ON THE VACUOLAR SYSTEM IN MAIZE ROOTS

by

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This thesis represents the candidate's own original work (stated in compliance with University regulations).

for my parents who mean so much to me, with love

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ABSTRACT

Root-cap cells of Zea mays L proliferate by division in the cap meristem, and subsequently differentiate and mature as they move towards the periphery of the cap, where they undergo autolysis and are sloughed. Vacuolar ontogeny has been shown to be complex, several different mechanisms operating not only within the root cap tissue, but within the single cells. Vacuolar initials (provacuoles) are formed in the meristem by the pinching off of single- or double-membrane bound vesiculations of the E.R. In some instances large vacuoles appear to be formed in the mature region of the cap through the sequestering of large organelle-free regions of cytoplasm by vesicles and small cisternae, thought to be of E.R. origin. Further development of provacuoles comprises their expansion and extensive fusion, this process culminating in the formation, in a mature cell, of just one large vacuole. The vacuoles of the mature region are

autophagically active, engulfing all types of cytoplasmic organelle which are subsequently lysed; these vacuoles show a positive cytochemical reaction for acid-phosphatase, further indicating that they are lysosomal in nature. The dictyosomes of the late mature cells are hypersecretory and autoradiographic and cytochemical evidence indicates that the vesicles contain an accumulation of polysaccharide. These vesicles appear to follow two secretory pathways; firstly fusion with the plasmalemma, with secretion of their content into the extra-protoplasmic space where it accumulates, finally penetrating the cell wall and middle-lamella and forming viscous polysaccharide slime on the exterior of the cap. Secondly, these vesicles appear to be engulfed by and broken down within the vacuoles. At this stage the vacuole expands considerably, and it has been postulated (Berjak and Villiers, pers. comm.) that hydrolysis of the dictyosomally-derived polysaccharide within the vacuole to monosaccharide units results in osmotic changes leading to an influx of water into the vacuole, and its consequent expansion. Autoradiographic, cytochemical and chromatographic evidence is not inconsistent with an accumulation of monosaccharide units being at least partially responsible for the osmotic uptake of water into the swelling vacuole. Finally, the vacuolar membrane becomes discontinuous, allowing hydrolytic enzymes presumably contained within the vacuole to come into contact with the cytoplasm, which consequently undergoes autolysis. At this stage the cell is sloughed from the cap.

PART ONE

INTRODUCTION

Cell biology began when Robert Hooke, an Englishman and curator of the Royal Society, first described cells in his 'Micrographia', published in 1665. With the aid of an early version of the compound microscope he was able to trace out small compartment-like units in the woody tissues of plants - these he named 'pores' or 'cells'. Subsequent studies of cells have been dependent on improvements in the resolving power of the microscope. Initially knowledge of the internal structure of the cell was limited - Hooke 'plainly enough discover'd these cells or pores fill'd with juices'. The cell fluid was named 'protoplasm' by J.E.

Purkinje in 1840, and as improvements in light optics occurred, substructural compartments within the protoplasm were recognised (see Wolfe, 1972). The vacuole, being one of the most conspicuous components of the plant cell, was one of the earliest to be noticed. It appeared as a fluid-filled, translucent region surrounded by the jelly-like cytoplasm and had the property of accumulating basic vital stains such as neutral red. This criterion, introduced by Dangeard in 1916 (see Dangeard, 1956; *loc. cit.* Matile 1974) recognises that the vacuole often accumulates organic acids and phenolic compounds capable of binding basic stains.

Further elucidation of the structure and development of the vacuole had to await the advent of the electron microscope, which went into commercial production in 1938. With the aid of this instrument it was discovered that plant cells are subdivided into several morphologically distinct compartments, separated from one another by membranes. The vacuome (all the vacuoles present within a cell) apparently occupied most of the volume of the cell, consisting either of a number of small vacuoles or, in the case of parenchymatous cells, a single large vacuole. The vacuole, the interior of which appeared relatively featureless, was always surrounded by a single membrane, termed the tonoplast.

Initially the vacuole was regarded as having rather a limited role in cell metabolism. The term vacuole (reminiscent of vacuum) is perhaps symbolic of this approach although it undoubtedly refers to the featureless, watery appearance of the vacuolar content or 'cell sap'. Vacuoles were considered to represent the sites of temporary or final deposition of waste products, and Matile (1975) stresses that the term waste product means that the substance has no *presently known* function. By use of histochemical tests and vital stains, early plant cytologists deduced a vast amount about the chemical composition of the vacuolar fluid (cell sap). Inorganic salts, sugars, organic acids, amino acids, amides, lipids, mucilages, gums, tannins, anthocyanins and flavones have been detected within the vacuoles by such means.

1.1 SUBSTANCES DEPOSITED IN VACUOLES AND THEIR POSSIBLE FUNCTIONS.

1.1.1 *Metabolic intermediates*

A large number of the intermediates of plant metabolism are formed in excess, and if stored within the cytoplasm may interfere with ongoing metabolism. For example squeezed lemon juice has a pH of approximately 2.5, which is well below the pH optimum of the enzymes involved in cytoplasmic metabolism. This acidity is thought to be the result of the accumulation of tricarboxylic acid cycle intermediates. It must therefore be postulated that such substances formed in excess are compartmentalised to prevent their interference with cytoplasmic metabolism. The existence of discrete pools of intermediates not in equilibrium with metabolised pools has often been demonstrated (Beevers *et. al.*, 1966). It is assumed that such storage pools exist within the vacuole, this observation being based on the well known acidity of the vacuolar contents as indicated by the colour of anthocyanins, natural indicators present within certain cell saps, or by vital indicator stains such as bromophenol blue (Matile, 1976). There are many examples of such storage pools, a striking one being the accumulation of amino acids within the vacuoles of Candida utilis cells. Wiemken and Nurse (1973a,b: *loc. cit.* Matile, 1976), after stepwise extraction of cells of Candida utilis, reported that only 5 - 10% of the total amino acid was to be found in the cytoplasm, the remaining 90% being stored within the vacuole. Moreover the vacuolar amino acids were predominantly nitrogen-rich, and if the cells were supplied with inorganic nitrogen, this pool was filled with endogeneously produced amino acids. If, however, nitrogen-rich amino acids were supplied as the sole nitrogen source, they were accumulated preferentially and accounted for 50% of the vacuolar pool. Matile (1976) states that 'vacuolar storage pools of metabolic intermediates represent an important device for maintaining homeostasis within the cytoplasm. As pointed out by Wiemken and Nurse (1973b) plant cells are more or less directly exposed to environmental changes; in this situation the vacuole

with its nutritious cell sap represents a large internal environment that buffers the cytoplasm against environmental changes'.

1.1.2 *Reserve substances*

Most plants convert large quantities of reserves into osmotically inactive macromolecules such as protein, starch and glycogen. In many cases these macromolecular reserves are stored within modified vacuoles, one of whose chief functions is storage. For example the reserve proteins of many plant seeds are stored within aleurone vacuoles. Tombs (1967) found that 70% of the total protein as well as the major reserve of glycinin was located within the protein bodies of soybean meal. Spherosomes, or oil vacuoles are ubiquitous in plant cells and appear to contain a large fraction of the lipid reserve of the cell. Jacks *et al.*, (1967) demonstrated that over 90% of the total lipid is contained within the spherosomes of peanut seeds.

1.1.3 *Inorganic substances*

Approaches to the problem of accumulation of inorganic solutes within the vacuoles have been largely indirect, owing to the difficulty of obtaining uncontaminated isolates of vacuolar fluid. Evaluations have thus been based on radio-isotope losses from labelled tissues. Only the vacuoles of giant coenocytic algal cells, such as those of Chara, are large enough to allow the insertion of micro-electrodes and micropipettes, therefore lending themselves to studies of the chemical composition of the cell sap or the ion-transporting capacity of the tonoplast (Matile, 1976).

It has been shown that certain inorganic ions accumulate within the vacuole against a concentration gradient. In this connection Ribaillier *et al.*, (1971) have reported that Mg^{2+} was accumulated 90-fold, Ca^{2+} 60-fold and Cu^{2+} 2-fold in the vacuoles of Hevea latex as opposed to the cytoplasm. Equal concentrations of K^{+} were found in the vacuole and cytoplasm. The vacuoles were also found to contain an accumulation of acid-soluble inorganic

phosphates.

Possible mechanisms of solute accumulation within the vacuole

The accumulation of ions within the vacuole against a concentration gradient has led to the speculation that the tonoplast functions not only as a barrier, but also as a 'gate and pump', controlling the entry of specific ions into the vacuole to maintain them at the metabolically required levels within both the cytoplasm and vacuole (Bidwell, 1974). Indeed active permease-mediated transport across the tonoplast appears in many cases to be a requirement for the accumulation of higher solute concentrations in the cell sap than in the cytoplasmic matrix.

Boller *et al.*, (1975), working on isolated vacuoles of Saccharomyces cerevisae characterised a specific transport system which catalyses the exchange of arginine added to the medium with arginine present in the vacuoles. This does not, however, explain the accumulation of arginine within the vacuole.

According to Matile (1976) charged solutes may be trapped by non-diffusable counter-ions present within the vacuole. For example, cationic vital stains are thought to accumulate within the vacuole as a result of binding to vacuolar anions such as organic acids, flavones, tannins and other phenolic compounds. Donnan charge-equilibria and the precipitation of salts may be responsible for their accumulation within the vacuole. A striking example of this is provided by the studies of Matile *et al.*, (1970), on accumulation of the alkaloid sanguinarine into the vacuoles of Chelidonium latex. They reported that isolated vacuoles rapidly absorbed sanguinarine dissolved in the surrounding medium. The process did not depend on an energy source although the alkaloid was transported against a concentration gradient. However, as the alkaloid may be in a bound form, the transport may not be against an activity gradient. Ion exchange appeared, however, to take place in the cell sap, as the

entry of sanguinarine into the vacuole was accompanied by the release of other vacuolar alkaloids into the surrounding medium. The capacity of the vacuole to absorb sanguinarine from the medium appeared to be limited by the amount of exchangeable ions present within the cell sap.

Thus it appears that, apart from permease-mediated transport into the vacuole, compounds may be accumulated within the vacuole as a result of properties of the vacuole (e.g. accumulation of anions) established before the accumulation began. Other methods of solute accumulation within the vacuole will be dealt with later in this chapter.

1.2 TURGOR

The role of the vacuole in the maintenance of cell turgidy in plants is fundamental, and was recognised early on by plant physiologists.

The state of turgor of the plant cell depends on the amount of water contained within the cell vacuole which in turn depends on the water potential of the vacuole. A brief discussion of the factors contributing to and affecting water potential seems warranted before a description of the rôle of the vacuole in the maintenance of cell turgidity.

To simplify matters, the typical plant cell may be regarded as consisting of a vacuole, a cell wall, and a thin layer of cytoplasm between the vacuole and cell wall. The vacuole contains an aqueous solution. The cell wall is capable of pressing on the cell contents and thereby inducing pressure on the vacuole. The layer of cytoplasm and cell wall represent the cellular matrix.

Water Potential

To move requires energy. Water, like any other substance, will move down an energy gradient until it has reached the lowest possible energy level. At this stage equilibrium can be said to have been reached and further movement of water will not result in any further loss of energy.

Free energy is defined as the energy available at a particular temperature to do work.

The Chemical Potential of a substance is the free energy per mole of that substance and is a measure of the energy with which a substance will react or move.

The Water Potential (ψ^*) is the chemical potential of water and is a measure of the energy available for reaction or movement. The net movement of water is always from a region of higher potential to a region of lower potential. The potential of pure water is, by definition, zero at atmospheric pressure. The presence of a dissolved substance within water lowers its potential, which will therefore be negative. If two solutions of differing concentration are separated by a differentially permeable membrane, water will move by osmosis from the region of higher water potential (least dissolved solutes) to the region of lower potential.

The water potential of a cell consists of three component potentials:

The Matric Potential (ψ_m) expresses the adsorptive affinity of water to colloidal substances and surfaces in plant cells (for example cytoplasmic and cell wall materials). The matric potential may be of considerable value in tissues comprising largely non-vacuolated cells, but in herbaceous plants, whose cells contain large vacuoles and very little cytoplasm, it is almost negligible and may be disregarded.

The Osmotic Potential (ψ_π) depends on the total number of solute particles in solution (in the vacuole). The nature of these solute particles has been previously discussed. The osmotic potential is always less than zero and is a measure of the pressure that can be generated within a cell by water diffusing in by osmosis.

The Pressure Potential (ψ_p) When turgor pressure develops within a cell water is 'squeezed out' of the cell. In other words it moves out of the cell down a pressure gradient. In such a case the water within the cell (vacuole) is said to have a positive pressure, that is a pressure higher than that of the solution outside the cell.

The water potential is the sum of the three major component potentials

$$\psi_w = \psi_m + \psi_\pi + \psi_p$$

and as ψ_m is negligible for highly vacuolate cells,

$$\psi_w = \psi_\pi + \psi_p.$$

Changes in the solute concentration within the vacuole will cause changes in the osmotic component of the water potential, and thus cause water to flow either into or out of the cell. If the solute concentration within the vacuole is increased, the osmotic potential (ψ_π) within the vacuole will decrease, resulting in a corresponding decrease in vacuolar water potential (ψ_w). Water will enter the vacuole from the surrounding medium down a gradient of water potential in an attempt to establish a new equilibrium. The vacuole will consequently swell and become turgid, exerting a pressure on the cell wall. The cell wall exerts an equal and opposite pressure, known as turgor pressure, back on the cell contents. Water inflow ceases when equilibrium has been achieved. Similarly a decrease in solute concentration within the vacuole will result in an efflux of water from the vacuole and cell, causing the protoplasm to shrink. Turgor pressure is thus decreased. In extreme cases this condition may result in plasmolysis of the cell.

The turgidity of a cell has a considerable effect on its physiological well being. Were it not for the fact that a plant cell is usually swollen and distended throughout most of its life-time, its internal structural integrity could not be maintained. In the absence of turgidity, organelles within a cell would not be able to maintain the special spatial relationship necessary for normal metabolic functioning. Turgor pressure ensures the maintenace of normal rigidity in a plant cell - indeed the normally upright position of the shoot of a very young seedling whose cells are not yet lignified depends largely on positive turgor pressures developed

within the individual cells of the shoot.

A second rôle of turgor pressure appears to be in cell enlargement during cell growth and involves the irreversible stretching of the primary cell wall. The process of irreversible stretching is thought to start in response to a metabolic event in which the chemical bonds of the primary cell wall are weakened or loosened. As a consequence the balance between inwardly acting turgor pressure, which is lessened through weakening of the cell wall, and outwardly directed pressure by the vacuole is disturbed. The cell wall yields to the latter pressure and plastic stretching takes place. At the same time it is thought that solute molecules may be secreted into the vacuole (perhaps by one of the mechanisms discussed earlier), so inducing the osmotic uptake of water from neighbouring cells and ultimately from external water sources, thus maintaining the osmotic potential of the vacuole at its original value. Studies by Green *et al.*, (1971) have shown that although irreversible growth is a function of turgor, the regulation of cell elongation is not primarily exercised by the osmotic properties of the cell sap. They found the resumption of growth in Nitella to be connected with increased extensibility of the cell wall rather than increased turgor. Hall and Cocking (1974) working on Avena, reported that auxin-induced bursting of protoplasts occurred even if they were kept under conditions of incipient plasmolysis. It therefore seems that turgor pressure represents one of the pre-requisites of growth which is subject to hormonal regulation (Matile, 1976).

Reversible changes in cell volume, known as turgor movements, occur periodically and recurrently in the plant cell and appear to depend upon the elastic properties of the cell wall. A good example of this is the opening and closing of stomata brought about by the guard cells under the influence of changes in cell turgor. Guyot and Humbert (1970) have observed that in the dark, when the stomata are closed, the vacuome consists of numerous tiny

vacuoles. The stomata are open in the light and in this case the vacuome consists of a single large vacuole formed by the coalescence of smaller vacuoles. This behaviour points to a cyclic, reversible deposition of solute materials within the vacuole/s. During daylight hours deposition is maximal and the single large vacuole is turgid, resulting in the opening of the stomata. The reverse appears to occur at night, causing flaccidity of the vacuoles and closing the stomata. It is now widely accepted that K^+ fluxes are involved in this cyclic change in osmotic potential (Mansfield, 1976).

For many years the plant vacuome was thought of as a repository for cellular waste material, a storage area for metabolic intermediates and large macromolecules, and an organelle playing a significant role in the maintenance, if not regulation, of cell turgor. Research did not really progress in other areas and the concept of the vacuome consequently remained static.

1.3 THE LYTIC COMPARTMENT

1.3.1 *The need for a lytic compartment within the cell*

Metabolism may be divided into two processes, anabolism and catabolism, both of which occur simultaneously in the cell. Anabolic (building-up) processes involve the synthesis of complex macromolecules, and are mediated by a wide variety of specific enzymes. Catabolic processes involve the enzyme-mediated breakdown of such complex macromolecules into their constituent building blocks. If occurring at the same time in the same place these two processes are antagonistic and may be said to be mutually exclusive, as the one cannot proceed in the presence of the other and vice versa. The need for both processes to occur within the cell is obvious, as the products of anabolic metabolism are subject to wear and tear (i.e. are metabolically labile), and economy demands that damaged cell components be broken down and re-used. In addition, turnover prevents excess and damaged macromolecules and organelles from cluttering up the cytoplasm and interfering with ongoing metabolism. As anabolic and catabolic process are not usually separated in time, theory predicts that by their mutually exclusive nature they must be separated in space. Matile (1975) has stated that 'turnover requires compartmentation of processes which would otherwise interfere with one another'. In other words hydrolytic enzymes operating within a cell must be compartmentalised.

1.3.2 *Lysosomes*

Lysosomes were first identified in animal tissue by de Duve (1969). These 'suicide bags' as he termed them were deduced to consist of non-specific digestive enzymes or hydrolases, spatially segregated from the rest of the cytoplasm by bounding membranes. The original definition was based exclusively on biochemical data gathered from the study of rat-liver homogenates. A significant proportion of acid phosphatase and other hydrolase activity is not detectable in such homogenates unless the membranes have been disrupted - a phenomenon described as latency (of the enzymes). The bulk of the

latent rat-liver hydrolases can be sedimented in the same fraction as the mitochondria, which implies that the hydrolase-containing organelles are of similar size and density to mitochondria; indeed these organelles were originally thought to be a class of mitochondria. However, a distinct class of subcellular organelles, termed lysosomes, was later identified by electron microscopists in thin sections of rat liver and, further, acid phosphatase activity was localised to these particles with the aid of a cytochemical reaction (Gomori, 1952). Gahan (1967) suggested that at least two hydrolases should be associated with such an organelle before it can be termed a lysosome. The pH within the lysosomes is usually about 5, so lysosomal hydrolases usually have acid pH optima, although a few cases of hydrolytic enzymes with alkaline pH optima have been reported. de Duve (1969) has suggested that the lysosomes within a tissue or cell are heterogeneous with respect to hydrolase content, although not to the extent of 1 lysosome - 1 enzyme. He postulated two chief roles for the lysosome.

1. *Function in intracellular digestion:*

   Support for this role came from Novikoff *et al.*, (1964) who suggested that lysosomes coalesce with pinocytotic vesicles which contain material of extracellular origin destined for digestion.

2. *Cell death:*

   In some cases liberation of lysosomal enzymes within a cell may occur, resulting in autolysis of the cytoplasm and consequently death of the cell.

1.3.3 *Lytic compartments within the plant cell*

Wilson (1973) has stated that plant physiologists were 'reluctant to borrow or even consider' the lysosomal concept used in

animal cytology and physiology. Although this is perhaps putting it a little strongly it is certainly true that the search for lysosomes in plant tissue proved fruitless for a long while. The need for such organelles to exist in plants was accepted, although they could not be identified at ultrastructural or biochemical levels.

Histochemical investigations of plant tissue already carried out in the nineteen-fifties showed acid phosphatase-positive particles within the cytoplasm (e.g. Jensen (1956), working on root-tips of Vicia faba, Allium cepa and Pisum sativum). The resolving power of the light microscope was not, however, sufficient to allow detailed observations of the nature of these deposits. Gahan and Maple (1966) demonstrated that acid phosphatase was confined to particulate sites in the root meristem of Vicia faba, whereas the senescing cells at the periphery of the cap showed a diffuse reaction. In this connection Berjak (1968) has suggested that the senescence of these cells is accompanied by the release of hydrolytic enzymes normally confined within an organelle.

At ultrastructural and biochemical levels, however, attempts to identify plant lysosomes met with little success. Hydrolases appeared to be largely associated with the soluble fraction of plant homogenates and exhibited poor sedimentability and an absence of latency. For example, in extracts of cotyledons of germinated pea seeds, prepared and processed as for rat-liver lysosomes, only 10% of the total acid phosphatase activity was contained in the 'mitochondrial fraction' as opposed to 80% sedimentable activity in rat-liver homogenates. Moreover the sedimentable acid phosphatase activity showed no latency, as did that from rat-liver (Corbett and Price, 1967).

It therefore seemed likely that the lysosomes characteristic of rat-liver did not exist, at least in the same form, in plant tissues. According to Matile (1975) 'sedimentability and latency depend on whether or not the integrity of possible lysosomes is preserved on the

homogenization of tissues'. The plant vacuole is a large and fragile organelle which would not survive most conventional methods of homogenization. Poux (1963) working on shoot-apex meristem cells first demonstrated acid phosphatase within the plant vacuole at the ultrastructural level. Thus if the vacuole was indeed the seat of hydrolysis within the cell it was not surprising that conventional biochemical techniques had failed to reveal this. Berjak, in 1968, reported organelles present in the root cap and apex of Zea mays which conformed in appearance and enzyme activity to the lysosomes found in animal tissue. She further reported that these organelles showed a developmental sequence relating them to the more conventionally known plant vacuoles which had already been shown to exhibit acid phosphatase activity. Also in 1968, Matile and Moor reported organelles present in freeze-etched preparations of Zea mays root tips, which they suggested represented stages in the development of the lysosomal apparatus. Using cell fractionation methods, Matile (1968) succeeded in isolating three different types of lysosomal vacuole varying in density and hydrolase content, from the maize root tip. Thus it was suggested that the vacuole is one of the major seats of hydrolytic activity in plants.

Hydrolytic enzymes have been localised to several membrane-bound (or partially membrane-delimited) compartments apart from the vacuole, and these are consequently also classified as part of the lysosomal system.

1. The extracellular space (the region outside the plasmalemma) is the site of considerable acid phosphatase activity, this usually being associated with the cell walls(e.g. McLean and Gahan, 1970). Moreover acid phosphatase does not appear to be the only enzyme involved. The presence of acid phosphatase is not a constant feature of all cell walls and where present it appears to vary according to the nature of the tissue (Matile, 1975).

2. Spherosomes. Cytochemical evidence has accumulated that the organelles commonly known as the spherosomes may be sites of hydrolase activity. According to Matile (1975) spherosomes are a heterogeneous class of organelles having in common a spherical shape, a high lipid content and an anomalous bounding membrane. Waleck-Czernecka (1962, 1965) (*loc. cit.* Matile, 1975) has succeded in localizing a number of hydrolases including acid phosphatase to these bodies. Yatsu *et al.*, (1971) were not, however, able to detect acid phosphatase in spherosomes isolated from onion and other oily tissues. The picture thus appears confused. It is likely that the spherosomes of certain plants or tissues are hydrolytically active whilst those of others serve partly as inert lipid storage bodies, the hydrolases necessary for lysis of their content being manufactured elsewhere and later becoming associated with these subcellular particles (or vice versa).

3. Aleurone Bodies (also termed protein bodies or protein vacuoles). These organelles are present in the reserve tissues of many seeds and appear to contain protein and phosphate reserves. These reserves are mobilised on germination, apparently by hydrolytic enzymes contained within them. In this connection Matile (1968) has shown that aleurone bodies isolated from cotyledonary cells of germinating pea seedlings contain a number of acid hydrolases. Thus the aleurone bodies appear to have the dual function of accumulation and intracellular digestion of reserves. Hinkelmann in 1966 reported that aleurone bodies appear to inflate and fuse during germination and finally come to occupy most of the space formerly occupied by the cytoplasm. The enzymes localised within these bodies include proteases, phosphatases, RNAse, β-amylase and α-glucosidase, which suggests that other cell constituents apart from protein and phosphate reserves are broken down

within these lysosomes (Matile, 1969). In this connection Baumgartner and Chrispeels (1976), working on proteolysis in mung bean seedlings, reported that the protein bodies of the dry bean exhibited no enzyme activity and were not able to autodigest. Subsequent to imbibition and germination, however, carboxypeptidase and an endopeptidase were found associated with these bodies, and were apparently responsible for the rapid digestion of their protein reserves. These investigations suggested that the enzymes necessary for digestion were synthesised by the ER and transported via ER-derived vesicles to the protein bodies.

Thus it appears that a variety of different organelles and cell compartments are implicated in lysosomal activity. This applies to both plant and animal cells, for it is widely accepted that the auto- and heterophagic vacuoles of animal cells are also lysosomal in nature (e.g. Daems *et al.*, 1969; Ericsson, 1969). This contrasts sharply with the idea originally developed for rat liver that 'the lysosome' was a specific organelle with distinct morphological characteristics. For this reason, nomenclature represents something of a problem. Matile (1974), in his review on plant lysosomes, has considered the problem and made the following conclusions and suggestions. Firstly, the classical lysosome-like organelles represent merely a transient stage in the development of the vacuolar component of the lysosomal system, which also comprises other organelles, all of which are ontogenetically related in one way or another (see later in this Chapter). de Duve, who originally coined the term lysosome for animal cells, has subsequently recommended the use of the term vacuome as being more adequate for designating the totality of membrane systems involved in the lysosomal process. The vacuome includes the ER, Golgi complexes, the whole family of lysosomes, vesicles produced by the plasmalemma through pinocytosis and phagocytosis and, lastly, the extracellular space. According to Matile, de Duve's comprehensive term 'vacuome' applied to animal cells, has been familiar to the plant cytolgist since 1916 when Dangeard used it to designate the totality of cell compartments which had in common the ability to accumulate the vital stain, neutral red. He further

states that this convergence of terminology in animal and plant cytology is meaningful as the plant vacuole *is* lysosomal in nature. The term lysosome has, however, been introduced in plant cytology despite the fact that it refers to a group of organelles, all of which have other names. Matile considers it clumsy to abandon the classical terms for a currently fashionable one which will perhaps fall into disuse. He recommends that the term lysosome should be used exclusively in a biochemical sense and that the totality of membrane-bound structures containing lysosomal enzymes be termed the *lysosomal cell compartment* or *lytic compartment*. The morphologist dealing with this compartment should use the classical names for its individual constituents. The abovementioned system shall be adopted as far as possible in this work. In this regard it would appear consistent to refer to the vacuolar membrane as the tonoplast only when the osmotic properties of the vacuole are being discussed, and otherwise to use the term 'vacuolar membrane'.

1.4 DYNAMICS OF THE LYTIC COMPARTMENT

1.4.1 *The origin of vacuoles*

The picture as regards the origin of the vacuolar component of the lytic compartment is, at present, confused, with different investigators working on a variety of plant materials reporting a number of methods of vacuolation.

What appears to be the main school of thought is that vacuoles originate from the ER, although the mechanism of formation appears to vary from species to species and even within tissues and cells of a particular species. A convincing body of evidence has accumulated in favour of the origin of small vacuoles, termed provacuoles, from the ER in the meristematic regions of the roots of various plants. Matile, working on provacuoles isolated from the meristematic cells of Zea mays root tips, has shown them to contain enzymes that are normally associated with the membranes of the ER (Matile, 1968a). This suggests that provacuoles may be ontogenetically related to the ER. Vacuoles have been shown to originate from local dilations of ER cisternae (e.g. Buvat and Mousseau, 1960 (*loc. cit.* Matile, 1975); Poux, 1962 ; Mesquita, 1969). Berjak (1972) working on cress, has reported that such dilations may occur at either intermediate or terminal locations on the ER cisternae. In maize, vacuolation has been reported to occur by vesiculation of the ER (Matile and Moor, 1968; Berjak and Villiers, 1970). In all cases the newly formed provacuole has been described as a single-membrane bounded body. Berjak (1972) reported that acid phosphatase activity in the root cap of cress was associated with the vesicles being pinched off from the ER, rather than with the ER cisternae themselves. In the light of this Matile (1974) suggested that provacuoles represent a product of ER differentiation rather than a product of mere dilation, and on these grounds has proposed that it is not necessary to consider the reticulum as a constituent of the lytic compartment.

In certain plant tissues elements of ER have been observed to surround and completely sequester portions of cytoplasm, resulting in the formation of double-membrane bound vacuoles known as cytolysomes. Villiers, in 1967, working on long-dormant embryos of Fraxinus excelsior, first reported the existence of cytolysomes in plant tissue. He found acid phosphatase activity associated with these organelles and it therefore appeared that they formed part of the lysosomal compartment of the cell. Upon dilation of the surrounding ER cisternae, a vacuole containing one or several intravacuolar vesicles was formed. The membranes surrounding these vesicles were subsequently digested away, as was the sequestered cytoplasm, finally leaving a vacuole indistinguishable from any other vacuole.

Another school proposes that vacuoles are independent of any other organelle. In this regard Manton (1962) reported that the stellate vacuoles of the meristematic cells of the thallus of Anthoceros did not appear to originate from any other organelle. Barton (1965), studying the origin and development of the vacuoles in the root tip cells of Phaseolus vulgaris, reported that the vacuoles arise by expansion of small irregularly-shaped provacuoles present in the meristematic cells. On the basis of a differential staining effect of the membranes of these provacuoles compared with those of any other organelle she concluded that provacuoles were not derived from other cellular organelles. In the light of this she suggested that one or more provacuoles persist in the cytoplasm during cell divisions, thus giving continuity to these structures. There are indeed examples of organelles possessing a persistent system of vacuoles. Wiemken *et al.*, (1970), working on the budding cycle of the yeast Saccharomyces, found that initial budding cells contain numerous small vacuoles which inflate and fuse at late budding. Upon initiation of budding the large vacuoles appear to undergo extensive shrinkage and fragmentation. Guyot and Humbert (1970) have reported a similar cycle occurring in the guard cells of stomata.

Yet another school of thought was initiated by Mühlethaler (1966) who reported that vacuolar initials might arise *de novo* as a result of hydration of the cytoplasmic matrix. This idea did not initially find much favour, but has recently been brought up again by Khera and Tilney-Basset (1976). As a result of a study on the origin of vacuoles in young embryos of Pelargonium x Hortorum Bailey, they reported that some vacuoles are formed as differentiated zones of cytoplasm around which the tonoplast is gradually built up from vesicles and small cisternae.

The last school proposes that vacuoles originate from the Golgi apparatus. Marinos (1963) reported that vacuoles arose in cells of barley shoot apices by an enlargement of the entire space of a single Golgi cisterna. Ueda, in 1966, made similar observations after a study of vacuolation in the alga Chlorogonium. It has also been shown that the pulsating vacuoles responsible for the excretion of water in certain algae originate from the Golgi apparatus (Schnepf and Koch, 1966). Marty (1976) has put forward a somewhat novel idea concerning the origin of vacuoles from the Golgi apparatus of coenocytic lacticifers. He pointed out that in the youngest proliferating cells of root tips there are few, if any, vacuolar profiles. Employing a combination of conventional transmission electron microscopy, ultrastructual cytochemistry and very high voltage (3MV) electron microscopy, he found that an extensive system of smooth-membrane-bounded cavities arises from the mature face of the Golgi bodies. This reticulate network, which is termed GERL (from Golgi, ER, Lysosome) appeared to give rise to the provacuolar apparatus. It apparently contained acid phosphatase and was observed to form a cage-like structure (sequestration cage) in which a substantial volume of cytoplasm was trapped. Marty suggested that sequestration was completed by lateral fusion of the bars of the cage and that hydrolytic enzymes (typified by acid phosphatase) originally contained within the 'bars' migrated into the entrapped cytoplasm. Thus in the early stages of development the 'provacuole' was bounded by a double membrane. Vacuolation was said to be complete when both the sequestered cytoplasm and the inner of the two bounding membranes were digested away.

1.4.2 *Development of vacuoles*

The development of provacuoles has been widely shown to comprise their extensive swelling and fusion. In this connection Berjak and Villiers (1970) reported that provacuoles formed in the meristematic tissue of the maize root cap appeared to undergo swelling and fusion during their subsequent development. The end product of this process was a single large vacuole occupying most of the volume of the cell.

The autophagic engulfment of Golgi-derived vesicles by the vacuoles has been observed both in thin sections and freeze-etched preparations of root tip cells (Berjak and Villiers, 1970; Matile and Moor, 1968). Acid phosphatase activity has been localised cytochemically not only to vacuoles *per se*, but also to dictyosomal cisternae and vesicles of a variety of plants (e.g. by Brandes and Bertini, 1964 (*loc. cit.* Matile, 1975). Dauwalder *et al.* (1969), Ray *et al.* (1969) and Poux (1970) have found acid phosphatase activity associated with Golgi membranes. In this connection Matile (1974) has pointed out the possibility that the Golgi apparatus is involved in the intracellular translocation of at least some hydrolytic enzymes.

This mechanism is, however, by no means universal. Wiemken (1969), (*loc. cit.* Matile, 1975) demonstrated that polysomes were associated with the membranes of isolated yeast vacuoles which had been contrasted positively with uranyl acetate. It is possible that these polysomes are responsible for the synthesis of at least some vacuolar hydrolytic enzymes. Reports on the presence of acid phosphatases within the dictyosomes of the root cap cells of Zea mays are conflicting (Berjak, 1968; Kephart *et al.*, 1966). However the consensus of opinion seems to be that this enzyme is not associated with the dicytosomes of the cap under normal conditions.

It is well established that the hypersecretory dictyosomes of the mature and senescing cells of the root caps of certain grasses (e.g. maize, barley and wheat) contain a complex polysaccharide (e.g. Jones and Morré, 1967; Northcote and Pickett-Heaps, 1966; Floyd and Ohlrogge, 1971; Dauwalder and Whaley, 1974; Paull, Johnson and Jones, 1975). It is therefore possible that incorporation of dictyosomal vesicles into vacuoles represents a Golgi-mediated internal secretion of carbohydrates (perhaps acidic polysaccharides and also phenols). These compounds could be responsible for the accumulation of cations associated with the binding of anionic material within the vacuole as follows: Intravacuolar digestion of complex polysaccharides could be responsible for an increase in the number of micromolecules in the vacuolar fluid. This would result in an increase in the osmotic component of the water potential of the cell sap, causing water to enter the vacuole by osmosis. In this connection it has already been mentioned that the vacuoles at this stage of development in the root cap (e.g. maize) undergo a considerable expansion. Thus the Golgi-mediated secretion of complex polysaccharides into the vacuole may contribute to the osmotic stabilization of the expanding vacuoles. It has already been mentioned (see above) that other mechanisms may also be involved in this process.

Mature vacuoles may exhibit considerable autophagic activity apart from the engulfment of dictyosomally-derived vesicles. The vacuoles of the senescing cells situated at the periphery of the maize root cap provide a good example of this (Berjak and Villiers, 1970). Such vacuoles appear to engulf almost every kind of organelle present within the cell, many of these being damaged. In some cases the whole areas of cytoplasm, including their constituent organelles, are engulfed. Autophagy appears to begin with an invagination of the tonoplast, produced by local membrane ingrowth (Matile, 1975) surrounding the material to be incorporated, and is complete when the tonoplast pinches off, thus forming an intravacuolar vesicle surrounding this material. The bounding membrane of this vesicle is assumed to rupture, exposing the cytoplasm to the attack of hydrolytic enzymes presumably

present within the vacuole (Matile and Moor, 1968). Fineran (1970) has shown, using the freeze-etching technique, that the convex fracture face of the vacuole shows a higher density of globular particles at sites of invagination compared with inactive regions of the tonoplast. Matile (1970) has suggested in this regard that membrane differentiation is involved in autophagy, although whether it is a cause or effect is uncertain.

Senescence and autolysis

Vacuoles appear to play a major role in the autolysis of cells. Berjak and Villiers (1970), working on the root cap of Zea mays, reported extensive expansion and fusion of the vacuoles of the senescing cells at the periphery of the root cap. Cell death was finally accomplished through dissolution of the vacuolar membrane, allowing the release of hydrolytic enzymes from the vacuole into the cytoplasm which was consequently autolysed. On the completion of autolysis the cytoplasm was completely destroyed, leaving an apparently empty space surrounded by the cell wall. Before this stage dictyosomally-derived polysaccharides had accumulated to a great extent in the extra-protoplasmic spaces, finally moving into the regions between the outermost cells of the cap. The outermost cells were thus loosened from one another and appeared to slough off and float in the viscous mucilage surrounding the cap. It is possible that particularly pectinases within this secretion serve to degrade and thus weaken the middle lamellar region, thus aiding in the separation of the cells from one another (Berjak, personal communication).

1.4.3 *Origin and development of spherosomes and aleurone bodies*

Frey-Wyssling *et al.*, (1963) demonstrated the origin of spherosomes from the ER. Swartzenbach (1971) reported that the lipid is stored in the hydrophobic central layer of the triple-layered membrane of prospherosomes. During the process of lipid accumulation the inner layer of the bounding membrane appeared to be relegated to

the centre of the spherosome, which was consequently bounded by only the outer half of the usual triple-layered membrane.

At early stages of development aleurone bodies are indistinguishable from provacuoles, and appear to originate similarly through vesiculation of the ER (e.g. Yatsu, 1965, *loc. cit.* Matile, 1974).

1.4.4 *Origin and development of the lysosomal exospace*

In higher plants the formation of the extracellular space coincides with cell plate formation. At late anaphase numerous dictyosomal vesicles appear to contribute to the formation of the new cell plate : the newly formed plasma membrane represents the product of fused Golgi membranes. (Frey-Wyssling *et al.*, 1963). The secretory activity of the dictyosomes associated with wall development appears to continue as the cell plate grows in thickness, and has also been correlated with the later growth of the cell walls. According to Matile (1974), if the hypothesis of Golgi-mediated secretion of hydrolases into the vacuome is correct, the secretion of the dictyosomal material into the exospace could also account for the presence of hydrolases there. In this connection Gahan and McLean (1969) have demonstrated acetyl-esterase in the Golgi complex and cell wall, as well as in the ER cisternae lying in close proximity to the plasmalemma. Vesicles which are suggested to be ER-derived have also been observed to behave in a manner similar to Golgi-derived vesicles, and may be involved in the secretion of cell wall material and extracellular hydrolases (Cortat. *et al.*, 1972).

The Golgi apparatus appears to be involved in the secretion of the mucilaginous material which adheres to the surface of the root tips of many grasses (e.g. maize, wheat, barley). Hypersecretory dictyosomes are formed in the mature and senescing cells of the root caps of such plants. As has been previously mentioned these vesicles

appear to contain a complex polysaccharide (e.g. Jones and Morré, 1967). They fuse with the plasmalemma, thus secreting their contents into the extra-protoplasmic space where it accumulates (see e.g. Berjak and Villiers, 1970). After build up has reached a certain level the polysaccharide appears to penetrate the cell wall and to accumulate between the outermost cells of the cap, eventually forming a macroscopically visible mucilaginous droplet on the outside of the root tip. Thus in addition to the secretion of extracellular hydrolases and cell wall material during differentiation and development, the dictyosomes appear to be implicated in the production and secretion of slime in the older root cap cells of some plants.

1.5 THE ENDOMEMBRANE CONCEPT

It appears that the lytic compartment in its entirety comprises a major part of the endomembrane system, the theory of which has been reviewed by Mollenhauer and Morré (1974). They define the endomembrane system to be a developmental continuum of membranous cell components functioning along two main pathways of membrane differentiation.

1. To the cell surface (plasmalemma).
2. To the vacuolar apparatus.

It is proposed that this system accounts for the biogenesis and multiplication of most of the cytoplasmic membranes of the eukaryotic cell. Continuities among the separate endomembrane components demonstrate that these cytoplasmic membranes occur as localised specializations of an inter-associated system. Endomembrane components are thought to be biochemically distinct from the inner membranes of semi-autonomous organelles such as chloroplasts and mitochondria by the absence of DNA and cytochrome oxidase, the inability to generate ATP through respiratory-or photosynthetic-chain-linked phosphorylation, and by a high degree of functional inter-dependence.

The endomembrane system comprises:

1. The outer membrane of the nuclear envelope and its extensions, including the rough ER which functions in protein synthesis and nucleocytoplasmic exchanges. Berjak (personal communication) is of the opinion that the rough ER functions as the parent membrane (and as such the site of *membrane* protein synthesis) with the nuclear envelope as one line of specialization).
2. Systems of transition elements, incapable of protein synthesis, which function in one or both of the two

major pathways of membrane differentiation.

(a) one pathway is towards the plasmalemma, where the membranes become progressively more like the plasma membrane,

(b) the other pathway is to the interior of the cell, as ER-like structures differentiate to form vacuolar membranes

The term 'transition element' denotes endomembrane components with properties intermediate between those of the rough ER and outer membrane of the nuclear envelope on the one hand, and the plasmalemma or tonoplast on the other. A form of the smooth ER and the Golgi apparatus are major transition elements. The criteria for membrane differentiation are based on relative membrane thickness, functional associations and biochemical characteristics. Reversals of differentiation are not excluded, but it is assumed that membranes differentiated in one direction are no longer compatible with membranes differentiated in the other direction. For example, the vesicles of the hypersecretory dictyosomes of the maize root cap differentiate to become plasmalemma-like; they ultimately fuse with the plasmalemma, but are incapable of fusing with the tonoplast, which is a product of differentiation in the opposite direction. Incorporation of such vesicles into the vacuole is thus through the invagination of the tonoplast (autophagic activity of the vacuole).

1.6 THE PRESENT INVESTIGATION

The present investigation has involved the study of aspects of the origin and development of the vacuolar system in particular, and the lytic cell compartment in general, in the root cap of Zea mays L. The project arose as an attempt to find answers to some unresolved questions raised as a result of the study of Berjak (1968) into senescence processes occurring in the root cap of Zea mays L. She found the root cap to be ideal for a study of that nature as it exhibits a complete developmental sequence, culminating in the senescence and death of the outermost cells, within a small volume of tissue. Cells produced in the cap meristem differentiate, mature, senesce and finally die as they reach the periphery of the cap, where they are sloughed off. The root cap turns over completely once every 24 hours, and approximately 10 000 cells are sloughed off during this time period (Clowes, 1970). The development of the vacuoles appeared to parallel the developmental sequence observed for the other organelles within the cap. Vacuoles were generated in the meristematic tissue and appeared to enlarge and fuse during differentiation and maturation, this process culminating in the production of generally just one large vacuole occupying most of the volume of the cytoplasm. The dictyosomes of the mature and senescing cells, as has previously been mentioned, are hypersecretory and appear to manufacture a complex polysaccharide which is transported via the dictyosomal vesicles in two directions.

1. The dictyosomal vesicles fuse with the plasmalemma, ejecting their content into the extra-protoplasmic space where it builds up and eventually forms a slime droplet on the surface of the cap.
2. The dictyosomal vesicles appear to be autophagically engulfed by the vacuoles, in which case they may play a role in osmotically stabilizing the rapidly-expanding vacuoles of the mature and senescing cells.

To this end it is thought that the polysaccharide contained within such vesicles may be broken down to monosaccharide units through the action of hydrolytic enzymes contained within the vacuole. The increase in the number of particles in solution would increase the osmotic component of the water potential, resulting in the entry of water into the vacuole by osmosis and its consequent swelling.

No attempt has been made in this investigation to characterise exhaustively every aspect of the development of the vacuolar system and lytic compartment, as such a task is beyond the scope of this work. Instead the sequence of development described by Berjak (1968) was verified ultrastructurally, after which some ultrastructural, cytochemical and biochemical techniques were applied to the investigation of a few specific aspects of the developmental sequence.

The origin of the root cap vacuoles was of special interest in view of the uncertainty as to the origin of plant vacuoles in general. In this regard ultrastuctural techniques combined with the accepted test for acid phosphatase (Gomori, 1952) were employed in an attempt to characterise further the origin of the root cap vacuoles.

Also of interest was the theory that osmotic swelling of the vacuoles of the mature and senescing cells occurs after autophagic incorporation of hypersecretory dictyosome vesicles, and hydrolysis of their polysaccharidic content within the vacuole.

Paull *et al.*, (1975), after an analysis of the monosaccharide composition of the root-cap slime (which is of dictyosomal origin) of Zea mays, reported that fucose was one of the major constituents of the dictyosomally-manufactured polysaccharide. As fucose is found on relatively few occasions in the cells of higher plants, and is not

widely involved in cell matabolism, it would appear to be an ideal specific marker by which to follow the fate of the polysaccharide manufactured in the dictyosomes. Various experiments involving the incorporation of radioactive fucose into the root cap were thus performed, and the pattern of incorporation was followed autoradiographically at light and electron microscope levels. The level of incorporation of label into the vacuoles was of particular interest as it was thought that this would reflect the dynamics of polysaccharide incorporation into the vacuoles.

If polysaccharide of dictyosomal origin is hydrolysed within the vacuole to monosaccharide units, it would appear feasible to expect an accumulation of such monosaccharides within the vacuole if they are to function in osmotically stabilising the swelling vacuole. In this regard vacuoles were isolated from the root tip and their monosaccharide content analysed by gas and paper chromatography. As the root cap slime also appears to originate within the dictyosomes, it might be expected to contain a similar range of monosaccharide units. Root cap slime was thus isolated, purified, hydrolysed and analysed by gas and paper chromatography. The monosaccharide composition of the slime was used as a reference with which to compare the monosaccharide content of the vacuoles.

The presence of polysaccharide within the dictyosomal vesicles was verified using a cytochemical technique for polysaccharide localization. It was hoped to employ this technique to investigate the fate of the dictyosomally-derived polysaccharide within the vacuole. Techniques for polysaccharide localization at ultrastructural level have only recently been developed, and what is the best method does not yet appear to have been agreed on. Many variations of such techniques have appeared in the literature, all yielding results of differing quality. In view of this, several of these techniques have been tested in the present work with the aim of developing a method suitable for the study of dictyosomally-derived polysaccharide in the root cap of Zea mays.

PART TWO

MATERIALS AND METHODS

2.1 THE MATERIAL

Initial investigations were carried out on Zea mays L. var. SA_4 caryopses obtained from the Pioneer Seed Company, Greytown, Natal, South Africa, who are specialist producers of hybrid maize. Owing to the subsequent unavailability of SA_4 further investigations, constituting the bulk of this report, were carried out using a closely related variety, Zea mays L. var. PNR 44, obtained from the same source.

PNR 44 is a double-cross hybrid bred originally from four parental lines. This variety of a yellow maize has a medium growth period with flowering occurring approximately 76 days after planting. It is not as vigorous a germinator as SA_4, but exhibits fairly uniform germination characteristics and has a medium yield potential. PNR 44 is fairly stable and is adaptable to a wide range of climatic conditions. It exhibits resistance to many cob and leaf diseases, being particularly resistant to Helminthosporium turcicum, a fungus prevalent in Natal, and thus well suited to local conditions. On the debit side PNR 44 is very susceptible to tassel smut (Sphacelotheca reiliana) and boil smut (Ustilago zeae) and is prone to lodging (falling over).

The caryopses (from now on referred to as seeds, as they are more commonly known) were allowed to dry on the parent plant until the moisture content reached 14 per cent. They were then harvested, coated with fungicide (Captan, Malathion and sodium molybdate) and offered for sale in this condition. Upon reaching our laboratory the seeds were stored in sealed plastic containers at $5^{\circ}C$ until required for use.

2.2 SEED GERMINATION

The seeds were removed from storage at 5°C and allowed to reach room temperature in an air-tight container in order to prevent condensation of moisture on their exteriors. They were then rinsed with running tap water to remove the fungicide coating and soaked in tap water at 25°C for 12 hours. (This will be referred to as 12-hour imbibed material). At this stage the embryo is fully imbibed but growth by elongation has not yet commenced. The radicle is still surrounded by the coleorhiza and has not yet penetrated through the seed coat. The 12-hour imbibed seeds were placed with their embryo sides in contact with moist paper towel within a loosely-closed plastic container and maintained at 25°C for a further 36 hours. At this stage the coleorhiza had emerged from the seed coat, and the radicle had broken through the coleorhiza; the root had an approximate mean length of 2 cm. (This will be referred to as 48-hour imbibed material).

2.3 METHODS USED IN THE PREPARATION OF MATERIAL FOR ELECTRON MICROSCOPY

2.3.1 *Postfixation with osmium tetroxide*

The following solutions were prepared:

Cacodylate Buffer (approximately 0,05 M)

Solution A: 0,2 M sodium cacodylate

Solution B: 0,2 M hydrochloric acid

50 ml solution A were diluted to 196 ml with distilled water. Solution B was added to the above until a pH of 7,2 was obtained (approximately 3 ml).

Glutaraldehyde Fixative (6%) in Cacodylate Buffer

glutaraldehyde (Merck, Microscope Grade, 25%)	24 ml
cacodylate buffer, pH 7,2	76 ml

Osmium Tetroxide (2%)

0,1 g solid osmium tetroxide was dissolved in 5,0 ml cacodylate buffer on a rotating wheel overnight and stored at 5°C. It was found that the osmium tetroxide recrystallised from solution if stored for long periods of time. In this event it was necessary to redissolve the osmium at room temperature as described above before use.

Epoxy Resin Embedding Medium (Spurr, 1969)

vinylcyclohexene dioxide (V.C.D.)	5,0 g
diglycidyl ether of polypropylene glycol (D.E.R.)	2,5 g
nonenylsuccinic anhydride (N.S.A.)	13,0 g
dimethylaminoethanol (S1)	0,2 g

The terminal 1,5 mm of the root-tips of both 12 and 48-hour imbibed maize seeds were excised and subjected to the procedures described below. All steps were carried out at room temperature

unless otherwise stated. The root tips were:

(a) fixed with 6% glutaraldehyde in cacodylate buffer for 2 hours at 5°C,

(b) washed with cacodylate buffer for 2 hours with four changes to ensure removal of glutaraldehyde,

(c) postfixed with 2% osmium tetroxide for 2 hours at 5°C,

(d) rinsed briefly with distilled water,

(e) stained with a 2% aqueous solution of uranyl acetate for 30 minutes,

(f) dehydrated with a graded alcohol series (25, 50, 75, 100% ethanol, 30 minutes in each with two changes),

(g) infiltrated with propylene oxide for 40 minutes with two changes,

(h) retained in a 50/50 Spurr's epoxy resin/propylene oxide mixture for 4 hours,

(i) allowed to infiltrate overnight in pure epoxy resin,

(j) placed in embedding moulds containing fresh epoxy resin and polymerised at 70°C for 8-16 hours.

2.3.2 *Postfixation with potassium permanganate*

The following solutions were prepared:

Acetate Buffer (0,1 M)

Solution A: 0,1 M sodium acetate (anydrous)

Solution B: 0,1 M acetic acid

Solution B was added dropwise to solution A until a pH of 7,2 was obtained. The buffer was stored at 5°C to minimise microbial contamination.

Luft's Permanganate Fixative (modified after Mercer and Birbeck,1961)

Solution A:

sodium barbitone	14,7 g
sodium acetate trihydrate	9,7 g
distilled water to	500 ml

Solution B: 0,1 M hydrochloric acid

2 g potassium permanganate were added to a mixture of 20 ml solution A and 22 ml solution B and the whole made up to a volume of 60 ml with distilled water. Dissolution of the potassium permanganate was achieved by rotating the vessel on a wheel overnight. The solution was stored in the dark at 5°C.

Glutaraldehyde Fixative (6%) in Acetate Buffer

glutaraldehyde (Merck, Microscope grade, 25%)	24 ml
acetate buffer, pH 7,2	76 ml

The terminal 1,5 mm of the root-tips of both 12 and 48-hour imbibed maize seeds were excised and:

(a) fixed with 6% glutaraldehyde in acetate buffer for 2 hours at 5°C,

(b) washed with acetate buffer for 2 hours with four changes to ensure the removal of the glutaraldehyde,

(c) postfixed with Luft's Permanganate Fixative for 45 minutes at 5°C,

(d) rinsed briefly with distilled water.

Staining with uranyl acetate, dehydration and embedding were carried out according to Section 2.3.1, steps e - j.

2.3.3 *Use of piperazine-NN' bis-2-ethane-sulphonic acid (PIPES) buffer fixation (modified after Salema and Brandao, 1973).*

The following solutions were prepared:

PIPES Buffer (0,03 M)

1,0 M sodium hydroxide (approximately 30 ml) was added to 0,9 g of PIPES until a pH of 8,4 was obtained. (The PIPES dissolved at approximately pH 5,5). This solution was made up to 100 ml with distilled water and stored at 5°C.

Glutaraldehyde (3%) in PIPES Buffer (0,03 M)

glutaraldehyde (Merck, Microscope Grade, 25%)	2 ml
PIPES buffer, 0,03 M, pH 8,4	14 ml

Osmium Tetroxide (2% aqueous)

0,1 grams of solid osmium tetroxide were dissolved in 5 ml distilled water on a rotating wheel overnight. The solution was stored at 5°C. Salema and Brandao (1973) recommended that postfixation should take place in a PIPES-buffered osmium tetroxide solution. It was found, however, that no matter how scrupulous the conditions of cleanliness, the osmium tetroxide appeared to react with the PIPES, thus effectively removing a lot of the osmium from solution. In view of this it was decided to use aqueous osmium tetroxide as a postfixative.

The terminal 1,5 mm of the root-tips of 12 and 48 hour-imbibed maize seeds were excised and:

(a) fixed with 3% glutaraldehyde in PIPES buffer (0,03 M, pH 8,4) for 1 hour at 5°C,

(b) washed with distilled water for 1 hour at 5°C to ensure the removal of both the free glutaraldehyde and the PIPES,

(c) postfixed with 2% aqueous osmium tetroxide for 1 hour at 5°C,

(d) rinsed briefly with distilled water.

Staining with uranyl acetate, dehydration and embedding were carried out according to Section 2.3.1, steps e - j.

2.3.4 *2,2-dimethoxypropane as a rapid dehydrating agent*

10 ml 2,2-dimethoxypropane were activated with three drops of 33% hydrochloric acid. Instead of the conventional use of a graded alcohol series for dehydration, the fixed and stained tissues were

(a) dehydrated with activated dimethoxypropane solution for 10 minutes,

(b) left in pure epoxy resin overnight,

(c) placed in embedding moulds containing fresh epoxy resin and polymerised at 70°C for 8 - 16 hours.

2.3.5 *Preparation of collodion-coated grids*

A 250 ml beaker was filled with distilled water and a piece of lens tissue gently pulled across the surface to remove any dust particles. One drop of a 2% solution of collodion in amyl acetate was deposited carefully onto the surface of the water with a finely drawn Pasteur pipette. The beaker was covered with a Petri dish to prevent air currents interfering with the formation of the collodion film, and then allowed to stand until the film showed slight wrinkling. The grids were dropped gently onto the film. A piece of filter paper was dropped carefully onto the film and grids, which adhered to it; the filter paper was then removed, and laid grid-side up in a Petri dish lined with fresh filter paper. The grids were allowed to dry for 24 hours before use and were used within a week.

2.3.6 *Ultramicrotomy and electron microscopy*

Tissue sections exhibiting pale gold interference colours when stretched with xylene (400 - 600 Å thick) were cut on an LKB Ultratome 111 ultramicrotome using glass knives and collected on uncoated 50 µm copper grids which had been briefly flamed to ensure removal of grease.

Sections were viewed in a Philips EM200 electron microscope operating at an accelerating voltage of 60 kV. All photographs were taken on 35 mm Kodak Safety-Positive film.

2.3.7 *Post-staining*

2.3.7.1 *Post-staining with lead citrate*

Lead Citrate Solution (Reynolds, 1963)

lead nitrate	1,33 g
sodium citrate	1,76 g

The above ingredients were placed in a 50 ml volumetric flask and 30 ml freshly boiled and rapidly cooled, distilled water added. The flask was shaken vigorously for 1 minute and intermittently for 30 minutes to enable the lead citrate to form. 8 ml of 1 M sodium hydroxide, freshly made up in boiled, cooled distilled water, were added and the resulting clear solution made up to 50 ml with boiled distilled water.

The lead citrate solution was routinely centrifuged at 8 000 g (at room temperature) before use, in order to remove any lead carbonate crystals which happened to have formed in the solution.

Staining Procedure

(a) Several pieces of filter paper were fitted into the base of a glass Petri dish and saturated with a concentrated (> 1 M) solution of sodium hydroxide.

(b) Small drops of lead citrate solution were deposited on a piece of dental wax which had been placed on top of the filter papers in the Petri dish.

(c) The grids were floated, section side down, on the drops of lead citrate solution and allowed to stain for 45 minutes in the closed Petri dish.

(d) The grids were then removed and washed gently in 0,01 M sodium hydroxide after which they were well rinsed with distilled water and allowed to dry on a piece of filter paper.

Except where otherwise mentioned, all sections were post-stained with lead citrate in this manner.

2.3.7.2 *Post-staining with uranyl acetate*

Sections were sometimes stained with uranyl acetate, after staining with lead citrate, in order to further enhance specimen contrast. A 1% solution of uranyl acetate in 70% ethanol was prepared and filtered.

Staining Procedure

(a) Grids were floated section-side down on drops of uranyl acetate solution which had been deposited on a piece of dental wax. Staining was allowed to proceed for 10 minutes.

(b) The sections were washed with distilled water and allowed to dry on filter paper.

2.3.8 *Preparation of carbon-coated grids*

Grids containing sections were coated with a layer of carbon 70 - 100 Å thick using a Jeol JEE 4C vacuum evaporator. Shadowing was normal to the section.

2.4 LIGHT MICROSCOPY

Median longitudinal sections of the root tip and cap were cut from epoxy resin-embedded material (see 2.3.1, 2.3.2, 2.3.3) with glass knives on an LKB Ultratome 111 ultramicrotome. The sections, 1µ in thickness, were spread with xylene before being transferred to a drop of water on a clean glass slide and dried down on a slidewarmer in a dust-free atmosphere.

If the sections were to be stained they were immersed in a toluidine blue solution (0,05%) buffered with phosphate at pH 6,8 (Berjak and Lawton, 1972) and stained for 15 minutes at 60°C. Excess stain was removed by irrigation with water. After drying on a hot plate at 40°C the sections were mounted in a drop of immersion oil and viewed with a Zeiss GFL photomicroscope using phase contrast optics and a yellow or blue filter.

Black and white photographs were taken on Ilford Pan F (ASA 50) film. Colour photographs were prepared commercially from slides taken on Kodak Ektachrome film (ASA 64).

2.5 CYTOCHEMICAL LOCALIZATION OF ACID PHOSPHATASE

The following solutions were prepared:

Acetate Buffer (0,1 M)

Solution A: 0,1 M sodium acetate

Solution B: 0,1 M acetic acid

Solution B was added dropwise to solution A until a pH of 7,2 was obtained.

Incubation Medium (Gomori, 1952)

(a) 3,4 g sodium acetate were made up to 500 ml with distilled water.

(b) The pH was adjusted to 5 using acetic acid.

(c) 0,6 g lead nitrate were dissolved in the above solution.

(d) 1,5 g sodium-β-glycerophosphate were dissolved in 50 ml distilled water and added to the above solution.

(e) The medium was incubated for 24 hours at 40°C and filtered before use.

Incubation Medium (Control) (Gahan, 1967)

The control medium consisted of the above with the addition of 0,42% sodium fluoride as an enzyme inhibitor.

The terminal 1,5 mm of the root tip of 12 and 48-hour imbibed material was excised and treated as follows:

(a) fixed with 6% glutaraldehyde in acetate buffer (pH 7,2) for 2 hours at room temperature,

(b) washed with acetate buffer for 3 hours with five changes,

(c) the controls only were incubated in 0,42% sodium fluoride in acetate buffer (pH 7,2) for 90 minutes with two changes,

(d) (i) The experimental sample was maintained in the incubation medium for 90 minutes at 40°C.

(ii) The controls were incubated in the control medium (containing 0,42% sodium fluoride as an enzyme inhibitor) for 90 minutes at 40°C.

(e) both experimental and control samples were washed with cacodylate buffer (pH 7,2) (see Section 2.3.1) for 30 minutes with two changes,

(f) postfixation, dehydration and embedding were carried out as described in Section 2.3.1.

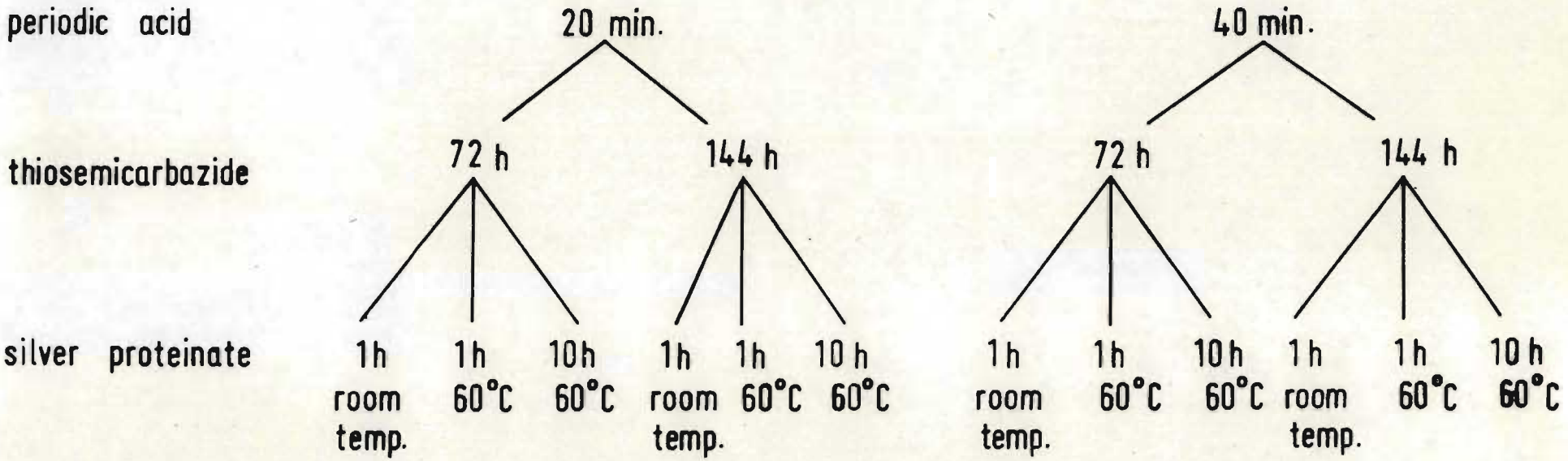
2.6 CYTOCHEMICAL LOCALIZATION OF POLYSACCHARIDE

A wide variety of techniques for the localization of polysaccharide at the ultrastructural level have been reported in the literature (e.g. Rambourg, 1967; Thiéry, 1967). Although all the techniques work on the same basic principle, they yield somewhat differing results. This section may be regarded as a catalogue of the author's attempt to find a technique most suited to the problem at hand (see Results Section).

Unless otherwise stated, all work was carried out on 48-hour imbibed root tips postfixed with either osmium tetroxide or potassium permanganate (Sections 2.3.1 and 2.3.2). Sections were picked up on 50µm gold grids. Post-staining was not carried out (unless indicated to the contrary).

The techniques employed involve treating the sections with a variety of oxidising and staining agents. This was accomplished by floating the grids section-side down on the particular solution contained in a closed, sterile plastic Petri dish. Where it was necessary to heat the solution, a glass Petri dish was used. Grids were manipulated with jewellers fine forceps. When transferring a grid from one solution to another it was necessary to take particular care not to allow the grid to flip over, as can happen readily as a result of surface tension forces, as once this has occurred it is difficult to tell to which side of the grid the section is adhering; this leaves the possibility that the remainder of the process will be carried out on the wrong side of the grid, with obviously unsatisfactory results. Thus in this event the grid was discarded, or, if it could not be spared, was sunk so as to expose both sides of the section from then on to the action of the chemical agents.

Figure 1 VARIATIONS TESTED, BASED ON THE METHOD OF THIÉRY (1967)



2.6.1 *Preliminary investigations*

2.6.1.1 *Starting point - The method of Thiéry (1967) (loc. cit. Baker and Evans, 1972).*

Sections were:

(a) oxidised with 1% periodic acid in distilled water for 20 minutes (controls were run without periodate oxidation),

(b) washed with three changes of distilled water overnight,

(c) treated with 1% thiosemicarbazide in 10% acetic acid for 72 hours,

(d) washed with descending concentrations of acetic acid (10,5 and 2%, 10 minutes in each),

(e) washed with distilled water for 20 minutes with two changes,

(f) treated with 1% aqueous silver proteinate (Merck, 'Albuminose silber, für die Mikroscopie') for 35 minutes in the dark,

(g) washed with distilled water for 30 minutes with two changes, and allowed to dry on filter paper.

2.6.1.2 *The effect of altered temperature and duration of incubation on staining intensity*

The Thiéry technique did not yield results which were useful in the context of this investigation, therefore several variations were tried. All solutions were used at the same concentrations as before, but durations and temperatures of incubation were varied. In addition the effect of post-staining with lead citrate (see 2.3.7.1) before the polysaccharide localization procedure was investigated.

Figure 1 illustrates the permutations and combinations tested. The steps were carried out in the same order as previously

(see 2.6.1.1). The controls in all cases were run without periodate oxidation.

2.6.1.3 *The effect of thiocarbohydrazide on staining intensity*

The procedure was carried out according to Thiéry (see 2.6.1.1) except that the thiosemicarbazide was replaced with thiocarbohydrazide, which was reported (Maruyama, 1974) to give a greater intensification of the staining reaction.

The sections were:

(a) oxidised with 1% periodic acid in distilled water for 20 minutes,
(b) washed with three changes of distilled water overnight,
(c) treated with 0,2% thiocarbohydrazide in 10% acetic acid for 72 hours (modified after Maruyama, 1974),
(d) washed with descending concentrations of acetic acid (10, 5, and 2%, 10 minutes in each),
(e) washed with distilled water for 20 minutes with two changes,
(f) treated with 1% aqueous silver proteinate for 35 minutes in the dark,
(g) washed with distilled water for 30 minutes with two changes and allowed to dry on filter paper.

2.6.1.4 *Silver methenamine as a staining reagent*

At this stage it was decided to attempt an alternative technique (Rambourg, 1967) which utilized silver methenamine, as opposed to silver proteinate, as a staining reagent.

Silver methenamine reagent was prepared as described below:

Glassware

All glassware was soaked in hot chromic acid (90°C) for 1 hour, left to stand in the chromic acid for a further 24 hours at room temperature, and then rinsed thoroughly with tap water followed by distilled water. The same vessel was always used to contain the same solution, and in cases where a precipitate appeared on the walls of the vessel it was replaced.

The following solutions were prepared:

(a) methenamine (hexamethylene tetramine) - freshly made up as a 3% solution in distilled water,

(b) silver nitrate - freshly made up as a 5% solution in distilled water,

(c) sodium borate - a 2% solution in distilled water which could be kept for 1 week at room temperature (Rambourg, 1967).

Silver Methenamine Reagent

The following procedure was carried out in the dark room under a yellow-green safelight:

(a) 5 ml silver nitrate solution were added to 45 ml methenamine solution. A white precipitate appeared which dissolved on shaking.

(b) 5 ml sodium borate solution was thoroughly mixed with the above.

(c) The resulting clear silver methenamine solution was filtered through two thicknesses of Whatman No. 42 filter paper into an Erlenmeyer flask. The solution was used immediately.

Preparation of the Sections for Staining

Sections were:

(a) oxidised with 1% periodic acid in distilled water for 20 minutes,

(b) washed with three changes of distilled water overnight.

Staining Procedure

(a) A covered glass Petri dish (60 mm in diameter) was preheated to a temperature of 60°C and maintained at this temperature.

(b) Approximately 30 ml of the silver methenamine solution was poured into the dish.

(c) The grids were floated section-side down on the surface of the solution and allowed to stain for 30 minutes in the dark within the covered Petri dish.

(d) The grids were removed and some were allowed to stain for a further 30 minutes in a fresh silver methenamine solution.

(e) All sections were washed with distilled water (30 minutes with three changes), and some were further washed on 5% sodium thiosulphate for 10 minutes before being washed again with distilled water.

2.6.1.4.1 The procedure in 2.6.1.4 was repeated but the grids were allowed to stain for 30 minutes only, at a temperature of 40°C.

2.6.1.4.2 As problems were still being experienced with the silver methenamine technique it was decided to try a variation of the method employing a more dilute silver methenamine solution (de Martino and Zamboni, 1967).

The following solutions were prepared:

Methenamine (hexamethylene tetramine) -
freshly made up as a 3% solution in distilled water,
Silver nitrate -
freshly made up as a 5% solution in distilled water,
Sodium borate -
a 5% solution in distilled water which could be left at room temperature for 1 week.

The silver methenamine was prepared under the conditions described in Section 2.6.1.4.

Silver Methenamine

5 ml silver nitrate solution were added to 100 ml methenamine solution and shaken to dissolve the white precipitate which formed. This was diluted with an equal volume of distilled water. 4 ml sodium borate solution was added to 50 ml of the above and the resulting clear silver methenamine solution filtered through two layers of Whatman No. 42 filter paper into an Erlenmeyer flask.

The staining procedure was carried out according to Section 2.6.1.4 with the exception that the above silver methenamine solution was used in place of the standard reagent (Rambourg, 1967).

2.6.2 *The final series of experiments*

These were based on the conclusions reached in the preliminary experiments but a few further modifications were carried out.

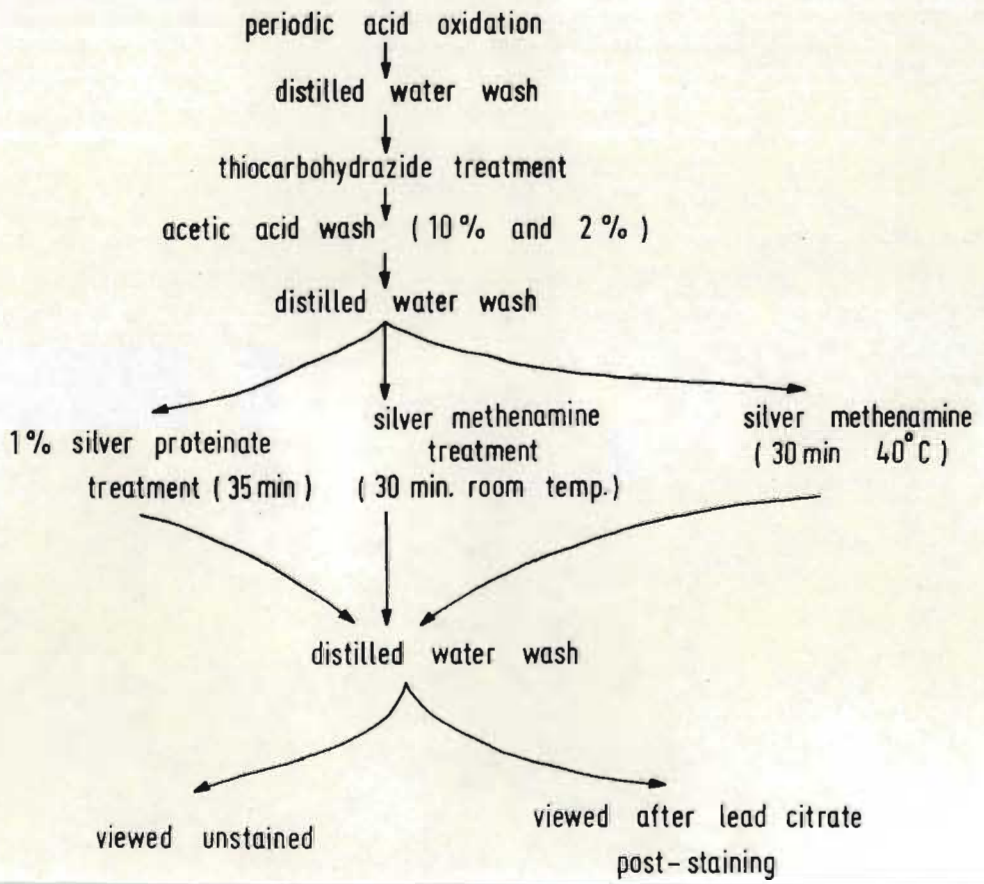
Sections were:

(a) oxidised with 1% periodic acid in distilled water for

Figure 2

DIAGRAMMATIC REPRESENTATION OF THE FINAL PROCEDURES USED TO LOCALIZE POLYSACCHARIDES AT THE ULTRASTRUCTURAL LEVEL

SECTIONS MOUNTED UPON 50μ GOLD GRIDS WERE TAKEN THROUGH THE FOLLOWING STEPS :



20 minutes,

(b) washed overnight with three changes of distilled water,

(c) treated with 0,2% thiocarbohydrazide in 10% acetic acid for 72 hours,

(d) washed with descending concentrations of acetic acid (10,5 and 2%, 10 minutes in each),

(e) washed with distilled water for 20 minutes with two changes,

(f) treated in the dark with

either: 1% silver proteinate for 35 minutes,

or : silver methenamine (Rambourg, 1967) for 30 minutes at room temperature,

or : silver methenamine for 30 minutes at 40°C.

(g) washed with distilled water for 30 minutes with two changes,

(h) either: allowed to dry on filter paper

or : post-stained with lead citrate for 45 minutes and then allowed to dry on filter paper.

For a diagramatic representation of the above see Figure 2.

Controls

(a) In order to determine what effect (if any) the various postfixatives might have on the cytochemical reaction, material which had not been postfixed with either osmium tetroxide or potassium permanganate was subjected to the above procedure. Fixation, dehydration and embedding of this material was carried out according to 2.3.1 but postfixation with osmium tetroxide and staining with uranyl acetate (Steps c, d and e) were omitted.

(b) A second set of controls were run according to the procedure described in this section, but without periodic

acid oxidation, in order to ascertain the extent of the reaction produced by free aldehyde groups already existing within the material.

The Method of Choice

Sections were:

(a) oxidised with 1% periodic acid in distilled water for 20 minutes,

(b) washed overnight with three changes of distilled water,

(c) treated with 0,2% thiocarbohydrazide in 10% acetic acid for 72 hours,

(d) washed with descending concentrations of acetic acid (10,5 and 2%, 10 minutes in each),

(e) washed with distilled water for 20 minutes with two changes,

(f) treated in the dark with 1% silver proteinate for 35 minutes at room temperature,

(g) washed with distilled water for 30 minutes with two changes,

(h) post-stained with lead citrate for 45 minutes before being allowed to dry on filter paper.

2.7 AUTORADIOGRAPHY

2.7.1 *Incubation with isotope*

2.7.1.1 Root-tips were excised from 48-hour imbibed maize embryos (6 samples of 5 each) and incubated in L-(1-^{3}H) fucose (5,3 Ci/m mole, 0,75 mCi/ml) for the following periods at room temperature: 5, 10, 20, 30, 60 and 180 minutes. In the case of the 5, 10 and 20 minute incubations root tips were bisected along the median longitudinal axis to facilitate penetration of the isotope into the inner tissues. The incubation was carried out in a pill vial (2,5 cm in diameter) and sufficient isotope-containing solution was added to just cover the material. Five further 48-hour imbibed seeds were allowed to grow for 12 hours at room temperature with the tip of the root immersed in a small volume of L-(1-^{3}H) fucose of the same specific activity and radioactive concentration.

2.7.1.2 The radicle tips of 12-hour imbibed maize embryos were excised and incubated in L-(1-^{3}H) fucose (5,3 Ci/m mole, 0,75 mCi/ml) for 90 minutes at room temperature.

2.7.1.3 The root tips were excised from twenty 48-hour imbibed embryos and incubated with L-(1-^{3}H) fucose (5,3 Ci/m, 0,7 mCi/ml) for three hours at room temperature. They were then washed briefly with distilled water and divided into three samples of five each which were chased with a cold L-fucose solution of equivalent concentration (0,03 g/l) for periods of 0, 30, 90 and 135 minutes respectively.

2.7.1.4 Twenty 48-hour imbibed seeds were allowed to grow for 6 hours at room temperature with their root tips immersed in a solution of L-(1-^{3}H) fucose (5,3 Ci/m m mole, 0,75 mCi/ml). The roots were then washed with distilled water and the root-tips excised and chased for 3 hours in a cold L-fucose solution (0,03 g/l).

In all cases control material was incubated in a cold L-fucose solution (0,03 g/l) in place of incubation with isotope.

2.7.2 *Preparation of material for autoradiography*

Following incubation with isotope the root tips were rinsed briefly with distilled water before being fixed and embedded according to the procedure described in Section 2.3.1.

Tissue sections to be processed for electron microscope autoradiography were picked up on collodion-coated copper grids (2.3.5), and post-stained with lead citrate (2.3.7.1) before being coated with a thin layer of carbon (approximately 100 Å thick) (2.3.8). 1µ thick sections intended for light microscope autoradiography were cut from the same blocks and dried down on slides coated with gelatine chrome-alum adhesive.

2.7.2.1 *Preparation of gelatine chrome-alum adhesive-coated slides*

Gelatine Chrome-Alum Solution

0,50 g gelatine were added to 100 ml distilled water and the mixture heated at 45°C in a water bath to dissolve the gelatine. This solution was allowed to cool, after which 0,05 g chrome-alum was added. The final solution was double filtered through Whatman No. 1 filter paper and used within 48 hours.

Unused microscope slides were washed with hot soapy water, soaked in chromic acid for 12 hours and finally rinsed well with distilled water. The clean grease-free slides were dipped into the gelatine chrome-alum solution and excess solution allowed to drain off. The slides were dried in an oven at 60°C for 12 hours and and stored in a slide box under dust-free conditions.

2.7.3 *Autoradiographic procedures for light microscopy*

All procedures were carried out in a dark room lit with a yellow-green safelight only when necessary. Ilford L4 Nuclear Research Emulsion was diluted 50/50 (v/v) with distilled water and incubated in a water bath at 45°C for 10 minutes with gentle stirring in order to achieve a homogeneous solution of emulsion. The slides were dipped into the emulsion and any excess emulsion drained off, after which their undersides were wiped free of emulsion and they were laid flat for 30 minutes and allowed to dry.

One emulsion-coated experimental slide was taken out of the dark room and fogged with light to serve as a control against latent image fading. In addition one adhesive coated slide containing no sections was coated with emulsion to serve as an added control for background irradiation.

The coated slides were exposed in a light-tight box containing silica gel as a desiccant, for 6 days at 5°C.

Development

Development took place in total darkness. Slides were:

(a) developed in Ilford Phen-X developer (undiluted) for 4 minutes at 20°C,
(b) washed with distilled water for 1 minute,
(c) fixed in Amfix ('M & B' (Maybaker)) for 2 minutes at 20°C,
(d) washed with distilled water for 1 minute (at this stage they were removed from the dark room),
(e) dehydrated in a graded alcohol series 25, 50, 75, 100% ethanol, 2 minutes in each),
(f) further dehydrated in fresh 100% ethanol for 2 minutes,
(g) immersed in xylene for 2 minutes,
(h) mounted in Canada Balsam.

Slides were viewed by phase-contrast microscopy with a Zeiss GFL photo-microscope and photographs taken on Ilford Pan F film using a green filter.

2.7.4 *Autoradiographic procedures for electron microscopy (modified after R.N. Pienaar, personal communication)*

2.7.4.1 *Application of photographic emulsion by the wire loop method*

(a) Double-sided adhesive tape (3M) was applied to the tapered ends of corks, and perforations slightly smaller than the diameter of an electron microscope grid were drilled through the adhesive tape and cork. The grids were mounted gently over the holes so that only their edges adhered to the adhesive tape. The following procedures were carried out in the dark room lit with a yellow-green safelight only when necessary.

(b) Ilford L4 Nuclear Research Emulsion was diluted 1/2 (w/v) with distilled water and incubated at 45°C for 15 minutes with occasional gentle stirring in order to achieve a homogeneous solution of emulsion.

(c) The emulsion was allowed to stand in an ice bath for 4 minutes and stirred gently.

(d) It was then kept at 20°C until it formed a viscous semi-gel (5 - 10 minutes). In these conditions, when a wire loop (3 cm in diameter made of 20 gauze tungsten wire) was dipped into the emulsion and withdrawn, a firm elastic membrane was formed and showed no tendency to flow when the loop was rotated. Care was taken to withdraw the loop edge first to avoid gross overloading.

(e) The loop was gently lowered over the upright cork holding the grid, producing an initial tent-like appearance. Only on further lowering of loop did the layer of emulsion break away from the loop.

(f) Two corks were inserted one into either end of each length of plastic tubing (into which a small air hole had been made) to protect the coated grids from dust. Exposure took place for two months at 5°C in a light-tight box, with silica gel as a desiccant.

2.7.4.2 *Development of electron microscope autoradiographs*

Development took place in total darkness. The exposed, coated grids, still on the corks, were:

(a) developed in a stock solution of Kodak D19B developer for 2 minutes at 20°C,

(b) washed with distilled water for 1 minute,

(c) fixed with Amfix for 2 minutes at 20°C,

(d) washed with distilled water for 2 minutes with one change,

(e) allowed to dry before being removed from the corks.

2.8 ISOLATION OF VACUOLES

The procedure was modified from Matile (1968) by Matile and Berjak (personal communication).

The following solutions were prepared and stored at 5°C.

A. Medium

0,5 M sorbitol
in 0,05 M Tris-HCl buffer (pH 7,6)
in 0,001 M EDTA (this component was necessary to prevent inter-membrane bonding between divalent cations).

B. Sucrose (density gradient quality)

Solutions of the following concentrations: 20%, 25%, 30%, 35%, 40%.

C. An equivalent series of sorbitol solutions, a hydrometer being used to obtain densities exactly equal to those of the sucrose series.

Procedure

(a) The seeds were imbibed and set to germinate and the basal 1 cm of the root cut off and placed in distilled water in an ice bath (at least 1 gm of tissue was used).
The following steps were carried out in a cold room at 5°C.

(b) The material was washed in distilled water, blotted dry quickly and weighed.

(c) An equivalent weight of quartz sand (0,1 to 0,8 mm in diameter) was weighed out and added to the root tips.

(d) This mixture was placed in a pre-cooled mortar together with a volume of pre-cooled medium equivalent to the weight of material.

(e) The sand, medium and material were mixed lightly and then ground very gently from the edges of the mortar inwards

in order to avoid breaking up the sand particles and thus rupturing the vacuoles.

(f) Once a brei had been obtained another equivalent volume of medium was added. The mixture was ground gently with a round and round action but no pressure until the end-product was 'egg soupy' (at this stage no recognizable tissue remained).

(g) The homogenate was poured off into a beaker; the mortar was washed out twice with an equivalent volume of medium and the washings added to the homogenate. At this stage four equivalent volumes of medium had been used.

(h) The homogenate was centrifuged for 10 minutes at 500 xg to precipitate tissue debris, nuclei, starch and all the sand present.

(i) The cloudy supernatant was decanted with a pasteur pipette and centrifuged for 20 minutes at 20 000 xg in an angle under refrigeration. The supernatant was discarded. The pellet consisted of four concentric bands, the vacuoles being contained in the white, outermost band.

(j) The outermost (vacuolar) band of the pellet was scraped away using a small spatula and gently resuspended in approximately 5 ml medium, by homogenising very gently with a teflon piston.

(k) Stepwise sorbitol density gradients (equivalent to 20% to 40% sucrose) were prepared, using 2 ml of each of the five solutions of different density in a 5 cm cellulose nitrate centrifuge tube.

(l) One millilitre of vacuolar suspension was layered on top of each gradient which were then centrifuged for 150 minutes at 124 000 xg in a swinging-bucket rotor.

(m) Each band obtained was removed from the gradient with the aid of a Pasteur pipette and washed twice in 0,05 M Tris-HCl buffer (pH 7,6). After each washing the vacuoles were centrifuged down at 20 000 xg for 20 minutes. The washings were retained.

(n) The resulting vacuolar pellet was taken up in a small volume (approximately 1 ml) of distilled water.

The well-washed vacuolar samples were sonicated in distilled water until they lysed (approximately 24 hours in an 80/40 watt Transistorised Soniclean). A check under the light microscope revealed whether or not lysis had occurred. If lysis was incomplete the vacuolar sample was frozen and thawed several times to effect final lysis.

Both the lysed vacuolar sample and the washings, which had been retained, were concentrated down under vacuum at 40°C. The samples were analysed chromatographically for monosaccharides (Section 2.10).

2.9 ISOLATION OF ROOT CAP SLIME

2.9.1 *Accelerated production of slime (Paull, Johnson and Jones, 1975)*

Seeds were rinsed in running tap water for 15 minutes to remove the fungicide and then soaked in tap water for 12 hours. They were surface sterilized with a 5% 'Jik' (sodium hypochlorite) solution for 2 minutes and then washed well with sterile, distilled water. Seeds were laid embryo-side down in sterile petri dishes containing sterile distilled water and allowed to grow until the roots reached a length of approximately 2 centimetres.

Modified Hoagland's Solution (0,5 M)

This contained

0,5 mM KH_2PO4	)
2,5 mM KNO_3	) Hoagland's Solution
2,5 mM $Ca(NO_3)_2$	)
1,0 mM $MgSO_4$	)
25 µM boric acid	
40 mM sucrose	

The above medium was autoclaved at 15 psi and 120°C for 10 minutes. On cooling 20 µg/ml chloramphenicol and 5 µg/ml streptomycin were added as an extra safeguard against microbial contamination.

Twenty five excised root tips (approximately 2 cm long) were incubated in the dark at 25°C in 10 ml modified Hoagland's medium in a sterile 50 ml Erlenmeyer flask plugged with cotton wool. The incubation was carried out on a reciprocating shaker operating at 80 cycles per minute and lasted for 30 hours. Sterile microbiological procedure was observed at all times during the above manipulations.

The incubation was terminated by making up the medium to 75% (V/V) ethanol by the addition of absolute ethanol and keeping it at 5°C for 12 hours. Under these conditions the slime formed a fibrous white precipitate which was spooled onto a Pasteur pipette and stored in 75% ethanol. The extent of microbial contamination was checked under the light microscope. This slime was purified as detailed in Method 2.9.2 below.

2.9.2 *Harvesting and purification of root cap slime*

12-hour imbibed seeds were surface sterilised with a 5% 'Jik' (sodium hypochlorite) solution for 2 minutes and washed well with distilled water. They were placed embryo side down on two layers of paper towelling (moistured with distilled water containing 20 µg/ml chloramphenicol and 5 µg/ml streptomycin) in loosely closed plastic dishes and allowed to grow until the roots reached a mean length of 2 cm.

The slime was aspirated from the root tips and purified according to the method of Jones and Morré (1966) as follows:

(a) The slime was diluted with distilled water to reduce its viscosity,

(b) centrifuged at 20 000 g for 1 hour to remove sloughed cells, cell walls and other debris,

(c) the supernatant was made to 75% (V/V) ethanol and allowed to precipitate overnight at 5°C,

(d) the white fibrous precipitate was spooled onto a glass rod, transferred to cold 95% ethanol, and washed eight times with 95% ethanol,

(e) the washed slime was resuspended in water and precipitated again from 75% ethanol, after which it was dried down under vacuum at 40°C.

2.9.3 *Hydrolysis of root cap slime*

The dried slime was solubilised in 96% sulphuric acid at room temperature and immediately diluted to a final acid concentration of 3% after which it was autoclaved at 15 psi and 130°C for 1 hour before being allowed to cool. The hydrolysate was neutralised with a saturated aqueous solution of barium carbonate and concentrated under vacuum at 40°C to a volume of approximately 0,2 ml.

2.10 CHROMATOGRAPHY

2.10.1 *Paper chromatography*

The presence of individual monosaccharides in a sample was detected using one dimensional descending paper chromatography. 5 µl aliquots of the samples were spotted onto Whatman No. 1 chromatography paper and the chromatograms run with a 4/1 isopropanol/water mixture as solvent for 36 hours at 25°C, in a sealed tank saturated with solvent. The bottom edge of the paper was serrated to assist the flow of solvent off the paper. 1% aqueous solutions of representative monosaccharides were used as standards.

The sugars were visualised by spraying the dried chromatograms with aniline-diphenylamine location reagent and then heating briefly at 100°C.

Aniline-diphenylamine reagent (Plummer, 1978)

1% aniline	5 volumes
1% diphenylamine	5 volumes
85% phosphoric acid	1 volume

2.10.2 *Gas-liquid chromatography*

2.10.2.1 *The columns*
(3% silicone SE-30 on chromasorb GHP 100/120 mesh support phase)

0,9 g silicon SE-30 were dissolved in approximately 100 ml chloroform and added to 30 g Chromasorb GHP (Supelco.) (100/120 mesh). Chloroform was evaporated off in a rotary evaporator at 40°C until a wet slurry was left. The slurry was kept at room temperature until all the chloroform had evaporated (approximately 48 hours; at this stage the smell of chloroform could no longer be detected).

The coated support phase was packed into twin glass columns (170 centimetres in length) under constant vibration to ensure firm packing.

2.10.2.2 *The monosaccharide derivatives. Monosaccharide-trimethylsilyl ethers (after Nurock, personal communication)*

Silylating Reagent

trimethylsilylimidazole	12 ml
pyridine	3 ml

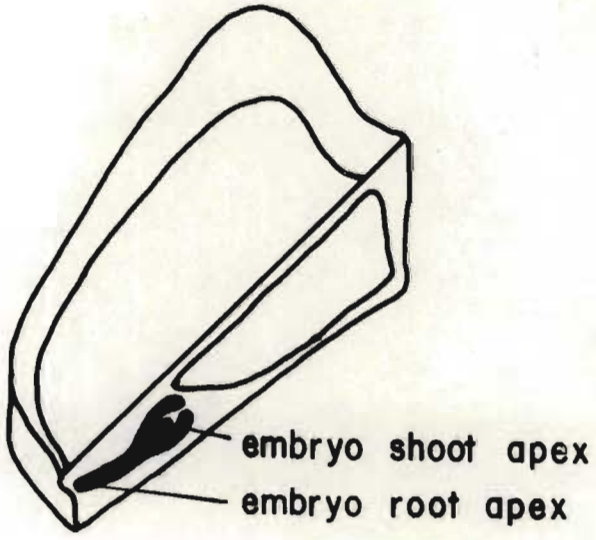
10 mg dried material or 10 µl aqueous material were dissolved in 0,6 ml silylating reagent and shaken vigorously for 10 minutes at room temperature. Dilute samples were concentrated by saturating the above solution with imidazole, adding 0,1 ml dodecane and shaking. The monosaccharide-trimethylsilyl ethers will concentrate in the dodecane phase.

2.10.2.3 *Operating conditions*

Gas-liquid chromatography was carried out in a dual column Pye Unicam Series 104 Chromatograph with nitrogen as carrier gas flowing at 30 ml/min. Hydrogen flame ionization detectors were kept at 250°C with a hydrogen flow rate of 30 ml/min and an air flow rate of approximately 200 ml/min. 2 µl samples were injected with a Hamilton microlitre syringe into the injection ports,which were not heated. The columns were either operated isothermally at 170°C or programmed from 140°C to 210°C with a temperature increment of 6°C per minute. 10 mm per minute was found to be a suitable chart speed. Peaks on the chromatograms were identified by comparison with known standards run under similar conditions.

FIGURE 3

SCHEMATIC REPRESENTATION OF A 12 HOUR-IMBIBED MAIZE CARYOPSIS (L/S)



PART THREE

RESULTS

3.1 LIGHT MICROSCOPY

3.1.1 *12-Hour imbibed material*

After twelve hours of imbibition the radicle of the maize embryo was still surrounded by the coleorhiza and had not yet penetrated the seed coat (Figure 3). Imbibition had, however, been completed and the previously quiescent embryo was about to begin normal metabolism (Berjak and Villiers, 1970). Plate 1 illustrates

the anatomical structure of the root cap of the 12-hour imbibed maize embryo in median longitudinal section as seen with the light microscope.

The root cap may be divided into three zones:

1. The cap meristem (Plate 2)

The root cap produced by division of the cells of the meristematic region of the cap. These cells appeared compact and approximately square in section and were virtually devoid of conventional translucent plant vacuoles. Vacuoles appeared instead as numerous small, darkly stained provacuoles reminiscent of the lysosomes (with which they have been equated by Berjak (1968)) which may be observed in animal cells under the light microscope.

2. A central zone of non-dividing cells (Plate 3)

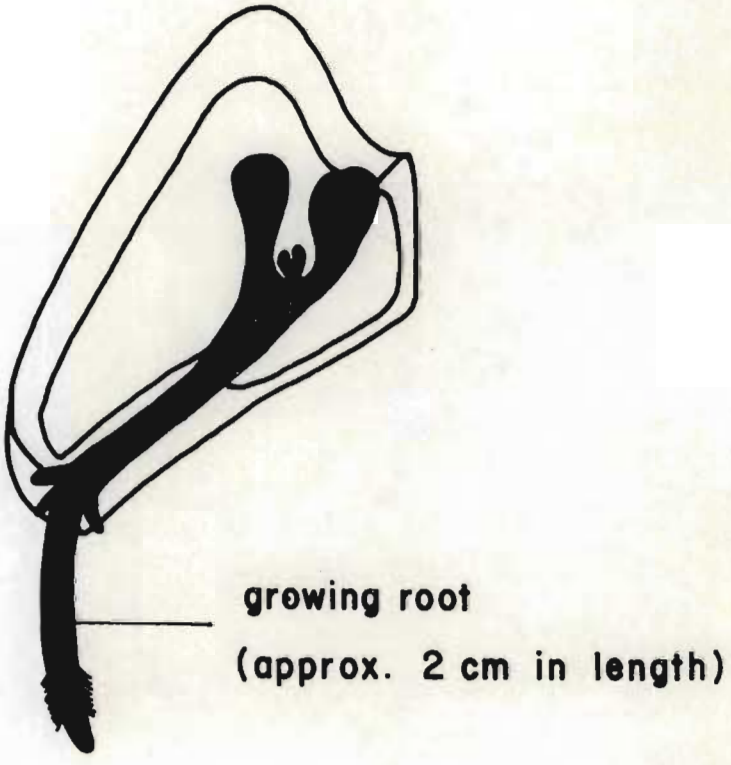
These cells appeared larger than those found in the meristematic region. Provacuoles were present, but careful examination revealed that many of these were surrounded by a translucent envelope and gave the impression of having expanded. In this state they were more reminiscent of the usual form of plant vacuoles.

3. A narrow peripheral zone of apparently highly vacuolate cells (Plate 4)

There was a sharp distinction between the apparently normal cells of the central zone and the apparently highly vacuolate peripheral cells which appeared virtually devoid of cytoplasmic content. The impression was one of

FIGURE 4

SCHEMATIC REPRESENTATION OF A 48 HOUR-IMBIBED MAIZE CARYOPSIS (L/S)



precipitous senescence having occurred at the outer edge of the root cap, producing apparently dead cells which sloughed off from the cap.

3.1.2 *48-hour imbibed material*

After 48 hours of imbibition (and at least 36 hours of normal ongoing metabolism) the radicle had broken through the coleorhiza and seed coat, and the young root had attained an approximate length of 2 cm (Figure 4). Plate 5 illustrates the anatomical structure of the root cap of the 48-hour imbibed maize root cap as seen in median longitudinal section under the light microscope. Development of the root cap proceeds in a direction distal to the meristematic region where the cells of the cap are produced by continuous division. For clarity the cap may be divided into four separate zones, but it should be noted that the development of the cap is a continuous process and that there is therefore no sharp dividing line between these zones. These zones are:

1. The meristematic zone (Plate 6)

   The meristematic cells appeared small and approximately square with the nucleus occupying a large volume of the cell in relation to the cytoplasm. Very young, dense provacuoles, as seen in the corresponding cells of 12-hour imbibed material, could not be observed within 48-hour imbibed meristematic cells. Vacuoles, where present, appeared as small translucent structures scattered throughout the cytoplasm.

2. The zone of differentiation (Plate 7)

   The zone of differentiation is situated distal to the meristem; differentiating tissue may be distinguished

from meristematic tissue by the presence of numerous darkly-stained, globular amyloplasts at the distal end of each cell. The vacuoles were concentrated in the proximal portion of the cytoplasm, appeared translucent, and were larger in size and more numerous than those observed in the meristematic tissue.

3. The zone of mature cells (Plate 8)

The zone of mature cells is situated distal to the zone of differentiation. The two zones appeared similar under the light microscope; the difference was that the mature cells were larger in size and more vacuolated.

4. The zone of senescing cells (Plate 9)

The zone of senescing cells was situated at the outer or peripheral edge of the root cap. The vacuoles appeared to have coalesced, this process culminating in the formation of one or several large translucent vacuoles occupying most of the cytoplasm. In sections treated with toluidine blue, a stain specific for polysaccharides, an accumulation of darkly stained material, apparently polysaccharide in nature, seemed to have accumulated between these outermost cells (Plate 10). The outermost cells, which appeared highly disorganised, separated from the body of the cap and appeared to 'float' in the slimy mucilage which adhered to the outside of the cap. The cytoplasm, of many of these cells appeared to have shrunk away from the cell wall (Plate 11) and it seemed as if there was an accumulation of some substance between the cell wall and cytoplasm.

3.2 ELECTRON MICROSCOPY

3.2.1. *Preparation of material for viewing in the electron microscope*

Although most methods used to prepare material for viewing in the electron microscope yield similar results, each has its advantages and disadvantages. Several methods were tried in the present study with a view to obtaining the best preservation of the experimental material. As the work focuses largely on vacuoles and, to a lesser extent, on the Golgi apparatus, it was particularly important that these organelles should be well preserved and stained. It would appear useful to present the results obtained by the various procedures even though they are not directly related to the subject of study.

It should be remembered that the appearance of the material in the electron microscope depends not only on the preparative methods used, but also on the physiological state of the tissues at the time of fixation. For example the diffuse, disorganised appearance of an ageing cell should not be interpreted as being a result of poor fixation.

3.2.1.1 *Glutaraldehyde/osmium tetroxide fixation*

This widely-used method gave a pleasing overall result. All cytoplasmic components appeared well preserved - Plates 12, 13 and 14 show nuclear material, ribosomes, mitochondria, plastids, endoplasmic reticulum and lipid droplets (spherosomes?) in a good state of preservation. Lipoprotein membranes were usually clearly visible (Plate 14), however in some cases the vacuolar membrane appeared indistinct (Plates 15, 16). Dictyosomes were well preserved and on occasion their vesicles appeared to contain a *slightly* electron-opaque matrix (Plate 16).

3.2.1.2 *Glutaraldehyde/potassium permanganate fixation*

This generally harsher method of fixation differed from the above in that ribonucleoprotein was not well preserved. In fact ribosomal material could only rarely be distinguished even faintly (Plate 17) and generally appeared completely absent from the cytoplasm, which took on a diluted, featureless appearance (Plate 18). Lipoprotein membranes stained very distinctly and thus vacuolar membranes, where present, were clearly visible (Plate 19). Owing to the intense membrane stain and the featurelessness of the cytoplasm, micrographs, especially at low power, tended to take on an outline appearance (Plate 20). In a few cases the vacuolar membranes appeared to have been disrupted during fixation (Plate 21) and lay in part in the centre of the vacuole and not apposed to the cytoplasm, as was usually the case (Plate 22). As was the case with osmium tetroxide post-fixed material, the dictyosomes appeared well preserved with the vesicles of the hypersecretory dictyosomes showing slight electron-opacity (Plate 23). The contents of the lipid droplets appeared to have been etched away, leaving only a shrunken, stellate outline (Plate 17).

3.2.1.3 *The use of PIPES buffer in fixation*

Primary fixation was with glutaraldehyde buffered with piperazine-NN'-bis-2-ethane sulphonic acid (PIPES) and was followed by postfixation with 2% aqueous osmium tetroxide. The results obtained were similar to those obtained using cacodylate-buffered glutaraldehyde and osmium tetroxide as fixatives but overall preservation was far finer, giving the cytoplasm an extremely dense, detailed appearance (Plate 24). Membranes were well preserved although not always easy to distinguish. The contents of the dictyosomal vesicles appeared in many cases to be quite distinctly stained (Plate 25), although this was not always the case.

3.2.1.4 *2,2-dimethoxypropane as a rapid dehydrating agent*

The use of 2,2-dimethoxypropane as a dehydrating agent had no gross adverse effect on cellular ultrastructure. However lipoprotein membranes, although well preserved, appeared almost completely electron-translucent and could only be distinguished by virtue of an apparent 'negative staining' effect in comparison with the dense surrounding cytoplasm (Plate 26).

3.3 ULTRASTRUCTURAL OBSERVATIONS

The ultrastructure of the root cap will be discussed in terms of the zones loosely defined in Section 3.1 (Light Microscopy). No attempt will be made to completely characterise all the organelles present in each cell zone (see Berjak, 1968) as this is not relevant to the subject of this study. Attention will be focused on the vacuolar and, to a lesser extent, Golgi systems, with mention of other organelles where pertinent.

3.3.1 *The origin of vacuoles*

Vacuole formation in the root cap appeared to occur in cells at all stages of development, from the meristematic to senescing regions. However this phenomenon was not readily observed in the meristematic and newly differentiating cells, particularly of the 12-hour imbibed embryo. While the caryopsis is still attached to the cob the embryo develops to completion and thus the cells of the embryo contain an accumulation of relatively newly-formed and forming organelles in readiness for the commencement of growth at germination. It is at this stage that the caryopses become naturally quiescent as they are allowed to reach air-dryness on the cob before harvesting. After 12 hours of imbibition the previously quiescent seeds have commenced active metabolism but at this stage their ultrastructure still largely reflects the state of development of the embryo prior to quiescence.

3.3.1.1 *Vesiculation of the endoplasmic reticulum*

The most commonly observed method of vacuole formation was through vesiculation of the endoplasmic reticulum and appeared to occur in cells at all stages of development. At least three distinct mechanisms appeared to be involved.

FIGURE 5

DIAGRAMMATIC REPRESENTATION OF THE FORMATION OF UNEXPANDED ER-DERIVED PROVACUOLES

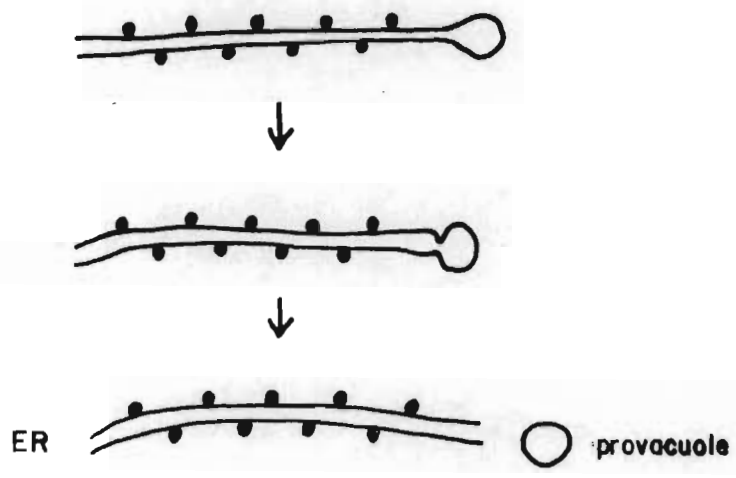
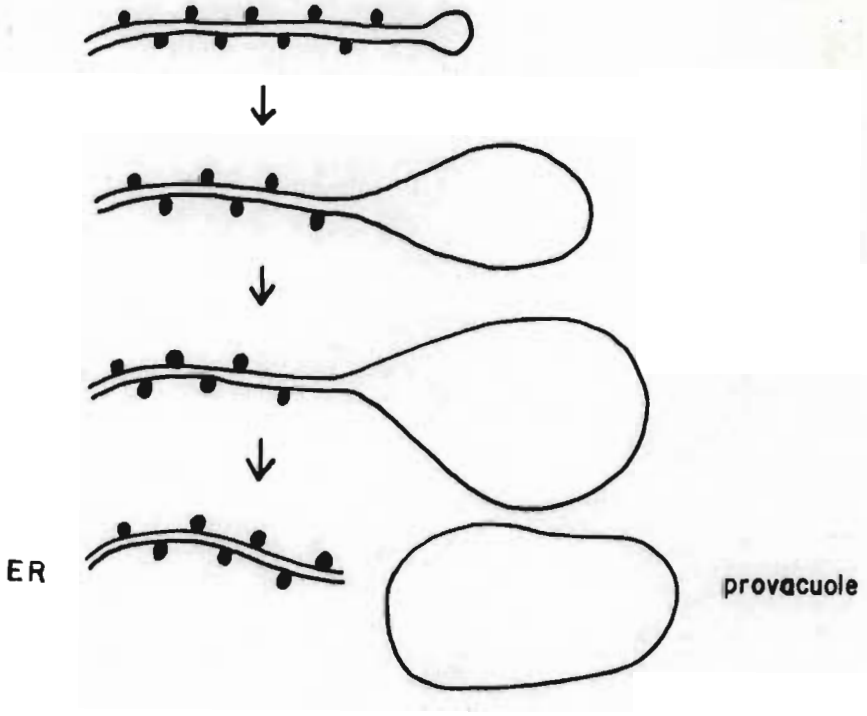


FIGURE 6

DIAGRAMMATIC REPRESENTATION OF THE FORMATION OF EXPANDED ER-DERIVED PROVACUOLES

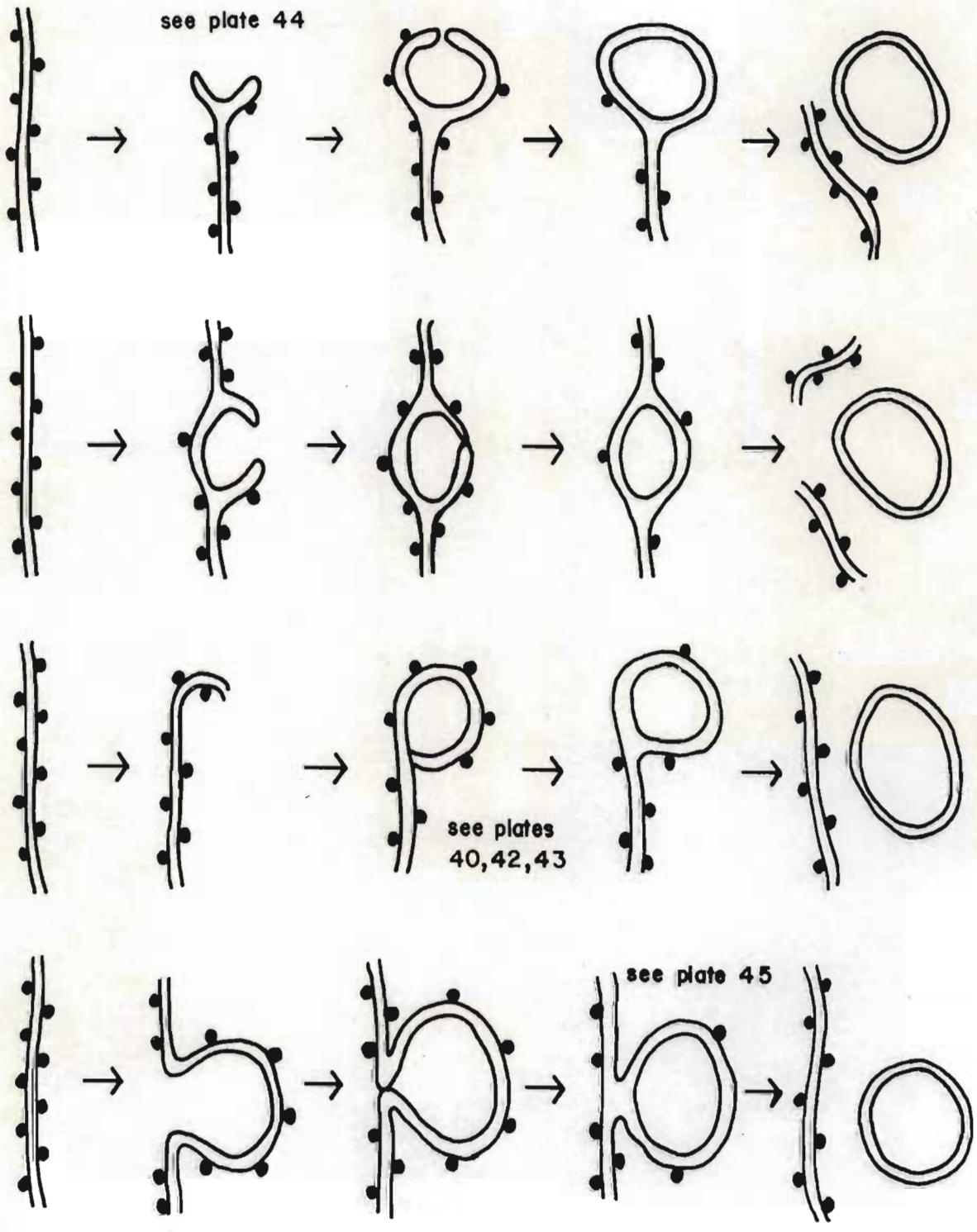


1. Small electron-opaque swellings, not much larger in diameter than the width of the ER lumen, appeared to form on the terminal portions of the ER cisternae (Plates 27, 28, 29) (See Figure 5). The interiors of these swellings were continuous with the lumen of the ER. The ER appeared to constrict behind each swelling, thus pinching it off to form an independent cytoplasmic vesicle surrounded by a single membrane (Plates 30, 31, 32, 33). Once these swellings had separated from the body of the ER they were regarded as independent organelles and termed provacuoles. Initially the provacuoles could be seen in close proximity to the ER (Plate 34), but they later appeared to become distributed throughout the cytoplasm (Plate 30). Observations on material postfixed with osmium tetroxide verified that the rough endoplasmic reticulum was involved in this process.

2. The terminal portion of the lumen of the rough ER appeared to swell, giving rise to vesicles morphologically continuous with the lumen (See Figure 6). This phenomenon was similar to that previously described except that the vesicles appeared to expand considerably before they actually separated from the ER to become independent vacuoles (Plates 35, 36). The interiors of such vesicles appeared either entirely or only partly electron-opaque. The ER appeared to differentiate adjacent to the swelling - in this regard the lumen appeared to have constricted slightly and contained electron-opaque material similar in appearance to that observed within the vesicle itself (Figure 35). This phenomenon was observed less frequently than was the budding off of the smaller vesicles described previously, but it should be remembered that the likelihood of the plane of section passing through the point of attachment of a large vesicle to the ER is small. Many similar vesicles within the cytoplasm appeared to be separate from the ER

FIGURE 7

DIAGRAMMATIC REPRESENTATION OF PROPOSED METHODS FOR THE FORMATION OF DOUBLE-MEMBRANE BOUND VESICLES FROM THE ER



(Plate 34) but may in fact still have been continuous with it although this was not obvious from the section. Once separation from the ER has occurred these organelles will be referred to as expanded provacuoles.

3. Another method of vacuolation appeared to involve the formation of expanded, double-membrane bound vesicles attached to the ER (see Figure 7). These vesicles appeared either terminal (Plates 37, 38) or intermediate (Plate 39). Terminal portions of the ER cisternae appeared to wrap round portions of cytoplasm, eventually enclosing them into vesicles which consequently appeared double-membrane bound. The space in between the two membranes in such cases corresponded to the lumen of the ER (Plates 40, 41, 42). The interior of some of these vesicles appeared empty, whilst others appeared to contain a matrix which varied in electron-opacity. Such vesicles may also have had their origins in cup-shaped invaginations or outgrowths of the ER (Plates 43, 44) which eventually enclosed portions of cytoplasm to form double-membrane bound bodies. These vesicles eventually separated from the ER and distributed themselves throughout the cytoplasm (Plates 45, 46, 47, 48).

The nature of the material contained both within the vesicles and between the two membranes bounding each vesicle appeared to vary. In some cases the vesicles appeared to contain material similar to the surrounding cytoplasm (Plate 47) whilst in other cases they appeared empty (Plate 38), or contained a featureless matrix of varying electron opacity (Plates 38, 44). The material occupying what was once the lumen of the ER appeared to vary from being non-evident (Plate 47) to being intensely electron-opaque (Plates 38,45). It should be noted that if both electron-transparent and electron-opaque material were present within a single lumen the plane of section might have shown either only

FIGURE 9

DIAGRAMMATIC REPRESENTATION OF VACUOLE FORMATION THROUGH REGIONAL DIFFERENTIATION OF THE CYTOPLASM

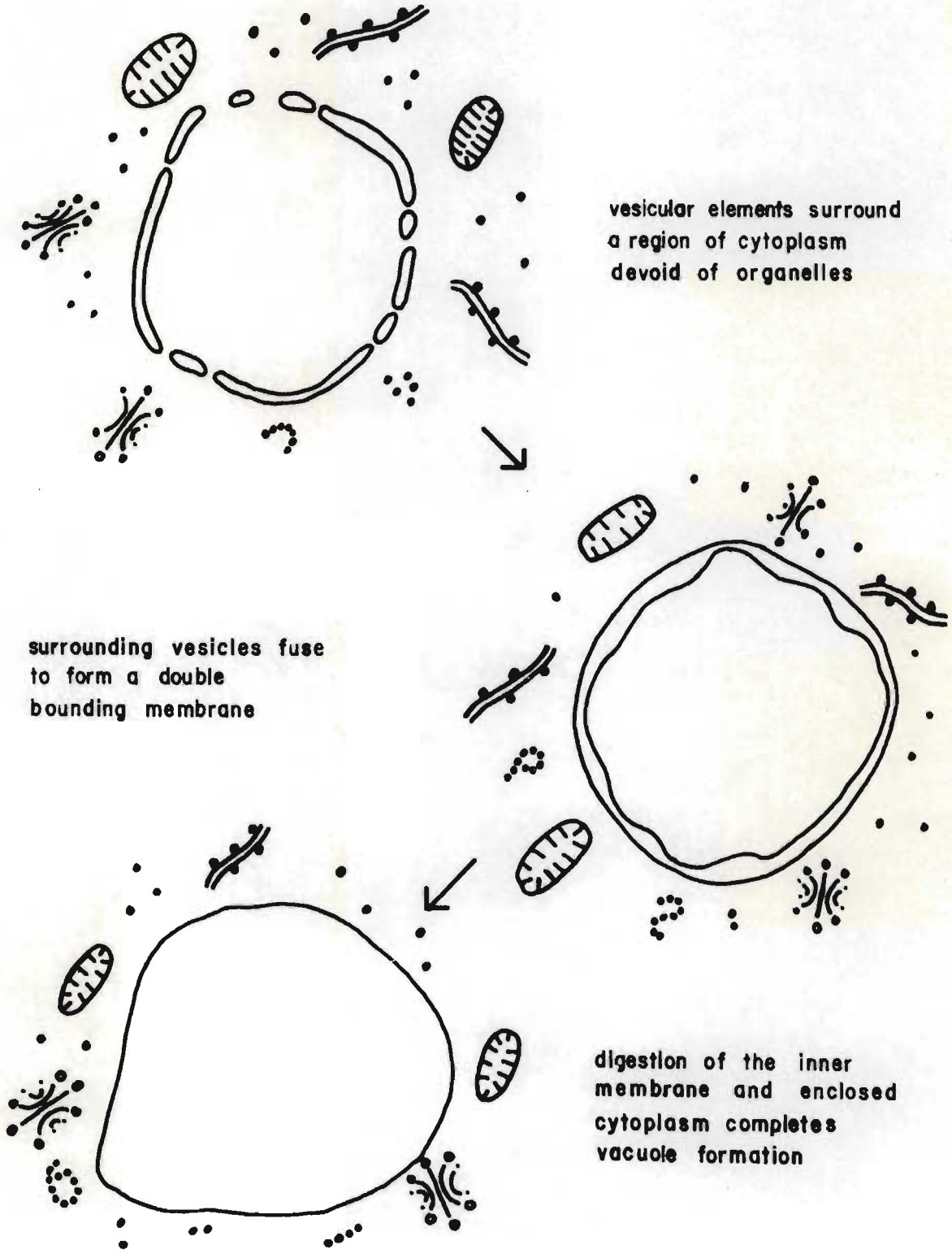
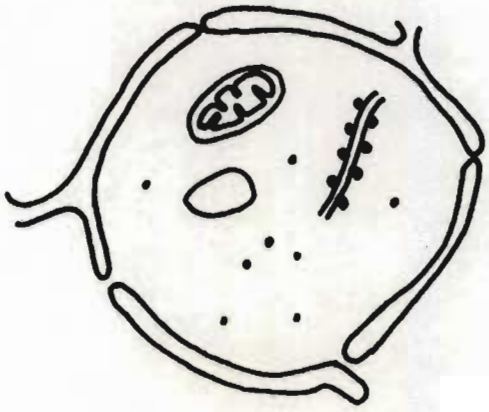
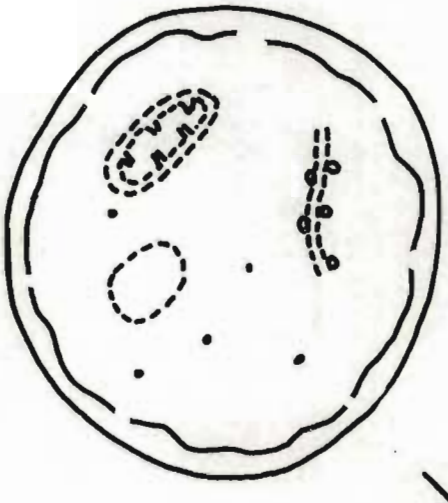


FIGURE 8

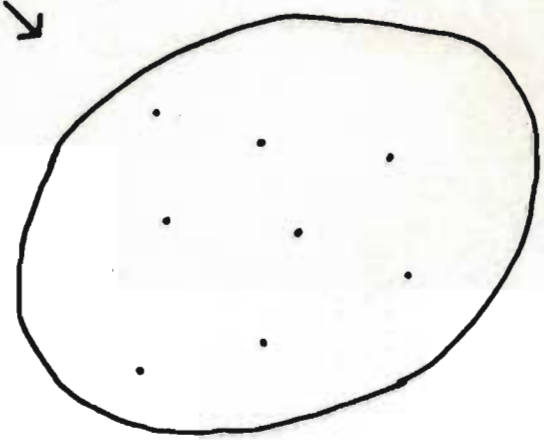
DIAGRAMMATIC REPRESENTATION OF CYTOLYSOME FORMATION AND DEVELOPMENT



ER elements surround a portion of cytoplasm



formation of discontinulties in the inner membrane precedes degradation of the cytoplasmic contents



vacuole

one, or the other, or both together.

3.3.1.2 *Formation of cytolysomes (Figure 8)*

Double-membrane bound vesicles which could be described as cytolysomes were occasionally found in the cytoplasm of both 12 and 48-hour imbibed root cap cells (Plates 49,50). The interior of such vesicles appeared to contain ground cytoplasm and various organelles such as mitochondria, ER elements, ribosomes and other unidentified vesicles. The surrounding double-membrane was ER-like, although the lumen appeared to have expanded in places. As such vesicles did not occur frequently their development could not be followed fully, but it appeared that the inner membrane became discontinuous, after which the cytoplasmic content was digested away (Plate 50), eventually leaving a single-membrane bound electron-translucent vesicle indistinguishable from any other vacuole. As the cytoplasmic contents occupied most of the volume of the vesicle it is felt that this phenomenon does not represent autophagic engulfment of cytoplasm by preformed lysosomal vacuoles, an example of which is presented in Plate 51.

3.3.1.3 *Vacuole formation through regional differentiation of the cytoplasm (Figure 9)*

This phenomenon was observed chiefly in differentiating and mature cells of the 48-hour imbibed maize root cap. Although by no means a common occurrence, it was observed in both the SA_4 and PNR_{44} varieties of Zea mays. In some caps it was not evident at all, while in others it appeared relatively frequently although not in every cell. The cells where it did occur did not appear unusual in any way and contained other apparently 'normal' looking vacuoles.

The process appeared to begin by a regional differentiation of a portion of the cytoplasm - that is, all organelles appeared to migrate away leaving a clear area of cytoplasm devoid of any organelles except perhaps ribosomes. The situation as regards ribosomes was not clear as the cytoplasm of cells in this state of differentiation did not appear to contain many ribosomes. In the initial stages of this process the originally-differentiated cytoplasm appeared identical in consistency and density with the surrounding non-differentiated cytoplasm. During the differentiation process the differentiating portion of cytoplasm became surrounded by numerous vesicles (Plates 52, 53). It should be pointed out that some of the micrographs presented here represent material which has been cytochemically treated in order to localise polysaccharide at ultrastructural level. As cytochemistry was carried out after sectioning of the embedded tissue (as opposed to in the block) it could not have had any effect on cellular ultrastructure prior to fixation. In any event this phenomenon was also observed in material which had not been cytochemically treated, and is therefore not thought to be an artefact of fixation. The origin of these vesicles is unknown, but they are suggested to be ER-derived. Certainly, although many dictyosomes were present in the region, these vesicles (Plate 52) in no way resembled the vesicles of dictyosomal origin. The vesicles appeared to expand, taking on the appearance of small irregular vacuoles (Plate 53) and to fuse (Plates 53, 54) until they completely encircled the differentiated portion of cytoplasm. The final appearance was that of a large, featureless portion of cytoplasm, encircled by two membranes with an apparently empty gap occurring between them. This gap varied in width, in some places appearing ER-like and in others being much wider and irregular in shape (Plate 54).

The fate of the inner membrane was not entirely clear, but by inference it will be assumed that it became discontinuous and, together with the enclosed cytoplasm, was digested away as many similar

vacuoles were observed within the cytoplasm with their contents in various stages of digestion.

In some cases the cytoplasm appeared to differentiate to a far greater extent before becoming membrane-bound. The differentiated cytoplasm appeared dilute and lacked density in comparison with the surrounding cytoplasm. At this stage only a few vesicles had come to surround the differentiated cytoplasm which largely remained unenclosed (Plate 55). Subsequent development was as described above, and appeared to culminate in the formation of an irregularly-shaped entire membrane surrounding the enclosed cytoplasm (Plate 56). The already diffuse cytoplasmic content appeared to be digested away until the vacuole appeared almost completely electron translucent (Plate 57) and similar to any other vacuole.

Pinocytosis

Occasionally apparently inward vesiculations of the plasmalemma into the cytoplasm were observed in 48-hour imbibed material. These vesicles on occasion appeared empty (Plate 32) but usually contained vesicular material similar to that found in the cytoplasm (Plate 58). These apparent pinocytotic vesicles appeared to break free of the plasmalemma to form independent cytoplasmic vesicles (Plate 59) indistinguishable from any other vesicle or vacuole found in the cytoplasm. It should be noted that it was difficult to tell whether such vesicles were formed by extension of the plasmalemma or whether in fact they were the result of fusion of a cytoplasmic vesicle or vacuole with the plasmalemma. In this regard the small vesicles found within the 'pinocytotic vesicles' appeared similar to others found in the cytoplasm, whereas similar vesicles were never observed within the cell wall or extra-protoplasmic space.

3.3.2 *Development of vacuoles*

3.3.2.1 *12-hour imbibed material. The meristem and surrounding regions.*

Plate 60 illustrates a group of cells typical of the region just distal to the meristematic zone. As not much difference in ultrastructure could be observed between the meristematic region and those cells immediately distal to it these two regions will be discussed together.

Many provacuoles varying both in size and electron opacity, could be seen within the tissue (Plate 61). The diameter of these provacuoles varied from 30 nm, or just larger than the width of the ER lumen, to approximately 824 nm. If the size distribution of the provacuoles was plotted (Table 1, Figure 10, overleaf) it appeared that the curve approximated very roughly the normal distribution curve; most provacuoles had diameters between 298 and 418 nm, whilst some were smaller and some larger. It should be noted that the plane of section may not pass through the largest diameter of a provacuole and that the figures may consequently be somewhat underestimated. Thus if most provauoles are formed by vesiculation of the ER, these provacuoles would appear to expand a great deal and accumulate within the cells in which they were formed. The smaller provacuoles which were not present in great numbers can be assumed to be newly formed. The larger ones appeared to have either undergone expansion or may have expanded whilst still attached to the ER and only recently have become independent organelles.

FIGURE 10

SIZE DISTRIBUTION OF PROVACUOLES WITHIN IMMEDIATELY POST-MERISTEMATIC CELLS

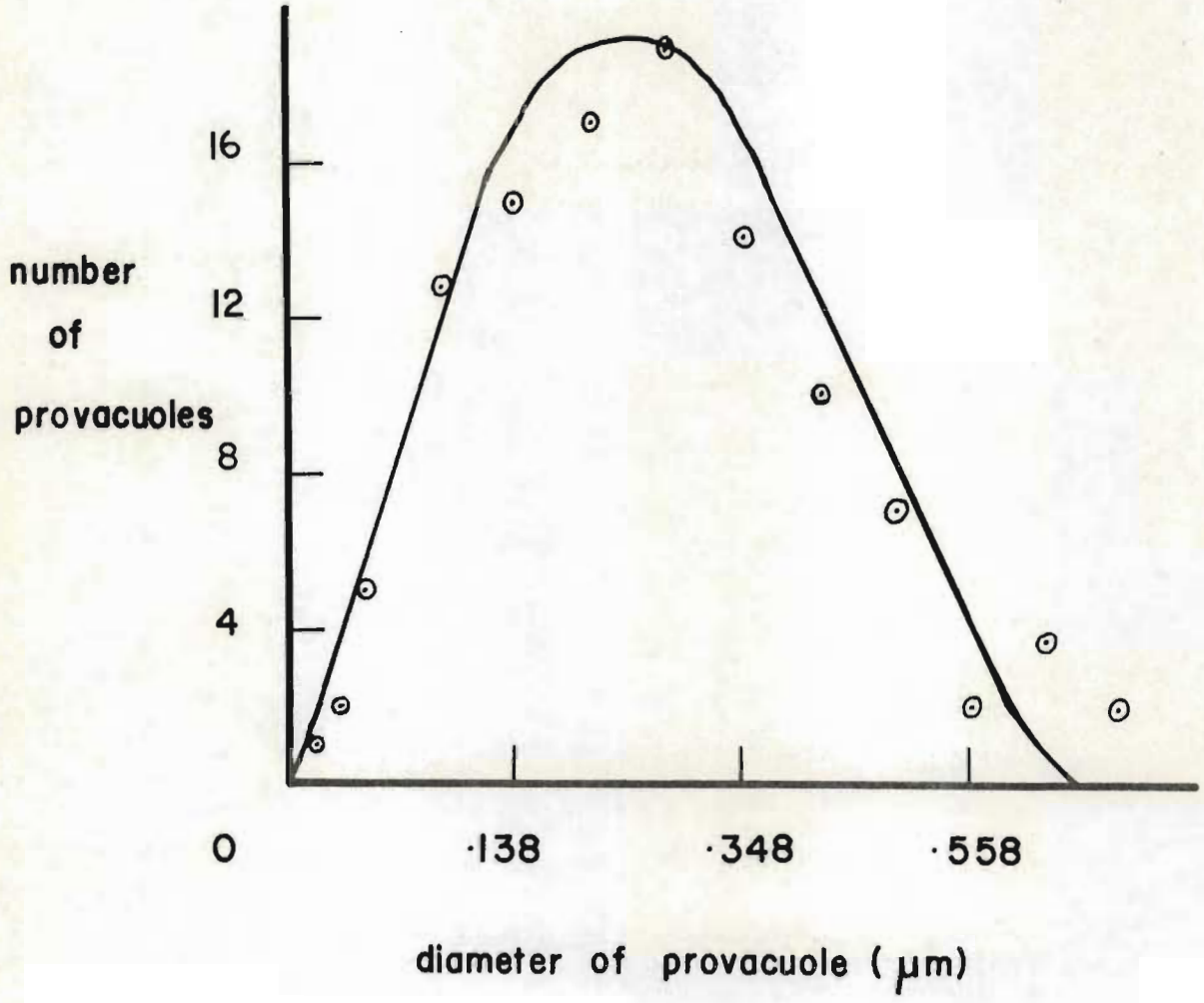


Table 1

SIZE DISTRIBUTION OF PROVACUOLES WITHIN IMMEDIATELY POST-MERISTEMATIC CELLS (See Figure 10).

Spectrum of provacuoles per cell cross section

Diameter of provacuoles (µm)	No. of provacuoles
0,030	1
0,040	2
0,068	5
0,138	13
0,208	15
0,278	17
0,348	19
0,418	14
0,488	10
0,558	7
0,628	2
0,698	4
0,768	2

ER Lumen diameter	0,024 µm
Mean diameter of provacuole	0,320 µm
Mean number of provacuoles per cell cross section	121

Assuming

1. The cell to be a rectangular box of dimension 9 750 × 9 750 × 15 250 nm.
2. The mean diameter of a provacuole is 320 nm. If the cell is divided into a number of 'slices', each 320 nm thick, any 50 nm thick section will cut through a representative sample of the provacuoles in the slice and noting that there were approximately 26 provacuoles per section per cell.

The number of provacuoles per cell would be given by

$$\frac{\text{Volume of cell}}{\text{Volume of 320 nm-thick slice}} \times 26$$

$$= \frac{9\ 750 \times 9\ 750 \times 15\ 250}{320 \times 9\ 750 \times 15\ 250} \times 26$$

$$= 790.$$

Thus there appeared to be approximately 790 provacuoles per cell in the region just distal to the meristem of the 12-hour imbibed maize embryo. Development of the provacuoles must be considered in terms of their apparent origin.

Single-membrane bound provacuoles

The interior of such a provacuole was once continuous with the lumen of the ER and contained an intensely electron-opaque matrix thought to have been manufactured by the ER (Plate 62). Owing to the intensely electron-opaque nature of the matrix, the bounding membranes tended to be obscured and were not always easy to distinguish. These dense organelles may initially have expanded as such, but after a certain point further expansion appeared to occur by a lifting away of

bounding membrane from the electron-opaque matrix, thus forming an electron-translucent 'halo' around it (Plate 62). Provacuoles of varying diameters appeared to undergo this type of expansion. It should be remembered that, owing to the plane of the section, the apparent diameter of a provacuole might be less than its actual diameter. It is felt, however, that this cannot entirely account for the apparent difference in size of the provacuoles at the stage when they begin expansion. It should also be remembered that in some cases such expansion appears to occur whilst the provacuole is still attached to the ER.

Double-membrane bound provacuoles

The space between the two membranes bounding such provacuoles was thought to correspond to the lumen of the ER, whilst the interior apparently contained cytoplasm and/or an electron-opaque matrix possibly secreted by the ER (see Plates 38 - 48). During development the space between the two ER-derived membranes appeared to expand (Plates 63, 64, 65, 66, 38) and an intensely electron-opaque matrix (analogous to that contained within the single-membrane bound provacuoles) developed within this space (Plates 38, 64). In some cases the central matrix also appeared intensely electron opaque (Plates 63, 66). It was difficult to follow the fate of the inner membrane as this was generally obscured by the electron-opacity of the vacuolar content, but as double-membrane bound vacuoles did not persist in more developed tissue it can be inferred that the inner membrane was broken down, resulting in the mixing of the contents of the two compartments it once separated. At this stage the provacuole would appear very similar to those provacuoles originally only bounded by a single membrane.

Association of provacuoles with the ER

Some provacuoles, both expanding and unexpanded, appeared to be secondarily encircled by one or more concentric layers of rough ER (Plates 63, 66, 67). There appeared to be a constant gap, similar in dimension to that of the ER lumen, between the encircling layers of ER and the vacuolar membrane. Although this phenomenon did not appear to occur in the case of the very small provacuoles (up to 100 nm in diameter) provacuoles of all larger sizes and states of expansion appeared involved in this association at some stage during their development. The association occurred only rarely in truly meristematic cells, being more characteristic of the zone of cells distal to the meristem.

The Golgi apparatus

The Golgi apparatus was not prominent in the zone of meristematic cells and took the form of isolated dictyosomes scattered in the cytoplasm. Such dictyosomes as were present consisted of 2 - 5 stacked cisternae and produced only very small vesicles, if any (Plate 45).

The zone of mature, non-dividing cells

The zone of mature, non-dividing cells occupied the major portion of the root cap of the 12-hour imbibed maize embryo and was situated distal to the meristematic region. Very few *un*expanded provacuoles were present within the mature cells, and where present appeared to be undergoing fusion and subsequent expansion (Plates 68, 69). In most cases the expansion of provacuoles which began in the region immediately distal to the meristem appeared to have continued. The expanded vacuoles varied in diameter from 1 800 nm to 7,2 µm, which is considerably larger than even the largest

provacuole (824 nm in diameter). The expanded vacuoles appeared largely electron-translucent, although they contained intensely electron-opaque material (reminiscent of that contained within provacuoles) in the form of dispersed globules associated with the vacuolar membrane (Plate 70). Although the expanded vacuoles did not appear to fuse to any great extent, considerable fusion of provacuoles must have occurred within the younger mature cells as calculations revealed that there were only an average of 240 expanded vacuoles per mature cell, as opposed to 790 provacuoles per immediately post-meristematic cell.

The expanded vacuoles of the mature cell appeared to autophagically engulf cytoplasmic vesicles of unknown origin (see Berjak, 1968). Additionally the larger vacuoles contained many membranous elements (possibly sheet-like ER) (Plate 71). In some cases the plasmalemma appeared to have shrunk away from the cell wall (Plate 72) and an accumulation of membranous material appeared within the extra-protoplasmic space.

The Golgi apparatus was not prominent in the mature cells and appeared as isolated dictyosomes, consisting of 2 - 4 cisternae, distributed within the cytoplasm. These dictyosomes showed little evidence of secretory activity.

The zone of senescing and dead cells

This zone occupied a narrow region at the extreme peripheral edge of the root cap of the 12-hour imbibed maize embryo. The senescence process appeared to occur very rapidly, as in many cases apparently normal mature cells were found adjacent to completely dead cells (Plate 70). In the senescing cell the expanded provacuoles appeared to have coalesced (Plate 73) to form a few much larger vacuoles. The membranes of the larger vacuoles were discontinuous,

resulting in the mixing of the vacuolar contents with the cytoplasm and its presumably consequent autolysis (Plate 74). The process culminated in the death of the cell, the dead cell containing only lipid droplets, a few ribosomes and the membranous remains of organelles (Plate 74).

3.3.2.2 *48-hour imbibed material*

After 48 hours of imbibation the embryo has commenced active growth, the first meristematic cell divisions having occurred by 48 hours after the start of imbibation. Development of the vacuoles proceeds in much the same way as for 12-hour imbibed embryo although certain differences and added features may be observed.

The Meristem

Vacuoles appeared to originate in the meristem chiefly by vesiculation of the ER in the manner previously described. However in 48-hour imbibed tissue the provacuoles remained small, and did not appear to accumulate in the enlarged form within the meristematic cells as was the case with 12-hour imbibed material. Plate 75 illustrates a typical meristematic cell containing many small ER-derived provacuoles and only a few larger ones (see also Plate 76). Expansion of the provacuoles by the lifting away of the vacuolar membrane from the electron-opaque content was not observed in *truly meristematic* cells of the 48-hour imbibed material and close association between the ER and provacuoles did not appear to occur *at all* in the root cap cells of 48-hour imbibed material at any stage of development.

It should be noted that the vesicles produced by the ER (Plate 77), at least some of which appeared to develop into provacuoles, were almost indistinguishable from dictyosomally-derived vesicles (Plate 78). In this connection it seemed that both

types of vesicle contributed to phragmoplast formation during cytokinesis. In newly divided cells microtubules could be observed radiating out into the cytoplasm at rightangles to the forming phragmoplast (Plate 79). Small vesicles, presumably having their origin from both the dictyosomes and the ER, appeared to be guided by the microtubules towards the phragmoplast, where they coalesced to form larger vesicles and eventually the cell plate and plasmalemma.

In osmium tetroxide post-fixed material lipid droplets (spherosomes?) could be distinguished from provacuoles on the grounds that they were fairly uniform in size, less electron-opaque than provacuoles and their contents appeared 'smooth' and not very granular (Plate 76); their bounding membranes also were usually indistinct. It appeared that these organelles were produced as vesiculations of the ER which subsequently separated from the ER and became independent (Plates 80, 81). The situation as regards membranes was not clear but initially it would appear that the lipid droplets were single-membrane bound. Observations on potassium permanganate post-fixed material, (in which the lipid content was etched away, leaving only a shrunken outline), tended to confirm the above (Plates 82, 83). The membranes surrounding such lipid droplets on occasion appeared 'double'. This could be due to the separation of the inner and outer layers of a single bounding membrane from one another.

The zone of differentiation

The zone of differentiation was situated distal to the meristem. Its cells were characterised by the presence of a number of amyloplasts containing large starch grains. The amyloplasts were usually situated at the distal end of the cell, whilst the vacuoles seemed to occupy the proximal portion (Plate 84).

Very few compact provacuoles were present within the

differentiating cells, which appeared however to contain many newly expanded provacuoles (Plate 85). The bounding membranes of the newly expanded provacuoles appeared to have lifted away from the electron-opaque matrix, which still however featured prominently within the vacuole. Expansion appeared to continue until the vacuole reached a diameter of approximately 450 nm, which is ten times larger than the diameter of the average unexpanded provacuole. At this stage the electron-opaque matter did not occupy a significant volume of the vacuole and was usually associated with the vacuolar membrane. Ribosomes did not appear to be associated with the outer surface of the vacuolar membrane. Fusion of the expanded lysosomal vacuoles appeared to begin with the vacuoles forming localised protrusions (reminiscent of amoeboid pseudopodia) which interlocked with similar protrusions on neighbouring vacuoles (Plate 86). This process appeared to culminate in the formation of a sphere of interlocking vacuoles almost completely surrounding the nucleus of the cell (Plate 87).

The Golgi apparatus took the form of isolated dictyosomes, consisting of 3 - 5 cisternae, distributed throughout the cytoplasm (Plate 88). Some of the small secretory vesicles produced by the dictyosomes were autophagically engulfed by the vacuoles, although this phenomenon was not marked.

The zone of mature cells

The zone of mature cells occurred distal to the zone of differentiation, approximately in the centre of the root cap. The expansion and fusion of vacuoles which began in the differentiating zone continued in the mature zone. Vacuolar fusion was especially pronounced - fusing vacuoles appeared to interlock much more deeply and the vacuolar membranes in the interlocking region appeared in a state of dissolution (Plates 89, 90). As fusion progressed these membranes disappeared completely (Plate 91). At this stage there

was continuity between the interiors of both vacuoles, and the region of fusion was marked merely by a constriction in the fused vacuoles (Plate 92).

The dictyosomes of the mature cells appeared more active in a secretory capacity than those of the differentiating cells. In this regard they produced many vesicles slighly larger in size than those produced within the differentiating cells. Dictyosomes were often seen in close proximity to the vacuoles (Plates 93, 94, 95) and their vesicles appeared to be autophagically engulfed by the vacuoles. In this regard the vacuolar membrane appeared to invaginate around the vesicle and subsequently to pinch it off into the interior of the vacuole (Plate 96). At this stage the vesicle (or vesicles) were surrounded by a portion of the tonoplast (Plate 95) which then disintegrated (Plate 97), leaving them free within the vacuole (Plate 98). Later still the membranes of these vesicles appeared to disintegrate resulting in a mingling of their contents with those of the vacuole. In this regard it has been established that these vacuoles contain hydrolytic enzymes (typified by acid phosphatase) (Berjak, 1968) and may thus be described as lysosomal vacuoles. It would appear that the degeneration of the dictyosomal vesicles within the vacuole is a result of the action of vacuolar hydrolases.

The zone of senescing cells

Cell degeneration was initiated about three cells from the outermost edge of the cap. However the early signs of cellular senescence are subtle (Plate 99), but degeneration proceeds apace, these cells soon being autolysed and sloughed off at the cap surface. Plate 99 illustrates a portion of a cell typical of the proximal region of this zone. Expansion and fusion of vacuoles appeared to continue (Plate 100). In addition the production of provacuoles appeared to begin afresh (Plate 99). The newly formed provacuoles expanded, as

described previously, and fused not only with one another but also with the much expanded vacuoles already present within the tissue (Plate 101). Although it was suspected that these newly-formed provacuoles might in fact be microbodies, their enzymological characteristics categorise them as provacuoles (Berjak, personal communication) (refer also to Chapter 4: Discussion).

The dictyosomes of the senescing cells underwent a marked change and began to produce many large secretory vesicles: such dictyosomes are known as hypersecretory. They appeared to consist of 4 - 6 cisternae and the forming and maturing faces situated at opposite ends of the stack of cisternae, could be clearly discerned. Vesicle size appeared to increase progressively across the stack from the forming to the maturing face where the cisternae and associated secretory vesicles appeared to separate from the stack and move into the cytoplasm, (Plate 102). Once free in the cytoplasm the secretory vesicles appeared to separate from the cisternae and follow one of two paths.

1. The major path by far leads towards the plasmalemma, with which the vesicles apparently fused, thereby ejecting their contents into the extra-protoplasmic space (between the plasmalemma and cell wall) (Plate 103). The membrane of the vesicle then becomes part of the plasmalemma which took on a convoluted appearance owing to the increase in its surface area (Plate 104). The contents of the secretory vesicles built up within the extra-protoplasmic space and, in some cases, depending on the intensity of post-staining, appeared as an accumulation of pockets of electron-opaque material approximately the size of the secretory vesicles (Plates 105, 106). Once build-up had reached a certain level the secretion apparently penetrated the cell wall and accumulated in the middle-lamella region,

thus aiding in the separation of the cells from one another.

Varietal differences in this process tended to occur. In the case of var. SA_4 secretory vesicles occasionally appeared not to fuse with the plasmalemma, but were passed through the plasmalemma by apparent reverse pinocytosis (Plate 107), after which they accumulated within the extra-protoplasmic space (Plate 99).

2. The dictyosomally-derived vesicles also appeared to move in the direction of the vacuoles, where they were autophagically engulfed (Plates 108,109). This process was far more pronounced than in the cells of the mature zone.

Autophagy

The lysosomal vacuoles of the senescing cells exhibited a great deal of autophagic activity apart from the engulfment of dictyosomally-derived vesicles. Whole dictyosomes, mitochondria (Plate 110), ER (Plate 111), plastids, lipid droplets (Plate 111), small vacuoles and membranous whorls (possibly sheet-like ER) (Plate 112), in damaged and apparently undamaged states, were autophagically engulfed. These organelles subsequently appeared within the vacuole in various stages of degradation (Plates 113, 114, 115, 116). In some cases (e.g. Plate 110) the organelle being engulfed appears to be deformed, the deformed region protruding into the vacuole. This may be indicative of the active participation of the organelle in its own engulfment (see e.g. Villiers, 1971).

Note: As has been stated previously, the zones into which the root cap has been divided are artificial, the development of the root cap being a continuous process with no really sharp transition regions. It is therefore a moot point as to when a cell can actually

be described as having begun senescence or degeneration. The cells described above as being senescing, could equally well be thought of as being at the peak of maturity. Development of the root cap, however, is aimed at the final destruction of the cells as they reach the periphery of the cap, where they are sloughed. In this regard the zone described here as senescing, has begun to show the distinctive changes geared specifically towards the final destruction of the cytoplasm and sloughing of the cell. These are:

1. Secretion of dictyosomally-derived polysaccharide into the extra-protoplasmic space and ultimately the intramural region.
2. Autophagy, which can be thought of as minimising the actual events of final autolysis and implies the redundancy of many cellular components no longer necessary for metabolism.
3. A high degree of vacuolar swelling prior to membrane dissolution and the onset of autolysis, or senescence proper.

Autolysis and cell death

The zone of autolysing and dying cells was situated at the periphery of the root cap. Such cells generally contained only one large vacuole (Plate 117) or a number of smaller vacuoles all interconnected through fusion which probably occurred during maturation (Plate 118). The tonoplast appeared to become discontinuous, resulting in a mixing of the vacuolar contents with the cytoplasm and apparent autolysis of the cytoplasm (Plate 119). Cytoplasmic detail became indistinct as the constituent organelles were digested away. Still later the plasmalemma appeared to become discontinuous, exposing the extra-protoplasmic portion of the cell to the vacuolar contents. Cell wall and middle-lamella degeneration combined with the accumulation of dictyosomally-derived secretion between the cells apparently caused the autolysing and autolysed cells to slough off from the cap (Plate 117).

3.4 CYTOCHEMICAL LOCALIZATION OF ACID PHOSPHATASE

The experimental samples subjected to the procedure of Gomori (1952) for the localization of acid phosphatase gave a reaction clearly different from that observed in the controls, in which sodium fluoride was present as an enzyme inhibitor (Plates 120, 121).

12-hour imbibed tissue

The cell walls, nuclei and provacuoles of the meristematic cells contained dense, intensely electron-opaque lead phosphate deposits, the morphology of which appeared to vary according to the subcellular localization (Plates 120, 122). Large needle-like crystals were associated with the nuclei, whereas the lead phosphate over the provacuoles and cell walls appeared as finely crystalline clumps. (it should be noted that the apparent nuclear reaction is generally regarded as an artefact (see Chapter 4, discussion)). Reaction product did not appear to be associated with every provacuole, and where the association did occur only a portion of the electron-opaque region appeared to give a positive reaction (Plates 123, 124). In view of this it is possible that at least some of the provacuoles which did not appear to react did in fact contain reaction product which was not situated in the plane of the section. Reaction product did not appear confined to provacuoles of any one size (and thus stage of development). The association of acid phosphatase would appear to confirm the lysosomal nature of the provacuoles, and thus justify the use of the term lysosomal in this connection.

There was no apparent change in the distribution of acid phosphatase activity in the mature and senescing cells towards the periphery of the root cap. Cell walls, provacuoles and expanded vacuoles appeared to account for most of the acid phosphatase activity,

which occasionally also appeared associated with the mitochondrial and plastid membranes and the cytoplasm (Plates 125, 126). The apparent association of acid phosphatase activity with the mitochondrial and plastid membranes in the very mature and senescing zones may represent a preparation for organelle self-destruction; on the other hand it may be an artefact of preparation (see Chapter 4, for further discussion). The vacuolar membranes of the senescing cells appeared to become discontinuous with the result that acid phosphatase activity (of presumably vacuolar origin) was associated with most organelles in a dispersed, granular reaction. Most organelles, including the cell wall, appeared in a state of disorganization and breakdown, with lead phosphate crystals being associated with the regions undergoing dissolution (Plate 127). In completely autolysed cells acid phosphatase activity was associated with the dispersed membranes and confluent lipid droplets which were all that remained of the cytoplasm.

48-hour imbibed tissue

The meristematic cells of 48-hour imbibed tissue showed very little acid phosphatase activity when compared with those of 12-hour imbibed tissue. Plate 128 shows deposits of lead phosphate at cytoplasmic sites thought to be provacuoles. Such findings are in agreement with the results presented in the previous section, viz. that relatively large, compact provacuoles do not appear to accumulate to any great extent in the meristem of the 48-hour imbibed root cap. No acid phosphatase activity was observed in the control material (Plate 129).

Needle-like and granular crystals of lead phosphate were associated with the expanding vacuoles of the differentiating and mature cells. Most of the vacuolar activity appeared associated with the vacuolar membrane and electron-opaque material present within the

vacuole (Plates 130, 131, 132). Such activity appeared absent from the control material (Plate 133). The dictyosomes at this stage did not give a positive acid phosphatase reaction except where vacuolar membranes had become discontinuous, releasing the vacuolar contents into the cytoplasm (Plate 134). Heavy lead phosphate deposits (absent from the controls) were associated with the vacuoles of the senescing cell (Plates 135, 137). Material autophagically engulfed into the vacuole and in an apparent state of breakdown gave strongly positive reaction (Plate 136). The question of the association of acid phosphatase activity with the vesicles of the hypersecretory dictyosomes was difficult to assess. It is clear, however, that reaction product was never associated with the contents of the dictyosomal vesicles.

Intensely electron-opaque lipid deposits, smaller than the conventional lipid droplets or spherosomes, appeared associated with most cytoplasmic membranes (particularly the dictyosomal and vacuolar membranes and the plasmalemma) in the senescing cell. This phenomenon could be observed in material *not* subjected to any cytochemical procedure (Plate 103), material subjected to the cytochemical reaction for acid phosphatase (Plate 138) and also the control material in which sodium fluoride was used as an enzyme inhibitor (Plate 139). It would thus appear that this material was not a positive reaction product of the cytochemical method employed. According to Berjak (personal communication) these lipid-like deposits are probably representative of localised sites of membrane degradation, and are unlikely to be mediated by vacuolar acid phosphatase in the non-autolysing situation. As the cell is programmed to die they could thus be described as *pre-autolytic* but *senescent* changes. In this regard Berjak (1968) has reported a massive increase in protein synthesis in early senescence; she is of the opinion that hydrolases of non-vacuolar origin, also manufactured at this stage, may be responsible for localised membrane degradation.

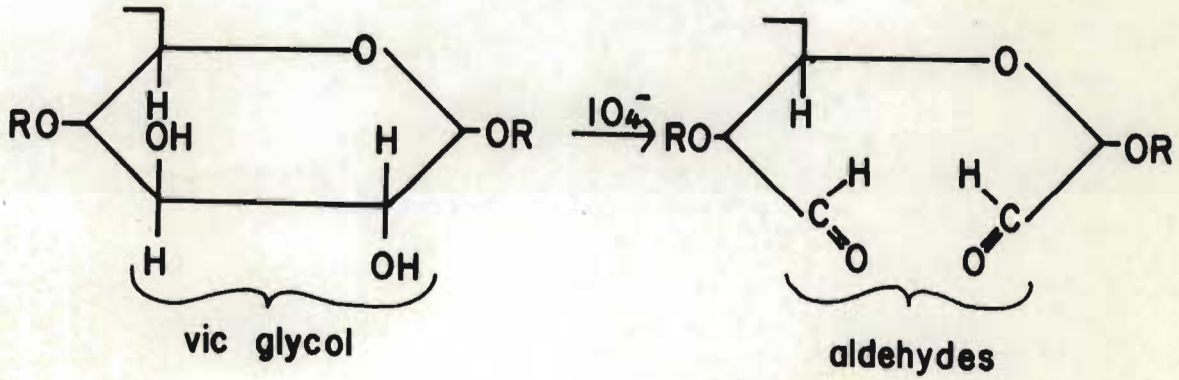
However these deposits appeared far more intensely electron-opaque in experimental than in control and untreated material, and in a few instances took on a crystalline appearance similar to that of lead phosphate deposits found in the vacuoles (Plates 140, 141, 142). On these grounds it is possible that they may indeed have given a positive reaction for acid phosphatase. The picture is further complicated by the fact that, although the tonoplast visible in the plane of section is apparently intact, vacuolar discontinuity may well have occurred out of the plane of section. Thus any cytoplasmic reaction (for acid phosphatase) could still be due to the vacuolar origin of the hydrolase.

In the final stages of senescence the vacuolar membranes became discontinuous, releasing the contents of the vacuole into the cytoplasm. Concentrated deposits of lead phosphate appeared throughout the cytoplasm, which was in a generally degraded state, and were associated with almost every organelle (Plate 148).

3.5 CYTOCHEMICAL LOCALIZATION OF POLYSACCHARIDE

Those carbohydrate molecules which are oxidised by periodic acid may be detected in hisotological sections by the periodic acid Schiff technique. Histological localization of polysaccharide however, lacks precision as organelles as small as dictyosomes cannot be resolved with light optics. It would therefore appear desirable to locate the site of periodic acid-reactive carbohydrates in the electron microscope. In this regard alkaline silver solutions have replaced the Schiff reagent as an electron stain so as to detect aldehyde groups produced by periodic acid oxidation. van Heydingen (1965), applying such a technique to formalin-fixed thyroid tissue, observed that cellular constituents which were stained with the Schiff reagent in the light microscope were also stained with silver methenamine in the electron microscope.

A variety of methods operating on this principle have been developed to localise polysaccharide at ultrastructural level. All methods involve the oxidation of vic-glycols (1,2-glycols) (contained within carbohydrates) to aldehydes with periodic acid.



Subsequent to periodic acid-oxidation, the sections may be treated in a number of ways to effect final staining.

1. The most direct method appears to involve the treatment of the section with an alkaline silver methenamine solution, (Rambourg, 1967) probably resulting in the binding of silver moieties to the freed aldehyde groups.
2. In other cases, sections may first be treated with either thiocarbohydrazide or thiosemcarbazide (e.g. Thiery, 1967). This is thought to result in the reaction of the thiocarbohydrazide or thiosemicarbazide groups with aldehydes produced by periodic acid-oxidation. Subsequent to this the section is usually treated with silver proteinate, the silver moieties then binding to the thio-groups and forming an end-product visible in the electron microscope.

Although the basic result is similar in most cases, many variations of the above techniques have been tried, all yielding somewhat differing results. Variations appear to occur in the specificity of the reaction, the texture of the staining (coarse or fine), and the degree to which polysaccharides of differing complexities and compositions are stained. Variations appear to be related not only to the particular chemical system used to effect staining, but also to the duration of staining and the temperature at which it was allowed to occur.

In the context of the present investigation it was necessary to find a repeatable method by which polysaccharide of dictyosomal origin could be stained with a minimum of side-reactions and background silver deposition. In addition it was desirable to be able to visualise not only the polysaccharide, but also ultrastructural detail, in order to be able to follow the fate of the polysaccharide

at subcellular level. To this end, the following variations of the method were tested:

1. The original Thiéry method, periodic acid, thiosemicarbazide - silver proteinate (Section 3.5.1.1).
2. The effect of alterations in temperature and duration of incubation on the staining reaction obtained with the above method (Section 3.5.1.2).
3. The effect of treatment with thiocarbohydrazide (in place of thiosemicarbacide) on staining intensity i.e. periodic acid - thiocarbohydrazide - silver proteinate (Section 3.5.1.3).
4. The reaction obtained with periodic acid - silver methenamine. (The effect of alterations in temperature and silver methenamine concentration were also tested here). (Section 3.5.1.4).
5. The reaction obtained with the periodic acid - thiocarbohydrazide - silver methenamine system at differing temperatures of incubation (Section 3.5.2).

To check the extent to which post-fixation with either osmium tetroxide or potassium permanganate interfered with the staining reaction, cytochemistry was carried out on material which had been post-fixed as well as material which had not. In the latter case, ultrastructural detail was not at all clear. Material was post-stained with lead citrate both before and after cytochemistry to check whether or not this clarified substructural detail.

3.5.1 *Preliminary investigations*

3.5.1.1 *The Thiéry method (see Section 2.6.1.1)*

The starch grains of the amyloplasts appeared to stain most intensely, the silver deposits being finely granular and localised chiefly to the periphery of the grains (Plate 144). Such deposits were not observed in the controls, which were not oxidised with periodic acid (Plate 145). The cell walls reacted to a lesser degree, appearing more electron-opaque than the surrounding cytoplasm and giving a faint indication of granularity (Plate 146). In general, staining could not be detected over the dictyosomal vesicles, even where the dictyosomes exhibited hypersecretory activity. In a few instances, however, isolated vesicles originating from the hypersecretory dictyosoms stained relatively intensely, as did the accumulated dictyosomal secretion within the extra-protoplasmic space (Plate 147). It was difficult to distinguish cytoplasmic detail as, except in rare instances, cytoplasmic membranes appeared unstained (Plates 146, 148). In this regard the vacuoles could not be distinguished at all. Background cytoplasmic stain was apparent, as well as apparently random deposition of small silver grains over the whole section (Plate 149).

3.5.1.2 *The effect of alterations in temperature and duration of incubation on staining intensity (see Section 2.6.1.2)*

Except for the harshest treatment, none of the treatments to which the tissue was subjected produced a staining reaction clearly different from that produced by any other treatment, including the original Thiéry method. The most intense staining was observed in sections which had been oxidised with periodic acid for 40 minutes, treated with thiosemicarbazide for six days and stained with silver proteinate for ten hours at room temperature or one hour at 60°C (the harshest treatment). The most significant difference was that

the vesicles of the hypersecretory dictyosomes appeared to react to a far greater degree than the surrounding cytoplasm, as did the dictyosomal secretion accumulated within the extra-protoplasmic space (Plate 150). Despite the fact that sections were post-stained with lead citrate prior to cytochemistry the resolution of cytoplasmic detail was scarcely greater than if this had not been the case although on occasion vacuoles could be distinguished (Plate 151). Concomitant with the overall increased staining intensity came an increase in general cytoplasmic staining, imparting to the sections a dingy, messy appearance (Plate 150). *Random* background silver deposition did not appear to increase significantly with harsher treatment (Plate 156).

3.5.1.3 *The effect of thiocarbohydrazide on staining intensity (see Section 2.6.1.3)*

Treatment of sections with thiocarbohydrazide in place of thiosemicarbazide produced a clearly more intense staining reaction. Plastids, cell walls, dictyosomal vesicles and secretion of dictyosomal origin gave an intense, finely granular reaction (Plates 152, 153, 154). The cytoplasm appeared almost completely unstained and resolution of cytoplasmic detail was poor, but the sections were completely free of random background deposition of silver.

3.5.1.4 *Silver methenamine as a staining reagent (see section 2.6.1.4)*

Treatment of sections with silver methenamine for 30 minutes at 60°C following periodate oxidation produced a very intense staining reaction. The reaction was far more intense and coarsely granular than that obtained by treatment with thiocarbohydrazide and silver proteinate instead of silver methenamine. Staining was again localised to the plastids, dictyosomes, extra-protoplasmic dictyosomal secretion and cell walls (Plate 155). In some cases it was possible to distinguish the apparent engulfment of dictyosomal vesicles by the vacuoles (Plates 156, 157). Resolution of cytoplasmic detail was poor, and background

deposition of silver was heavy.

Staining was even more intense in sections treated with silver methenamine for 60 minutes at 60°C. No added cytoplasmic detail could be distinguished, in fact extremely heavy deposition of silver over the cytoplasm tended to obscure any cytoplasmic detail which may have been visible (Plates 158, 159).

Staining with silver methanamine for 30 minutes at the lower but still elevated temperature of 40°C did not lessen the heavy cytoplasmic and background deposition of silver (Plate 160, compare Plate 157).

The quality of staining produced by use of the more dilute silver methenamine solution of de Martino and Zamboni (1967) as a staining reagent was not noticeably different from that obtained using the standard (Rambourg, 1967) (Plate 161).

3.5.2 *The final series of experiments (see Section 2.6.2)*

All experiments conducted (see Section 2.6.2) appeared to give satisfactory results. Post-staining with lead citrate after cytochemistry served to improve the resolution of cytoplasmic detail (Plate 162) and did not appear to alter the staining reaction. The quality of staining (3.5.2.1) obtained using the different methods will be reported for sections not post-stained with lead citrate. However, for clarity, ultrastructural observations (3.5.3) will be made where possible, from post-stained sections.

3.5.2.1 *Quality of staining*

Use of silver proteinate as a staining reagent after periodate oxidation and treatment with thiocarbohydrazide

These results are reported in Section 3.5.1.3. It should be further noted that treatment with silver proteinate at 40°C did not produce a reaction noticeably different from that obtained when silver proteinate was used at room temperature (Plate 163). Post-staining of sections with lead citrate, which allowed the visualization of cytoplasmic detail, revealed that the lipid droplets gave an intense positive reaction comparable to that of the plastids and far darker than that over the dictyosomal vesicles (Plate 171).

Use of silver methenamine as a staining reagent after periodate oxidation and treatment with thiocarbohydrazide

Staining of sections with silver methenamine at room temperature after pretreatment with thiocarbohydrazide produced a finely granular staining of polysaccharide comparable to that obtained when silver proteinate was used as a stain under similar conditions. In both cases a finely granular electron-opaque matrix appeared over the polysaccharidic portions of the cytoplasm and the lipid droplets. The plastids stained more intensely than did polysaccharide of dictyosomal origin or cell wall material (Plates 164 - 167). Specific staining in sections treated with silver methenamine appeared slightly more intense than was the case with silver proteinate (Plates 166, 167). In both cases the cytoplasm was lightly stained with little random background deposit: however, on occasion, heavy deposits of silver appeared to precipitate randomly over the cytoplasm of silver methenamine treated sections (Plate 168).

Staining with silver methenamine at the elevated temperature of 40°C even after treatment with thiocarbohydrazide resulted in the

precipitation of heavy flocculent deposits of silver over the cytoplasm, thus obscuring cytoplasmic detail (plate 169).

Controls

1. To check the extent to which post-fixation in osmium tetroxide, a powerful oxidising agent, affected the staining reaction, material was fixed in glutaraldehyde alone and then subjected to the cytochemical procedure. The pattern of staining was entirely similar to that obtained with post-fixed material, with the exception that the lipid droplets, which stained intensely in post-fixed material, did not appear stained and were in fact difficult to distinguish at all in non post-fixed material (Plates 170, 171, 172). Fixation of material which had not been post-fixed was, however, inferior to that obtained with a combination of glutaraldehyde and osmium tetroxide as fixatives. In this regard, in non post-fixed material, the stained material within the dictyosomal vesicles appeared to have shrunken into the centre of the vesicle and was surrounded by an electron-translucent halo (Plates 173, 174). Such shrinkage also occurred with the extra-protoplasmic material of dictyosomal origin. In material not post-fixed in osmium tetroxide, cytoplasmic detail was difficult to distinguish even when the sections were post-stained with lead citrate.

2. Cytochemistry was carried out on osmium tetroxide post-fixed material, but oxidation of the sections with periodic acid was omitted. No specific staining reaction comparable to that obtained with oxidised material was observed in these controls (Plates 175, 176).

3.5.3 *Ultrastructural observations*

Ultrastructural observations were made on osmium tetroxide post-fixed material which had been

(a) oxidised with periodic acid,
(b) treated with thiocarbohydrazide,
(c) stained with either silver proteinate or silver methenamine,
(d) post-stained with lead citrate,

as this procedure appeared to give the best results (see Section 2.6.2).

3.5.3.1 *12-hour imbibed material*

Apart from intense staining of the lipid droplets (Plate 177) the only organelles to react with any specificity were the cell walls (Plate 178). The dictyosomes of 12-hour imbibed material produced very small vesicles which did not give a positive reaction (Plate 178). Neither the provacuoles nor the expanded vacuoles contained silver deposits over and above those seen in the controls (Plates 179, 180).

3.5.3.2 *48-hour imbibed material*

The meristem

Apart from intense staining of lipid droplets the only other regions to react were the cell walls and dictyosomal vesicles. Staining over the dictyosomal vesicles was not heavy and appeared very finely granular and similar to that over the wall (Plate 181, 182). In cases where a phragmoplast was being formed, the ER and dictyosomally derived vesicles contributing to the forming phragmoplast gave a positive reaction similar to that of the phragmoplast and adjacent cell wall (Plate 183).

Cytoplasmic detail was not clear in material not post-stained with lead citrate, and very small, less intense, deposits of silver were not readily visible. Very heavy or large deposits of silver could be discerned and as they tended to take on the shape of the polysaccharide-containing organelle, the organelle could also be identified. Thus although lead citrate brought up contrast in the cytoplasm and might be said to confuse the picture as regards the cytochemical reaction, its use was essential when the staining characteristics of small localised regions of the cytoplasm (e.g. dictyosomal vesicles) were under investigation.

No specific granular staining was observed in the unoxidised controls (Plate 184).

The differentiating and mature zones

The pattern of staining in these regions was similar to that observed over the meristem, the notable exception being the amyloplasts, the starch grains of which gave an intensely positive reaction (Plate 185). As was the case in the meristem the dictyosomal vesicles gave a positive reaction (Plate 186). Although incorporation of dictyosomal vesicles into vacuoles is known to occur, especially in the mature cells, the vacuoles did not appear to contain positively stained material similar to that contained within the vesicles. The vacuoles did, however, contain electron-opaque material dispersed within them. However, similar material was observed in sections not cytochemically treated, as well as in the unoxidised controls, and thus could not be identified as polysaccharide (Plates 187, 188).

The zone of senescence

A marked change in the pattern of polysaccharide metabolism appeared to occur in the senescing cells of the 48-hour imbibed root

cap. The dictyosomes at this stage were hypersecretory and produced many large vesicles which separated from the mature face and dispersed into the cytoplasm. Although the vesicles in some cases appeared to increase in size as they progressed from the forming towards the mature face of the dictyosome the quality of staining did not appear to change much (Plate 189). In some cases staining of the mature vesicles appeared more intense, which perhaps indicates an increase in concentration of polysaccharide or a change in the nature of the polysaccharide during maturation across the stack (Plate 190). Once the vesicles were free in the cytoplasm staining appeared in some instances to intensify further (Plate 191) until it resembled the reaction of the extra-protoplasmic slime.

The dictyosomal vesicles apparently followed one of two paths:

1. Either:
   They fused with the plasmalemma, secreting their contents into the extra-protoplasmic space (Plates 192, 193, 194). This secretion was observed to build up within the extra-protoplasmic space until it occupied a large proportion of the cell volume (Plates 195, 196, 197) after which it appeared to penetrate the cell wall and accumulate in the middle lamella region (Plate 198) and finally at the exterior of the root cap (Plates 199, 200).
2. Or:
   The dictyosomal vesicles were autophagically engulfed by the vacuoles. In this connection positively stained dictyosomal vesicles could be seen closely apposed to the vacuolar membrane and in various stages of engulfment (Plates 201, 202, 203, 204). Thus polysaccharide of dictyosomal origin apparently entered the vacuole in substantial quantities.

Lipid droplets, which react more strongly than the dictyosomal vesicles, also appeared to be engulfed by the vacuoles (Plate 205).

The situation as regards the accumulation of positively stained material (polysaccahride) within the vacuoles was confused. In osmium tetroxide post-fixed material stained aggregates were visible within the vacuole, the quality of the staining being similar to that encountered within the dictyosomal vesicles (Plates 206, 207). However, similar aggregates appeared in the unoxidised controls (Plate 209), and coarsely-granular aggregates were evident in material not cytochemically treated (Plate 208). It cannot therefore be said conclusively that this material *is* polysaccaharide in nature. Material not post-fixed in osmium textroxide but cytochemically treated showed aggregates of stained material within the vacuoles and in this case the only heavy metal to come into contact, as it were, with the tissue was silver. Staining here was not intense, but differed a little from cytoplasmic staining (Plate 210). Although the background staining may be a function only of the polysaccharide groups within the cytoplasm, the silver may also act as a general cytoplasmic stain, similar to lead citrate. Thus it is possible that the stained aggregates within the vacuoles contain polysaccharide, but they may equally well consist of other chemical species. Both dictyosomal vesicles and what appeared to be lipid droplets were observed within the vacuoles in various stages of degradation (Plates 205, 211). In the early stages of degradation the membranes of the dictyosomal vesicles appeared to have been hydrolysed with the result that the contents of the vesicles were exposed to the action of the vacuolar enzymes. Initially the positively stained contents of the vesicles were visible (Plate 211), but they appeared to disperse rapidly. Careful examination at this later stage revealed a finely granular electron-opaque matrix filling the whole vacuole (Plate 212). This could represent a dilute polysaccharide matrix stemming from dispersion of the more concentrated polysaccharide once contained within the dictyosomal vesicles.

3.6 AUTORADIOGRAPHY

3.6.1 *Light microscope autoradiography*

The following measurements and estimates were made for each of the three experiments carried out (see Sections 2.7.11, 2.7.13, 2.7.1.4):

1. The number of grains per cell (excluding the cell wall and material accumulated outside the plasmalemma) were counted for meristematic, mature and senescing cells over five sections and averaged. Owing to the limited resolution obtainable with thelight microscope silver grains could not be localised to any organelle but the vacuole. Bearing in mind the approximate doubling in size as the cell progresses from the meristematic towards the senescing region, it may have been pertinent to measure grains per unit area as opposed to grains per cell. The advantage of the latter measurement is that it permits a direct comparison of the level of labelling in the different cell zones.
2. The extent of wall labelling was visually estimated on a scale of 1 - 6 to show the general trend in this regard. The level of accumulation of labelled material in the extra-protoplasmic space was estimated in the same way.

Incorporation of tritiated fucose into the root cap (see Section 2.7.1.1)

48-hour imbibed maize roots were incubated in L-(^{3}H) fucose for periods of time increasing from 5 minutes to 12 hours. The following were plotted against duration of incubation for the meristematic and/or

senescing cells (Table 2, Figures 11 and 12, overleaf).

1. grains per cell,
2. estimated level of wall labelling, and
3. estimated level of accumulation of label in the extra-protoplasmic space.

Labelling over the meristematic cells did not reach a high level up to the first 20 minutes of incubation, but increased markedly after 1 hour of incubation (Figure 11). At this stage not much label appeared to have been incorporated into the cell wall (Plates 213 - 216). Over the following 11 hours the level of cellular incorporation in the meristem appeared to increase only slightly, however a large amount of label had accumulated over the walls by this stage (Plates 217, 218).

The pattern and intensity of labelling over the senescing cells was similar to that over the meristematic region up to the first 3 hours of incubation (Plates 219 - 222). During the next 9 hours, however, incorporation rose sharply, approximately doubling itself (Plates 223, 224). Label appeared to accumulate over the vacuoles, in the cytoplasm around the periphery of the vacuoles, and as clumps (probably collections of dictyosomes)in the latter two cases adjacent to the plasmalemma (Plates 223 - 228).

The pattern of incorporation of label into the cell walls and extra-protoplasmic space of the senescing cells appeared to follow the trend observed for cellular incorporation, also reaching a maximum at the end of the incubation period (Figure 12). For shorter durations of incubation it appeared that label accumulated within the extra protoplasmic space more quickly than it did over the cell walls (Plates 229, 230).

FIGURE 12

RELATIVE LEVELS OF INCORPORATION OF (^{3}H)-FUCOSE INTO THE SENESCING ZONE OF THE ROOT CAP

(see table 2B)

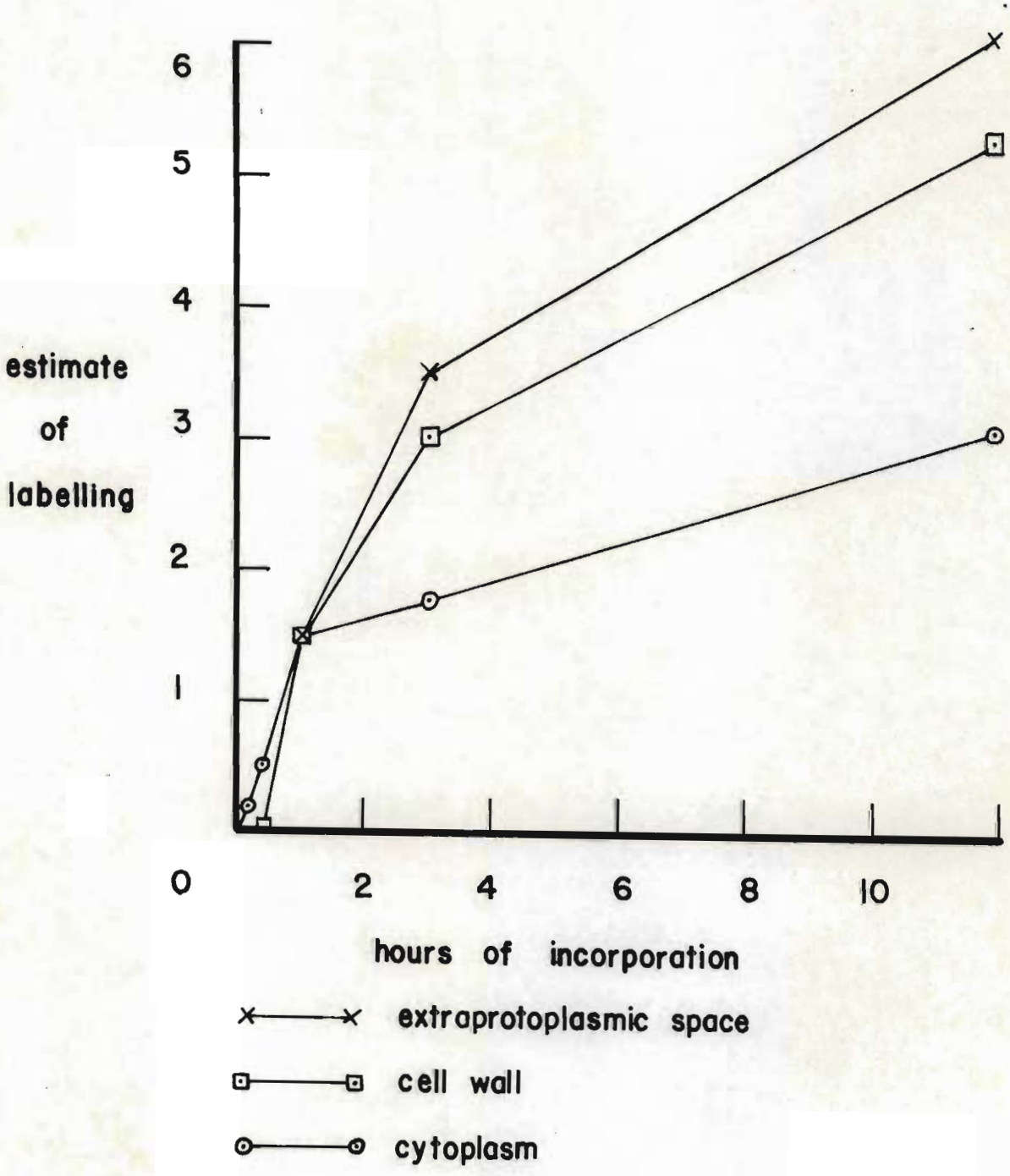


FIGURE II

LEVELS OF CYTOPLASMIC INCORPORATION OF (^{3}H)-FUCOSE INTO THE ROOT CAP (see table 2A)

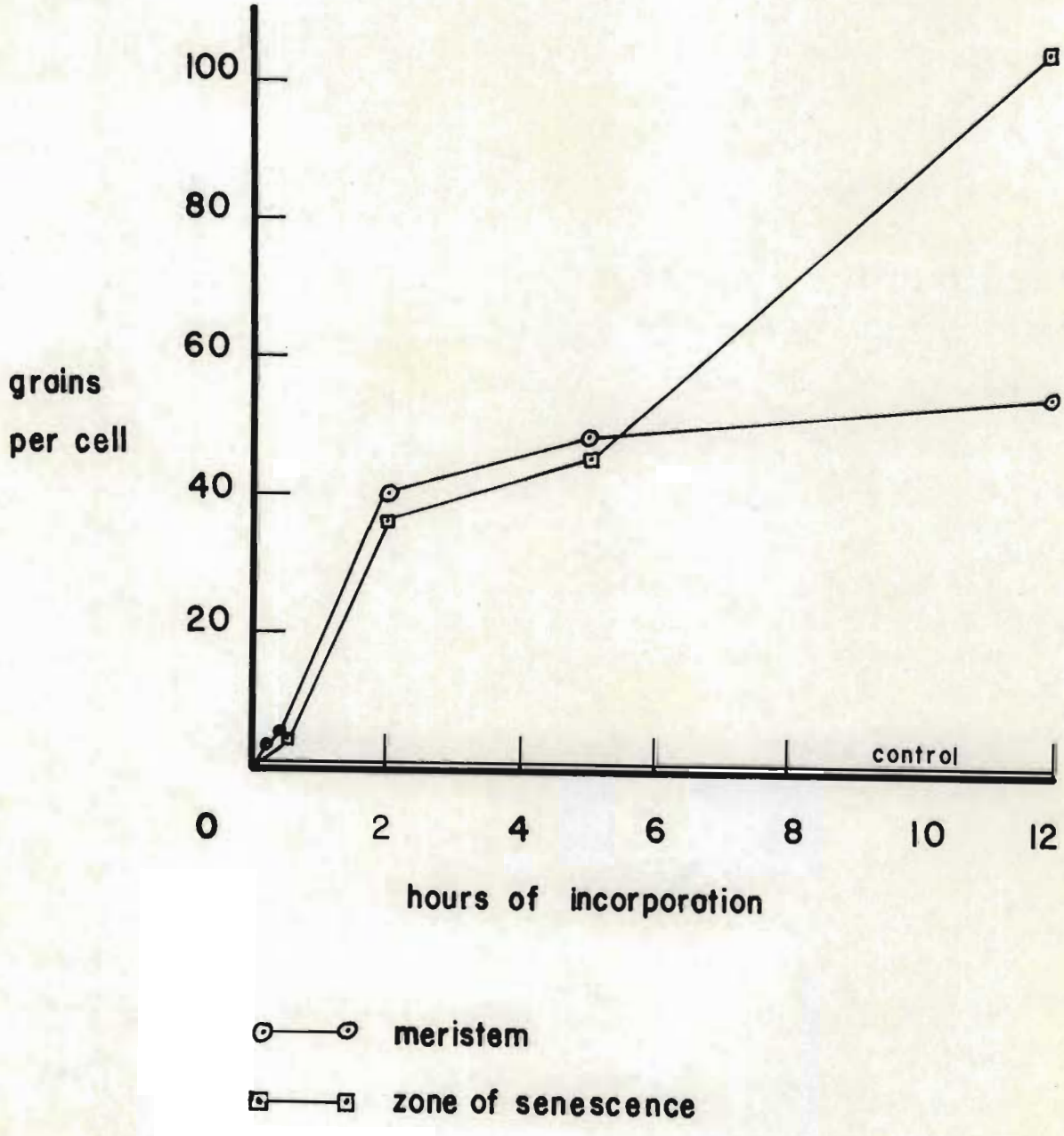


Table 2A

LEVELS OF CYTOPLASMIC INCORPORATION OF (^{3}H)-FUCOSE INTO THE MERISTEMATIC AND SENESCING ZONES OF THE ROOT CAP (see Figure 11)

Incubation period	Grains per cell (mean)	
	Meristem	Zone of senescence
5 min	1	1
10 min	3	2
20 min	5	4
60 min	40	36
3 hr	48	45
12 hr	54	104

Table 2B

RELATIVE LEVELS OF INCORPORATION OF (^{3}H)-FUCOSE INTO THE SENESCING ZONE OF THE ROOT CAP (see Figure 12).

Incubation period	Estimated level of labelling (on a scale of 1-6)		
	Cytoplasm	Extraprotoplasmic space	Cell wall
5 min	0,1	0	0
10 min	0,2	0	0
20 min	0,5	0	0
60 min	1,5	1,5	1,5
3 hr	1,7	3,5	3,0
12 hr	3,0	6,0	5,3

Chase experiments

(a) 48-hour imbibed root caps were incubated in tritiated fucose for 3 hours, after which they were chased with a cold fucose solution for periods varying between 0 and 2,25 hours (see Section 2.7.1.3).

The following were plotted against duration of incubation for the meristematic and/or senescing cells (Table 3, Figures 13, 14, overleaf).

1. Grains per cell,
2. estimated level of wall labelling,
3. estimated level of accumulation of label within the extra-protoplasmic space.

The levels of incorporation of isotope into both the meristematic and senescing regions of the cap appeared to decrease with increase in length of the chase (Figure 13). In the case of the meristematic cells incorporation was reduced to 30% of its original level after 30 minutes and during the next 1,75 hours reduced to 21% of the starting level.

The level of incorporation into the senescing region was consistently greater than that of the meristematic region. Furthermore incorporation did not decrease as dramatically, reducing to 67% of the original level after 30 minutes, and to 54% after 2,25 hours. There was an apparent increase in labelling intensity between 1,5 and 2,25 hours, but its significance is doubtful.

As the level of labelling over the protoplasm of the senescent cells decreased there was a concomitant rise

FIGURE 14

RELATIVE LEVELS OF INCORPORATION OF (^{3}H)-FUCOSE INTO THE SENESCING ZONE OF THE ROOT CAP AS A FUNCTION OF DURATION OF CHASE (see fig. 3b)

(material pulsed with (^{3}H)-fucose for 3hr prior to the chase)

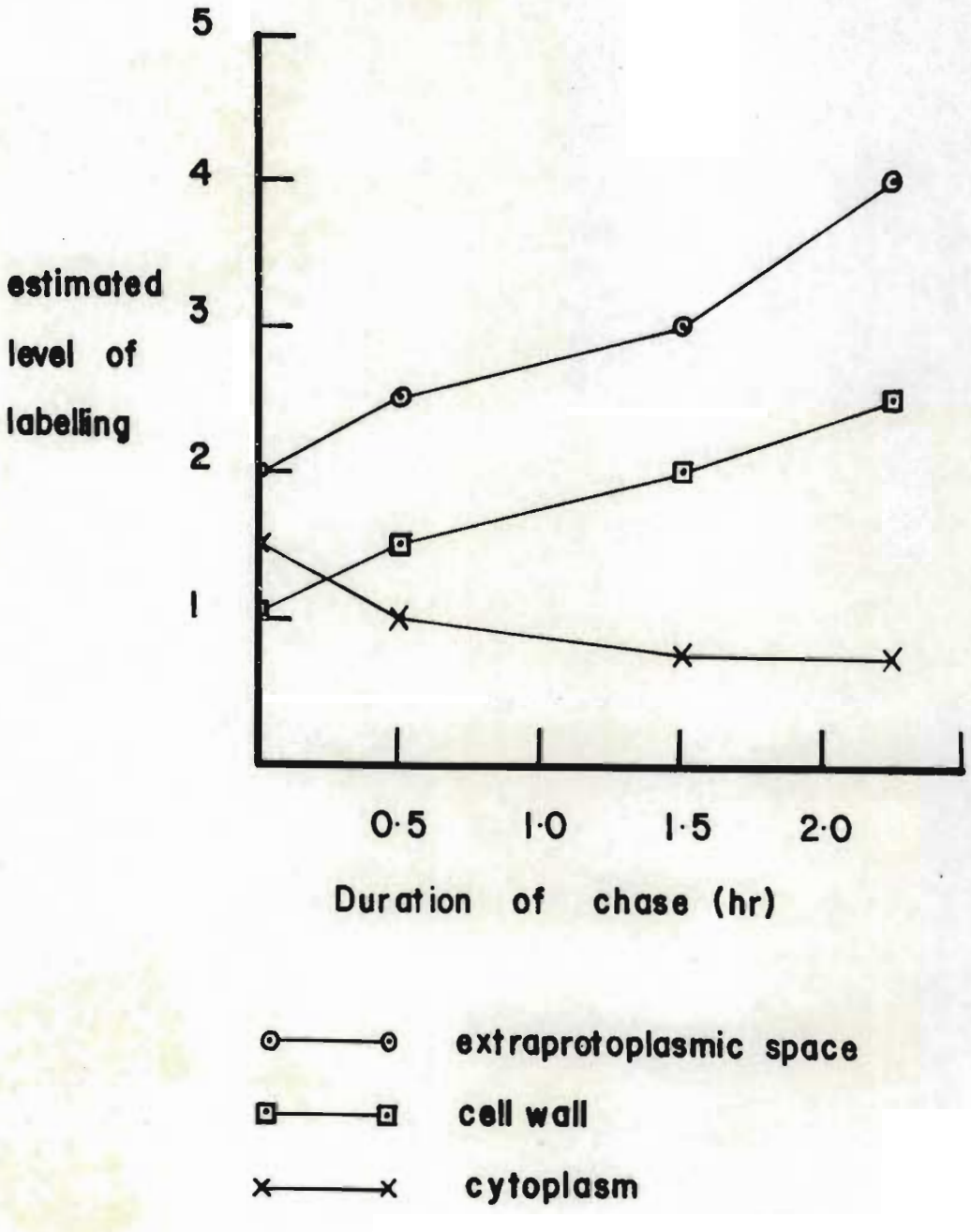
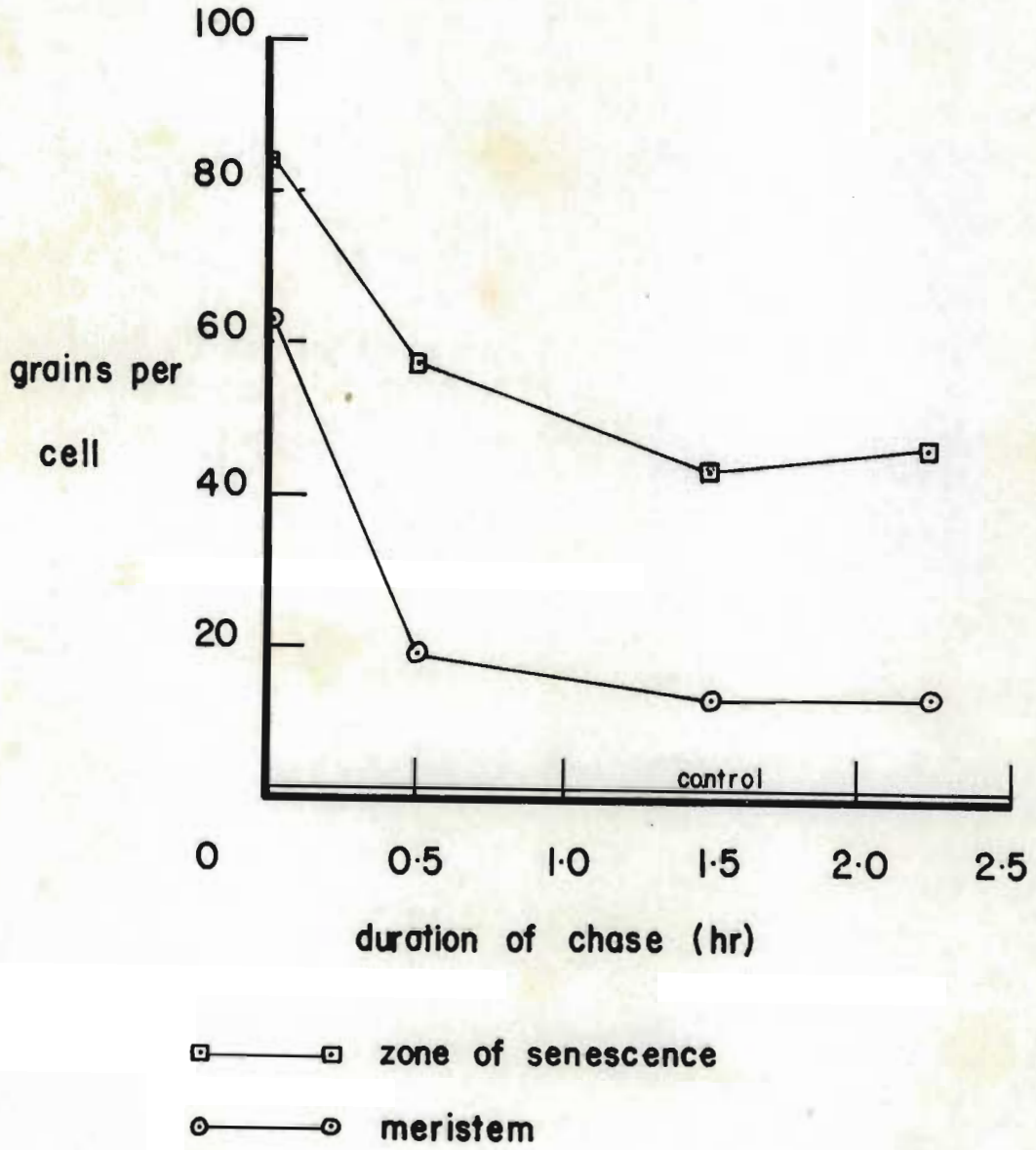


FIGURE 13

LEVELS OF CYTOPLASMIC INCORPORATION OF (^{3}H)-FUCOSE AS A FUNCTION OF DURATION OF CHASE (see table 3A)



material pulsed with (^{3}H)-fucose for 3hr prior to the chase

Table 3A

LEVELS OF CYTOPLASMIC INCORPORATION OF (^{3}H)-FUCOSE AS A FUNCTION OF DURATION OF CHASE (see Figure 13)

MATERIAL INCUBATED WITH (^{3}H)-FUCOSE FOR 3 HR PRIOR TO CHASE

Duration of chase (hr)	Grains per cell (mean)	
	Meristem	Zone of senescence
0,0	63	84
0,5	19	57
1,5	13	43
2,25	13	46

Table 3B

RELATIVE LEVELS OF INCORPORATION OF (^{3}H)-FUCOSE INTO THE SENESCING ZONE AS A FUNCTION OF DURATION OF CHASE (see Figure 14)

Duration of chase (hr)	Estimated level of label		
	Cytoplasm	Extraprotoplasmic space	Cell wall
0,0	1,5	2,0	1,0
0,5	1,0	2,5	1,5
1,5	0,75	3,0	2,0
2,25	0,75	4,0	2,5

in the amount of label associated with the cell wall and extra-protoplasmic space (Figure 14) (Plates 231 - 232). Label also appeared to accumulate between the outermost cells and on the exterior of the cap (Plate 233). The level of label in the vacuoles did not appear to decrease at all during the chase, but if anything to increase.

(b) 48-hour imbibed root tips were incubated in tritiated fucose for 6 hours, after which some of them were chased with a cold fucose solution for 3 hours (see Section 2.7.1.4).

The results obtained appear to confirm the trend shown in the previous chase experiment in that the level of incorporation into the meristematic cells decreased to 33% of the original level after 3 hours chase, while incorporation into the senescing cells decreased to only 66% of the original level.

Incorporation of tritiated fucose into 12-hour imbibed material

12-hour imbibed root tips were incubated in tritiated for a period of 90 minutes (see Section 2.7.1.2). Incorporation into the meristem was approximately twice as heavy as into the peripheral mature and senescing cells of the cap (an average of 14 grains per cell as opposed to 7). Cell wall labelling was not noticeable; the label was approximately three times lower than was the case for correspondingly incubated 48-hour imbibed material.

3.6.2 *Electron microscope autoradiography*

48-hour imbibed root-tips were incubated in an L-(^{3}H) fucose solution for 12 hours before being prepared for autoradiography.

Incorporation was divided between the various organelles in the cytoplasm of the senescing cells as follows:

TABLE 4

Organelles	% incorporation of radioactivity	
Vacuoles	30	
Dictyosomes	39	
Plastids	3	31
Mitochondria	6	
Lipid droplets	2	
ER	8	
Ground cytoplasm	12	

Thus the greatest proportion of cytoplasmic radioactivity (39%) was associated with vesicles of the hypersecretory dictyosomes (Plate 238). Label appeared to accumulate within the vacuoles and was associated with the electron-opaque material of the vacuole as well as with the apparently featureless electron-translucent portions (Plates 239 - 243). The other organelles, including the the ground cytoplasm, accounted for the remaining 31% of the cytoplasmic incorporation. By far the greatest proportion of radioactivity was, however, associated with the cell walls and especially the extra-protoplasmic space where it appeared to accumulate as a result of fusion of the dictyosomal vesicles with the plasmalemma (Plates 244 - 246). No incorporation of radioactivity into the unincubated controls was observed.

3.7 CHROMATOGRAPHY

3.7.1 *Paper chromatography*

Rg values were calculated from the chromatograms using galactose as a reference standard.

$$Rg = \frac{\text{distance moved by compound X}}{\text{distance moved by galactose}}$$

(a) The monosaccharide composition of root cap slime

The results are represented in Table 5 and Figure 15, overleaf.

The position of a diffuse spot on the chromatogram was taken as its centre and the error calculated as half the diameter of the spot. Spots were identified by comparison of their Rg values and colours with those of known standards. Although no attempt was made to quantify the results it appeared from the intensity of colour developed, and the size of the spot, that fucose, glucose, galactose and arabinose were the major constituents of hydrolysed, purified root cap slime. Galacturonic acid and xylose appeared to be present in lesser amounts. The presence of glucuronic acid and fructose could not be discounted but, if present, were masked by a large galactose spot.

The above separation was obtained using a mixture of isopropanol and water as a solvent. A mixture of butanol, glacial acetic acid and water as solvent did not give a significantly different separation. In view of this it appeared pointless to attempt a two dimensional separation as this would probably have resuled in further diffusion of the already diffuse spots and not improved the separation.

FIGURE 15

DESCENDING PAPER CHROMATOGRAPHY OF HYDROLYSED, PURIFIED ROOT CAP SLIME

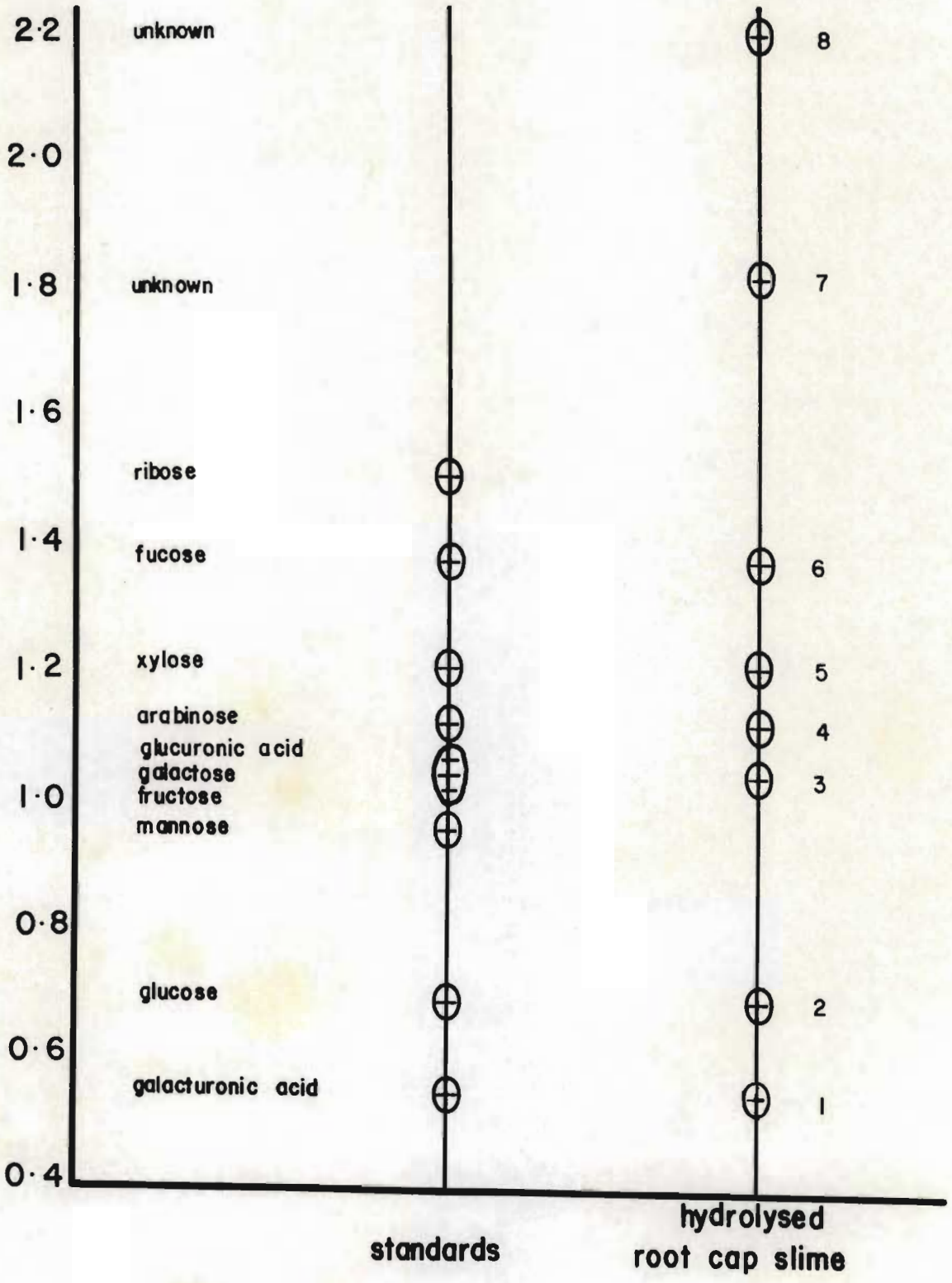


TABLE 5

DESCENDING PAPER CHROMATOGRAPHY OF HYDROLYSED, PURIFIED ROOT CAP SLIME (See Figure 15)

Monosaccharide standards			Hydrolysed slime		
	Colour of spot	Rg(±.03)	Rg(±.03)	Colour of spot	Spot number (See Figure 15)
galacturonic acid	uv	0.49	0.48	uv	1
glucose	brown/uv	0.63	0.64	brown/uv	2
mannose	uv	0.92	-	-	-
fructose	uv	0.99	1.00?	uv	?
galactose	pink/uv	1.00	1.00	pink/uv	3
glucuronic acid	uv	1.01	1.00?	uv	?
arabinose	pink/uv	1.07	1.06	pink/uv	4
xylose	pink/uv	1.18	1.17	pink/uv	5
frucose	brown/uv	1.33	1.31	brown/uv	6
ribose	brown/pink/uv	1.46	-	-	-
	-	-	1.77	1	7
	-	-	2.16		8

uv: Fluoresces under u.v. light.

(b) The monosaccharide composition of the vacuolar sap

The mixed vacuolar isolate appeared to contain a spectrum of vacuoles of varying sizes (Plates 247, 248, 249). Density gradient cenrifugation of this isolate yield three bands of differing density. These will be denoted as

L - upper, or least dense band,
M - middle, or medium density band,
H - lower, or most dense band.

The following samples were chromatographed:

1. A mixed, lysed vacuolar isolate.
2. Bands isolated by density gradient centrifugation and then lysed:
   (a) L
   (b) M
   (c) H.
3. An unlysed mixed vacuolar sample.
4. Supernatant retained after final washing of the mixed vacuolar isolate with distilled water.
5. Various monosaccharides and sorbitol as standards.

The results are represented in Table 6, overleaf. It appeared that:

1. Fucose, galactose and glucose were present in a mixed vacuolar isolate.
2. No monosaccharides could be detected in the three vacuolar bands isolated by density gradient centrifugation.

TABLE 6

DESCENDING PAPER CHROMATOGRAPHY OF ISOLATED ROOT CAP VACUOLES: MONOSACCHARIDE COMPOSITION OF VACUOLAR SAP

Monosaccharide standards	Colour	Rg (±.03)	Experimental samples - Rg(±.03)					
			1	2a	2b	2c	3	4
galacturonic acid	uv	0.49	-	-	-	-	-	-
glucose	brown/uv	0.63	0.64	-	-	-	-	0.64
mannose	uv	0.92	-	-	-	-	-	-
fructose	uv	0.99	-	-	-	-	-	-
galactose	pink/uv	1.00	1.00	-	-	-	-	-
glucuronic acid	uv	1.01	-	-	-	-	-	-
arabinose	pink/uv	1.07	-	-	-	-	-	-
xylose	pink/uv	1.18	-	-	-	-	-	-
fucose	brown/uv	1.33	1.35	-	-	-	1.34	-
ribose	brown/uv	1.46	-	-	-	-	-	-

KEY:

1 mixed, lysed vacuolar isolate

2a light) Vacuolar bands isolated
2b medium) on sorbitol density
2c heavy) gradients

3 mixed, unlysed vacuolar isolate

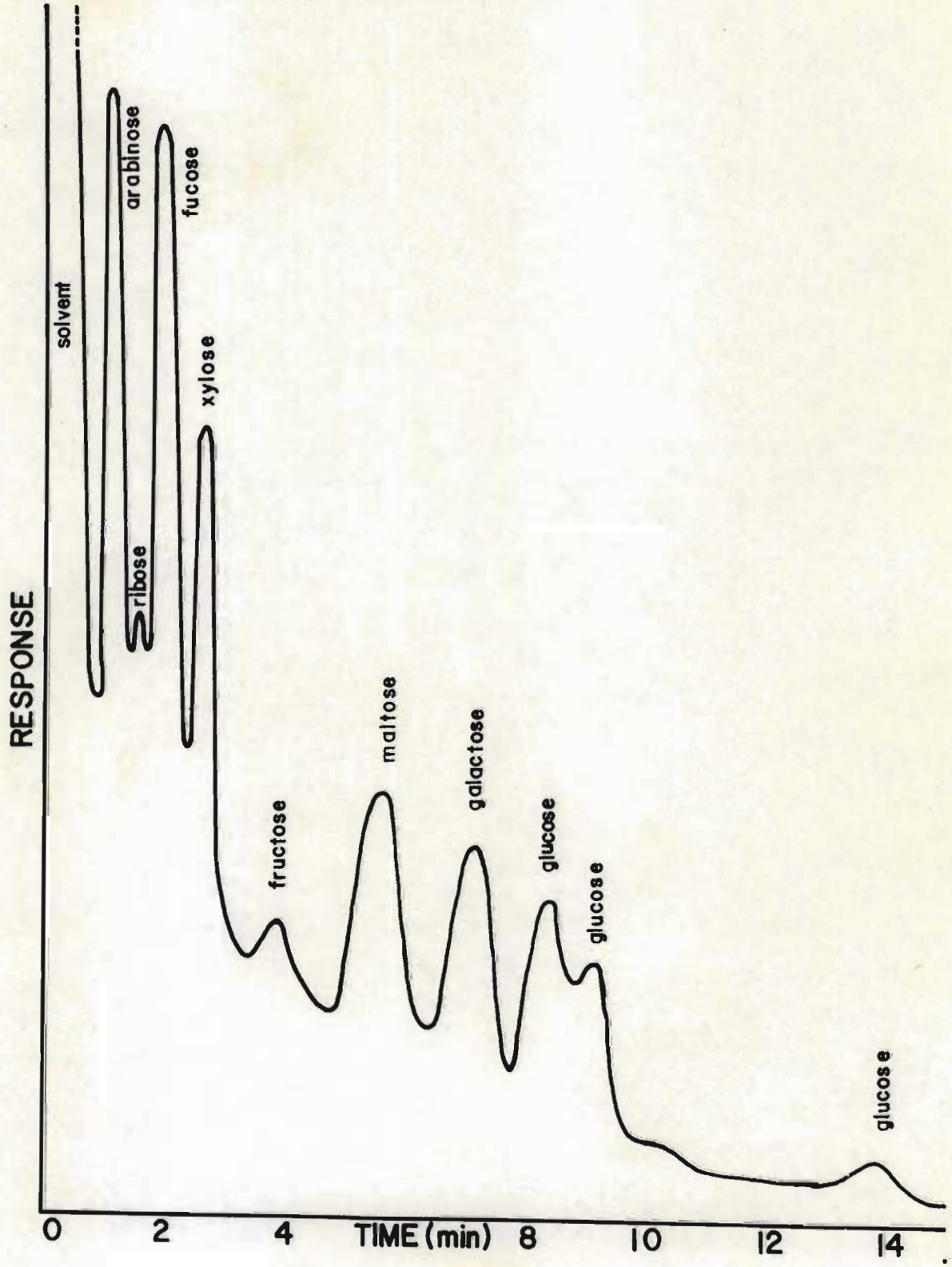
4 washings

uv: Fluoresces under uv light.

FIGURE 16

GLC ELUTION PATTERN OF TRIMETHYLSILYL ETHERS OF A MIXTURE OF MONOSACCHARIDES

Column run isothermally at 170°C



3. Fucose was present in the unlysed vacuolar sample.
4. Glucose and sorbitol were present in the supernatant retained after the final washing of the mixed vacuolar sample with distilled water.

3.7.2 *Gas - liquid chromatography*

Separation of a mixture of monosaccharide standards was achieved on a column with 3% SE30 as liquid stationary phase under the conditions described in Section 2.10.2.3. Experimental samples known to contain polysaccharide (this had been established from previous paper chromatography) were derivatised (reacted with trimethylsilylimidazole) and run through the gas chromatographic system. Monosaccharides were not detected in these samples even at very high detector sensitivity levels. It is perhaps still pertinent however to discuss details of the standard separation as the system was definitely operational and may be of use in future work.

Figure 16 illustrates the separation attained with the column running isothermally at 170°C. The solvent peak (trimethylsilylimidazole and pyridine) was large but did not appear to mask any of the derivative peaks although at high sensitivities arabinose appeared as a shoulder on the tail end of the solvent peak. Peak spread appeared to increase with retention time. Peak tailing was not a problem. Glucose, galacturonic acid, fructose, galactose, mannose and xylose separated into two anomeric peaks each, with the first isomer to be eluted giving a peak 5 - 10 times greater in area than the second. These peaks are thought to represent α and β isomers of the sugars, although pure samples of each isomer were not available to establish the isomeric identity of each peak. During separation many of the minor isomeric peaks were masked by the presence of other derivatives with similar retention times. Retention times of arabinose and ribose were very similar, causing ribose to appear as a shoulder on the arabinose peak. Fructose and mannose appeared

FIGURE 18

GLC ELUTION PATTERN OF TRIMETHYLSILYL ETHERS OF OLIGOSACCHARIDES IN THE VACUOLAR FLUID

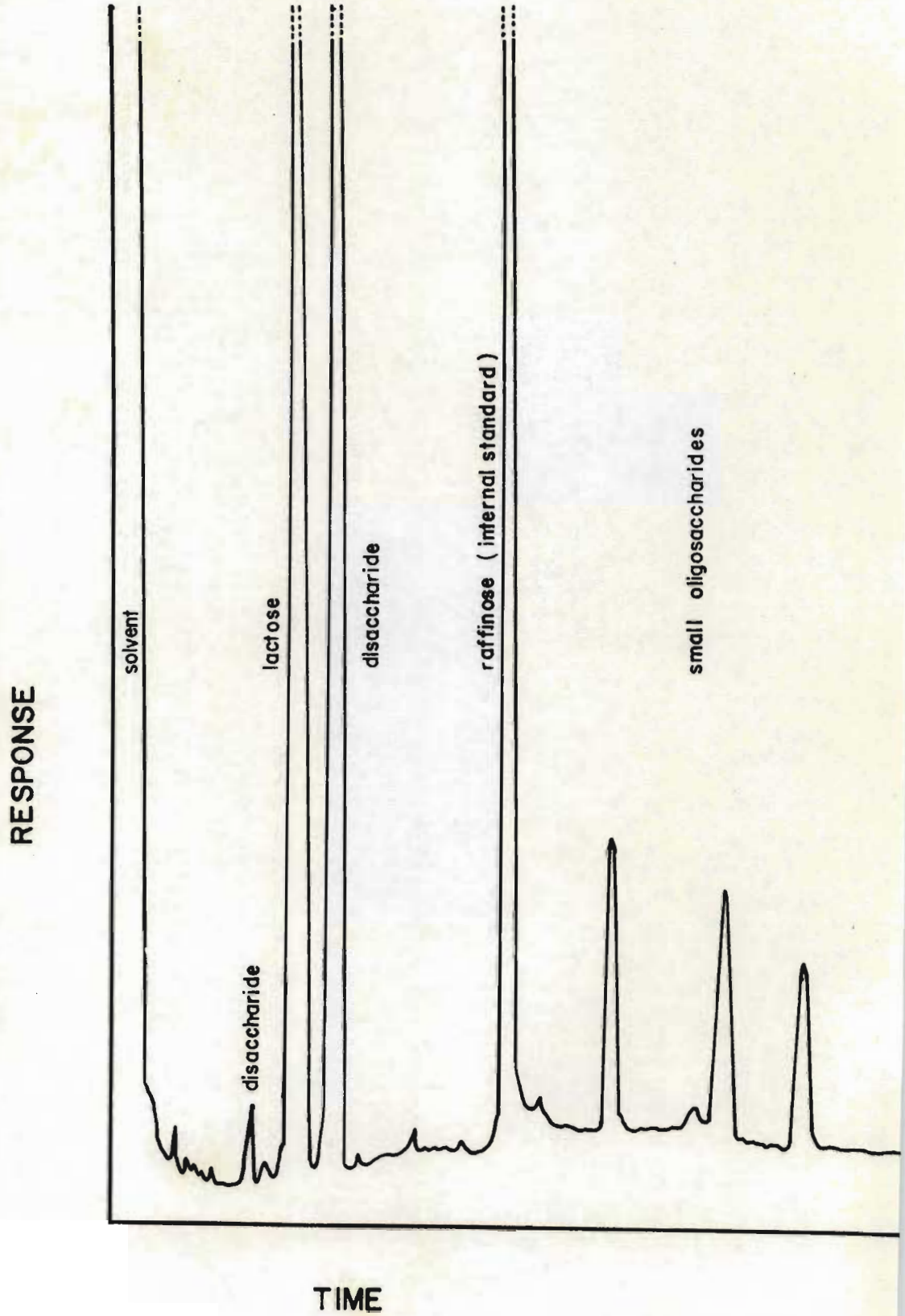
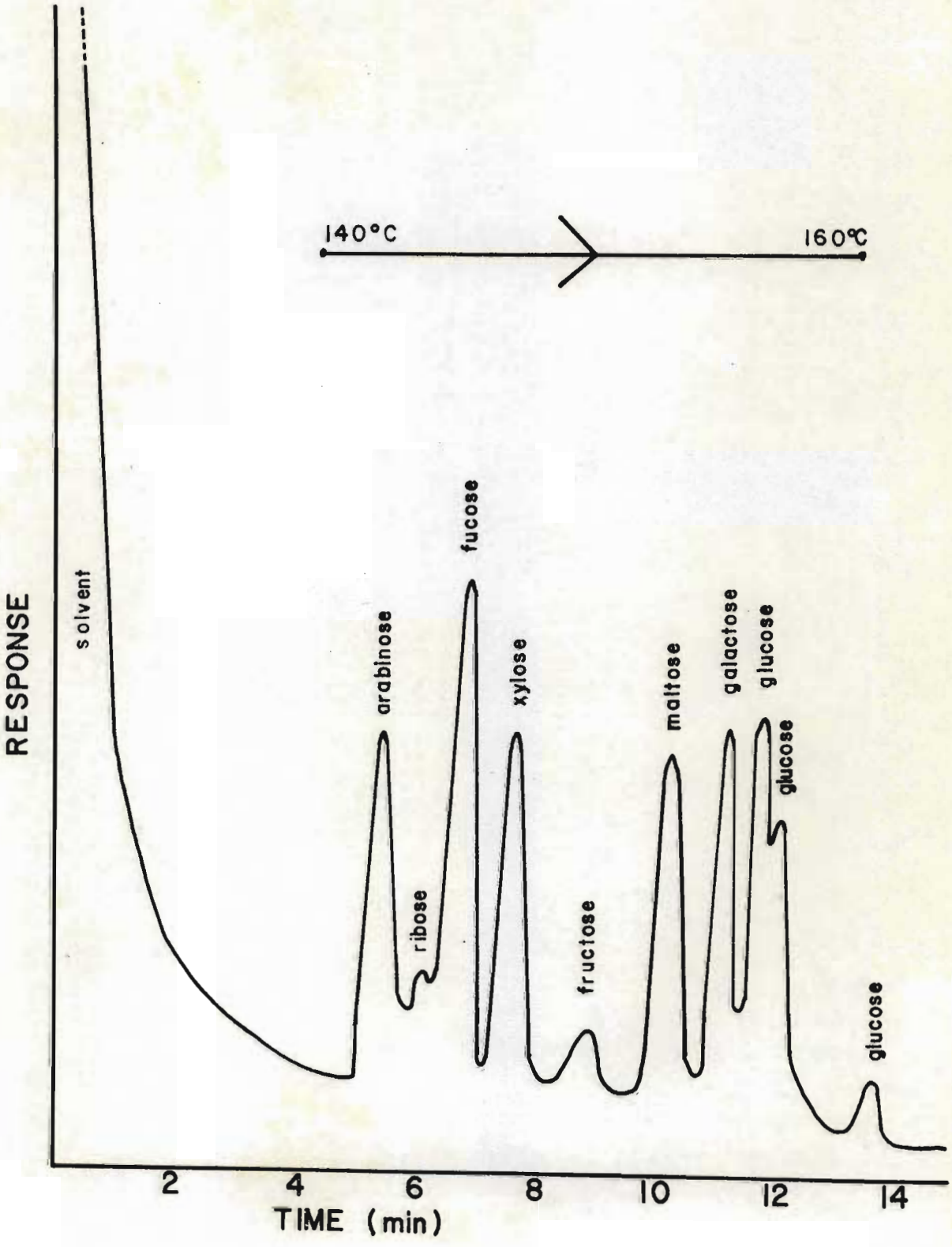


FIGURE 17

GLC ELUTION PATTERN OF TRIMETHYLSILYL ETHERS OF A MIXTURE OF MONOSACCHARIDES

Column temperature programmed from 140°C to 160°C at 6°/min



to have identical retention times, however the presence of mannose could be distinguished as its minor isomer formed a shoulder on the glucose peak.

Figure 17 illustrates the separation obtained when the column temperature was programmed from 140°C - 210°C with temperature increment of 6°C/minute. Although separation was not significantly improved the problem of peak spread was eliminated.

A mixed vacuolar isolate was analysed for the presence of small-chain saccharides in the laboratories of the Sugar Milling Research Institute (a division of the Council for Scientific and Industrial Research) by Dr. A. Nurock. Separation took place on a Beckman GC-55 Gas Chromatograph using a 40 metre stainless steel capillary column coated with OV 17 as stationary phase. The oven was programmed from 210°C to 265°C at a temperature increment of 10°C per minute. Raffinose, a trisaccharide, was used as an internal standard. Results showed that two disaccharides, one of these being lactose, were present as major components, with a third disaccharide being present in lesser amounts (Figure 18). Also present were three peaks consistent with four and five chain saccharides. The chain length of the saccharides was established from their retention times, but standards were not available to allow their exact monosaccharide composition to be established.

PART FOUR

DISCUSSION

4.1 PREPARATION OF MATERIAL FOR ELECTRON MICROSCOPY

Several different types of fixation were tested during the course of the present study with a view to obtaining the best possible preservation of the experimental material. As the work focuses largely on the vacuole and other components of the endomembrane system, it was particularly important that these organelles should be well preserved and stained.

Vacuoles are diverse structures, and as such may contain a variety of different solute materials depending on the nature of the

plant and the location and function of the tissue within which the vacuole is found. It is therefore not surprising that difficulty may be experienced in obtaining satisfactory preservation of all vacuoles within a cell, in different cells and in root tips at different stages of development. Preservation of vacuoles involves a reaction between the fixative, the vacuolar membrane (tonoplast), the surrounding ground plasma and the vacuolar sap (Fineran, 1971). The chief variable is probably the composition and concentration of the vacuolar contents, and this may lead to differences in preservation.

When a cell is bathed in fixative, several changes may occur. Early stages of fixation probably destroy the differential permeability of the vacuolar membrane (McLean, 1960) so that a new osmotic relationship is established between the vacuole and fixative. According to Fineran (1971) vacuoles might be expected to shrink, burst or maintain a rounded shape depending on whether hypotonic, hypertonic or isotonic conditions prevail.

As the vacuole is so sensitive to the conditions of fixation it is particularly important to find a method of fixation which preserves it adequately. It is the opinion of Fineran (1971) that the diverse interpretations of the form of vacuoles in electron micrographs (e.g. Buvat, 1958, 1967; Manton, 1962; Poux, 1962; Mesquita, 1969) probably reflect a lack of knowledge of vacuolar behaviour during fixation. Studies using the freeze-etching technique (Branton and Moore, 1964; Matile and Moor, 1968; Northcote and Lewis, 1968) suggest that many of the irregular forms in chemically fixed material are apparently artefacts of specimen preparation, as freeze etching, which usually does not involve chemical fixation, reveals vacuoles with smoothly contoured, if not spherical forms. These are believed to represent the normal shapes of vacuoles (Matile and Moor, 1968; Fineran, 1971). In the light of the above the criteria for good preservation of the vacuole are an intact tonoplast and a

smoothly contoured shape. This appears reasonable, especially if describing the *final* form of the vacuole. It will, however, be argued later (see section on the origin of vacuoles) that irregular forms may exist during the early developmental phases of some types of vacuole.

All preparative methods employed in the present study involved two stages of fixation. In all cases glutaraldehyde was used as a primary fixative, whereas a variety of postfixatives and buffering systems were employed. According to Fineran (1971) the type of fixative, rather than its concentration or other variables such as buffering systems used, temperature, pH and duration of incubation, is the most important factor in obtaining good fixation. It would thus appear that the differences observed are due mainly to the nature of the post-fixative, although the other variables mentioned may also play a rôle.

4.1.1 *The use of PIPES buffer during fixation*

The most satisfactory overall preservation of the material was achieved with the primary use of PIPES-buffered glutaraldehyde (pH 8.4), followed by post-fixation in aqueous osmium tetroxide. This represents a modification of the procedure recommended by Salema and Brandão (1973) who employed PIPES-buffered osmium tetroxide as a post-fixative after primary fixation with PIPES-buffered glutaraldehyde. This modification was necessary as it was found that, even under the most scrupulous conditions of cleanliness, the PIPES reacted with the osmium tetroxide to give a deep-violet precipitate and thus removed most of the osmium from solution. It is not known whether this effect was due to the PIPES itself, or resulted from the pH(8.4) of the PIPES solution, although the former seems more likely.

Buffering during fixation

It would appear that the lack of buffering during post-fixation did not affect fixation adversely as the material was well preserved. It would seem more essential for buffering to occur during primary fixation, when most of the crosslinking between macromolecules takes place - it should however, be borne in mind that Fineran (1971) has reported that an entirely aqueous fixation schedule produces results not noticeably different from one in which a buffer is employed. The present results are in agreement with this.

A buffer may have two functions:

1. it may serve as an 'indifferent salt' - in other words its ionic strength should be comparable to that within the vacuole so as to minimise shrinkage or swelling of the vacuole during fixation,
2. it should serve to maintain the pH within the tissue at a particular value during fixation.

Fixation solutions are usually buffered at pH's between 6.8 and 7.4. There do, however, appear to be two schools of thought on this issue. On the one hand it is maintained that the pH of the buffer should correspond to the pH of the tissue (e.g. Millong, 1962), and it is for this reason that pH's between 6.8 and 7.4 are commonly selected. This line of thought stands to reason, but the narrowness of the pH range selected is subject to question as the pH within different intracellular compartments may vary considerably, being as low as 5.5 within the vacuole. The other school maintains that pH plays little part in fixation (e.g. Baker, 1965; Fineran 1971), and that satisfactory preservation of the material may be obtained at any pH between 5.5 and 9.0. The present results are in agreement with this, as PIPES, buffered

at pH 8.4 gave excellent preservation of cellular material, including the vacuole. It would thus seem that, if indeed buffering during fixation does lead to better preservation of the material, the rôle of the buffer as an indifferent salt may be more important than its rôle in maintaining a constant pH within the tissue.

It is inevitable that a certain amount of protoplasmic material, particularly micromolecules, is leached from the tissue during fixation. Salema and Brandão (1973) have reported that significantly less leaching occurred when PIPES, as opposed to cacodylate, phosphate, acetate or collidine, was used as a buffer during fixation. This would perhaps account for the fine detail and the apparent denseness of the cytoplasm observed with this method of fixation. In this regard the polysaccharide contained within the vesicles of the hypersecretory dictyosomes and constituting the extracellular slime was particularly well preserved in material fixed according to this method. In view of the low rate of leaching of protoplasmic material from the tissues, this method would appear ideal for use in cytochemical work, although the pH of the buffer would probably have to be varied according to the nature of the molecules to be localised. For example, fixation at pH 8.4 would be likely to damage and thus inactivate hydrolytic enzymes whose pH optima are in the region of 5.

A drawback of this method was that membranes, although well preserved, did not always stain clearly. This presented a problem as it was important, especially in the case of ageing cells, to be able to visualise the vacuolar membrane clearly so as to be able to establish whether or not it had become disrupted as a result of the ageing process.

4.1.2 *Glutaraldehyde/osmium tetroxide fixation*

Good overall preservation, although not quite as fine as that obtained with the PIPES method, was achieved using cacodylate-buffered glutaraldehyde (pH 7.2) as the primary fixative, followed by post-fixation with cacodylate-buffered osmium tetroxide. This method, however, had the advantage that the vacuolar membranes were usually easy to distinguish. In this regard Fineran (1971) has recommended this method as being most suitable for the preservation of plant material in general, and vacuoles in particular, although he did not investigate the properties of PIPES as a buffer.

4.1.3 *Glutaraldehyde/potassium permanganate fixation*

The poorest overall quality of fixation, although by far the best membrane staining, was obtained with the use of acetate-buffered glutaraldehyde as a primary fixative followed by post-fixation in an acetate-buffered potassium permanganate solution (after Mercer and Birbeck, 1961). Although clear staining of membranes was desirable, this method presented three major problems.

1. The potassium permanganate apparently effectively destroyed ribonucleoprotein, with the result that the ribosomes were not readily recognisable as such.
2. This method of post-fixation resulted in removal of most of the lipid material from the lipid droplets (spherosomes), in some cases rendering them almost indistinguishable from other cytoplasmic vesicles, including small vacuoles.
3. In many cases post-fixation with potassium permanganate appeared to cause the artefactual disruption of the vacuolar membrane. This is particularly undesirable when the vacuole is the primary subject of investigation.

Such disruptions of the vacuolar membrane appeared to be the result of bursting. Certainly, the lesions in the membrane were large in comparison to the size of the vacuole, as would be expected if the vacuole had burst, and seem unlikely to have resulted from molecule-molecule interactions (between the membrane and the $KMnO_4$) which would be more likely to cause small-scale lesions (of molecular dimensions). It is, of course, possible that harsh oxidation of membrane proteins or lipids may have resulted in the overall weakening of the membrane which, if then stressed, would be likely to rupture.

The interactions between buffer, permanganate and vacuolar fluid are complex, and it is therefore difficult to determine exactly why bursting (or shrinkage) should occur. Primary fixation with glutaraldehyde seems to stabilise the vacuolar membrane to a large degree and thus reduce the liklihood of its subsequent disruption. The fate of the vacuole during post-fixation may depend on whether the permanganate or the buffer ions enter the vacuole first. In the former case, precipitation reactions between $KMnO_4$ and the vacuolar contents may serve to lower the osmotic component of the water potential within the vacuole, causing efflux of water and subsequent shrinkage of the vacuole. If the buffer ions reach the vacuole first the resultant increase in ionic concentration within the vacuole might result in an influx of water into the vacuole which would then swell and might burst, especially if the vacuolar membrane had been weakended by prior reaction with fixative. The above argument would, however, apply to cases where either OsO_4 or $KMnO_4$ had been used as post-fixatives, and yet vacuolar bursting is not common when osmium tetroxide is used as a post-fixative.

Although OsO_4 and MnO_4^- ion are very similar in structure and chemical behaviour, the difference could perhaps be attributed to differences in oxidising strength and rate of movement of these two chemical species in relation to one another. Bearing in mind the previous discussion on buffering of fixatives, it would seem unlikely that the use of acetate buffer with the permanganate system (as opposed to cacodylate with the osmium) could be responsible for the observed difference in behaviour.

Thus all of the methods of fixation tested had their advantages and disadvantages. Cacodylate-buffered glutaraldehyde/osmium tetroxide was found to embody the best combination of fixation on the one hand, and membrane staining on the other and was therefore used preferentially throughout the work although other methods were also employed where appropriate.

4.1.4 *The use of 2,2-dimethoxypropane as a dehydrating agent*

Muller and Jacks (1975) have reported that acid-activated 2,2-dimethoxypropane (DMP) may be employed as a dehydrating agent (in place of the usual graded alcohol/acetone series) where rapid dehydration is desirable. This method was investigated as it would appear to be particularly useful in cytochemistry, where rapid processing is necessary to prevent diffusion of the end product away from the reaction site. It was found, however, that the membranes of DMP-treated tissue often appeared completely unstained and were only visible by virtue of an apparent 'negative staining' effect in comparison with the surrounding cytoplasm. A similar effect was observed by Berjak (pers. comm.) working on the seminal roots of barley. Also in this connection Barnes (pers. comm.) found that DMP treatment completely removed the osmium black from maize root tips fixed anhydrously in osmium vapour. It would therefore seem that DMP, at least in some cases, has the effect of removing osmium associated with cytoplasmic membranes and prevents any further binding of heavy metal (for example lead, during post-staining)

to the membranes. In view of the above the use of DMP as a dehydrating agent was avoided in the present investigation. However, since the completion of this work, Lawton (personal communication) has suggested that the difficulties described might have arisen through over-acidification of the DMP. She suggests that addition of 1 drop of 33% HCℓ to 100 ml DMP, instead of 3 drops of 10 ml, should be employed. This modification remains to be tested.

4.2 CRITERIA FOR IDENTIFICATION OF COMPONENTS OF THE LYTIC COMPARTMENT

There are two major methods used by cell biologists to identify an organelle as belonging to the lytic compartment of the cell.

4.2.1 *Ultrastructural observations*

Observation of the ultrastructure within a particular tissue allows particular organelles to be recognised by their morphological appearance and classified on this basis. One may thus recognise e.g. a mitochondrion, or a vacuole. Without further (cytochemical and biochemical) information, however, it is difficult to assign a function to a particular organelle or cell component (see 4.2.2). However once such information is available, it is possible to assign functions to what were before only shapes - in this way it has been discovered that the plant vacuole is a major component of the lytic compartment of the plant cell (see Introduction).

The next thing to consider is the formation of the organelle. An organelle in its early stages of development may not always resemble a miniature version of the final product, but careful observations of ultrastructure may allow the two to be related through the establishment of a developmental sequence leading to the final form. The plant vacuole is generally held to be lytic in nature, but ultrastructual studies alone cannot reveal the exact stage during its development at which it acquires lytic properties. For this, chemical information is required.

4.2.2 *Cytochemical tests*

Cytochemical tests provide the cell biologist with a powerful tool enabling him to correlate the function of an organelle with its

structure. In cases such as the lytic compartment, which embodies many morphologically diverse portions of the cell (such as the vacuole, developing vacuoles, and extra-protoplasmic space) it would not have been possible to identify the various components without the aid of cytochemical techniques. The most commonly used test for lytic enzymes is the reaction for the enzyme acid phosphatase (Gomori, 1952). As this test has been employed in the present study to confirm ultrastructual observations concerning the lytic compartment it would perhaps be pertinent to consider the validity and applicability of the test before drawing any conclusions from it.

Acid phosphatase has been widely used as a general marker-enzyme for lysosomes, but several researchers have cast doubt on the validity of this assumption. According to Matile (1969):

> 'Acid phosphatase seems to represent a doubtful marker enzyme of lysosomes; the simplicity of its assay and its easy cytochemical demonstration have raised the popularity of this enzyme out of all proportion to our knowledge of its functional significance'.

He further states (Matile, 1974) that the widespread idea that lysosomes are characterised by specific acid-β-glycerophosphatase activity is erroneous and emphasises that plant acid phosphatases are extremely unspecific, using as an example the work of Shaw (1966) who purified an acid phosphatase from tobacco leaves which catalysed the hydrolysis of a wide variety of phosphate esters as well as of anydrous bonds of phosphoric acid. It further appears that the inability to demonstrate the presence of acid phosphatase in a particular cell compartment is not sufficient grounds for rejecting it as a lytic compartment. For example the yeast vacuole 'which is the logical site for the hydrolytic enzymes of yeast, does not appear to contain acid phosphatase, unless in a repressed form' (Matile, 1969).

In this regard de Duve (1963) has suggested that there may be enzymic differences between lysosomes, although not to the extent of one lysosome - one enzyme. Thus Gahan's suggestion that at least two distinct hydrolytic enzymes should be found associated with a membrane-bound body before it can be identified as being lysosomal in nature, appears sound (Gahan, 1967). In the present investigation the Gomori test for acid phosphatase has been used to at least partially confirm that membrane-bound bodies designated as being part of the lytic compartment are indeed so, but unfortunately the scope of the investigation has not allowed for the testing of a second hydrolytic enzyme.

In addition to the aforementioned limitations, the acid phosphatase test itself has some practical limitations which should be considered.

The test for acid phosphatase

With cytochemical methods, under appropriate conditions of fixation and incubation, a particulate distribution of the reaction product of an enzyme or enzyme system can be obtained, and its location visualised with the electron microscope. Cytochemical procedures for localising lysosomal enzymes are, however, highly selective: most of the enzymes, including 'acid phosphatase' exist in a variety of molecular forms (isoenzymes) as a family of enzymes showing overlapping, if not identical substrate specificities, or as multi-enzyme complexes (Beck and Lloyd, 1969). The Gomori reaction utilises sodium-β-glycerophosphate (buffered at pH 5.0) as a substrate, in the presence of lead nitrate - the released ions are thus precipitated as lead phosphate. The studies of Holt and Hicks (1961) and of Essner and Novikoff (1961), based on methods for the demonstration of enzyme activity developed for light microscopy, opened the way for the identification of individual lysosomes by taking advantage of the fact that the final product of the enzyme reaction, an insoluble complex

of lead and phosphate ions (Gomori, 1952), scatters electrons sufficiently to make this product visible as electron-opaque in ultra-thin sections.

A disadvantage of the localisation procedures at ultra-structural level is the necessity for fixation of the specimen prior to incubation. As a consequence of prefixation, some loss of enzyme activity has to be accepted (e.g. Holt and Hicks, 1961). In the light of this, the fixative giving the least diminuition of enzyme activity should be chosen. According to Anderson (1967) (*loc. cit.* Daems *et al.*, 1969) the purity of the fixative also appears to influence the degree of retention of enzyme activity. Unfortunately, however, the price of a higher retention of enzyme activity is often poor preservation of morphology. In general, a compromise between these conflicting requirements must be accepted. As a result of the diminuition of enzyme activity by fixation prior to incubation, it must be accepted that only the actual demonstration of enzyme activity can have a positive value. According to Daems *et al.*, (1969) the absence of demonstrable enzyme activity could mean one of three things:

1. The enzyme is absent.
2. Enzyme activity has been so greatly diminished by the prefixation procedure that the reaction is not sufficiently sensitive to detect the remnants of activity.
3. In the case of the demonstration of lysosomal enzymes the pre-fixation may not have made the lysosomal membrane sufficiently permeable for the incubation medium to penetrate.

A disadvantage of this method for the localisation of acid phosphatase is that reaction product is often found in the nuclei, in association with the chromatin, or may be scattered throughout the

cytoplasm (Daems *et al.*, 1969). Nuclear precipitation of lead phosphate crystals was common and marked in the present investigation. Such precipitation is generally considered to be an artefact produced by the affinity of the chromatin for lead salts, resulting in non-enzymatic reactions (Gomori, 1952). Holt (1959) considered the lead concentration of the medium to be critical, such artefacts being produced only at critical concentrations of lead. Such deposits of lead may be removed by a brief acetic acid rinse following incubation (Gomori, 1952), thus perhaps confirming that they are artefacts. Quantitative and semi-quantitative experiments, however, suggest that this procedure not only removes the aberrant precipitate, but also removes some precipitate in places where it would be expected to be found on the basis of light microscope histochemistry (Goldfischer *et al.*, 1964). On these grounds such procedures do not appear desirable and were not used in the present investigation.

Cytoplasmic lead phosphate deposits were also observed during the present investigation, as well as apparent deposits with the mitochondrial and plastid membranes. Such staining is usually regarded as an enzyme - dependent artefact resulting from the diffusion of reaction intermediates, reaction product or enzyme (Daems *et al.*, 1969). It is possible, however, that chemical fixation may have rendered the vacuolar membranes highly permeable and thus allowed reaction intermediates, product or enzyme to escape from the vacuole and distribute itself within the cytoplasm. It should also be borne in mind that physical bursting of a lysosomal or vacuolar membrane (as a result of adverse experimental conditions or of the physiological status of the cell, e.g. as a consequence of the ageing process) may result in the release of hydrolytic enzymes into the cytoplasm. If the plane of an ultra-thin section does not pass through the discontinuity in the membrane the reason for the cytoplasmic deposits would not be evident. Finally, when considering the localisation of acid phosphatase at the ultrastructual level the

following further points should be borne in mind:

1. The site of the reaction product may not always coincide with the site of enzyme activity. In this connection the diffusion of stain precursors before the final trapping reaction can seriously influence the location of the final reaction product (Koenig, 1967).
2. Loss of enzyme reaction product can occur as a result of osmium-tetroxide post-fixation (Peale and Luciano, 1964) or during the staining of ultra-thin sections (Katino *et al.*, 1968).
3. Incubation at an acid pH and relatively low osmotic values of incubation medium may damage the fine-structural detail of cell components (Daems *et al.*, 1969).

Thus as long as the limitations of the method are borne in mind it still appears that the Gomori reaction for acid phosphatases can be very useful for the *in situ* identification of lytic cell compartments at ultra-structural level.

4.3 THE ORIGIN OF VACUOLES

4.3.1 *Vesiculation of the endoplasmic reticulum*

Present observations on Zea mays indicate that the chief method of vacuolation in the meristematic region is through vesiculation of the endoplasmic reticulum; it appears that this may occur in more than one way.

4.3.1.1 *Provacuoles bounded by a single membrane*

The development of the root cap has been previously described (see Introduction). Meristematic cells undergo differentiation, mature and finally age and die as they reach the periphery of the cap, where they are sloughed. Most vacuoles appear to be formed within the meristematic region, and develop progressively within the cell in which they were formed, becoming recognisable as typical large 'plant vacuoles' by the time the cell has reached maturity. Vacuoles appear to be formed within the meristem through the budding off of small, single membrane-bound vesicles, from the cisternae of the rough ER. These vesicles, termed provacuoles, whose interiors were once continuous with the lumen of the ER, appear to be formed at terminal (rather than intermediate) locations on the ER cisternae. These observations are in agreement with those of Matile and Moor (1968) obtained using the freeze-etch technique, as well as with the ultrastructural findings of Berjak and Villiers (1970).

An intensely electron-opaque substance appears to be deposited within the interior of the forming provacuole, having its origin apparently within the lumen of the ER cisternae. Once the provacuole has separated from the ER this substance persists and appears to increase in volume. Acid phosphatase activity has been localised to this electron-opaque matrix not only in the present study, but also by

Berjak and Villiers (1970). The present results, however, indicated that not every organelle having the appearance of a provacuole reacted positively for acid phosphatase, and where a positive reaction was observed, the enzyme was often localised to only a portion of the electron-opaque matrix (usually near the edge). Interpreting these results in the light of the previous discussion on the acid phosphate reaction, it cannot be concluded that those provacuole-like bodies were not in fact provacuoles merely because they did not give a positive reaction for acid phosphatase. If, indeed, acid phosphatase was absent, these bodies may well have contained other hydrolytic enzymes. It is also possible that at very early stages of development the enzyme may have been present in a latent form. When present, the enzyme activity was often localised only to a portion of the content of the provacuole. This may represent a localised distribution of enzyme within the matrix (the activity being only at the edge of the matrix) although there is no precedent for this described in the literature. It is also possible that such a localised distribution may be brought about either by limited penetration of the incubation medium into the provacuole, or through diffusion of reaction product, stain precursors or enzyme to the edge of the provacuole during washing. Where such a localised distribution exists, it is likely that in many cases the plane of a thin section may not pass through that region of the provacuole containing reaction product, which could account for some of the apparently acid phosphatase - negative provacuoles.

Many other lines of investigation support the proposed origin of provacuoles from the ER. The freeze-etching studies of Matile and Moor (1968) have indicated the homology of the vacuolar membrane and ER. They observed that both the surface of the ER facing the cisternae, and the vacuolar membrane are densely sculptured, with particles 110-120 Å in diameter, whereas the surfaces adjacent to the ground cytoplasm carry

far fewer particles of equivalent size. This is in agreement with the theory that the interior of the provacuole was once continuous with the lumen of the ER. In addition Matile (1968) has demonstrated that fractions of provacuoles isolated from maize root tips contain enzymes that may be found associated with the ER.

Barton (1965) has proposed that the provacuoles of meristems represent persistent organelles which multiply by division. He based his hypothesis on the different contrasting of ER and provacuole membranes, when stained with uranyl acetate. Matile (1974) has pointed out that it is at least equally likely that this phenomenon is a result of membrane differentiation during provacuole formation.

It is interesting to ask when exactly the provacuole becomes functional as a vacuole, and this question depends on the definition of the word vacuole. The plant vacuole appears to have at least a dual function in that it plays a rôle in the water relations of the plant as well as serving as a major part of the plant lysosomal system. Considering the former criterion, the provacuole, as soon as it has separated from the ER and is an independent entity, can be established through developmental studies as a direct precursor of the large vacuoles of the mature root cap cell. It is, however, unlikely that the provacuole in its early stages of development plays any rôle in the water relations of the plant. Perhaps more interesting is the question of when the provacuole becomes functional in the lytic sense. As provacuoles originate from the ER can the ER be regarded as part of the lytic compartment of the cell?

Present results show that the provacuoles in the meristematic cells of the maize root cap only show acid phosphate activity once they have separated from the ER to become independent. Thus if acid phosphatase is used as a marker enzyme, it would appear that the

provacuole only becomes functional in a lytic sense once it has separated from the ER, which would not then be regarded as part of the plant lysosomal system. In this regard provacuoles may be thought of as a product of differentiation, rather than of mere dilation of the ER cisternae (Matile, 1974).

Present results indicate, however, that the electron-opaque substance contained within the provacuole is synthesised by the ER (perhaps by the ribosomes associated with the ER) and passed within the lumen to the forming vacuole. This substance gives a positive reaction for acid phosphatase in many provacuoles, but never whilst it is in the ER lumen. It could be that this electron-opaque substance is at least partially protein in nature, and represents hydrolytic enzyme synthesised by the ER and passed into the developing provacuole. (The above is pure speculation but the opaque provacuoles do resemble protein storage vacuoles). Further, if the hydrolytic enzymes were synthesised within the ER, it would have to be postulated that they were in a inactive form and only became activated once the provacuole was an independent entity. This would explain why acid phosphatase activity was never found associated with the ER itself. In addition the membranes of the ER have not been shown to be resistant to the attack of hydrolytic enzymes, whereas those surrounding active lysosomal bodies obviously must be. It would thus be undesirable to have an active hydrolytic enzyme in contact with the ER membrane, and is probable that the differentiation of the ER into a provacuole includes differentiation of the ER membrane into one resistant to the attack of hydrolytic enzymes. In this respect Dürr *et al.*, (1976) have shown the inner surface of the tonoplast to have associated saccharide residues which it might be postulated, protect this membrane against hydrolytic activity from within.

It should be noted that the exact time at which the provacuole becomes typically active may differ from species to species. For example Berjak (1972), working on cress, localised acid phosphatase to the incipient provacuole whilst it was still attached to the ER cisternae. At this stage, however, the forming provacuole could be clearly distinguished from the rest of the cisternae, which exhibited no acid phosphatase activity. In this case, it would have to be assumed that the terminal portion of the ER cisterna had differentiated sufficiently to allow it to resist attack by the hydrolytic enzymes it contained, and further, that there was no back-diffusion of enzyme into the cisternae. If this is so, then the difference may be regarded as being of degree rather than kind in a similar process occurring in both cress and maize.

In maize separation of the incipient provacuole from the ER usually occurred before the former had increased in size (i.e. whilst it was still only slightly larger in diameter than the ER itself). On occasion, however, the incipient provacuole appeared to undergo considerable expansion before it actually pinched off to become independent. The above was found to occur in both 12 and 48-hour imbibed material. The observations of Berjak (1972), working on cress, and Mesquita (1969), working on Lupinus provide a precedent for this, although in those plants this method of vacuolation was common and appeared to occur predominantly, if not to the exclusion of the budding off of small vesicles (provacuoles) from the ER. Matile and Moor (1968) reported, however, that local inflations of the ER have never been observed in freeze-etched root meristems. Matile (1974) quoted Fineran (1970), who demonstrated by comparative chemical and freeze-fixation that 'the irregular shapes of vacuoles in thin sections are apparently caused by shrinkage during fixation. When shrinkage is severe, portions of the tonoplast become apposed and superficially resemble profiles of the ER'. Matile thus believes the vesiculation theory to be correct (Matile, 1974). Certainly in maize, on which

Matile and Moor carried out their investigations, vesiculation occurs far more often than 'ballooning' of the provacuole whilst still attached to the ER. It is also undoubtedly the case that shrinkage during fixation would lead to apposition of portions of the vacuolar membrane, causing them to superficially resemble ER profiles. One would, however, expect to be able to observe other manifestations of shrinkage, apart from this. For example, if shrinkage occurred during fixation one might expect the vacuolar membrane to pull away from the surrounding cytoplasm, giving the regions surrounding the shrunken portion a diluted or even featureless appearance. In no case, however, was this observed, the surrounding cytoplasm appearing densely packed and similar to any other portion of cytoplasm.

It appears that the difference between the two processes is one of degree rather than kind and lies merely in the stage of development at which the provacuole separates from the ER cisterna. Berjak (1972) is of the opinion that provacuole formation by vesiculation of the ER might be more characteristic of monocots in general. The results of Berjak (unpublished observations) working on barley roots, and of Mesquita (1970) working on Allium are in agreement with this. ER vesiculation followed by expansion of the vesiculation whilst the ER interconnections persist might in turn be more characteristic of dicots, this pattern of development having been observed in Lepidium (Berjak, 1972), Glechoma (Bowes, 1965) and in Lupinus (Mesquita, 1969). Present observations indicate that this division may not be absolute, and that both types of vesiculation may occur in a particular issue, be it monocot or dicot, although one type may predominate over the other.

4.3.1.2 *Provacuoles bounded by a double membrane*

Present observations indicate that double membrane-bound provacuoles may be produced from the ER in a variety of ways: (see 'Results' for diagrams). These appear to involve:

1. The envelopment of a portion of cytoplasm by the wrapping around of the terminal portion of an ER cisterna on itself.

2. The development of cup-shaped outgrowths at terminal portions on the ER cisternae, these outgrowths eventually enclosing a portion of cytoplasm into a double membrane-bound vesicle (provacuole).
3. The formation of cup-shaped invaginations in the cisternae of sheet-like ER, these invaginations eventually pinching off to form double membrane-bound vesicles.

The above three processes are largely similar, resulting in the formation of double membrane-bound cytoplasmic vesicles, thought to represent a form of provacuole. It appears that the space in between the two bounding membranes represents what was once the lumen of the ER. In this regard intensely electron-opaque material, similar to that observed within single membrane-bound provacuoles (which were once continuous with the ER lumen) accumulates between the two membranes. As has been discussed for single membrane-bound provacuoles, this material is thought to represent, at least in part, hydrolytic enzyme manufactured by the ribosomes associated with the rough ER, and transported (possibly in inactive form) to the site of vesicle formation. The interior of such double membrane-bound provacuoles may either contain material clearly recognisable as ground cytoplasm, or a matrix which may be of varying electron opacity. In some cases the central enclosed space contained intensely electron-opaque material (resembling that found between the two bounding membranes or within single membrane-bound provacuoles). It is not clear how this material accumulated within the central space (as it does not resemble cytoplasm unless already very degraded) but it is possible that it was secreted into the interior of the provacuole from the space between the two bounding membranes, either whilst the provacuole was still attached to the ER, or after it had separated to become a distinct entity. It is not clear, however, what the significance of this process would be, as subsequent development of the double membrane-bound provacuole appears to involve the enlargement of the space between the two bounding membranes, followed by the digestion of the

inner membrane, leaving a partially expanded provacuole indistinguishable from any other provacuole derived in the more conventional way. If indeed the inner membrane is digested away, or disintegrated, it is interesting to speculate on how this is accomplished, for the membranes surrounding components of the lytic compartment must be resistant to the attack of hydrolytic enzymes. It is possible, however, that direct communication with the cytoplasm is necessary for maintenance of such resistance (see Matile, 1975). If this is so the inner membrane, which is not in direct contact with the cytoplasm, may lose resistance and become susceptible to the attack of hydrolytic enzymes contained either between the two bounding membranes or within the central lumen. However, in the light of the discovery that the tonoplast is asymmetric (Dürr *et al.*, 1976) it is quite possible that association of the saccharide residues, which presumably must take place prior to *activity* of the hydrolases developing, occurs on the inner surface of the outer membrane only.

It is possible that some of the bodies described as double-membrane bound provacuoles may represent misinterpretations of the three-dimensional structure of single-membrane bound provacuoles as seen in this section. For example, if a single-membrane bound provacuole was not spheroidal in shape, but had flattened into a concave disc (similar to the shape of a red blood cell), a section through the disc may give the appearance of a double-membrane bound provacuole containing electron-opaque material between the two bounding membranes and cytoplasm within the central lumen.

It is unlikely, however, that such misinterpretations were commonly made as:

1. If provacuoles were commonly flattened as described, sections in other planes would reveal this flattened shape - and this was never observed in the present investigation.

2. The bodies designated as double-membrane bound provacuoles were not observed as isolated entities, but as part of a complete developmental sequence beginning with the ER and ending with the formation of apparently normal expanding single-membrane bound provacuoles.

It should also be borne in mind that the intensely electron-opaque nature of the content of a single-membrane bound provacuole tends to obscure the bounding membrane. On expansion of such a provacuole the bounding membrane appears to lift away from the electron-opaque content, which remains in the centre of the vacuole. In some cases the outer rim of this electron-opaque material appears different from the rest, sometimes giving the appearance of a membrane. If this occurs, the provacuole may appear to have two bounding membranes. Care was taken in the present investigation to avoid such confusion and the bodies designated as double-membrane bound provacuoles generally contained inner membranes whose tripartite structure was visible, as such.

It should also be pointed out that single-membrane bound provacuole encircled by a piece of ER could be confused with a double-membrane bound provacuole. Careful observation, however, would reveal three bounding membranes (as opposed to two) and thus eliminate the possibility of such confusion.

Owing to the relatively rare occurrence of this phenomenon and the fact that it was best observed in potassium permanganate-postfixed material (on which the test for acid phosphatase was not carried out), acid phosphatase activity has not been localised to these bodies. It is clear that hydrolytic enzymes must be localised cyto-chemically to these bodies before they can positively be identified

as being lysosomal in nature. If, however, this is the case (as seems likely on the basis of ultrastructural evidence) it would appear to represent a modification in kind, of the process of production of provacuoles by vesiculation of the endoplasmic reticulum, and remains compatible with the idea of the rough ER as parent membrane giving rise to vacuoles as one specialisation of the endomembrane system. The significance of the process is, however, not clear as in general evolution has tended towards cellular processes occurring by the simplest and most direct method, thus saving energy and materials. Thus unless there is some advantage to vacuolation occurring by this method, it is difficult to see why it would occur. One may speculate that the formation of provacuoles in this way allows the early inclusion of smaller or larger volumes of cytoplasm within the central lumen. Contact between this cytoplasm and the hydrolytic enzymes once contained between the two membranes allows hydrolysis of the former to occur, leading perhaps to an increase in the concentration of osmotically-active particles within the provacuole and providing an explanation for the initial expansion of such provacuoles (before they become autophagically active).

4.3.1.3 *Spherosomes*

Spherosomes are a confused class of organelles, not only with regard to whether or not they belong to the lysosomal cell compartment, but also from the morphological point of view (Matile, 1974). Reports on the presence of acid phosphatase within spherosomes are conflicting (see Introduction). It is likely that various classes of spherosomes exhibiting different functions may exist within different plants, different tissues of the same plant or even within a single cell. Acid phosphatase does not appear to be present within the spherosomes of the maize root cap (present observations: Berjak, 1968). In addition the studies of Murray (unpublished), also on the maize root cap, reveal there to be no lipase present within the spherosomes. It

thus seems likely that these bodies in the maize root cap function merely in lipid storage, and do not belong to the lysosomal cell compartment. It is, however, possible that these bodies contain other hydrolytic enzymes (not tested for).

It was with the possibility in mind that the spherosomes of the maize root cap do belong to the lysosomal cell compartment that their origin was investigated by other workers. Frey-Wyssling *et al.*, (1963) reported that spherosomes originate by vesiculation of the ER. Swartzenbach (1971) has proposed that the development of newly-formed prospherosomes proceeds by the accumulation of lipid between the two layers of the normal bounding membrane of the prospherosome. As the lipid accumulates to a greater extent the inner layer of the membrane is relegated to the centre of the spherosome, which is then bounded by only the outer half of the original bounding membrane. The present results indicate (in agreement with those of Frey-Wyssling *et al.* reported above) that spherosomes originate as vesiculations of the ER. Glutaraldehyde/osmium tetroxide fixed material did not allow the membranes of the spherosomes to be observed with much clarity. However, when the material was fixed with potassium permanganate the lipid content of the spherosomes was etched away, leaving inly the bounding membrane plus possibly a small amount of residual lipid. In such cases it often appeared as if the two halves of the bounding membrane of prospherosomes were separating from one another, although the situation was confused by the shrinkage of the spherosomal membrane. The observations, in general however, were not inconsistent with those reported by Schwartzenbach (1971).

4.3.1.4 *A note on microbodies*

There is a certain similarity between provacuoles and microbodies in thin section, and on these grounds it would perhaps be possible to confuse the two if they exist within the same cell at the same time.

Microbodies include membrane-bound organelles associated with peroxide metabolism (key-enzyme catalase) and are also termed peroxisomes. Although microbodies have been observed in a wide range of plant tissues including roots (Mollenhauer *et al.*, 1966; Huang and Beevers, 1971), biochemical and cytochemical characterization has been largely restricted to leaf and seed storage tissues. In the case of maize and barley, with carbohydrate as the major storage material, a class of microbody termed the glyoxysome (containing enzymes of the glyoxylic acid cycle in addition to catalase and other enzymes) has been found only in the scutellum (Longo and Longo, 1970 a,b) and aleuron tissue (Jones, 1972). It is noteworthy that these tissues form only a small fraction of the total seed of these species, but contain high proportions of lipid (Breidenbach, 1976). As microbodies have never been characterised in the root cap of maize it seems unlikely that the organelles designated as provacuoles could actually represent microbodies. This is confirmed by the fact that acid phosphatase, a key lysosomal marker enzyme, which is not associated with microbodies, has been localised to the organelles designated as provacuoles (present observations: Berjak, 1968).

4.3.2 *Localised hydration of the cytoplasm*

Another view of vacuole formation expressed by Muhlethaler (1966), which is mentioned by Clowes and Juniper (1968), but not by Matile (1974) is that vacuoles are formed by local hydration of the cytoplasm, and that the tonoplast develops later. It is perhaps because Muhlethaler (1966) did not describe how the tonoplast could be formed around the differentiated area, that his suggestion was not further pursued for a long while. Khera and Tilney-Basset (1976), working with young embryos of Pelargonicum X Hortorum Bailey, reported that some of the vacuoles of the cotyledonary and radicle cells appeared to be formed in this manner. During cytoplasmic differentiation all

organelles appeared to migrate away from the differentiating zone and distribute themselves within the surrounding undifferentiated cytoplasm, leaving only ground cytoplasm and ribosomes within the differentiated region. Vesicles and small cisternae, thought to be originated from the ER, subsequently surround the differentiated zone and fuse with each other, the process ending with complete sequestration of the differentiated cytoplasm. As the bounding membrane appeared double in some places, but only single in others, these workers have proposed that the two parallel membranes fuse with one another to give rise to a single bounding membrane. Subsequent to this event the sequestered cytoplasm appeared to be digested away, thus giving rise to a large vacuole.

During the course of the present study a similar process was found to occur within some of the differentiating and mature cells of the root cap of Zea mays. Cytoplasmic differentiation, as described above, was found to occur, with the differentiated region becoming surrounded by vesicles and small cisternae. Sequestration of the enclosed cytoplasm was accomplished through fusion of the surrounding vesicles. Subsequently the space between the two bounding membranes was observed to enlarge, followed by the disappearance of the inner of the two membranes. The origin of the vesicles which come to surround the cytoplasm is uncertain, the two major possibilities being their derivation from the ER or the Golgi apparatus. It is felt, however, that the vesicles were not derived from the Golgi apparatus, which (in these cells) took the form of discrete dictyosomes having no observable developmental connection with the process described here. It would thus appear more likely that such vesicles had their origin in the ER.

Khera and Tilney Basset (1976) proposed that the two membranes surrounding the differentiated portion of cytoplasm fused, thus giving rise to a single bounding membrane. In the present study the position as regards the bounding membranes was not clear-cut, but there was no evidence to support the fusion of the two bounding membranes. Equally, the evidence for the digestion or disintegration of the inner bounding membrane is circumstantial, although it is assumed that this is what occurred.

The cytoplasm, at the time at which it became completely sequestered, appeared (in different cases) to be at different stages of development. Although always devoid of organells the differentiated cytoplasm appeared to be in what might be interpreted as different stages of hydration upon sequestration; in some cases it appeared as dense as the surrounding undifferentiated cytoplasm whilst in others it was far more dilute. In this regard it is interesting to consider whether the decrease in density of the cytoplasm is due to a dilution or hydration process, or whether, in fact, it is the result of enzymatic digestion. If the apparent decrease in density occurred only after the cytoplasm had become completely surrounded by membraneous elements, it would seem likely that the decrease in density was the result of digestion of the cytoplasm by hydrolytic enzymes once contained within the surrounding vesicles, and subsequently released into the central enclosed cytoplasm. However, as the decrease in density in some cases appeared to begin before sequestration was complete, it would appear that, at least in these instances, dilution other than enzymatic digestion was involved. The basis for the above assumption is that hydrolytic enzymes are unlikely to be released into regions not surrounded by membranes, as any diffusion away from the site of action (namely the differentiating region of cytoplasm) would result in widespread cytoplasmic destruction. If, however, the decrease in density is as a result of enzymatic

digestion, it is possible that the necessary enzymes are synthesised by the ribosomes found within the differentiated region. If such enzymes were very short-lived they would perhaps not have time to diffuse and cause any significant destruction in the rest of the cytoplasm. The above is pure speculation, but could be confirmed by cytochemical tests for relevant hydrolytic enzymes. As this phenomenon was not found to occur in every root cap examined, and because the tests for phosphatase were only carried out on a limited sample size, these results are not comprehensive enough to allow comment on the present or absence of acid phosphatase during this type of vacuolation.

Marty (1976) reported that vacuoles are formed in coenocytic laticifers through the sequestration of large portions of cytoplasm by an extensive system of smooth membrane-bounded cavities arising from the mature face of the Golgi bodies. The sequestration process itself was similar to that described above, although it appeared that, in the case of the maize root cap, the ER rather than the Golgi apparatus was the source of the surrounding membranes. In addition the processes differed in that the cytoplasm in Marty's system did not appear to undergo differentiation. It should be noted that the process of cytolysome formation is essentially similar to the process described above for maize, the difference again being that cytolysome formation does not involve differentiation of the cytoplasm.

It is interesting to consider why vacuoles should be formed in this way in some of the cells of some root caps. Parallels have been drawn above to the process of vacuolation observed by Marty (1976) in coenocytic laticifers, and also to cytolysome formation. Both cytolysome formation and the process described by Marty involve the sequestration and digestion of large portions of cytoplasm, including organelles, resulting in the direct formation of relatively large

vacuoles. In the former case this may be a mechanism for the removal of damaged organelles and the subsequent recycling of their component macromolecules (as described by e.g. Villiers, 1967) whilst in the latter (coenocytic laticifers) the purpose would appear to be the large-scale removal of the cytoplasm of particular cells in order that they may serve as latex-conducting elements. The process described here differs from the above in that the organelles themselves appear to be conserved, only the cytoplasm being enclosed and digested. As the process was not observed in every root cap sectioned, but only in certain batches, fixed at particular times, it may be that it is characteristic of a particular state of differentiation of the root cap (as has been suggested by Khera and Tilney-Basset, 1976), or is, perhaps, a response to some pathological condition of the tissues, not observable at the macroscopic level. The fact that the process occurred within the differentiating and mature regions of the cap, where vacuolar *expansion* commonly occurs, suggests that it could function in the quick formation of large vacuoles, whilst preserving intact and functional organelles. However, as this process was not observed in every case it would seem that it is not the commonly-occurring one. If, however, the process occurs in response to a pathologival condition of the tissues (not observable at macroscopic level, or even at ultra-structural level apart from the phenomenon described), its function would presumably be involved in counteracting such a condition; however, it is not possible to speculate further, the existence or nature of such a condition being purely conjectural.

4.3.3 *Cytolysomes*

Villiers (1967) was the first to report the existence of cytolysomes in plant tissue; this report has since been followed by a number of others (e.g. Mesquita, 1972; see also Introduction). From these reports it appears that cytolysomes are formed as a result of pathological conditions in plant (and animal) cells, and are especially

numerous in plant embryo tissues held in the dormant state for an unusually long period of time (Villiers, 1967). Membrane-bound bodies conforming in appearance and showing a similar developmental sequence to cytolysomes have been observed in limited numbers in apparently normal 12-and 48-hour imbibed root cap cells (see Results Section). It is possible that these organelles arose in response to a pathological condition which developed within the root cap tissue during seed-storage. The seeds used were stored for at most 12 months after harvest and before germination, and would not therefore have been subject to large-scale deterioration - this would perhaps explain why these organelles if developed as a pathologic response, were present in such limited numbers.

4.3.4 *Pinocytosis*

The process of pinocytosis is not uncommon within the cells of the maize root cap (see Results Section). It is doubtful, however, whether or not pinocytotic vesicles should be classified as vacuoles. These vesicles, which originate as inward vesiculations of the plasmalemma and contain material originally found within the extra-protoplasmic space, apparently move across the cytoplasm and eventually are engulfed by a vacuole, thus ejecting their contents into the vacuole. They are thought to contain material (small bodies or large macromolecules) which could not otherwise cross the plasmalemma, and function in transporting this material to the vacuoles, which presumably contain the hydrolytic enzymes necessary for their digestion. There is no evidence to show that pinocytotic vesicles contain hydrolytic enzymes, although it should be borne in mind that, once separate from the plasmalemma, it is difficult to distinguish such a vesicle from any other small vesicle or vacuole. Whether or not pinycytotic vesicles should be considered as vacuoles may be a question of semantics, but as they have neither been shown to play a major role in the plant-water relations, nor to contain hydrolytic enzymes, they are probably best considered as transport vesicles.

In some cases it is difficult to distinguish between pinocytosis and exocytosis. This is particularly so when the apparent pinocytotic vesicle contains smaller vesicles similar to those found within the adjacent cytoplasm, and not observable within the extra-protoplasmic space. It therefore sometimes appears as if small vacuoles are fusing with the plasmalemma and ejecting their contents into the extra-protoplasmic space. According to Matile (1975) this has never been known to occur, and is unlikely as the tonoplast and plasmalemma represent differentiation of the ER in opposite directions and are therefore non-compatible according to the endomembrane theory. If small vacuoles were involved in exocytosis it would have to be postulated that their bounding membranes dedifferentiated and then redifferentiated to become plasmalemma-like. Alternatively, and perhaps more likely, the vesicles apparently involved in exocytosis may be transport vesicles which originated from the ER or the Golgi bodies(and not vacuoles).

4.3.5 *General*

Thus it appears that, at least in the root cap of maize, vacuoles may be formed by a variety of methods and it may be that one ontogenetically distinct class of vacuole is also distinguished from another by the spectrum of associated hydrolases. In this context Matile (1968) has shown that individual classes of lysosomes (provacuoles and vacuoles of differing density) appear to contain different spectra of hydrolytic enzyme. The major pathway of vacuole formation appears to be the formation of provacuoles through single or double membrane-bound vesiculations of the ER. Other pathways involve the sequestering of portions of cytoplasm (be they differentiated or undifferentiated) to form large vacuoles or cytolysomes. These are thought to occur respectively as a result of a special state of differentiation of the tissues, or in response to localised pathological conditions arising in the cytoplasm. In all cases the vacuolar membrane appeared to originate from the endoplasmic reticulum.

The intravacuolar space was in some cases continuous with the lumen of the ER, and in others appeared to be formed as a result of digestion of the enclosed cytoplasm. In the event of the intra-vacuolar space once having been continuous with the lumen of the ER, it seems likely that at least the initial supply of hydrolytic enzymes, typified by acid phosphatase, was manufactured by the ER and transported via the lumen of the ER to the forming vacuole. Where sequestering of portions of cytoplasm by vesicles (probably of ER origin) occurred, it is likely that the hydrolytic enzymes were contained within the sequestering vesicles, although the present results provide no direct evidence of this. Thus it appears that the ER plays a central rôle in most aspects of vacuole formation, this being in keeping with its rôle as the 'parent membrane' of the cell.

Other methods of vacuole formation

There is no evidence from the present studies on maize, to support the theory of Manton (1962) that provacuoles arise *de novo* in the cytoplasm of the meristematic cells, or that of Barton (1965) who maintained that certain provacuoles persist in the meristematic cells, multiplying by division, and are independent of any other organellar system for their origin.

Similarly, vacuoles were not observed to arise from the Golgi apparatus either

1. by expansion of a single dictyosomal cisterna or vesicles (e.g. Marinos, 1963; Ueda, 1966), or
2. through the formation of a GERL system (e.g. Marty, 1976).

In the case of 1, above, it has been demonstrated that the dictyosomes of the maize root cap (other than in certain pathological states) do not contain hydrolytic enzymes. Other researchers,however,working on different plant material, have localised acid phosphatases to the

vesicles of the Golgi apparatus. However, the fact that the dictyosomal vesicles do contain hydrolytic enzymes does not necessarily imply that they function as lysosomal vacuoles *per se* - they may function merely in the intercellular translocation of hydrolytic enzymes (Matile, 1975) which contribute to the vacuolar hydrolase complement when such vesicles are engulfed by the vacuoles.

It should also be remembered that certain techniques may not be suitable for revealing particular types of situation. For example, freeze-etching may not be suitable to reveal the initial stages of the process of surrounding a portion of cytoplasm by small vesicles (Khera and Tilney-Bassett,1976).Similarly serial sections, or the viewing of thicker sections with a high-voltage electron microscope, may be better than isolated thin sections for revealing three-dimensional reticulate networks or interconnections between the Golgi apparatus. Thus the limitations of the techniques used should always be borne in mind.

In conclusion, it appears that vacuolation may occur in a variety of ways, not only according to the plant, tissue, cell or state of differentiation, but also according to the purpose for which the vacuole is being formed. Differences observed between plants should be looked at not only on a functional basis, but also in an evolutionary context (Berjak, 1972).

4.4 CYTOCHEMICAL LOCALIZATION OF POLYSACCHARIDE

4.4.1 *The effect of glutaraldehyde fixation on the specifity of the cytochemical reaction*

The cytochemical methods used in this investigation to localise polysaccharide all involve:

1. The oxidation of 1,2 glycols (vic glycols, found in carbohydrate molecules) to aldehydes, with periodic acid.
2. Reaction of the oxidised materials with an alkaline silver reagent (silver methenamine or silver proteinate); this is thought to result in the formation of an electron-opaque silver-aldehyde complex, visible in the electron microscope.

Prior to cytochemistry the material is chemically fixed with glutaraldehyde. Glutaraldehyde functions as a fixative by forming chemical cross-links between adjacent protein molecules in the tissue, the aldehyde groups being the reactive groups. It is inevitable that some of the glutaraldehyde groups will only react monofunctionally, leaving the unireacted aldehyde group free within the tissue (Tramezzani *et al.*, 1964). These aldehyde groups would then be free to react with the silver stains during the cytochemical procedure, resulting in a non-specific cytoplasmic deposition of silver. Such a background was observed in the present investigation, as well as in investigations by other workers (e.g. Rambourg and Leblond, 1967; Pickett-Heaps, 1967). Swift (*loc. cit.* Pickett-Heaps, 1967) has reported, however, that glutaraldehyde reacts almost completely bifunctionally during fixation, and Pickett-Heaps (1967) after using specific aldehyde-blocking reagents such as dimedone and iodo-acetate, came to a similar conclusion. It therefore appears that the possible monofunctional reaction of glutaraldehyde is not a substantial problem

and that the explanation for the background staining may lie elsewhere.

In this regard Fahini and Drochmans (1965) (*loc. sit.* Rambourg, 1967) have reported that commercial solutions of glutaraldehyde are likely to be contaminated with the acrolein from which they are prepared. Acrolein is an unsaturated aldehyde which is known to react rapidly through its ethylene linkage with -SH, -NH and $-NH_2$ groups of proteins and with the imidazole group of histidine (van Duijn, 1961). Thus some of the non-specific staining observed after glutaraldehyde fixation may be due to the complexing of silver with the free aldehyde group of acrolein.

As non-specific staining did not present a major problem in the present investigation, it was thought desirable to use glutaraldehyde (as opposed to formaldehyde, which is a mono-aldehyde) as a fixative, as it gives a better preservation of ultrastructure. In addition it was felt that at least some of the background staining may have been due to the polysaccharides naturally occurring within the cytoplasm, as well as to the reactivity of lipids, the effect of post-staining, and the type of coupling agent and silver-stain used (see below).

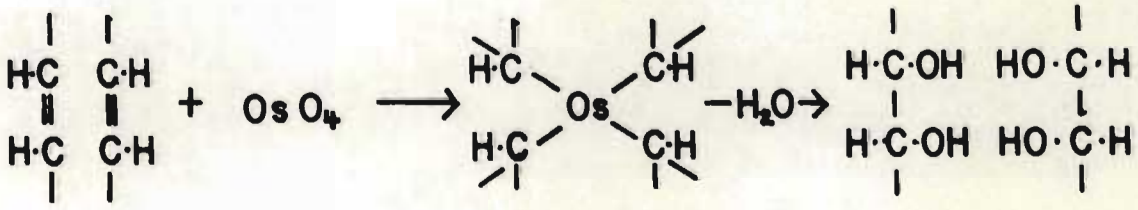
4.4.2 *The effect of post-fixation of the specificity of the reaction*

The presence of osmium in a section has been reported to result in an argentophilic reaction (Marinozzi, 1961). Thus post-fixation with osmium tetroxide prior to cytochemistry could conceivably lessen the specificity of the cytochemical reaction. However, treatment of the sections with hydrogen peroxide or periodic acid has been reported to substantially decrease the overall non-specific reaction with the silver-stain (Pickett-Heaps, 1967). As the cytochemical procedures used in the present investigation involve the treatment of the section with periodic acid, the above would not appear to present a problem. Further,

background staining (presumed to be the result of the monofunctional reaction of glutaraldehyde) has been reported to be greatly reduced if osmium tetroxide is used as a post-fixative. The reactive groups (free aldehydes and sulphydral groups) are thought to be modified or rendered inactive by exposure to the osmium post-fixative, and would thus not be free to react with the silver reagent (Pickett-Heaps, 1967).

Post-fixation with either O_sO_4 or $KMnO_4$, both powerful oxidising agents, introduces artefacts in the form of diols (Baker, personal communication).

Unsaturated lipid bonds are especially prone to such oxidation, which is thought to occur as follows (Papaphilipou, personal communication):



In this regard it was found that the lipid droplets within the cytoplasm gave an intense positive reaction after cytochemistry. They could, however, be clearly distinguished as lipid, as they gave a qualitatively different reaction (ungranular) from those other cytoplasmic constituents known to contain polysaccharide. Also, silver deposition was often found associated with membranes; this could either be due to the reactivity of the membrane lipid, or to the association of carbohydrate with the membranes (Pickett-Heaps, 1967) (it is well known that the plasmalemma has carbohydrates associated with it, perhaps in the form of glycoproteins (e.g. Whaley *et. al.*, 1972)).

This 'side-reaction' would appear to be a major disadvantage as it reduces the specificity of the reaction. It was found, however, that material not post-fixed with osmium tetroxide was poorly preserved and appeared to have undergone extensive shrinkage. In addition, owing to the lack of heavy-metal staining, cytoplasmic detail was difficult to distinguish. In the present work, in the

and better preservation, osmium tetroxide was used as a post-fixative where necessary; controls, fixed with glutaraldehyde only, were always run for comparison and gave an essentially similar pattern of silver deposition, the only difference being that the lipid did not stain.

4.4.3 *The cytochemical reaction*

The cytochemical reaction involved three basic steps:

1. Treatment of the sections with periodic acid, resulting in the oxidation of vic-glycols to aldehydes.
2. Treatment of the oxidised sections with either of the coupling agents, thiosemicarbazide or thiocarbohydrazide.
3. Staining of the treated sections with either silver methenamine or silver proteinate.

4.4.3.1 *Periodic acid oxidation*

Pickett-Heaps (1967) has reported that minor variations in the length of treatment with periodic acid do not appear to affect the cytochemical reaction. The present results are in agreement with this, as even doubling the length of the oxidation period did not appear to have any noticeable effect on the pattern of staining. It is, however, likely that marked increases in either the concentration of the periodic acid solution used or the length of the treatment will result in the oxidation of chemical groups other than vic-glycols, thus decreasing the specificity of the reaction. In this regard Pearse (1968) has suggested that oxidation times no longer than 10 minutes should be used to ensure the sole oxidation of polysaccharides, and that longer oxidations will result in the formation of PAS-positive glycolipids, phospholipids and unsaturated lipids. In the present investigations some lipid was indeed observed to react positively,

although it is felt that this may have been due to the effects of post-fixation in osmium tetroxide in addition to the fact that the oxidation time used was 20 minutes, as recommended by Thiery (1967). In fact the present experiments have revealed little observable difference for all times of oxidation up to 40 minutes, and it is thus felt that the oxidation is not a major cause of non-specific staining.

It is noteworthy that the different carbohydrate-containing organelles gives a qualitatively and presumably quantitatively different staining reaction. For example the starch of the amyloplasts reacts far more intensely than the contents of the vesicles of the hypersecretory dictyosomes; differences in granularity and intensity of stain can be seen between dictyosomal vesicles at opposite ends of the dictyosomal stack. It is felt that these differences are related to the type of monosaccharide units of which the polysaccharide is made as well as to whether or not the polysaccharide is complexed with another chemical species, as in glycoproteins or glycolipids. Chain lengths, and the concentration of the polysaccharide may also play a rôle. Monosaccharides, by themselves, contain vic-glycols, but would not give an observable positive reaction if isolated. It would appear necessary for them to be held in close proximity, as in a polysaccharide chain, and for a group of polysaccharide chains to be contained (e.g. within a vesicle) at a sufficiently high concentration before a silver aggregate, visible in the electron-microscope, would be formed. The type and linkage of the monosaccharide units constituting a chain of N-acetyl glucosamine units in a 1,4 linkage contains no vic-glycols (although a polysaccharide) and would therefore not be oxidised by periodate. The same chain, if in a 1,6 linkage, would contain one vic-glycol per monosaccharide unit, and would probably give a strong reaction (See Figure 19).

Thus the type and arrangement of the monosaccharide of the polysaccharide probably affects the quality of the staining reaction. Starch, a simple α 1,4-linked glucose chain reacts very strongly, as was noted in the present results (intense amyloplast staining). It should thus be borne in mind, when using this technique, that the final appearance of the section will depend not only on the reaction conditions used, but also on the structure of the polysaccharides present.

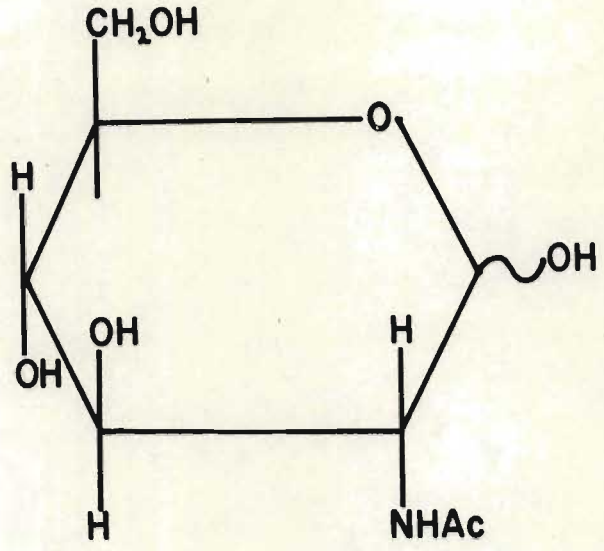
4.4.3.2 *The use of coupling agents (thiosemicarbazide and thiocarbohydrazide*

The use of thiosemicarbazide or thiocarbohydrazide as coupling agents after periodic acid-oxidation serves to increase both the specificity and the intensity of the cytochemical reaction. These compounds, in their rôle as coupling agents, are thought to react with the aldehyde groups (primarily produced by periodic acid-oxidation). The thio-radicals of these groups are then allowed to react with the silver-stain, resulting in the deposition of silver at periodic acid-oxidised sites. The apparent advantage of using these coupling reagents lies in the fact that they react *specifically* with aldehyde groups, and, after reaction, leave two reducing groups for every aldehyde reacted (Freundlich and Robards, 1974). It thus appears that they have the potential to give a two-fold intensification of the reaction.

Baker (personal communication) has reported that thiocarbohydrazide and thiosemicarbazide may be used as coupling agents with equal effect. Present results, and those of Maruyama (1974), indicate that thiocarbohydrazide gives a far greater intensification of the staining reaction than does thiosemicarbazide. It is felt, however, that the specificity of the reaction may have suffered slightly, although this is arguable. It appears that the choice of reagent

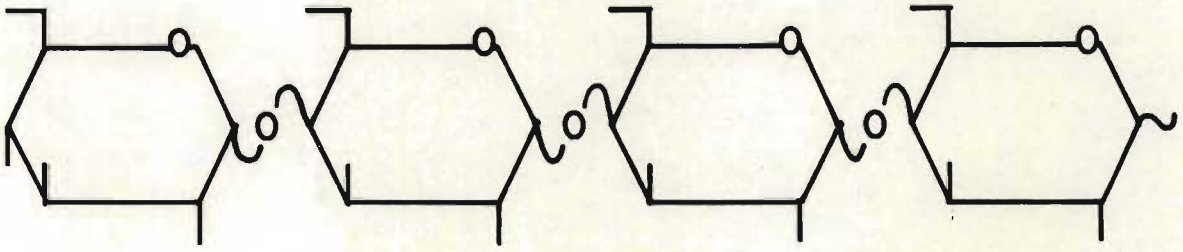
FIGURE 19

N-acetyl glucosamine

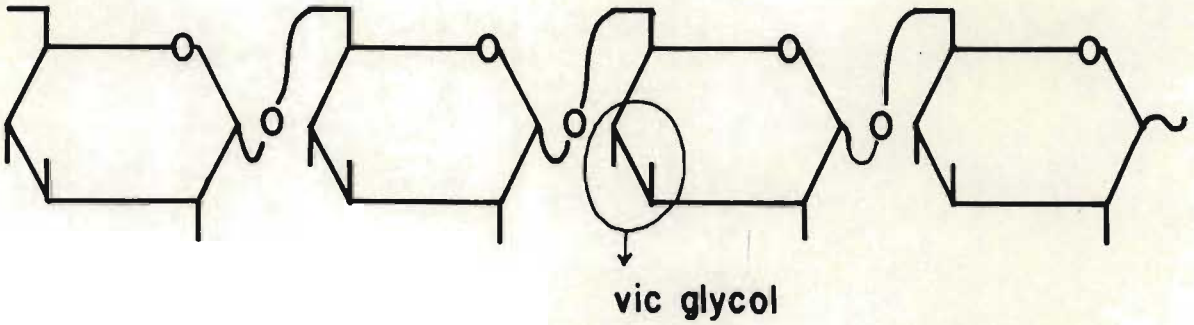


a 1,4-linked N-acetyl glucosamine chain

(contains only a terminal vic glycol)



a 1,6-linked N-acetyl glucosamine chain



vic glycol

depends on the type of polysaccharide being studied; Baker was investigating the properties of the starch-grains and extra-cellular polysaccharide of the alga Ectocarpus, both of which stain relatively intensely. If the polysaccharide is present in low concentrations or difficult to visualize, thiocarbohydrazide would be the preferred reagent.

The duration of the treatment with the coupling reagent appears to be critical up to 3 days, after which no noticeable differences in the quality of staining were observed (present results, and those of Baker, personal communication). A treatment of 1 hour is sufficient to allow the starch grains of the amyloplasts to react intensely, whereas a 3 day incubation is necsssary before the contents of the dictyosomal vesicles will stain. It is thought that this effect may be related to the complexity of the polysaccharide and the number and stereochemical availability of the free aldehyde groups. Complex mucopolysaccharides and glycoproteins, for example, require a 3-day treatment for their localization (Thiery, 1967). In this regard it would seem that the vesicles of the hypersecretory dictyosomes must contain a complex polysaccharide, or perhaps glycoprotein. The present, and other investigations (e.g. Paull, Johnson and Jones, 1975) have revealed that the dictyosomal vesicles contain a polysaccharide composed largely of underivatised monosaccharides, with a small proportion of uronic acids. Paull, Johnson and Jones (1975) found no protein associated with the isolated polymer. It would thus seem that it is not a glycoprotein; the relatively long treatment needed to reveal the presence of this polymer may be related to the types of linkages between the monosaccharide units and the degree of branching, cross-linking and folding it contains.

4.4.3.3 *The silver-stains: silver proteinate and silver methenamine: Staining with silver methenamine without prior treatment with a coupling agent*

Earlier methods for the cytochemical localization of polysaccharide using silver as an electron-stain did not involve the use of coupling agents prior to the staining step (e.g. Rambourg, 1967). Staining with silver methenamine was carried out at temperatures of between 40 and 60°C, and invariably resulted in the formation of a heavy, flocculent silver deposit over all the cellular material. Silver was not, however, deposited over the portions of the section consisting only of epoxy-resin. It is thus felt that the staining reaction must have been initiated by reactive groups (aldehyde and perhaps others) occurred within the cytoplasm; it is possible that the silver methenamine began to crystallise out, using the silver deposits resulting from the initial, presumably specific, silver deposition as 'seeds'. In this regard it was found that solutions of silver methenamine tended to crystallise out on the walls of the glass vessel in which they were stored, especially if left for more than a day. There are many precautions mentioned in the literature designed to prevent this heavy deposition of silver (e.g. Pickett-Heaps, 1967; Rambourg, 1967); however, despite observing the most scrupulous conditions of cleanliness and observing reaction times and temperatures very carefully, it was found to be almost impossible to satisfactorily control the staining reaction. The concentration of the silver methenamine did not appear to affect its behaviour to any degree either; use of a more dilute silver solution (de Martino and Zamboni, 1967) did not result in any improvement in the quality of the staining. It is the author's opinion that silver methenamine is too unstable a reagent to give consistently good results with this type of work.

Use of silver proteinate and silver methenamine as stains in conjunction with a coupling agent

After treatment of the oxidised sections with a coupling agent, it was found that silver proteinate and silver methenamine could be used as stains with almost equal effect at room temperatures (approximately 20°C). Both resulted in the formation of a finely granular, apparently specific deposition of silver over regions thought to contain polysaccharide, although the silver methenamine was prone to give a slightly more intense reaction, with more background clumping, than was the silver proteinate. However, when staining was carried out at elevated temperatures (up to 60°C) there was only a slight intensification of the silver proteinate reaction whereas the silver methenamine-treated sections contained very heavy, apparently random deposits of silver over the cytoplasm. It therefore appears that prior treatment with a coupling agent does nothing to stabilise the silver methenamine, which has an inherent tendency to crystallise out, especially at elevated temperatures.

Silver proteinate would thus seem to be the preferable reagent to use in this reaction as it is stable for long periods at elevated temperatures. The advantage of silver methenamine is that it can be readily prepared from bench-chemicals. In addition, not all sources of silver proteinate are reliable; Baker (personal communication) has recommended 'Albuminose silver', für die Mikroskopie', made by Merck (and used in this investigation). The only conditions under which it would seem reasonable to use silver methenamine would be at room temperature, preferably in conjunction with a coupling agent, if silver proteinate is not available.

4.4.3.4 *The use of grids during the cytochemical procedure*

Pickett-Heaps (1967), working with periodic acid/silver methenamine technique, reported a substantial increase in the level of random background silver deposition if the cytochemistry was carried out with the sections adhering to grids. It is thought that this could be the result of instabilities created by surface-tension forces induced by the grid bars. The present results are taken chiefly from sections treated with silver proteinate, where random background silver deposition was not a significant problem. The alternative to the use of grids, is transferring the free-floating sections from solution to solution with a wire loop; it was felt that it was unwarranted to carry out this difficult procedure when there was only the possibility of a very slight improvement in results.

Note

The reason for using gold, as opposed to copper electron microscope grids is that copper is readily oxidised by periodic acid.

4.4.4 *Post-Staining*

For the purpose of the present investigation it was necessary to be able to resolve clearly both the dictyosomal vesicles and the vacuoles. To this end most sections were post-stained with lead citrate both before and after cytochemical treatment. In the former case the resolution of cytoplasmic detail was not significantly improved, probably because the lead deposits were leached from the section during the cytochemistry (especially during the periodate oxidation). In the latter case, however, the cytoplasm could be visualised in detail. As long as the results were interpreted by considering them in conjunction with the images seen on sections which had not been post-stained, it was felt that little confusion would arise owing to the added presence of lead atoms on the section. An added

disadvantage of post-staining is that it tended to decrease slightly the intensity of the silver-stained regions; this effect was not, however, very marked.

4.5 DEVELOPMENT AND FUNCTIONS OF THE VACUOLAR SYSTEM

4.5.1 *The early development of provacuoles: The accumulation of provacuoles in the meristem of 12-hour imbibed tissue*

The average meristematic cell of a 12-hour imbibed embryo contained a large number (approximately 790) of relatively large (average diameter 320 nm) provacuoles. This is in marked contrast to the meristematic region of the 48-hour imbibed embryo, where the cells appeared to contain approximately one tenth of this number of provacuoles; the provacuoles that were present were also slightly smaller. This is thought to reflect the fact that the embryos were stored in the quiescent state; it would appear logical for the quiescent embryo to contain an accumulation of organelles so that, on imbibition, active growth and development can commence quickly. The quiescent embryo only becomes fully imbibed after 12 hours of soaking in water (Berjak, 1968) - thus at this stage active metabolism is probably only just beginning, and one would expect to see an accumulation of provacuoles within the meristem. After 48 hours the radicle has attained a length of approximately 2 cm and to the root cap is undergoing rapid development (it turns over completely every 24 hours. (Clowes, 1970)). It is possible that the accumulation of provacuoles in the 12-hour meristem at least in part (because of vacuolar implication in expansion growth) served to lay the foundation for the sudden rapid development of the radicle and root cap, which would enable the seed to become quickly established within the soil.

Association between provacuoles and the ER

Many of the provacuoles of the meristematic and especially immediately post-meristematic region of the root cap of the 12-hour imbibed embryo appears to be encircled by a single ER cisterna. The provacuole

and ER appear to be separated by a constant gap, approximately equal to the diameter of the ER cisterna itself (Berjak, 1968; Matile and Moor, 1967; Fineran, 1971; and present observations). Further, this phenomenon has not been observed in 48-hour imbibed maize embryos. The fact that this association can be observed with regularity in root cap cells at a particular stage of development suggests that there is a functional relationship between these two cell components, and that this function is related to the stage of development of the root cap. Several suggestions have been put forward:

1. Fineran (1971) has suggested that hydrolases, synthesised by the ER, are transported in this way to the developing vacuole. This suggestion would appear feasible, although a mechanism would have to be postulated whereby the enzyme molecules secreted from the ER enter the provacuole in the apparent absence of any direct connections between the two.

   In this regard the surrounding sheath of ER could prevent diffusion of (possibly) active hydrolytic enzymes into the cytoplasm. Lodish *et. al.*, (1975) postulated that proteins intended for cytoplasmic use are synthesised on membrane-free polysomes whereas proteins intended for export (either out of the cell or to specific membrane-bound organelles) are synthesised on membrane-bound polysomes. Dobberstein, Blobel and Chua (1977), using *in vitro* translation techniques, have found that the secretory proteins of a variety of organisms all contain a similar N-terminal 'leader' sequence consisting of 15 to 16 hydrophobic amino acids. In his 'Signal Hypothesis' Blobel has postulated the existence of a receptor, specific for the 'leader' sequence, within the membrane through which the protein is to be transported. Binding of this sequence to the receptor, subsequent cleavage of the leader sequence from the rest of the protein by membrane-associated protease and a conformational change

would result in the transport of the protein through the membrane. The close association between the ER (with membrane-bound polysomes) and the provacuole seems to suggest that hydrolytic enzymes manufactured on the ER may be transported in this way into the vacuole. It is clear, however, that no evidence exists as yet to support the presence of such a leader sequence on the hydrolytic enzymes (if they are indeed synthesised during this developmental phase) or the presence of a receptor within the provacuolar membrane. This mechanism, however, would account for the *specific* transfer of enzymes to the provacuole, and for the prevention of back-leakage of hydrolytic enzymes into the cytoplasm.

It should be remembered that, in maize, the association between ER and provacuoles, occurs only in 12 (and not 48)-hour imbibed embryos. Thus if this is a mechanism for transport of hydrolases, it is specific to early imbibition stages. This implies that there must be at least one other mechanism for transport of hydrolases to the vacuoles, as this process may also occur in the later imbibed embryo. It is possible that the provacuolar enzymes undergo degradation or loss of activity during maturation and/or quiescence of the seed, and that the close association allows for the quick synthesis and transport of enzymes to the preformed provacuoles once the necessary water-content has been reached. This would be an alternative to the presumably much slower process of forming provacuoles *de novo* by vesiculation of the ER and then allowing for them to increase in size.

For a further discussion of the transport of hydrolases to the vacuoles (see Section 4.5.3).

2. Fineran (1972) has also postulated that ER-ensheathing of provacuoles may function as a transport system for non-protein substances to and/or from the provacuole. In this regard he reports that the ER may possibly supply water to enlarging vacuoles during periods of rapid cell-expansion. This may be possible although there appears to be no clear precedent for the transport of water via the ER; in addition present observations indicate that ensheathing of expanding provacuoles occurred only seldom, and ensheathing of obviously expanding vacuole was not observed. Vacuolar expansion occurs to a far greater extent in 48-hour imbibed embryos, where ensheathing does not occur. It would seem more likely that osmotic changes within the vacuole are responsible for the influx of water and increase in size of the vacuole. Fineran also postulates that the 'extensive area of ER in close proximity to the vacuole may also serve as a 'collecting centre' for substances withdrawn from the vacuole and their subsequent transport to sites of utilization elsewhere. He further states that this could be important for vacuoles functioning as lysosomes as a means of rapid redistribution of products derived from lysosomal activity. However, this is unlikely in the case of the provacuole/ER association during early imbibition, as functioning of the former in the capacity of a lytic compartment (judging from autophagic activity) seems not to occur until a measure of expansion has taken place.

3. Berjak and Villiers (1970) have suggested that ER-enwrapping of provacuoles may function in providing a localised supply of energy to provacuoles. They cited Bonner (1961), who has

suggested that plant cells have a pathway of electron transport alternative to cytochrome oxidase, and linked with cytochrome b_7, which is associated with the ER. This would perhaps explain why the enwrapping phenomenon is restricted to the 12-hour imbibed seed, where active metabolism is presumably only just commencing. It is strange, however, that the other organelles were very seldom observed in close proximity to the ER, although they would, presumably, be just as much in need of energy. It has been subsequently suggested, however, that the ER-associated cytochrome b_7 does not function in energy transfer (Woolhouse, personal communication). In this regard too, Novikoff and Shin (1964) who observed an association between ER and hepatic microbodies, suggested that this occurred because the microbodies themselves were formed from the ER. Provacuoles are also ER-derived, and it seems likely that the enwrapping phenomenon represents a renewal of 'supplies', diminished during quiescence (but originally supplied to the provacuole by the ER during its genesis) to the provacuole. However, the present work has shown that it is unlikely that this relationship has to do with the actual formation of the provacuole from the ER.

Expansion of provacuoles

It is likely that an increase in the number of osmotically active (solute) molecules would precede the uptake of water into the provacuole, thereby maintaing its stability. It is interesting to speculate on the means by which this solute-accumulation might occur:

1. Hydrolysis of macromolecules would result in an accumulation of their 'building blocks' within the provacuole. Acid phosphatase is known to be present within these provacuoles, presumably in an active form (present results and, e.g. Berjak, 1968). Other hydrolytic enzymes are also likely to be present. If, however, the active enzyme was present

before the expansion began, it must be postulated that the macromolecular substrate was either not present at that stage, or was present in a form not accessible to enzymatic attack. In both cases difficulties arise, as in the former case it would be necessary to postulate the transport of macromolecules to the provacuole (perhaps by ER-enwrapping?), whilst in the latter it would be necessary to explain the nature of the signal causing a presumed change in conformation of the macromolecules.

The reverse argument also applies - if the substrates were present before expansion begam it would be necesary to explain the transport of hydrolytic enzymes to the provacuole (perhaps by ER-enwrapping), or, if they already existed within the provacuole, their activation.

2. Alternatively, small molecules and charged species might be transported across the membrane into the provacuole, perhaps by an active, permease-mediated process (Bidwell, 1974). As has been mentioned in the Introduction, a few specific exchange-transport systems within the vacuolar membranes of certain plants have been characterised (e.g. by Boller *et. al.*, 1975). The presence of a one-way 'pump' for the transport of molecules into the provacuole has not, however, been reported. According to Matile (1976), it is possible that charged solutes may be trapped by non-diffusible counter-ions present within the vacuole, thus resulting in a further accumulation of solutes within the vacuole. Clearly, however, no specific evidence exists as yet to explain the means by which early expansion of provacuoles occurs.

4.5.2 *Fusion of vacuoles*

Partially expanded vacuoles, formed by expansion of provacuoles, unergo extensive fusion, particularly in the differentiating region of the 48 hour-imbibed root cap.

Membranes undergoing fusion should have similar chemical compositions (de Duve and Wattiaux, 1966). This would appear to pose no problem, as most vacuoles are derived from the ER, and would thus have compatible membranes. Fusion occurs between vacuoles of similar size; it is likely that their membranes would be at an equivalent stage of development or differentiation (to a tonoplast-like membrane) and thus chemically compatible. It is interesting that the large vacuoles of the mature region appear to engulf, rather than fuse with provacuoles. These two types of vacuole represent late and early stages in vacuolar development, and it may be that fusion between them is not possible owing to chemical incompatibility.

A change in the physical properties of the membrane is necessary in order to accelerate the fusion process (Lucy, 1969). Dingle (1968) has proposed that individual membrane-bound vesicles will fuse only at high values of surface tension. The smaller the vesicle, the greater will be the degree of curvature of the membrane and the higher the surface tension. Vacuoles, being relatively large, would not have a great degree of curvature. A greatly increased curvature in a portion of the vacuolar membrane would result in a local reduction in the stability of that membrane, and thus possibly allow the initiation of membrane fusion. In this regard, fusion of vacuoles in the maize root cap appeared to begin with the formation of small protruberances, of high curvature, on neighbouring vacuoles. Subsequent contact between these protruberances resulted in the fusion of the two membranes. Perhaps localised stress caused the formation of perturbations in the lipid bilayer, disorganising

the structure sufficiently to allow intermingling of lipid moieties from the two membranes. Dingle (1968) has further explained the physical process of membrane fusion in terms of a micellar model for membrane lipids, not currently accepted. The real mystery is what causes the perturbation to form.

Possible (indirect) involvement of the vacuole in geotropism

The partially-expanded, fusing vacuoles of the differentiating region of the 48-hour imbibed root cap were located in the portion of the cell proximal to the meristem, whereas the amyloplasts appeared to be located in the distal region of the cell. It would appear that the least dense and perhaps most dense cell components adopt these relative positions, and, in this respect, e.g. Iversen (1969) has shown the root tip amyloplasts to function as statoliths determining geotropic response.

4.5.3 *Transport of hydrolases to the vacuole*

Matile (1968) has shown that at least four populations of lysosome (characterised on the basis of differing buoyant densities) can be obtained from a cell-free extract of maize root tips. Using the freeze-etch technique he has shown that these populations correspond to different developmental stages of the vacuoles (lysosomes) within the root tip. Each class of lysosome appears to contain a different spectrum of hydrolytic enzymes - this implies that additional hydrolases must be synthesised by, or transported to the vacuole during the course of its development.

It is at least possible that the initial supply of vacuolar hydrolytic enzymes is synthesised by the ER and transported to the incipient provacuole as it is being formed from the ER. In this regard acid phosphatase activity has been localised to the newly formed provacuoles (although this does not indicate that they contain other hydrolases as well). Most enzymes are, however, labile, and may need to be replaced

during the lifespan of the vacuole. The maize root-cap may be an exception to this, as each cell has a lifespan of only 24 hours (Clowes, 1971). Thus if the vacuolar hydrolases were stable for this relatively short time-span, they may not need to be replaced. It is still, however, necessary to explain how different types of hydrolases are synthesised and transported to the vacuoles during their development.

1. Possible synthesis of hydrolases within the vacuole

Vacuoles have been shown to contain at least some of the chemical species necessary for protein synthesis. For example Matile (1974) has reported that they contain RNA and amino-acids. However, if protein synthesis were to occur within the vacuole one would expect to find intact ribosomes, mRNA, initiation and elongation factors and a supply of GTP within the vacuole. It is, additionally, extremely unlikely that hydrolytic enzymes are synthesised within the vacuole, as the pH (approximately 5.5) (Matile, (1974) would probably preclude any synthetic process and proteases present within the vacuole would be likely to degrade any protein product newly-synthesised, unless it was somehow protected.

2. Possible synthesis of hydrolases by polysomes associated with the vacuolar membrane

The present investigation did not reveal polysomes associated with the vacuolar membrane. The thin-sectioning techniques used may not have been suitable for the detection of a perhaps relatively small number of polysomes on the membrane. Matile (1968), using the freeze-etch technique, has shown the existence of ribosome-like particles on the cytoplasmic side

of the vacuolar membranes of the root-tip cells of maize (although these might have been cytoplasmic) and Wiemken (1969; *loc. cit.* Matile, 1975), working on isolated yeast vacuoles, has reported a similar association. Thus it may be possible that vacuolar hydrolases are synthesised on the exterior of the vacuolar membrane, and then transported into the vacuole. However, Berjak (personal communication) is of the opinion that the ER becomes abruptly degranulated at a position shortly behind its vesiculation to form a provacuole, the latter appearing consistently free of associated ribosomes.

3. Possible dictyosomal transport of hydrolases

Hydrolases, synthesised by the ER, may be transported via the dictyosomes (a transitional derivative of the ER) to the vacuoles. Although acid phosphatase activity has been localised to the dictyosomal vesicles of the root tip of a number of plants (e.g. by Coulomb and Coulomb, 1973, working on Curcurbita) it has not been found associated with the dictyosomes of the healthy maize root cap. Equally, there is no evidence for the presence of any other hydrolytic enzyme within the dictosomal vesicles of the maize root cap. It is possible, however, that such enzymes are present in a nonactive precursor form awaiting activation within the vacuole, perhaps by a specific enzymatic cleavage. Certainly there is ample evidence that dictysomal vesicles are engulfed by the maize root-cap vacuoles from the early-mature stage onwards (e.g. Matile and Moor 1968; Berjak, 1968, present observations).

Many vacuolar hydrolases, particularly the glycosidases, are glycoproteins (Matile, 1975). The Golgi apparatus is known to be involved in synthesis, storage and secretory aspects of

carbohydrate metabolism (e.g. Mollenhauer and Morré, 1966; Maruyama, 1974). It is thus possible that hydrolases synthesised by the ER, are glycosylated within the dictyosomal vesicles during transport to the vacuole. The yeast vacuole has not, however, been observed to engulf dictyosomal vesicles (Wiemken, 1969, *loc. cit.* Matile, 1975). Matile thus considered it likely that *in yeast* vacuolar hydrolases are synthesised solely by tonoplast-associated polysomes, and concludes that a Golgi step is not essential for their glycosylation.

Some interesting work has recently been done with animal lysosomal hydrolases. It appears that the terminal sugar residues attached to lysosomal glycosidases (all of which are glycoproteins) are essential for their recognition by and uptake into cultured fibroblasts (Neufield, *loc. cit.* Hughes and Sharon, 1978). Inhibitor studies indicate that the terminal sugar residues are recognised by a specific membrane receptor. It has been postulated that this recognition system is the major pathway for the cycling of lysosomal enzymes from synthetic sites to the outside of the cell, and their re-entry into lysosomal vesicles (Hughes, 1977). This theory may be interesting if applied to plants, although there is no evidence to support the existence of receptors on vacuolar membranes. For example, precursor hydrolases might be synthesised by the ER, glycosylated by a series of specific enzymes (not necessarily within the Golgi apparatus) and then diffuse through the cytoplasm until they recognise a specific receptor on the vacuolar membrane. *In vitro* studies on enzyme uptake by plant vacuoles might be used to investigate this coupled with e.g. periodate oxidation to degrade the sugar residues on the glycoprotein, which could then be tested for

ability to be taken up by the vacuole. Inhibitor studies with sugars may also prove useful.

4.5.4 *The integrity of the vacuolar membrane*

The plant vacuole is an organelle containing a wide variety of hydrolytic enzymes, including proteases (see e.g. Matile, 1975). The vacuolar membrane must, therefore, have some special property which prevents it from being digested by the very enzymes it contains.

Matile (1975) has postulated that the maintenance of tonoplast integrity depends on a continuous supply of membrane constituents and energy from the surrounding cytoplasm. This seems likely in view of the fact that, after autophagy, the portion of the tonoplast which has become separated from the main body of this membrane undergoes degradation within the vacuole.

There is evidence to suggest that many, if not all, of the lysosomal enzymes may normally be attached in some way to the inside of the vacuolar membrane (see review by Lucy, 1969). Such a non-catalytic interaction may prevent the formation of an enzyme-membrane complex that would lead to hydrolysis (of the membrane).

The present investigation, and others (e.g. Berjak, 1968) have shown that acid phosphatase appears to be present within the interior of the vacuole, apparently associated with the electron-opaque matrix commonly found within the vacuole. This may be an artefact due to chemical fixation and cytochemical procedures having loosened the enzymes from their attachment to the tonoplast. On the other hand the electron-opaque matrix may represent some non-hydrolyzable conglomerate within the vacuole, to which the enzyme is attached, thus protecting it from hydrolytic attack. There is, however, a substantial

body of evidence (see Matile, 1975) to show that, on tissue homogenization (which involves disruption of the vacuolar membrane) many lysosomal enzymes can be recovered chiefly in the soluble fraction. This probably indicates that they were not bound, although, had they been bound, the fractionation procedure may have released them.

Durr *et. al.* (1976) investigated the binding of the lectin, Concanavalin A, to the yeast vacuolar membrane. Concanavalin A is a widely used marker for the localization of saccharide residues on membrane surfaces. They found that tritium-labelled Con. A bound only to the *inside* of the vacuolar membrane and the *outside* of the plasmalemma, which suggests that both membranes are asymmetrical with respect to saccharide distribution. It is notable that both the interior of the vacuole and the extra-protoplasmic space are recognised lytic compartments (Matile, 1974). It is possible that the saccharide residues protect the membrane from hydrolysis, although it would have to be postulated that there are no hydrolases present in the vacuole which are capable of breaking down those saccharide chains, or than they are sterically protected from hydrolysis through their attachment either to the membrane or each other.

4.5.5 *Autophagy*

In the late-mature and early-senescing regions of the root cap vacuolar fusion has proceeded to a great extent; the cells contain a few large vacuoles which are very active autophagically.

Autophagy appears to begin with an inward invagination of the vacuolar membrane, surrounding the organelle to be engulfed. This invagination subsequently separates from the vacuolar membrane, forming an intravacuolar vesicle which subsequently undergoes degradation, presumably by hydrolysis, within the vacuole. Coulomb (1973) has

suggested that invaginations of the vacuolar membrane, especially when they appear empty, or contain myelin-like membraneous whorls, are artefact of fixation. This phenomenon, however, is equally evident in freeze- etched root-tip cells, which are not chemically fixed and therefore not subject to artefacts of fixation. In this regard the convex fracture-face of invaginations contains a much more concentrated population of globular particles (approximately 10 nm in diameter) when compared with the non-invaginating portions of the membrane. Although the function of these particles is unknown, their crowding at sites of invagination suggests that the invagination is a real phenomenon, and perhaps indicates an active rôle for the vacuolar membrane in autophagic engulfment (Matile, 1975).

Autophagy in the mature and senescing regions of the root cap is an extensive phenomenon, with the complete spectrum of cellular organelles, both damaged and apparently (visibly) undamaged , being subject to engulfment. This raises several questions:

Firsly, what is the significance of autophagy in rélation to the development of the root-cap? It should be remembered that the ultimate fate of a root-cap cell is autolysis, after which it is sloughed from the cap. Matile (1975) states that the significance of autophagy in the root tip is not clear, as the biochemical correlate of this process has not been elucidated. He speculates that autophagy is the morphological counterpart of what, in biochemical investigations, appears as the degradative phase of turnover. In this regard, Berjak and Villiers (1970) have reported a sharp increase in the rate of protein synthesis (as measured by the incorporation of tritrated leucine) in the senescing region of the maize root cap. They are of the opinion that this could at least in part, reflect a dramatic increase in the synthesis of hydrolytic enzymes, in preparation for autolysis. If the amino acid pool was relatively small, it would be

necessary to obtain a fresh supply of precursor amino acids before any major synthetic process could begin. While autophagy may, in part, be thus involved, it would seem, however, that the (autophagic) process is too extensive to occur merely as a supply of amino acids for this purpose. In this regard it is at least possible that autophagy functions in part as a salvage process. Rather than losing all the cellular building-blocks during the sloughing of the cell, they may diffuse upward into the younger regions of the root cap, where they may be recycled.

Autolysis occurs in under 3 hours, thus necessitating extensive enzymatic degradation within a relatively short time-span. The occurrence of autophagy, prior to autolysis, may serve to reduce the number of hydrolytic events necessary for the ultimate completion of autolysis (Berjak, personal communication).

The second question is - how is authophagy controlled? Both damaged and apparently undamaged organelles are engulfed - is there any specificity in this process, or are organelles selected for engulfment by random contact with the vacuolar membrane? As autophagy occurs extensively in the senescing region of the cap, it seems unlikely that it is a random process as far as cell type is concerned. In some cases (present results) an organelle being engulfed appeared to form a protrusion which became surrounded by an invagination of the vacuolar membrane before the rest of the organelle was engulfed. Villiers (1971) observed a similar phenomenon in Fraxinus seeds subjected to long periods of artificially-imposed domancy. He interprets this as active participation by the organelle in its own engulfment. Matile (1975) has speculated that such damaged organelles may release denatured protein molecules into the cytoplasm, and be recognised for elimination in this way. It may be that organelles which appear morphologically undamaged do in fact have a biochemical malfunction, and are similarly recognised for autophagy. In the context of the maize root-cap, however, the

engulfment and breakdown of undamaged organelles can be rationalised in terms of the preparation for autolysis and possibly the need to recycle cellular building blocks to younger regions of the root-cap.

4.5.6 *Vacuolar swelling in the zone of senescence*

Vacuolar fusion in the zone of senescence has proceeded to the stage where the cells contain generally just one, or a few large vacuoles. As was discussed in the previous section, these vacuoles are extremely active autophagically. The dictyosomes of the late-mature and senescing regions are hypersecretory, and produce many large vesicles which apparently contain polysaccharide (see below). These vesicles appear to follow two paths:

1. They move towards the plasmalemma, with which they fuse and, by a process of exocytosis, secrete their content into the extra-protoplasmic space. The secretion (polysaccharide slime) builds up in the extra-protoplasmic space, ultimately penetrating the cell wall and accumulating as a droplet on the outside of the root cap.
2. Dictyosomal vesicles appear to be engulfed by and broken down within the vacuole. At this stage the vacuole undergoes a noticeable increase in size. It has been postulated (Berjak and Villiers, 1970) that hydrolysis of the polysaccharide contained within the vesicles to mono- or oligosaccharide units results in an increase in solute concentration within the vacuole, thus lowering the water potential and leading to an influx of water into the vacuole and its consequent swelling. In order to test this theory an attempt has been made to analyse the sugar content of the vacuoles, particularly of the senescing region. The techniques employed were cytochemistry,

autoradiography, and chromatography; the results obtained will be discussed under the heading of the technique used to obtain them. It should be noted that a lot of data, not directly relevant to the theory of vacuolar swelling, was obtained during the investigation. Although these data have been reported in the Results section for the sake of clarity and completeness, they will not be discussed here unless directly relevant.

4.5.6.1 *Cytochemical localization of polysaccharide*

Polysaccharides were localized cytochemically using periodic acid-oxidation in conjunction with silver as an electron stain (see Section 4.4).

Although the dictyosomal vesicles, even in the meristematic region, were observed to react positively, substantial amounts of polysaccharides were observed within the vesicles only when the dictyosomes became hypersecretory. This is in agreement with the result of Paull and Jones (1975). Further, it was apparent that by far the major pathway was towards the secretion of the dictyosomal polysaccharide into the extra-protoplasmic space - this was confirmed by the autoradiographic results (see below). At no stage of development was there an obvious accumulation of polysaccharide within the vacuoles, although positively-stained dictyosomal vesicles were engulfed by the vacuole in substantial numbers. It was possible to observe the polysaccharide immediately after engulfment, whilst the dictyosomal vesicle was still membrane-bound, but it appeared to be rapidly hydrolysed once the bounding membrane had undergone dissolution. The large vacuoles of the senescing region were sometimes observed to contain a very light, general silver deposition, which may have been indicative of the

presence of a very dilute polysaccharide matrix within the vacuole. The results, however, would tend to confirm the dictyosomal polysaccharide is broken down very quickly after entry into the vacuole, and does not accumulate as polysaccharide to any great extent, as it does in the extra-protoplasmic space and on the exterior of the root cap.

4.5.6.2 *Incorporation of (^{3}H) L-fucose into the root cap*

Previous studies (Paull, Johnson and Jones, 1975) have indicated that fucose is the major component of the maize-root polysaccharide-slime, although other sugars are present in substantial amounts. Glucose and galactose, however, are readily metabolised by plant cells, and would thus be unlikely to provide specific labelling of this polysaccharide. Kirby and Roberts (1971) utilised tritiated fucose in an autoradiographic study of slime secretion, and reported that very few silver grains occurred in regions other than the extra-protoplasmic space, and over the dictyosomes. Tritrated fucose was therefore chosen as a label with which to follow specifically the fate of the dictyosomal polysaccharide. Autoradiography is probably not suited to the detection of an accumulation of monosaccharides within the vacuole, as it is likely that small molecules will be washed out of the tissue during preparation for electron microscopy. Thus any label detected within the vacuole is probably incorporated into oligo- or polysaccharides. Light-microscopic autoradiography indicates that cellular incorporation of label in the senescing region of the cap had reached approximately half its maximal level after 3 hours, the incorporation approximately doubling during the following 9 hours. Further, autophagic engulfment of polysaccharide-containg dictyosomal vesicles begins in the mature region of the cap and continues until autolysis begins, a period of approximately 3 hours. Hence if an accumulation of label within the vacuoles (apparently the minor pathway), was to be detected, it was thought desirable to use incubation periods of between 3 and 12 hours, and to use label of as high a specific activity as possible.

Light microscope autoradiography was useful in estimating the general level of cytoplasmic label, but resolution was not good enough to allow silver grains to be assigned unequivocally to any particular organelle. Thus although an attempt has been made to quantify the results, the figures reported should be regarded as estimates, rather than absolute values.

A low level of cytoplasmic label can be detected over both the meristematic and senescing regions of the cap after only 10 minutes of incorporation. Northcote and Pickett-Heaps (1966), who followed the incorporation of tritrated glucose into the maize root cap at electron microscopic level, have reported that the initial label is associated almost extensively with the dictyosomes. It seems likely that the label has been incorporated into the dictyosomal polysaccharide. After 30 minutes of incorporation label can be detected over the extra-protoplasmic space. In this regard Paull and Jones (1975) have reported that there is a 30-minute delay between the first appearance of label over the dictyosomes, and its accumulation over the extra-protoplasmic space. After one hour of incorporation, the level of cytoplasmic label over both the meristematic and senescing regions of the cap has increased considerably. This is thought to reflect an increase in the amount of label incorporated into the dictyosomal polysaccharide. In addition, after 1 hour, a considerable accumulation of label is visible over the extra-protoplasmic space, and label has begun to appear in the cell walls.

The level of cytoplasmic label over the meristematic region was observed to reach a plateau after one hour, whereas incorporation into the senescing region approximately doubled during a following 11 hours of incubation. Although the dictyosomes of the meristematic region produce polysaccharide (see previous section - Cytochemistry), it is chiefly destined for incorporation into the cell wall, and the dictyosomes remain relatively small. The dictyosomes of the senescing

region, however, produce large vesicles and are active in polysaccharide secretion, both into the vacuoles as well as into the extra-protoplasmic space and cell wall. After prolonged incubation (12 hours) light microscopy revealed that a considerable amount of the cellular label was associated with the vacuoles. Thus the apparent accumulation of cytoplasmic label over the senescing, but not the meristematic, region could be due, at least in part, to the accumulation of polysaccharide within the vacuole. At the electron microscope level it appeared that the largest proportion of the cytoplasmic label (39%) was associated with the dictyosomes after 12 hours of incorporation. Roughly 30% of the cytoplasmic label was vacuole-associated. Cytochemical results (see previous section) have indicated that the dictyosomal polysaccharide is hydrolysed very quickly after incorporation into the vacuole. This label is thought to represent, at least in part, unhydrolysed polysaccharide, as well as oligosaccharide units within the vacuole. As was previously mentioned, it is likely that any labelled monosaccharides would have been washed out of the tissue during preparation for electron microscopy. If the chemically-fixed vacuolar membrane was resistant to the free passage of monosaccharides out of the vacuole, some of the label would represent monosaccharide units. Although they may have been derived at least in part from hydrolysis of the dictyosomally-secreted polysaccharide, it is possible that these monosaccharide units entered the vacuole through a transport mechanism in the vacuolar membrane, presumably for storage in the vacuolar pool.

Pulse-chase experiments

Material was pulsed with tritrated fucose for 3 hours, and then chased with cold fucose for periods of up to 2,25 hours in an attempt to follow the temporal fate of the dictyosomally-synthesised polysaccharide.

The levels of incorporation of label into both the meristematic and senescing regions of the cap appeared to decrease with increase in length of the chase (see Figure 13). The level of cytoplasmic label over the meristem was reduced to 30% of its original level after 30 minutes, and during the next 1,75 hours to 21% of the initial level. Incorporation into the senescing region was consistently higher than into the meristem, as would be expected on the basis of large population of hypersecretory dictyosomes in this region. The level of cytoplasmic label over the senescing region did not appear to decrease as dramatically as was the case in the meristem, reducing to 60% of the original level after 30 minutes of chase, and to 54% during a further 1,75 hours of chase.

The significant decrease in cellular labelling which occurs after a 30 minutes chase period is in agreement with the results of Paull and Jones (1975), who, in a similar experiment, found that it took 20 to 30 minutes for label to travel from its site of synthesis (dictyosomal vesicle) to the extra-protoplasmic space. The fact that the level of cytoplasmic label did not decrease as dramatically on chasing in the senescing region as it did in the meristem, may reflect the trapping of fucose within the vacuoles of the senescing region. Present results indicate that the fucose- containing dictyosomal-polysaccharide enters the vacuole in substantial quantities. Thus, as has been previously mentioned, vacuolar label may represent unhydrolysed polysaccharide, or oligosaccharide units.

Further, it should be noted that, as the level of cytoplasmic label decreased during the chase, a corresponding increase in label associated with the cell wall and extra-protoplasmic space was observed (Figure 14).

This observation has been made by other investigators (e.g. Paull and Jones, 1975) and probably reflects the major pathway of the dictyosomal vesicles - towards fusion with the plasmalemma and secretion of their content into the extra-protoplasmic space.

4.5.6.3 *Chromatography*

Root tip vacuoles were isolated and lysed and their contents analysed chromatographically in an attempt to detect a possible accumulation of monosaccharide within them. Owing to lack of time the experiments had to be terminated before the systems could be perfected, thus only a limited amount of information is available.

Isolated root tip vacuoles were fractionated into 3 bands, on sorbitol density gradients according to the procedure of Matile and and Berjak (personal communication). The 3 bands contained vacuoles of different sizes (and densities) and are thought to correspond to provacuoles (most dense), partially-expanded vacuoles and fully expanded vacuoles (least dense) (Berjak, personal communication). The purpose of the experiment was to compare the relative monosaccharide contents of the three classes of vacuole, in an attempt to detect a possible accumulation of monosaccharides within the largest vacuole class. Such an accumulation, if it existed, may have reflected hydrolysis of dictyosomally-derived polysaccharide within the vacuoles of the senescing region.

A mixed, unfractionated vacuolar isolate was lysed, and the monosaccharide content analysed in a paper-chromatographic system (see Results section). Fucose, glucose and galactose were detected in this sample (Table 6). Glucose and sorbitol were detected in the final washings carried out during vacuolar purifications. The presence of

sorbitol in the washings is to be expected as the homogenization medium contained a high concentration of this sugar alocohol. The presence of glucose in the washings, however, indicates that this sugar may have been a contaminant, and was not really derived from the contents of the vacuoles. Further, fucose was detected in an unlysed vacuolar sample. This could represent fucose which had leaked out of the vacuole, or otherwise could mean that the fucose was a cytoplasmic contaminant. Results obtained were thus unsatisfactory; only galactose, and perhaps fucose and glucose were detected in an unfractionated vacuolar sample. In addition, no monsaccharides at all were detected in the 3 bands obtained after density-gradient fractionation. It is possible that the vacuolar membranes were permeabilised during isolation, and that the solute content of the vacuoles, including monosaccharides, leaked out into the isolation medium. Secondly, it may be that insufficient vacuoles were isolated to yield sufficient quantities of monosaccharides detectable in a paper-chromatographic system. This is especially true of the samples obtained after fractionation on density gradients.

The monosaccharide composition of hydrolysed, purified root cap slime was analysed using the same chromatographic system in order to provide a standard with which to compare the spectrum of monosaccharides detected within the vacuole (both the slime and at least some vacuolar monosaccharides are thought to be dictyosomally-derived). The slime was found to contain a high proportion of fucose, glucose, galactose and arabinose, with lesser amounts of galacturonic acid and xylose. This result is in substantial agreement with the results of both Jones and Morré (1967) and Paull, Johnson and Jones (1975), although there are discrepancies between all three sets of results, which may perhaps be attributed to varietal differences. Jones and Morré found very little fucose in the polysaccharide, whereas present results and those of Paull, Johnson and Jones (1975) indicate a high percentage of fucose.

Analysis of a mixed, lysed vacuolar sample for small oligosaccharide using a gas chromatographic system revealed 3 peaks corresponding to disaccharides, one of them being lactose, and 3 other peaks consistent with tetra- and penta-saccharides of unknown composition. It is possible that oligosaccharides were easier to detect than monosaccharides, as the vacuolar membrane might be less permeable to the large oligosaccharides. These results, however, give no evidence to support an accumulation of monosaccharides within the large vacuoles of the senescing region of the root cap.

In summary, cytochemical evidence suggests that the dictyosomal polysaccharide undergoes hydrolysis very soon after being incorporated into the vacuole, as no heavy silver deposits were observed to accumulate within the vacuoles of the senescing region of the cap. A fine, general deposition of silver was, however, apparent over the whole of the vacuole, possibly being indicative of a very dilute polysaccharide matrix, or an accumulation of oligosaccharides. An accumulation of oligosaccharides would be expected if the particular glycosidases needed to break down the monosaccharide linkages of the oligosaccharides were not present within the vacuole. As the chemical structure of the dictyosomal polysaccharide is as yet unknown, this remains a possibility. Autoradiographic evidence reveals an accumulaion of isotope within the vacuoles after prolonged incubation with isotope of very high specific activity. This isotope is thought to be incorporated into unhydrolysed polysaccharide, as well as into oligosaccharide units existing within the vacuole. Although no evidence is available concerning the exact monosaccharide composition of the vacuoles of the senescing region of the cap, the evidence presented above is not inconsistent with the theory that the hydrolysis of dictyosomally-derived polysaccharide within the vacuole results in an accumulation of smaller solute molecules and a consequent influx of water into the vacuole.

4.5.7 *Autolysis*

The extreme distal edge of the root cap consists of a layer of cells (approximately 3 deep) undergoing autolysis and sloughing from the cap. The process of autolysis appears to begin with the development of discontinuity in the vacuolar membrane, presumably allowing hydrolytic enzymes to come into contact with, and degrade, the cytoplasm.

An interesting question is - how is the degradation of the vacuolar membrane brought about? Matile (1975) has postulated that a continuous supply of energy is necessary for the maintenance of the integrity of the vacuolar membrane. It is possible that a shut-down of nuclear information at this stage is responsible for a slow-down in energy-requiring metabolic processes (including ATP synthesis), thus gradually reducing the supply of energy to the vacuolar membrane, causing it to become susceptible to attack by vacuolar hydrolytic enzymes. For example, the onset of autolysis in the gills of the basidiomycete Coprinus lagopus coincides with a sharp decline in respiratory enzyme activity (Iten and Matile, 1970).

It is just possible (Berjak, personal communication) that there may be a measure of cytoplasmic synthesis of hydrolases, in the senescing region of the cap, this being responsible for the initiation of autolysis prior to actual tonoplast degradation. In this regard dense deposits (but apparently not unequivocal indications of acid phosphatase activity *per se*) have been found associated with the mitochondrial and plastid membranes of cells whose vacuoles appear, at least in thin-section, to be intact. If there is a cytoplasmic synthesis of hydrolases it is possible that they can degrade the vacuolar membrane by attacking it from the outside, which does not bind Concanovalin A (see section Tonoplast integrity), and may not be

protected from enzymatic degradation. However, there is no conclusive evidence for this suggestion.

Matile and Winkenbach (1971), working on the senescing corolla of Ipomea purpurea, have observed a shrinkage of the vacuoles and apparent dilution of the cytoplasma, immediately prior to autolysis. They interpret this as resulting from severe changes in membrane permeability - perhaps hydrolases may leak through the membrane and begin to digest the cytoplasm even before rupture of the vacuolar membrane. Another possibility is that the postulated accumulation of monosaccharides within the senescing vacuoles ultimately generates a water potential so low that membrane growth and expansion can no longer keep pace with the influx of water, and the vacuole bursts. This may provide a rationale for the sudden increase in vacuolar size, the reason for this phenomenon in swiftly turning-over root cap cells being otherwise difficult to explain.

The question remains - why is autolysis necessary? Secretion of dictyosomal polysaccharide into the extra-protoplasmic space and ultimately into the intramural region is likely to loosen the outer cells from one another, thus facilitating their being sloughed from the cap. It may be that the eventual leakage of specific hydrolytic enzymes (normally contained within the vacuole, and not found outside of the cell) into the intramural region aids in the digestion of the middle-lamella and thus in the freeing of the outer cells from one another. In this context autolysis may be necessary in speeding up cell loss in terms of the function of the root cap (in protecting the root apical meristem) and the maintenance of the overall streamlined shape of this tissue. Further, it is possible that the molecular precursors produced during autolysis could diffuse back into the younger region of the cap, for reuse, thus avoiding their loss as a consequence of sloughing.

4.6 RETROSPECT

The vacuolar system plays a vital rôle in the development of the maize root cap. Indeed, vacuolar development can be said to parallel the development of the root cap. In the younger, differentiating regions of the cap vacuoles are no doubt responsible for the maintenance of cell turgor, as well as being the centres for intracellular molecule turnover, thus ensuring that molecular building-blocks are recycled to enable growth and development to continue. The ultimate fate of a root cap cell is its senescence and autolysis, the autolysed cell being sloughed off from the exterior of the cap thus presumably reducing friction during growth of the root through the soil, and protecting the root meristem proper. The vacuolar system plays a central rôle in the development of the ageing root cap cell towards autolysis. In this regard the vacuoles of the senescing region of the cap are very active in autophagy, perhaps serving to minimise the final events of autolysis, as well as to allow the recycling of hydrolysed macromolecular constituents through their diffusion back to the younger regions of the cap. Finally, the disruption of the vacuolar membrane and the consequent release of hydrolases brings about cellular autolysis, as well as perhaps aiding in the loosening of the outermost cells from one another by hydrolysis of the middle lamella.

PART FIVE

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