

AN ELECTRON MICROSCOPICAL EXAMINATION OF THE  
INFLUENCE OF LOW TEMPERATURE AND CHEMICAL INHIBITORS ON  
PHLOEM STRUCTURE, WITH SUPPORTING PHYSIOLOGICAL WORK

CYNTHIA HOGBen

Department of Botany

Bedford College

University of London

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## ABSTRACT

After a general introduction to previous work in this field of research the thesis divides naturally into two parts.

Sieve elements are notoriously difficult to prepare for ultrastructural examination and considerable time was spent in somewhat novel attempts to overcome the problems of turgor pressure release which have bedevilled phloem microscopists for many years. The methods tried are described and evaluated. Working on the principle that artefacts of preparation are less likely to mislead where several methods are employed, the ultrastructure of the normal sieve elements was thoroughly investigated, and it is hoped, established. One novel method which emerged was a procedure designed to facilitate correlation of micrographs of thin sections made by transmission electron microscopy with others taken in the scanning electron microscope. This proved especially useful for identification of structures, such as sieve plates, presenting an unfamiliar appearance under the scanning microscope. This method appears worthy of further application.

Having thus established the normal appearance of sieve elements from the stolons the second part of the programme was based on a firm foundation. Administration of inhibitory treatments was carried out as simply as possible, and emphasis was laid on physiological confirmation that active translocation was proceeding normally before the treatment, and more slowly (generally) subsequently.

Electron microscopic examination showed that certain differences of ultrastructure were present in the variously inhibited materials. These differences are described. However, it seemed unlikely that the features found were responsible for the inhibition observed.

There is obviously a continuing field for further investigation into the reasons for the effect of inhibitors on phloem transport.

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## GENERAL INTRODUCTION

### A. PREFACE

For about 140 years, since Hartig's first description of phloem tissue in 1837 started a detailed interest in the problems of translocation two important questions have remained unsettled. The first concerns the in vivo structure of the sieve elements, and the second the motive power of the assimilate stream. Answers to one of these questions would contribute considerably towards solving the other.

Any investigation into the structure of the sieve elements requires that the interval pressure be discharged in some way. This is liable to be a traumatic operation and controversy has raged for years as to whether sieve tube contents, as seen in microscopic sections, around the sieve plate are natural, or whether they are the result of displacement during pressure release.

This thesis presents a study of the fine structure of sieve elements after the axis has been treated with inhibitors of translocation. In an effort to achieve a minimum disturbance of the structural contents of the sieve elements much work has been done to find the best method of preparation. Many methods of fixation and further preparation have been tried and either rejected, or incorporated into the technique finally used.

## B. THE STRUCTURE OF THE ANATOMICALLY MATURE SIEVE TUBE

A sieve tube which has reached anatomical maturity must be defined as one which has completed obvious differentiation, and which is apparently undergoing no further constructive ultrastructural changes. At this stage many organelles which were present in immature sieve elements have disappeared and others have been modified.

It is generally agreed that a thin layer of parietal cytoplasm, bounded by a plasmalemma lines the sieve tube. This contains endoplasmic reticulum which is no longer associated with ribosomes as in the immature tube. It appears to occur in various forms. It has frequently been observed in orderly stacks or cisternae, but systems of tubules, frequently convoluted, have also been recorded. The question of the extent of the endoplasmic reticulum is controversial, and may be variable according to the plant species studied. It has been seen passing through the sieve plate pores, and Deshpande (1974) noted that it appeared to penetrate the plasmodesmata of developing sieve pores of Saxifraga sarmentosa (L).

Mitochondria have been frequently reported as inclusions of mature sieve elements, found in the parietal cytoplasm close to the plasmalemma. Some are reported to have normal appearance, but they have also been observed to present a more rounded appearance than those normally seen (Johnson, 1969, and Turgeon, Webb and Evert, 1975), and have inflated cisternae. Many reports of sieve element structure include no mention of mitochondria and there must be speculation as to whether they are absent, damaged beyond recognition, or merely outside the authors context at the time. Certainly the mature sieve element of Saxifraga sarmentosa (L) appears to contain few mitochondria but this will be considered in more detail in a later chapter.

Many workers report the disappearance of the nucleus during the development of the sieve tube and mature sieve elements are generally considered to be enucleate. Dictyosomes, ribosomes and microtubules are normally absent, as is a tonoplast.

Very early in the study of phloem the presence of a proteinaceous substance, formally referred to as 'slime', but since about 1967 called phloem-protein, abbreviated universally to P-protein, has been recognised as a characteristic component of sieve elements, particularly in dicotyledons. Many forms of P-protein have been found broadly fitting into four morphological categories viz: granular, tubular, fibullar and crystalline. Deshpande (1974) studying Saxifraga sarmentosa (L) found the tubular form present during the late stages of maturation, but it is apparently the fibullar form that is found in the mature element, in common with most other dicotyledons species.

The aspect of P-protein most in dispute concerns not its occurrence, but its distribution within the lumen of the mature sieve element.

Callose is universally found lining the pores of the sieve plates, certainly at some stage in their life history, but there is much controversy regarding how much, if any lines the pores of the mature sieve plate pores, particularly if the sieve tubes concerned are considered to be functional when prepared for examination. Deshpande (1974), describing the differentiation of sieve plate pores in Saxifraga sarmentosa (L) states that the removal of the callose laid down early in differentiation appears to be an essential process of pore formation in this species. She found pores in mature sieve elements both having callose linings and without. Her speculation was concerned with whether this callose was residual, or whether it had been freshly laid down as a wound response.



Eschrich (1965) and Engleman (1965 a) certainly suggest that wound callose is laid down very rapidly. 30 sec - 5 min has been suggested, depending on the distance of the plate from the wound. It has therefore been postulated that callose may be laid down during preparation for ultrastructural examination.

It is generally agreed that plastids occur in the parietal cytoplasm of mature sieve elements. The state of these plastids, together with the quantity of callose present, and the distribution of P-protein within the sieve element gives much information concerning the care taken during the preparation of the tissue for electron microscopy, and this whole question has become very controversial. Many methods have been tried in order to avoid surge effects, and consequently movement of structure within the sieve element during their preparation, and this quest has inevitably linked with a search for rapid fixation methods. The importance of this was realised as early as 1885 when Fischer plunged Cucurbita tissue into boiling water in the hope that the sieve element contents would be stabilised in their in vivo position. Since then numerous other methods of rapid or especially careful fixation have been employed, usually when some particular aspect of sieve element structure was under investigation.

Frequently the plastid membrane appears ruptured in electron micrographs, with the inclusions scattered in the cell lumen. This state is believed to be due to osmotic shock during fixation and/or the embedding procedure. This view is taken by Tamulevich and Evert (1966) who state categorically "in well fixed elements, the outer membranes of plastids are almost always intact and the starch granules contained within the plastids". Evert (1977) suggests that the condition of the plastids is "a valuable indicator of disturbance in sieve tube members". Certainly very careful preparation, employing





## PLATE 1

x 4,150

Stolon placed over 30% chloroform solution before cutting. Although the plastid has ruptured the starch grains have moved hardly at all, indicating that turgor pressure had been relieved prior to preparation.

methods which reduce or eliminate shock in the sieve elements, produces images showing intact plastids. Bouck and Cronshaw (1965) immersed their entire pea seedlings in osmotically active solution prior to cutting into segments, and intact plastids are shown in their micrographs.

Hogben and Spanner (1977) treated Saxifraga sarmentosa (L) stolons with chloroform vapour prior to fixation. By weakening membranes and permitting lateral leakage from the sieve elements pressure within the elements was reduced before they were cut. As seen in plate 1, the plastid membranes were not intact, but this was thought to be due to the effect of the chloroform vapour, rather than to shock, as the contents were not displaced.

Many of the methods, however, which have been used to reduce shock must also have prevented translocation for a long period prior to fixation. Wilting (Anderson and Cronshaw, 1970), starvation (Evert, Eschrich and Eichhorn, 1973), as well as tissue cultures (Anderson and Cronshaw, 1970 and Cronshaw and Anderson, 1971), all illustrated intact, apparently unshocked plastids, but also inactive sieve elements.

Tissue frozen prior to examination shows intact plastids. Although some expansion of the sieve element contents has probably taken place on freezing (Spanner 1978) this is more marked in the central rather than the parietal position occupied by the plastids. This is one of the most convincing indications for intact plastids being the natural state.

As callose is not a fragile component of the sieve element freezing techniques probably give an accurate picture of how much callose normally lines the sieve plate pore. Cronshaw and Anderson (1969) froze whole plants into liquid nitrogen. Although the subsequent fixation technique may be suspect with regard to mobile structures with-

in the sieve element, further callose is unlikely to have been laid down as its monomer molecules would in all probability have leaked through damaged membranes as the temperature rose. The quantity of callose shown in their plates is variable, but never is it absent, nor present as a really thick cylinder.

Fixation as rapid as possible appears to give the most natural picture of the callose deposit. Fischer's early observations concerning his *Cucurbita* plunged into boiling water indicated that such plants contained less callose than those fixed chemically. Somewhat later A W Siddiqui repeated the experiment with the narrower axes of Helianthus stem and the stolon of Saxifraga sarmentosa (Siddiqui and Spanner, 1970), and showed only narrow cylinders of callose lining the pores.

The chloroform treatment of Saxifraga sarmentosa stolons previously quoted (Hogben and Spanner, 1977) must have emptied the sieve tubes to some extent of the soluble carbohydrate required to synthesise the callose, and indeed only narrow or negligible callose cylinders were seen lining the pores (their plates 1C and 1D). All this evidence bears out the conclusions reached by Weatherley and Johnson (1968) that some callose is normally present on the sieve plate of translocating sieve tubes, but not a great deal.

Some work has been done indicating that temperature has an influence on callose formation in the living sieve element. Experimental results adduced by Webster and Currier (1968) and McNairn (1972) suggested that callose deposits increase with temperature, especially when this rose above 35°C. They also note that by morning callose was at a minimum, regardless of how high the temperature had been previously, some indication that, especially with greenhouse grown plants, the amount of callose observed may vary with time of sampling.

Prolonged periods of darkness (over 16 h) also appear to increase callose deposition (Eschrich, 1963 a). Because the occurrence of callose is such a variable factor conjectures concerning its purpose are somewhat precarious. It is usually suggested that the major function is one of protection and closing, both as a response to injury, but also in the regulation of the exchange of material through cells. Eschrich (1965) has also proposed that callose may take up water from the translocate, thus regulating osmotic conditions in the sieve element.

P-protein is essentially a mobile structure in the sieve element and this makes its *in vivo* disposition in the lumen of the sieve tube very controversial. Obviously methods of preparation which negate, or at least limit surge effects must be employed when P-protein is under investigation. It is also becoming recognised that it is essential to ensure that the sieve element is translocating immediately prior to fixation. These prerequisites limit preparation methods which can be used. Fixation by quick freezing must be suspect after a recent strong criticism (Spanner, 1978 a) suggesting that the large expansion of water on freezing may cause violent axial displacement. Methods which have been used in attempts to observe the distribution of P-protein include pre-wilting (Anderson and Cronshaw, 1970), starvation, (Evert et al, 1973), and preparation of pith cultures, (Cronshaw and Anderson, 1971). As previously discussed, there is unlikely to be any translocation proceeding prior to fixation in such plants, rendering results questionable. Where methods to reduce turgor release in material likely to be actively translocating have been used the results reported have been variable. The experiments of Fischer, Siddiqui and Spanner, Hogben and Spanner have been already quoted with relation to callose. They also gave results regarding P-protein. Although Fischer found no P-protein in sieve plate pores

the later experiments showed P-protein blocking the pores. When Engleman (1965) fixed unexcised portions of phloem, P-protein was observed in the pores, as well as scattered in the lumen of the sieve tube. Thaine proposed (1964, 1967) that transcellular strands traversed sieve tubes, passing through the sieve plate pores. His suggestion led to much work on the subject and Johnson (1973, 1976) proposed that the strands were bundles of P-protein filaments, probably passing through sieve pores.

The interpretation of the distribution of the P-protein in the sieve elements, and especially within pores seems to depend on the theory of translocation favoured by the reporter. Where the pressure flow theory is advocated any P-protein found plugging the pores is assumed to have reached them after pressure surge, whatever precautions were taken during the preparation. Spanner (1978 b) in a comprehensive review of the situation notes that "an occluded pore is by far the commonest of all sieve plate images". He also argues that if surge effects were as unavoidable as has been claimed it would be at least as likely that in vivo P-protein plugs would be blown out of the pores as that luminal material would be blown in. It seems inconceivable, he suggests, that the precisely aligned fibrils that Benuke, for instance, found in Aristolochia (1971), and shown in his figure 1, arrived there as a result of a surge artefact; they are far more likely to be natural.

Although P-protein is almost universally present in Dicotyledons, it is absent in many Monocotyledons and Gymnosperms. This lack of constancy implies that if P-protein is active in translocation different plants must have different mechanisms.



### C. ANATOMICALLY MATURE SIEVE TUBES AND TRANSLOCATION

An anatomically mature sieve tube has already been defined, but the stage of which it is physiologically mature ie translocating has been the subject of a certain amount of controversy. There is, however, fairly general agreement that the anatomically mature sieve elements are the functioning ones. The main voice of dissent in this area belongs to Wark and Chambers (1965), who, after their investigation of the phloem of Pisum sativum declared that the immature stages of the sieve tube are the functional ones, an opinion not shared by Bouck and Croushaw working on the same plant at about the same time. Some evidence from work on Monocotyledons indicates that the condition of physical maturity described for Dicotyledons may not always apply in Monocotyledons. Melaragno and Walsh (1976), working with Lemna, Walsh and Evert (1975), using Zea, and also Walsh and Popovich (1977) with Epifagus (not a Monocotyledon, but a holoparasitic angiosperm) show electron micrographs of sieve elements having their lumens occupied by vacuoles, with typical tonoplasts. All these plants lack P-protein. In none of these papers has any attempt been made to demonstrate translocation in the sieve tubes in question.

Certainly in Saxifraga sarmentosa, used for the work undertaken in this thesis the overwhelming majority of sieve elements found fitted the description of the anatomically mature element. If the view is taken that only the anatomically immature elements function, very little, if any, translocation could take place in the stolon, a fact not borne out by the experimental results which will be presented later.

It is extremely difficult to make a positive identification of a sieve tube as simultaneously anatomically and physiologically mature as in the senses defined above. Two methods have been employed to

demonstrate that translocation was taking place in sieve elements just before preparation for electron microscopy.

- (a) Microautoradiography
- (b) Concurrent demonstration of the passage of radioactive material through the axis.

Fisher (1975) used microautoradiography in Soybean, correlating light microscope autoradiographs with montages of electron micrographs of a single isolated vascular bundle. All his material was prepared by freeze substitution, and as already noted, criticism has suggested that volume changes in the lumen of the sieve elements may have displaced the labelled contents. Most preparation methods indeed, could also permit some movement of soluble labelled material.

The second method involves the gross physiology of suitable organs chosen for the simplicity of their vascular anatomy. It verifies that at least some of the sieve tubes between source and sink are functioning but gives no indication of which ones are active. The method has been used by Giaquinta and Geiger (1973 and 1977), also Hogben and Spanner (1977). This method has also been used for the experimental work presented in this thesis. Since the stolon of Saxifraga sarmentosa is ephemeral and its longitudinal growth is very rapid, and all primary, it is assumed that most, if not all, the observed sieve elements are functioning. Care has also been taken to ensure that a high proportion of the elements present in any one segment have been examined.

Neither of these methods is totally satisfactory but, to quote Melaragno and Walsh (1976) "We are observing a 'slice in time' and until we are able to communicate with the sugar molecule, we have no way of knowing with absolute certainty that a sieve element was actually conducting when the 'stop action' took place".

#### D. THE EFFECT OF INHIBITORS ON ULTRASTRUCTURE

Very little work has been undertaken to date concerning the ultrastructural effects of inhibitors of translocation.

Giaquinta and Geiger (1973) studied the effects of chilling on sugar beet (Beta vulgaris (L)) and bean (Phaseolus vulgaris (L)), described as chilling resistant and chilling sensitive species respectively. In a statement of their findings with regard to the chilling sensitive species they say that in treated plants "the pores are generally plugged with cytoplasmic material which is often continuous with the material lining the walls". They further suggest, and present a micrograph (their plate 5I) to show, that "the walls of cold treated sieve tubes seem generally to contain less material, suggesting that chilling has disrupted the cytoplasmic material lining the walls causing it to be swept into the pores. Flow apparently causes streamers to protude through many of the plates". These workers proceeded to 'measure' pore blockage by demonstrating the degree of movement consequent on cold treatment of the characteristically flagellated protein crystals present in the sieve tubes of Phaseolus. They excised uniformly the sieve tubes of a number of samples, kept at different temperatures, and measured the distance of the crystals from representative sieve plates. They stated that "the inability of these crystals to respond to the pressure release suggests that chilling has physically blocked the sieve tubes, either by gelling of the sap or by obstruction of the sieve plates". Taken in isolation this suggestion appears valid, but taken in conjunction with the statement quoted earlier regarding the sweeping of the cytoplasmic material into the pores, it appears decidedly contradictory. If some parietal sieve tube contents are swept into the sieve plate pores might it not be reasonable to suggest that other central sieve tube contents (such as the protein

crystals) would be swept in the same direction at the same time? Such contradictory suggestions render the interpretation of other observations suspect. Further suspicion is cast on the validity of their conclusions by the fact that much of the material was prepared by freeze substitution. The use and value of this method has already been discussed together with reasons for suggesting that it may be unsatisfactory in the present context.

Several other physiologists have studied the effect of low temperature inhibition on phloem transport, but few have looked at phloem tissue using the electron microscope to find out whether any changes in ultra structure yield a clue as to the mechanism of low temperature inhibition. One who mentions that he has done so is Webb (1971). Although no micrographs are published in the paper he states that "inhibition of translocation at 0°C (in Cucurbita melopepo) is brought about by some other means than a plugging of the sieve plate pores with either protein fibrils or callose". In their paper quoted above Giaquinta and Geiger make no comment on the callose lining except to note that in the controls "callose is generally minimal", a statement which does not seem consistent with their micrographs which give a varied picture, both in controls and experimental material.

The condition of the plastids in the sieve tubes is not discussed by Giaquinta and Geiger and in their published micrographs none is observed. Surely it might be expected that if the displacement of parietal sieve tube contents towards the sieve plate on treatment is as marked as they claim it is, such vulnerable structures as plastids (whole or disrupted) should be conspicuous near the sieve plates?

The same workers also presented a paper (Giaquinta and Geiger 1977) describing the effect of cyanide as an inhibitor of translocation in Phaseolus vulgaris (L). Essentially the results they reported were the

same as for their chilled material. They found sieve plate pores blocked with cytoplasmic material, somewhat thin cell walls indicating structural damage, and only a thin layer of callose lining the sieve plate pores. Only two micrographs are presented in their paper and the appearance of the experimental material pictured indicates considerable damage. There is some evidence of ice crystal formation, but the general appearance also indicates other structural damage during preparation. The image is so poor that their conclusion that "the lateral walls are relatively free of this material (cytoplasmic material) which characterizes the controls", is impossible to concede. The fibullar material in the lumen of the sieve tube shows no features to enable its identification as 'cytoplasmic material' and indeed shows more resemblance to fibrils of P-protein. Their paper does not stress the use of specifically young petioles (as were used in their chilling work) so this is not an improbable identification. Another possible reason for the poor condition of the experimental material as shown in the micrographs may be the extremely high concentration of the potassium cyanide ( $100mM$ ), used to inhibit translocation. Such a high concentration may possibly have produced an osmotic effect on the tissue, as well as disturbance of general metabolism. In the corners of such companion cells as are visible there is indication of plasmolysis.

Giaquinta and Geiger make the suggestion that "cyanide may also cause loss of selective permeability of the sieve tube plasmalemma causing sudden pressure release and loss of structural integrity of the sieve tube contents". When the present investigation was being undertaken this suggestion was kept in mind. They also made observations about the recovery of translocation rate after inhibition with potassium cyanide. "Recovery seems to depend on re-establishing the structural integrity of the conducting channels by partial reversal

and displacement of the cytoplasmic material in the pores" is their conclusion, but it remains highly tentative.

Another piece of work, using cyanide as a translocation inhibitor and using (as in the present work) the stolon of Saxifraga sarmentosa (L), was undertaken by F Qureshi (Qureshi and Spanner 1973 d and e). He found that submersion of part of the stolon in a concentration of potassium cyanide of  $5 \times 10^{-3}$  M (ie 5mM), was sufficient to show an inhibitory effect pronounced, but falling short of complete stoppage. Qureshi (1973 e) particularly studied the effect of inhibition by cyanide on callose deposition. He used different fixatives and found little callose when a 3:1 mixture of 95% alcohol and glacial acetic acid was used to fix the material, but somewhat more when 6% glutanaldehyde, suitably buffered, was the initial fixative. He showed that the alcohol and acetic acid did not dissolve callose by first fixing in glutanaldehyde then in the alcohol/acid mixture; callose was still fairly abundant. His finding of minimal callose lining the sieve plate pores coincides with the observations of Giaquinta and Gieger. During the course of his work Qureshi (1973 d) also experimented with different methods of applying the cyanide. He found that gaseous cyanide was a more effective inhibitor than the solution, as apparently a much higher concentration was taken up by the stolon. His methods, and indeed the apparatus which he describes in this paper, were used for the experimental work reported in this thesis.

It is apparent from the work reported that ultrastructural observations on inhibited material have been restricted. Some investigation has been made into callose deposition. The distribution of the P-protein in the sieve tube has received a passing mention (Qureshi and Spanner 1973 e) and such labile structures as plastids have been more or less totally ignored, although they, or their contents, appear in

Qureshi's micrographs. Many of the reservations regarding the validity of Giaquinta and Geiger's work arise out of the preparative methods they used. Passing comment has already been made regarding freeze substitution techniques. Various methods of freeze substitution have been the subject of further investigations undertaken in this work.

The inhibitory work presented here seeks to throw light on the ultrastructural characteristics of all the sieve tube contents, in phloem tissue prepared by the most satisfactory fixation methods so far found.







PLATE 2

x  $\frac{1}{2}$

Whole plant of Saxifraga sarmentosa (L) showing  
short stolons.

## PROLOGUE

The structure of the stolon of *Saxifraga sarmentosa* (L).

The plant used for all the work reported in this thesis was *Saxifraga sarmentosa* (L), an herbaceous plant having a rosette habit, and bearing stolons which eventually give rise to daughter plants at their distal ends, (see plate 2). The stolons frequently grow to a length of 50 cm making them particularly suitable for experimental work, especially translocation studies. Previous work (Qureshi and Spanner, 1971) has demonstrated that, through these stolons, long distance transport is unidirectional, ie towards the tip, under normal growing conditions. The sucrose being translocated appears to show little lateral movement from the sieve elements (Qureshi and Spanner, 1973).

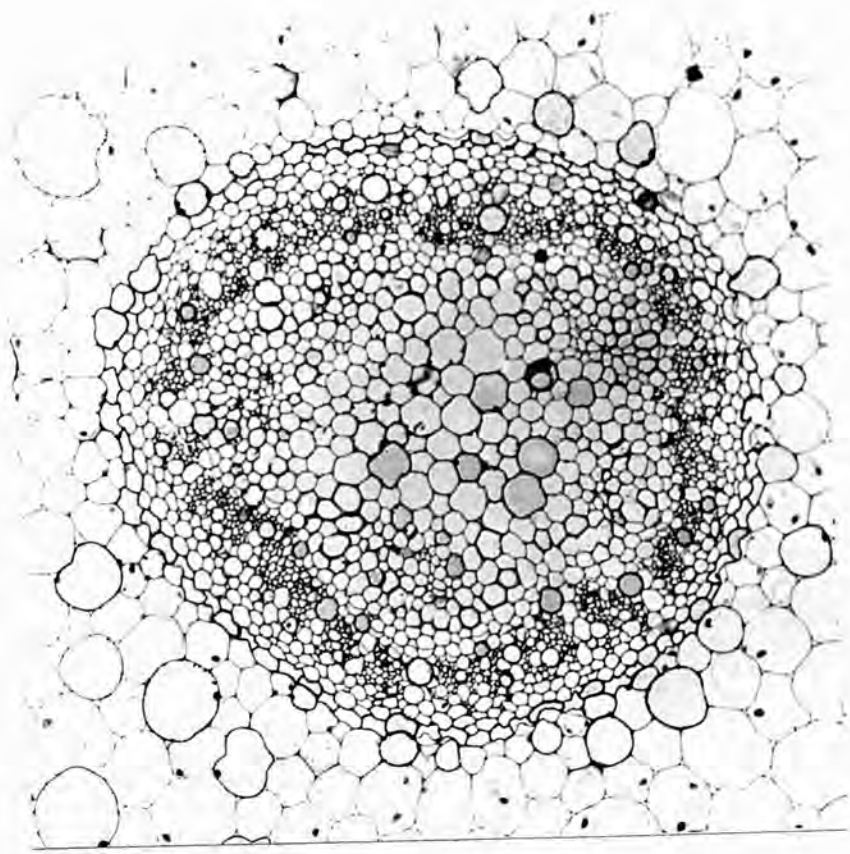
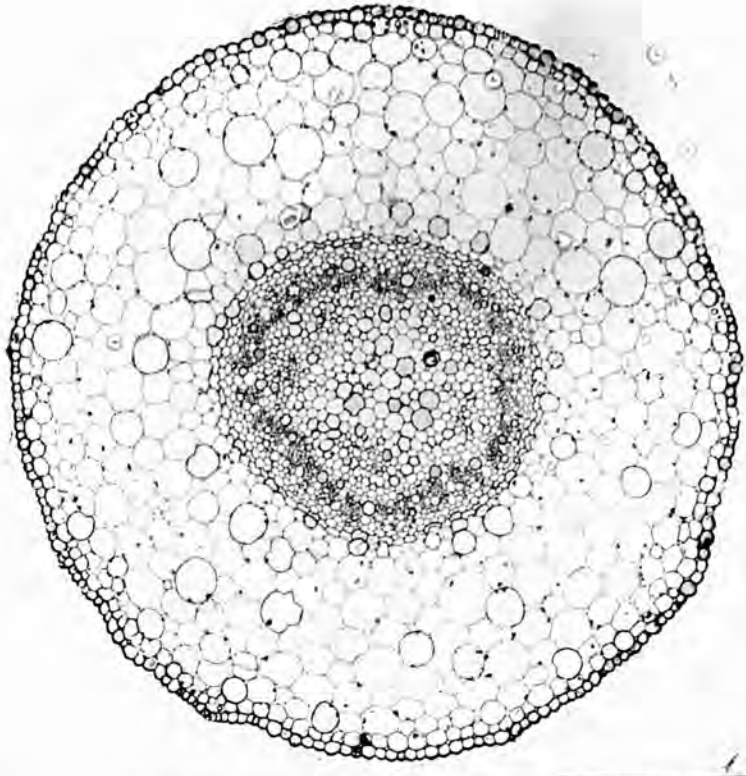
A preliminary investigation of the stolon structure was made using both optical and electron microscopy.

Methods. (a) Thick sections (1 - 2 $\mu$ m thick) of conventional resin-embedded material were cut, and stained with toluidine blue to show the general arrangement of tissues under the light microscope (see Appendix E).

(b) Portions of stolon were also fixed and wax embedded (see Appendix F) and both transverse and longitudinal sections were cut from this material using a Cambridge rocking microtome. These were stained by established methods to define the cellulose, starch and lignin structures (Appendices B, C and D).

(c) Lipids are dissolved in the dehydrating fluids used in these procedures and so remained unidentified. A fixation method (Ciaccio) employed primarily by zoologists (Appendix G) to fix lipids was therefore also tried. However, the Ciaccio fixative apparently failed to preserve the lipids in this plant material. Fresh hand sections were





## PLATE 3

x 80

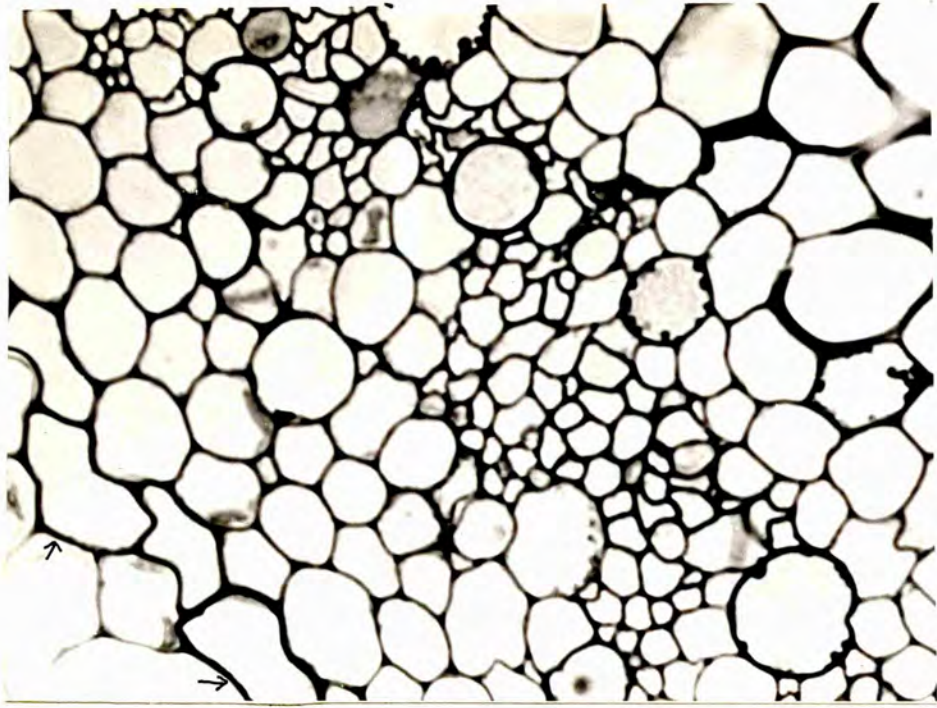
T.S. of entire stolon showing the proportions of the stele and cortex.

## PLATE 4

x 210

Stele of the stolon showing the ring of vascular tissue, and no discrete bundles.







## PLATE 5

x 1080

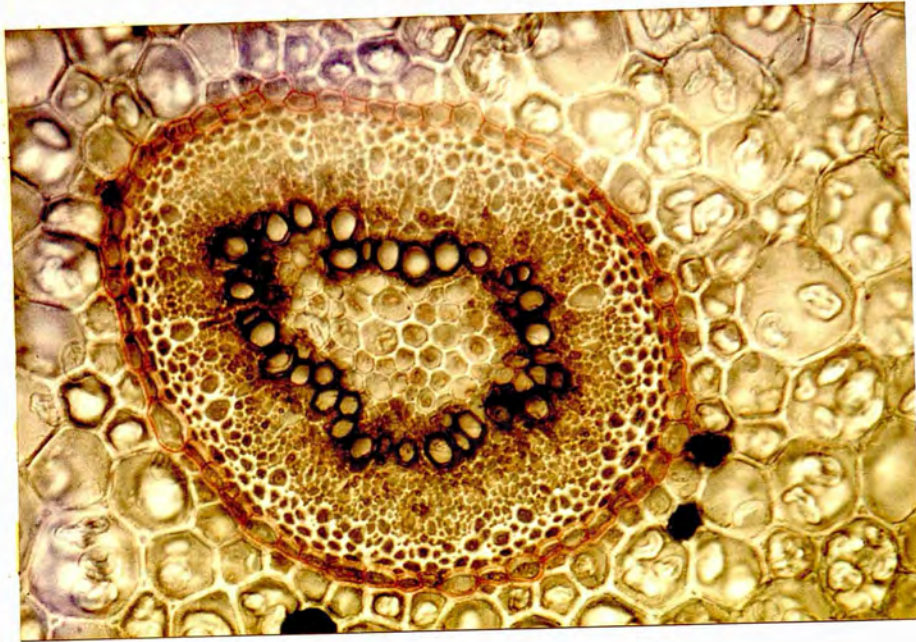
T.S. of the vascular tissue of the stolon. The arrows indicate the thickened endodermal walls. The small cells of the phloem are interspersed with larger parenchyma cells. There is no suggestion of a cambial region.

## PLATE 6

x 60

T.S. of the stolon stained with Sudan IV. The stain has been taken up by the cuticle and endodermis.





## PLATE 7

x 250

Stele showing the endodermis stained with Sudan IV.

The parenchyma cells with thickened walls show clearly between the endodermis and the phloem.

## PLATE 8

x 820

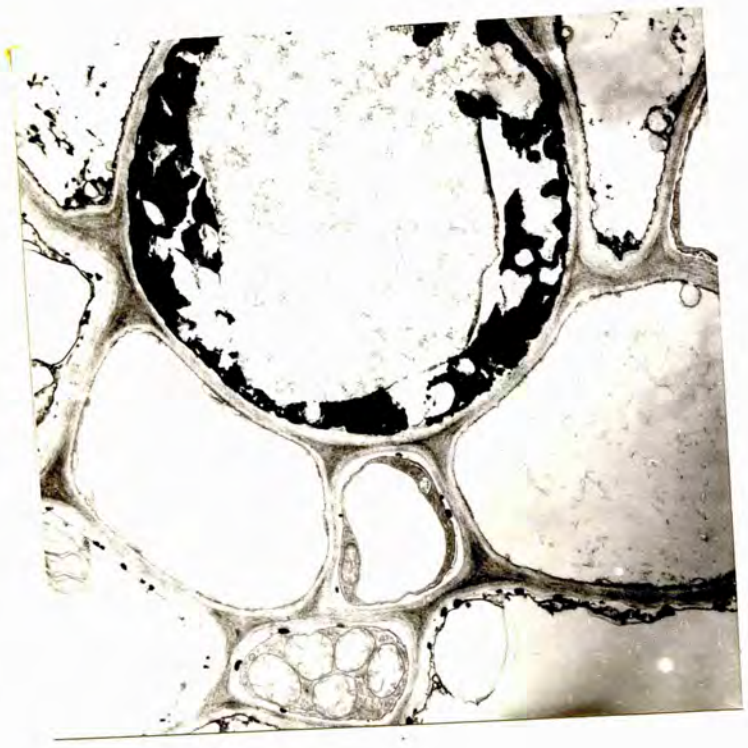
Endodermis stained with Sudan IV. The lipid-containing cell walls are confined to one cell layer. The lipid deposition appears to be irregular, the inner tangential walls having less.

then cut and prepared according to Appendix A.

(d) Different regions of the stolon were studied ultra-structurally by initially cutting segments 5 cm long into fixative. Before completing the preparation (see Appendix J) the central 1 cm was excised from each segment and further cut into 1 mm portions under fixative. Such material was taken from the proximal and distal ends of the stolon, as well as from the middle.

Results and Observations. Thick sections of resin embedded material cut transversely revealed a more or less circular profile (see plate 3) with the epidermal cells having thicker walls than the cortical cells. The external epidermal walls appeared no thicker than the radial and inner walls. The stele showed as a compact region with a diameter about  $\frac{1}{3}$  of that of the stolon (see plate 4). Since the middle region of the stolon from which this material was obtained has an unusual diameter of about 1 mm, the diameter of the stele is approximately 0.3 mm. The cells of the layer surrounding the stele have thickened walls which are irregularly shaped. These are indicated by arrows on plate 5. They appeared to form a continuous layer, making a boundary for the stele at which the cortex readily separates. This layer stained red with Sudan IV, similarly to the outer sheath over the epidermal cells. This shows clearly in plate 6. This indicates that lipid was present probably as cutin or wax over the epidermis and as suberin in the layer bounding the stele, identified as the endodermis. The suberin appeared to be evenly distributed in the cell walls (see plates 7 and 8), unlike the differential suberisation found in the root endodermis which directs the passage of water through the cells. This suggests that the stolon endodermis is a more waterproof structure. Plate 8 shows layers of parenchyma cells under the endodermis which have slightly thicker walls than those of the cortex and pith. The radial extent of this layer is variable, sometimes





## PLATE 9

x 140

L.S. of part of the middle region of the stolon stained with safranin and fast green. The annular and/or spiral thickening in the xylem vessels has taken up more fast green than safranin showing how little they are lignified.

## PLATE 10

x 9,750

T.S. of the proximal region of the stolon showing a tannin cell at the top of the micrograph, also there is one of the companion cells packed with mitochondria which are frequently observed in this region.



it is only one cell deep but in other places its depth may be up to four cells. These thicker walled cells were shown to possess starch - containing plastids, as were the parenchyma cells of the pith and cortex. Advantage has been taken of these structural features in the experimental work, for much of which short lengths of stolon (5 - 10 cm) were decorticated along the endodermal line of weakness. In such a state it was shown (and a report is given later in this thesis), that translocation proceeded more or less normally for many hours after surgery. Sometimes it appeared to slow down for a short period during the actual operation, but it returned to normal soon afterwards. The suberised endodermis protects the stele from dehydration. The removal of the cortex was designed to help the rapid penetration of fixatives, and also of the inhibitors used in the experimental treatments.

No cambium was apparent, suggesting that the vascular tissue is all primary in origin. Correspondingly xylem elements showed only spiral or annular thickening typical of primary xylem (see plate 9).

The optical microscopy gave an overall picture of the relative positions of the different cells composing the phloem tissue, as well as the spatial association between the xylem and phloem. This information later proved valuable when identifying cells seen under the scanning electron microscope.

Ultrastructural observations. Comparison of structure was made between the proximal region, ie that very close to the parent plant, the middle region, which was the portion used for the physiological treatments, and the distal region just behind the daughter bud. There were distinct ultrastructural differences between the three regions of the stolon looked at.

The proximal region of the stolon showed a particularly interesting feature. Many of the companion cells were packed with mitochondria (plate 10) and few large vacuoles were found. This type of cell appears to have





## PLATE 11

x 25,000

Montage showing part of a companion cell in the proximal region of the stolon in L.S. The cytoplasm is dense, and mitochondria abound. Vacuoles appear to be small and scattered. In the periplasmic space there are many vesicles.



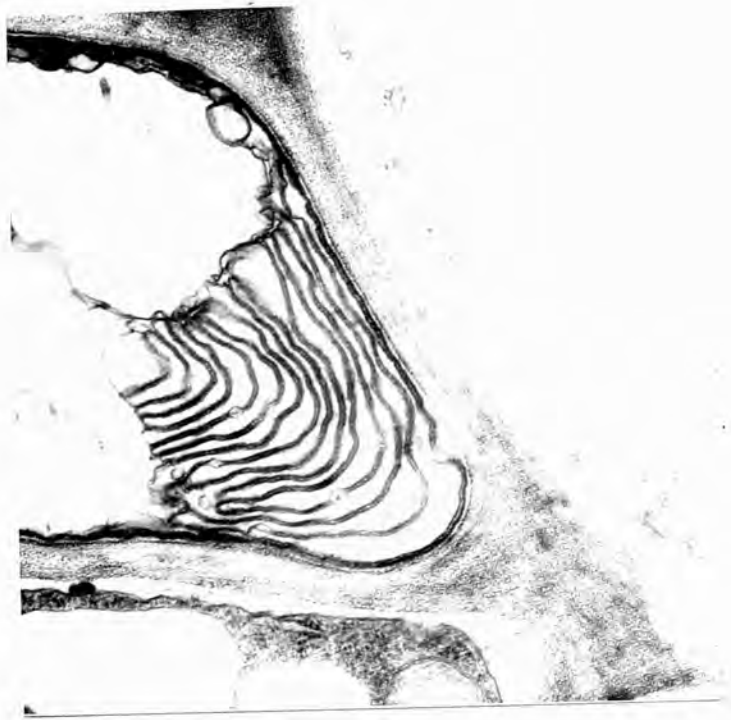


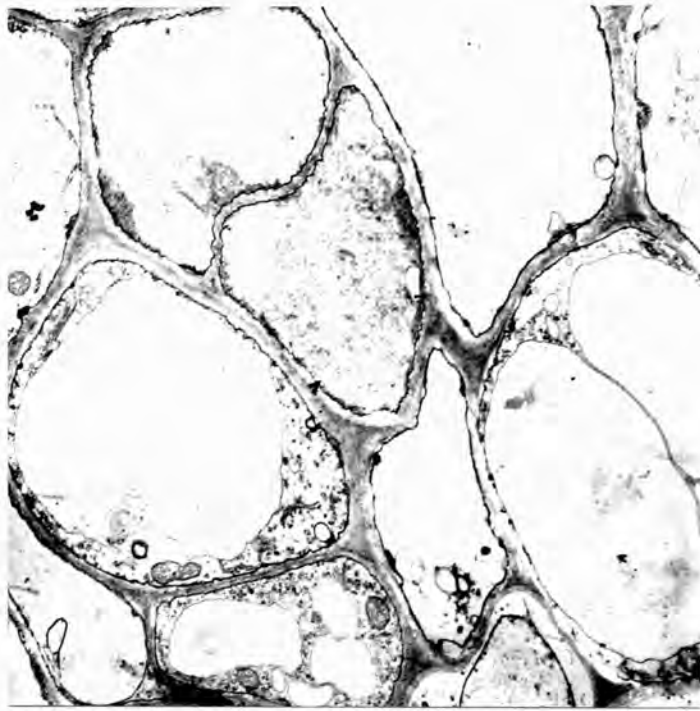
PLATE 12

x 33,500

T.S. of a corner of a sieve element in the proximal region of the stolon showing the membrane stacks which are a fairly common feature.







## PLATE 13

x 9,750

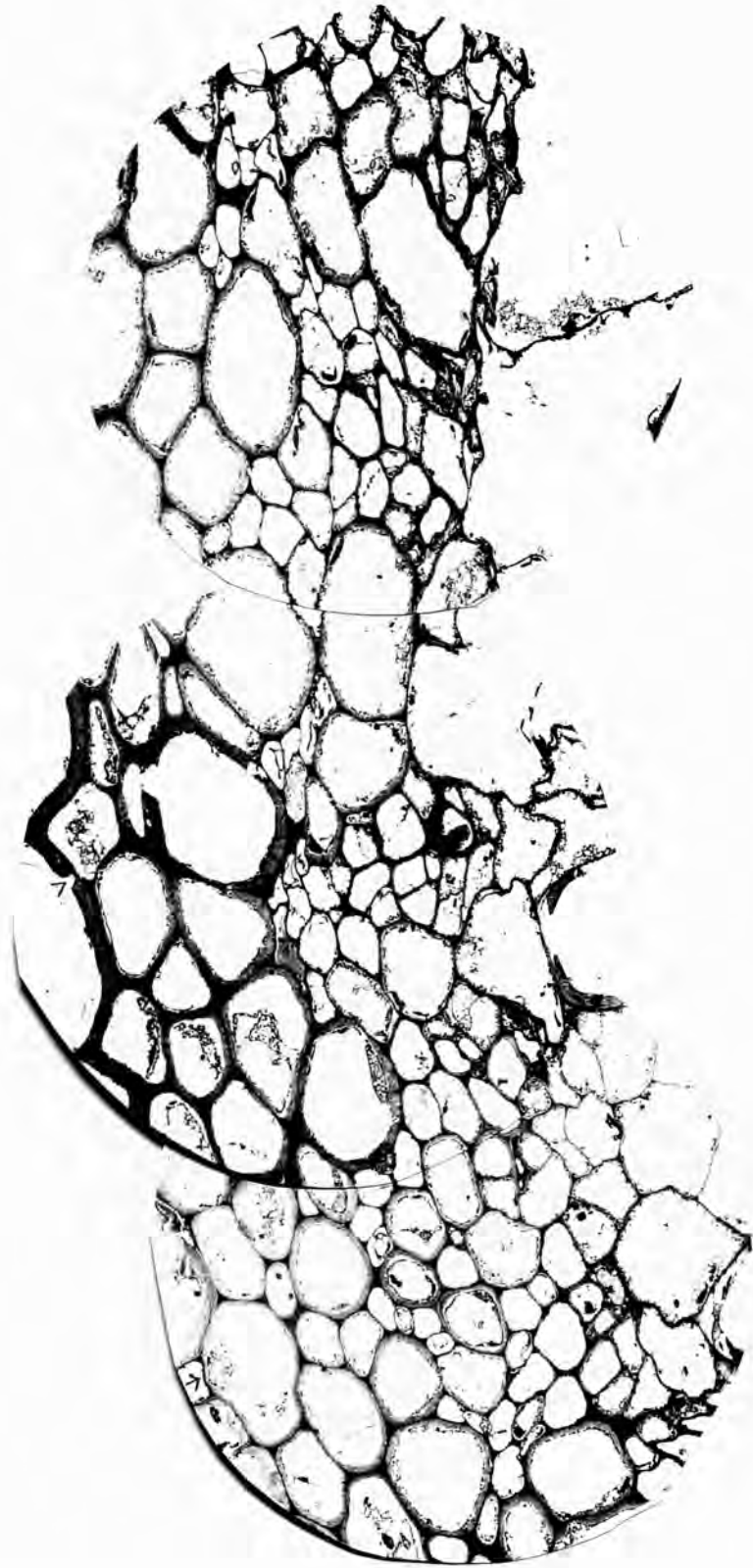
T.S. of the middle region of the stolon showing rather wider phloem elements having a somewhat empty appearance.

## PLATE 14

x 6,700

L.S. of the middle region of the stolon. The companion cell in the lower part of the picture lacks the very dense cytoplasm found in the proximal region. The sieve plate is callosed, but no rapid fixation methods were used.





## PLATE 15

x 1,330

Montage of the outer region of the stele showing  
thick-walled cells, up to four cell-layers in depth.

The outer layer (endodermis) appears to be irreg-  
ularly thickened.



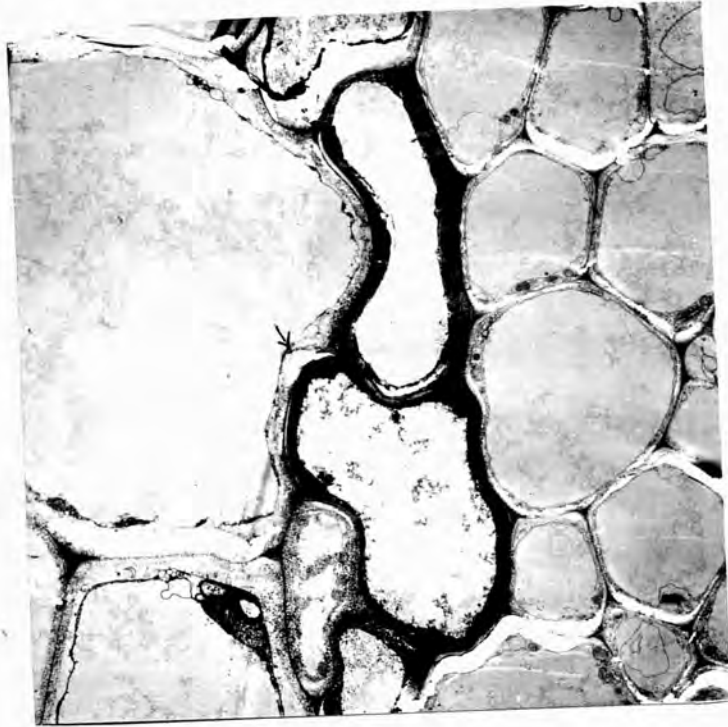


PLATE 16

x 3,300

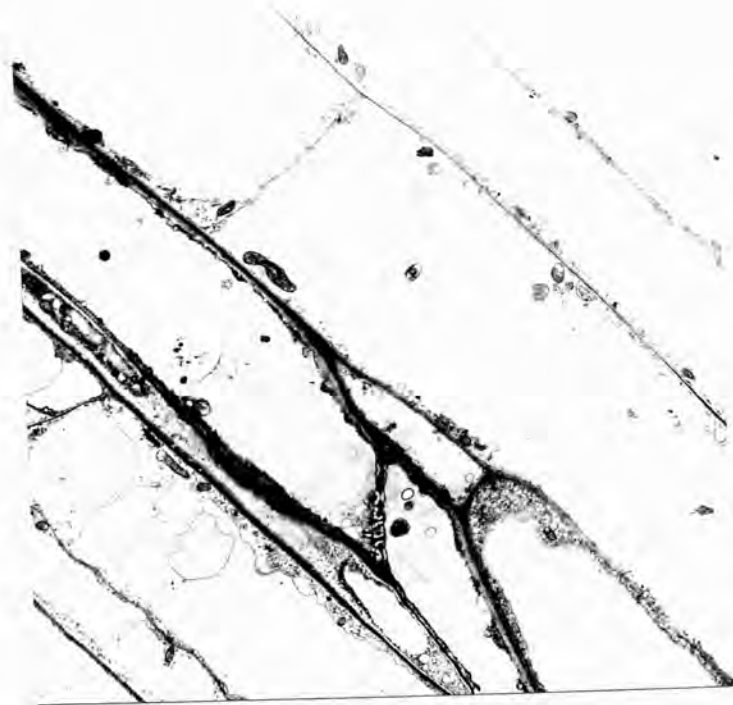
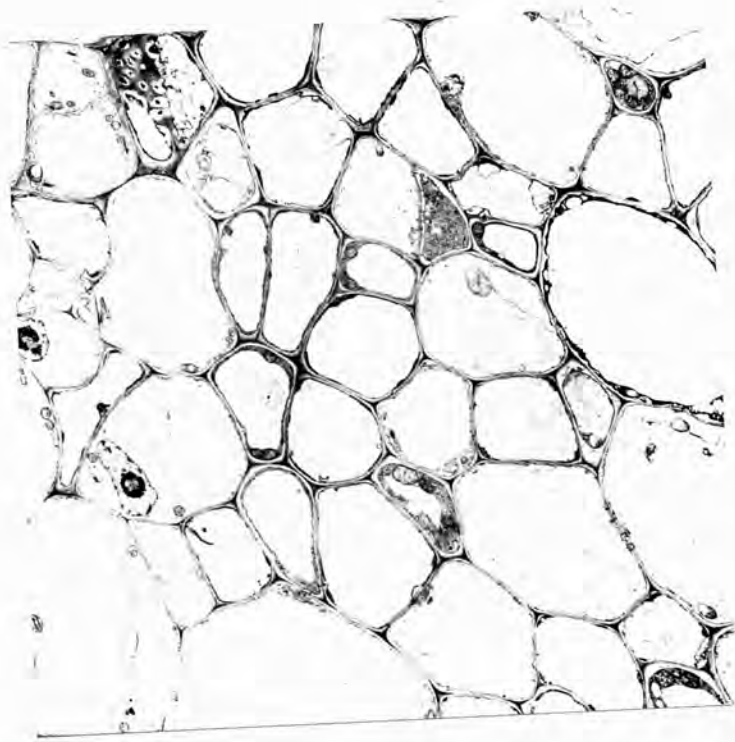
T.S. of the thickened endodermal region which corresponds more closely with light microscope observations.



numerous small vacuoles and much dense cytoplasm. These features are further illustrated in plate 11. The tannin cell at the top of plate 10 is a regular feature of the phloem tissue in all regions of the stolon. Within the sieve elements convoluted membranes were frequently observed. These are shown as stacks in plate 12. Such membrane complexes have been regularly reported in many species (Johnson, 1969). Mitochondria were rarely seen within the sieve elements in any region.

In the middle region, on the other hand, the companion cells were all well vacuolated, contributing to the generally empty appearance of the tissue seen clearly in plates 13 and 14. The cytoplasm of the companion cells, however, is somewhat more dense than that of other cells in the phloem. Mitochondria are present in these companion cells, but not so abundantly as in the proximal region indicating perhaps, a lower energy requirement in the middle region. The endodermis of this region, when studied at ultrastructural level yielded further information to that gained from observations made using the optical microscope. Plates 15 and 16 show cells with thickened walls. The radial depth of these ranged from one to four cells from the endodermal layer. Arrows on plate 15 show where the cortex has separated during surgery, and the outer layer of cells shown here appear to be irregularly thickened. The outer layer appears to correspond with the endodermis of the optical micrographs, and the layers of thick walled cells underneath, to the thick walled parenchyma cells shown clearly in plate 8. Arrowheads on plate 15 point out thin regions in the thick walls which were not observed at optical level. Plate 16 shows the same region, but the portion on this micrograph more closely resembles the observations made on the thick sections. The cortex was still attached to this sample, but at the arrow it can be seen to have pulled away slightly. The underlying phloem parenchyma cells of this sample do not appear to have thickened walls.





## PLATE 17

x 3,300

T.S. of the distal region of the stolon. Cells of phloem elements are smaller than in the middle region and the companion cells are more highly vacuolated than in the proximal region.

## PLATE 18

x 3,300

L.S. of a sieve element in the distal region.

The structure of the distal region of the stolon closely resembled that of the middle region. The low power micrograph shown in plate 17 shows highly vacuolated tissue giving the whole region its typical empty appearance noted again in plate 18. This distal region, because of the method of preparation, where the central portion only of a larger segment, was examined, was not really very close to the growing point, where more activity and therefore more organelles might have been expected.

There seemed to be no differences in the occurrence of plastids, or in the distribution of the P-protein within the sieve elements, in the different regions.

## PART 1 INTRODUCTION

The search for the best methods of preparation

It is generally agreed that if a similar image is observed after using a wide variety of preparative methods for electron microscopy the result is likely to present a truer picture of the living structure than if only a narrow range of methods is used. The same artefacts are less likely to occur consistently where methods vary. With this in mind a series of preliminary programmes was run to find the best methods of dealing with the phloem of the Saxifraga stolon.

The presumed or real artefact of sieve element preparation which is the most disturbing, and difficult to overcome is the 'surge effect' which plausibly arises from violent longitudinal movement of the sieve element contents when a breach is made in the integrity of the cell wall. To establish a sound basis for later discussion such artefacts were at the outset created deliberately in this work so they could be the better recognised or eliminated when their presence was debatable.

Sorensen's phosphate mixture of strength M/15, buffering at 7.2, is routinely used in this laboratory for all fixation. However, it is frequently suggested that plant tissues, being generally more acid than animal tissues, should be buffered at a lower pH. A series of initial trials was performed therefore to test whether this was so, and to see whether significantly better preparations could be obtained at other pH values.

At the commencement of this research a paper appeared by two Portuguese workers who made rather extravagant claims regarding the efficiency of a new buffer they had used in fixation (Salema and Brandao 1973). This was the Zwitter ion buffer Piperazine N-N bis (2 ethanol sulphonic acid), commonly referred to as Pipes buffer. It

seemed worthwhile to test this, and several experiments were run to test its potentialities.

Obviously the more rapidly fixatives penetrate the better the chance of good fixation. Previous observations in this department showed that translocation continues in the decorticated stolon (da Cruz 1976). Since the phloem lies only a few cells under the endodermis, penetration of fixative into the decorticated stolon would be expected to be very rapid, so this line of attack was also followed up.

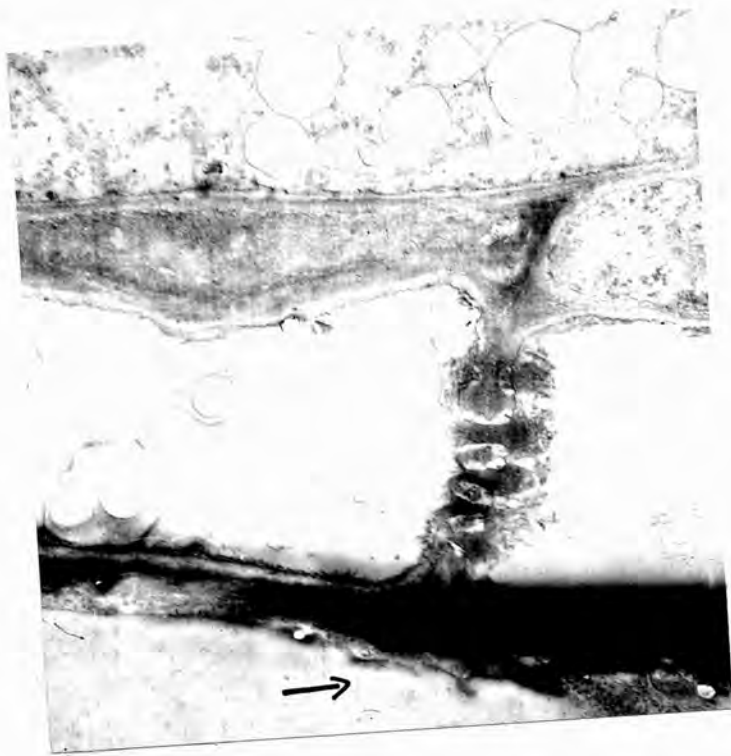
Earlier work had been undertaken in this laboratory in an attempt to minimise surge effect using chloroform vapour to effect a preliminary weakening of the sieve tube membranes and allow lateral leakage of the sieve tube contents before fixation (Hogben and Spanner, 1977). This approach was extended in this research by including small amounts of chloroform in the fixative itself. It was also considered that with the decorticated stolon, movement of the fixative through the suberised endodermis might be speeded up by the chloroform.

Scanning electron microscopy had, until recently, been used very little in the study of sieve tube structure. It was thought that further useful information concerning sieve tube condition could be gained if this technique was employed.

Finally many preparative methods involving fixation by freezing have been employed in ultrastructural investigations. Three of these methods have been retried, and in the light of later thought on the subject (Spanner, 1978), reassessed as to their value in sieve element preparation.







## PLATE 1.1

x 9,750

A section from the second segment from the distal end of the prepared part of the stolon. Surge effects might be expected. Although the P-protein bulges as though some pressure had been exerted, the plastids away from the surge are unbroken.

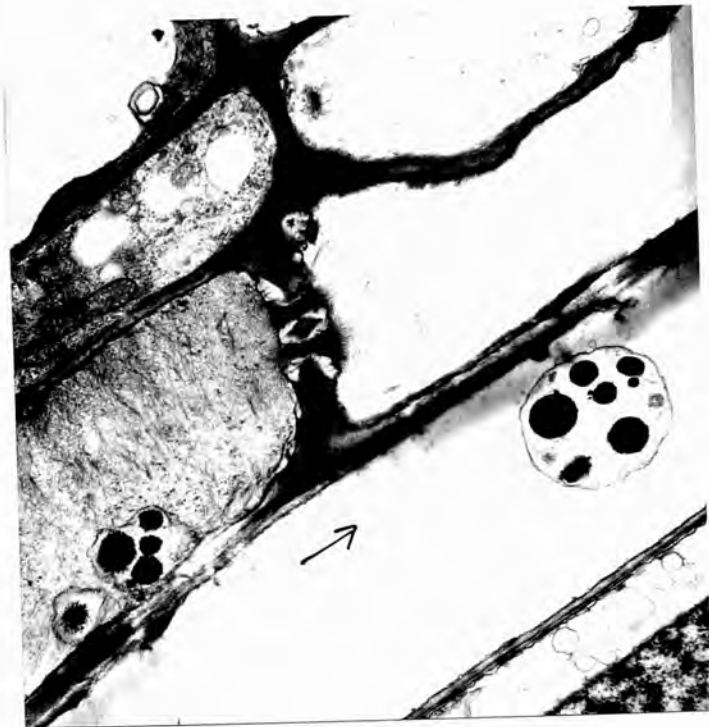
## PLATE 1.2

x 13,300

A section from the segment (no. 1) nearest the distal end of the prepared part. The P-protein shows some bulbous effect on the surge side of the plate, and some streaming away from it.

On all the micrographs in this chapter the arrows indicate the probable direction of the flow of the translocate, ie. towards the distal bud.





## PLATE 1.3

x 6,700

Another section from segment 1. There is little or no evidence of pressure release effects.

## PLATE 1.4

x 9,750

No real evidence of pressure release effects are obvious either in this view of segment 1.

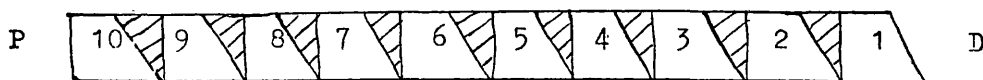
## Chapter 1

Distribution of P-protein with intentional surges

When segments of stolon are cut from the entire organ without careful precautions surges undoubtedly occur in the vicinity of the cut ends. Does the distribution of P-protein on the sieve plates change in any recognisable or regular way as plates are examined further and further away from the cut ends? Does the distribution show any obvious relation to the direction of the previous translocation? These questions were the first to be examined.

Method. Pieces approximately 2.5 cm long were cut abruptly from the middle region of stolons, and placed, after a delay of 2 - 3 seconds, in fixative (appendix J) for 1 - 1½ h. To record the presumed direction of translocate flow the proximal end of the piece was cut transversely and the distal end obliquely. The initial cuts, removing the segment, were made simultaneously using a razor blade assembly and the trimming to shape done under the fixative. After preliminary fixation the piece was further subdivided into small segments each bounded by a transverse cut proximally and an oblique cut distally (fig 1). The small segments were further processed individually in numbered phials and embedded.

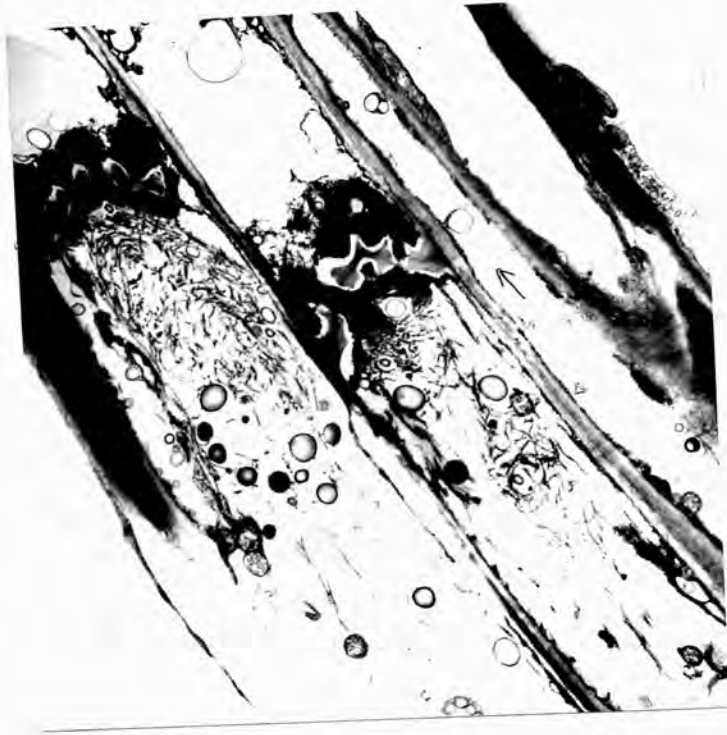
Figure 1



Before sectioning the blocks were trimmed so that the shape of the sections again indicated the proximal-distal direction. The distance from the original cut ends was of course also known.

Results and Observations. Micrographs from segments 1 - 3 (Plates 1.1 - 1.5), ie from near the distal cut end of the stolon







## PLATE 1.5

x 6,700

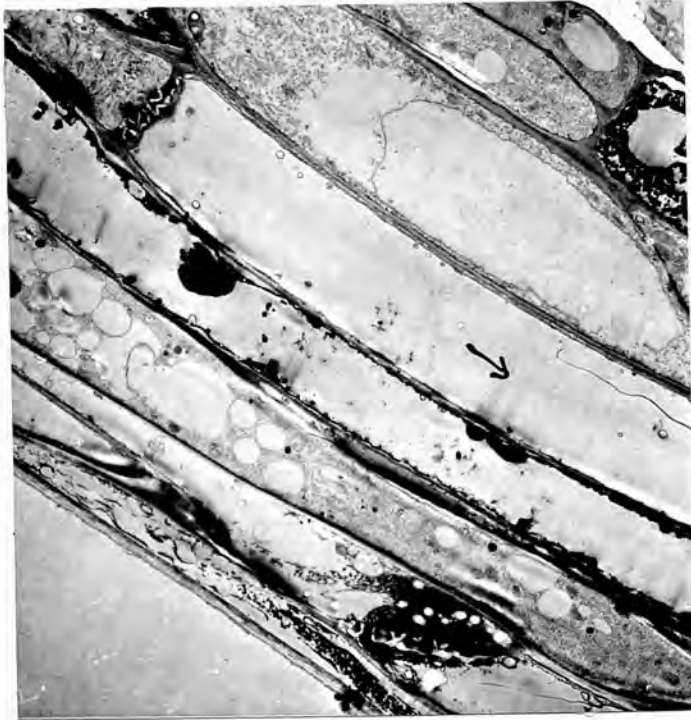
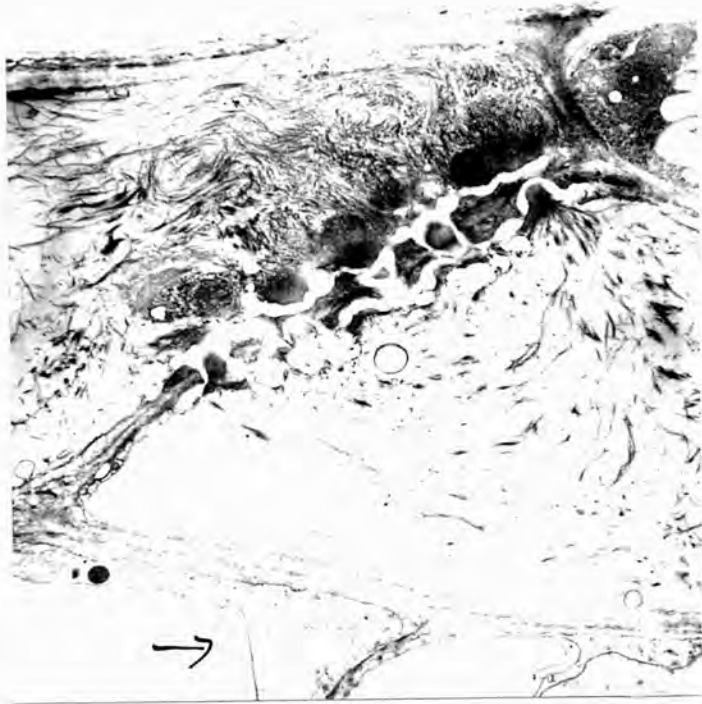
Segment 3. Although 3-4mm removed from the cut end of the prepared region surge effects are seen here. P-protein is piled up against the sieve plate on the side from which the pressure release effects would be expected, but broken plastids are seen on the other side of the sieve plate.

## PLATE 1.6

x 3,300

Segment 10. Some evidence of surge effect although this was the fourth segment from the end. Surge in this segment might be expected from the proximal end, but the ruptured plastids are on the other side of the plate.





## PLATE 1.7

x 9,750

Segment 12, near the proximal end of the prepared region. Evidence of surge effects are noticed in the dense P-protein on the surge side of the plate.

## PLATE 1.8

x 3,300

Segment 7, ie. towards the middle of the whole segment. Even in this section surge effects are seen in the dense protein at the plate and ruptured plastids.

typically showed plugs of P-protein in the pores of the sieve plates. The direction of the arrows on all the micrographs in this chapter point towards the distal end, ie in the presumed direction of translocate flow.

Plate 1.1 shows plugs of P-protein in the sieve plate pores which appear bulbous on one side of the plate, the side from which surge might be expected, and having fibrils entering the lumen of the sieve tube on the other side of the plate. Plate 1.2 shows bending of the P-protein fibrils on the 'surge' side of the plate, with 'streamers' of fibrils on the other side. The condition of the plastids was somewhat variable. Although many appeared ruptured others were intact (plates 1.1, 1.3, and 1.4). Plates 1.3 and 1.4 show typical images seen at this end of the stolon, with P-protein in the sieve plate pores, as well as distributed in the lumen of the sieve elements, also intact plastids. These plugs show structured fibrils, but at many other sieve plates observed an ill-defined mass of loose starch grains, and dense masses P-protein were seen blocking the pores. Plate 1.5 is typical of sieve plates showing this image. At the proximal end of the stolon (plate 1.6) similar images were also obtained. Plates 1.3 and 1.4, even though they were within 1 mm of the cut surface, show very little movement of the sieve element contents, whilst 1.5 and 1.6, also taken from within 1 mm of a cut end show evidence of far more movement. Plate 1.7 shows bent over fibrils of P-protein on the other side of the sieve plate from which surge may have been expected, with streaming fibrils towards the surge!

In the centre of the cut segment, ie at over 1 cm from the cut surface, the resulting micrographs showed essentially similar images to those already discussed.



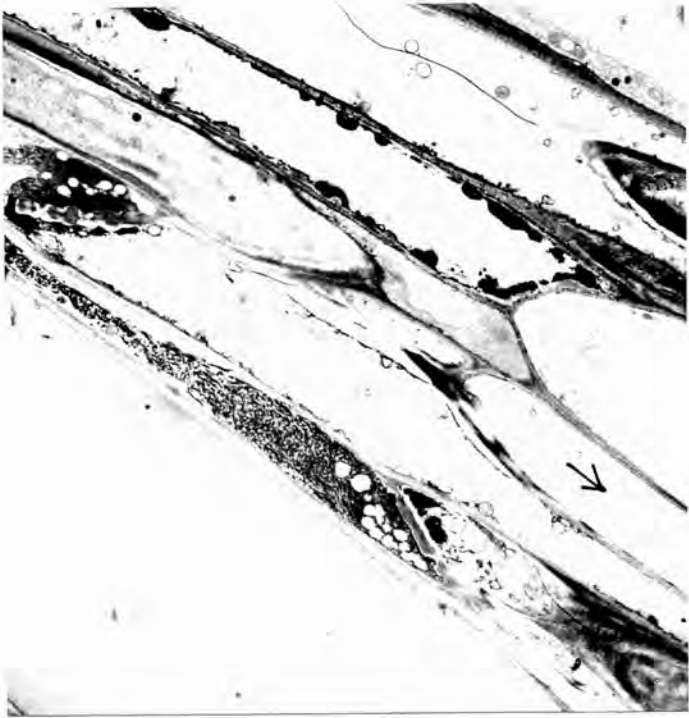


PLATE 1.9

x 3,300

Segment 7. Again surge effects are seen.

PLATE 1.10

x 9,750

Segment 6. Rather less surge effect observed.



The range of images, from those showing obvious disturbance of sieve element contents (Plates 1.8 and 1.9) to those showing little evidence of movement (Plate 1.10), was still seen.

From this work it appeared therefore that the incidence of surge, with the consequent displacement of the sieve element contents was not as unquestionable as frequently indicated. When surge, to any great extent, occurs, the effects are recognisable (cf micrographs already quoted) by the dense masses of P-protein and starch grains trapped unsymmetrically at the sieve plate, and where these are found in any ultrastructural work on sieve elements they may be regarded as indicative of poor preparation, ie no care taken to alleviate the effects of turgor pressure release.

There is no evidence from the micrographs to show whether the P-protein has been forced into previously empty pores by the surge, or whether it was already present.

The micrographs presented in this section are only a very low proportion (less than 0.03%) of those taken, but were chosen as typical representatives of the total.



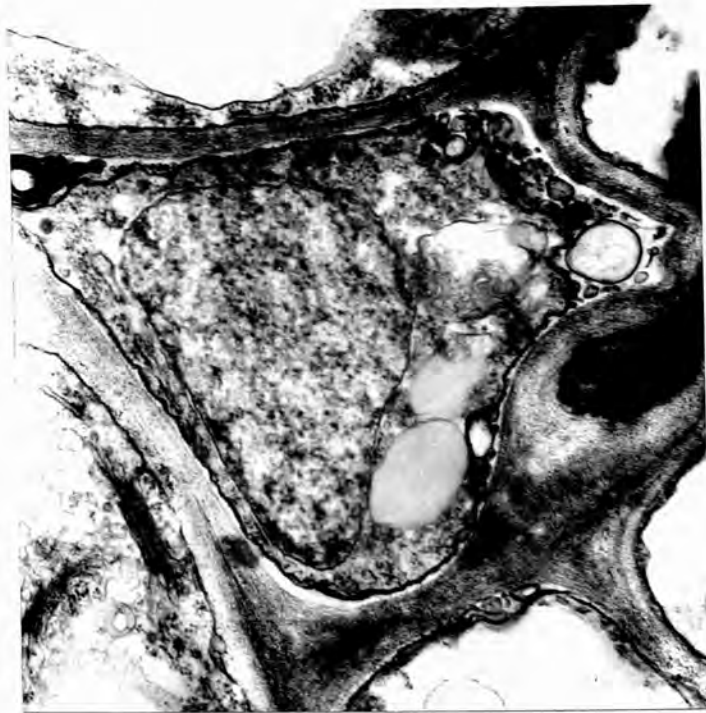
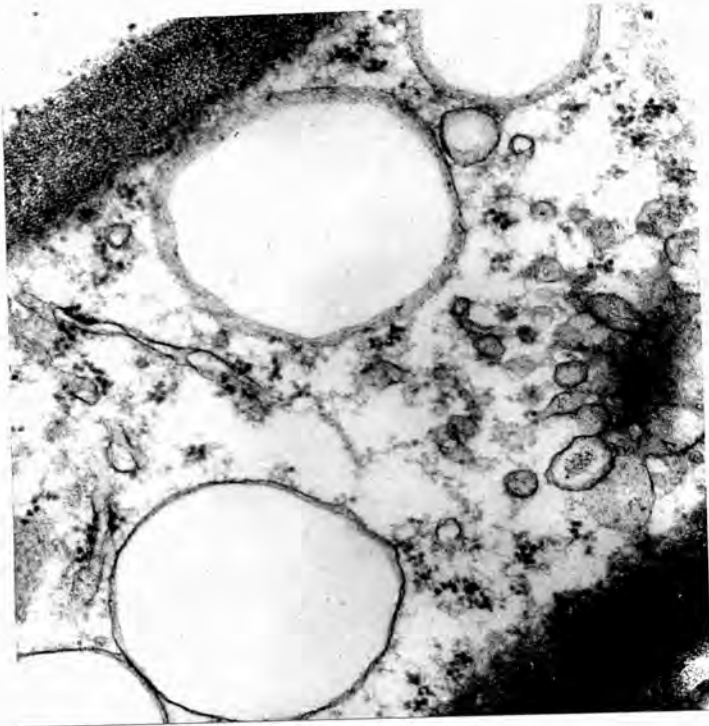


PLATE 2.1

x 54,000

Fixed at pH 6.4. Although ribosomes can be seen  
the ground cytoplasm is very empty.

PLATE 2.2

x 33, 500

Fixed at pH 6.4. The nucleus is hardly more dense  
than the cytoplasm in this companion cell.

## Chapter 2

## CHEMICAL FIXATION

## (a) Experiments with standard fixation at various pH values.

Method. In the first series of observations Sorensen's phosphate buffer was prepared at various pH values (Appendix H). It was used to make up 5% glutaraldehyde fixative with pHs of all subsequent washings, post fixation etc. Portions of stolon about 2 cm long were excised from the middle of the length, and after 1h in fixative the central 1 cm was cut out and subdivided into samples 1 mm long to complete the fixation. Apart from pH variation routine preparation methods were employed throughout (Appendix J).

Results. Well established criteria were used to assess the quality of fixation of the different treatments. It is generally agreed that the cytoplasmic ground substance should appear finely granular, showing no empty spaces. Obviously there should be no plasmolysis. Double membraned organelles should have the membranes intact and parallel. Mitochondria should be neither shrunken nor swollen, having parallel cristae membranes and a dense matrix. The nuclear ground substance should appear uniformly dense between scattered, more electron opaque masses of chromatin. Judged by these criteria, and contrary to what is frequently recommended for plant material, better fixation was apparent at the higher pH values used.

At pH 6.4 the cytoplasmic ground substance appeared very empty. This is very obvious in plate 2.1. Ribosomes were apparent, but rather more empty space was present than is usually seen in well fixed material. The double membranes appeared fairly parallel and a well formed stack of dictyosome membranes is seen in plate 2.2. The nuclear material of the same plate was not so dense as is normally seen. Odd vesicles and



## PLATE 2.3

x 81,000

Fixed at pH 6.4. P-protein fibrils show a clearly banded structure.

## PLATE 2.4

x 27,000

Fixed at pH 6.8. In this companion cell the ground cytoplasm is dense, and the mitochondria look normal.

## PLATE 2.3

x 81,000

Fixed at pH 6.4. P-protein fibrils show a clearly banded structure.

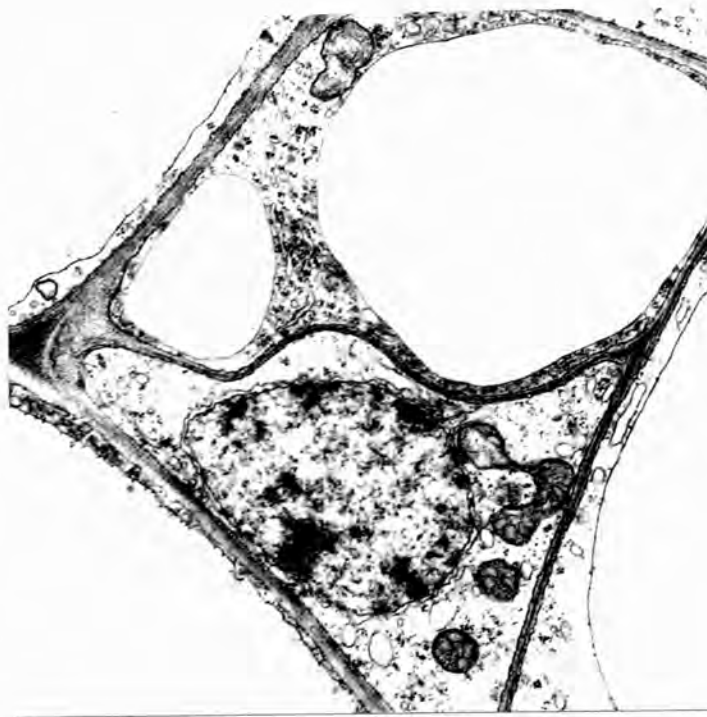
## PLATE 2.4

x 27,000

Fixed at pH 6.8. In this companion cell the ground cytoplasm is dense, and the mitochondria look normal.







## PLATE 2.5

x 20,000

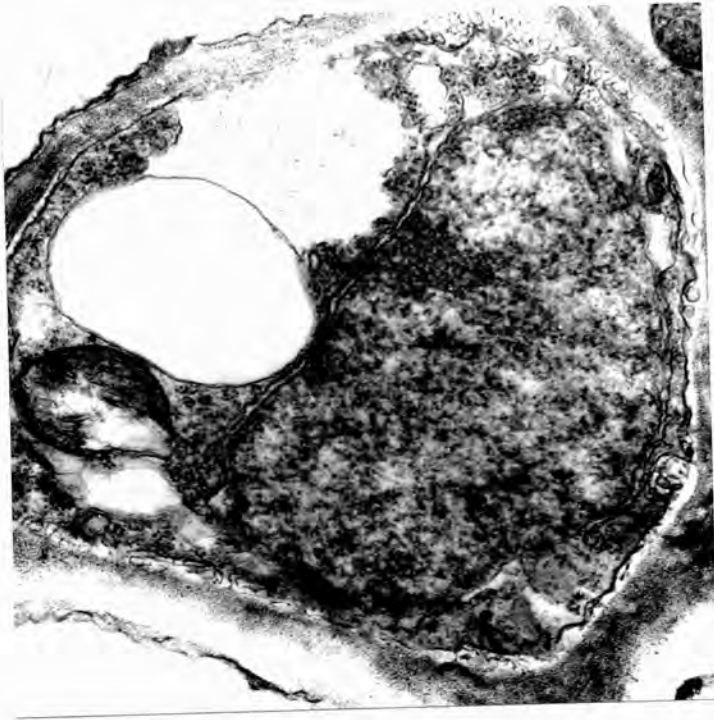
Fixed at pH 6.8. Probably a young, differentiating sieve element. The nuclear membranes are not parallel and the cytoplasm is rather empty.

## PLATE 2.6

x 33, 500

Fixed at pH 6.8. The companion cell appears fairly well fixed, but the mitochondrion on the right has rather more space than is usual in a well-fixed subject.





## PLATE 2.7

x 33,500

Fixed at pH 7.2. The fixation image differs little from those fixed at pH 6.8. Although nuclear membranes are parallel a mitochondrion looks little different from the one in 2.6.

## PLATE 2.8

x 20,000

Fixed at pH 7.2. This is apparently well-fixed although the ground cytoplasm is not so dense as is often observed.

1

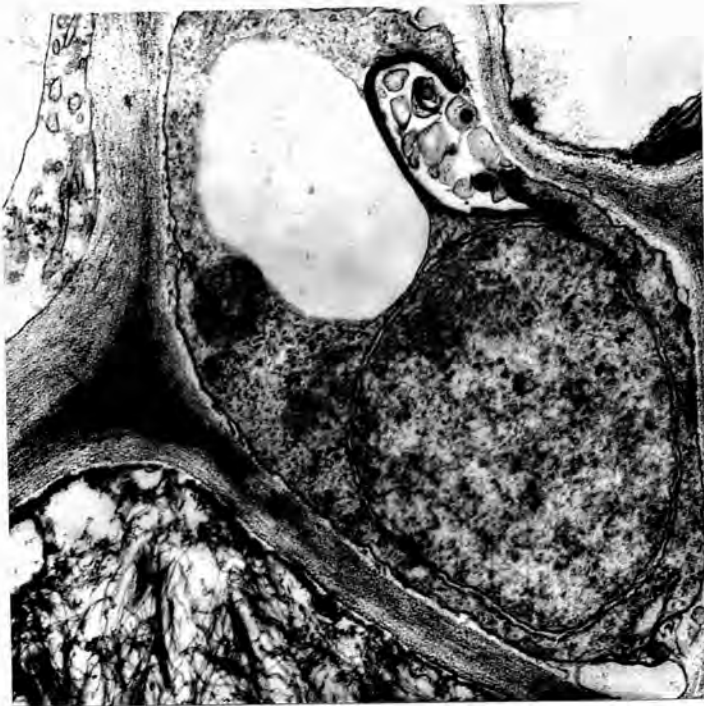
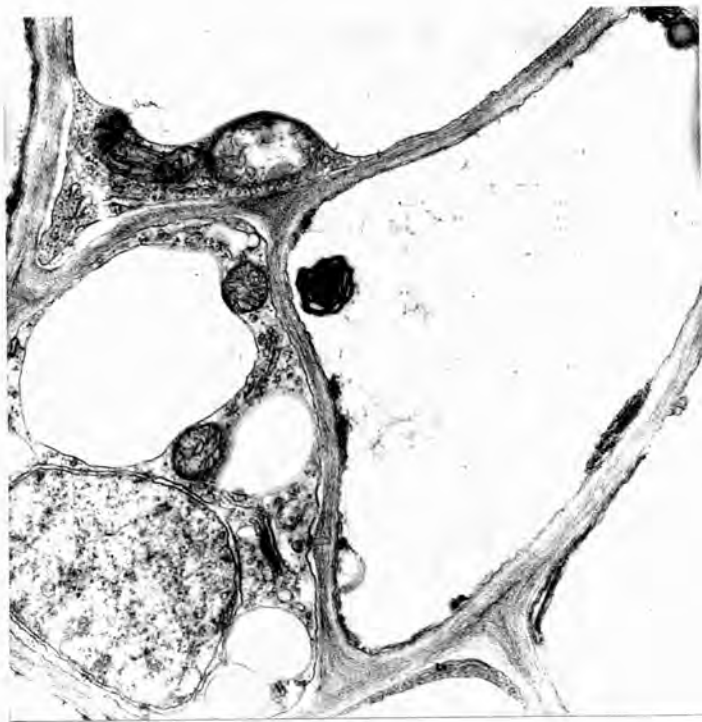




PLATE 2.9

x 20,000

At pH 7.6 good fixation is apparent.

PLATE 2.10

x 33,500

This also seems well-fixed at pH 7.6.

membrane complexes abounded and are evident in both these plates. These are often thought to be artefacts of poor fixation. Thus it appeared that cell contents had been reached out at this pH, and perhaps membranes disturbed. P-protein, however, appeared to have fixed fairly well, showing the clearly banded structure seen in plate 2.3.

At pH 6.8 fixation appeared somewhat better. Both the cytoplasmic ground material and the nuclear material were more dense than at the lower pH, and the mitochondria looked more normal (compare plates 2.4, 2.5 and 2.6 with 2.1 and 2.2). Double membranes were usually parallel as seen in plate 2.6. Vesicles were often observed between the cell wall and plasma membrane again shown in 2.6, but this is a frequent feature of the plant whatever the treatment. The general appearance of this tissue differed little from that fixed at the next pH ie 7.2. This is shown in plates 2.7 and 2.8. However, one of the mitochondria in 2.6 has space in it.

At pH 7.6 membrane preservation appeared to be excellent. The nuclear membranes in all three plates illustrating this pH series are well defined, and parallel as are those of the mitochondria visible in plates 2.9 and 2.11, and also in the dictyosome of plate 2.9. Membrane stacks are also seen in the portions of sieve elements showing in plates 2.9 and 2.10. The ground cytoplasm looked normally granular and and ribosomes were distinct.

As a result of this work, where little apparent difference in the fixation quality showed at pH values of 6.8, 7.2, and 7.6, it was deemed unnecessary to alter the pH of the buffer that was regularly used.



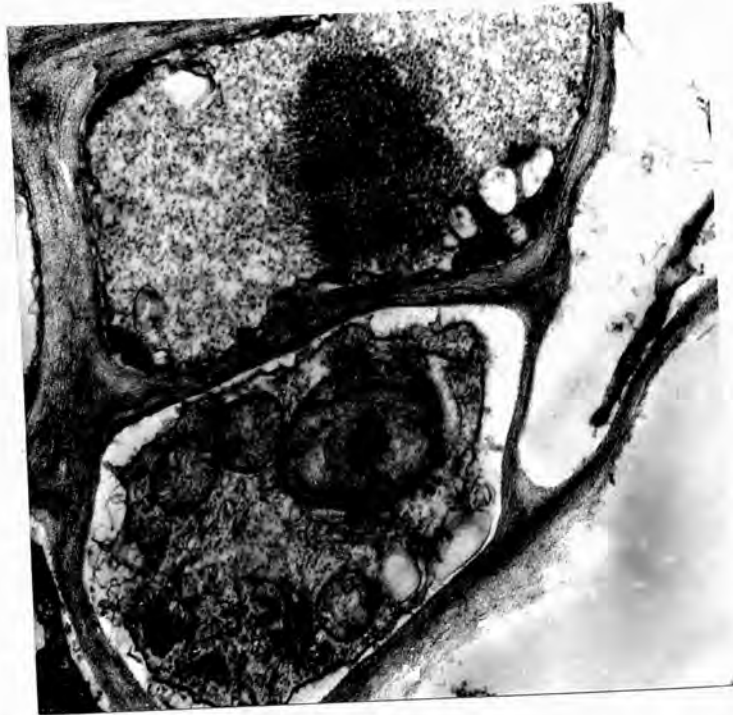


PLATE 2.11

x 27,000

Good fixation again observed at pH 7.6.

PLATE 2.12

x 27,000

Fixed in Pipes buffer. The companion cell is plasmolysed. It is possible that the protein crystal, never observed with other fixatives, may be a fixation artefact.

(b) Comparison of Pipes buffer with the conventional phosphate buffer.

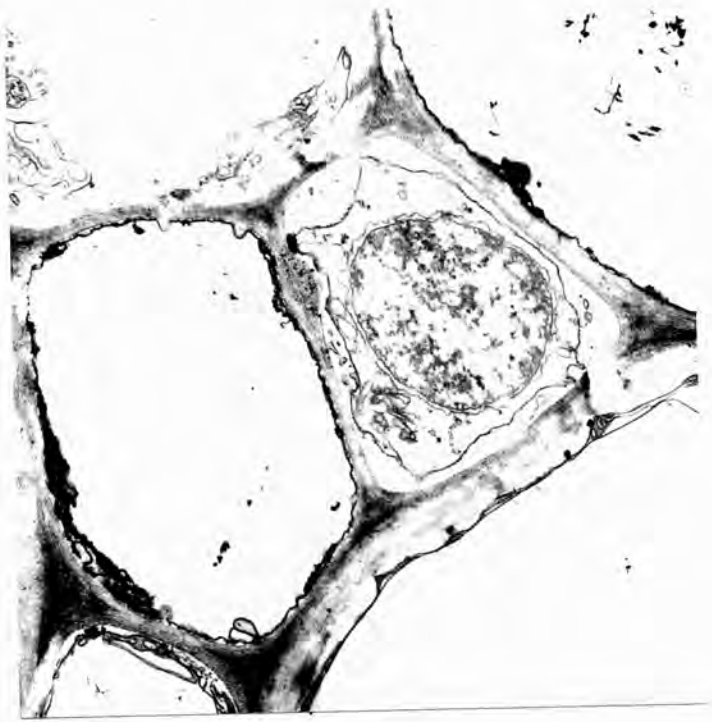
Salema and Brandão's report (1973) on the use of Pipes buffer stressed the importance of adjusting the osmolality of both fixative and rinsing solutions to that of the vascular sap, and also the necessity of using a strong buffer, so that when the somewhat acid fluid is released from the vacuole buffering power in the desired range is not lost. They examined various plant tissues including meristematic and parenchyma tissue of Beta vulgaris, Allium cepa and Solanum pseudocapsicum. They assessed this buffer as being of particular value "in the quality of preservation and the stabilisation of all components", so that loss by leaching was reduced. This last point they also established biochemically. They also considered that good preservation of ground cytoplasm, organelles, and membranes was obtained at a wide range of pH values, from 6.0 - 8.4.

It seemed worthwhile therefore to test this buffer using Saxifraga sarmentosa (L), and three test series were run, making up fresh buffer each time. As the primary interest was in sieve tubes it was necessary to remember the peculiar preparation difficulties, but phloem tissue also contains parenchyma and companion cells, and it was with reference to these cells that the quality of fixation was primarily judged.

Method. The buffer was made up according to the formula given in Appendix K, taken directly from the paper quoted. Excision procedures used were the same as before.

Results. These were frankly disappointing and often very variable, companion cells even in the same section showing widely different degrees of preservation. Evidence of plasmolysis was seen in many of the companion cells. This is seen in plates 2.12 and 2.13. It was noted in a previous chapter that the space between the plasma membrane and cell wall, some-







## PLATE 2.13

x 20,000

Fixed in Pipes buffer. There is every evidence of poor fixation, with the companion cell showing leaching of cell contents, plasmolysis, and having anything but parallel nuclear membranes.

## PLATE 2.14

x 33,500

Fixed in Pipes buffer. This shows better fixation than 2.13 although some leaching of cell contents is apparent. The membrane stack in the sieve tube is more elaborate than is usually seen.





PLATE 2.15

x 27,000

Fixed in Pipes buffer. Apparently well fixed, although the nuclear membranes are not parallel.

PLATE 2.16

x 27,000

Fixed in Pipes buffer. This is taken from the same section as 2.13, but shows far better fixation.

times called the periplasm or extra mural space frequently contains vesicles, and appears to be a definite, non-artefactual structure. Using Pipes buffer many of the periplasmic spaces were more or less empty. The outline of the plasmalemma seen in these two plates was irregular, apparently anchored to the cell wall at intervals, and the space was often large, as seen in plate 2.14 where widely spaced vesicles are present. Often, too, the membranes surrounding the mitochondria and nuclei were not smooth and parallel and the cristae appeared bulbous (plate 2.15). These signs are usually accepted as criteria of poor fixation. Membranes and other sieve element contents appeared much less affected. Ruptured plastids, however, were more frequently seen than when phosphate buffer was used, and since similar steps had been taken to minimise surge, this may have been a significant inferiority. Leaching of ground cytoplasm was observed in many companion cells, giving a rather empty effect, but in other cells, even in the same section fairly good fixation was seen. Plates 2.13 and 2.16 were taken from a single section and fairly close together, and whereas 2.13 is obviously poorly fixed, 2.16 shows well fixed material. The latter type of cell was in the minority however. It has been suggested (Lehmann and Mancuss, 1957) that a granular appearance of cell cytoplasm is indicative of precipitation, and it should be empty. This view is not now generally held, and in this study it is the empty cells which show other fixation artefacts such as irregular membranes and a multiplicity of vesicles especially in the cytoplasm.

The Pipes buffer seems to have had peculiar fixation effects on proteins, although whether it preserves these in more or less natural conditions was difficult to assess.

In many sieve elements P-protein aggregations were observed in apparently crystalline condition, with the components arranged in a

lattice (see plate 2.12). Such crystalline arrangements have only been seen on one other occasion when other fixation methods were used.

Elaborate configurations of membrane stacks were also frequently observed in sieve elements fixed with this buffer. A sample is seen in plate 2.14. Again, such configurations are not unique to this method of fixation, but here they occurred with much greater frequency. Why this was so is not easy to suggest, and it seems unfair to hold it against the buffer.

As a result of these findings using Pipes buffer it was concluded that for phloem tissue, at any rate, this expensive buffer showed no marked advantages over the phosphate buffer normally used, and perhaps had some disadvantages. Its use was therefore abandoned.

## Chapter 3

### ATTEMPTS TO SECURE MORE RAPID FIXATION

#### (a) Decortication of the stolon

Removal of the cortex before fixation must enable more rapid penetration of the fixative. As the cortex of the stolon can be easily removed, with no apparent damage to the stele, and previous work done in these laboratories by da Cruz (1976) had shown that a decorticated stolon continues to translocate, the proposal to remove it seemed a sound one. It was also felt that flooding the stolon with fixative before excision might at least partially relieve the effects of turgor pressure release. A version of this technique has been tried before eg by Engleman (1965), but this author did not confirm that his plant was actively translocating immediately prior to fixation.

Method. The cortex of 5 cm of the middle of the stolon was dissected away from the stele under a binocular dissection microscope. The two regions separated readily at the endodermis. During this operation the stolon was still attached to the parent plant and the region being decorticated was kept immersed in 5% glutaraldehyde in phosphate buffer. The stele was then left in the fixative for a further 1h before the segment was excised and further cut up into 1 mm samples. These samples were postfixated and processed for electron microscopy as usual (Appendix J). Plate 3.1 shows a cleanly decorticated stele.

The effect of decortication on translocation was concurrently investigated. Some 3 - 4h before decortication the leaves subtending the stolons were fed with  $^{14}\text{CO}_2$  as described in chapter 9, and the rate of accumulation of radioactive translocate at the distal end of the stolon was monitored continuously using an end window G M tube.







PLATE 3.1

Approximately natural size

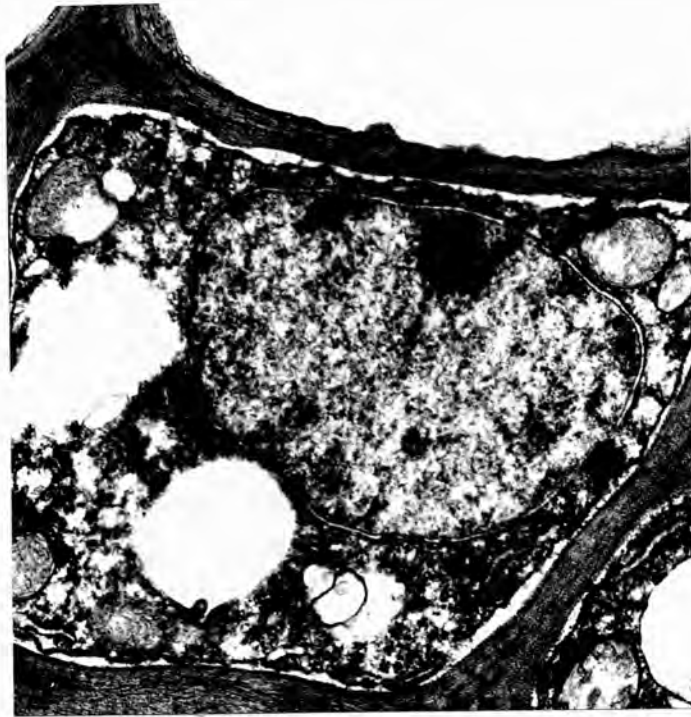
A decorticated stolon.

PLATE 3.2

x 20,000

A companion cell from a decorticated stolon showing  
good fixation.





## PLATE 3.3

x 27,000

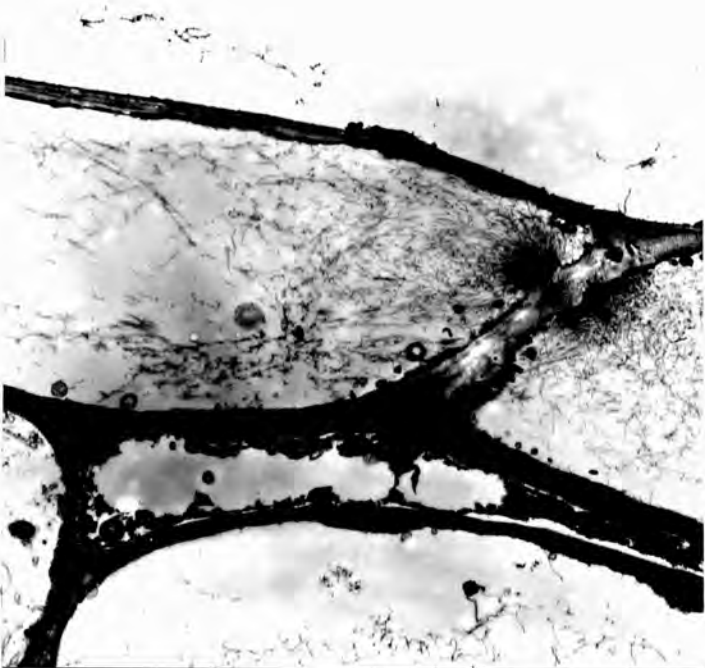
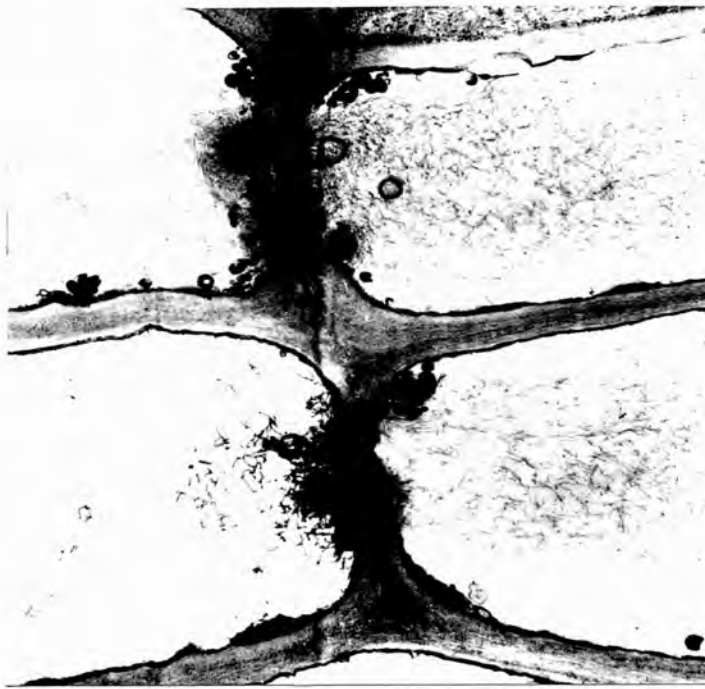
A well-fixed companion cell from a decorticated stolon.

## PLATE 3.4

x 20,000

L.S. of a sieve plate from a decorticated stolon. There is little callose lining the sieve plate pores, and the evenly distributed P-protein shows little sign of disturbance.





## PLATE 3.5

x 9,750

L.S. of sieve tubes of a decorticated stolon.

## PLATE 3.6

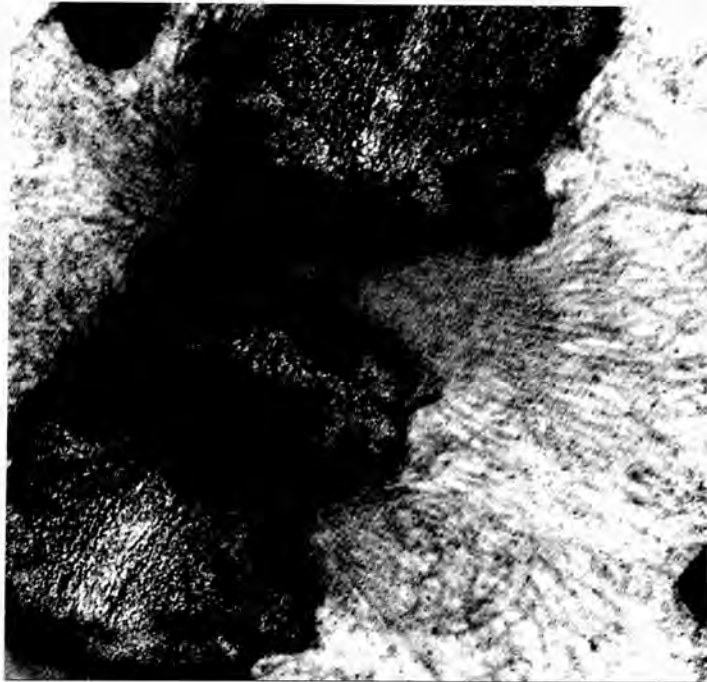
x 9,750

L.S. of a sieve tube of a decorticated stolon.

None of the sieve elements shown on these plates  
show real evidence of surge effects.







## PLATE 3.7

x 4,150

L.S. of decorticated stolon in the phloem region.  
No callose has been deposited on the sieve plate,  
nor is there any evidence of surge effects.

## PLATE 3.8

x 81,000

A sieve plate pore in L.S. from a decorticated  
stolon. There is no callose lining the pore, and  
the P-protein is aligned neatly through the pore,  
with the fibrils extending symmetrically on either  
side.

Results. The companion cells were the principal ones looked at when the quality of fixation was the factor being judged, but in the assessment of surge effects the observation of sieve elements was of course central.

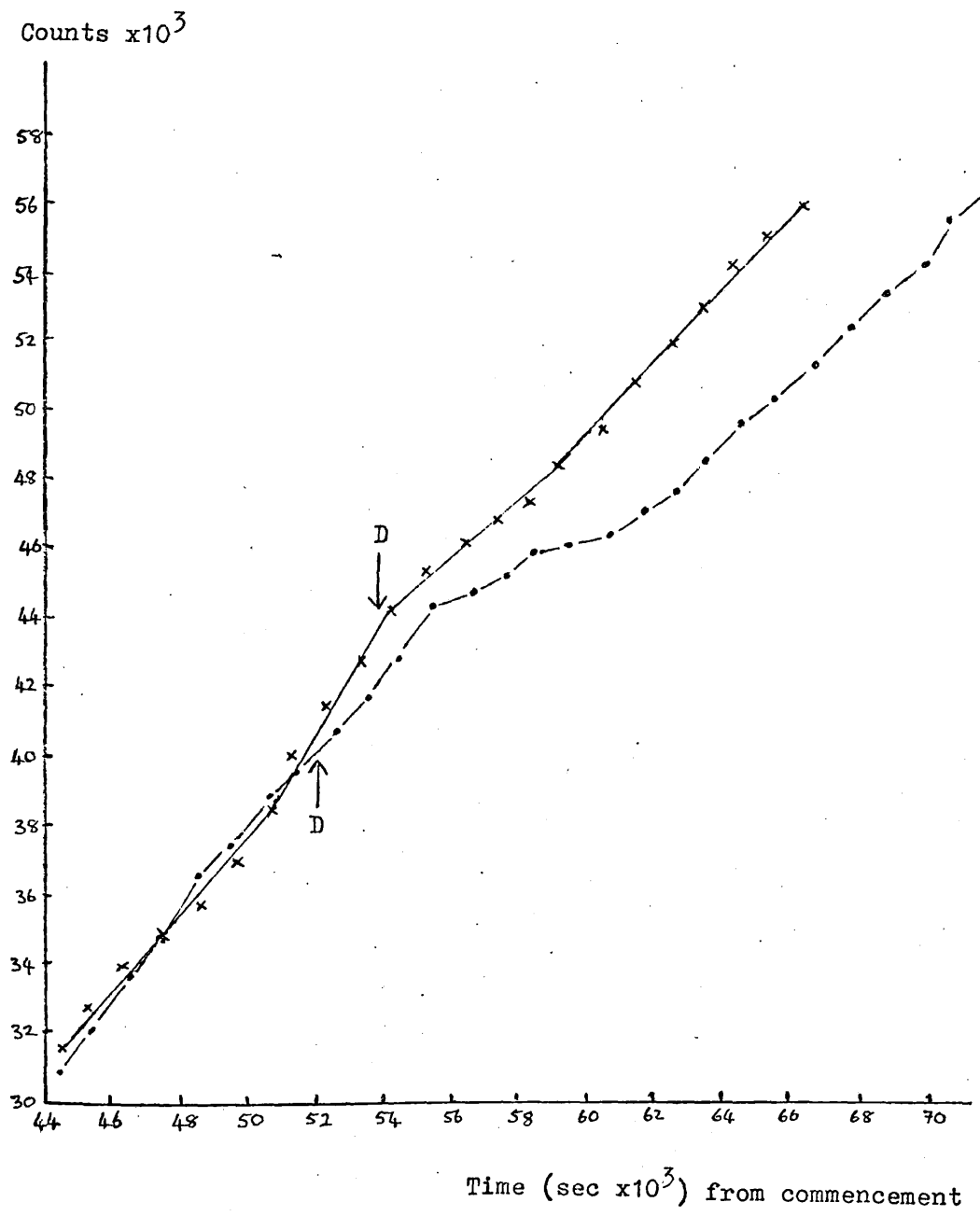
Companion cells showed dense granular cytoplasm. Nuclear membranes were parallel and there was no evidence of plasmolysis (see plates 3.2, 3.3 and 3.7). In the sieve elements shown in longitudinal section in plates 3.4 - 3.8 very little callose is seen lining the sieve plate pores. P-protein was generally evenly distributed through the pores and on either side of the sieve plate (plates 3.4, 3.6 and 3.8). Plastids in the sieve elements were frequently intact, or if the membrane was broken the starch grains had not moved far. Most remained within the plastids indicating that little flow had taken place subsequent to their rupture. The results of this inevitably more rapid fixation on unexcised tissue show every evidence of material being well fixed, with surge effects kept at a very low level. It was therefore decided, that provided normal translocation was maintained for some hours after decortication, this method of fixation was worth pursuing in preparing the material used in the subsequent work.

The physiological work was as follows: after feeding the subtending leaf with about 50  $\mu\text{Ci}$  of  $^{14}\text{CO}_2$  translocation was allowed to proceed in the light for some hours, until the print out of the radioactive counts at the stolon apex showed a steady build up of radioactivity. The centre of the stolon was then carefully decorticated for 5 - 7 cm and covered with fine, vaselined polythene to prevent drying out.

The graphs included in chapter 10 show that in some instances translocation appeared to slow up for a short time during the actual operation, but normal translocation rates soon resumed and were

The effect of decortication  
on translocation.

Fig.2



maintained for as long as any experimental work was likely to continue, as shown in figure 2.

From this time forward therefore, material for examination was taken from decorticated stolons treated with fixative before excision, as described above. Care was always taken in this to allow time for recovery from surgery before inhibitory treatments were started.

(b) Use of chloroform in the fixative.

Work undertaken some years previously had, as mentioned earlier, indicated the value of chloroform applied to the functioning stolons in small concentrations, in reducing surge effects by permitting slow lateral leakage of the sieve tube contents (Hogben and Spanner, 1977). In this work entire stolons had been exposed to the vapour. It was decided that if a low concentration of chloroform was included in the fixative itself, the latter might penetrate the tissue more rapidly, and at the same time reduce longitudinal surge even further, as membranes were weakened gradually, but uniformly. Contributing to this conclusion was the discovery of the nature of the water resisting properties of the endodermis, owing to its suberised nature. If the lipids of the suberm could be loosened very rapid penetration might ensue.

Method. Initially a test series using different strengths of chloroform in the fixative was run. The chloroform delutions were prepared as previously described (1977) using phosphate buffer instead of distilled water. Solutions were used with 10%, 30%, 50%, 70% and 90% saturated chloroform water in the buffer, but these were diluted by  $\frac{1}{5}$  of their volume when used to buffer the glutaraldehyde. A control using no chloroform was also used. Twelve similar stolons were matched in pairs and decorticated for about 3.5 cm. One of each pair was decorticated in the fixative containing chloroform whilst it was still attached to the plant, and the other was







## PLATE 3.9

x 13,300

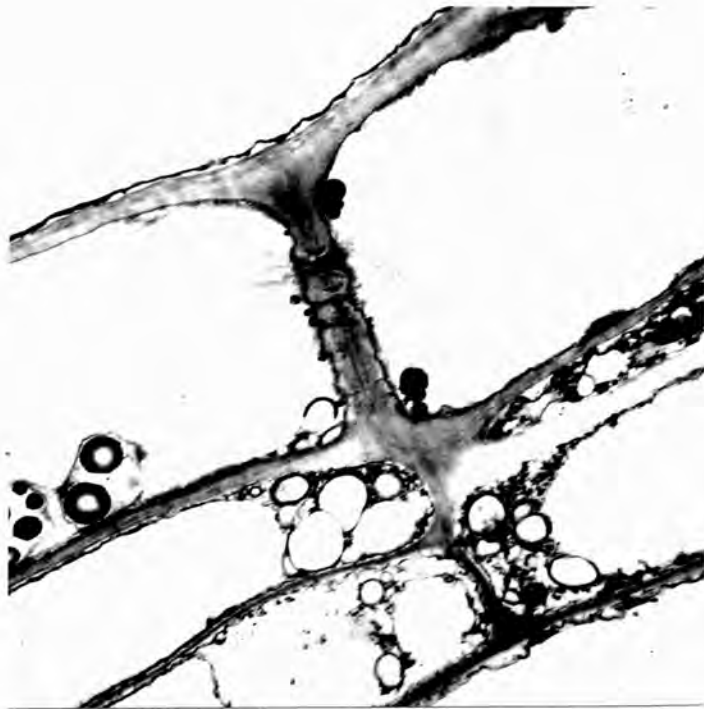
Decorticated and fixed in 5% glutaraldehyde containing 70% chloroform water before being excised from the stolon. In this high concentration of chloroform the P-protein is de-natured.

## PLATE 3.10

x 6,700

Decorticated but excised from the stolon before fixation in 5% glutaraldehyde containing 10% chloroform water. Again the P-protein is de-natured.





## PLATE 3.11

x 9,750

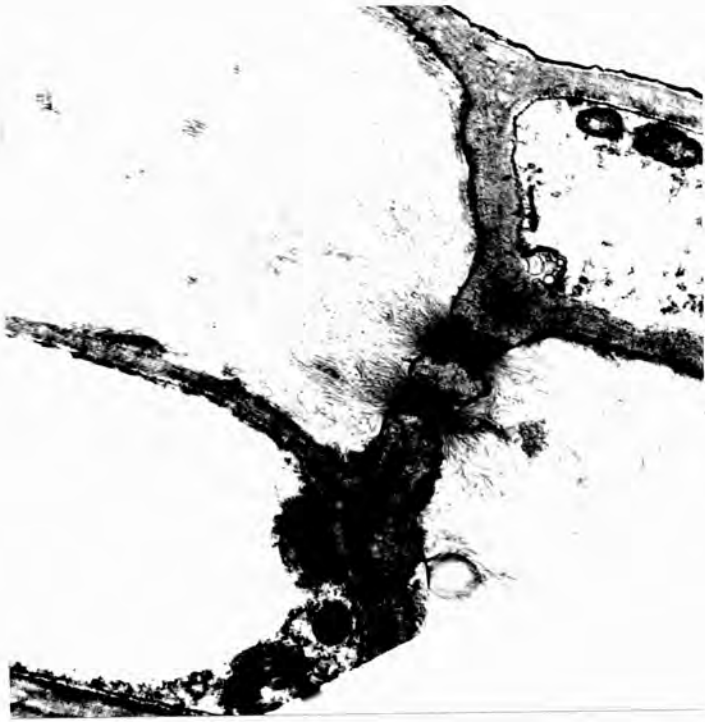
Stolon excised before fixation in 5% glutaraldehyde + 30% chloroform water. The P-protein is in fairly good condition, but surge effects are evident, also decorticated.

## PLATE 3.12

x 6,700

Stolon decorticated and fixed in 5% glutaraldehyde + 10% chloroform water before being excised. No surge effects are seen, and plastid membranes are intact.





## PLATE 3.13

x 20,000

Both the micrographs on this page show decorticated stolons fixed before excision in 5% glutaraldehyde + 30% chloroform water. No surge effects or denaturing of the P-protein are seen here.

## PLATE 3.14

x 54,000

The P-protein shows no denaturing. No callose lines the sieve plate pores.

excised within the decorticated region before placing in similar fixative. After  $1\frac{1}{2}$ h in fixative the decorticated stolons were cut into 1 mm segments and the normal preparation routine was completed.

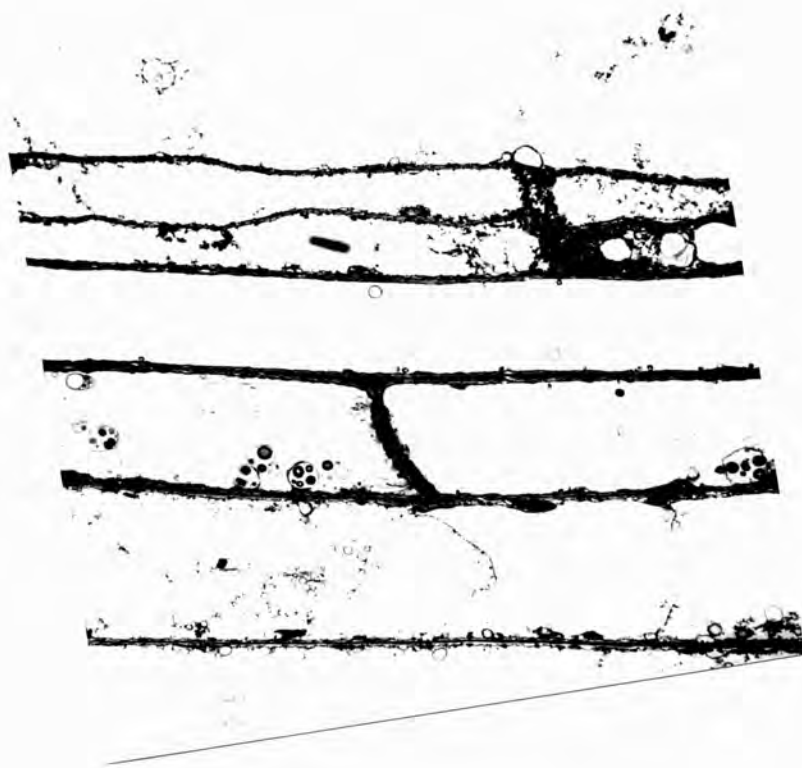
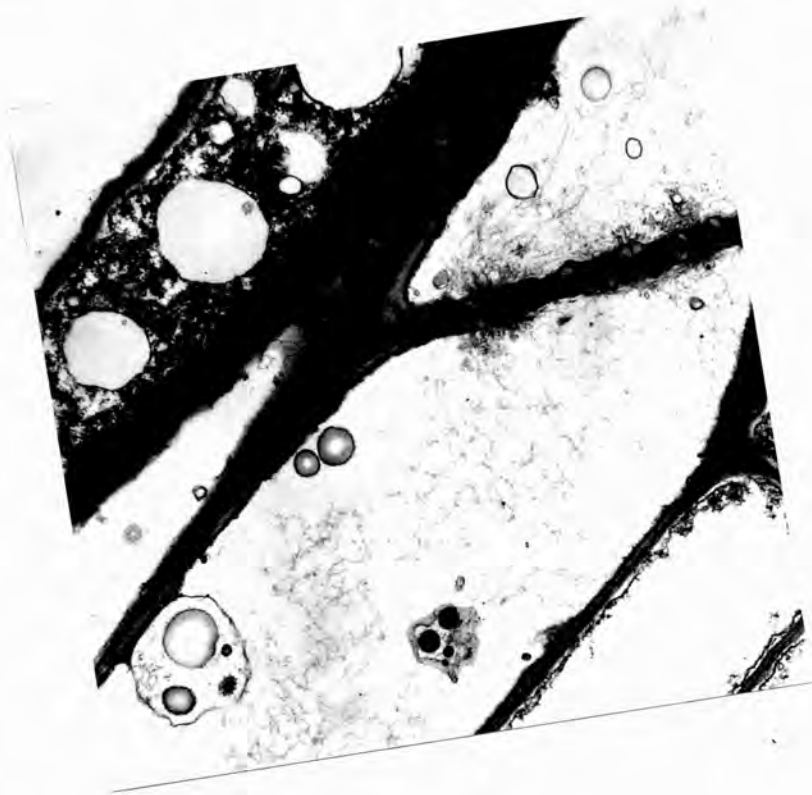
Results. The fixative containing chloroform at the two lowest concentrations showed the best fixation when judged by the criteria previously cited in chapter 2. At higher concentrations the P-protein showed signs of coagulation (plate 3.9). Those stolons which had been cut from the plant prior to fixation showed similar signs of coagulated protein, even at lower concentrations (plates 3.10 and 3.11), but possibly the sections had been obtained from nearer the cut ends. Broken plastids and non-uniform distribution, even of coagulated protein indicated surge effects (plate 3.10). Even where surge effects were not pronounced (plate 3.11) in these predetached stolons there were still more than were seen in stolons unexcised before fixation. Incidentally the strength of chloroform in the buffer of the stolon fixed whilst attached in plate 3.9 was 70%, whereas the coagulated material shown in the predetached stolons of plates 3.10 and 3.11 were subjected to 10% and 30% respectively.

Those stolons fixed in low chloroform concentrations whilst still attached to the parent plant showed every evidence of good fixation. The small portion of companion cells shown in plate 3.12, having 10% chloroform in the buffer, displayed no sign of plasmolysis and plastids were entire. More controversially the P-protein fibrils were evenly distributed through the sieve plate pores (plates 3.13 and 3.14), and as in the previously reported experiments with chloroform (Hogben and Spanner, 1977) little callose was seen lining the sieve plate pores.

In view of the results obtained from this preliminary series of experiments a second, rather more elaborate series was using inter-







## PLATE 3.15

x 9,750

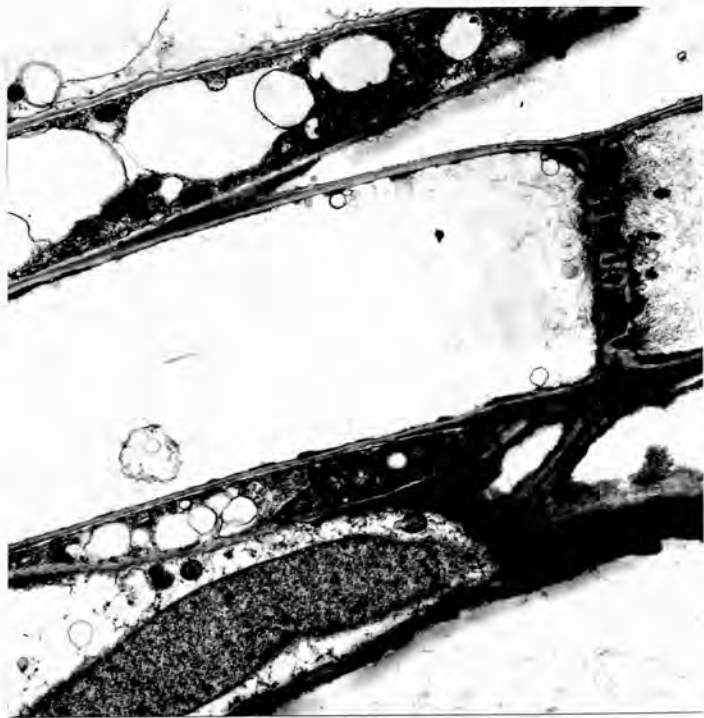
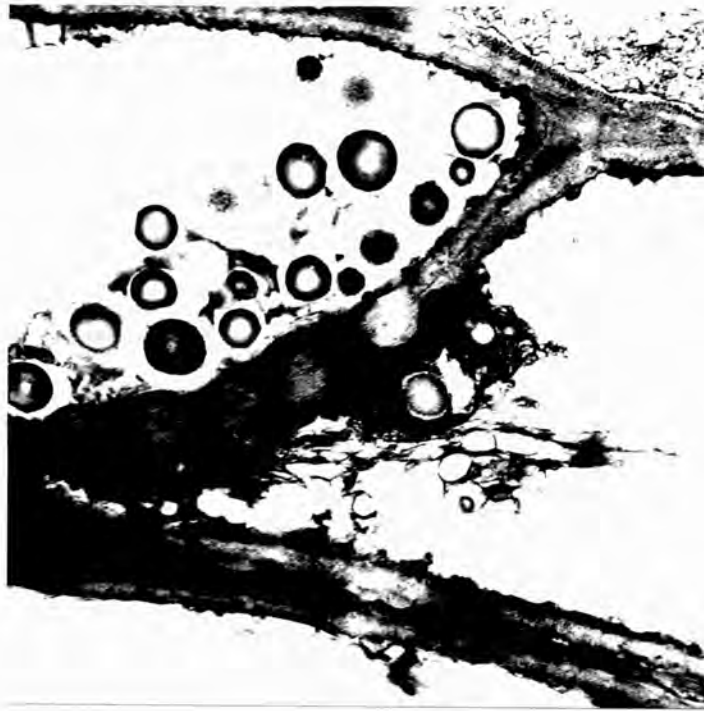
Although both the micrographs shown on this page are of sections excised before fixation in 5% glutaraldehyde + low concentrations of chloroform water there is no sign of pressure release effects.

## PLATE 3.16

x 3,300

The plastids are intact on either side of the sieve plate.





## PLATE 3.17

x 17,500

Fixed before excision as 3.15 and 3.16, but showing evidence both of surge and de-naturing of the P-protein. Possibly the section was taken from very close to the cut surface.

## PLATE 3.18

x 6,500

Decorticated and fixed prior to excision in 5% glutaraldehyde + low chloroform concentration. The companion cell is well fixed, and the sieve tube is apparently unaffected by surge.

mediate strengths of chloroform in the buffer used to make up the fixative (15% and 25%), and comparing the effects shown in the central portion of the stolon segment with the outer region, ie near the cut ends.

Method. Again, paired stolons were fixed as before, one of each pair being cut before fixing. Additionally the centre of each decorated segment, its length being about half the total ie about 1.5 cm, was separated after fixation from the ends which together comprised about 1.5 cm and the two regions were further prepared separately.

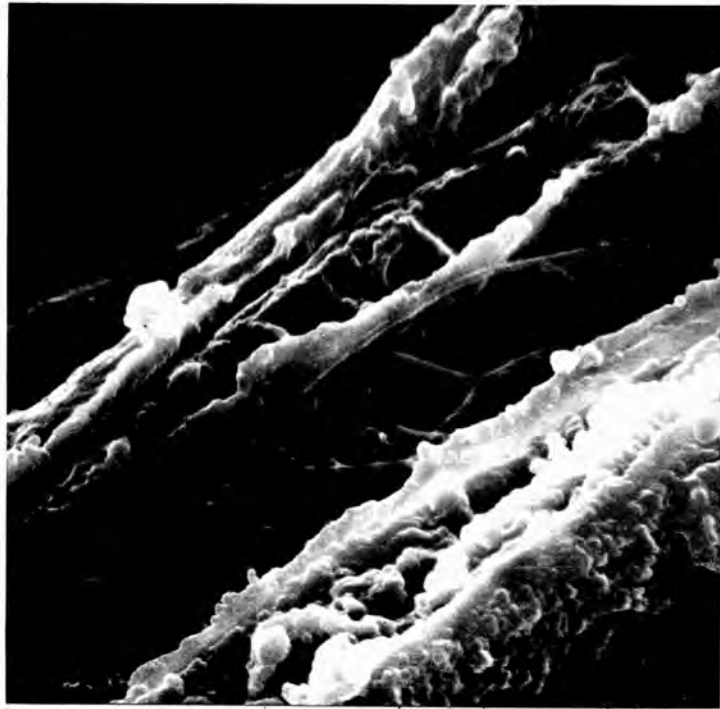
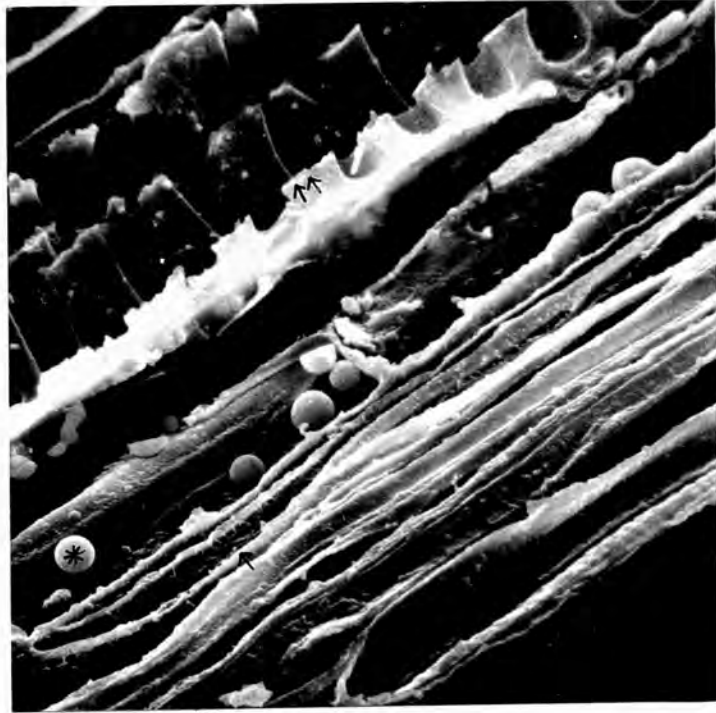
Results. Plate 3.15 shows the central region of the stolon which was fixed in buffer containing 25% chloroform before adding the glutaraldehyde. There was little or no evidence of surge effects. Plastids were intact and the P-protein was evenly distributed through the pores and within the lumen of the sieve tubes. Plates 3.16 and 3.17 were taken from near the ends of pre-detached stolons and more variable images were observed. There was no evidence of surge effect, but every indication of good fixation in plate 3.16, but 3.17 showed ruptured plastids, with the P-protein flattened on one side of the sieve plate, and streaming through on the other, typical of turgor pressure release. All the material in this experiment, which had been fixed whilst still attached to the plant showed excellent fixation, with little obvious surge effects. Plate 3.18 shows part of a companion cell and almost certainly another one as well, which were apparently very well fixed. The strength of chloroform solutions used in this series appeared to make little difference, the important factor being fixation before excision.

These results thus showed that even better fixation of the phloem of an attached stolon could be attained when the fixative had a low concentration of chloroform in it. All the experimental work

subsequently performed therefore included chloroform (less than 30%)  
in the buffer used to prepare the fixative.







## PLATE 4.1

x 1,500

Scanning electron micrograph of the phloem region.

Xylem appears obviously across the top left hand corner.

## PLATE 4.2

x 5,000

The cobwebby effect is probably P-protein. The dense material in the cell across the bottom right hand corner may well be tannin.

## Chapter 4

### SCANNING ELECTRON MICROSCOPY

In the search for information on functioning sieve plates it was felt that the image shown by the scanning electron microscope might be useful in clarifying their condition after experimental treatment. Identifying sieve plates at all however, proved very difficult in this mode, but it led on to further interesting work reported in chapter 6.

Method of Preparation. The material first tried was decorticated stolon, in fact the same material as was prepared for the work reported in chapter 3 (a). It was chemically fixed, post-fixed and dehydrated. At this stage some of the material was removed from the phial and split longitudinally. After critical point drying (see Appendix M) the material was mounted, cut surface uppermost, on stubs before being gold sputter-coated (Appendix N). The specimens were then viewed using a Cambridge S4-10 Stereoscan microscope.

Results. The low power micrograph shown in plate 4.1 demonstrates the relationship of the tissues in the stele as seen in this instrument. When this is compared with optical micrographs, especially plate 9, it is evident that elongated elements in the position expected for sieve elements frequently show a network of filaments inside the lumen of the cells, see arrows on plate 4.1. This network was only observed in long, narrow elements and none was seen in obviously xylem tissues (double arrows), nor in the wider parenchyma cells. The identification of the last as parenchyma is aided by the fact that they contain plastids (see \* on plate 4.1). This was also noted in the optical micrographs. Some of the more 'crusty' looking cells, apparently having either dense contents, or some deposit on the tissue



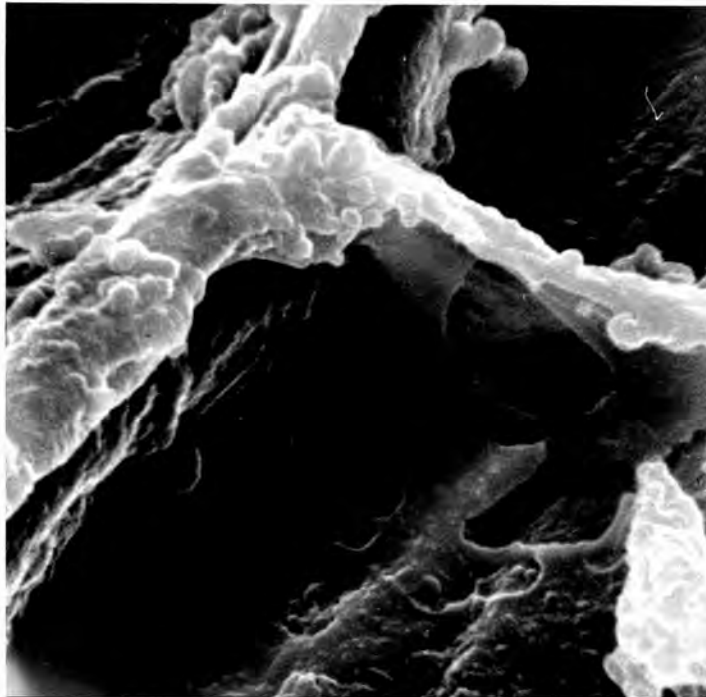


PLATE 4.3

x 3,900

Cobwebby P-protein in a sieve tube.

PLATE 4.4

x 11,000

Probably a dried down tonoplast.

may well be tannin cells which abound in the vascular tissue of this plant. Such cells are seen adjacent to the sieve elements in plates 4.2 and 4.3.

Within most of the phloem elements is seen a cobwebby network (plates 4.1 - 4.3). This appeared to be composed of bundles of filaments, probably P-protein, as described by Johnson et al (1976) in Nymphoides peltata. Suggestions that they might be made up of dried down, ruptured membrane material have been discounted after finding cell contents (plate 4.4) which appear indubitably composed of membrane material, dried and ruptured. This could well be showing a tontoplast, as seen from the vacuole side. In no way do these resemble the aforementioned network. If this cobwebby material does consist of bundles of filaments of P-protein it must be assumed that the transverse wall of an element containing it must be a sieve plate although no pores may be distinguishable. Such putative sieve plates were viewed from all angles in the attempt to identify pores, making full use of the tilt and rotation of which the instrument was capable. However, the results were not altogether satisfying. In order to clarify this point two further lines of investigation were therefore pursued.

(a) Digestion of the protein. It was thought that pore identification might be hindered precisely because the fibres thought to be P-protein densely occluded them. Digesting the protein away suggested itself as a possible solution.

Method. Three enzymes, pronase, papain and pepsin were prepared as 0.5% solutions in phosphate buffer. The first two were buffered at pH 7.2, but unfortunately no record was kept of the pH for the pepsin. As the methods used by Deshpande (1976) was closely followed it was probably buffered at pH 3.5 using citrate buffer. Stolon segments about 1 mm long were incubated in these solutions for 2h





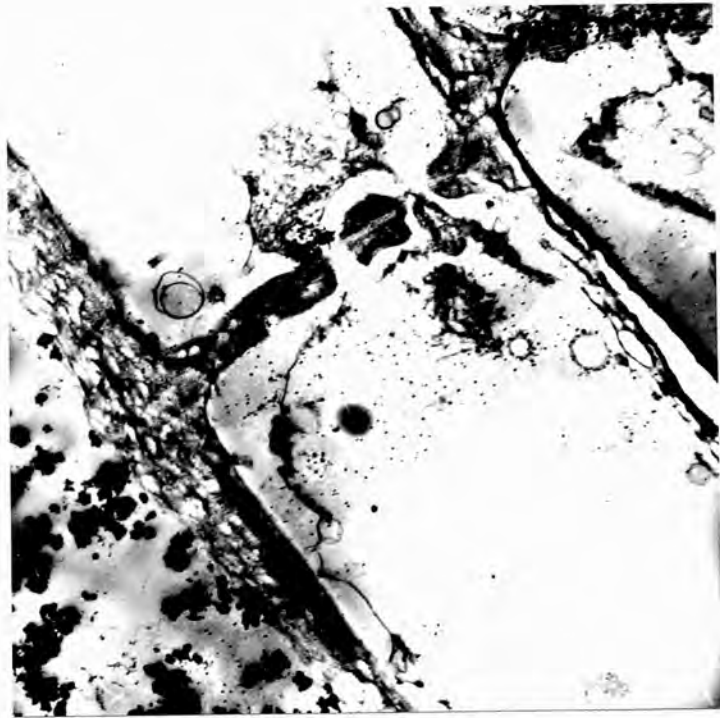


PLATE 4.5

x 13,300

P-protein digested in papain for 2h.

PLATE 4.6

x 13,300

P-protein digested with pronase for 2h.



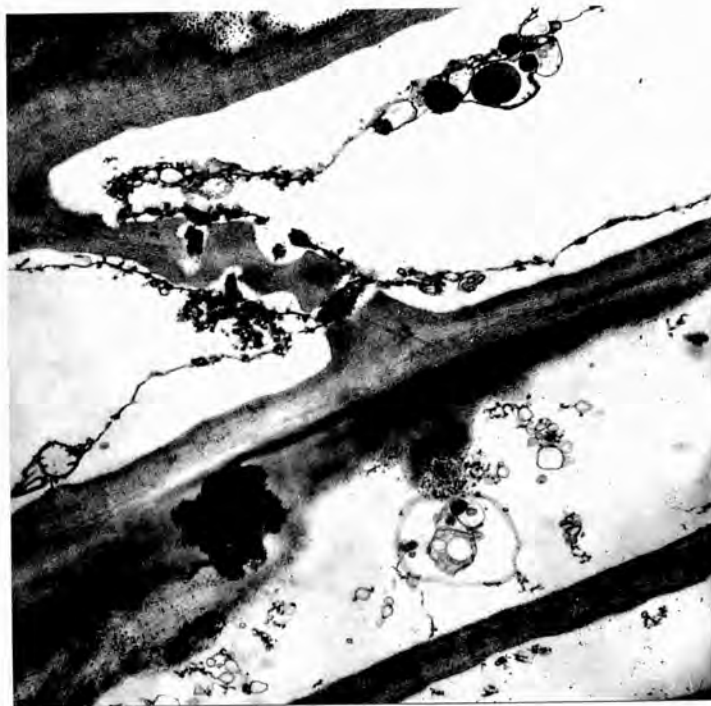
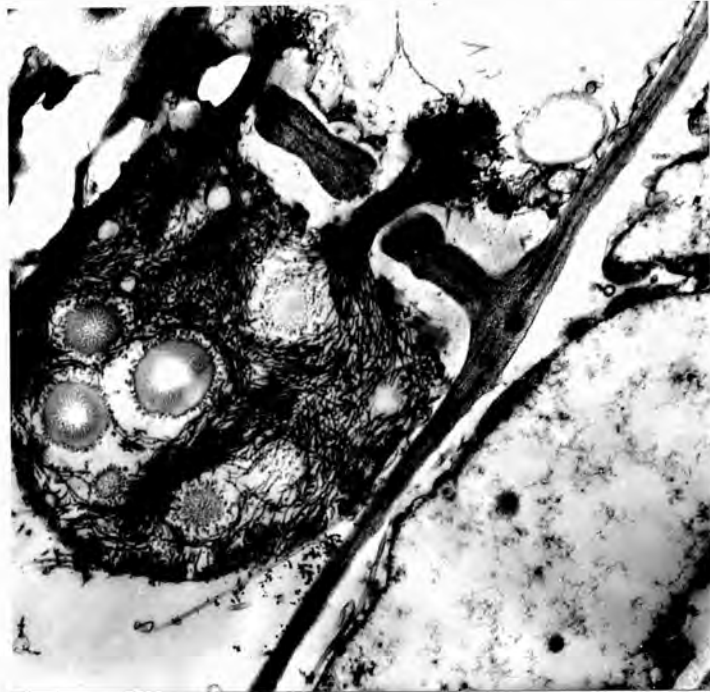


PLATE 4.7

x 13,300

P-protein digested with pepsin for 2h.

PLATE 4.8

x 13,300

P-protein digested in pronase for 14h.



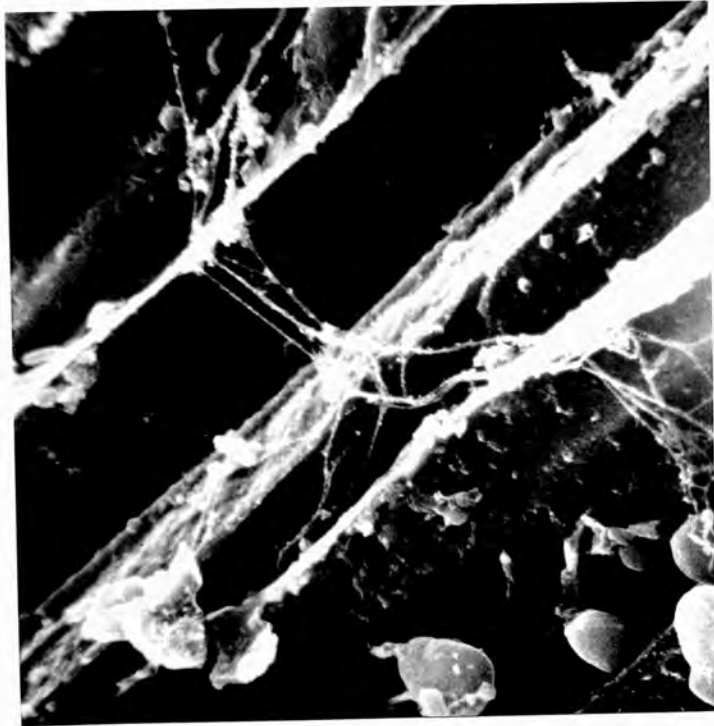
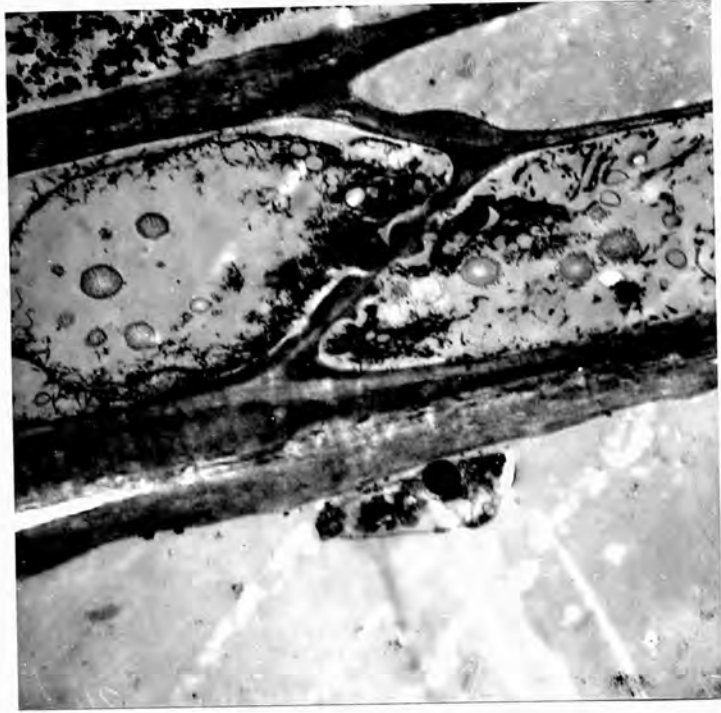




PLATE 4.9

x 9,750

P-protein digested with papain for 14h.

PLATE 4.10

x 5,000

After 14h. digestion with pronase fine filaments are seen extending between cells. It is thought they may be fungal hyphae grown during the incubation.

at 37°C. The material was then prepared for ultrastructural examination.

Results. T.E.M. pictures of the material made it evident that little protein digestion had taken place. Plates 4.5, 4.6 and 4.7 show the results of this series of treatments. Plate 4.5 shows material treated with papain, and a certain amount of digestion appears to have taken place. Some fragments of protein can be seen, but not many. Plate 4.6 and 4.7, for which pronase and pepsin were the proteolytic enzymes used respectively show less evidence of protein digestion. The protein fibrils in 4.6 appear shorter than those normally observed so some digestion by pronase may have occurred, but in plate 4.7 long fibrils are apparent, an indication of little or no digestion by the pepsin.

It was decided that digestion probably needed a longer time than had been allowed in the previous trial, so a further experiment was set up using only two of proteases, papain and pronase. Pepsin was abandoned as previously no protein digestion appeared to have taken place. The material was incubated at 37°C in these proteases for 24 h, and the tissue was evacuated to aid enzyme penetration.

Further results. The protein in the sieve tubes shown in plates 4.8 and 4.9 as seen in the T.E.M. was certainly denatured by was still very much in evidence, having been only partially digested. This time the pronase (plate 4.8), appeared to have been the more efficient protease. When viewed in the S.E.M. (plate 4.10), it was evident that the general condition of the material was poor. It did not split cleanly, probably due to denaturing of cell wall material by the protease, and the tissue was difficult to recognise. Crystals, probably from the buffer, were plentiful. Filaments, finer than previously seen were observed, but as they passed from cell to cell they were assumed to be contaminants, possibly fungal which

may have entered as spores, germinated and grown during the long incubation period.

Comments. Although it proved unsatisfactory on this occasion the digestion of the P-protein should be possible and more time developing the technique could be well spent. There are several possible improvements to the methods used which could be tried. More careful preliminary experimentation as to the optimal pH required for each protease used, as well as the most satisfactory buffer for each might improve the technique. It is possible that the sieve plates were not very accessible to the large enzyme proteins. Use of fairly thick (0.25 mm) longitudinal sections of stolon, or even segments split longitudinally in half would give better enzyme accessibility. It may also be of more value if sieve plates known to be near the end of treated segments were examined.

Such improvements might reveal sieve plates in the S.E.M. but are very time consuming and were not further pursued in the present investigation.

(b) Etching blocks previously used for T.E.M. A rather different attempt to identify sieve plates under S.E.M. was made by using fluids to etch the surface of blocks previously used for transmission work.

It was assumed, and subsequently the assumption proved correct, that the corrosive fluid would at least partially destroy the P-protein. In theory this should help to expose any sieve plate pores.

Method. The etching fluid used was made up according to the recipe of Lane and Europa (1965) given in Appendix L. The tips of the blocks were allowed contact with the fluid for times ranging from 15 seconds - 5 minutes. The longer times ie 3 - 5 minutes, proved most satisfactory, but varied, apparently according to the hardness





PLATE 4.11

x 500

Etched block

PLATE 4.12

x 500

Etched thick section

of the resin in the block. The material was then cut from the top of the block, rather roughly, but usually including the whole sample in the block. It was mounted on a stub and gold sputter-coated (Appendix N) before viewing in the S.E.M.

Results. These were unexpectedly variable. Sometimes the actual tissue structure appeared to have been eroded, whilst others etched for the same length of time showed much resin still obscuring part of the specimen as in plate 4.11. Once again searching the specimens failed to reveal any obvious sieve plates.

Although this method did not prove very helpful in sieve plate identification, the idea of dissolving resin, not just from a block surface, but from a thick (approximately  $2\mu\text{m}$ ) section, was born from it.

Such sections cut from tissue which had been digested with protease for 24h before fixing and embedding were first used. Clear micrographs of the material were obtained, with easily recognisable elements (plate 4.12). This micrograph shows the xylem across the top right hand corner. The irregularly shaped cells (arrowed) across the bottom left hand corner are part of the endodermis. Many of the other cells are parenchyma cells. Those filled with irregularly globular material may well be tannin cells, whilst those long narrow cells towards the bottom of the micrograph are almost certainly sieve elements. This technique showed so much promise that no further etching of blocks was carried out, but the section etching was carried further in an attempt to correlate T.E.M. work directly with the S.E.M. observations. The results are described in the next chapter.

## Chapter 5

## CORRELATION OF TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

Since the scanning electron microscope gives a picture of the surface of a tissue it has been widely used to demonstrate the structure of naturally exposed surfaces, or those exposed by fracture or dissection. Much of the early work done on plants concerned wood, which being a relatively robust material is easy to prepare. A good deal of the later work done on internal structures of plants has employed freeze-drying together with freeze-fracturing. Excellent S.E.M. results have been obtained using these techniques (Johnson et al, 1976, their plates 4, 5 and 6, and Troughton and Donaldson, 1972), but once used the same specimen cannot be subsequently examined in the T.E.M. The nearest comparison which can be made involves replicate specimens.

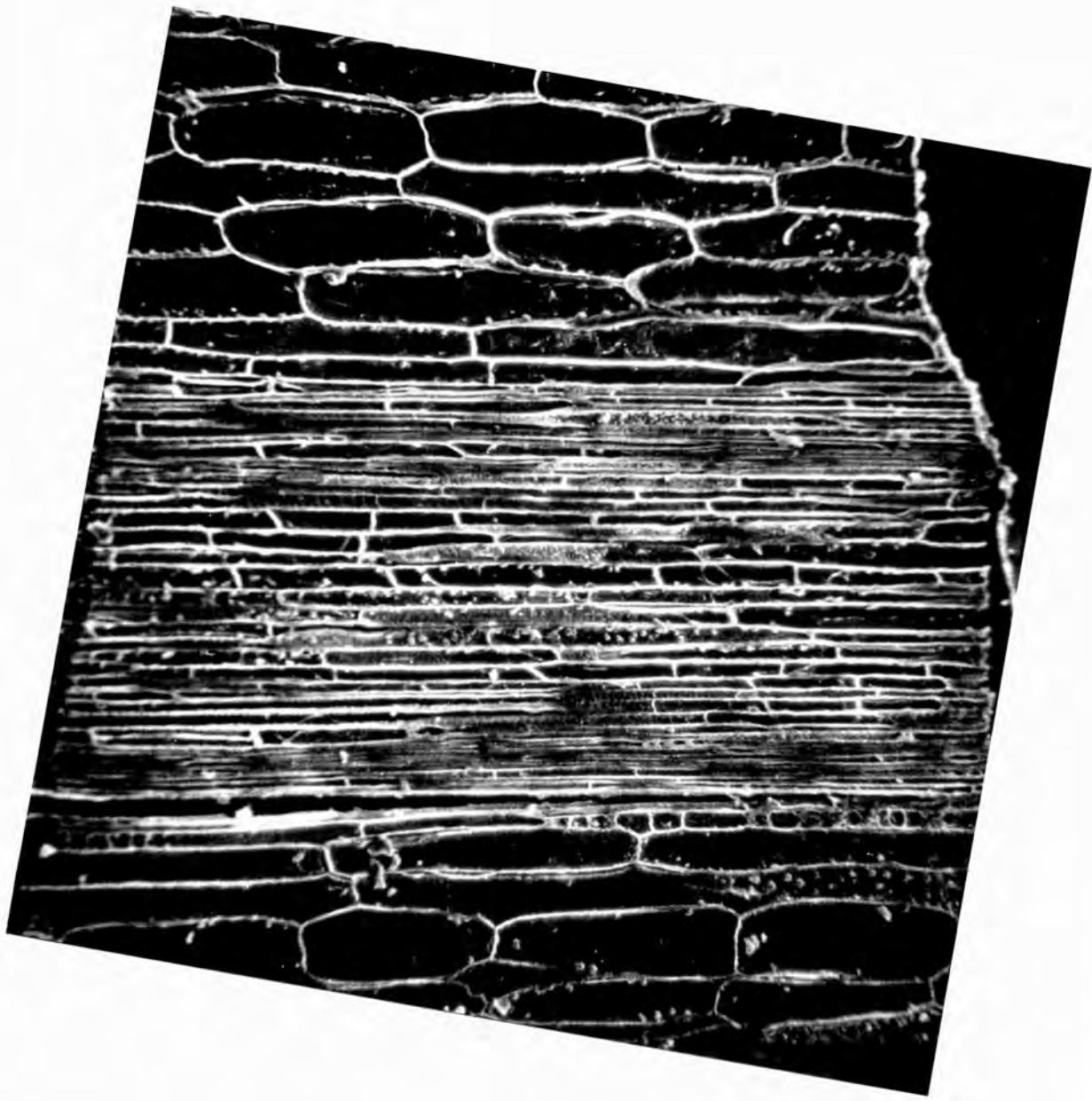
Attempts have been made to first section blocks for T.E.M. work, and then to etch the face of the block for the S.E.M. (Shih 1974), but although some correlation between the two techniques is possible only one valid comparison can be made per block.

In the last chapter it was described how thick sections were etched for S.E.M. work. This suggested the possibility of cutting alternate thin sections on a grid for the T.E.M., and thick ones for etching and viewing with the S.E.M. The unexpected difficulty in identifying sieve plates using the more conventional S.E.M. methods prompted the idea of finding sieve plates on the thin sections, mapping their position on the section, and then looking for corresponding transverse walls in similar positions on the thick sections with the S.E.M. Their appearance should at least provide a clue as to the aspect they present in this mode.

The material used for this work was that already prepared when the geography of the stolon was investigated, as reported in the prologue.







## PLATE 5.1

x 135

Etched section. Micrograph enlarged to allow phloem regions to be identified with those taken at higher power.





PLATE 5.2

x 500

Part of montage used to identify the positions  
of cells in higher power micrographs.







PLATE 5.3

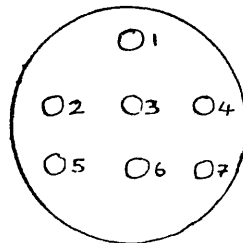
x 125

SEM in transmission mode used to identify the section  
on the grid which was then used for thin-section  
microscopy in the transmission microscope.

Special methods. Thin sections were cut and picked up on a grid. The next section was cut 2 - 3  $\mu$ m thick and picked up in a wire loop. It was transferred to a coverslip 13 mm in diameter, exactly the diameter of the stubs which fit the S.E.M.

Sections were arranged on the grid in such a way that their order could be recognised (see fig. 3).

Figure 3



They were slowly dried on the coverslip over a hotplate at 18 - 20°C. The sections were deresinated using the same etching fluid described in chapter 5, but usually only about 1 minute was required. It was found later that the process could be monitored under a binocular microscope. They were then gold sputter coated as before.

Sections were photographed as follows:-

(a) The thick sections were photographed at low power (x 180) under scanning mode to show the geography of the section (Plate 5.1).

(b) The phloem regions of the same sections were photographed at higher power (x 500) and montages of the whole region were prepared. An example of this is shown in Plate 5.2.

(c) The S.E.M. was then used in the transmission mode to photograph the thin section on the grid prepared for transmission work at low power (x 125). This is shown in Plate 5.3.

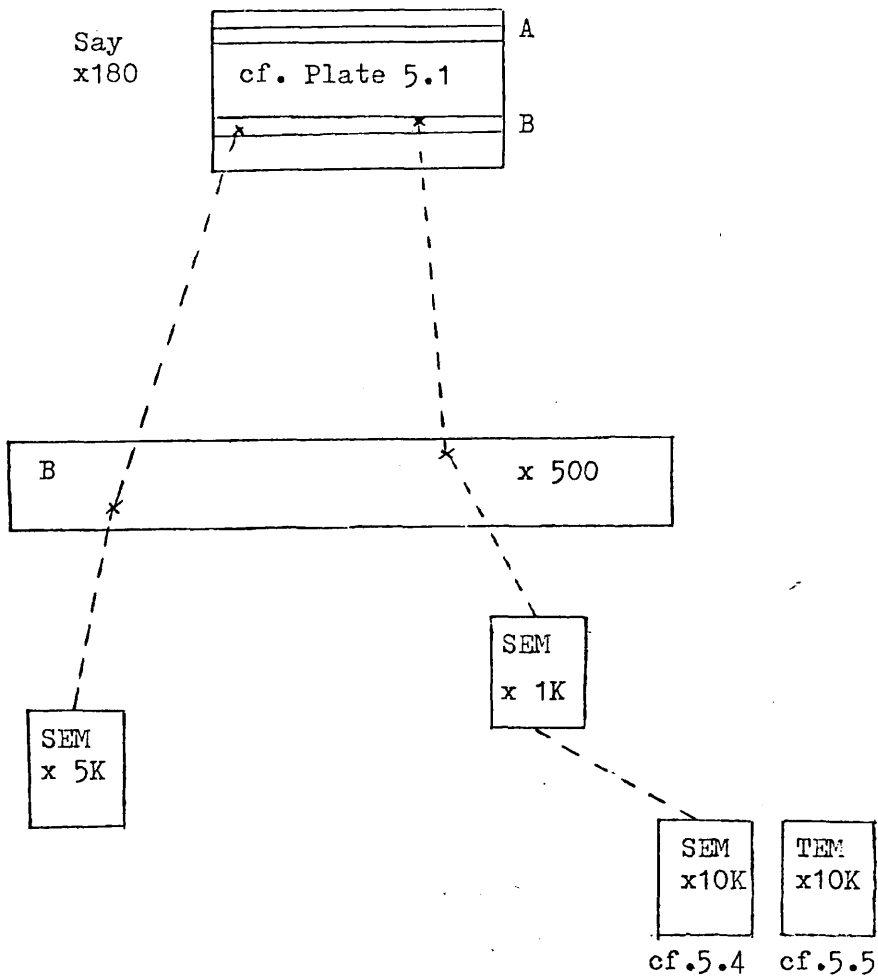
(d) The same section was similarly photographed at higher power (x 510) and a large montage of the whole region was made. This made comparisons of thin and thick sections relatively easy.

(e) The S.E.M. micrographs (x 500) prepared as montage (see (c)),

Plan for correlation work

Fig.4

Montage of phloem region A x 500 cf. Plate 5.2







## PLATE 5.4

x 10,000

Transmission micrograph showing a curved sieve plate  
and a companion cell with an oblique cell wall.

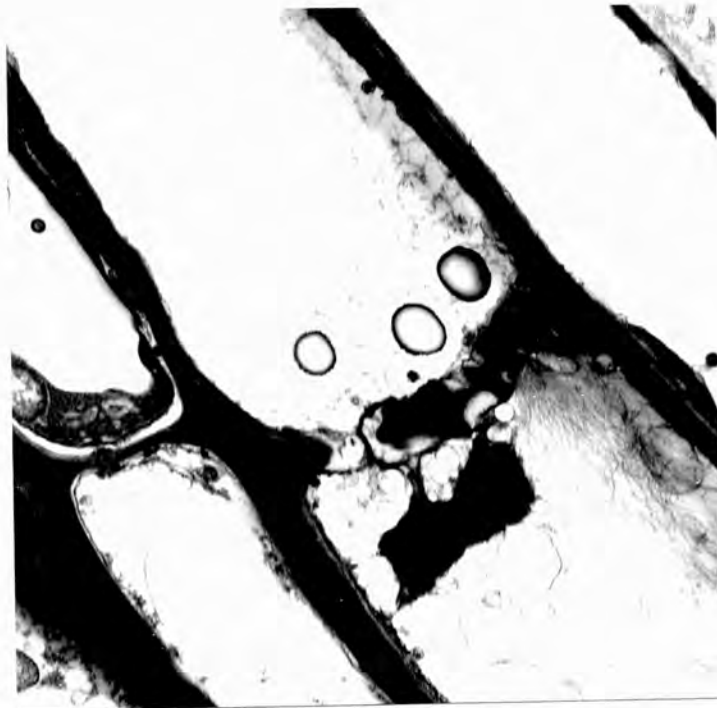
## PLATE 5.5

x 10,000

Scanning micrograph of adjacent thick section.

These micrographs superimpose well.







## PLATE 5.6

x 13,300

Even at different magnifications these sections show similarity. Compare with 5.8 for the SEM micrograph at the same magnification.

## PLATE 5.7

x 5,000

Scanning electron micrograph of the adjacent thick section.

were used as 'map references' for higher power scanning work (x 5,000 and x 10,000). When transverse walls thought to be potential sieve plates were found and photographed their position was marked, both on an enlargement of 5.1 and on the montage (5.2).

(f) Detailed examination of the thin sections in the transmission microscope followed. Using the T.E.M. initially in scanning mode the sections were selected to correspond with those already mapped. Sieve plates were photographed and their positions on the section were marked on the montage made of the same thin section (x 510) described in (d).

A large plan, using the micrographs, was built up, and pins attached to cotton were used to identify potential sieve plates photographed at the various magnifications. This plan is illustrated in fig. 4.

Results. Eventually two unmistakable sieve plates, and another plate observed in the transmission microscope were correlated with structures seen in the scanning micrographs. The sieve elements were matched initially by their relevant sizes, cell wall shapes, and from their known positions with relation to the whole section. The plates correlated had definite idiosyncrasies of shape which facilitated their identification. Many sieve plates found on the thin sections lacked such distinguishing features and the only available method of correlation was from their position (over which it is rather difficult to be precise) on the section. Later corroborative evidence was added as the micrographs were enlarged for inclusion in this work. An enlargement of the S.E.M. photograph was placed under the lens of the enlarger. The image of the relevant T.E.M. micrograph was superimposed on it. Those shown in 5.4 and 5.5, also 5.6 and 5.7 fitted admirably. Thus the sieve plate shown on 5.4 and 5.5 has a somewhat curved shape whilst the adjacent companion cell has an unusually oblique wall. These features are fairly obviously matched on the two photographs. Due to the curvature of the sieve plate the pores shown in 5.4 appear to penetrate only incompletely. The indentations seen on the





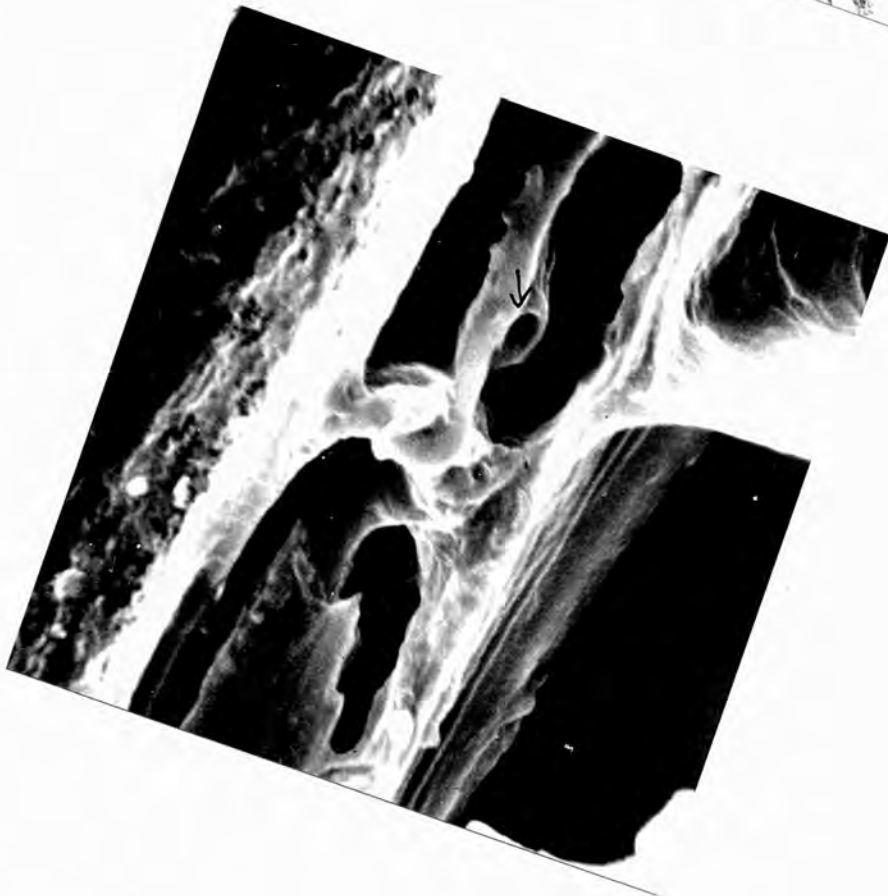
PLATE 5.8

x 13,300

Enlargement of 5.7 by superimposition over 5.6.

Obviously the same sieve plate.





## PLATE 5.9

x 9,750

Transmission micrograph showing an oddly curved sieve plate.

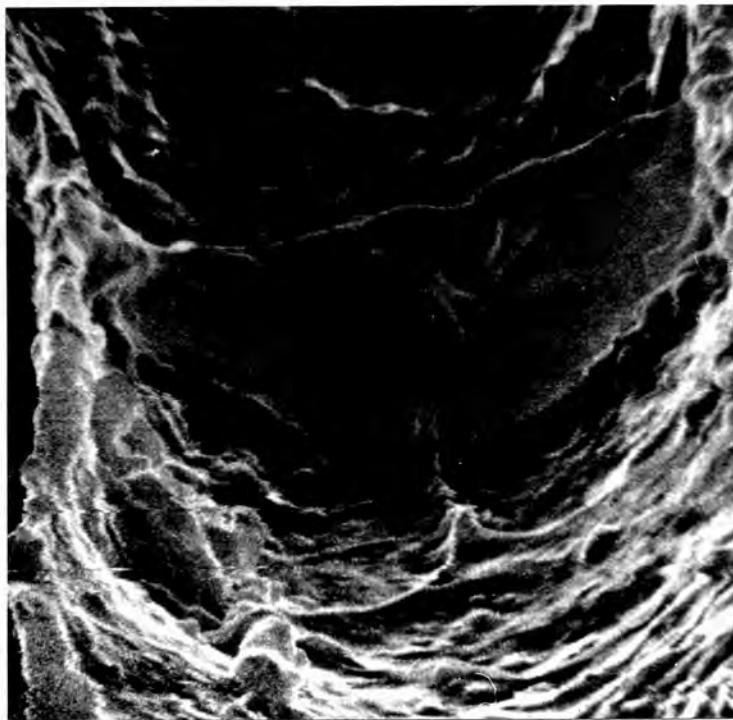
## PLATE 5.10

x 5,000

Electron micrograph of a sieve plate resembling, in part, that shown in 5.9. Two parts of the sieve plate shown here lie in different planes, which would account for only half being shown in the thin section.







## PLATE 5.11

x 10,000

Scanning electron micrograph with arrowed indentations originally thought to be sieve plate pores. The wall is very thin, and the cell is wide. It is almost certainly the transverse wall of a phloem parenchyma cell.

transverse wall of 5.5 seem to represent these pores.

In the sieve plate shown on 5.6 the pores seen in the original S.E.M. picture (5.7) are rather less convincing. The relative shapes and sizes of the cells on both the micrographs correspond well however, and it was shown by the subsequent enlargement of 5.7 shown in 5.8, and the superimposition of this and 5.6 gave an excellent match.

The micrographs shown in plates 5.9 and 5.10 present a similar, though less convincing, comparison. From the position on the section, the general shape, and the corresponding locus of the lateral sieve plate pores it seems very probable that they represent the same sieve plate. The photographs did not superimpose well, but they form however, a possible matching pair. Since the S.E.M. is capable of tilt and rotation not paralleled by the T.E.M. it was considered that they could be the same plate viewed from a somewhat different orientation. Note was taken of this and in future use of this correlation technique care must be exercised not to tilt the S.E.M. specimen unduly.

Seen in isolation, ie without the correlated transmission micrographs it is doubtful whether the sieve plates found by scanning microscopy would have been recognised as such. The only distinguishing features are their sectional position, thickness (somewhat more than other transverse walls), together with a suggestion of indentation indicating possible pores.

These points are illustrated by plate 5.11 where the transverse cell wall is very thin. However, rounded indentations (arrowed) can be faintly distinguished. Nevertheless, the cell is rather wide for a sieve tube, and its known position right at the extreme outer edge of the phloem indicates that it is almost certainly the end wall of a parenchyma cell.

Although the sieve plates illustrated here are not obscured by P-protein they are still somewhat difficult to identify positively. The most characteristic features, ie the pores, which it might be expected

would be obvious are not strikingly in evidence. Some obscuring callose had undoubtedly been laid down during preparation, as seen in the T.E.M. micrographs. This might be expected to appear as raised rings perhaps in the S.E.M. micrographs, and in fact it is arguable that it does (arrows in 5.11).

The limited experience with this technique proved very interesting. There is clearly scope for future work with it in the identification of somewhat elusive structures. Had time permitted it would have been developed further in the present work.





PLATE 6.1

Approximately natural size

The copper block apparatus used for freezing.



## Chapter 6

## FIXATION BY QUICK FREEZING

To date all the methods of fixation used in the present work have been chemical. In view of the many interesting and successful published researches in which physical fixation methods involving quick freezing it was decided to prepare some tissue in this way. The principle that artefacts are less likely to remain unnoticed where widely different preparative methods are used is a well established one. In what follows both freeze drying and freeze substitution techniques are described.

(a) Quick freezing combined with freeze drying.

General method. Loosely held copper blocks attached to surgical forceps formed the cooling surfaces. Their flat inner surfaces were slightly spaced by means of adjustable screws so that the decorticated stolon was not crushed when clamped between them during the freezing process (see Plate 6.1). The quenching fluid used was Freon 12 cooled in a bath of liquid nitrogen.

Special method. The freeze drying was completed in an Edward's freeze drier, the Pearse Tissue Drier, Model EPD 2, following the method advised in the makers handbook, noting especially their suggestion that plant material appeared to require far longer to dry than the animal material described in the handbook. After freezing in the quenching fluid the portion of stolon was transferred immediately to liquid nitrogen in a shallow container in the freeze drier. As advised, dehydration was carried out at 30°C for periods of up to 60 h before the vacuum was reduced. The first material prepared in this way proved impossible to embed. Initially the material was placed in propylene oxide prior to infiltration with resin. It was noted that the stolon fragments failed to sink in the propylene oxide, and when embedding was apparently complete the blocks were full of air or vapour bubbles. In an attempt to over-come

this problem partial rehydration of the material prior to embedding was tried on the supposition that really hard drying of the cellulose cell walls had compacted them too thoroughly to allow the liquid media to penetrate. A series of sulphuric acid dilutions was accordingly prepared to give relative humidities ranging from 10 - 100%. The fragments of freeze dried material were placed in the atmosphere above the acid for 18 h before infiltration by propylene oxide and then embedding was attempted.

Results. Embedding appeared to be more successful, especially with the fragments subjected to the higher humidities, but when the material was subsequently cut the state of preservation was so appalling that the method was abandoned.

Comments. Plant material has apparently previously been found difficult to embed after freeze drying (Jensen 1962). Where the method has been used successfully the plant material has not been enclosed in cellulose cell walls. It seems to be the cell walls that cause the problems. A study by Branton and Jacobson (1961) suggests that the first part of the plant to dehydrate is the cell wall. This renders it impervious to water so that the inside of the cell is unable to dry out. Early experiments by Hermans (1938) showed that the speed at which water molecules can diffuse through cellulose decreases very rapidly as the water content of the cellulose lessens. Branton and Jacobson suggested that the problem can be overcome using controlled drying in a gas stream containing water vapour. It must be noted that this early work was concerned with the infiltration of wax as the embedding medium. Jensen (1962), suggested that the problem can be overcome if the material is dried in a gas stream containing between  $2.6 - 4.0 \mu\text{g H}_2\text{O/l}$  so that water is drawn out of the cells before the walls are dehydrated. The apparatus for this is somewhat elaborate and was not available so the rehydration method seemed the only alternative.





PLATE 6.2

x 20,000

Freeze substitution using acetone.

PLATE 6.3

x 13,300

Freeze substitution using acetone for the longer period. Ice crystal damage is evident, especially in the phloem elements other than the obvious sieve tube.

(b) Quick freezing combined with freeze substitution in acetone.

Hereward and Northcote (1972) described a method of freeze substitution with acetone which appeared from their report both simple and successful. Their method was followed using a fine decorticated stolon of Saxifraga sarmentosa (L), the only differences being the substitution of Freon 12 for the Freon 22 they used as a quenching fluid, and the use of T.A.A.B. resin where they had used araldite. Quick freezing was carried out as previously described using the copper block apparatus. The material was quickly transferred to the substitution fluid viz: 2% osmium tetroxide in acetone dried with molecular sieve. It was kept in dry ice, the first series for 3 days as recommended by Hereward and Northcote and the second series for 5 days, at  $-30^{\circ}\text{C}$ . This later series was maintained at the low temperature using just the temperature control of the Edward's Freeze Drier.

Results. The condition of the material was not very impressive. Plate 6.2 shows the state of a sieve plate from the first substitution series which followed Hereward and Northcote's method precisely. There is considerable denaturing of the P-protein and such as can be seen of the contents of the adjacent cells indicates plasmolysis and every other evidence of poor fixation. There is no very obvious ice crystal damage. The reason for embarking on the second, slightly different series was the outcome of a conversation with Dr. Stanley Bullivant who suggested that although Hereward and Northcote's pictures showed well fixed tissue, that fixation image may be solely chemical, the timing being too short for low temperature substitution to complete. The results from the second series as shown in plate 6.3 were still poor and here ice crystal damage is evident.

(c) Freeze substitution using Ethylene Glycol.

Another method of freeze substitution recommended by Pease (1973),







PLATE 6.4

x 3,300

Freeze substitution in ethylene glycol.

PLATE 6.5

x 6,700

Frozen material fixed in warm glutaraldehyde.

using aqueous ethylene glycol as the substitution fluid was also tried. The method described by Pease is far from complete, giving, for example, no detail of the length of time allowed for the frozen material to return to room temperature. A stage which was also incompletely described concerned the dilution of the ethylene glycol using a balanced salt solution + 5% glutaraldehyde.

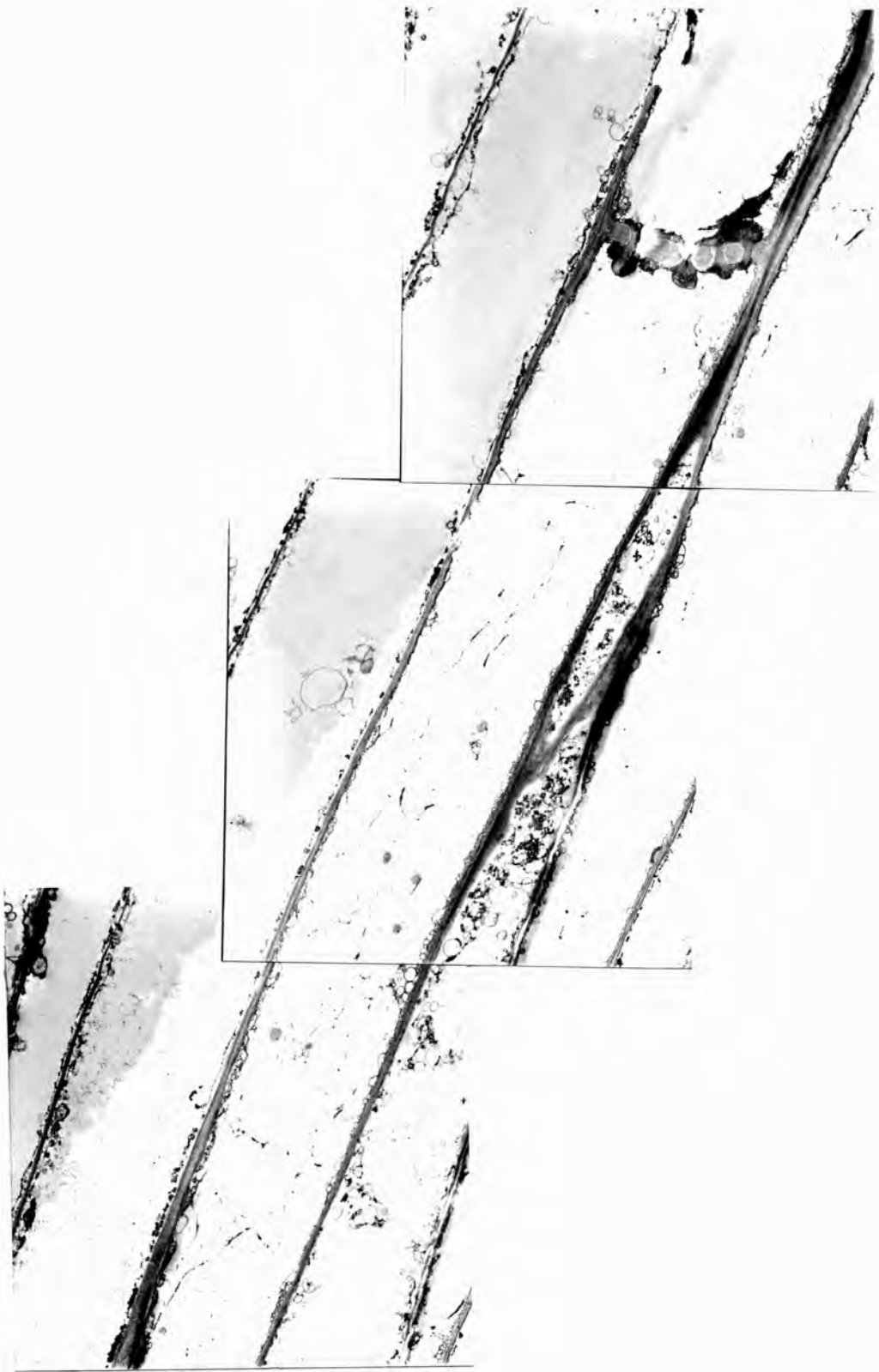
Method. Pease's method was followed as closely as the details given permitted. Freon 12 was used instead of his Freon 22 as the quenching fluid. The ethylene glycol was diluted to 70% with phosphate buffer, and this was used to dilute the glutaraldehyde to 5%. The substitution procedure was accomplished at  $-50^{\circ}\text{C}$  in the Edward's Freeze Drier, again only using the temperature, and not the evacuation control. The material was kept at this low temperature for 7 days, then allowed to rise slowly to room temperature over 7 h. The material was passed slowly through a series of ethylene glycol and acetone mixtures, with the acetone proportion steadily increasing to 100%, thence it was passed to propylene oxide and T.A.A.B. resin for embedding.

Results. Once again the fixation image, as depicted in Plate 6.4, was unsatisfactory and there is evidence of ice crystal damage. The promise of ethylene glycol proving a good substitution agent for plant tissue seemed unfulfilled, and the attempt to use it was accordingly abandoned. The work being described was, as a matter of fact, undertaken during a period of a priore ie appraisal of quick freezing as a suitable fixation method for sieve tubes (Spanner, 1978). This reappraisal seemed so discouraging that the whole approach was discontinued. Further discussion is deferred until later.

(d) Quick freezing followed by chemical fixation.

Before doubt was cast upon quick-freezing as a useful method of fixation for sieve elements it was thought that 'instant' fixation by freezing





## PLATE 6.6

x 5,000

Montage of part of a sieve tube, frozen and then fixed in warm glutaraldehyde, to show the disposition of the P-protein in the lumen of the tube.

would not only preserve the in vivo disposition of the sieve tube contents, but membranes would be so damaged that internal pressures would vanish and subsequent chemical fixation could be accomplished without risking longitudinal surge. It was reasoned that, if after quick-freezing the material was plunged into warm glutaraldehyde ice crystal formation might be avoided. This procedure would also avoid the practical problems experienced in maintaining suitably low temperatures for freeze substitution, and also the difficulties of infiltration found after freeze drying.

Method. A decorticated region of stolon was quick frozen using the copper block apparatus as previously described. The segment was then plunged into 5% glutaraldehyde at 40°C. The temperature of the fixative sank rapidly to 17°C, but it was returned to 30°C in about 30 seconds by the addition of hot water to the water bath. The sample remained in the fixative for 2 h before being processed as usual (appendix J).

Results. The fixation image was poor, cell contents being clumped and obviously damaged (plate 6.5). The P-protein shows evidence of coagulation which may have been due to the temperature of the glutaraldehyde. Plastids were ruptured and both they, and the P-protein showed predominantly on one side of the sieve plate. Plate 6.6 shows the P-protein passing through the sieve plate pores, but is also distributed in the lumen of the sieve element.

This method appeared to be useful for the corroboration of P-protein disposition in vivo; and it was thought for the glutaraldehyde the fixation of the P-protein could be improved. However, before further work was undertaken, a priori doubts had been cast, as noted above, on the validity of quick freezing techniques for sieve tubes, so no further work along these lines was undertaken.

## Chapter 7

A FURTHER APPROACH TO THE GENERAL PROBLEM: IMMOBILISATION OF P-PROTEIN  
IN BOILING WATER

It is impossible to separate the quest for the best methods of fine cytoplasmic fixation of sieve elements from other investigations designed to throw light on the gross in vivo situation of the sieve element contents, especially the disposition of the P-protein. Only by including information obtained by such coarse means is it possible to know what 'good' fixation of sieve tubes is.

Inevitably, since Fischer's early work in 1885, investigations into the anatomy of sieve elements have cast doubt on the validity of structures seen, since it has not been known how much the release of turgor pressure during preparation affects what is observed. Fischer reasoned that material plunged into boiling water before fixing and sectioning must show, at least, the position of structures in the sieve elements as they were in life. He used Cucurbita sp. however, and as the diameter of the sieve elements in this genus is somewhat large, and each element is deeply buried in other tissue, the temperature of the phloem must have risen relatively slowly, thus rendering his conclusions somewhat dubiously relevant to the present day investigation. The method was used again by A. W. Siddiqui in the late 1960s, using material of much smaller diameter (Helianthus - young stems, and especially Saxifrage sarmentosa stolons), so that on plunging into boiling water the sieve element temperature would rise much more rapidly than with Fischer's Cucurbita. The results he noted were in definite contrast with Fischer's as he found the coagulated P-protein passing through pores in the sieve plates. Neither worker confirmed that translocation was proceeding in the tissue immediately prior to the boiling water treatment. There seemed reason therefore for repeating the work of Siddiqui with additional refinements.







## PLATE 7.1

x 7,500

Montage of part of a heat treated sieve tube to show the disposition of the P-protein and plastids in the lumen.



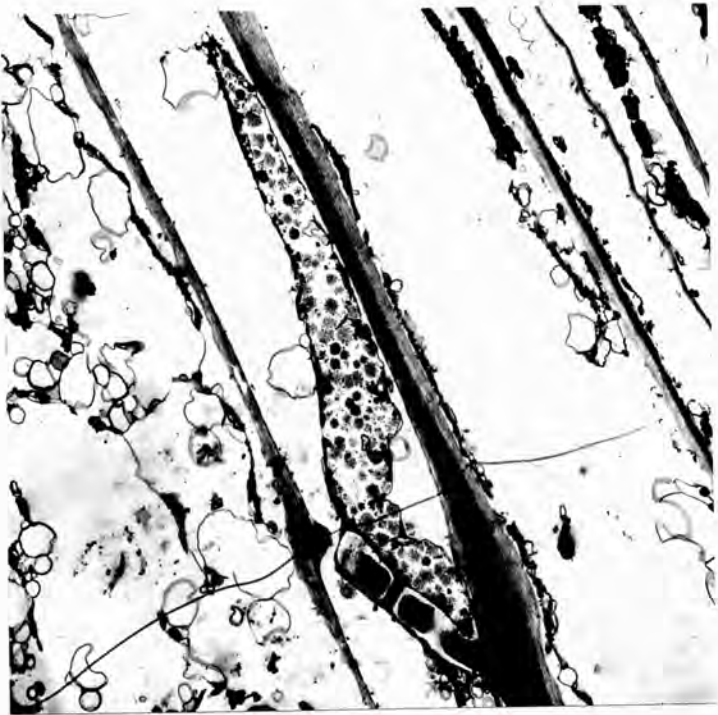


PLATE 7.2

x 6,750

Sieve plate of heat-treated sieve tube showing

P-protein passing through the pore.

These included using a decorticated stolon of Saxifraga to allow an even more rapid rise in the temperature of the sieve elements. In fact the latter must have reached boiling point almost instantly, so close are they to the surface. In addition prior translocation was confirmed by physiological means.

Method. The stolon was decorticated and an hour later the subtending leaf was fed with  $^{14}\text{CO}_2$ . The distal end of the 450 mm stolon was monitored with a G. M. tube, and some 3 h later a high level of radioactivity was detected in the apex. At this stage the decorticated region of the stolon was plunged horizontally into briskly boiling water whose heat was maintained with an immersion heater. After 1 minute the decorticated part of the stolon was cut out and subdivided into small segments into glutaraldehyde fixative prior to conventional preparation for electron microscopy.

Results. About forty sieve plates were observed. In every case the denatured P-protein was passing right through the sieve plate pores. The montage forming plate 7.1 shows the coagulated protein on both sides of the pore and continuing for a considerable distance along the sieve tube. Both plates 7.1 and 7.2 show that plastids are commonly found rather more abundantly on one side of the plate than the other. Starch grains are gathered into the axial region of the section by the collapse of the protoplast. There was an impressive unanimity in the results.

## Chapter 8

## DISCUSSION OF PART I

One of the primary objectives in this work on preparative techniques for electron microscopy was the identification and reduction of surge effects observed as turgor pressure in the sieve elements is released.

Artefacts of preparation judged to be surge effects were of course met, but they were not as widespread as the literature has indicated. Masses of dense, ill-defined P-protein, including many starch grains from broken plastids, and completely clogging the whole sieve plate, were observed as a general rule, only within a short distance, say 0.5 cm, of the primary cuts. When found occasionally in other circumstances they seemed to be indicative of sudden localised penetration of chemical fixative. Such disordered masses may be regarded as evidence of poor techniques except where they were intentionally sought. When measures were taken to reduce turgor first these dense masses plugging the sieve plates were not observed. The best method found for reducing their incidence was the addition of small quantities of chloroform water to the glutaraldehyde fixative in normal use.

The problem, so frequently posed, still remains unsolved: are the sieve plate pores naturally occluded with P-protein or is this material carried into them during preparation? Evidence is accumulating here however to indicate that the pores are plugged in vivo, but a final statement on this must be delayed until later in this thesis.

In a paper reporting earlier work on the subject (Hogben and Spanner, 1977) it was noted that the use of chloroform vapour to reduce turgor in sieve tubes, by permitting lateral leakage through weakened membranes, still showed P-protein occluding the pores. Evidence of only slight longitudinal movement was adduced from the fact that although plastid membranes were ruptured, possibly as a result of the action of the





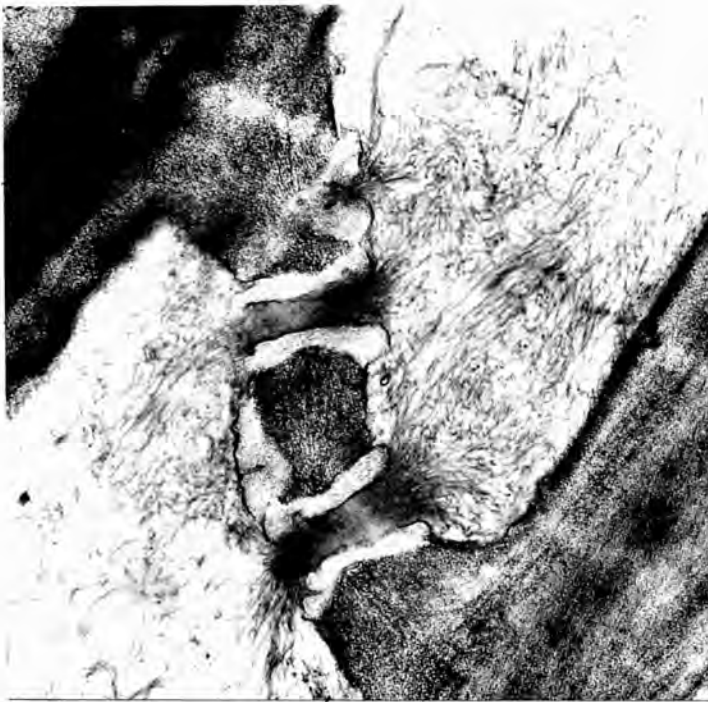
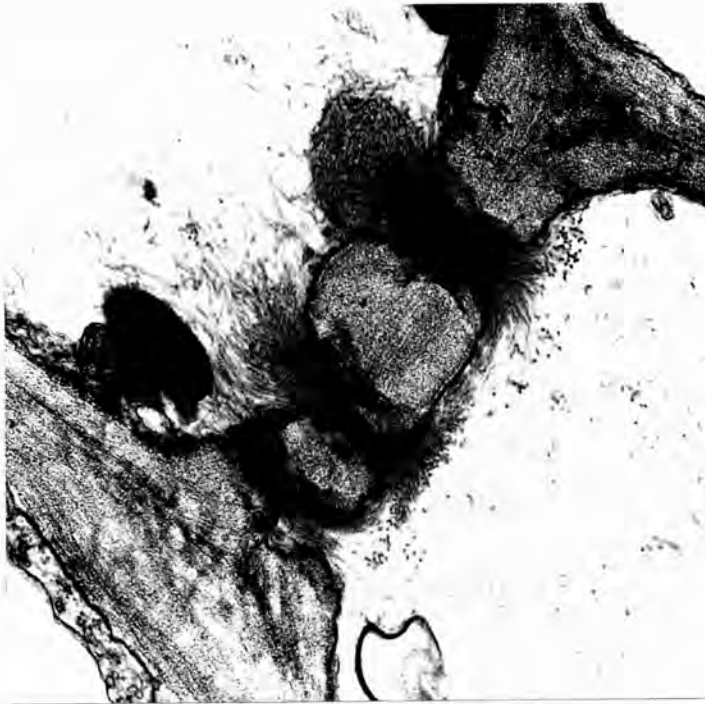


PLATE 8.1

x 40,500

P-protein showing the disposition indicated by  
fig. 5b.

PLATE 8.2

x 33,500

P-protein showing the disposition illustrated by  
fig. 5a.

chloroform, starch grains had not moved out of them. Very similar results were noted in this work where chloroform was added to the fixative.

P-protein fibrils passing through pores are often shown axially orientated into one side of the pore, but emerging somewhat bulbously on the other. This feature is usually cited as due to the release of turgor pressure carrying the protein into the pores. From the observations made during this work it is suggested that the 'bulge' is probably the result of only very mild surge.

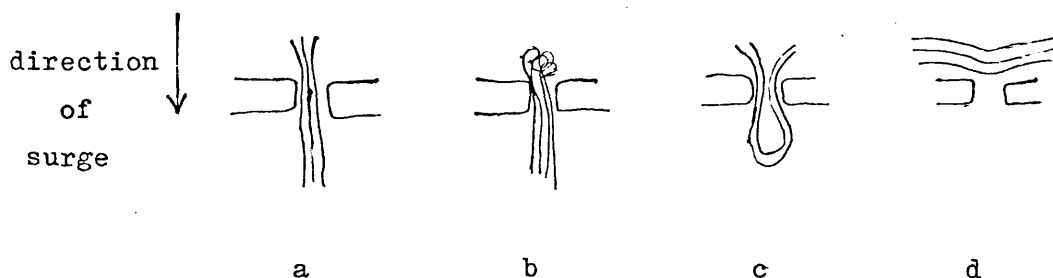


Figure 5. Diagrammatic appearance of common images.

All the images illustrated by figure 5 have been observed during this work with the possible exception of 5c, and all (except 5d) have been seen together with only mild surge effect as judged by the unbroken plastids. The configuration in 5b is present in plate 1.2 from chapter 1, taken from a region of the stolon where some surge might have been expected. It appears even more clearly in plate 8.1 where more care was taken to alleviate pressure effects during preparation. Only if the protein fibrils were already present in the pores could turgor pressure release have caused the collapse of the fibrils on to the plate seen on the upstream side of the surge. Figure 5c represents a common configuration of P-protein fibrils appearing in electron micrographs, where surge is likely to have occurred. It is suggested that as a result of surge the unsupported length of the fibrils is forced through the pores

with the ends tailing on the other side. If this interpretation is correct the 'bulge' is likely to be on the downstream side. The likelihood of the ends of the fibrils being neatly aligned through the pores on the downstream side is remote, and the more probable disposition of fibrils carried bodily towards the pore as a result of surge is illustrated in 5d. This is frequently observed in micrographs of preparations where no care to mitigate surge has been taken (see plate 1.6). Micrographs illustrating diagram 5c were difficult to find in this work with certainty, but plate 1.1 where the direction of surge is known, looks the most likely candidate for this interpretation. A similar conclusion was reached by Siddiqui, Jones and Spanner (1974), and is illustrated in their figure 1B.

These conclusions can be correlated with the theory proposed by Spanner (1975) regarding the state of the P-protein distribution in sieve tubes according to whether they are quiescent or actively translocating. Quiescent tubes, on being cut, may well show the effect illustrated in 5c, whilst an actively translocating sieve tube may be expected to resemble 5b. Where an image of diagram 5d type is found it must be concluded that poor surgery of the material has rendered it useless for interpretation. Figure 5 a is represented only in micrographs where fixation has been most careful, and yet it is frequently observed, see plates 3.8, 3.14 and 8.2.

The methods of preparation using cryofixation were abandoned, not so much because of inevitable problems encountered, but as a result of thoughts emerging at the time concerning the value of quick freezing with special reference to sieve elements (Spanner, 1978). His speculations regarding the effect of dielectric constants of substitution fluids, both on charged protein fibrils, as well as ions in the sap, cast doubt on the value of pursuing such methods; further his calculations

regarding the volume changes of water, and the axial displacement of the sieve tube sap consequent on freezing, appeared sufficiently conclusive for all methods of fixation using freezing to be called seriously in question.

Some considerable time was spent trying to find sieve plates using scanning electron microscopy. This work proved to be of little value, but led to the development of the technique for correlating it with the transmission work described in chapter 5. Although not found outstandingly useful in this particular project it has already proved valuable in other fields (zoological) where it has been tried.

Although so many of the preparative methods tried during this research were dismissed as having little or no special value, this was largely because they contributed nothing new to what older, well tried methods already did. Their merit lay in their corroborative evidence, making it less likely that accepted structures were artefacts. Perhaps the most important of these was the periplasmic space observed, especially in companion cells. This frequently contains vesicles, the presence of which are often thought to be artefacts of preparation. As they are seen in almost every method of fixation used they must rather be considered to be an integral part of the structure.

Lamellar stacks, and the odd configurations found in the sieve elements have often been described as artefacts. Again, these are found so consistently that they must be considered real.

Other consistent features of the phloem of Saxifraga sarmentosa (L) are the small companion cells with their dense cytoplasm and small vacuoles. Within the sieve elements starch containing plastids abounded. Mitochondria were only infrequently observed. P-protein was regularly found, both in the lumen of the elements and occluding the pores. In good preparations callose was frequently absent from the pores, or only appeared as a thin layer.

The appearance of the sieve plates was remarkably uniform. Very few young sieve tubes, with unperforated plates were seen in the middle region of the stolon. This observation indicates that the anatomically 'mature' stage of the sieve element must also be the physiologically mature one.

## PART II INTRODUCTION

The effect of inhibitory treatments on phloem ultrastructure.

The stolon of Saxifraga sarmentosa (L) has already been used successfully in these laboratories for physiological work involving inhibitory treatments. Its unidirectional transport of sucrose has been studied and thoroughly documented (Qureshi and Spanner, 1971, and Qureshi and Spanner, 1973 b). The information gained from this work was made use of in the present study, particularly with regard to the length of time it takes for radioactive tracer to start accumulating in the daughter plant. Initially the same methods of administering the labelled tracer were employed; but as in this work apparatus was available for monitoring the mass delivery rate in the sink, continuous feeding of the source leaf with labelled carbon dioxide was later adopted.

The same plant material was used by F. Qureshi for his physiological work with chemical inhibitors notably dinitrophenol (Qureshi and Spanner, 1973 c), nitrogen (Qureshi and Spanner, 1973 a) and cyanide (Qureshi and Spanner 1973 d). His finding regarding the best method of application of the cyanide (ie in gaseous form) and the most satisfactory concentration of the gas (viz. over  $5 \times 10^{-3}$  M potassium cyanide solution activated with sulphuric acid) were followed. His work included a series of experiments on the length of stolon which required inhibition for any marked effect on translocation to be noticed. About 20 cm appeared to be the most satisfactory length. The actual perspex chamber which he used and described on P 752, fig. 1 (Qureshi and Spanner, 1973 d) was again employed.

Saxifraga sarmentosa (L) was also used by Irene Chamberlain in these laboratories for experiments involving inhibitory chilling. She found that satisfactory inhibition occurred around 0°C (Chamberlain and

Spanner, 1978) and so this was the temperature used in the rather simpler experiments described here.

Some of the experiments using both methods of translocation inhibition were run concurrently. During the period of the experimental work the method of administering the labelled carbon dioxide was changed and improved, so this aspect of the physiological work is described separately.



## Chapter 9

## ADMINISTRATION OF LABELLED CARBON DIOXIDE AND MEASUREMENT OF ITS EFFECT

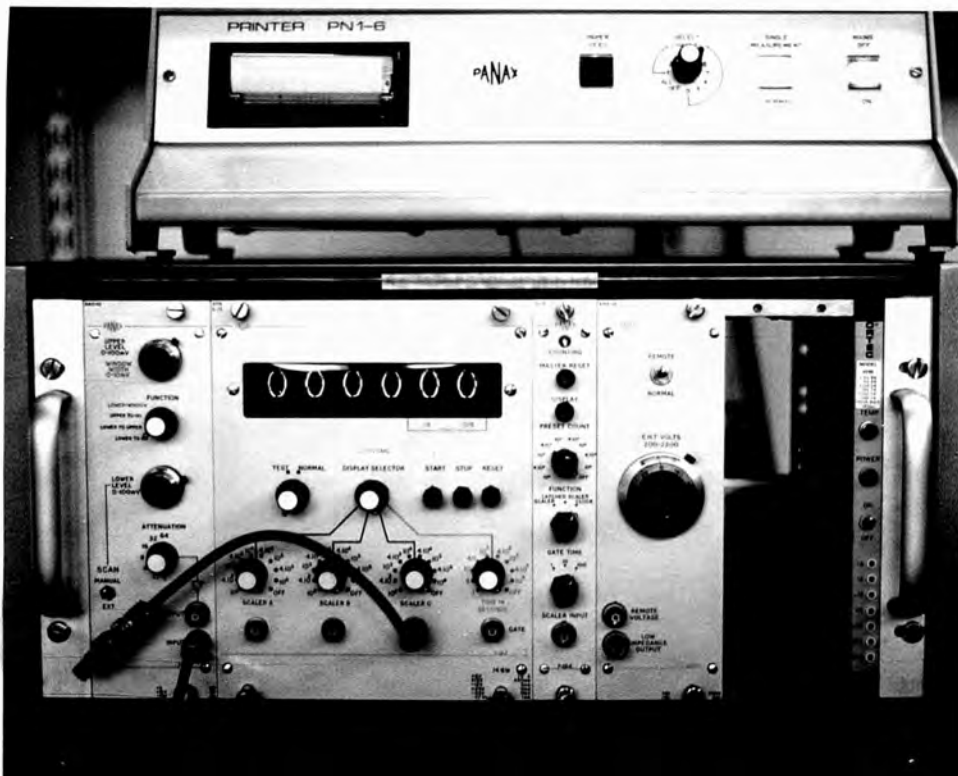
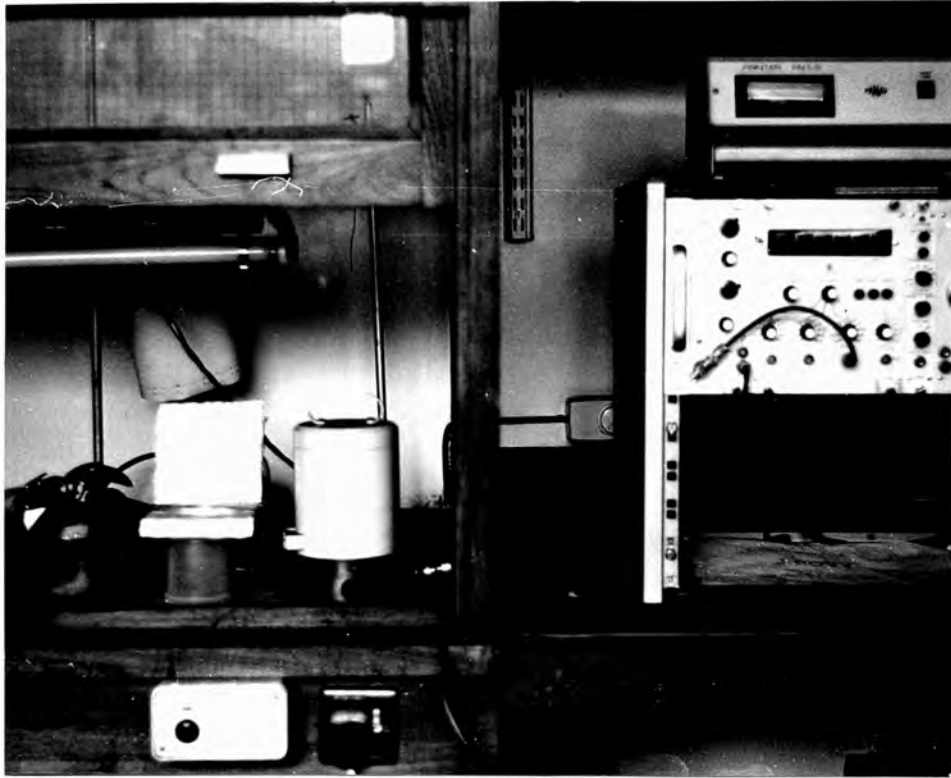
The plants used for all experimental work were grown in a heated greenhouse at about 18°C in 10 cm plastic pots. The temperature was not closely controlled and summer temperatures reached 25 - 30°C, in spite of shading, for part of the period of the experimental work. They were watered with a dilute proprietary nutrient. Stolons at least 35 cm long were required for the experiments and were used before the distal buds showed any obvious signs of developing into daughter plants. Any stolons bearing lateral branches were discarded. Plants selected were vigorous, having healthy, mature leaves subtending the stolon to be used.

Before feeding with  $^{14}\text{CO}_2$  the subtending leaf was enclosed in a chamber made from heavy gauge lay-flat polythene tubing 10 cm wide and about 15 cm long. The lengths were folded to make gussets and heat sealed at one end. They were made airtight round the petiole with double sided adhesive tape.

For the first series of experiments a small piece of indicator paper, on which a few drops of  $\text{Na}_2^{14}\text{CO}_3$  solution containing 50  $\mu\text{Ci}$  had been dried down, was attached to the inside of the leaf chamber with a fragment of adhesive tape. Subsequently a drop or two of 3%  $\text{H}_2\text{SO}_4$  was introduced with a fine hypodermic needle to moisten the paper and release the  $^{14}\text{CO}_2$ . The hole made in the chamber was then sealed with petroleum jelly.

A first trial run was set up during the morning of 9th May 1977, using a stolon 41 cm long. The stolon cortex was removed between 12 and 14 cm from the parent plant, and the decorticated area was wrapped in fine greased polythene. The plants were fed with  $^{14}\text{CO}_2$  at 12.05h and allowed to translocate in natural light until 5.00h the next morning when a 250v, 150w Tungsten filament lamp, used in conjunction with a water filter was switched on. This gave an illumination of 2,000 - 2,400 lx according to





## PLATE 9.1

The arrangement of the apparatus used for a chilling experiment with the stolon passing into a Panax tower enclosing an end window GM tube.

## PLATE 9.2

The equipment used for counting and printing out the count for accumulated  $^{14}\text{C}$ .





## PLATE 9.3

The polythene chamber attached to the leaf subtending the experimental stolon, in which the phial containing the  $\text{Na}_2^{14}\text{CO}_3$  is placed.

whether the light meter was placed at the top or bottom of the plant. The tip of the stolon was fitted into a Panax lead tower (Plate 9.1) under an end window G.M. Tube. This was connected to the counting and print out units shown in plate 9.2 which recorded the accumulation of  $^{14}\text{C}$  in the stolon tip. Counting was started before decortication. The rate of accumulation was slow, but continued to rise steadily until the experiment was stopped at about 16.45h on the second day, ie some 30h after its commencement. Satisfied that sufficient  $^{14}\text{CO}_2$  was being transported early inhibition experiments were conducted under these conditions still showing the somewhat slow rate of accumulation of  $^{14}\text{CO}_2$ . After the initial trail run the stolon was decorticated later, after a steady accumulation rate had been observed, but well before inhibitory treatment was applied.

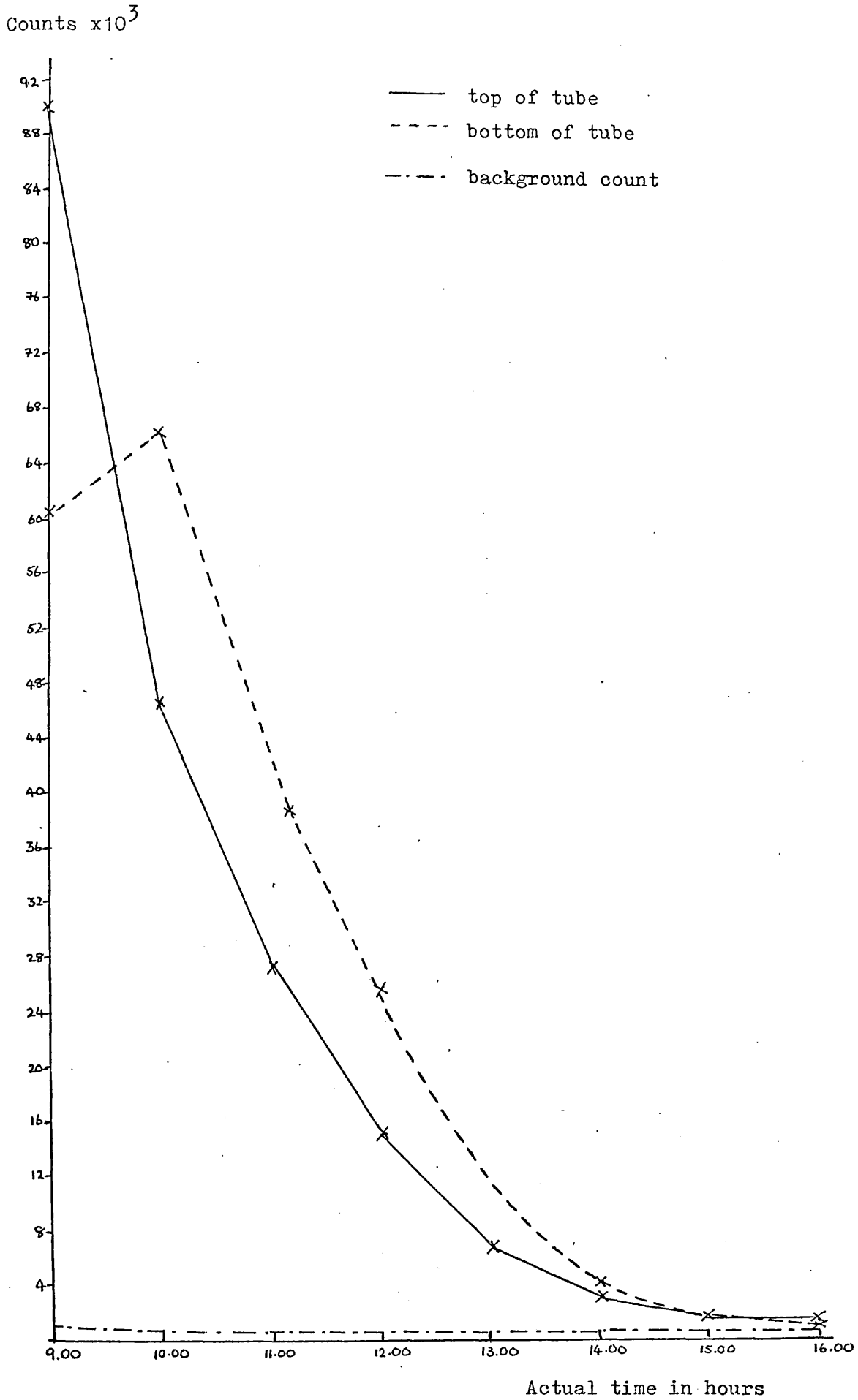
To try to improve transport it was decided to change the lighting to give a higher intensity ie approximately 3,500 lx. Accordingly a set of four 20W fluorescent tubes were arranged. These were left on continuously. This promoted considerably more rapid translocation. This, combined with the fact that by early June temperatures were also rather higher provoked a situation where the supply of  $^{14}\text{CO}_2$  was exhausted before the experimental work was completed, necessitating provision of a longer lasting supply of the tracer.

To provide more carbon dioxide 1 ml. M  $\text{Na}_2\text{CO}_3$  was mixed with each 0.1 ml. (ie  $50\mu\text{Ci}$ )  $\text{Na}_2^{14}\text{CO}_3$  in a glass phial. This was placed in the leaf chamber adhering to it by double sided adhesive tape, and balanced on a firm base as shown in plate 9.3. A few drops of the indicator B.D.H. 4460 were added before the sodium carbonate was activated with enough 3%  $\text{H}_2\text{SO}_4$  to make it just acid. This appeared to release plenty of  $^{14}\text{CO}_2$ , but very rapidly so that its translocation was again finished before the experiment was completed. To effect a slow, steady, more



Reduction of  $^{14}\text{C}$  in the phial with time.

Fig. 6



continuous release a plastic cap was put on the phial, having a hole pierced in it with a hot mounted needle. Activation of the  $\text{Na}_2^{14}\text{CO}_3$  was easily accomplished through the hole using a hypodermic syringe. The hole also permitted slower release of the  $^{14}\text{CO}_2$ . This method proved very successful. A test was done, alongside one of the actual experiments to ascertain that the  $^{14}\text{CO}_2$  continued to be released throughout the period of the experiment.

0.1 ml. samples from a phial of activated sodium carbonate were taken hourly from both the top and the bottom of the solution and placed in scintillation fluid in the dark before being assayed in a liquid scintillation counter. The average of the three counts for each sample was used to plot the graph shown in figure 6. Although the level of tracer remaining in the phial by the end of the experiment was low, there was still plenty remaining at such a time as it would have been absorbed by the plant. This experiment showed that the desired aim had been achieved, viz. a plentiful supply of  $^{14}\text{CO}_2$  for the duration of the experiment.

Having found a satisfactory way of administering  $^{14}\text{CO}_2$  continuously this method was used for the majority of the inhibitory experiments i.e. those that had not previously been completed.

Measurements of the accumulation of the  $^{14}\text{CO}_2$  at the distal end were made continuously during the course of all experiments. The counter was set to print out every 4,000 sec. for the first few hours - usually 12 - 16h, after the commencement of the experiment. The count rate was graphed for this period and usually (unless there was some fairly obvious physical reason against it) a steadily rising count soon supervened. As soon as the steady rate of accumulation had been established the experimental inhibitor treatment was applied. In the earlier experiments decortication was carried out at this stage, and further monitoring of the accumulation rate was carried out before inhibitory treatments were

given. The timer setting was altered at this stage to effect print out every 1,000 sec. so that changes in the rate of accumulation could be more precisely noted. When plotting the graph the important consideration was the slope of the curve which indicated the rate of translocation. To maintain a comparative curve the earlier counts were plotted as their average counts per 1,000 sec. Once the inhibitory treatments had been applied, and a slower rate of accumulation had been observed, the material was fixed and processed for ultrastructural examination.





PLATE 10.1

The insulated apparatus used in the chilling experiments.

## Chapter 10

## INHIBITION OF TRANSLOCATION BY CHILLING

Method. The plants were grown, selected and fed with labelled carbon dioxide as previously described. Once a steady accumulation rate was being recorded in the stolon tip a 10 cm length in the centre of the stolon was cooled using finely crushed ice. The apparatus used in this procedure is shown in plate 10.1. The stolon passed through slots cut in the rim of a plastic petri dish and rested on a bed of dental wax. This served both as a surface on which decortication could be carried out, as well as aiding thermal insulation. The whole petri dish was encased in a box of expanded polystyrene, cut to fit closely. This formed the main insulation. The petri dish lid also had corresponding slots, and the polystyrene box had narrow grooved channels (arrowed) so that the stolon was held gently and not strained. Normally the whole assembly rested on the stage of a binocular microscope so that decortication, as well as observations on the condition of the stolon could be made with facility. Before crushed ice was placed over the stolon the latter was covered with extra thin polythene film so that the decorticated stolon did not dry out, and so that water from the melting ice did not come into contact with the exposed stele.

The measurement of the temperature of the stolon was made periodically using a fine copper constantan thermocouple. The reference junction was placed in a Thermos flask of melting ice and the other touched the stolon. Readings were taken on a high impedance electronic microvoltmeter, and were always about 1 v. Since the thermoelectric power of the couple used is about  $40 \text{ v deg.}^{-1}$  this indicates a stolon temperature constantly very close to  $0^{\circ}\text{C}$ . Much of the work was done in very hot weather so constant checking of the ice supply and of the stolon temperature was maintained. Water from melting ice was withdrawn with a pipette and

Decorticated stolon chilled

Fig. 7A

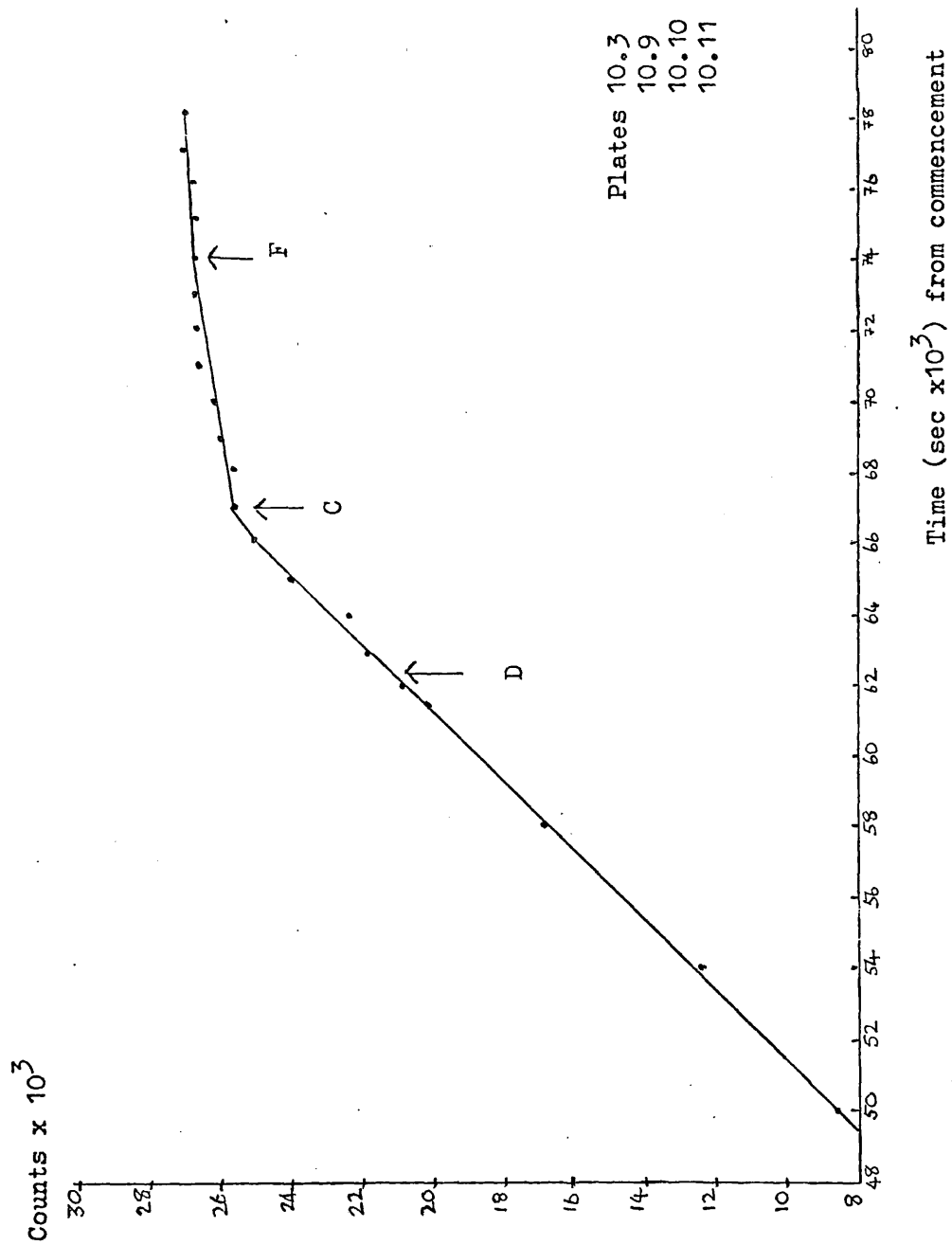




Fig. 7b

Decorticated stolon chilled

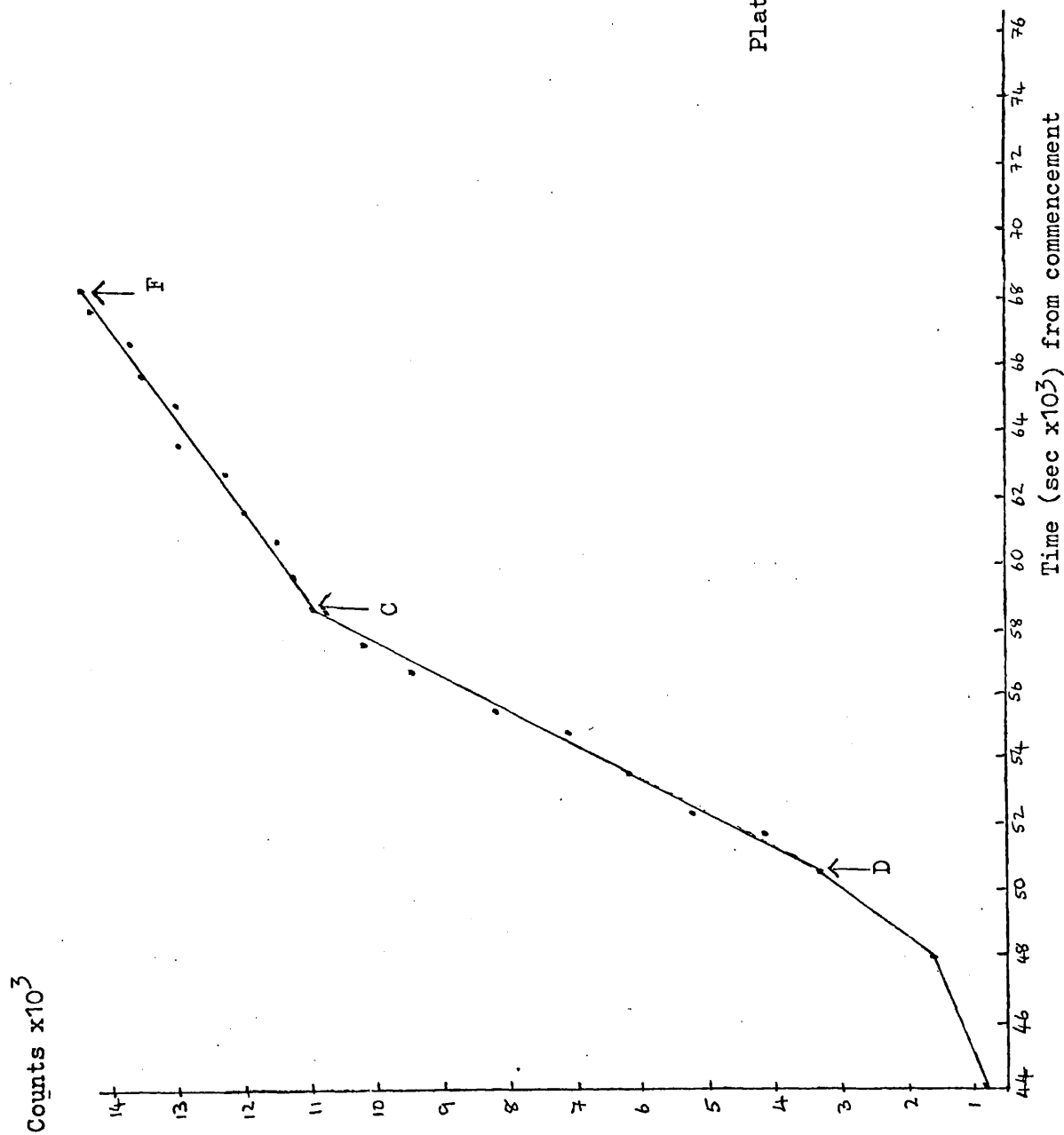


Plate 10.7

Fig. 8a

Intact stolon chilled

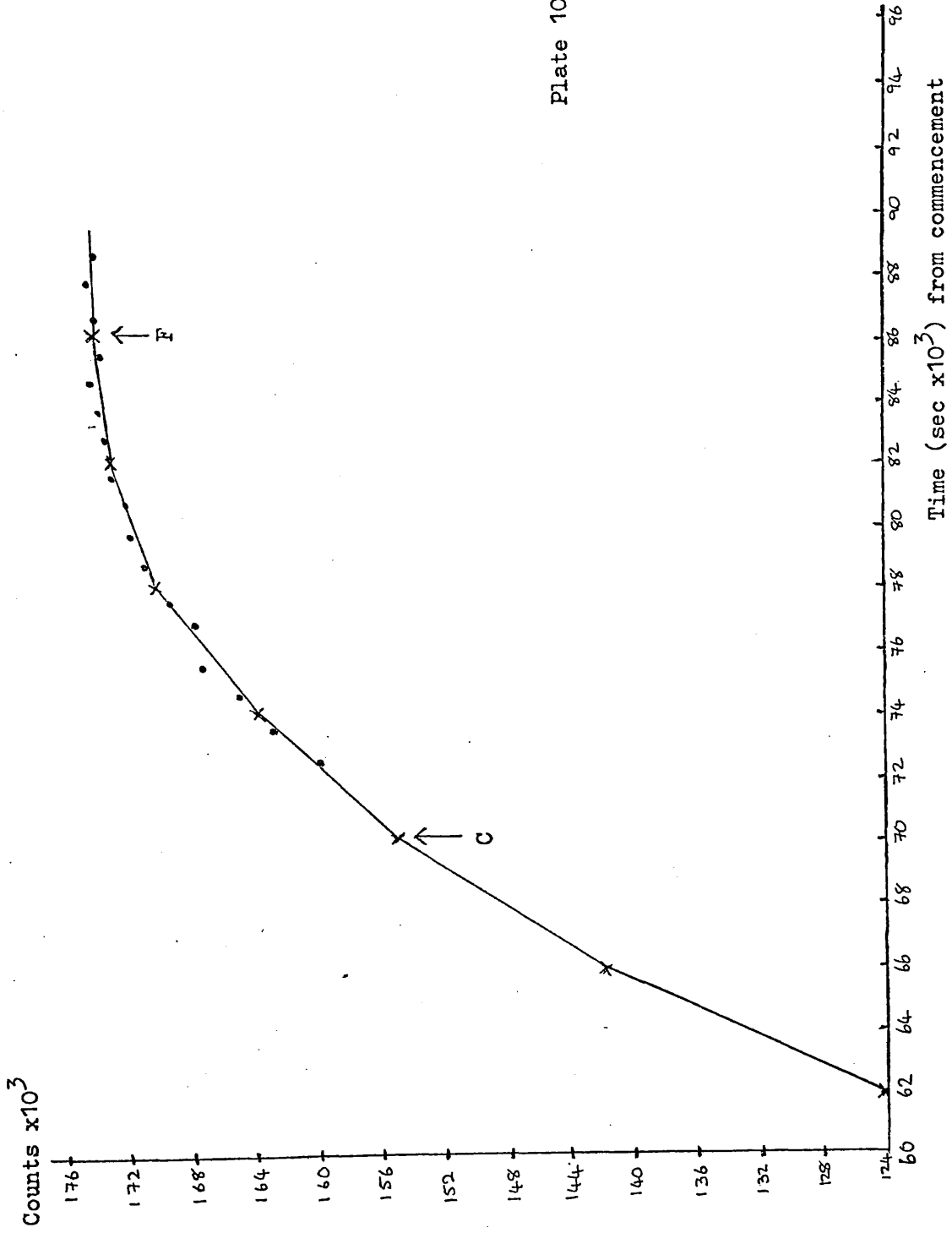


Plate 10.6

Fig. 8b

Intact stolon chilled

Counts  $\times 10^3$

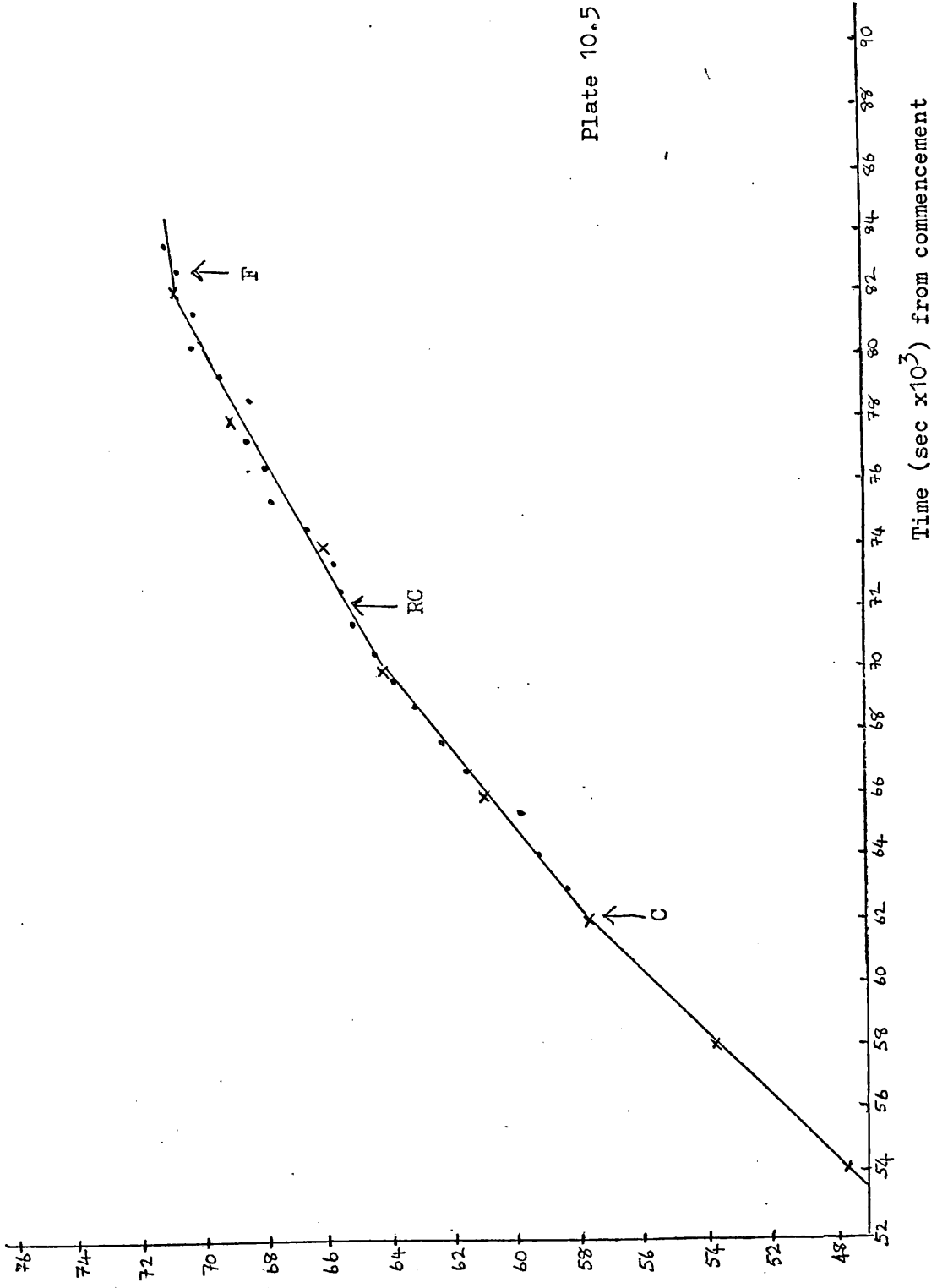
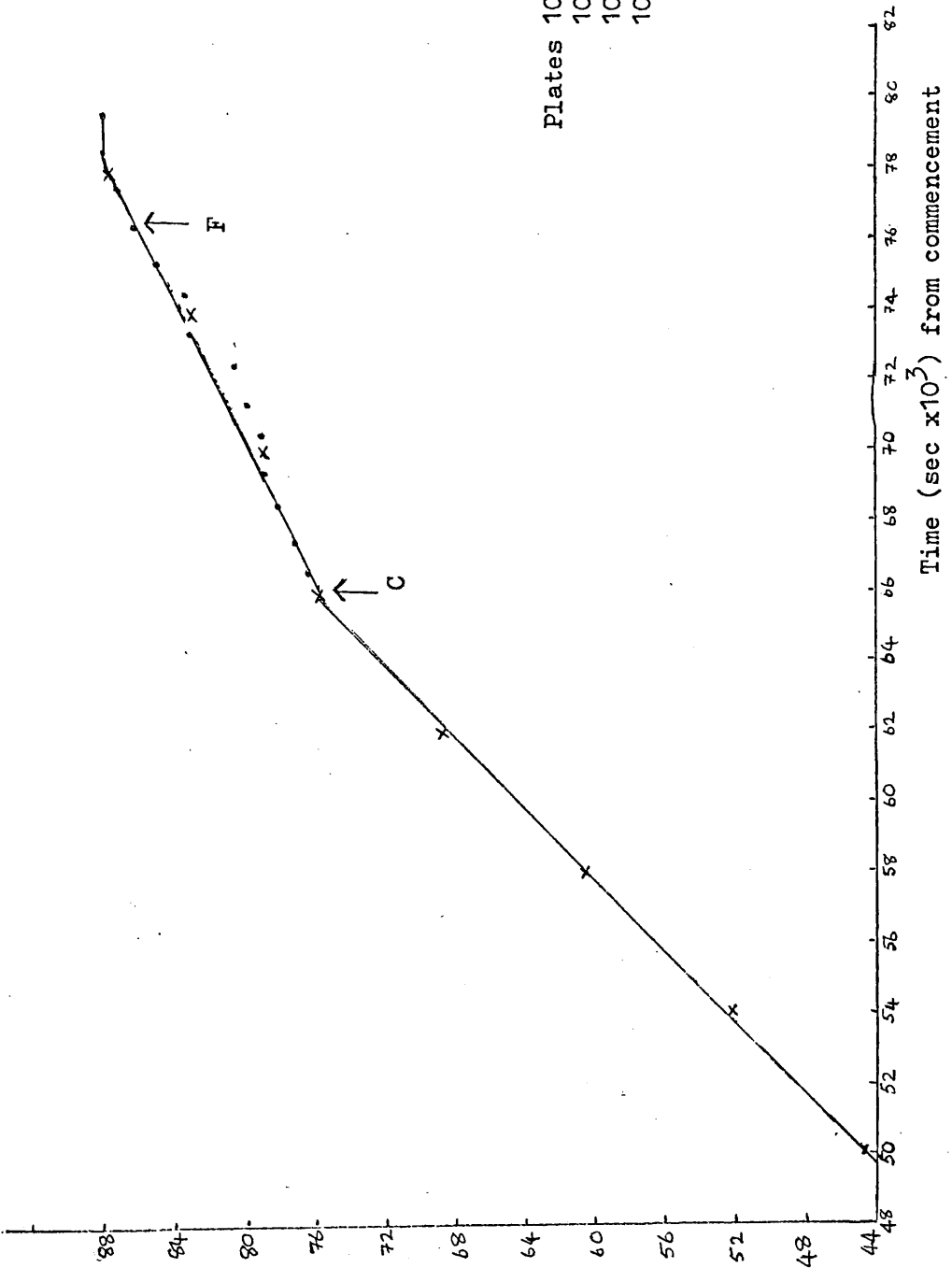


Fig. 8c

Intact stolon chilled

Counts  $\times 10^3$



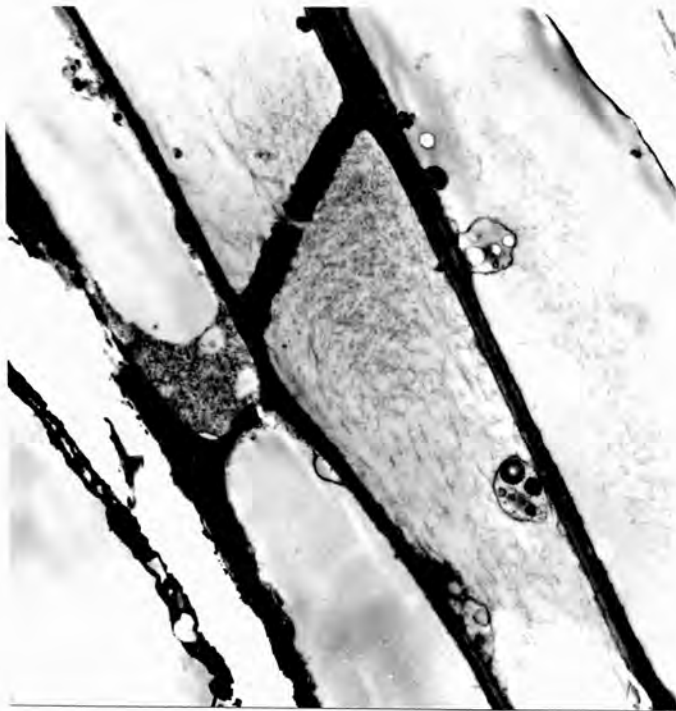
and the ice replaced as necessary. It was a measure of the success of the insulation that this was required only infrequently, even at a greenhouse temperature of 30°C!

Some of the chilling experiments were performed on the decorticated stolons, and others with the stolon in their natural state. The reason for this was entirely one of timing. After decortication it was necessary for experimental validity, to monitor the rate of accumulation of  $^{14}\text{CO}_2$  for some time. Frequently the operation caused slight slowing in the accumulation rate. Inhibitory treatments could not be started until this rate had stabilised (as it usually did to nearly its previous level). It often required several hours before this was shown with certainty (see figure 7). The inhibitory treatments also needed to be applied for several hours (often 5 or 6) before a suitable length of graph could be plotted to show unequivocal inhibition. After several 14h long working days it was regretfully decided to abandon decortication in order to complete the experimental manipulations within the compass of a less exacting schedule. Figure 8 shows how stolons left entire responded to chilling treatments.

Fixation was always completed before the segment of stolon to be processed for microscopy was cut from the experimental plant. In the later experiments (which included some of those inhibited by chilling) chloroform was included in the glutaraldehyde fixative.

Results. Giaquinta and Geiger had concluded that the major effect of cooling on chilling - sensitive plants showed as a disruption of cytoplasmic material within the sieve tube, together with its displacement into the sieve plate pores. They had also noticed "increased vesiculation in the sieve tube cytoplasm". They used young Phaseolus petioles to illustrate their work and no specific observations were made on P-protein distribution, only on the movement of the discrete crystal.





## PLATE 10.2

x 13,300

Stolon decorticated before chilling. Fixed before excision in 5% glutaraldehyde. It was damaged during decortication. The P-protein is denatured and the adjacent cell shows plasmolysis.

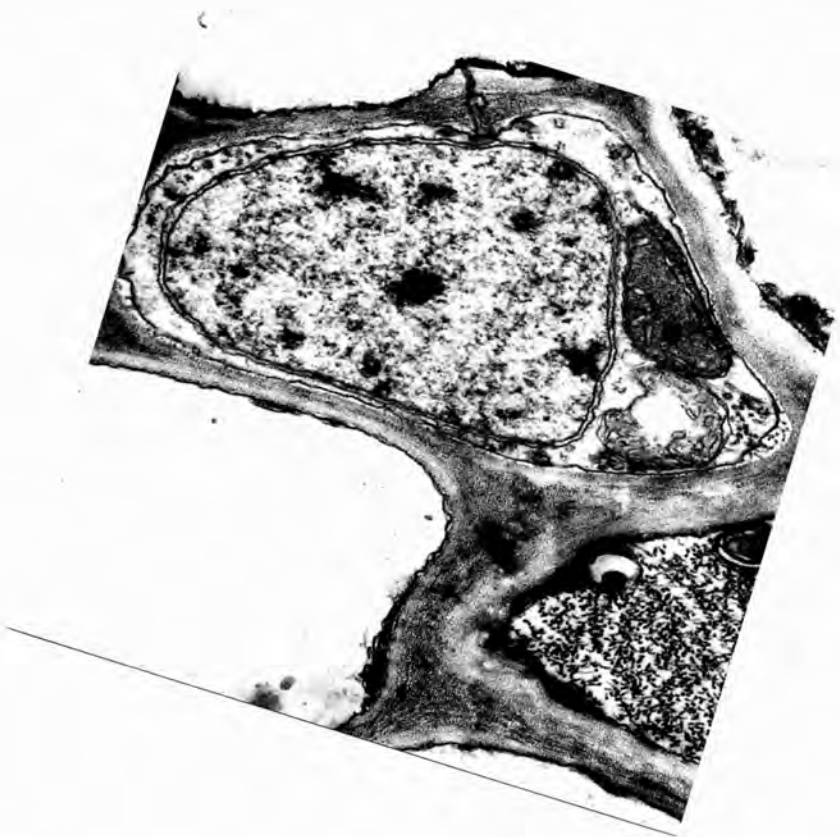
## PLATE 10.3

x 6,300

Stolon decorticated before chilling and then fixed before excision in 5% glutaraldehyde.







## PLATE 10.4

x 6,700

The companion cell shows good fixation, and membranous material adheres to the sieve tube wall.

## PLATE 10.5

x 27,000

T.S. of a companion cell. The ground cytoplasm looks less dense than usual.

Both micrographs are from stolons chilled whilst intact then fixed in 5% glutaraldehyde + chloroform.

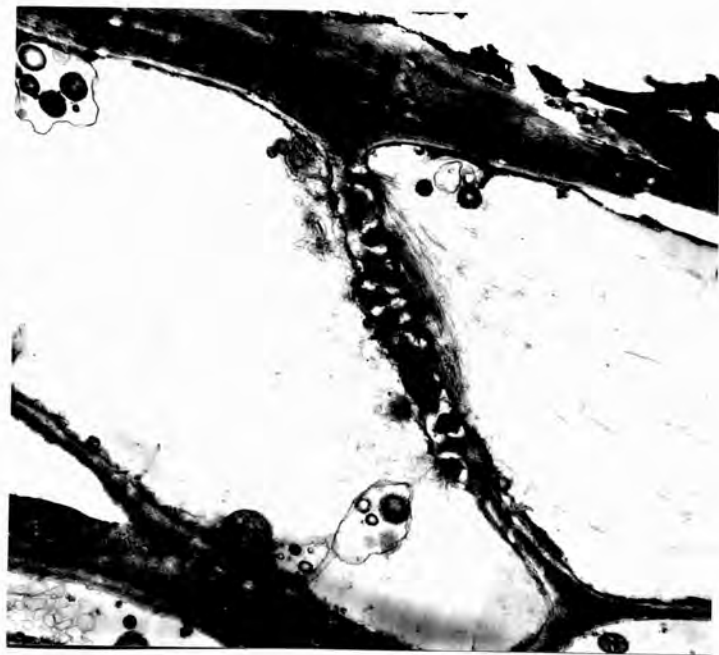
It is more surprising to find they made no observations as to the state of the plastids or callose, especially as in a paper published a little before theirs, Webb (1971) had pointed out the likelihood of minimal callose deposits being observed, as enzymatic action promoting its formation is also inhibited by low temperature.

The results of this study bear out this latter deduction. None of the micrographs obtained show heavy callose deposits, even in material known to have been physically damaged during preparation (plate 10.2). Here the cell adjacent to the sieve tube shows plasmolysis, sieve tube plastids are ruptured, and the P-protein is coarse and fragmented, but callose is minimal. In these circumstances it would usually be found where no chilling has been given. In general the degree of callose deposition coincides with that normally found where rapid fixation has been ensured, but where no chilling has been given.

That chilled tissue is not ipsofacto already damaged and incapable of good fixation is evidenced by plates 10.3, 10.4 and 10.5. The companion cells in the micrographs show no plasmolysis. The ground tissue is granular and even at the low magnifications of 10.3 and 10.4 polysomes can be identified. Membranes in 10.5 are smooth and intact, plastids of 10.3 and 10.5 are unruptured, and the mitochondria appear normal. These three micrographs also illustrate examples of the two major experimental procedures employed. Plate 10.3 shows a decorticated stolon fixed in glutaraldehyde without chloroform. Plates 10.4 and 10.5 show entire stolons fixed in glutaraldehyde and chloroform. The presence of the latter probably accounts for the starch grains not enclosed in plastids seen in plate 10.4.

The micrographs of chilled material show little structural difference from ordinary well-fixed material. It may be argued that the intact plastids generally found in the chilled material may have remained





## PLATE 10.6

x 9,750

Intact stolon chilled and fixed in 5% glutaraldehyde + chloroform. Neither the mobile plastids nor the mitochondrion shown have moved towards the sieve plate.







## PLATE 10.7

x 5,000

Stolon decorticated and fixed in 5% glutaraldehyde.

The montage shows the disposition of the P-protein in the lumen of the sieve tube, and also unbroken plastids.





## PLATE 10.8

x 10,000

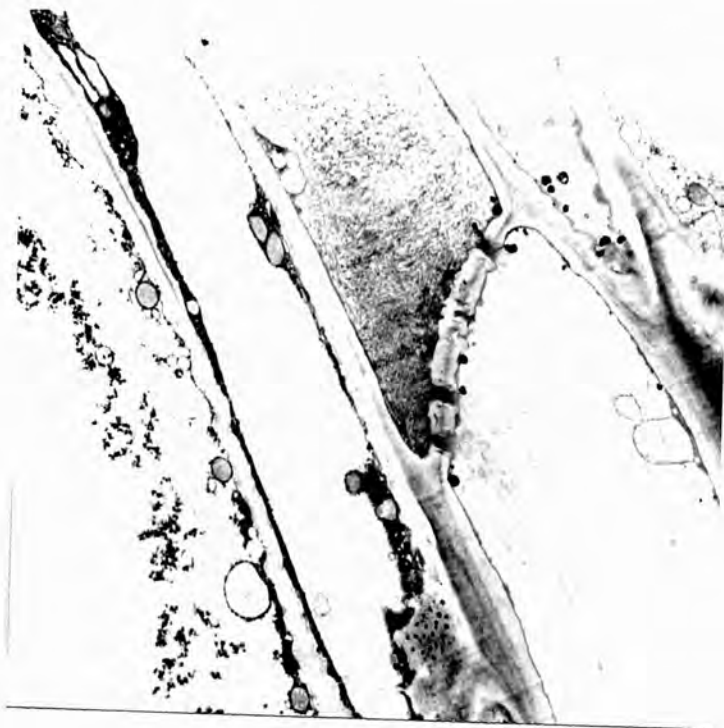
Montage of part of a sieve tube from a chilled,  
intact stolon fixed in 5% glutaraldehyde + chloroform.  
The starch grains from ruptured plastids do not have  
appeared to have moved far from their original positions.

unruptured due to the increased viscosity of the cell sap. This could have slowed down turgor pressure responses, or low temperatures may have strengthened the plastid membranes. The pictures, however, differ little from those illustrating chapter 3.

The only material which may suggest that the intactness of the plastids may be due to increased sap viscosity or strengthened membranes after chilling is shown in plate 10.6. Several intact plastids, as well as a small, but normally preserved mitochondria are present in the sieve tube, but this is taken from chilled material which had not been decorticated, neither was there any chloroform in the fixative. Both these circumstances would tend to make fixation less rapid than in other micrographs shown. The material, however, was completely fixed before it was cut from the parent plant, which makes even this evidence rather tenuous.

Some micrographs (plates 10.7 and 10.8) show odd aggregations of cytoplasmic material, and possibly some vesiculation, within the sieve tubes, as suggested by Giaquinta and Geiger. There is no indication, though of such inclusions being swept towards the sieve plates, indeed as the montages show, they are well distributed in the lumen of the sieve tubes. There are no indications of thin, disintegrating cell walls as also suggested by Giaquinta and Geiger and plenty of cytoplasmic material lines the cell walls. The distribution of the P-protein is somewhat variable, but the overall impression is that the variations found are typical of those normally seen in the sieve elements of uninhibited Saxifraga sarmentosa (L). Plate 10.9 shows fairly sparse P-protein in the pore, indeed a similar degree of blockage has been used to illustrate 'open' pores. There is however a definite blockage but nothing on the scale described by Giaquinta and Geiger. No membranous material is evident, and the P-protein is present on both sides of the







## PLATE 10.9

x 13,300

Decorticated stolon, chilled and fixed in 5% glutaraldehyde. The P-protein is evident near the plate, but no detached membranous material.

## PLATE 10.10

x 6,700

Decorticated stolon, chilled and fixed in 5% glutaraldehyde. The P-protein is seen mainly on one side of the pore. The plastids are intact.





## PLATE 10.11

x 27,000

Decorticated stolon chilled and fixed in 5% glutaraldehyde. The P-protein fibrils are aligned parallel to one another through the pores.

## PLATE 10.12

x 54,000

Intact stolon fixed in 5% glutaraldehyde + chloroform after chilling. The P-protein fibrils are arranged as indicated in fig. 5b, possibly showing mild surge effect.





## PLATE 10.13

x 81,000

Chilled, intact stolon fixed in 5% glutaraldehyde + chloroform. The P-protein fibrils are aligned parallel to one another through the pores. Membranous material adheres to the cell wall.

pore. Plate 10.10 also illustrates a condition of P-protein distribution frequently found and thought to be typical of an actively translocating sieve element (Spanner, 1979), having most of the P-protein on one side of the plate. Plates 10.11 and 10.13 show the equally frequently observed position of P-protein in the pores. The fibrils appear to be aligned in a parallel position through the pores, and as already discussed in chapter 8, present a picture unlikely to occur as a result of any imposed force, be it release of turgor pressure or chilling as implied by Giaquinta and Geiger. Plate 10.12 is a further illustration of the configuration shown in figure 5b of chapter 8 and is at most thought to represent a very mild surge, nothing like that envisaged by Giaquinta and Geiger. The P-protein in these pores appears to be in a natural position and not likely to have been forced in there. Plate 10.12 also shows membrane material adhering to the walls of the sieve element (arrowed), but with no evidence of any movement having occurred, and certainly not showing the disintegration noticed by Giaquinta and Geiger.





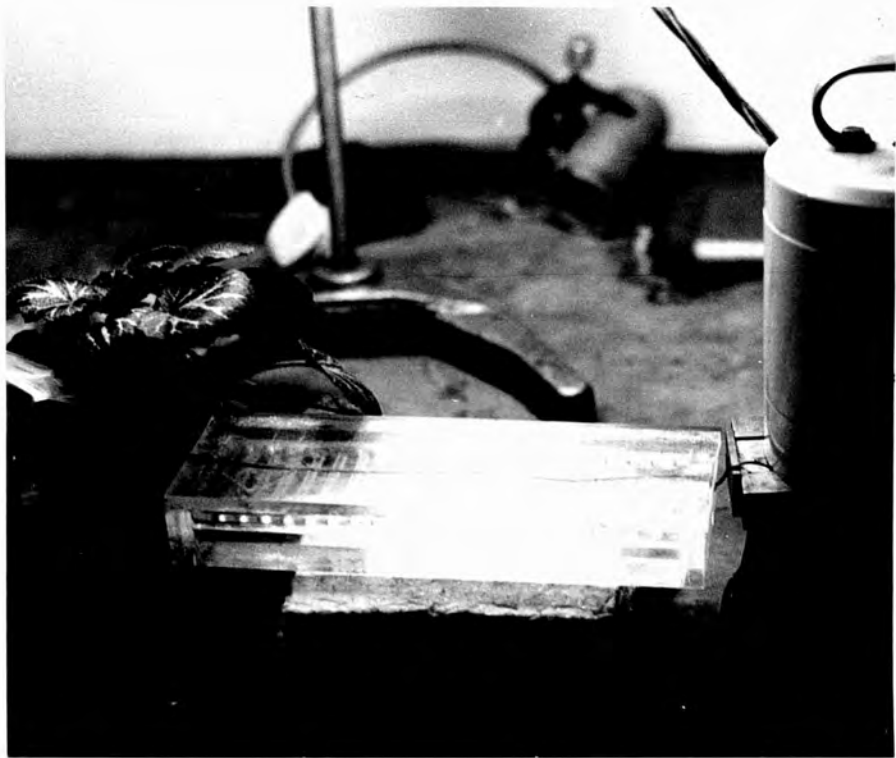


PLATE 11.1

Apparatus used for the administration of HCN gas  
to the stolon.

## Chapter 11

## INHIBITION OF TRANSLOCATION WITH CASEOUS CYANIDE

Method. Plants of Saxifraga sarmentosa (L.) grown under conditions similar to those used for the chilling experiments were also utilised for this series. Especially long stolons, not less than 45 cm were needed so that an adequate length could be treated. Qureshi and Spanner, (1973 d) found that marked inhibition was readily observed when the cyanide gas was applied over a length of about 25 cm. The subtending leaves of the stolons used for the experiments were fed with  $^{14}\text{CO}_2$  as previously described. Before inhibitory treatment was started a steady accumulation rate of  $^{14}\text{C}$  was recorded at the daughter plant. The first experiments were conducted using decorticated stolons, but in view of the time involved, naturally entire stolons were subsequently used. The apparatus used for the application of cyanide gas is shown diagrammatically in Qureshi's paper (1973 d, his figure 1), and also in plate 11.1. It consisted of a perspex chamber approximately 25 cm long. The stolon fitted into a groove in the perspex and was held gently by cotton wool soaked in petroleum jelly to render the whole chamber gas-tight. The stolon rested on a perforated platform above a reservoir containing 25 ml of  $5 \times 10^{-3}\text{M}$  KCN + methyl orange indicator. A perspex lid was sealed to the chambers with petroleum jelly. The KCN was acidified with 9% sulphuric acid (the indicator turning purplish) using a hypodermic syringe, the needle passing through a small hole in the lid which was subsequently sealed with a greased cover slip. The same hole was used at the end of the experiment for the injection of 1M NaOH to stop further HCN production. These experiments were conducted in a fume cupboard with the extractor fans full on, under the same lighting conditions as described in chapter 9. The accumulation of  $^{14}\text{C}$  at the distal end of the stolon was plotted as shown in figures 9 and 10. When a

Enhanced rate of translocation after HCN

Fig.9

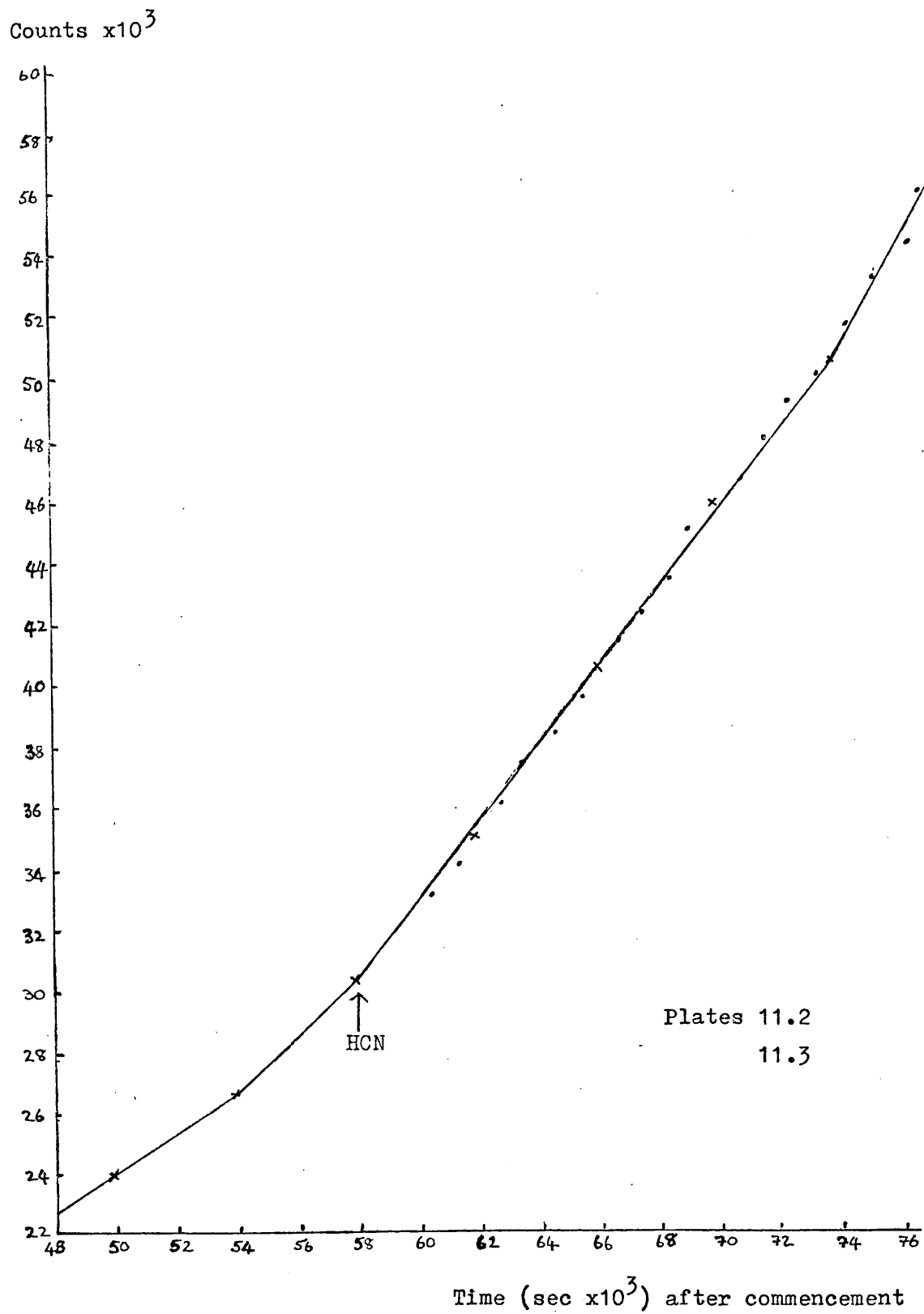


Fig. 10a

Inhibition with HCN

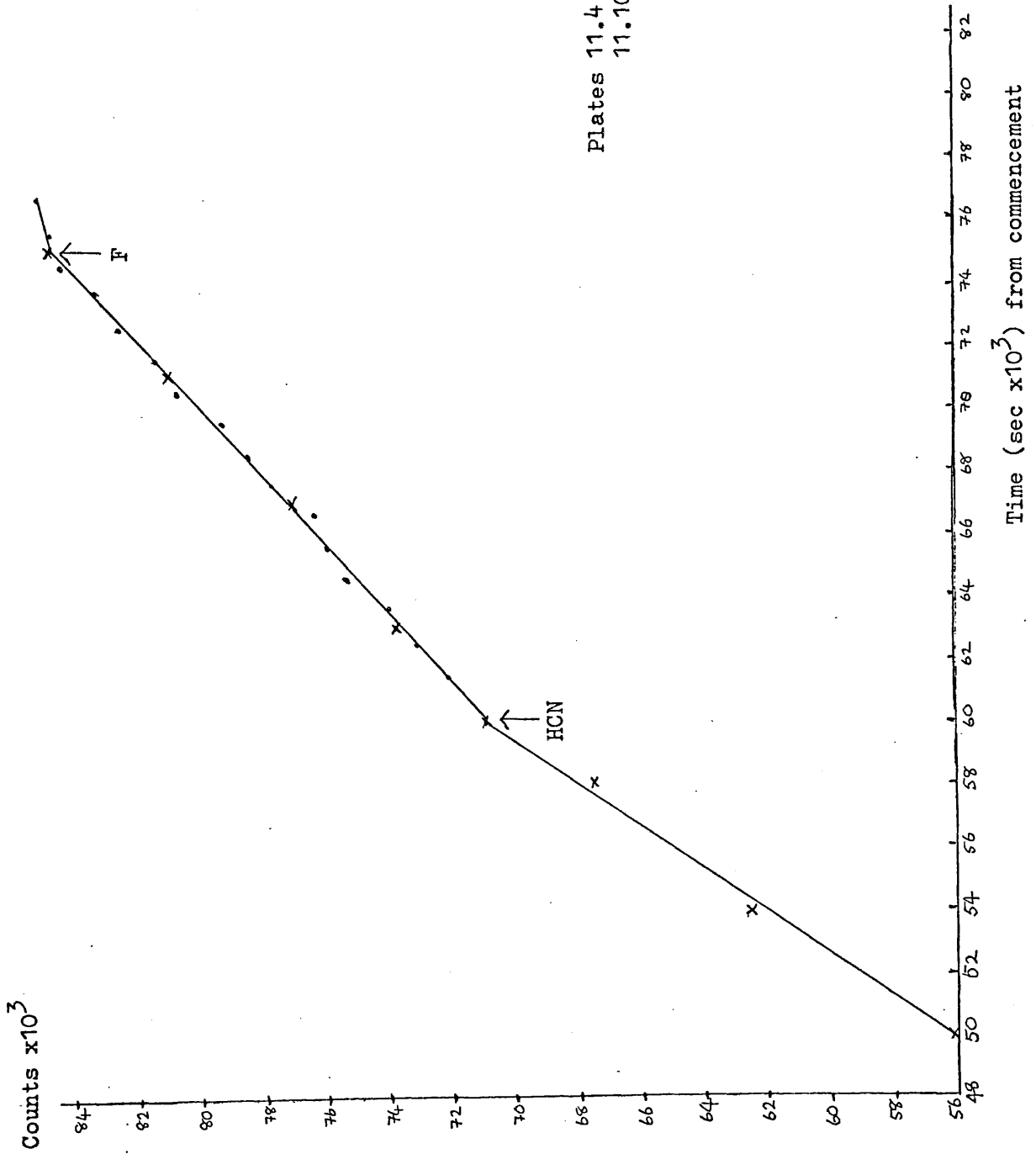
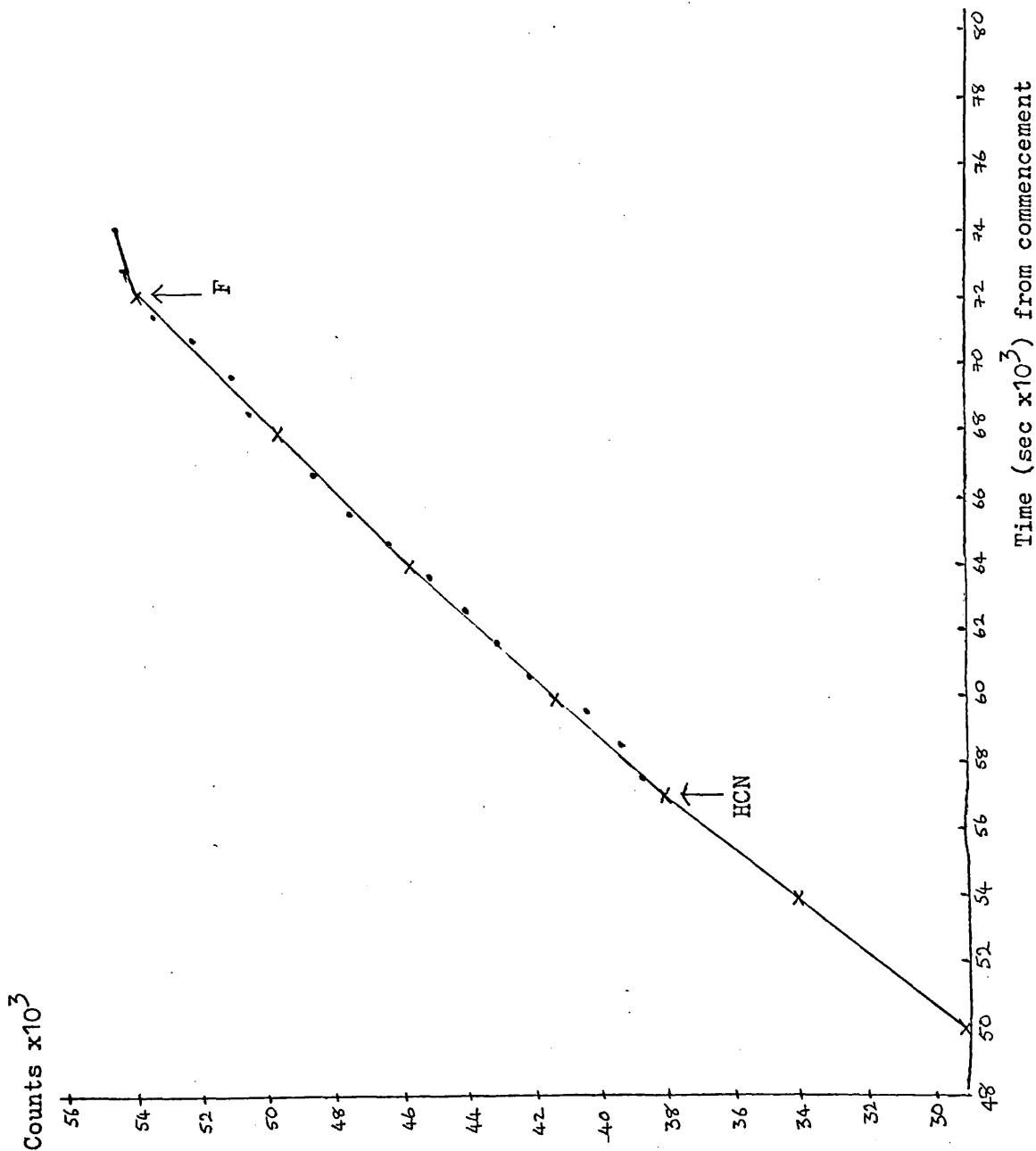


Fig. 10b

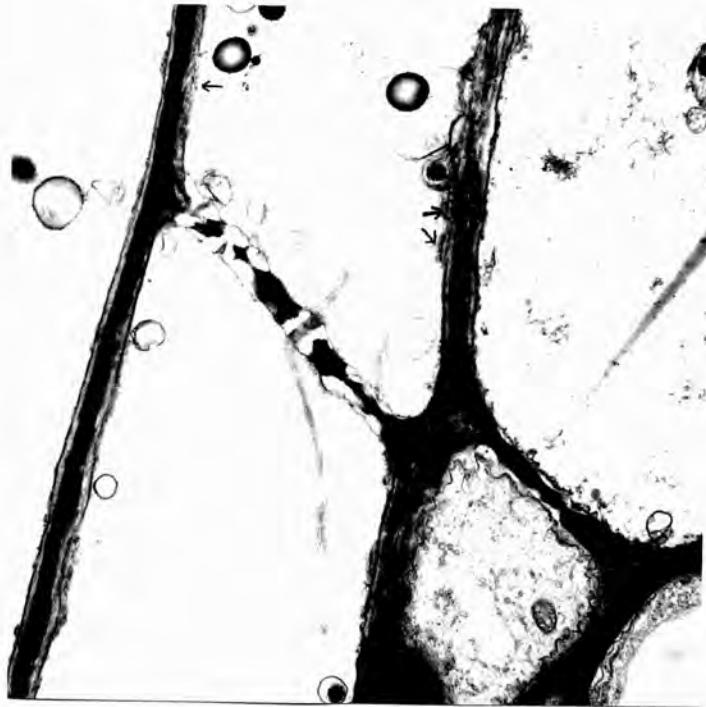
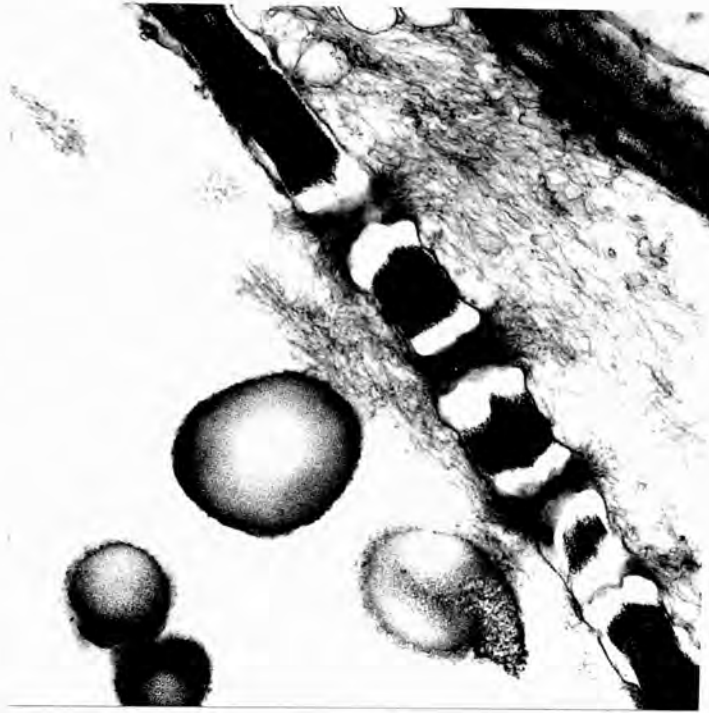
Inhibition with HCN



Plates 11.5  
11.9







## PLATE 11.2

x 33,500

The plastids are ruptured and callose lines the sieve plate pores.

## PLATE 11.3

x 9,750

The starch grains have moved little from the ruptured plastid.

Both micrographs show stolons, inhibited whilst intact and whose translocation rates were enhanced by the cyanide. Fixed in 5% glutaraldehyde + chloroform.

steady rate of accumulation was indicated the KCN was acidified, and the accumulation rate was monitored for several hours further. When a new, steady rate showed the alkali was injected into the chamber so that gas production ceased. The chamber was opened immediately and a grooved petri dish 10 cm in diameter containing phosphate buffered 5% glutaraldehyde, latterly containing chloroform as well, was placed under the stolon and covered. The stolon portion prepared for ultrastructural examination was not excised until fixation was complete (several hours). Further preparation was carried out as described in Appendix J. To complete the picture material was similarly prepared from stolons which had been shown to have had their translocation inhibited by gaseous cyanide, but which had at least partially recovered from the inhibition on removal of the treatment. The stolons used were from another worker's physiological experiments, and unfortunately the relevant graphs could not be obtained. One stolon was fixed half an hour after the HCN had been stopped, when slight improvement in the translocation rate was already showing. The other was fixed some 5h after inhibition was stopped, when the rate had apparently returned to normal.

As can be seen from the graphs in figure 9 the application of HCN gas sometimes produced an enhanced rate of translocation. The results shown appeared after the chamber had been thoroughly checked for leakage and fresh KCN had been prepared. Other workers' concurrent experiments running in the same laboratory using the same subject, but alternative apparatus also demonstrated an enhanced translocation rate in some experiments. Because this occurred with some frequency stolons exhibiting this feature were also prepared for ultrastructural examination.

Results. Plates 11.2 - 11.3 are taken from plants whose translocation rate was not inhibited by the HCN. Plates 11.2 and 11.3 show sieve plate pores which have a moderate amount of callose lining them. There is little evidence of pressure release surge. Many of the plastids are



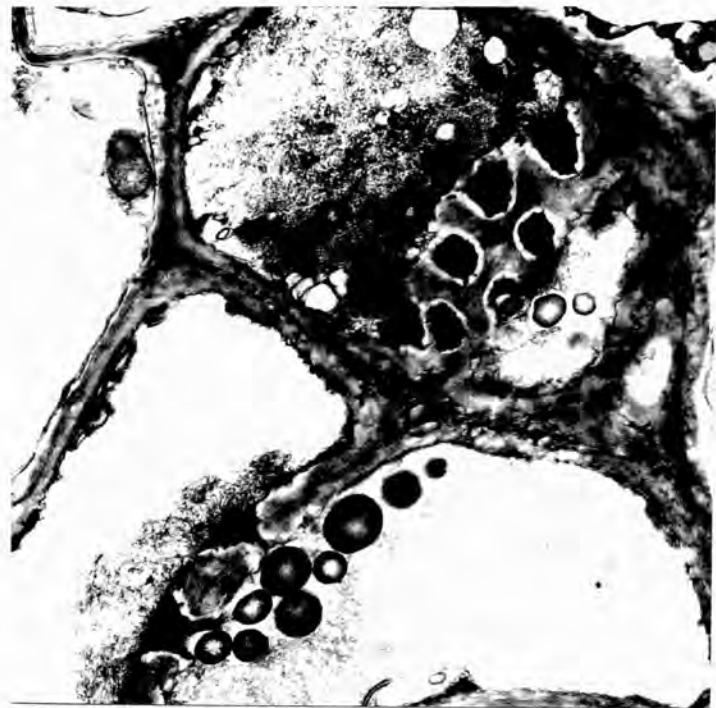
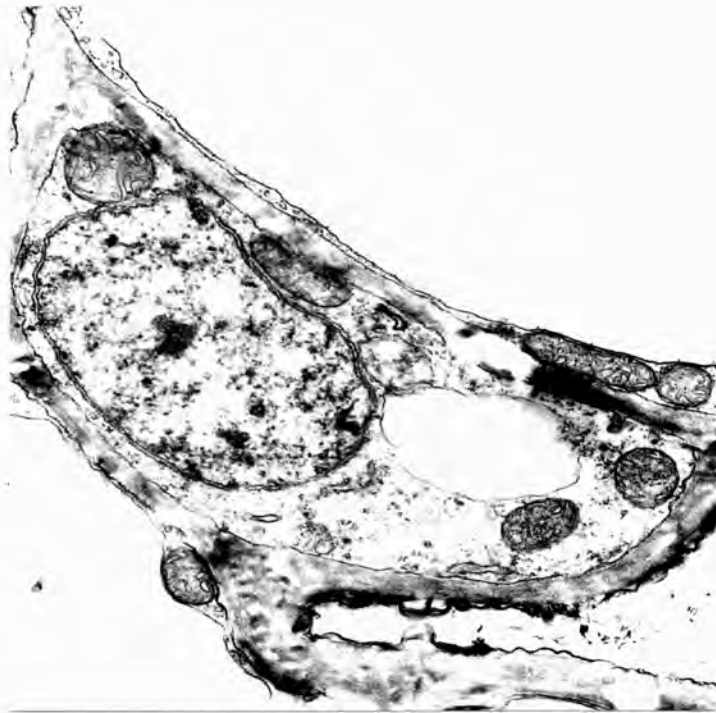


PLATE 11.4

x 20,000

Companion cell showing good fixation.

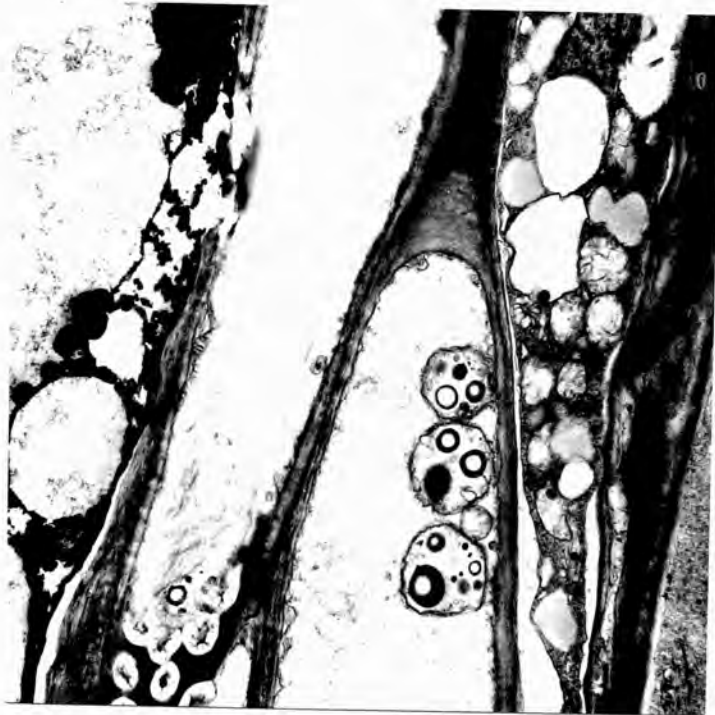
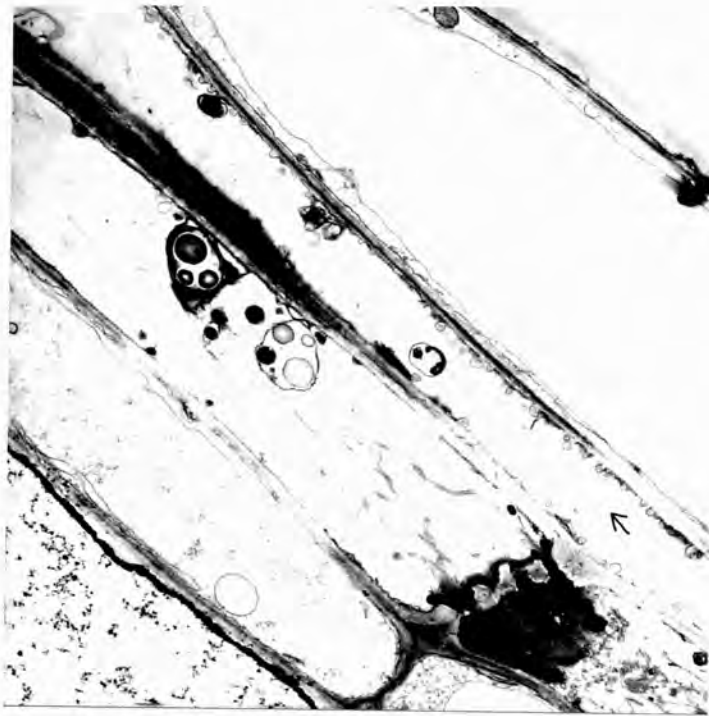
PLATE 11.5

x 13,300

T.S. showing a sieve plate and a lateral sieve plate  
with some callose lining the pores.

Micrographs from stolons inhibited when intact and  
fixed in 5% glutaraldehyde + chloroform.







## PLATE 11.6

x 6,700

Unbroken plastids downstream of surge, (direction is indicated by the arrow).

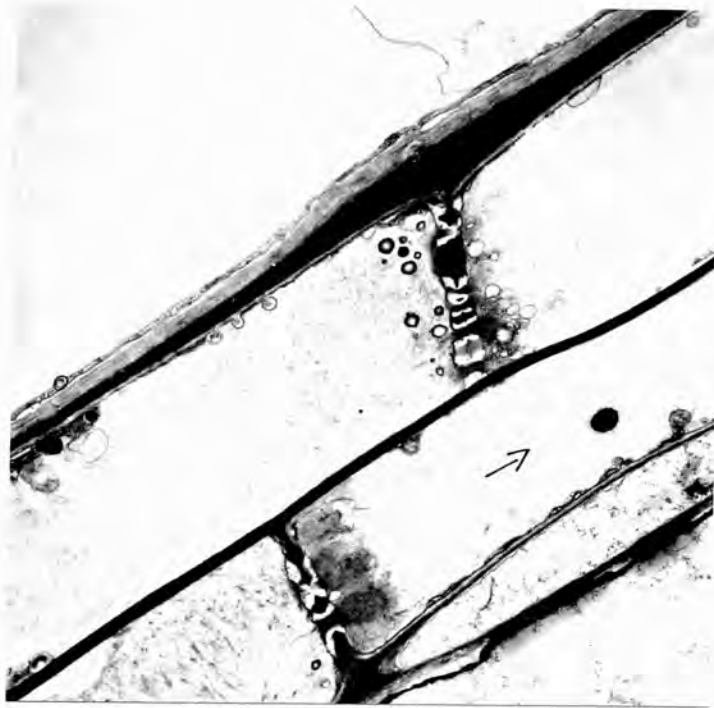
## PLATE 11.7

x 9,750

Intact plastids at the end of a sieve tube.

Both micrographs are from material inhibited as intact stolons before fixing in 5% glutaraldehyde + chloroform.





## PLATE 11.8

x 6,700

Although there are signs of material at the sieve plate which might be interpreted as membranous, the plasma membrane is still attached to the sieve tube wall, and activity is indicated by the pinocytotic vesicles.

## PLATE 11.9

x 33,500

Sieve plate pores lined with callose.

Both micrographs are taken from intact, inhibited stolons fixed in 5% glutaraldehyde + chloroform. The arrows indicate the probable direction of surge.

broken, and in 11.2 some released grains have apparently moved towards the sieve plate. There is evidence of trailing P-protein through a pore in 11.3, although in this micrograph the plastid contents show little sign of movement. There are cytoplasmic materials within the lumens of both sieve elements illustrated, but there is also plenty of cytoplasmic material lining the cell wall as arrowed in 11.3.

Micrographs from tissue in which translocation was definitely inhibited (see figure 10) show little differences from 11.2 and 11.3. The companion cell shown in 11.4 does not appear to have suffered any damage as a result of the inhibition. Membranes are smooth and parallel to one another and mitochondria appear normal. There is no evidence of plasmolysis having taken place. Callose deposits are variable. The portion of the sieve plate caught transversely in plate 11.5 appears to have only a little callose lining the pores. The lateral sieve plate shown at the bottom of the same plate also shows little callose, but P-protein is seen on both sides of the pore, together with the contents of broken plastids. This appears to be a constant feature in sieve elements of material treated with cyanide. The only unbroken plastids were either downstream of a pressure release as shown in 11.6, or in what may well be a young, imperforate sieve tube as shown in 11.7. The presence of the contents of broken plastids near the sieve plate is also a feature of the micrographs of sieve elements whose translocation had been inhibited by cyanide, and by various other methods (Qureshi and Spanner, 1973 e). There is also evidence of surge having taken place. The movement of the contents of plastids towards the sieve plate, so frequently observed in this work with cyanide, is an indication of this. The position of the P-protein fibrils in the pores shown in plates 11.8 and 11.9 also suggests that there has been some surging movement within the sieve element. The direction of the surge in both micrographs appears to be in the direction of the arrows. The disposition of the P-protein in the lower



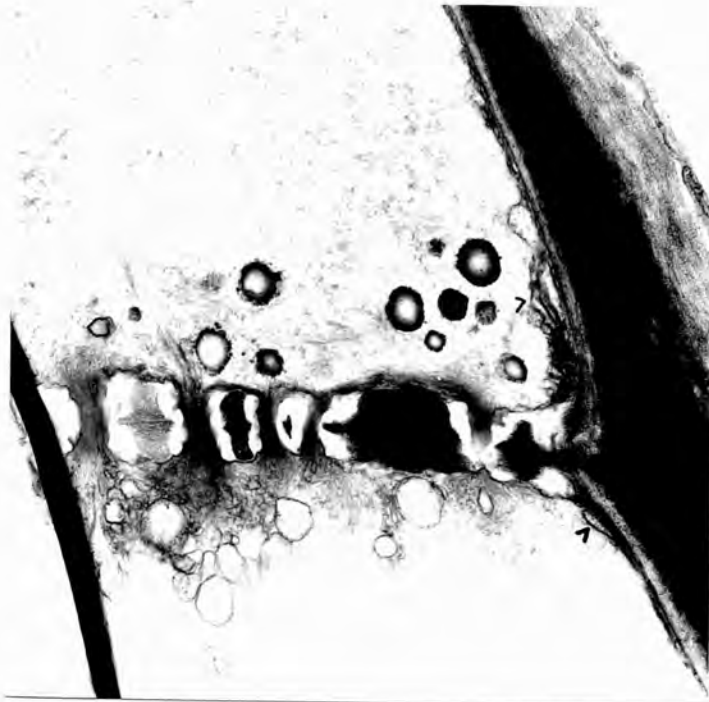


PLATE 11.10

x 9,750

Streaming P-protein and free starch grains, but  
no loose cytoplasmic material.

PLATE 11.11

x 20,000

Enlarged area from the same section as 11.8, but  
here attached membrane material is visible (arrowhead).

Intact, inhibited stolons fixed in 5% glutaraldehyde  
+ chloroform.





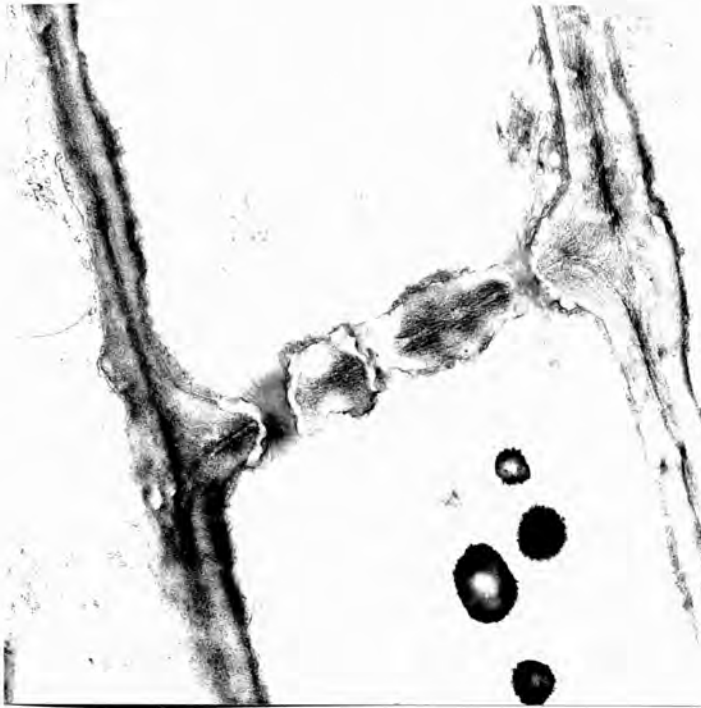


PLATE 11.12

x 20,000

Very little evidence of P-protein disturbance although plastids are ruptured.

PLATE 11.13

x 20,000

Loose material is seen on both sides of the plate!

Intact, inhibited stolons fixed in 5% glutaraldehyde  
+ chloroform.



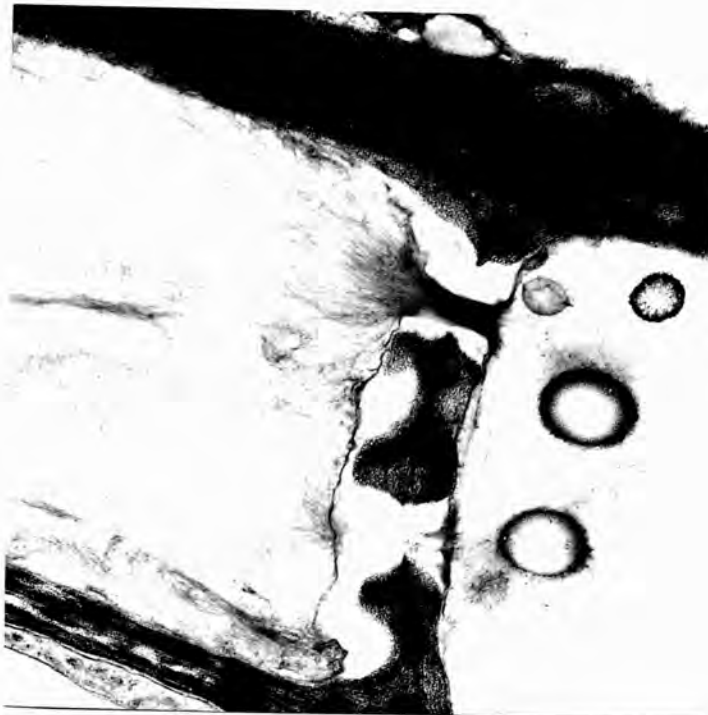
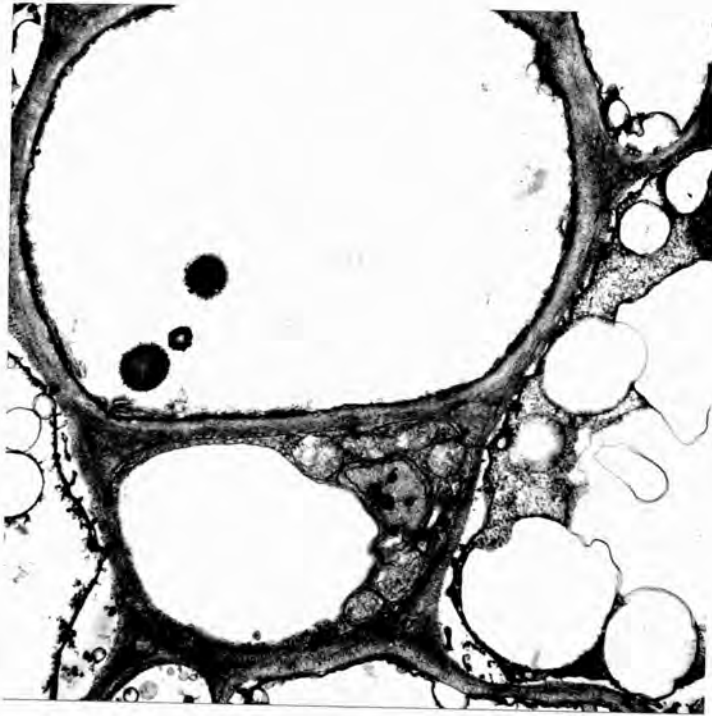


PLATE 11.14

x 13,300

T.S. of a sieve element and a well fixed companion cell.

PLATE 11.15

x 27,000

Evidence of mild surge?

Micrographs from intact stolons whose cyanide inhibition had been stopped 30min. before fixation in 5% glutaraldehyde + chloroform.



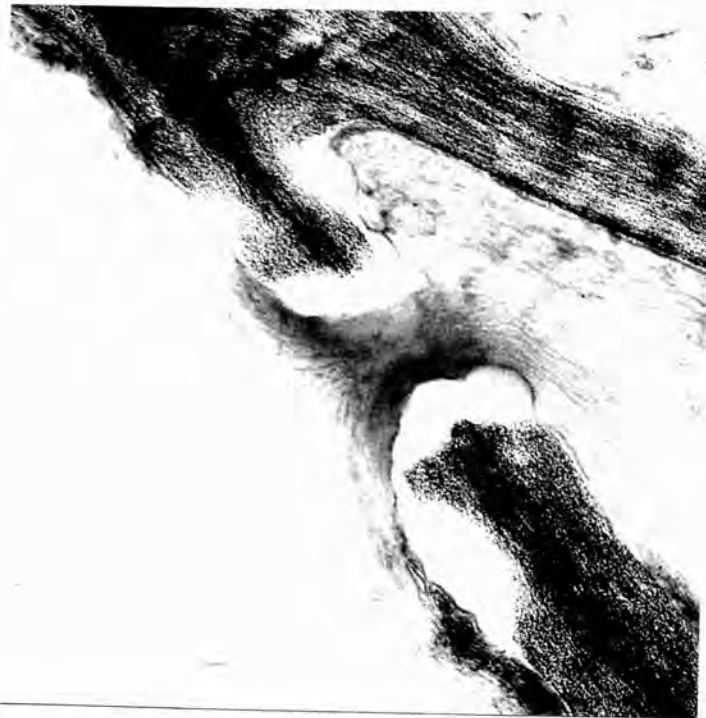




PLATE 11.16

x 20,000

Free starch grains on either side of the plate,  
and yet appearing too large to have passed through!

PLATE 11.17

x 54,000

P-protein fibrils apparently bent by mild surge.

Inhibitory treatment removed from the intact stolon  
5h. prior to fixation in 5% glutaraldehyde + chloroform.



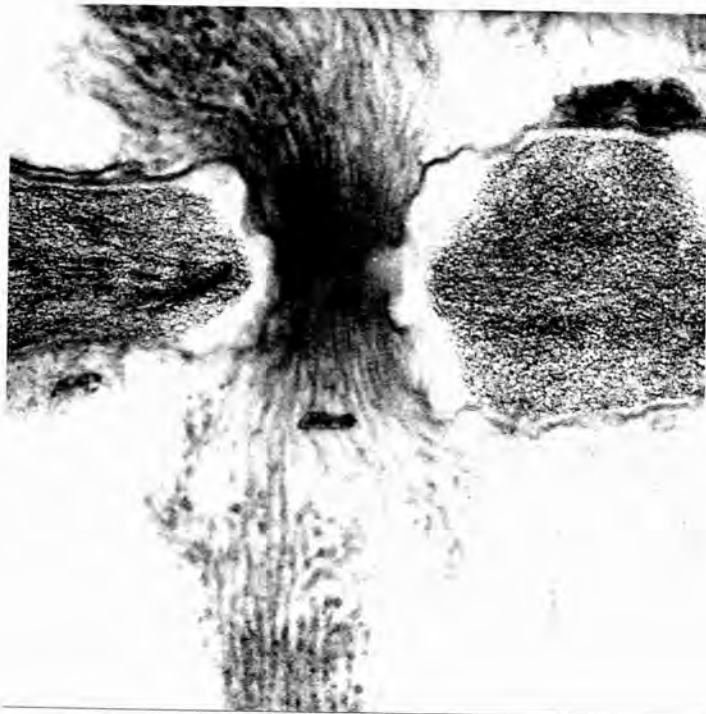


PLATE 11.18

x 81,000

Alignment of P-protein through the pore from  
material recovered from inhibitory treatment for  
5h. Fixed in 5% glutaraldehyde + chloroform.

sieve element of 11.8 shows the type illustrated in figure 5c. The flattening of the fibrils upstream of surge seen in 11.9, together with trailing fibrils downstream is also indicative of mild surge, but the dense P-protein seen against the sieve plate in 11.6 indicates stronger surge. Fairly mild surge is also indicated in the remaining micrographs of material whose translocation was being inhibited at the time of fixation by cyanide (plates 11.10 - 11.13), but the plugging of the pores with P-protein is no more dense than that usually found, and the alignment of the banded fibrils through the pores of 11.12 is noteworthy. All these micrographs show cell walls apparently lined normally with cytoplasmic material, but in 11.11 - 11.13 there is what appears to be cytoplasmic material in the lumen of the sieve elements as well.

The image presented by stolons whose translocation rate had just started to recover from cyanide inhibition is shown in plates 11.14 and 11.15. The normal-looking companion cell in 11.14 and the broken plastid in the adjacent sieve tube, present a very similar picture to those sampled under full inhibition. 11.15 shows more callose lining the sieve plate pore than was frequently seen, but this was a variable condition. Both these micrographs feature cytoplasmic material lining, apparently naturally the cell walls of the sieve elements.

Plates 11.16 - 11.18 are taken from the stolon whose translocation rate had apparently returned to normal 5h after inhibitory treatment had been removed. The overall picture varies little from that seen during inhibition. Broken plastids are seen on either side of the sieve plate in 11.16, but evidence of mild surge, ie slight flattening of the P-protein fibrils was also observed. Both in plates 11.17 and 11.18 the beautifully aligned P-protein fibrils through the sieve plate pores give no evidence of being anything but in their natural position.

The protein in these micrographs shows more evidence of streaming, as though some movement of the sieve element contents may have resumed,

and is the only obvious structural difference from sieve elements which were fixed while inhibited. In none of these micrographs is there a heavy deposit of P-protein on one side of the sieve plate as seen, for example in plate 11.6.

The images of the recovered material are so little different from those of inhibited material that it is difficult to believe that structural changes account for the slowing of the translocation rate.

## Chapter 12

## DISCUSSION

Giaquinta and Geiger (1973 and 1977) contend that in the sieve elements of material inhibited by chilling or cyanide, the sieve plate pores are plugged unnaturally with cytoplasmic material displaced by the treatment. Their definition of cytoplasmic material is that it is matter which originated from the former lining of the lateral walls (1977), but in their earlier paper (1973) it appears to include P-protein and organelles also. In this report their later definition has been adopted, and P-protein and organelles have been distinguished from membranous and vesicular material which could have been derived from cytoplasmic lining material.

The statement which opened this discussion concerning the state of the sieve plate pores of inhibited material has not been borne out by this study. Pores were not observed where cytoplasmic material could conceivably have caused any blockage. Giaquinta and Geiger suggested in both their papers that the cytoplasmic material found at the sieve plate was derived from "material that formerly lined the lateral walls". This suggestion has not been confirmed by this work. Micrographs of both chilled and cyanide inhibited material show no more cytoplasmic material within the sieve tube lumen and pores than is normally found there. The lateral walls of the sieve elements appear to have the usual amount of lining. Correspondingly Giaquinta and Geiger's suggestion that the cytoplasmic material they observed "is often continuous with the material lining the walls" could not be corroborated. It is possible that the material which they observed had their linings detached from the wall, either by the surge set up as sieve tubes freeze (Spanner, 1978), or as a result of poor chemical fixation. There may be evidence for the former hypothesis in the region of plate 6.5 which

is arrowed. Here the somewhat denatured, and therefore not readily identifiable material may be detaching from the cell wall. Fisher (1975) also refers to the presence of stacks of endoplasmic reticulum on either side of the sieve plate. The tissue he used had not been subjected to any form of inhibition, but he did use cryofixation techniques.

Lack of callose lining the sieve plate pores of inhibited material is reported, both by Giaquinta and Geiger, and by Qureshi (Qureshi and Spanner, 1973 e), although this lack is not always corroborated by published micrographs. The conclusion derived from the present study is that the quantity of callose generally seen is rather a function of the rapidity of fixation. When methods of rapid fixation are used the amount of callose observed is usually minimal. Qureshi (Qureshi and Spanner, 1973 e) found that the quantity of callose he noted depended on the fixative used. The alcohol/acetic acid fixative, which was the one he used when little callose was seen, is very drastic and a lipid solvent; the chloroform employed in the present study attacks membranes. It is reasonable to suppose that in both cases membranes were weakened allowing rapid entry of the fixative, as well as possible lateral leakage of sieve element contents. This lends weight to the argument that in the in vivo state a functioning sieve tube has little callose lining the pores.

Although little callose was seen lining the sieve plate pores in the chilled material studied, rather more was generally observed in material inhibited by cyanide, in spite of the rapid fixation methods employed. This does not concur with Qureshi's conclusions. It is suggested that the difference may be due to the influence of temperature. Work has been published on the effects of heat on callose formation (McNairn, 1972). This author found that when cotton was subjected to temperatures above 40°C callose deposits increased considerably. Webster



and Currier (1968) had previously reached similar conclusions and quoted evidence from other published work that in "wheat root hairs callose deposition increased above 30°C". Many of the experiments reported in this present study were conducted in conditions in which the afternoon air temperatures exceeded 30°C and were occasionally in excess of 35°C. Fortunately for the validity of the cyanide experiments concerned these high temperatures only occurred in the later afternoon when inhibition due to cyanide was well charted. Nevertheless fixation often took place at high temperatures. It is therefore concluded that this factor, rather than the cyanide treatment accounts for the greater than usual callose deposits. On this assumption it is not surprising to find that the chilling experiments showed no extra callose.

The position of plastids and their contents were used in judging whether mobile sieve element contents had been swept towards the sieve plate pores. Most workers who have observed phloem ultrastructurally agree that they are amongst the most fragile and their starch grains amongst the most mobile of the sieve element contents. Giaquinta and Geiger do not mention plastids in their papers. The chilled material studied here showed no variation either in condition or disposition of the plastids from those found in the sieve elements of other well fixed material. The plastids in the sieve elements of cyanide inhibited tissue were commonly broken, and frequently starch grains were close to the sieve plate.

The P-protein within the sieve plate of chilled tissue was dispersed throughout the sieve tube. All the pores contained its fibrils, which showed little evidence of disturbance and none of any indubitable surge effects. In cyanide inhibited material also the P-protein showed fibrils well-aligned through the pores (plate 11.12). However 'streamers' were frequently seen (plate 11.10) on one side of

the pore, usually the side opposite the starch grains, indicating some evidence of surge.

The problem, so frequently posed, as to whether sieve plate pores are naturally occluded with P-protein, or whether this material is carried into them during preparation has had more light shed on it during this study. In spite of the many different fixation methods tried, and the multiplicity of treatments given to the material, including inhibition, no pores have been seen where protein is not present. Indeed, so frequently are the banded fibrils neatly aligned in the pores, see plates 3.4, 3.8, 3.14, 8.2, 10.9, 10.12 and 11.18, that it has become impossible to believe that this disposition is an artefact of preparation. The overwhelming evidence suggests that the pores are plugged in vivo.

Webb (1971), after apparently having made some ultrastructural studies concludes that chilling has no obvious anatomical effect on the tissue. He suggests that some other reason for translocation inhibition must be sought. This is essentially the conclusion reached in the present study. The pictures of chilled tissue particularly are very similar to those of normally translocating, uninhibited material. If the translocation inhibition is not the result of an unnatural physical blockage of the sieve plate pores three other possibilities seem to remain, viz:-

- (a) changes in the characteristics of the membranes
- (b) inhibition of essential metabolism in the translocating cells
- (c) changes of sap viscosity within the sieve elements.

The first two suggestions could point to active mechanisms of translocation being involved, and indeed the last does not rule this out. Giaquinta and Geiger (1973) made the suggestion that "chilling causes the lipid portion of the plasma membranes, or perhaps the sieve tube reticulum to undergo a phase change". They suggest that this phenomenon could be the reason for the vesiculation and detachment of the cytoplasmic

material from the wall, which they observed. Such vesiculation has not been seen in this work, nor the thin cell walls. Their suggestion of phase change, although their reasons for suggesting it are not supported by this work, is not ruled out by an electro-osmotic theory of translocation which involves ion transport through the membranes.

More obvious disruptions of the sieve element is seen in the micrographs of tissue inhibited by cyanide. Indeed, so much is this the case that the conclusion could be reached that the mechanisms of inhibition in the two cases may differ. In Qureshi's micrograph (Qureshi and Spanner, 1973 e), his plate 1G shows a heavy deposit of P-protein which appears to block the pores. This, and ruptured plastids are constant features of micrographs taken during this study, see plates 11.6 and 11.8. Few unbroken plastids were ever observed.

Giaquinta and Geiger (1977) make the suggestion that "cyanide may cause loss of selective permeability of the sieve tube plasmalemma causing a sudden pressure release and loss of structural integrity of the sieve tube contents". It is known that cyanide is highly reactive with metallo-proteins forming stable complexes with many of them (Wishnick and Lane, 1969) but further study is needed to elucidate its full effect on membranes.

Giaquinta and Geiger (1977) also state that "the inhibition of transport (by cyanide) was correlated with a disruption of the structural integrity of the sieve tube (sieve pore blockage) rather than the impairment of a metabolic process in the translocation path driving translocation". This is unconvincing, especially as the 'recovered' sieve element presented very similar images to those observed in inhibited tissue. The statement is not verified either by other workers.

Qureshi and Spanner (1973 d) quote their findings to make the following suggestion, "the fact that tracer does not leak out and accumulate at the entry of the poisoned region indicates that the inhibitor has not

merely destroyed the integrity of the sieve tube membranes". They add "the most likely interpretation therefore ..... is that cyanide interferes with the supply of energy to the transport mechanism within the sieve tubes". In spite of their statement nothing in Giacinta and Geiger's work contradicts these findings, and the generally good condition of the tissues observed in this study tend to support it, see plate 11.4.

Furthermore Barclay (1978), in his observations using Nomarski optics found that "the assumption that P-protein in the form of slime candles or plugs, has a function in stopping flow through the sieve plate is suspect because slime candles do not necessarily stop surge flow through the sieve plate."

The main effect seen on membranes was in the notoriously sensitive plastid membranes. Barclay (1978) in his attempt to find out how much ruptured plastids could be used in assessing sieve element damage found that "most of the sieve elements examined in the present work were damaged at least to the extent that the plastids had burst", and this from an examination of living tissue! In this work no plasmolysis of companion cells was observed, and surely, if a plasmamembrane integrity is involved lateral leakage of the sieve element contents would have occurred, as noted in previous observations (Hogben and Spanner, 1977) so that less blockage of sieve plate pores, not more would have resulted.

No evidence has ever been put forward to suggest that cyanide (or other chemical inhibitor) has any effect on the viscosity of the sieve element contents. To a small extent at least, of course, this must be a consequence of chilling.

Once again the effect of cyanide on the cell metabolism remains the most probable candidate for the role of inhibitor in the search for the mode of action of this poison in transport inhibition.

Neither inhibitory treatment appeared to produce ultrastructural

effects sufficient to inhibit transport. Reasons for the slowing of the translocation rate must be sought in causes which produce no ultrastructural effect and it seems probable that these are metabolic. More work is required to elucidate this.

## APPENDIX

Methods used in preparation of the stolons of Saxifraga sarmentosa (L).

1. Stains and Techniques used for Optical Work.

A. Identification of lipids.

A saturated solution of Sudan IV was prepared in 70% ethanol and filtered.

Hand transections of fresh material were warmed in the Sudan IV until the alcohol boiled. They were then mounted in glycerine jelly.

B. Staining lignified tissue.

A saturated solution of Safranin 'O' was prepared in 50% ethanol and filtered. Parafin wax embedded sections, both L.S. and T.S. were de-waxed in xylene and then taken down a descending alcohol series to 50%. After 10 minutes in the Safranin they were then re-dehydrated. Usually when 90% ethanol was reached the sections were counterstained in fast green before the dehydration was completed. After clearing in xylene the sections were mounted in D.P.X.

C. Staining cellulose tissue.

A saturated solution of fast green was prepared in 90% ethanol and filtered. Wax embedded sections were taken to 90% ethanol before staining, dehydrating, clearing and mounting in D.P.X. This stain was used as a counterstain after Safranin 'O' or iodine in potassium iodide solution.

D. Identification of Starch.

Wax embedded sections were re-hydrated to distilled water through a descending alcohol series and then stained with iodine in a potassium iodide solution. Sections were usually dehydrated and counterstained with fast green, and some with Safranin 'O' as well.

E. Staining of thick (1 - 2 m) resin embedded sections for Optical work.

The thick sections were cut with a wet knife and the sections picked out of the boat using a nichrome wire loop. The sections were

dried down on warm slides on a hot plate set at 18 - 20°C. The sections were stained in a 1% solution of toluidine blue in 1% borax solution.

F. Preparation of material for wax-embedding.

Fixation. This was carried out in either 5% glutaraldehyde in phosphate buffer, pH 7.2, or, in an attempt to preserve the lipids, in Ciacco fixative.

Dehydration. An acetone series was used, starting with 7.5% acetone, and after transfer to 10% acetone, increasing concentrations in steps of 5% up to 100% acetone were used. The whole process was carried out slowly over a period of three days.

Clearing. The clearing agent and wax solvent used was chloroform.

Embedding. Parafin wax was added slowly until sufficient had dissolved, when the chloroform was then allowed to evaporate. The material was then embedded in blocks.

G. Ciacco fixative for lipid preservation (Lison 1936).

7.5% $K_2Cr_2O_7$	53 ml
40% Formaldehyde	16 ml
Formic acid	0.4 ml
Distilled water	30 ml

Small pieces (1mm lengths) of stolon were fixed for 2 days in the solution and post fixed for 7 days in 3%  $K_2Cr_2O_7$ . The material was washed in running water for 24 h before dehydrating, clearing and wax embedding in the usual manner.

Methods used in the preparation of stolons of Saxifraga sarmentosa (L) for electron microscopy.

H. Preparation of Phosphate buffers for glutaraldehyde fixation.

M/15 ie. 0.066M buffer solution

To prepare 100 ml. Sodium salt.  $Na_2HPO_4 \cdot 2H_2O$ . 2.37g.

Potassium salt.  $KH_2PO_4$ . 0.91g.

Relative proportions used for buffers of various pH values.

pH	ml. 0.066M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	ml. 0.066M $\text{KH}_2\text{PO}_4$
6.4	3.0	7.0
6.8	5.0	5.0
7.2	7.0	3.0
7.6	8.5	1.5

Glutaraldehyde was used as 5% solution in the buffer of relevant pH.

ie. 2.5 ml 25% glutaraldehyde: 10 ml buffer.

J. Normal fixation routine.

Fix in 5% glutaraldehyde  $1\frac{1}{2}$  - 2 h.

Wash in buffer. Two rinses and overnight at 4°C.

Postfix in 1%  $\text{OsO}_4$  in buffer. 2 h.

Rinse in distilled water.

Dehydration.	30% Acetone	15 min.
	50% Acetone	15 min.
	70% Acetone	15 min.
	90% Acetone	15 min.
	100% Acetone	2 x 20 min.
	50:50 acetone: propylene oxide	10 min.
	Propylene oxide	2 x 15 min.
Infiltration	10% resin	overnight
	50% resin	4 - 6 h
	100% resin	overnight
	100% resin	4 h
Embedding	Fresh 100% resin in moulds.	
Polymerisation	60°C for 24 - 48 h.	
Resin used	TAAAB resin	25.0g
	DDSA hardener	13.5g
	MNA plasticizer	11.5g
	BDMA accelerator	1.0g



K. Pipes buffer (Piperazine-N-N'-bis-(2ethanol sulphonic acid)).

0.3 solution of NaOH - Pipes.

- (i) 9g Pipes powder added to 50 ml distilled water.
- (ii) Using a pH meter 1N(M)NaOH was added dropwise until pH 8.0 was reached and Pipes powder had dissolved.
- (iii) The volume was made up to 100 ml with distilled water. From this stock 0.2M Pipes buffer was prepared which had pH 6.8 for diluting  $\text{OsO}_4$ .

Osmolality Buffer	mos. M	
	pH 6.8	pH 8
0.3M Pipes		813
0.3M Pipes	760	
0.2M Pipes	660	
5% glutaraldehyde + 0.08M Pipes		805
2% $\text{OsO}_4$ + 2M Pipes	750	

After the method of Salema and Brandão (1973).

L. Fluid for etching blocks and sections.

A saturated solution of sodium hydroxide was prepared in absolute ethyl alcohol and allowed to stand for several days until brown in colour. When used for etching it is important to keep the material covered so that sodium hydroxide crystals do not form.

M. Critical point Drying for Scanning Electron Microscopy.

This was carried out using  $\text{CO}_2$  in a Polaron CPD apparatus from both acetone and ethyl alcohol at a temperature of  $32^\circ\text{C}$ .

N. Gold Sputter Coating for Scanning Electron Microscopy.

A Polaron E5100 series II cool sputter coater was used to gold coat the specimen to a thickness of 100 nm.

## REFERENCES

- ANDERSON, R. and CRONSHAW, J. (1970). 'Sieve Plate Pores in Tobacco and Bean.' *Planta (Berl.)* 91, 173 - 180.
- BARCLAY, G. F. (1978). Ph.D. Thesis.
- BEHNKE, H. D. (1971). 'The Contents of the Sieve-Plate Pores in Aristolochia', *J. Ultrastruct. Res.* 36, 493 - 498.
- BOLE, B. and PARSONS, E. (1973). 'Scanning Electron Microscopy of the Internal Cellular Structure of Plants', *J. Microscopy* 98, 1, 91 - 97.
- BOUCK, G. B. and CRONSHAW, J. (1965). 'The Fine Structure of Differentiating Sieve Tube Elements', *J. Cell Biol.* 25, 79 - 96.
- BRANTON, D. and JACOBSON, L. (1961). 'Freeze Drying of Plant Material', *Exp. Cell. Res.* 22, 559 - 568.
- CRONSHAW, J. and ANDERSON, R. (1969). 'Sieve Plate Pores of Nicotiana', *J. Ultrastruct. Res.* 27, 134 - 148.
- CRONSHAW, J. and ANDERSON, R. (1971). 'Phloem Differentiation in Tobacco Pith Culture', *J. Ultrastruct. Res.* 34, 244 - 259.
- DA CRUZ, G. (1976). Ph.D. Thesis
- DESHPANDE, B. P. (1974). 'Development of Sieve Plates in Saxifraga sarmentosa (L)', *Ann. Bot.* 38, 151 - 158.
- DESHPANDE, B. P. (1976). 'Observations on the Fine Structure of Plant Cell Walls I', *Ann. Bot.* 40, 433 - 437.
- ENGLEMAN, E. M. (1965 a). 'Sieve Elements of Impatiens sultanii. I. Wound Reaction', *Ann. Bot. N. S.* 29, 83 - 101.
- ESCHRICH, W. (1963). 'Beziehungen Zwischen dem Auftreten von Callose und der Feinstruktur des Primären Phloems bei Cucurbita ficifolia', *Planta (Berl.)* 59, 243 - 261.
- ESCHRICH, W. (1965). 'Physiology of the Sieve Tube Callose', *Planta (Berl.)* 65, 280 - 300.

- EVERT, R. F., ESCHRICH, W. and EICHORN, S. E. (1973). 'P-protein Distribution in Mature Sieve Elements of Cucurbita maxima', *Planta* (Berl.) 109, 193 - 210.
- EVERT, R. F. (1977). 'Phloem Structure and Histochemistry', *Ann. Rev. Plant Physiol.* 28, 199 - 222.
- FISCHER, A. (1885). 'Ueber den Inhalt der Siebröhren in der Unverletzten', *Amer. J. Bot.* 51, 552 - 559.
- FISCHER, A. (1886). 'Neue Beiträge zur Kenntniss der Siebröhren', *Ber. Sächs Ges. (Akad.) Wiss.* 38, 291 - 336.
- FISHER, D. B. (1975). 'Structure of Functional Soybean Sieve Elements', *Plant Physiol.* 56, 555 - 569.
- GIAQUINTA, R. T. and GEIGER, D. R. (1973). 'Mechanism of Inhibition of Translocation by Localised Chilling'. *Plant Physiol.* 51, 372 - 377.
- GIAQUINTA, R. T. and GEIGER, D. R. (1977). 'Mechanism of Cyanide Inhibition of Phloem Translocation'. *Plant Physiol.* 59, 178 - 180.
- HEREWARD, F. V. and NORTHCOPE, D. H. (1972). 'A Simple Freeze-substitution Method for the Study of Ultrastructure of Plant Tissues', *Exp. Cell Res.* 70, 73 - 80.
- HOGBEN, C. and SPANNER, D. C. (1977). 'The Effect of Chloroform Vapour Treatment on the Appearance of the Sieve Plates of Saxifraga sarmentosa (L)', *Ann. Bot.* 41, 661 - 663.
- JENSEN, W. A. (1962). 'Botanical Histochemistry', W. H. Freeman and Co.
- JOHNSON, R. P. C. (1969). 'Crystalline Fibrils and Complexes of Membranes in the Parietal Layer in Sieve Elements', *Planta* (Berl.) 84.
- JOHNSON, R. P. C. (1973). 'Filaments but no Membranous Transcellular Strands in Sieve Pores in Freeze-etched, Translocating Phloem', *Nature* 244, 464 - 466.
- JOHNSON, R. P. C., FREUNDLICH, A. and BARCLAY, G. F. (1976). 'Transcellular Strands in Sieve Tubes; What are They?', *J. Exp. Bot.* 27, 1117 - 1136.

- LANE, B. P. and EUROPA, D. (1965). 'A Method for Etching Blocks',  
J. Histochem. Cytochem. 13, 579.
- LISON, (1936). Histochemie Animale. Methodes et Problemes.
- MCNAIRN, R. B. (1972). 'Phloem Translocation and Heat Induced Callose  
Formation in Field Grown Gossypium hirsutum (L)', Plant Physiol.  
50, 366 - 370.
- MELARAGNO, J. E. and WALSH, M. A. (1976). 'Ultrastructural Features of  
Developing Sieve Elements in Lemna minor - the Protoplast', Amer.  
J. Bot. 63, 8.
- PEASE, D. C. (1973). 'Substitution Techniques'. Advanced Techniques in  
Biological Electron Microscopy. Koehler.
- QURESHI, F. A. and SPANNER, D. C. (1971). 'Unidirectional Movement of  
Tracers along the Stolon of Saxifraga sarmentosa (L)', Planta (Berl.)  
101, 133 - 146.
- QURESHI, F. A. and SPANNER, D. C. (1973). 'Movement of <sup>14</sup>C Sucrose along  
the Stolon of Saxifraga sarmentosa (L)', Planta (Berl.) 110, 145 - 152.
- QURESHI, F. A. and SPANNER, D. C. (1973). 'Cyanide Inhibition of Phloem  
Transport along the Stolon of Saxifraga sarmentosa (L)', J Exp.  
Bot. 24, 751 - 762.
- QURESHI, F. A. and SPANNER, D. C. (1973). 'The Effect of Inhibitors on  
Callose Formation in the Sieve Tubes of Saxifraga sarmentosa (L)',  
Ann. Bot. 37.
- SALEMA, R. and BRANDAO, I. (1973). 'The use of Pipes Buffer in the  
Fixation of Plant Cells for Electron Microscopy', J Submicro.  
Cytol. 5, 79 - 96.
- SHIH, C. Y. (1974). 'SEM Studies of the Internal Organisation of Plant  
Organs', Scanning Electron Microscopy II', IIT Res. Inst.
- SIDDIQUI, A. W. and SPANNER, D. C. (1970). 'The State of Pores in  
Functioning Sieve Plates', Planta (Berl.) 91, 181 - 189.

- SIDDIQUI, A. W., JONES, R. F. and SPANNER, D. C. (1974). 'Translocation in the Stolon of Saxifraga sarmentosa (L). The Ultrastructural Background', Ann. Bot. 38, 145 - 149.
- SPANNER, D. C. (1975). 'The Electro-osmotic Theory', Phloem Transport, Plenum Press.
- SPANNER, D. C. (1978), 'The Münch Hypothesis, Freeze-substitution, and the Structure of Sieve Plate Pores', Ann. Bot. 42, 485 - 488.
- SPANNER, D. C. (1978). 'Sieve Plate Pores, Open or Occluded? A Critical Review', Plant, Cell and Env. 1, 7 -20.
- SPANNER, D. C. (1979). 'The Electro-osmotic Theory of Phloem Transport: A Final Statement', Plant, Cell and Env. 2, 107 - 121.
- TAMULEVICH, S. R. and EVERT, R. F. (1966). 'Aspects of Sieve Element Ultrastructure in Primula obconica'. Planta (Berl.) 69, 319 - 337.
- THAINE, R. (1964). 'Protoplast Structure in Sieve Elements', New Phytology 63.
- THAINE, R., PROBINE, M. C. and DYER, P. Y. (1967). 'The Existence of Transcellular Strands in Sieve Elements', J. Exp. Bot. 18, 110 - 127.
- TROUGHTON, J. and DONALDSON, L. A. (1972). 'Probing Plant Structure', Chapman and Hall.
- TURGEON, R., WEBB, J. A. and EVERT, R. F. (1975). 'Ultrastructure of Minor Veins in Cucurbita pepo leaves', Protoplasma 83, 217 - 232.
- WALSH, M. A. and EVERT, R. F. (1975). 'Ultrastructure of Metaphloem Sieve Elements in Zea mays', Protoplasma 83, 365 - 388.
- WALSH, M. A. and POPOVICH, T. M. (1977). 'Some Ultrastructural Aspects of Metaphloem Sieve Elements in the Aerial Stem of the Holoparasitic Angiosperm Epifagus virigiana (Orobanchaceae)', Amer. J. Bot. 64, 326 - 336.
- WARK, M. C. and CHAMBERS, T. C. (1965). 'Fine Structure of the Phloem of Pisum sativum. I The Sieve Element Ontogeny', Aust. J. Bot. 13, 171 - 183.

- WEATHERLEY, P. and JOHNSON, R. P. C. (1968). 'The Form and Function of the Sieve Tube: a Problem in Reconciliation', *Int. Rev. Cytol.* 24, 149 - 192.
- WEBB, J. A. (1971). 'Translocation of Sugars in Cucurbita melapepo. VI The Reversible Low Temperature Inhibition of  $^{14}\text{C}$  Movement and Cold Acclimation of Phloem Tissue', *Can. J. Bot.* 49, 717 - 733.
- WEBSTER, D. H. and CURRIER, H. B. (1968). 'Heat Induced Callose and Lateral Movement of Assimilates from Phloem', *Can. J. Bot.* 46 1215 - 1220
- WISHNICK, M. and IANE, M. D. (1969). Inhibition of Ribulose diphosphate carboxylase by Cyanide', *J. Biol. Chem.* 244, 55 - 59.