FINE STRUCTURE OF PLANT CELLS IN RELATION TO SALT ACCUMULATION

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The abbreviations used on the figures are as follows:

c	crystalloid body	n	nucleus
cw	cell wall	og	osmiophilic globule
er	endoplasmic reticulum	p	plastid
g	Golgi body	st	starch
m	mitochondrion	v	vacuole
mb	microbody	ve	vesicle

The marker on each figure indicates 1μ.

SUMMARY

Several aspects of the process of ion uptake in plant storage tissues were studied using techniques of electron microscopy. During aging of beetroot disks in aerated solution, changes in length of endoplasmic reticulum (ER) lamellae were observed. The lamellae, which were reduced to vesicles 2 hours after cutting the tissue into disks, reappeared during the following 48 hours, and thereafter continued to increase in length for some time. This observation is discussed in relation to similar changes reported in other plant tissues, and in connection with a possible effect on ion movement, as the capacity to accumulate ions develops during the first 12-24 hours of aging. After a period of 48 hours, crystalloid bodies appeared within the cisternae of the ER and the number of these bodies increased during further aging. The crystals were found to contain protein, and the possible nature of this protein is discussed.

The importance of calcium for membrane integrity and its effect on the uptake of monovalent ions are well recognised phenomena. Hence, the effect of addition or withdrawal of calcium was investigated. No conclusive ultrastructural changes resulted with the exception of the apparent enlargement of ER cisternae caused by calcium removal, an observation suggesting the formation of vacuoles.

Because of a possible involvement of membrane ATPases in sodium and potassium movement, as found in animal cells, the cellular localisation of ATPase was attempted using histochemical methods. This work

is discussed in relation to published criticisms of the method, and also recent evidence suggesting that ATP formation may be an alternative to ion movement.

Studies of developmental processes following slicing of beetroot tissue by using inhibitors of RNA and protein synthesis produced interesting ultrastructural results. The re-formation of long ER lamellae during aging was inhibited by treatment with cycloheximide, puromycin and p-fluorophenylalanine but unaffected by actinomycin, and thus seemed dependent on protein synthesis but not on the formation of m-RNA. Actinomycin and p-fluorophenylalanine promoted the onset of crystal production indicating that the protein synthesizing system associated with the ER is relatively stable and is concerned with the production of non-functional protein. On the other hand, actinomycin inhibited the development of the ion accumulation mechanism, although it did not affect the developed ion uptake process. The development is then dependent in some way on the synthesis of m-RNA after slicing. Either the required m-RNA is destroyed at the time of slicing, or a different mechanism for ion movement is operative in sliced tissue.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference is made in the text.

Signed

I INTRODUCTION

Metabolic changes initiated by the cutting of storage tissue into disks are generally considered to constitute a developmental process which takes place in response to a new environment. Cells which were originally embedded within a large storage organ believed to be dormant, are now more or less exposed to the surrounding aerated solution, and it is to be expected that some cellular processes are affected.

The nature of the initial event which triggers off the sequence of metabolic changes is unknown. Perhaps the most commonly observed effect of slicing is an immediate and progressive increase in respiration rate, linked to phosphorylation. It has been suggested that the energy thus made available may be used in a variety of endergonic reactions such as salt uptake and the synthesis of various cell constituents e.g. proteins and lipids. Oxygen supply for this respiration is undoubtedly important, but the idea that the changes during 'aging' are the result of increased oxygen tension has been shown to be untenable (Laties 1957; MacDonald 1968). It is interesting to note that the tricarboxylic acid (TCA) cycle is inoperative or markedly depressed in fresh potato tissue, and develops after slicing, but mitochondria from fresh tissue show full TCA activity. Hence some internal factor seems to be responsible for the lack of activity in whole tissue. Laties (1957) suggested the presence of a volatile metabolic inhibitor, for which there is, as yet, no experimental evidence.

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In addition to the increased respiratory rate, most storage tissues when cut into disks and subjected to washing in aerated solution develop, over a period of one or more days, a capacity to accumulate ions. It has been argued that the mechanism of this induced salt uptake may not resemble that operating in intact roots. But storage tissue has been widely used in studies of the mechanism of salt accumulation because such tissues can provide a supply of disks with identical morphological characteristics and with repeatable changes in the sequential development of absorbing processes for specific ions during a period of washing or 'aging' following cutting. Factors which are found to modify the time-course of development or decay of any of these ion absorption processes may provide information about their respective mechanisms.

It should be noted that in any studies of ion accumulation it may be necessary to consider fluxes rather than net transport. Van Steveninck (1962) demonstrated that, in beetroot tissue, the K^+ influx in fresh tissue did not change appreciably during aging, but the K^+ efflux decreased together with a progressive increase in Cl^- influx. Thus there appears to be a mechanism for K^+ uptake in freshly cut tissue whereas the anion uptake mechanism is induced by the new environment.

Early workers showed that ion accumulation was quite distinct from the physical processes of diffusion, adsorption and ion exchange, i.e. the uptake of ions consists of at least two phases - a relatively rapid non-metabolic phase and an active accumulation process. Experimental evidence has shown that the cytoplasm is relatively inaccessible to ions

and it is agreed that the plasmalemma presents a significant barrier to free ion movement, while the intercellular spaces and cell walls are considered to be part of the water free space. The exchange or Donnan free space (DFS) which results from the establishment of an equilibrium between cations and fixed anions in the tissue is not located with such certainty, although a majority seems to favour the exclusion of the cytoplasm. Pitman (1965) provided details of the DFS of beetroot tissue and considers that 95% of the total DFS charge is associated with the wall space and 5% with the cytoplasmic phase, probably the surface, which may include ion carriers. In general terms, some part of the DFS seems to be indirectly dependent on metabolism for maintenance of negative charges at the surface of the cytoplasm. Also, the permeability or physical properties of the plasmalemma can easily be modified by environmental conditions. For example, Handley, Vidal and Overstreet (1960) found that anaerobiosis enhanced the non-metabolic entry of ions into corn root meristems indicating that the impermeability of the plasmalemma may not be maintained under anaerobic conditions.

Likewise, the active process of ion accumulation will be influenced by the direct effects of the external solution on the physical and chemical properties of the cytoplasm or the plasmalemma. Ionic composition of the external solution may also have profound effects e.g. the effect of calcium on membranes has been a subject of speculation for years. Early explanations were given in terms of the colloidal nature of cytoplasm, viz. Ca^{++} , as

a divalent ion, would tend to coagulate the colloids and thus decrease permeability, an effect opposite from colloid dispersal by univalent ions. Thus a certain ratio of univalent to polyvalent ions is required for normal membrane function (Clowes 1916; Dixon and Bennet Clark 1930; Mazia 1940). It was also found (Collander 1957), that Ca^{++} could bind anionic groups of proteins and phosphatidic acids, providing a more rigid and less permeable structure than when univalent ions are present. An effect on hydration may also be important, as various multivalent cations have been shown to have specific effects in preventing the hydration of nucleoprotein films (Ambrose and Butler 1953). In this connection, the degree of contact between plasmalemma and cell wall may be important. Bennet and Rideal (1954) suggested that the interface between protoplasm and external solution is situated within the cell wall, its area being only a fraction of the total area of the cell wall. Thus cations in the external solution could profoundly affect the exposed protoplasmic surface area.

More directly, Weber (1932) showed that Ca^{++} is essential for plasmalemma formation, and Marinos (1962, 1963) found that plasma and vacuolar membranes disintegrate when Ca^{++}, specifically, is lacking. More recently, Van Steveninck (1965a) showed that in cells of beetroot storage tissue, Ca^{++} is mainly associated with the cell surface, and suggested that Ca^{++} is specifically involved in bonding negative charges of the protoplast surface and the cell wall, the unspecific effect of divalent

cations on the protoplasm being important only when a large proportion of the Ca^{++} is withdrawn from the cell. Thus it appears that Ca^{++} is required in the synthesis and maintenance of membranes.

These direct membrane effects cannot yet be separated from other observed effects of Ca^{++} on the ion uptake processes. A stimulatory effect of polyvalent cations on the uptake of other ions was first reported by Viets (1944). This phenomenon has been widely confirmed with an almost corresponding number of different explanations, relating Ca^{++} to all possible aspects of carrier function e.g. Ca^{++} enhances the formation or turnover of an ion carrier (Marschner 1961); Ca^{++} functions as a co-factor in the formation of the carrier (Jacobson et al 1950); Ca^{++} increases the rate of breakdown of the ion-carrier complex (Overstreet et al 1952; Leggett 1956); Ca^{++} enhances the affinity between carrier and ion (Kahn and Hanson 1957); more specifically, Ca^{++} protects ribonucleo-protein as a carrier moiety from breakdown by endogenous enzymes (Hanson 1960; Tanada 1962).

Calcium is also considered to play an important role in ion selectivity probably through its effect on the cell surface. Epstein (1961) states that the mechanism responsible for the discrimination between K^{+} and Na^{+} depends on the presence of Ca^{++} and locates the carrier mechanism in the plasmalemma because of the immediately reversible loss of specificity when Ca^{++} is removed from the ambient medium. Some workers (Jacobson et al 1960, 1961; Waisel 1962) consider that Ca^{++} blocks the entrance of interfering ions. Hooymans, using excised roots (1964),

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concludes that the effect of Ca^{++} on anion uptake on the one hand and cation uptake on the other, is based on two completely different mechanisms viz. screening of the negative charges in the close neighbourhood of carrier sites (most likely to be found on a membrane) in the former case, and lowering of the permeability of the plasmalemma in the latter. Similarly, Pitman (1964) considers that Cl^- uptake limits K^+ uptake in beetroot cells and that Ca^{++} tends to increase the Cl^- uptake. He suggests that uptake of the anion is limited by diffusion through a negatively charged surface or membrane which will be more permeable to anions when divalent, rather than univalent, cations are present.

Although there is as yet no clear insight into the mechanism of ion transport, recent quantitative information on electrochemical potential gradients for various ions and their location across specific cell membranes has given further support to the concept of ion pumps. These pumps are clearly dependent on metabolic energy which could be provided by adenosine triphosphate (ATP) as bond energy, or more directly, by charge separation across the membranes. Robertson (1968) suggests that there are three ways in which energy may be transferred from its source (mitochondria, chloroplasts or from cytoplasmic glycolysis) to the site of an ion pump, viz. (1) transfer of ATP, (2) transfer of reducing power by means of hydrogen or electron carriers, or (3) transfer of H^+ or OH^- after charge separation. In addition, mitochondrial movement, or for that matter any cytoplasmic movement, could contribute towards the availability of energy at different sites in the cell.

The phenomenon of salt respiration (Robertson et al/1955) in washed slices of storage tissue, has revealed a close relationship between ion uptake and the oxidative electron transport system mediated by the cytochromes in mitochondria. Robertson (1960) emphasised that the inhibition of ion uptake by the uncoupler, dinitrophenol, does not necessarily implicate ATP as the energy source for ion transport. Further evidence was given by Millard et al (1964) who consider that phosphate uptake in mitochondria is driven by cation uptake. During substrate oxidation, hydrogen ions are released and exchanged for either a monovalent cation or for a divalent cation + monovalent anion. Thus charge separation, with a loss of hydrogen ions to the medium is viewed as the essential act in both the ion transport system and in phosphorylation.

Mechanisms proposed for active transport of ions involve either a direct coupling to electron transport (Lundegardh 1945; Robertson 1960; Mitchell 1966) or coupling to salt-stimulated ATP breakdown. In the latter case, the energy from hydrolysis of ATP by adenosine triphosphatases (ATPases) is used to transport ions, perhaps through conformational changes of a carrier system in the membrane (Opit and Charnock 1965). In animal cells which possess a coupled active transport of Na^+ and K^+, the plasma membrane contains a Mg^{++} requiring, Na^+ and K^+ stimulated ATPase. The fact that the cardiac glycoside, ouabain, specifically inhibits the coupled transport of Na^+ and K^+ and also inhibits the Na^+ and K^+ stimulated ATPase indicates the involvement of this enzyme with the transport process

(Skou 1965). Evidence for this so-called sodium pump comes from a wide variety of cells and tissues such as crab nerves, mammalian erythrocytes, kidney, brain and muscle (see Farquar and Palade 1966).

Most ATPases which have been detected in plant tissues differ from the animal transport ATPases in one or more respects. Brown and Altschul (1964) and Brown et al (1965) described an ATPase from peanut seedlings that is stimulated by ouabain and inhibited by high salt concentrations. Bonting and Caravaggio (1966) reported the absence of ouabain sensitivity and Na^+-K^+ synergism in ATPase from Ulva lactuca; and Brown and Chattopadhyay (1966), Dodds and Ellis (1966), Gruener and Neumann (1966), McLurkin and McLurkin (1967) have reported salt-stimulated ATPases from barley roots, carrot cell walls, bean roots and pine seedling root tips respectively. Atkinson and Polya (1967) described salt stimulated ATPases from carrot, beetroot and Chara australis, and showed no ouabain inhibition or synergistic effect of K^+ and Na^+. Hence, it appears that there is, at present, no conclusive evidence for the ATP-linked transport system in plants. In a recent publication Fisher and Hodges (1969) investigating a Mg^{++} (or Mn^{++}) dependent, Na^+ and K^+ stimulated ATPase in cellular fractions from oat roots, claimed that, although a relationship between ATPase activity and ion transport is not determined, sufficient membrane associated ATPase exists to account for K^+ transport.

Another aspect of ion uptake which has provoked interest is the role of protein synthesis. In 1941, Steward and Preston showed that

potato slices in aerated salt solutions converted soluble nitrogen compounds to insoluble protein. On the basis of this and later work, Steward stressed that the capacity for renewed growth as evidenced by protein synthesis is essential for salt accumulation (Steward and Millar 1954), but Sutcliffe (1954) concluded from his observations on beet disks that the disks consisted of mature non-growing cells in which salt uptake was independent of protein synthesis.

No sign of cambial activity was found in beetroot by Bennet Clark and Bexon (1943) or Sutcliffe (1952), or in carrot (Turner 1940), and swede tissue is without cambial initials. On the other hand, cell division was observed in potato disks by Steward, Wright and Berry (1932) and Hackett and Thimann (1952), but Thimann and Loos (1957) were unable to confirm this observation.

More recently, MacDonald, Knight and de Kock (1961) have shown that protein synthesis occurs when disks of sugar beet, red beet, carrot and swede are maintained in running water containing nitrate. Even in distilled water at 25°C, the protein content of beetroot disks doubled in four days while the soluble nitrogen content fell. They concluded that the changes occurring in the disks during aging are best described in terms of renewed growth, and looked upon the development of a capacity for salt uptake as a property of cells which have undergone rejuvenation, rather than cells which are either dormant or non-growing. They also suggested that the protein synthesised might be present and operative in more than one form e.g. as protoplasm (biochemically inert) or in association with

carriers or enzymes.

More evidence in support of a relationship between ion absorption and protein turnover in plant cells came from experiments using protein synthesis inhibitors, but results of such experiments should be treated with caution. In 1960, Sutcliffe suggested that chloramphenicol prevented salt uptake by inhibiting protein synthesis. Ellis (1963) pointed out that only the D-threo isomer of chloramphenicol possessed antibiotic activity, and yet the L-threo isomer inhibited ion uptake almost as effectively. Further criticism of Sutcliffe's work came from MacDonald et al (1966). They found that high concentrations (3mM) of chloramphenicol inhibited both protein synthesis and the development of ion absorption capacity in beetroot disks, but at low concentrations (0.1mM), development of ion uptake capacity was inhibited and protein synthesis stimulated. They also drew a distinction between the two isomers on the basis of their contrasted effects on the development of ion uptake capacity, as distinct from the uptake process itself, and concluded that ion uptake and protein synthesis are affected independently. Chloramphenicol was previously thought to specifically inhibit protein synthesis by preventing the association of m-RNA with ribosomes. But Hanson and Krueger (1966) indicated that the major site of action of chloramphenicol was on oxidative phosphorylation, and Ellis and MacDonald (1967) showed that incorporation of leucine into protein by beetroot microsomes was insensitive to chloramphenicol.

The effects of some other inhibitors of protein synthesis on ion

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uptake have recently been determined. MacDonald et al (1966) found that ion uptake in beet was insensitive to puromycin but the development of ion uptake capacity was inhibited. Polya (1968), using cycloheximide and cryptopleurine, found a lag between the inhibition of protein synthesis and the inhibition of ion uptake in aged beet slices. He concluded that his results did not support the hypothesis of obligatory coupling between the two processes.

Recent interpretations of the effects of these inhibitors have led to a growing realisation of their effect on the synthesis of specific proteins. Several workers have studied the development of activity of particular enzymes e.g. invertase in disks of storage tissue. It has been shown that protein synthesis is involved in the case of artichoke disks (Edelman and Hall 1963, 1965) and some ribonucleic acid (RNA) component has been implicated in carrot (Leaver and Edelman, 1965). Vaughan and MacDonald (1967b) found that development of invertase activity in beetroot disks during aging was inhibited by actinomycin D (which inhibits DNA-dependent RNA synthesis, Laszlo et al 1966) and by p-fluorophenyl-alanine which either effects protein synthesis directly or by becoming incorporated to produce an abnormal protein (Nooden and Thimann 1965). Both these substances also inhibited the normal increase in RNA content. In potato disks, Click and Hackett (1963) found that puromycin added at the commencement of aging inhibited uracil incorporation into RNA. Willemot and Stumpf (1967) studying the development of fatty acid synthetase in aging potato slices, found an accompanying rise in protein and RNA synthesis, and Sampson and Laties (1968) reported a burst of ribosomal RNA synthesis induced in potato

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tissue by slicing. All these results are consistent with the concept of a derepression occurring at the time of slicing which leads to increased synthesis of specific proteins or enzymes.

It is interesting to note the early implication of RNA in ion uptake (Lansing and Rosenthal 1952; Tanada 1956). RNA was then thought to be a possible ion carrier, some of the physical and chemical properties of which would be controlled by the nature of the associated protein. An indirect involvement of RNA in ion uptake through the synthesis of some enzyme or specific protein now seems more likely.

Remarkably few observations have been made at the ultrastructural level and it seemed possible that some changes in the structure, number, or organisation of cell membranes or organelles might occur when the ion uptake capacity of storage tissue disks is developing. Hence particular attention was paid to the following aspects which are reported in this thesis:

(a) Changes in ultrastructure of the large parenchymatous cells of storage tissues during a period of aerated washing (with special consideration of techniques for fixation).

(b) The effect of Ca^{++} on cellular structure by inducing leakage of beetroot cells with ethylenediamine-tetra-aceticacid (EDTA), followed by addition of Ca^{++} to stop leakage.

(c) Attempts to localise ATPase activity in beetroot and swede tissue using a modified Wachstein-Meisel method.

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(d) Studies of cellular changes due to the effect of inhibitors of RNA and protein synthesis on both the development of ion uptake capacity and on the existing ion uptake mechanism.

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II MATERIALS AND METHODS

1. Plant material

Beetroot, carrots and swedes were obtained fresh, as required, from one commercial source in Adelaide, and, later, from one commercial source in Brisbane.

(a) Beetroot

The root of Beta vulgaris L shows the usual type of primary and early secondary development. Later, a series of super-numerary cambia arise outside the normal stele of the hypocotyl and upper part of the root and produce several rings of vascular tissue each consisting of a layer of collateral strands of xylem and phloem embedded in parenchyma and separated by bands of large thin-walled parenchymatous cells (Esau 1965). Material for ion uptake studies was taken from this region of anomalous development around the central core. Tissue sampled for electron microscopy was cut from the parenchyma layers between the rings of vascular tissue. Initially samples were taken from other parts of the root so that specialised non-parenchymatous cells would be recognised at magnifications greater than ten thousand times.

(b) Carrot

The storage organ of the carrot (Daucus carota L) also develops from the hypocotyl and upper part of the tap root. After sloughing the cortex in the usual way a prolific development of parenchyma occurs in both xylem and phloem. Material from the xylem was used for ion uptake

studies and electron microscopy.

(c) Swede

Like carrot, the fleshy hypocotyl root of the swede (Brassica napobrassica Mill.) consists of a large amount of storage parenchyma associated with an otherwise normal arrangement of tissues, but in this case the proliferation occurs in the pith. The pith parenchyma was used in this work. The ultrastructure of cells constituting the cortex and xylem was also investigated.

2. Ion measurements

For preliminary experiments to study changes in ultrastructure during aging and the development of the capacity for ion accumulation, cylinders of beetroot and swede tissue 15 mm in diameter, and cylinders of carrot tissue 6 mm in diameter cut parallel to the long axis of the root with cork borers, were sliced into disks 1 mm thick (average fresh weight 190 mg, 200 mg and 35 mg respectively). The disks were kept moist with wet paper during cutting and were then washed in deionised water (with aeration) in the proportion of approximately 1500 large disks to 4 litres of water, or 1200 small disks to 500 ml, for $1\frac{1}{2}$ to $2\frac{1}{2}$ hours with three changes. Leakage of ions into these rinses was measured. Batches of disks (usually 3 replicates) were then added to flasks containing water in the proportion of 1 gm tissue to 25 ml water (usually 200 or 250 ml). The flasks were placed in a water bath at 24° C and aerated continuously. Samples (10 ml) of the external solutions were taken at intervals, and replaced by an equal volume of

deionised water. K^+ and Na^+ concentrations were determined using an EEL flame-photometer, and Ca^{++} and Mg^{++} concentrations with a Techtron atomic absorption spectrophotometer.

In later experiments where various treatments were applied to fresh disks, the initial rinses were carried out as described above. Batches of disks were then added to flasks containing the treatment solution in the proportion of 1 g of tissue to 25 ml solution, and the flasks aerated as above.

In experiments where disks were aged prior to treatment, after the initial rinses, the disks were kept in aerated deionised water (in one flask) with daily changes and daily measurement of ion leakage. After the required time interval, disks were transferred to flasks containing the treatment solutions (3 or 4 replicates). Samples of the external solutions for flame photometry were taken at intervals and replaced by an equal volume of the original solution.

Details of the age of disks at the commencement of treatment, and the concentrations of all solutions are included in the results section.

3. Sampling for electron microscopy

Disks for electron microscopy were removed from the flasks at the required times, either two large disks from different replicate flasks, or alternatively, 4 small disks, two from each of two replicates, contributing to each sample. No attempt was made to adjust the

solution volume to correct for changes in the amount of tissue present in flasks during the course of an experiment, because the number of disks in all cases was large compared with the number removed even during the course of a long experiment requiring several samples for electron microscopy.

4. Preparation procedures for electron microscopy

At the commencement, and as new methods were published during the course of this work, many variations of the preparatory procedure were tried and adopted if found to be satisfactory or advantageous.

(a) Fixation

The fact that plant material is difficult to fix satisfactorily has been recognised since the beginning of biological electron microscopy, and is thought to be due to the presence of large turgid vacuoles not found in animal cells. McLean (1960) supposed that the destruction of the differential permeability of the tonoplast as the fixative penetrates results in the sudden release of vacuolar sap into the incompletely fixed cytoplasm causing disruption of the cytoplasmic matrix and, possibly, some organelles. In addition, when fixing large parenchymatous cells such as those of storage tissues, the fixative at the site of fixation may be significantly diluted by the vacuolar contents.

The procedures for fixation used during this work are listed in Table I (page 18) and Appendix I. In the majority of experiments two, or sometimes three, different fixatives were employed so that adequate

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Table I Procedures for fixation (for further details see Appendix I (a))

	Fixative	Buffer	Duration	Notes
	1% OsO_4	veronal acetate pH 7.3 + 0.05 M sucrose	2 hr	Sucrose added to increase tonicity (Caulfield 1957)
	"	" " " + 0.5 M sucrose	2 hr	
*	"	" " " + 0.4 M sucrose	2 hr	
	"	1% K dichromate pH 7.0 + 0.25 M sucrose	2 hr	Chromate and dichromate ions thought to act as both buffer and fixative (Dalton 1955, Köllman 1960)
*	2% OsO_4	0.1 M cacodylate pH 7.2	1½ hr, 2 hr	
	4% formaldehyde	phosphate pH 7.3 + 0.4 M sucrose	2 hr	Post fixation in 2% OsO_4+ for 1 hr (Holt and Hicks 1961)
	"	" " " "	3 hr	sole fixative
	"	veronal acetate pH 7.4 + 0.4 M sucrose	2 hr, 20 hr	Post fixation in 1% OsO_4+ + 0.3 M sucrose for 1 hr
	"	" " " " "	3 hr	sole fixative
*	4% glutaraldehyde	0.1 M cacodylate pH 7.2	1 hr	Post fixation in 2% OsO_4+ for 1 hr, 1½ hr, 2 hr (Sabatini, Bensch and Barnett 1963)
	"	"	2 hr	" " in 2% OsO_4 for 1½ hr
	"	"	3 hr	" " in 2% OsO_4 for 2 hr
	"	"	12 hr	" " in 2% OsO_4 for 1 hr
	5% glutaraldehyde	"	1 hr	" " in 2% OsO_4 for 1½ hr
	"	phosphate pH 7.3	1 hr	Sole fixative or post fixation in 2% OsO_4 for 1 hr
	"	phosphate pH 7.2 + 0.4 M sucrose	30 min	Post fixation in 2% OsO_4+ for 30 min (Holt and Hicks 1961)
	"	" " " "	2 hr	Sole fixative
	"	veronal acetate pH 7.3 + 0.4 M sucrose	30 min	Post fixation in 2% OsO_4+ for 30 min
	"	" " "	2 hr	Sole fixative
	12½% glutaraldehyde	phosphate pH 7.3	1 hr	Sole fixative or post fixation in 2% OsO_4 for 1 hr
	2% $KMnO_4$		30 min	(Mollenhauer 1959)
*	2% $KMnO_4$	veronal acetate pH 7.2 - 7.4	30 min, 45 min, 1 hr	
	2% $NaMnO_4$	"	45 min	
	2% $Ca(MnO_4)_2$	"	45 min	(Afzelius 1962)

* denotes fixatives most frequently used
+ OsO_4 used for post fixation was made up in the same buffer as the preliminary fixative

comparisons could be made. In all cases, fixation was carried out at low temperature (0-5°C) because of the general reduction in cellular activity.

A section or disk of tissue 1 mm thick was placed on a narrow strip of dental wax (in later experiments the dental wax rested on ice or a cold surface in a refrigerated cabinet) and, using a new, cleaned razor blade was trimmed on three sides (being held by the fourth), leaving only the required part of the tissue as a strip about 3 mm wide by 8-10 mm long. If fixing with OsO_4 or glutaraldehyde, a few drops of ice-cold fixative were added to the tissue at this stage: if with $KMnO_4$, the tissue was chopped without addition. The 3 mm wide strip was cut lengthwise into 3-4 strips and then crosswise to give 1 mm (approximately) cubes, discarding the end by which the strip was held during cutting. The cubes were then transferred to a tube (3" x 0.5") containing 1-2 ml of cold fixative, by immersing the end of the wax strip in the fixative. This procedure took only a few seconds. The small tubes containing fixative and tissue blocks were immersed in ice during the period of fixation, with occasional agitation.

The duration of fixation depends on both the fixative and the tissue, the rate of penetration varying considerably. Fixation periods of 30 mins to 2 hours were used for OsO_4 (see Table I), as usually recommended. Similar periods were generally used for aldehyde fixation whether followed by fixation with OsO_4 or not. Sabatini et al (1963)

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showed that aldehyde-fixed tissues can be stored for weeks in aqueous solution before post-fixation with OsO_4 without deleterious effect. In this work glutaraldehyde-fixed tissue blocks were rinsed three times in the appropriate buffer solution for periods ranging from 1 hour to 15 hours (overnight), but not longer, before post-fixation with OsO_4. Likewise, when fixation was complete, the tissue was rinsed at least 3 times sometimes over a period of 1 hour, but frequently overnight, before dehydration.

In the case of permanganates, the fixation is of shorter duration (30 mins to 1 hour), because these fixatives are progressive oxidants. In a small cube of tissue, only a relatively narrow zone will be well-preserved. After permanganate fixation the tissue was rinsed rapidly several times in deionised water and dehydration commenced immediately to minimise the swelling which often occurs at this stage.

Attempts were made to increase the rate of penetration of the fixative by vacuum infiltration. At the beginning of the period of fixation, the tubes containing tissue in fixative were placed (in an ice bath), in a vacuum chamber and the pressure lowered gradually for a few minutes often causing the tissue to float. When the vacuum was gradually released, the tissue sank. But this treatment made little, if any, difference to the quality of the preservation.

The following considerations led to the use of three main

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groups of fixatives as indicated in Table I.

(i) Osmium tetroxide

The original veronal-acetate buffered OsO_4 fixative was developed by Palade in 1952. Inorganic buffers were not used because of the likelihood of forming precipitates with divalent ions present in the tissue; veronal acetate or cacodylate buffers are commonly employed.

In this work, fixatives containing OsO_4 were generally made up in small quantities, the OsO_4 being allowed to dissolve in the required buffer overnight. Occasionally larger amounts were dissolved at higher concentrations and subsequently diluted with the required buffer.

(ii) Aldehydes

When used alone, aldehyde fixatives preserve proteins and carbohydrates, but lipids are not rendered insoluble in organic solvents and hence may be extracted during the dehydration process. Primary fixation with aldehydes is advantageous because of their effective penetration and a post-fixation can be used to reduce leaching of lipids. The appearance of tissues after post-fixation in OsO_4 is very similar to that of material fixed in OsO_4 alone, but the mitochondrial matrix, ground plasm, and structural contents of the ER often appear denser after the double fixation, suggesting that a greater proportion of the material is retained (Sabatini et al 1963). Although a wide range of aldehydes has been compared (especially by Sabatini et al 1963), formaldehyde for particularly rapid penetration, and glutaraldehyde

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seem to be the most useful, and are usually buffered with either phosphate or cacodylate. Holt and Hicks (1961) claim that veronal reacts with formaldehyde so that all buffering capacity in the required range is lost. Because of its two aldehyde groups, glutaraldehyde is capable of crosslinking compounds with which it reacts. Thus even though its molecule is larger than formaldehyde it tends to give better fixation of proteins, particularly when used as the sole fixative (Pease 1964).

Care must be taken to use pure forms of both these aldehydes. Commercially available formalin generally contains some added methanol rendering it unsuitable for use as a fixative. Pure formaldehyde is readily prepared by dissociating the polymer paraformaldehyde, under slightly alkaline conditions at about 60°C (Pease 1964). Glutaraldehyde is supplied commercially as a 25% solution but is somewhat unstable giving acidic breakdown products. Hence the pH of the glutaraldehyde was checked frequently and when found to be low, the stock solution was shaken vigorously with anhydrous barium carbonate to raise the pH to 5.5,centrifuged,and the supernatant stored under refrigeration (Glauert 1965).

Aldehydes have been widely used as primary fixatives for histochemistry where the requirements are slightly different in that maximum preservation of cytological detail should be coupled with maximum preservation of enzyme activity. This aspect will be discussed further in Section III Part D.

(iii) Permanganates

These fixatives introduced by Luft (1956) complement the aldehydes in that the membrane lipoproteins are rendered insoluble whereas almost all other parts of the cell are lost. For this reason, the permanganates should be regarded as special purpose fixatives, and have been used with advantage, for example, in studying ER in botanical material (Whaley et al 1959; Jackman and Van Steveninck 1967). Slight differences in appearance of membranes have been observed with variation of the cation (Wetzel 1961; Afzelius 1962). Afzelius found that calcium permanganate more effectively preserved the triple-layered structure of the unit membrane.

The value of including a buffer in the fixative solution is doubtful (Mollenhauer 1959); veronal acetate is frequently used.

(b) Dehydration and embedding

After rinsing briefly in water, tissue blocks were taken successively through 25%, 50%, 75% and 100% acetone - 5 min. each in 25% and 50%, 5 min. or longer (frequently overnight) in 75% and 3 rinses of 10 min. each in 100%. Initially AR acetone was distilled over calcium chloride and stored over anhydrous copper sulphate, but later the distillation was omitted. As it is impractical to move small tissue blocks from one tube to another, each dehydrating solution was removed with a Pasteur pipette and the next poured onto the tissue remaining in the tube.

Araldite (see Appendix I (b) for mixture used) was then

added to the tissue blocks in 100% acetone to give a 50:50 mixture, and the containers rotated at an angle of 45° at 50°C for 30 min. It can be expected that this fluid mixture will exchange fairly rapidly with pure solvent within the tissue. As much of this mixture as possible was removed with a Pasteur pipette and replaced with the undiluted resin mixture. The tubes were again rotated at 50°C. This replacement procedure was repeated twice. Selected tissue blocks were then transferred to Araldite-filled gelatine capsules (size 3, dried for several days before use) and allowed to harden for several days at 50°C.

Recently this embedding procedure has been modified in some respects, mainly to produce blocks with better cutting properties. Propylene oxide was introduced (2 rinses of 15 min. duration) to the final stage of dehydration and as an intermediate solvent (Luft 1961). An equal quantity of Araldite mixture containing proportionately less dibutyl phthalate (see Appendix I (b)) was added to the third propylene oxide rinse and the tubes rotated with lids on at room temperature for 20-30 min., before being left standing (lids removed) overnight. Selected tissue blocks were placed in gelatine capsules next day.

On some occasions the tissue blocks failed to sink through the Araldite in the capsules. This difficulty was experienced more often with freshly cut tissue than with aged disks, and after glutaraldehyde followed by OsO_4 fixation. Attempts were made to ensure complete infiltration by exposing the tissue to reduced pressure while in both

diluted and normal strength Araldite. No improvement was observed, and it was assumed that the blocks failed to sink because of the near-equal densities of resin and resin-infiltrated tissue, and the viscosity of the resin. The problem could usually be overcome by placing the tissue blocks in empty, dried gelatine capsules and then filling with Araldite.

Epon (see Appendix I (b)) was also tried because of its fluid nature and superior penetrating qualities. The embedding schedule was then abbreviated slightly: 15 min. in 50:50 mixture of Epon and acetone; this mixture withdrawn and replaced by Epon for 30 min. before placing tissue in Epon-filled capsules. The tubes were rotated constantly at 50°C during infiltration.

Despite its comparatively high electron density, Araldite was perhaps found to be a slightly more satisfactory embedment, although a strict comparison was not made. Its thermal stability is a distinct advantage, and the sectioning properties were generally found to be excellent, especially when propylene oxide was used to ensure uniform and relatively rapid infiltration of the resin into the tissue. Probably the greatest drawback to the use of Epon is its hygroscopic nature which necessitates additional precautions especially in a humid environment.

(c) Sectioning

Blocks were prepared for sectioning by trimming the portion containing the tissue under a binocular microscope which allowed cell

outlines to be clearly seen. A cutting face about 0.5 mm square was commonly used.

The early stages of this work were carried out using a Siroflex ultramicrotome; this was followed by a Sorvall Porter-Blum MT-2, and more recently by an LKB Ultrotome. Cutting speeds varied around 2 mm per sec.

Glass knives were found to be generally more convenient to use than a diamond knife. These were made from $\frac{1}{4}$ in. plate glass, at first by hand using glass pliers with attached strips of adhesive plaster, and later, using an LKB knifemaker. The knife angle was in the vicinity of 50° and knives were mounted at about 4° from vertical. Various troughs were used at different stages; a line drawn with a wax pencil $\frac{1}{4}$ in. from the knife edge and parallel to it; adhesive tape ('Sleek') sealed with wax; or aluminium or bronze troughs attached with wax.

The liquid used in the knife bath was 10% acetone throughout, and the level was maintained by means of a hypodermic syringe. Sections were manipulated on the surface using an eyelash mounted on the end of a toothpick, and expanded with chloroform or dichloroethylene vapour. Silver or gold sections (600-1000 Å thick) were picked up from above on 2.3 mm grids coated with a Parlodion supporting film and a film of evaporated carbon for heat stabilisation. Occasionally the Parlodion film was removed from the grids by placing in contact with acetone-soaked filter paper, but this was not usually necessary.

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(d) Staining

Araldite sections are perhaps the most difficult to stain due to the hydrophobic nature of the embedment. Alkaline lead citrate (Reynolds 1963; see Appendix I (c)) was found to be the most useful stain in this work. Small drops of the stain were placed on dental wax (or Parafilm) in a Petri dish containing filter paper soaked with N NaOH, and the grids floated on these drops. In this way the formation of lead carbonate crystals on the surface of the drops was kept to a minimum. Contamination could be further reduced by touching the grids onto the surface of a drop of water before placing on the drop of stain. Permanganate fixed sections usually required 5-12 min. staining and OsO_4 fixed sections (with or without preliminary aldehyde fixation) 30-45 min. Grids were then rinsed in 0.02 N NaOH followed by deionised water, or simply held in a jet of deionised water for a few seconds. Grids were allowed to dry on filter paper and stored in LKB gridboxes.

Uranyl acetate was also used either alone or in conjunction with the lead stain. Ethanolic solutions were found to stain Araldite-embedded tissues more readily than aqueous solutions. Uranyl acetate is more soluble in ethanol, but ethanolic solutions are less stable forming a precipitate gradually, and hence must be made fresh or centrifuged immediately before use. In the early stages of this work, 1-2% uranyl acetate in 50-96% ethanol was used following OsO_4 fixation when lead was found to be less effective. The period of staining

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varied up to one hour and was carried out in a moist chamber in a manner similar to that described previously. With such long periods of exposure of grids to alcoholic solutions, contamination could not be entirely avoided.

A double stain i.e. a brief exposure to lead citrate after staining with uranyl acetate has proved more effective for OsO_4-fixed tissues. Grids are floated on drops of uranyl acetate, either a saturated solution in 50% ethanol for 25-35 min., or 2% in 96% ethanol for 7-12 min., rinsed in 50% ethanol, then water, and floated on lead citrate for 1-2 min. before rinsing thoroughly.

Staining 'en bloc' (Terzakis 1968) with 0.5% aqueous uranyl acetate for 20 min., prior to dehydration, showed no particular advantage.

(e) Electron microscopy

All electron microscopy was carried out using Siemens I or IA microscopes operating at an accelerating voltage of 60 KV.

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III RESULTS AND DISCUSSION

Part A - Ultrastructure of storage tissues

(a) Beetroot

When sampled as described the tissue blocks consist almost entirely of large cells with thin walls (1-3μ wide) lined by a layer of cytoplasm often as thin as 0.3μ (Fig. 1 and 2). Hence the outer membranes of organelles are frequently in close proximity to both plasmalemma and tonoplast, and the tonoplast maybe displaced around organelles (Fig. 2). Very occasionally cytoplasmic protrusions are seen (Fig. 3), probably indicating the presence of strands across the vacuole which might be expected to collapse at the time of fixation, but, in any case, would appear incomplete in section.

The cytoplasmic volume of each cell is small, and although few organelles are visible in each section, plastids, mitochondria, Golgi bodies, microbodies and endoplasmic reticulum lamellae are generally seen. The nucleus (Fig. 4) is often deeply lobed, and nearly always associated with numerous mitochondria and plastids. The nuclear membrane is occasionally seen to be continuous with the endoplasmic reticulum (Fig. 5). The mitochondria appear to be relatively uniform in size but the plastids show more variation. After $KMnO_4$ fixation plastids are surrounded by the usual double membrane and contain various other internal membranes, 'star' bodies (Weier, 1961) and starch, (Figs. 2, 4, 5, 6, 7). Single membrane bound bodies of various shapes and sizes

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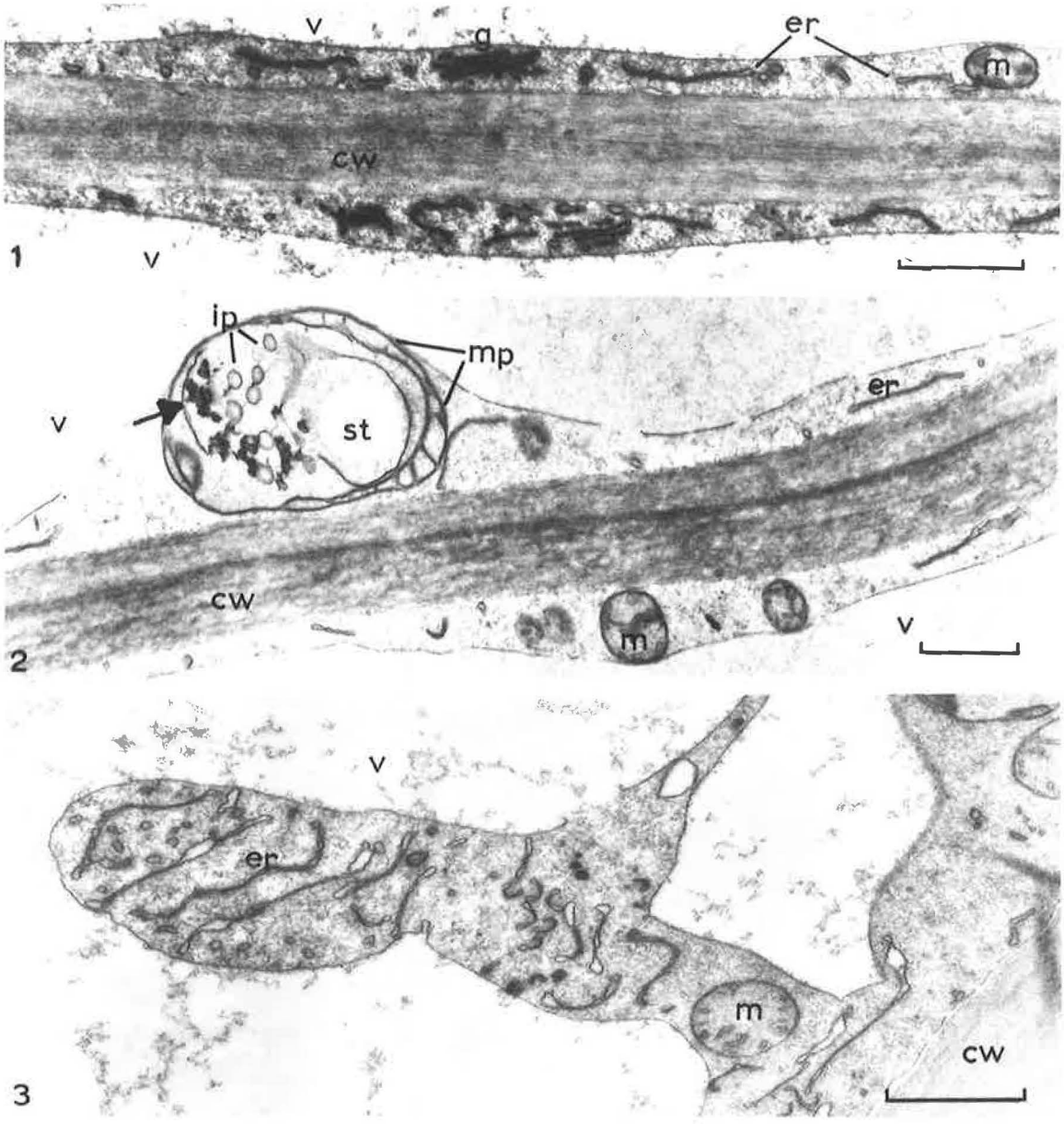
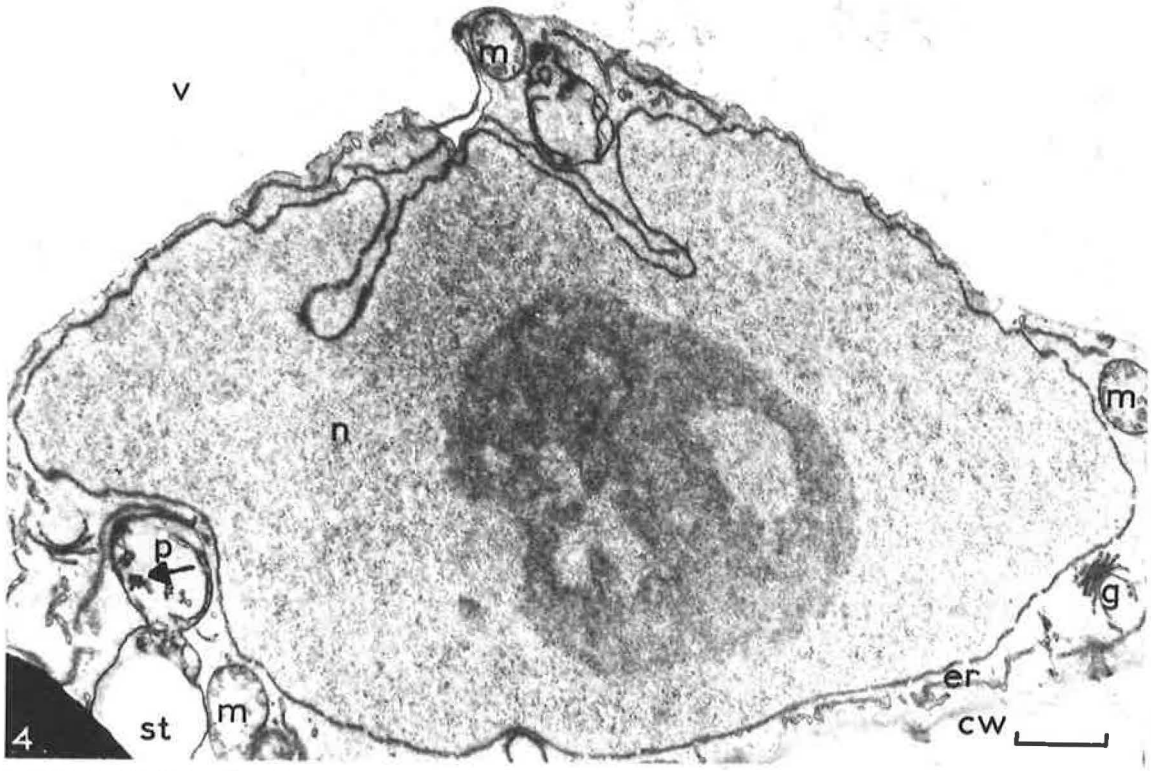


Fig.1-3. Parenchyma cells of beetroot showing thin layer of cytoplasm and mitochondria, plastids, endoplasmic reticulum and Golgi bodies. Cytoplasmic strands occur occasionally (Fig. 3). The plastid (Fig.2) includes marginal bodies (mp), intraplastid bodies (ip), and 'star' bodies (arrow), $KMnO_4$ fixation.



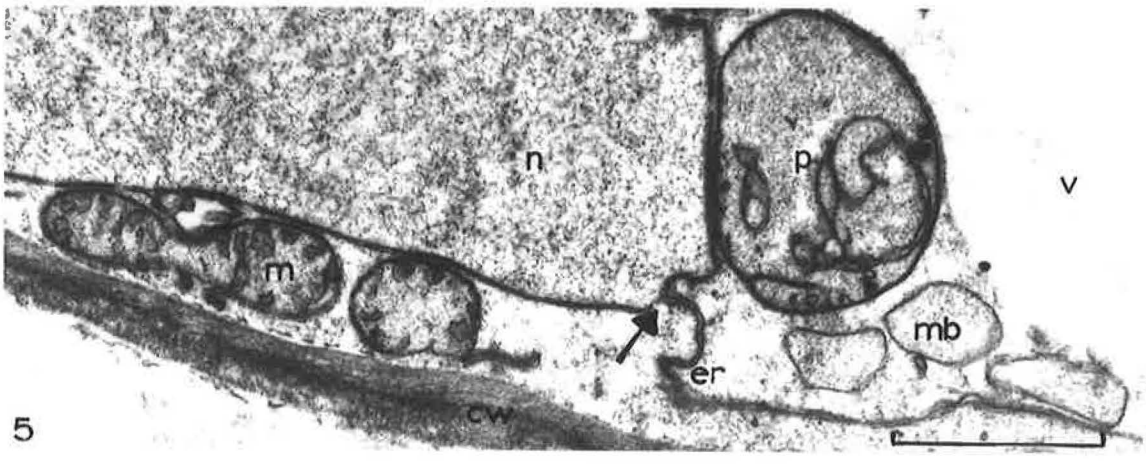


Fig.4-5. Nucleus of beetroot parenchyma cell showing close association with other organelles and continuity between nuclear membrane and endoplasmic reticulum (Fig.5 arrow). The arrow (Fig.4) indicates plastid 'star' bodies. $KMnO_4$ fixation.

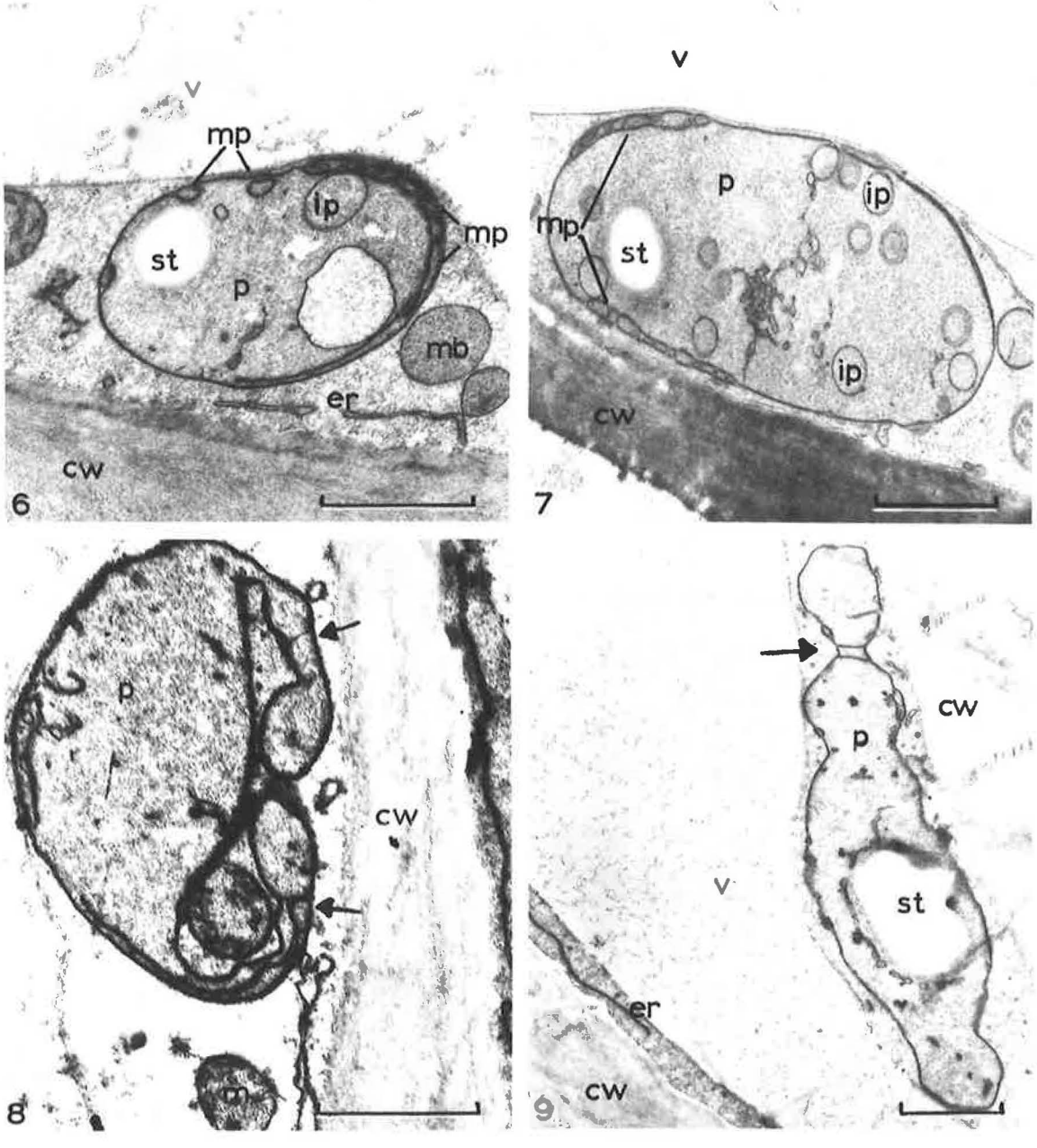


Fig.6-9. Plastids in beetroot parenchyma cells showing double membrane of envelope, starch, intraplastid bodies (Fig.6,7 ip), marginal bodies (Fig.6,7, mp), and subdivided protrusions (Fig.8,9, arrows). Fig.6-8, $KMnO_4$ fixation; Fig.9, $NaMnO_4$ fixation.

within the matrix of the plastid appear to be similar to those described in potato tuber by Marinos (1967) who concluded that these intraplastid bodies consist largely of proteins and nucleic acids. Often another membrane which appears to arise from an invagination of the inner membrane of the plastid envelope, extends as a double membrane parallel to the envelope (Fig. 6). The outer of these membranes joins frequently with the inner membrane of the envelope producing numerous separate marginal bodies (Fig. 7). It seems unlikely that this process constitutes the origin of intraplastid bodies, as these should then be bounded by double membranes (Fig. 6). If considered in three dimensions, Figure 6 suggests that cytoplasmic microbodies may originate in this way from the plastid matrix. Plastids frequently appear to contain internal bodies of similar density to the surrounding cytoplasm, and cytoplasmic microbodies (Frederick et al 1968) are common. Alternatively these areas may represent deep invaginations or isolated pockets of cytoplasm within the plastid.

The shape of the plastids is extremely variable. Plastids with long subdivided 'arms' are often seen but generally in inadequately fixed cytoplasm. This appearance suggests the budding of proplastids or mitochondria (Fig. 8 and 9), as observed in the cytoplasm of tobacco leaf hairs in the film of Honda, Hongladarom and Wildman. Their evidence for the production of mitochondria in this way seems insufficient, as the small bodies leaving the plastid envelope could be proplastids or microbodies. Greatly elongated plastids are seldom

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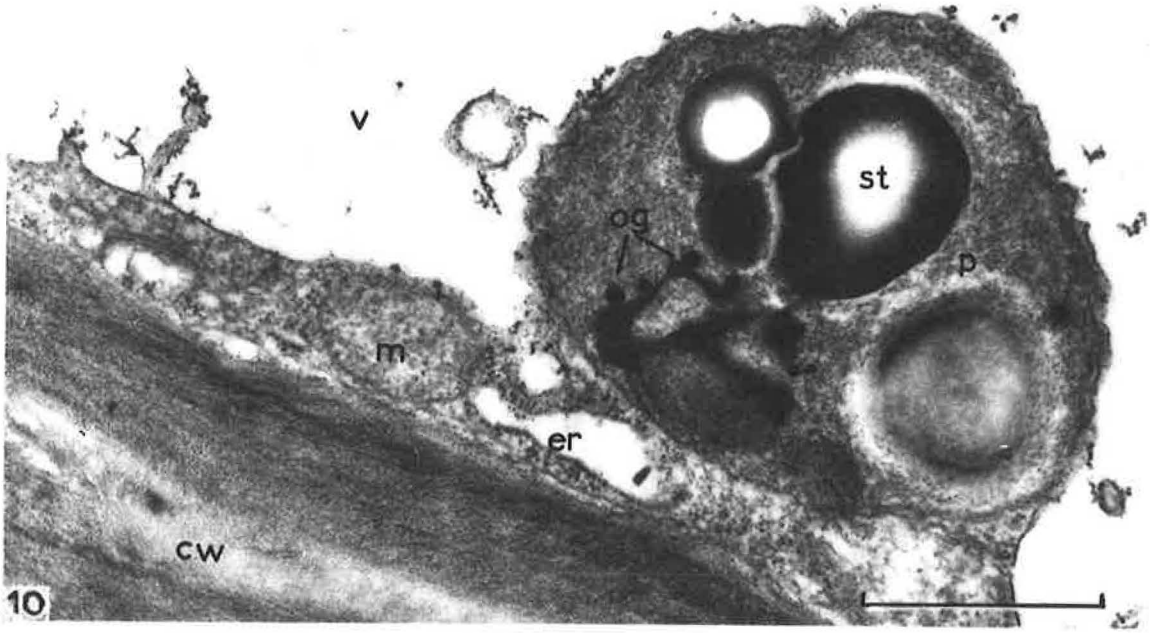
seen in well-fixed beetroot cells (occasionally after glutaraldehyde-OsO_4 fixation), but this is probably due to the speed of fixation. The retractions after division shown in the film were extremely rapid, and would most likely occur during penetration of the fixative, resulting in the oval shape frequently observed in section.

Glutaraldehyde-osmic acid fixation produces a different appearance of the plastids (Fig. 10 and 11) which is sometimes difficult to reconcile with the $KMnO_4$ picture. The 'star' bodies now appear as black osmiophilic globules, and various dark internal bodies in a variety of shapes become visible. Ferritin which may be identical with the stromacentre of Gunning (1965) is often seen (Fig. 11). In the ground cytoplasm, ribosomes become visible, some of which are aligned along the endoplasmic lamellae or grouped to form polysomes.

Initially the vascular rings of intact roots were also investigated so that cells other than the large parenchyma would be detected at magnifications higher than ten thousand times. Heavily lignified xylem vessels are immediately recognisable in both transverse and longitudinal sections (Fig. 12-14). Adjacent small thin-walled cells, probably phloem sieve tubes, companion cells or parenchyma, often contain a relatively large amount of cytoplasm, many organelles and plasmodesmata (Fig. 15). Intercellular spaces are common. Nuclei are seen frequently (Fig. 14), also plastids, mitochondria, Golgi bodies, long ER lamellae and vesicles. Some cells contain unusually large mitochondria (Fig. 12 and 16) in which the cristae sometimes do not



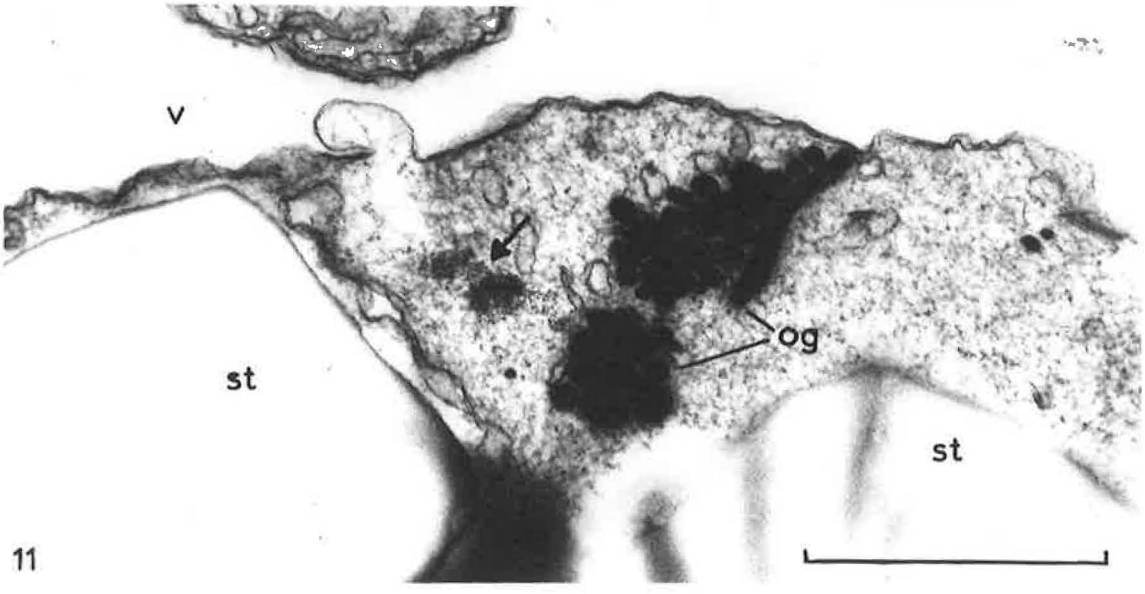
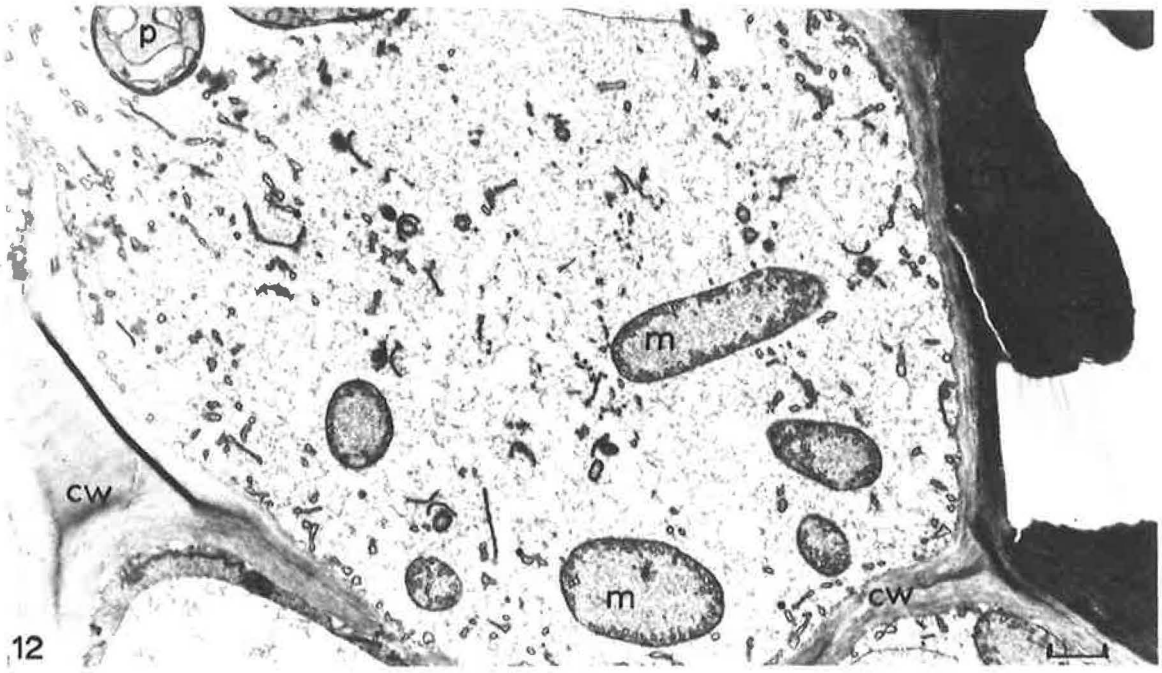


Fig.10-11. Plastids in beetroot parenchyma cells showing starch, osmiophilic globules and ferritin (Fig.11, arrow). Glutaraldehyde-OsO_4 fixation.



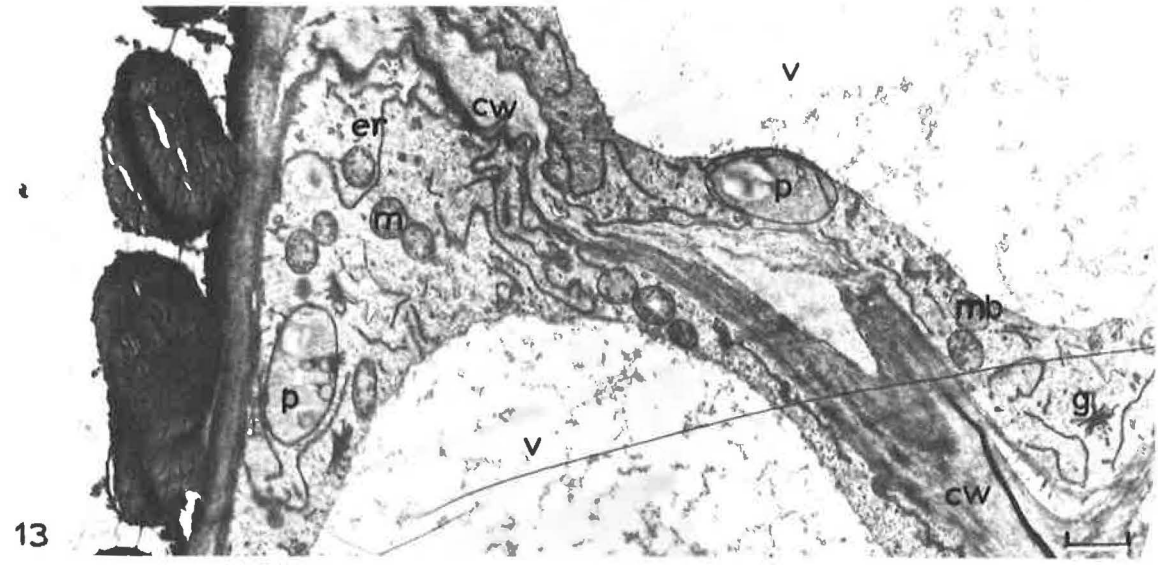


Fig.12-13. Vascular tissue of beetroot in transverse (Fig.12) and longitudinal (Fig.13) section. Cells adjacent to xylem vessels are possibly sieve tubes containing large mitochondria (Fig.12) and elongate parenchyma cells with many organelles (Fig.13). $KMnO_4$ fixation.

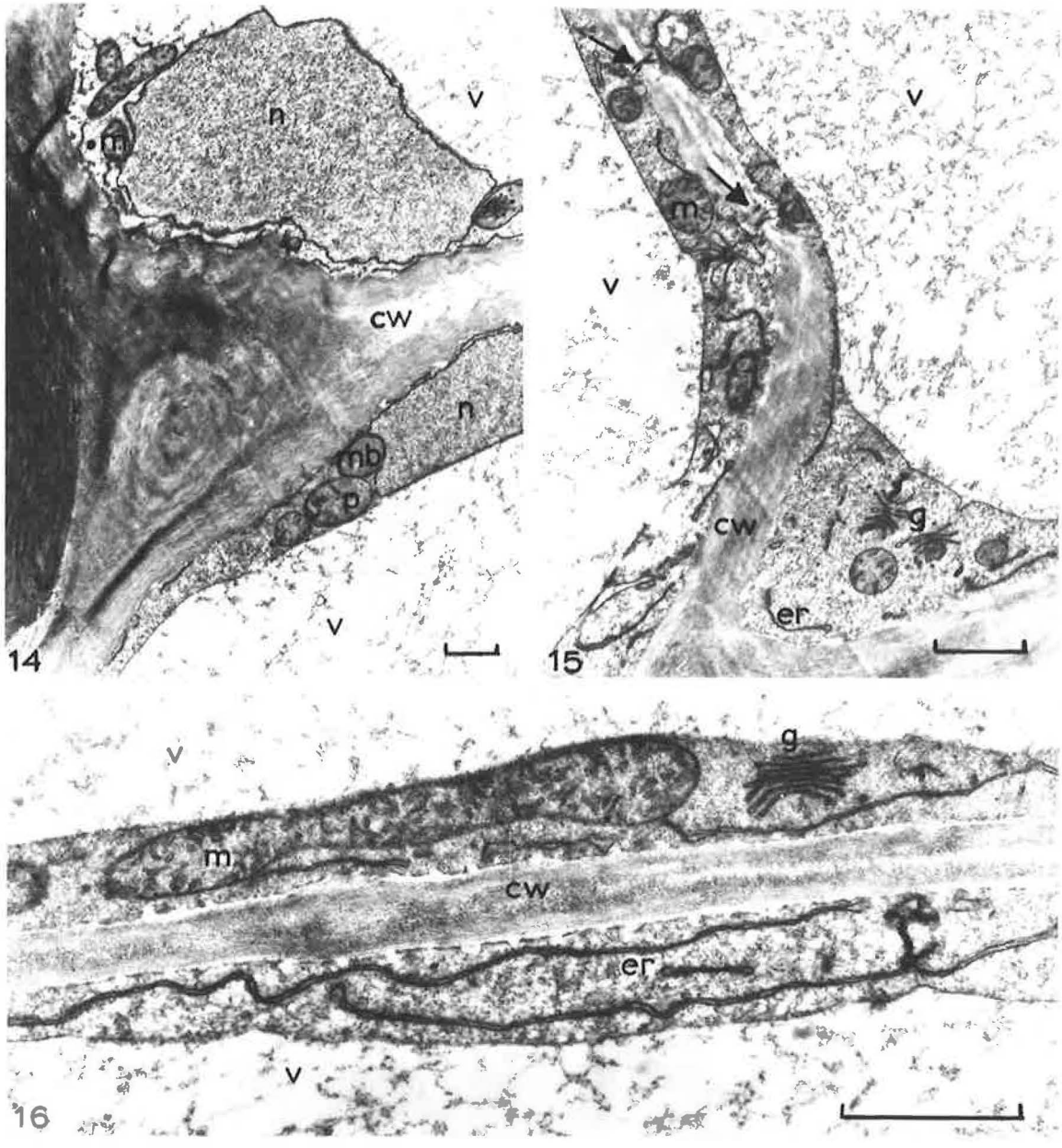


Fig.14-16. Vascular tissue of beetroot showing nuclei in cells adjacent to xylem vessels (Fig.14), plasmodesmata (Fig.15 arrows) and large mitochondria with long ER lamellae (Fig.16). $KMnO_4$ fixation.

extend deeply into the matrix. Thus vascular tissue is easily distinguished from parenchyma by cell size, wall thickness and amount of cytoplasm.

Occasionally batches of beetroot had rings of white colourless tissue concentric with the vascular rings. This tissue proved to consist of normal living parenchyma cells, but apparently lacked the colored pigment, betacyanin.

(b) Carrot

The general appearance of carrot cells depends largely on the fixative used. As might be expected, the walls vary in thickness (0.3-2μ) and sometimes appear to separate in the region of the middle lamella, especially when fixed with $KMnO_4$ (Fig. 17). This fixative is also unsatisfactory because of the large amount of sugar stored in the tissue, but it does provide the best picture of the plastids and chromoplasts (Frey Wyssling and Schwegler 1965). The carotene-containing chromoplasts develop from cup-shaped proplastids by the production of lipoprotein lamellae as needles, sheets or tubes (Fig. 17 and 18) and the complete breakdown of the plastid stroma (Frey Wyssling and Schwegler 1965). Glutaraldehyde - OsO_4 fixation also reveals the lamellar structure (Fig. 19).

Plastids of carrot often show marginal bodies similar to those described in beetroot (Fig. 18). Within the matrix, there are usually large numbers of osmiophilic globules of variable size (Fig. 20 and 21), together with other membranes and vesicles and occasional pro-lamellar

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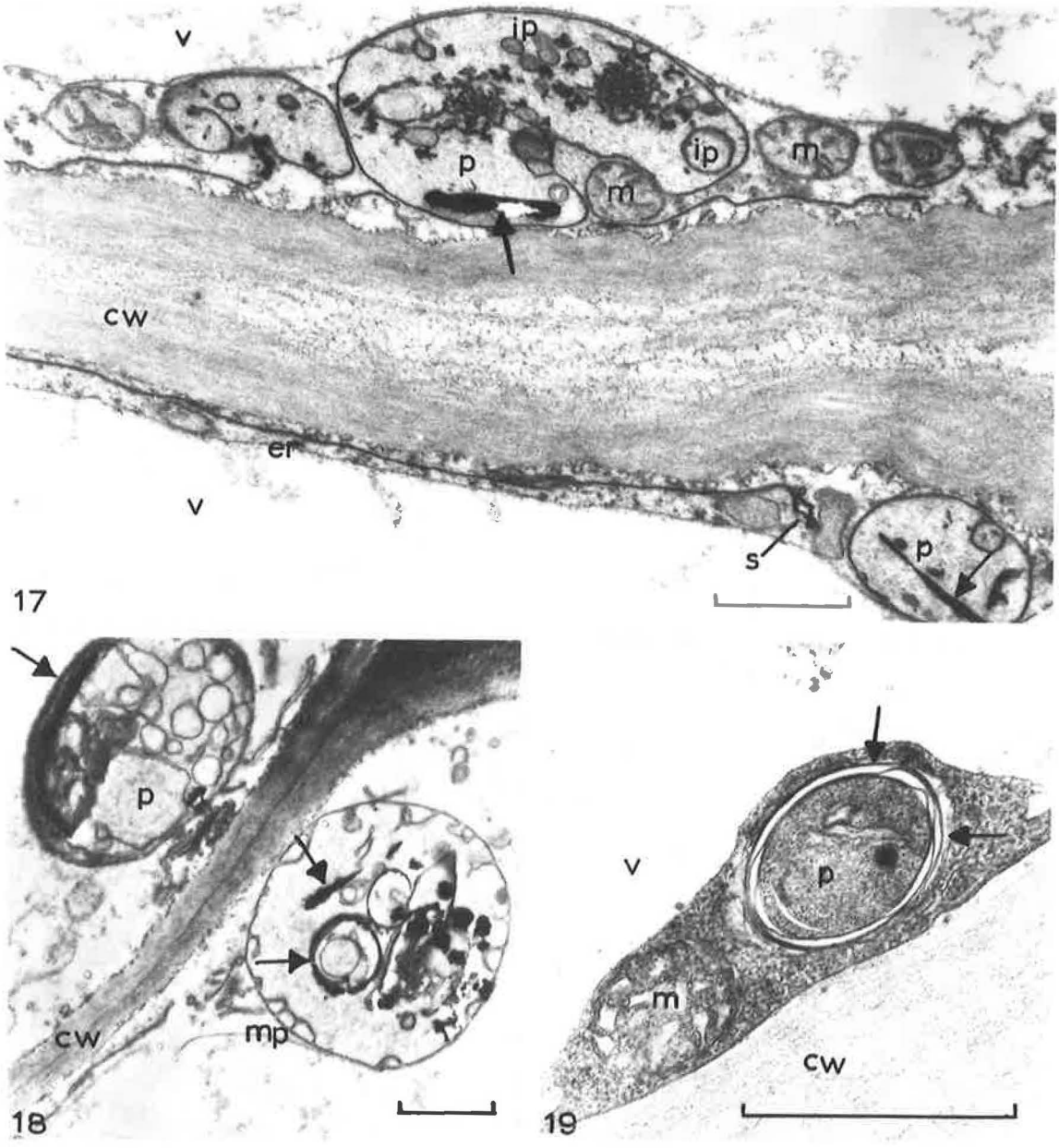


Fig.17-19. Xylem parenchyma of carrot. The plastids show lipoprotein lamellae in various stages of chromoplast development (arrows), 'star' bodies and intraplastid bodies (Fig.17 ip), and marginal bodies (Fig.18 mp). Fig.17, fresh disks (rinsed 2 hr), $KMnO_4$ fixation showing characteristic appearance of spherosome(s); Fig.18, aged disks (148 hr), $KMnO_4$ fixation; Fig. 19, aged disks (170 hr), glutaraldehyde-OsO_4 fixation.

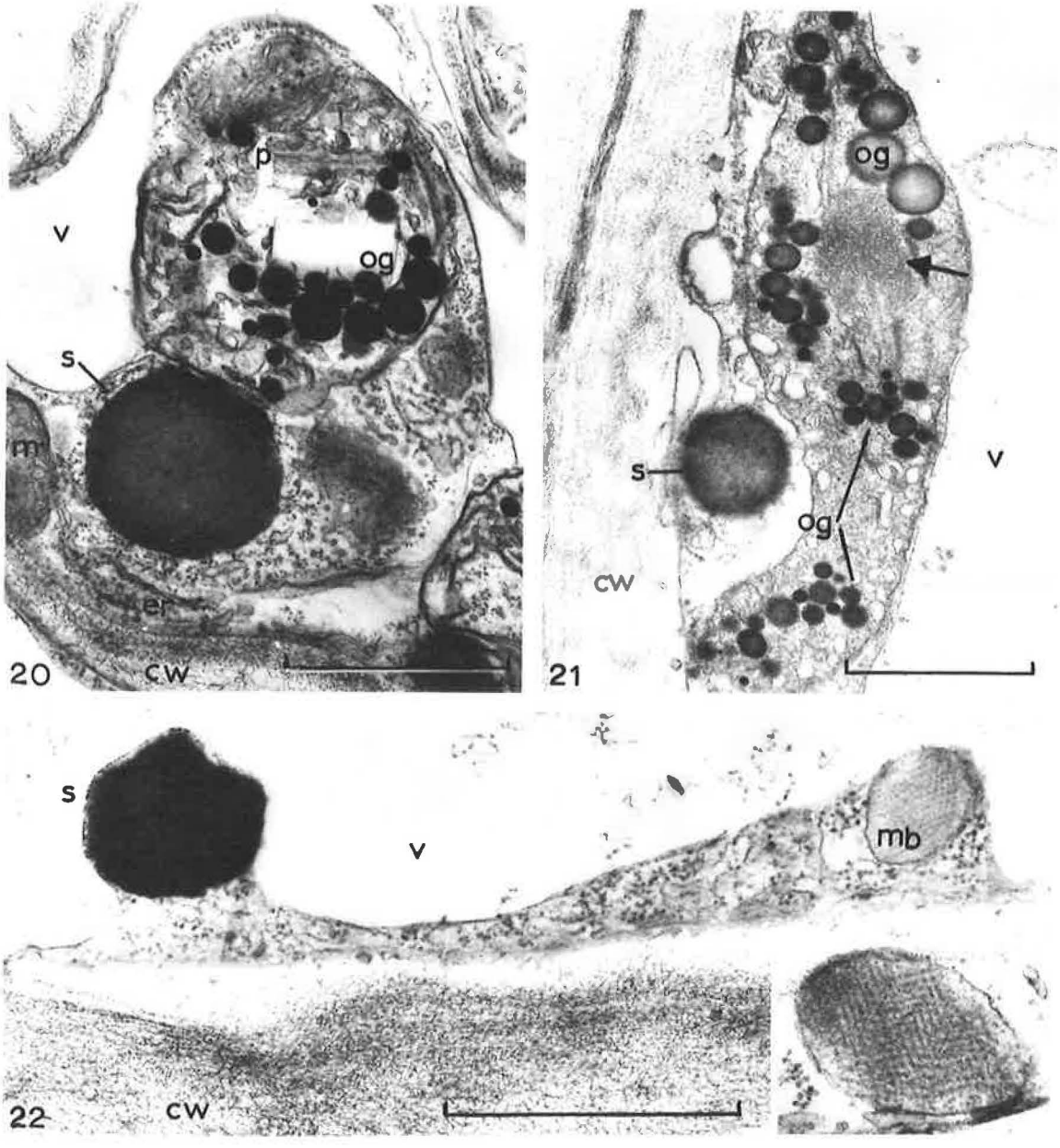


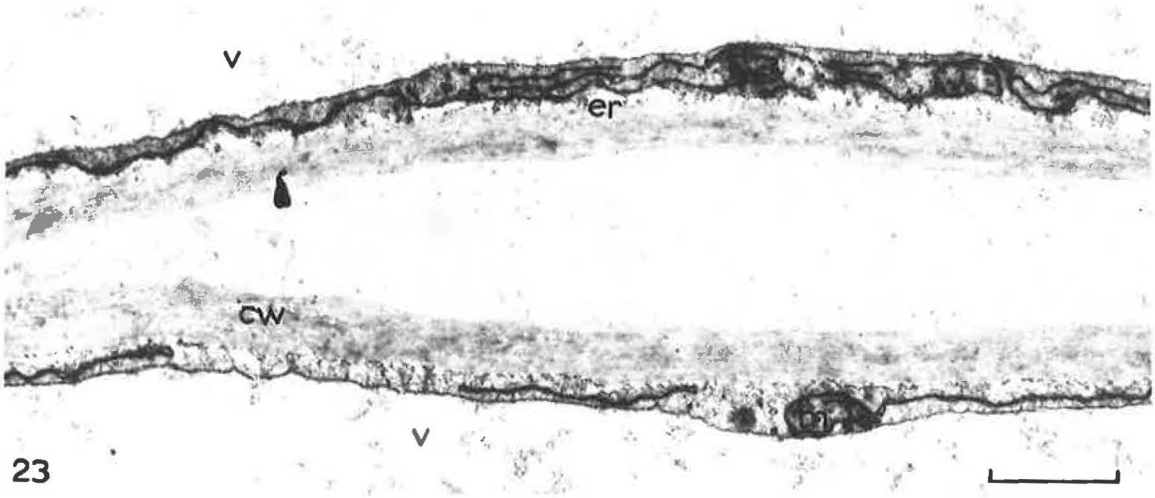
Fig.20-22. Xylem parenchyma of carrot showing spherosomes(s), microbodies with crystalline structure (Fig.22 and inset), and plastids containing numerous osmiophilic globules (Fig.20,21) and a prolamellar body (Fig.21, arrow). Fig.20, aged disks (96hr), OsO_4 fixation; Fig.21, whole tissue, glutaraldehyde-OsO_4 fixation: Fig.22, aged disks (196 hr), OsO_4 fixation.

bodies. Cytoplasmic bodies less than 1μ in diameter, which stain darkly with OsO_4 (Fig. 20-22) and are unstained by $KMnO_4$ (Fig. 17) are thought to be spherosomes. In OsO_4-fixed tissue, another organelle is seen, not infrequently which is similar in size to the microbodies of beetroot (about 0.5μ in diameter). It is membrane bound and the matrix shows a crystalline lattice structure (Fig. 22). Frederick et al (1968) interpreted similar crystal containing bodies as a specialised type of microbody characteristic of metabolically less active cells.

(c) Swede

The selected cells of swede are generally circular in outline with walls about 1μ thick and cytoplasmic lining 0.2-0.3μ wide (Fig. 23). Some wider pockets of cytoplasm are seen especially where organelles are grouped around the nucleus (Fig. 24). Frequent intercellular spaces occur.

The cytoplasm of whole swede contains numerous small mitochondria, long twisted endoplasmic lamellae, numbers of plastids and more Golgi bodies than either of the other storage roots studied. Within the plastids (Fig. 24-27) are large starch grains, many osmiophilic globules ('star' bodies after $KMnO_4$ fixation), some ferritin, and few to many marginal bodies of the type already described (Fig. 25). In several instances, groups of microbodies (or proplastids or promitochondria) were seen adjacent to plastids with these marginal bodies, again suggesting a possible method of biogenesis of these organelles (Fig. 26). Another inclusion, apparently crystalline and possibly within cisternae of the



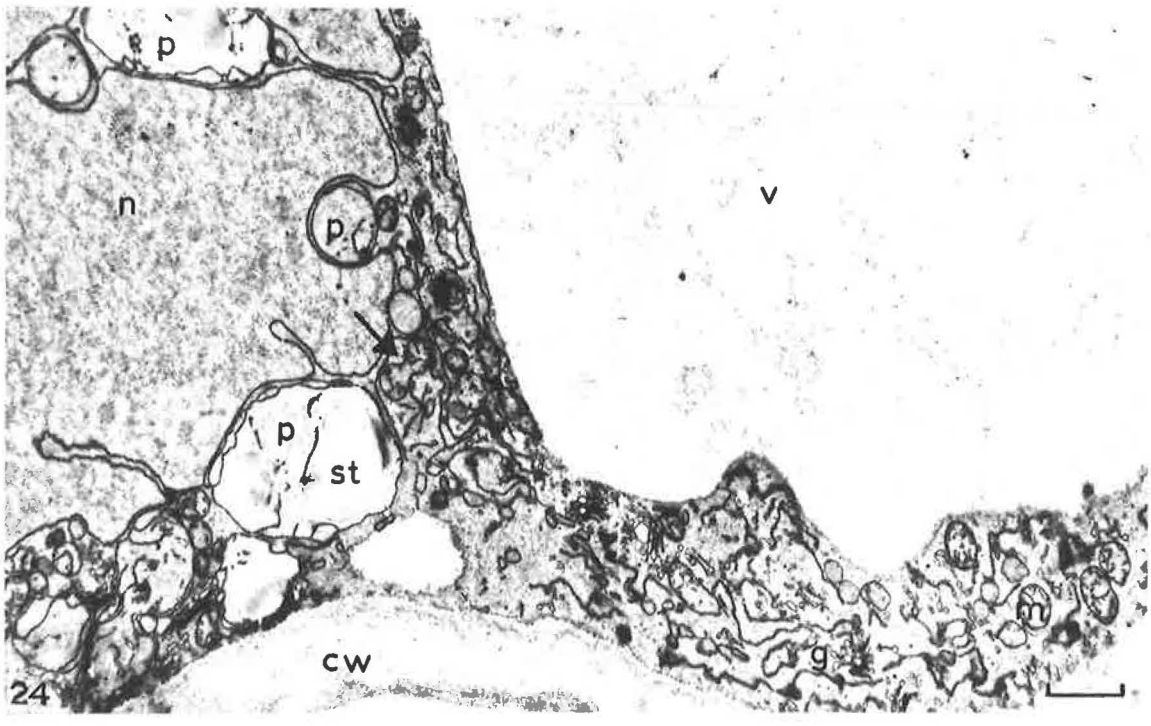
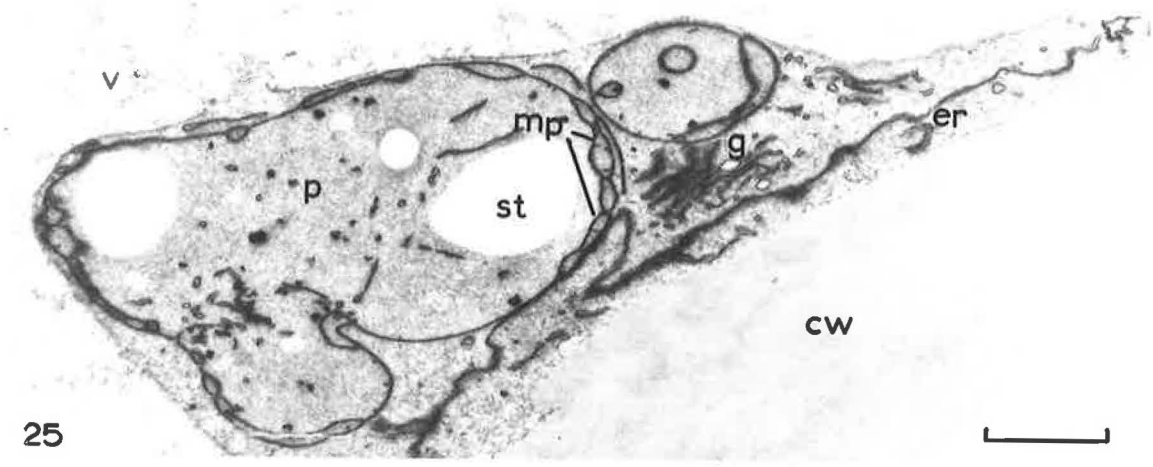


Fig.23-24. Swede storage parenchyma showing thin cytoplasm with long ER lamellae (Fig.23) and large number of organelles closely associated with the nucleus (Fig.24). Constricted mitochondrion (Fig.24, arrow). $KMnO_4$ fixation.



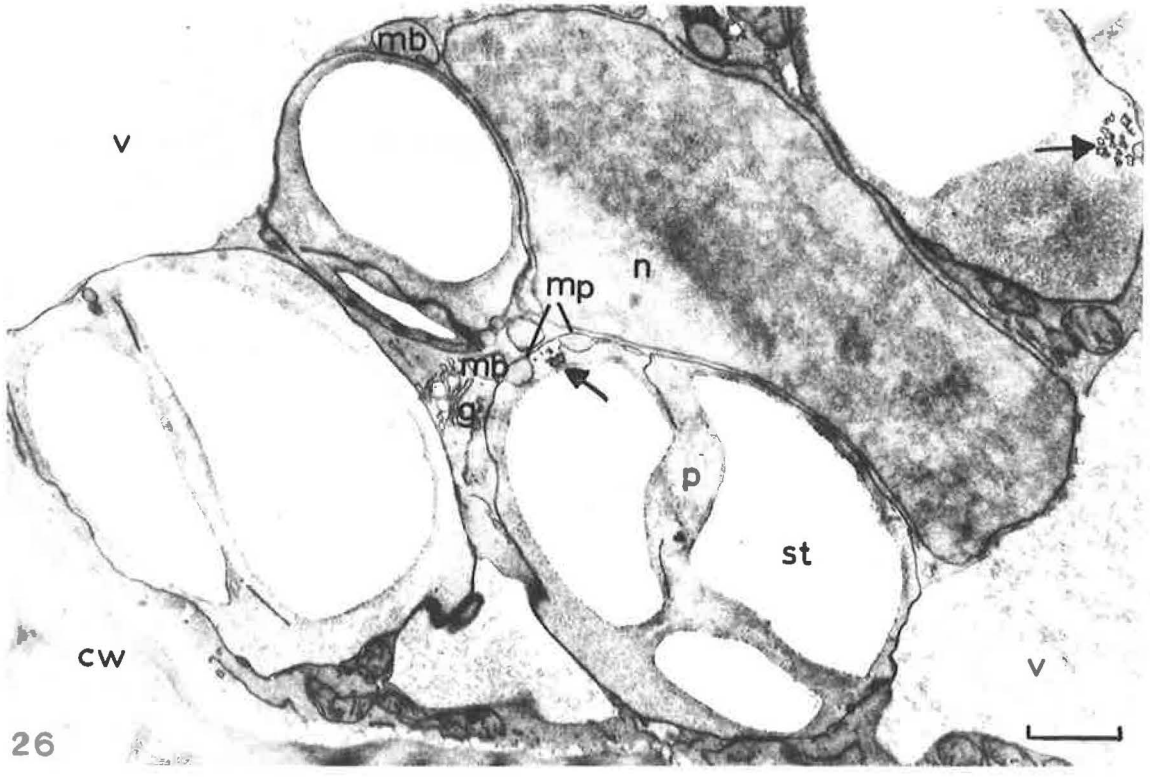
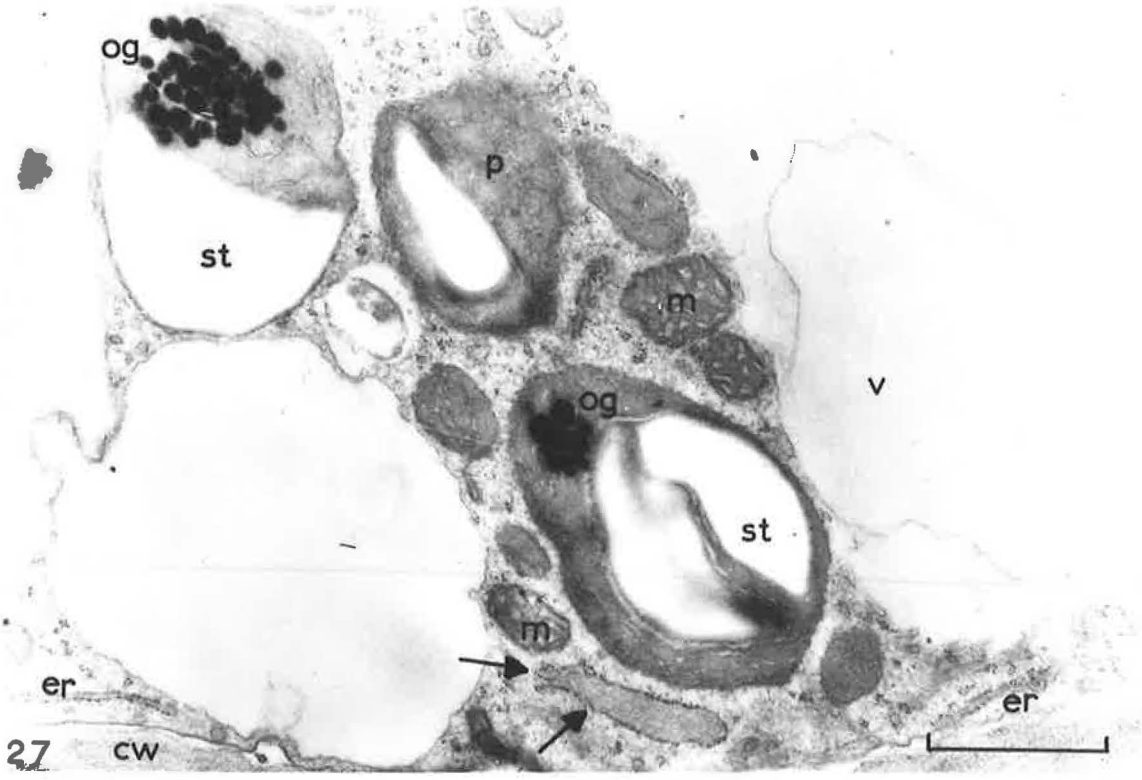


Fig. 25-26. Swede storage parenchyma. Plastids containing starch, 'star' bodies, (arrows) and marginal bodies (mp). $KMnO_4$ fixation.



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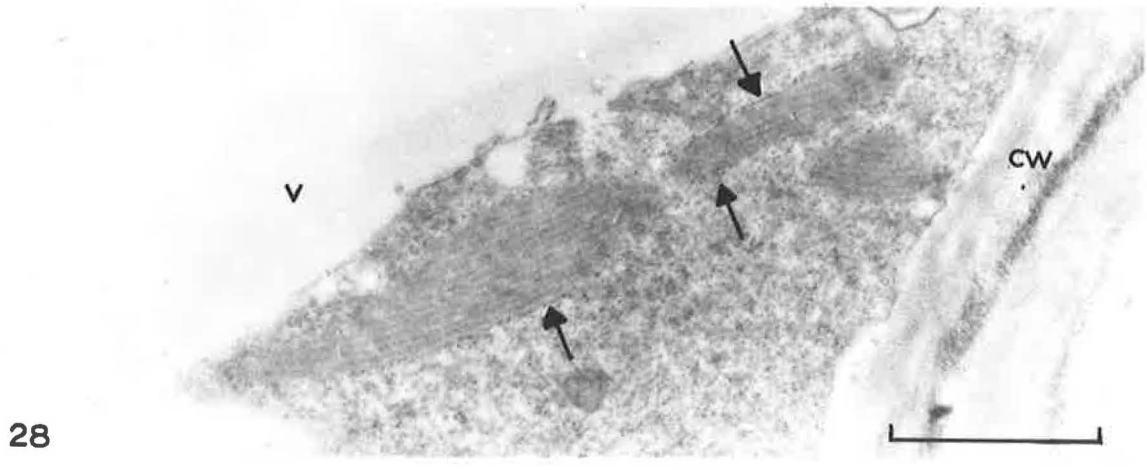

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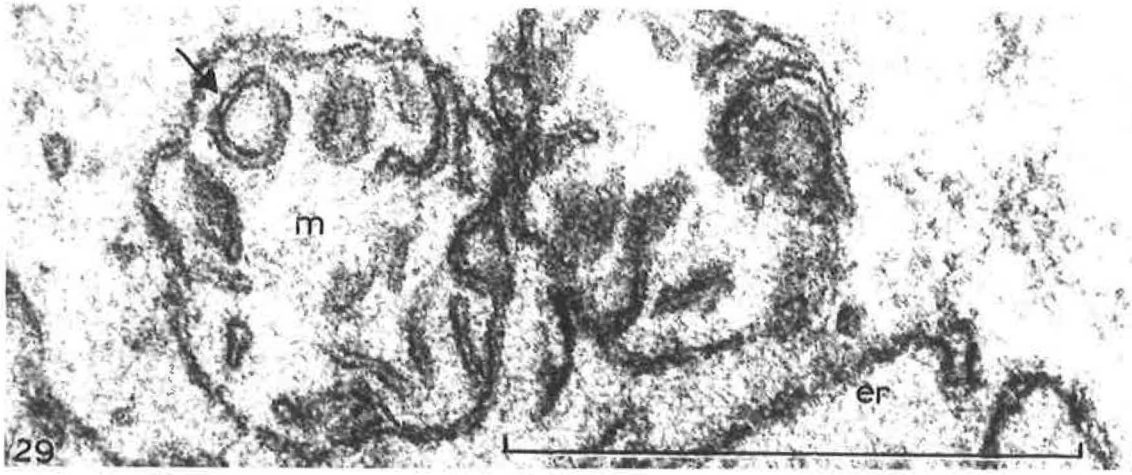
Fig. 27-28. Swede storage parenchyma showing inclusion lined with ribosomes (arrows). Glutaraldehyde-OsO_4 fixation.

ER was observed occasionally (Fig. 27 and 28). Rarely, mitochondria were seen with looped cristae (Fig. 29) suggesting the formation of promitochondria by a process similar to that observed in isolated swede mitochondria (Van Steveninck and Jackman 1967).

Initially the xylem and cortex of swede were also investigated. The walls of cells in the xylem varied in thickness (up to 5μ) and the cells contained much cytoplasm and ER. In the cortical region large intercellular spaces were evident and cell walls were usually less than 1μ wide. Some small cells, possibly phloem, were seen with thin walls (0.5μ), a large nucleus, and large numbers of starch free plastids (Fig. 30).



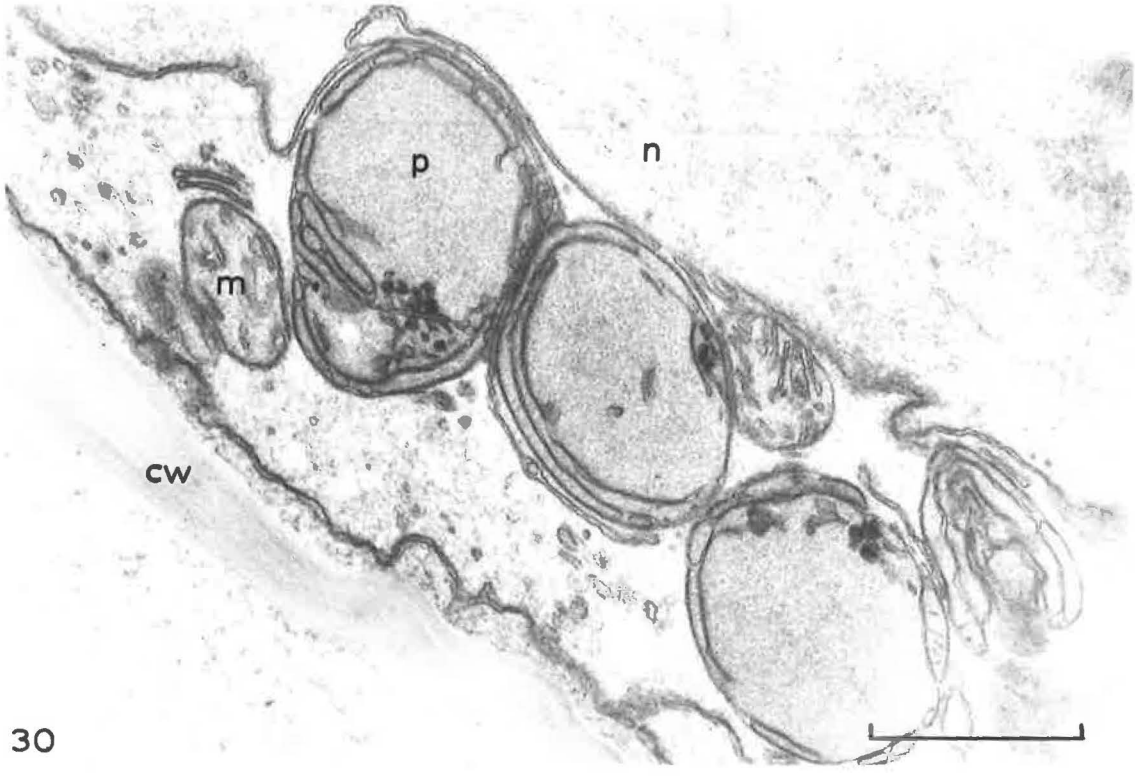


Fig.29. Swede storage parenchyma. Mitochondria with looped cristae (arrow). $KMnO_4$ fixation.

Fig.30. Vascular tissue of swede. Small, thin-walled cell with large nucleus and numerous starch-free plastids. $KMnO_4$ fixation.

Part B - Changes in aging disks of storage tissue

1. Ion movements

The pattern of net Na^+ and K^+ movement between beetroot slices and the external solution is shown in Fig. 31. Rapid leakage of Na^+ and K^+ from the tissue was followed by a phase of rapid net accumulation of Na^+ at 20 hr, while a phase of slow net accumulation of K^+ beginning at 10 hr was followed by a rapid phase of uptake at about 50 hr. This pattern of ion movement is typical for beetroot slices (Van Steveninck 1961).

Fig. 32 shows the pattern of net Na^+ and K^+ movement between carrot slices and the external solution. Initial leakage of both Na^+ and K^+ from the tissue is followed at about 20 hr by a period of rapid net uptake.

The pattern of net Na^+ and K^+ movement between swede disks and the external solution is shown in Fig. 33. This pattern is similar to that for carrot, except that swede tissue shows a greater selectivity for K^+ and this may account for the incomplete uptake of Na^+ in this experiment.

Table II further emphasises the difference in selectivity between these three storage tissues. Beetroot, generally, shows little selectivity for K^+ or Na^+, while swede shows a strong preference for K^+, and carrot takes an intermediate position. Significant losses occur during preliminary rinsing, but these are mainly from cells cut or damaged during slicing.

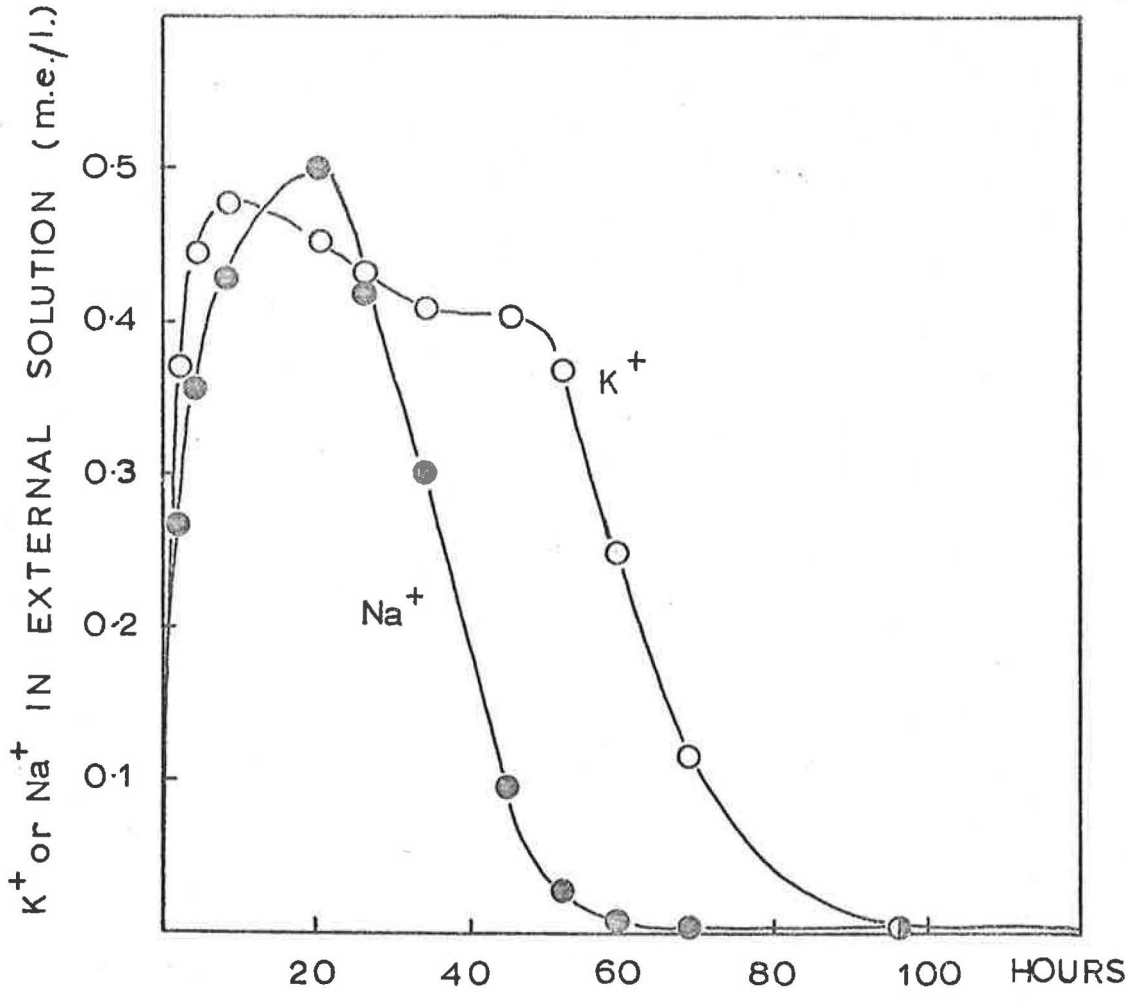


Fig. 31. Changes in Na^+ and K^+ concentration of external solution due to release or uptake of ions by beetroot disks (15 mm diam.; 1 mm thick; 50 disks per 250 ml water; 24°C)

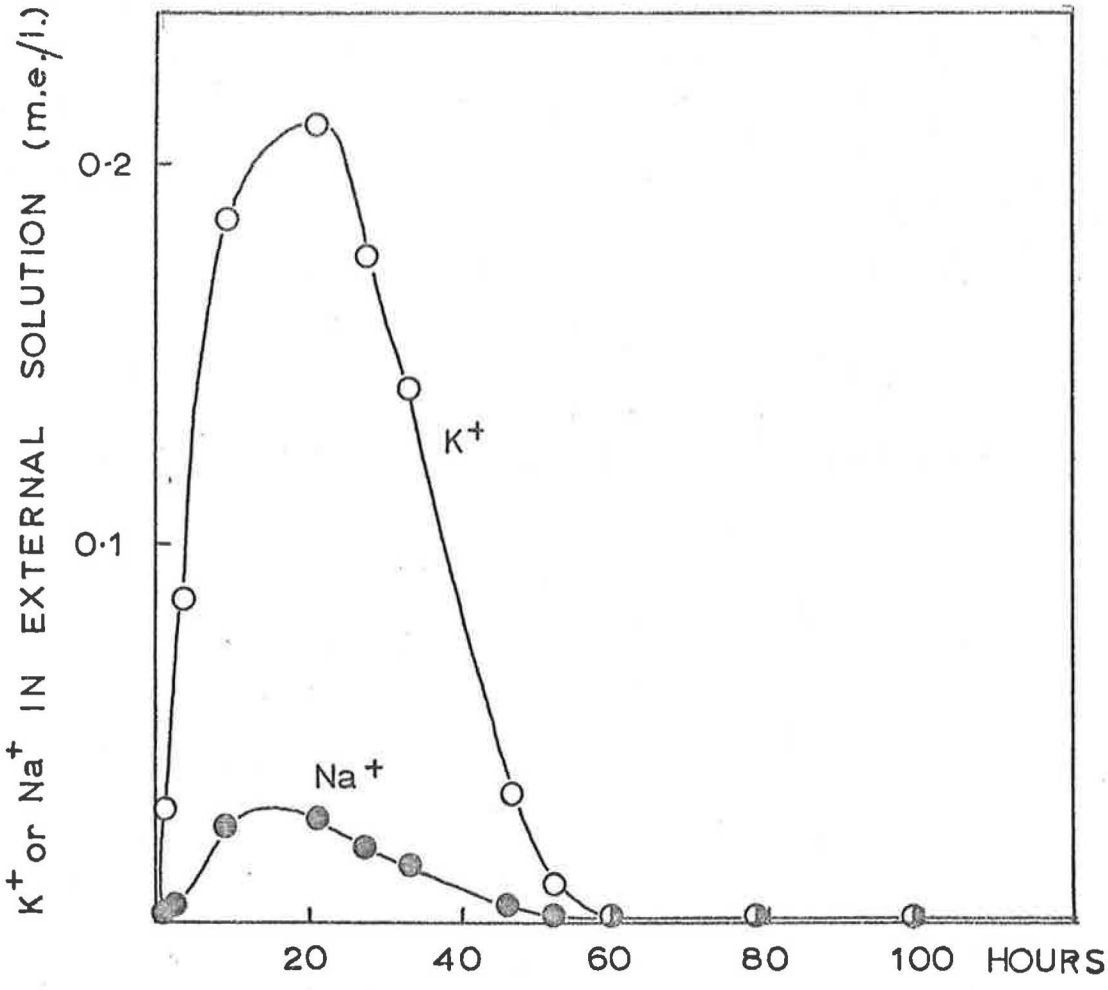


Fig. 32. Changes in Na^+ and K^+ concentration of external solution due to release or uptake of ions by carrot disks (6 mm diam.; 1 mm thick; 8 g disks per 200 ml water; 24°C)

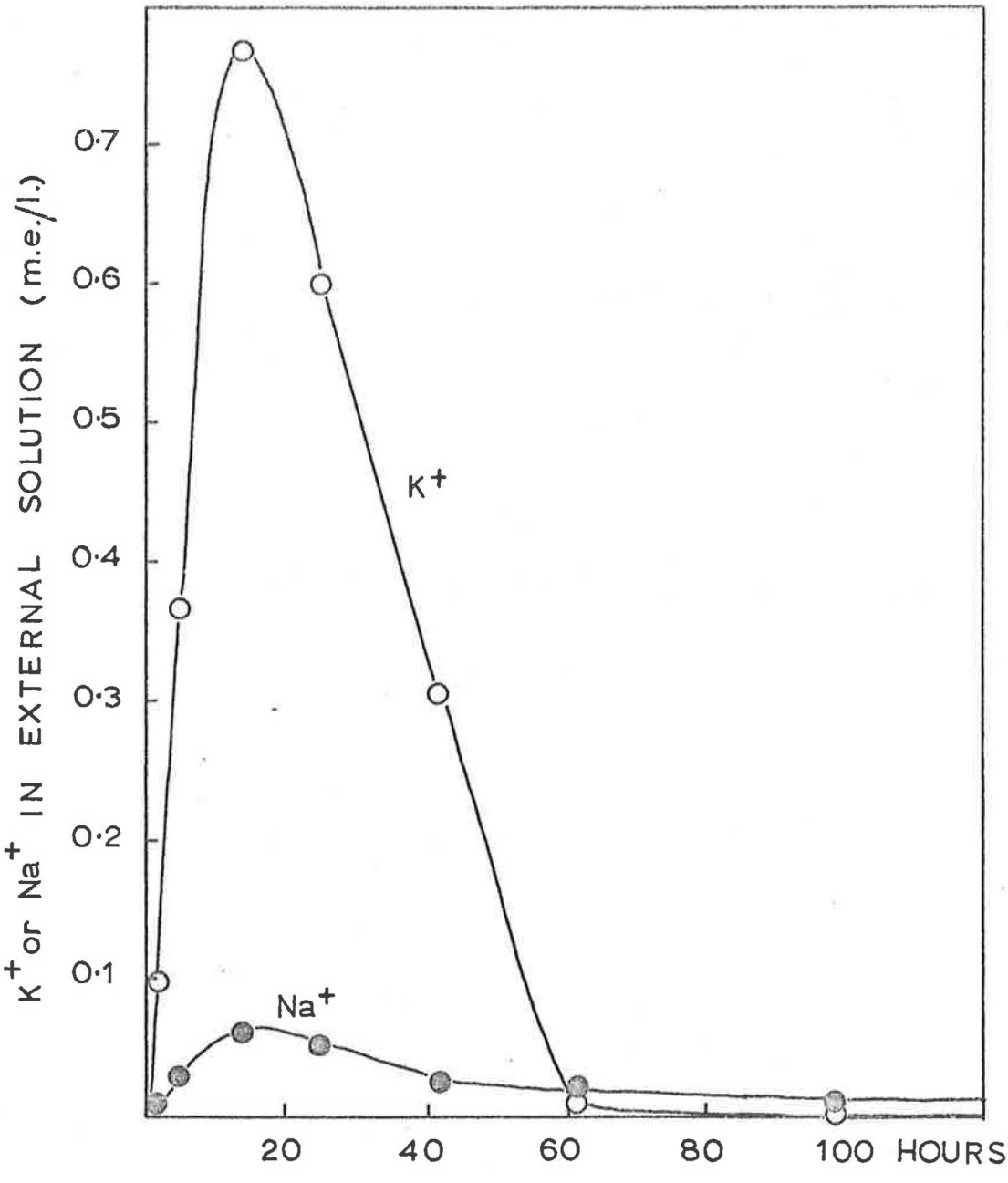


Fig. 33. Changes in Na^+ and K^+ concentration of external solution due to release or uptake of ions by swede disks (15 mm diam.; 1 mm thick; 50 disks per 250 ml water; 24°C)

Table II Sodium and potassium content of beetroot, carrot and swede tissue (m.e. per kg)

	Total		Lost in preliminary rinses		Disk content at 0 hr	
	K^+	Na^+	K^+	Na^+	K^+	Na^+
Beetroot	71.4	76.5	28.6	21.5	42.8	55.0
Carrot	96.3	31.5	16.6	6.6	79.7	24.9
Swede	90.0	3.6	13.4	0.7	67.6	2.9

2. Ultrastructural changes

(a) Beetroot

During the period of aerated washing the most marked change is shown by the endoplasmic reticulum. The lamellar ER present in the tissue fixed at the time of cutting (Fig. 1) is no longer visible after the preliminary rinses, but is reduced to a few cytoplasmic vesicles (Fig. 34 and 35). After washing for approximately 24 hours, the lamellae are again present to the original extent, and thereafter continue to increase in length until an almost continuous layer is formed in the cytoplasm by about 48 hours (Fig. 36-38, and Appendix III, Plates 1 and 2). This membrane persists until at least 192 hours. The changes are more readily observed after $KMnO_4$ fixation, but there

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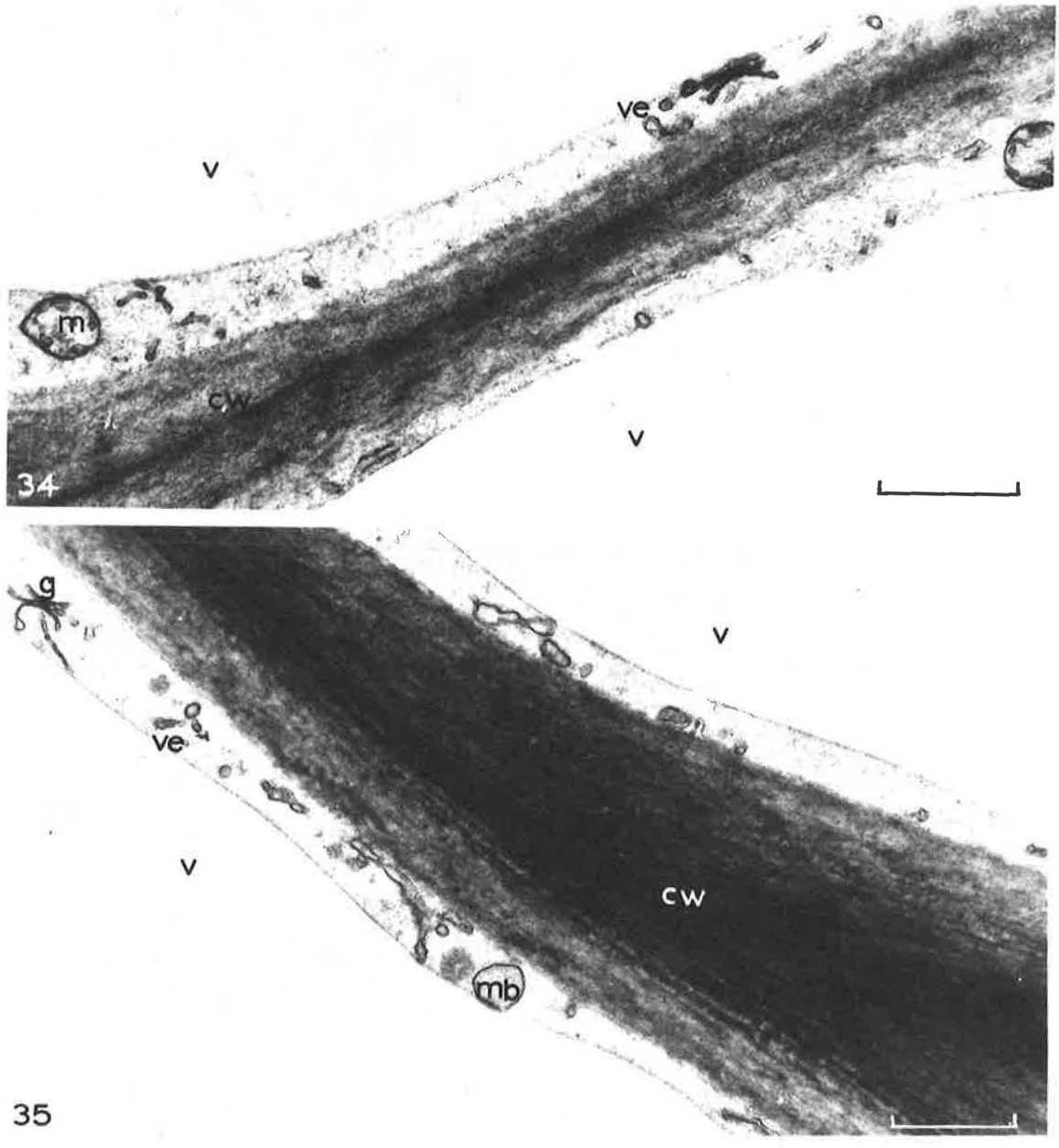

Fig.34-35. Parenchyma from beetroot slices following preliminary rinses (4 hr.). ER lamellae reduced to cytoplasmic vesicles. $KMnO_4$ fixation.

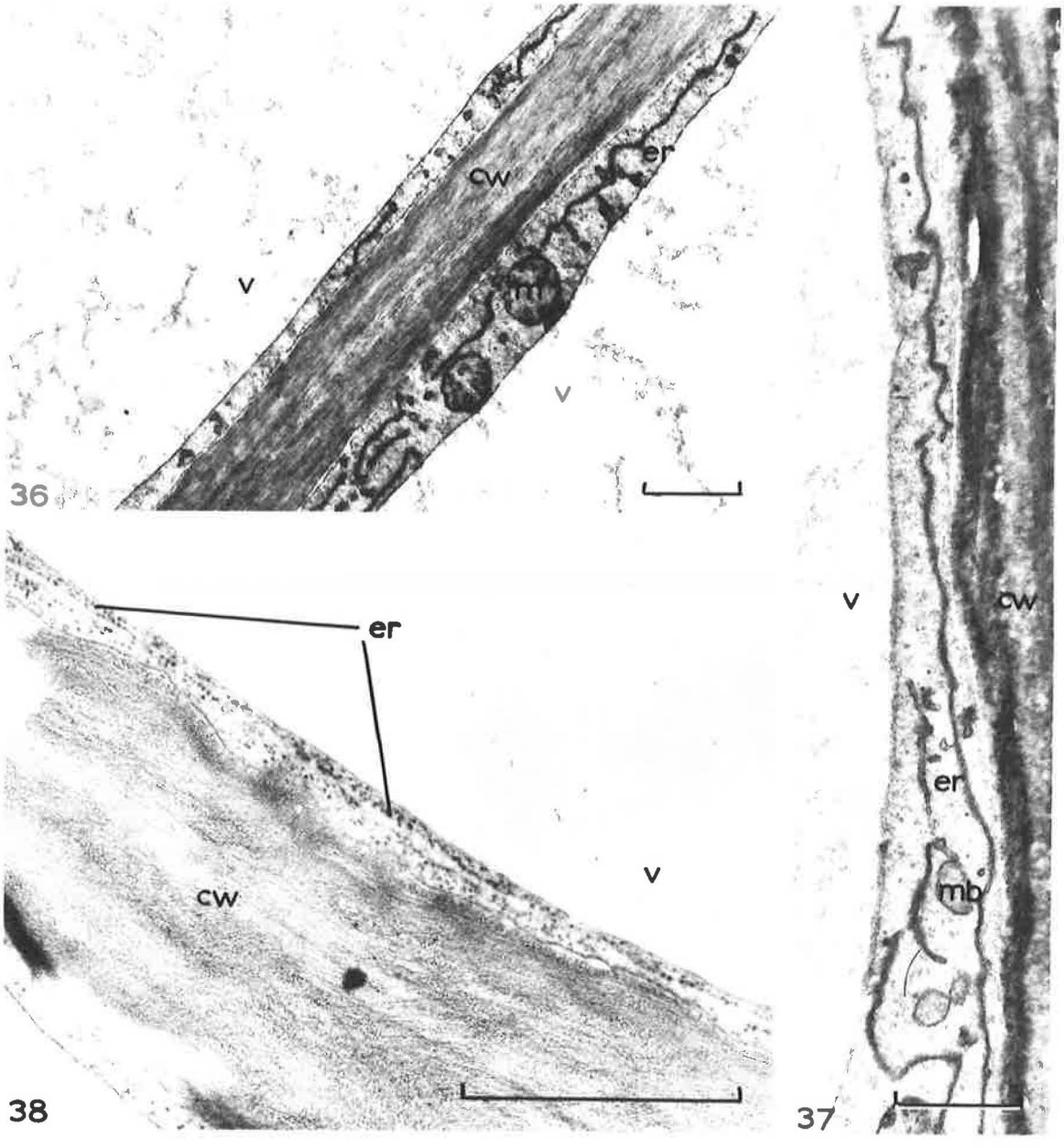

Fig.36-38. Parenchyma from beetroot slices washed for 50 hr. (Fig.36), 74 hr. (fig.37), and 144 hr (Fig.38) showing increase in length of ER lamellae. Fig.36,37, $KMnO_4$ fixation; Fig.38, glutaraldehyde-OsO_4 fixation.

is clearly an increase in ER in glutaraldehyde - OsO_4 fixed tissue.

Occasionally by 48 hours, and frequently by 96 hours, the ER contains crystals which become more numerous with time (Fig. 39-46). The outlines of these crystals sectioned in various planes, indicate a rhombahedral form. The crystal matrix is either uniformly granular with no visible lattice structure, or shows one or two concentric bands (Fig. 39 and 40). These crystals are generally single, but occasionally two are observed enclosed in the same portion of the ER (Fig. 41). Frequently one long endoplasmic membrane shows a number of crystals along its length (Fig. 42 and 43). The membrane is usually closely appressed to the crystal outline, but, on rare occasions, small membrane-bound bodies may be seen enclosed with the ER adjacent to a crystal (Fig. 44). After an extended period of washing (14 days), similar microbodies may be seen outside the ER membrane adjacent to crystals which have lost their linear outline (Fig. 45). When fixed with glutaraldehyde - OsO_4, ribosomes are generally visible along the endoplasmic membrane surrounding the crystal (Fig. 46).

During this work crystals were not seen in whole beetroot or freshly cut disks. Pellets of mitochondria isolated from whole tissue, however, always contained a small but significant number of crystals, which were probably present in parts of the root not usually selected for electron microscopy. Hence samples were taken from above the centre of the root i.e. below the base of the leaves, presumably in the centre

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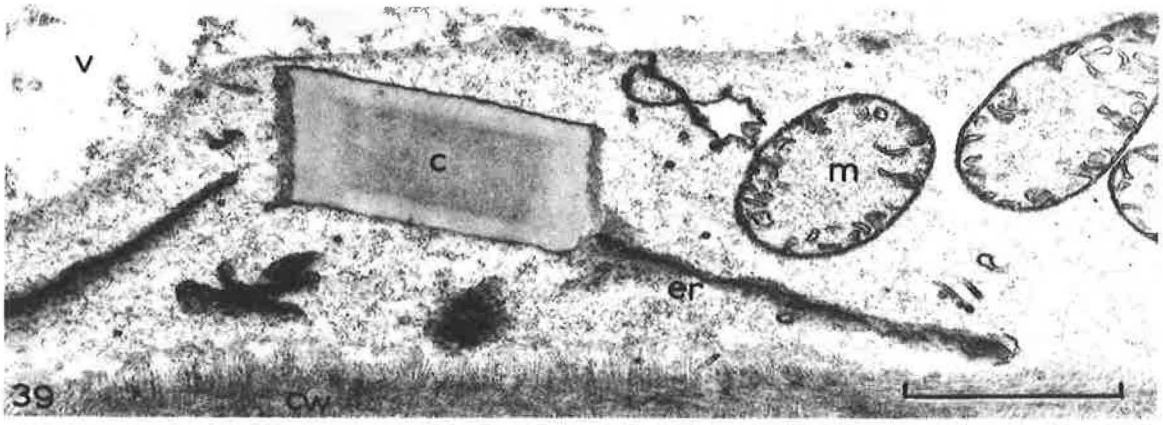
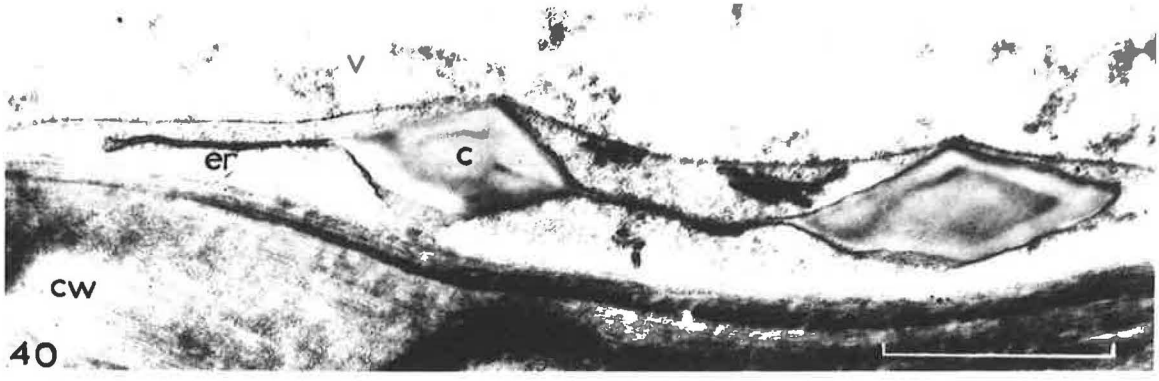




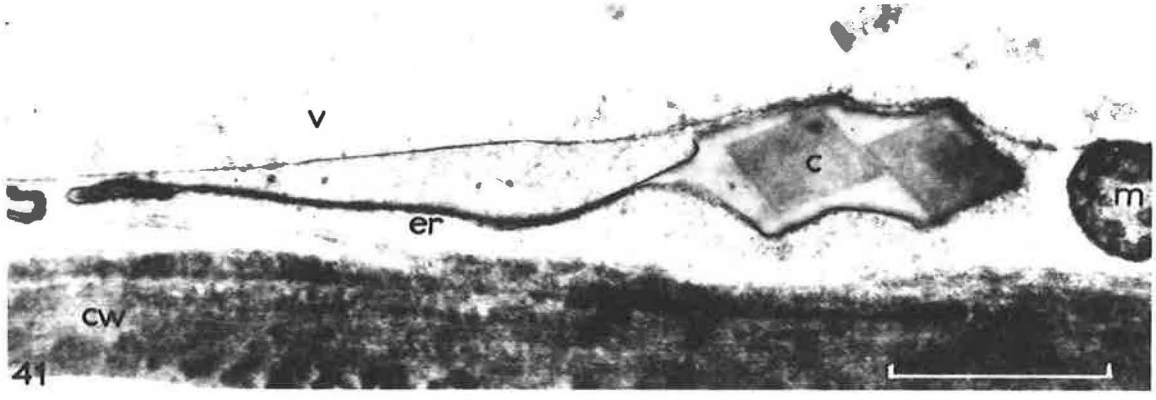


Fig.39-41. Parenchyma from beetroot slices washed for 192 hr showing crystalloid bodies enclosed singly within cisternae of the ER, or rarely with two crystals within the same portion of the ER (Fig.41). Concentric bands in the crystal matrix are sometimes visible (Fig.39,40). $KMnO_4$ fixation.

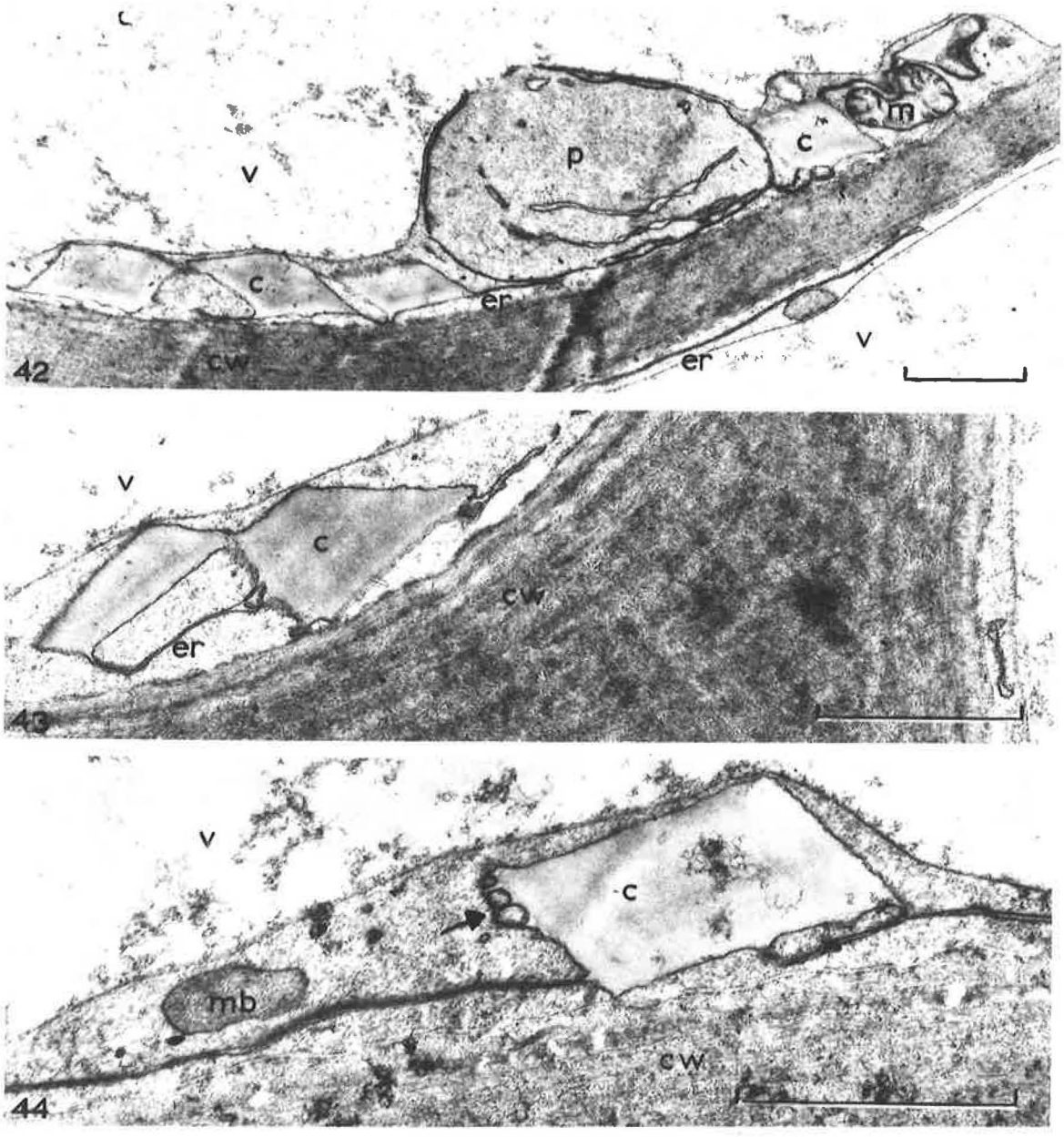
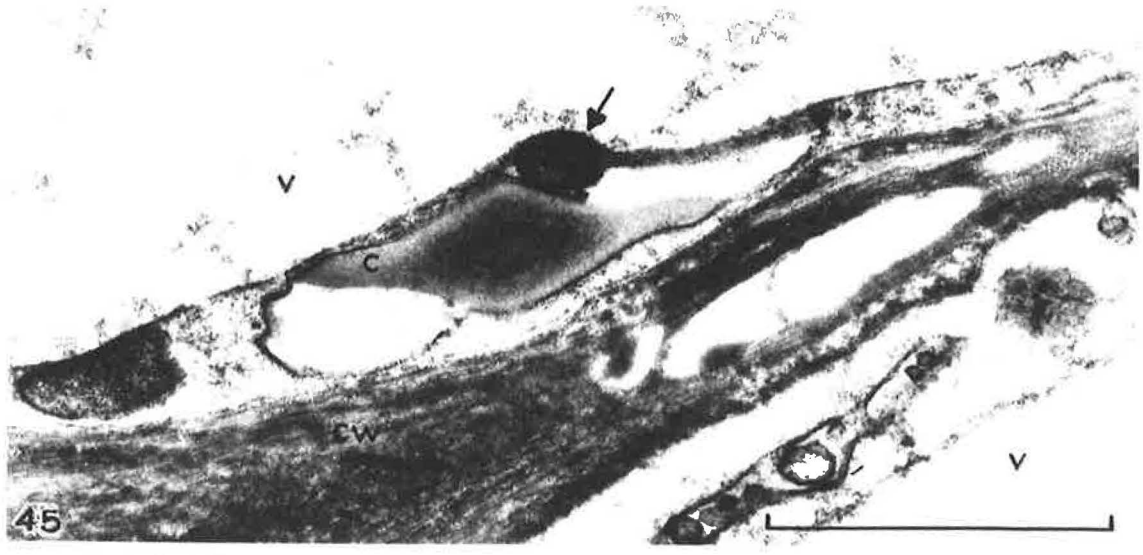


Fig.42-44. Parenchyma from beetroot slices washed for 192 hr showing crystalloid bodies. Several crystals may be enclosed along the length of one ER cisterna (Fig.42,43) and occasionally small membrane-bound inclusions are seen adjacent to the crystal (Fig.44, arrow). $KMnO_4$ fixation.



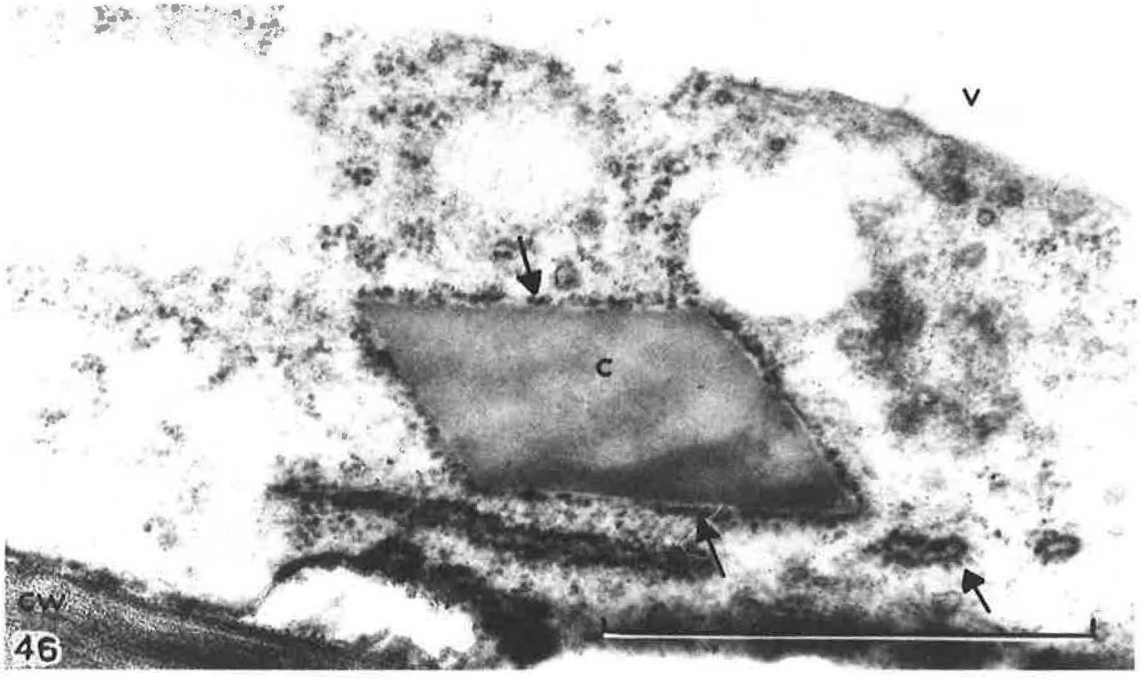


Fig.45. Parenchyma from beetroot slice washed for 336 hr showing irregular outline of crystal and adjacent microbody (arrow). $KMnO_4$ fixation.

Fig.46. Parenchyma from beetroot slice washed for 48 hr showing ribosomes along crystal margin and ER (arrows). Glutaraldehyde-OsO_4 fixation.

of the hypocotyl tissues, and from the base of the swollen part of the root. Cells from the hypocotyl showed apparent poor fixation with little cytoplasmic content, and it is possible that these cells are senescent. Several cells contained mitochondria (some abnormally large), short ER lamellae, and occasional plastids. Crystals showing partial digestion, similar to those in mitochondrial preparations (Van Steveninck and Jackman 1967) were seen occasionally. Cells from the base of the root, as expected, consisted of functional vascular tissues.

Sometimes changes are observed within the plastids during this period of aging. Firstly a prolamellar body appears which gives rise to strings of vesicles (Fig. 47). After 3-4 days these vesicles begin to resemble grana thylakoids (Fig. 48 and 49) of a chloroplast (seen in section or face view). This process is probably a response to light which is essential for grana development in etiolated leaves (Muhlethaler and Frey-Wyssling 1959). It is interesting to observe that plastids in an underground storage organ appear to have, or acquire within a few days, the capacity to form grana (Gerola 1960).

Observations of changes in other organelles or membranes were generally inconclusive, but further observations on material fixed in OsO_4 alone are included in Part C. Large irregularly shaped mitochondria were perhaps found more frequently in intact tissue and nearly all mitochondria in aged disks were small and circular in section.

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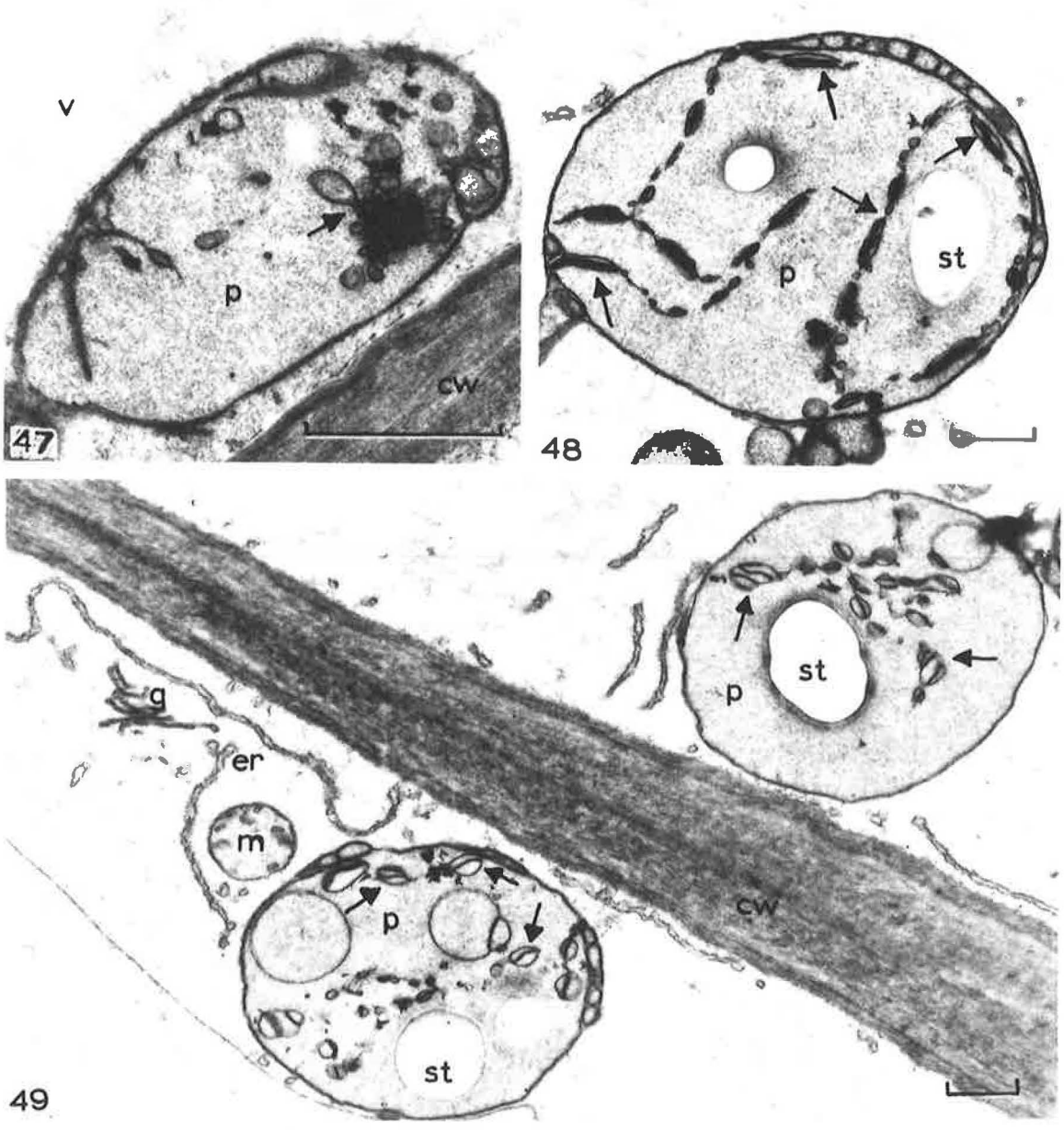

Fig.47-49. Plastids in parenchyma from beetroot slices washed for 98 hr showing vesicle formation from prolamellar body (Fig.47, arrow) and development of grana thylakoids (Fig.48,49, arrows). $KMnO_4$ fixation.

(b) Carrot

There were no convincing changes in these cells during the aging period. From general observations in three experiments, it seemed possible that the number of mitochondria increased almost immediately after cutting. So an attempt was made to count the number of mitochondria in many fields of the electron microscope at a particular magnification, giving the following preliminary results for one experiment: Whole carrot tissue gave an average of one mitochondrion in 8 fields at a magnification of 24,000 times. At the same magnification an average of 1 or 2 mitochondria per field were found in disks immediately after the preliminary rinses or after a further 28 or 170 hours of washing. This result was not confirmed by another experiment, but in this case the tissue fixed for electron microscopy was taken from a large number of disks cut over a period of $1\frac{1}{2}$ hours into water and then rinsed once in distilled water. Hence any disk sampled at 0 hr could have been in water for any time from a few minutes to $1\frac{1}{2}$ hr.

Another frequently observed difference was the absence of a well-defined plasmalemma in whole and freshly sliced tissue whether fixed with $KMnO_4$ or glutaraldehyde-OsO_4 (Fig. 17, 18, 21). In aged disks (28 hours or more), a clear three-layered membrane was frequently visible with the latter fixative (Fig. 19). But this observation, although it was not confined to one experiment, may be a result of slight differences in fixation.

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(c) Swede

Again there were no consistently found ultrastructural changes during the period of aerated washing. The tonoplast, which was often broken in whole tissue (Fig. 50) became more apparent and was usually intact in aged tissue (Fig. 51), but this change is thought to be an artifact of fixation ($KMnO_4$) and possibly staining. It could, however, reflect the stability of this membrane.

In contrast to the ER in beetroot, the intact swede usually has extensive lamellae. During cutting and rinsing these remain unchanged but the ER in tissue aged for more than 48 hours was found to consist almost entirely of vesicles (Fig. 51).

3. Discussion

It may be too ambitious to expect any obvious ultrastructural change in storage root slices which can be directly or indirectly correlated with the development of the capacity to accumulate ions. It is quite possible that there is a change in number of organelles e.g. mitochondria. The first requirement for any quantitative electron microscopy, especially when working with large cells of which such a small part is selected by sectioning, is reliable fixation and specimen preparation. Until this can be achieved, only very marked changes will be detected.

It is possible that fixation which is suitable for one stage of cell development or senescence may be less suitable for another stage. Similarly, it should be taken into consideration that the actual slicing

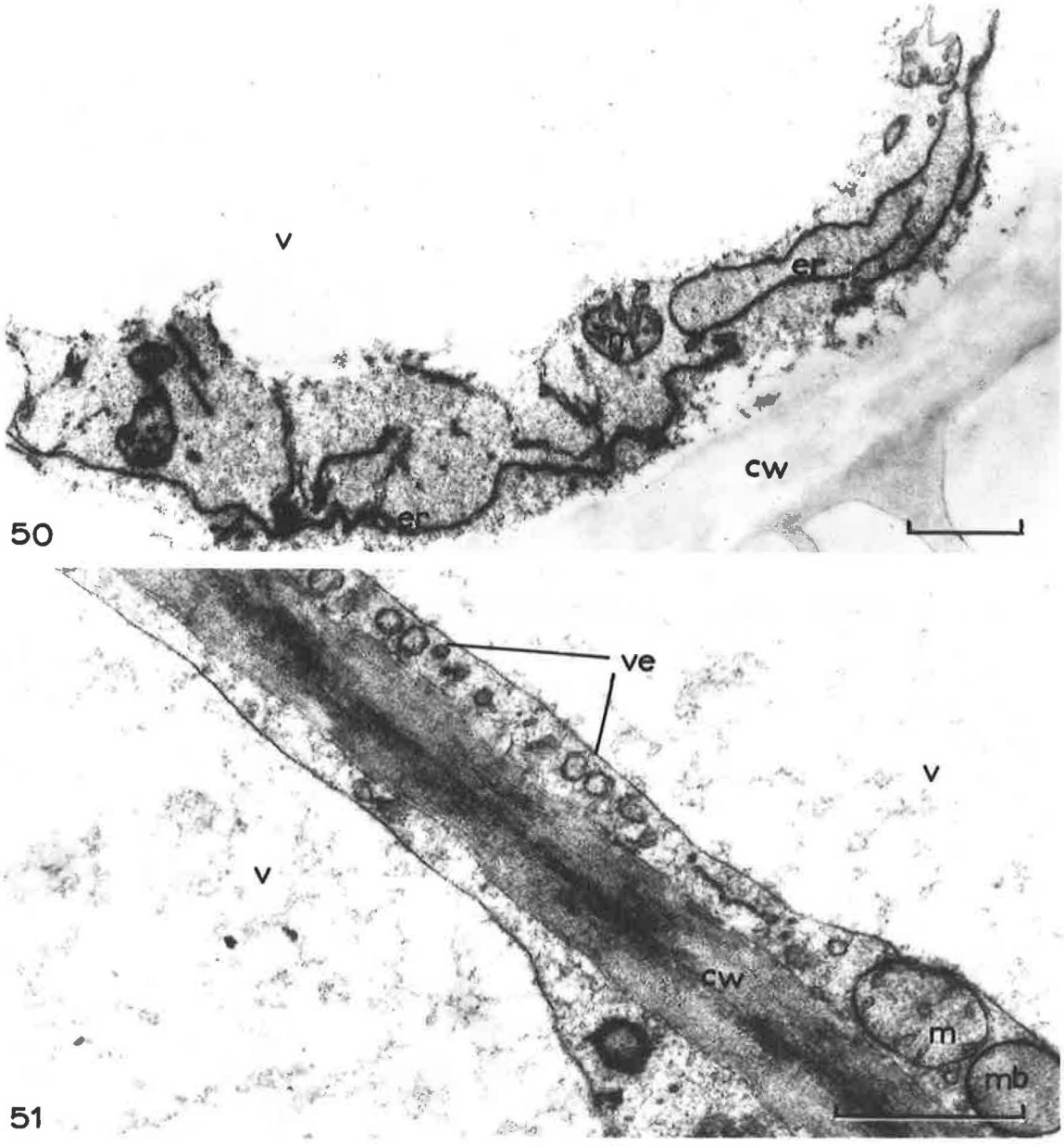

Fig.50. Parenchyma from whole swede showing long ER lamellae and absence of intact tonoplast. $KMnO_4$ fixation.

Fig.51 Parenchyma from aged swede disks (118 hr) showing numerous cytoplasmic vesicles and intact tonoplast. $KMnO_4$ fixation.

and washing of the tissue may alter the cellular response to fixation. Thus the apparent absence of lamellar endoplasmic reticulum in freshly sliced beetroot tissue could be regarded as a secondary effect due to a lack of preservation of cellular detail at the moment of fixation. However, no significant changes in the appearance of other organelles were found, indicating that the cutting and washing had not changed their response to fixation.

Other workers have observed changes in amount of ER lamellae apparently caused by external factors such as mechanical injury (Mollenhauer et al 1960), increased pressure (Honda et al 1961), wilting, oxygen deficiency (Wrischer 1960), cyanide, high carbondioxide or carbonmonoxide tension, colchicine, and high intensity radiation (Whaley et al 1964). But such treatments usually induce an almost instantaneous build up of lamellae rather than the breakdown seen in beetroot disks. This leads to the idea that the vesicles or component elements of the lamellae may be present or remain in the cytoplasm until the necessary condition is reached for their reconstitution. Some in vitro membrane systems have been dispersed by detergents (Razin et al 1965) or removal of salt (Brown 1965) and reorganised in the presence of di- or multivalent cations into structures similar to the original. Based on these observations, Green and Perdue (1966) developed the concept of macromolecular repeating units in membrane structure.

There have also been numerous reports of changes in form or

amount of ER in plant cells associated with stages in development and differentiation. For example, in the developing cotyledons of Pisum, and again in the cotyledons during germination, there are marked changes in the form of the ER. An increase in lamellae occurs at a time when the germinating cotyledons are temporarily accumulating carbohydrate (Bain and Mercer 1966). In the differentiation of root cells of maize, also, an increase in lamellae during the secretory stage is followed by a reduction (Whaley et al 1962). Other changes of lamellae to vesicles occur in embryonic leaf primordia of maturing seeds (Nougarede 1963), differentiating sieve elements (Kollman and Schumacher 1962) and tracheids (Esau et al 1963; Srivastava and O'Brien 1966), root cap cells (Hrsel 1966), and senescing leaves (Shaw and Manocha 1965). Such observations are sometimes considered to be an indication of degeneration of the protoplast (Esau 1963), and this may be the case in freshly cut beetroot slices. However, it seems that the slicing and washing in some way causes developmental processes to commence and the term rejuvenation is then more appropriate.

The finding that net accumulation of K^+ by beetroot at the completion of the lag phase is due to a decrease in apparent efflux rather than an increased influx (Van Steveninck 1962) suggests the development of some barrier to leakage of ions. The highest rate of efflux measured within 4 hours of slicing the tissue, corresponds with the observed reduction of the endoplasmic reticulum to small vesicles, and when the efflux is much reduced after 48 hours of washing, the ER forms an almost continuous lamellar system. It may be suggested that these

lamellae would constitute a barrier to direct ion movement between the external solution and the vacuole. But alternatively the reduced efflux could result from a reduction of free ion movement in the cytoplasmic phase, and the ER could possibly play a role in achieving this effect.

Ion transport in these tissues and others, by a process similar to pinocytosis is a possibility. Bennett (1956) proposed such a mechanism in which ions become bound to the outer surface of the plasmamembrane. A small vesicle is then formed by invagination and separation from the rest of the membrane and it carries the ions into the cytoplasm where they are released. Transport by such a mechanism would obey the enzyme kinetic analysis proposed in support of a carrier-operated mechanism (Epstein and Hagen 1952). On the basis of competition for space and for other reasons, the implication of carriers in ion transport based on this type of analysis has been criticised (Briggs et al 1961, De Wit et al 1963). MacRobbie (1964) has shown that chloride ions do not enter the vacuole of a Nitella cell at a steady rate but discontinuously in small packages of about 50 ions. This observation suggests the possible movement of ions in minute vesicles. The cytoplasm generally appears as a complex net-work of minute channels and particles, but the chances of positively observing such transport vesicles with the electron microscope at the present time are remote because the appearance of the cytoplasm may be modified greatly by the fixative used. Osmic acid used alone for example, tends to reveal a larger number of

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cytoplasmic vesicles of all sizes than other fixatives and these may not be artifacts. It would be a relatively simple matter to select a series of electron micrographs of OsO_4-fixed tissue to illustrate a proposed mechanism for ion transport by vesicles but it is impossible, of course, to determine the direction of movement. Any such vesicles observed in the cytoplasm are bounded by single membranes and are morphologically similar to, and may be identical with, Golgi or ER produced vesicles. In the future, however, improved methods of fixation used in conjunction with high resolution ionic reagents to localise particular ions, might give some information as to the content of these cytoplasmic vesicles. Possible evidence for this type of mechanism comes from freeze etching which reveals the appearance of membrane surfaces without the use of chemical fixation. Moor and Muhlethaler (1963) have shown by this technique, uniform folds in the plasmalemma of yeast which could become vesicles.

In considering cellular transport, a continuation of the endoplasmic reticulum system with either the plasmalemma or the tonoplast would be of some significance. Frey-Wyssling and Muhlethaler (1965) distinguish at least two series of homologous membranes in the cell: (i) the Golgi vesicles and plasmalemma, and (ii) the nuclear membrane, ER, mitochondria and plastids. The ontogenetic relationships of the tonoplast are still in doubt, but Buvat (1962) favours a relationship with the ER. There is no evidence for a continuation of the plasmalemma with the ER in plant cells although this has often been illustrated because of a schematic diagram by J.D. Robertson in support of his unit membrane concept (1959).

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In general, the development of a membrane organisation in the cytoplasm may have a bearing on the concept of "filling the cytoplasm with ions" which explains the absorption shoulder phenomenon (Laties, 1959; Van Steveninck, 1964; Osmond and Laties, 1968). Cytoplasmic vesicles, the intracisternal spaces of the ER, or of other organelles, which would then represent regions of high ionic concentration may be involved.

Apart from the energy required for actual ion movement, processes involving the building, maintenance and movement of membrane structures by the cell also require energy and must be important in cellular metabolism. Various membranes have been shown to differ widely in chemical composition, especially in lipid to protein proportions and lipid composition, and this leads to important differences in structure and hence function. For membranes such as plasma membranes containing high proportions of cholesterol and lipids containing saturated long chain fatty acids, it is reasonable to assign the bimolecular lipid structure. But membranes containing high proportions of unsaturated fatty acids will preferably form a more loosely organised molecular arrangement as, for example, in mitochondria and chloroplasts. In this case, evidence from several diverse techniques favours a globular protein structure with lipid molecules inserted within it (Benson 1964). Thus one type of membrane operates effectively as a permeability barrier, while the inner membranes of organelles are concerned with solid state reactions e.g. electron

transport, protein synthesis (O'Brien 1967). It seems likely that membranes of the ER lamellae combine the properties of both types of membrane.

The ER, because of its associated ribosomes, is generally thought to be concerned with synthesis of proteins. In animal cells there is abundant evidence for production of amorphous protein in the intracisternal space, and transport within the ER to eventual secretion from the cell. Recently, Csillik and Knyihar (1968) reported the existence of 'endoplasmic units', non reacting globular particles (320-350 Å) within the cisternae of the ER, and suggest that they are responsible for the tertiary structure of the synthesised protein molecules.

In plants there are several records of inclusions within the lamellae of the ER (Buttrose et al 1960; Buvat 1961; Jensen 1963; Newcomb 1963; Bonnett and Newcomb 1965), none of which appears to be crystalline. On the other hand, crystals do occur in other regions of plant cells: frequently in chloroplasts (Engelbrecht and Esau 1963; Sun 1965; Price et al 1966; Price and Thomson 1967; Shumway et al 1967; Manton 1966; and Bain 1968); occasionally in mitochondria (Arnott 1967); or cytoplasm (Sitte 1958; Cronshaw 1964, ~~1965~~; Thornton and Thimann 1964; Bouck 1965; Marinos 1965; Petzold 1967; Arnott and Dauwalder 1967; Jensen and Valdovinos 1968). Many of these cytoplasmic crystal-containing bodies have recently been interpreted as a specialised type of microbody characteristic of metabolically less active cells by Frederick et al (1968), who reported a close association between plant microbodies and the rough-

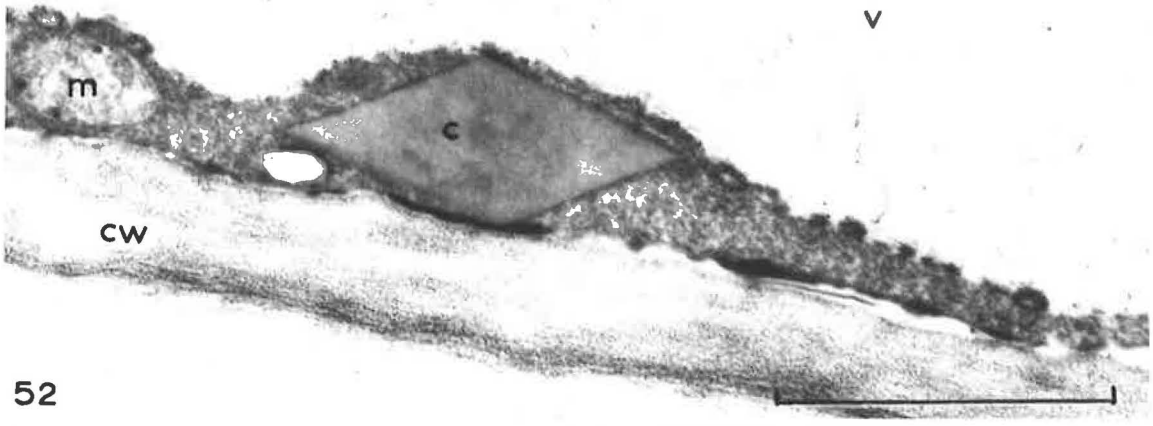
surfaced cisternae of the ER. Although the ER often appears to merge with the microbody, the membrane continuity between the two structures was never clearly defined, in contrast with the non-crystalline protein accumulation which is clearly within the cisternal swellings of the ER in radish root cells (Bonnett and Newcomb 1965). Frederick et al (1968) believe that there is some functional connection between the ER and plant microbodies mainly because they observed that crystallisation of the matrix of the microbody frequently starts in the margin adjacent to the ER.

Several functional roles have been proposed for plant microbodies containing crystalline nucleoids, which are generally believed to consist of protein or lipoprotein (Cronshaw 1964; O'Brien and Thimann 1967; Petzold 1967). Because of the similarity with animal lysosomes and their occurrence generally in cells of degenerating tissues, Cronshaw (1964) and Jensen and Valdovinos (1968) suggested that crystalline inclusions were probably storage sites for hydrolytic enzymes. Marinos (1965) also suggested that the crystalline bodies in dormant potato tubers were sites of protein storage.

In an attempt to determine the constitution of the crystalline bodies within the cisternae of the ER in aged beetroot disks, small blocks of tissue (0.5 mm^3) were incubated at 37^{o}C with 1% pronase, a nonspecific proteolytic enzyme, for periods up to 24 hours, prior to fixation. Although the appearance of the cytoplasm and the release of numerous fibrils (probably cellulose) into intercellular spaces and vacuoles indicated the activity of the enzyme, this treatment was without effect on

the crystals (Fig. 52), possibly because the large enzyme molecules were unable to penetrate the ER membrane. Subsequently thin sections of tissue fixed in glutaraldehyde only were floated on 0.5% pronase at 37° and this treatment usually caused the complete disappearance of the crystals within 1-2 hr (Fig. 53), indicating that they are composed largely of protein. It is not yet known whether the crystals constitute a store of enzyme protein or merely non-functional protein, but some evidence is available in support of the latter.

An increase in numbers of mitochondria in storage tissues immediately following slicing is reported by several workers. Verleur and Uritani (1965) suggested that the amount of mitochondria in sliced potato tissue was higher than in intact tubers on the basis of changes in nitrogen content and cytochrome oxidase activity. Asahi et al (1966) similarly found an increase in the mitochondrial fraction in aging sweet potato root slices by counting the number of Janus green B stained particles. Lee and Chasson (1966) found an increasing yield of mitochondrial material from aging potato disks, using mitochondrial nitrogen as a measure of yield. They did not examine the mitochondrial preparations electron microscopically and the pellet may have contained a large amount of non-mitochondrial membrane material (Van Steveninck and Jackman 1967). However they claimed, from electron microscope studies that the aged tissue contained a much greater mitochondrial population than the fresh tissue. If the mitochondrial number does increase, this could be achieved by division involving the production of septa (Fig. 54), constrictions



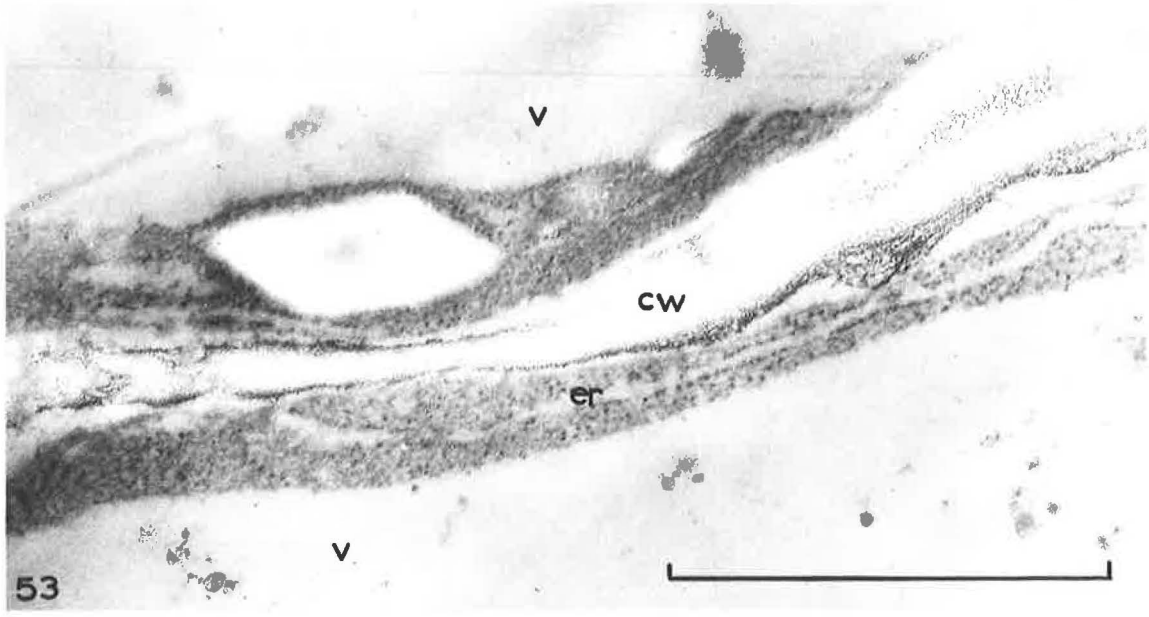


Fig.52. Effect of incubating small blocks of aged beetroot tissue with pronase for 24 hr prior to fixation in glutaraldehyde-OsO_4. Note fibrils in intercellular space and vacuole.

Fig.53. Effect on crystalloid bodies of floating thin sections of glutaraldehyde fixed aged beetroot tissue on pronase solution for 3 hr.

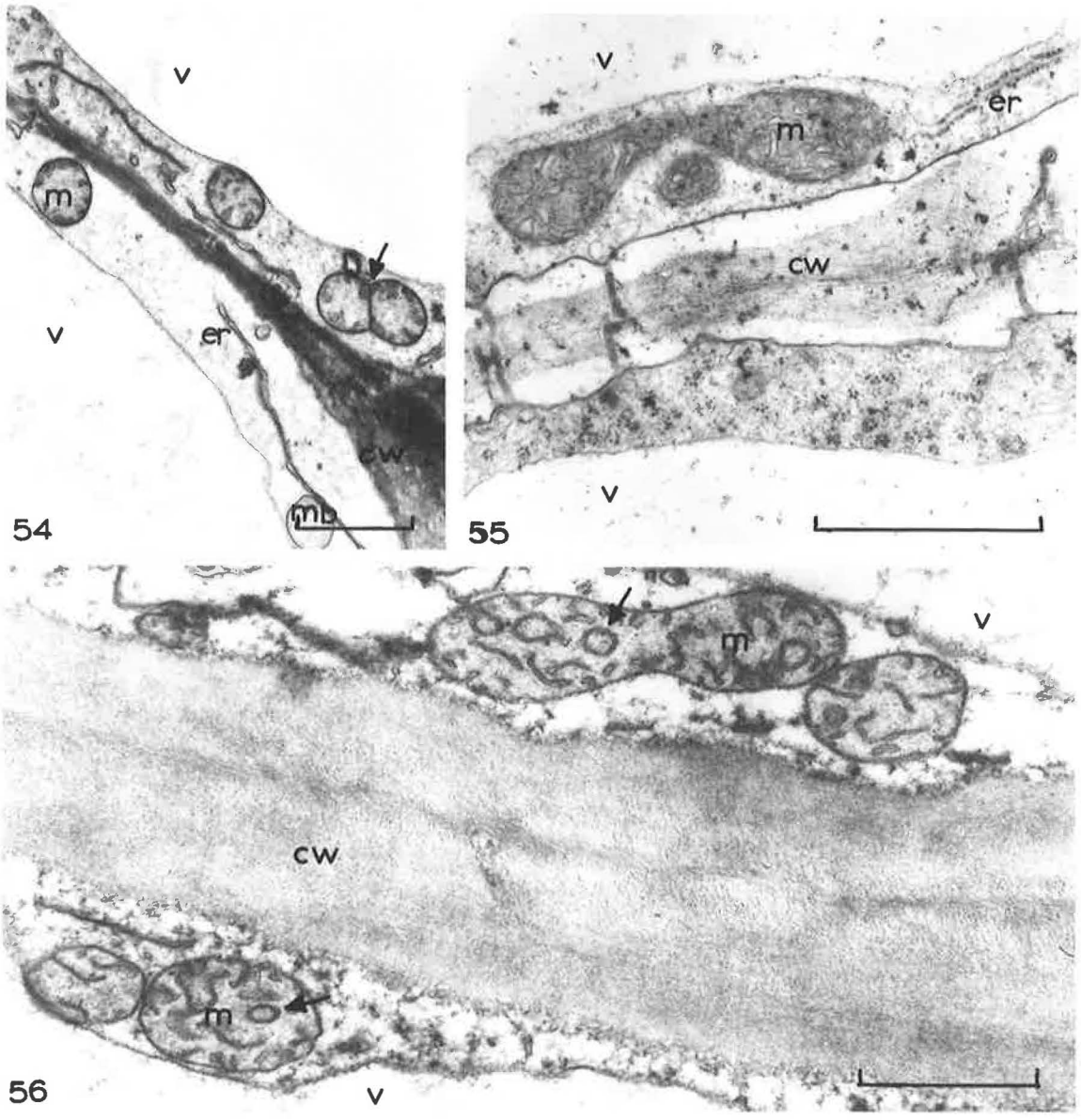


Fig.54-56. Possible means of increase in number of mitochondria. Production of septa (Fig.54, arrow), constrictions (Fig.55), or initials from looped cristae (Fig.56, arrows). Fig.54, aged beetroot disks (68 hr), $KMnO_4$ fixation; Fig.55, aged swede disks (118 hr), glutaraldehyde-OsO_4 fixation; Fig.56, fresh carrot disks, $KMnO_4$ fixation.

(Figs. 5, 24, 55), or alternatively by the production of initials from looped cristae (Fig. 29 and 56) as suggested for isolated swede mitochondria (Van Steveninck and Van Steveninck 1969). A possible origin from plastids has been described previously (Fig. 8 and 9). Other pathways have been suggested but those relating to de novo synthesis (Lee 1964) should be regarded with caution.

Since phospholipids are universal components of membranes, any change in content of membranous materials will involve net synthesis of phospholipid (unless membrane units pre-exist in the cytoplasm). Following reports of increases in membranes in disks of storage tissues during aging, several workers have recently studied changes in lipid metabolism. Willemot and Stumpf (1967) followed the development of fatty acid synthetase activity in potato tuber disks and found a 6- to 12-fold increase in the first 6-8 hours. Tang and Castelfranco (1968) found a similar increase in phospholipid synthesizing ability in potato in the first 6 hours of aging. Galliard et al (1968) reported the rapid increase of a lipid synthesizing system reaching full development in 2-4 hours in disks of peel from immature apples. These results indicate a very rapid response to slicing, and it may be that changes in cellular membranes, or synthesis of membrane units, take place much more rapidly than anticipated. But, membrane synthesis is also dependent on the supply of protein and this may control the process.

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Part C - Structural Aspects: treatment with Ca^{++}, EDTA

The effects on disks of beetroot of either added Ca^{++}, or addition of Ca^{++} following its removal with EDTA have been reported by Van Steveninck (1961, 1965a, 1965b). The addition of relatively high concentrations of Ca^{++} (10^{-2}M) was shown to shorten the lag phase in net K^{+} absorption in beetroot tissue. The apparent K^{+} influx was immediately depressed for a period of 6 to 20 hours (at pH8 and pH6 respectively), and then increased to a value 3-4 times that of the control. At the same time (20-40 hr), apparent K^{+} efflux was decreased.

Exposure to EDTA solutions of a concentration (10^{-4}M) sufficient to remove by chelation about 70% of the tissue Ca^{++} produced a marked leakage of K^{+} from beet disks. Smaller amounts of EDTA had little or no effect, the EDTA: tissue ratio being the controlling factor rather than EDTA concentration. The remaining 30% of the total tissue Ca^{++} was not readily extracted with EDTA unless the tissue was killed, and may have been present as a complex more stable than Ca^{++}-EDTA. This EDTA-induced leakiness was immediately and completely reversed by the addition of Ca^{++} and this result was most likely due to a direct effect of Ca^{++}, although Mn^{++} and, to a lesser extent, Sr^{++}, were also effective.

In this investigation some of the above experiments were repeated and a sampling procedure was included to study possible ultrastructural changes.

1. Effect of Ca^{++} added at the beginning of or during the lag phase in net K^{+} uptake.

In this experiment beet disks (50 per 250 ml) were placed in

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10^{-2}M $CaCl_2$ after initial rinsing, or alternatively at 26 hr sufficient 0.2M $CaCl_2$ was added to the solution in the flasks to give a final concentration of 10^{-2}M (immediately after sampling).

Figure 57 shows the effect of added Ca^{++} on the duration of the lag phase in net K^+ uptake. In the control, slow uptake began at about 10 hr, and rapid uptake at about 50 hr. Whether added at 0 or 26 hr, Ca^{++} shortened the lag phase as expected. However, the lack of an immediate response to Ca^{++} by washed disks did not confirm earlier findings (Van Steveninck 1964), and may have been due to a certain amount of Na^+ being added as an impurity in the calcium chloride. Even though the increased period of K^+ leakage in Ca^{++} solution was an atypical result, samples of disks for electron microscopy were taken as follows: from control flasks at 0, 2, 26, 48, 96 and 192 hr; from flasks receiving Ca^{++} at 0 hr at 2, 26, 48, 96 and 192 hr; and from flasks to which Ca^{++} was added at 26 hr at 2 and 22 hr after the addition.

There appeared to be no effect of added Ca^{++} on the ultrastructure of beet cells. The reassembly of ER lamellae was found to occur in controls and Ca^{++} treated tissue at about the same rate, the amount and length of ER being similar at 2 hr, 26 hr and 48 hr whether Ca^{++} was added or not. No other organelles or membranes appeared to be affected.

Considering that addition of a relatively high concentration of Ca^{++} had no apparent long term effects on ultrastructure, no further experiments of this type were carried out.

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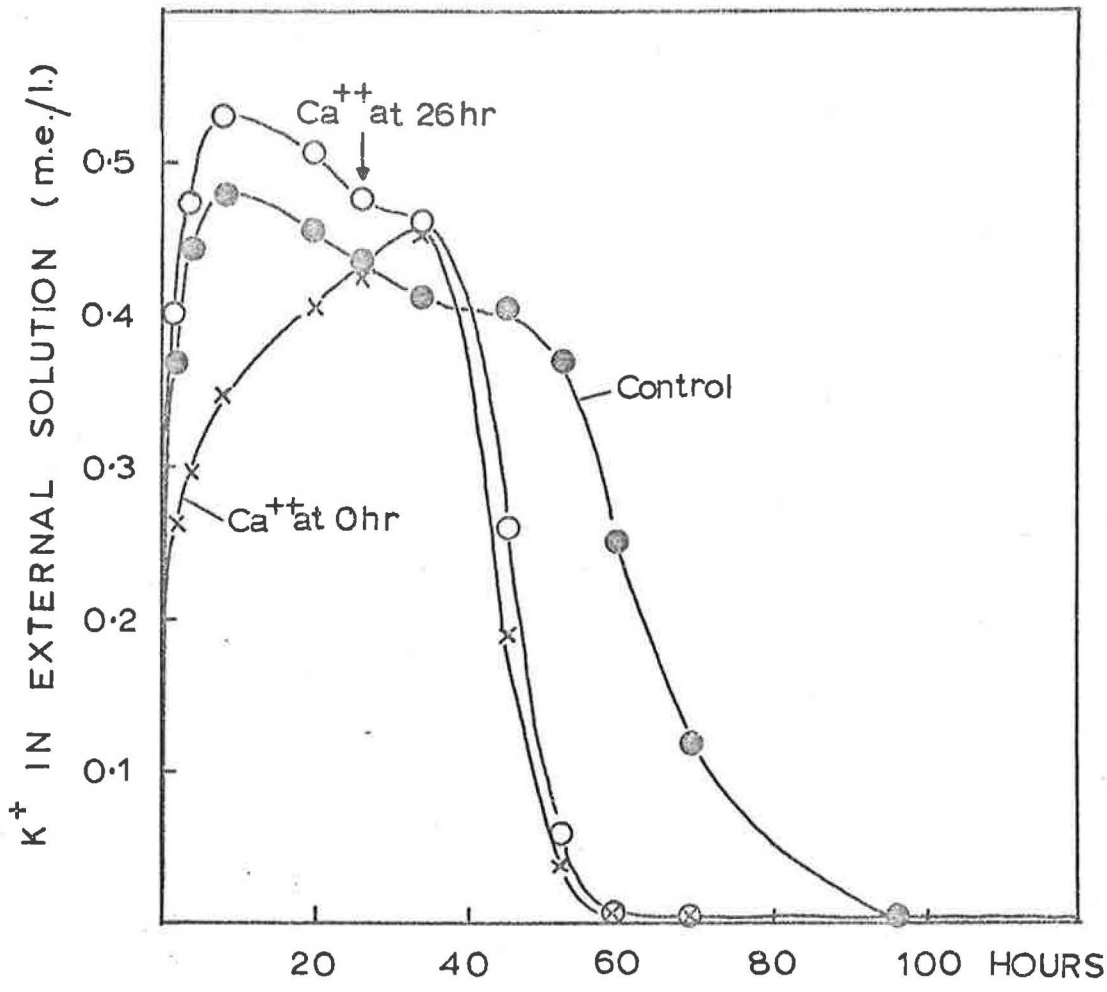

Fig. 57. Changes in K^+ concentration of external solutions due to release or uptake of ions by fresh beetroot disks (50 per 250 ml solution) after addition of Ca^{++} (final concentration 10^{-2}M) at 0 hr and at 26 hr

2. Effect of Ca^{++} added to rinses immediately after cutting beetroot into disks.

Because significant changes in ultrastructure take place during slicing and preliminary rinsing of disks, the short term effects of added Ca^{++} were investigated. A small number of disks was cut in the usual way, rinsed momentarily three times in distilled water or 10^{-2}M $CaCl_2$ and placed in either water of 10^{-2}M $CaCl_2$ solution with aeration. Samples were taken for electron microscopy immediately after the preliminary rinses, and after 1, 2 and 4 hours of washing. Fixation was in $KMnO_4$ only.

Again, little difference, if any, was detectable due to the inclusion of Ca^{++} in the rinsing solution. Fixation of cytoplasm and preservation of plasma membrane and tonoplast appeared to be slightly improved by the Ca^{++} addition. There was also a slight indication that the breakdown of ER cisternae was delayed by Ca^{++} treatment, but after 4 hr the ER was reduced to vesicles in both cases. More definite observations or conclusions could not be made.

3. Effect of removal of Ca^{++} with EDTA and subsequent addition of Ca^{++} after induced leakage.

(a) Unbuffered EDTA: In an initial experiment, aged disks (113 hr) which had already developed the capacity for net uptake of K^+ and Na^+ from the external solution were used. Although 1.25×10^{-4}M EDTA (free acid because K^+ would interfere with ion measurements) at pH 5-6 caused a withdrawal of Ca^{++} from the tissue (disks at 113 hr contained 2.8 m.e.Ca^{++} per kg; by 14 hr, the external solution contained 0.054 m.e.Ca^{++} per l,

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representing a withdrawal of approximately 30% of the Ca^{++} present), even after 48 hr no leakage of K^+ or Na^+ into the external solution was detected. Evidently, in comparison with fresh disks, a larger proportion of the Ca^{++} is not readily extractable after aging. In the same experiment, leakage could be induced by the addition of more EDTA, but this lowered the pH to approximately 4. The pH of the control solution was 6.8 - 6.9.

Samples for electron microscopy were taken 8 hr after the commencement of the experiment. Even though no general leakage was induced, compared with the control, the fixation of tissue treated with EDTA was poor, there being little cytoplasmic preservation. Occasionally some remnants of organelles and the tonoplast could be seen. A plasmalemma was usually not visible whereas the control showed a distinct wavy membrane ($KMnO_4$ fixation).

(b) Tris buffered EDTA: In order to maintain the pH of solutions at 7.8 - 8.0, 10^{-2}M tris (tris (hydroxymethyl)-amino methane) buffer was used in all subsequent experiments. At this pH Ca^{++} is more efficiently chelated by EDTA.

Tris buffer itself has been shown to affect the lag phase of net K^+ uptake in beetroot tissue (Van Steveninck 1961, 1965b). At pH 8.0, 10^{-2}M tris-HCl caused the immediate onset of net K^+ uptake by fresh beetroot disks i.e. the lag phase is shortened, an effect similar to but more immediate than that of Ca^{++}. Thus if tris is used, fresh disks can also be used to study EDTA induced leakage, but it is essential

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to realise the possible effect tris may have on cellular ultrastructure. Using either fresh or aged disks several experiments were carried out in order to achieve fixation for electron microscopy at optimal times.

The results of one experiment using disks aged for 65 hr are shown in Fig. 58. The effect of each treatment on ion movement was, briefly as follows:

(i) Tris has no effect on net K^+, Na^+ and Ca^{++} movement in 65 hr old disks, but may induce an initial leakage of Mg^{++} which is taken up again by the tissue in 10 hr.

(ii) Tris + EDTA causes continuous leakage of K^+ and Na^+ and initial withdrawal (complete in 2 hr) of Ca^{++} and Mg^{++}. This amount of Ca^{++} is calculated to be equivalent to approximately 80% of the total Ca^{++} present in the disks at the commencement of the experiment.

(iii) The further addition of Ca^{++} at 8 hr, stops leakage of K^+ and Na^+ and induces uptake of K^+ by 2-6 hr and Na^+ by 6 hr. The Ca^{++} addition was equivalent to 0.16 m.e. per l, hence it caused the immediate uptake of approximately 0.035 m.e. Ca^{++} per l and 0.075 m.e. Mg^{++} per l from the solution. Samples were taken for electron microscopy at 0, 8, 10 and 23 hr.

Some experiments however yielded unexpected ion movements. For example, in one experiment using aged disks in tris-buffered solutions, after leakage was induced by EDTA, Ca^{++} added at 22 hr prevented further loss of K^+ and Na^+ but failed to promote net uptake. On another occasion

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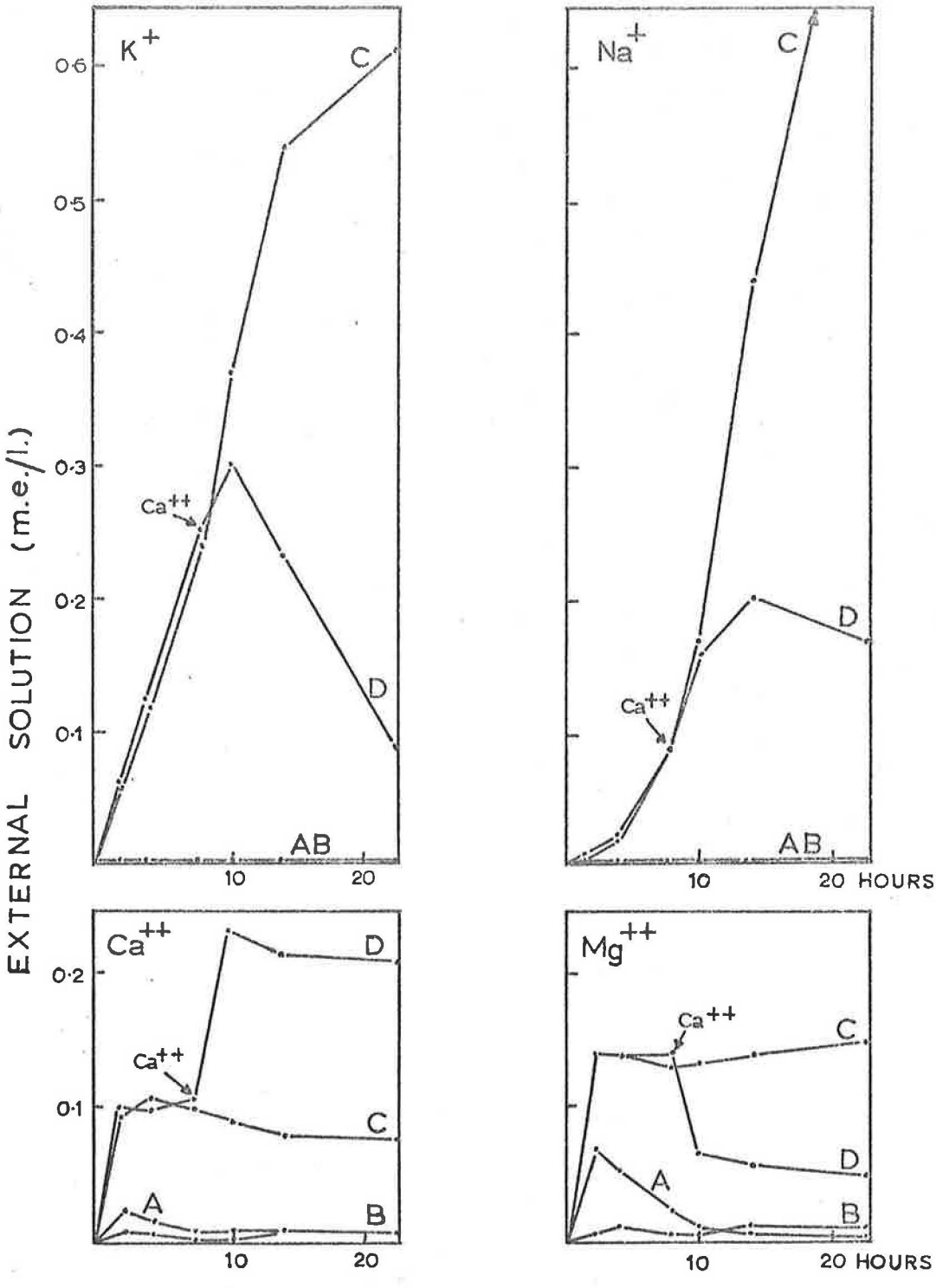

Fig. 58. K^+, Na^+, Ca^{++} and Mg^{++} concentrations of external solutions due to release or uptake by beetroot disks (50 per 250 ml solution) aged for 65 hr prior to commencement of experiment. A, control; B, 10^{-2}M tris-HCl; C, 10^{-2}M tris-HCl + 1.25 x 10^{-4}M EDTA; D, 10^{-2}M tris-HCl + 1.25 x 10^{-4}M EDTA + 1.6 x 10^{-4}M $CaCl_2$ added at 8 hr

using fresh disks, Ca^{++} added with tris buffered EDTA at 0 hr reduced the rate of net leakage of K^{+} and Na^{+} until about 6 hr, whereas Ca^{++} added at 5 hr did not reduce leakage. Evidently in this type of experiment, it is essential to add the correct amount of EDTA which is determined by the initial Ca^{++} content of the tissue. Ca^{++} determinations could not be readily carried out during later experiments.

In comparing ultrastructural observations from such experiments, the net movement of ions at the time of each sample was taken as a basis. Although the importance of Ca^{++}, or divalent ions in general, in the maintenance of membrane structure has frequently been inferred, there were disappointingly few general conclusions which could be drawn from these experiments. The observations are summarised below, but there is still a degree of uncertainty regarding any general conclusions, largely because of the variability in the experimental results which await substantiation.

In experiments with aged disks, special attention was given initially to observations of changes in the long ER lamellae. Tris alone was found to have no effect (Fig. 59) but after 8 hours treatment with tris + EDTA there was a marked change in appearance of $KMnO_4$ fixed material. The cytoplasm seemed to become aggregated in some regions of the cell leaving thin cytoplasmic connections between the 'clumps'. The amassed cytoplasm was highly vesiculated i.e. it contained numerous large (up to 5 μ) single membrane bound electron transparent areas and this appearance persisted until the end of the experiments (approx 24 hours) (Fig. 60, 61).

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The origin of these vesicles is open to speculation. It seemed possible that they might have originated simply by expansion and possible segmentation of ER bound areas. The addition of Ca^{++} after 8 hours aeration in tris + EDTA produced little obvious change (Fig. 62) but by 23 hours some parts of the cytoplasm at least were seen to be free of large vesicles and the long ER lamellae had reappeared (Fig. 63). By this time net uptake of K^{+} and Na^{+} was occurring, so that there seemed to be a possible relation between vesiculation of cytoplasm and net leakage.

An attempt was made also to note the number of small cytoplasmic vesicles (0.1 - 0.15μ) present in each treatment at different times. In the control and tris-treated at 8 hr (Fig. 59) and at 23 hr these were few. Vesiculation of the cytoplasm interfered with the comparison but the number seemed to increase markedly sometime after the addition of Ca^{++}, again coinciding with the rapid uptake phase (Fig. 63).

The plasma membrane ($KMnO_4$-fixed) was generally not visible as a discrete membrane after treatment of aged disks for 8 hr or more in 10^{-2} M tris HCl (Fig. 59). In control disks of the same age, it usually appeared as a broken line, but occasionally became indistinct. Some treatments (e.g. tris + EDTA at 8 hr) also caused the plasma membrane to withdraw from the cell wall giving an appearance of slight plasmolysis (Fig. 60).

In fresh disks, the long ER lamellae were not generally apparent until 25-46 hr, and although by 9 hr the tris treatment in which active uptake was taking place showed some lengths of ER (Fig. 64, glutaraldehyde - OsO_4 fixation), none of the treatments were conclusively shown to affect

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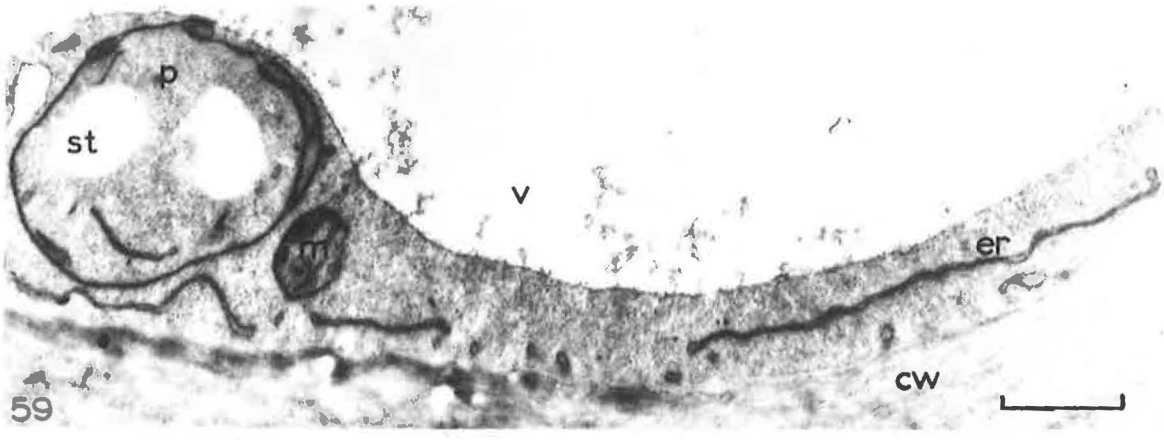

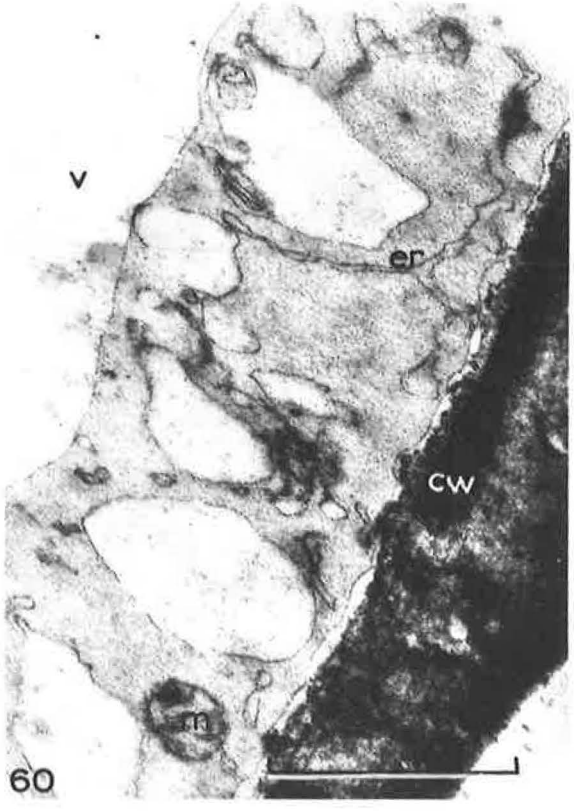

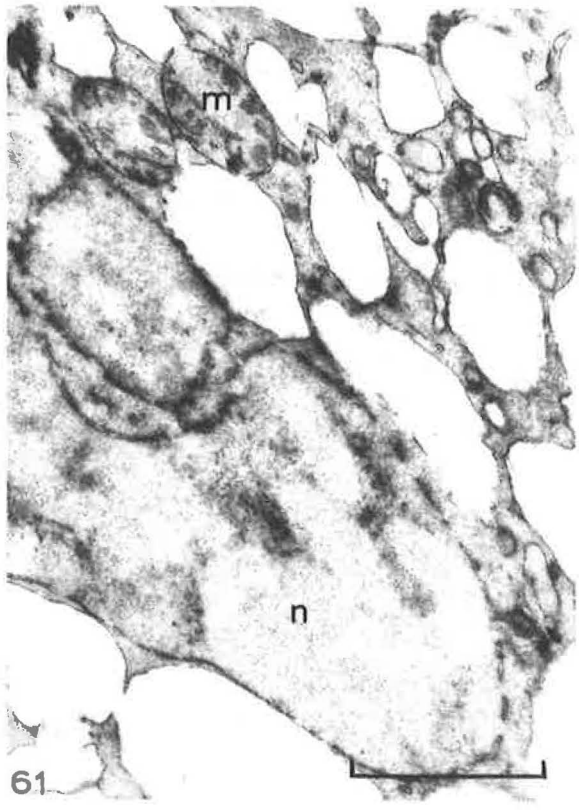

Fig.59. Aged disks of beetroot (65 hr) treated with tris for 8 hr. No obvious effect on ER lamellae. $KMnO_4$ fixation.

Fig.60. Large electron transparent areas in cytoplasm of aged (65 hr) disks of beetroot after treatment with tris + EDTA for 8 hr. $KMnO_4$ fixation.

Fig.61. Electron transparent areas in cytoplasm of aged (113 hr) disks of beetroot after treatment with tris + EDTA for 24 hr. $KMnO_4$ fixation.

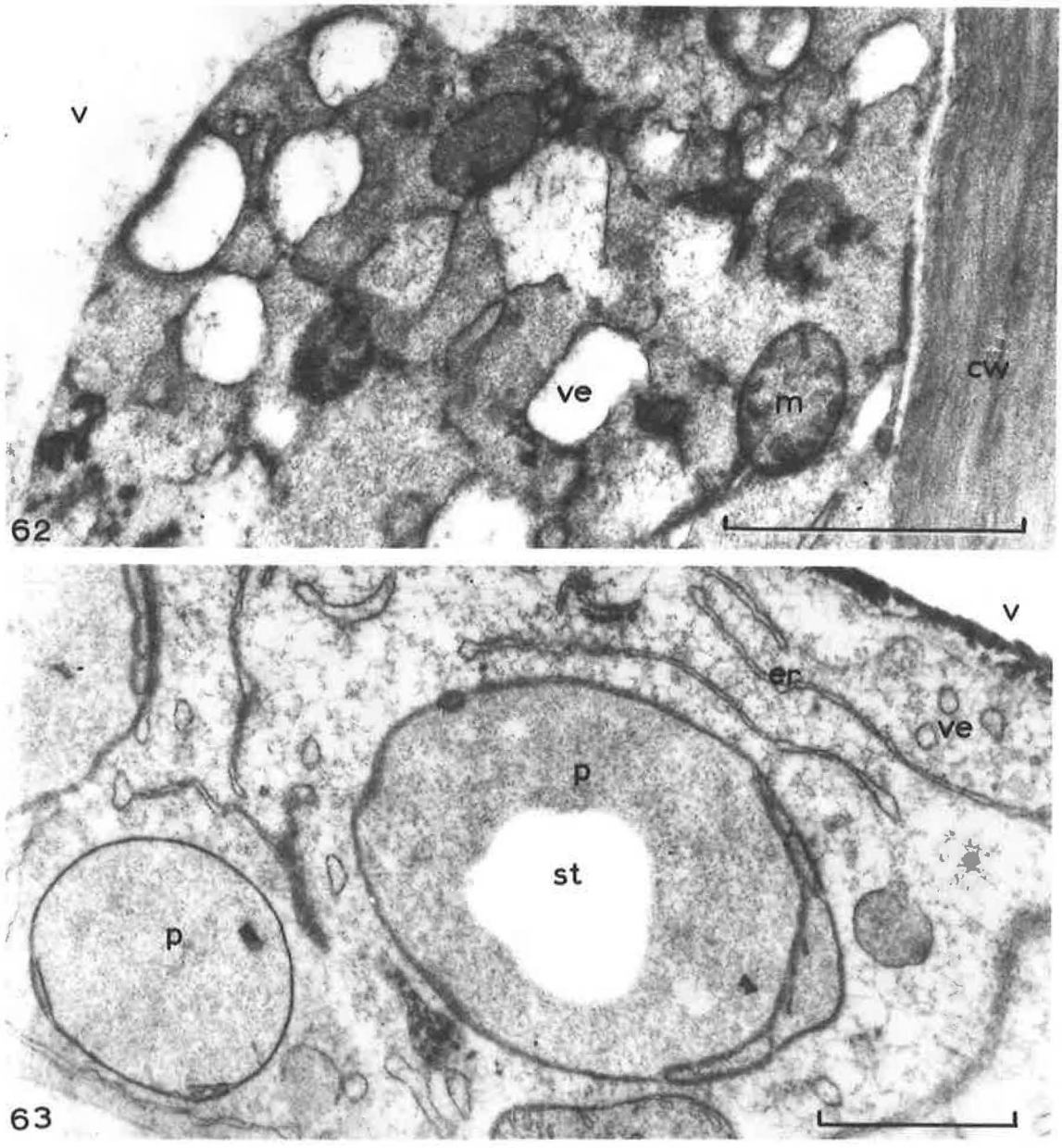

Fig.62-63. Appearance of cytoplasm after treating aged (65 hr) beetroot disks with tris + EDTA for 8 hr and then adding Ca^{++} for 2 hr (Fig.62) or 23 hr (Fig.63). Prolonged treatment with Ca^{++} reduced the number of large cytoplasmic vesicles and apparently increased the number of small vesicles. $KMnO_4$ fixation.

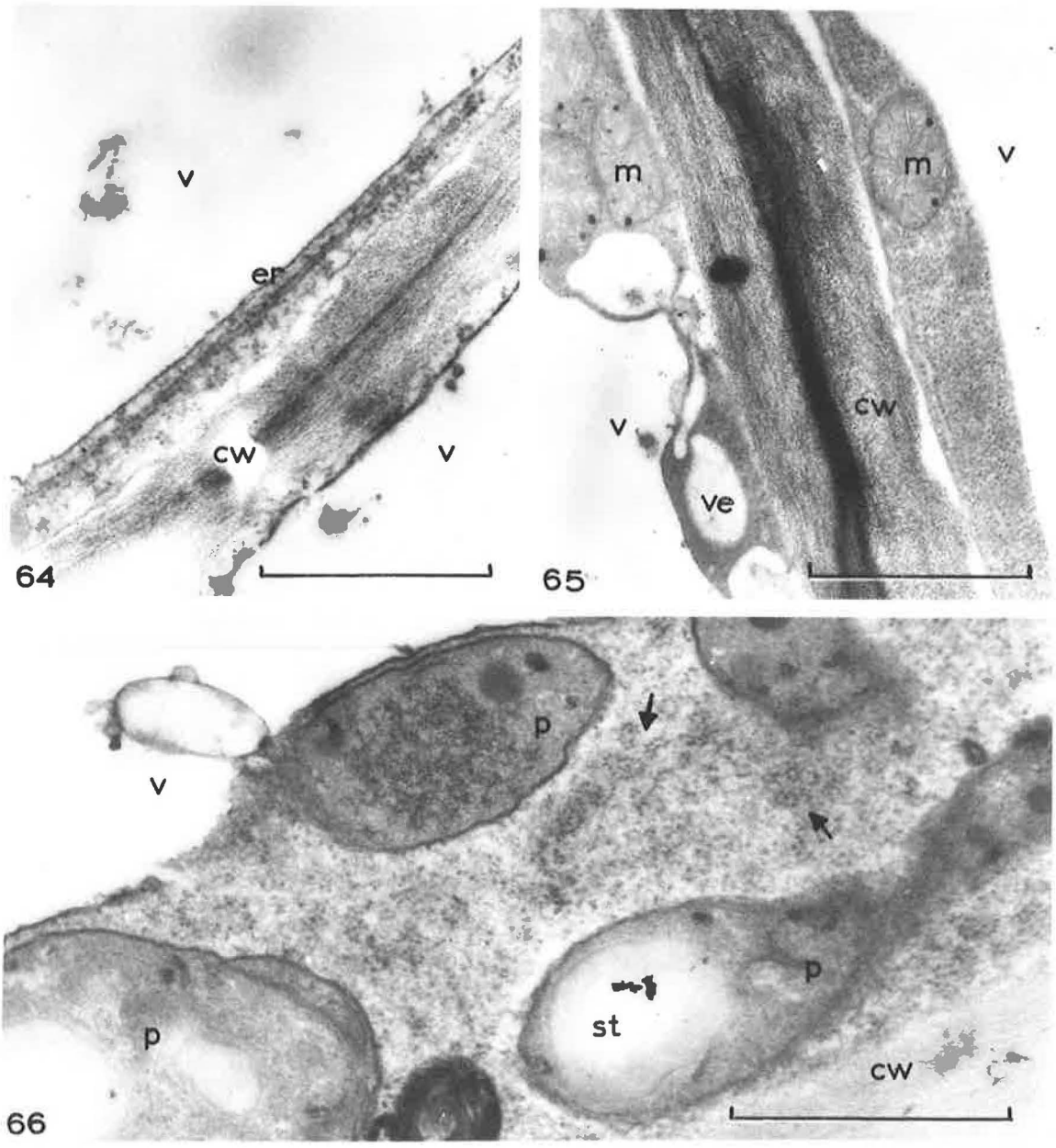

Fig.64. ER in fresh disks of beetroot after treatment with tris for 9 hr. Glutaraldehyde-OsO_4 fixation.

Fig. 65. Fresh beetroot disks treated with tris + EDTA for 9 hr. Note uniform distribution of ribosomes and large cytoplasmic vesicles. Glutaraldehyde-OsO_4 fixation.

Fig.66. Control showing cytoplasmic polysomes (arrows). Glutaraldehyde-OsO_4 fixation.

the reassembly of the lamellae.

The effect of tris + EDTA was similar to that observed in aged disks. The cytoplasm contained some large vesicles by 4 hours and many by 9 hours. In glutaraldehyde-osmium fixed tissue, the cytoplasm surrounding these vesicles frequently appeared to be denser than usual, consisting largely of uniformly distributed ribosomes and some ribosomes aligned along ER (Fig. 65). In controls the cytoplasm showed the normal density and polysomes were often clearly visible (Fig. 66).

The number of small cytoplasmic vesicles ($KMnO_4$ fixation) again appeared to increase in the control and tris treatment during 25 hours of washing fresh disks i.e. when net uptake was proceeding (Fig. 67-69). These vesicles were sometimes associated with the Golgi apparatus.

As in aged disks, 9 hr treatment of fresh disks with tris alone seemed to alter the normal appearance of the plasma membrane (Fig. 69, $KMnO_4$ fixation). Tris treated disks at this time were undergoing rapid uptake while control disks were in the lag phase, before the onset of rapid net uptake. After 25 hours in tris, small discontinuous segments of the membrane were again visible coinciding with the completion of uptake of available K^+ and Na^+.

Again, plasmolytic movement of the plasma membrane away from the cell wall was sometimes apparent but this movement was found to be more frequent after glutaraldehyde - OsO_4 fixation (Fig. 70, 71) and was commonly seen in all treatments incorporating tris.

In some instances, where glutaraldehyde - OsO_4 fixation was used, the tonoplast showed as a heavily stained dark line, which was shown not

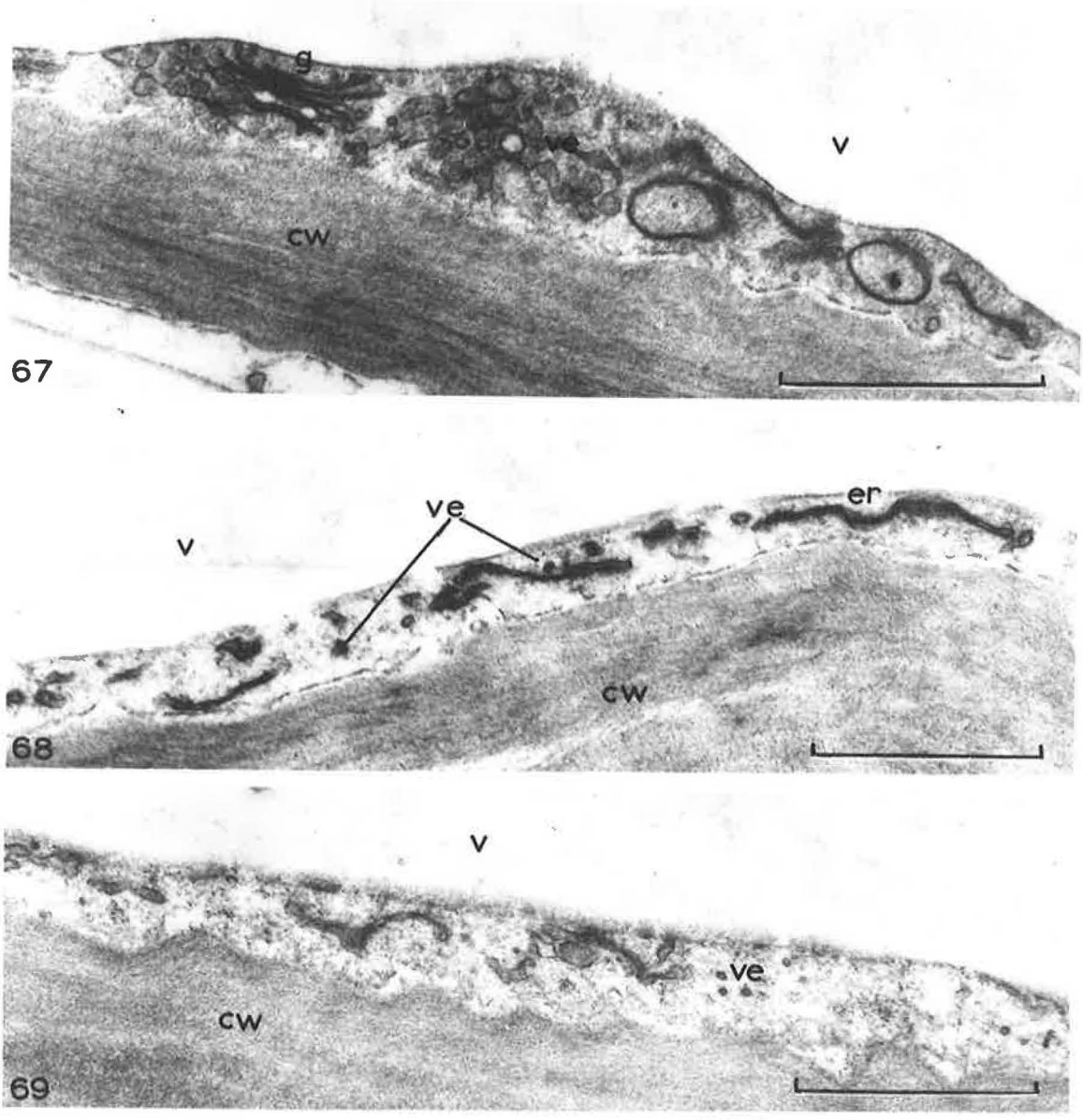

Fig.67-69. Fresh disks of beetroot after 9 hr washing in water (Fig.67,68) and tris (Fig.69) showing numerous cytoplasmic vesicles. $KMnO_4$ fixation.

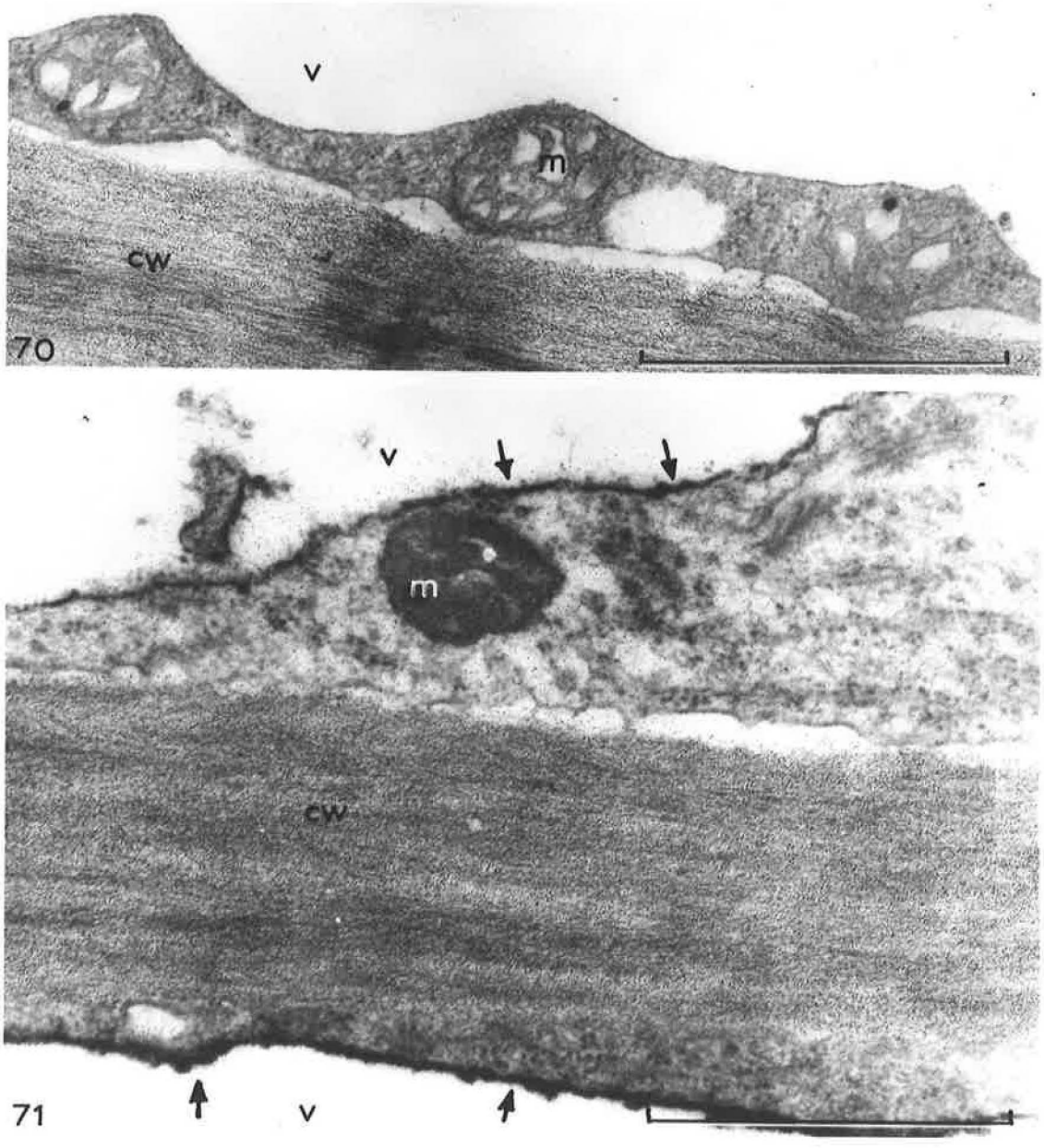

Fig.70-71. Fresh disks of beetroot treated with tris + EDTA + Ca^{++} for 4 hr (Fig.70) or tris for 9 hr (Fig.71) showing movement of plasma membrane away from cell wall and dark staining of tonoplast after tris treatment (Fig.71, arrows). Glutaraldehyde-OsO_4 fixation.

to be due to post staining. The intensity of the stain was reduced in cells treated with Ca^{++} (tris + EDTA + Ca^{++}) at the beginning of the experiment (compare Fig. 70 and 71). In EDTA treatments, the effect was not consistently observed (Fig. 65).

Some interesting observations of cell membranes and ground plasm were made in tissues fixed with OsO_4 alone. Fixation of controls (to 46 hr) showed reasonable preservation of intracellular membranes (Fig. 72, 73). The plasma membrane was clearly visible as a wavy line and the tonoplast was often densely stained (as in glutaraldehyde - OsO_4 fixation). Cell organelles were reasonably well preserved and often ribosomes and polysomes were present. Small, appearing spotted, cytoplasmic inclusions may correspond with the minute vesicles observed with $KMnO_4$ fixation (Fig. 72). Cells treated with tris and fixed with OsO_4 alone always showed a wide electron transparent area in the region of the plasma membrane (Fig. 74, 75). The tonoplast was sometimes intact although the structure of the ground plasm was severely affected. The inclusion of EDTA or EDTA and Ca^{++} in the treatment solution did not alter this appearance. Thus further observations on OsO_4 fixed material were not possible.

During dehydration and embedding it was observed that all tissue blocks of the controls became dark in colour after OsO_4 fixation, but all the remaining treatments containing tris remained colourless or almost so.

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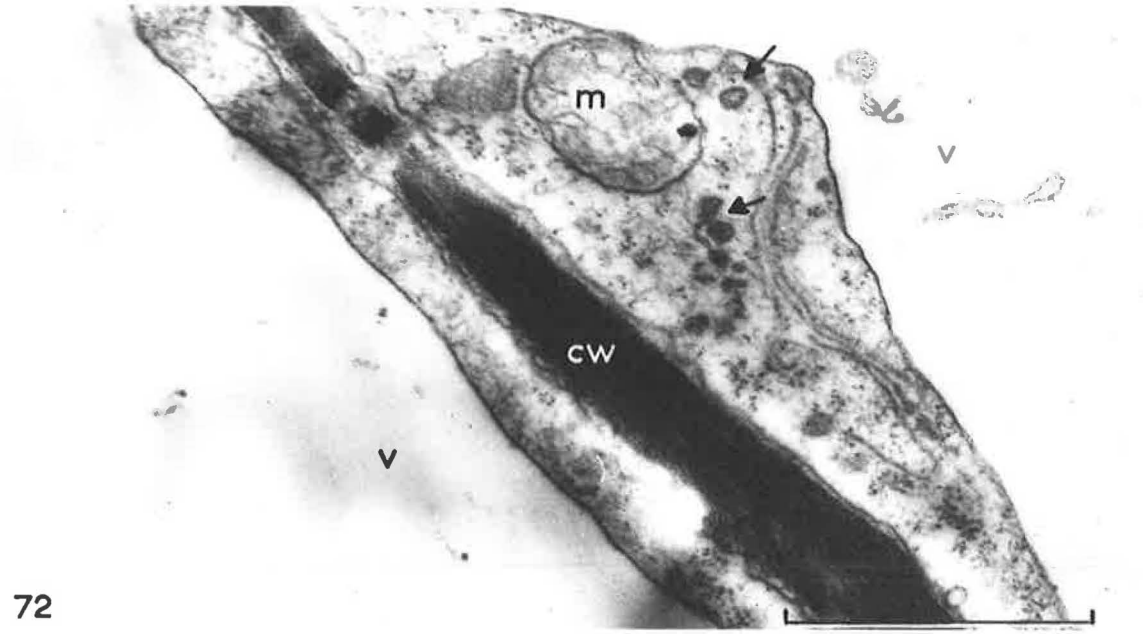

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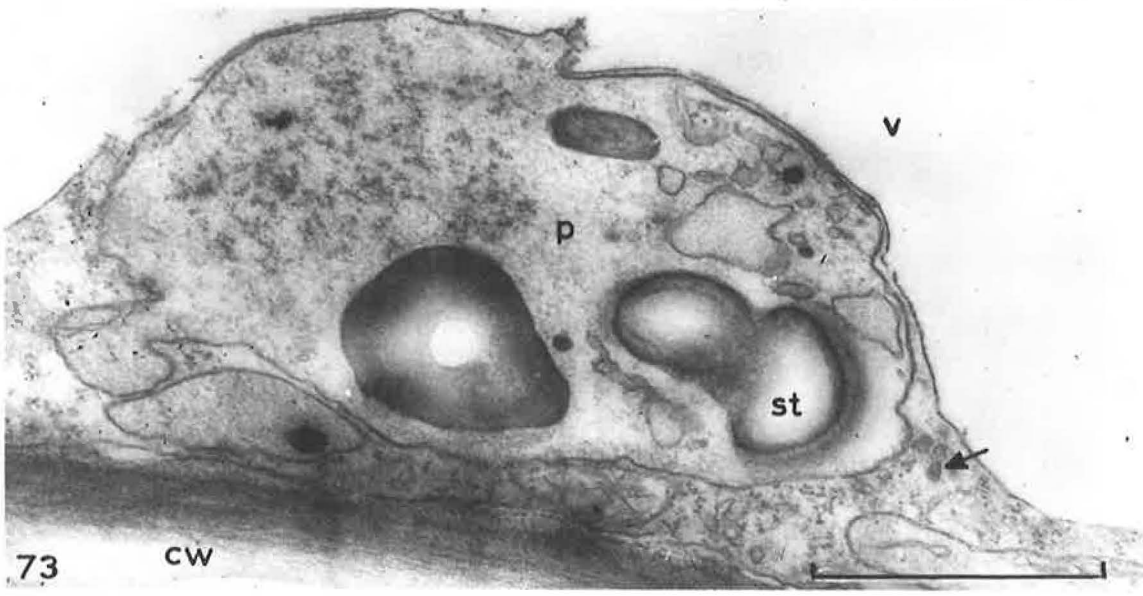

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Fig.72-73. Fresh disks of beetroot aerated in water for 4 hr (Fig.72) or 9 hr (Fig.73) and fixed in OsO_4 alone. Note preservation of cellular membranes, organelles, ribosomes and small cytoplasmic inclusions (arrows).

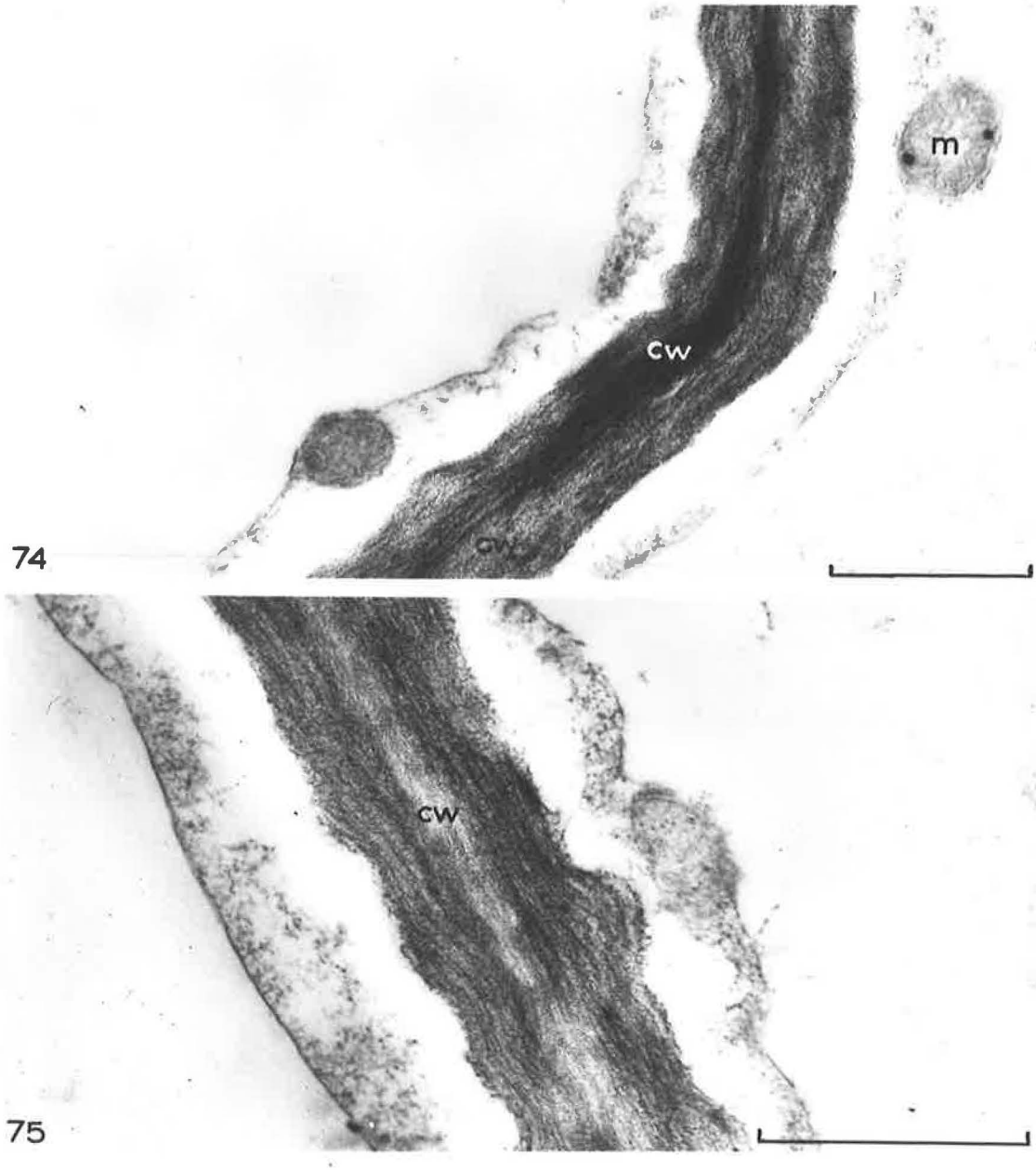

Fig.74-75. Fresh disks of beetroot treated with tris for 4 hr (Fig.74) or 9 hr (Fig.75) and fixed in OsO_4 alone. Note appearance of ground plasm and wide electron transparent areas on either side of the cell wall.

4. Discussion

The important role of Ca^{++} in maintaining cytoplasmic structure and membrane stability suggests the possibility of structural changes when external Ca^{++} concentration is altered. But there are few reports of attempts to find ultrastructural changes in plant cells which might accompany the well-documented physiological responses to addition or removal of Ca^{++}.

The addition of Ca^{++} brings about the well known but, as yet, unexplained stimulation of monovalent cation uptake. An interaction of Ca^{++} with a particular cell membrane or organelle, or even an indirect effect of Ca^{++} on some aspect of cell metabolism might be expected to cause a morphological change. But, apart from a possible effect on the rate of breakdown and reassembly of ER lamellae, no differences between the control and Ca^{++} treated beetroot disks were detected. However, these experiments are preliminary only and with improved cellular preservation it would be possible to make a more careful comparison particularly of cell membranes.

The establishment of conditions of Ca^{++} deficiency seems more promising for ultrastructural studies, and under these conditions subcellular changes have been observed by several workers. Meristematic tissues developing in the absence of Ca^{++} are perhaps an obvious choice for this work especially as good preservation can be readily achieved with non-vacuolate or slightly vacuolate cells. Thus Marinos (1962) working with barley shoot apices, and Marschner and Gunther (1964) with barley

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root tips found that Ca^{++} deficiency first causes the breakdown of the tonoplast and plasma-membrane and loss of structure in the ground cytoplasm. A sudden breakdown of the tonoplast due to Ca^{++} removal was also observed by Frank (1962) in vacuolated cells of two algae, Hydrodictyon and Sphaeroplea.

In beetroot tissue, treatment of disks for 8 hr with unbuffered EDTA prevented satisfactory fixation. It is possible that the low pH (4.2) rather than the lack of Ca^{++} was responsible for this effect. Other work (Peterson 1962) suggests that an increase in $[H^+]$ causes the increased dissociation of calcium proteinate and thus the ground cytoplasm, internal cell membranes or surface membranes, if Ca^{++} is evenly distributed between the cell wall and cytoplasmic fractions (Van Steveninck 1965a), may become disorganised. Recently it has been shown that artificial asymmetric phospholipid membranes are unstable under conditions of asymmetric distribution of Ca^{++} or H^+ with respect to the membrane (Papahadjopoulos and Ohki 1969). Such a condition is very likely attained when using unbuffered EDTA solutions.

Treatment of disks with tris buffered EDTA to remove Ca^{++} caused net leakage of Na^+ and K^+ and caused some alteration to cell ultrastructure. The cytoplasm became thicker in some parts of the cell and within this cytoplasm, large membrane bound vesicles appeared which seemed to have originated from the ER lamellae. This process may be similar to that described by Buvat (1962) for the origin of vacuoles.

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Treatment of beetroot disks with EDTA in this way has been shown to remove 69-76% of the total Ca^{++} present in the tissue possibly equal proportions being withdrawn from the cell wall and protoplasmic fractions (Van Steveninck 1965a). The removal of Ca^{++} might allow some relaxation of structural elements within the ground cytoplasm and could possibly also be translated into the apparent distortion of the ER system. It is not known whether this visible morphological change is directly connected with the leakage of ions.

Another observed effect of tris+EDTA treatment of disks was a change in the distribution of ribosomes in glutaraldehyde - OsO_4 fixed material. The cytoplasm frequently took on a dense appearance, and the ribosomes, except for those which remained attached to the ER membranes, appeared to be uniformly distributed. The in vitro breakdown of polysomes to free ribosomes by EDTA treatment has been reported previously (Loening 1968).

The addition of Ca^{++} to disks treated with tris-buffered EDTA could be shown to induce net K^+ and Na^+ uptake. Ultrastructural effects were not apparent, except in one experiment when the inclusion of Ca^{++} in the solution prevented or reduced the formation of an electron dense product seen in association with the tonoplast in the controls and other treatments when fixed with glutaraldehyde - OsO_4.

Tris-HCl buffer which was used to correct the low pH of the EDTA solutions, shortens the lag phase in Na^+ and K^+ uptake by freshly cut beetroot disks. The buffer was also shown to have some influence on cellular ultrastructure depending on the fixative used. With $KMnO_4$ and

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glutaraldehyde - OsO_4 there was little effect. The ground cytoplasm appeared normal, but usually the plasma membrane was not visible. After OsO_4 fixation alone, tris-treated tissue was characterised by wide electron transparent areas in the region of the plasma membrane and a marked lack of ground plasm structure. Further investigation is required to explain this apparent blocking by tris of sites usually bound by OsO_4 during fixation. In this connection, Van Steveninck (1965b) compared the effects of tris and Ca^{++} on the lag phase and interpreted his results to indicate competition for a common site. Ca^{++} was shown to be more efficient in this respect than the univalent tris ions. But in this work with OsO_4 as sole fixative the inclusion of Ca^{++} in the tris buffered EDTA even when added at the beginning of the experiment had no effect distinguishable from that of tris alone. In other words the inclusion of tris apparently obscured any visible effects of other additions when fixed with OsO_4.

It seems possible that further studies of these treatments and/or fixatives which produce visible effects at the protoplasmic surface might prove interesting. Recent work has revealed that plasma membranes are indeed more complex than was previously thought. Firstly freeze etching showed the presence of folds or pores such as those reported by Moor and Muhlethaler (1963), in the plasmalemma of yeast cells. More recently, Wallach (1966) found that the disruption of Ehrlich ascites carcinoma cells leads to fragmentation of the plasma membrane into diverse vesicle types each bearing a different complement of the functions of the intact cell surface. This result implies that the surface of these cells is organised

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as a mosaic of relatively large macromolecular assemblies with discrete functions. A further indication of the variability of the cell surface was obtained by Mayo and Cocking (1969) who reported that certain areas of isolated tomato fruit protoplasts stained with phosphotungstic acid, and these areas were associated with pinocytotic activity. In this connection, several theories to explain the active transport of ions have been based on mechanisms similar to pinocytosis. Recently, MacRobbie (1969) attempting to explain measurements of ion fluxes into Nitella, suggested a dynamic membrane model involving entry of salt by pinocytotic vesicles at the plasmalemma and transfer to the vacuole in micro-vacuoles. In $KMnO_4$ fixed beetroot tissue there was a preliminary indication that minute vesicles were more frequent in cells undergoing rapid uptake. Further work at higher magnification is required before any conclusions can be drawn, as well as a careful comparison of the appearance of such vesicles after different fixation processes.

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Part D - Energetic aspects: ATPase localisation

1. Histochemical techniques

Histochemical methods aim to produce a visible product at the end of a controlled enzyme reaction. The original procedure for localisation of non-specific acid or alkaline (mono) phosphatases (Gomori 1952) was modified for ATPase by Wachstein and Meisel (1957) and this method has been widely used. Sections, cut on a freezing microtome, are fixed for 16-24 hr in formaldehyde prior to incubation for periods ranging from 5 min to 3 hr in a buffered medium (pH7.2) containing ATP, $Pb(NO_3)_2$ and $MgSO_4$. The sections are then washed and immersed in $(NH_4)_2S$ to produce a visible precipitate of PbS at the sites of activity of ATPase.

Histochemical methods used at the ultrastructural level have generally been derived from those used in light microscopy. If a reaction product, preferably particulate, with adequate electron scattering properties can be created in situ, the electron microscope should enable localisation to cytoplasmic membranes or to organelles beyond the resolving power of the light microscope. The final conversion to sulphide is unnecessary since the reaction product lead phosphate, is itself electron opaque.

The product must resist solution or displacement by subsequent treatments such as dehydration and/embedding. It is also important that the deposit at the active sites should not obscure the morphology of the structures present so that it remains possible to distinguish reaction

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product on membranes from that between membranes e.g. in the case of ER cisternae or Golgi vesicles. Lead phosphate methods may not be capable of indicating enzyme sites at this level because, when the deposit is abundant it fills spaces, when less, it is seen on the membrane. This can mean either that the membrane is the actual site of the enzyme, or that lead phosphate is attracted to the membrane from the adjacent space.

Thus cellular morphology must be preserved as well as enzyme activity and the true localisation of the enzyme. Histochemical reactions are usually carried out before dehydration for two main reasons. Firstly, the absolute amounts of enzymes generally present in thin sections are too low to give sufficient reaction product, and secondly, enzyme activity is usually lost during dehydration and embedding, particularly when epoxy resins are used. However, preservation of enzyme activity at the highest possible level is not always advantageous. If the reaction by which the primary product of the enzyme reaction is immobilised is slow, it will be effective only if the release of the product is slow. Thus lowered enzyme activity may give apparently improved localisation. This can be achieved by incubating at non-optimal pH or alternatively by partial fixation before incubation.

Preliminary fixation is also advantageous for ultrastructural histochemistry because of the morphological damage suffered by unfixed tissue during incubation. Even a brief exposure to OsO_4 has been found to seriously inhibit the activity of many enzymes. On the other hand, fixation in cold formalin allows a variable amount of activity of a number of enzymes,

but often fails to give adequate morphological preservation. Accordingly, Kaplan and Novikoff (1959) introduced a post-fixation in OsO_4 after the incubation for enzyme action of formalin-fixed tissue. Sabatini et al (1963) tried a wide range of aldehyde fixatives varying the concentration, the buffers and the duration of fixation. Different aldehydes were found to preserve and inhibit different enzyme systems; for example, many systems were inhibited more by 60-90 min in cold 5-6% glutaraldehyde than overnight in 4% formaldehyde.

A brief survey of some of the methods used for ultrastructural localisation of ATPases in animal cells is given in Appendix II. Generally, small tissue blocks are fixed, and cryostat sections (25-80μ) cut prior to the incubation. When larger blocks are carried through, the possibility exists that the substrate and other reagents may penetrate the tissue at different rates and hence the reaction may be restricted to the outer layers of cells. In addition, the preliminary fixative (e.g. glutaraldehyde) may not completely penetrate the tissue block in a short time interval and hence the diffusion of soluble enzymes or reaction products from an inadequately fixed central core may occur giving a false localisation (Goldfischer et al 1964). Holt and Hicks (1962) reported excellent preservation of fine structure and discrete staining throughout the tissue when frozen sections, 25-60μ thick were used. Kaplan and Novikoff (1959) however, observed structural damage which they attributed to freezing and thawing. Such observations may depend on the tissue under investigation.

The period of incubation may also be important. In light

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microscopy over-incubation is usually desirable in order to produce sufficient product to be visible, but for electron microscopy the deposit at the active sites should not obscure morphology. Over-incubation can usually be recognised as some diffusion of the reaction product will occur and the density of the precipitate will decrease with increasing distance from the active site.

Salt stimulated ATPases from beetroot and carrot have been described by Atkinson and Polya (1967). When these storage tissues were disrupted, a minor component of the salt-stimulated ATPase was found to be inactive in salt uptake. The main activity was found in the soluble fraction, and some activity was also located in a particulate fraction corresponding to the microsomes derived from cell membranes (sedimenting at 25,000g). The relative amount of activity associated with this fraction was found to change during the aging of beetroot disks, a six-fold increase in amount being measured (Polya, personal communication). At the same time, the ATPase remaining in the supernatant (200,000g) decreased markedly. A similar change was not observed with swede disks. The possibility existed of determining the site of this membrane-attached ATPase in the cell.

In this study, an attempt was made to localise ATPase activity in swede and beetroot tissue of different ages: whole tissue and freshly cut disks (rinsed 3 hr) of both roots, and disks of beetroot aged for 148 hr.

Slices or disks of tissue 1 mm thick were trimmed to strips about 3 mm wide on dental wax. Two longitudinal cuts were made to give strips

approximately 1 mm wide, followed by transverse cuts to give 0.5 - 1 mm cubes, discarding both ends of the original strips. These tissue blocks were immediately plunged into ice cold 4% formaldehyde containing 10% sucrose and buffered with veronal acetate (pH 7.4). Preliminary fixation for either 2 or 20 hr was followed by two brief rinses in cold 0.1M tris-maleate buffer containing 10% sucrose and stored overnight (2 hr fixation) or 3 hr (20 hr fixation) in the same buffer. The incubation medium for the histochemical reaction is shown in Table III. In preparation of the medium, the ATP solution was added to the buffer, the $Pb(NO_3)_2$ added dropwise, and the $MgCl_2$ added finally before filtering through Whatman No. 42 paper.

TABLE III Incubation medium for histochemical localisation of ATPase

	Reaction medium	Control
ATP(di Ksalt), 125mg/100ml	20ml	-
Trismaleate buffer, 0.2M, pH 7.3	20	20
$Pb(NO_3)_2$, 2%	2	2
$MgCl_2$, 0.5M	5	5
Distilled water	3	23
Final pH (after filtration)	7.06	7.26

Tissue blocks were incubated, with shaking, in these media at 37°C for either 15 - 20 min or for 60 min. Two rinses in cold veronal acetate buffered 10% sucrose (pH 7.4) over 25 min. followed before post-

fixation for 1 hr in cold 1% OsO_4 buffered with veronal acetate and containing 10% sucrose. Dehydration and embedding were carried out in the usual way. Because of the inherent difficulty in satisfactorily fixing these tissues, samples were also post-fixed in cold 2% veronal acetate buffered $KMnO_4$ (pH 7.4) for 45 min.

Sections were viewed both unstained and stained with either saturated uranyl acetate in 50% ethanol for 45 min or with Reynolds lead citrate for 10 min.

2. Results and Discussion

On the whole, the preservation of cellular morphology was poor, and for this reason no firm conclusions regarding localisation of enzyme action could be made. In the case of aged beetroot disks only, the preservation was sometimes fair and some preliminary observations were possible. Several blocks of each preparatory treatment were sectioned and studied and the lead deposit was found to be most widely distributed in tissues which had been incubated with ATP for 15 or 60 min. after preliminary fixation for only 2 hr. The deposit generally occurred in intercellular spaces, and along cytomembranes expecially the plasma membrane and the tonoplast (Fig. 76, 77, 78, 79). Occasionally mitochondrial cristae showed a well-defined precipitate especially where mitochondria were not contained within well-preserved cytoplasm (Fig. 77). In many instances the cytoplasm included numerous vesicles or vacuoles, some large, and it is possible that some of these resulted from enlargement and fragmentation of ER cisternae such as those seen in Fig. 78 and 79. The

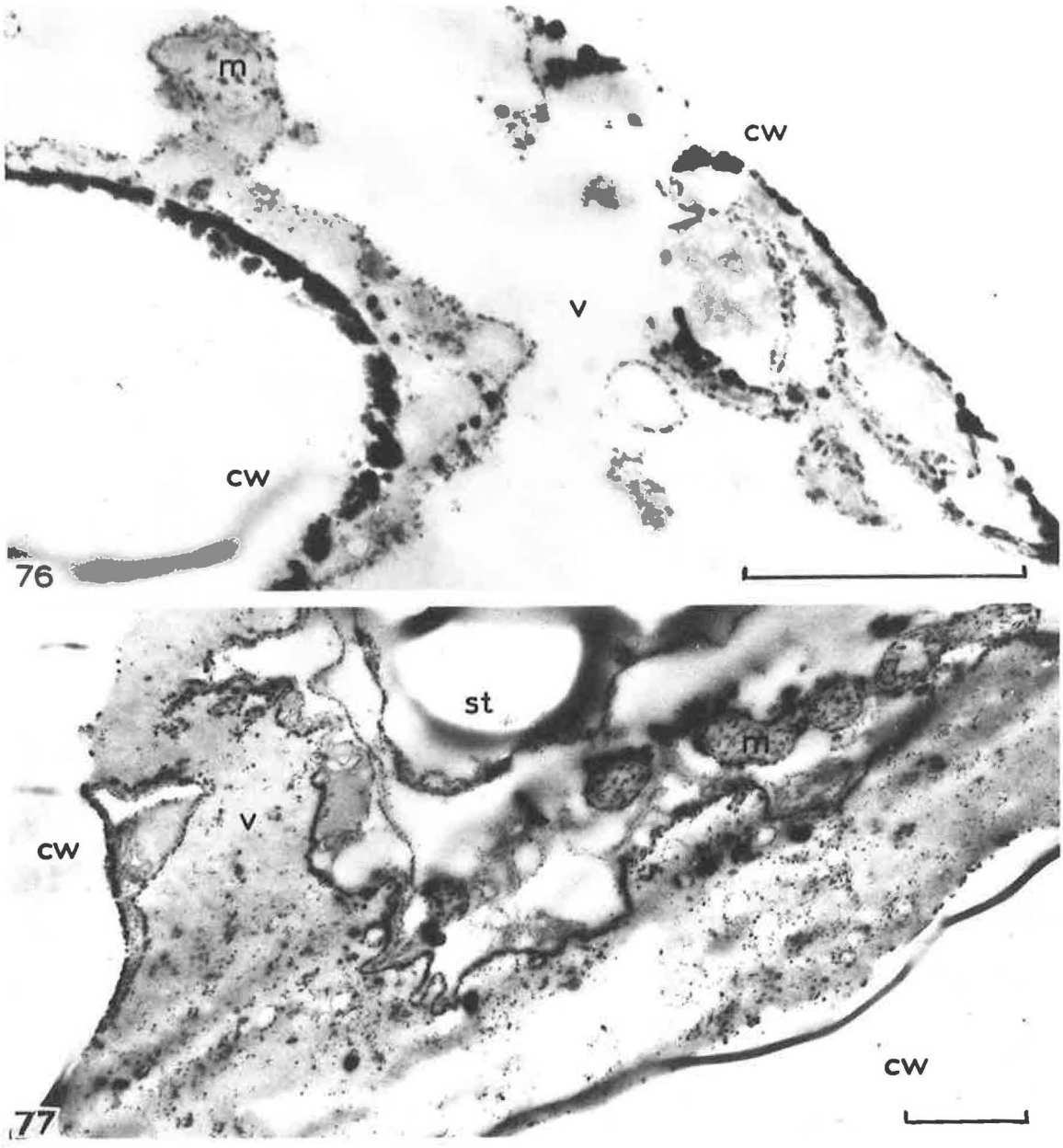
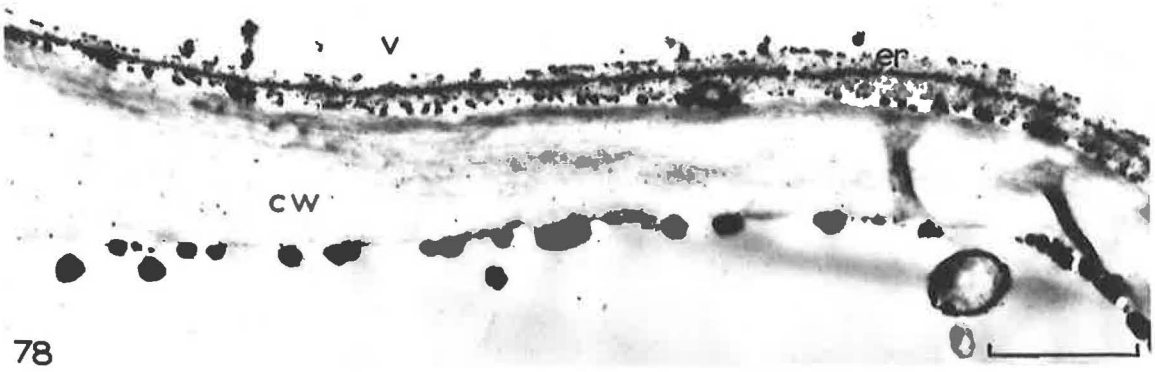


Fig.76-77. Small cubes of aged (148 hr) beetroot disks incubated in modified Wachstein-Meisel medium for 15 min after preliminary fixation with formaldehyde for 2 hr. Post-fixation in OsO_4 for 1 hr. Unstained. Lead deposit associated with cytomembranes, particularly the plasma membrane and tonoplast, and with the mitochondrial cristae (Fig.77).



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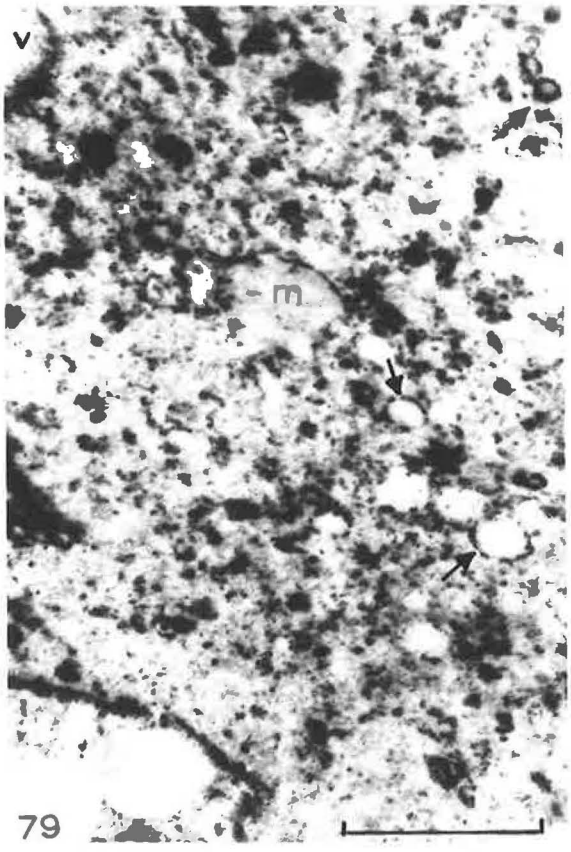

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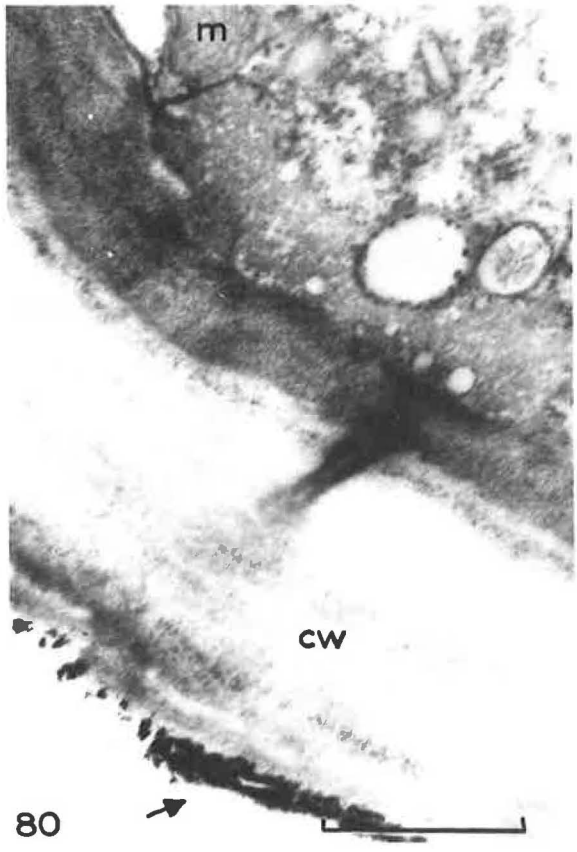

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Fig.78-80. Small cubes of aged (148 hr) beetroot disks incubated in modified Wachstein-Meisel medium for 1 hr (Fig. 78,79) or in medium with ATP omitted for 15 min (Fig.80) after preliminary fixation with formaldehyde for 2 hr. Post-fixation in OsO_4 for 1 hr. Fig.78, uranyl acetate stained; Fig. 79,80, lead citrate stained. Lead deposits in intercellular spaces and along plasma membrane, tonoplast, ER (Fig.78), and cytoplasmic vesicles (Fig.79, arrows). The control shoed deposit in intercellular spaces (Fig.80, arrow).

controls, without added ATP, whether incubated for 15 or 60 minutes, showed relatively small amounts of precipitate located mostly in the intercellular spaces or cell wall (Fig. 80). This deposit was considered at that time to be most likely due to insufficient rinsing of the tissue to remove the incubation medium or possibly due to non-specific phosphatase activity.

In the tissue fixed for 20 hours in formaldehyde, the deposit was confined to intercellular spaces or the region of the plasma-membrane, (Fig. 81, 82), indicating that most enzyme activity, with the possible exception of that associated with the plasma-membrane, was lost as a result of the longer preliminary fixation.

Post fixation with $KMnO_4$ was not satisfactory. The cytoplasm appeared to be disrupted (probably due to the preliminary fixation) with many large vesicles and little ground plasm. The only visible deposits were around intercellular spaces and occasionally a slight deposit in the region of the plasmalemma.

Several attempts were made to determine whether differences in fixation and lead deposition occurred at different depths within blocks of beetroot tissue usually an estimated 8-10 cells in width. As accurately as possible, half the tissue was removed from the top of the block and the four sides trimmed. Sections were then cut from the approximate centre, and at four successive depths towards the outside of the block, about 50μ being discarded between each series of thin sections. Cytoplasmic preservation seemed to be slightly better in an intermediate position, and was poorer towards the outer surface of the block where no intact cytoplasm was visible, probably because of damage during the initial

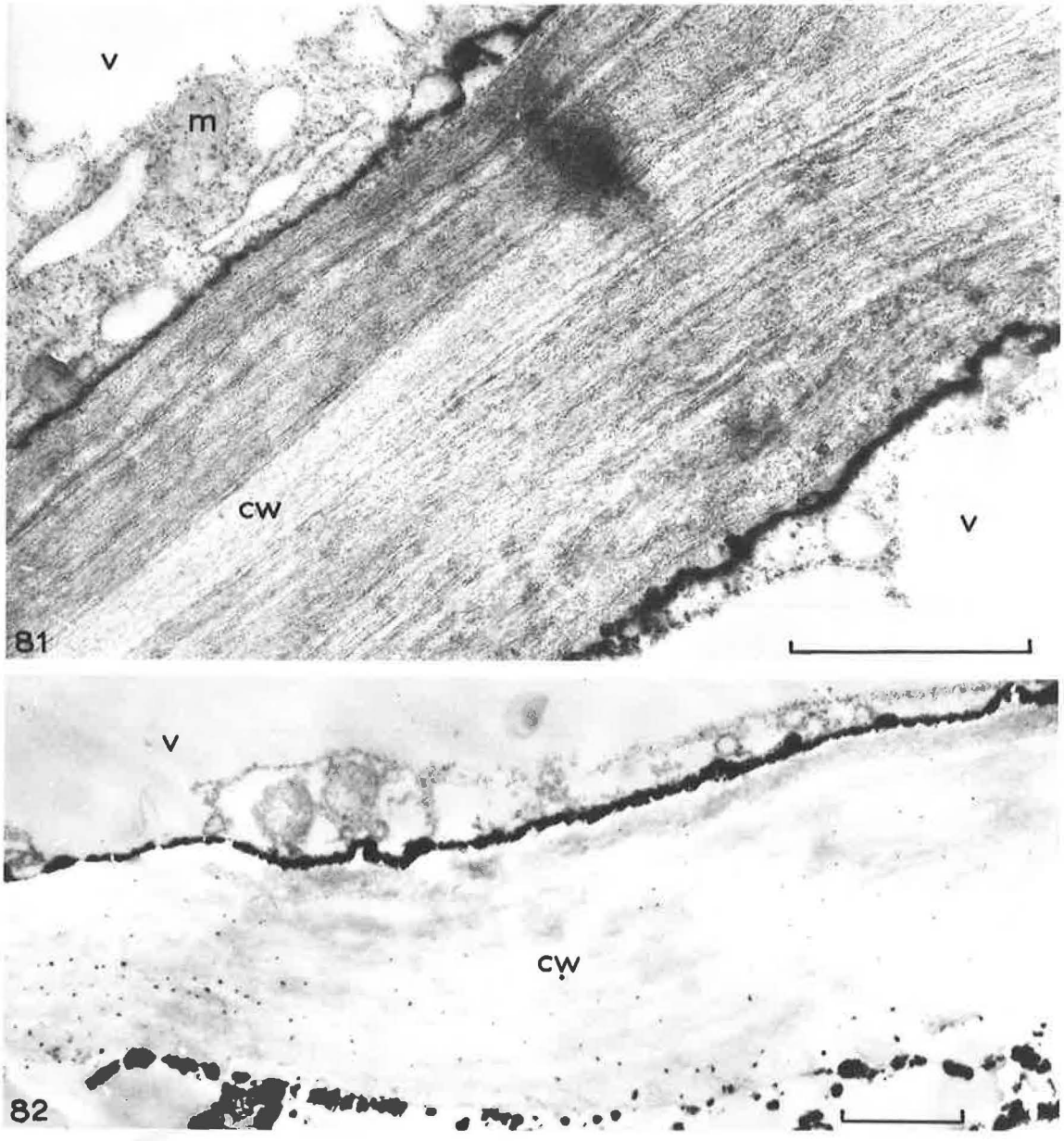

Fig.81-82. Small cubes of aged (148 hr) beetroot disks incubated in modified Wachstein-Meisel medium for 15 min (Fig.81) or 1 hr (Fig.82) after preliminary fixation with formaldehyde for 20 hr. Post-fixation in OsO_4 for 1 hr. Uranyl acetate stained. Deposit largely confined to cell walls and region of the plasma membrane.

cutting of the tissue into blocks. No differences in lead deposition were consistently found at different depths within the tissue.

Thus the most obvious problem in this histochemical work is the difficulty of adequately preserving these large vacuolate cells, and improved fixation is the primary requirement. Then more ambitious experiments could be carried out to determine the effect of various inhibitors using a wide range of nucleoside mono-, di-, and tri-phosphates as substrates. With animal tissues, histochemical methods have indicated that the plasma membrane is capable of hydrolysing all the nucleoside phosphates tested, and that the phosphatases associated with the ER and Golgi membranes are more specific (Novikoff et al 1962). But in plants, no ATPase has yet been localised histochemically. Non-specific acid phosphatases have been reported in wheat meristem (Poux 1963), Cucumis root meristem (Poux 1966), and in sieve elements of Pisum (Zee 1969).

In the last few years several workers have doubted the significance of their findings using the Wachstein-Meisel method after obtaining non-specific depositions of lead (Behnke 1966; Tormey 1966; Moses et al 1966). These observations have led to critical examinations of the method. The lead ion, which was known to inhibit ATPase activity in homogenates of unfixed rat liver (Novikoff et al 1958), was found to further inhibit the activity remaining in fixed tissue when added in concentrations used in the Wachstein-Meisel reaction mixture (Moses et al 1966). On the other hand it was shown that the lead ion may catalyse the non-enzymatic hydrolysis of ATP and other nucleoside phosphates (Rosenthal et al 1966). Localisation of lead deposits to the plasma membrane,

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the most common site, were found to occur only with concentrations of lead and ATP in the mixture which also gave significant non-enzymatic hydrolysis of ATP. And, changing the relative concentrations of lead and ATP caused localisation of the reaction product to entirely different subcellular sites (Moses et al 1966, Abel 1969). Such changes can be brought about by interaction between constituents of the medium, for example, the chelation of lead by ATP (Tice 1969). Thus, variation of one constituent may result in unexpected changes in the effective concentration of other constituents of the reaction mixture (Jacobsen and Jorgensen 1969), or changes in the amount of reaction product bound at the site (Rosenthal et al 1969). The reaction product deposited in tissues is not simple lead phosphate as the nucleotide is also bound. Thus some of the deposits observed in beetroot tissue may have been due to an unbalanced reaction mixture, or simply to unequal rates of penetration by the various components of the mixture.

It has been suggested that under some conditions lead phosphate will be lost to the medium (Rosenthal et al 1966), and if this is so it could penetrate fixed tissues via intercellular channels and may even be bound to specific sites on the cytoplasmic surface to give the impression of a positive reaction (Berridge and Gupta, 1968). The amount of such deposits may depend on the effectiveness of washing following incubation. This phenomenon may explain the large deposits seen in intercellular spaces and along the plasma membrane in some beetroot preparations (Fig. 78). A recent publication (Ganote et al 1969) reports the formation of significant

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amounts of lead phosphate in the reaction medium by lead catalysed hydrolysis, and the deposition of lead phosphate and lead at tissue sites parallel to those observed with the complete Wachstein-Meisel reaction mixture.

Problems also arise with the use of inhibitors. Side reactions of the inhibitor can occur, e.g. sodium fluoride precipitates lead from the reaction mixture, thus altering its effective concentration. Tissues may contain a variety of ATPases with different responses to inhibitors. For instance, the Na^+ and K^+ dependent ATPase of animal cells is more susceptible to inhibition by fixatives or by Pb^{++} ion than other ATPases, and the activity remaining in fixed tissues has not been shown to be significant in active transport (Tormey 1966).

Despite these problems many workers continue to justify their interpretations of histochemical localisation of ATPase, often to supplement biochemical data on enzyme activity of various sub-cellular fractions (Jacobsen and Jorgensen 1969). Usually some precautions are taken to overcome the published objections referred to above e.g. by shortening the time of preliminary fixation (to 5 min), incubating at 20°C instead of 37°C, lowering the Pb^{++} concentration to a level where its inhibitory effect is reduced, or raising the substrate concentration to reduce non-enzymic hydrolysis (Novikoff 1967; Abel 1969). It is often difficult to believe that small deposits which appear in intimate and restricted association with a specific unit membrane could arise by a non-enzymic process (Tice and Engel 1966, Farquar and Palade 1966, Berridge

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and Gupta 1968). But these studies of the reactions involved indicate that in using a lead salt deposition method for enzyme localisation great care must be taken to choose the optimum mixture and conditions for a particular histochemical reaction, and that, at present, it is unwise to rely on histochemical results alone.

Because of the current doubts regarding the reliability of such histochemical localisations, this aspect of the work was not continued. Further, as indicated in the introduction, although several ATPases have been reported in plants, to the present time, there is limited evidence for the direct involvement of these enzymes in ion transport. Hodges (1966) reported oligomycin inhibition of ion uptake by plant roots and considered that his results supported the idea that ATP formation is a primary requirement in providing energy for ion transport. Atkinson and Polya (1967) speculated on the possible role of salt stimulated plant ATPases, basing their ideas on the correlations between the properties of these enzymes and the properties of the higher concentration (0.5 - 50 mM) absorption isotherm of kinetic analyses of ion uptake (Epstein 1966). They suggested that the ATPases may be concerned, either alone, or in conjunction with an electron transport system, in the transport of salt by this low affinity mechanism. Fisher and Hodges (1969) found also that the concentrations of monovalent salts required to activate the ATPase from oat roots were quite high, and thus in the range of mechanism 2. Torii and Laties (1966) suggested the tonoplast as the site of this transport mechanism, while Welch and Epstein (1968) believe the low affinity mechanism to be

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located at the plasmalemma.

At present, evidence seems to be accumulating to show that ATP is not directly involved in ion transport, an opinion first proposed by Robertson (1960). After investigating the effect of specific inhibitors on ATP content, respiration rate and salt accumulation rate in carrot tissue, Atkinson et al (1966) favoured the theory that ion transport is directly coupled to the electron transport system, and may be an alternative to ATP formation. Further evidence was obtained using L-ethionine as an ATP-trapping system in aged carrot xylem tissue (Atkinson and Polya 1968), when it was found that salt uptake continued for some time despite a rapid drop in ATP content and respiration rate. These workers suggested that the integrity of structures involved in salt accumulation may depend on ATPase activity since ATP: ADP: AMP ratios regulate the activities and conformations of many enzymes. Recently, considerable discrepancies between the times taken for the effects of anaerobiosis and inhibitors on influx of ions, and on ATP content were found in beetroot parenchyma (Polya and Atkinson 1969), again supporting the direct involvement of electron transport rather than ATP hydrolysis in ion accumulation. The similarities between the characteristics of ion uptake into isolated mitochondria and ion uptake by disks of storage tissue, leads to the implication of mitochondria in ion accumulation processes. It is, however, interesting to note the possibility that other organelles or membrane systems, e.g. nuclei, which have been shown to synthesise ATP aerobically, may in the future be found to possess electron transport systems and also play a role in salt accumulation.

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Part E - Developmental aspects: Effect of inhibitors of protein synthesis

1. Ion movements

Several experiments were carried out to determine the effects of adding inhibitors of RNA and protein synthesis* to beetroot disks of various ages. As this thesis is mainly concerned with ultrastructural aspects, the ion transport measurements are not reported in full, but are summarised in Table IV. Ion measurements in most experiments were made in collaboration with R.F.M. Van Steveninck.

From the results shown in Table IV it appears that actinomycin D has no inhibitory effect on ion movement when it is added after the development of the ion uptake mechanisms. Puromycin is not effective if added when uptake of ions from the external solution is almost complete (at 72 hr), but it causes some inhibition when added earlier in the uptake period (at 44 hr). Cycloheximide added at any stage, on the other hand, causes eventual inhibition of ion uptake either at once or after a lag of variable duration.

If an inhibitor is found to affect the process of ion uptake, then, clearly, it will be difficult to determine its effect on the development or generation of mechanisms for ion uptake. Thus the apparent effects of cycloheximide, puromycin and p-fluorophenylalanine

* Actinomycin was supplied by Merck, Sharp and Dohme, Sydney; puromycin and cycloheximide by Nutritional Biochemicals Corporation; and p-fluorophenylalanine by Sigma Chemical Company.

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Table IV Effect of inhibitors of protein synthesis on ion movement (a) before and (b) after the generation of ion uptake capacity

Freshly cut beetroot disks or disks aged in deionised water for 44 or 72 hr were transferred to solutions of the inhibitors (actinomycin D, 2×10^{-5}M; puromycin, 10^{-4}M; cycloheximide, 3.5×10^{-6}M; p-fluorophenylalanine, 2×10^{-3}M) to which 0.5mM KCl + 0.5 mM NaCl was added.

(a) Period required for generation of uptake capacity

Treatment	K^+	Na^+	Cl^-
Control	25 hr	18 hr	32 hr
actinomycin at 0 hr	> 50 hr	> 30 hr	> 50 hr
puromycin at 0 hr	Ion leakage for duration of experiment (80 hr) (some leakage of pigment occurred)		
cycloheximide at 0 hr	Little net movement for duration of experiment (80 hr)		
p-fluorophenyl-alanine at 0 hr	10 hr followed by leakage at 35 hr	8 hr followed by slow leakage at 44 hr	70 hr

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(b) Effect on uptake capacity

Treatment	K^+	Na^+	Cl^-
actinomycin			
at 44 hr	no effect	no effect	no effect
at 72 hr	no effect	no effect	no effect
puromycin			
at 44 hr	inhibition after 5 hr	uptake completed before 44 hr. No leakage	inhibition after 5 hr
at 72 hr	no effect	no effect	no effect
cycloheximide			
at 44 hr	inhibition after 15 hr	uptake completed before 44 hr	inhibition after 7 hr
at 72 hr	inhibition after 25 hr	inhibition after 5 hr	immediate inhibition
p-fluorophenyl-alanine at 72 hr	inhibition for 20 hr, followed by uptake	slight inhibition	——

on the development of the process is complicated by their effect on the uptake process itself. But actinomycin D inhibits only the development of the uptake mechanisms for the three ions measured, net uptake of K^+ and Cl^- being inhibited for longer than Na^+ uptake. The developmental process seems to involve an initial stage which can be inhibited by actinomycin D, followed by a stage when net uptake of ions is just beginning which is still sensitive to puromycin but not to actinomycin.

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Results such as these must be interpreted with caution, for several reasons. Some of the observed differences may be due to differences in the times required by the various inhibitors to penetrate to their sites of action. This may be overcome by pre-incubating the tissue with the inhibitor at 1°C (Gayler and Glasziou 1969). Apparent recovery after a period may indicate that the concentration of inhibitor is no longer effective, or that bacterial contamination has occurred. The latter is difficult to eliminate. Bacon et al (1965) described the preparation of aseptic disks by pre-soaking whole beetroots and blocks cut from them in 0.1% $HgCl_2$ for $1\frac{1}{2}$ hours and 20 minutes respectively, but it is possible that this treatment could have some unknown effects on the cells in addition to those which have been measured. The addition of chloramphenicol during aging greatly decreases bacterial contamination, but also affects ion uptake. In one experiment using carrot tissue, 45 mg/l chloramphenicol was found to decrease the initial rate of uptake slightly, thus confirming earlier findings (Van Steveninck 1961). Simultaneous ultrastructural studies, of course, provide an immediate check for bacterial infection.

The results of these experiments are in general agreement with those reported by other workers. MacDonald et al (1966) found that puromycin at a concentration of 5×10^{-6}M was effective in inhibiting the development of chloride absorptive capacity in beetroot, but

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had no effect on chloride uptake itself at concentrations up to 1×10^{-4}M. They interpreted their results in terms of a difference in affinity of puromycin for the ion uptake mechanism which they considered to involve ATPase and the synthesis of ATPase proteins. Several reports indicate that actinomycin D (Laszlo et al 1966) and puromycin (Appleman and Kemp 1966) do not act specifically as protein synthesis inhibitors. There is some evidence from this work that puromycin added to freshly cut disks causes leakage of pigment indicating a possible direct effect on cell membranes. A similar observation was made by MacDonald et al (1966).

Cycloheximide in concentrations as high as 5×10^{-3}M does not inhibit protein synthesis in a number of bacteria (Whiffen 1948) while at concentrations of about 5×10^{-5}M it almost completely inhibits protein synthesis in higher plant tissues (Venis 1967). In this work, ion uptake was markedly affected by 3.5×10^{-6}M cycloheximide. Polya (1968) in experiments of shorter duration, and using a higher concentration (3×10^{-4}M) observed that cycloheximide inhibited protein synthesis within $\frac{1}{2}$ hr in aged (24 hr) beet disks, although net Na^+ uptake was not affected until $1\frac{1}{2}$ hr. He concluded that, because of this lag, the results did not support the idea of a direct link between the two processes. It seems then that the effect of these inhibitors on the process of ion uptake may be an indirect one. It is interesting to note that the uptake of the three ions measured in these experiments is affected at different times suggesting the existence of separate mechanisms or unequal rates of decay of some component.

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The effect of actinomycin D is the most conclusive and strongly suggests that the development of salt uptake mechanisms depends on the synthesis of new RNA after slicing. Either the required RNA is completely destroyed by the cutting of the disks, or alternatively the cells of the disks take up ions by a mechanism different from that of the intact root.

It has been suggested for various enzyme systems which are activated by slicing (Willemot and Stumpf 1967, Vaughan and MacDonald 1967a, Rhodes et al 1968, Galliard et al 1968, Gayler and Glasziou 1969) that the cutting of the tissue in some way causes a derepression allowing the production of new m-RNA's and hence new proteins or enzymes. Similarly the production of specific proteins or carriers may be an essential step in the development of a capacity for salt uptake in beet disks. Further, it seems that the RNA produced is relatively stable since actinomycin added at a later stage is not effective.

2. Ultrastructural observations

In experiments to detect changes in fine structure of beetroot cells caused by treatment with inhibitors of RNA and protein synthesis, the appearance of all cell membranes and organelles and the ground cytoplasm were initially recorded. The changes which were consistently observed are summarised in Tables V, VI and VII, each of which represents a different experiment.

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Table V Effect of actinomycin D, puromycin and cycloheximide on fine structure of beetroot tissue (first experiment)

Freshly cut beetroot disks or disks aged in deionised water for 44 hr were transferred to solutions of actinomycin D (2×10^{-5}M), puromycin (10^{-4}M), and cycloheximide (3.5×10^{-6}M) to which 0.5mM KCl + 0.5mM NaCl was added. Disks treated at 0 hr were sampled at 50 hr and disks treated at 44 hr were sampled 30 hr later (at 74 hr). All samples were fixed with $KMnO_4$. (0 denotes not seen; + occasional; ++ average; +++ more than average).

Treatment	Time Sampled	ER lamellae	Crystals	Plastid bodies	
				Internal	Marginal
control	50 hr	++	0	++ (large)	++
actinomycin at 0 hr	50 hr	++	++	++ (large)	++
puromycin at 0 hr	50 hr	+	0	0	+
cycloheximide at 0 hr	50 hr	+	0	0	++
control	74 hr	+++	++	+	++
actinomycin at 44 hr	74 hr	+++	++		++
puromycin at 44 hr	74 hr	+++	++		
cycloheximide at 44 hr	74 hr	+++	0 (?)	+	+ (small)

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In the first experiment uptake of K^+, Na^+ and Cl^- by the control disks was almost complete at the time of the first sampling for electron microscopy (50 hr). Uptake was inhibited by puromycin and cycloheximide but the actinomycin D treated disks had commenced slow net uptake by this time. Ultrastructural observations showed that puromycin and cycloheximide (Fig. 83 and 84) prevented the normal appearance of ER lamellae seen in the control and actinomycin D treatment (Fig. 85). Thus it seems that resynthesis of the ER into lamellae can be correlated with net accumulation of ions.

By the time of the second sample (74 hr), nearly all ions were taken up from the external solution by the control and actinomycin D treated (at 44 hr) disks, while little ion movement occurred in disks given puromycin or cycloheximide at 44 hr. There was no apparent difference in amount of ER suggesting that after the reassembly of lamellae has commenced, the inhibitors have no effect on this process.

Actinomycin D added at 0 hr appeared to promote the formation of crystals (Fig. 86). No crystals were seen (at 50 hr) in the control or in disks treated with puromycin or cycloheximide at the beginning of the experiment. Cycloheximide at 44 hr may also inhibit crystal formation.

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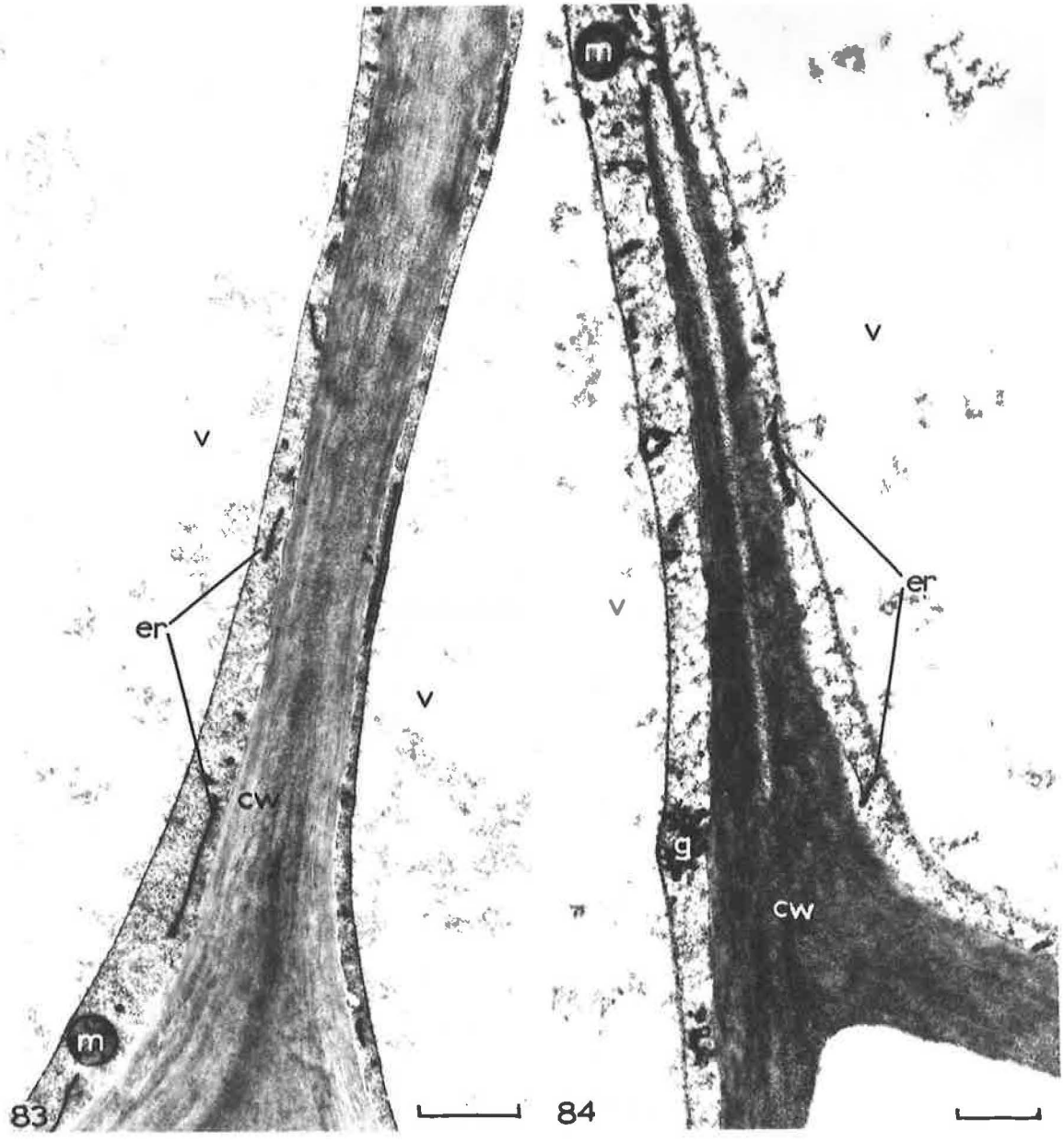

Fig.83-84. Cells from fresh beetroot disks aged for 50 hr in puromycin (Fig.83) or cycloheximide (Fig.84) solution showing short ER lamellae. $KMnO_4$ fixation.

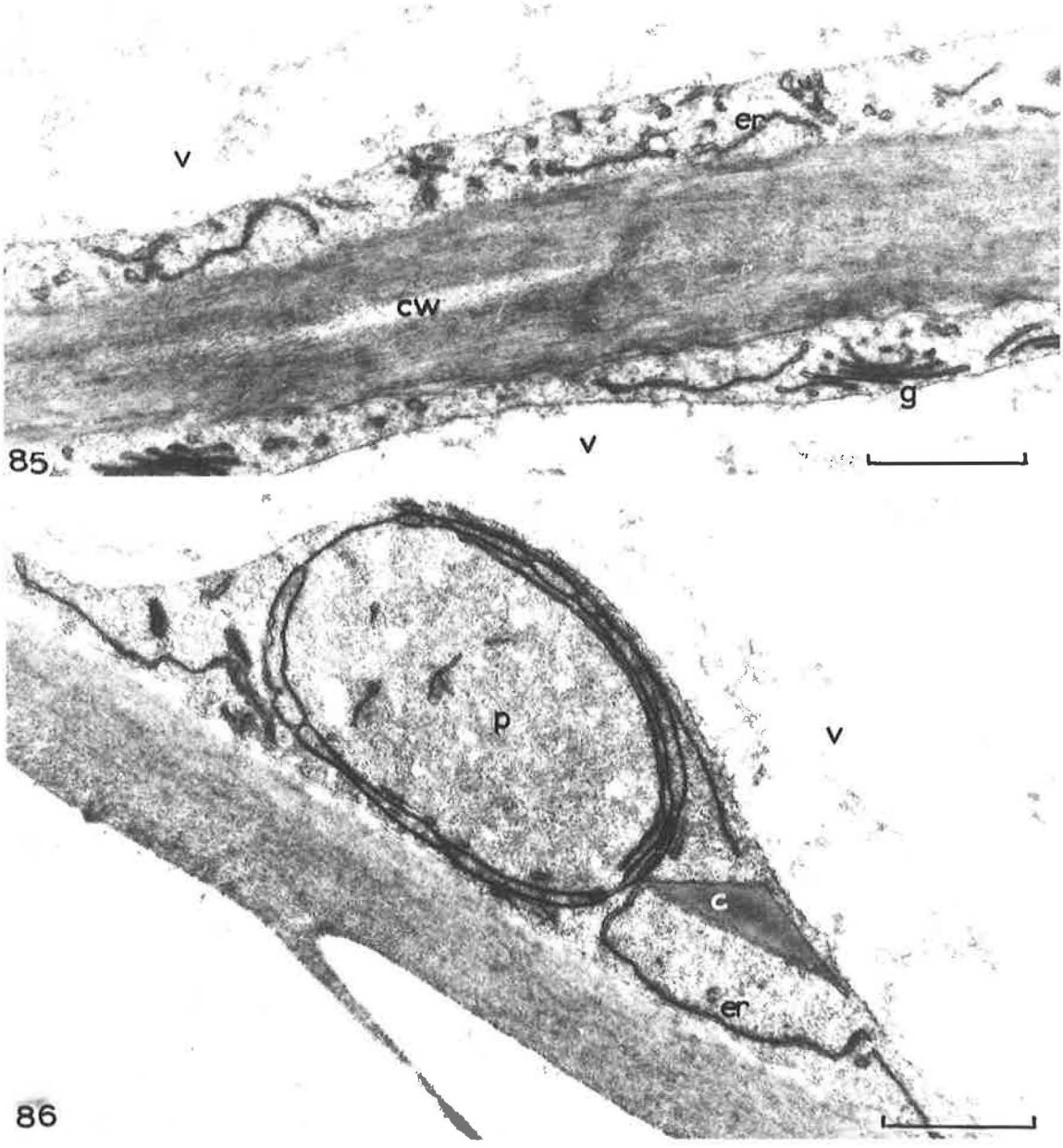

Fig.85-86. Cells from fresh beetroot disks aged for 50 hr in actinomycin D solution showing the ER lamellae and the presence of crystals. $KMnO_4$ fixation.

Table VI Effect of actinomycin D, puromycin, cycloheximide and p-fluorophenylalanine on fine structure of beetroot tissue (second experiment)

Freshly cut beetroot disks or disks aged in deionised water for 68 hr were transferred to solutions of actinomycin D (2×10^{-5}M), puromycin (10^{-4}M), cycloheximide (3.5×10^{-6}M) and p-fluorophenylalanine (2×10^{-3}M) to which 0.5mM KCl + 0.5mM NaCl was added. Samples were fixed at 64 hr and 94 hr respectively with both $KMnO_4$ and glutaraldehyde - OsO_4. (Symbols as in Table V).

Treatment	Time sampled	ER lamellae	Crystals	Plastid bodies ($KMnO_4$ fixation) Internal	Marginal
Control	64 hr	+++	+	++	++
actinomycin at 0 hr	64 hr	+++	+++	+	++
puromycin at 0 hr	64 hr	+	0	+	+
cycloheximide at 0 hr	64 hr	+	0	++	++
p-FPA at 0 hr	64 hr	+	+++	++	++
control	94 hr	+++	++	++	++
actinomycin at 68 hr	94 hr	+++	++	++	++
puromycin at 68 hr	94 hr	+++	++	+	++
cycloheximide at 68 hr	94 hr	++	0?	++	++
p-FPA at 68 hr	94 hr	+++	+++	++	++

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In the second experiment (Table VI), the reassembly of ER lamellae appeared to be limited by the addition (at 0 hr) of puromycin, cycloheximide and p-fluorophenylalanine (p-FPA), but unaffected by actinomycin D (Fig. 87-90). Further increase in length of lamellae after 68 hr may be inhibited by cycloheximide.

By the time of the first sampling (64 hr), the actinomycin treated disks had commenced a delayed uptake of all three ions, puromycin had caused some leakage, cycloheximide, after initial leakage, prevented net uptake, and p-FPA caused no net uptake of K^+ but rapid Na^+ uptake. Twenty-six hours after adding inhibitors to aged (68 hr) disks, rapid net uptake was occurring in all treatments except cycloheximide. Thus it appears that net ion uptake can again be roughly correlated with length of ER lamellae.

In this experiment crystal synthesis was enhanced by the addition of either actinomycin D or p-FPA at 0 hr (Fig. 89 and 90), but appeared to be inhibited by cycloheximide when added at either 0 or 68 hr.

Thus actinomycin D at 0 hr appears to enhance crystal synthesis and possibly stimulate ER synthesis. The other three substances added at 0 hr inhibit the build up of ER lamellae, and p-FPA promotes crystal formation. Cycloheximide added at 68 hr seems to prevent further lengthening of ER lamellae and inhibit crystal synthesis, while p-FPA again stimulates crystal formation.

In these two experiments puromycin added at 0 hr usually reduced the cytoplasm to a thin layer (Fig. 83 and 87). In addition it allowed

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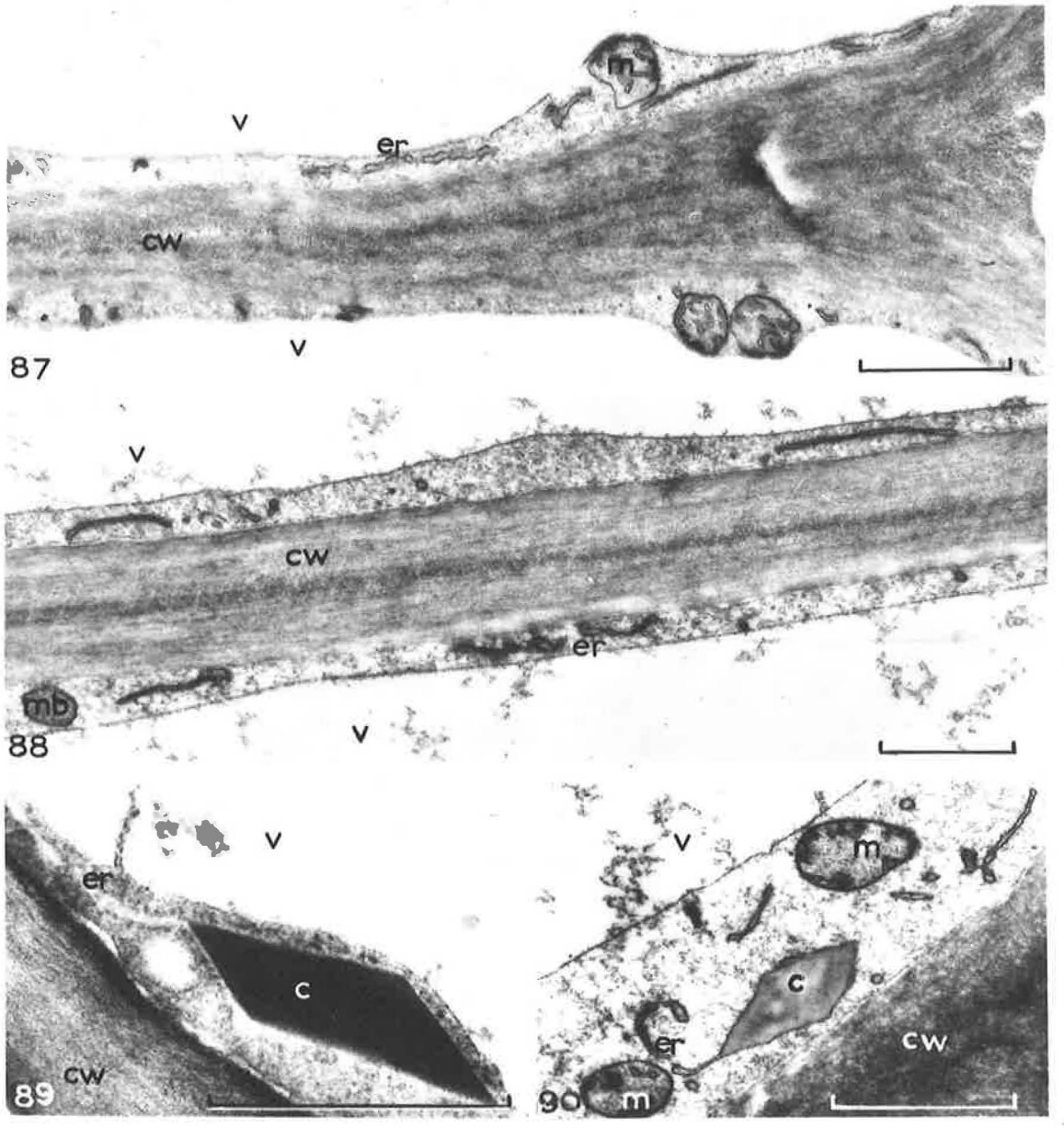

Fig. 87-90. Cells from fresh beetroot disks aged for 64 hr in puromycin (Fig.87), p-fluorophenylalanine (Fig.88,90), or actinomycin D (Fig.89) solution. Note short lengths of ER in puromycin and p-fluorophenylalanine treated cells and crystals in actinomycin and p-fluorophenylalanine treatments. Fig.87,88,90, $KMnO_4$ fixation; Fig.89, glutaraldehyde-OsO_4 fixation.

the development of only a few membrane bound internal bodies or marginal bodies in the plastids, seen after $KMnO_4$ fixation. These bodies are probably identifiable with the osmiophilic areas seen in glutaraldehyde-OsO_4 fixed cells.

A further experiment was carried out to confirm some of the previous observations with actinomycin D and p-FPA. Samples for electron microscopy were taken more frequently in the early stages, and the experiment was continued for a longer period. Results from the later samples should be accepted with caution for the reasons outlined previously in the section on ion movements.

As can be seen in Table VII, no distinction can be made between actinomycin D treatment (Fig. 91-94) and the control, but p-FPA again inhibited the formation of long ER lamellae (Fig. 95-98). Both actinomycin D and p-FPA advanced the onset of crystal formation, a few crystals being apparent after 24 hr, and many by 48 hr in both treatments. Some regions in p-FPA treated disks contained a large number of crystals (Fig. 96 and 99) which occasionally may link up (Fig. 100).

In this experiment ion uptake cannot be readily correlated with changes in the ER. At 96 hr when ER lamellae were long in the control and actinomycin D treatment, the uptake of K^+, Na^+ and Cl^- was complete in the control and either complete or proceeding rapidly in the actinomycin D treatment. The effect of p-FPA on ion movement was variable (Table IV). It is possible that Cl^- uptake is related to length of ER lamellae, but more experimentation is required to study these changes in more detail.

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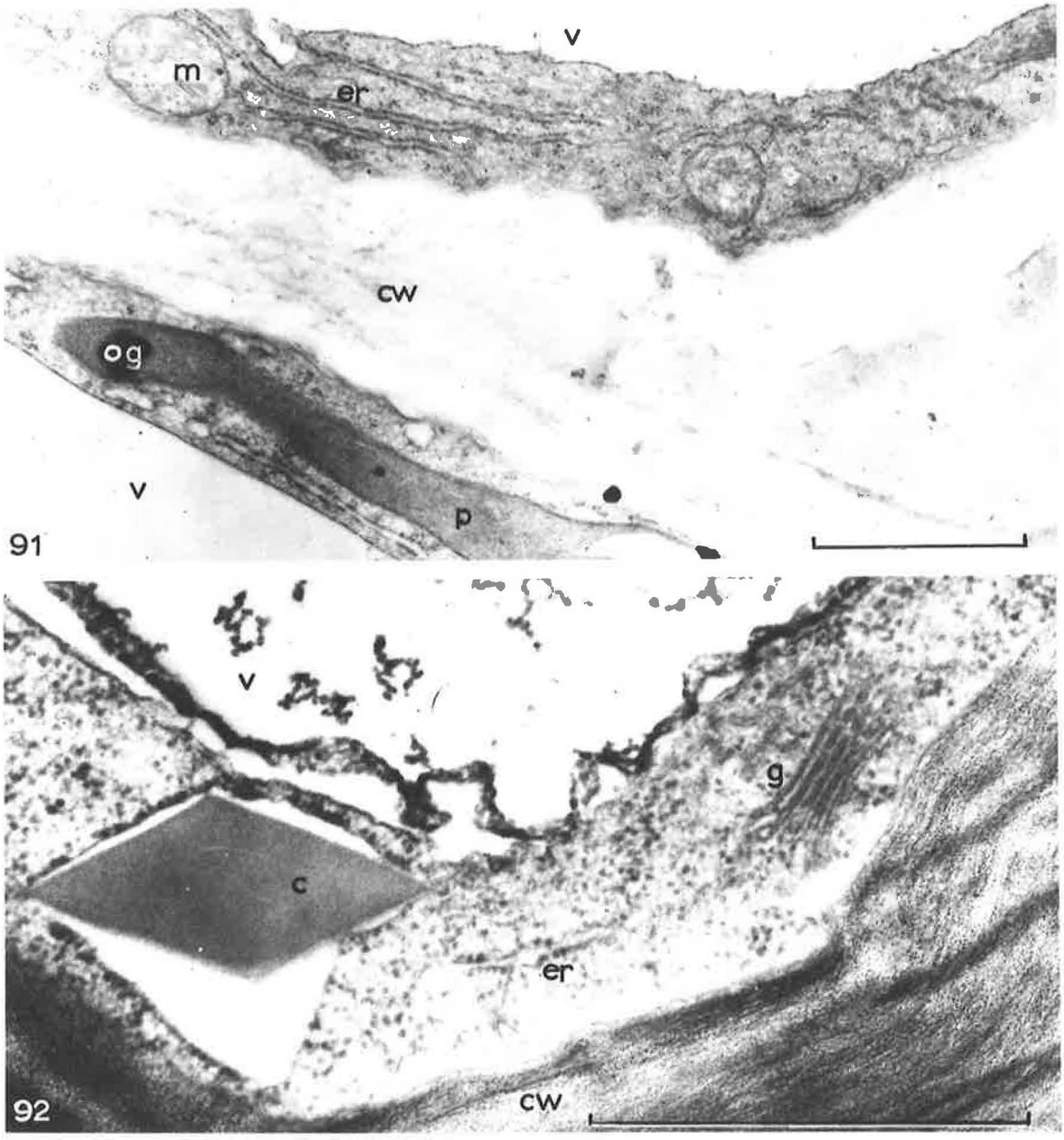


Fig.91-92. Cells from fresh beetroot disks aged for 48 hr in actinomycin D solution. Note length of ER lamellae similar to that of untreated cells, and presence of crystals induced by actinomycin treatment. Glutaraldehyde-OsO_4 fixation.

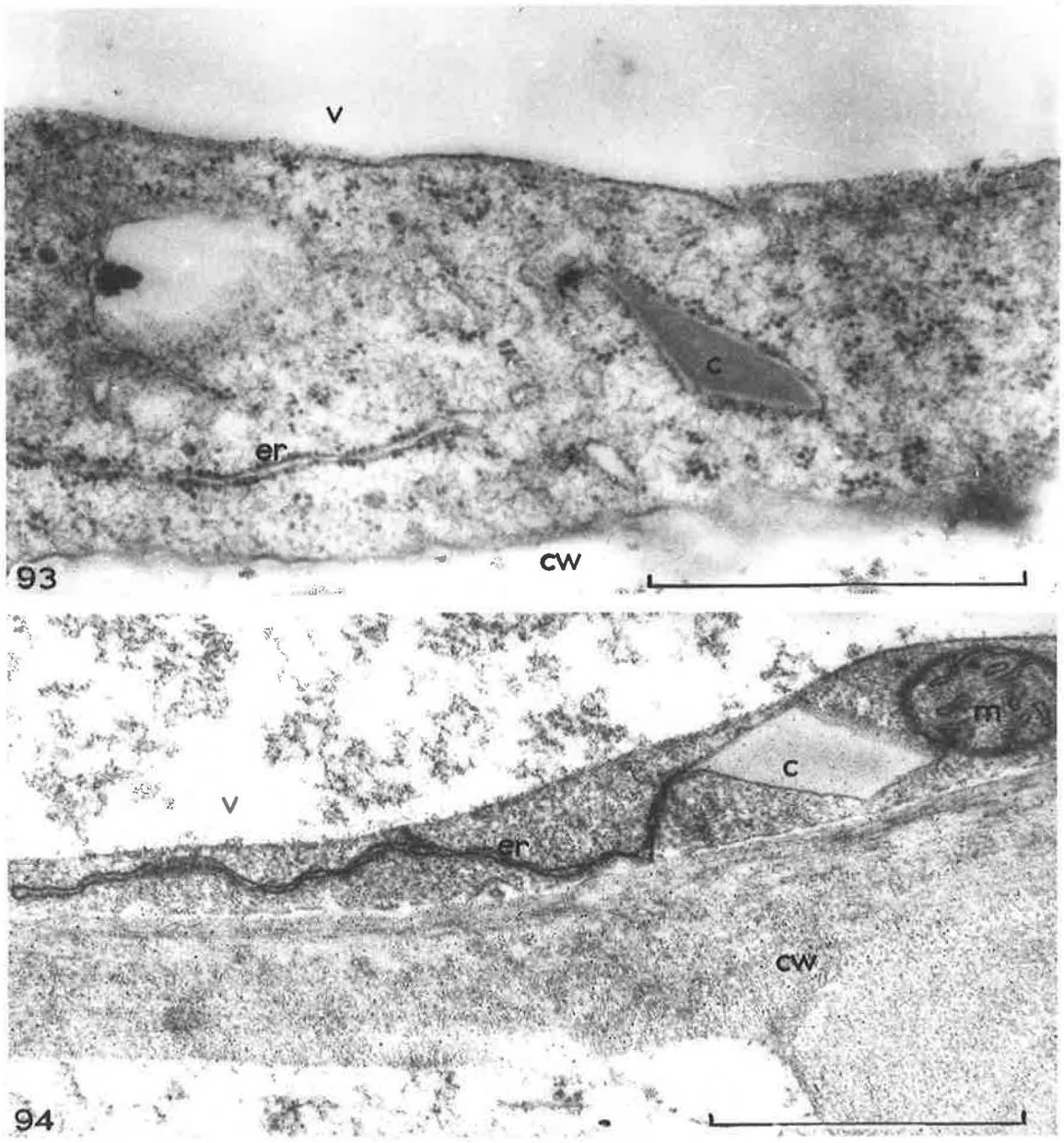
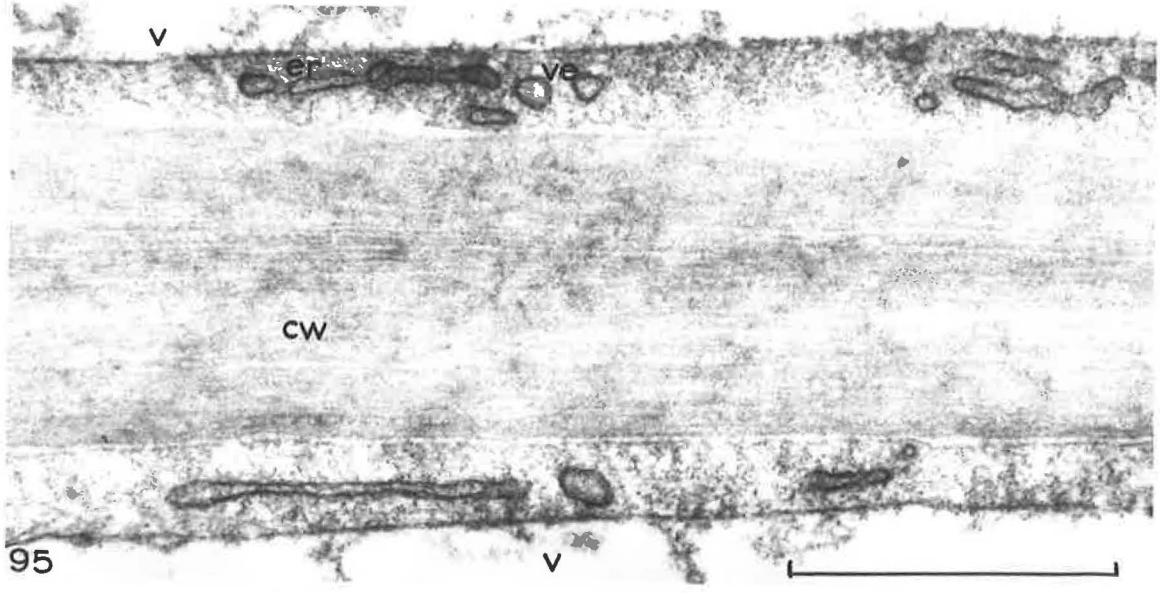


Fig. 93-94. Cells from fresh beetroot disks aged for 48 hr (Fig.93) and 96 hr (Fig.94) in actinomycin D solution. Note length of ER lamellae and presence of crystals. Fig. 93, glutaraldehyde-OsO_4 fixation; Fig.94, $KMnO_4$ fixation.



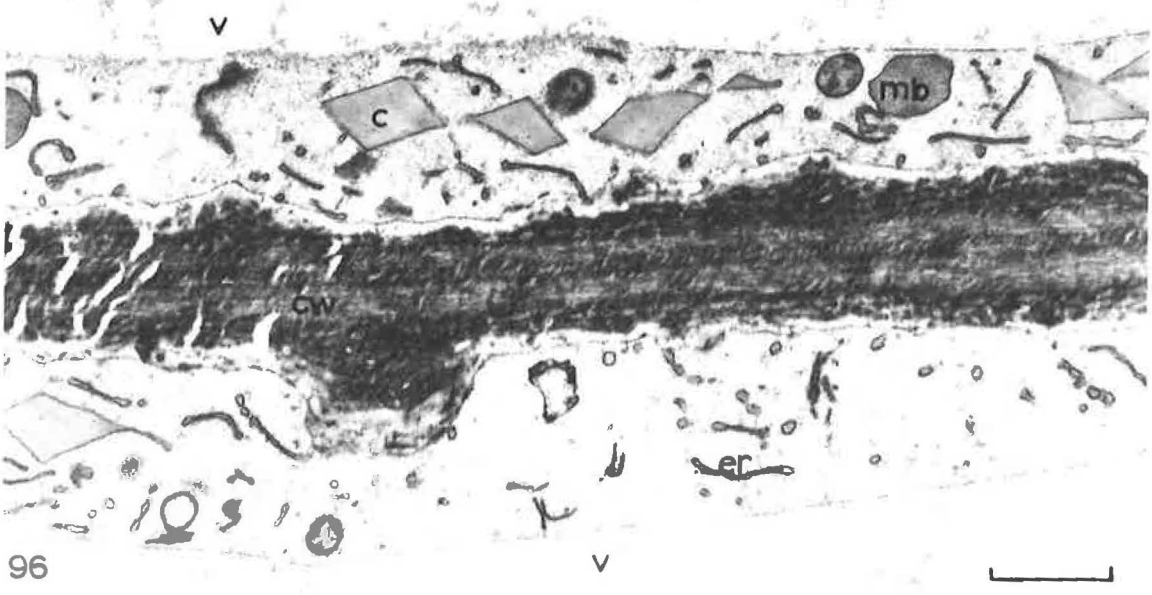


Fig.95-96. Cells from fresh beetroot disks aged for 96 hr in p-fluorophenylalanine solution. Note absence of long ER lamellae and presence of numerous crystals (Fig.96). $KMnO_4$ fixation.

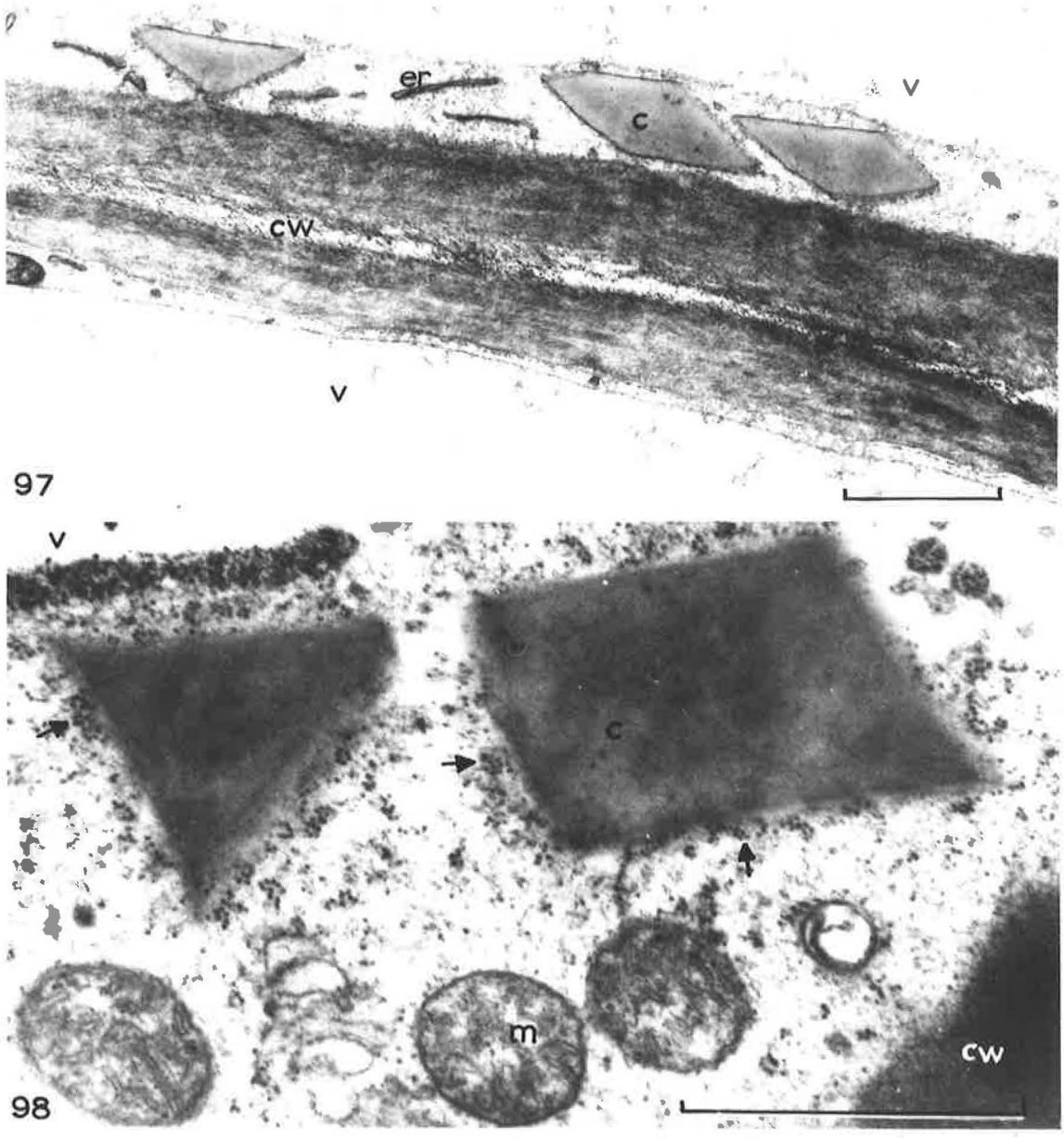


Fig.97-98. Cells from fresh beetroot disks aged for 96 hr (Fig.97) and 144 hr (Fig.98) in p-fluorophenylalanine solution. Note absence of long ER lamellae and presence of polysomes adjacent to the crystals (arrows). Fig.97, $KMnO_4$ fixation; Fig. 98, glutaraldehyde-OsO_4 fixation.

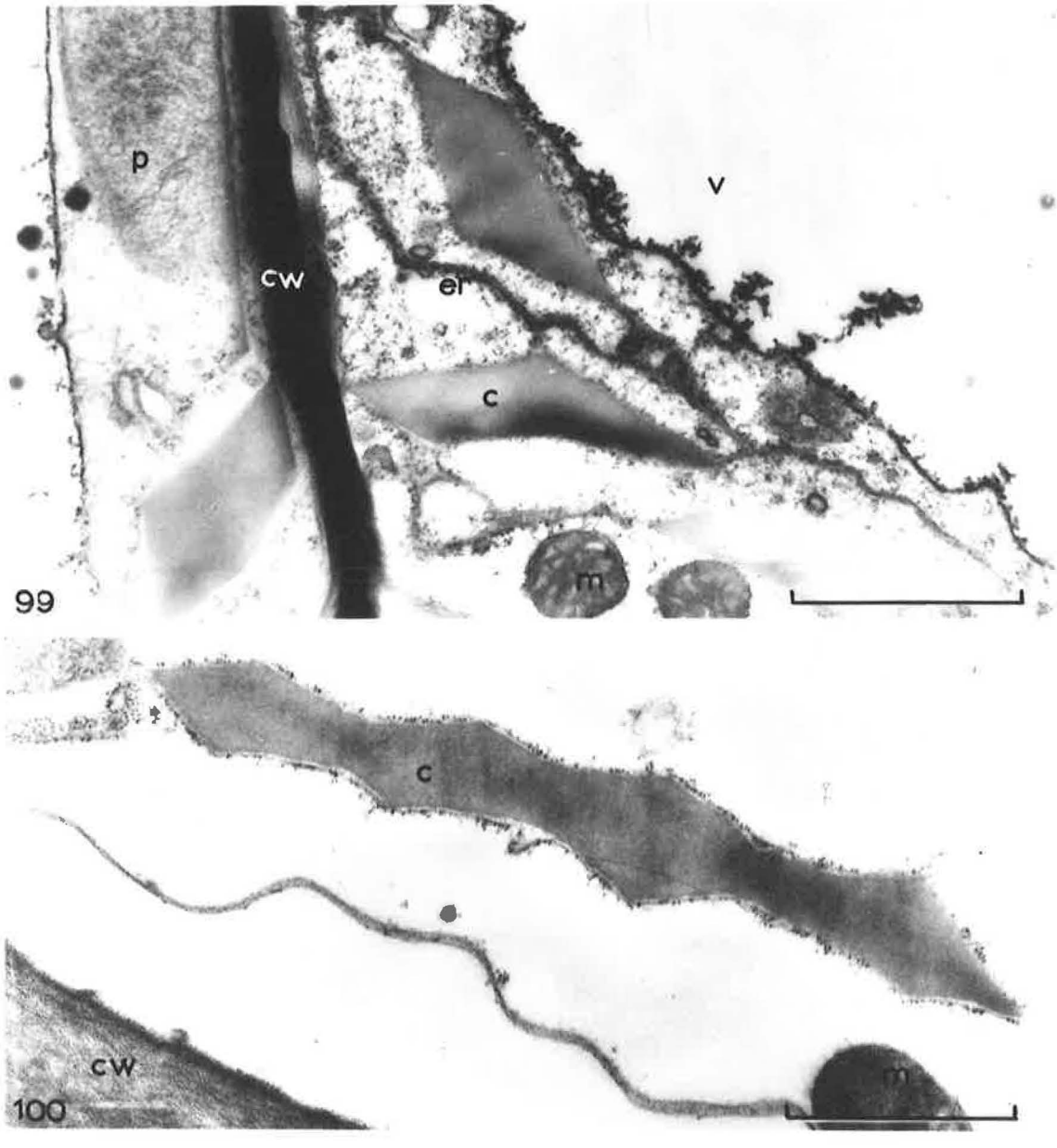


Fig.99-100. Cells from fresh beetroot disks aged for 96 hr (Fig.99) and 144 hr (Fig.100) in p-fluorophenylalanine solution showing presence of many crystals. Glutaraldehyde-OsO_4 fixation.

Table VII Effect of actinomycin D and p-fluorophenylalanine on fine structure of beetroot tissue (third experiment)

Freshly cut beetroot disks were transferred to solutions of actinomycin D (2×10^{-5}M) and p-FPA (2×10^{-3}M) to which 0.5mM KCl + 0.5mM NaCl was added. Samples were fixed at 0, 24, 48, 96, 144 and 336 hr with $KMnO_4$ and glutaraldehyde - OsO_4. (Symbols as in Table V).

Treatment	Time sampled	ER lamellae	Crystals
Control	0 hr	+	0
control	24 hr	++	0
actinomycin	24 hr	++	+
p-FPA	24 hr	+	+
control	48 hr	++	0
actinomycin	48 hr	++	++
p-FPA	48 hr	+	+++
control	96 hr	+++	+
actinomycin	96 hr	+++	++
p-FPA	96 hr	+	+++
control	144 hr	+++	+
actinomycin	144 hr	+++	+++
p-FPA	144 hr	+	+++
control	336 hr	++?	+
actinomycin	336 hr	+++	+++

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An attempt was made, using glutaraldehyde-OsO_4 fixed tissue, to observe any possible effects of these inhibitors on the formation of cytoplasmic and ER bound polysomes. All ER membranes appeared to be rough. Cytoplasmic polysomes seemed unaffected by actinomycin D for at least 48 hr (Fig. 93). In p-FPA treated tissues, cytoplasmic polysomes were evident throughout the experiment and often seemed to be closely associated with the margins of crystals (Fig. 98).

Since the first two experiments had given conflicting results (Tables V and VI), further observations were made on the numbers of membrane bound internal bodies within the plastids. Actinomycin D again seemed to cause a reduction in the number. But, since it is possible to cut fifty or more sections from a single plastid, many sections must be viewed before conclusions are drawn regarding presence or size of relatively small internal bodies. For this reason, all the observations of changes in plastid contents await further confirmation.

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3. Discussion

Changes occurring in freshly cut slices of storage tissue could be due to either activation of pre-existing enzymes or de novo synthesis of enzyme proteins, as found, for example, in the early stages of germination (Marré et al 1965). It is likely that both processes occur, but several workers have demonstrated that the rate of RNA and protein synthesis increases after slicing.

Click and Hackett (1963) were the first to show that development of enhanced respiration during aging of potato is dependent on the synthesis of new RNA. Setterfield (1963) showed by autoradiographic techniques that a rapid synthesis of RNA occurs in the nuclei of freshly cut artichoke slices. The parallel development of RNA synthesis and invertase activity was shown in disks of carrot by Leaver and Edelman (1965), and in beetroot by Vaughan and MacDonald (1967b), and an increase in fatty acid synthetase activity was shown to be related to a temporary rise in rate of RNA synthesis in potato disks (Willemot and Stumpf 1967). Sampson and Laties (1968) found that a burst of ribosomal RNA synthesis is induced in potato tissue by slicing and this continues for about 12 hours. This ribosomal RNA synthesis is sensitive to puromycin, in contrast to non-ribosomal RNA synthesis, and they conclude that puromycin is only effective during the first 12 hours following slicing.

Cell-free preparations were then investigated. Chapman and

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Edelman (1967) using preparations from artichoke tubers found more active incorporation of amino acid into protein by fractions from aged disks than from freshly cut disks. Maximum activity was obtained at about 24 hours, and this activity was found in two fractions one of which corresponded to the mitochondria and the other to the microsomes.

Ellis and MacDonald (1967) described differences in the ability of microsomal fractions from fresh and aged beet disks to incorporate leucine into protein. They found that microsomes consisting of membranes and ribosomes, from disks aged for 1 or 2 days had a higher incorporating capacity than those from fresh disks. Furthermore, it seemed that the low activity of fractions from fresh disks was attributable to a deficiency in the microsomal fraction itself, rather than a deficiency in the supernatant fraction, which was essential for activity, or to a dissociable inhibitor. Since the RNA content of the microsomal fraction increased during aging, they suggested that the increase in incorporating ability is due to a synthesis of new m-RNA induced by slicing, rather than an activation or increase in availability of pre-existing messenger. Puromycin* and cycloheximide* inhibited microsomal activity. This suggestion was supported in later experiments (Ellis and MacDonald 1968). The high speed supernatant fraction, which is necessary for

* Puromycin is thought to inhibit protein synthesis by prematurely terminating the unfinished polypeptide chain, and cycloheximide by interfering with the transfer reaction in peptide bond formation.

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microsomal activity was found to incorporate leucine into a product with the properties of amino-acyl RNA. Further, addition of poly-U to microsomes from fresh disks stimulated the incorporation of phenylalanine but had no effect on leucine incorporation. Thus low activity of the microsomes appears to be due to lack of m-RNA.

Leaver and Key (1967) also interpreted poly-U-stimulated phenylalanine incorporation by carrot root ribosomes in terms of a shortage of m-RNA. Using sucrose density gradients they found that, during aging of carrot disks single ribosomes were converted to polyribosomes and presumed that this is due to appearance of m-RNA. The work was not accompanied by electron microscopy, and our observations of fine structure in aging carrot disks did not substantiate their findings. But, just as alterations are possible during the isolation of cellular fractions, there may be variation, during fixation, of some factor controlling polysome stability.

In beetroot, cytoplasmic polysomes as well as monomers and membrane bound ribosomes are visible in freshly cut disks, and it seems that neither slicing nor fixation causes disintegration of existing polysomes. With actinomycin D treatment preventing the production of new m-RNA, the number of polysomes is expected to decrease with time. Sarma et al (1969) using sucrose density gradients, found a marked disaggregation of non-membrane bound hepatic polysomes 12 hours after administration of actinomycin to mice. In beetroot, some polysomes were visible up to four days after addition of actinomycin D.

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These experiments using inhibitors of RNA and protein synthesis combined with ultrastructural observations lead to some interesting tentative conclusions. Firstly the production of long ER lamellae seems to be dependent on protein or polypeptide synthesis since it is inhibited by puromycin, cycloheximide and p-FPA. The reassembly is apparently not affected by actinomycin D and hence does not seem to be dependent on the synthesis of new m-RNA afterslicing.

The crystals are probably formed by the continued production of polypeptides in the cisternae of the ER until a concentration is reached which leads to deposits of crystalloid protein bodies. Because actinomycin D was found to promote crystal formation, it seems that the m-RNA required for this particular synthesis is present in the cell together with the other essentials i.e. amino acids, t-RNA, ribosomes etc. If actinomycin D treatment blocks the synthesis of new m-RNA, these essentials may be diverted towards the existing protein synthesising system of the ER-m-RNA-ribosome complex. The continued synthesis of these crystals indicates that this complex must be stable. In the untreated tissue, crystals are found only in aged tissue, which may be interpreted as a further indication of the stability of the ER protein synthesising system.

Such a difference in stability has been observed in liver tissue by Blobel and Potter (1967) who reported that membrane bound polysomes

are more resistant to exogenous RNA-ase than free polysomes. In plants the existence of a long-lived m-RNA is postulated in germinating cotton embryos where actinomycin D does not inhibit amino acid incorporation or cause a loss of polysomes during the first 16 hours of germination (Dure and Waters 1965). The observation suggests that this protein synthesis is directed by m-RNA which exists in the mature seed and which is not rapidly degraded and resynthesised.

Treatment of disks with p-FPA seemed to inhibit the formation of long ER lamellae and yet led to an increased synthesis of crystalloid protein. The effect of p-FPA on protein synthesis is not precisely known, but it is generally thought that it becomes incorporated in the polypeptide chains resulting in the production of abnormal or non-functional protein. At present it seems that this crystal formation can continue for some time without detrimental effects on the general metabolism of the cell, an observation which suggests that these bodies consist of a non-specific, non-functional protein. To put these conclusions on a firmer basis, it may in future be possible to check the incorporation of p-FPA using electron autoradiography, and to isolate the crystals and check for enzyme activity.

Thus, in beetroot tissue, there seem to be two distinct protein synthesising systems. One, the cytoplasmic or non-membrane bound system, may depend on the production of new m-RNA after slicing, and is therefore sensitive to actinomycin D. The other more stable protein synthesising system involving ER-bound ribosomes and m-RNA is in existence at the time of

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slicing and committed to the production of structural or relatively inert protein. Normally, competition between these two systems for some at least of the essential components of proteins might be expected, but when the cytoplasmic system is not operating the membrane-bound system could operate with less restriction.

Free and membrane-bound polysomes in other tissues have been suspected of synthesising different groups of proteins. The synthesis of glycoproteins (Hallinan et al 1968) and serum proteins (Redman 1968) in liver is thought to be carried out exclusively by the ER system. In plants, Opik (1968) and Payne and Boulter (1969) suggested that the membrane-bound ribosomes preferentially synthesised the storage protein in developing seeds while the free polysomes synthesise other proteins.

A remarkably similar observation on crystal formation was reported for crystal containing cells in the cecal epithelium of mice (Wesley and Jensen 1969). Treatment with actinomycin increased both the number of crystal containing cells and the number of crystals within them. As the m-RNA existing in the cells at the time of treatment was shown to be sufficiently stable to permit protein transcription in adjacent secretory cells, it was suggested that the same stability of m-RNA might account for the increased number of crystals.

IV GENERAL DISCUSSION

Recent physiological studies show that changes in storage tissue immediately following slicing are consistent with the concept of activation of genetic material allowing the synthesis of new types of m-RNA. The synthesis of RNA and enzymes may be required for the onset of various metabolic processes. Evidence presented in this thesis indicates that the development of a capacity to accumulate ions may also be interpreted in this way. Concentrations used in these experiments (0.5 mM K^+ or Na^+) achieved either by initial leakage or by adding salt in the case of aged disks, are in the range of the high affinity uptake mechanism (system 1) as defined by Epstein et al (1963). On the basis of evidence presented, it can be concluded that this study concerns the generation of a system 1 mechanism. The cellular location of the two uptake mechanisms is at present controversial. Welch and Epstein (1968) favor the plasma membrane as the site of both systems operating in parallel, while Torii and Laties (1966) believe that system 1 (high affinity) operates at the plasma membrane and system 2 (low affinity) functions at the tonoplast.

Calcium is found to be essential for the maintenance of ion selectivity of both systems (Rains, Schmid and Epstein 1964), and an investigation of the role of Ca^{++} may give some indication of the location of these ion transport systems. The removal of Ca^{++} from beetroot disks

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caused a marked, but reversible, leakage of ions. Ultrastructural studies after Ca^{++} removal revealed apparent enlargement of ER cisternae, but gave no conclusive evidence of the sites of transport. Indirect confirmation of an effect of Ca^{++} at the cell surface was perhaps given by the observation that preservation of the plasma membrane region with OsO_4 was prevented in the presence of tris buffer. Competition between tris and Ca^{++} for a common site has been suggested previously (Van Steveninck 1965b).

Studies of the energetic aspects of ion movement in beetroot disks have led to speculation on the possibility of an ATPase, either alone or in conjunction with an electron transport system, being associated with a membrane such as the tonoplast (Atkinson and Polya 1968) and playing a part in ion transport. Attempts to localise the sites of ATPase activity in beetroot cells failed to give convincing results. But ion transport may be coupled directly to electron transport without intermediate ATP formation, and electron transport systems may prove to be a common feature of other membranes besides those of mitochondria and chloroplasts. The enzymes of electron transport and a number of associated reactions are known to be situated in microsomes isolated from animal cells (Dallner and Ernster 1968). The idea of different regions of a membrane surface having specialised functions fits in with current concepts of membrane organisation in terms of a mosaic of macromolecules. Histochemical methods might then be expected to reveal differences in apparent selectivity of various

parts of a membrane for particular reagents (Mayo and Cocking 1969). In ion transport the selective attachment of ions to specialised areas of the plasma membrane may precede the formation of minute vesicles which penetrate the cytoplasm carrying the ions inwards. MacRobbie (1969) has suggested such a mechanism to explain her results. Her hypothesis involves the entry of salt by formation of pinocytotic vesicles, fusion of these vesicles with the ER, and the further production of minivacuoles for transfer to the vacuole.

A dramatic change in the length of ER lamellae was the most important observation in aging beet disks. This change may be the result of either de novo synthesis of the membrane components or simply the reassembly of subunits already existing in the cytoplasm. The components of the ER in plant cells are relatively unknown. Phospholipids are constituents of all membranes, and in animal microsomes the relatively large phospholipid content controls the membrane conformation and may regulate permeability. The ER is known to be the site of synthesis of phospholipids which may then be transferred to other cellular membranes or perhaps be incorporated in the ER itself. The presence of two groups of phospholipids, possibly representing metabolically active and inert membrane components, is suggested by half life studies (Dallner and Ernster 1968). Neutral lipids such as cholesterol are also present in most membranes indicating some fundamental function, possibly concerned with either

membrane structure and the stabilisation of molecular arrangements or an effect on the surface negative charge. The existence in membranes of purely structural proteins as distinct from enzymes has not been shown conclusively. Recent work indicates that the protein component of ER membranes may be synthesised by the ER itself (Campbell and Lawford 1968). The small component of RNA which is associated with the membranes of microsomal fractions is presumed to be bound to the ER. This RNA is different from both ribosomal and cytoplasmic RNA and it is now generally accepted that m-RNA is associated with membranes of the rough ER.

In connection with the question of de novo synthesis of membrane components, it is interesting to note that inhibitors of protein synthesis were found to delay the appearance of long ER lamellae in aging beetroot disks. This observation suggests that the protein components may be synthesised de novo during this period and may be essential for the assembly of the membrane. Phospholipid synthesis is apparently regulated by the amount of protein available and the lipid composition may be dependent on the peptides present through their specific interactions. Also the tertiary structure of the membrane protein may be determined by the phospholipids and attained only after combination with the lipids (Wallach and Gordon 1968).

Whether the alteration in the ER is induced directly by slicing and washing, or indirectly by the effect of changes in other cellular components brought about by the treatment, the change must

inevitably modify its protein synthesising function. The type of protein synthesised by the ER and the actual mechanism of synthesis is, for the most part, not accurately known. In the case of glycoprotein synthesis in liver cells, the polypeptides are transferred from the ribosomes into the endoplasmic cisternae and the synthesis is completed during passage through the rough and smooth ER (Redman and Sabatini 1966). But there is no real evidence that proteins are not produced on either side of the membrane. An argument in favor of this hypothesis is the observation that the ER membrane, unlike the plasma membrane for example, is not asymmetrical (Wallach et al 1966).

Because of the large number of cistrons coding for ribosomal RNA in higher organisms, a heterogeneous population of ribosomes probably exists in the cytoplasm. The specificity of attachment of these ribosomes to m-RNA is not understood, but evidence is accumulating that free cytoplasmic polysomes and ER bound polysomes are responsible for the synthesis of different proteins. The possibility also exists that the ER, for example, synthesises several classes of proteins either in adjacent areas or over widely separated parts of the membrane surface.

The function of proteins synthesised by the ER in plant cells, has not been clearly established. Previous reports suggest either the production of storage protein to be 'secreted' into the vacuole, as in maturing cotyledons (Bain and Mercer 1966; Opik 1968) or alternatively the production of hydrolytic enzymes (Schnepf 1963; Opik 1966). Fragments of ER carrying hydrolases were also isolated from corn seedlings

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(Semadeni 1967). Moreover, some functional relationship between the ER and plant microbodies seems likely and again most of the enzymes localised in these organelles are hydrolases (Frederick et al 1968). These observations suggest that the ER may synthesise enzymes which when released bring about breakdown of cytoplasmic components or perhaps even parts of the cell wall. Further, the enzymes produced may vary during cellular differentiation or senescence. Thus the production of crystals within the ER cisternae in aged beetroot disks may be the result of a developmental change in ER protein synthesis. This work has shown the ER-m-RNA-ribosome protein synthesising system to be stable, but the actual half-lives of the components are unknown. Further incorporation of m-RNA into the membrane would be necessary to account for such developmental changes, and if ER sub-units are incorporated into the membrane by intussusception, then this m-RNA could be introduced at the same time.

Future work rests on the development of two techniques. Firstly, electron autoradiography could provide information regarding the origin of sub-units, both protein and lipid, of the ER membrane, and also the mode of incorporation of these sub-units. The same technique might provide further information about the constituents of the crystals. Secondly, the study of the ion transport systems in beetroot at the electron microscopy level would be greatly assisted by the development of techniques for intracellular localisation of ions. The latter method has already shown promising results.

Appendix I

(a) Buffers and fixatives

Veronal acetate buffer

2.94 g sodium barbitone

+ 1.94 g sodium acetate ($3H_2O$) dissolved in 100 ml water.

0.2M cacodylate buffer

10.701 g sodium cacodylate

+ 50 ml 0.1 N HCl; dilute to 250 ml.

1% OsO_4 in veronal acetate buffer with added sucrose.

4 ml 2% OsO_4 solution

+ 1 ml veronal acetate

+ 1 ml 0.1 N HCl

+ 2 ml water; dissolve 1.10 g sucrose (0.4 M).

1% OsO_4 + 1.25% $K_2Cr_2O_7$ + 0.25 M sucrose

4 ml 2% OsO_4

+ 2 ml M sucrose

+ 2 ml 5% $K_2Cr_2O_7$ solution containing 0.3 ml 2.5 N KOH.

2% OsO_4 in 0.1 M cacodylate buffer

0.1 g OsO_4 dissolved in 2.5 ml 0.2 M cacodylate buffer

+ 2.5 ml water

2% OsO_4 in 0.05 M phosphate buffer

0.1 g OsO_4 dissolved in 2.5 ml 0.2 M NaH_2PO_4 + 2.5 ml 0.2 M Na_2HPO_4

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4% formaldehyde in veronal acetate buffer with sucrose

0.4 g paraformaldehyde heated in 2 ml veronal acetate + water;

+ 2.2 ml 0.1 N HCl; dilute to 10 ml;

+ 1.37 g sucrose (0.4 M)

4% formaldehyde in phosphate buffer with sucrose

0.4 g paraformaldehyde dissolved in

1.7 ml 2.52% NaOH at about 60°C

+ 8.3 ml 2.26% NaH_2PO_4

+ 1.37 g sucrose (0.4 M)

5% glutaraldehyde in veronal acetate buffer with sucrose

8 ml 25% glutaraldehyde

+ 5 ml veronal acetate

+ 5 ml 0.1 N HCl

+ 22 ml water

+ 5.5 g sucrose (0.4 M)

5% glutaraldehyde in 0.05 M phosphate buffer

5 ml 25% glutaraldehyde

+ 6.25 ml 0.2 M phosphate buffer; dilute to 25 ml

5% glutaraldehyde in phosphate buffer with sucrose

5 ml 25% glutaraldehyde

+ 8.3 ml 2.26% NaH_2PO_4

+ 1.7 ml 2.52% NaOH

+ 2.74 g sucrose (0.4 M); dilute to 20 ml.

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4% glutaraldehyde in 0.1 M cacodylate buffer

8 ml 25% glutaraldehyde

+ 25 ml 0.2 M cacodylate buffer; dilute to 50 ml.

2% $KMnO_4$ (or $Ca(MnO_4)_2$ in veronal acetate buffer

1 g $KMnO_4$

+ 10 ml veronal acetate

+ 11 ml 0.1 N HCl; dissolved and made up to 50 ml.

2% $NaMnO_4$ in veronal acetate

2 g $NaMnO_4$

+ 20 ml veronal acetate

+ 0.1 N HCl to give pH 7.2 (approx. 13 ml); dilute to 100 ml.

Embedments

Araldite mixture

10 ml casting resin M

10 ml hardener 964 B

0.4 ml accelerator 964C

1.0 ml dibutyl phthalate (later changed to 0.6 ml)

In measuring the components of resin mixtures volumetrically care was taken that the highly viscous materials did not come in contact with the upper wall of the measuring cylinder used. Accelerator was measured with a pipette allowing time for drainage and then using the same pipette for the dibutyl phthalate. Accelerator must be uniformly distributed to prevent local variations in hardness of the block. Hence the components were mixed in a graduated cylinder by pumping a glass plunger, almost fitting the internal diameter of the

cylinder, up and down vertically for 10-15 min.

Epon

Mixture A: 62 ml Epon 812

+ 100 ml DDSA (dodecenyl succinic anhydride)

Mixture B: 100 ml Epon 812

+ 89 ml MNA (methyl nadic anhydride)

Just before use, equal amounts of A and B were mixed and the accelerator (DMP30) added in the proportion of 1.5 - 2%.

(c) Reynolds lead citrate stain

1.33 g $Pb(NO_3)_2$

+ 1.76 g sodium citrate; dissolve in 30 ml water, shake 1 min and allow to stand for 30 min. Add 10 ml N NaOH and make up to 50 ml.

Appendix II Survey of methods used for histochemical localisation of ATPase activity using electron microscopy

Author and Material	Preliminary fixation	Incubation	Post-fixation	Control treatments
Essner et al 1958 - rat liver	Thin slices cut in cold, buffered 1% OsO_4 (pH 7.2); 7 min; several rinses in veronal acetate buffer	Wachstein-Meisel (1957) medium; 37°C; 15-30 min; wash in cold veronal-acetate	Cold 1.6% formaldehyde in 0.28 M veronal-acetate; overnight	(a) cold, buffered 1% OsO_4, 15 min; no incubation (b) ATP replaced by 5-adenylic acid (30 min incubation
Kaplan and Novikoff 1959 - rat kidney	Tissue; overnight in cold formol-calcium	25μ frozen sections in Wachstein-Meisel medium; 3 min at 37°C or 1½-2 hr at 2°C	brief, buffered OsO_4	
Persijn et al 1961 - mouse liver and kidney	1 mm^3 blocks, cold 1% OsO_4 in 0.05M tris-HCl (pH 7.3) + 10% sucrose, 2-3 min	0.05M tris-HCl (pH 7.3) 1.3 x 10^{-3}M ATP 0.18M potassium sodium tartrate 1 x 10^{-3}M $MgCl_2$ 5 x 10^{-3}M $Pb(NO_3)_2$ pH 6.8 + 6% sucrose	cold OsO_4 in veronal acetate + 0.22M sucrose; 1 hr	ATP omitted or replaced by phenyl phosphate
Lansing and Lamy 1961 - rotifer cilia	cold, 1% OsO_4, 6 min	as Essner et al 1958	as Essner et al 1958	(a) ATP replaced by adenylic acid (b) 1% cold OsO_4, no incubation
Tice and Barrnett 1962 - rat heart muscle myofibrils	0.5 mm^3 muscle blocks unfixed, isolated myofibrils fixed in cold 6.5% hydroxy-adipaldehyde in 0.05M cacodylate (pH 7.2) + 0.4M sucrose; 1 hr; wash in cacodylate buffer	0.0025M ATP + 0.04M $CaCl_2$ or 0.05M $MgCl_2$ + 0.005M $Pb(NO_3)_2$ pH 6.5; (after filtering, 3-4 x 10^{-4}M ATP) 30-40 min	1% buffered OsO_4 + sucrose 4°C, 1-2 hr	(a) ATP replaced by AMP, ADP, IDP, ITP, β-glycerophosphate, thiamine pyrophosphate (b) pre-incubation in 0.1M N-ethyl-maleimide, (1 hr, pH 7.4) or 10-3M Cu^{+++}
De Beyer et al 1962 - mouse heart muscle	1 mm^3 tissue blocks, 1% OsO_4 in 0.05M tris-HCl (pH 7.3) + sucrose; 4°C; 3-4 min	0.05M tris HCl (pH 7.3) 1.3 x 10^{-3}M ATP 0.18M potassium sodium tartrate 10^{-3}M $MgCl_2$ 5 x 10^{-3}M lead nitrate pH 6.8; room temp., 20-30 min	buffered OsO_4 + 4% sucrose pH 7.2; 4°C; 1 hr	(a) ATP replaced by AMP or monophenyl-phosphate (b) 0.003M PCMB

Author and material	Preliminary fixation	Incubation	Post-fixation	Control treatments
Ashworth et al 1963 - rat small intestine, kidney and liver	25μ cryostat sections, 10% buffered formalin + 4.9% sucrose (pH 7.2); 4°C; 30-90 min	Wachstein-Meisel, 15 min	veronal acetate buffered 1% OsO_4 + 7.5% sucrose (pH 7.2)	ATP omitted
Sabatini et al 1963 - rat liver etc.	1 mm^3 blocks; 4-6.5% glutaraldehyde in 0.1M phosphate or cacodylate (pH 7.2), ½-2 hr; 4% formaldehyde in 0.1M buffer + 0.22M sucrose (pH 7.4), 2-4 hr; stored in buffered 0.2M sucrose at 4°C	Wachstein-Meisel medium 30-60 min	1-2% buffered OsO_4 + sucrose, 2-4 hr	ATP replaced by glycerophosphate
Marchesi and Barrnett 1963 - blood capillaries of rat heart	1-2 mm^3 blocks; or 12% hydroxyadipaldehyde 6.5% glutaraldehyde in 0.1M cacodylate (pH 7.4); 4°C; 2 hr. 4% formalin in 0.06M phosphate + 7.5% sucrose (pH 7.5); 4°C; 10 hr; rinsed in buffered sucrose	Wachstein-Meisel medium; at room temp. or at 10°C; 15-90 min; brief wash	Cold buffered OsO_4-sucrose, 2 hr.	(a) pre-fixation in 1% buffered OsO_4 + sucrose 10 min (b) ATP replaced by ADP, AMP, β-glycerophosphate, phenyl-phosphate
Epstein and Holt 1963 - Hela cells	cell suspension, 5% glutaraldehyde in 0.067M cacodylate (pH 7.2), 0°C, 30 min; rinsed twice 2-3 min in 0.25M sucrose in 0.1M cacodylate	Wachstein-Meisel modified (Pearse, 1961), room temp.; 10 min; washed twice 2-3 min in tris buffered sucrose	phosphate buffered OsO_4-glucose, few min.	
Otero-Vilardebo et al 1963 - colonic epithelial cells	5 mm^2, 4% formalin in 0.05M cacodylate + 1% $CaCl_2$ ± 10% sucrose (pH 7.4-7.6); 4°C, 16 hr; rinsed 6 times (2 hr) in 10% sucrose in 0.05M cacodylate, 4°C	cryostat sections 30-40μ; 8.3 x 10^{-4}M ATP; + 0.08M tris-maleate; + 2.5 x 10^{-3}M $Pb(NO_3)_2$ + 0.05M $MgCl_2$ filtered, pH 7.3; 37°C; 5-90 min	2% 0.1M phosphate buffered OsO_4; 4°C; 45-60 min	(a) ATP omitted (b) 10^{-3}M N-ethyl maleimide in medium

Author and material	Preliminary fixation	Incubation	Post-fixation	Control treatments
Nagano 1965 rat sperm tail	$3mm^3$ blocks, cold 6% glutaraldehyde; in 0.1M cacodylate (pH 7.2) 1-2 hr; washed	50μ frozen sections; Wachstein-Meisel, room temp., 5-60 min; washed	cold 2% OsO_4 in s-collidine, 1 hr	(a) ATP replaced by AMP creatinine phosphate, β-glycerophosphate, phenyl phosphate (b) 5×10^{-4}M PCMB (c) fixation 2% OsO_4, 1 hr
Tice and Smith 1965 - blowfly flight muscle myofibrils	6.5% hydroxy-adipaldehyde (pH 6.8) in 0.05M cacodylate + 0.4 M sucrose, 4°C, 30 min; washed cacodylate-sucrose, muscles dissected out	0.002M ATP + 0.04M $CaCl_2$ or 0.05M $MgCl_2$; + 0.005M Pb $(NO_3)_2$ (pH 6.5) 30 min	1% OsO_4 in veronal-acetate, 4°C	(a) ATP replaced by ADP, ITP, IDP, thiamine pyrophosphate (b) pre-incubation in 0.1M N-ethyl maleimide (pH 7.2) 30 min.
Gauthier and Padykula, 1965 - gas bladder muscle of toad fish	Narrow strips of muscle; 5% glutaraldehyde in cacodylate; 60 min	frozen sections 40-80μ Wachstein-Meisel (1957) + cystein, 37°C 30 min	2% OsO_4	ATP replaced by ADP, AMP or glycerophosphate
Farquar and Palade 1966 - amphibian epidermis	strips 1 x 5 cm 1.5% glutaraldehyde in 0.067M cacodylate, 4°, 2-4 hr or Bakers formol-calcium, 16-24 hr; washed and stored in 7% sucrose in 0.1M cacodylate	50μ sections (not frozen) modified Wachstein-Meisel containing Pb $(NO_3)_2$ concentration reduced to 2.4 mM or with adjusted cation content; 25° or 37°; 10-90 min; rinsed in 7% sucrose in veronal acetate buffer	1% OsO_4 in veronal acetate or 0.1M phosphate 45 min	(a) ATP replaced by ITP, ADP, AMP or GP (b) ouabain
Yasuzumi and Tsubo 1966 - mouse choroid plexus epithelial cells	6.25% glutaraldehyde in 0.075M cacodylate + 0.33M sucrose, 4°C, 1 hr. Washed 1 hr cold buffered sucrose	Wachstein-Meisel; 37°C, 15-30 min.	1% OsO_4 in veronal acetate (pH 7.2), 1 hr	(a) incubated in absence of Pb (NO_3); subsequently immersed in 2% Pb $(NO_3)_2$ 37°C, 10 min (b) 5×10^{-4}M PCMB

Appendix III

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CHANGES IN THE ENDOPLASMIC RETICULUM OF BEETROOT SLICES DURING AGING

By Margaret E. Jackman* and R. F. M. Van Steveninck*

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Summary

Ultrastructural changes occurring in beetroot parenchyma were studied from the time of cutting into disks and throughout the following 192 hr of aerated washing. The most marked change was the reduction of the endoplasmic reticulum to small cytoplasmic vesicles immediately after cutting (when leakage of ions is greatest), followed by a reorganization into lamellae (coinciding with the onset of net ion accumulation) and subsequent extension of the lamellar system. The possible relationships between these observations and others on plant cells are discussed.

I. Introduction

Freshly cut slices of beetroot, like most other storage tissues, lack the ability to accumulate salts from dilute solutions before the completion of an initial period of aerobic washing (Stiles and Dent 1946). Thereafter, a marked increase in the capacity to both absorb and retain salts occurs. It is generally assumed that this sequence of events is related ultimately to the coincident changes in respiratory metabolism, and more particularly in oxidative phosphorylation on which many cellular activities depend directly or indirectly (Robertson 1960).

The ultrastructure of parenchyma cells of beetroot slices during aging was investigated to detect possible changes in number, form, or distribution of organelles which might be related to the progressive increase in salt uptake capacity.

II. Materials and Methods

(*a*) *Ion Uptake*

From cylinders of beetroot (*Beta vulgaris* L.) 15 mm in diameter, disks 1 mm thick (average fresh weight 190 mg) were cut, and rinsed for 2 hr in aerated distilled water with three changes. Batches of 50 disks (three replicates) were placed in 250 ml distilled water and aerated at 24°C. Samples of the solutions were taken at intervals during the following 192 hr, and K^+ and Na^+ concentrations determined using an EEL flame-photometer.

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(b) Electron Microscopy

Samples of two disks were withdrawn after the preliminary washing period, after a further 2 hr aerated washing, and after 26, 48, 96, and 192 hr. Another sample was taken from an intact beetroot and fixed immediately. In the development of the fleshy hypocotyl root, additional cambia arise outside the normal primary and secondary vascular tissues, and these produce several concentric rings of parenchymatous xylem and phloem separated by bands of large thin-walled parenchyma (Esau 1953). In this work, care was taken to avoid, as far as possible, the tissue containing vascular elements. Strips of parenchyma 2–3 mm wide were cut from the disks, quickly cut into three strips, chopped into blocks less than 1 mm^3, and dropped into cold fixative. As difficulties were expected in achieving satisfactory fixation of these large vacuolate cells, the following fixatives were tried in preliminary tests:

(1) 2% $KMnO_4$ in veronal acetate buffer, pH 7·3, for 45 min at 0–2°C.

(2) 2% $NaMnO_4$ in veronal acetate buffer, pH 7·2, for 45 min at 0–2°C.

(3) 2% $Ca(MnO_4)_2$ in veronal acetate buffer (Afzelius 1962) for 45 min at 0–2°C.

(4) 1% OsO_4 in veronal acetate buffer and 0·4M sucrose, pH 7·3 (Caulfield 1957, modified), for 2 hr at 0–2°C.

(5) 1% OsO_4 and 1·25% $K_2Cr_2O_7$ in 0·25M sucrose, pH 7·0, adjusted with KOH (Kollman 1960) for 2 hr at 0–2°C.

(6) 4% formaldehyde in sodium phosphate buffer and 0·4M sucrose, pH 7·3, for 2 hr at 0–4°C followed by 1% OsO_4 in veronal acetate and 0·4M sucrose, pH 7·2, at 4°C (Holt and Hicks 1961).

(7) 5% glutaraldehyde in veronal acetate buffer and 0·4M sucrose, pH 7·2, for 30 min at 4°C followed by 1% OsO_4 in veronal acetate for 30 min at 4°C.

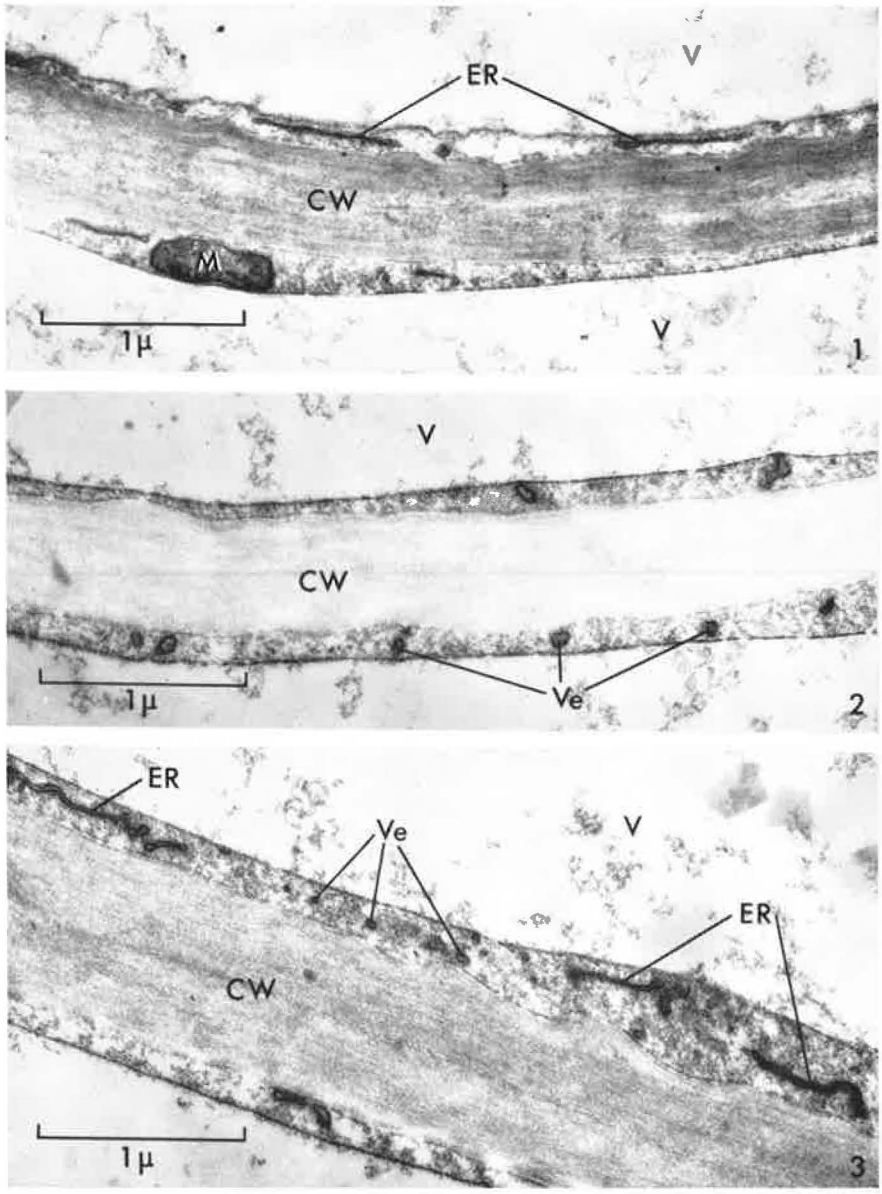
(8) 5% glutaraldehyde in sodium phosphate buffer and 0·4M sucrose, pH 7·2, for 30 min at 4°C followed by 1% OsO_4 in veronal acetate for 30 min at 4°C (Sabatini, Bensch, and Barrnett 1963).

In comparison with the other fixatives, permanganate fixation resulted in a more even precipitation of the cytoplasm, more complete preservation of the plasma membrane and tonoplast, and, in general, a smoother appearance of cell membranes. There was no appreciable difference in fixation with either potassium, sodium, or calcium permanganate. Hence, tissue blocks were fixed in 2% $KMnO_4$ buffered with veronal acetate for 45 min at 0–2°C, and, as a check, in 1% OsO_4 in veronal acetate with 0·4M sucrose for 2 hr at 0–2°C. After fixation, the blocks were rinsed twice with distilled water, dehydrated in acetone (5 min in 25% and 50%, 20 min or overnight in 75%, then 30 min in 100% with three changes), and embedded in Araldite. Thin sections cut with a Si-Ro-Flex ultramicrotome were stained in lead citrate (Reynolds 1963) for 20 min if permanganate-fixed, or in saturated 96% ethanolic uranyl acetate for 1 hr if fixed in OsO_4, and examined in a Siemens Elmiskop I electron microscope.

III. Results

The pattern of net Na^+ and K^+ movement between beetroot slices and the external solution is shown in Figure 1. Rapid leakage of Na^+ and K^+ from the tissue

CHANGES IN ENDOPLASMIC RETICULUM OF BEETROOT SLICES



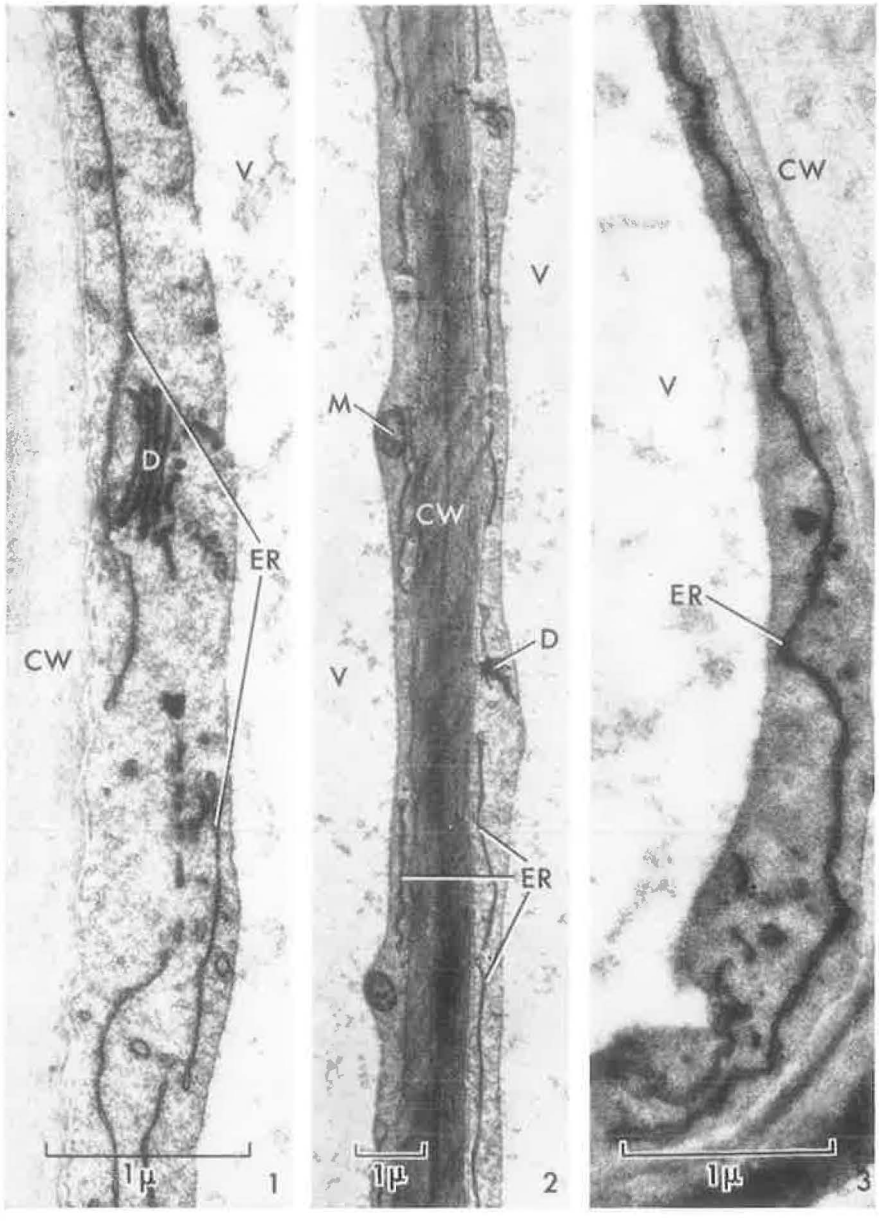
All figures in Plates 1 and 2 are electron micrographs of beetroot tissue which was fixed in buffered 2% $KMnO_4$, and embedded in Araldite. Sections were stained with lead citrate. Abbreviations used on the plates are as follows: *CW*, cell wall; *D*, dictyosome; *ER*, endoplasmic reticulum; *M*, mitochondria; *V*, vacuole; *Ve*, vesicle.

Fig. 1.—Parenchyma cells from intact beetroot cut at the time of fixation. The amount of endoplasmic reticulum (*ER*) usually observed in whole tissue is shown.

Fig. 2.—Cells from beetroot disks washed for 2 hr prior to fixation. The lamellar endoplasmic reticulum (*ER*) has almost disappeared, but vesicles (*Ve*) are present in the cytoplasm.

Fig. 3.—Cells from beetroot disks washed for 26 hr prior to fixation. After 26 hr the lamellar endoplasmic reticulum (*ER*) has partially reappeared, while some vesicles (*Ve*) persist.

CHANGES IN ENDOPLASMIC RETICULUM OF BEETROOT SLICES



Figs. 1–3.—Cells from beetroot disks washed for 48, 96, and 192 hr respectively prior to fixation. The lamellae of the endoplasmic reticulum (*ER*) have increased in length by 48 hr and are generally longer than those found in the intact beetroot. After 48 hr the lamellae show further extension, and now form an almost continuous layer within the cytoplasm, which persists until at least 192 hr.

was followed by a phase of rapid net accumulation of Na^+ at 20 hr, while a phase of slow net accumulation of K^+ at 10 hr was followed by a rapid phase of uptake at about 50 hr. This pattern of ion movement is typical for beetroot slices (Van Steveninck 1961).

The most marked ultrastructural change in the parenchyma cells during this period of aerated washing was shown by the endoplasmic reticulum, readily observed in $KMnO_4$-fixed tissue, but also evident when fixed with OsO_4. The lamellar endoplasmic reticulum present in whole tissue (Plate 1, Fig. 1) was reduced to a number of small cytoplasmic vesicles during the cutting and preliminary washing period (Plate 1, Fig. 2). On further washing, the lamellae were reconstituted to the original extent by 26 hr (Plate 1, Fig. 3) and thereafter continued to increase in amount until 96 hr (Plate 2, Figs. 1 and 2). By this time an almost complete cell lining of endoplasmic reticulum was present, which persisted until at least 192 hr (Plate 2, Fig. 3). These changes in the extent of endoplasmic reticulum during the process of aging of beetroot disks were confirmed in several separate experiments.

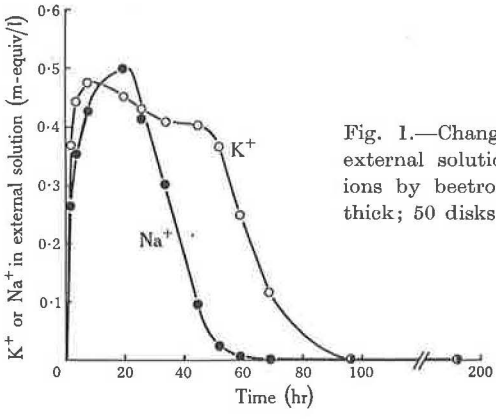


Fig. 1.—Changes in Na^+ and K^+ content of external solution due to release or uptake of ions by beetroot disks (15 mm diam.; 1 mm thick; 50 disks per 250 ml water; 24°C).

After 96 hr, and rarely after 48 hr, occasional crystal-shaped bodies were observed inside the endoplasmic reticulum. Further work on the nature of these crystals is in progress.

It should be noted that the actual slicing and washing of the tissue might alter the cellular response to fixation. The apparent absence of the lamellar endoplasmic reticulum in freshly sliced tissue then could be regarded as a secondary effect due to a lack of preservation of detail at the moment of fixation. However, no significant changes in the appearance of other organelles were found, indicating that the various treatments had not changed their response to fixation.

Observations of changes in number or nature of other cell organelles or membranes during aging were inconclusive. It is possible that some changes in size and shape of mitochondria occurred, since large irregularly shaped mitochondria were found only in the whole tissue, whereas mitochondria in parenchyma of aged disks were small and circular in section, or almost so.

IV. Discussion

It has been shown (Van Steveninck 1962) that the net accumulation of K^+ by beetroot at the completion of the lag phase is due to a decrease in apparent efflux rather than an increased influx. This observation suggests that some barrier to leakage develops during the lag phase. The highest rate of efflux, measured within 4 hr after slicing the tissue, coincides with the observed reduction of endoplasmic reticulum to small cytoplasmic vesicles, but the endoplasmic reticulum forms an almost continuous lamellar system when the efflux is greatly reduced after 48 hr. It seems likely that this extensive endoplasmic reticulum in aged beetroot parenchyma would act as a barrier to ion movement in addition to the selective resistances of the tonoplast and plasma membrane.

Numerous authors have reported marked changes in the amount of endoplasmic reticulum in plant cells. These changes can be classified into two groups:

(1) Changes caused by external factors, e.g. wilting (Schnepf 1961), mechanical injury (Mollenhauer, Whaley, and Leech 1960), increased pressure, oxygen deficiency, cyanide, high carbon dioxide or carbon monoxide tension, colchicine, and high intensity radiation (for references see Whaley, Kephart, and Mollenhauer 1964). Such treatments usually induce an almost instantaneous build-up of lamellar and often coiled reticulum. Rapid, and in some cases reversible, changes of this kind suggest the transformation of molecular or larger units already present in the cytoplasm into an organized lamellar system. This concept has been elaborated by Green and Perdue (1966) on the basis of the spontaneous reconstitution of dispersed membrane units observed by Razin, Morowitz, and Terry (1965) and Brown (1965).

In contrast to the apparent increase in the amount of endoplasmic reticulum caused by mechanical injury, increased pressure, or wilting, the cutting and initial washing of beetroot disks produced a breakdown of lamellae into small vesicles. It is possible that the elements of the lamellar membrane were still present in the cytoplasm, but the reconstitution was not instantaneous, and required several hours.

(2) Changes occurring during normal development and aging (Whaley, Mollenhauer, and Kephart 1962; Juniper 1964; Bouck and Cronshaw 1965; Bain and Mercer 1966*a*, 1966*b*). Some of these have been associated with a specific functional state of the cells, e.g. the increases observed in maize root cells at the beginning of vacuolation (Whaley, Mollenhauer, and Kephart 1962), and in pea cotyledons during germination when the tissue is temporarily accumulating soluble carbohydrate (Bain and Mercer 1966*b*). The reappearance and development of an extensive lamellar endoplasmic reticulum during aging of beetroot may be similar in nature.

Differentiation and aging may also be accompanied by morphological modifications of the endoplasmic reticulum, usually from flat cisternae or tubules to discrete vesicles, e.g. in embryonic leaf primordia of maturing seeds (Nougarede 1963), maturing and aging cotyledons (Bain and Mercer 1966*a*, 1966*b*), differentiating sieve elements (Kollmann and Schumacher 1962), and tracheids (Esau, Cheadle, and Risley 1963), root calyptra cells (Hršel 1966), and senescing leaves (Shaw and

Manocha 1965). Morphological changes of this sort are sometimes considered to be an indication of degeneration of the protoplast (Esau 1963). The vesicles apparently arising from the endoplasmic reticulum during cutting and preliminary washing of the beetroot tissue may be equivalent to spherosomes. Spherosomes are considered to originate from the ends of the endoplasmic reticulum in certain tissues (Frey-Wyssling, Grieshaber, and Mühlethaler 1963) and, when isolated, have been shown to contain hydrolases, which are thought to be responsible for the breakdown of cytoplasm in mature detached tobacco leaves (Balz 1966). Furthermore, the production of hydrolytic enzymes has been ascribed to the endoplasmic reticulum in other plant tissues (Schnepf 1963; Öpik 1966).

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