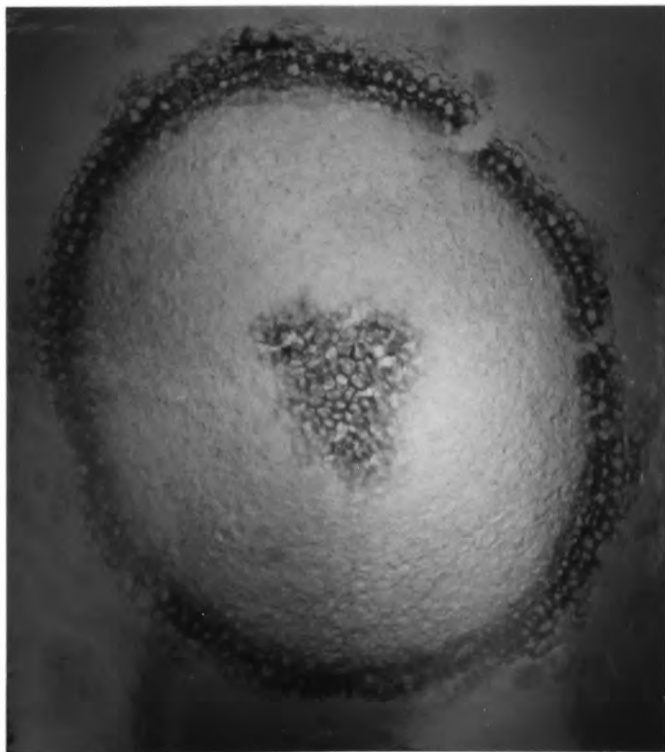


Fig.III.6a)

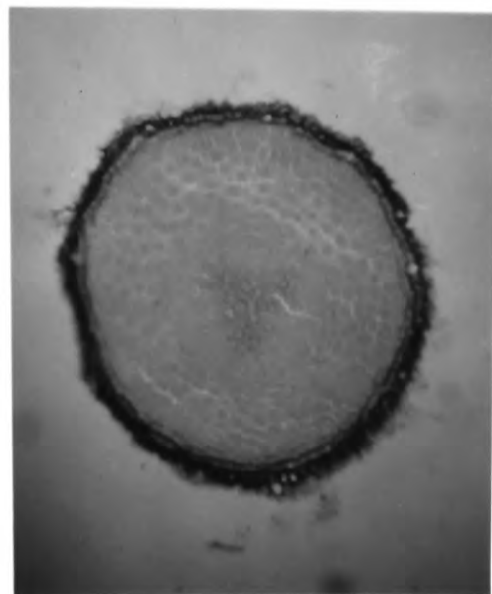


Fig.III.6b)

- Fig. III.7: Sections approximately 700-800 μm from the apex. Root cap activity still high in both fixed and unfixed roots. End-product is localised in the 4 arms of the procambium. Innermost layers of the cortex begin to stain at this stage of differentiation. Magnifications: Fig. III.7a) x150; III.7b) x120. (XIII).
- Fig. III.8: Distance from apex approximately 1300 μm . Staining of the endodermoid cells as a ring outside the vascular tissue is very obvious in both roots. Protophloem poles begin to stain for esterases. Fixed root micrograph shows normal colour of stained section. Magnifications: Fig. III.8a) x150; III.8b) x145. (XVIII).
- Fig. III.9: Approximately 1400 μm from the root apex. Epidermal and root cap end-product staining is beginning to diminish. Root cap now only 1 cell thick. Most vascular tissue is stained for esterases. Magnifications: Fig. III.9a) x150 III.9b) x 105. (XVIII).

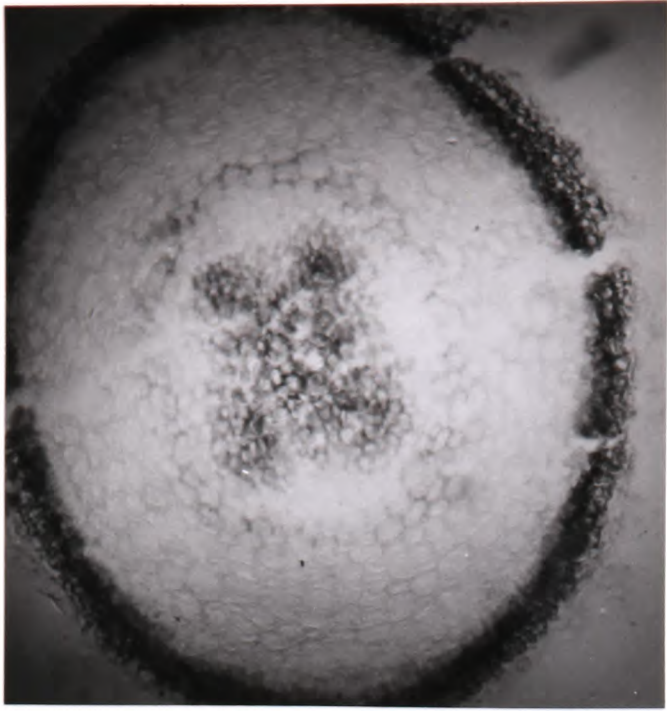


Fig. III.7a)

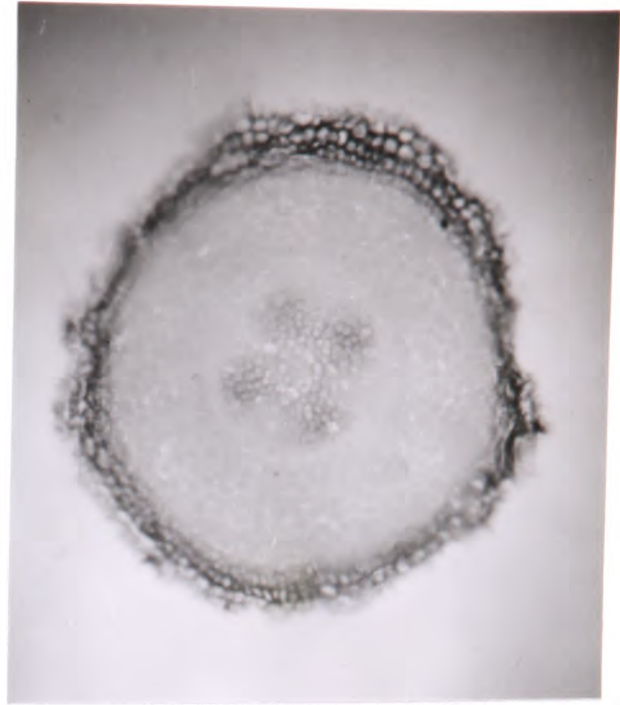


Fig. III.7b)

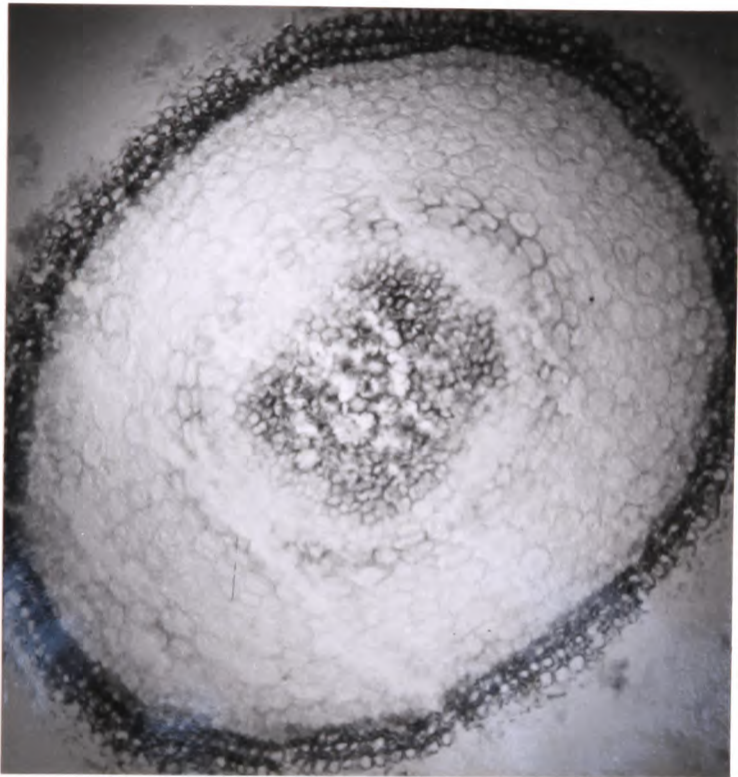


Fig. III.8a)

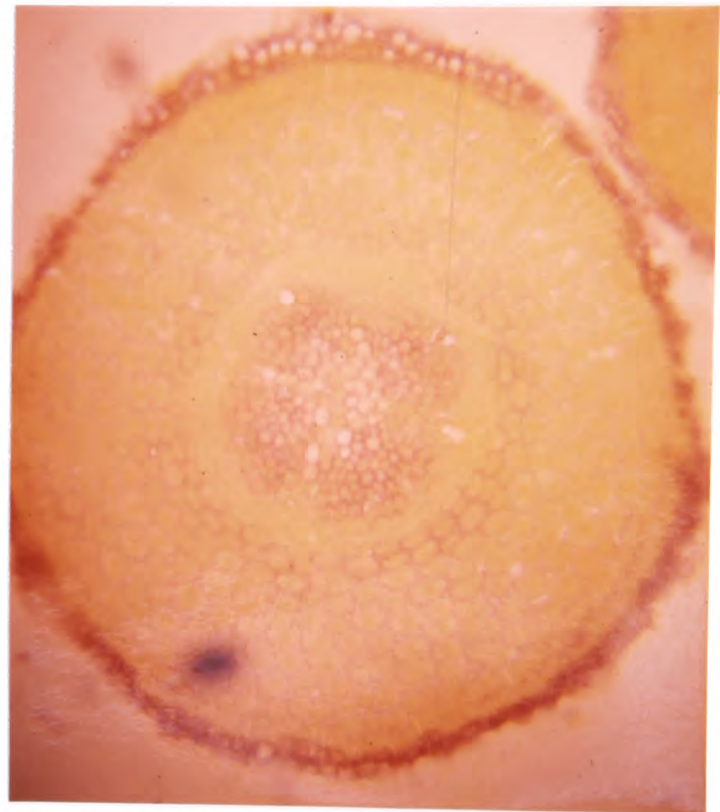


Fig. III.8b)

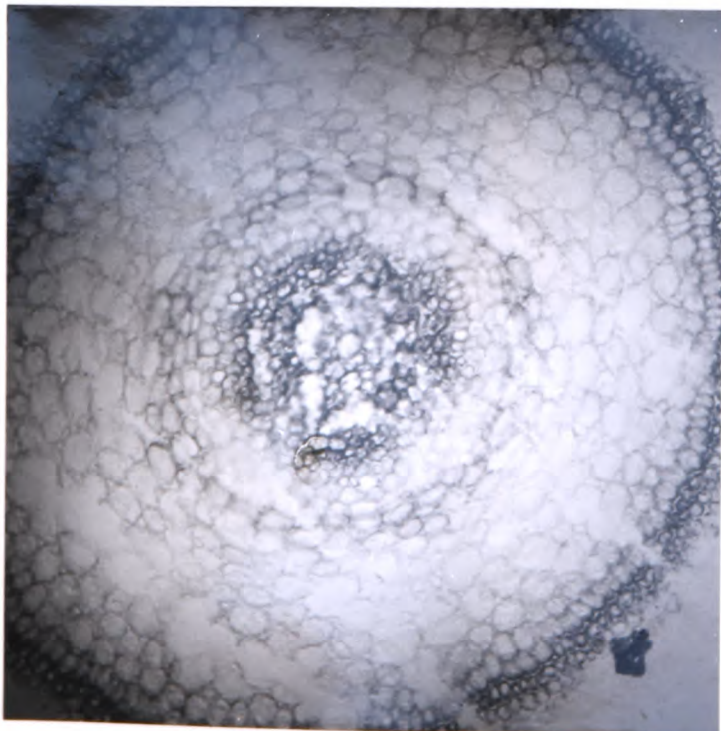


Fig. III.9a)

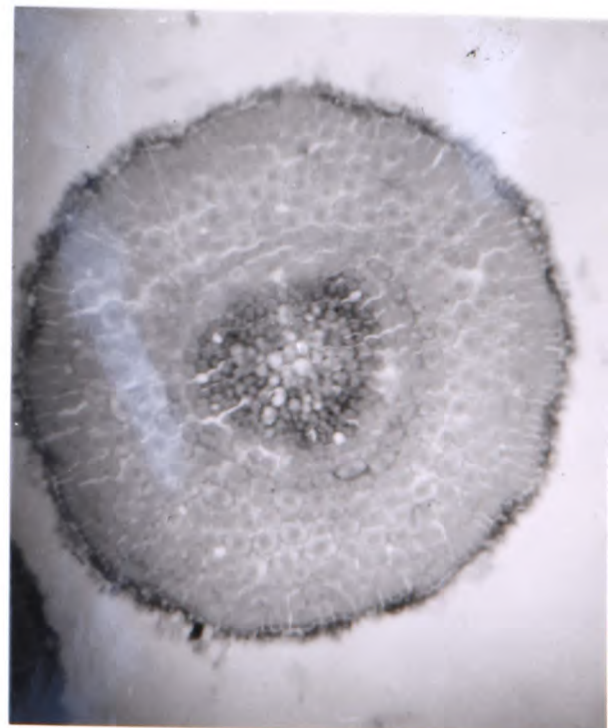


Fig. III.9b)

The esterase end-product in fixed tissues occurred mainly on the longitudinal walls in longitudinal sections and on the tangential walls when seen in transverse sections. The outer tangential wall contained both particulate and diffuse stain (Fig. III. 10.b). The same was true of unfixed tissue, but the effect was less striking (Fig. III. 10.a). The stain for the enzyme in unfixed material, however, was only intense at about 800 μ m from the tip, after which it declined. It was, nevertheless not lost completely, and was evident in sections taken several mm away from the root tips of both unfixed and fixed tissues.

Cortical tissues.

The cortex, throughout its differentiation, stains lightly for enzyme end-product when compared with other tissues. Where the cortical cells had vacuolated extensively, and increased their original size several times, particles could be seen within the cytoplasm adhering to the periphery of the cell. It was rare to see any stain within the area that corresponded to the position of the vacuolar sap, possibly because of the loss of vacuolar material during tissue sectioning and preparation, or simply because it was absent.

It was possible to detect a decrease in the frequency of the particles beyond about 2 mm from the tip in both fixed and unfixed root sections. However, in the region of endogenous lateral root growth, several mm behind the apex, considerable esterase activity was indicated in the neighbouring cortical cells, so that the capacity for synthesis of these enzymes does not appear to be lost during differentiation.

Fig.III.10: Approximately 1500 μ m from the root tip. Endodermoid ring of staining cells is still obvious. Compare states of cortical vacuolation in Fig.III.10b) with Fig.III.11b). Magnifications: Fig.III.10a) x 150, Fig.III.10b) x 120. (XX).

Fig.III.11: Sections approximately 1800 μ m from the root apex. The root cap is no longer present. Vascular tissues of unfixed roots still stain strongly for esterases unlike corresponding fixed tissues at this stage of development. 'Endodermoid ring' still evident. Cortical cells nearly all fully vacuolate. Magnifications: Fig.III.11a) x 150, Fig.III.11b) x 120. (XXIV).

Fig.III.12: 2100 - 2200 μ m from the apex of the root. Endodermoid ring of stain has faded in both unfixed and fixed tissues but note marked difference in staining of the stelar tissues after glutaraldehyde fixation. Magnifications: Fig.III.12a) x 150 Fig.III.12b) x 120. (XXIX).

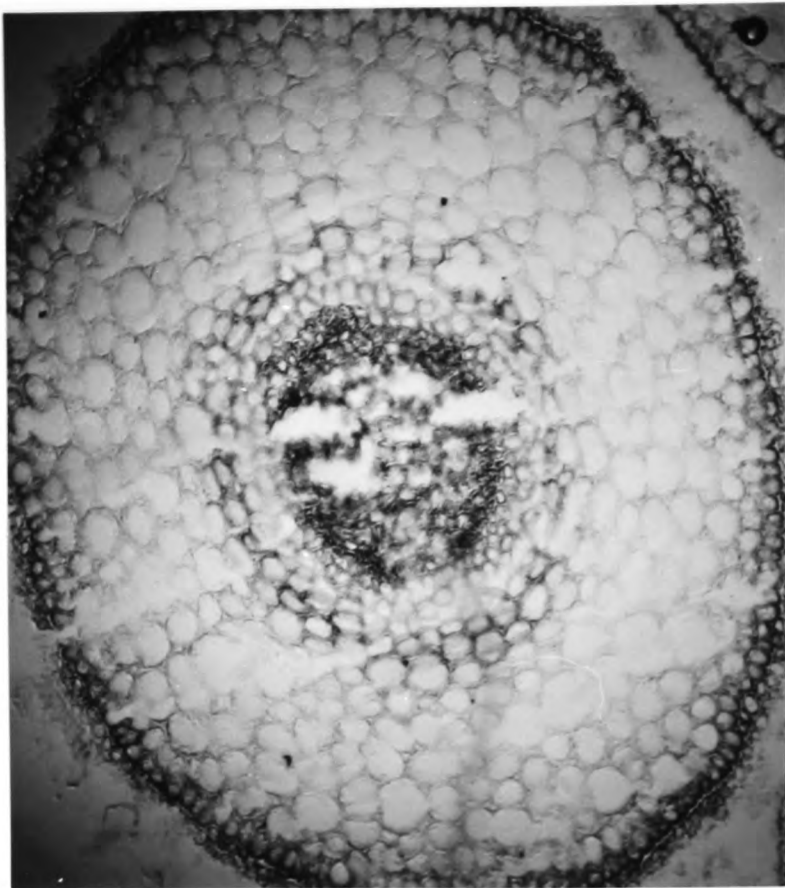


Fig. III.10a)

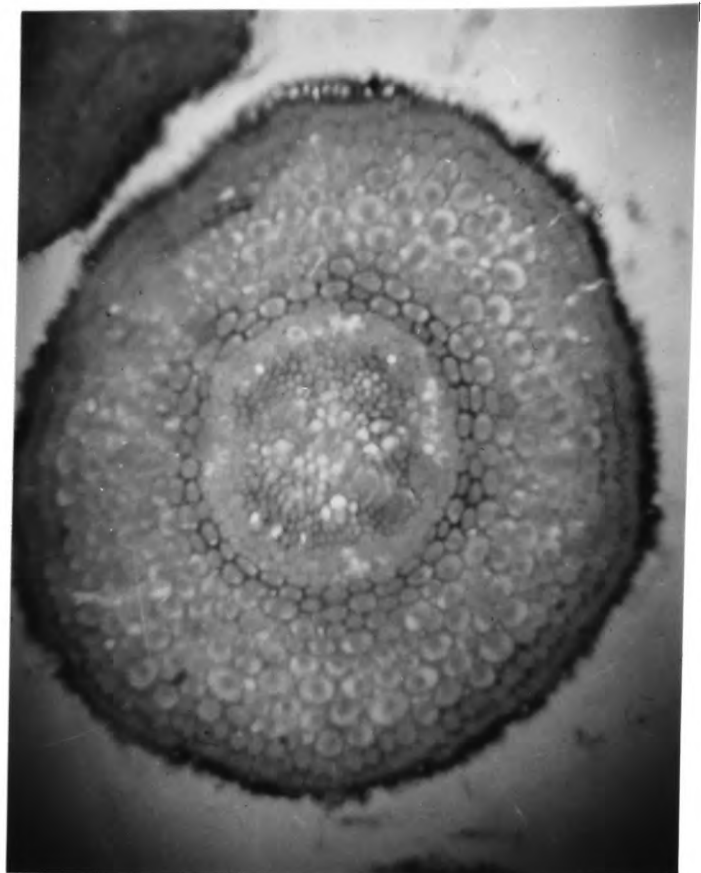


Fig. III.10b)

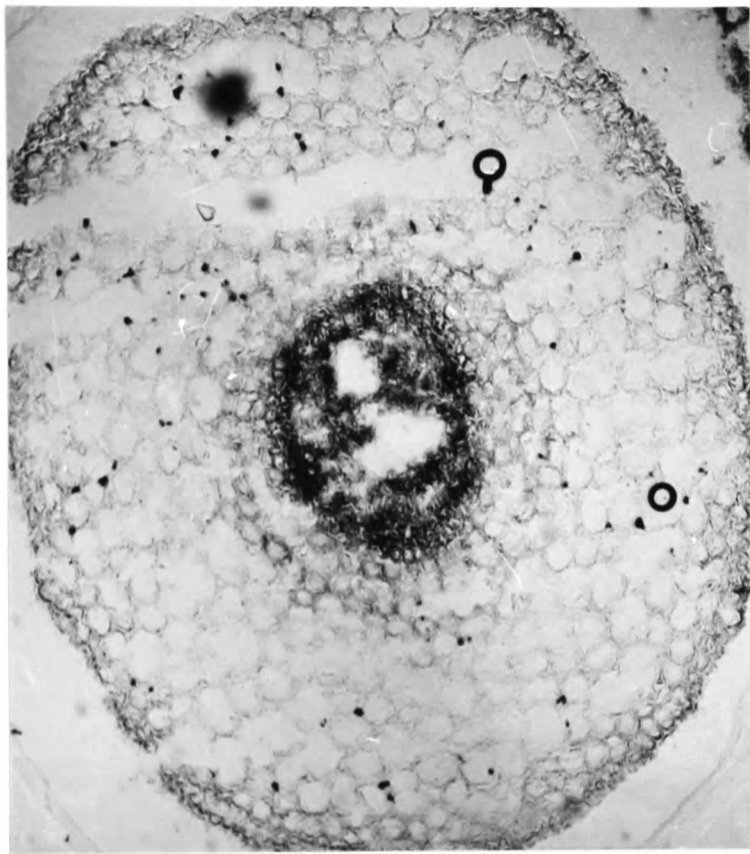


Fig. III.11a)

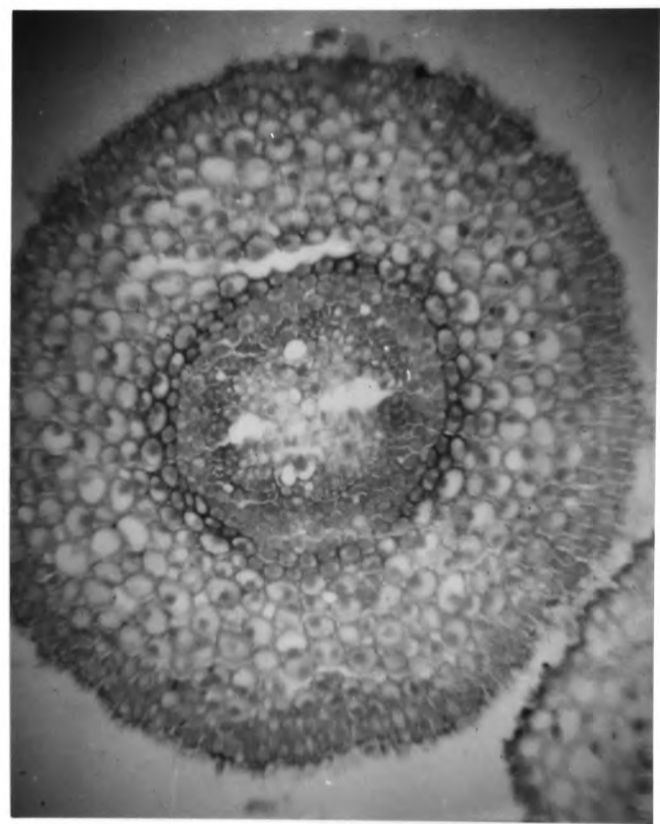


Fig. III.11b)

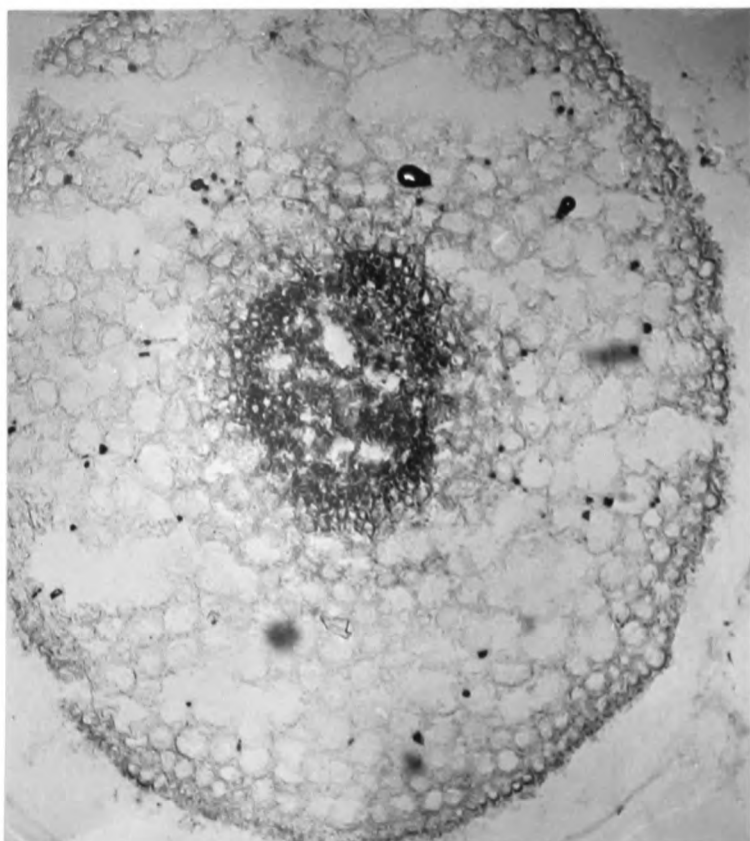


Fig. III.12a)

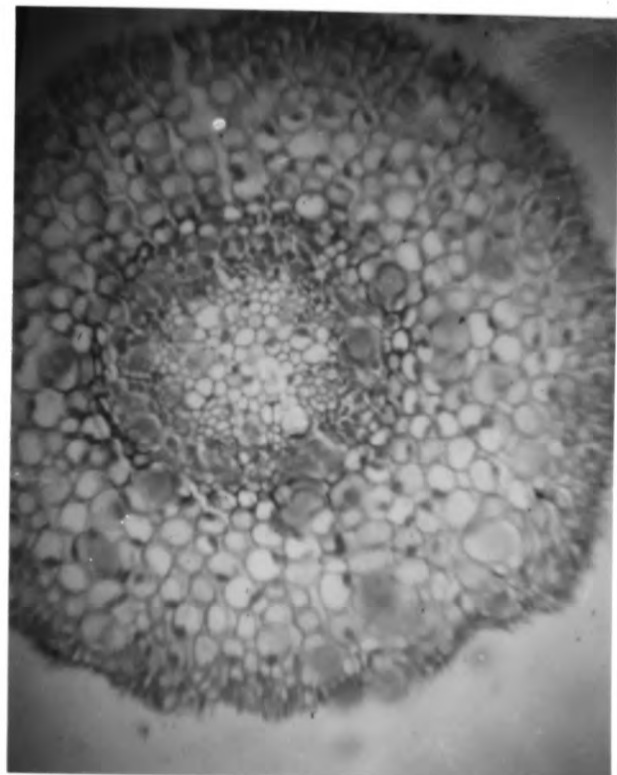


Fig. III.12b)

- Fig.III.13: 2.5-3mm from the root tip. Esterase activity has decreased in unfixed tissue when compared with Fig.III.12a). No tissues of fixed roots show any enzyme staining at this magnification. Magnifications: Fig.III.13a) x150; Fig. III.13b) x120. (XXXIV).
- Fig.III.14: Appearance of no-substrate control section of unfixed root showing absence of activity from all tissues. Approx. 1mm from the root apex. Magnification: x 150.
- Fig.III.15: Longitudinal section of unfixed Vicia faba lateral root showing absence of activity from apical initials (a.p.) and cortical cells but intense particulate activity in the procambium and root cap. Magnification: x260;.
- Fig.III.16: Longitudinal section of fixed root. Intense activity is associated with the tangential walls of procambial cells. Magnification: x 260.
- Fig.III.17: High power magnification of the region 'apical initials' from Fig.III.16. Only particulate activity can be seen. The nuclei are unstained. Magnification: x 640.

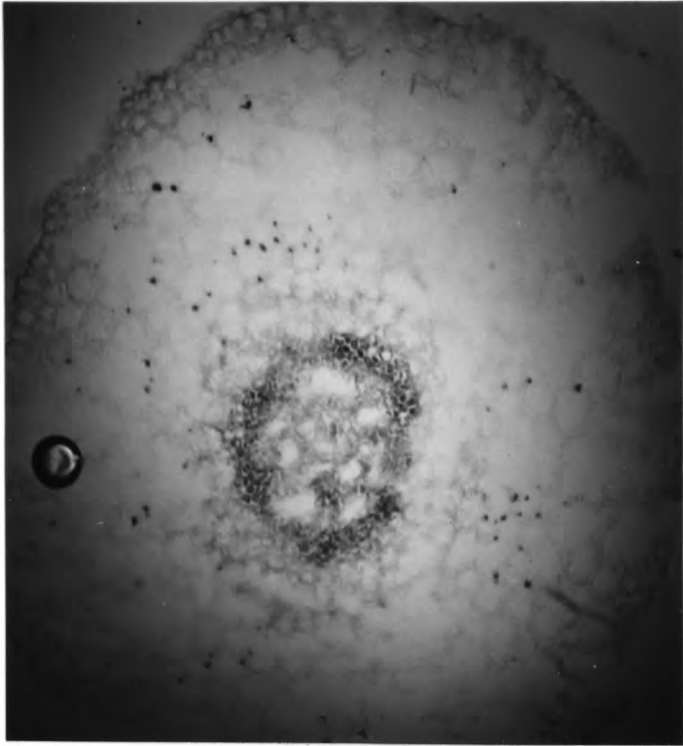


Fig.III.13a)

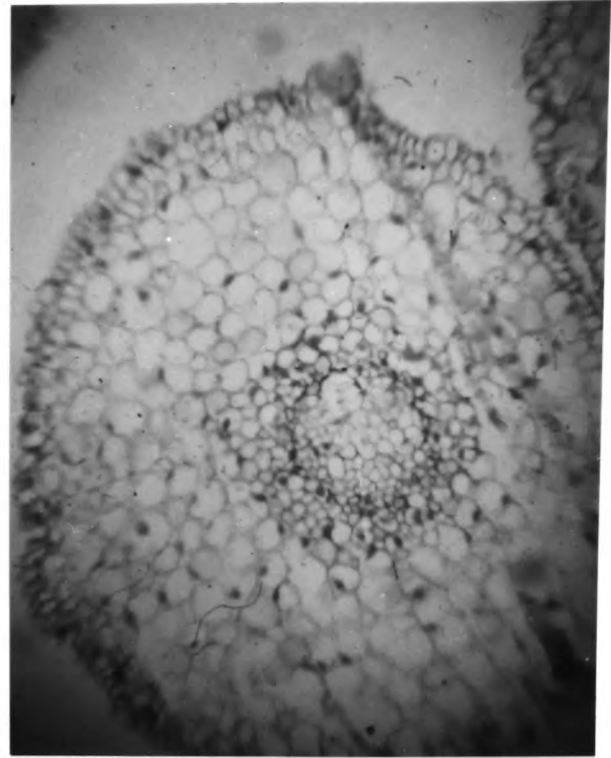


Fig.III.13b)

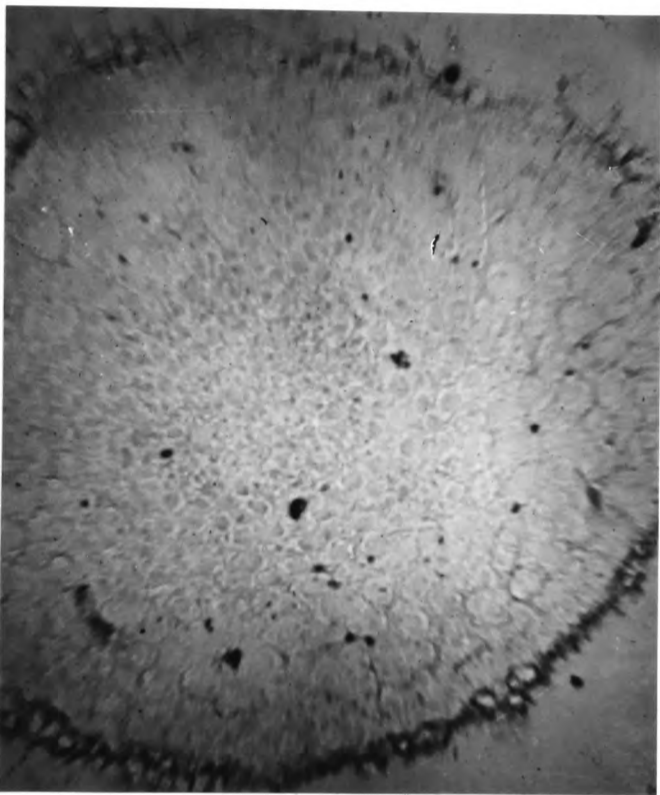


Fig.III.14

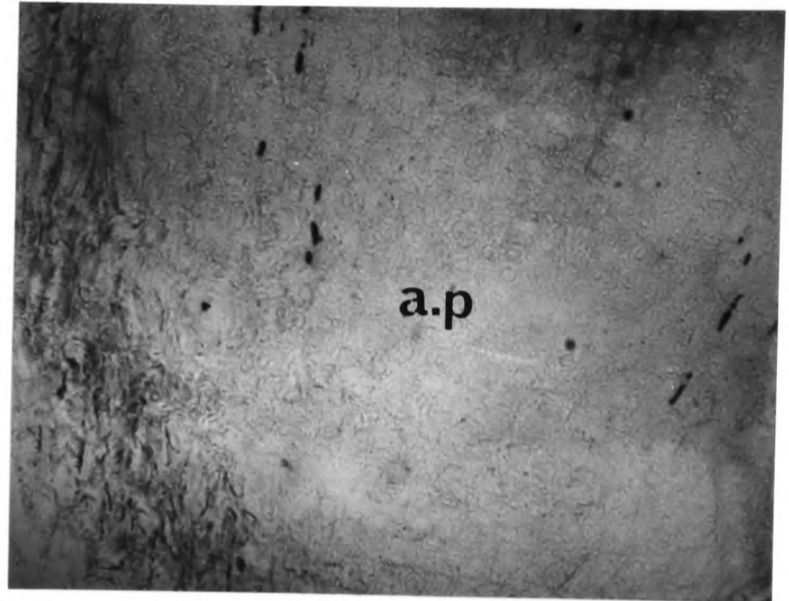


Fig.III.15

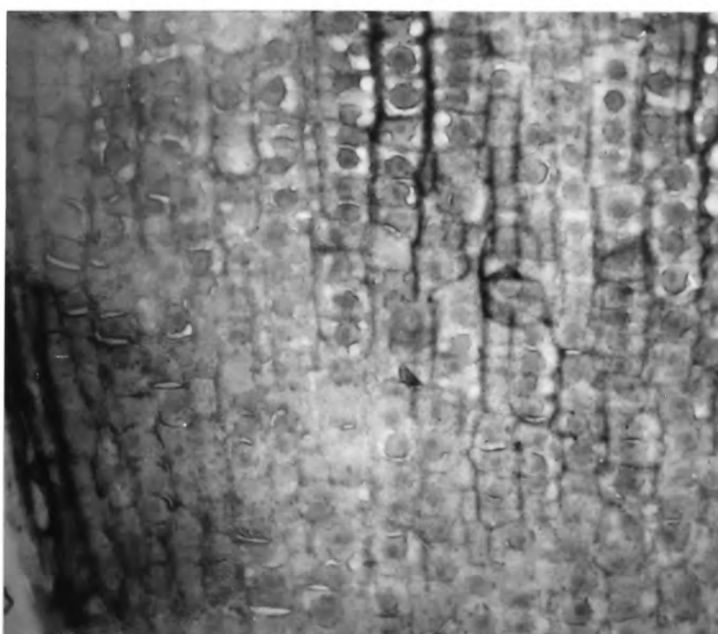


Fig.III.16



Fig.III.17

Fig. III. 18: High power magnification of transverse section of Fig. III. 8b). Enzyme activity is seen associated with stelar tissues (S) and endodermoid cells (E) as both diffuse and particulate forms of azo-dye staining. Magnification: x 640.

Fig. III. 19: Fixed cells of the endodermoid tissues (E) showing the presence of particulate staining only. Cells of the pericycle (pe) also contain enzyme end-product. Magnification: x 1600.

Fig. III. 20:
& III. 21 High power detail of Fig. III. 13a) showing end-product in diffuse form associated with the cell walls of fully differentiated protoxylem tissue (p) and in both particulate and diffuse form in the large central metaxylem elements (M). Magnification: x 390.

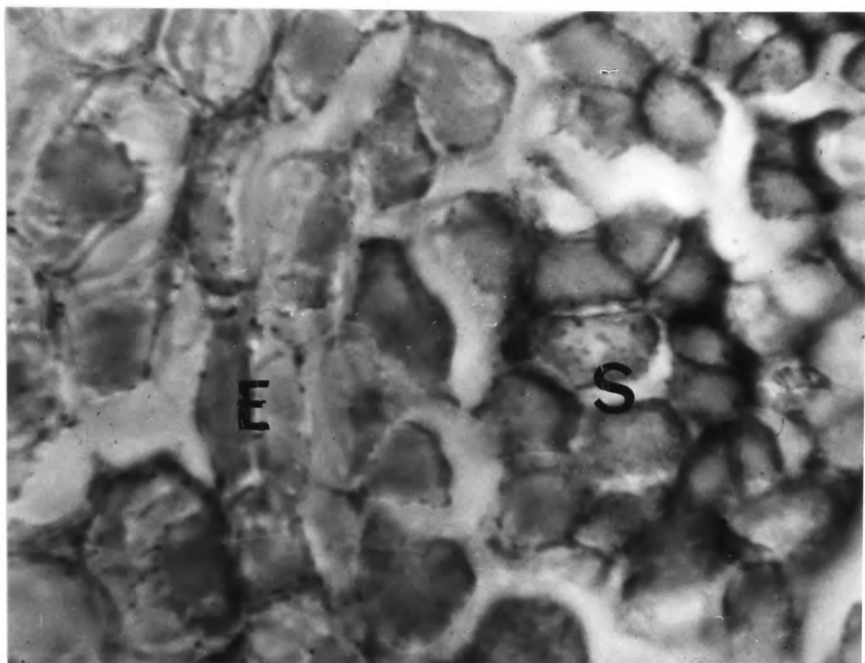


Fig.III.18

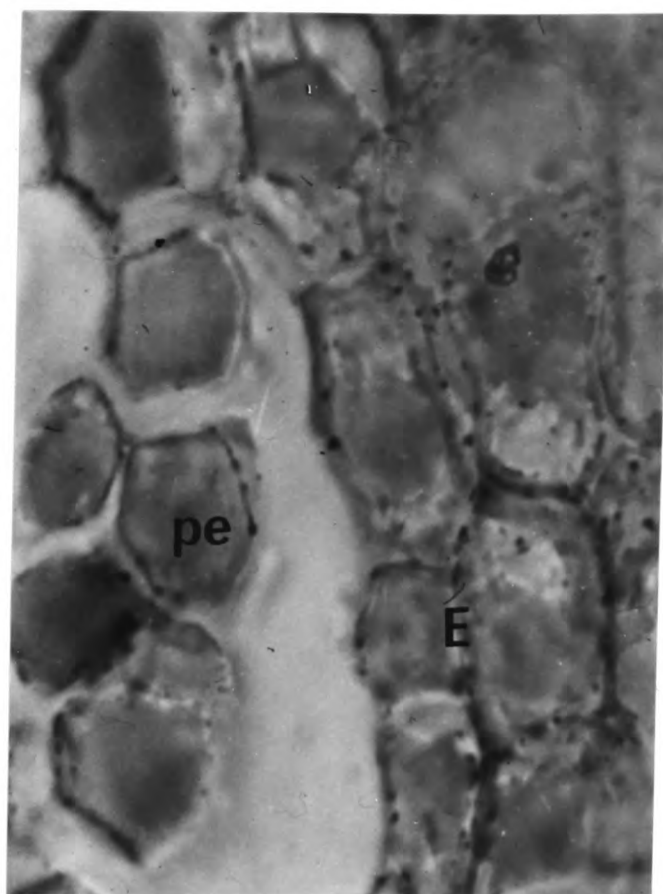


Fig.III.19

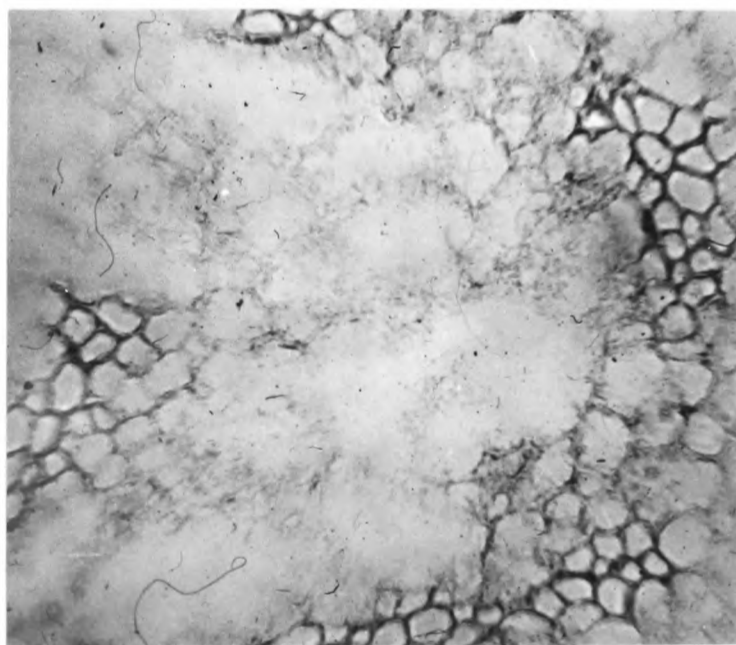


Fig.III.20

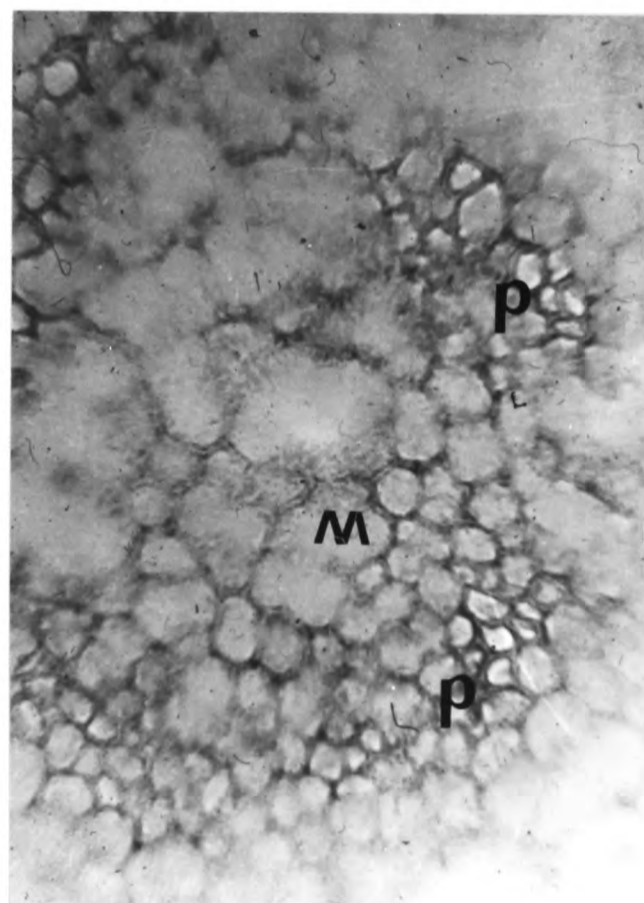


Fig.III.21

Endodermoid Cells.

In the series of photomicrographs shown (Fig. III.4a. to III.13b.) the sudden appearance at about 900 μ m of the ring of staining due to esterase activity outside the vascular tissue and its equally sudden disappearance at about 2,000 μ m in fixed roots, or diminution in unfixed roots, was one of the most characteristic events seen during the differentiation of these enzymes in root tissues. High power microscopical examination of this intense ring of activity in fixed and unfixed material showed both a diffuse and a particulate staining pattern (Figs III.17. and III.18.). It was the innermost cortical layer that was usually the more intensely stained, although all endodermoid cells possessed a similar pattern of activity. The particulate stain was most evident in a peripheral cytoplasmic position and the diffuse form in the walls of the cells.

The significance of changes in enzyme activity in the endodermis has been discussed by Van Fleet (1952, 1961) and further examination of the significance of esterase staining in this tissue is made later.

Pericycle.

In the early stages of cell differentiation (0-700 μ m) the pericycle was conspicuously devoid of enzyme end-product under low power magnification in both fixed and unfixed roots, an observation enhanced by the appearance of an intense ring of esterase stain in the innermost cortical layer (e.g. Figs.III.11.a. and III.11.b.). In longitudinal sections of unfixed tissue the entire vascular cylinder including pericycle tissues, stained intensely for esterase

activity up to about 2 mm from the tip, beyond which the reaction diminished. In contrast, cells of the pericycle in fixed longitudinal sections of the root contained only scattered azo-dye particles.

Vascular Tissue.

i) The Procambial Cells.

Procambial tissue arises in the root some 500 μ m from the tip and is characterised in both unfixed and fixed sections by a heavy deposition of esterase end-product on both longitudinal and transverse walls. In fixed tissue diffuse staining was particularly apparent on the longitudinal walls (Fig. III.16.) although the cytoplasm of both fixed and unfixed cells contains numerous, close-packed azo-dye particles.

At about 700 μ m from the tip enzyme end-product is localised in 4 'arms' (Figs. III.7.a and III.7.b) with the intervening cells almost devoid of end-product. In later stages of development these arms become the protoxylem poles (Figs. III.8.a and III.8.b) and the intervening cells the protophloem poles.

ii) Stelar Tissues.

Between 1000 to 1200 μ m from the root tip transverse sections of tissues showed the beginning of differentiation of the four protoxylem poles (Figs. III.8.a and III.8.b). Esterase activity was very intense in the four arms of the differentiating protoxylem and the pattern was similar for fixed and unfixed roots. Close microscopical examination revealed that particulate and diffuse end-product was associated with cytoplasm and cell walls respectively. In contrast the intervening protophloem poles were almost devoid of stain. By about 1,400 μ m from the tip, there was equal staining throughout the stelar tissues (Figs. III.9.a and

III.9.b.) in the case of unfixed tissue and in all vascular tissues with the exception of the pericycle in fixed material. At a distance of 1.8 mm from the tip early metaxylem elements were obvious in transverse sections and high power microscopical examination showed that particulate esterase end-product was associated with the cell periphery and diffuse end-product with the cell wall, (Fig. III.20 and III.21.).

Serial transverse sections showed that maximal staining intensity was reached in the vascular tissues between 1.0 mm and 1.5 mm from the tip, after which the reaction appeared to weaken. Such changes were not so obvious in longitudinal sections, although even naked-eye observations showed that in unfixed tissue the stain intensity diminished slightly after 2 mm but achieved a constant level for several more millimetres towards the base of the root.

An almost complete ring of diffuse stain was sometimes seen in transverse sections of the stelar tissues, with particulate stain in the central and conspicuously enlarged metaxylem elements alone.

Differentiation of the phloem and xylem tissues continued beyond the 3 mm of root studied but longitudinal sections of fixed and unfixed roots showed that there was no marked change in the level of staining beyond this point. In unfixed roots, mature lignified xylem elements were observed some 4.5 mm from the tip in longitudinal sections and were seen to contain particulate esterase end-product in the unthickened regions of the cell. Scott et al., (1960) have shown that at the ultrastructural level the remains of the cell protoplast lines the mature tracheid. Esterase sites are therefore presumably associated with this layer.

III.3.4. Biochemical Studies.

Sonication of tissue sections from fixed root tips obtained by irradiation for up to 5 min show that it was still possible to observe groups of cells, sometimes 20-30 in number, with intact contents. When unfixed tissues were sonicated, however, no intact cells were observed and only cell fragments were visible.

The graph of esterase release plotted against sonication time for unfixed material yields an approximately asymptotic curve approaching a plateau at about 2 min (Fig. III. 22.). Measurements of total protein released as a function of sonication time showed no detectable differences between 15 and 120 sec of sonication. However, esterase molecules were still being released since the specific activity of the enzymes continued to increase beyond 15 sec. It is probable therefore that the esterases form only a very small percentage of the total protein since their release after 15 sec of sonication did not alter estimates of total protein content.

In addition these data suggest that the enzymes are structurally bound to some component(s) in the cells.

Fig. III. 23. shows the difference observed between the esterolytic activities of fixed and unfixed material when serial root segments from the root tip to approximately 3.4 mm behind it were assayed separately. The three curves for unfixed tissue were obtained from experiments carried out on separate batches of lateral roots. The dates of the experiments are given for reference purposes.

The peak value for esterase activity per whole segment in unfixed roots is almost 8 times that of fixed roots in segment IV. (Fig. III. 23.). In all other segments of unfixed roots esterase

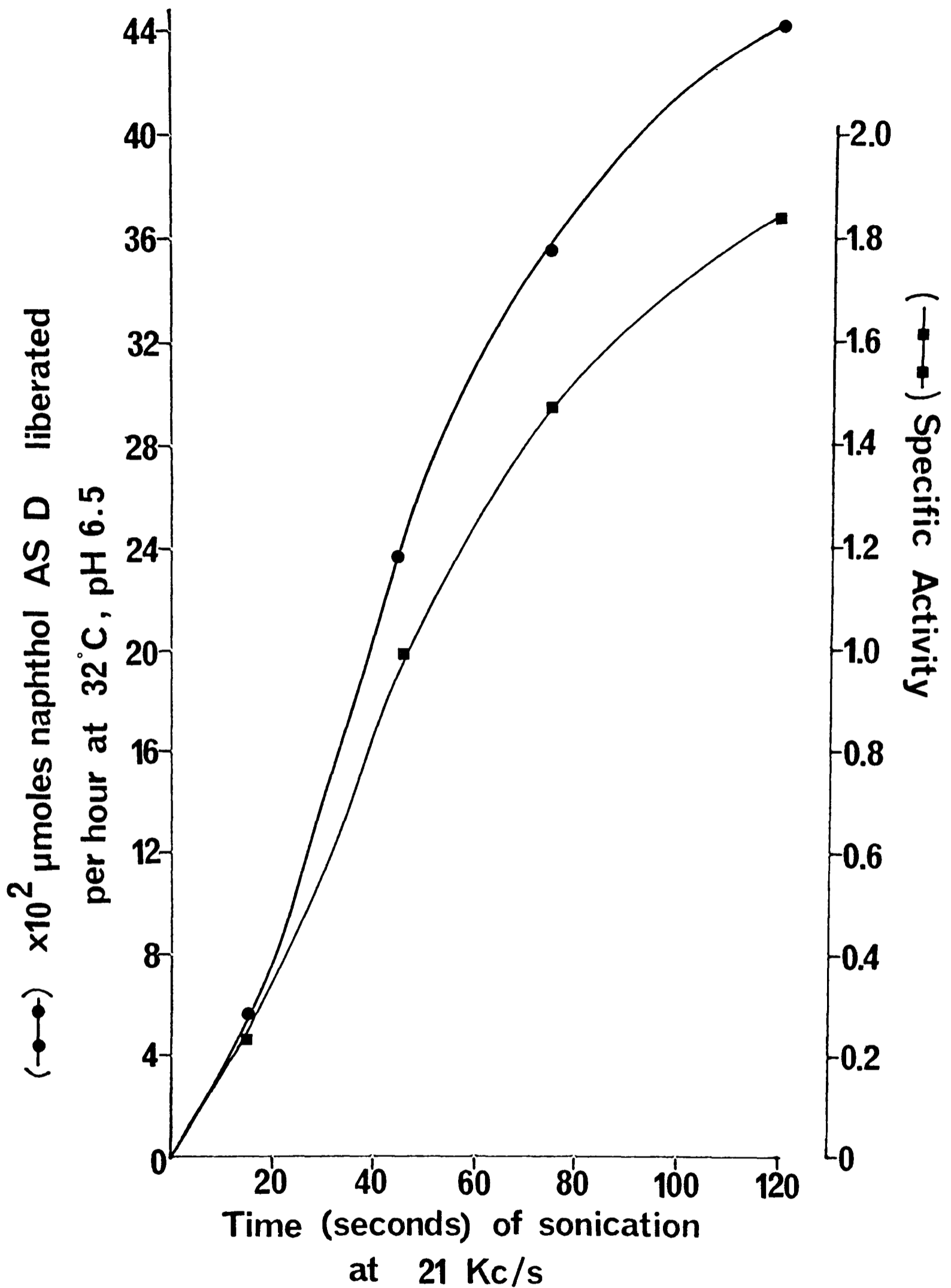


Fig.III.22: The release of esterases from whole 3mm lengths of unfixed lateral roots cut into 20 μm sections, as a function of sonication time. Specific activity(\blacksquare) was determined as ' $\mu\text{moles naphthol AS D liberated per h per mg protein}$ '.

Fig.III.23: Changes in esterase activity per whole root segment in fixed and unfixed roots in relation to tissue differentiation. Each segment contained 120 x 20 μm thick sections except for experiment 22-4-72 where 96 x 20 μm thick sections were used. All sections were sonicated for 2min at 21 Kc/s.

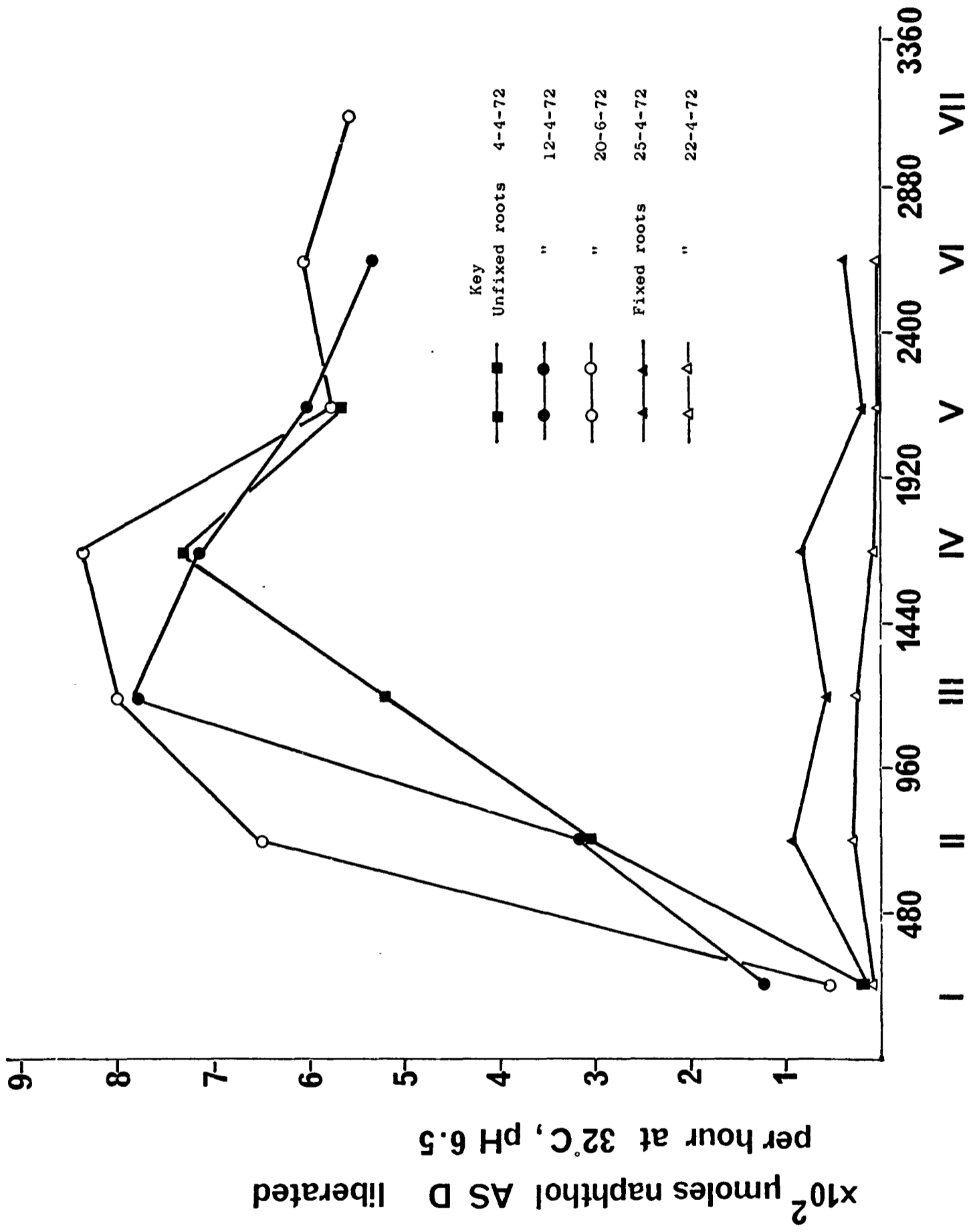


Fig. III.23

activity was also markedly higher than correspondingly fixed segments (with the possible exception of Segment I). In both fixed and unfixed roots sonication was for 2 min but microscopical observations showed that cell-free extracts could not be obtained from fixed roots. Presumably, therefore, the large differences in esterase activity between the two types of root are partly due to the inability of the irradiation treatment to disrupt fixed cells. Other causes may be inactivation of the enzymes by the fixative and the failure of the ultrasonication treatment to release the enzymes from membrane-bound cell organelles. A fuller discussion of these points is given in Section III.4.

The difference in the two curves for fixed tissues is probably a reflection of the total numbers of roots used for sectioning and sonication. In the experiment of 25-4-72, 50 roots were used (the same as that for experiments on unfixed roots), whilst only 40 roots were used in the experiment of 22-4-72.

All 3 curves for unfixed tissue show a similar steep increase in esterase activity up to Segment IV, the zone from 1440-1920 μ m behind the root tip. The next segment, V, showed a sharp decrease in activity and this level was maintained in the final two segments. Means and standard errors of esterase activity were calculated from these results on a) a per whole segment basis, b) a per unit weight of protein basis and c) a per unit area of average-sized section basis, (Fig. III. 24.). The last-mentioned estimation was carried out by photographing all 168 of the 20 μ m sections from a single unfixed root, projecting the negative image on to standard weight paper, drawing round the boundaries of the images, cutting out the areas so circumscribed

Fig.III.24: Changes in esterase activity in relation to cell differentiation in unfixed roots. Activity is expressed on i) per whole root segment basis, ii) per unit protein basis (specific activity) and iii) per unit area of average sized root section. Except where indicated (Table III.6) each point is the mean of 3 determinations with its corresponding standard error.

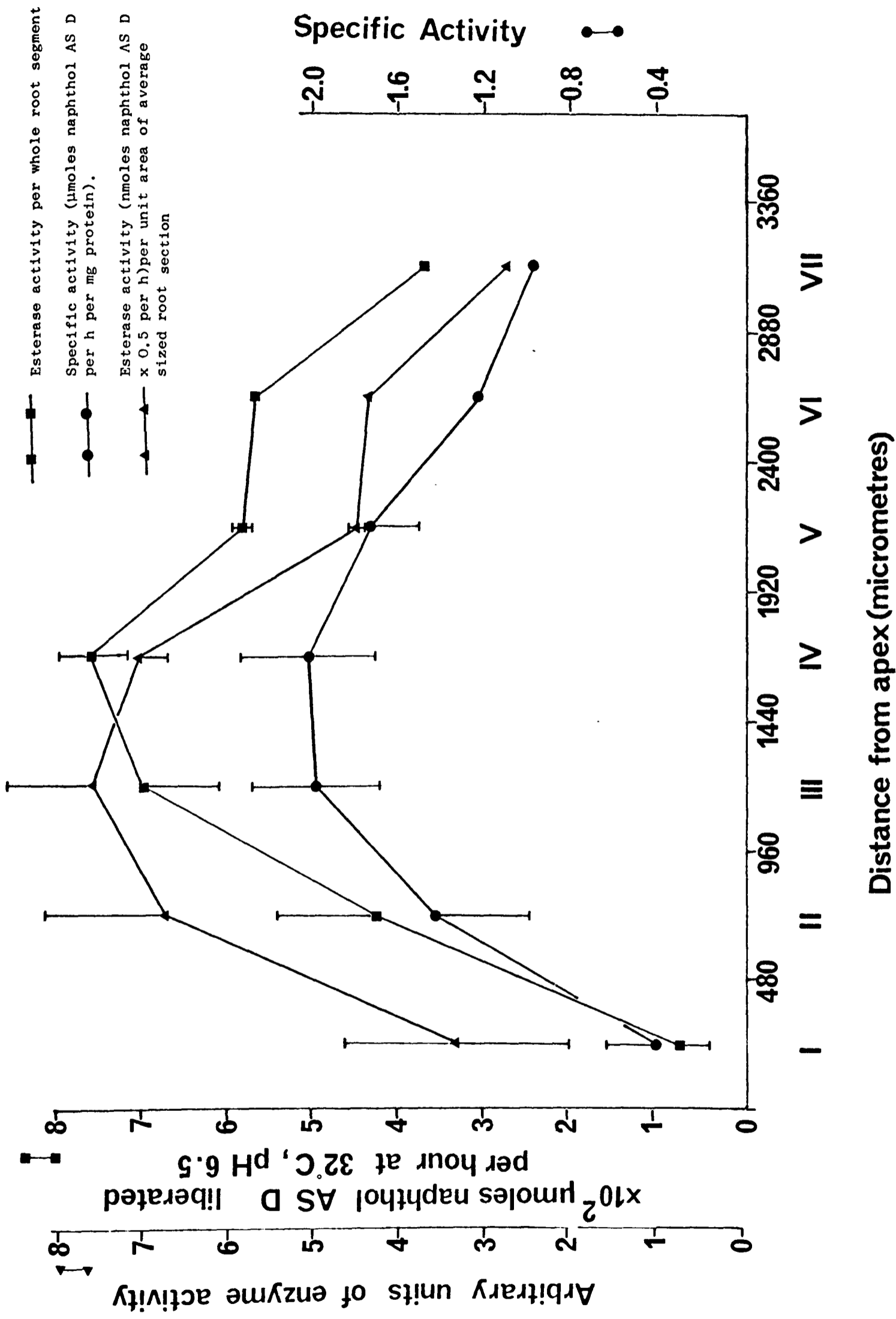


Fig. III. 24

and finally weighing each piece of paper. Thus 120 pieces of paper from each segment were weighed and the 7 means calculated. The relative cross-sectional area of each segment was then calculated by using the smallest value (Segment I) as the divisor. All results are shown in Table III.5.

By comparison of the results expressed graphically in Fig. III. 24 with Figs. III.2 and III.3., it can be seen that the initial peak of activity expressed on a per unit area of average-sized section basis occurs at about $1200\mu\text{m}$ from the root tip (i.e. the mid-section of segment III). Photomicrographs (Figs. III.8a and III.8b.) of this region show that the staining in the ring of endodermoid cells is obvious, stelar tissues also stain intensely as do the 3 or 4 layers of the root cap. Sections in this region therefore appear, at least cytochemically, to contain the maximum amount of esterase activity.

However data expressed on a 'per whole segment' basis show a peak of activity in segment IV between 1440 and $1920\mu\text{m}$ from the tip. Cytochemical staining patterns show that although enzyme activity is intense in the younger cells of this segment (e.g. Figs. III.9a and III.9b) by about $1900\mu\text{m}$ from the root apex, staining of all tissues had diminished, and significantly root cap cells are no longer present. The high level of esterase activity in this segment in biochemical assays may therefore be due to greater cell number. The figures for protein content for this segment are only marginally greater than that for segment III and this factor is unlikely to contribute to the greater activity.

The information from the curve of enzyme activity per unit protein is enlightening since increases in esterase activity between

Table III.5. Esterase activity (mean of 3 determinations and standard error) in different segments of the unfixed root. 50 roots were used for the determination.

Segment	Esterase Activity ^a per whole segment	Protein (g) per whole segment	Specific Activity †	Esterase Activity ^b per unit area of average-sized root section	Relative Cross-sectional area
I	0.66 ± 0.31	168	0.38 ± 0.23	3.30 ± 1.56	1.00
II	4.25 ± 1.13	258	1.43 ± 0.45	6.79 ± 1.79	3.15
III	6.99 ± 0.91	314	1.81 ± 0.29	7.58 ± 0.99	4.61
IV	7.62 ± 0.38	317	2.02 ± 0.31	7.09 ± 0.35	5.41
V	5.83 ± 0.12	277	1.74 ± 0.23	4.49 ± 0.09	6.48
VI	5.68**	252**	1.23**	4.35**	6.52
VII	3.70*	304*	0.97*	2.26*	6.54

** Mean of 2 determinations.

* One determination.

† μ moles naphthol AS D liberated per h per mg protein.

a $\times 10^2$ μ moles naphthol AS D liberated per h.

b nMoles naphthol AS D $\times 0.5$, liberated per h.

segments I and III are not due solely to increases in protein content. Although increases in the cross-sectional area of the root are greatest over the first 3 segments, increases in esterase activity per whole segment are sufficient to give a maximum activity on a per unit area of section basis in segment III, with only a slight decrease in segment IV.

Cell and tissue differentiation beyond about 2 mm from the tip show marked decreases in enzyme activity when expressed on a per whole segment or per unit area of section basis. These decreases coincide with the loss of end-product staining from the endodermoid cells and the diminution of staining intensity in many of the stelar tissues (Figs. III.12.a and III.12.b). Increases in cell volume, cell number and the development of large intercellular spaces, particularly between enlarging procortical cells of segment V, VI and VII mean that the cross-sectional area of the root continues to increase whilst activity per whole segment declines. This must therefore account for the large decrease in activity based on per unit area of section, between segments V, VI and VII.

Total protein per whole segment declines between segments IV and V and this may partly account for the drop in esterase activity per whole segment. Some of the decrease, however, must be due to a real decline in the activity of the enzymes since specific activity also began to fall between segments IV and V.

Although enzyme activity continued to fall in segments V, VI and VII on all three bases it is noticeable that protein content per segment appeared to remain constant, (Table III.5.).

There are good correlations between these results and the cytochemical observations which revealed that many cells had low stain

intensities and had completed their differentiation in segments VI and VII.

III.3.5. A note on enzyme activity expressed on a per cell basis.

When measuring changes in enzyme activity in differentiating roots data expressed on a per cell basis are usually regarded as the most reliable since increases in activity over the meristematic zone of the root may merely reflect the greater number of cells in the more mature sections of tissue. Unfortunately cell counts using the chromic-acid maceration technique (Brown and Rickless, 1949) were impracticable in the present work because of the small size of the sections (20 μ m thick). A 20 μ m thick transverse section will at most contain a layer of 3 cells, the outermost 2 of which will probably be ruptured by the sectioning process. When the cells are macerated in chromic acid, therefore, the percentage of whole cells will be extremely small whilst the numbers of cell fragments will be very high. Little reliability could therefore be placed on cell counts made on such sections.

Because of this limitation in the present technique it is not possible to determine the relative changes in enzyme activity per cell during the differentiation of the various cell types.

III.4.

DISCUSSION

These studies have shown that a close relationship exists between changes in the levels of non-specific esterases and the processes of differentiation in the terminal 3 mm of young root tips of Vicia faba.

A most striking feature of the study has been the close agreement between cytochemical observations and the biochemical estimations of non-specific esterase activity.

The rise in activity in the region 0-1200 μ m of the root tip is not merely a reflection of increases in overall protein content or increases in cross-sectional area of the root, although the possibility that it is due to greater cell number per segment has to be considered. The cytochemical studies show that within this region enzyme activity is particularly intense in the procambial tissue and particularly with that part of it which later differentiates into the protoxylem cells. Activity in the root cap cells of the first millimetre or so of root tip is also intense, consequently making it difficult to assess the contribution of this tissue to the overall enzyme activity in biochemical assays. It is noticeable, however, that in the two segments containing the highest esterase activity, III and IV, the root cap is restricted to a single layer of cells and its expected contribution to the total esterase activity in these segments would be small.

The ring of staining associated with the endodermoid cells in segments III and IV is a rather puzzling feature of this study but verifies the claim of Van Fleet (1961) that a large number of hydrolytic enzymes, including esterases, are present at the boundary of cortex and stele, where, he maintains they are involved in

producing fatty oils that are later used in the formation of the Casparian strip.

It may be taken then that the rise in the levels of these enzymes is associated mainly with the differentiation of the vascular tissues and with the protoxylem in particular. The esterases, appearing in cells at discrete particulate sites, are most active in cells that are elongating, vacuolating and, in their later stages of differentiation, undergoing gross structural changes, involving in the latter case the controlled autolysis of their components.

Matile and Winkenbach (1971) have proposed that during the growth of the vacuole in plant cells undergoing cytoplasmic autolysis, parts of the cytoplasm are sequestered into the vacuole where they are then digested by several acid hydrolases. Biochemical assay of the same tissues showed that activity of several enzymes increased markedly whilst these processes were going on. In the case of differentiating protoxylem we have a comparable situation since large numbers of subcellular organelles are lost during the differentiation of these cells and in the present study the process is matched by increases in non-specific esterase activity.

Moreover very large scale structural changes in these cells such as the removal of the end-walls of adjacent protoxylem cells would seem to require a variety of hydrolytic enzymes to effect the process. McLean (1970) has in fact observed intense acid phosphatase and esterase activity at these sites. Other workers (Sutcliffe and Sexton, 1969; Benes and Opatrna, 1964) have also shown cytochemically that differentiating xylem stains intensely for acid hydrolases.

The present cytochemical studies have also shown that phloem cells contain high esterase activity. In addition McLean (1970) has shown that acid phosphatase activity is intense in differentiating protophloem cells and is particularly associated with the sieve pores of forming sieve plates in the later stages of their differentiation. Phloem cells, like xylem cells, also undergo gross structural changes at the subcellular level, although complete autolysis of the cytoplasm does not occur. It must be borne in mind, however, that other cells of the differentiating stele do not undergo such marked structural cytoplasmic changes but still have a high level of staining with esterase end-product. The evidence for the involvement of esterases, and other acid hydrolases, in these specific subcellular phenomena is therefore only circumstantial and it is more likely that they are involved in the whole process of cellular differentiation. This is emphasised by the fact that the decline in esterase activity after 2 mm from the apex coincides with the completion of the differentiation process for many of the cells of the primary tissue, although it was interesting to observe that fully differentiated cells several millimetres away from the root tip still contained particulate activity. Presumably, therefore other peaks in esterase activity may have been recorded if the study had been extended to include say the terminal 10 or even 15 mm of the root.

In this connection it is interesting that Robinson and Brown (1952) working with Vicia faba roots have recorded peaks of activity for a number of enzymes, including acid phosphatase on a per cell basis at about 8 mm from the tip. Sutcliffe and Sexton (1969) also found this for invertase activity in pea roots.

However, these groups of workers invariably used much thicker sections of tissue and studied changes in enzyme levels over greater lengths of the root than in the present study, thus making comparison between the two sets of results rather difficult. Nonetheless, Robinson and Brown did report that acid phosphatase activity was higher in the first mm of the root tip on a per cell basis than in the subsequent two one mm sections. Plotting the activity of the enzymes on a per section basis however showed that maximum activity was recorded in the first 2 mm, a result also recorded by Sutcliffe and Sexton (1969) for β -glycerophosphatase in pea roots, and for non-specific esterase in the present work.

Whilst expressing results of enzyme activity on a per unit area of section basis in studies of cellular differentiation yields useful information when compared with data expressed in other ways it is usually a poor guide to changes in enzyme levels since changes in its dimensions can be due to changes in cell number, cell volume and increases in size of the intercellular spaces between the cells. It is unfortunate therefore that it was not possible to present data on a per cell basis, although the work of Brown and Broadbent (1951) on pea roots may indicate the relative changes in cell number expected during root differentiation, since they made cell counts on comparable sized segments (400 μ m thick) as those used in the present study. These investigators showed that cell counts per segment were at a maximum between 800 and 1200 μ m from the tip, whereafter there was a progressive decline to about 5 mm from the tip. On the basis of this evidence it is conceivable that on a per cell basis esterase activity may have been at a maximum in older segments of the broad bean root, possibly segments VI or VII or even older uninvestigated segments of the root.

Other physiological and biochemical parameters such as respiration rate, RNA content, DNA content were not measured in relation to cell differentiation although many investigators have done so in the past for Vicia (Robinson and Brown, 1952; Jensen, 1955, 1958; Holmes et al., 1954) and other roots (Brown and Broadbent, 1951; Heyes and Brown, 1956; Sunderland and McCleish, 1961; McCleish and Sunderland, 1961). In future investigations the use of the present methods would permit the detection of small changes in the quantity of any of these parameters, changes that may otherwise be missed when thicker segments of tissue are used.

Sahulka and Benes, (1969), when using polyacrylamide gel electrophoresis to detect changes in the isoenzyme populations of non-specific esterases during cell differentiation in the terminal 10 mm of Vicia faba roots, used extracts from only 3 segments of the root i.e. those corresponding to cell division, enlargement, and maturation. Using the substrate naphthol AS acetate they found no marked differences between electrophoreograms from the 3 zones. If extracts from sonicated, smaller segments of tissues had been used, clearer differences between electrophoreograms from the 3 zones may have been visible, as the biochemical data from this work suggest.

Presumably all previous recorded serial sectioning techniques involving studies of root cell differentiation, have been performed at room temperatures. It is quite likely, however, that controlled low temperature freeze-sectioning produces less shrinkage and expansion of the tissues during the cutting process than similar work carried out at higher uncontrolled room temperatures. During the course of cutting many sections over an

extended period of time this factor may assume considerable importance in reducing the sometimes, large variation in results reported in work of this sort, (Robinson and Brown, 1952).

In fixed roots only about one-eighth of the activity obtained from unfixed material could be detected in biochemical assays, whereas apparently comparable levels could be detected cytochemically. It is well known that the activity of a number of enzymes is reduced by glutaraldehyde (see Hopwood, 1972, for review) although non-specific esterases have not been assessed in this respect. Different staining patterns in unfixed and fixed root sections are discernable at about 2 mm from the root tip, where esterase end-product in the stelar tissues of fixed roots is very much reduced when compared to corresponding unfixed root sections. Some enzyme losses must therefore be due to glutaraldehyde fixation. Other factors are also likely to have produced the observed differences in the biochemical assays for fixed and unfixed roots.

Probably most important of these is a different sonication effect on the two types of tissues. This is almost certain to be the case since, for fixed roots, cell-free extracts could not be obtained in the time of sonication given. Also Nachlas et al., (1956) have shown that fixation of tissue sections decreases the amount of 'lyo' - or readily-diffusible form of the enzymes - lost to the surrounding medium. This would further reduce the amount of esterase released into the buffer in fixed tissue sections, and help to account for the very low levels of the enzymes in sonicated extracts. Furthermore Ellar et al., (1971) showed that glutaraldehyde-treated membranes of Micrococcus lysodeikticus contain more protein

than untreated ones after polyacrylamide gel electrophoresis, whilst Hopwood (1969) obtained similar results with glutaraldehyde-fixed liver slices. In the latter case unfixed control slices lost significant amounts of their proteins during electrophoresis as evidenced by gel staining.

Further investigations are necessary, therefore, to determine the length of sonication time necessary to produce cell-free extracts of glutaraldehyde-fixed tissues containing levels of esterase activity, comparable with those detected in unfixed roots.

Other useful information on the subcellular origin of esterases has been gained using ultrasonication as a method of disrupting cells and tissues. Most protein is released very quickly (within 15 sec) by sonication of unfixed tissue sections at 21 Kc/s, but esterases continue to be released up to 2 min as evidenced by continuing increases in their specific activity with time of sonication given. Since the cytochemical studies show the enzymes to be present in small discrete particles, which electron microscope evidence suggests are small vacuoles (Chapter V), it is reasonable to suppose that the enzymes are rather tightly bound to some membranous component of the cells.

CHAPTER IV

A CYTOCHEMICAL AND BIOCHEMICAL STUDY OF NON-
SPECIFIC ESTERASES DURING SOFT FRUIT RIPENING

IV.1.

INTRODUCTION

A number of acid hydrolases, many of them known to be lysosomal in animal cells, have been shown to increase in activity during the ripening of a variety of fruits (see review by Dilley, 1970). It is possible therefore that one of the major causes of the softening of fruits as ripening and senescence proceed is the controlled synthesis or activation of enzymes concerned in the degradation of the structural components of cells and tissues.

In these circumstances, acid hydrolases, perhaps residing in discrete cytoplasmic structures such as vacuoles (Matile, 1969) may play a significant role in the autolysis and hydrolysis of macromolecular cell constituents as fruits ripen and the fleshy tissues finally soften and senesce.

Often the terms 'ripening' and 'senescence' are used synonymously but in the present work some definition of the two terms was thought necessary. Ripening may simply be defined as the series of changes in fruit colour, flavour and texture that lead to a state in which the fruit is edible and ripe (e.g. Rhodes, 1970). Clearly, then, the selection of fruit at the different stages of ripening in the present work is an arbitrary procedure since it is not based on objective measurements of physiological parameters such as the respiration rate, as is frequently done to determine the climacteric in fruits (Biale 1960, 1964).

Unlike ripening, senescence may not manifest itself visually, although textural and structural changes in the consistency of the fruit flesh may indicate that the organ is approaching death.

For this reason plant senescence is a more difficult concept to define when it refers either to individual plant organs

or whole plants. However, a useful definition, relating to individual organs, has been offered by Woolhouse (1967) who states that it is "a series of apparently programmed cell and tissue changes that lead to the death of that particular organ". In the context of the present work it may therefore be best regarded as representing the terminal stages of ripening. Consequently the point at which ripening ends and senescence begins is extremely difficult to define and it is possible that the two processes overlap.

Other authors have associated senescence with such events as the protoplasmic disintegration of fruit cells (Bain and Mercer 1964), changes in cell permeability (Sacher, 1966, 1967), organelle vesiculation, and the loss of ribosomes (Butler and Simon, 1971). In view of the known functions of lysosomal acid hydrolases in tissue regression and cell death in animal cells (de Duve and Wattiaux, 1966), ripening and senescence of fruits may represent a comparable situation in plant cells for the activation and/or synthesis of acid hydrolases.

Biochemical studies of acid hydrolases during fruit ripening are numerous but they have rarely, if ever, been carried out in association with cytochemical studies. The nearest approach has been that of Clements (1965, 1966, 1970) who used the techniques of gel electrophoresis and enzyme histochemical staining to show changes in the iso-enzyme patterns of a number of acid hydrolases, including non-specific esterases, during the ripening of several fruits.

Biochemical assays of fruit extracts have shown that several acid hydrolases increase in activity with ripening.

Notable examples of these are the results of Rhodes and Wooltorton (1967) who detected increases in acid phosphatase and ribonuclease as soon as the respiration climacteric of apple fruits was reached. In addition Maris McArthur Hesse (1956) showed an increase in amylases and phosphorylases during pear maturation as did Mattoo and Modi (1969) in mango and Biale (1964) in banana.

With this in mind it was the initial intention of this work to carry out light microscope cytochemical investigations of the subcellular localisation of non-specific esterases in the pulp tissues of a variety of soft fruits during the ripening process. Using cytochemical techniques it was hoped to determine whether the enzymes were particle-bound throughout the ripening stages or whether they were released into the cytoplasm at a critical stage, thereby bringing about widespread cell death and consequent softening of the tissues. Cytochemical and biochemical work on potato tuber cells (Pitt and Coombes, 1968, 1969) has already shown that the release of the hydrolytic enzymes, acid phosphatase and non-specific esterase, from the particle-bound state into the cytoplasm can occur, although in this case it was observed only in tissue infected with Phytophthora erythroseptica.

Of the three soft fruits examined initially (viz., raspberry, blackberry and strawberry) the strawberry was singled out for further biochemical and ultrastructural study since preliminary work suggested that in the ripening tissue esterase activity might be contained in distinct subcellular structures. For cytochemical work 3 ripening stages were chosen from each of the 3 fruits. In both the strawberry and the raspberry these covered the change in flesh colour from green to red and in the blackberry from red to black.

It is here relevant to add that as the strawberry receptacle is not a true fruit an examination of the involvement of non-specific esterases during its ripening process entails a study of changes in fleshy tissues that are of a completely different embryological origin from those in true fruits, although in all soft fruits the succulent tissues are parenchymatous.

At present knowledge concerning enzyme changes in developing strawberry receptacles is sparse and whilst a few workers have made attempts to investigate physical and chemical changes during ripening (Wade, 1964; Neal, 1965), only Gizis (1964) has been concerned with enzyme studies. Even here the enzymes chosen, pectinesterases and polygalacturonases, were not studied with reference to ripening, emphasis being laid on the techniques of isolation and characterisation.

The intention of the biochemical investigations of non-specific esterases in strawberry ripening was to measure the esterolytic activity from pulp extracts of the 3 ripening stages - fully-formed green or green-white immature berries, white-red maturing berries and red, ripe berries, and to express the results on a per fruit, per g fresh weight and per unit protein basis. (To complete the study it would have been preferable to carry out cytochemical and biochemical studies on obviously overripe fruit, but this could not be done since the berries were obtained in bulk from a commercial source, and the likelihood of damage and infection of very soft fruits during harvesting and transport was too great to merit experimentation).

This approach allowed the biochemical estimates of esterase activity to be correlated with cytochemical findings relating to

changes in the subcellular localisation of esterase activity and with cytological changes at the ultra-structural level.

In addition, changes in the Michaelis constants and pH optima of the different enzyme extracts were to be monitored during ripening and related to the biochemical data.

To provide a more complete picture of strawberry receptacle maturation 2 much younger stages of berry development were chosen for light microscope cytochemical studies in the final season (1972) in order to complement those of the 3 later ripening stages that had been examined in the previous 3 seasons. In this way the ontogeny of the esterases could be recorded throughout the post-fertilisation development of the strawberry receptacle. Biochemical studies of these two youngest stages of receptacle development could not be carried out because of the practical difficulty of separating achenes from the very small receptacles, a procedure thought necessary because of the likelihood that programmed enzyme changes do not proceed simultaneously in both structures. Only when berries had reached a certain handling size could the achenes be prised off without unduly disorganising the tissue underneath.

Although it was decided from the outset, in both the cytochemical and biochemical studies to concentrate attention on enzyme changes during the development of the receptacle it was accepted that the growth of the receptacle is fundamentally influenced by the developing achenes (Nitsch, 1950, 1955). Therefore, for comparative purposes only, some preliminary cytochemical and biochemical studies were performed on achenes taken from the 3 ripening stages of receptacle development.

Since the present work deals with non-specific esterases, the possibility needed to be examined that pectin methyl esterase (PME) can hydrolyse the histochemical substrate, naphthol AS D acetate. McLean and Gahan (1970) believed this to be a distinct possibility since they observed esterase end-product staining in plant cell walls when using this substrate in cytochemical studies, an observation endorsed by the findings described in Chapter III. During the course of the present work, therefore, the suggestion of McLean and Gahan was tested in relation to the investigation of fruit ripening, a process in which changes in the activity of pectic enzymes may play a critical role because large increases in cell size and accompanying changes in tissue texture are probably associated with alterations in the physico-chemical properties of the pectinous middle lamella. In this connection Neal, (1965) has shown that, as strawberries ripen, the middle lamellae of the cortical parenchyma cells split and other workers have shown that pectin methyl esterase activity increases several fold as tomatoes ripen and change colour from green to red (Hobson, 1963, 1964; Markarkis, 1969).

IV.2. CYTOCHEMISTRY

MATERIALS AND METHODS.

IV.2.1. Growth and selection of fruits.

Strawberry.

All fruits grown for this work were of the variety Cambridge Vigour. Post-fertilisation receptacles, representing various stages in development, were selected by the criteria shown in Table IV.1. Very young fruits, stages A and B, for the cytochemical work only, were selected on the basis of receptacle size. Cell-size measurements of stages A, B, and C were also made after the initial selection to characterise further each stage of ripening, since overall berry size is mainly dependent on cell size (Havis, 1943) and such data may prove useful for reference purposes to future investigators. Those receptacles that were used for both cytochemical and biochemical studies, however, were selected on a mean fresh weight basis. The procedure was as follows:-

Between 70 and 100 berries from each stage of ripening (C, D and E) were weighed individually after removal of the calyx, care being taken not to damage the pulp.

From the assembled data the means, the standard error of the means and the standard deviations (with 95% confidence limits) were calculated for each stage of ripening (Table IV.1.). This was carried out each season from 1969 to 1972 even though biochemical work was not started until 1971. The data shown in Table IV.1. as an example, are those of season 1969. The standard deviations (with 95% confidence limits) showed that there was a good deal of

Table IV.1. Criteria adopted for the selection of the different ripening stages of the post-fertilisation receptacle

Stage of receptacle development	Receptacle size (mm)		Mean fresh weight (g) +S.E.M	Standard deviation (95% confidence limits)	Appearance of fruit
	h* Height	w* Width			
A	2.5	1.7	—	—	Hard, green
B	5.0	3.2	—	—	Hard, green
C (Immature)	—	—	5.70 [±] 0.37 (94)	3.68	Flesh colour green or green- white. No red pigment
D (Maturing)	—	—	8.37 [±] 0.26 (72)	4.35	Skin of receptacles estimated as possessing 20-80% red colouration. Tissues firm.
E (Mature)	—	—	10.84 [±] 0.23 (83)	4.13	Uniformly coloured red receptacles. Flesh firm to soft but not overripe.

h* maximum height was measured from the apex of the receptacle through the mid-section to the nearest basal vascular bundle (see Fig.IV.1.)

w* maximum width through the mid-section

* 1969 data. In later seasons mean fresh weight did not vary significantly from the values given above.

Figures in brackets denote the number of berries used in calculations of the means and standard error of the means and of the standard deviations.

variation in berry weight at each of the stages of ripening.

Because only 3 berries from each ripening stage were needed for cytochemical work, it was possible to choose them within the range of berry weight covered by the standard errors of the mean. However for biochemical work this was not always possible since within the individual batches supplied (see below) only inconveniently small numbers of the berries came within this range.

The procedure in 1971 and 1972 for biochemical work therefore was to choose the desired number of berries as close to the mean as possible.

For convenience each stage of fruit development was given a letter as indicated in Table IV.1., which is used throughout this Chapter.

Berries from stages C, D and E (i.e. the ripening stages) were obtained from a local farm (Halsted, nr. Sevenoaks, Kent) where they were grown under commercial conditions, whilst fruits at stages A and B were from plants grown on a plot of garden soil at Thames Polytechnic, and initially obtained from W.G. Hardy,* New Romney, Kent.

A programme of the cytochemical and biochemical work on strawberry receptacles undertaken for each season is given in Table IV.2.

Raspberry. (Rubus idaeus)

Fruits from 3 ripening stages, corresponding to stages C, D and E of strawberry development were selected on the basis of colour differences i.e.

* Stocks approved as pathogen-free by Ministry of Agriculture, Fisheries and Food.

Table IV.2. A programme of the cytochemical and biochemical studies of strawberry receptacles over 4 seasons.

Stage of receptacle development	Type of study	No. of receptacles examined cyto-chemically each season.	No. of seasons examined	Years
A	Cytochemical	3	1	1972
B	Cytochemical	3	1	1972
C (Immature)	Cytochemical	3	4	1969-1972
	Biochemical	-	2	1971, 1972
D (Maturing)	Cytochemical	3	4	1969-1972
	Biochemical	-	2	1971, 1972
E (Mature)	Cytochemical	3	4	1969-1972
	Biochemical	-	2	1971-1972

- a) Green, unripe drupelets. Fruit hard.
- b) Green-red, part-ripe drupelets. Fruit firm and the skin surface possessing between 20-80% red colouration. (assessed visually).
- c) Red, ripe drupelets. Fruits uniformly red with the flesh firm and not overripe.

Cytochemical localisation studies were extended to the receptacle tissues for comparison with the observations on strawberry fruits. 2 drupelets from each of 3 raspberry fruits were examined during one season only - 1969. A total of 3 receptacles from each of the stages a), b) and c) were examined cytochemically.

All the raspberry fruits were obtained from the same commercial source as the strawberries.

Blackberries. (Rubus fruticosus)

Ripening stages were again selected on the basis of colour differences only.

- a) Unripe. Drupelets red and firm.
- b) Part-ripe. Skin of drupelets estimated visually as possessing between 20-80% black colouration.
- c) Ripe. Drupelets black and shiny. Flesh soft but not overripe.

The source of fruit was the same as that for strawberries and raspberries.

2 drupelets from each of 3 fruits were used for cytochemical investigation in one year only - 1969.

IV.2.2. Measurements of cell size in strawberry receptacles.

Cell size measurements were made on a Zeiss microscope using an eye piece graticule and a stage micrometer on sections that had been frozen according to the method of Gahan et al., (1967). This procedure was used throughout for the freezing of all fruit material for cytochemical investigation.

Measurements were taken from the cortex of the strawberry receptacle at the point of maximum girth. The maximum length and breadth of each cell was recorded and the mean and standard error calculated for each series of measurements (Table IV.3.). The cortex was chosen since it has previously been shown that cell division in the pith tissues of strawberry receptacles may proceed throughout maturation whereas it ceases before anthesis in the cortex (Havis, 1943).

It was only possible to measure cell sizes in stages A, B and C of receptacle development since in unfixed frozen sections of stages D and E the cells and tissues disintegrated after freezing and sectioning.

IV.2.3. Tissue preparations for the cytochemical localisation of non-specific esterases.

Strawberry.

Fruits from stages A and B were small enough to be frozen whole on the block holder. (Achenes could not be removed at this stage of development). For stages C, D and E achenes were first removed with forceps and a cork borer then used to take 1 cm diameter tissue cores at right angles to the main axis and at the point of maximum girth. When sections of achenes were required

Table IV.3. Measurements of average cell size cortical parenchyma from strawberry receptacles taken from the 1972 crop.

Stage of receptacle development	Cell size* of cortical parenchyma (Mean \pm S.E.M.)	
	Length (μm)	Width (μm)
	A	41.1 \pm 1.7
B	59.9 \pm 5.3	38.2 \pm 1.7
C	109.5 \pm 6.9	73.6 \pm 3.6
D	-	-
E	-	-

* Cell size measurements based on 10 observations.

for comparison it was found best to leave them attached to the receptacle and to freeze both structures simultaneously.

Fruits from stages A and B were frozen sideways on to the block holder to enable a sequential series of sections to be cut through the long axis. For stages C, D and E, tissue cores were first cut in half transversely (since they were inconveniently long otherwise), and each portion frozen in turn, with the long axis of the original fruit at right angles to the face of the block holder.

In some cases where good preservation of cell structure was the ultimate aim, small pieces of receptacle tissue from stages C, D and E were first fixed in 2.5% glutaraldehyde as described in Section I.2.2.1.1. Fruits at stages A and B did not need such treatment, the cells being sufficiently small and containing enough cytoplasm per unit of cell volume to remain intact during the sectioning process. Disruption occurred frequently in older receptacles where the cells were highly vacuolate.

10 to 20 μ m thick sections of fresh or fixed strawberry material were cut in a Bright's cryostat at -20°C to -25°C . Sections were picked up from the cold knife by bringing a warm slide or coverslip close to the cold section and allowing the section to 'jump the gap'.

Sections were allowed to dry in air at room temperature before incubating for non-specific esterases.

Raspberries.

Drupelets could be cleanly removed from the receptacle by plucking with a pair of forceps. The drupelets were then

mounted in pairs either upright or on their sides on the cryostat chuck for freeze-sectioning. Before preparing receptacles for longitudinal sectioning in the cryostat, all drupelets were removed. Details of the sectioning process were the same as those given for strawberry.

Blackberries.

Although drupelets could be removed from the receptacle of this fruit, the receptacle's tissues were often damaged in the process and so were not used further.

Once removed the drupelets were mounted and sectioned in the way described for raspberry.

IV.2.4. Enzyme localisation procedure for non-specific esterases.

In all the fruits, this was carried out using the conventional azo-dye method of Burstone (1962), with naphthol AS D acetate as substrate and fast red violet LB salt as the coupling reagent. The details of the procedure have already been described (see III.2.7.).

For all fruits and tissues the range of incubation times was 8-30 min and control material was incubated in the absence of substrate. Following incubation, sections of tissues were rinsed briefly in distilled water and mounted directly in Farrant's medium. Sections for light microscopy were viewed with bright field and phase contrast optics.

IV.2.5. Photomicrography.

Colour transparencies of strawberry tissue were made at magnifications ranging from x38 to x 1000 on an Agfachrome 50L film using a Zeiss microscope with bright field optics.

IV.3.

RESULTS. - (CYTOCHEMISTRY)

At all stages of strawberry and raspberry development a common pattern of tissue arrangement was discernible. The receptacle consisted of a central pith region, encased in a cylinder of vascular traces from which emerged branches that traversed the cortical region and connected with the base of each achene in the case of the strawberry, (Fig. IV.1. and IV.2.) and with the base of each drupelet in the raspberry. Entire sections of blackberry receptacles could not be prepared for the reasons already stated.

IV.3.1. Strawberry.

Stage A receptacles.

Esterase activity was confined largely to the cells of the pith. The reaction product occurred either as particles 0.5-1.0 μ m in diameter, or as a diffuse red colouration. The particles were most often seen in the thin layer of cytoplasm adhering to the cell wall (Fig. IV.4.), but not in the wall itself.

(Colour photomicrographs of Fig. IV.3. - IV.17. are not as clear as the original transparencies from which they were printed. For this reason the transparencies are provided in an envelope at the back of the top copy of the thesis).

A small proportion of cells showed diffuse azo-dye staining in large membranous bodies within the cells, (Fig. IV.3.). The largest of these bodies measured 30 μ m in diameter, but the usual size was less than half this value. The most obvious feature of these bodies was their angularity and the lack of uniformity in shape from cell to cell.

FIGS.IV.1. - IV.18: Photomicrographs of strawberry receptacles.

Abbreviations:

- a = achene
- a.p = amyloplast or starch-containing body
- b = membranous cell inclusion with esterase staining
- c = cortex of receptacle
- d = druse
- E = embryonic tissue (cotyledon)
- e.p = particle with esterase activity (0.5 - 1.0 μ m)
- f₁f₂ = files of cells with particulate esterase activity
- N = nucellus
- n = nucleus
- p = pith of receptacle
- p.s = procambial strand
- R = receptacle tissue
- T = testa
- v = vesicle containing esterase activity
- v' = vesicle devoid of esterase activity
- vt = vascular trace

Fig.IV.1: Longitudinal section through young post-fertilisation receptacle(Stage A). Magnification: x 48.

Fig.IV.2: High power magnification of Fig.IV.1 showing esterase activity in vascular traces (vt) entering young achenes (a). Magnification: x 122.

Fig.IV.3: High power detail of pith region of receptacle showing membranous body (b) staining heavily for esterases (Stage A receptacle). Magnification: x 768.

Fig.IV.4: High power detail of cells from the pith showing presence of nuclei (n) with esterase activity associated with a membranous body (b) and occurring in a thin layer of cytoplasm, in particulate form (e.p). Magnification: x 1920.



Fig.IV.1

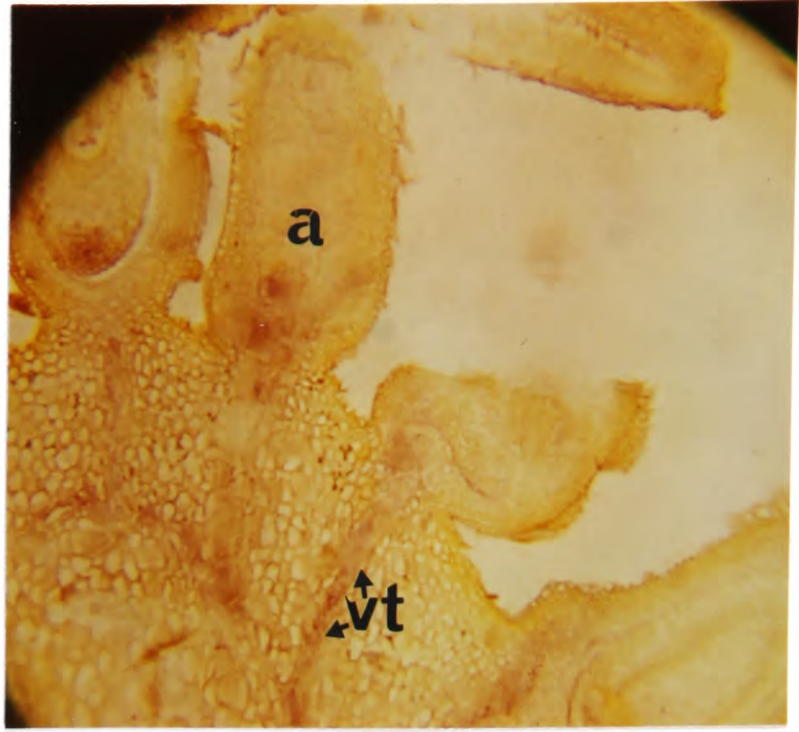


Fig.IV.2

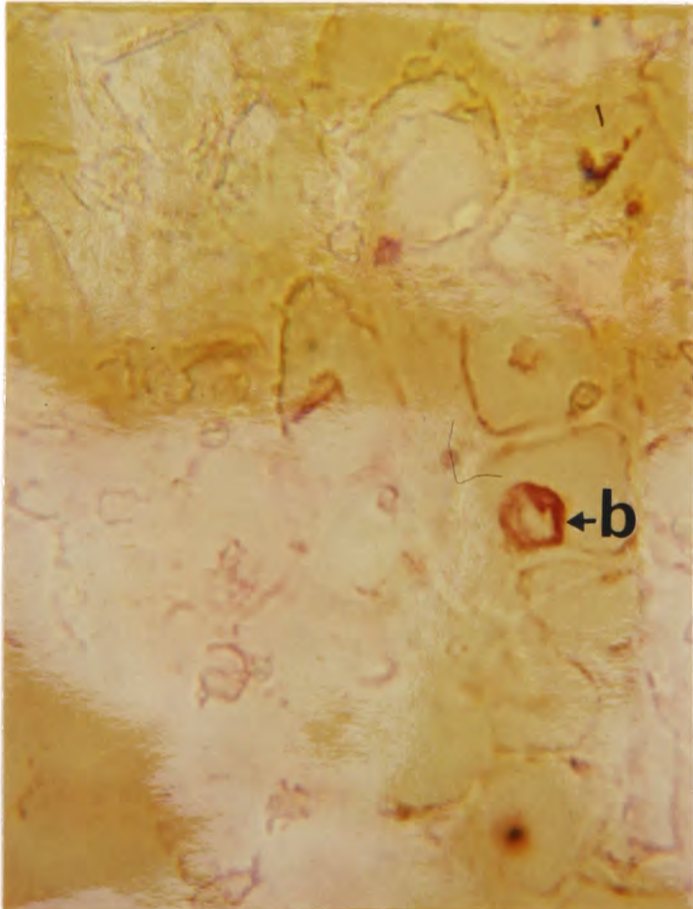


Fig.IV.3

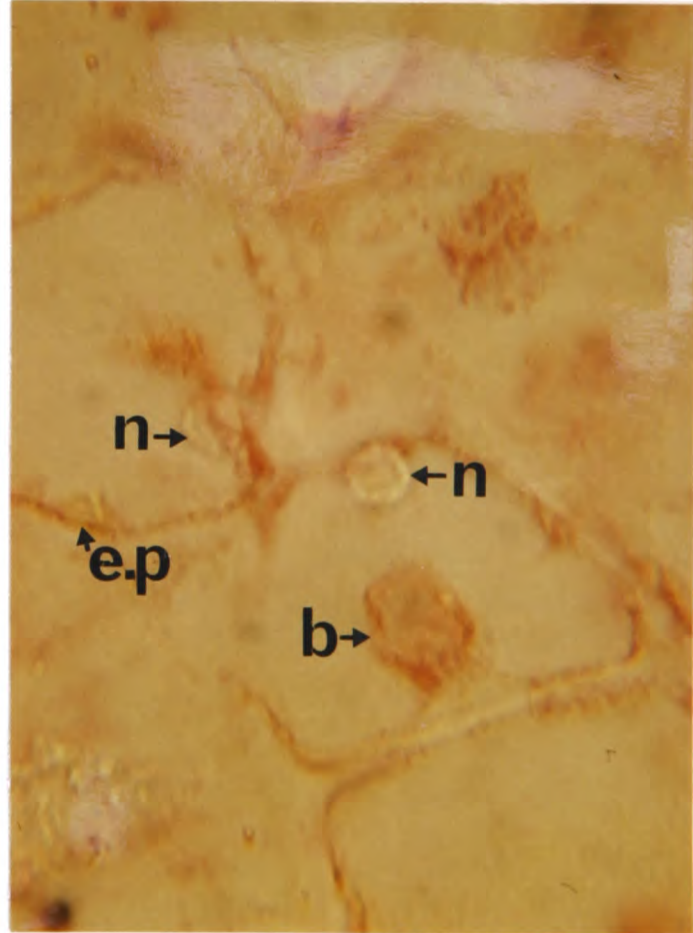


Fig.IV.4

Fig.IV.5: Pith of young receptacle (Stage A) showing esterase end-product in particulate form and associated with several membranous bodies (b). Large arrows denote the long axis of the receptacle. Magnification: x 307.

Fig.IV.6: Pith of young receptacle (Stage A). Particulate form of the azo-dye is intense in two adjacent files of cells ($f_1 f_2$). Magnification: x 307.

Fig.IV.7: Section of stage A receptacle incubated without substrate. No staining for esterase activity can be seen. The vascular trace (vt) separates the pith (right) from the cortex (left). Magnification: x 307.

Fig.IV.8: Stage B receptacle. Membranous bodies (b) are evident within pith cells and stain diffusely with azo-dye. Magnification: x 307.

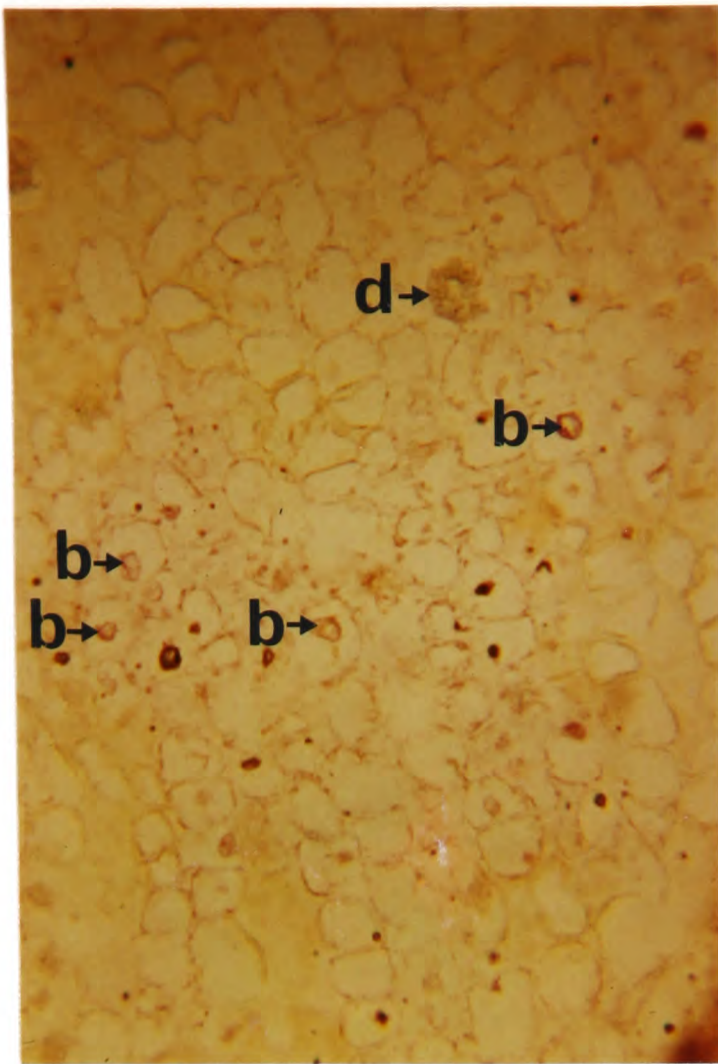


Fig.IV.5

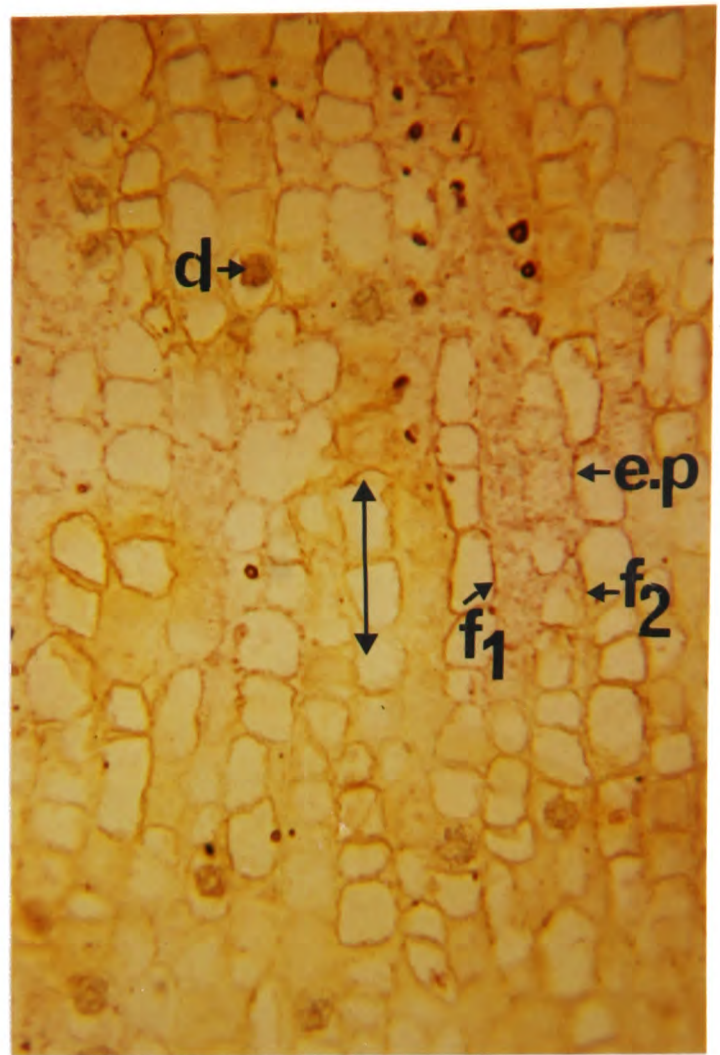


Fig.IV.6

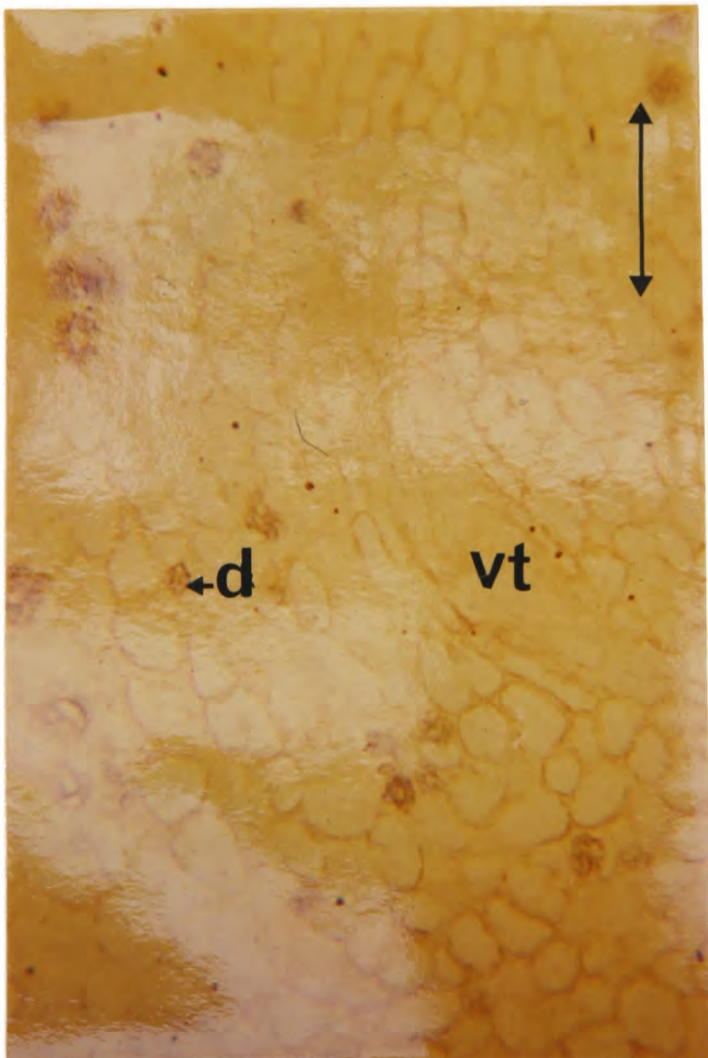


Fig.IV.7

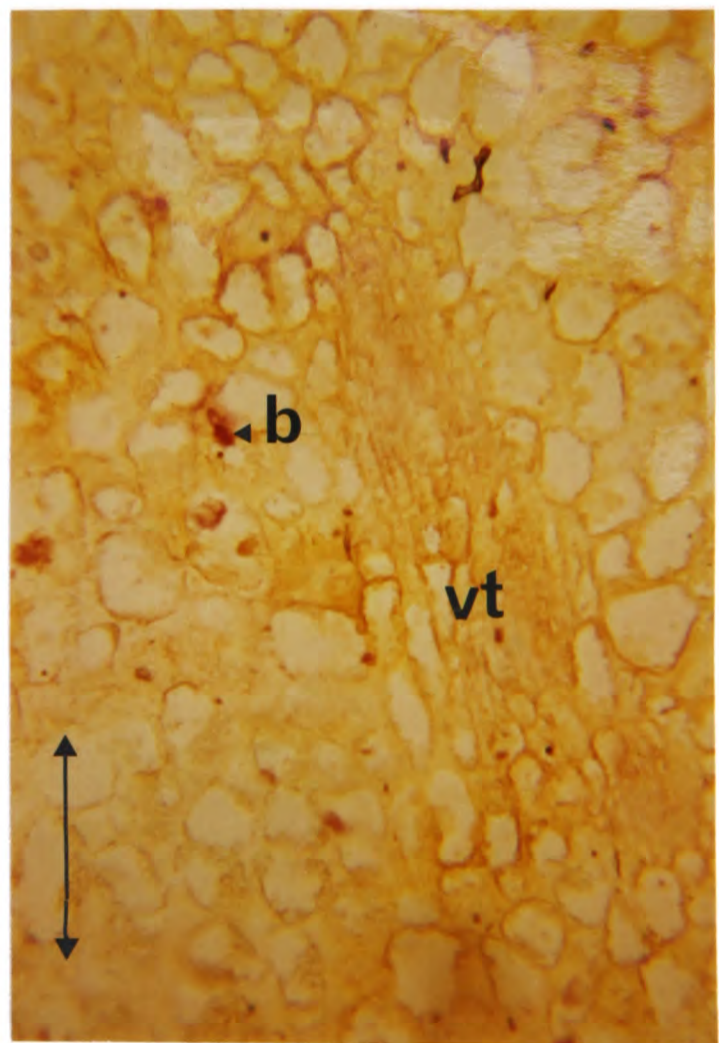


Fig.IV.8

It is difficult to understand the nature of such large structures although possibly they represent the focal points of acid hydrolysis within the cell. In this case several subcellular organelles may exist within a common membrane and the end-product staining may represent autolysis of the constituents. Another possibility is that they are artefacts caused by injury to the cytoplasm during low temperature sectioning. The intense staining may then be no more than the result of several layers of cytoplasm, containing azo-dye particles, superimposed one upon another.

Nuclei were seen in most cells of the receptacle, often being encased in a layer of cytoplasm that stained for the enzymes, (Fig. IV.4.). The nuclei themselves, never exhibited esterase activity.

Among cells of other tissues, particulate staining was very noticeable in cells of the vascular traces (Fig. IV.2.). Staining of the cell walls with esterase end-product was not observed in either cortical or pith cells.

Druses, large crystals, presumably of calcium oxalate (Esau, 1953), were obvious in receptacle cells at this stage of development. Their occurrence is probably unrelated to esterase activity since they were seen in some cells that lacked and some that contained enzyme end-product. The crystals themselves did not stain.

The no-substrate control receptacles showed that no end-product in either the particulate or diffuse form was present in any of the cells. (Fig. IV.7.).

Stage B Receptacles:

In these older and larger receptacles the amount of dye product in either particulate or diffuse form was much reduced, although some diffuse stain, associated with large membranous cell inclusions, could still be seen (Fig. IV.8.). Particulate material was found only infrequently in the cells of the pith and cortex, but it could still be identified in vascular tissue. Here also, it was reduced when compared with stage A receptacles.

A considerable number of cells in both the pith and cortex showed a yellow staining of the cell walls. This could be due to the coupling of the diazonium salt with low-molecular weight compounds such as phenols and anthrols, manufactured in response to changing physiological and biochemical conditions. Druses were still observed but reduced in number.

A noticeable morphological change had occurred in the cells of the receptacle at this stage in that amyloplasts were present in large numbers, (Fig. IV.10.). They were identified by their size (approx. $5 \times 2 \mu\text{m}$), and at high power magnifications a number of individual starch grains within single amyloplasts were visible.

In addition to their considerable increase in size, compared with cells of stage A receptacles most cells had become more spherical in shape, (Figs. IV.10., IV.11.). Cell walls were again devoid of enzyme end-product.

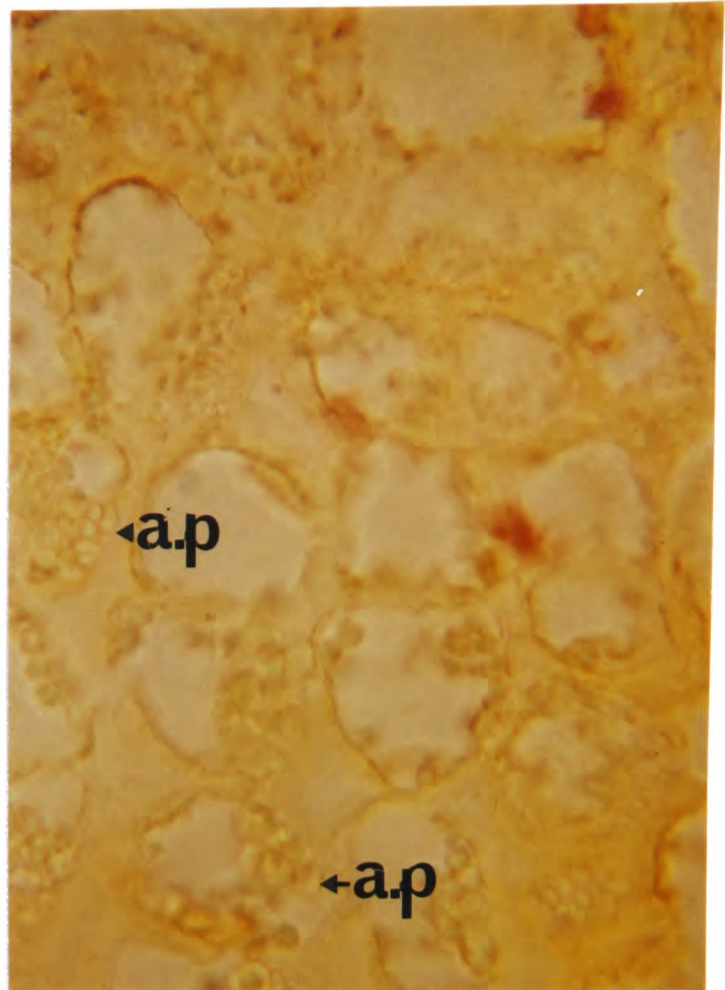
Receptacles of Stages C, D and E:

Receptacles from these 3 stages of ripening were examined in material grown in 4 consecutive seasons. All 3

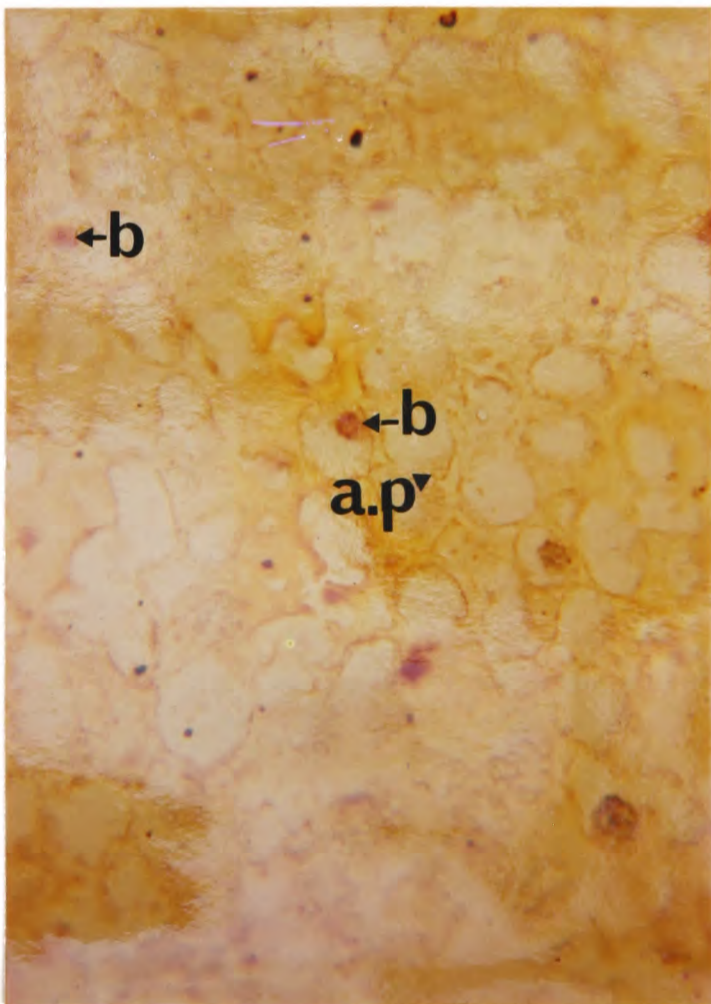
- Fig.IV.9: Pith cells of stage B receptacles with large numbers of amyloplasts (a.p). A membranous body (b) is out of the plane of focus and the outline of an amyloplast underneath is obvious. Magnification: x 1920.
- Fig.IV.10: Stage B receptacle - Pith. There are several amyloplasts (a.p) per cell. Cells are now markedly spherical. Magnification: x 768.
- Fig.IV.11: Stage B receptacle - Cortex. In the centre of the micrograph yellow staining of cell walls is obvious. Magnification: x 307.
- Fig.IV.12: Disrupted pulp tissues from maturing (Stage D) receptacles (cortex) - 1969 season. Note the range in vesicle size, and that after this incubation time (15min) the structures were completely filled with azo-dye. Magnification: x 307.



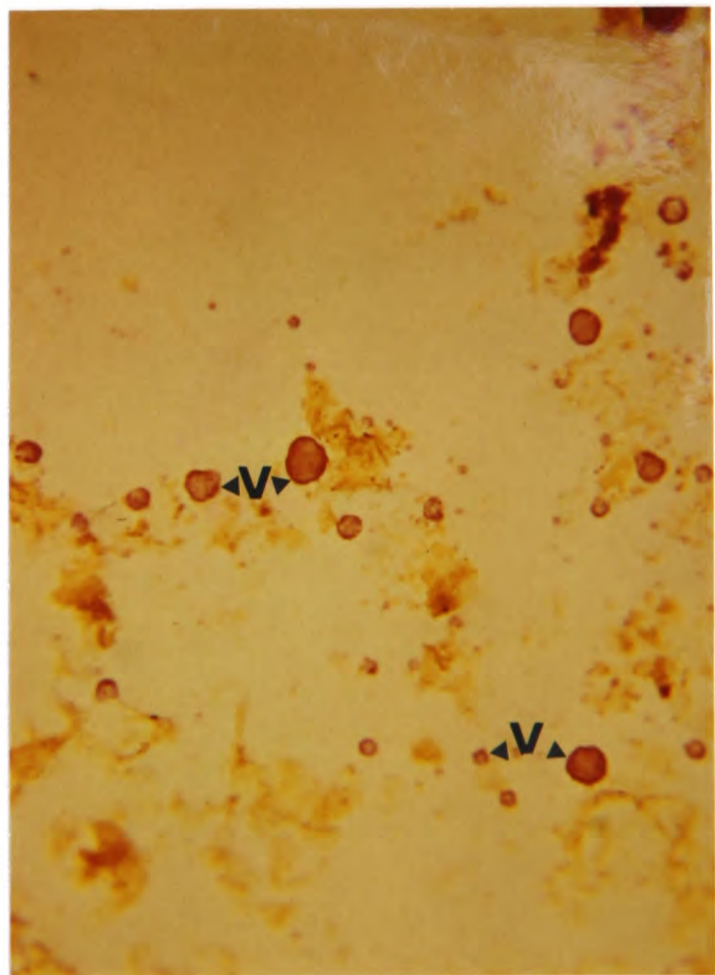
FigIV.9



FigIV.10



FigIV.11



FigIV.12

stages of receptacle development contained esterase-staining bodies but the nature and frequency of occurrence of these bodies in tissue sections fluctuated from season to season. It was not practicable to assess quantitatively the frequency of such bodies. Consequently, in Table IV.4., only the presence (+) or absence (-) of esterase-staining structures is indicated. The extent of their occurrence is reported separately for each season's observations.

Table IV.4. Cytochemical tests for the presence (+) or absence (-) of non-specific esterases in ripening strawberry fruits,* stages C, D and E over 4 consecutive seasons.

Date of examination	Stage of Ripening of Fruits		
	C (Immature)	D (Maturing)	E (Mature)
1.7.69	+	+	+
11.7.69	+	+	+
23.6.70	-	-	-
8.7.70	-	+	+
3.7.71	-	-	-
5.7.72	-	+	+

* The number of receptacles examined was 3 in each case.

Marked morphological changes were again evident when comparisons were made with younger receptacles.

Only a few amyloplasts were found in cells from stage C receptacles and by the time berries had begun to go red they were no longer to be seen.

The sizes of cortex cells had almost doubled from stages B to C. (Table IV.3.). Although data are not available for unfixed frozen strawberry tissue, Havis, (1943) reported cell diameters of $200\mu\text{m}$ for ripe strawberry cells in fixed wax-embedded tissues. This means that cell diameters had, probably, at least doubled again by the time berries were fully ripe, (Stage E). At the same time the mean fresh weight had also doubled (Table IV.1.).

Receptacles from the 1969 season: (Stages C, D and E)

On first inspection, the most obvious feature was the extremely poor preservation of cellular structure after cryostat sectioning (Fig. IV.12. to IV.16.). Preservation could be markedly improved by glutaraldehyde fixation but the fixative made it difficult to assess corresponding losses of enzyme activity (cf. Section III.4.). In all ripening stages C, D and E, the presence of enzyme reaction product was observed in spherical structures varying in size from small particles $0.5\mu\text{m}$ in diameter to extremely large bodies up to $45\mu\text{m}$ in diameter, (Fig. IV.15.).

The cutting of frozen sections of extremely large cells no doubt produces widespread cell rupture. This probably accounts for the effect seen in Fig. IV.15., where the small esterase-staining particles appear to be within the cells of the ruptured tissue whereas the much larger esterase-containing structures appear to have been released.

- Fig.IV.13: Section from same receptacle as Fig.IV.12 incubated for esterases for 8min only. Patches of enzyme end-product can be seen within the larger vesicles (v). Magnification: x 1024.
- Fig.IV.14: Many esterase-containing vesicles have been separated from the tissues of the receptacle (R). Mature berry (Stage E) - 1969 season. Magnification: x 410.
- Fig.IV.15: Small esterase particles (0.5-1.0 μ m diameter) within pulp cells from the pith region. Larger vesicles lie free in the cytoplasm (v). Maturing berry (Stage D) - season 1969, section incubated for 20min. Magnification: x 1024.
- Fig.IV.16: Control section from maturing berry (1969) incubated without substrate for 20min. Vesicles (v) show no evidence of end-product staining for esterases. Magnification: x 1024.

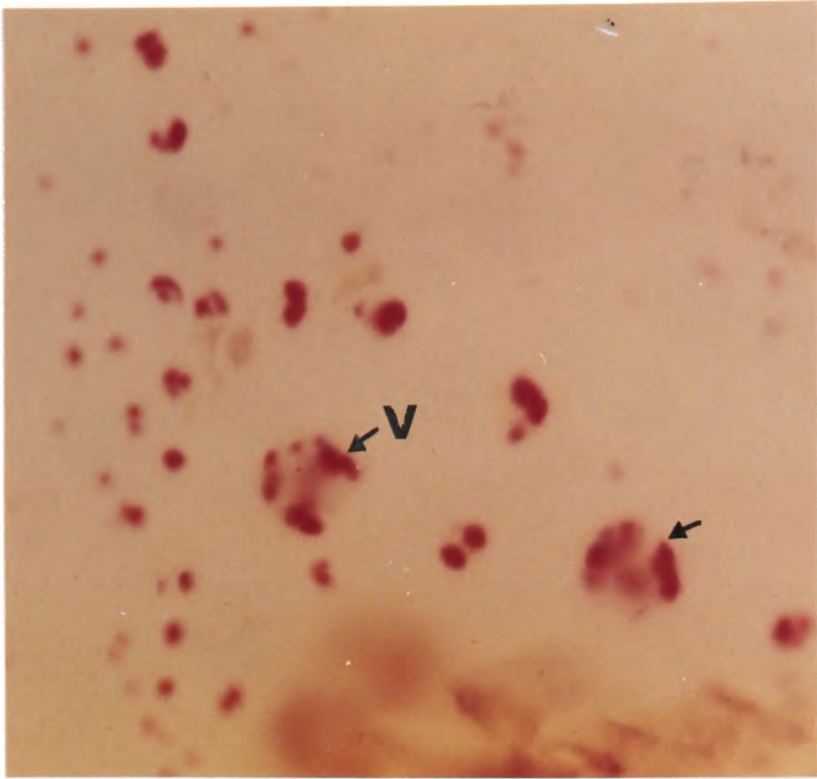


Fig.IV.13

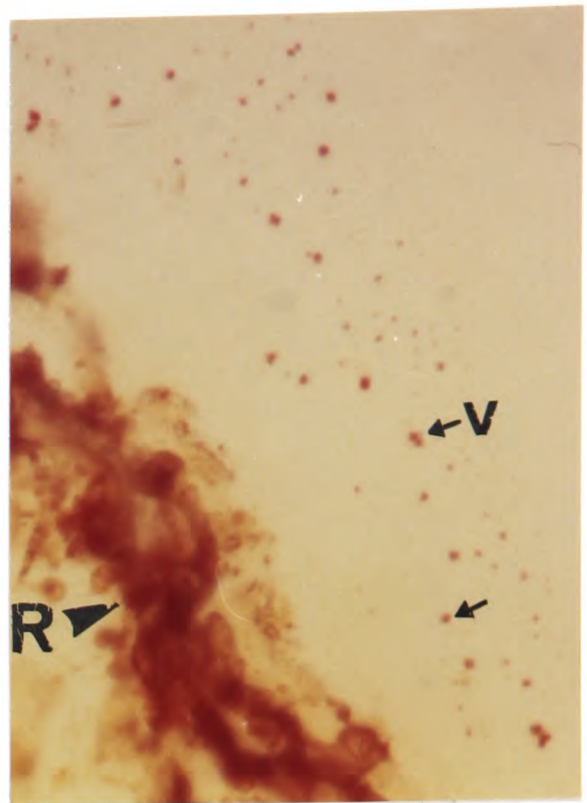


Fig.IV.14

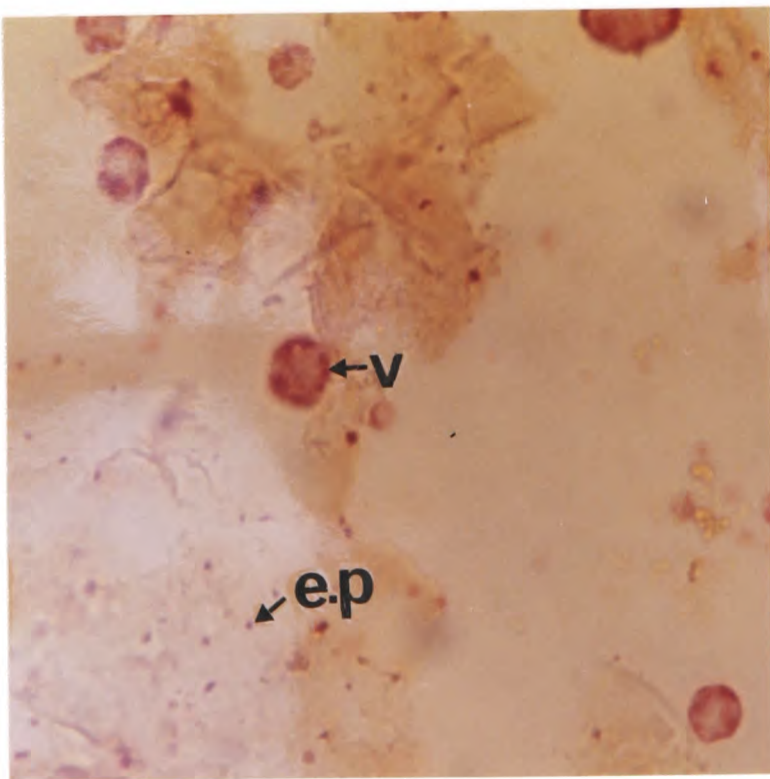


Fig.IV.15

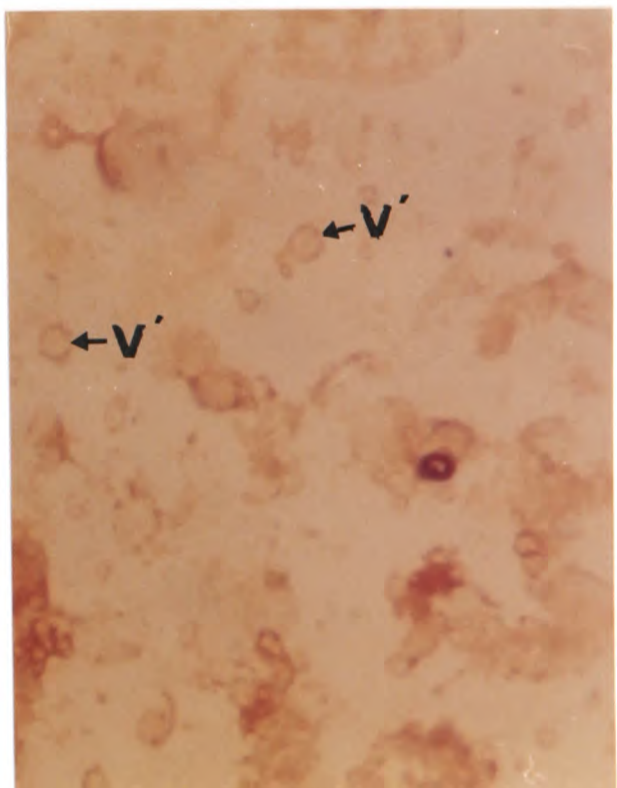


Fig.IV.16

Particulate and diffuse staining of vascular traces was still apparent in tissues from all 3 stages of ripening but it was not as frequent as in younger receptacles. Vascular traces generally tended to be stained deep-brown, in both test and no-substrate control sections, presumably due to the presence of the diazonium salt.

1970 Data: (Stages C, D and E)

In marked contrast to the previous season, fresh strawberry tissue examined towards the end of June failed to show the presence of any end-product in the cells under the same conditions of enzyme incubation as employed in 1969. Some two weeks after the first attempt, however, similar vesicles containing end-product were observed in maturing and mature pulp cells but their number was considerably reduced when compared with the 1969 data. No staining could be observed in immature fruit (stage C) despite prolonged searching of a number of sections, although apparently 'empty' vesicles, of a size and appearance comparable with those containing end-product at the other stages of ripening, were evident. These 'empty' vesicles were indistinguishable from those seen in no-substrate controls. (Fig. IV.16.).

1971 Data: (Stages C, D and E)

Esterase end-product could not be found in any of the pulp tissues although several receptacles from each stage of ripening were examined.

1972 Data: (Stages, C, D and E)

Vesicles containing enzyme end-product were found in this season's tissue, although their frequency was still much lower than

in the 1969 fruit and compared more closely with that in the 1970 fruit, even to the extent that there was no esterase activity detectable in the immature pulp.

A full consideration of the factors which might have produced such variability in the results is given in the discussion,(IV.6).

IV.3.2. Esterase activity in developing achenes.

Esterase reaction-product was detected in achenes from receptacles at all stages of development in each year.

The stain was particularly dense in the embryos, although in very young achenes it could be seen associated with the fruit wall also. Figs. IV.17. and IV.18., show that dye-product was particularly concentrated in procambial strands, seen in a longitudinal section of an achene. Very often the dye-product was seen as large feather-like crystals, indicating very high enzyme activity and some diffusion of the primary reaction product before capture by the coupling reagent. Azo-dye deposits were also visible as discrete particles 0.5 to 1.0 μ m in diameter.

IV.3.3. Raspberry:

An examination of the epicarp and fleshy mesocarp of several drupelets from all 3 ripening stages failed to reveal the presence of any enzyme end-product over the ranges of incubation time used, viz. 8-30 min. It did occur, however, in the form of particles, 0.5 to 1.0 μ m in diameter in association with vascular strands entering the endocarp, and with some of the so-called 'transition' cells (Reeve, 1954) adjacent to the sclereids of the endocarp.

In green, unripe receptacles, particulate end-product was frequently seen in the cells of vascular tissue and lining the

Fig. IV.17: Part of an achene from a maturing receptacle (Stage D). Section incubated 20min for esterase activity. Intense activity (high enzyme concentration) has resulted in the deposition of large crystals of the azo-dye. Many small esterase particles (approx. $1\mu\text{m}$ diameter) are localised mainly in the procambial strand (p.s). Deep brown-yellow staining of some tissues, notably the nucellus (N) by the diazonium salt, is apparent. Magnification: x 307.

Fig. IV.18: High power magnification of Fig. IV.17. Magnification: x 768.

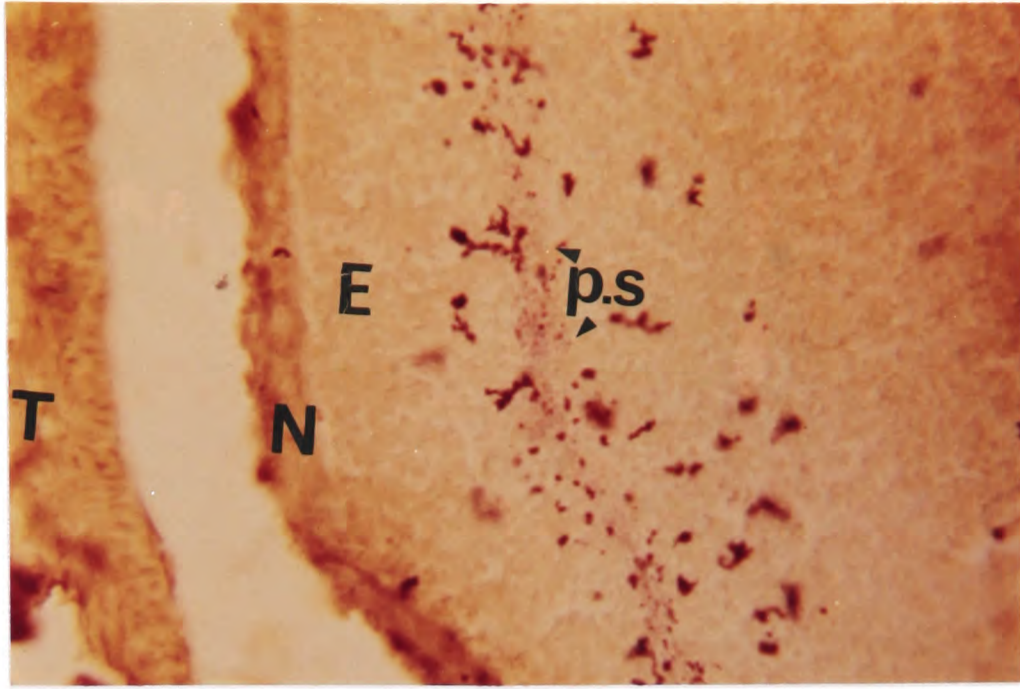


Fig.IV.17

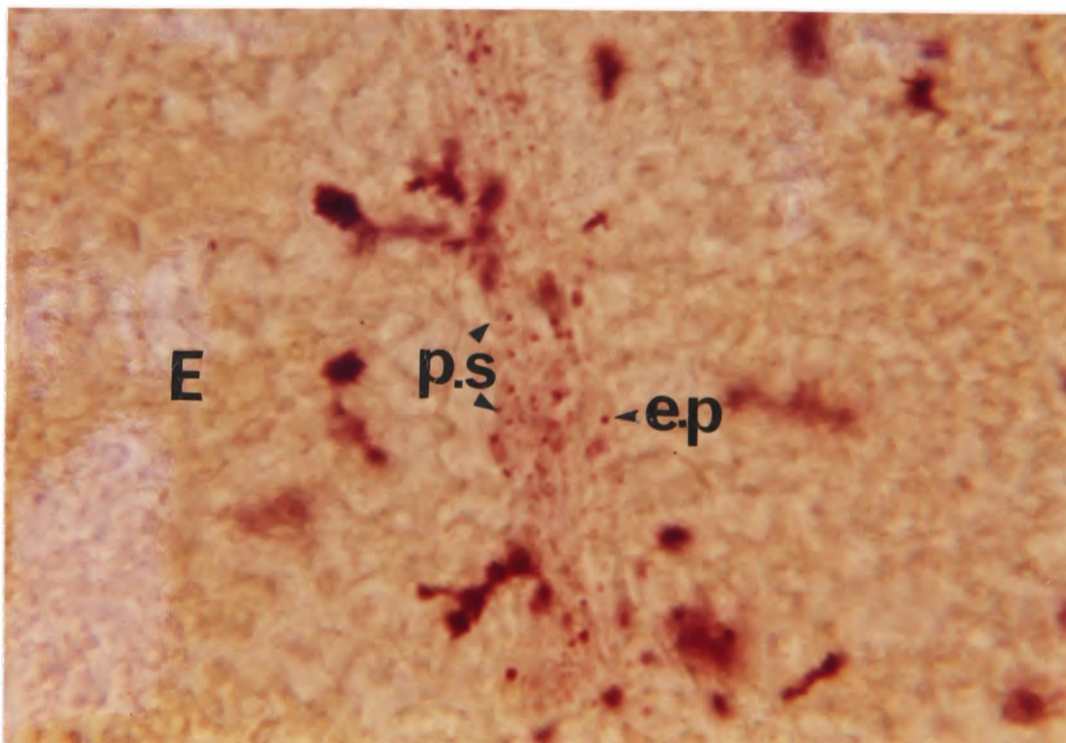


Fig.IV.18

cytoplasm of many vacuolating pith and cortical cells. Nuclei were unstained.

The amount of end-product did not increase appreciably between 10 and 30 minutes of incubation.

At later stages of receptacle maturation the nature and pattern of end-product deposition did not change markedly.

Particulate end-product deposition was still characteristically associated with vascular tissues and with some cells of the pith and cortex, although as in strawberry receptacles these cells became more spherical and many cell walls stained yellow with the coupling salt.

Large membranous bodies, staining diffusely for end-product, could be seen at all 3 stages of receptacle development but they were never as frequent as those observed in young strawberry receptacles. They were more like the angular, irregularly shaped bodies seen in younger strawberry receptacles than the spherical structures seen in the later stages of ripening.

High esterase activity was associated with the procambial strands in the developing embryo tissues. Both the 'feathery' type of end-product deposition and the particulate forms of the azo-dye (0.5 - 2.0 μ m diameter) were readily detected (cf. strawberry achenes). Cell walls of either receptacle cells or of the fleshy mesocarp of the ripening drupelets did not exhibit end-product staining.

Control sections lacked enzyme end-product in all tissues of all fruits examined.

IV.3.4.

Blackberry.

Esterase-staining patterns for the drupelets of the fruit were essentially the same as those for raspberry drupelets; end-product only being found in vascular tissues entering the endocarp and in the procambial strands of the embryos. The fleshy mesocarp and the skin of the drupelets (epicarp) were devoid of esterase activity, either in the cell walls or in the cytoplasm of the tissues.

IV.4.

BIOCHEMISTRY

MATERIALS AND METHODS

IV.4.1. Selection of fruit.

The 3 stages of strawberry development green, or green-white immature, white-red, maturing, and red mature (i.e. C, D and E) were obtained in sequence during each season. The period over which fruit was harvested varied slightly in the two seasons but never lasted for more than 4 weeks, (between mid-June and mid-July).

With the exception of the work on liquid nitrogen-frozen berries, all the experiments were carried out on fresh fruit, and the period between harvesting, transportation and the start of experimentation on any sample was never more than 12 h.

The biochemical work extended over 2 seasons only; 1971 and 1972. A stock of fruit was stored from the 1971 season by plunging fresh fruit into liquid nitrogen, and subsequently maintaining them at -20°C until needed.

In both the 'Materials and Methods' and 'Results' sections the work on the 3 different sets of fruit will be dealt with separately and in chronological order.

After fruit was sorted into the appropriate category of ripeness (section IV.2.1. and Table IV.1.) the methods for the selection of a desired number of berries at each stage of ripening from the total populations were as described in Section IV.2.1. The amount of fruit used depended on the season, and varied between 96 g fresh weight (1972) to 500 g fresh weight, (1971) per ripening stage (see Table IV.8.).

IV.4.2. Enzyme extraction procedures.

In the light of practical experience some changes were made in the procedures adopted for the extraction of esterases from pulp tissue during the two years, 1971 and 1972 and these are explained below.

IV.4.2.1. The dialysing medium.

In the preparation of extracts of esterases from fresh strawberry pulp tissues in the season 1971, the procedure and techniques were essentially the same as those described by Mendoza et al., (1969), (and see Section IV.4.2.3.). In this technique, dialysis of the extract is carried out against double-distilled water. However, in the following autumn and winter months of 1971 work with broad bean lateral root extracts showed that dialysis of esterase preparations against 10mM sodium phosphate buffer containing 5mM MgCl₂ pH 6.5 (Section II.2.2.) increased the specific activity of the enzymes fourfold over replicate extracts dialysed against double-distilled water (see section II.3.2.7.). Consequently with the freeze-stored berries from season 1971 and fresh fruit from 1972 purification of the extracts was carried out using buffer - MgCl₂ mixtures for dialysis, rather than distilled water.

IV.4.2.2. Removal of achenes from receptacles.

At the close of the 1971 strawberry season, several hundred achenes from each ripening stage had been obtained. The achenes were separated by first blending whole berries in a thin PVP paste (see p.45), then filtering off the liquid enzyme extract and later picking and sieving the achenes from the powder that remained, after drying at room temperature the paste retained at the filter.

Cytochemical studies have already shown that esterase activity in achenes appeared to be intense (Section IV.3.2.). Some preliminary attempts were therefore made to measure the specific activities of enzyme extracts of achenes from different ripening stages for comparison with extracts of the pulp. It was during the preparation of these extracts that it was discovered that esterase activity was readily extractable from whole achenes in a manner that could have influenced estimations of enzyme activity and protein content made on pulp tissues in the season 1971. The method of preparing this achene extract was as follows:

2 g amounts of achenes from each of the 3 ripening stages were weighed and washed in 40 ml of 0.1M sodium phosphate buffer at pH 6.5. Achenes were separated from buffer by filtration through Whatman No. 1 filter paper. This procedure was repeated 4 times with the same 40 ml of buffer. In the washings from achenes of immature receptacles a pronounced yellow orange colouration developed. This became progressively less intense in washings from achenes of later ripening stages. These buffer extracts were used directly for assays of esterolytic activity and protein content.

Absorbance readings showed that between 0.18 and 0.20 μ mole of naphthol AS D was liberated per h at 32°C and pH 6.5, per ml of extract whilst measurements with the Folin-Ciocalteu reagent showed that 1.2 mg protein per g achenes were extractable from immature achenes, 0.7 mg from maturing and 0.2 mg from mature achenes. However it is known that a variety of non-proteinaceous compounds may influence colour development when using the Folin-Ciocalteu reagent (Lowry et al., 1951).

Likewise it is possible that substances such as aromatic amines and phenols were present in the leached material which could couple with the diazonium salt used in the assay of esterolytic activity. Chemical tests for the identification of such interfering substances were not carried out since it is clear that contamination could be prevented by removing achenes from the receptacle before the initial homogenisation. This procedure was therefore adopted for fresh fruit obtained in the 1972 season, but could not be used for the freeze-stored tissue. With frozen fruit removed from -20°C storage after 4-7 months and placed at room temperature, only 20 or 30 achenes per berry could be removed before the pulp was reduced to a mush. This was true of fruit from all ripening stages. Consequently there was unavoidable contact between pulp liquids and achene surfaces.

Furthermore, freezing and thawing of achenes would have increased the possibility of leakage of materials from them. It was therefore considered that since the receptacles of snap-frozen strawberries might unavoidably have already undergone contamination it would be easier to separate the achenes in this case by blending rather than by removing them individually from the receptacle mush.

The presence of interfering low-molecular weight compounds in achene extracts was likely to be of minimal importance in final assays for esterolytic activity and protein content from pulp extracts since most of them would have been removed in the purification procedures. Any extracted proteins however would not have been removed during the purification and dialysis procedures, thereby making it difficult to assess the effect of these substances on assays for protein or esterolytic activity.

For the reasons stated, data from the fresh fruit of season 1971 can be compared with those of later work to gauge the effect of making changes in the purification procedure (i.e. dialysing against buffer - MgCl_2 rather than water). Specific activity determinations from ripe pulp, 1971, is compared with corresponding data from 1971 freeze-stored fruit and fresh fruit 1972, in Table IV.6.

IV.4.2.3. Purification of enzyme extract.

Fresh fruit - 1971 season.

500 g amounts of berries from each of the 3 ripening stages were washed under jets of tap water to remove surface contaminants and then a final rinse in distilled water before allowing the fruit to dry on paper towelling at room temperature. Berries were then blended in an 'Atomix' blender (MSE) for 1 min at $\frac{1}{2}$ speed followed by 2 min at full speed in the presence of 1.2 ml/g of a thin PVP paste made up 24 h in advance with the following constituents:

- | | | |
|----|-----------------------------------------|---------|
| a) | Insoluble polyvinylpyrrolidone (PVP) | 110 g |
| b) | 0.1M sodium phosphate buffer, pH 6.5 | 500 ml |
| c) | 0.05M MgCl_2 | 50 ml |
| d) | L-ascorbate to a final concentration of | 100 mM. |

PVP and ascorbate were added for the reasons mentioned in Section II.2.2. All homogenising liquids and apparatus were precooled to $+4^\circ\text{C}$, and all extraction procedures carried out in a cold room at $+4^\circ\text{C}$.

The homogenate was adjusted to pH 6.0-6.5 by the addition of 1N NaOH and then filtered through 2 layers of cheesecloth to retain the achenes. The material retained was then washed with 2 volumes of double-distilled water and the slurry of the combined filtrates was transferred to a shallow dish with a large surface

area/volume ratio to allow relatively rapid freezing and thawing. All extracts were frozen at -20°C at least overnight before thawing to $+25^{\circ}\text{C}$ in a water bath. The cycle was repeated 4 times. The thawed pulpy extract was then centrifuged at $3000 \text{ xg}_{\text{av}}$ at $+4^{\circ}\text{C}$ for 10 min to remove cell debris. The supernatant was then fractionated by adding ammonium sulphate to 0.8 saturation and the mixture allowed to stand for at least 30 min before centrifuging at $10,000 \text{ xg}_{\text{av}}$ at $+4^{\circ}\text{C}$ for 30 min. The precipitate was then taken up in 10 volumes of 0.1M sodium phosphate buffer, containing 5mM MgCl_2 (pH 6.5) over a period of 1-2h. Sucrose was then added to the extract to a final concentration of 0.5M, and the mixture dialysed against 2 x 2 litres of double-distilled water, in the cold for 6h. The extract was then emptied from the dialysis bags and centrifuged at $10,000 \text{ xg}_{\text{av}}$ at $+4^{\circ}\text{C}$ for 30 min. The supernatant was again dialysed against 2 x 2 litres of double-distilled water under the same conditions and finally centrifuged at $10,000 \text{ xg}_{\text{av}}$ at $+4^{\circ}\text{C}$ for 30 min. The supernatant was then lyophilised by rotating the enzyme extracts in 'Quickfit' round bottomed flasks, partly immersed in a freezing mixture of 'dry ice' and absolute ethanol. The frozen extracts in each flask were then fitted to a 'cold finger' apparatus on a 'Genevac' High Vacuum pump. Lyophilisation was achieved under a pressure of 10-25 millitorr. The lyophilisates from each stage of ripening were then stored in stoppered flasks at -20°C until needed.

Liquid nitrogen-frozen berries - 1971 season.

500 g amounts of fresh berries from each stage of ripening were snap-frozen by immersion in liquid nitrogen in a Dewar flask.

The tissue subsequently used as the enzyme source was stored for up to 7 months at -20°C before rapid thawing to $+25^{\circ}\text{C}$ in a water bath. After homogenising whole berries, the extraction then followed the procedure detailed in section IV.4.2.3. with the exception that dialysis was performed against 10mM phosphate buffer, containing 5mM MgCl_2 at a final pH of 6.5 instead of double-distilled water.

Fresh fruit - 1972 season.

Due to the time and labour involved in removing achenes from receptacles by hand (each berry bore some 300 achenes) only 21 berries from each of the 3 ripening stages were used in the 1972 season's tests. It was easier to remove achenes from immature fruit than from either of the other two stages since the flesh was firm and did not break when forceps were applied to remove the achenes. Unavoidably, the mature berries, (and maturing berries to a lesser extent) often lost their entire skin during the process of achene removal.

After achene removal, the pulp was placed directly in chilled hydrated PVP paste. A lyophilisate was then prepared as already explained in IV.4.2.3.

The basic differences in the enzyme extraction procedure over the two seasons 1971-1972 are summarised in Table IV.5.

IV.4.3. Preparation of a crude enzyme extract from achenes removed from the 3 stages of receptacle development C, D and E.

Achenes removed from the 1972 berries at stages C, D and E were used for enzyme extraction. 1.0 g of achenes was

Table IV.5. The differences in technique for the extraction of esterases from fruit pulp in the seasons 1971 and 1972.

Year	State of receptacles	Time of removal of achenes	Dialysing medium
1971	Fresh	After homogenisation in Atomix blender	Double distilled water
1971	N ₂ frozen for 4-7 months	"	10mM phosphate buffer + 5mM MgCl ₂ , pH 6.5
1972	Fresh	Before homogenisation in Atomix blender	10mM phosphate buffer + 5mM MgCl ₂ , pH 6.5

ground in a mortar with a pestle for 10 min at +4°C, in the presence of 40 ml of 0.1M phosphate buffer, pH 6.5, containing 2 g of hydrated insoluble PVP and 1.0 g of acid-washed sand. A further 80 ml of the same buffer were then added to wash out residue from the mortar. The extract was then made to a final volume of 150 ml, and decanted directly into centrifuge tubes and centrifuged at 3,000xg_{av} for 15 min at +4°C to remove cell debris.

The supernatant was assayed immediately for esterolytic activity and protein content. Specific activities were determined with initial substrate concentrations of 0.0835mM and 0.167mM (Section II.3.2.1.). Esterase activity was expressed on a per achene basis, by counting the number of achenes in 1.0 g fresh weight of tissue at each stage of ripening and calculating the result as 'specific activity x protein per achene' (Table IV.9.). Although this experiment was carried out once only each of the readings for specific activity is the mean of 3 readings from each extract. Control tubes were prepared as described in Section II.2.3.3.

IV.4.4. The affinity of pectin methyl esterase for naphthol AS D acetate.

10 mg of purified tomato pectin methyl esterase (Sigma Chem. Co. Ltd.) were stirred into 50 ml of 0.1M sodium phosphate buffer, pH 6.5 for 1 h at 4°C. The solution was then centrifuged at 2,000 xg_{av} for 10 min to remove fine particles in suspension. The supernatant was adjusted to pH 6.5 and divided into 2 equal aliquots, one of which was dialysed against 2 litres of 0.1M sodium

oxalate (to destroy pectin methyl esterase activity Jansen *et al.*, 1947), containing 0.01M phosphate buffer (pH 6.5) and 5mM MgCl₂ over a period of 24 h at 4°C. The other aliquot was dialysed under identical conditions except that oxalate was omitted. After removing each aliquot from its dialysis bag the extracts were cleared by centrifugation at 2,000xg_{av} for 10 min and the pH of the oxalate-dialysed solution adjusted to 6.5 with NHCl. The supernatants were then assayed immediately for non-specific esterase activity as described in Section II.2.3.2.

Initial substrate concentrations of naphthol AS D acetate were 0.0835mM and 0.167mM, and incubation was allowed to proceed for 1 h at 32°C pH 6.5.

Control solutions measuring non-enzymic hydrolysis were as described in Section II.2.3.3.

The possibility that sodium oxalate could interfere with azo-dye coupling was tested by coupling naphthol AS D with the diazonium salt, fast red violet LB salt, in the presence of 0.02M sodium oxalate at pH 6.5. Control solutions contained 0.1M sodium phosphate buffer, pH 6.5, in place of sodium oxalate. The details of the procedure for estimating the effect of interfering substances on azo-dye coupling has been described in Section II.3.1.4. Both extracts were estimated for soluble proteins using the colorimetric method of Lowry *et al.*, (1951).

IV.4.5. Biochemical assay.

The procedure for the spectrophotometric assay of non-specific esterases, with naphthol AS D acetate as substrate and fast red violet LB salt as coupling reagent, was used. (See Section II.2.3.2) Except where estimations of K_m were attempted,

substrate concentrations of 0.0835mM and 0.167mM were used throughout the work.

IV.4.6. Protein estimation.

Soluble proteins were estimated using the colorimetric method of Lowry et al., (1951) with bovine serum albumen (BSA) as standard. Solutions were read at 500nm.

IV.5. RESULTS (BIOCHEMISTRY)

IV.5.1. Changes in esterolytic activity during the ripening of strawberry receptacles.

IV.5.1.1. A comparison of mean specific activities from different ripening stages over 2 seasons.

'Specific activity' is expressed throughout as μ moles naphthol AS D liberated per h per mg protein, at 32°C, pH 6.5.

The mean specific activities from each of the 3 different ripening stages, with standard error calculations, are presented in Table IV.6. Each result is the mean of a number of assays performed on lyophilisates obtained from fruit harvested on the same day.

Reference has already been made to an alteration in the extraction procedures between 1971 and 1972 (Section IV.4.2.) and a rearrangement of the data in Table IV.6. is shown in Table IV.7. to reveal how a change in the dialysing medium affected specific activity.

It is clear from these tables that the greatest decrease in specific activity from fresh fruit in season 1971, compared with 1972, occurred in enzyme preparations from red, mature berries.

Because decreases in specific activity were quite high in extracts from fresh fruit of 1971, they are excluded from the calculations in which esterolytic activity is expressed on a per berry basis or per g of fresh weight (Fig. IV.20. and IV.21.).

The data from Table IV.6. for frozen receptacles (1971) and for fresh fruit (1972) are plotted as histograms in Fig. IV.19, A - B. In 'A' (the lower substrate concentration) data from 1972

Table IV.6. Mean specific activities of esterases in strawberry pulp extracts, measured over 2 seasons.

Stage of Ripening	Fresh Fruit		Liquid N ₂ Frozen Fruit		Fresh Fruit		Substrate Concentration
	1971		1971		1972		
	Mean±S.E.		Mean±S.E.		Mean±S.E.		
Immature	0.40 ± 0.04 (9)		0.62 ± 0.215 (10)		1.01 ± 0.06 (9)		0.0835mM
Maturing	1.25 ± 0.45 (4)		2.65 (1)		2.29 ± 0.08 (10)		"
Mature	0.18 ± 0.02 (6)		0.78 ± 0.07 (14)		0.54 ± 0.06 (9)		"
Immature	0.72 ± 0.09 (14)		4.29 ± 1.06 (5)		2.26 (1)		0.167mM
Maturing	1.53 ± 0.25 (8)		4.65 ± 0.65 (5)		3.61 ± 0.10 (6)		"
Mature	0.17 ± 0.04 (6)		2.11 ± 0.35 (4)		1.00 ± 0.04 (7)		"

S.E. = Standard error. Figures in brackets denote number of assays on which the mean is based.

Table IV.7. Percentage reduction in mean specific activity of esterases extracted from 1971 fresh fruit compared with 1971 liquid N₂ frozen fruit and 1972 fresh fruit, as a result of dialysis against distilled water.

Stage of Ripening of 1971 fresh fruit	Reduction compared with 1971 liquid N ₂ frozen fruit	Reduction compared with 1972 fresh fruit	Substrate concentration
Immature	35.5	60.0	0.0835mM
Maturing	52.8	45.5	"
Mature	77.1	66.6	"
Immature	83.2	68.2	0.167mM
Maturing	67.1	57.7	"
Mature	91.9	83.0	"

Fig.IV.19: Mean specific activities of esterases in pulp extracts at the different stages of ripening, (I = Immature, MG = Maturing, M = Mature). A = Substrate concentration of 0.0835mM, B = Substrate concentration of 0.167mM. Figures at the base of each histogram are the number of assays performed to produce the mean value and standard error. (○ ○) = Fresh fruit - 1972, (□ □) = Freeze-stored fruit - 1971.

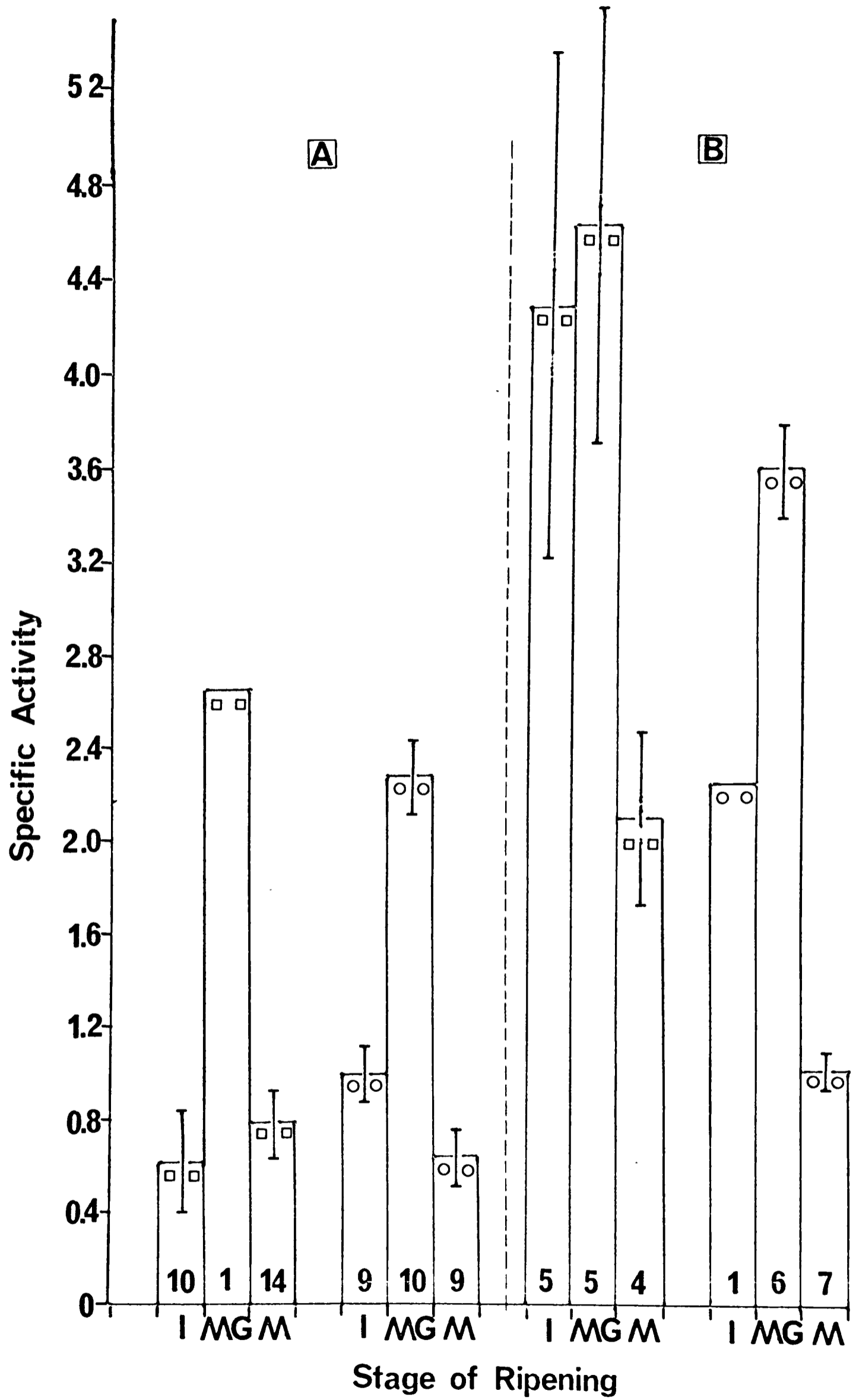


Fig. IV.19

fruit clearly show that specific activity values were highest when the berries were at the white-red stage of development. For freeze-stored fruit (1971) the one value recorded for berries from the same ripening stage is 3 - 5 times greater than the specific activity of lyophilisates from other ripening stages.

At the higher substrate concentration this pattern was repeated with the extracts from maturing berries having the greatest esterase activity per mg of protein, although the high standard errors recorded for frozen berry extracts from immature and maturing receptacles probably means that no significance can be attached to the higher mean in a comparison of the two. Both values however are much higher than that for the extract from ripe fruit. The 1972 data repeat this pattern; the difference between maturing and mature extracts being markedly widened.

The overall pattern suggests that specific activities of the esterases are at their highest during the maturing (stage D) of receptacle development.

At the lower substrate concentration the freeze-stored fruit data can be superimposed almost exactly on the 'fresh fruit - 1972' data, but at the higher substrate concentration (B) specific activity values for freeze-stored tissue are higher at all ripening stages, although standard errors for the maturing stage (MG) of the two sets of data overlap.

IV.5.1.2. Changes in esterolytic activity expressed on a
'per receptacle' or 'per g. fresh weight' basis.

Specific activity estimations are not the best guide to changes in enzyme levels where differential rates of synthesis of individual proteins may be changing quite markedly.

Changes in specific activity may then only reflect a dilution or concentration of existing enzyme molecules in a growing or diminishing total protein population. Changes in absolute levels of the enzyme are thus not detected. It is better, therefore, to express activity on either a 'per average-sized receptacle' basis or a 'per g fresh wt' basis. The total protein extracted from each ripening stage and the number and weight of receptacles used are known, therefore such an estimate is possible as shown in Table IV.8.

Table IV.8. Protein contents at each stage of fruit ripening over 2 seasons.

Stage of Ripening	No. of Receptacles	Total Fresh Weight (g)	Total Protein (μ g)	Protein (μ g) per average-sized receptacle	Protein (μ g) per g fresh weight of fruit
a) <u>Liquid nitrogen frozen fruit - Season 1971.</u>					
Immature	105	500*	6,969	66	14
Maturing	58	500*	19,623	338	39
Mature	51	500*	40,346	791	81
b) <u>Fresh fruit - Season 1972.</u>					
Immature	21	96 [†]	2,148	102	22
Maturing	21	167 [†]	5,660	270	34
Mature	21	193 [†]	26,769	1,275	139

* For reasons already stated fresh weight here includes the achenes.

[†] Fresh weight is pulp tissue only. Achenes removed prior to weighing.

Enzyme activity can be expressed as enzyme units per average-sized receptacle by multiplying each specific activity measurement (Table IV.6.) by the figure for 'protein per average-sized receptacle' (Table IV.6.).

The same principle was used when expressing enzyme activity on a 'per g fresh weight' basis; the figures for protein per g fresh weight being used instead.

The mean activities at both substrate concentrations expressed on either basis are plotted with standard error calculations for each ripening stage in Figs. IV.20. and IV.21.

IV.5.1.2.1. Frozen, stored fruit - 1971 season.

Activity expressed on a 'per receptacle' basis.

Activities at both substrate concentrations show a similar rise in activity during the transition from green or green-white immature to the white-red maturing berry and then a 'levelling off' once the berry is fully red. Although there is good general agreement between these figures and those for fruit from the 1972 season it is apparent that there was a good deal of variability amongst the individual estimates of activity. Since achenes were not removed before freezing some protein contamination may have occurred during subsequent extraction from this source.

Activity expressed on a 'per g fresh weight' basis.

These data show no large differences from those expressed on a per receptacle basis.

IV.5.1.2.2. Fresh fruit - Season 1972.

These data can be considered more reliable than those for freeze-stored tissue since there was no freeze-storage of the berries to increase the fragility of the achene tissues and thereby increase the likelihood of achene contamination.

The similar nature of the data in Fig. IV.20. and IV.21. expressed on either basis make it possible to consider them together.

The rise in activity from immature to maturing berries was again most noticeable, being of the order of 400-500% greater on a 'per receptacle' basis, and 200% greater on a 'per g fresh weight' basis for the lower substrate concentration.

At the higher substrate concentration increases were of a slightly lower order. The degree of variability within the samples was noticeably less than that observed in corresponding samples from freeze-stored tissue.

It will be seen from figures IV.20. and IV.21. that in all cases enzyme activity was greater where fruit had been freeze-stored for several months rather than where it had been used fresh. The possible significance of this is discussed later.

IV.5.2. Changes in esterolytic activity of crude extracts
from developing achenes of receptacles at stages C,
D and E .

Esterase activity per achene for the 3 ripening stages is presented in Table IV.9. A four-fold increase in esterase activity per achene was noted as the receptacles changed from green-white to white-red. Achenes also tended to change colour but not as uniformly as the receptacle. The achenes from stage C were green to pale-yellow in colour but at stages D and E they began to

Fig.IV.20: Esterase activity of ripening strawberry pulp extracts expressed on a per berry basis (A) and per g fresh weight basis (B). Initial substrate concentration was 0.0835mM for all determinations of activity. (○ ○) Fresh fruit 1972, (□ □) Freeze-stored fruit 1971.

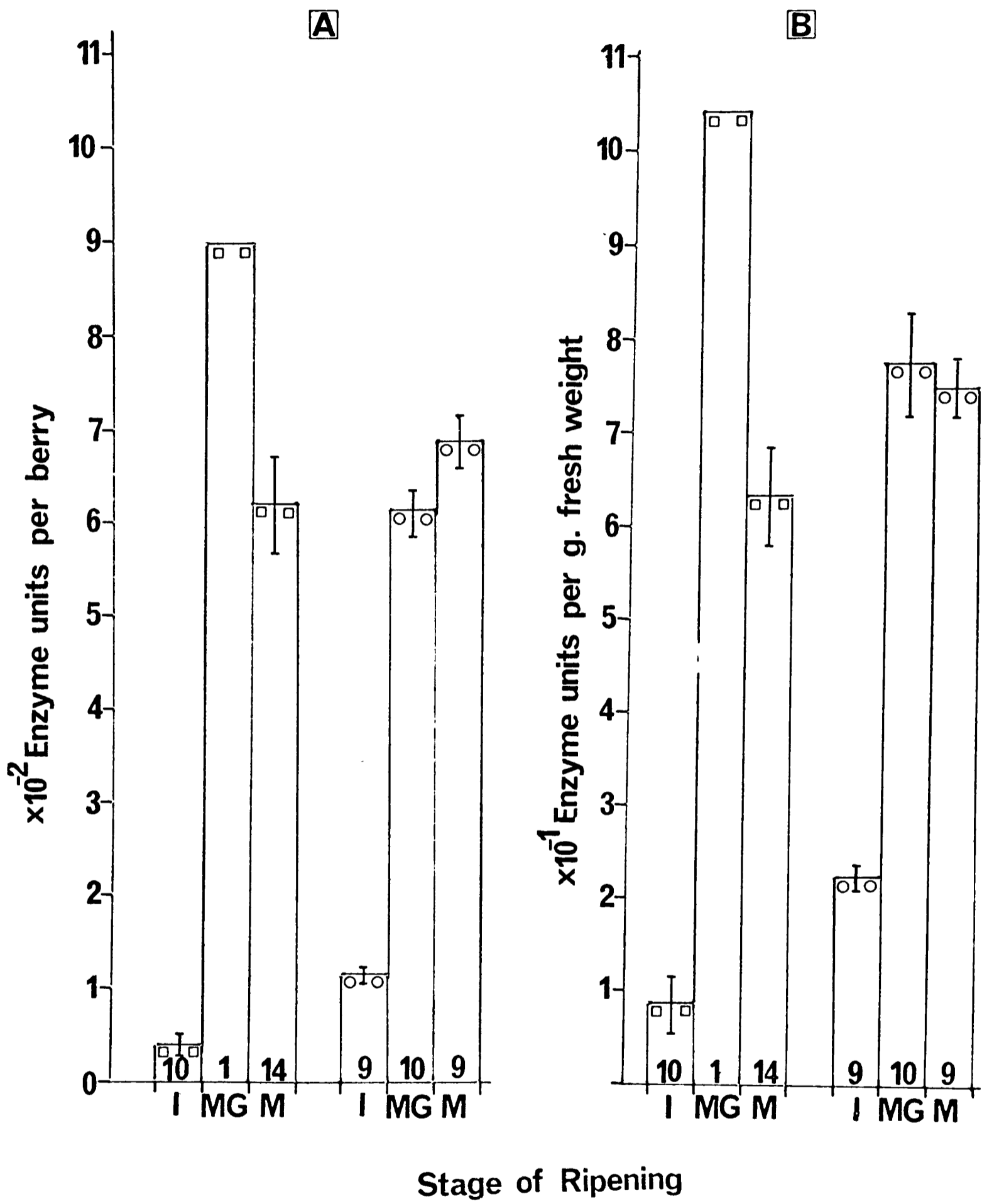


Fig. IV. 20

Fig.IV.21: Legend as Fig.IV.20 except that initial substrate concentration was 0.167mM for all determinations of estero-lytic activity.

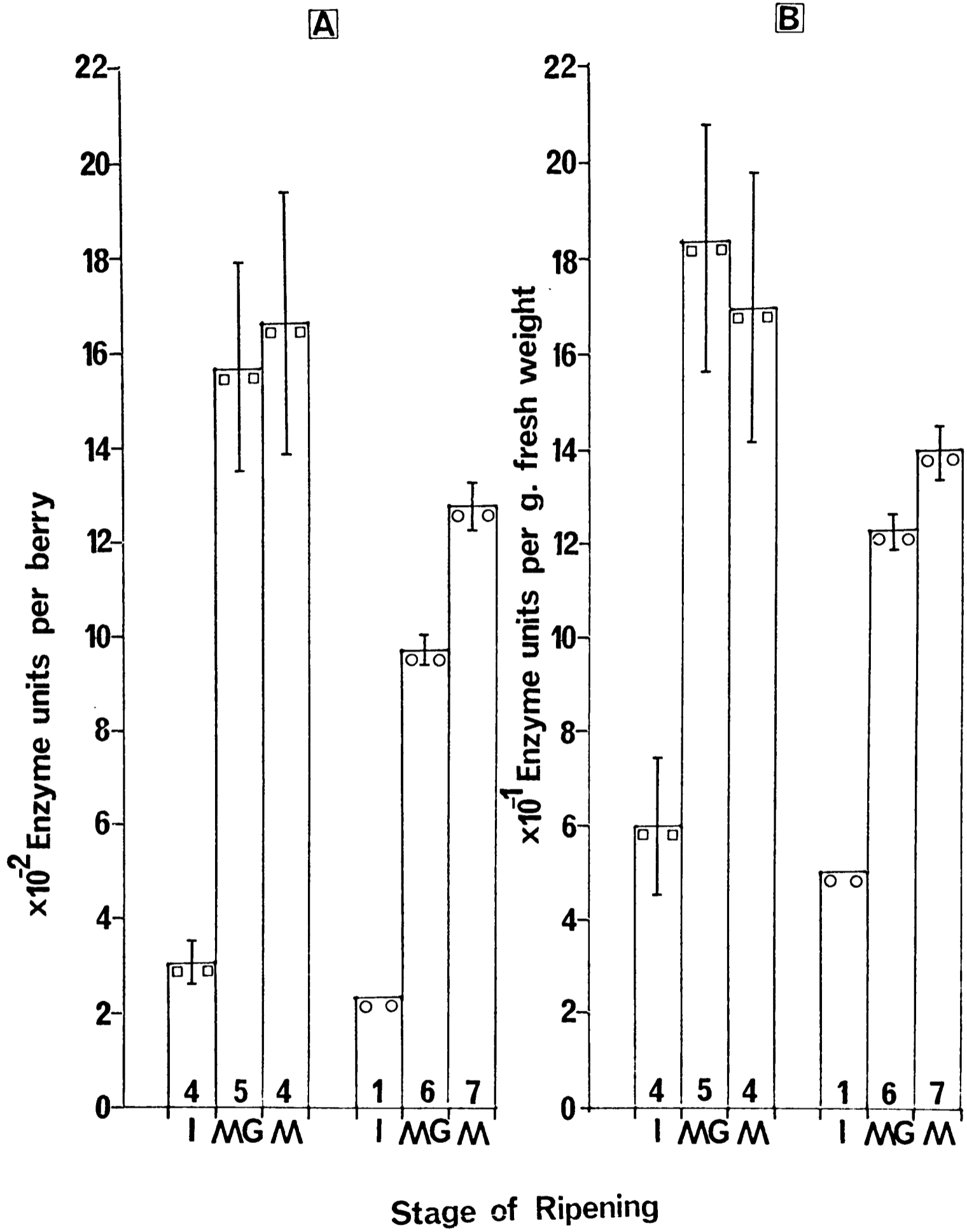


Fig.IV.21

Table IV. 9.

Esterase activity of ripening achenes, expressed on a specific activity and per achene bases.

Stage of ripening	Fresh Weight (g)	No. of** achenes	Protein per achene (μg)	Total protein extracted (mg)	Substrate concentration (mM)	Esterase activity* per achene
					0.835 mM	0.835 mM
					0.167 mM	0.167 mM
					(initial substrate concentrations)	
Immature (C)	1.0	2878	6.46	18.60	0.786	1.234
					5.08	7.97
Maturing (D)	1.0	2106	10.06	21.18	1.933	3.086
					19.44	31.03
Mature (E)	1.0	2110	7.64	16.12	1.513	2.306
					11.56	17.62

† μmoles naphthol AS D liberated/h/mg protein at pH 6.5, 32°C.* nanomoles naphthol AS D liberated/h/ μg protein x protein/achene (μg).

** Only 1 count per stage of ripening

acquire some red colouration, although completely red achenes were never seen.

This large increase mirrors the results obtained for corresponding changes in pulp extracts (Fig. IV.20. and IV.21.). However there was a drop in activity per achene by a half as the berries ripened from the white-red to the red, ripe stage, although the activity of ripe achenes was still approximately twice as great as that of immature achenes.

Enzyme units per berry for achene extracts were calculated from the data of Fig. IV.9. by multiplying the figures for esterase activity per achene by 300 (the average number of achenes per berry). These figures were then compared with those for pulp extracts from the 1972 season taken from Fig. IV.20. and IV.21. and presented in Table IV.10.

It can be seen that pulp tissues contributed very little esterase activity (5-20% of the total) to the berry as a whole at all ripening stages. This confirms the cytochemical work which shows that each achene possesses considerable esterase activity.

These results cannot be interpreted too literally since estimations of esterolytic activity from achenes were carried out on crude extracts only, whilst those from the pulp were extensively purified.

A better guide is given by a comparison of the specific activities for achene and pulp extracts taken from 1972 season (Table IV.11.). Specific activities are, with the exception of ripe fruit, notably higher in pulp extracts than achene extracts. Expressed this way, the results show that in all cases specific activity of non-specific esterases declined from the white-red to the red stage of berry development.

Table IV.11. also shows that specific activity measurements of pulp extracts tended to be lower in ripe berries than in immature berries, whereas the reverse was true for achene extracts.

Table IV.10. Total esterase activity per berry for pulp and achene extracts taken from fruit of season 1972, at the different ripening stages.

Enzyme units per berry				
	Pulp		Achenes	
Stage of Ripening	Substrate concentration (mM)		Substrate concentration (mM)	
	0.0835	0.167	0.0835	0.167
C	115	231	1524	2391
D	616	974	5832	9309
E	689	1279	3468	5286

Table IV.11. A comparison of the specific activities of pulp and achene extracts taken from the season 1972.

Stage of Ripening	Specific Activity			
	Pulp*		Achenes	
	Substrate concentration		Substrate concentration	
	0.0835mM	0.167mM	0.0835mM	0.167mM
C	1.01 ± 0.06 (9)	2.26 (1)	0.786	1.234
D	2.29 ± 0.08 (10)	3.61 ± 0.10 (6)	1.933	3.086
E	0.54 ± 0.06 (9)	1.00 ± 0.04 (7)	1.513	2.306

* These figures are taken from Table IV.6 and are the means and standard errors of a number of estimations (given in brackets).

IV.5.3. The affinity of pectin methyl esterase for naphthol AS D acetate.

Commercially purified pectin methyl esterase dialysed against either sodium oxalate-phosphate-MgCl₂ or phosphate-MgCl₂ mixtures exhibited no catalytic activity for naphthol AS D acetate over the 1 h incubation period. Control solutions, measuring non-enzymic hydrolysis, produced almost equivalent amounts of azo-dye. Estimation of protein content was performed on 1 ml aliquots of the enzyme extract after dialysis, (Table IV.12.).

Table IV.12. The activity* of PME tested against naphthol AS D acetate.

Substrate concentration (mM)	Solution	Commercially purified PME dialysed against:-		Protein concentration (µg/ml)
		a) Sodium oxalate-phosphate-MgCl ₂	b) Phosphate-MgCl ₂	
0.0835	Test	91	88	147
	Control	91	88	
0.167	Test	140	134	127
	Control	141	148	

* nanomoles naphthol AS D liberated per h.

Each value is the mean of 3 readings

PME = pectin methyl esterase.

Tests showed that 0.02M sodium oxalate had no capacity to interfere with the coupling of free naphthol AS D and the diazonium salt, fast red violet LB salt.

Gizis (1964) has shown that the pH optimum for the pectin methyl esterase extracted from the strawberry variety 'Northwestern' is 7.5, but the activity at 6.5 (the pH used here), is 92% that of the optimum.

IV.5.4. Biochemical characterisation of non-specific esterases from the 3 stages of ripening.

For reasons already explained only enzymes extracted from fresh pulp in 1972 after prior removal of the achenes were used in the studies on enzyme characterisation.

IV.5.4.1. Enzyme concentration and the velocity of hydrolysis.

At both 0.167mM and 0.0835mM substrate concentration, the velocity of the reaction appeared to be proportional to the amount of enzyme added. This was true of all 3 stages of ripening (Fig. IV.22. - IV.24.).

IV.5.4.2. Time course study of enzyme-catalysed hydrolysis.

The progress of enzyme-catalysed hydrolysis with respect to time was measured using only 0.0835mM as the initial substrate concentration with protein concentrations varying between 23 - 288µg/ml. Fixed time assays were performed after 0.25, 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 h of incubation. Fig.IV.27. shows that for mature pulp the reaction proceeded linearly for 60 min, but there was a slight deviation from linearity in extracts from immature and maturing berries, (Fig.IV.25 and IV.26.). Calculations showed that determinations of esterolytic activity by fixed-time assay over

Fig.IV.22 - IV.24: Enzyme concentration and the velocity of esterase hydrolysis at the three stages of ripening. In the assay of immature pulp the initial substrate concentration was 0.0835mM.

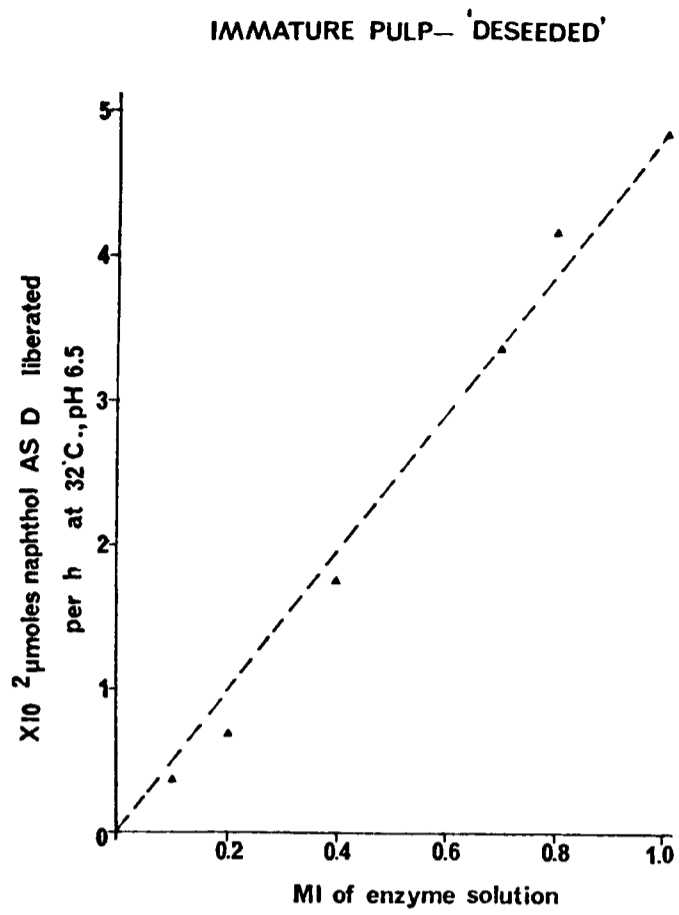


Fig. IV.22

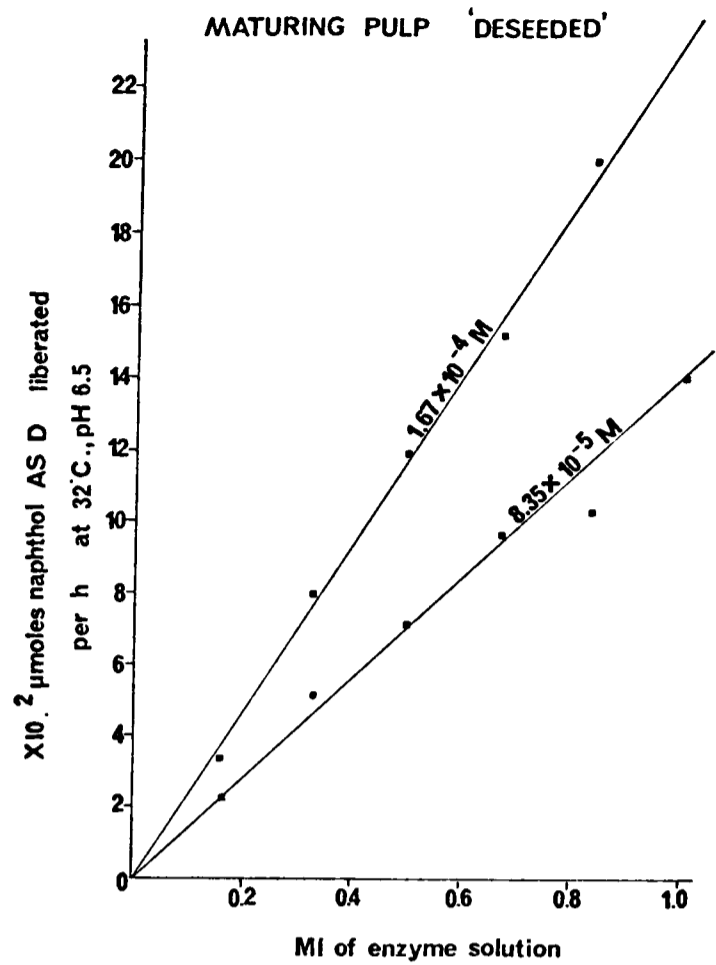


Fig. IV.23

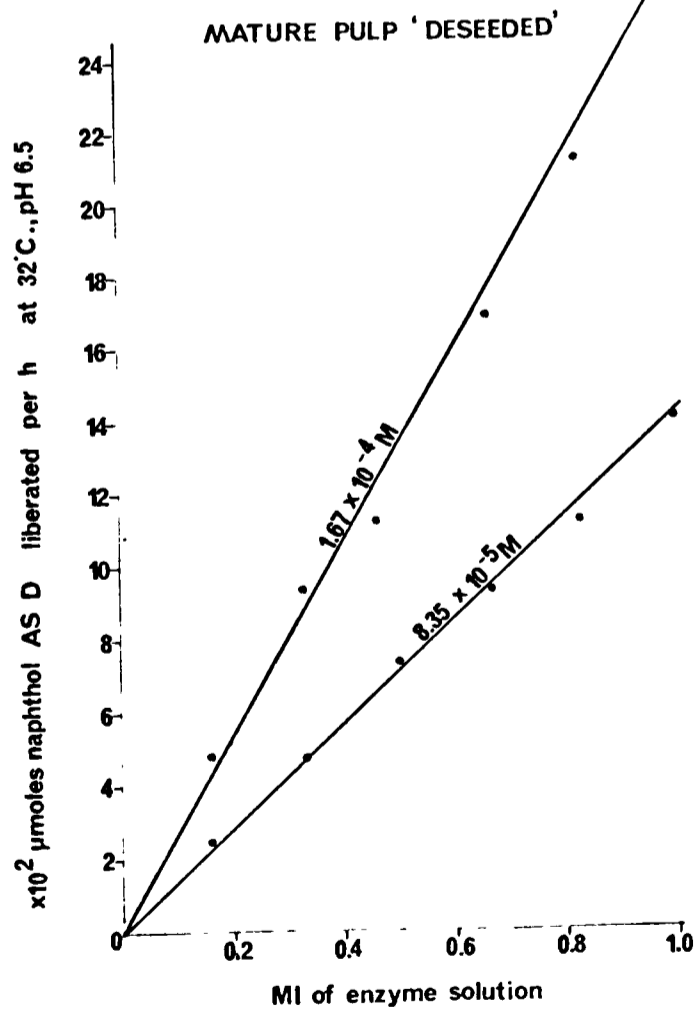


Fig. IV.24

Fig.IV.25-IV.27: Esterase activity as a function of time in pulp extracts from the 3 ripening stages. Substrate concentration at zero time was 0.0835mM.

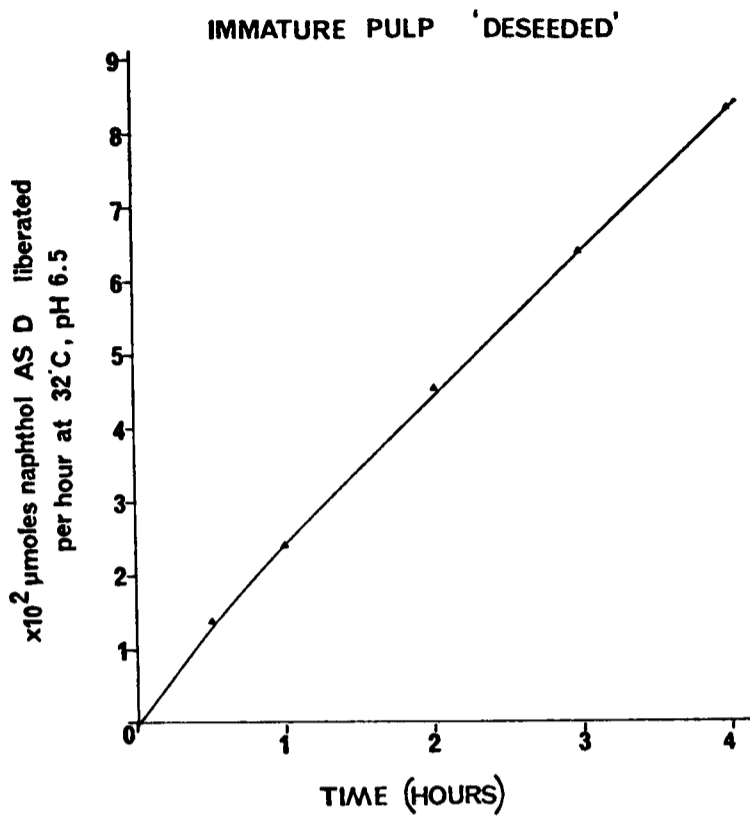


Fig.IV.25

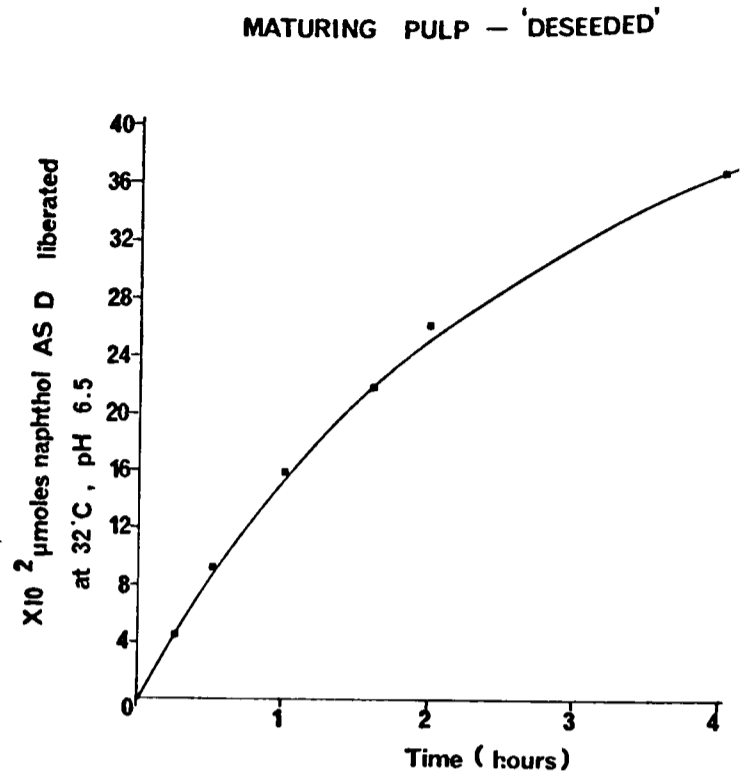


Fig.IV.26

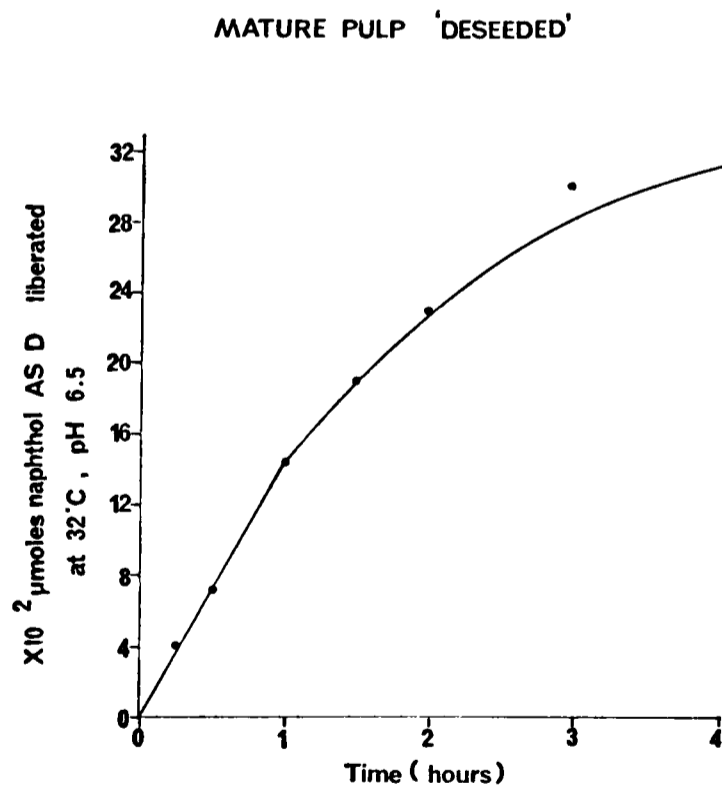


Fig.IV.27

a 1 h period thus underestimated the initial rates of hydrolysis by approximately 6% for immature extracts and 7% for maturing extracts. The reliability of fixed-time assays at 60 min for esterase activity at the various ripening stages (Figs. IV.19, IV.20, IV.21) are not materially affected by this slight difference, but the results suggest nonetheless that strict zero-order kinetics were not followed.

When the time course data were plotted according to the equation describing 1st order reaction kinetics a straight line fit was obtained up to 90 min for both maturing and mature pulp extracts, whereas linearity was maintained during the complete 4h for immature fruit, although the curve did not pass through the origin (Fig. IV. 28.). In addition when the data were plotted according to the equation describing 2nd order reaction kinetics then maturing and mature extracts yielded a straight line over 2 h in the case of maturing fruit, over 3 h in the case of mature fruit, and over the full 4 h in the case of immature pulp extract, (Fig. IV.29.).

Such findings indicate that, using a 60 min incubation time for assays of esterolytic activity complex 'mixed' reaction kinetics may be involved. However for the reasons already stated (Section II.3.2.4.) it was not practicable to use shorter assay times.

IV.5.4.3. Estimation of the Michaelis constants.

Figs. IV.31. and IV.32. show that the esterase-catalysed hydrolysis of the substrate follows the typical Michaelis-Menten relationship for maturing and mature pulp extracts, whilst the curve for immature tissue approaches more closely to a linear reaction. (Fig. IV.30.) Using the method of double reciprocal plotting ($\frac{1}{v}$ versus $\frac{1}{s}$) (Lineweaver and Burk, 1934) apparent K_m values

Fig.IV.28: 1st. order reaction plot of the data from Fig.IV.25 - IV.27. (\blacktriangle) Immature pulp extract, (\blacksquare) Maturing pulp extract, (\bullet) Mature pulp extract. Graph derived from the equation $t = 2.303/k \cdot \log (a/a-x)$ where a = initial substrate concentration, x = amount of substrate consumed after time t , and k = specific reaction rate.

Fig.IV.29: 2nd. order reaction plot of the data from Fig.IV.25 - IV.27. (\blacktriangle) Immature pulp extract, (\blacksquare) Maturing pulp extract, (\bullet) Mature pulp extract. Graph derived from the equation $t = 1/k \cdot (x/a(a-x))$, where the terms a, x, t and k have meanings equivalent to the definitions in the legend of Fig.IV.28.

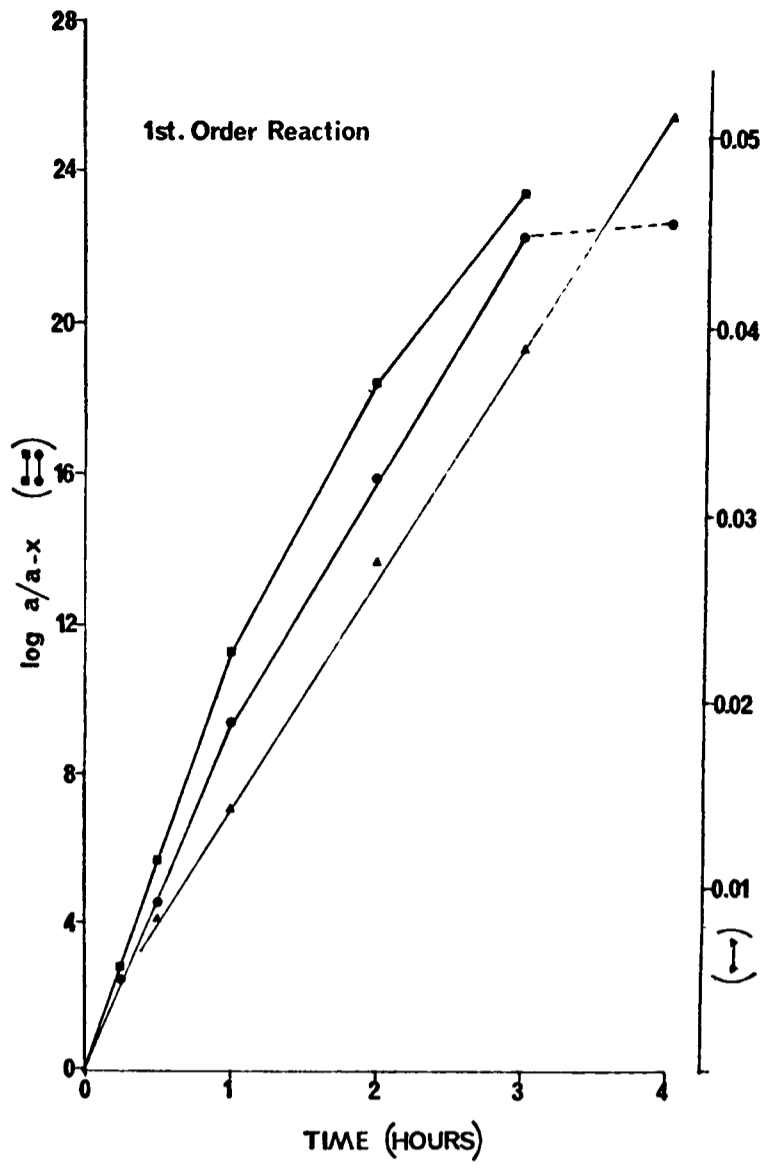


Fig. IV.28

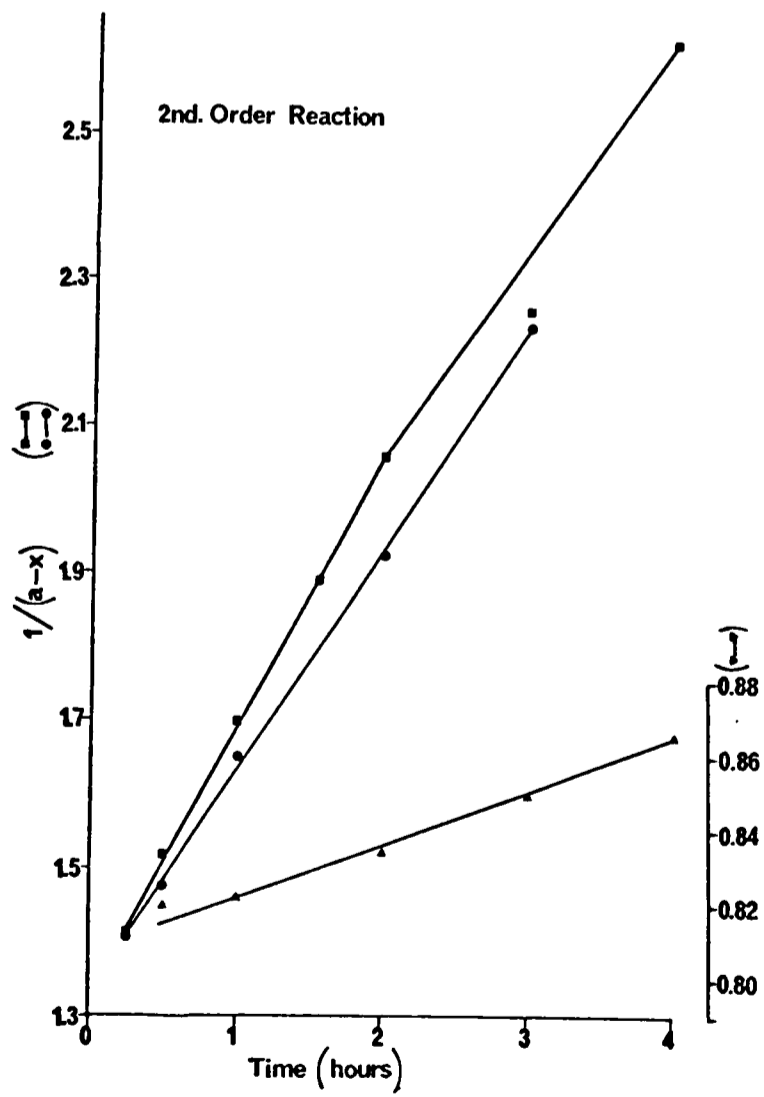


Fig. IV.29

Fig. IV.30-IV.32: Dependence of enzyme velocity on substrate concentration at the three ripening stages.

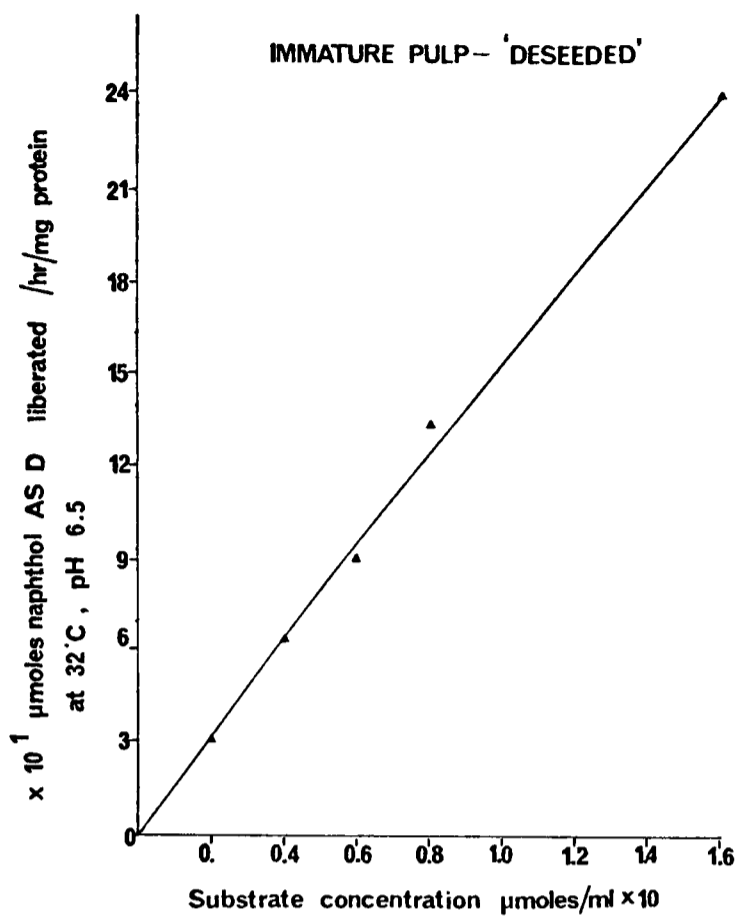


Fig.IV.30

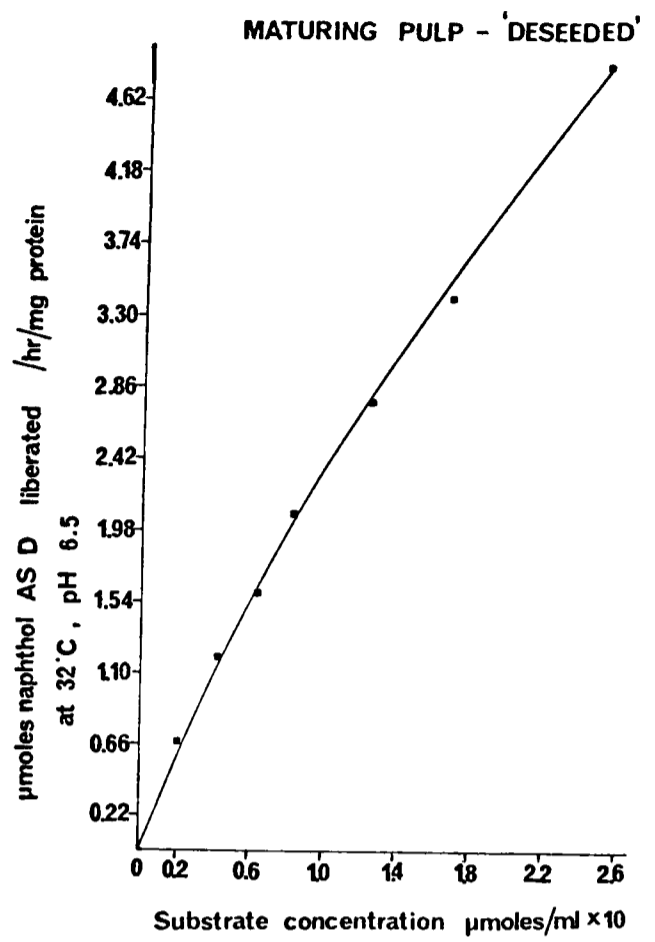


Fig.IV.31

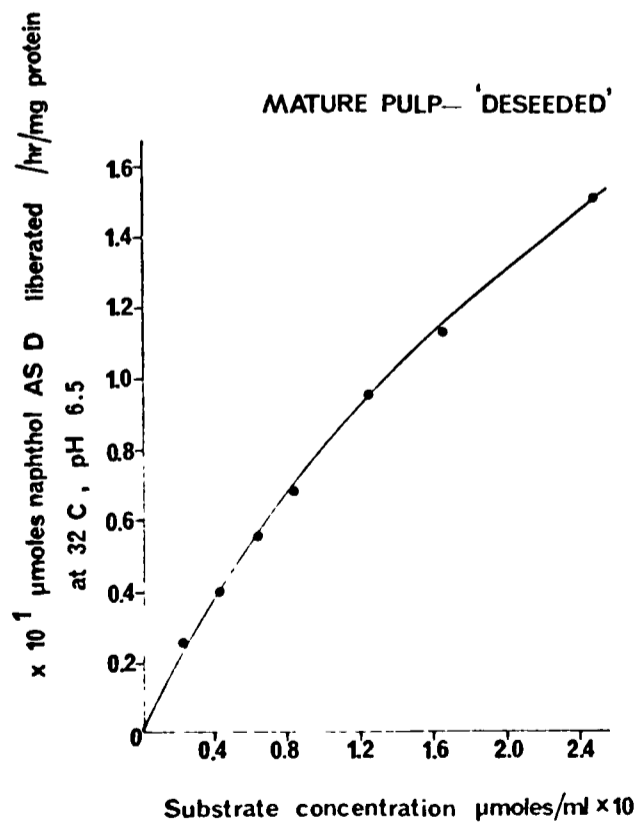


Fig.IV.32

were determined (Figs. IV.33. - IV.35. and Table IV. 13.). Other estimations of K_m from the previous year's fruit, (1971), is included for comparison in Table IV.13. For the graphical determination of K_m values all velocities were calculated on a per mg of protein basis.

Table IV.13. Estimations of K_m from the three stages of strawberry pulp ripening.

Source	Stage of Ripening	K_m (M)	V_{max} (μ moles/h)
Fresh fruit - 1972	Immature	1.66×10^{-3}	33.30
	Maturing	0.26×10^{-3}	8.33
	Mature	0.30×10^{-3}	3.45
N_2 -frozen fruit - 1971	Immature	1.66×10^{-3}	22.20
	Maturing	1.00×10^{-3}	14.28
	Mature	0.71×10^{-3}	3.07

In all cases the enzymes show very little affinity for their substrate and in the case of immature fruit a substrate concentration equal to K_m is clearly unattainable since the value extrapolated as 1.66×10^{-3} M exceeded the practical solubility of the substrate by a factor of approximately 10. K_m calculations for the other two stages (maturing and mature) produced values that corresponded with the upper solubility limits of the substrate. As noted in Chapter II, (section II.3.2.4.),

Fig.IV.33 - IV.35: Lineweaver-Burke plots ($1/v$ versus $1/S$) of the data in Fig.IV.30 - IV.32 to give apparent K_m values for esterases at the 3 different stages of ripening.

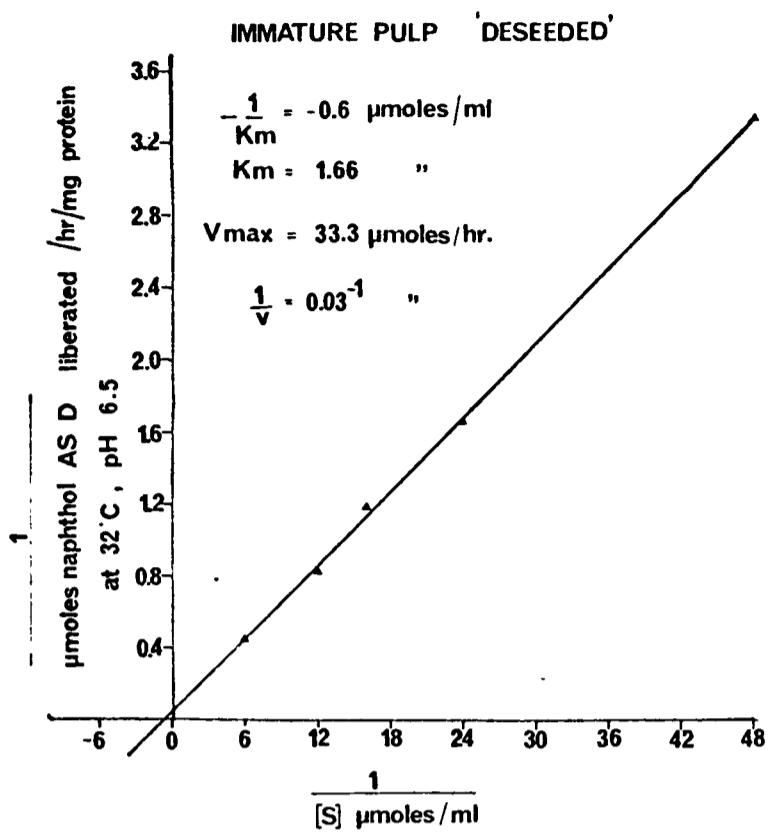


Fig. IV.33

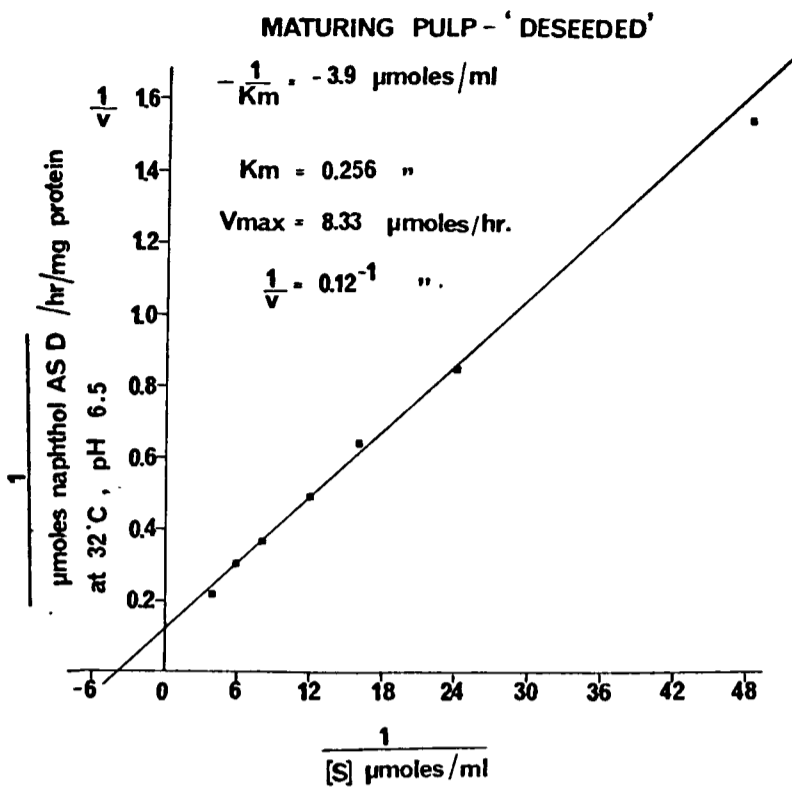


Fig. IV.34

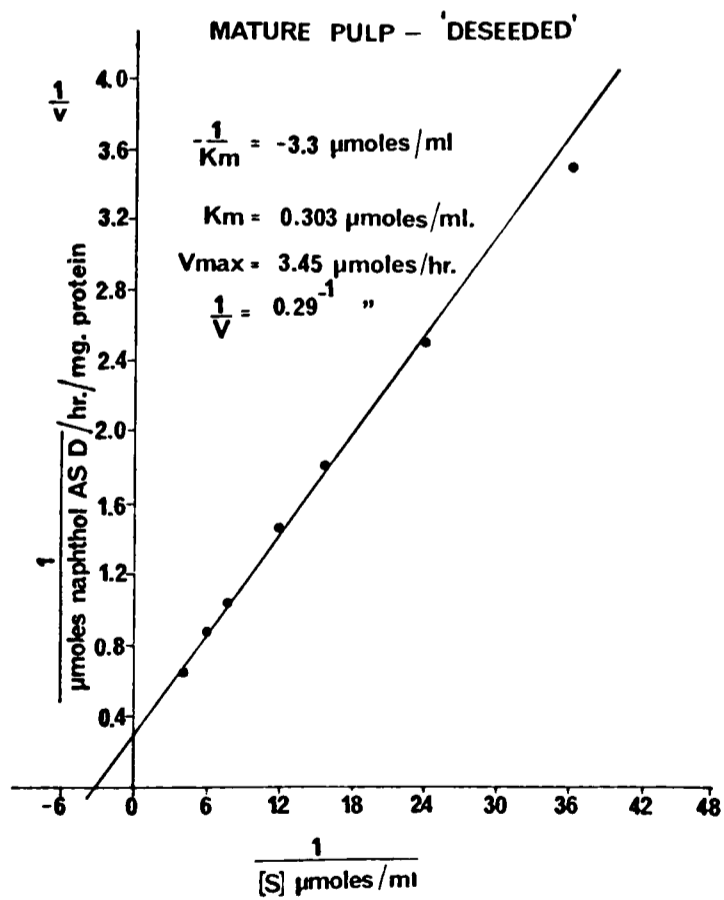


Fig. IV.35

V_{max} is achieved in a hydrolytic reaction only when the substrate concentration approaches 10 x K_m. Clearly these values are of little significance when considered as absolute measurements but serve to show that there is a fall in apparent K_m values by a factor of about 5 in the transition from immature to maturing berries, denoting a possible increase in affinity of the enzymes for their substrate as ripening proceeds i.e. a change in the isoenzyme population.

Table IV.14. % V_{max} determinations of the various pulp extracts for 2 substrate concentrations and 2 enzyme concentrations after 1 h of hydrolysis.

Stage of Ripening	Enzyme* concentration	[S ₀]	[S ₆₀]	% V _{max} .
Immature fruit	100	0.0835	0.0696	4.02
	10	"	0.0747	4.31
	100	0.167	-	-
	10	"	-	-
Maturing fruit	100	0.0835	0.0575	18.3
	16	"	0.0697	21.4
	100	0.167	0.1255	32.9
	16	"	0.1440	36.0
Mature fruit	100	0.0835	0.0606	16.7
	16	"	0.0740	19.6
	100	0.167	0.1246	29.1
	16	"	0.1493	33.0

[S₀] Initial Substrate concentration

[S₆₀] Substrate concentration after 1 h of incubation.

* 100 = arbitrarily chosen greatest enzyme concentration taken from Figs. IV.22 - IV.24.

IV.5.4.4. pH profiles of the 3 ripening stages.

A series of solutions was prepared of varying pH, employing McIlvaine's citrate-phosphate buffer, from 3 - 5.5 and Tris-maleate from 5.0 - 7.3.

The esterases extracted from ripening pulp exhibited fairly broad pH optima, although there was a slight shift to a more alkaline pH as fruits ripened, (Fig. IV.36.).

An overlap in pH values between the two buffers, citrate-phosphate and Tris-maleate, were in practice difficult to achieve because of the effect of DMF (See also Section II.3.2.5.).

Fig.IV.36: pH profiles of strawberry pulp extracts from the three stages of ripening. (\blacktriangle — \blacktriangle) Immature pulp, (\bullet — \bullet) Maturing pulp, (\blacksquare — \blacksquare) Mature pulp. Dotted lines signify the change-over point of the buffer systems.

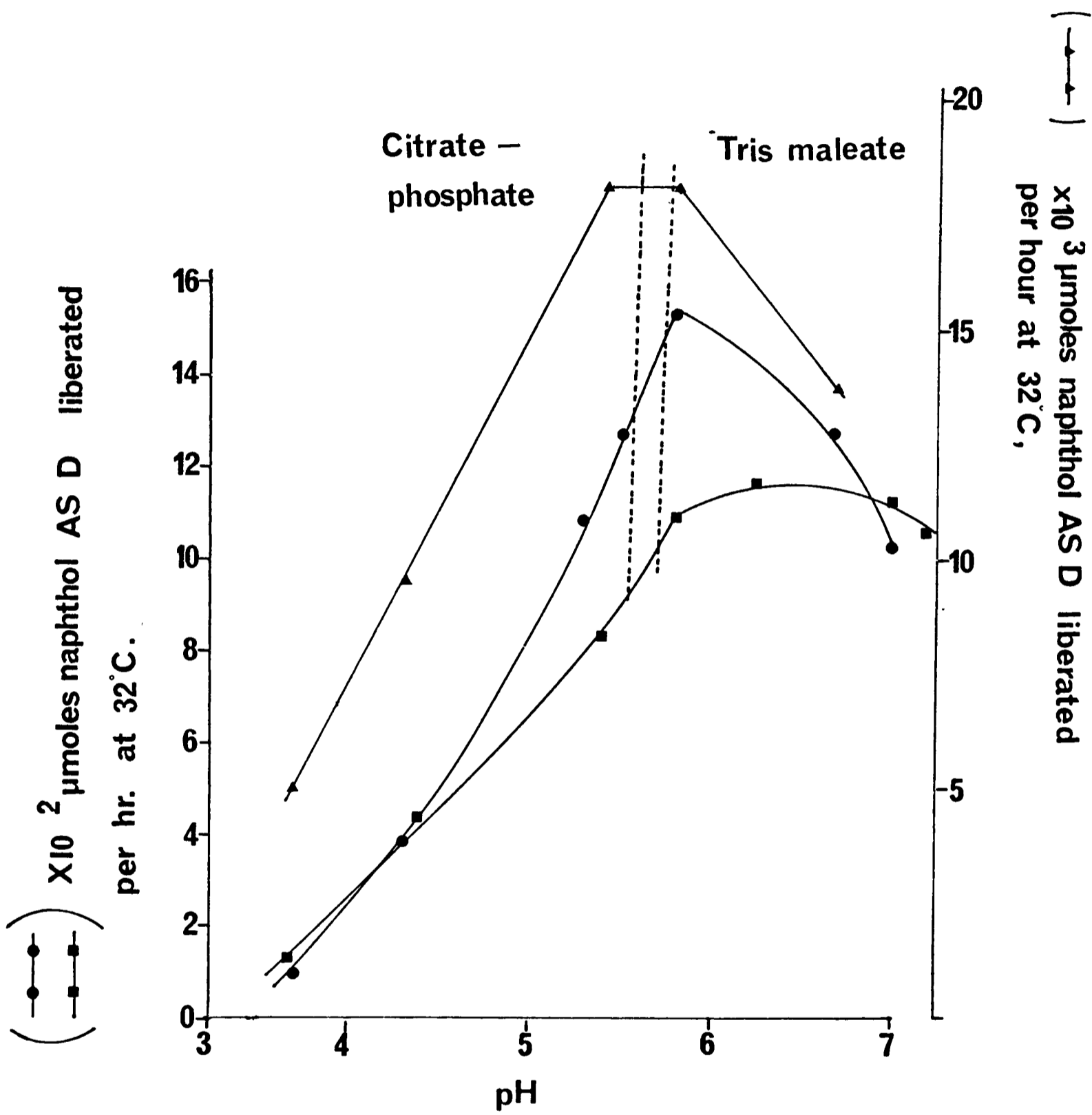


Fig. IV, 36

IV.6.

DISCUSSION

The recent discovery (James and Smith, 1972) that L-ascorbic acid can interfere with the diazo-coupling of hydrolysed substrate and the coupling reagent imposes a severe limitation on any conclusions that can be drawn from the cytochemical studies of soft fruit ripening. Fruits such as strawberry and blackberry, when ripe, have extremely high endogenous levels of L-ascorbic acid (mean value of ca. 60 mg/100 g fresh wt of fully ripe strawberry pulp, - Mapson, 1970) and the lack of enzyme end-product in fruit tissue sections after incubation does not necessarily mean that the enzyme being localized is absent. This may also account for the lack of enzyme staining in the drupelet mesocarps of raspberry and blackberry.

It is unfortunate too, that endogenous levels of ascorbate can fluctuate so markedly between individual fruits from the same plant, or from different plants, between the different stages of ripening, between years, and between the regions where grown, (Olliver, 1938). These factors may well explain the variability of the cytochemical data for ripening strawberry pulp over the 4 seasons examined.

Although these factors must be borne in mind distinct patterns of esterase staining were still observed in all 3 soft fruits.

The tissues that stained most noticeably in all soft fruits were the procambial strands of developing embryos, mature differentiated vascular traces and the receptacle parenchyma.

Since the ripening strawberry receptacle is known to be high in endogenous ascorbate (Mapson, 1970) and staining of some

cells and tissues still occurred, it is difficult to see how tissue sections of raspberry and blackberry epicarps and mesocarps could have remained free of any enzyme end-product, unless the enzymes were absent or the endogenous ascorbate levels in the drupelet flesh were much higher than those in the receptacles of either the strawberry or the raspberry.

In the strawberry receptacle non-specific esterases have been shown to exist in a variety of subcellular locations throughout the 5 developmental stages of the post-fertilisation receptacle. The particulate form of the azo-dye, existing as structures 0.5 - 1.0 μ m in diameter, was evident in very young receptacles and those undergoing ripening, whilst the huge, vesicular, bodies with esterase activity, were only present in ripening tissue (stages C, D and E).

It is not certain whether the large membranous-looking structures staining diffusely with enzyme end-product in young strawberry receptacles (A and B) and ripening raspberry receptacles are artefacts caused by freezing and sectioning or whether they represent true sites of heightened esterase activity. Only reliable electron microscope cytochemistry and additional cell fractionation studies would determine their cytological nature and whether they represent the foci of lytic activity and the autolysis of degenerating subcellular organelles (Matile and Winkenbach, 1971).

The subcellular localisation of esterases in ripening strawberry pulp (stages C, D and E) was considerably hindered by the poor morphological preservation and extremely large cell size. However it is probable that, judging from their spherical shape and continuous range in size, the larger vesicles result from the expansion of much smaller esterase-containing particles, possibly

even of the smallest ones observed, (0.5 μ m). Certainly the ultrastructural evidence from Chapter V, and other work (Butler and Simon, 1971) suggests that organelle vesiculation is a characteristic feature of plant cell senescence.

Structures of about the same size (up to 30 μ m in diameter) and shape containing both acid phosphatase and esterase activity have previously been seen by Pitt and Coombes, (1969) in potato tubers infected with Phytophthora erythroseptica. In this case cells displayed a great deal of diffuse staining. However neither very large particles nor diffuse staining were visible in uninfected tissues. Furthermore their biochemical evidence showed that upon infection a greater proportion of the total acid phosphatase activity could be extracted from the supernatant fraction than from the particulate fraction, almost the reverse of the results for uninfected tissue. These workers were therefore probably justified in concluding that the diffuse staining resulted from the disruption of the host cytoplasmic particles, a situation leading to the rapid death of the host cells and later softening of the tissues.

In strawberry pulp cells diffuse stain was never seen and there was never any evidence of fungal infection. However, it is unfortunate that a study of obviously overripe, and therefore late senescent, tissues was precluded since the observed particles may at these later stages have released their enzymes into the surrounding cytoplasm, thus causing widespread cell death and fruit softening. This would accord with the view of Butler and Simon (1971), who have proposed that in any senescing plant cells lysosomal enzymes would only be released in the final stages of senescence since these enzymes have a very low degree of specificity

and therefore once activated would cause total cell destruction rather than the more gradual sequence of changes that have been observed in early senescence.

The implication is therefore, that at some critical and possibly programmed time, the membranes of these large cytoplasmic acid hydrolase-containing particles undergo some modification in order that the enzyme contents are 'spilled out'. This could conceivably be the case for the esterase-containing bodies in strawberry pulp cells but evidence from the sonication of root cells (Section III.3.4.), suggests that a certain proportion of the enzymes would still be tightly bound. However, it is possible that changes in the structure of the bounding membrane permits both easy escape of the enclosed 'soluble' hydrolases, and a 'loosening' of any structurally attached enzymes.

This loss of differential membrane permeability, as it applies to cell membranes generally, has been cited as one of the contributing factors in the initiation of fruit ripening and senescence (Bain and Mercer, 1964; Sacher, 1966, 1967). According to this school of thought the loss of the membranes' structural and functional integrity leads to the random mixing of enzymes and substrates, and is one of the reasons for the increases in enzyme activity during ripening, so often observed in biochemical studies (Dilley, 1970).

The point needs to be made, however that large increases in esterase activity during strawberry ripening did not appear to be accompanied by the release of the enzymes from their particle bound state, at least as far as the cytochemical results show. The increases in esterase activity per fruit with ripening therefore probably represent an increase in the number of particles per fruit.

There is some evidence from the biochemical data that deep-freezing of strawberry pulp leads to some increases in measurable esterase activity. If this were not due to achene damage then it could be argued that the latency of the esterases residing in particulate forms is released more efficiently by prolonged freezing combined with short periods of freeze-thaw than by short periods of freezing and thawing alone.

The pH profiles show that esterases from all 3 ripening stages have a broad pH optimum of 5.5 - 6.5, values very similar to those for tomato non-specific esterases recorded by Heftmann (1971) as 5.0 - 7.0.

Non-specific esterases from strawberry pulp cells therefore may display latency, have acid pH optima and be particle-bound. In addition, Heftmann (1971) has shown that tomato non-specific esterases are sedimentable in a sucrose density gradient and are therefore also particle-bound. Together with these lysosome-like characteristics, the evidence from this and other work (Pitt and Coombes, 1969; Heftmann, 1971; Matile and Winkenbach, 1971) suggests that plant acid hydrolases are intimately involved in cell senescence, cell injury and cell death; known physiological functions of these enzymes in animal cells (de Duve and Wattiaux, 1966).

The above observations lead naturally to an examination of the biochemical data. Measurements of esterase activity during ripening of the strawberry show that, like many other hydrolases in other fruits, there are considerable increases during the change from the green, immature berry to the maturing white-red stage. This is so whether activity is expressed on a per berry, per g fresh

weight or per unit protein basis. Although activity remained high in ripe fruits on either a per berry or a per g fresh weight basis it fell substantially on a per unit protein basis.

Before deciding on the significance of these findings a number of other factors affecting these results, encountered during enzyme and protein extraction, must be considered.

Enzyme activity units are expressed on any of the 3 bases previously mentioned, criteria which are all ultimately dependent on the total amounts of protein extracted from the various stages of ripening. Both sets of data from the frozen and stored 1971 fruit and the fresh fruit 1972 season show a similar increase with ripening in the quantities of protein extracted. These increased yields could be the result of at least 3 factors:

a) A true increase in the quantity of protein synthesised during ripening. This has already been shown to be the case with apple (Hulme et al., 1968) pear, (Frenkel et al., 1968,) tomato, (Davies and Cocking, 1967) and banana (Brady et al., 1970).

b) The presence of inhibitor substances in fruit at earlier stages of ripening that may mask the presence or activity of enzymes. Weurmann (1953) has provided evidence for a pectinase inhibitor in developing Pyrus fruits. In particular, the presence of protein-complexing agents e.g. phenols, in immature fruit decreased as ripening proceeded (Williams 1959; Craft 1961; Young, 1965) so that artificially low yields of protein may be obtained from less mature fruits, and

c) As ripening proceeds a greater proportion of membrane-bound proteins may be solubilized as membranes and organelles are autolytically digested, thereby facilitating protein extraction from riper fruit (Miller and Romani, 1966). Romani

and Fisher (1966) noted a decrease in mitochondrial protein of pear during maturation, as did Dickinson and Hansen, (1965) for tomato tissue.

Besides the possibility of differential enzyme and protein extraction, it is apparent that the amount of protein extracted per berry and per g fresh weight at each ripening stage varied considerably between the two seasons 1971 and 1972.

The variability in fruit generally of, for instance, the amount of protein nitrogen, is well documented (Robertson and Turner, 1952). In the present study there are very obvious sources of variation. Probably the greatest of these may be ascribed to the difficulties in assessing, through colour changes only, the stage of ripeness of each berry. The other stems from the need to remove achenes from the pulp before homogenisation. As fruits generally contain more protoplasm per cell in the outer layers of their tissues (e.g. Hulme, 1936; Hansen, 1955; Bain, 1958), it is likely that the figures for protein extraction in maturing and mature tissue from 1972 season are lower than their true values since there was an unavoidable loss, (increasing with progressive ripening) of epidermal tissue during achene removal.

Despite these problems of interpretation there are a number of reasons for believing that both enzyme and protein increases were real and not due to the artefacts mentioned above.

By far the most convincing arguments are provided by the data showing enzyme activity expressed on a per unit protein basis (i.e. specific activity). Highest values were always recorded for berries that had begun to go red (the 'maturing' stage) followed by a substantial drop in the ripe fruit. This must essentially

represent a dilution of the esterase molecules in the total protein population, since total protein per berry continued to increase whilst esterolytic activity, expressed on either a per berry or per g fresh weight basis, was of the same order in white-red and red, ripe berries.

Closely related to these observations is the fact that K_m values of the esterases decreased by a factor of about 5 as strawberries changed from green or green-white to white-red. The inference is then that the increase in enzyme activity between these two stages of ripening is triggered by a change in the affinity of the esterases for their natural substrates, possibly by a progressive loss of enzyme inhibitor(s) as ripening proceeds.

Two other factors that might explain the greater protein recovery from riper stages are the use of large amounts of PVP in the extraction medium (Loomis and Bataille, 1966) and the loss of protein from the skins of riper strawberries during achene removal. The first suggests that figures for protein and enzyme recovery in immature green fruits are not underestimates and the second that those for the two later stages of ripening are underestimates. Consequently one might expect differences in protein and enzyme levels between immature fruits and the two older stages of ripening to be somewhat greater than those observed.

The pattern of growth of strawberry receptacles during ripening, plotted either as fresh or dry weight gain, follows a single sigmoid curve (Crane and Baker, 1953). Evidence presented here suggests that increases in fresh weight and cortex cell size are matched by parallel increases in esterase activity and protein synthesis up to the time the berry begins to acquire a red

colouration ('maturing' stage). As the berry becomes uniformly red, enzyme activity expressed on both bases continues to increase but at a much reduced rate. The arbitrarily designated maturing 'phase' may therefore mark the end of the growth phase and the switch to senescence.

Cytological evidence recorded in Chapter V supports this idea; such subcellular events as organelle vesiculation and loss of ribosomes were more noticeable in the pulp cells from maturing receptacles than in immature pulp cells.

However, the cytochemical evidence has already indicated that the red, ripe fruits chosen for this study do not represent berries in the stages of late senescence, since the esterase activity is still particulate and not diffuse. It may be convenient, therefore, to regard white-red berries as representing early senescing fruit; red, ripe firm berries as mid-senescing fruit, and overripe berries as late senescing fruit.

As far as is known only Heftmann (1971) has examined non-specific esterases biochemically, in relation to fruit ripening; greater esterase activity in cell fractions from red, ripe tomatoes was demonstrated compared with that in green, immature fruit. Heftmann also showed that enzyme activity was not associated with any particular fraction after separation of the tissue homogenates on a sucrose density gradient, thereby denoting that the particles containing esterase activity were of diverse bouyant densities and therefore probably, of differing size also.

The lack of esterase end-product staining on fruit cell walls throughout the increase in cell size, combined with the observed inability of tomato pectin methyl esterase to hydrolyse

the histochemical substrate naphthol AS D acetate, suggest that cell wall staining seen in other plant cells (McLean and Gahan, 1970) is not due to this enzyme.

However, the biochemical evidence is far from unequivocal in that a variety of factors could have produced the results observed; denaturation of pectin methyl esterase during dialysis; absence of any catalytic activity in the preparation supplied, or perhaps, most likely of all, destruction of the enzyme by one or more components of the assay system, could have been responsible for the final results. The data of Deuel and Stutz (1958) and Gizis (1964) suggest that pectin methyl esterase is specific for pectin and so one might expect that it would display no catalytic activity towards naphthol AS D acetate. Clearly this particular problem is unresolved and merits more extensive investigation.

Despite the preliminary nature of the biochemical data for achene extracts it is interesting that esterase activity reached a peak in both achenes and receptacles at the 'maturing' stage of fruit development. Furthermore the magnitude of this increase was of approximately the same order in both structures.

The decline in specific activity of the achenes as they passed from the white-red to the red stage of receptacle development was however, less than the concomitant decline in the specific activity of the receptacle. Clearly further investigation in this direction is needed to establish the nature of the relationships existing between esterase levels in the achenes and the receptacle of the strawberry fruit.

CHAPTER V

THE DEVELOPMENT OF METHODS FOR THE ULTRASTRUCTURAL
LOCALISATION OF NON-SPECIFIC ESTERASES IN PLANT
CELLS

V.1.

INTRODUCTION

The accurate cytochemical detection of acid hydrolases (and enzymes generally) at the subcellular level enables the sites of enzyme activity and their relationships to specific cell organelles to be defined. The cytochemical findings may be verified with appropriate tissue fractionation techniques, followed by biochemical assay of the enzymes under test. However, at present the ultrastructural detection of enzymes is fraught with a number of technical and chemical problems (Shnitka and Seligman, 1971). The main obstacles are: i) loss of enzyme activity through lengthy fixation procedures which are vital for adequate cellular preservation; ii) the lack of suitable electron-dense coupling salts forming stable, insoluble, complexes with their hydrolysed substrates.

The aim of the present study was to explore the use of ultracryotomy to circumvent the use of fixatives and secondly to extend the findings of Livingston and coworkers (1969), Beadle et al., (1971) and Gahan and McLean (1969), who used different electron dense coupling salts for the ultrastructural localisation of a number of acid hydrolases in both animal and plant cells. The first of these salts, diazotised lead phthalocyanin, was used by Gahan and McLean (1969) to define sites of non-specific esterase activity in root tip cells of Vicia faba. In the present work the same diazotate was used to localize esterases in developing strawberry fruits, and attempts have been made to follow morphological changes in subcellular organelles during the ripening process.

Later, an improved coupling salt, diazotised triphenyl-p-aminophenethyl lead (LPED) was kindly made available by Dr. D.C. Livingston, (Imperial Cancer Research Fund) to detect esterases in various tissues of Vicia faba roots, bearing in mind the particulate nature and characteristic location of the enzymes revealed by optical microscope studies, (Chapter III).

Also, since conventional alcohol dehydration of tissues for electron microscopy has been shown to remove end-product from tissues (Livingston et al., 1969) water soluble Durcupan has been used as an alternative dehydrating agent. A preliminary study was made of alcohol and Durcupan-treated root tip tissues and consequent changes and differences in the appearance of subcellular organelles were noted.

The main aim of this work was to extend some of the existing techniques for the ultrastructural detection of non-specific esterases in plant cells. In no way was it meant to be an exhaustive study but, rather an exploratory approach was used because of the limitation of time available.

The work may be conveniently divided into 4 sections.

A) Ultracryomicrotomy of plant cells, circumventing the use of fixatives:

Work on this aspect has been reported elsewhere (Gahan et al., 1970). A copy of this publication is kept at the back of the thesis.

B) A comparison of the effects of Durcupan and ethanol-induced dehydration in root tip cells.

C) The ultrastructural detection of non-specific esterase in developing strawberry fruits, using diazotised lead phthalocyanin as coupling salt.

- D) The ultrastructural detection of non-specific esterase in root tip cells of Vicia faba, employing diazotized LPED as coupling salt.

V.2.

MATERIALS AND METHODS

V.2.1.

Durcupan vs. Ethanol Dehydration. (B)

One week-old lateral root tips of Vicia faba, measuring 1 cm in length were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.1, for 3 h at +4°C. Root tips were cut longitudinally and transversely with the aid of forceps and razor blade. Areas corresponding to the root cap, apical initials, procortex and central cylinder were then dissected out from the terminal 3 mm of the tip. The operations were carried out with the aid of a binocular microscope under fluorescent light. Pieces of tissue were never larger than 1 mm³.

All material was then washed in 3 changes of cacodylate buffer, before post-fixing in 2% osmium tetroxide in 0.1M veronal acetate buffer, pH 7.1, at +4°C for 1 h.

Specimens were further washed in 3 changes of distilled water before dehydrating in either i) a graded series of ethanols or ii) a graded series of Durcupan (Fluka)/water mixtures.

i) Ethanol dehydration:

Tissue was dehydrated by immersing in cold (+4°C) ethanol of increasing concentrations, i.e. 30%, 50%, 70%, 90%, for 1 h each change, before subjection to 2 changes, 1½ h each change, of absolute ethanol, previously dried over silica gel. All material was then further dehydrated at room temperatures. Because of the high viscosity of the embedding agent, araldite, 1, 2-epoxypropane was used as a transitional solvent for infiltrating tissue with the Araldite (Glauert, 1963). The procedure was as follows;

specimens were placed for 1 h in epoxypropane and absolute ethanol, followed by a further hour in epoxypropane alone. The next step involved using equal volumes of epoxypropane and Araldite and then finally 2 changes of 100% Araldite. Material was left in the final change of Araldite at least overnight in an unstoppered container to permit evaporation of any remaining epoxypropane. The tissue was finally embedded in Araldite in shallow tin foil dishes by heat storing at +60°C for at least 2 days. Small pieces of polymerised resin containing the tissue were then glued onto wooden dowelling in the desired orientation.

ii) Durcupan dehydration:

Dehydration was here performed in a graded series of Durcupan/water mixtures of 50%, 70%, 90%, aqueous Durcupan for 1 h at each concentration. The tissue was then immersed in 2 changes of pure Durcupan, the first change after 1 h and the second overnight. Dehydration was performed at +4°C up to this stage but on placing the material in the first Durcupan/Araldite I (Glauert, 1963) mixture (70/30 v/v) the material was brought to room temperature. Subsequent infiltration required the use of increasing concentrations of Araldite I, in the Durcupan/Araldite mixtures. The gradation was as follows:

- 1) Durcupan/Araldite I (70/30 v/v) 2 h at room temp.
- 2) Durcupan/Araldite I (50/50 v/v) 2 h at room temp.
- 3) Durcupan/Araldite I (30/70 v/v) overnight at room temp.
- 4) Pure Araldite I 2 h at room temp.
- 5) 3 changes of Araldite II 1 h (each change) at room temp.
- 6) Material embedded in Araldite II was left at 60°C for at least 2 days.

*Araldite II, see Glauert, 1963 for details.

Hardened tissue blocks were finally mounted on wooden dowelling as previously described.

V.2.2. Esterase localization in strawberry fruits. (C)

V.2.2.1. Tissue preparation and fixation.

Small pieces of commercially grown strawberry fruits (var. Cambridge vigour) measuring not larger than 1 mm³ were dissected from the pulp tissue only, at the various ripening stages already defined (see Chapter IV.). Achenes and epidermal tissue were avoided. Tissue was then fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, at +4°C, for 3 h and then washed in several changes of fresh cacodylate buffer.

V.2.2.2. Incubation.

Dissected and fixed specimens of tissue were incubated for 25 min for non-specific esterases as described in Chapter IV, except that the diazo salt was omitted. Control tissue was incubated in the absence of substrate. After thorough washing in several changes of distilled water, the tissues were post-coupled with 10 mg/ml of lead phthalocyanin diazotate in 0.1M acetate buffer, pH 4.8, for 10 min at +2°C, since the normal pH values employed in the cytochemical detection of non-specific esterases (6.5 - 7.1) are not optimal for the coupling of the hydrolysed substrate with this particular diazotate (Livingston *et al.*, 1969). This prohibits the use of simultaneous coupling procedures, conventionally employed for the detection of enzymes at the optical microscope level.

After post-coupling, the tissues were washed in several changes of 0.1M acetate buffer pH 5.0 to remove non-specifically bound coupling salt.

The material was finally dehydrated in Durcupan and embedded in Araldite.

V.2.3. Tissue preparation and the localization of esterase activity employing diazotised LPED as coupling salt. (D)

Root tips were fixed as described in V.2.2.1. Lengths of root, approximately 1 cm long were then embedded in 15% gelatine and 50 μ m sections cut along the long axis, using a Smith and Farquhar chopper. The operation was performed at +4 $^{\circ}$ C. For enzyme incubation both simultaneous and post-coupling procedures were used. Pieces of tissue were incubated for 30 min when a simultaneous coupling procedure was used and for 10 min when a post-coupling procedure was adopted. All incubations for enzyme detection were at 37 $^{\circ}$ C. The substrate, naphthol AS D was present as a 12 mg-% solution in 0.05 ml of DMF with diazotised LPED at a final concentration of 2.5 mg %. 0.1M Tris-HCl, pH 6.8, was employed as a buffer for the incubation procedure, but 0.1M acetate buffer, pH 5.0 at room temperature was used for post-coupling of hydrolysed substrate. As a control, tissue was incubated in the absence of substrate. In addition, a number of glutaraldehyde-fixed, thin frozen sections (16 μ m) were cut on a freezing microtome and incubated in the same manner as for light microscopy observations. Both methods of coupling were used.

Material was finally dehydrated in Durcupan and embedded in araldite as described previously (V.2.1.).

V.2.4. Preparation of ultrathin sections for electron microscopy.

All ultrathin sections were cut on a Cambridge

Huxley Ultramicrotome. Only those that gave gold or silver interference colours (60 - 190 nm) under fluorescent lighting were used. The sections were picked up on to 200 mesh copper grids (Athene), coated with a film of 0.2% parlodion (Mallinck-rodt.) in amyl acetate. Appropriate sections were examined in an A.E.I. 6b electron microscope, with 25 μ m apertures and accelerating voltages of 40, 60 or 80 KV. When necessary some sections were post-stained in lead citrate (Reynolds, 1963), to increase contrast. Kodak E.M.5 plates were used to obtain electron micrographs.

V.3.

RESULTS

V.3.1. Durcupan vs. Alcohol dehydration.

Electron micrographs from ethanol and Durcupan dehydrated root cells of Vicia faba are presented in Fig. V.1. - V.14. A range of tissues and magnifications are shown, with electron micrographs presented in pairs to allow comparison.

Fig. V.1. shows the presence of spherosomes in Durcupan-treated root cap cells, where tissue was not post-stained in lead citrate. Post-staining can produce a different overall picture of organelle resolution as shown in Fig. V.2. which was post-stained.

Bowen (1971) maintained that in animal cells Durcupan-treated tissue often contained swollen mitochondria, whilst Staubli (1960) observed a shrinkage of nuclei. No such effects were noted here. Microtubules were observed in both Durcupan and ethanol-dehydrated tissue, despite maintaining tissue at +4°C during dehydration (Fig. V.11. and V.13.). Usually dehydration at room temperature is preferred if observations on microtubules are to be made, (Burgess and Northcote, 1967, 1968).

The osmiophilic matrix of developing vacuoles, found abundantly in Durcupan-treated material, was not so apparent in ethanol-dehydrated material although it was not entirely absent. This may suggest that the use of Durcupan improved the retention of lipoid substances in plant vacuoles.

V.3.2. Esterase localisation in strawberry fruits.

As noted by Livingston and coworkers (1969) the morphology of end-product obtained depended on the type of naphthol

FIGS.V.1 - V.30: ELECTRONMICROGRAPHS OF VICIA ROOT CELLS AND
STRAWBERRY FRUIT CELLS.

Abbreviations:

A = amyloplasts
C = chloroplasts or chromoplasts
c.w.f = cell wall forming region
G = golgi apparatus
gs = golgi saccules
gv = golgi vesicles
I.S = Intercellular space
M = mitochondrion
ML = middle lamella
mt = microtubules
N = nucleus
n = nucleolus
O = osmiophilic matrix
p = plastid
pl = plasmalemma
pld = plasmodesmata
RER = rough endoplasmic reticulum
seq = sequestration of cytoplasm
S = spherosome
T = tonoplast
U.I = unidentified inclusion
V = vacuole
W = wall
wi = cell wall inclusion

Fig.V.1: Adjacent root cap cells of Vicia faba. Tissue dehydrated in Durcupan. Post-stained in lead citrate. Bar = 1.0 μ m.

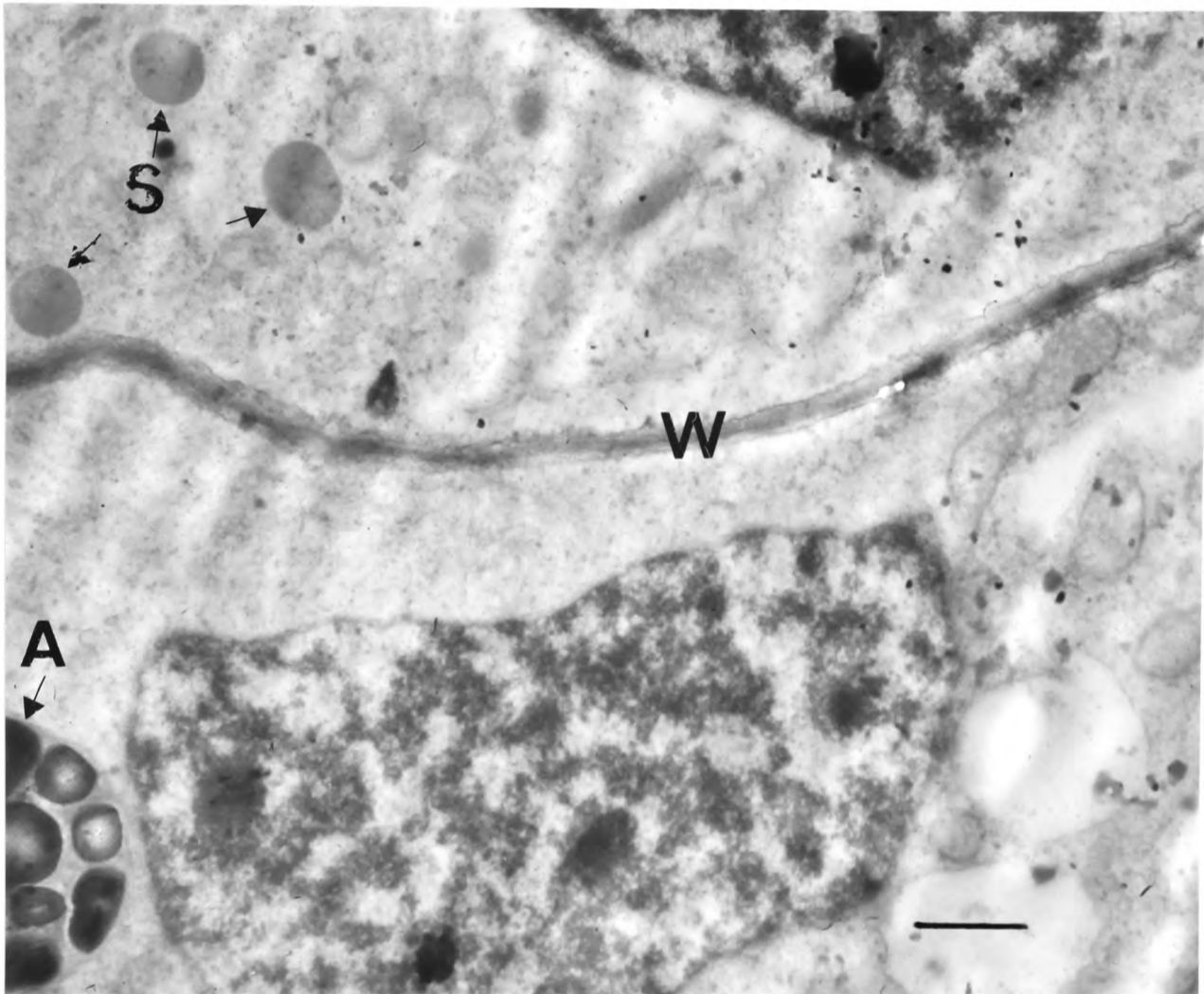


Fig.V.1

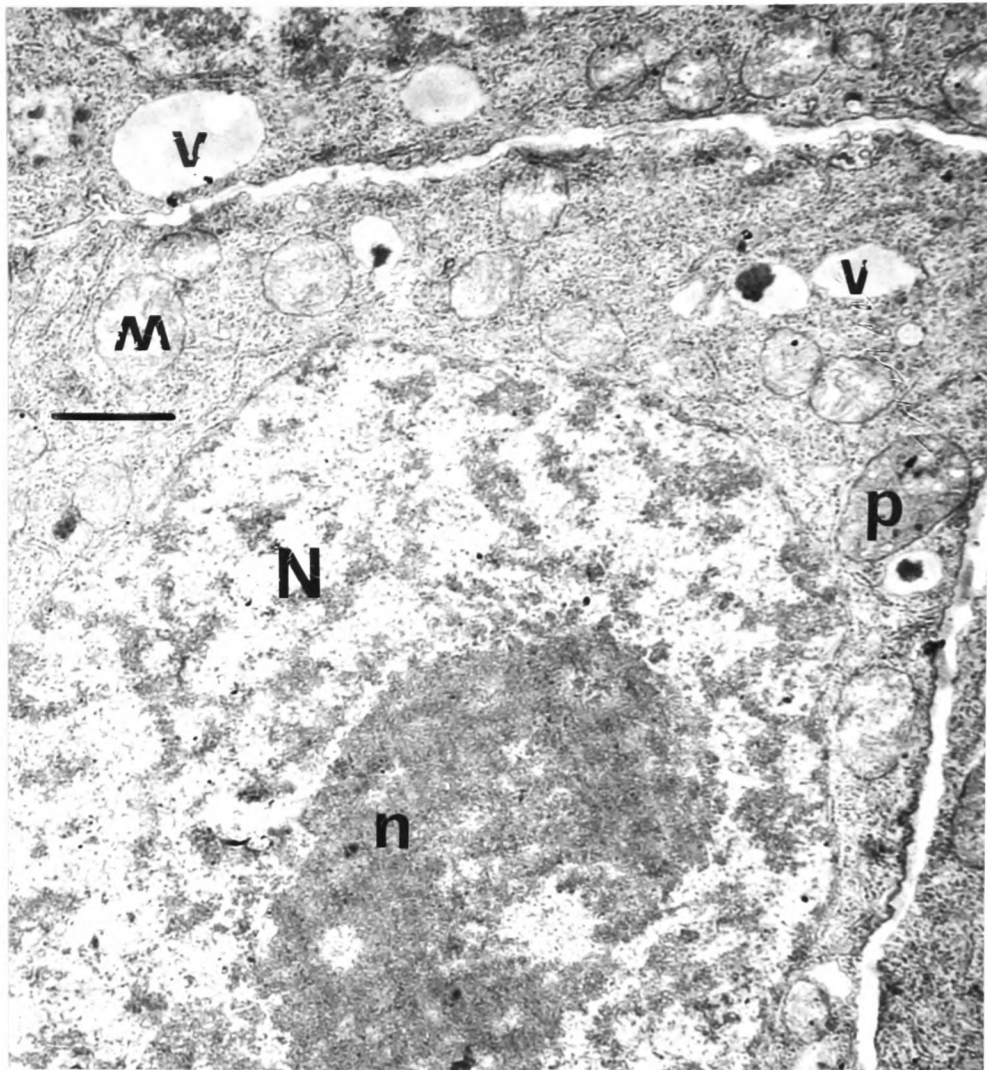


Fig.V.2: Cells from the apical meristem of Vicia root. Tissue dehydrated in Durcupan. Post-stained in lead citrate. Bar = 1.0 μm .

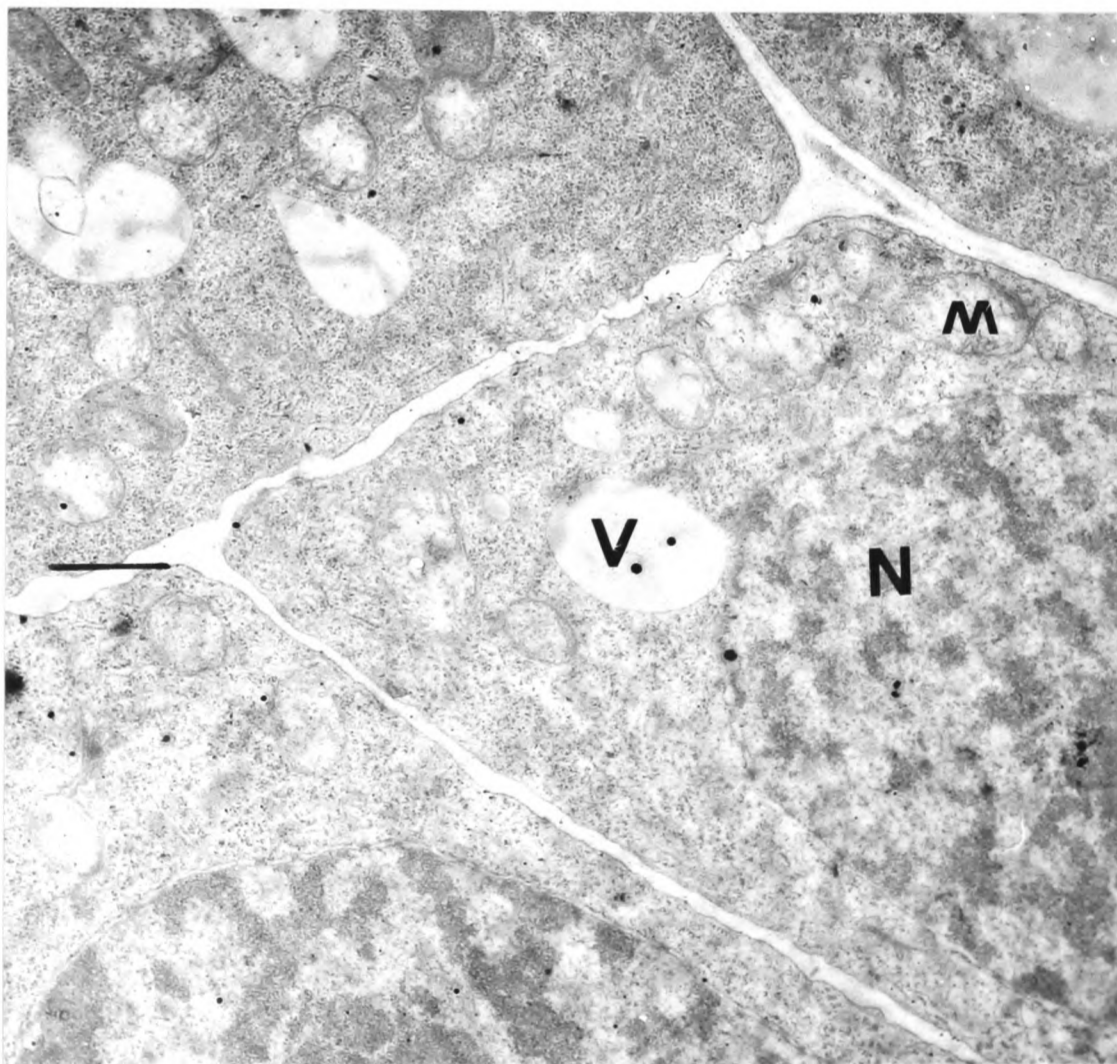


Fig.V.3: As Fig.V.2 but tissue dehydrated in ethanol. Post-stained in lead citrate. Bar = 1.0 μm .

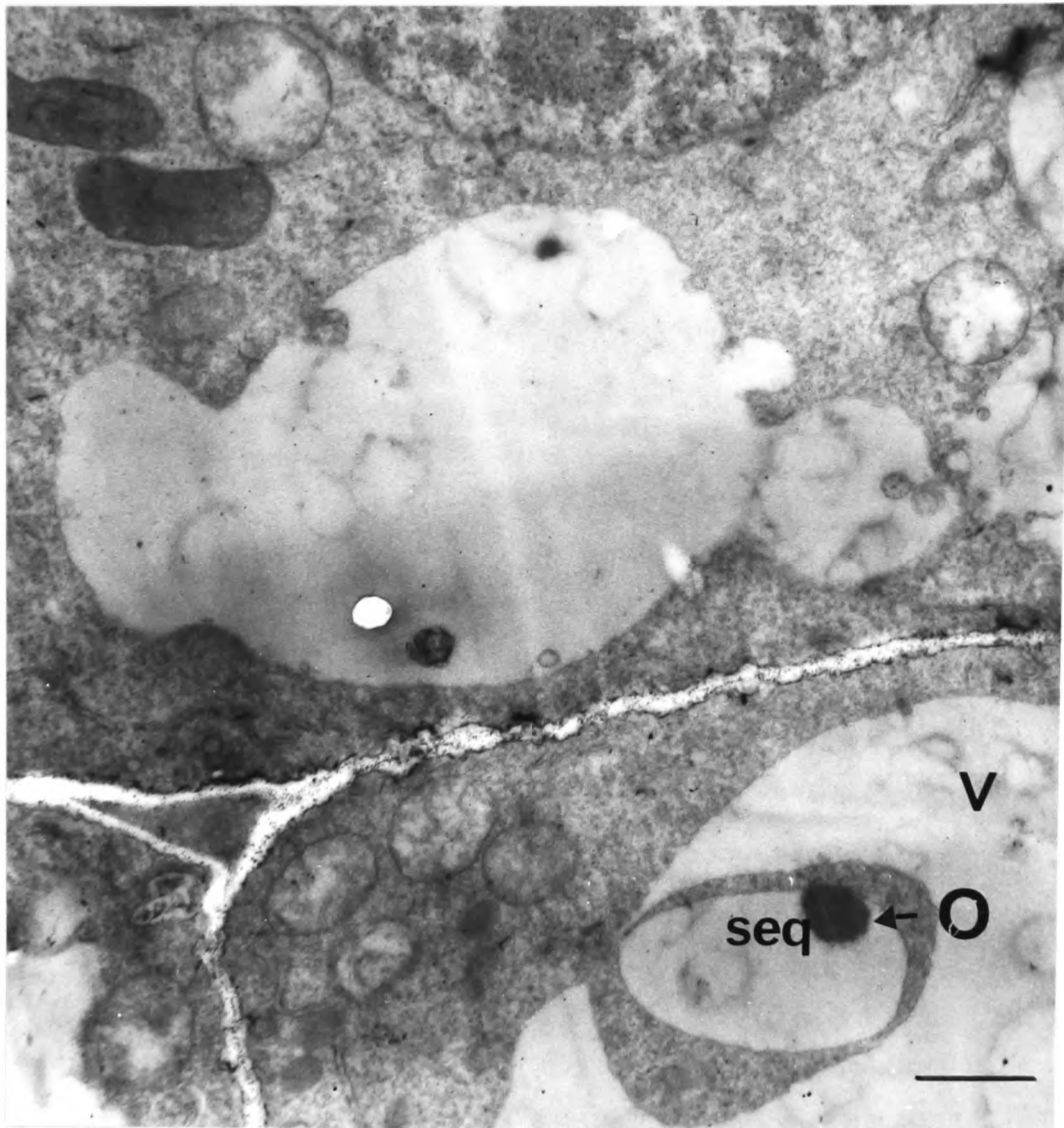


Fig.V.4: Apical meristem cells of *Vicia faba*. The cytoplasm has been sequestered into a vacuole (v) in the lower cell. A number of smaller vacuoles can be seen merging in the cell above. Durcupan dehydrated. Unstained. Bar = 1.0 μm .



Fig.V.5: Procortical cell, ethanol dehydrated. Post-stained in lead citrate. Bar = 1.0 μm .

Fig.V.6: Procortical cell. Durcupan dehydrated. A new cell wall is completing its formation (c.w.f) and at several points (open arrows) a number of minute vesicles can be seen within the new wall. Sequestered cytoplasm (seq) containing ribosomal material can be seen within a vacuole. Tissue post-stained in lead citrate. Bar = 1.0 μ m.

Fig.V.7: Procortical cell. Ethanol dehydrated. Golgi apparatus in glancing section is visible (G'). Post-stained in lead citrate. Bar = 1.0 μ m.

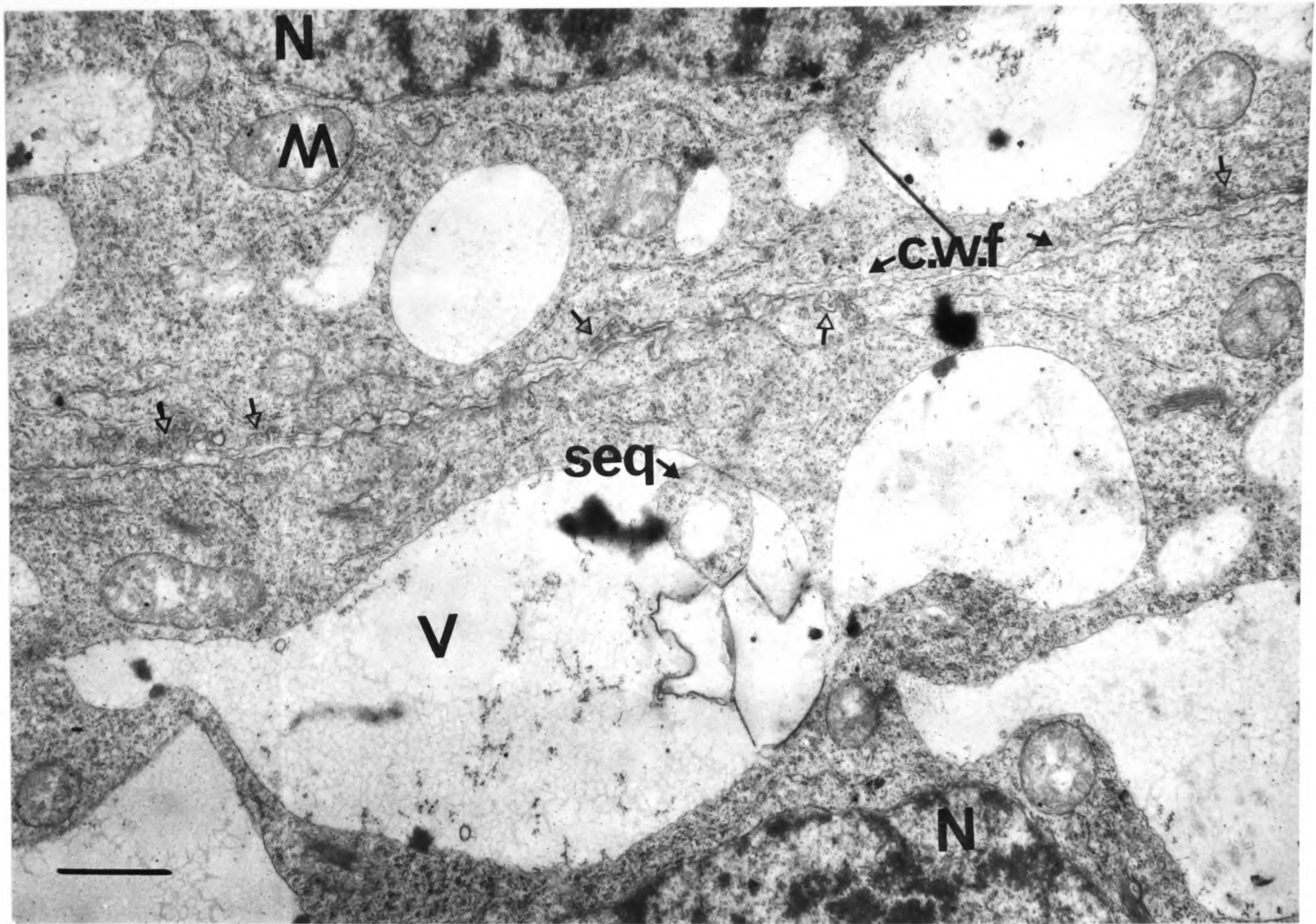


Fig.V.6

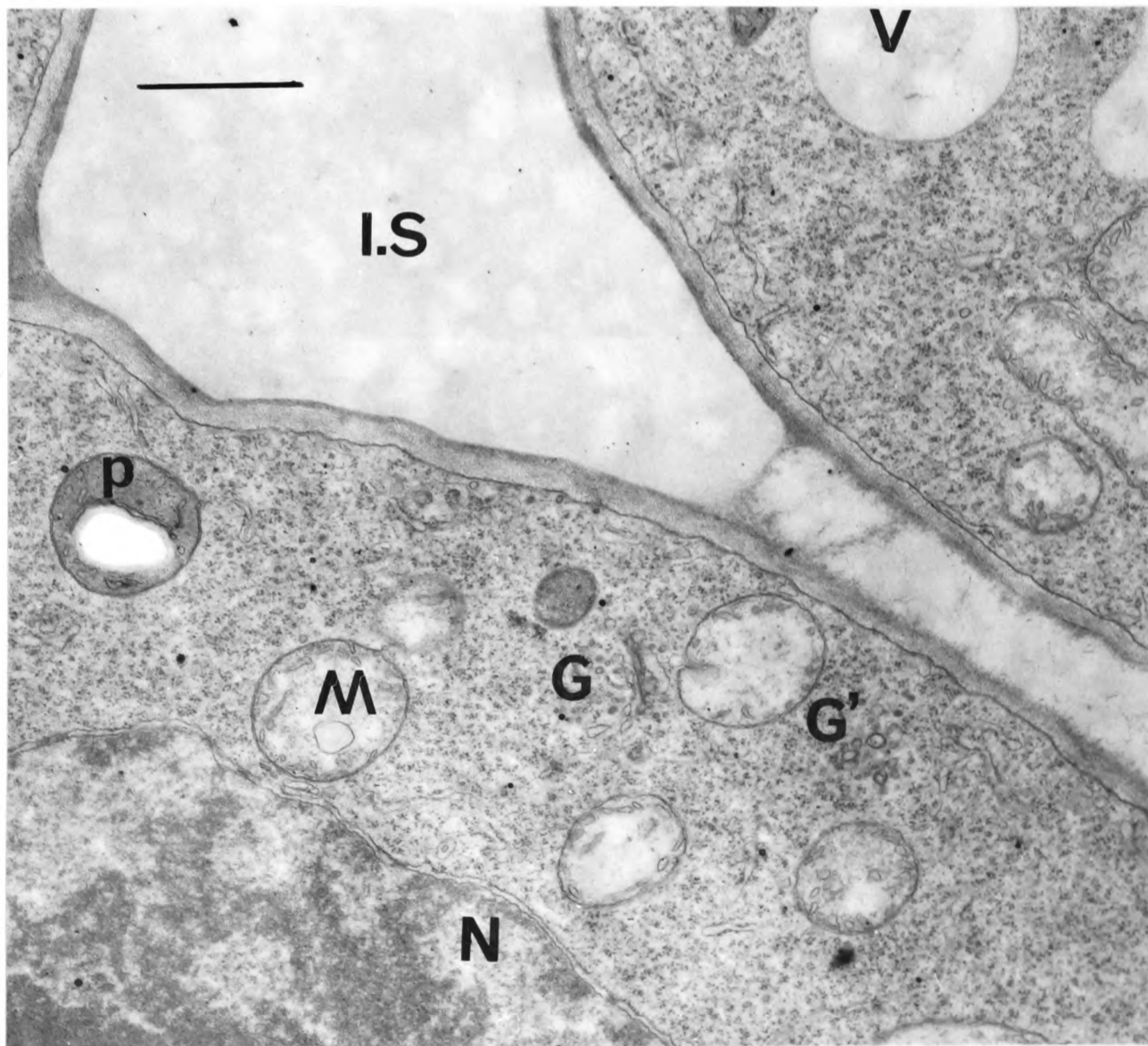


Fig.V.7

Fig.V.8: Meristematic cell. Durcupan dehydrated. Plasmodesmata (pld) and microtubules (mt) are visible. Osmiophilia are apparent in a number of vacuoles. Tissue post-stained in lead citrate. Bar = 0.5 μ m.

Fig.V.9: Meristematic cell. Ethanol dehydrated. The plasmalemma (pl) is indented at one point and a number of wall inclusions (w.i) are obvious underneath. Post-stained in lead citrate. Bar = 0.5 μ m.

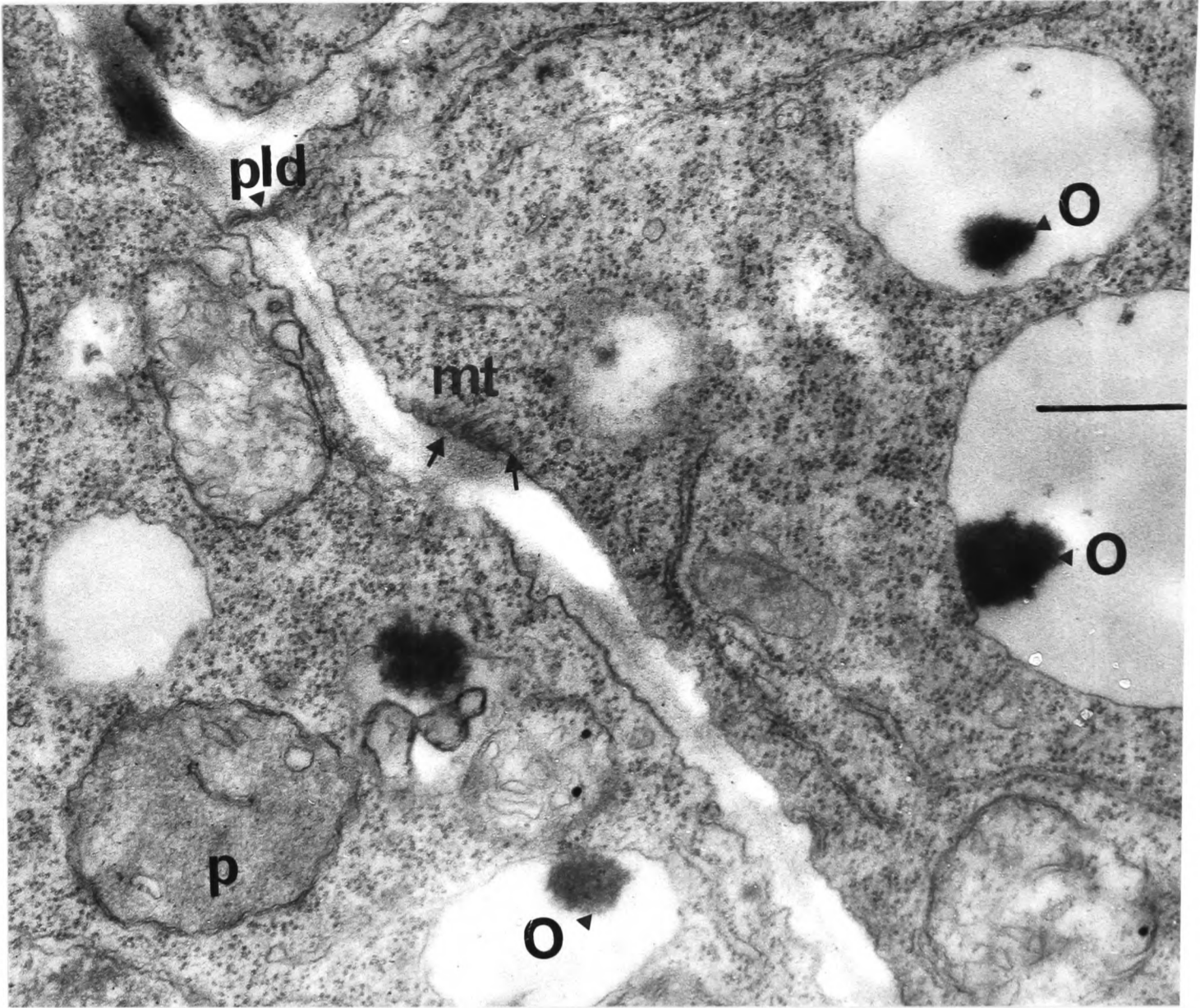


Fig.V.8

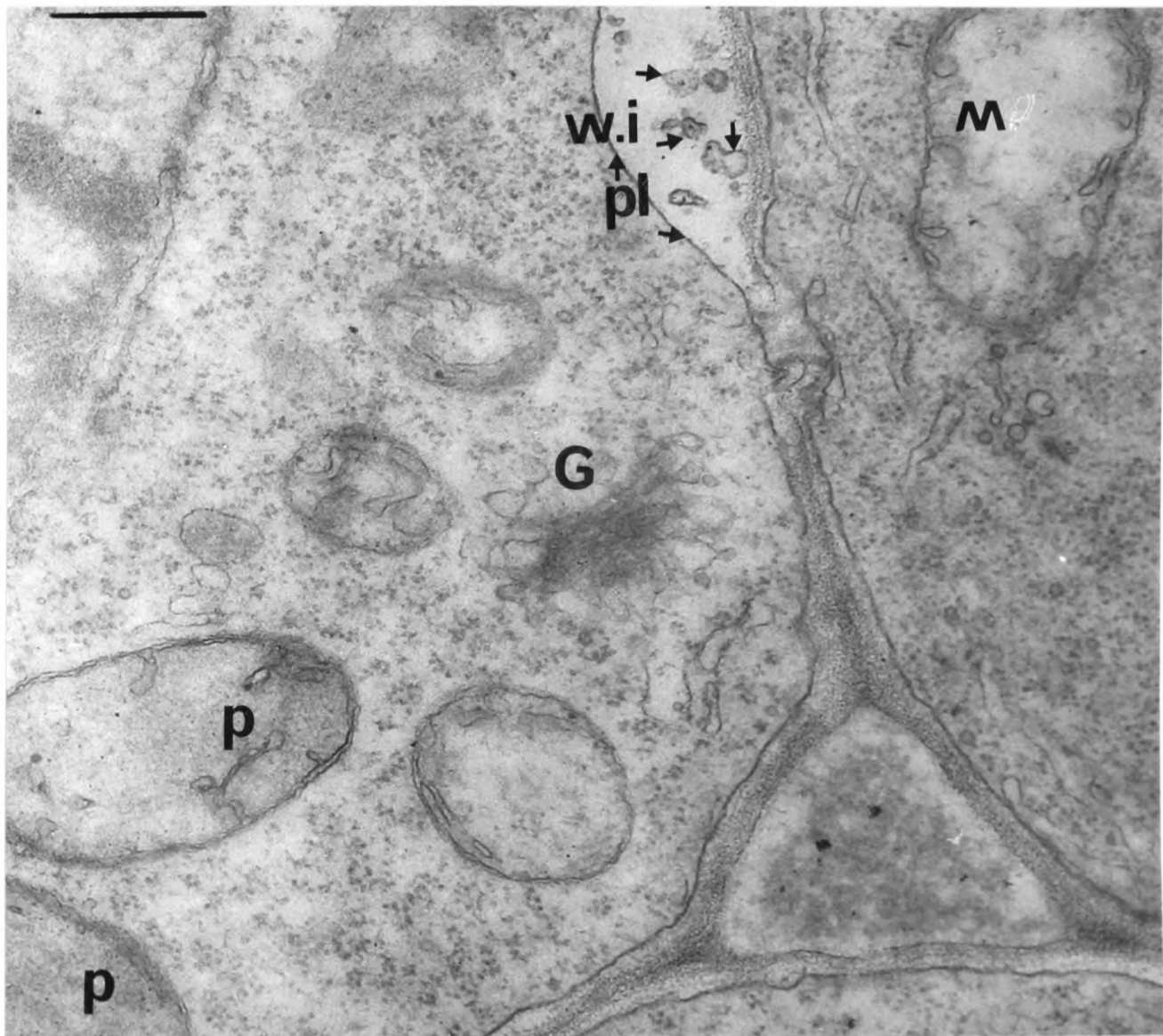


Fig.V.9

Fig.V.10: Meristematic cell. Durcupan dehydrated. Microtubules (mt and arrows) are visible in cross-section at the periphery of the cells. Post-stained in lead citrate. Bar = 0.5 μ m.

Fig.V.11: Procortical cell. Ethanol dehydrated. Microtubules are apparent in cross-section (mt). A spherosome (S) is present at top right. Post-stained in lead citrate. Bar = 0.5 μ m.

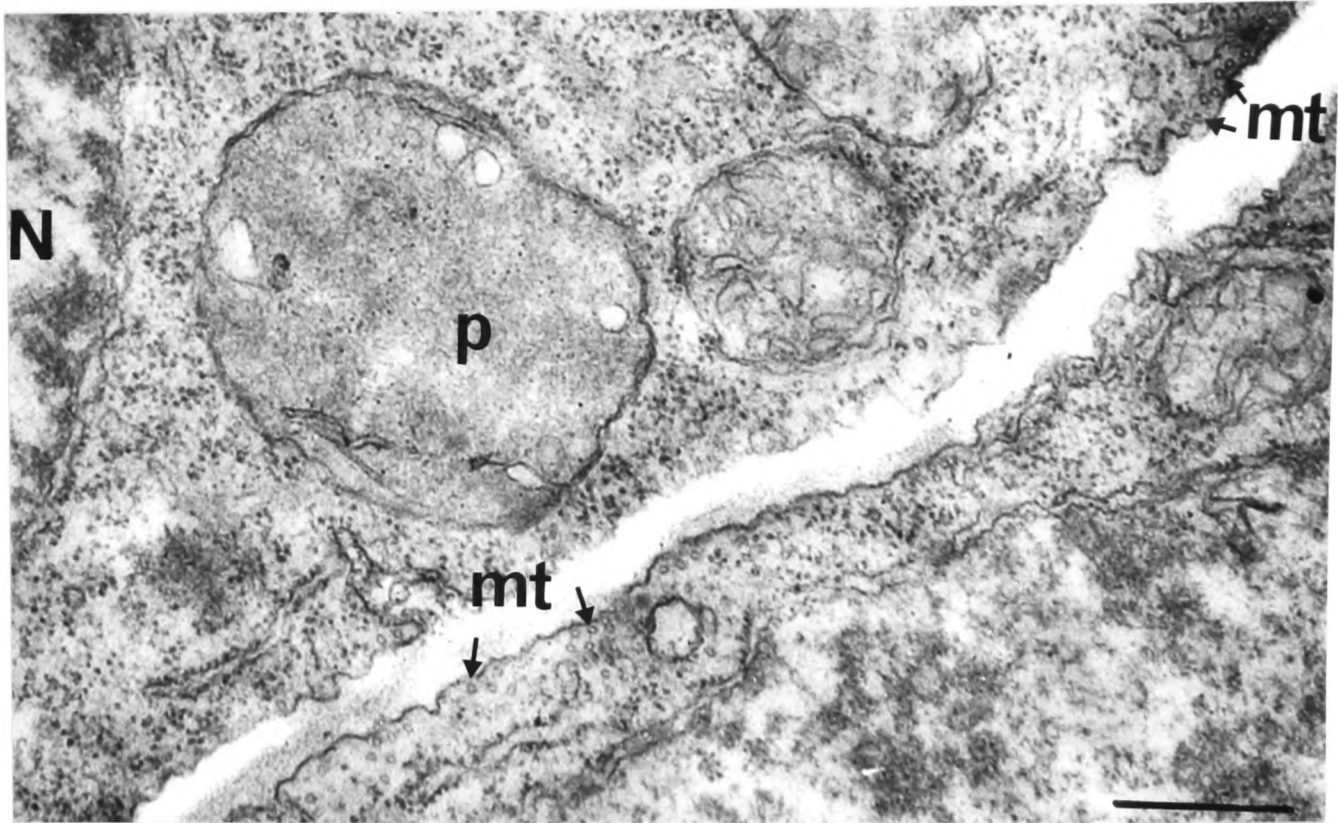


Fig.V.10

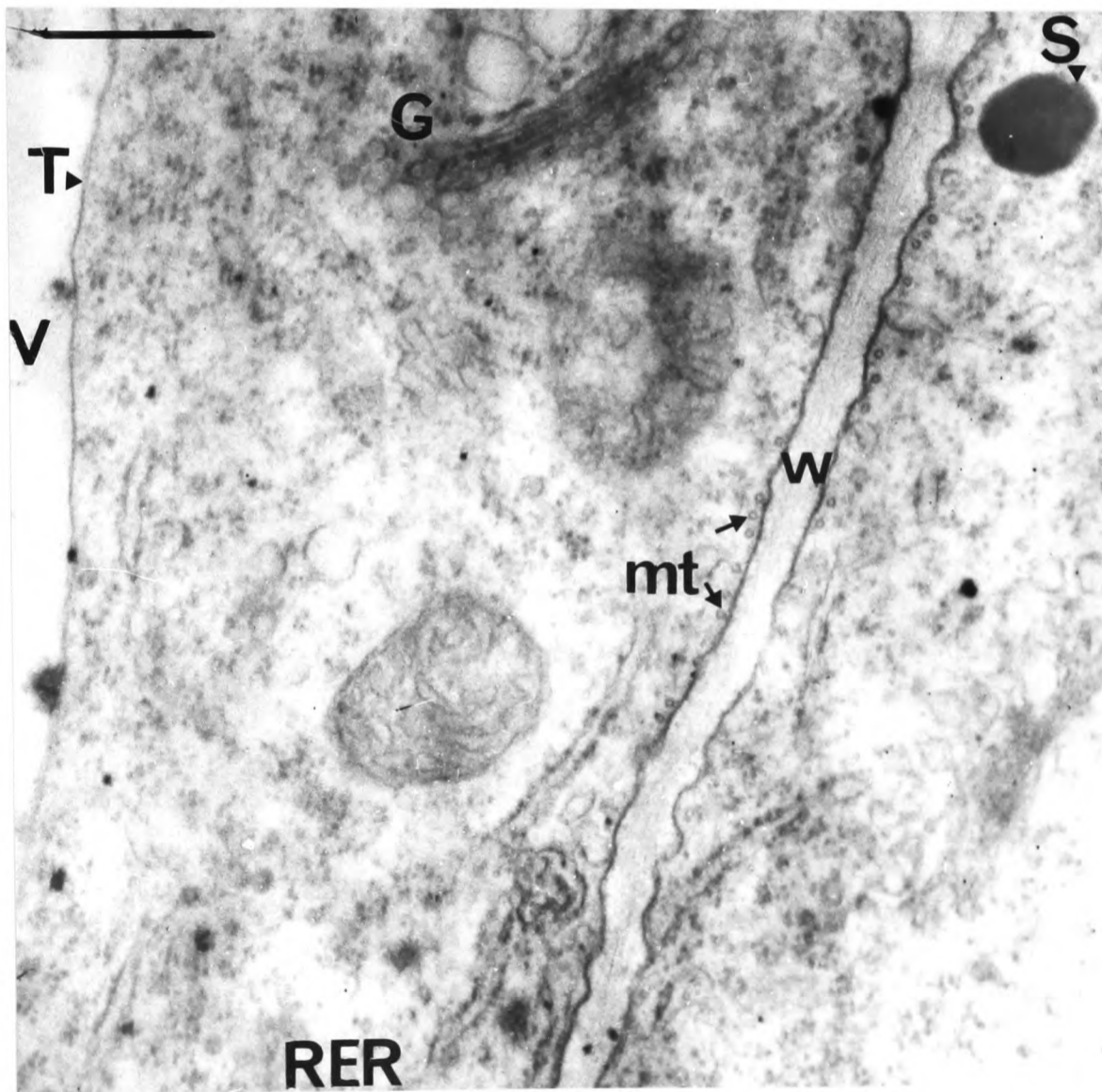


Fig.V.11

Fig.V.12: Meristematic cells. 3 sets of Golgi apparatus can be seen in tangential section, possibly indicating a common plane of alignment. Durcupan dehydrated. Post-stained in lead citrate. Bar = 0.5 μ m.

Fig.V.13: Procortical cell with microtubules (mt) cut in glancing section. Ethanol dehydrated. Post-stained in lead citrate. Bar = 1.0 μ m.

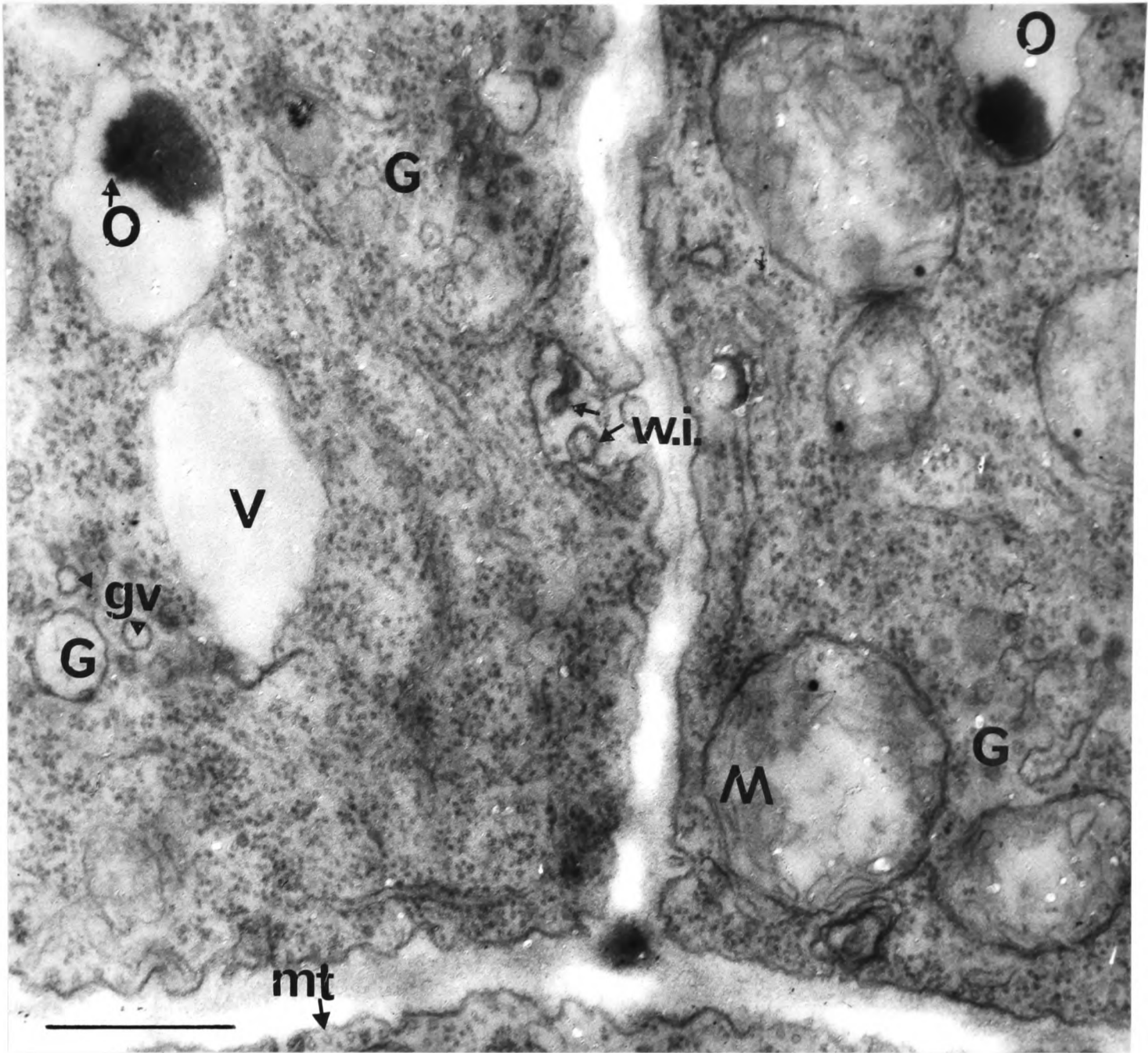


Fig.V.12

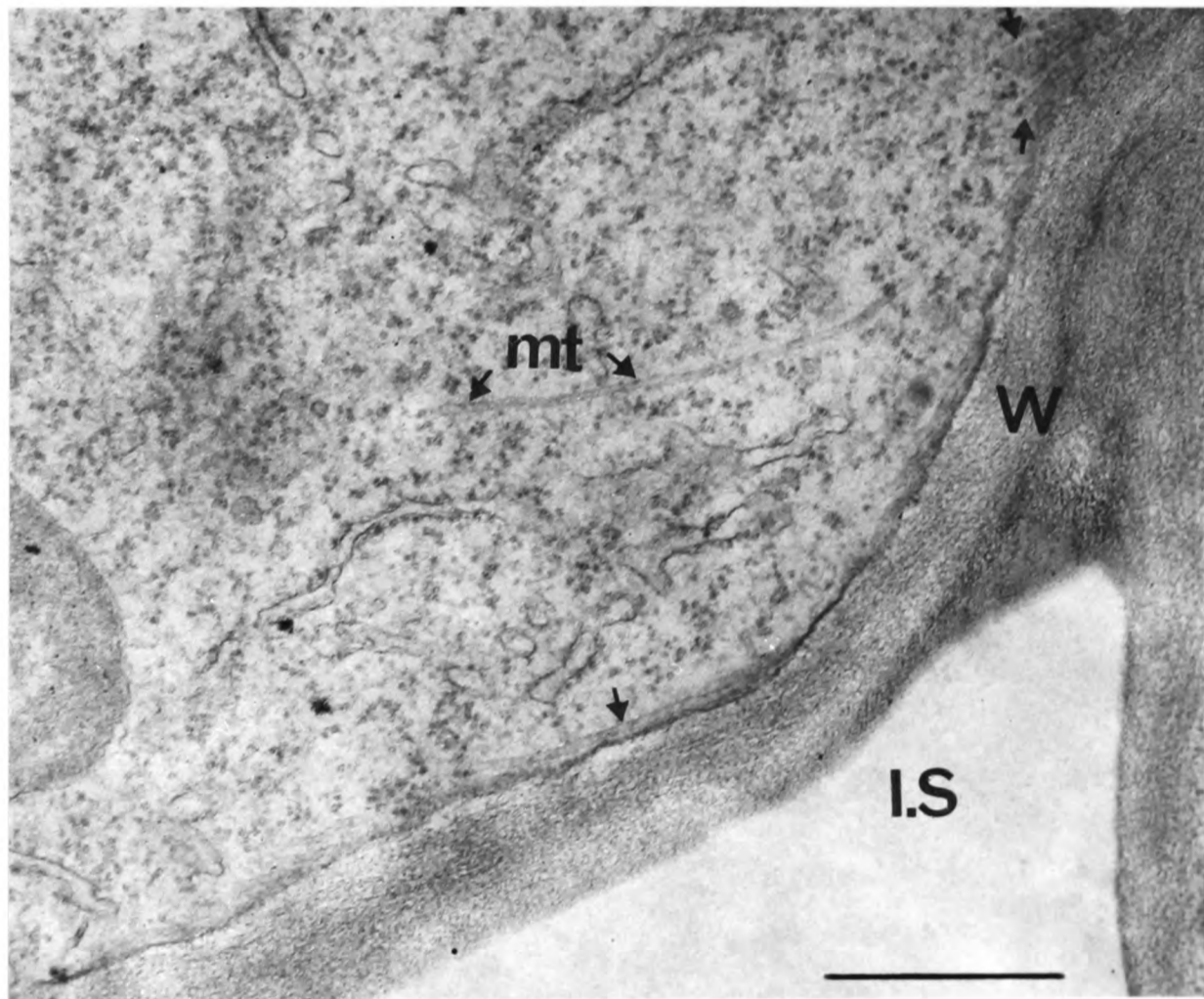


Fig.V.13

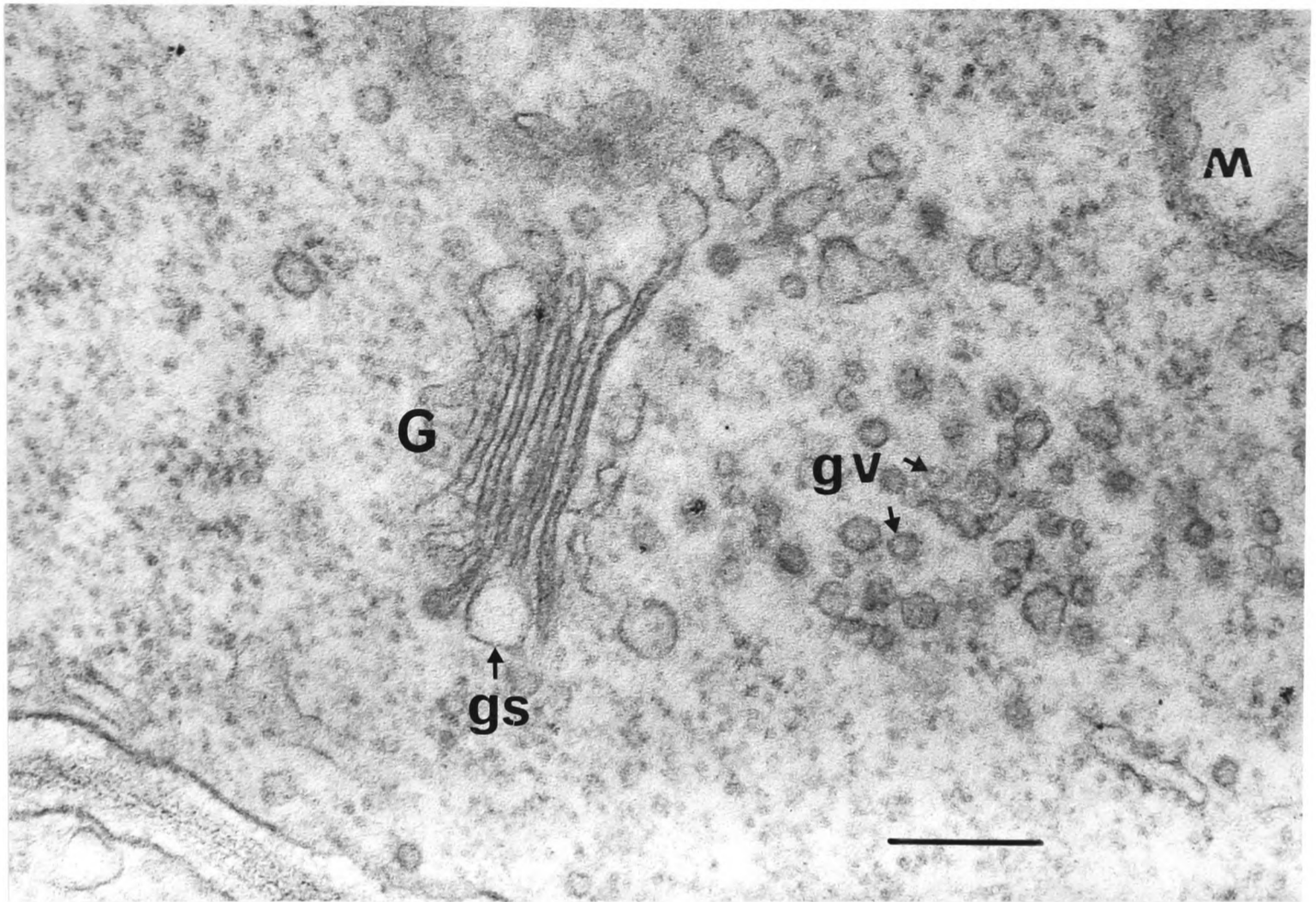


Fig.V.14: High power magnification of Golgi apparatus (G) with Golgi saccules (gs) and many vesicles (gv). Post-stained in lead citrate. Bar = 0.25 μm .

AS molecule employed. The end-product obtained using naphthol AS D produced a fine granular deposit, although it never occurred in a concentrated form to produce the dense black deposits as has been noted in animal cells.

The general impression gained from examining many sections from each stage of ripening was that it became increasingly difficult to locate recognisable organelles as ripening proceeded. There was a tendency for organelles to vesiculate or become distended so that in many sections observed from ripe tissue the few structural components remained in the form of many different-sized vesicles (Fig. V.15.). 'Vesiculation' of many subcellular organelles is a common characteristic of senescing leaf and fruit cells (see Butler and Simon, 1971, for review) and is normally regarded as a sign of general protoplasmic disintegration, (Bain and Mercer, 1964, 1966).

In unripe material, organelles such as Golgi apparatus, endoplasmic reticulum, mitochondria, plasma membranes, chloroplasts and vesicles of varying size were easy to recognise (Fig. V.16. and V.18.). Possibly because of the greater content of cytoplasmic constituents per cell at this stage of ripening, it was easier to observe the granular end-product. In Fig. V.16. it can be seen associated with Golgi cisternae and saccules, and what appear to be degenerating mitochondria (lower set of arrows).

It will be noted that free ribosomes are absent from the cytoplasm whilst they are still present attached to endoplasmic reticulum. Loss of ribosomal material is another characteristic ultrastructural feature of senescing plant cells and has been widely reported in wheat leaves and cucumber (Shaw and Manocha, 1965; Shaw *et al.*, 1965; Lewington *et al.*, 1967; Butler, 1967 and Eilam *et al.*, 1971).

- Fig.V.15: Pulp cell from the cortex of a mature, red berry. There is a lack of any recognisable cell organelles and only a heterogeneous assortment of vesicles is obvious. Cell wall material (W) appears to have undergone some physical change in appearance when compared to cell wall from immature fruit (Fig.V.17). Unstained. Bar = 1.0 μ m.
- Fig.V.16: Pulp cell from a green, immature berry. Lead deposits are associated with the Golgi cisternae, saccules and vesicles. Lead may also be seen attached to other cell organelles in the cytoplasm (lower set of arrows). Unstained. Bar = 0.5 μ m.

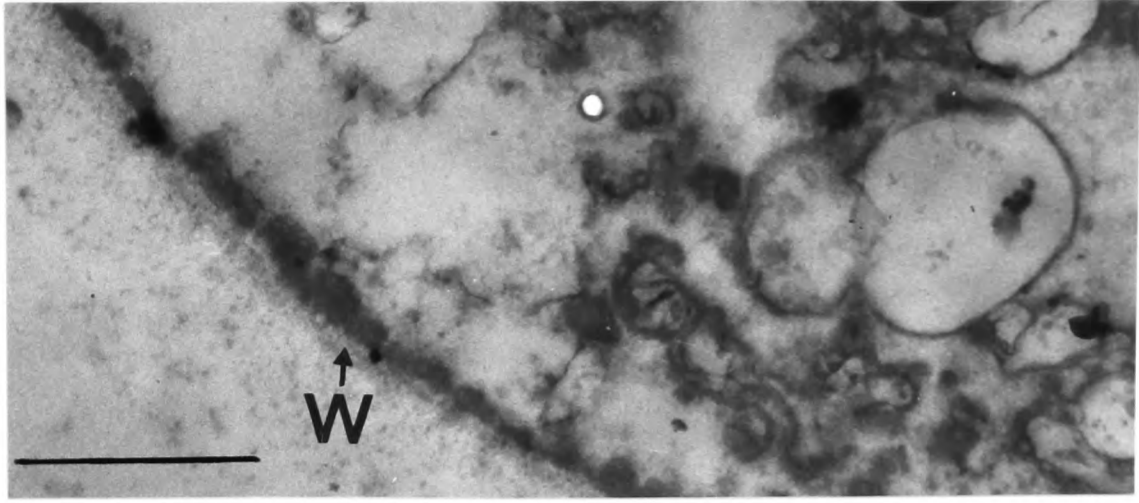


Fig.V.15

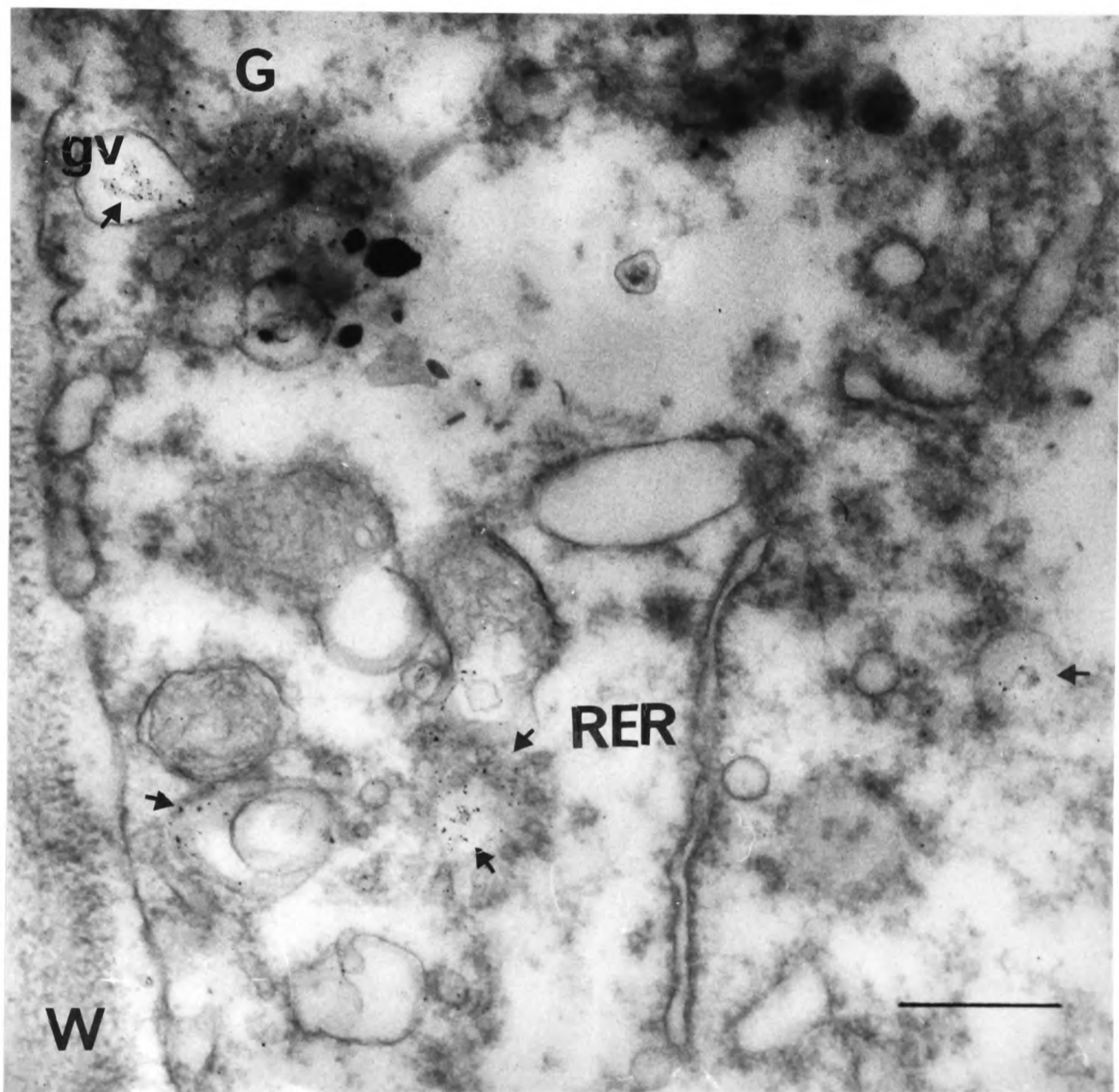


Fig.V.16

Fig. V.17. shows two cells, incubated for esterase activity in the absence of substrate, with a large vesicle (V) containing sparsely-deposited lead. The plasmalemmas of the two adjacent cells in this micrograph are almost completely divorced from their cell walls, indicating either a loss of adhesion at this particular physiological status of the cells or a fixation and embedding artefact.

Fig. V.19. shows the presence of a chloroplast embedded in a thin layer of cytoplasm. The thylakoid membranes appear to be disorganised, and the plastoglobuli or osmiophilic globules, clumped. One of the most conspicuous changes occurring in green tissues undergoing senescence is the breakdown of the chloroplasts with their attendant accumulation of osmiophilic globules. Butler and Simon (1971) in their review of the ultrastructure of plant senescence show numerous examples of this in micrographs from a variety of plant cells.

Chloroplasts, or chromoplasts, are recognisable in ripe tissue, although, since cells will have undergone many physiological and biochemical changes during the ripening process, it is to be expected that these and other subcellular organelles from this stage of ripening will also be very much changed. Unfortunately the chloroplast (chromoplast) in Fig. V.20. is poorly preserved and fine structure cannot be observed, although osmiophilic globules are still present.

In ripening tissue many organelles are still visible (Fig. V.21. and V.22.) but there is a marked tendency to vesiculation and in Fig. V.23. mitochondrial cristae are very much swollen. With cells undergoing gross alterations in cell

Fig.V.17: Green,immature berry.Pulp cell from the cortex.No-substrate control. A small amount of end-product is associated with a small vacuole (V and arrows) and the Golgi apparatus (G). Note that the cell walls are separated by unidentifiable bodies (UI).Unstained. Bar = 0.5 μ m.

Fig.V.18: Green,immature tissue.Cells incubated 25min for naphthol AS D esterase activity.No lead end-product is visible. Note that the plasmalemmas of both cells are almost entirely free from the cell walls. Unstained. Bar = 0.5 μ m.

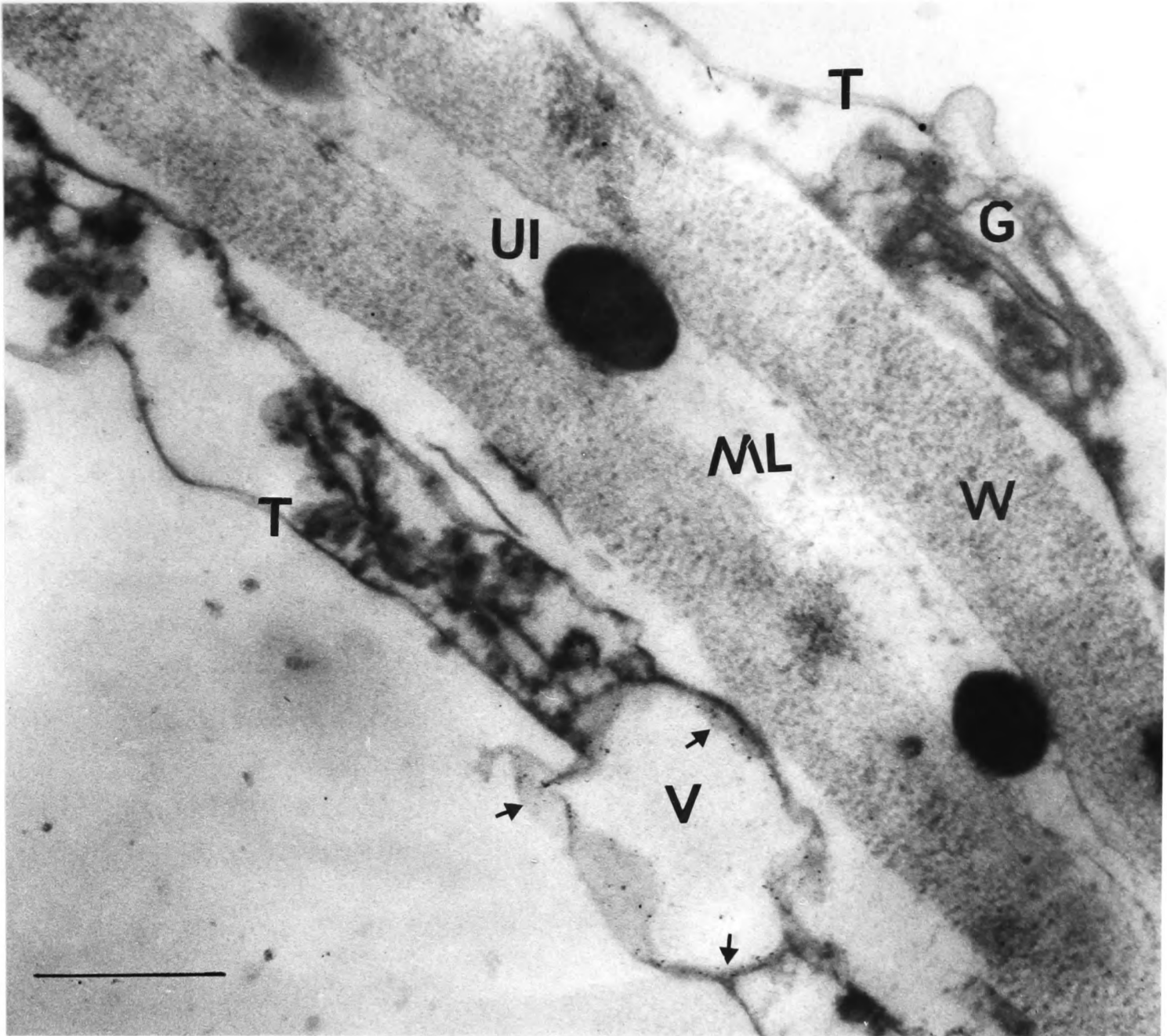


Fig.V.17

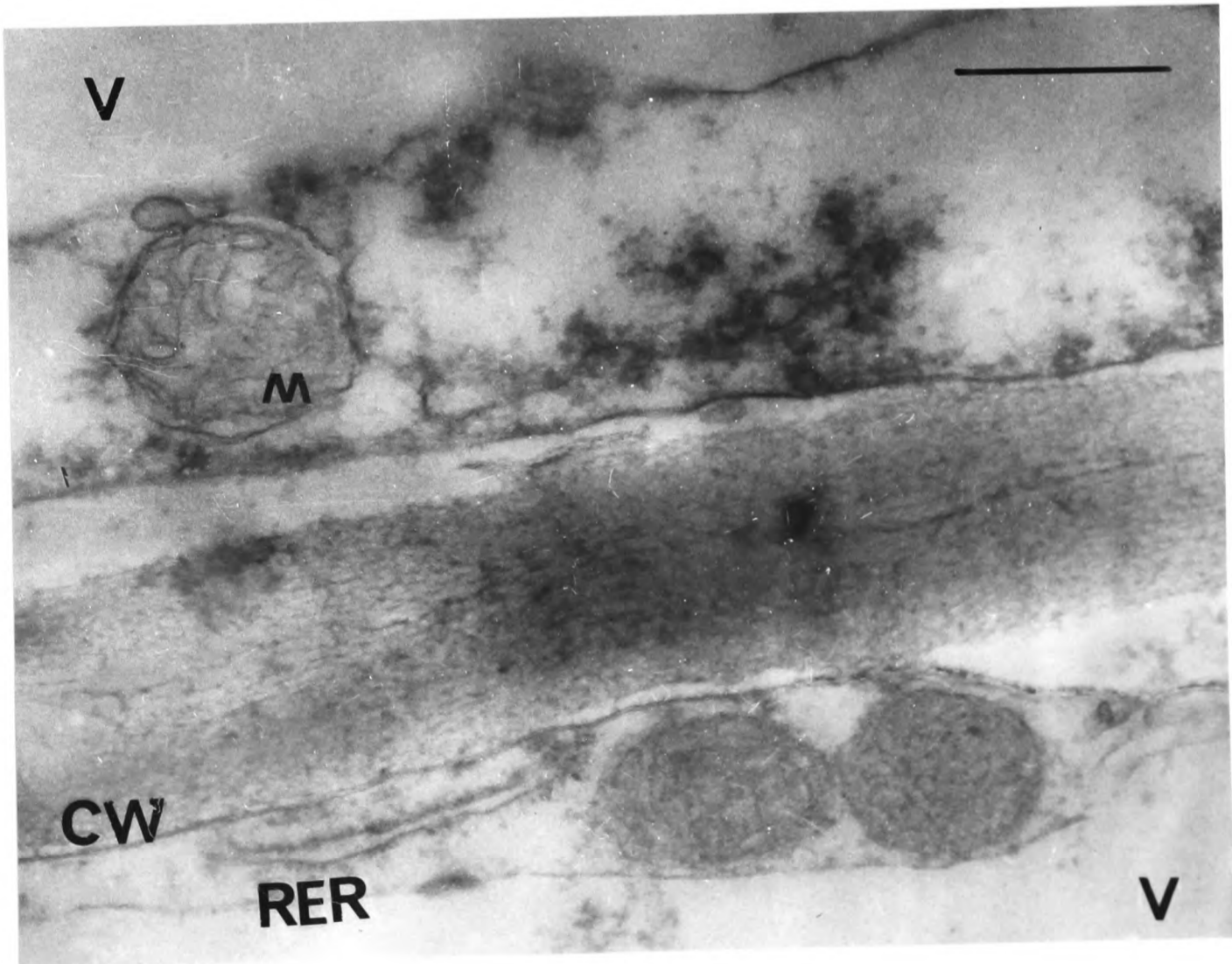


Fig.V.18

Fig.V.19: Green,immature tissue.Cells incubated for 25min for naphthol AS D esterase activity without substrate.No lead end-product is visible.Compare the degree of morphological preservation of the chloroplast (C) in this micrograph with that in Fig. V.20. Unstained. Bar = 0.5 μ m.

Fig.V.20: Ripe berry. No-substrate control. Chloroplast (or chromoplast?) still evident but poorly preserved although osmiophilic globules (O) are recognisable. Unstained. Bar = 0.5 μ m.



Fig.V.19

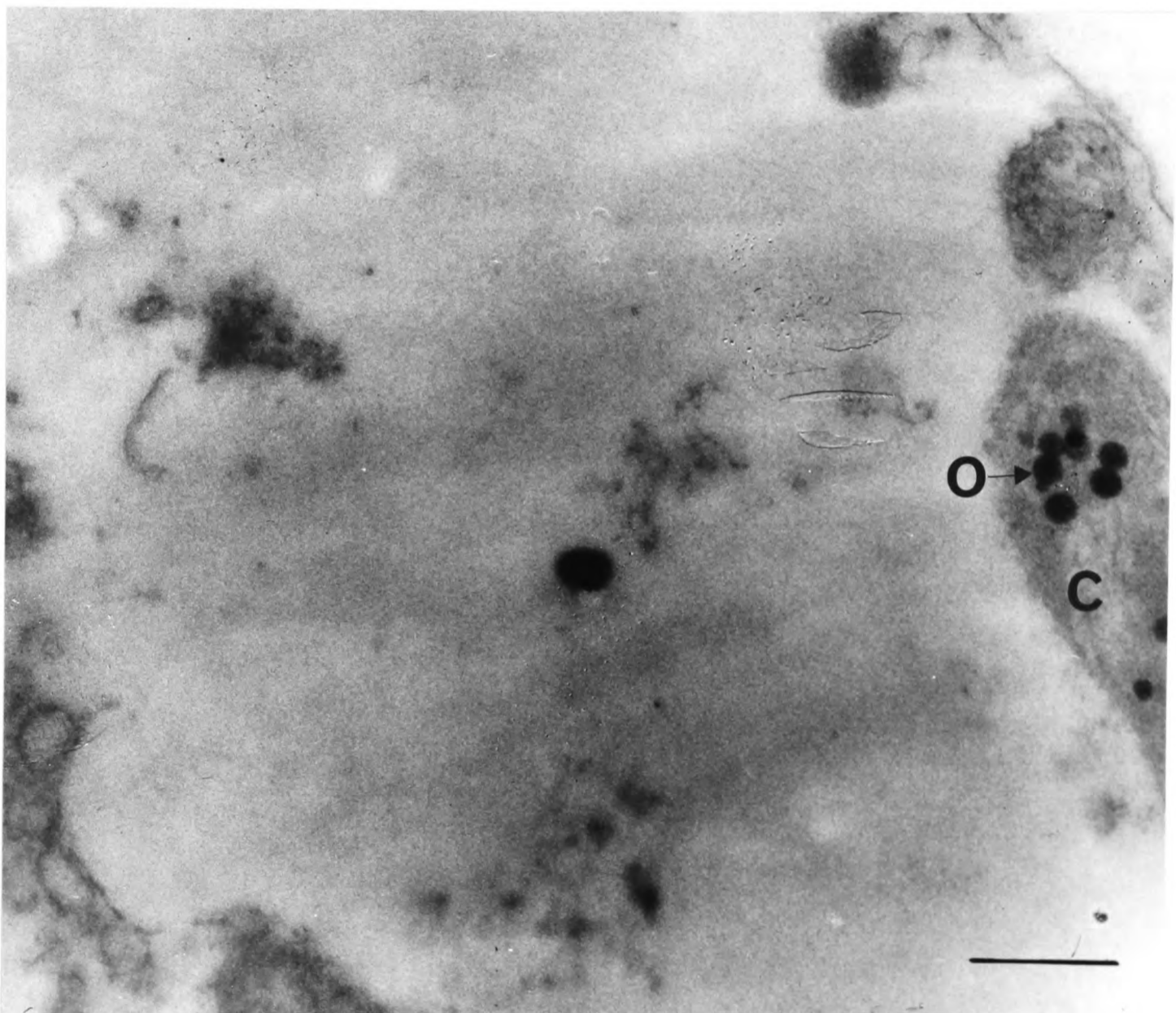


Fig.V.20

Fig.V.21: Maturing berry. No-substrate control. A number of organelles are visible at this stage of ripening (Stage D) but note the tendency to vesiculation. Several spherosomes (S) can be seen. Unstained. Bar = 1.0 μm .

Fig.V.22: Different area of the same cell as Fig.V.21 showing that Golgi bodies are still identifiable at this stage of ripening. Unstained. Bar = 1.0 μm .

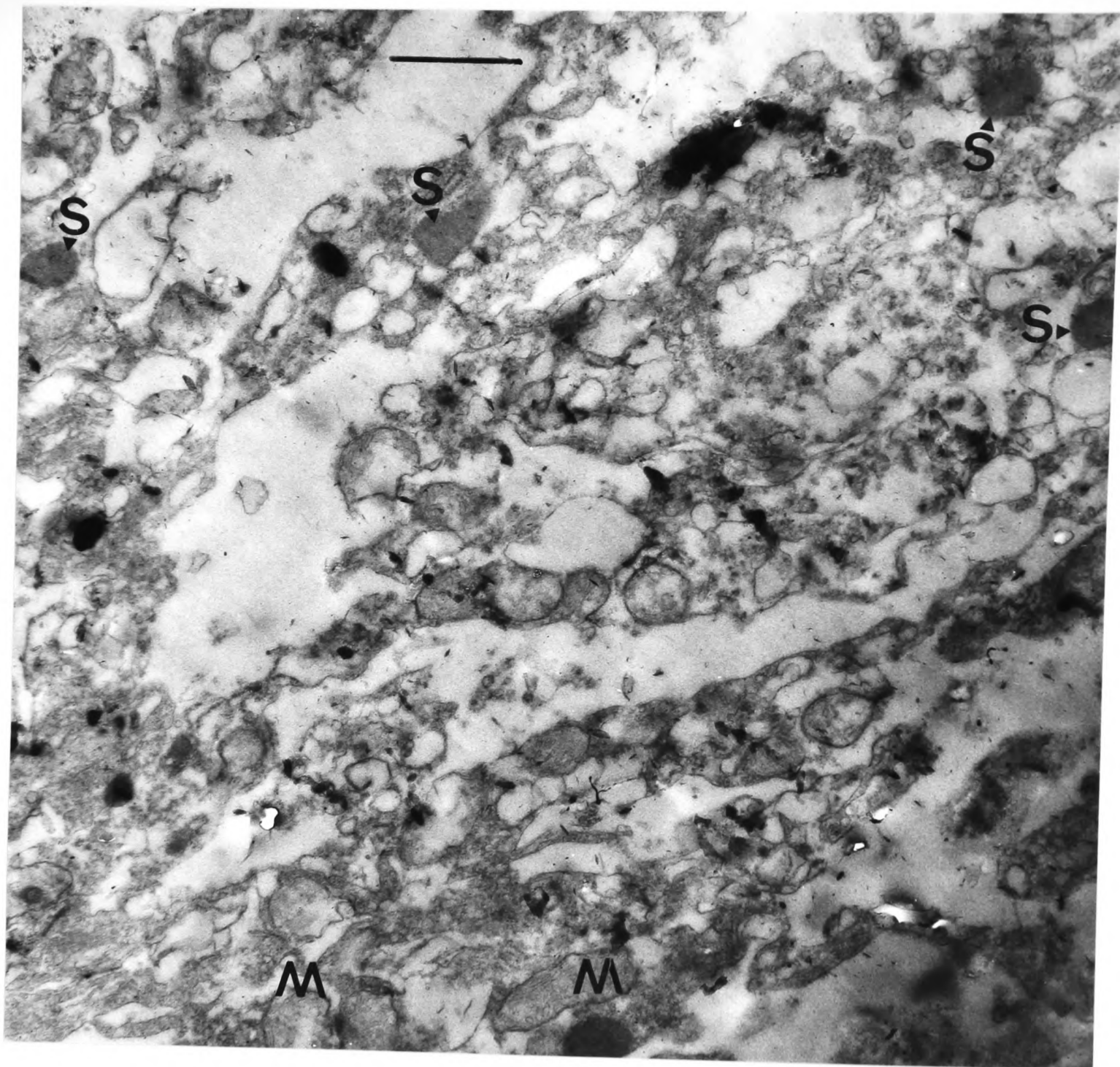


Fig.V.21

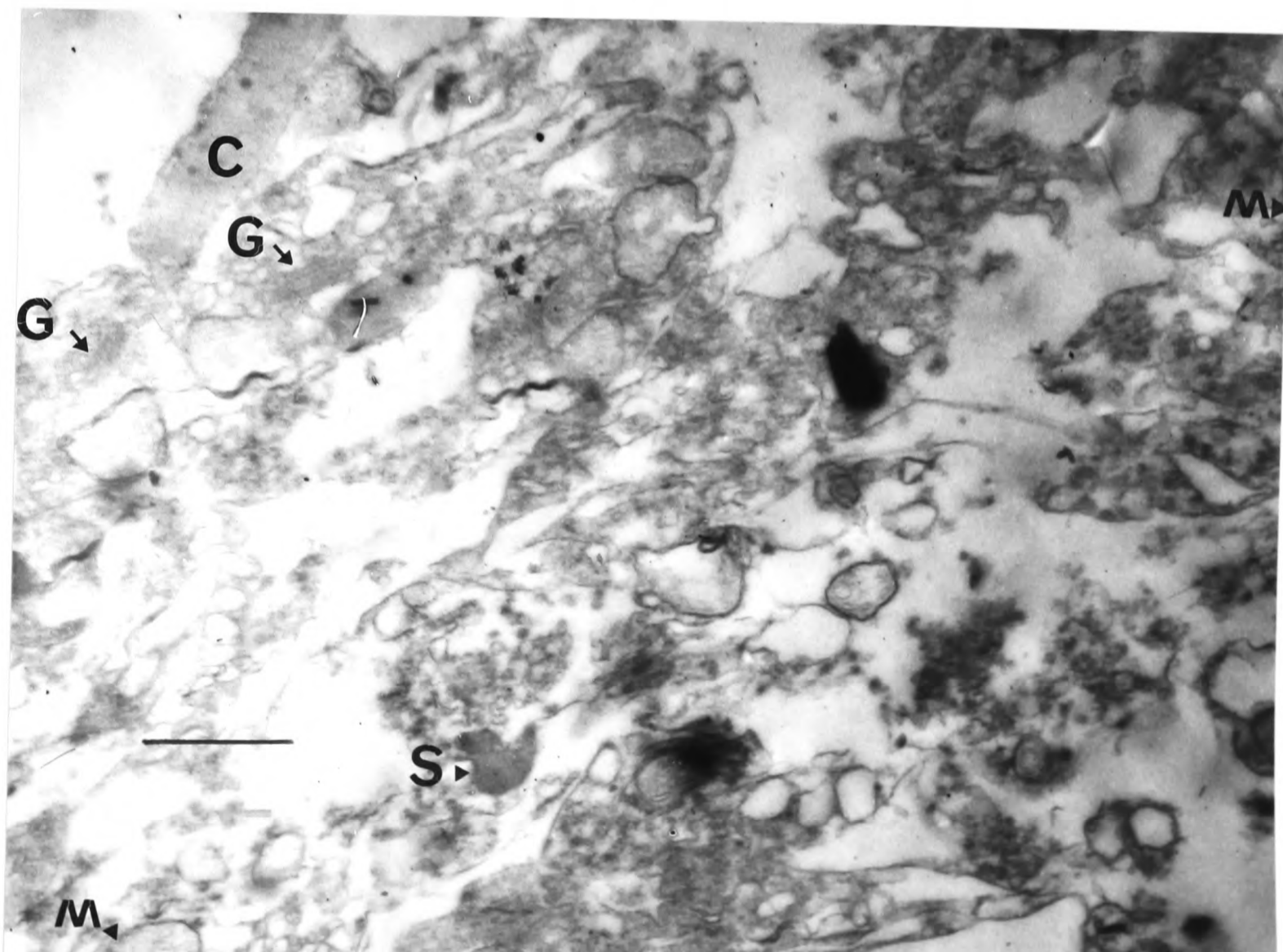


Fig.V.22

- Fig.V.23: White-red maturing berry. Pulp cell from the cortex. No-substrate control. Note the marked swelling of the mitochondrial cristae and the large numbers of vesicles. Unstained. Bar = 1.0 μ m.
- Fig.V.24: White-red maturing berry. Cell from the same area of tissue as Fig.V.23 but note that lead end-product is associated with a number of small vesicles (arrows). Unstained. Bar = 1.0 μ m.

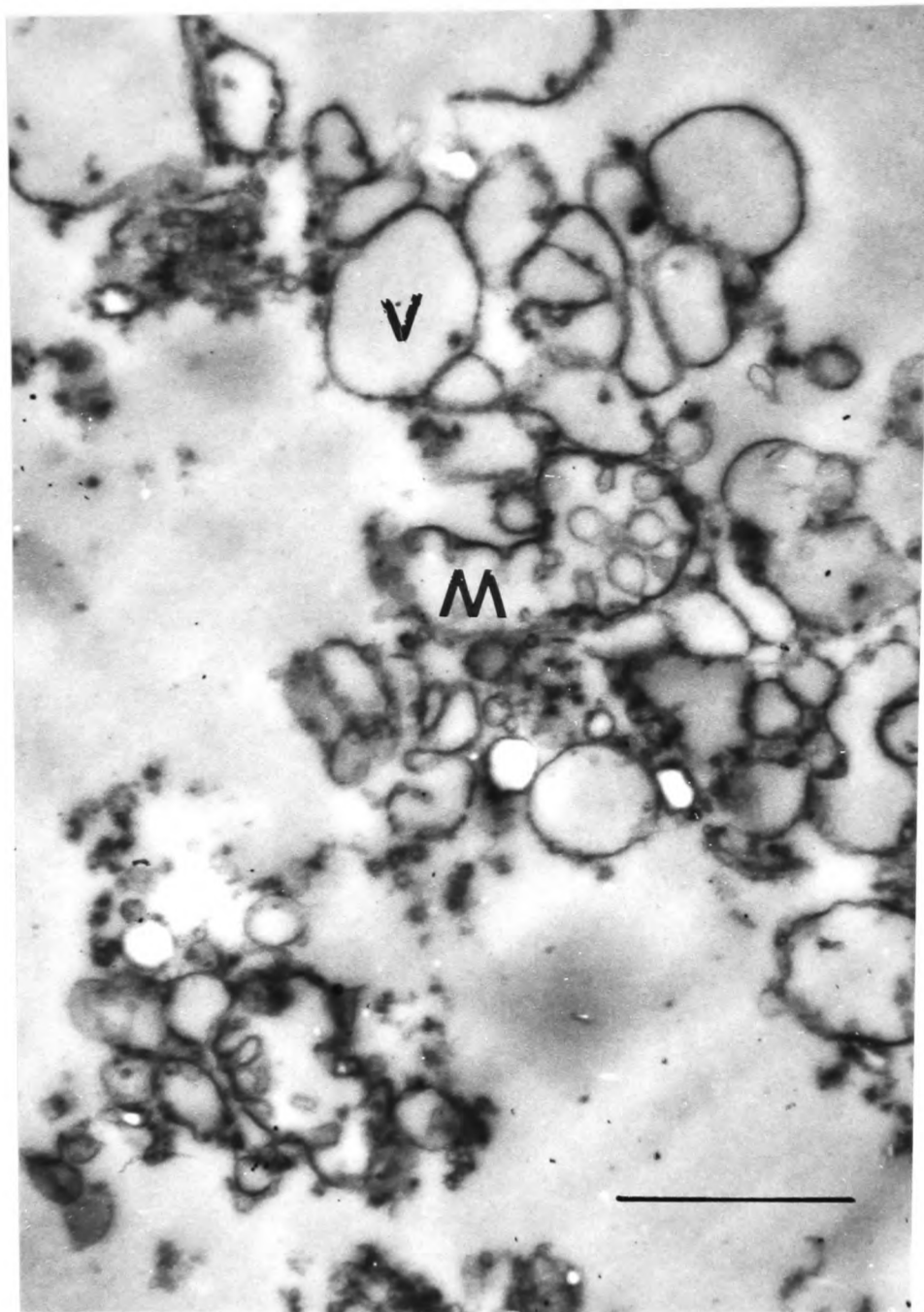


Fig.V.23

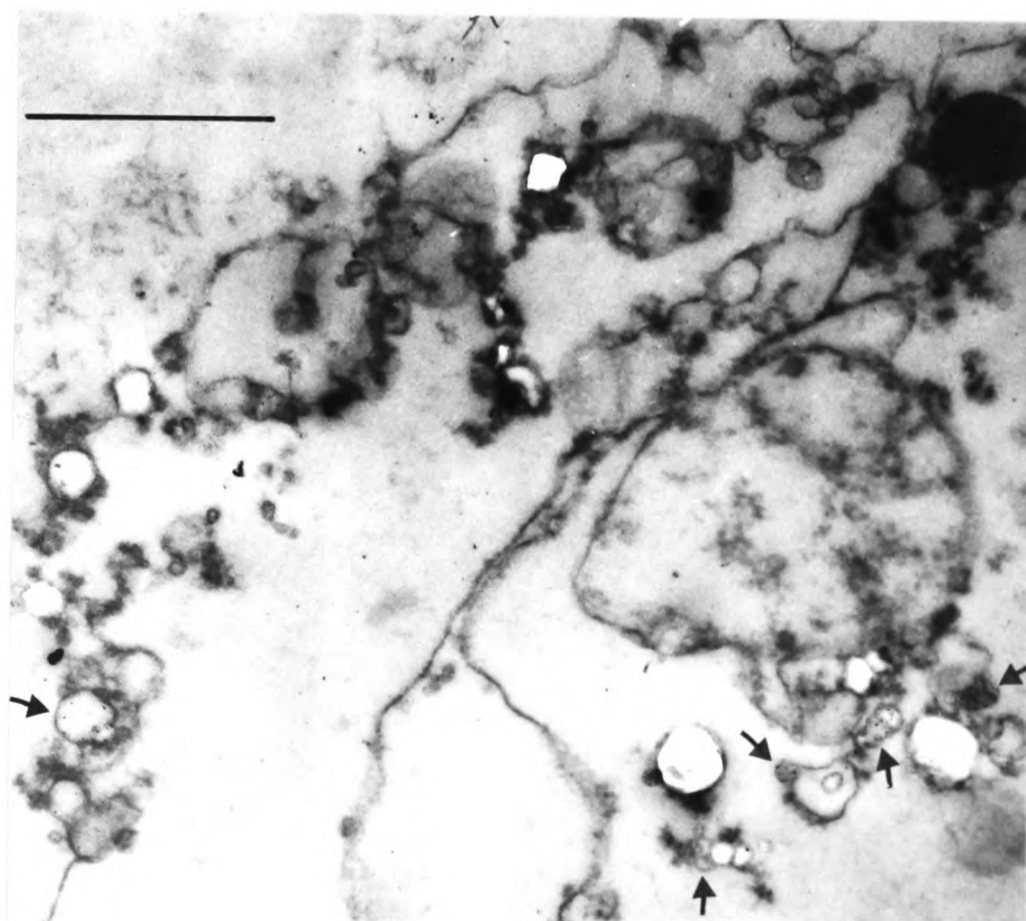


Fig.V.24

metabolism it is difficult to determine whether the observed ultrastructural changes are due to underlying physiological and biochemical differences or imperfections in the fixation and embedding techniques, since different tissues probably require different treatments to give optimal preservation. Comparisons of these micrographs with the earlier ones of Vicia faba root cells, in which the same conditions of fixation and embedding were employed, emphasize this point.

On the other hand, the ultrastructural changes noted in ripening strawberry fruits follow the same pattern as those recorded for other senescing plant tissue, where parallel biochemical studies have often shown that cytological changes such as ribosomal loss can be correlated with a loss of RNA and an increase in RNA ase. (Shaw and Manocha, 1965; Shaw et al., 1965).

Figs. V.24. and V.25. showed enzyme end-product attached to the membranes of variously-sized vesicles, although it was absent from the general cytoplasm and areas of the micrograph devoid of cell constituents. This is unlikely to represent the site of enzyme action, however, since the end-product was also present in the 'no-substrate' controls. Close scrutiny of several sections of ripe tissue failed to reveal the presence of lead end-product although this may merely reflect the poor preservation of the tissue and the sparsity of cytoplasmic structures.

Spherosomal structures may be noted in Fig. V.26., two of which appear to have clear areas at one side. They compare closely with the size and appearance of spherosomes identified by Yatsu et al., (1971) in other plant cells, fixed first in glutaraldehyde and then post-fixed in osmium tetroxide.

Fig.V.25: White-red maturing berry.No-substrate control. Lead end-product can be seen associated with a number of small vesicles (arrows). Unstained. Bar = 1.0 μ m.

Fig.V.26: Mature red berry. Tissue incubated 25min for naphthol AS D esterase activity. No end-product is visible.Ultrastructural preservation is poor but spherosomes are recognisable (S). Unstained. Bar = 1.0 μ m.

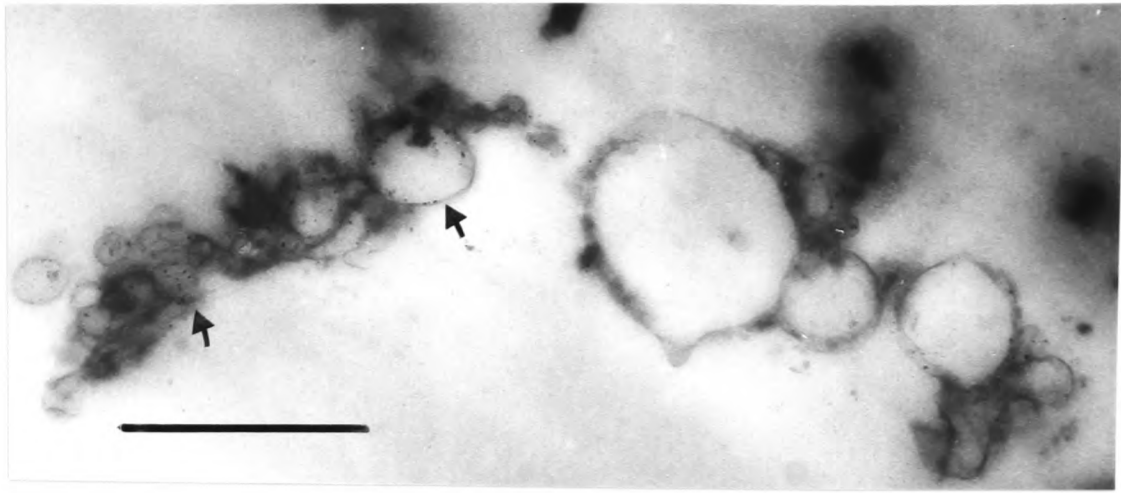


Fig.V.25

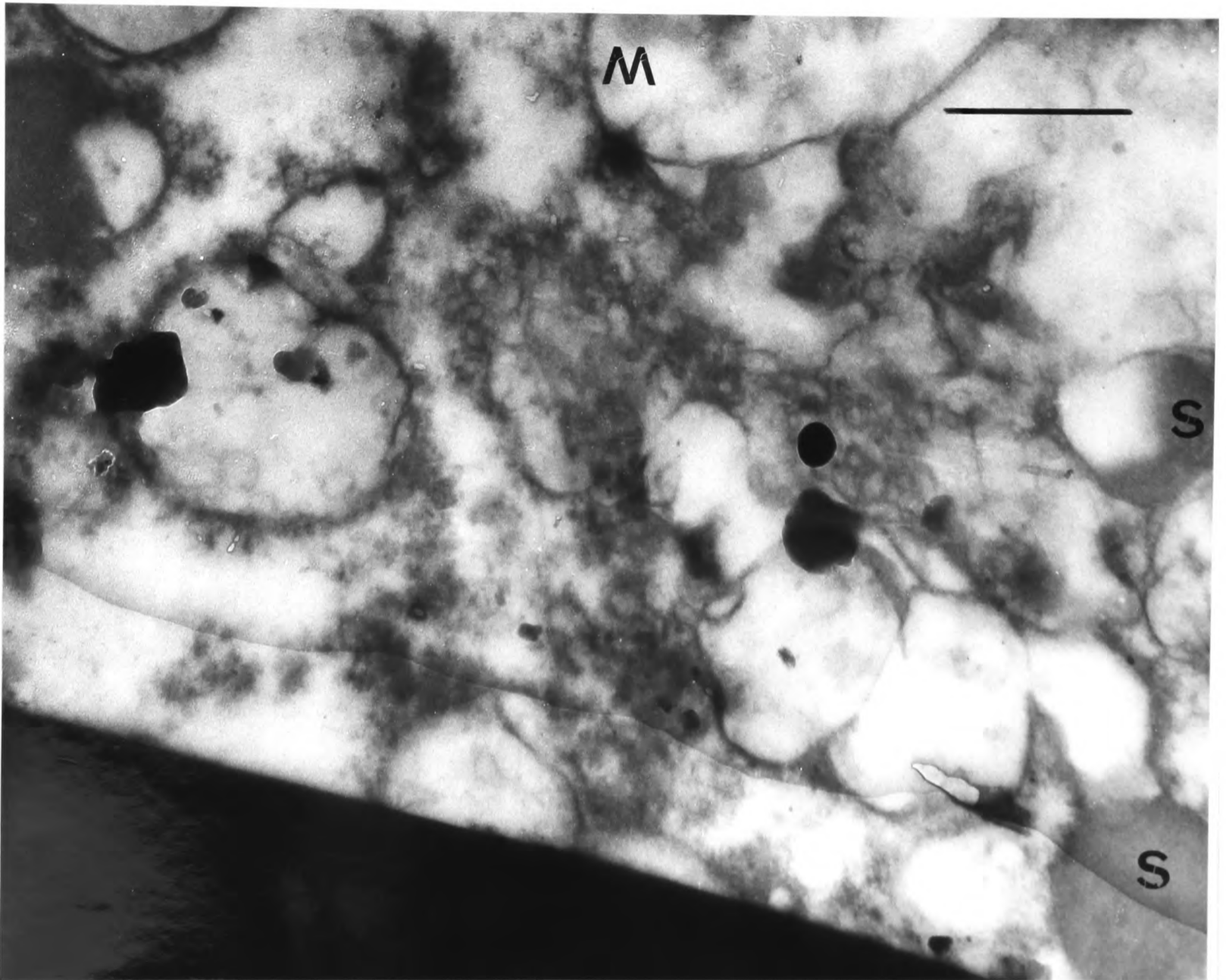


Fig.V.26

It is conceivable that the smaller particles staining for esterase at the light microscope level may correspond to spherosomal particles, whilst the larger structures may represent varying stages in the growth of spherosomes into mature vacuoles (Frey-Wyssling et al., 1963). The very large vesicles (5 μ m and more in diameter) observed under the light microscope did not appear to have their counterparts at the ultrastructural level. Indeed, the largest vesicles observed ultrastructurally were only 2 μ m in diameter, compared with the enormous 40 μ m-diameter bodies seen at the optical level. Since many of these large structures appear to lie free in the cytoplasm they may well have been lost in the washing procedures.

V.3.3. Esterase localisation in root tips using LPED as coupling salt.

The results obtained using LPED as the lead diazotate in root cells were somewhat improved when compared with those recorded for lead phthalocyanin in strawberry pulp tissue, in that no electron-dense end-product could be detected in control sections.

However, the validity of comparing two botanically distinct tissues with respect to their response to two different lead coupling salts must remain doubtful. In addition, it should be emphasized that, due to lack of time, the investigation of the use of these coupling salts was not as exhaustive as would have been wished.

Only simultaneously coupled material was examined at the electron microscope level since light microscope studies had already revealed that this procedure produced a pattern of coloured end-product that closely resembled the normal light microscope cytochemical tests employing non-lead bearing coupling salts.

The electron micrographs, Figs. V.27. - 29., show two epidermal cells that were about to complete cell division. Complete nuclei (N) with intact nuclear envelopes (ne) are distinguishable but the region labelled 'w.f.' on one of the radial walls shows files of rough endoplasmic reticulum (RER) aligned parallel to the cell plate, with a continuum present between the cells. Completed wall formation may be observed on either side of this region however (Fig. V.28.). The granular end-product may be observed in membrane-bounded small vacuoles predominantly disposed along the radial walls of the cells. The vacuole labelled 'a' in Fig. V.27. should be compared with that labelled 'b', the former containing concentrated end-product attached to what appears to be a roughly circular osmiophilic matrix (Fig. V.28. shows a higher magnification of this). This matrix measures approximately 0.2 μ m to 0.5 μ m in this part of the cell and is surrounded by a larger vacuolar membrane in some cases (a, b) but in others there is no vacuolar space (cf. 'y, z' Fig. V.27.). It is possible that this matrix may have shrunk away from the vacuolar membrane during the preparative procedures, but comparison with a variety of other plant and animal cells show that similar organelles have been observed, when material is both glutaraldehyde and osmium-fixed. (See for instance Berjak, 1972; Shoup, 1966). Where less concentrated, end-product may appear as a fine granular deposit (cf. vacuoles a₂ and a₁ in Fig. V.28.), in both vacuoles and in intimate association with rough endoplasmic reticulum (Fig. V.29., arrows), present at the new cell wall only. Significantly, end-product may not be seen on ER that is not adjacent to the cell plate. In Fig. V.29. a small vacuole (v) appears to be in the process of being incorporated into the new cell wall, in that it lies directly along the plane of cell division. It is reasonable to suggest that such vacuoles are

Fig.V.27: Epidermal cells of Vicia faba lateral root incubated for naphthol AS D esterase activity for 30min using a simultaneous coupling procedure. Karyokinesis is complete but a continuum remains between the two cells. Dense enzyme end-product is present in some vacuoles (a,z) but not in others (b,y). Unstained. Bar = 1.0 μ m.

Fig.V.28: High power magnification of the region of wall formation (w.f) in Fig.V.27 showing end-product as dense deposits in some vacuoles (a_1) and as a fine grain in others (a_2). Unstained. Bar = 1.0 μ m.



Fig.V.27

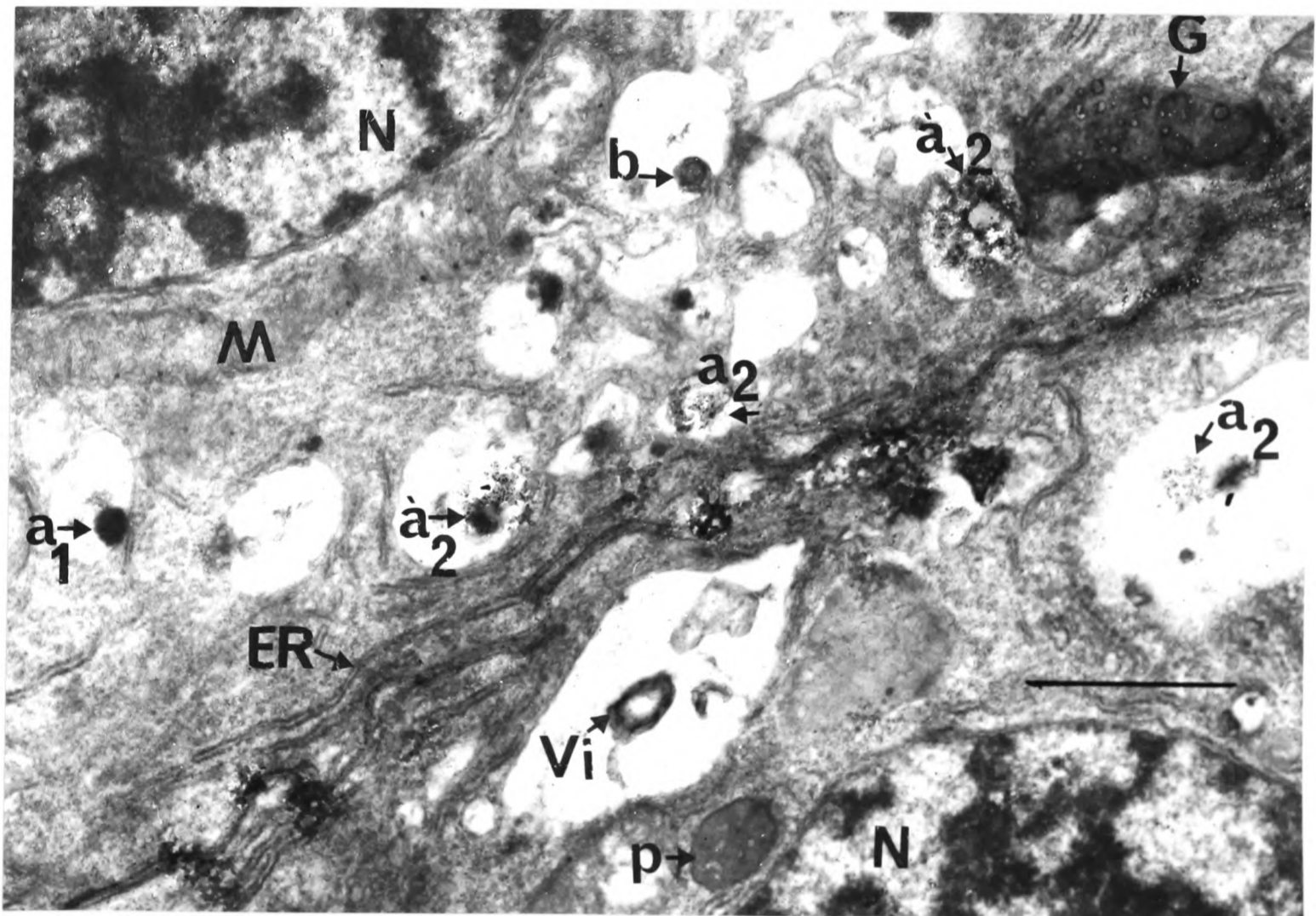


Fig.V.28

Fig.V.29: High power magnification of Fig.V.28. Rough endoplasmic reticulum (RER) appears to be intimately involved in new cell wall formation. Esterase activity is represented by lead end-product in both vacuoles and along lengths of ER, (arrows). Note absence of end-product from ER not at the plane of cell division. Dotted lines indicate the plane of cell division. Unstained. Bar = 0.5 μ m.

Fig.V.30: Procambial cells. Control tissues incubated without substrate. Osmiophilic matrices within vacuoles lack end-product (b). Unstained. Bar = 5.0 μ m.

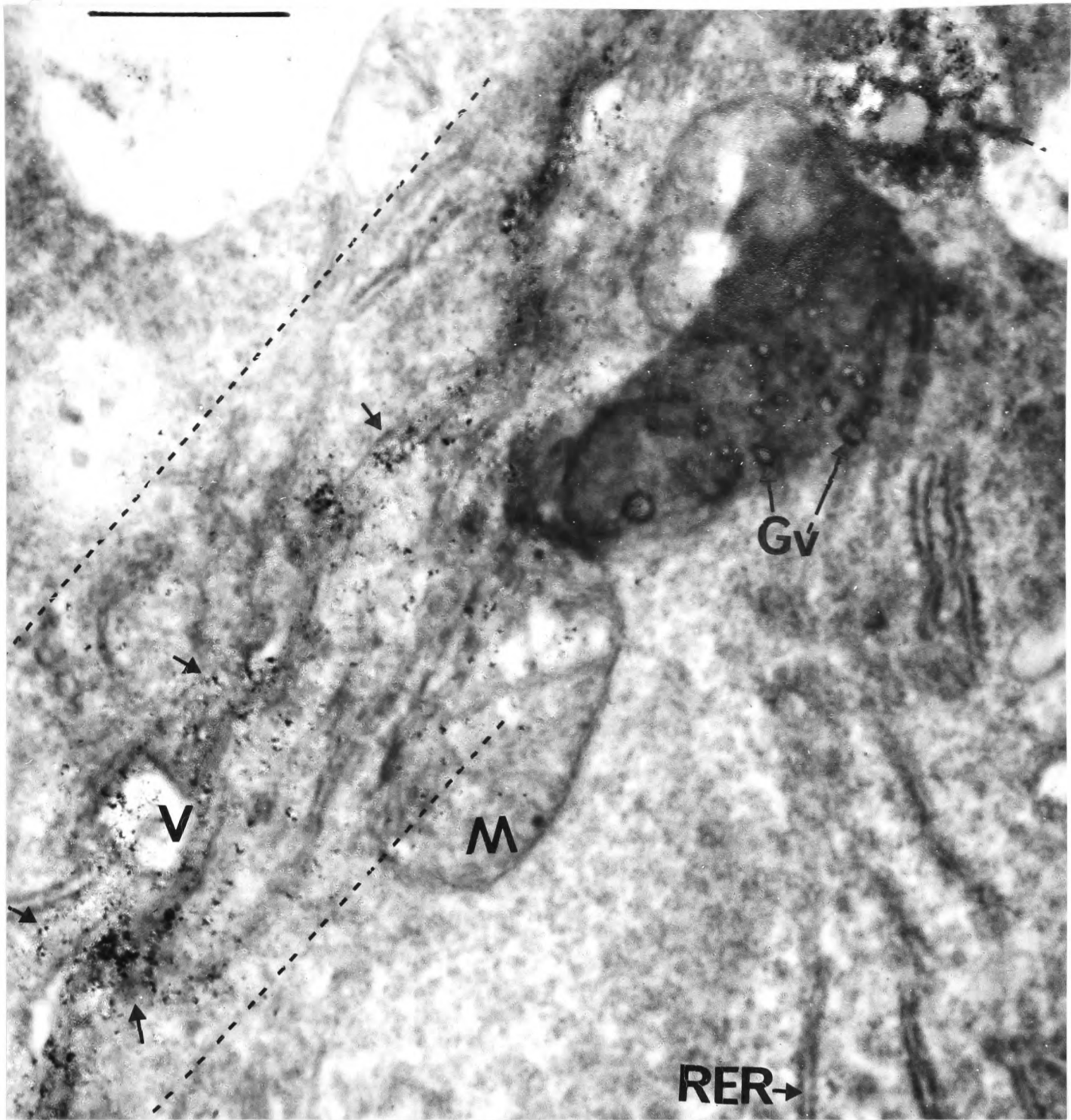


Fig.V.29

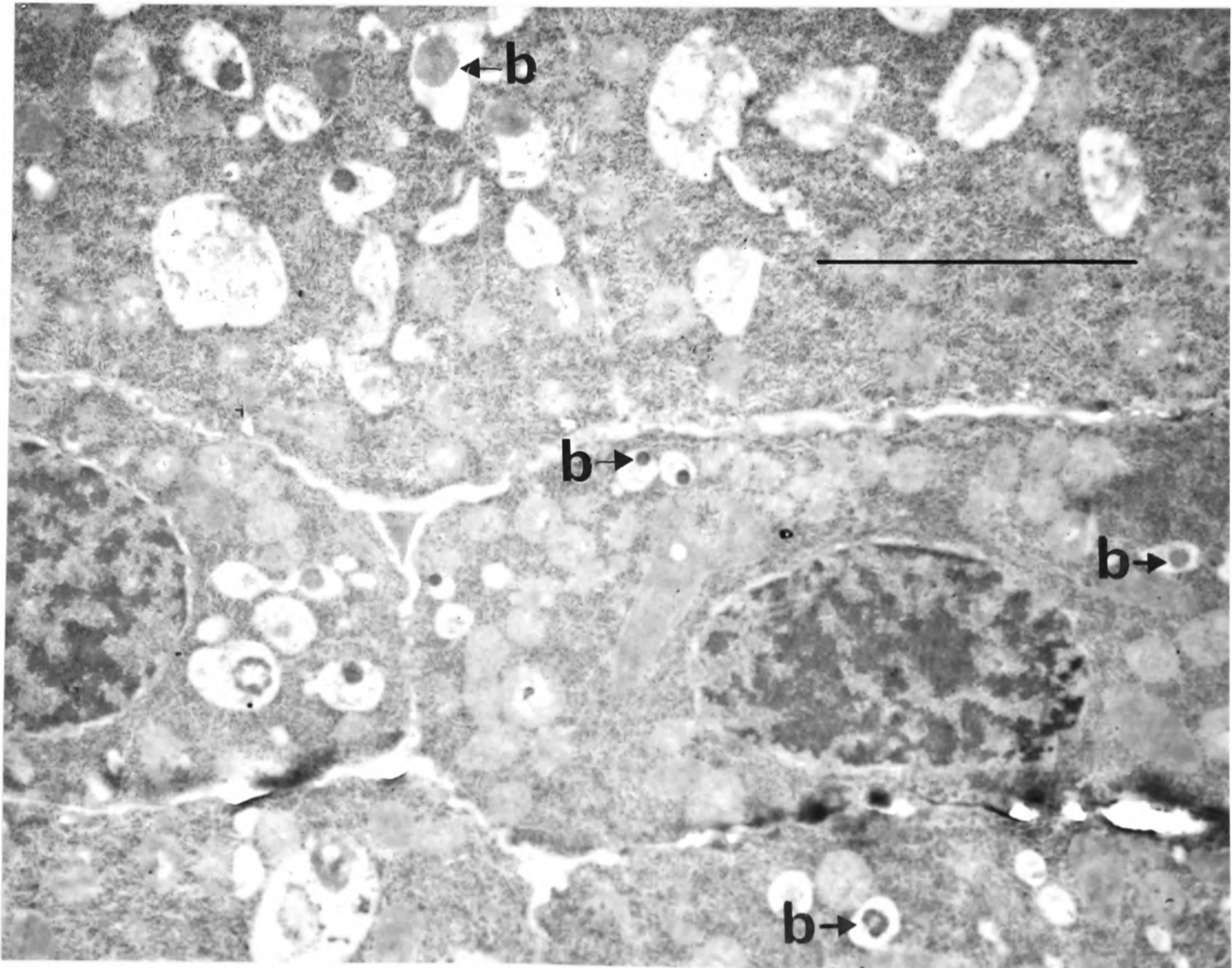


Fig.V.30

homologous with the 'phragmosomes' reported seen by Porter and Machado (1960) in dividing cells of onion root tip.

In a control section (Fig. V.30.), only grey osmiophilic centres in vacuoles ('b') were visible, although a cell at a stage of division comparable with that observed in test material was not found.

V.4.

DISCUSSION

All the above observations were made on a comparatively small number of cells, since time did not permit a more exhaustive examination of the disposition of end-product material in the various plant cells examined. Consequently detailed comparisons with light microscope observations are not possible.

Durcupan as a replacement for conventional dehydrating reagents in ultrastructural studies is not widely established and alcohol (e.g. Smith and Fishman, 1969; Bowen, 1972) and hydroxypropyl methacrylate (O'hare et al., 1971) still find favour. However, Beadle et al., (1971) and Bowen, (1971) have used water soluble Durcupan in their dehydration procedures for animal tissues and neither group of workers has reported a deleterious effect on enzyme localisation. Loss of lead-containing azo-dye may occur if the enzyme activity is very high or incubation times are too long (Livingston et al., 1970). Livingston et al., (1970) suggested that this loss of dye was possibly due to incomplete and inefficient post-fixation.

As far as is known only Tice and Barnett, (1965); Livingston et al., (1969), and Gahan and McLean, (1969), have used the diazotate of lead phthalocyanin for the ultrastructural localisation of non-specific esterases in plant and animal cells. The results of the present work do not bear out those of the last-mentioned workers for plant cells in that i) the morphology of the end-product was different; it being finely granular here as opposed to dense and globular in their studies, ii) although end-product was associated with Golgi cisternae and vesicles, variously sized vacuoles and endoplasmic reticulum in developing strawberry pulp cells it was not detected in spherosomes, nuclear membranes, or plasmodesmata as they noted in Vicia root cells and iii) the

present study showed that enzyme end-product was found to be associated with test and substrate-omitted material whereas Gahan and McLean could not detect end-product in control tissue. It is, however, likely that two such botanically distinct tissues as root tips and ripening strawberry pulp react in different ways to the effects of fixation, enzyme incubation, dehydration and embedding.

More work is necessary to determine whether the preservation of the various subcellular organelles of fruit cells can be improved by experimenting with different fixation procedures and to resolve the problem of non-specific lead adhering to control sections. Some possible explanations for this are given later.

The amount of loss of end-product from thin sections of tissue at each stage of the preparative procedures for electron microscopy was not examined. Despite this it was obvious that the amount of LPED in the cells examined did not correspond with that expected from light microscope studies. The methods employed here closely paralleled the procedures laid down by Livingston's group for animal tissue, and it is quite likely that these conditions are not optimal for plant material.

Cytochemical detection of non-specific esterases in animal cells at the ultrastructural level, have shown these enzymes to be associated with both lysosomal particles (Wachstein *et al.*, 1961) and the endoplasmic reticulum (Holt and Hicks, 1966). The arguments for considering the plant vacuoles as analagous to animal lysosomes have already been set out (see Chapters II, III and IV). Thus, these preliminary studies would corroborate both the cytochemical and biochemical findings of other workers for the subcellular localisation of non-specific esterases in both plant

and animal cells. For example Matile and Spichiger (1967), using biochemical methods, found esterases with high specific activity, associated with the spherosome fraction from tobacco endosperm, whilst Beadle et al., (1971) detected these enzymes cytochemically in both lipid droplets and membranous material concentrated in large membrane-bound bodies in the cells of the midgut epithelium of certain insects. Wigglesworth (1966) claims that in insect cells all lipid droplets possess a small zone of esterase activity at their surface, a structure he called 'esterase caps' or catalysomes. Pitt and Galpin (1973), found esterase activity associated with both 'heavy' and 'light' lysosomal fractions separated on a Ficoll density gradient. Their observations were made with potato shoot tissue.

Gahan and McClean (1969), using lead phthalocyanin diazotate as coupling salt, found esterases in broad bean root tips associated with a wide range of subcellular organelles including spherosomes. Their observation that the end-product was present on newly-forming cell walls and in surrounding vesicles is particularly relevant to the present studies. It is likely that these vesicles are homologous with the 'phragmosomes' seen by Porter and Machado (1960) in the dividing cells of onion root tips and with those seen by Burgess and Northcote (1968) in dividing cells of wheat roots.

The suggestion by Porter and Machado that phragmosomes "look most like small foci of lysis" is particularly relevant to the finding that vesicles associated with cell plate formation appear to contain hydrolytic enzymes, although there is no evidence that pectin-containing and enzyme-containing vesicles should be one and the same structure. Indeed not all vacuolar osmiophilia

contained end-product and it is probable that plant vacuoles are biochemically and physiologically heterogeneous.

Diemling and Madreiter (1972), have produced an up-to-date table of the available methods for the ultrahistochemical detection of non-specific esterases, including their own method whereby esters or derivatives of 8-hydroxyquinoline, were used as substrates. Yellow bismuth oxinate, formed by enzymic hydrolysis, is converted to electron-dense bismuth sulphide. Electron-dense material was present in lipid droplets and mitochondria, the only report so far of a mitochondrial esterase. Two further substrates, 8-acetoxyquinoline and 8-butyryloxyquinoline were used and a mitochondrial reaction was only obtained with the former, whilst staining of the lipid droplets occurred with both substrates. This work, and that of several others (Vatter et al., 1968; O'hare et al., 1971) has the advantage that hydrolysed substrate has characteristic absorption properties and can be quantitatively estimated by spectrophotometric methods, in tissue homogenates. This permits direct correlation with ultrahistochemical localization.

When Smith and Fishman (1969) were localizing acid hydrolases at the electron microscope level using diazotised acetoxymurcuric aniline as coupling reagent, they noted that in their 'no-substrate' controls, glutaraldehyde-fixed tissues developed a stronger affinity for the diazotate. In addition the ER cisternae showed little or no reaction when cacodylate instead of phosphate buffer was used with glutaraldehyde, suggesting greater enzyme inhibition with glutaraldehyde-cacodylate mixtures. This may well explain the presence of lead phthalocyanin diazotate in ripening fruit cells incubated without substrate.

Despite the good general agreement between the findings here and those reported by other workers for the subcellular localization of nonspecific esterase it would be prudent to suggest that much more work needs to be done to solve the problems of high resolution enzyme cytochemistry.

GENERAL DISCUSSION

In the study of plant cell differentiation, maturation and senescence, it has proved to be both possible and profitable to combine cytochemical and biochemical procedures in an investigation of the roles of non-specific esterase in these developmental processes.

In the studies of the role of acid phosphatase-staining particles in the process of cell division, however, it was not possible to use this approach. Although the use of statistical procedures appeared justified in the conditions of experimentation, perhaps a more satisfactory and simpler way of demonstrating any 'triggering' capacity of these enzymes would have been to turn to the use of synchronously dividing plant cell cultures such as those used by Yeoman and coworkers (Yeoman and Evans, 1967; Yeoman et al., 1968).

In this system small explants are cut from Jerusalem artichoke tubers and grown on a sucrose-mineral salts medium. These normally non-dividing cells can be stimulated to enter mitosis shortly after excision by the addition of the synthetic hormone, 2:4-dichlorophenoxyacetic acid (2:4-D) (Yeoman and Davidson, 1971). It will be recalled that in order for Allison and Mallucci to test their theory that phosphatases 'trigger' mitosis it was necessary to stimulate normally non-dividing cells into mitosis by adding phytohaemagglutinin. By using the synchronously dividing plant cultures a direct comparison can be made with animal cell studies.

Suitable numbers of explants treated with 2:4-D could be assayed for acid phosphatase (Hall and Butt, 1968) at each stage of the cell cycle (G_1 , S, G_2 , and M), and the results expressed on

per cell, per explant and per unit protein bases. A comparison could be made with both cultures lacking the hormone and those with the membrane-stabilizing agents, chloroquinone and prednisolone (as used by Hirschhorn and Hirschhorn, 1965) added to the growth medium.

In the present work significantly greater numbers of particles staining for naphthol AS BI phosphatase were found in mitotic cells as compared with interphase cells. This is the reverse of the results expected if these enzymes have any capacity to 'trigger' mitosis. In turn, these results pose another question. What are the reasons for mitotic cells possessing greater naphthol AS BI phosphatase activity than interphase cells? Some of these answers may be provided by the results from studies using synchronous cell cultures.

Providing that adequate consideration is given to possible kinetic problems that may arise as a result of using supersaturated solutions it has been possible to adapt enzyme cytochemical procedures using naphtholic substrates for biochemical assay and enzyme characterisation. This is particularly valuable with enzymes such as non-specific esterases where lack of specificity has led to problems in classification (Pearse 1973). Furthermore, a fuller understanding of esterase function might be obtained by the isolation of esterase isoenzymes by gel electrophoresis and the performance of kinetic studies on the purified enzyme.

Besides using whole root extracts for this work there is no reason why these characterisation studies could not be extended to studies of cellular differentiation in root tips. Using the procedures of serial sectioning and the assay method for naphthol

AS D esterases it is now possible to perform kinetic studies on small segments of root corresponding to the zones of division, elongation and differentiation. K_m determinations of esterases from each of these regions might prove rewarding since changes in binding characteristics of the enzymes may reflect changing patterns in synthesis and turnover that are related to the dynamic events of cell growth. Heyes and Brown, (1956) postulated that the types of protein synthesized along the root changed with the metabolic states of the cell so that in, for example, cells undergoing division the complex of catalytically-active protein molecules was different from that in cells undergoing vacuolation and expansion or maturation. Measurements of K_m values for esterases (and other hydrolases) could prove important in testing this theory.

In the absence of cell fractionation studies it has not been possible to determine whether the biochemical equivalents of animal lysosomes, plant vacuoles, are the sites of intensive esterase activity in the differentiating root cells or ripening cells of the strawberry. However the evidence that has been obtained shows that esterases from both root and fruit cells exhibit both acid pH optima and a degree of latency. Sonication of root tip cells showed that esterases are released more slowly than many other proteins and are therefore presumably membrane bound. Added support for this idea comes from electron microscope cytochemistry which has shown the enzymes to be associated with the membranes of small vacuoles and the rough endoplasmic reticulum. In fruit cells latency may have been revealed by the heightened esterase levels found in extracts from low-temperature stored

strawberries when compared with freshly harvested fruit.

Uncertainty arises in this case, however, because of complications due to possible losses of enzymes from damaged achenes.

Subjecting tissue sections of deseeded strawberry pulp to increasing sonication times may show a similar pattern of latent enzyme release to that observed for root tip cells. In addition the data from naphthol AS BI phosphatase suggests that at a critical time of incubation (between 40 and 50 min) the latency of the enzymes is released, as witnessed by the large increases in the number of azo-dye particles at 50 as opposed to 40 min.

During the differentiation of root cells both xylem tissues and to a lesser extent, phloem cells, are subjected to extensive intracellular changes involving the turnover of many macromolecules and the degradation and loss of several subcellular organelles, (Wooding and Northcote, 1964; Cronshaw and Bouck, 1965; Northcote and Wooding, 1968; Sheldrake, 1970 and O'Brien, 1970). The coincident rise in esterase activity assessed cytochemically and biochemically implies some fundamental relationship with either cell elongation or differentiation or both, the exact nature of which is still unknown.

Likewise, the cytochemical and biochemical studies of strawberry tissues revealed increases in esterase activity as the fruits ripened. The increase of esterase activity in cell differentiation, maturation and ripening may be due to one or more of the following; activation of the enzymes, loss of inhibitors or de novo synthesis. These changes may, in turn, be brought about by alterations in the physiological and biochemical states of the developing tissues. Distinction between these

alternatives would only be possible by carrying out appropriate protein synthesis inhibitor studies and radiochemical assays, perhaps in addition to electrophoretically separating proteins and esterases at different stages of cell differentiation and ripening.

From the above it is apparent that a large measure of control is needed to regulate the programmed synthesis and degradation of enzymes during such events as cell differentiation and cell senescence. Several investigators have attempted to explain the nature of these control mechanisms in slime moulds (Ashworth, 1971) and higher plant cells generally (Heslop-Harrison, 1971; Wareing, 1971). Heslop-Harrison (1971) has suggested that a gene-switching mechanism, along the lines proposed by Jacob and Monod (1961) for bacteria, may control the rates of protein and enzyme synthesis during normal plant cell differentiation, whilst Ashworth (1971) distinguishes between intracellular control mechanisms that regulate the activity ('fine control') or quantity ('coarse control') of enzymes synthesized and intercellular mechanisms that control the types of enzymes synthesized. In this connection Wareing (1971) has proposed that plant hormones control patterns of differentiation by acting at both transcriptional and translational stages of RNA and protein synthesis and by acting as effector substances in gene derepression. When the exact nature of these mechanisms is known there is no reason to suppose that they may not be of universal application.

Senescence as an extended process of differentiation would likewise be susceptible to these control mechanisms and they have been implicated by Butler and Simon (1971) as the agents

that programme the course of plant cell and tissue senescence. With reference to lysosomal enzymes these authors suggest that during the course of senescence the structural and functional organisation of cells is lost and with consequent loss of differential membrane permeability these enzymes are released into the cytoplasm. Certainly the large increases in esterase activity and changes in enzyme-substrate binding characteristics from the immature to the 'maturing' stage of strawberry development coupled with the ultrastructural evidence of ribosomal loss and organelle vesiculation suggests that a gene-switching mechanism, perhaps elicited by hormonal control, may be operative here.

The present cytochemical studies showed that esterases were particle-bound during the ripening process thereby indicating that the observed increases in biochemical assays of esterase activity were due to an increase in the number of particles. Whether the latency of these particles would be broken in the cells of overripe fruit is not known. This idea could be tested by homogenising tissues from each of the ripening stages so that the latency of the esterase-containing particles was not broken and subsequently assaying esterase activity in supernatant and particulate fractions after appropriate centrifugation (e.g. Pitt and Coombes, 1968). Increases in the supernatant fraction with ripening would indicate changes in the permeability of the particle membrane.

Cytochemical observations at the light and electron microscope levels of root and fruit cells reveal that non-specific esterases are associated with a range of subcellular particles. This accords with the observations made by other workers (Matile, 1968b; Heftmann, 1971; Pitt and Galpin, 1973) that non-specific

esterase activity can be found in several fractions after the differential density gradient centrifugation of cell-free extracts. Besides the biochemical evidence there is considerable evidence from morphological studies to suggest that there are several different subcellular organelles that could possibly be the sites of esterase activity in root and fruit cells. In view of current theories concerning plant lysosomes (Matile, 1969) the vacuoles are the first organelles to merit consideration. The origin and ontogeny of these structures have been discussed by many workers in the past (Clowes and Juniper, 1968; Matile, 1969; Berjak, 1972). Current opinion favours the view that they are formed either by terminal or intermediate inflations of the endoplasmic reticulum, thereby giving rise to small provacuoles 0.1 to 0.3 μ m in diameter, (Matile and Moor, 1968; Berjak and Villiers, 1970). This therefore means that the vacuolar membrane or tonoplast should be homologous with the ER membranes (Bowes, 1965; Buvat, 1961; Poux, 1962). Matile and Moor (1968) showed in their freeze-etch studies of root meristem cells from maize, a marked ultrastructural similarity between these membranes. Furthermore, Matile (1969) has suggested that, since the enzymes associated with vacuoles isolated from meristematic cells, also characterise the ER membrane (Matile, 1966, 1968b), then it is likely that acid hydrolases could be derived from the latter.

Some convincing evidence for this notion comes from the combined physiological and electron microscope studies of Vigil and Rudatt (1970) who showed that during the secretion of acid hydrolases from the aleurone layer of barley seeds the rough ER

becomes vesiculate, and there is an accumulation of dense globular material in terminal regions of the ER and in indentations of the plasmalemma facing the cell wall.

Other evidence suggests that small lipid droplets or spherosomes are also derived from the ER (Frey-Wyssling et al., 1963) and whilst some investigators claim that a variety of hydrolases are associated with isolated spherosome fractions (Matile et al., 1965; Balz, 1966; Semadeni, 1967), Yatsu et al., (1971) working with a highly purified spherosome fraction from onion and cabbage, could detect no acid phosphatase in these organelles, (although no other acid hydrolases were assayed).

Evidence from these studies (Chapter V) suggests that small vacuoles (0.3 - 1.0 μm) containing esterase end-product may find their way into newly-forming cell walls. This is to be contrasted with many other studies in plant cells which have shown that Golgi-derived vesicles carrying cell wall components are incorporated into cell walls (Porter and Machado, 1960; Northcote and Pickett-Heaps, 1966; Matile et al., 1967). Although these vesicles are much smaller than provacuoles (50 nm diameter, Clowes and Juniper, 1968) there is no reason to suppose that populations of Golgi vesicles or the Golgi apparatus themselves are not biochemically heterogeneous (Roberts and Northcote, 1970), nor that these vesicles have some propensity for fusing with other cellular vacuoles to form primary lysosomes, as some workers believe occurs in animal cells (Novikoff et al., 1964 and reviewed by de Duve and Wattiaux, 1966). Moreover the possibility must also be considered that the Golgi complex is capable of synthesizing enzymes directly or at least packaging them after

transport from their sites of synthesis on the ribosomes of the ER (Dauwalder et al., 1969). The cytochemical evidence from these studies that shows esterases to be present in cell walls of Vicia faba roots (particularly root cap cells) confirms the observations of Gahan and McLean (1969) and McLean (1970).

Other biochemical evidence also suggests that a high percentage of the total acid hydrolase activity may reside in plant cell walls (80% for acid phosphatase in cultured cells, Lamport and Northcote, 1960). Recent evidence for the original postulate (Sutcliffe, 1962) that some plant cells at least may carry out the process of pinocytosis (Wheeler and Hanchey, 1971; Wheeler et al., 1972) is interesting in this light, since if future evidence confirms this unequivocally then one way of accounting for plant cell wall enzymes would be to suggest that they are carried there by small vesicles or vacuoles budded off from the ER cisternae or Golgi apparatus. They would finally discharge their contents into the wall by fusion with the plasmalemma; i.e. exocytosis will occur. The processes of pinocytosis and exocytosis have, of course, already been verified in animal cells (Cohn and Benson, 1965a, 1965b).

From the foregoing discussion it is obvious that the esterase-staining particles seen at the light microscope level in root and fruit cells, 0.5 - 1.0 μ m in diameter, could be Golgi vesicles, swollen ends of the Golgi cisternae or Golgi saccules, ER-derived provacuoles, small vacuoles or spherosomal bodies. Depending on the physiological status of the cell, one or more of these organelles may be the esterase-containing body in root and fruit cells.

It is more difficult to assign a subcellular origin to the large membranous bodies of young strawberry receptacles that stained diffusely with end-product, and the enormous lipid-droplet-like bodies seen in ripening fruit cells. On the basis of size the membranous bodies could be analogous to the secondary lysosomes of animal cells (de Duve, 1969; Beadle et al., 1973), and represent the coalescence of a number of smaller esterase-staining organelles. Only reliable electron microscope cytochemistry and subcellular fractionation studies would reveal whether this is so. It might be logical to regard the immense lipid-droplet-like esterase-containing bodies as the result of general cell organelle vesiculation (possibly of ER cisternae) that is a characteristic feature of senescing plant cells (Butler and Simon, 1971).

In glutaraldehyde-fixed tissues, esterase-staining particles appeared slightly larger than in unfixed cells. This was true of both root and fruit cells, and may be due to the known effects of this fixative on the permeability characteristics of cell membranes (e.g. Jard et al., 1966; Grantham et al., 1971; Hopwood, 1972).

In these studies only infrequent reference has been made to the roles of plant hormones in differentiation and senescence, since no attempt has been made to assess the effect of these substances on acid hydrolase activity. However there is now a substantial body of evidence that many of these hormones are of fundamental importance in affecting the synthesis and stability of a range of plant acid hydrolases. For instance Gibson and Paleg (1972) showed that gibberellic acid can decrease the latency, and thereby increase the availability of α -amylase in tissue

homogenates of the barley aleurone layer. Filner and Varner (1967) and Jacobsen and Varner (1967) working with the same tissue have shown by density labelling methods, that gibberellic acid induced de novo synthesis of α -amylase and protease respectively.

Balz (1966) has demonstrated that kinetin, the phytohormone that delays senescence even in detached leaves, inhibits the breakdown of protein and chlorophyll, and also the formation of lysosomal enzymes such as ribonuclease. Similarly Srivastava and Ware (1965) had previously noted that the same hormone applied to excised barley leaves caused a decrease in the activity of ribonuclease.

There are no reported direct relationships between IAA or any of its derivatives, and acid hydrolase activity, although the fundamental importance of this hormone (in conjunction with cytokinins) in protein and RNA synthesis has been known for some time (Skoog and Miller, 1957). Perhaps some idea of the possible relevance of auxin to acid hydrolase production can be gauged by the work of Shelldrake and Northcote (1968) and Shelldrake (1970), who showed that autolysing cells of differentiating xylem and phloem in callus blocks of bean release auxin directly into the growth medium. In view of the proposed roles of acid hydrolases in autolytic phenomena a relationship, either causal or incidental, seems probable (Northcote, 1971). Moreover, many workers in the past (Jacobs, 1952; Wetmore and Rier, 1963; Sachs, 1972) have demonstrated the importance of IAA and its derivatives in the regeneration of vascular tissues in callus cells cultured from a variety of plants.

In the light of these comments it would be interesting to determine whether the association of intense esterase (and acid phosphatase) activity with the procambial and vascular tissues of the broad bean root and the vascular traces of developing fruit tissues is related to the formation of IAA during vascular differentiation.

The eventual relationships between hormone production, plant cell differentiation and senescence on the one hand and acid hydrolases on the other may only be revealed by the use of combined physiological, biochemical and cytochemical techniques, both on separated micro-dissected tissues and, perhaps more conveniently on undifferentiated cultured plant cells.

Methodological problems pertaining to the accurate localisation of β -glycerophosphatase in plant cells still remain, and in view of the past controversy over this enzyme in animal cells (e.g. Holt, 1959) a full-scale investigation of factors affecting lead staining of plant cells using the Gomori procedure is needed. Problems are also associated with azo-dye methods both at the light and electron microscope levels. At the optical level these methods are generally regarded as reliable (Burstone, 1962; Pearse, 1960, 1973) but the findings that reducing agents such as ascorbic acid can prevent the formation of azo-dye means that extra care must be taken when investigating material known to contain high endogenous concentrations of the reagent.

However it is likely that in the cytochemical conditions of test, endogenous ascorbate levels from thin tissue sections will have been sufficiently diluted by the large volumes of buffer in

the Coplin jar, to prevent the interference of the reducing agent in the formation of the azo-dye.

The problems of accurate, ultrastructural localisation of acid hydrolases are unfortunately numerous (see review by Shnitka and Seligman, 1971). Problems of substrate penetration of both fixatives and cytochemical reagents into tissue blocks can be overcome to a certain extent by either microdissection or by the use of tissue slices less than 50 μ m thick (Sexton *et al.*, 1971). More difficult to solve are the problems of the removal of enzyme end-product during dehydration and the embedding processes and the avidity of heavy metal diazotates for lysosomal structures (Beadle *et al.*, 1973). It was mainly for this reason that preliminary attempts were made to cut unfixed, frozen sections of plant tissues for the ultrastructural localisation of enzymes (Gahan *et al.*, 1970). The results indicate that much improvement needs to be made in the morphological preservation of subcellular structures before any confidence can be placed in the method.

If these problems can be overcome then direct confirmation of biochemical data from cell fractionation studies will be possible and more definitive statements made about the intracellular origin and function of these enzymes.

In conclusion, the evidence presented here has shown that acid phosphatases as detected by the azo-dye method have no capacity to trigger mitosis in plant cells, when differences between stained particle counts per cell in dividing and non-dividing cells are the criteria used to measure triggering.

Like several other enzymes, previously assayed by other workers, non-specific esterase activity increases markedly as plant cells expand, vacuolate and differentiate in the root tips of broad bean. A plateau of activity is reached by the time most cells have reached their fully functional differentiated state. Parallel cytochemical studies have verified this by showing that the most intense enzyme end-product was associated with cells undergoing growth processes.

Similarly the same hydrolases show several fold increases in activity as strawberries increase in size and fresh weight and the flesh undergoes a colour change from green to red. Increases in enzyme activity in this case have been matched by kinetic studies which showed an increased affinity of the enzymes for their substrate. Cytochemical studies have revealed that possibly greater esterase activity may exist in very young receptacles that are not amenable to biochemical assay due to the presence of achenes on the receptacle surface. Morphological studies at the ultrastructural level show that organelle vesiculation and loss of ribosomal material are the two predominant changes in ripening pulp cells, both being characteristic cytological features of senescing cells (Butler and Simon, 1971).

The non-specific esterases extracted from both root and fruit cells exhibit acid pH optima and show latency after ultrasonication or freeze-thawing. They are also compartmentalised within cells and may exist in a variety of subcellular organelles but appear to be particularly associated with small vacuoles and endoplasmic reticulum.

These studies have therefore shown that non-specific esterases in plant cells are intimately involved in the physiology and biochemistry of growth, differentiation and maturation on a scale comparable with their counterparts in animal cells.

PUBLICATIONS FROM THIS THESIS

1. Gahan, P.B., Greenoak, G and James D.J., (1970). Preparation of ultra-thin frozen sections of plant tissues for electron microscopy. *Histochemie*, 24, 230-235.
2. James, D.J., and Smith, A.R.W., (1972). The interference of L-ascorbic acid with a standard histochemical azo-dye coupling procedure. *Histochemie*, 30, 273-276.
3. Beadle, D.J., Dawson, A.L., James, D.J., Fisher, S., and Livingston, D.C., (1973) The avidity of heavy metal diazotates for animal lysosomes and plant vacuoles during the ultrastructural localisation of acid hydrolases. 2nd. International Symposium on Electron Microscopy and Cytochemistry, (Enschede, Holland) p.85. North Holland Publishing Company.
4. IN PRESS:
James, D.J., and Smith, A.R.W., (1974). A biochemical study of non-specific esterases from plant cells, employing the histochemical substrate, naphthol AS D acetate. *Histochem.J.* 4.

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Short Communication

**The Interference of L-Ascorbic Acid
with a Standard Histochemical Azo Dye Coupling Procedure**

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Summary. The reducing agent, L-ascorbic acid, has been shown to interfere with the chromogenesis of free Naphthol AS D, and the diazonium salt, fast red violet.

The findings reported here originate from studies in this laboratory on the cytochemistry and biochemistry of nonspecific esterases in developing plant tissues, notably the lateral root tips of the broad bean, *Vicia faba* and the maturing fruits of commercially grown strawberries.

In this work L-ascorbic acid was used routinely to prevent the darkening of plant extracts during and after homogenisation. The problems associated with the formation of coloured oxidation products upon cell and tissue breakage, the resultant denaturation of enzymes and the measures taken to prevent these effects are comprehensively reviewed by Anderson (1968).

Cytochemical studies of the esterases were performed using the azo dye method of Burstone (1962); the same enzymes were assayed biochemically, using a modification of Gomori's method (1953) developed by the authors (to be published elsewhere). Essentially this technique involves maintaining enzymically hydrolysed Naphthol AS D acetate in a clear, colloidal solution as free Naphthol AS D by the use of ethylene glycol and a detergent such as sodium dodecyl sulphate. Up to 0.033 μ moles per ml of free Naphthol AS D is then post-coupled with a standard excess of fast red violet LB salt, (diazotised 5-benzamido 4-chloro-*o*-toluidine; Sigma Chemical Co. Ltd., London, England). The absorbancy of the resulting soluble azo dye product is read spectrophotometrically at 520 nm. Standardisation is effected using solutions of Naphthol AS D (Sigma) of known concentration.

In the early stages of the work acetone-dried powder extracts of the tissues were prepared in which sodium L-ascorbate (Sigma) was added to a final concentration of 100 mM to prevent tissue discolouration. Such powders, when extracted with distilled water or buffer for the assay of enzyme content, produced lower colour intensities after assay than control solutions measuring non-enzymic hydrolysis.

The coupling process of fast red violet LB salt with added free Naphthol AS D in known concentration was then shown to be remarkably sensitive to the presence of small amounts of L-ascorbate. Fig. 1 shows the effect upon dye production of increasing the concentration of ascorbate in a standard system.

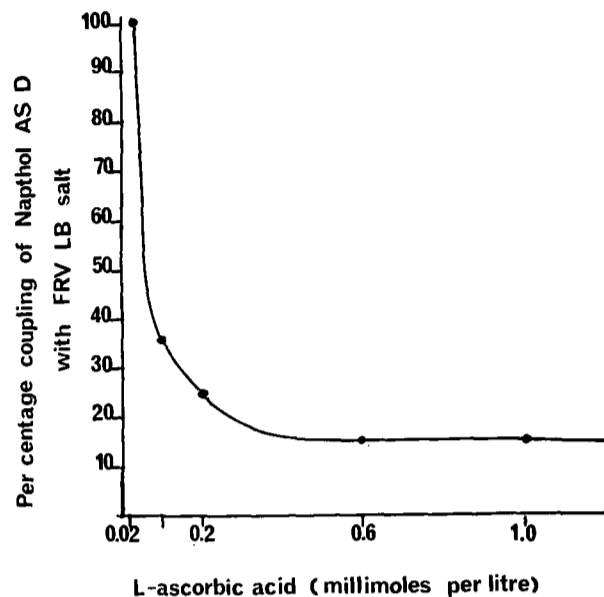


Fig. 1. The effect of L-ascorbate on the coupling of free Naphthol AS D with fast red violet LB salt. 3 ml. of Naphthol AS D solution, (0.1 μ mol/ml) was post-coupled with 5 ml of a 30 mg-% (w/v) solution of fast red violet LB salt in the presence of 1 ml of 5% (w/v) sodium dodecyl sulphate, 5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 1 ml of a 10 mg-% (w/v) solution of bovine serum albumen

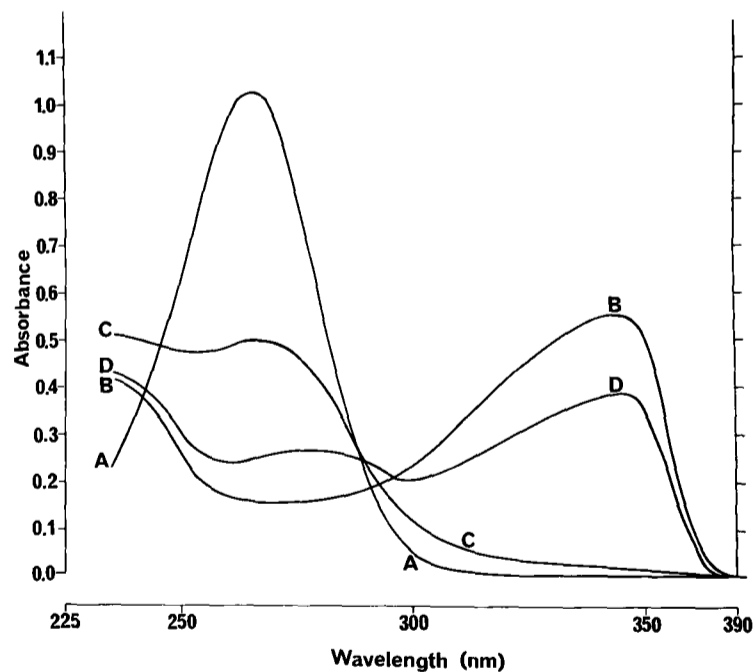


Fig. 2A—D. U. V. Spectra of L-ascorbate, fast red violet LB salt and mixtures thereof. Cuvettes of 1 cm. path length contained: A) 0.067 mM. L-ascorbate; B) 30 μ g/ml of fast red violet LB salt; C) 30 μ g/ml of fast red violet LB salt and 0.033 mM L-ascorbate; D) 30 μ g/ml of fast red violet LB salt and 0.008 mM L-ascorbate, all in McIlvaine's buffer pH 5.4. The reference cuvette contained the same buffer

The dye, once formed, was not bleached by subsequent addition of L-ascorbate. The free Naphthol AS D contains no bonds that are ordinarily considered to be sensitive to easy reduction, and so was not investigated further for reactivity. The diazo group of diazonium salts may, on the other hand, undergo readily a four-electron reduction to the corresponding arylhydrazine in the presence of a variety of reducing agents (*vide e. g.* Hickinbottom, 1957).

Fig. 2 shows the modifications in the u. v. spectra of solutions of fast red violet LB salt and ascorbate that are produced upon mixture at pH 5.4. At the λ max. of fast red violet LB salt of about 343 nm., and of ascorbate at 265 nm., absorbancy diminished rapidly. These results are consistent at least with the conversion of ascorbate to dehydroascorbate, and with removal of the diazonium salt. The nature of the 'stabilisers' used with this brand of fast red violet are not consistent with their possessing any absorbancy in the u. v., nor do they react with ascorbate. It would thus appear likely that ascorbate reduces the diazonium salt to the corresponding aryl-hydrazine, from which dye production is not possible.

The cytochemical significance of these findings has not yet been determined, but interpretation of localisation studies, using Burstone's method on tissues known to contain high levels of endogenous ascorbate, should be tempered by these findings.

In this respect, animal tissues such as liver and kidney, and many plant tissues, particularly fruits and vegetables, may pose problems in interpretation. Thus, in this laboratory, nonspecific esterases have been localised cytochemically in strawberry pulp in varying degrees of intensity over a period of three seasons. In one season no activity could be detected despite the examination of several fruits from all stages of ripening. In contrast it was always possible to measure esterolytic activity in the biochemical assay of purified, lyophilised extracts. The apparent disparity of these results might be reconcilable by the following reasoning: endogenous levels of ascorbate in fresh, frozen sections of strawberry pulp prepared for cytochemical tests may be sufficiently high at certain times of the season to interfere with the chromogenesis of the cytochemical reagents. Olliver (1939), discussed the influence of various factors, such as degree of maturity and date of picking, upon the level of L-ascorbate in any one berry. Furthermore, Jensen and Kavaljian (1956), when using the "silver method" to detect ascorbate cytochemically, in root tips, showed that the intensity of the stain varied from tissue to tissue. In the course of enzyme purification for biochemical assay however, extensive dialysis removes endogenous ascorbate, so allowing the detection of esterolytic activity in the assay procedure.

It is possible that other reducing agents may have a similar effect on chromogenesis and that coupling salts other than fast red violet, may be affected by them.

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Preparation of Ultra-thin Frozen Sections of Plant Tissues for Electron Microscopy

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Summary. A method is described, for the first time, by which ultra-thin frozen sections of plant tissues may be prepared for electron microscopy. Sections of both plant and animal tissues were prepared from either unfixed or fixed tissues, without prior dehydration or infiltration of the tissue with a support medium, and with the aid of a Reichert OmU2 ultra-microtome with freezing attachment.

Introduction

It is clear from a number of studies that the conventional methods of tissue preparation for electron microscopy result in the loss of lipids, proteins, and nucleic acids from both plant and animal tissues (Dallam, 1957; Cope and Williams, 1968; C. Cave and P. B. Gahan, unpublished data). In addition, the conventional methods of dehydrating and infiltrating with a resin often result in partial or total loss of the reaction product of a number of cytochemical reactions (e.g., Holt and Hicks, 1966; Livingston *et al.*, 1969).

Attempts have been made to improve this situation by the introduction of the use of a refrigerated ultra-microtome, enabling fixed or unfixed, ultra-thin frozen sections to be cut for electron microscopy whilst avoiding the use of organic solvents as dehydrating agents, and of embedding media (Bernhard and Leduc, 1967; Appleton, 1969; Christianson, 1969). Alternatively, ultra-thin frozen sections have been prepared from fixed or unfixed cryostat sections dried onto the outside of an Epon block and sectioned at room temperature with an ultramicrotome (Tranzer, 1965).

The methods of Bernhard and his collaborators had the disadvantage of the need to encapsulate the material in gelatin prior to cutting. This need has been overcome in the studies of Appleton (1969) and of Christianson (1969), in which fixed and unfixed frozen sections of mammalian tissues were prepared without the use of a support medium, and, in the case of Appleton (1969), directly onto the dry knife and with the omission of the flotation bath.

All such studies have concerned animal tissues, and there appears to be no such study with plant tissues. The present communication describes an approach to the problem of preparing ultra-thin frozen sections from embryos and root tissues of *Vicia faba*.

Materials, Methods and Results

1. Tissues. Lateral roots of *Vicia faba* were used when 1–2 cm in length. *V. faba* embryos taken from seeds soaked in running tap water for 24–48 hours were also employed.

As a control, and to test the cutting techniques already described, some animal tissues were employed, namely, small intestine, liver, kidney, and a transplanted tumour from a spontaneously occurring fibrosarcoma, all from male mice of the C57 strain.

2. Tissue Preparation. Animal tissue pieces (not larger than 0.5–1 mm, cubed) were used after fixation for 2 hours in 2.5 percent glutaraldehyde, in 0.1 M cacodylate buffer, at pH 7.1, at 2° C, followed by washing in cacodylate buffer at 2° C for 18 hours.

Plant tissues (not larger than 0.5–1 mm, cubed) were used either unfixed or fixed as described above for the animal tissues. The tissues were used directly or were subjected to treatments designed to protect the cells from freezing damage: a) infiltration with glycerol according to the method of Dolhoff *et al.* (1969 a, b); b) pretreatment with 5 percent aqueous polyvinyl alcohol (PVA) solution for 1 to 2 hours (Gahan *et al.*, 1967); or c) pretreatment with 1 percent gum acacia in 0.88 M sucrose solution for 30 minutes to 2 hours (Holt, 1958).

3. Preparation of the Sections. Small pieces of the various tissues, prepared by the methods described above, were frozen directly onto the precooled specimen holder of the microtome in the cutting chamber at different temperatures between –120 and –60° C.

Sections were cut on a Reichert OmU2 ultra-microtome with a freezing attachment, using glass knives having a cutting angle of 45 degrees. A concentration of 60 percent dimethylsulfoxide (DMSO) in water was used for the flotation solution, since lower concentrations of the DMSO tended to freeze at the lower temperatures employed.

During the cutting of the sections, variations were made in the cutting speed, the angle of the knife to the specimen, the temperature of the knife, and the temperature of the specimen itself (Table).

It was difficult to observe the very thin sections on the surface of the DMSO bath, there being no interference colours, and so the first section cut was always thick, to act as a marker for the subsequent sections.

Both the fixed and unfixed sections were picked directly onto copper grids. Initially, use was made of formvar-coated grids, but it was found that uncoated grids of 400-mesh size could be employed satisfactorily. At times, the DMSO did not drain completely from the sections, rendering them opaque in the electron beam. This difficulty was overcome by washing the sections briefly with distilled water and was reduced also by the use of uncoated grids.

4. Staining. Attempts to stain the sections conventionally with either uranyl acetate or lead citrate proved abortive, as has been described by other workers (e.g., Appleton, 1969). In an effort to introduce some contrast into the section, it was decided to try to react the tissue for acid β -glycerophosphatase activity by the method of Gomori (1950), since this technique should have resulted in the

Table. *Effects of the pretreatment and variation in temperatures on the production of ultra-thin frozen sections of embryos of *V. faba* fixed in 2.5 percent glutaraldehyde, in 0.1 M cacodylate buffer, at pH 7.1, for 2 hours, at +4°C. The microtome was employed with section-thickness setting at the gold position and the cutting speed at the diamond knife setting*

Pre-treatment	Freezing temperature (°C)	Knife temperature (°C)	Block temperature (°C)	Observations
None	-110	-40	-90	Good sections. No ribbons. Good sections in ribbons. Good preservation of the cytoplasm
	-60	-40	-90	
Glycerol	-110	-40	-90	No sections obtained
PVA	-110	-40	-110	Sections thick, compressed, but in ribbons. Cytology poor
	-110	-40	-90	Less compression and thinner sections
	-60	-40	-70	Sections thin
Gum-	-120	-40 to -30	-70 to -60	Ribbons of intact sections, but compression. Very bad ice damage
	-60	-40 to -30	-80 to -60	Good sections. Ice damage very much reduced (Fig. 1)

deposition of lead at specific and known sites in the plant cells (Gahan and McLean, 1969). The glutaraldehyde-fixed tissue blocks were incubated for 30 minutes at 37°C in the reaction medium normally employed for acid β -glycerophosphatase activity. For a normal cytochemical reaction, an incubation time of 8 minutes has been found to be satisfactory in meristematic tissues (Gahan and McLean, 1969), but, in order to be certain that lead would be deposited, the blocks were deliberately over-incubated for 30 minutes, which would be expected to yield a heavy nuclear deposition of lead. After incubation, the tissue blocks were rinsed in 1 percent acetic acid for 10 seconds and then in buffer for 30 minutes prior to immersing in the gum-sucrose medium described above.

5. Viewing the Sections. Sections were viewed, either unstained or reacted by the Gomori method, with an A.E.I. EM 6b, using accelerating voltages of 40 and 60 kv, and employing 25 μ m objective apertures.

Discussion

The better sections were obtained from the fixed material after treating the tissue blocks with gum-sucrose for 30 minutes, prior to freezing at 60°C, directly onto the block-holder. Sectioning was performed preferentially with a glass knife cutting angle of 45 degrees, employing the cutting speed recommended for the diamond knife by the manufacturers of the microtome, the section-thickness setting that would yield gold sections with resin-embedded material, a knife temperature of -40 to -30°C, and a specimen temperature of -80 to -60°C.

1. *Animal Tissues.* Using these preparation and cutting conditions, all of the animal tissues employed yielded very good sections, though similar difficulties to those described below with plant tissues occurred in attempts to stain the sections with lead citrate and uranyl acetate.

2. *Plant Tissues.* The use of glycerol as a pretreatment resulted in very poor preservation of the plant cytoplasm, presumably due in part to plasmolysis, but the nuclei were kept in very good condition. The pretreatment with PVA resulted in the production of good sections, but, possibly because of the presence of the PVA, even thin sections were very electron-dense and there was extreme difficulty in observing subcellular detail. Gum-sucrose proved to be the best of the pretreatments essayed, yielding good sections with well-preserved cytoplasm. Good sections were also prepared from tissue blocks which had received no treatment or fixation.

Changing the initial freezing temperature had severe effects upon the final preservation of the subcellular organelles. If a freezing temperature of -60°C was used, as described by Gahan *et al.* (1967) for cryostat preparations, then minimal freezing damage was observed (Fig. 1). If, however, the freezing temperature was lowered to -110°C , then the lower the temperature, the greater the damage, regardless as to whether or not the tissue was fixed and whether or not there had been some form of pretreatment.

The cutting speeds were of importance; the faster the speed, the more compression was observed and the greater the cutting damage. Due to the construction of the knife-holder, it was not possible to test the effects of the diamond knife available in the laboratory, and so the comment of Dolhoff *et al.* (1969b), concerning the superiority of diamond knives versus glass knives, could not be tested.

Section-thickness settings selected varied, but the best results obtained were those employing the thickness setting that would yield gold sections with tissues embedded in resin. Owing to the lack of interference colours when the sections were floating on the DMSO bath, it was not possible to gauge the thickness of the sections. An assessment of section thickness once the sections were in the electron microscope revealed them to be of the order of 80 nm under normal conditions of cutting.

Cutting temperatures were assessed on a trial-and-error basis, and optimal conditions were found to be (a) knife temperature at -40 to -30°C , and (b) specimen temperature at -80 to -60°C . These seemed surprisingly high in view of the heat likely to be generated at the cutting front but which did not appear to affect the good preservation of the sections. Lower temperatures resulted in much thicker sections, more compression, and a greater difficulty in obtaining ribbons.

The introduction of an electron-dense stain into the sections still remains a problem. Other workers have had similar difficulties, and it would seem that this condition may be due in part to the lack of disturbance of the tissue structures through the omission of organic solvents and resins from the processing, so reducing the number of binding sites for the heavy metals.

It is clear that good, ultra-thin frozen sections of plant and animal tissues can be prepared, and the way is now open for the application of this approach to studies of lipids, diffusible substances, and enzymes in plant tissues. It is



Fig. 1. Ultra-thin frozen section of root meristem of *Vicia faba*, which has been fixed in 2.5 percent glutaraldehyde for 2 hours and incubated for 30 minutes at 37° C for acid β -glycerophosphatase activity prior to freezing and sectioning. Note the heavy deposit of lead on the nucleus and the nucleolus and in the endoplasmic reticulum. No lead is associated with the mitochondria. *N* nucleus, *NU* nucleolus, *W* cell wall, *M* mitochondria with cristae. *NM* nuclear membrane, *ER* endoplasmic reticulum. Magnification: $\times 37000$

equally important to remember, however, that these conditions are satisfactory for root and embryo tissues of *V. faba*, but they may have to be modified slightly for other tissues.

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