A STUDY OF PHLOEM STRUCTURE AND FUNCTION BY ELECTRON MICROSCOPY AND OTHER MEANS.

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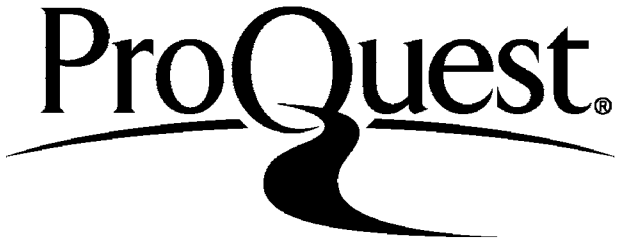

ABSTRACT

In view of the fact that function and structure, at cytological levels, cannot be separated, electron microscopic studies of the sieve tubes of *Nymphoides* petiole using different preparation techniques were carried out concurrently with physiological work. The structure of the sieve plate was investigated in detail.

Side by side some exploratory experiments to work out a method of measuring the velocity of transport and its temperature dependence were carried out using again the long petiole of *Nymphoides*.

The implications of the present findings are discussed in relation to the prevailing theories of transport mechanism.

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PHYSIOLOGICAL INVESTIGATION

GENERAL INTRODUCTION

In recent years emphasis has been laid on the problem of the relationship between the structure and function of the phloem, which has been established beyond reasonable doubt as the main channel of carbohydrate translocation in plants. In spite of this, there is as yet no general agreement as to the mechanism of this process. It was in this light that the present investigation was undertaken. Electron microscopical investigations of the sieve tube structure have been carried out along with physiological work on a single species. The two are dealt with separately in this thesis for the sake of convenience, the microscopical work being considered first.

CYTOLOGICAL INVESTIGATION

INTRODUCTION

The phloem has long been considered to be a complex tissue involved in the downward and upward translocation of organic materials and carbohydrates par excellence from the leaves to the other parts of the plant. As such it has attracted the attention of both the anatomists and the physiologists. It was Hartig (1837) who first discovered sieve-tubes in the phloem of higher plants. Since then they have most often been considered to be the specific tissue of carbohydrate transport, though other views have been held (for instance by Sachs (1863), who believed that sugars moved principally in the "conducting parenchyma" of the starch sheath). Mangham (1910-11) has reviewed much of the earlier work dealing with this conclusion. In the renewed phase of interest in the problem of translocation which began around 1920 the position of the phloem as the favoured tissue was challenged first by Birch-Hirschfeld (1919-20) and then by Dixon and Ball (1922) and again Dixon(1922). The basis of their objection was the extremely rapid rate of carbohydrate movement. Dixon and Ball (1922) were of the opinion that the sieve-tubes are structurally inadequate for such a rapid rate of movement to occur in them. They used dry weight changes of potato tubers in conjunction with the cross-sectional area of the phloem of the stolon to calculate the

rate of movement of sugars into the developing organ. Their value was 50cm/hr based on a sugar concentration of 10% in the entering stream - a rate which would traverse the field of a medium power (1/6") objective in about two seconds. Accordingly Dixon (1922) considered xylem to be the channel of transport though it must be mentioned that later (see Dixon and Gibbon (1932)) it seems he was converted to the view that the phloem is the tissue involved.

It was the classical work of Curtis (1920 and onwards) and Mason and Maskell (1928 and onwards) that established once again the ascendancy of phloem over xylem as the favoured channel of transport and the sieve elements as the most likely cells. Schumacher (1930) gave the first direct experimental evidence that the sieve elements are the actual cells involved in translocation. In 1933 he confirmed this by showing the movement of fluorescein in the sieve-tubes, though it must be conceded, a priori, that fluorescein transport is not to be considered as necessarily strictly analogous to sugar movement. More recent works with radio-active tracers by Biddulph and Markle (1944), Rabideau and Burr (1945), Biddulph (1956) and Biddulph, Biddulph and Cory (1958) have gone very far towards confirming this view.

In spite of the present general agreement over the pathway of translocation, controversy is still going on

regarding the mechanism of this movement. The reason for this is not far to seek. It has long been recognised that the structural peculiarities of the sieve element are related to their role in translocation. That is the reason why structural and functional studies of phloem have gone hand in hand. Differences in interpretation of the structural features of the sieve element are thus inevitably linked with different theories of translocation, though in fact the relationship between the two fields of studies has been a reciprocal one.

The cytological features which have been most debated are the general nature of sieve element protoplast and the particular structure of the sieve plate including the connecting strands. These have been assigned different roles in translocation by the advocates of different theories. Thus it is necessary to briefly review the theories current at the present time, and to see how their structural requirements differ.

Probably the most favoured hypothesis at the moment is that first put forward by Munch (1930). This supposes that the driving force of movement is a gradient in hydrostatic pressure acting longtitudinally in the sieve tube columns. This gradient is established osmotically, and consequently

is normally closely related to a gradient of osmotic potential in the sieve tube contents; further, it acts equally on all molecular species present, so that all of them partake in the resultant mass flow; though, it must be added, all do not necessarily achieve the same velocity. The structural requirements for the pressure-flow theory are therefore fairly clear: the resistance to longtitudinal viscous flow must be low, or the pressure gradient required would be prohibitively high; and this can be shown to imply either that the sieve plate pores have a fairly wide open channel through them, or that if filled with cytoplasm, this cytoplasm itself partakes in the rapid longtitudinal movement of the watery sap. A third alternative has been put forward by some workers, notably Crafts (1948): that the cytoplasm of the sieve tubes, though stationary, is denatured. Its polypeptide chains, he suggested, were straightened; some of the intermolecular bonds were broken; and there was a loss of the lipid material associated with permeability barriers. Coupled with thermal agitation, he believed a structure would result which would offer little resistance to viscous flow. In any case, the role of the sieve plates would be that of passive obstacles, even if they were not serious ones. Earlier (1931), Crafts had put forward the view that mass flow might occur within the oriented structure of the thick hydrated sieve tube walls.

However, as Curtis (1935) pointed out, these suggestions rest on a misconception of the mechanics of the problem; the implications of Poiseuille's Law are not avoided by merely straightening and aligning the stationary polypeptide or cellulose matrix through which flow is assumed to take place. The channels of movement remain excessively narrow, and the pressure gradient needed therefore correspondingly excessive. Thus the only possible alternatives if the pressure flow theory is to stand are the first two: a large open channel through each sieve pore, or rapid movement of the cytoplasm itself. On each of these two points the electron microscope has thrown valuable light, and the present study adds a little more.

The second theory, that of protoplasmic streaming, was first propounded by de Vries (1885), and has found a more recent champion in Curtis (1935-50). At the present time it cannot be said to be widely held. This is largely because no reliable reports of streaming in mature sieve tubes have ever appeared; further, the speed of cyclosis which would be necessary to sustain the known velocity of transport appears to be quite inconceivable. Structurally, the theory does not require the sieve plate pores to be open, as it has been commonly suggested that passage across the plates is by simple physical diffusion, a process to which the stationary

cytoplasm would offer very much less resistance than to viscous flow. However, it is very difficult to maintain that even with the help of cyclosis diffusion could be fast enough, and it is almost certainly necessary to assume that the cytoplasm must itself move rapidly through the pores. Further, it would be expected that if the protoplast were in rapid cyclosis this movement would somehow express itself at the level of submicroscopic morphology; and thus from both angles it might be expected that the evidence of an electron microscope study would be relevant.

Lastly, there is a group of theories, for the most part rather indefinite, which are often described as invoking "activated diffusion". This term was apparently first applied by Mason and Phillis (1934), who seemed to mean by it that the movement followed the pattern of a diffusion but was in some unspecified way speeded up by the expenditure of metabolic energy. The ideas of Kursanov (1952) seem to belong here. At the present time "activated diffusion" is often held to lead to a sort of mass flow in which the solvent water shares as well as the solutes; and the retention of the term "diffusion" to describe the process seems to be justified by the argument that the impulses which set the column moving are not general, like a pressure gradient, but extremely local. Thus if it is sugar that initially

experiences the impulses then these might be conceived of as arising at extremely numerous loci in the cytoplasm and as being of only molecular range. The sugar molecules as they diffused past would receive a 'kick' in the forward direction from a metabolising element in the stationary cytoplasm (and would of course transfer some of the energy received to solute and other mobile molecules); but these 'kicks', while all directed in substantially the same direction, might be considered as being otherwise administered "at random"; and this would justify calling the whole process an "activated diffusion", the individual sugar molecules, as it were, making their way separately down the column. Obviously, however, in very many important respects the whole process is very far from being of a diffusional nature.

Finally, the electroosmotic theory of Spanner (1958) suggests in a rather more explicit way a positive role for the sieve plates, which it supposes are complex structures with the pores filled with cytoplasm substantially stationary, and which are themselves the site of the motive forces. This suggestion, which the electroosmotic theory shares with some forms of the "activated diffusion" hypothesis, is made more likely by the recent discovery of Esau and Cheadle (1955) that in many dicotyledons anticlinal divisions in the secondary phloem mother cells lead to more

numerous sieve plates and shorter sieve tube elements than would be expected from the length of the cambial initials. In conformity with this Huber (1958), a supporter of the pressure-flow theory, seems to come to the conclusion that the sieve plates have a positive role to play in translocation; whereas previously he considered them to be obstacles.

Whatever the mechanism of movement, the moving solution must pass through the sieve plate. Therefore the understanding of the structure of the sieve plate seems essential to the understanding of its role in translocation. Here again there is no unanimity of opinion regarding the sieve plate structure, especially the structure of the connecting strands in the sieve pores. Up-to-date there are four views on its structure. Hill (1908) was of the opinion that the connecting strand is a hollow cylinder of cytoplasm, thus allowing for the vacuolar continuity of the superposed sieve tube elements. The presence of slime in the strand in cut materials was considered as an evidence for this view. Hill's view of the pore structure was accepted by Munch (1930) for his mass flow theory, though in a later communication in 1943 he claims it is not necessary. Mangenot (1926) holds the view that there is no cytoplasmic continuity from one sieve element to the other. The

connecting strand consists of two halves, each being invested by their own plasma membranes. Salmon (1946) agrees with Mangenot. A somewhat similar opinion has been expressed by Valz (1952) for plasmodesmata and the connecting strands in gymnosperms and some angiosperms on the basis of electron microscopic studies.

The third view of the structure of the connecting strands is due to Crafts (1939), who considers them to be solid cytoplasm, there being no vacuolar continuity through them. He considers the presence of slime as an artifact. Rouschal (1941) is also of the same opinion. He describes the connecting strand to be composed not of semi-permeable membrane alone but of inner cytoplasm delimited by plasmolemma. The result would be absence of a semi-permeable membrane. Absence of tonoplast would also add to the permeability of the sieve elements.

Lastly, Schumacher (1939) agrees with Crafts (1939) and Rouschal (1941) regarding the solid cytoplasmic nature of these strands, but adds that the sieve tube elements retain their individual tonoplasts even in mature stage.

These four views on the nature of the connecting strands are expressed diagrammatically in Figure 1.

This difference of opinion regarding the structure of the connecting strands thus demands for them further investigation. This is specially desirable in view of the

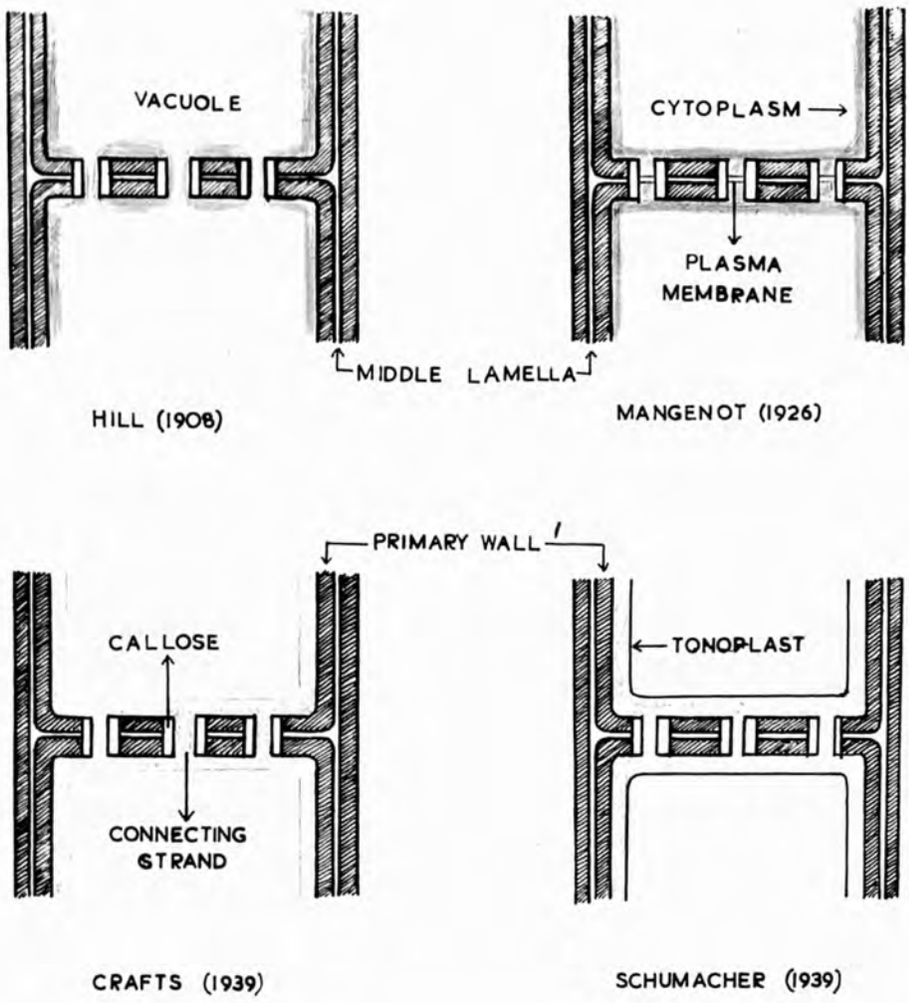

Figure 1. Diagrammatic representation of the structure of the sieve-plate according to different workers.

conviction, resulting from the more recent work in the physiological field, that the sieve plate with its pore content occupies a very important place in any mechanism of translocation. This need for renewed investigation of the sieve areas has been emphasised by Esau, Currier and Cheadle (1957).

As a welcome contribution to the solution of this problem a number of studies with the electron microscope have fairly recently appeared. Thus Hepton, Preston and Ripley (1955), Hepton (1957), Preston (1958) and Schumacher and Kollmann (1959) have published the results of electron microscopic investigations of the sieve plate region. It is only the latter workers who claim to have observed the presence of a tonoplast in mature sieve elements; but they all agree that the connecting strands are continuous from one sieve element to the next and that there is no such break as was postulated by Mangenot (1926). They all likewise agree that the cytoplasm in the pores is without the central vacuole figured by Hill (1908); on the contrary, it appears throughout its cross section to be particularly dense. These findings, deriving from such different types of material as Pinus, Cucurbita, Sorbus, Vitis and Passiflora, have such outstandingly important implications for translocation theory that it seemed worthwhile to carry on an

investigation on a subject very different again from these, and on which physiological work was being concurrently undertaken.

MATERIALS AND METHODS

1. Choice of the plant material

Having once defined the scope of the present investigation the matter to be settled first is the choice of the plant material. Correlation of the results obtained from the study of phloem structure and function could be attempted on a sounder basis if the same plant material is used in both electron microscopic and physiological investigations. Having this in mind, Nymphoides peltatum (Gmel.) O.Kunze was selected as the experimental plant and its petiole as the experimental material. It is commonly referred to as the fringed water-lily and belongs to the dicotyledonous family Menyanthaceae (Clapham, Tutin and Warburg, 1952). Its other synonyms are Limnanthemum peltatum (Gmel.) and L. nymphaeoides (L.) Link. It is a hydrophyte with long-petioled floating leaves and creeping rhizomes; and in fact it provides, in its petioles, long uniform uninterrupted organs which it was expected would be very suitable for studying the movement of tracer materials. The petiole has a simple anatomy with a large central vascular bundle and usually two to four smaller ones; further, the central bundle is surrounded by a broad zone of very open aerating tissue. Thus the abaxial segment of the petiole can be easily shaved off with a razor blade

leaving very little tissue still covering the phloem; this is important when scanning the living organ with an end window Geiger counter after the assimilation by the leaf of radioactive carbon dioxide. These features are shown in the transverse section. (Plate 1)

So far as its suitability for electron microscopic investigation is concerned, it can be mentioned that the petiole consists entirely of primary tissue. Hard and compact ground tissue is absent, and as a consequence the phloem is fairly easily accessible. As far as could be seen from preliminary investigations, there is no accumulation of the so-called slime at the sieve plate even in the somewhat less careful preparations made for optical microscopy. (Plate 2) Further, Nymphoides peltatum represents a plant type not so far investigated with the electron microscope; and as it turned out, it possesses to quite a marked degree the interesting feature of well-developed nacreous walls to the sieve tubes.

On the basis of the findings of Cheadle and Whitford (1941) and Cheadle (1948), it was expected that the sieve elements in the petiole of this hydrophyte would have simple and advanced type sieve plates on the transverse end walls. According to Esau and Cheadle (1959), simple sieve plates would have both large pores and large connecting strands.

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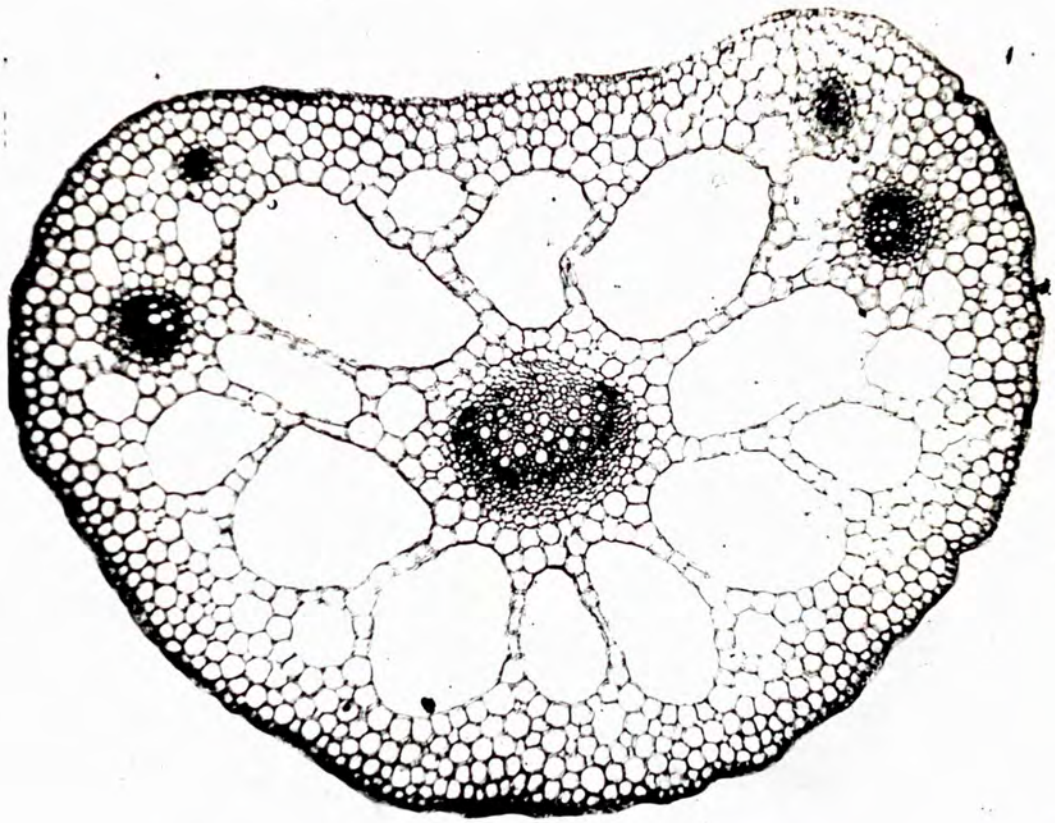

PLATE 1. T.S. petiole showing general features.
Stained in safranin and haematoxylin. X45

PLATE 2. L.S. vascular bundle showing absence of slime at the sieve plate with conventional preparation for optical microscopy. Stained in safranin and haematoxylin. X705

Such sieve plates, it was expected, would easily yield to electron microscopic examination.

Nymphoides was found fairly easy to grow in tanks about 18 inches deep in the laboratory window and in a large concrete tank under the greenhouse staging; artificial (tungsten) light of about 175-200 ft. candles was provided for about 16 hours per day. Growth during the winter months tended to be poor, some of the leaves remaining stunted and opening under water; and this naturally hindered physiological work. The individual plants were grown in seven-inch pots so that they could be easily moved when required. Further details of the method of growing them in the laboratory are given in the physiological section.

2. Fixation for electron microscopy

Fixation is the most critical part of the preparative technique for electron microscopy. Among botanical subjects this seems to be specially true in the case of sieve tubes, which have apparently tenuous, delicate, easily injured protoplasts. A further distinguishing feature of the sieve tubes is their high turgor pressure and the ease with which their contents can be displaced longtitudinally. Thus if the internal pressure is released too suddenly the contents suffer violent movement. These considerations demand special handling of the material during fixation. In view of this,

a part of the present investigation was devoted to finding a suitable technique. This, however, is a problem in itself and really demanded more time than could be spared.

It is considered desirable to discuss the fixation techniques so far available so as to form a background to what is to follow.

Bretschneider and Elbers (1952) tried physical fixation by freeze-drying the specimen. This did not prove satisfactory. Electron microscopists owe a great deal to Palade (1952), who gave the best general chemical fixative so far available. He recommended 1% OsO_4 solution buffered to pH 7.3 - 7.5, a near-neutral pH, with veronal-acetate buffer; and this has proved highly satisfactory for many animal tissues. It is not surprising, therefore, that attempts were made to improve this fixative rather than to find an alternative. As a result of further studies the following recommendations emerged:

(1) Fixation should be carried out at a temperature near zero.

(2) The fixative should preferably be nearly isotonic to the cell contents.

(3) Short fixation is to be preferred. Palade (1954) considers fixation times of 30 minutes to 2 hours to be most satisfactory.

(4) The entire procedure of fixation, dehydration and bringing the specimen to the embedding medium should be completed within 6 to 8 hours.

With this background in mind it was decided to use 1% OsO_4 buffered to pH 7 with veronal-acetate buffer and to carry out fixation at 0° - 4°C. The period of fixation, dehydration and soaking in methacrylate, the embedding medium, was limited to not more than 8 hours. Having decided these points, studies were carried out to determine the effect of duration of fixation and the tonicity of the fixative.

Two substances have been used to control the tonicity of the fixative. Rhodin (1954) used NaCl, whereas Caulfield (1957) used sucrose. The former is an electrolyte, the latter a non-electrolyte. Since it is known that fixation can be affected by specific ions, and the fixative already contained Na ions, it was decided to use sucrose to control the tonicity. Schumacher and Kollmann (1959) have also used sucrose in the fixative in connection with their recent study on the sieve tubes of Passiflora coerulea, and obtained very good results.

The choice of pH 7 for the fixative was decided on largely for reasons of precedent. Currier, Esau and Cheadle (1955) had used sucrose solution buffered to pH 7 in their plasmolytic studies of phloem, and Hepton (1957) had used OsO_4 at pH 7 for fixation of sieve tubes. He had also shown that fixation at relatively high pH was unsatisfactory. Schumacher and Kollmann (1959) had also carried out fixation

at pH 7. Finally, Biddulph and Cory (1957) have reported that the approximate pH of the phloem tissue is 7.2, slightly alkaline; at any rate, pH 7 gives the lowest point concentration of the physico-chemically very active H^+ and OH^- ions.

Thus the first fixative tried was 1% OsO_4 buffered to pH 7 with veronal-acetate buffer with or without the addition of either 0.5M or 1M sucrose. The fixation was carried out at 0° - 4°C either for ½ hour or 1. The material for fixation was obtained in the following way:

The petiole was first clamped near its basal end between the two arms of a spring clothes peg to collapse the sieve elements and prevent flow. It was then severed from the root-stock. The petiole was again clamped by another peg at a distance of about 3 inches from the first. A 0.5 to 1.mm. thick slice of the included petiole was cut from the middle of the 3-inch length, by making two cuts simultaneously with a razor blade assembly, and transferred immediately to the fixative. Three to four petiole sections 0.5-1 mm. thick were easily obtained by this technique from the whole length of the petiole. It was presumed that by the double cutting method longtitudinal surges of the contents due to sudden release of turgor would be avoided. It should be noted that the sieve tube elements of

Nymphoides peltatum are about 0.2 mm. long. Thus a section 0.5 mm. thick would contain at most two unopened sieve tube elements as shown in figure 2. Under these conditions it was

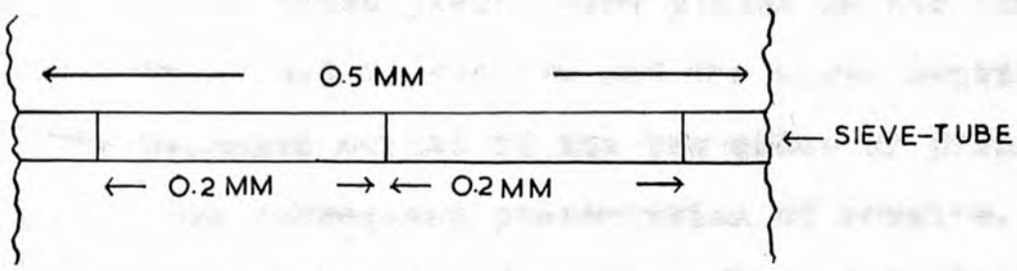

Figure 2.

expected that the fixative would penetrate rapidly in a longtitudinal direction, and this, it was thought, would be helpful with a substance like OsO_4 whose penetrating power for plant tissue has been reported to be low. Because such short lengths of the sieve tube columns were suddenly laid open to the fixative, the sucrose content of the latter was made equal to what the sap might be expected to contain, a figure of 10-25% (equal approximately to 0.3 to 0.7 M) having often been suggested for the latter. Towards the end of this investigation an approach to the problem of tissue preparation was tried more akin to that used by other workers (Currier, Esau and Cheadle, 1955; Hepton, 1957; Schumacher and Kollmann, 1959). Following the latter authors, 5-6 inch lengths of petiole were placed in 0.2 M sucrose buffered at

pH 7 with usually 0.01 M phosphate buffer also containing 0.001 M $Ca(NO_3)_2$ for 2 hours to reduce their turgor. They were then transferred to a similar solution with 0.25 M sucrose, and suitable pieces of the phloem cut out while immersed. Finally these pieces were placed in the osmium fixative containing 0.3 M. sucrose and the above mentioned buffer. The relative merits of the two modes of preparation will appear in the subsequent presentation of results.

The fixed materials were finally washed in distilled water and dehydrated in an ethyl alcohol series as usual. When using Schumacher's schedule the sections were washed with the phosphate buffer containing 0.3 M. sucrose and then dehydrated; at 70% alcohol the sections were transferred to alcohol containing 1% phosphotungstic acid and 0.5% uranyl acetate. The former is an electron stain whereas uranyl has been reported by Ward (1958) to reduce the phenomenon of cell explosion during methacrylate embedding. From absolute alcohol the sections were transferred to a 50 : 50 mixture of alcohol and methacrylate monomers for half an hour. They were then soaked in three changes of methacrylate for half an hour each prior to final embedding. In each case, the methacrylate contained 2% Luperco, and the monomer was usually a 10 : 1 mixture of the butyl and methyl esters. The details of the technique are given in Schedules 1 and 2.

Low and Freeman (1956), in an attempt to find an alternative to OsO_4 fixation, tried chromium compounds, but came to the conclusion that these were not as satisfactory as the former. At about the same time Luft (1956) experimented with $KMnO_4$ and came to the conclusion that, while it would not replace OsO_4 as a general purpose fixative, it gave excellent results with membrane systems within the cell; in fact, it seemed almost specific for the endoplasmic reticulum. Recently $KMnO_4$ has been used rather successfully by Whaley, Mollenhauer and Kephart (1959) in their study of maize root cap cells. The technique of $KMnO_4$ fixation has been discussed in a more recent paper by Mollenhauer (1959). Here he states that 5% unbuffered $KMnO_4$ gives excellent fixation with as short a fixation time as 2 minutes; however, fixation with 2% $KMnO_4$ at room temperature for 2 hours is to be preferred. As the result of $KMnO_4$ fixation appears very interesting, it was decided to try this technique as well, but using different fixation times of 5, 10 or 15 minutes. The method of obtaining material for fixation was the same as described previously. The details of fixation, dehydration and embedding are given in Schedule 3.

3. Fixation for optical microscopy

For optical microscopy material was fixed in Craf III for 17 hours, run up the alcohol series and finally embedded

in wax of melting point 52°C in the usual way. Craf III is one of the Nawaschin type of fixative having the following composition:-

1% chromic acid	30 ml.	Solution A
10% acetic acid	20 ml.	
40% aqueous formaldehyde	10 ml.	Solution B
water	40 ml.	

Solutions A and B are mixed in equal quantities just before fixation.

This fixative was used because it was considered by Cheadle, Gifford and Esau (1953) to be the most successful killing fluid for phloem preparation. Moreover, it had been used by Cheadle and Esau (1958) in their study of the secondary phloem of Calycanthaceae and again by Esau and Cheadle (1958) in their study of wall thickening in sieve elements. According to Sass (1940), a Nawaschin type fixative is an excellent hardening and preserving agent, and does not require washing out in water. This avoids the softening and pulping of material which might occur as a result of prolonged washing, specially if the material, like the petiole of N. peltatum, is intrinsically soft and delicate.

4. Embedding

A mixture of n-butyl and methyl methacrylate in the proportion of 10 : 1 was used as an embedding medium. The

embedding of the fixed and dehydrated sections was carried out by suspending them in the monomer mixture in gelatin capsules by means of a piece of very fine wire. This helped in the orientation of the section and in addition retained it in the centre of the block, a circumstance which gave greater scope when it was being finally excised for mounting. The free end of the wire was passed through a hole pierced in the capsule as shown in Figure 3.

Figure 3.

The use of the methacrylate as an embedding medium was first reported by Newman, Borysko and Swerdlow (1949); subsequently Borysko (1956) reported that curing of the monomer at 60°C reduced polymerisation damage. Polymerisation of the monomer mixture was therefore carried out by heating it at 60°C for 24 hours with the addition of 2% of Luperco CDB, the trade name of a catalyst consisting of

50% 2,4-dichlorobenzoyl peroxide in dibutyl phthalate.

Embedding of the sections in Araldite, as recommended by Glauert and Glauert (1958), was also tried. This proved difficult on account of the high viscosity of the resin. As it also ideally calls for the use of a diamond knife, and the prototype knife available proved insufficiently even and sharp, the use of this medium was abandoned. However, it would seem to offer advantages in the manipulation of plant material owing to its superior strength, adhesiveness, and thermal stability, as well as its reduced contraction on polymerisation. Araldite sections have also been reported to require no supporting film; their employment in botanical work, for a variety of reasons, might therefore be worth further investigation.

SCHEDULES FOR FIXATION, DEHYDRATION AND EMBEDDING

The earlier work which was done employed the following schedule:

Schedule 1

1. Sections fixed for ½ or 1 hour at 0-4°C in 1% OsO_4 buffered to pH 7 with veronal-acetate buffer with or without the addition of 0.5 or 1M sucrose.
2. Thorough washing in three changes of distilled water for 10 minutes each.
3. Dehydration in 20%, 50%, 70%, 90% and absolute alcohol for 30 minutes each.
4. Sections transferred to absolute alcohol, two changes of 30 minutes each.
5. Transference to 50/50 absolute alcohol/methacrylate for 30 minutes.
6. Soaking in three changes of methacrylate with catalyst for 30 minutes each.
7. Final embedding in methacrylate in gelatin capsules no.00 and polymerisation at 60°C for 24 hours.

As mentioned earlier, a rather different mode of preparation was tried later. This followed substantially the method used for plasmolytic studies by Currier, Esau and Cheadle (1955); and for microscopic examination by Hepton (1957), and by Schumacher and Kollmann (1959). The whole procedure may be outlined as follows:

Schedule 2

1. Four to five inch lengths of petiole pretreated for 2 hours in 0.2 M sucrose solution buffered to pH 7 with 0.01M phosphate buffer also containing 0.001 M $Ca(NO_3)_2$.
2. The central two inch portions excised and immersed for 1 hour in a similar solution containing 0.25 M sucrose.
3. Sections 0.5-1 mm. thick from the centre of the two inch lengths fixed for ½ hour at 0-4°C in 2% OsO_4 buffered to pH 7 with the above phosphate buffer containing 0.3 M sucrose.
4. Sections washed with plain buffer solution containing 0.3 M sucrose for 15 minutes.
5. Dehydration in 20%, 50% and 70% alcohol for 15 minutes each.
6. From 70% alcohol sections transferred to a solution of 1% phosphotungstic acid and 0.5% uranyl acetate in 70% alcohol for 15 minutes.
7. Dehydration continued in 90% and absolute alcohol for 15 minutes each.

8. Sections finally embedded in methacrylate as in the first schedule.

One final variant treatment was suggested by the successful use of permanganate by Whaley, Mollenhauer and Kephart (1959) for the fixation of maize root cells. These authors obtained very good preservation of membrane structures by this fixative; and it was felt that its use might reveal some of the fine structure of the normally electron-dense material in the sieve plate pores. The fixing schedule was as follows:

Schedule 3

1. Sections fixed for 5, 10 or 15 minutes at 24°C in unbuffered 5% $KMnO_4$.
2. Dehydration and embedding, without washing, as in first schedule.

5. Microtomy

Methacrylate-embedded blocks were cut, using glass knives (Latta and Hartmann, 1950) prepared in the conventional way, on a modified Cambridge Rocking microtome shown in Figure 4. Sections were first cut at 1µ and examined by phase contrast till they revealed some interesting features. Cutting was then continued at 250-500A, and sections were produced in ribbons. These were expanded by Xylene vapour as recommended by Satir and Peachey (1958) and mounted on formvar-coated "Athene" grids of the Sjostrand type.

The wax-embedded material was cut at a thickness of 10µ and processed as usual prior to staining. The sections were stained variously as described under "Results".

6. Microscopy

The sections on the grid were examined in an older pattern Siemens Ubermikroscop, with a beam voltage of 70 kilovolts and using an objective aperture of 50µ. In the beginning micrographs were made on Ilford contrasty special latern plates; subsequently it was found possible to use an improvised film camera with Ilford 35mm. unperforated safety positive film. The film camera had the advantage that it enabled eight exposures to be made instead of one before reloading. Considerable trouble was experienced at first

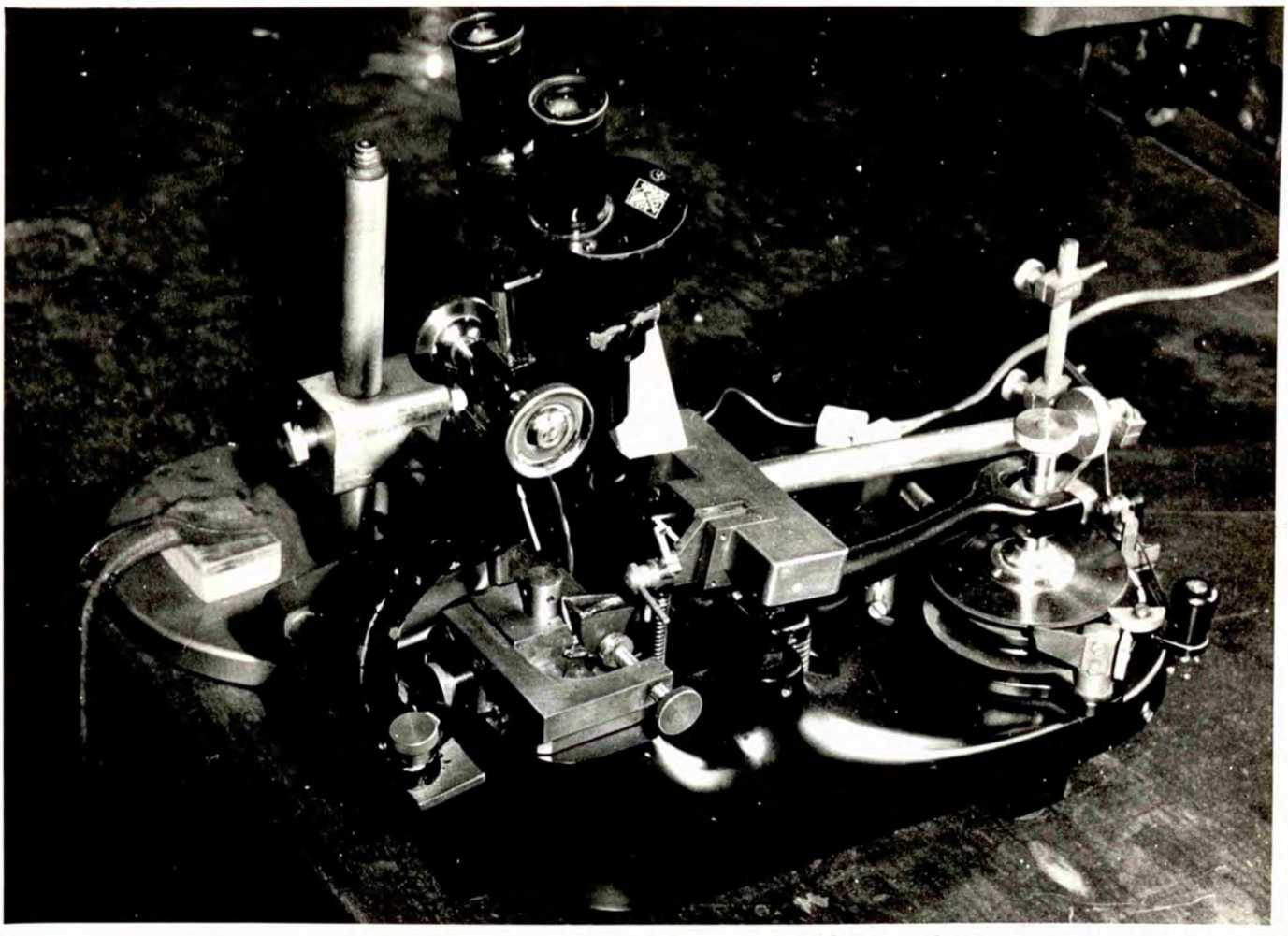

FIGURE 4. Modified Cambridge Rocker used for ultra-thin sectioning. The light beam is reflected from a mirror of Chance ON20 heat absorbing glass.

from static discharges on the film; the fact that these were registered with identical shape on two adjacent frames showed that they occurred as the film was being wound on to the take-up spool. This trouble is apparently still experienced in some newer instruments. It was completely overcome by arranging for the film to be carried on an endless strip of nylon. This limited the length which could be accommodated to eight frames, but by dispensing with the coiling of the film on spools it very greatly improved the ease of outgassing, and in fact it proved unnecessary to treat the films beforehand in a vacuum desiccator.

Optical micrographs were made, using a phase contrast microscope, on Ilford rapid process panchromatic plates.

RESULTS

1. General

Before describing the results of the present investigation it may be mentioned that in most cases sieve-tubes from the petioles of fully developed and healthy leaves were examined. It was hoped that such sieve-tubes would be mature and presumably functional. For the sake of comparison the results of the study of the sieve-tubes from the petioles of relatively young and of senescent leaves are also included. The materials for the former were obtained from leaves whose laminae were not fully unfolded and were still below the water surface (though it should be pointed out that the petiole would still have mature sieve elements); those for the latter were obtained from leaves in which the laminae were yellow and obviously dying. It is in this sense that the descriptions mature, young and senescent are used of the sieve-tubes throughout.

The petiole of Nymphoides, an aquatic angiosperm, contains typical sieve-tubes as will appear from later description. Each sieve-tube consists of a number of sieve-tube members or elements usually disposed end to end in long series, the common wall parts bearing the sieve-plates. This terminology is used in the sense of Cheadle and Whitford (1941). The terms sieve-tube element and sieve element have been used here synonymously.

The phloem in the petiole of Nymphoides is naturally primary in origin and would consist of protophloem and metaphloem, the latter being the subject of the present investigation. It consists of the usual three kinds of cells - sieve-tube elements, companion cells and phloem parenchyma cells as shown in Plate 3. This is an optical micrograph showing phloem in transverse section prepared by the method of Cheadle-Gifford and Esau (1953). A longtitudinal section through a sieve-tube prepared by the same method is shown in Plate 4. It shows a simple sieve-plate (having only one sieve area) localised on the transverse end wall or the common wall between the two superposed sieve-tube elements. Companion cells can also be seen. The section was prepared by staining the wall in tannic acid-ferric chloride and lacmoid (resorcin blue) was used to bring out callose which appears to be deposited on the plate. The sieve pores appear to be seen in surface view as well as in longtitudinal section; but owing to the small depth of focus in high power optical microscopy this may be partly deceptive. Pores in longtitudinal section show the callose cylinder extending throughout the plate. In this region a faint horizontal line can be seen extending between two callose cylinders. This may be the compound middle lamella. It is, in fact, a compound structure

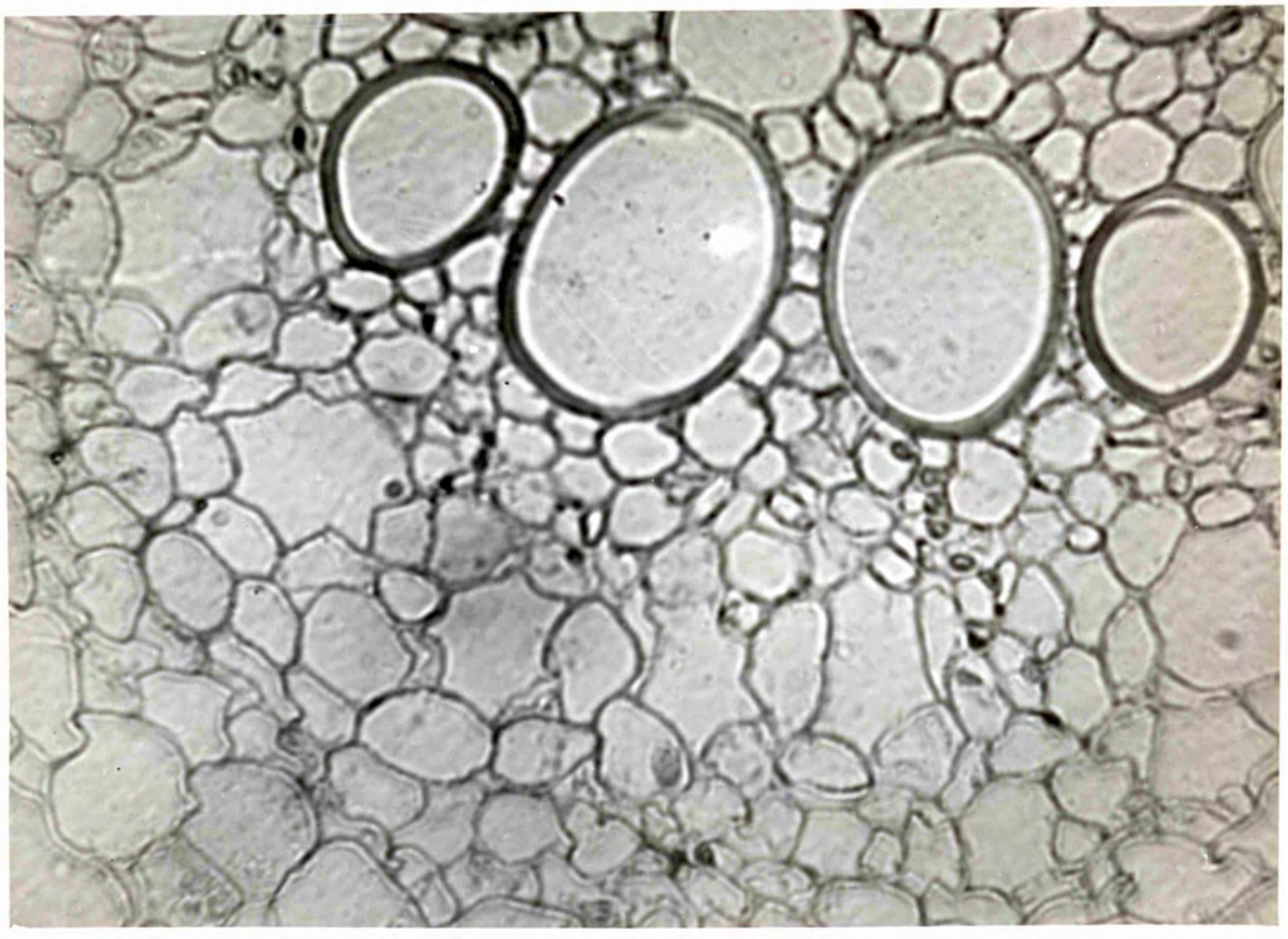

PLATE 3. T.S. vascular bundle showing different types of cells in phloem. A part of xylem can also be seen. Stained in tannic acid - ferric chloride and lacmoid. X800

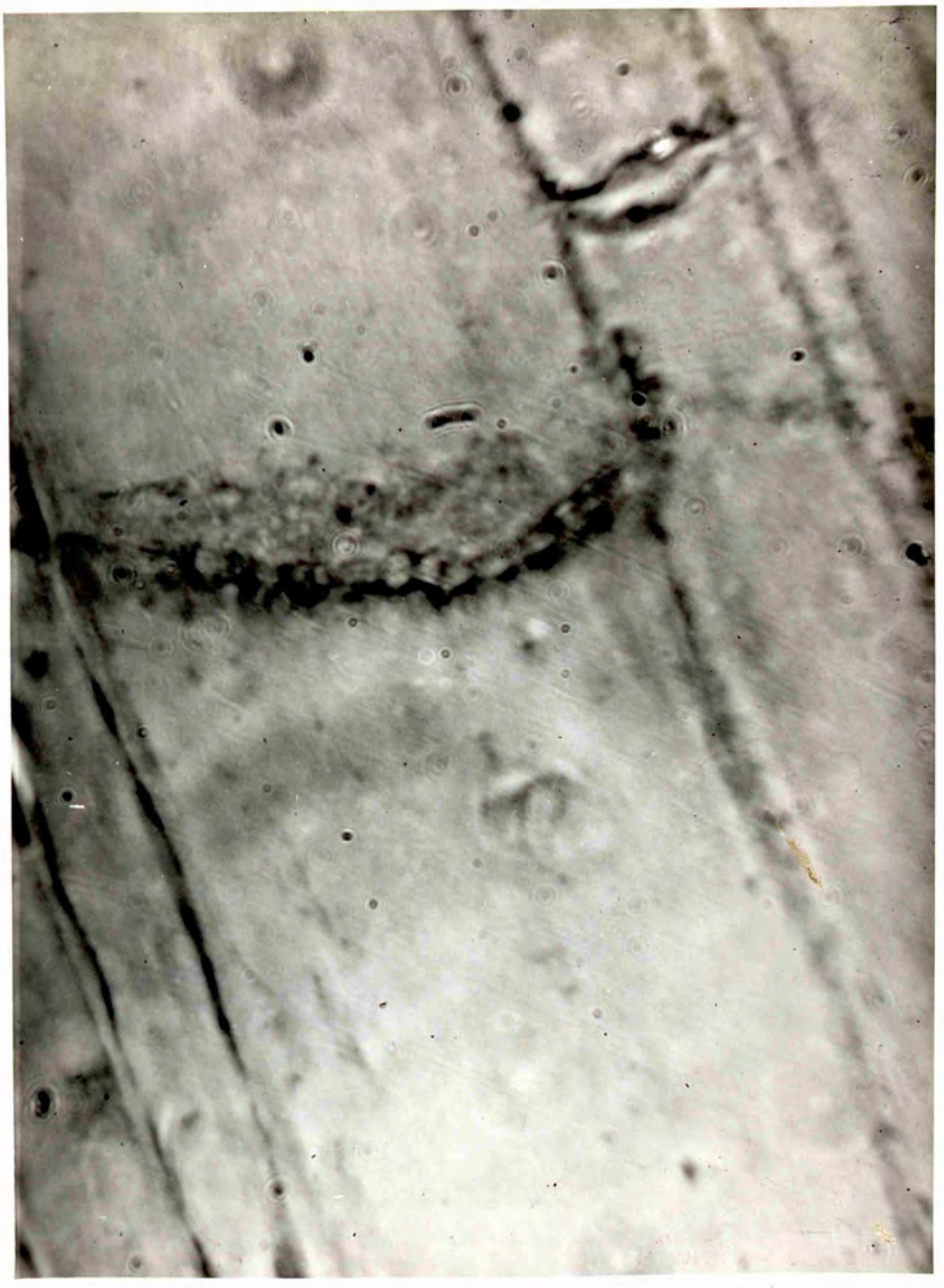

PLATE 4. L.S. through phloem showing a sieve tube with its companion cell to left. Simple sieve plate is localised on the transverse end wall. Stained in tannic acid-ferric chloride and lacmoid. X2250.

consisting of three layers: the two primary walls of the superposed sieve-tube elements and the intervening middle lamella. There does not seem to be any considerable accumulation of slime in the neighbourhood of the sieve plate.

A longtitudinal section of the sieve tube from an osmium-fixed methacrylate embedded petiole is shown in the optical phase contrast micrograph, Plate 5. In this section several adjacent sieve-tubes can be seen; two of which have simple sieve-plates on the end walls. A few plastids with starch grains also appear in the vicinity of the sieve-plates. A very interesting feature present in this micrograph is the marked thickening of the crenulated lateral wall of the sieve tubes, the adjacent walls of two sieve elements showing marked correspondence of their depressions which are probably the loci of pits or pit fields. The thickening of the wall was puzzling at first because it does not seem to show in the previous micrographs of conventionally stained sections. In fact it was considered to be an artifact of osmium fixation or methacrylate embedding. Later on however it proved to be a genuine feature of the tissue. In a recent paper Esau and Cheadle (1958) have reported similar wall thickening in the sieve elements of the secondary phloem in a large number of dicotyledonous plants and have referred to it by the older term "nacreous". In many of the cases they describe the

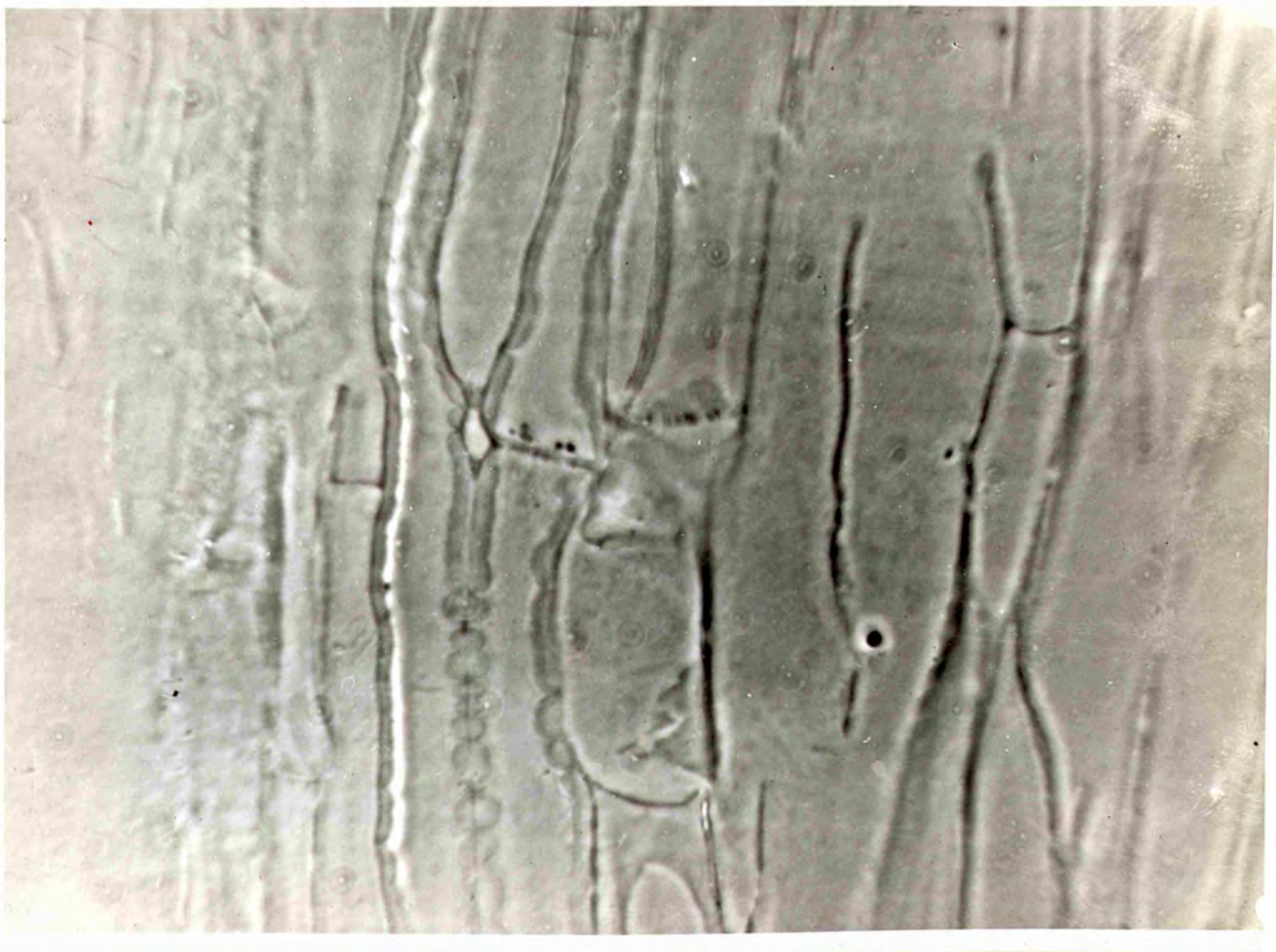

PLATE 5. L.S. through phloem showing several sieve tubes, two sieve plates and plastids. Lateral wall of the sieve tube is thick and crenulated. Fixed in osmic acid and embedded in methacrylate. X970.

thickening is quite excessive, sometimes almost occluding the lumen of the apparently functional sieve tube. The present state of affairs is therefore not exceptional, even when - as will appear in some of the micrographs - the thickening is very considerable.

2. The Nacreous Wall

According to Esau and Cheadle (1958), in many dicotyledons the wall of the sieve-tube consists of two parts: a thin outer layer next to the middle lamella and a thick inner layer next to the lumen. It is the latter which may develop into the typical nacreous wall. The sieve-tubes in the petiole of Nymphoides also show this division of their wall and the thickening of the inner layer is very marked. Plate 6 is an electron micrograph showing the nacreous wall from a specimen fixed in osmic acid without any sucrose addition. In the beginning it was considered a possibility that the wall might have undergone some degree of swelling in the fixative. Reports to this effect have been reviewed by Esau (1939). That this is not the case is rendered less likely by the next two plates. The nacreous wall from material fixed in OsO_4 with 0.5M and 1M sucrose is shown respectively in Plates 7 and 8. Addition of sucrose to the fixative does not seem to bring about any change in the general appearance of the nacreous wall. The last plate also shows the depressions in the wall

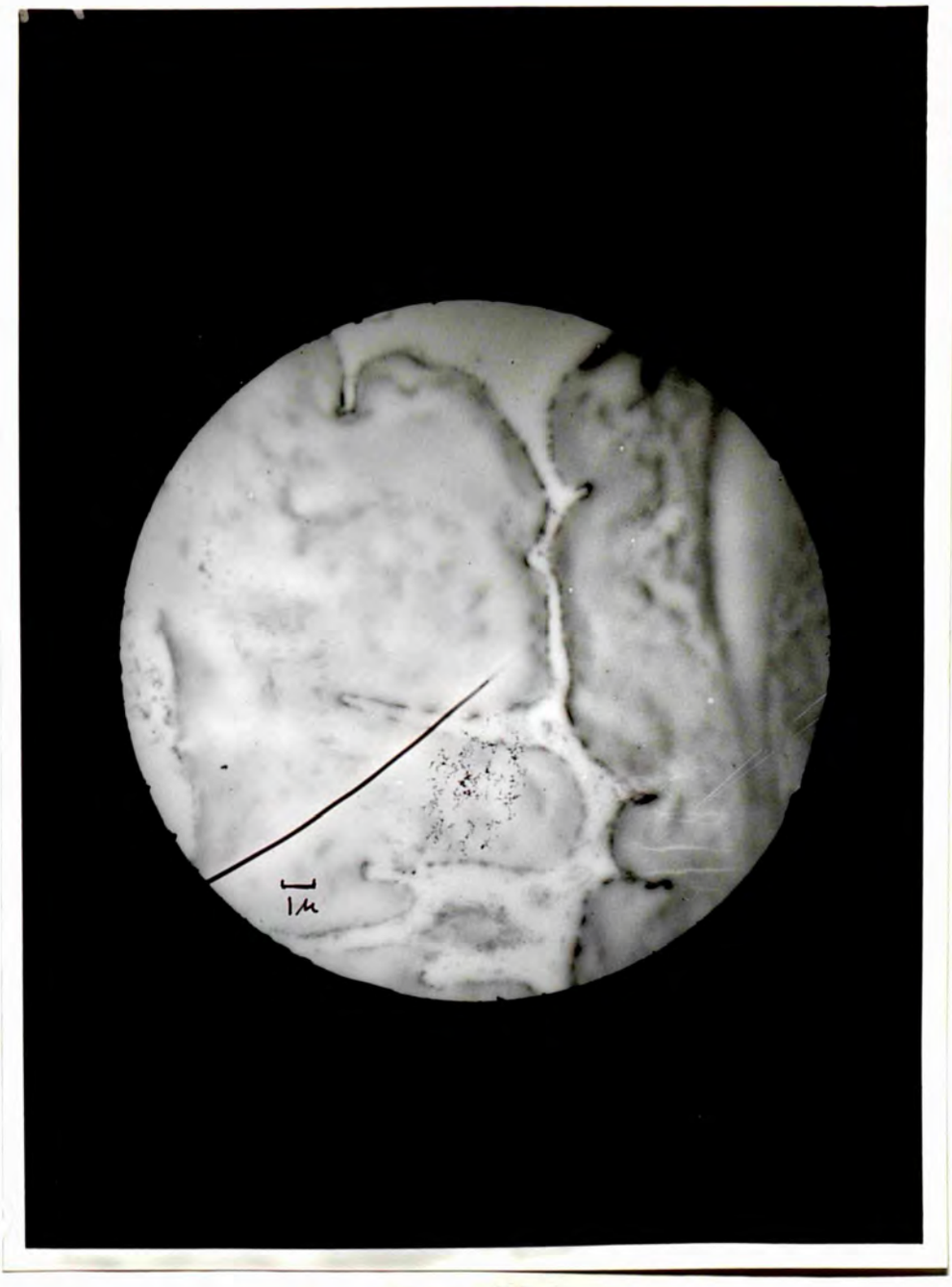

PLATE 6. Electron micrograph of sieve tube showing nacreous wall. Fixed in osmic acid without any sucrose.

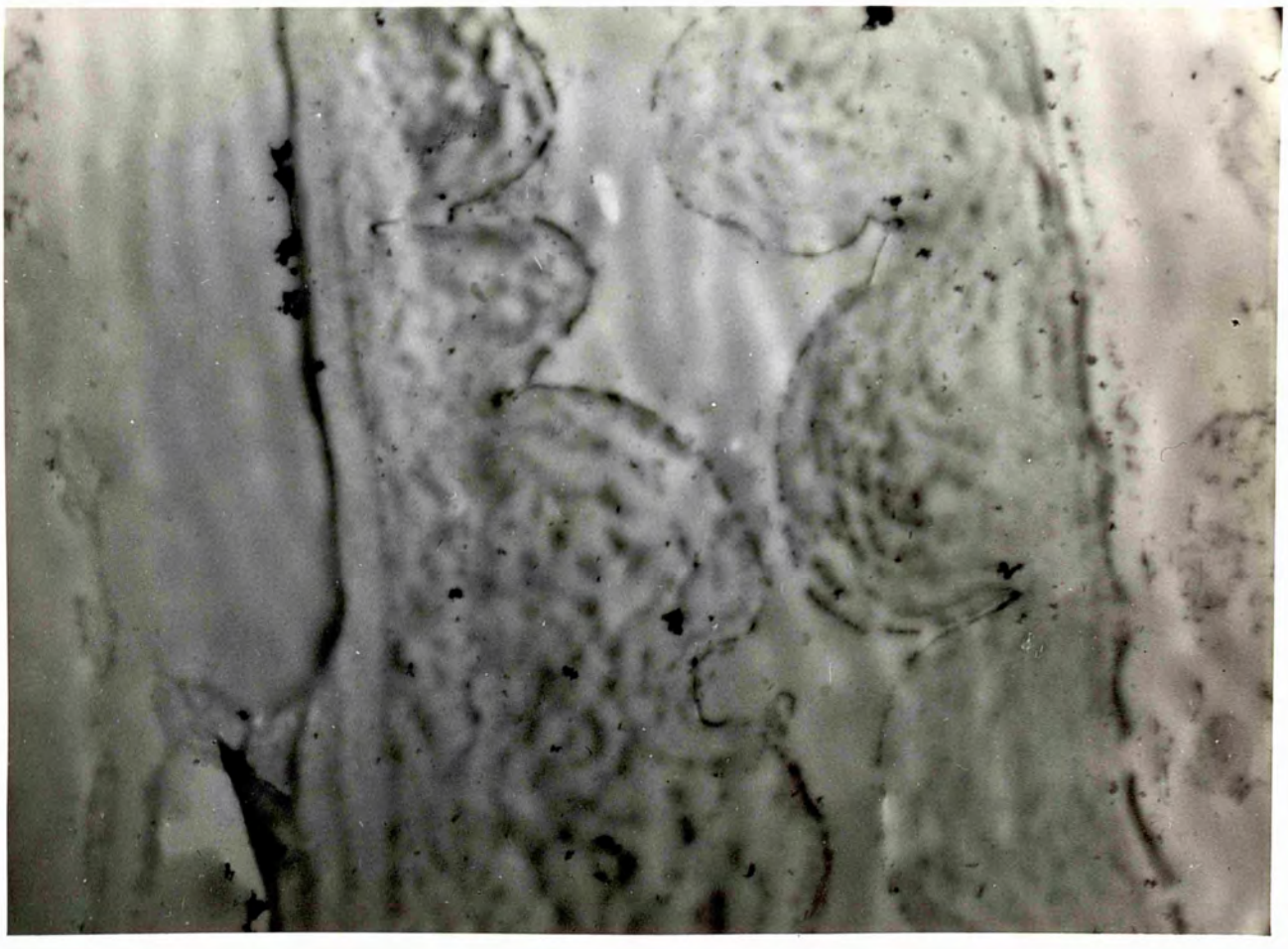

PLATE 7. Electron micrograph of sieve tube showing black lines in the matrix of the thick wall. Fixed in osmic acid with 0.5M sucrose.

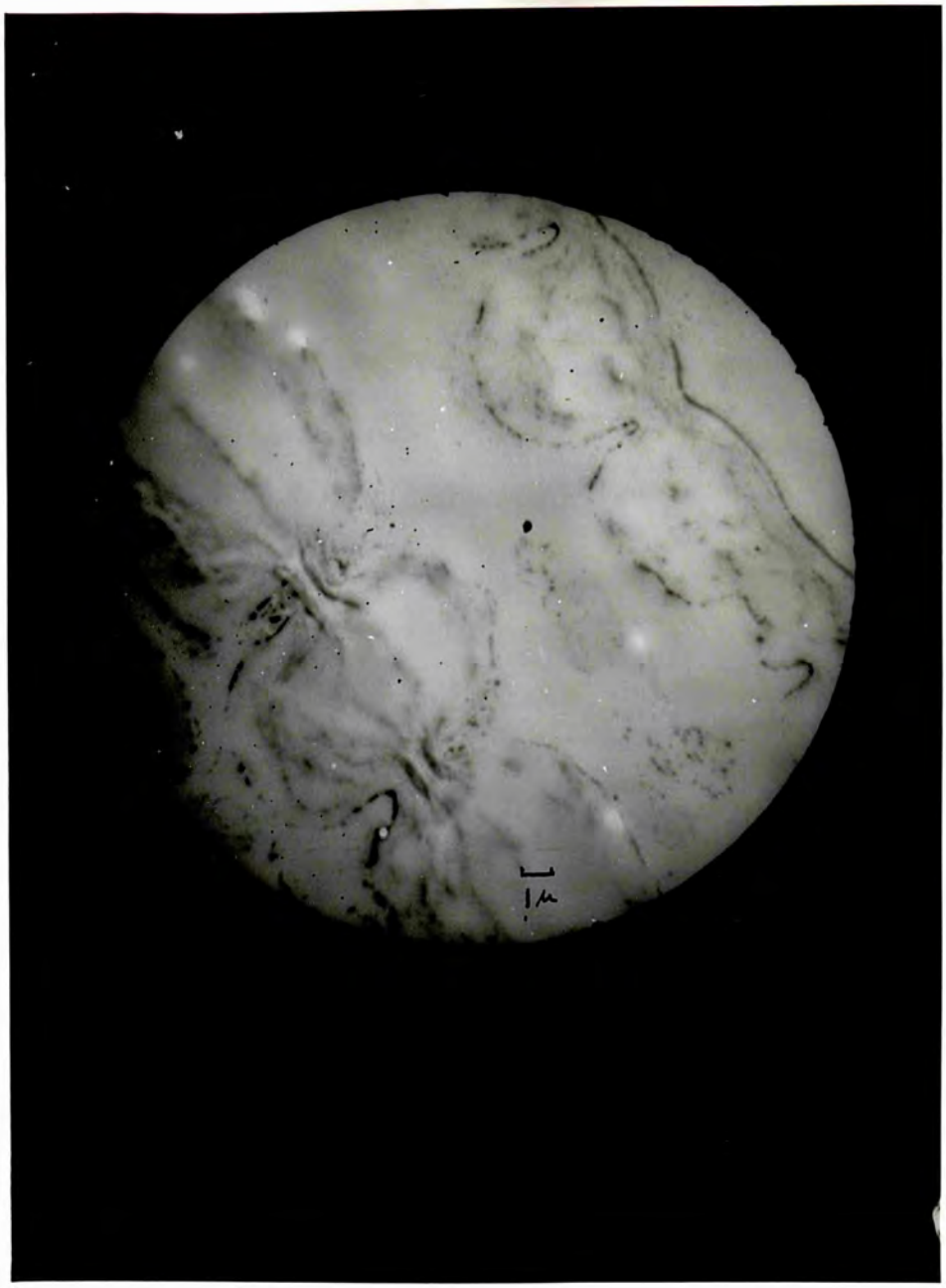

PLATE 8. Electron micrograph of sieve tubes showing nacreous wall. The two sieve tubes show corresponding depressions in their walls. Fixed in osmic acid with 1M sucrose.

of one sieve element corresponding with those of the adjacent element. These depressions probably represent pits or sieve areas.

The marked thickening of the sieve-tube wall reduces the lumen considerably. Its physiological implication will be considered later. The inner limit of the nacreous wall is so uneven that the wall appears lobed rather than crenulated, a term which frequently appears in the literature. Due to the uneven thickness of the wall, the outline of the lumen is very irregular. This can be seen from Plate 9, which shows a sieve element with its companion cell in a cross section. The difference in the thickening of the wall of the sieve element and the companion cell is very obvious.

In all of these micrographs the inner limit of the nacreous wall appears darker than the rest of the wall material. This may, according to Preston (1958) represent the wall/cytoplasm boundary; it is probably accentuated by poor fixation of the delicate cytoplasm. Further, in the matrix of the wall itself black lines can be seen, representing apparently two dimensional elements, since they appear more or less as lines in both longtitudinal and transverse sections. They are roughly parallel to the wall surface and resemble the apparent wall/cytoplasm boundary. They convey the impression that the nacreous layer grew so fast that it overtook and

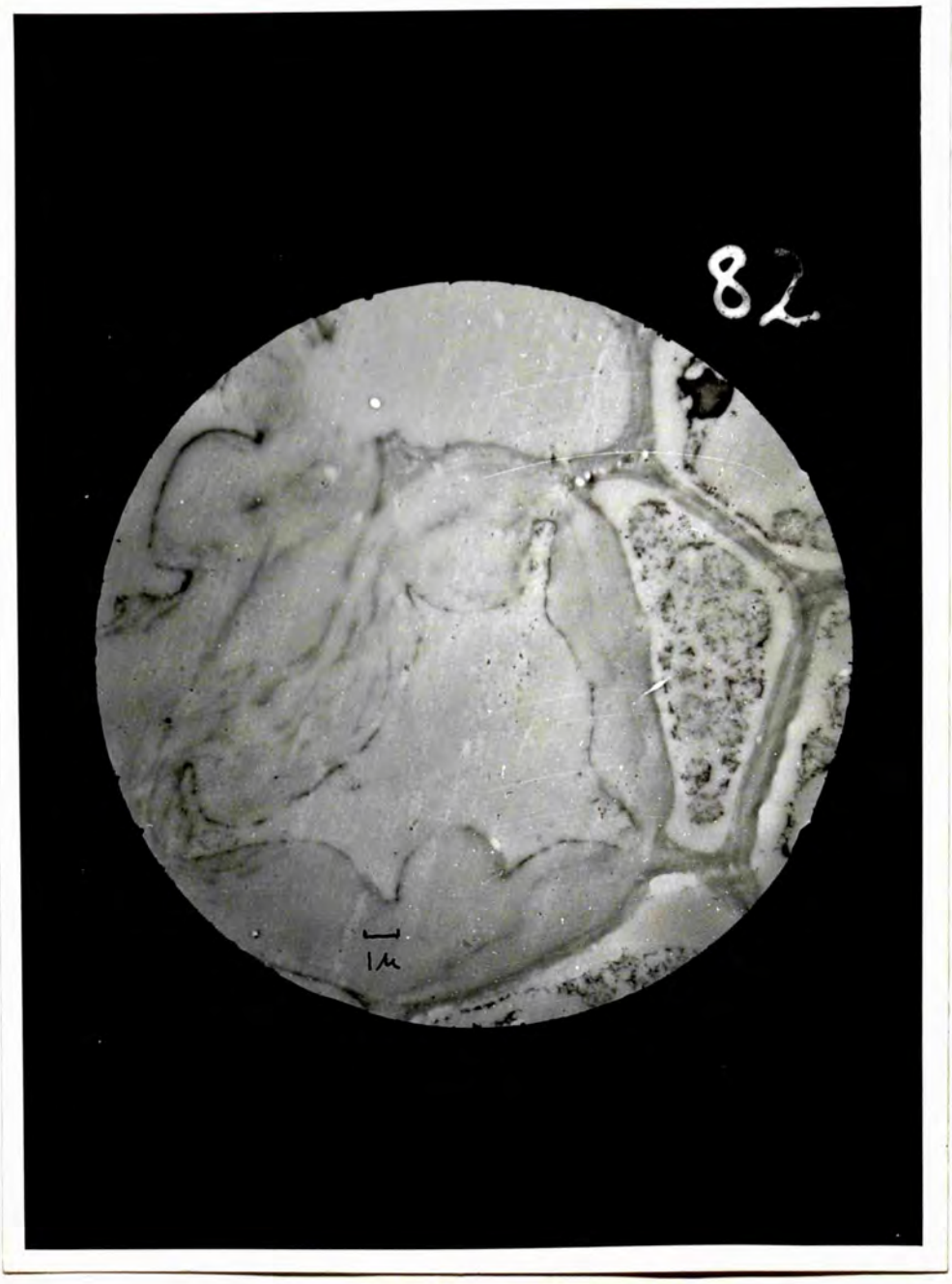

PLATE 9. Electron micrograph of sieve tube with companion cell in T.S. The lumen of the sieve tube is irregular in outline. Note the difference in the thickness of the wall of the sieve element and the companion cell.

enveloped some of the cytoplasm from those activity it arose. It can be noted that in many cases (see Plates 7, 8, 9, 10) the boundary between the cytoplasm and the nacreous wall has an appearance rather like a "dotted line" (- - - -). Sometimes this is very regular (Plate 10). This may in part be an artifact, but that is not entirely so is suggested by the fact that many of the dark inclusions within the wall thickness resemble it rather closely (see Plates 7, 10). It was hoped to investigate the structure of the nacreous wall by inter alia removing the methacrylate and shadowing with metal; but the evaporation unit, which was under construction in the Department, could not be finished in time.

All these micrographs are of the mature and presumably functional sieve elements. It was considered worthwhile, as mentioned earlier, to investigate further the thickness of the sieve-tube wall in the petiole of relatively young and senescent leaves. The nacreous wall of a young sieve-tube element is shown in Plate 11. In the case of a senescent leaf it was expected that the sieve-tube might have become non-functional after the decay of the lamina. Plate 12 shows the sieve element from a senescent petiole, the lateral wall showing its division into outer thin and inner thick layers. The latter is not materially different from the nacreous wall

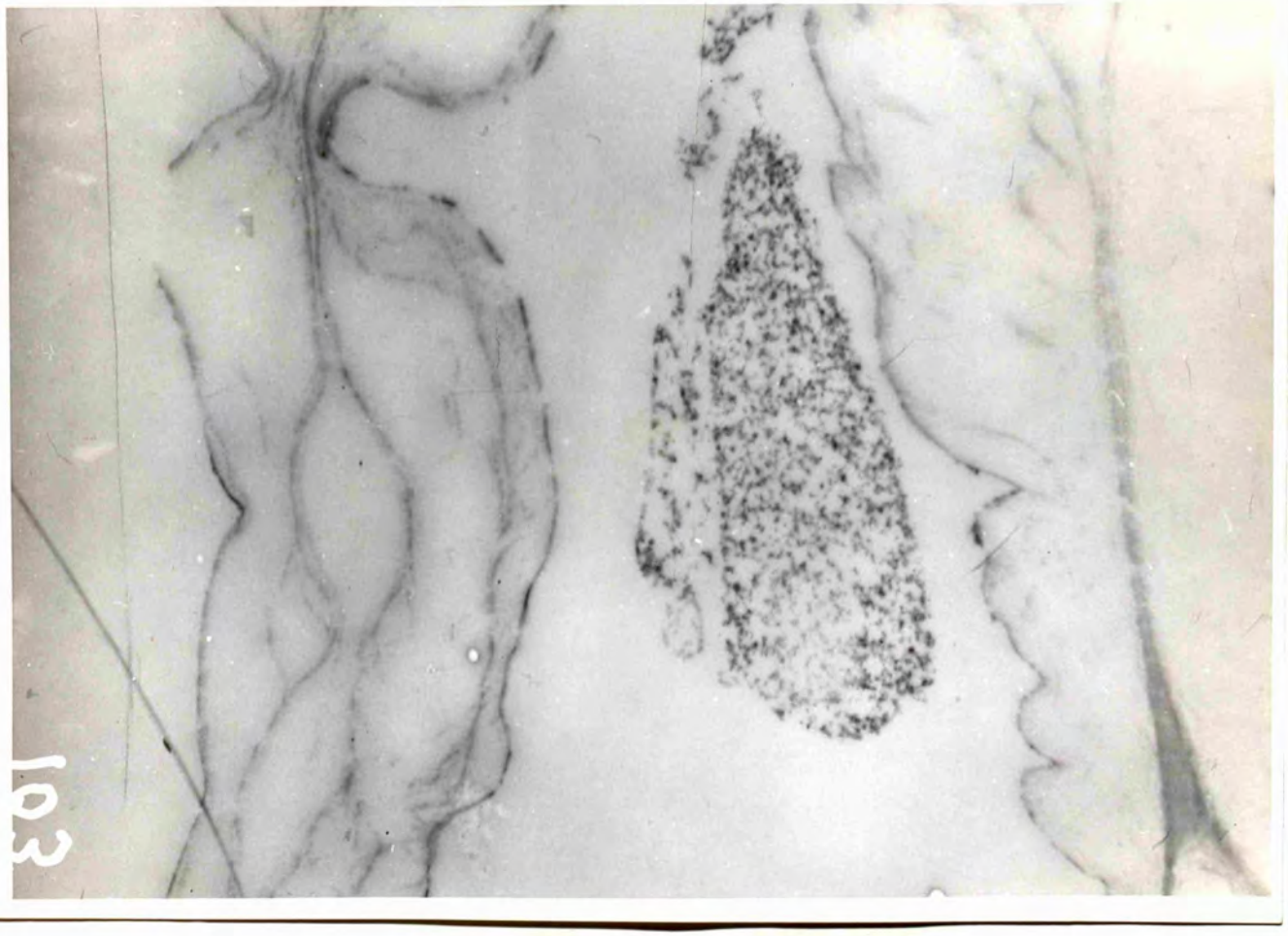

PLATE 10. Electron micrograph of sieve tube to show the "dotted line" appearance of the inner limit of the nacreous wall.

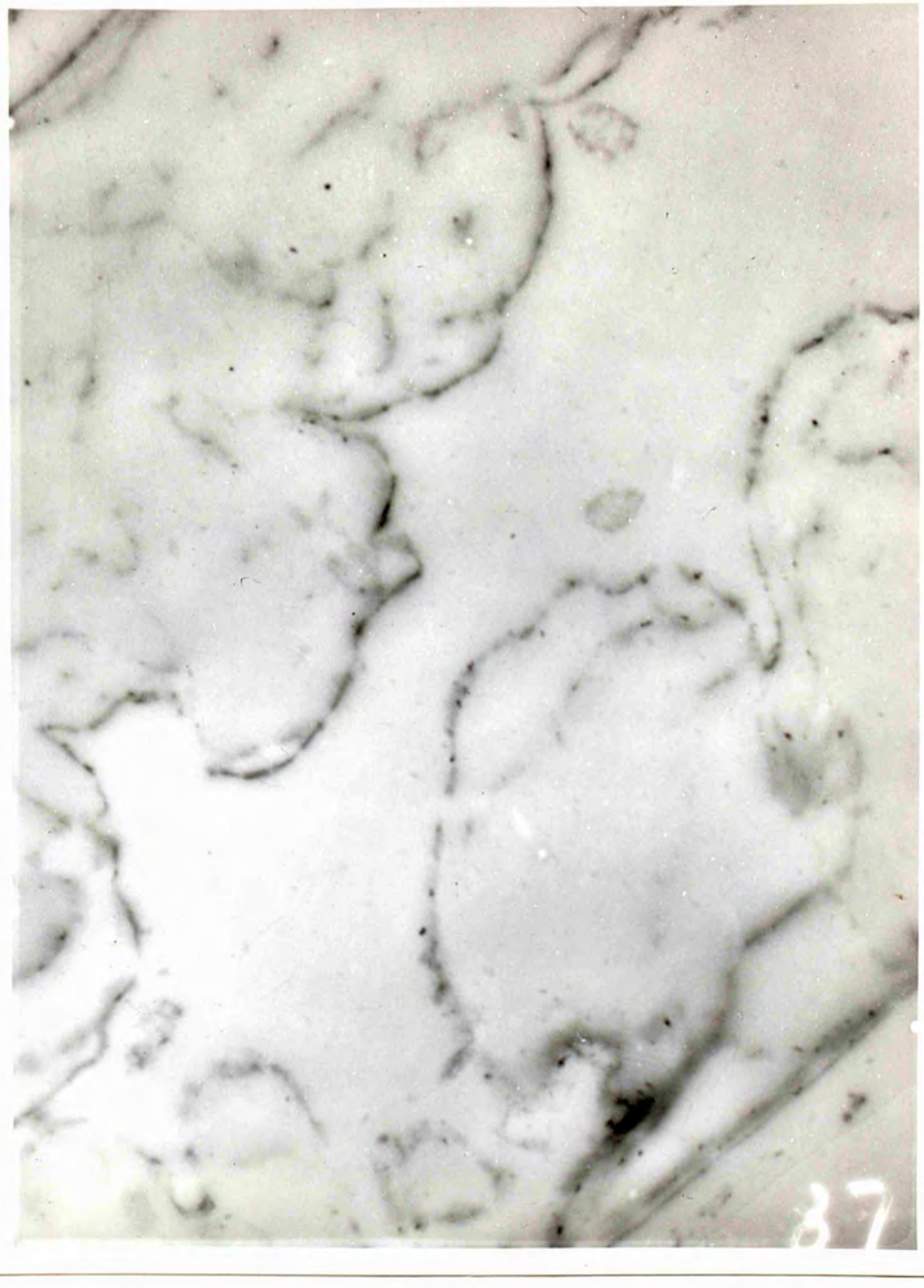

PLATE 11. Electron micrograph showing wall thickness in a young sieve tube.

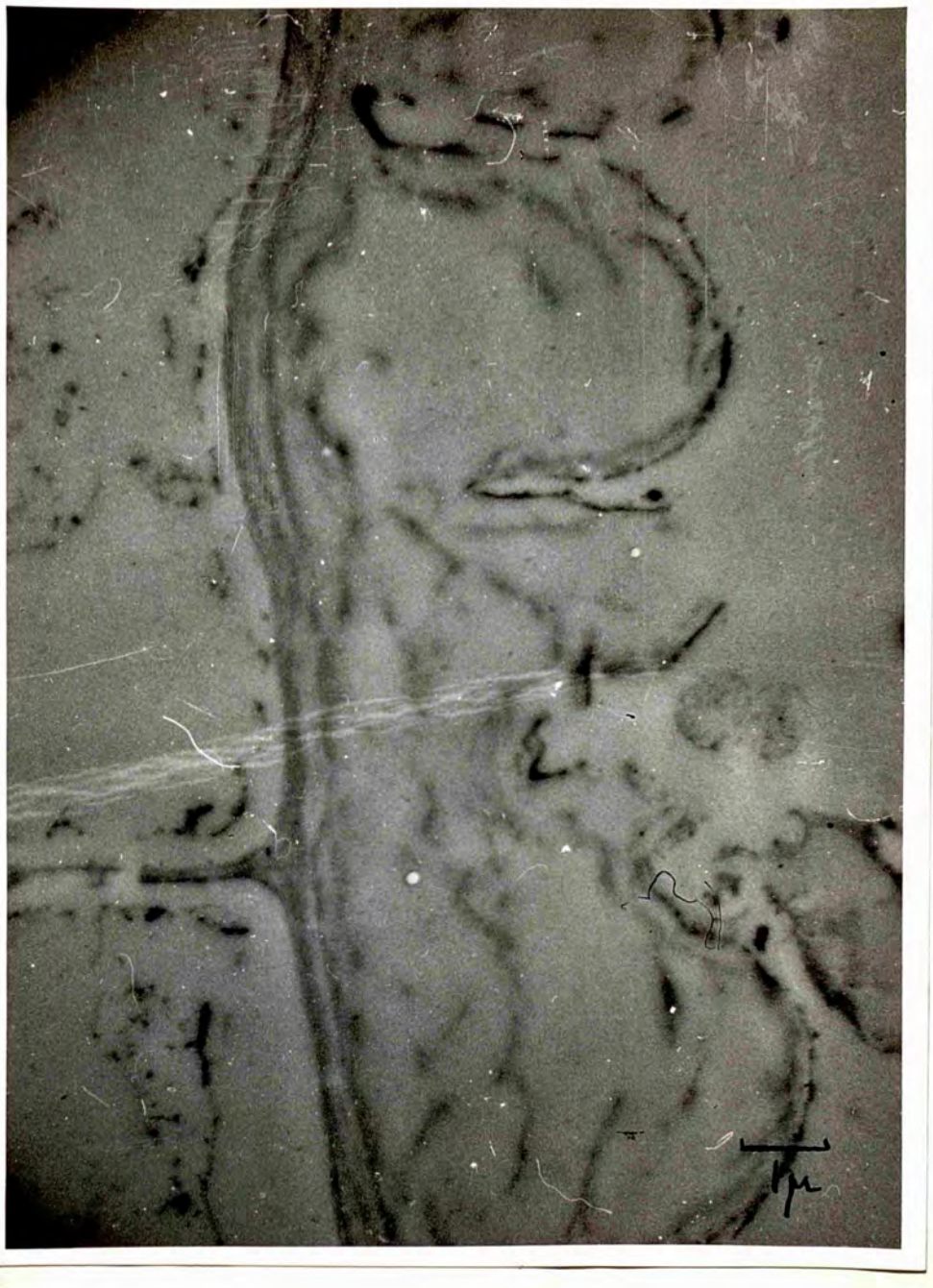

PLATE 12. Thick nacreous wall of the sieve tube obtained from senescent petiole.

shown earlier. This might suggest that in a primary organ the sieve elements cease to function with the organ itself without any change in their wall thickness; another possibility is that they are still in the last stage of their functional activity. That this may also be the case seems probable from Plate 13 of the xylem parenchyma from the same material still showing apparently intact cytoplasmic inclusions.

Earlier workers, notably Crafts (1931), considered the nacreous wall to be highly hydrated and to shrink during the preparative dehydration. Reduction of the wall thickening of primary sieve elements when the cell ages has also been reported, and the cause of this shrinkage is supposed to be dehydration. The present study has not yielded any evidence of such a shrinking in older elements; and so far as preparative treatments are concerned Esau and Cheadle (1958) reversed their former view that the wall in the fresh state was highly hydrated and shrunk on alcohol treatment, a view adopted by Crafts (1931) in his theory of translocation within the wall itself. Certainly the present micrographs do not suggest a shrinkage on preparation. It was hoped that metal shadowing might reveal the detailed structure of the nacreous wall, but the delay in the availability of the apparatus, as remarked earlier, destroyed this hope.

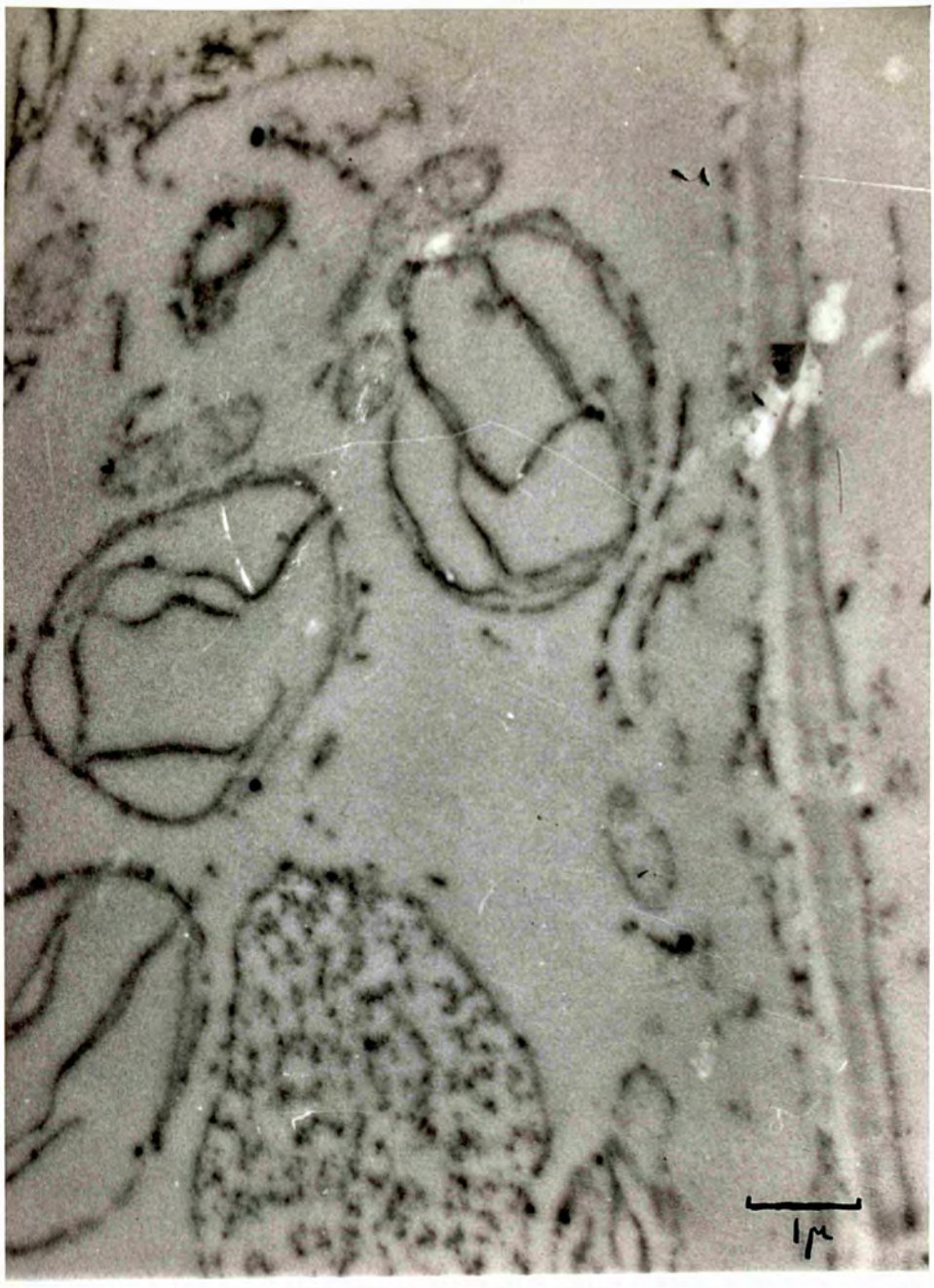

PLATE 13. Xylem parenchyma from senescent petiole showing inclusions. This shows the cell might be in a functional state.

The materials for the micrographs described above were prepared according to Schedule 1 with fixation time of ½ hour. Preparation of specimens according to Schedule 2 again involves OsO_4 as the fixative. As such this technique was not expected to yield any different result. Plate 14 shows a mature sieve element (with its companion cell?) in longtitudinal section prepared according to Schedule 2 but at pH5.8. The sieve element shows the same general features of the nacreous wall; the companion cell shows seven spherical bodies which may be poorly preserved mitochondria. They have an approximate diameter of 0.98μ. The preservation of companion cell cytoplasm at pH5.8 does not appear very satisfactory.

The next plate (Plate 15) shows a longtitudinal section of the sieve element fixed in $KMnO_4$ for ten minutes according to Schedule 3. Again the nacreous wall has the same appearance as that of the specimens fixed in OsO_4 according to Schedules 1 and 2, though it may possibly be somewhat swollen.

It has been mentioned earlier that the nacreous wall did not show in the optical micrographs 3 and 4. In an attempt to find out the reason for this, an optical investigation of the phloem was again undertaken. Since it

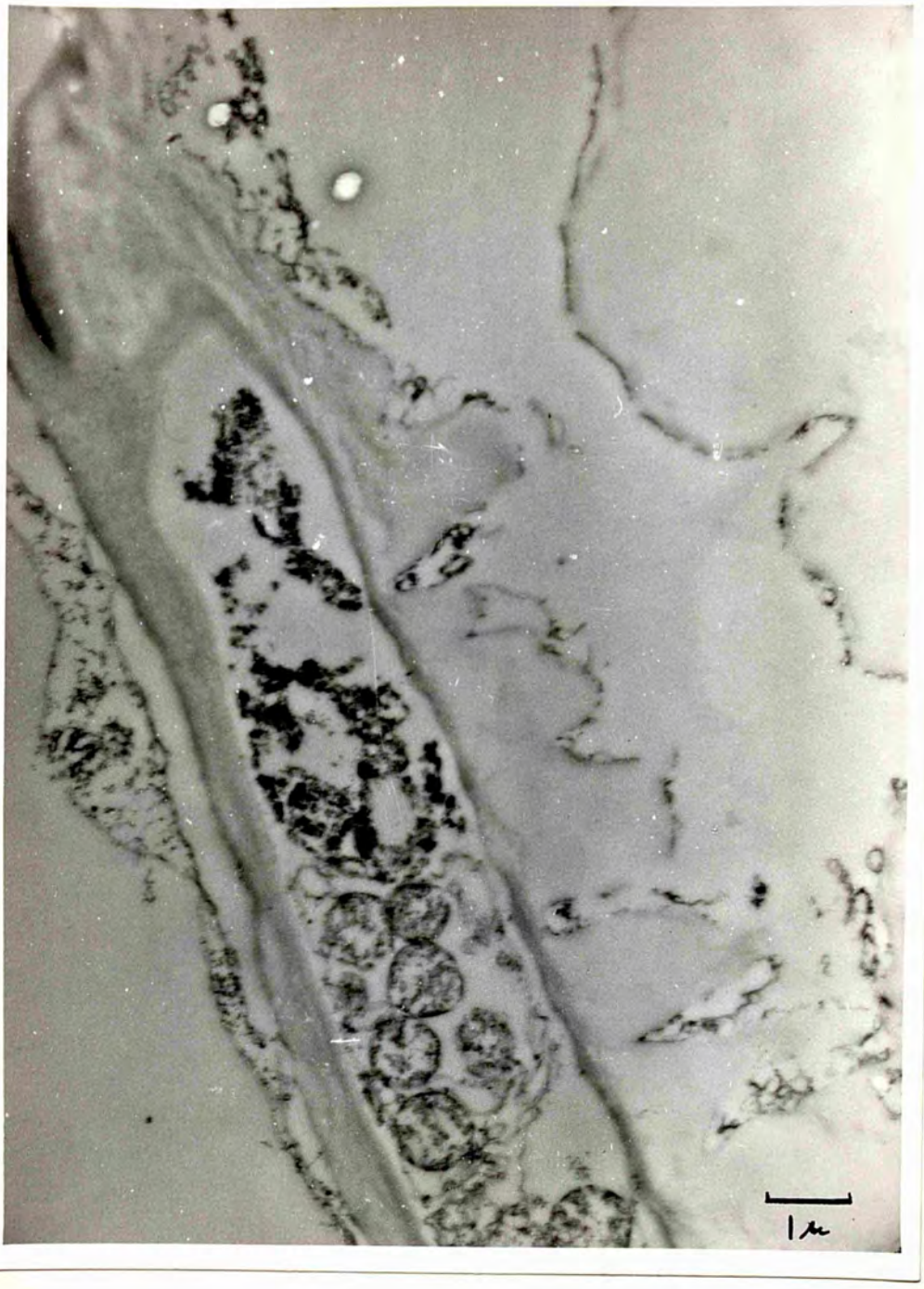

PLATE 14. Sieve tube (with its companion cell?) prepared according to Schedule 2 at p.H 5.8 showing nacreous wall. The preservation at PH 5.8 is not very satisfactory. The so-called companion cell shows a large number of mitochondrion-like inclusions.

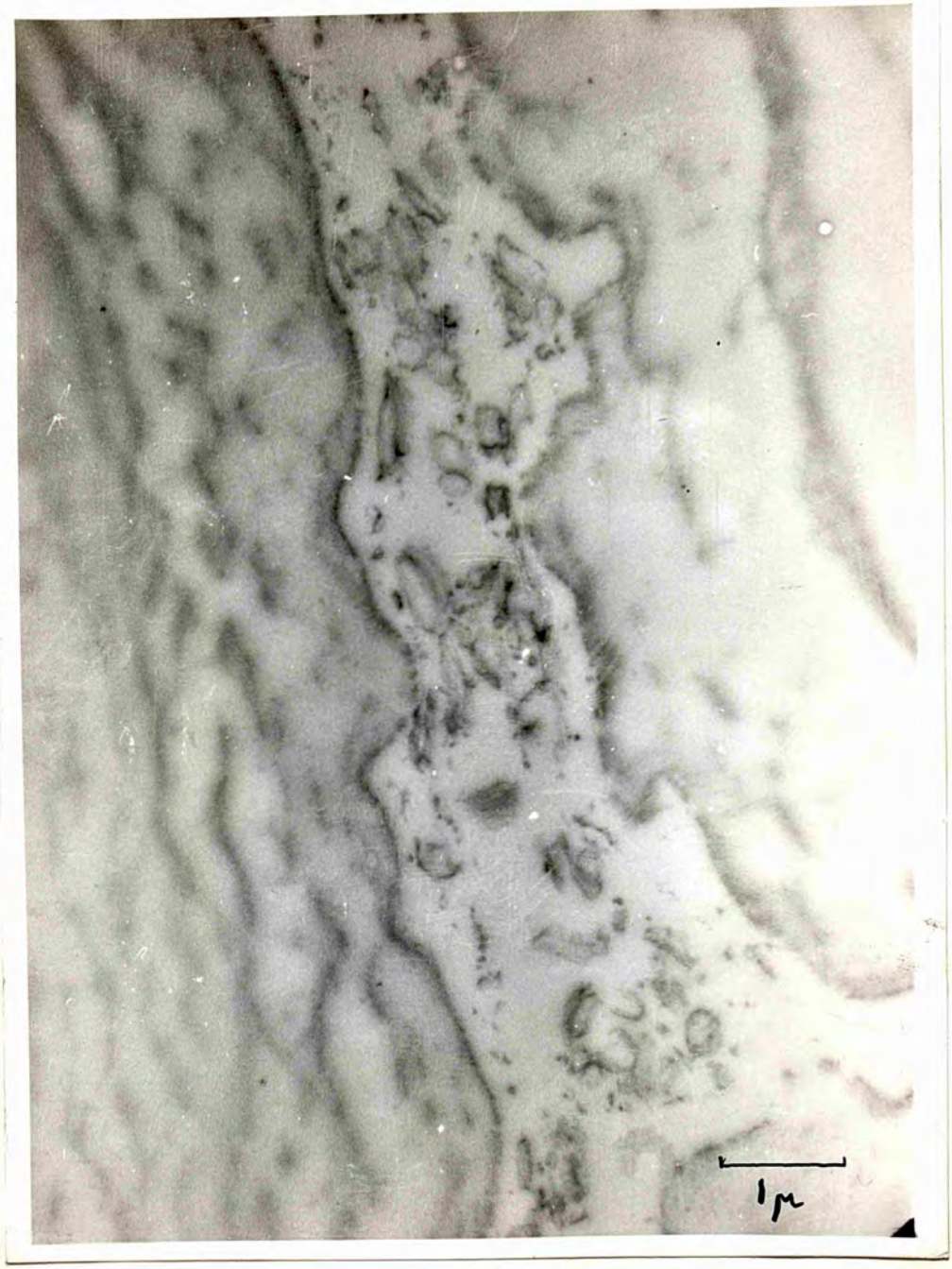

PLATE 15. Nacreous wall of the sieve tube fixed in $KMnO_4$.

is possible to get sieve elements in the very first transverse section of the petiole, sectioning in the longtitudinal plane was discarded in favour of transverse. The sieve-tube wall of protophloem, metaphloem and secondary phloem is reported to be mostly cellulose, hence stains suitable for cellulose were used to bring out the nacreous wall. Due to shortage of time only known staining techniques were employed in the optical investigation of the nacreous wall.

The first staining combination tried was safranin and haematoxylin, and the result is shown in Plate 16. It can be clearly seen that a nacreous wall is indeed present, but that only its inner limit is stained and that also only faintly; the matrix being optically clear. The two black areas probably represent obliterated sieve elements of the protophloem.

The result of staining in safranin and fast green is shown in Plate 17. The staining of the nacreous wall is very poor. Chang (1935) used light green to show the protophloem in the angiosperm shoot apex. Fast green, according to Conn (1940), stains very much like light green and is considerably less subject to fading. That is why fast green was used in place of light green; but the result was rather disappointing. One of the sieve elements shows the sieve plate.

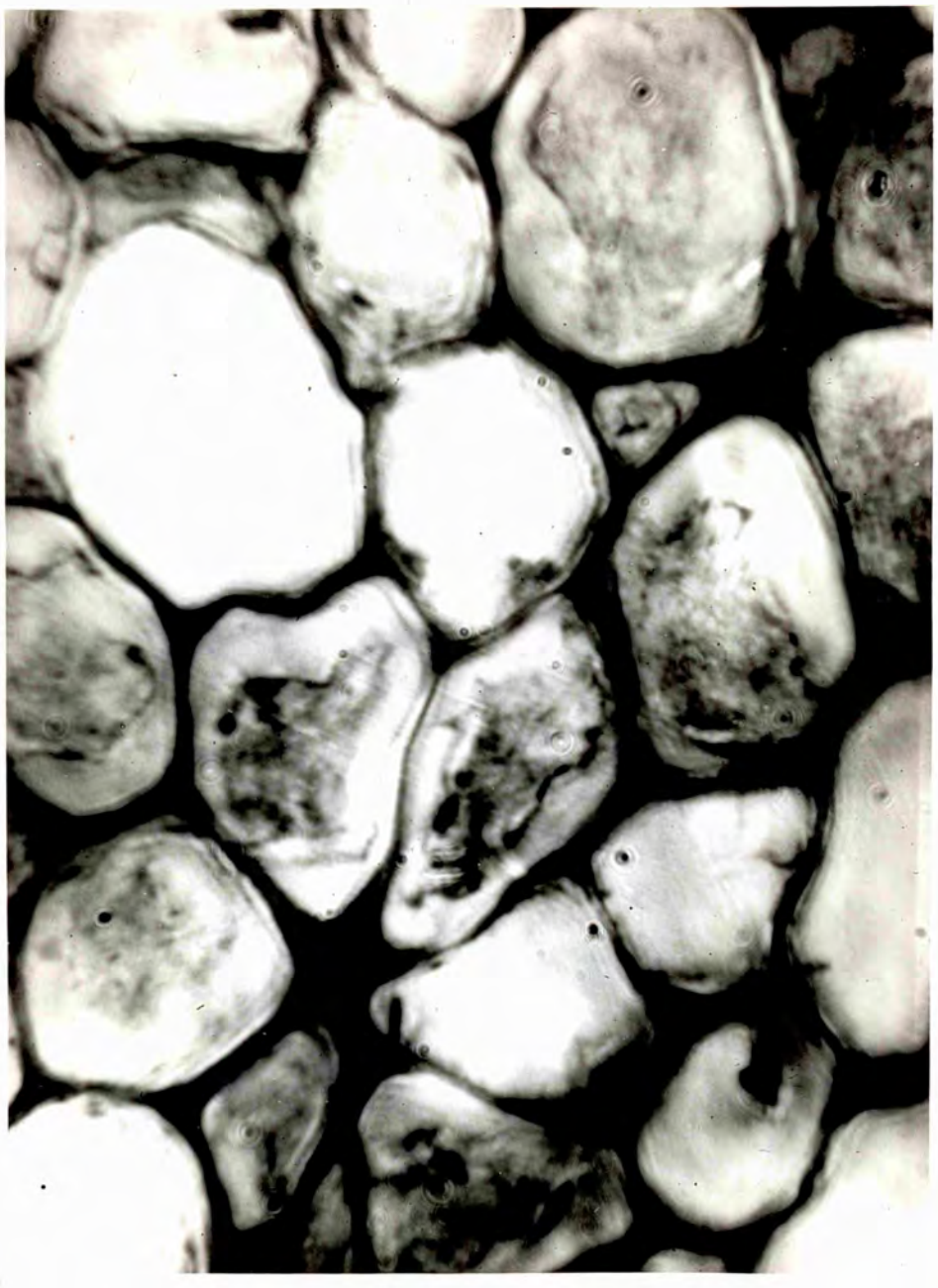

PLATE 16. T.S. phloem showing nacreous thickening in the sieve tubes. The two black areas represent obliterated protophloem sieve tubes. Stained in safranin and haematoxylin. X2250.

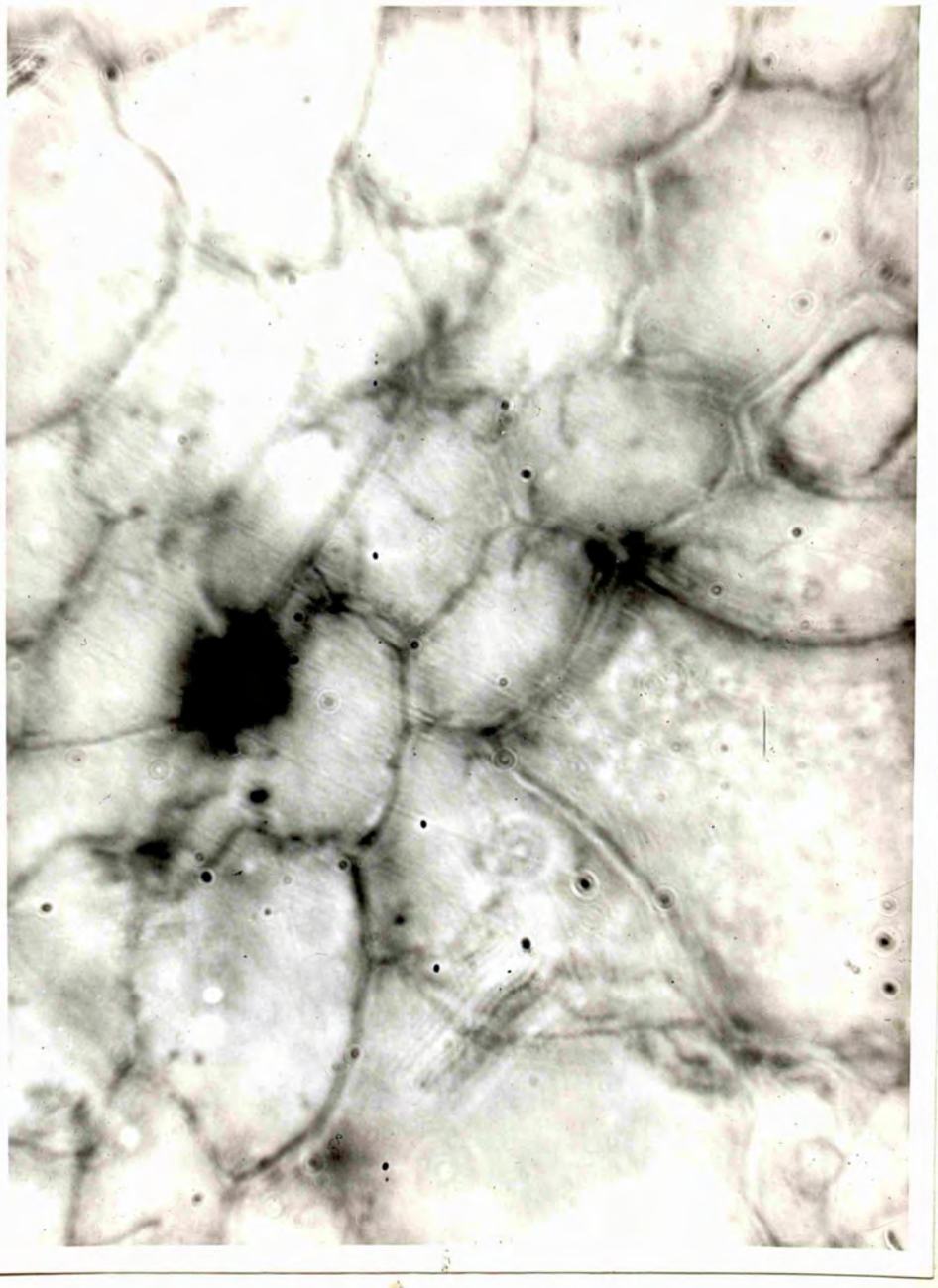

PLATE 17. T.S. phloem stained in safranin and fast green. Nacreous wall does not show up. One of the sieve tubes shows the sieve plate. X2250.

X1½

The next technique used in staining phloem was due to Cheadle, Gifford and Esau (1953). Tannic acid - iron chloride was used for staining wall and lacmoid (resorcin blue) to bring out callose. This staining method was also used by Esau and Cheadle (1958) in their study of wall thickening in sieve elements of secondary phloem. Though the callose was distinctly stained, response of the nacreous wall was poor, as seen from Plate 18. This is not entirely surprising, since Esau and Cheadle also experienced this difficulty. One of the sieve elements again shows the sieve plate.

The last staining technique tried consisted of staining the wall in tannic acid - iron alum after staining the cytoplasm in safranin and orange G, as recommended by Sharman (1943). This proved relatively successful in staining the nacreous wall and the result is shown in Plate 19. From the limited trial given to these staining techniques, it appears that the nacreous wall is difficult to stain; only the inner limit being faintly stained and the matrix being optically clear. This fact coupled with the poor response of the nacreous wall to tannic acid - ferric chloride is perhaps the reason why the nacreous thickening was missed in optical micrographs 3 and 4.

3. The sieve plate

As mentioned earlier, sieve-tubes of Nymphoides have simple sieve-plates. This point is clearly shown in the

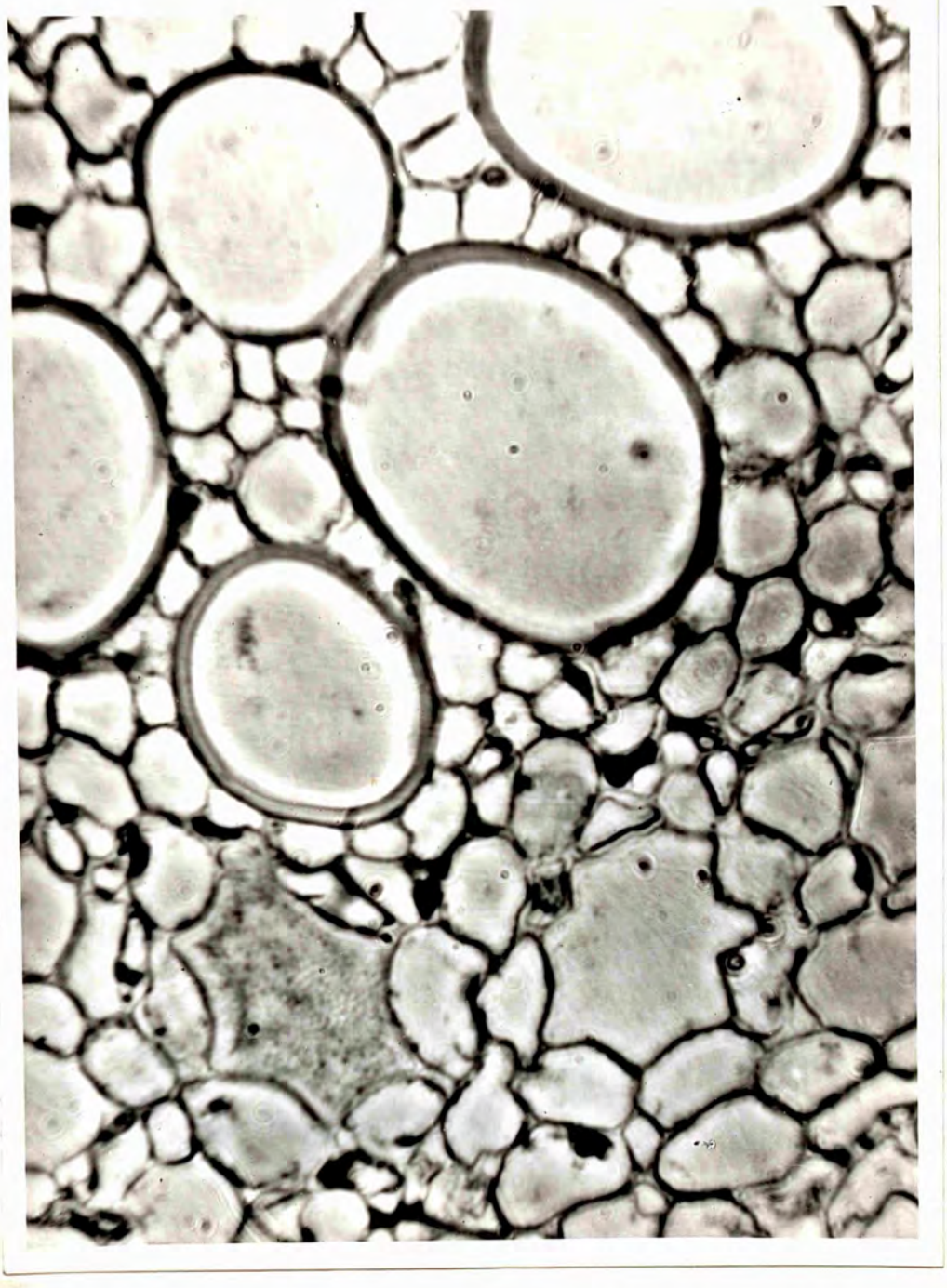

PLATE 18. T.S. phloem stained in tannic acid-ferric chloride and lacmoid. Nacreous wall does not show up. One of the sieve tubes shows its sieve plate. X970

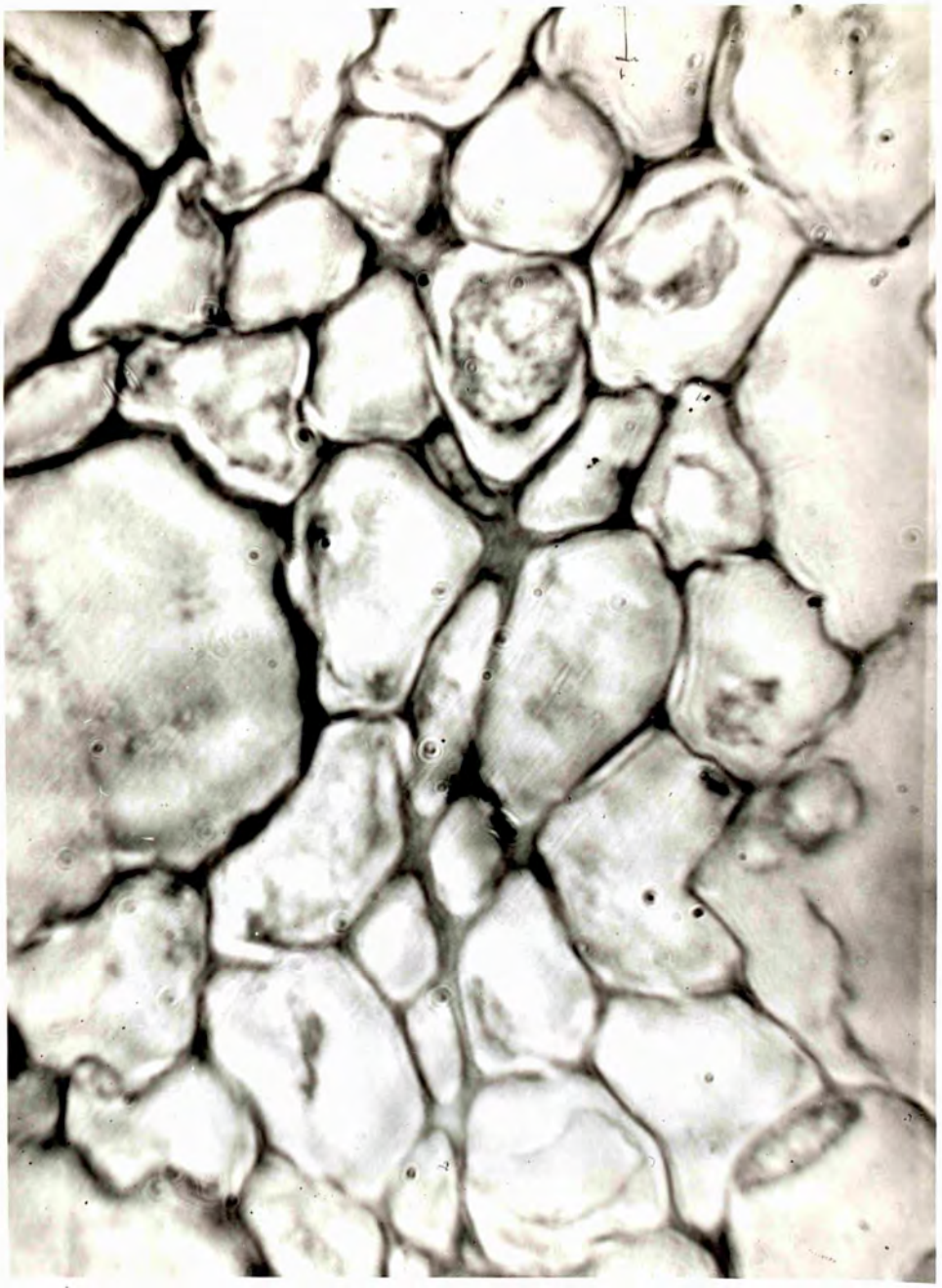

PLATE 19. T.S. phloem showing nacreous thickening in the sieve tubes. Stained in safranin-orange G and tannic acid-iron alum. The black area represents obliterated protophloem sieve tube. X2250.

optical micrographs 20 and 21 prepared according to the method of Cheadle, Gifford and Esau (1953). The plates show a single sieve area per plate. White circular areas probably represent spaces occupied by unstained connecting strands.

Passing on to the electron micrographs, the structure of the sieve plate is presented in Plate 22, which is a longtitudinal section of the sieve tube. The plate is localised to the transverse end wall which possesses a very obvious structure, the compound middle lamella, referred to in the optical Plate 4, being clearly resolved here into its three components. The double dark lines probably represent the inner limits of the primary walls. The plate shows five pores of which numbers 1, 4 and 5 show very definite contents (the "connecting strands"). These appear continuous with the surface cytoplasm. On either side of the strands can be seen a whitish collar which probably represents the callose cylinder. The section seems to have passed in pores 2 and 3 just through the boundary of the connecting strand and callose cylinder, pore 2 particularly showing the typical contents in the next serial section (Plate 23). One of the sieve tube elements shows a dark line in its what is apparently a lumen forming an elongated sac-like structure. It is difficult to tell its nature.

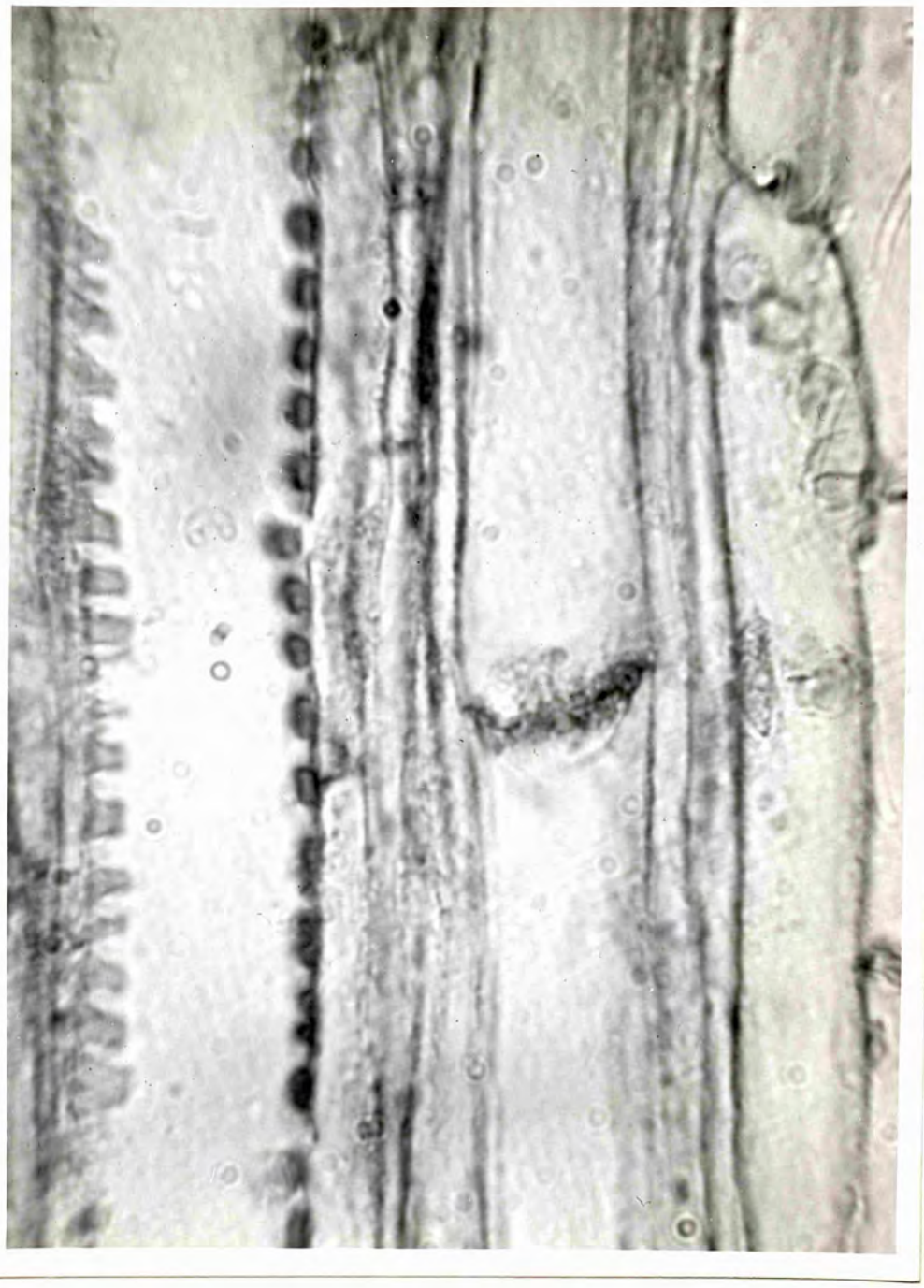

PLATE 20. L.S. vascular bundle showing xylem, phloem and starch sheath. Note the simple sieve plate with white circles representing spaces occupied by connecting strands. Callose cylinders appear as black rings. Stained in tannic acid-ferric chloride and lacmoid. X2000

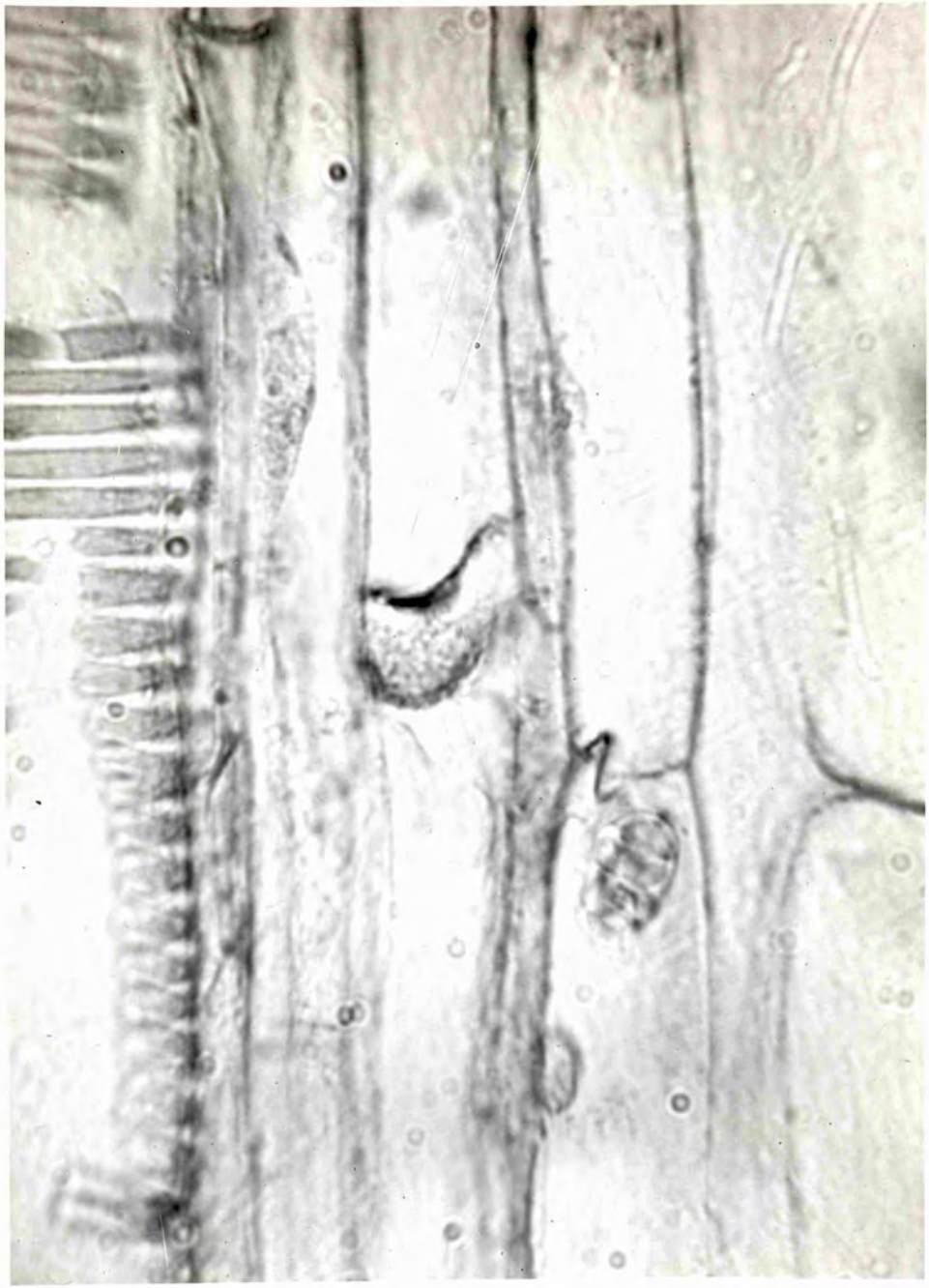

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PLATE 21. Another L.S. Note the single sieve area on the transverse end wall of the sieve tube. Companion cell is also seen. The cell of the starch sheath shows starch grains enclosed in the plastid. Stained in tannic acid - ferric chloride and lacmoid. X2000

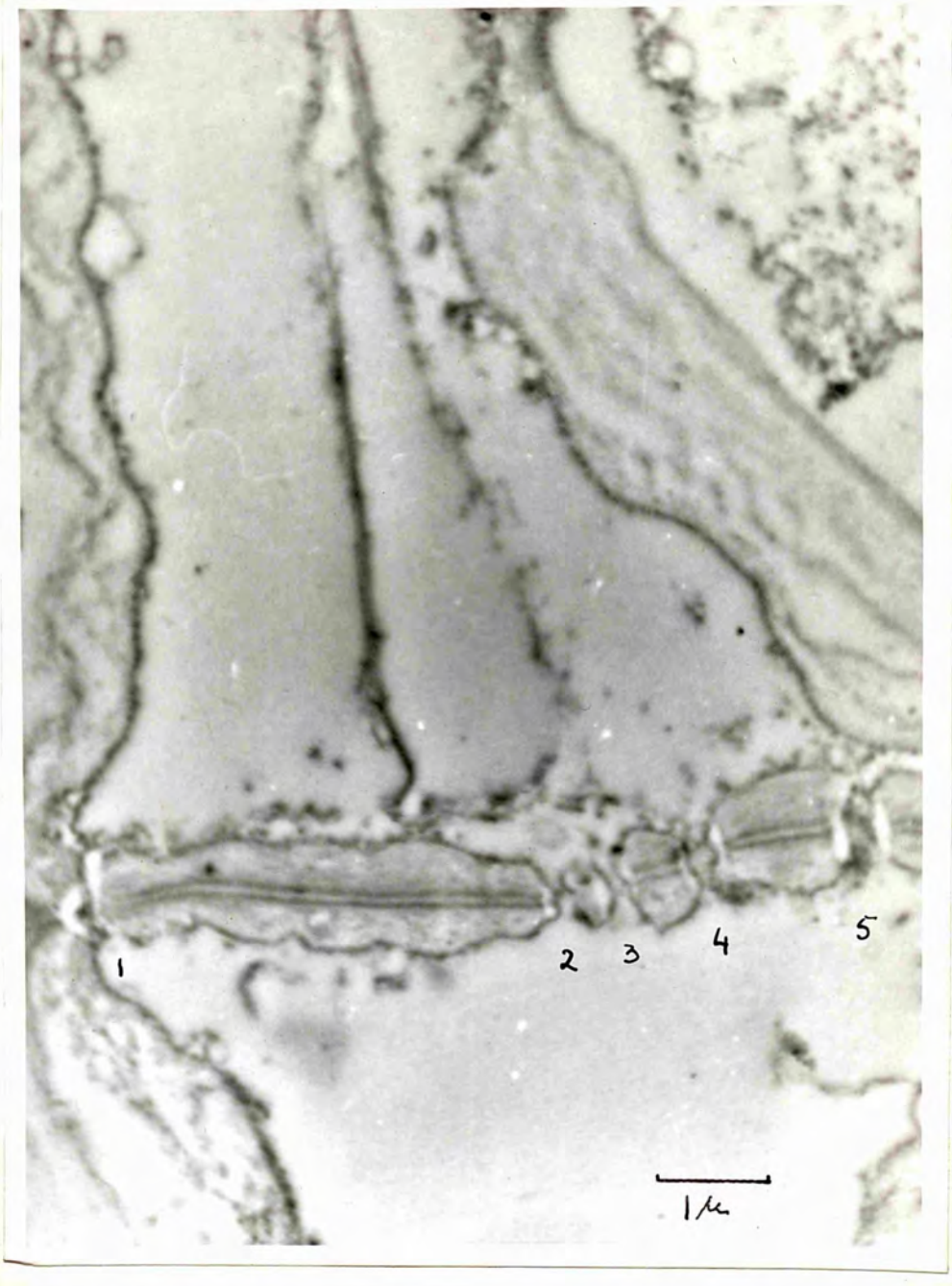

PLATE 22. Electron micrograph showing sieve tube with sieve plate. The structure of the plate is well resolved. Fixed in osmic acid without sucrose for 1 hour.

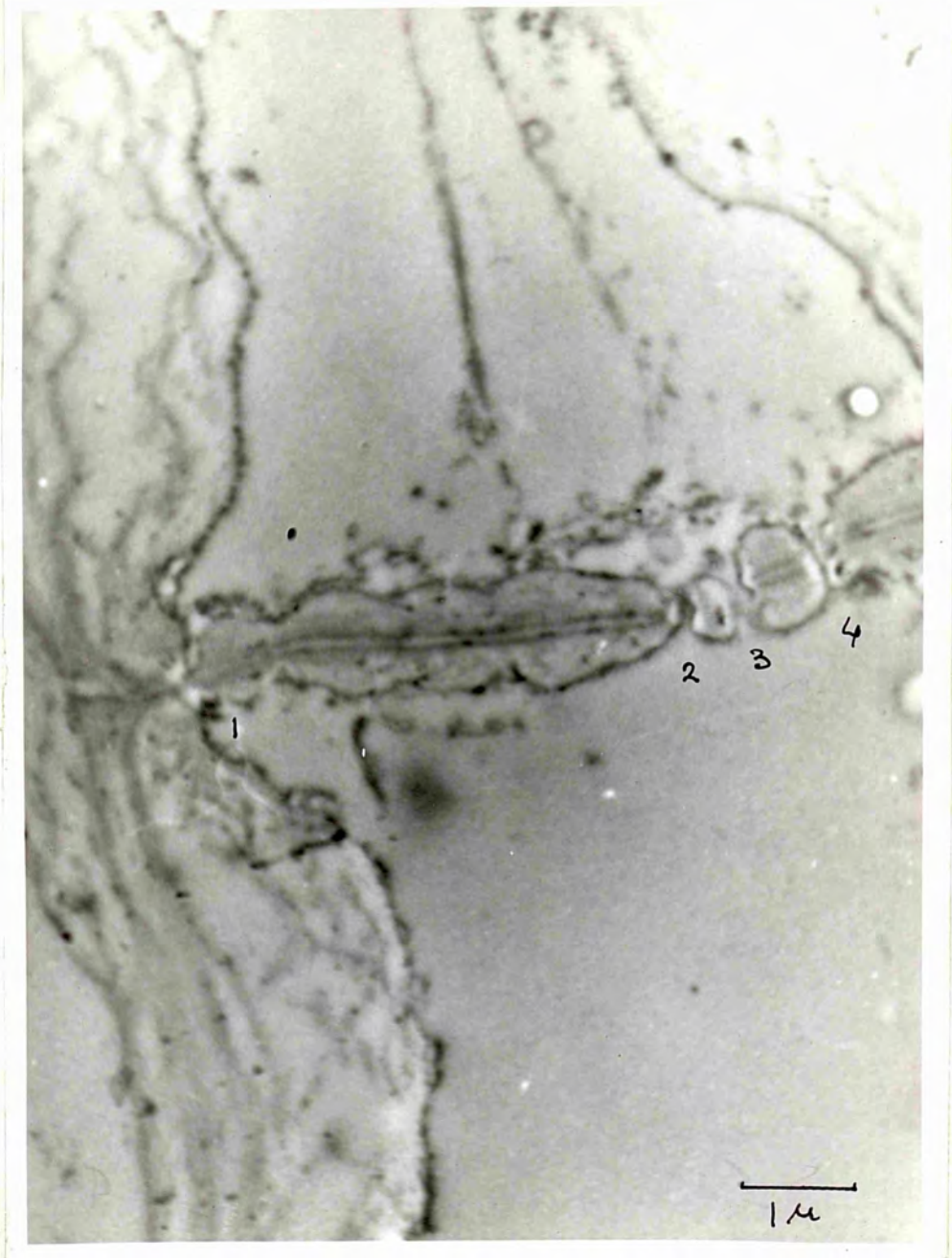

PLATE 23. Another section of the sieve plate of the previous micrograph. Note a structure in the lumen of one of the sieve element.

It does not seem to be a mere artifact, and it appears in another section of the same sieve tube shown in Plate 23. It does not represent the tonoplast even if there is one in the sieve tube, for it appears to be far too prominent to be such - compare with what is possibly the vacuolar membrane in a parenchyma cell shown in Plate 24. It may possibly be a consequence of the longtitudinal infolding of the nacreous wall, which in rare cases can even form a septum across the tube as shown in optical micrograph Plate 25. The materials for these plates were prepared according to Schedule 1 by fixing for one hour in the case of the former and half an hour for the latter in OsO_4 without sucrose. The probable plane of sectioning for Plates 22 and 23 is shown in Figure 5.

Another section from the material fixed similarly but only for half an hour appears in Plate 26. This shows a few features different from the previous two plates. The structure of the sieve-plate is not clear. This may be due to bad sectioning. In fact breaks in the lateral wall can be seen. The latter in this case is very thick, almost occluding the lumen of the tube, but absent from the sieve-plate region. The absence of nacreous thickening on sieve areas has also been noted by Esau and Cheadle (1958). The inner limit of the nacreous wall shows a very regular dotted appearance as remarked earlier. The connecting strands in the pores appear

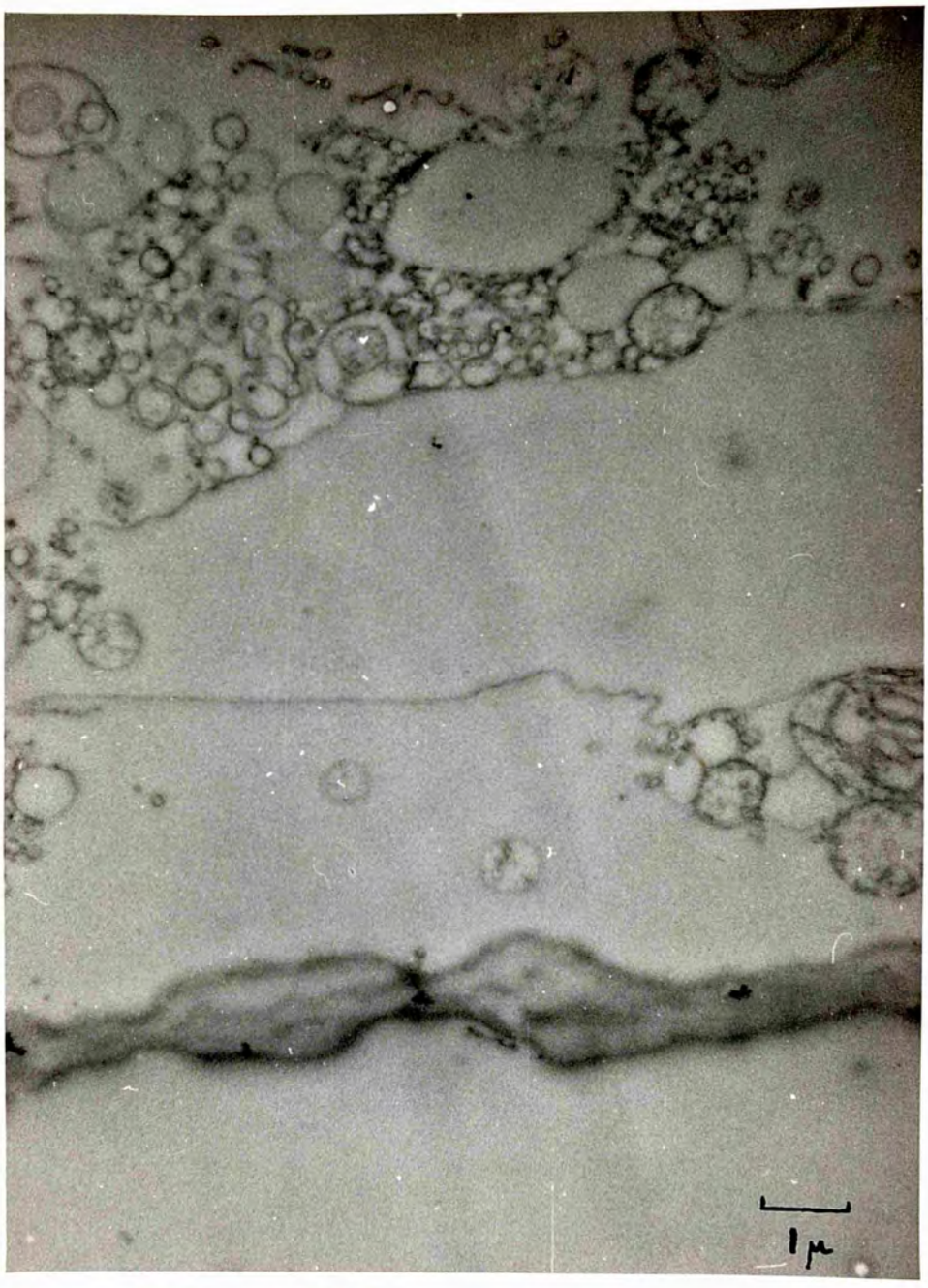

PLATE 24. A parenchyma cell showing possibly tonoplast.

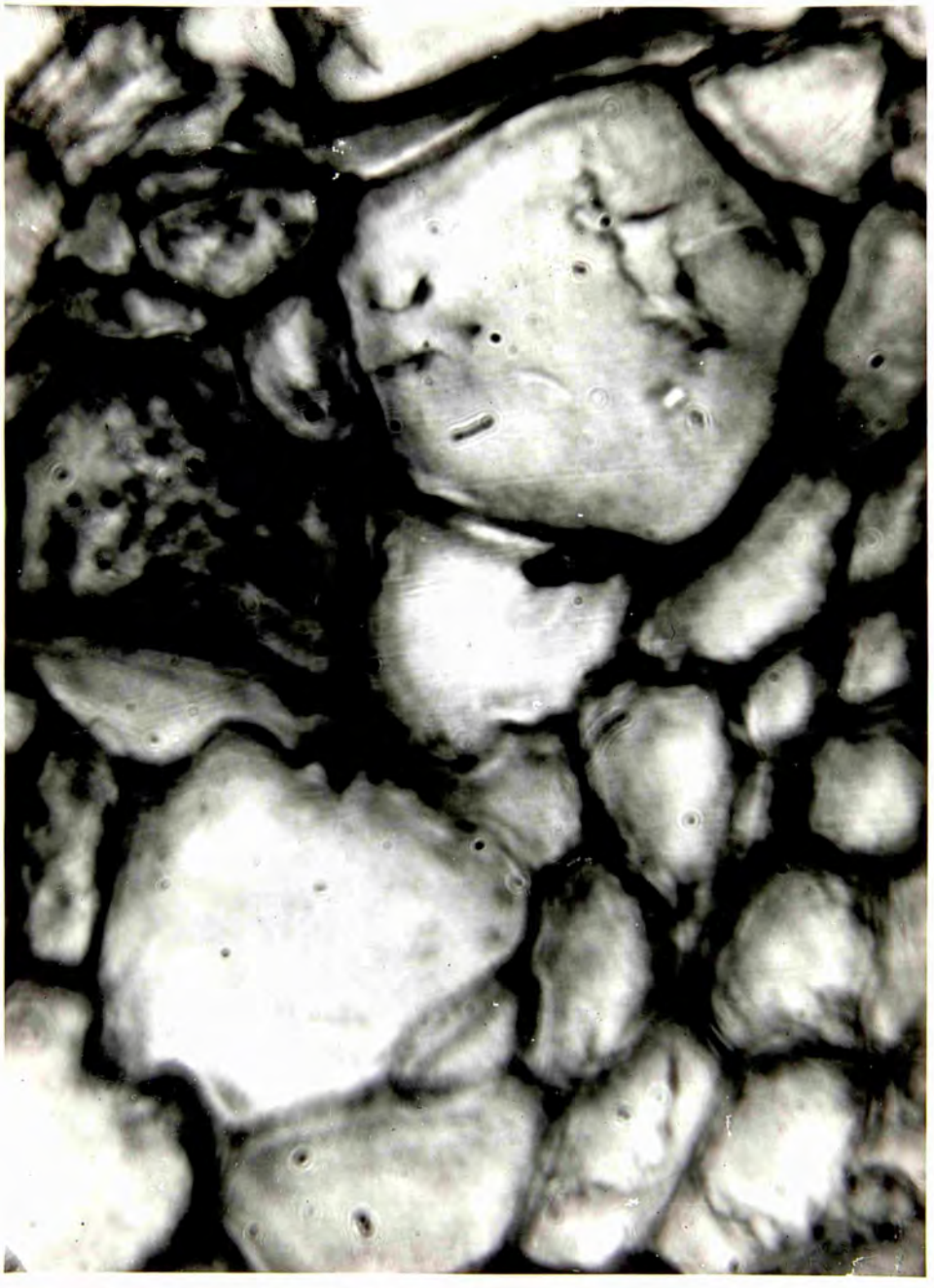

PLATE 25. T.S. phloem. One of the sieve tubes shows excessive development of its nacreous wall which divides the lumen in two parts. Stained in tannic acid - iron chloride and lacmoid. X2250

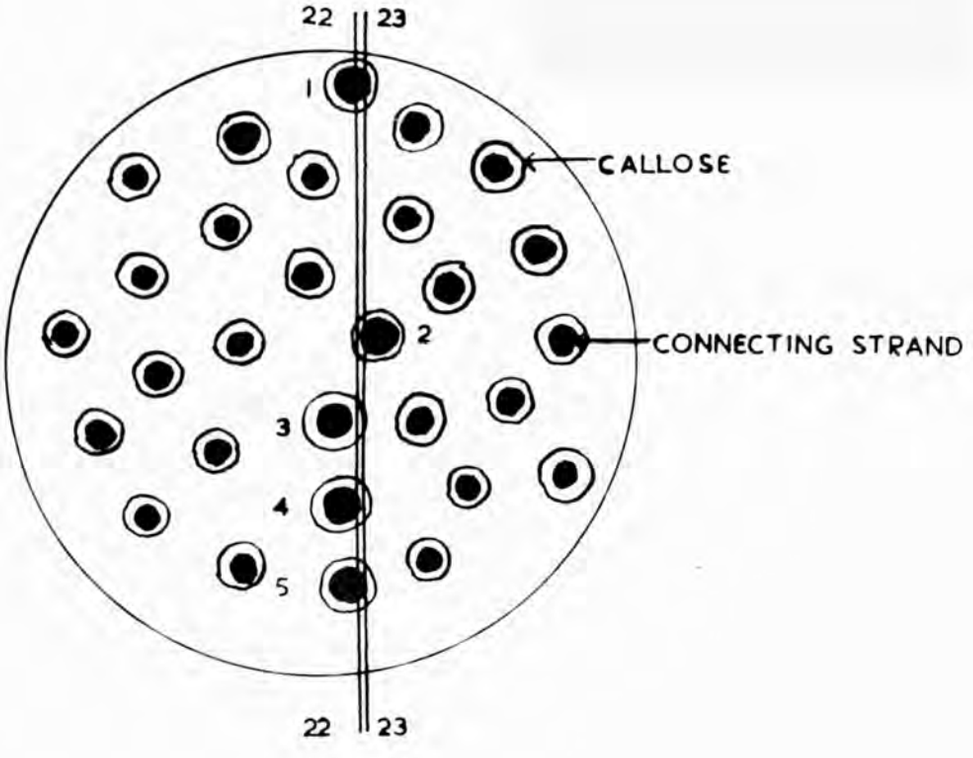

FIGURE 5

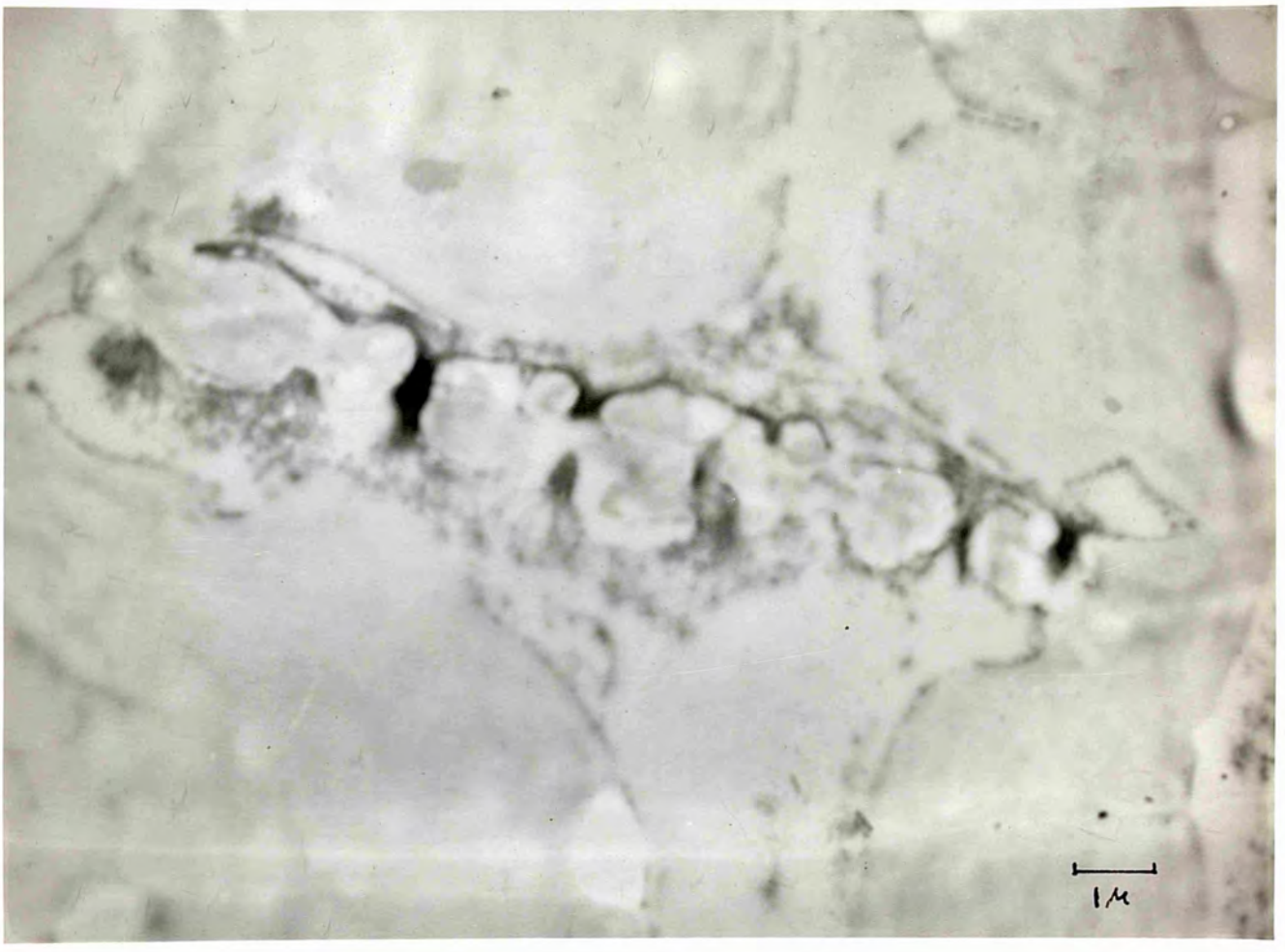

PLATE 26. L.S. Sieve tube. The sieve pores are filled with dark contents. The inner limit of the thick nacreous wall shows the "dotted-line" appearance. Fixed in osmic acid without sucrose for half an hour.

very dark in contrast to those in Plates 22 and 23, a feature noted by other investigators (see below). The reason for this is not clear. It may be mentioned, however, that the material of Plate 26 was fixed in July when the plant was growing vigorously whereas in the case of Plates 22 and 23 it was fixed in November when the growth, as mentioned earlier, was poor. Is the osmophily of the connecting strands then a function of seasonal variation? What relevance has this towards their role in translocation? These questions are difficult to answer without further investigation. The next plate (Plate 27) shows a part of the previous one at a higher magnification and indicates very clearly the continuation of the pore content with the surface cytoplasm. It is to be regretted that the present electron microscope is not capable of resolving the structure of the connecting strand; but there seems to be a suggestion of longtitudinal orientation of its fibrils as found by Schumacher and Kollmann (1959, private communication). Assuming that one of the pores has been cut in the median longtitudinal plane, the connecting strand measures about 0.2m in diameter.

From what has been said above, it appears that so far as the fixation of 0.5-1 mm. thick pieces of Nymphoides petiole are concerned an hour's fixation does not seem to be of any more advantage than half an hour's fixation in OsO_4.

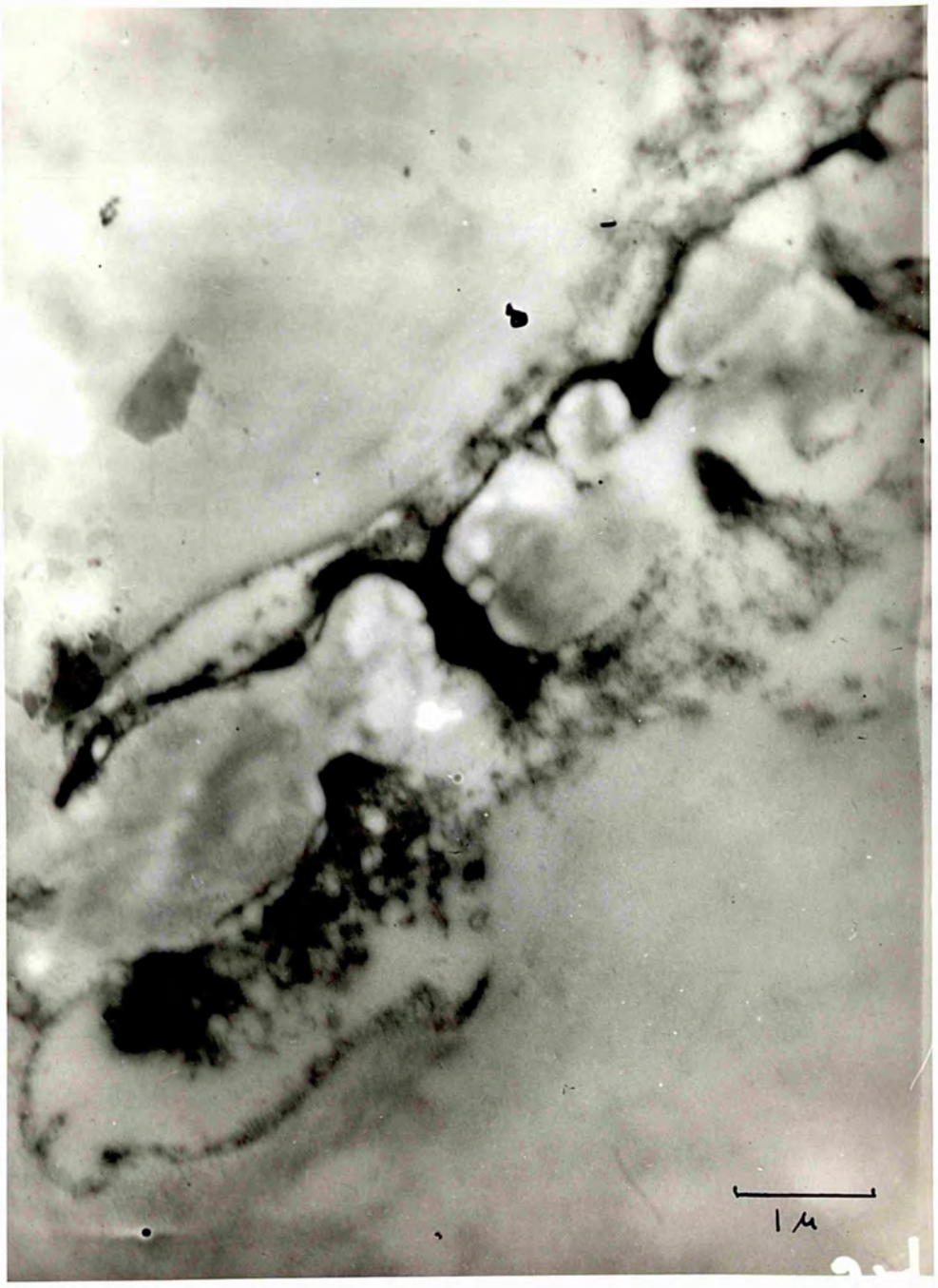

PLATE 27. A part of Plate 26 at higher magnification. Pore contents are continuous with the surface cytoplasm.

Consequently, fixation in OsO_4, whether in Schedule 1 or 2, was subsequently always carried out for half an hour. Further results of half an hour's fixation are shown in Plates 28 and 29. In the case of the former, the fixative had 0.5M sucrose incorporated; in the latter, 1M sucrose. None of the sections is good enough, yet they show some interesting features. Plate 28 shows the nacreous wall, pores with connecting strands and callose cylinder, and accumulation of cytoplasm on either side of the sieve-plate. The other plate shows even more clearly that the pores are filled with electron dense material which passes from one side of the sieve plate to the other, there being no discontinuity in the middle as postulated by Mangenot (1926) and Salmon (1946). Also there does not appear to be any vacuolar continuity through these darkly stained connecting strands as claimed by Hill (1908). As remarked earlier, the sections of these two plates are not good enough. This is certainly due to bad preparation and sectioning, but hardly to the addition of sucrose to the fixative. The latter claim is substantiated by Plate 30 obtained from a material fixed in a fixative containing 1M sucrose. This plate shows almost all the features of the sieve-plate discussed in the previous micrographs. In addition, the cytoplasm is better preserved than

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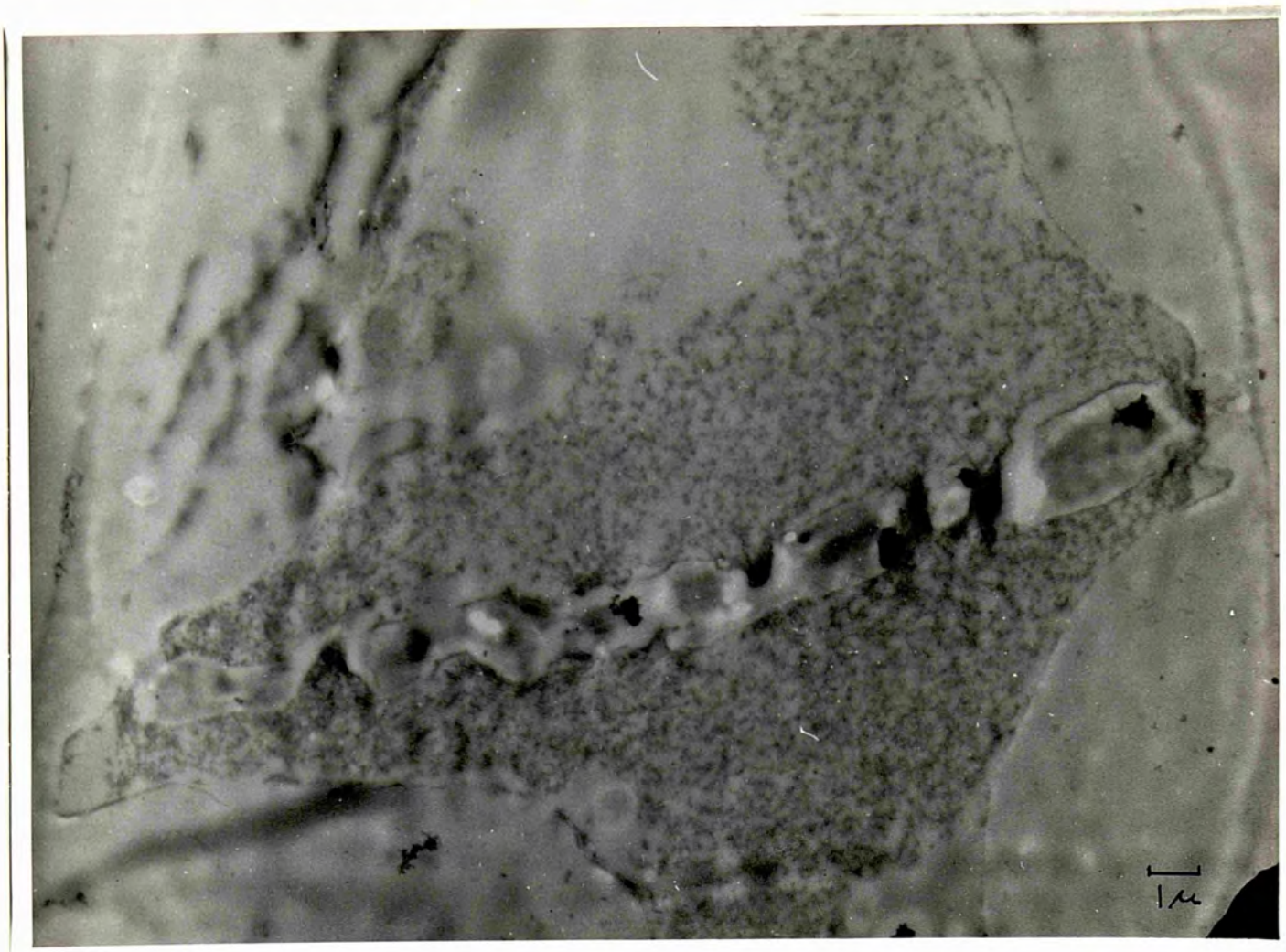

PLATE 28. L.S. Sieve tube showing accumulation of cytoplasm on either side of the sieve plate. Fixed in osmic acid with O.S.M. sucrose.

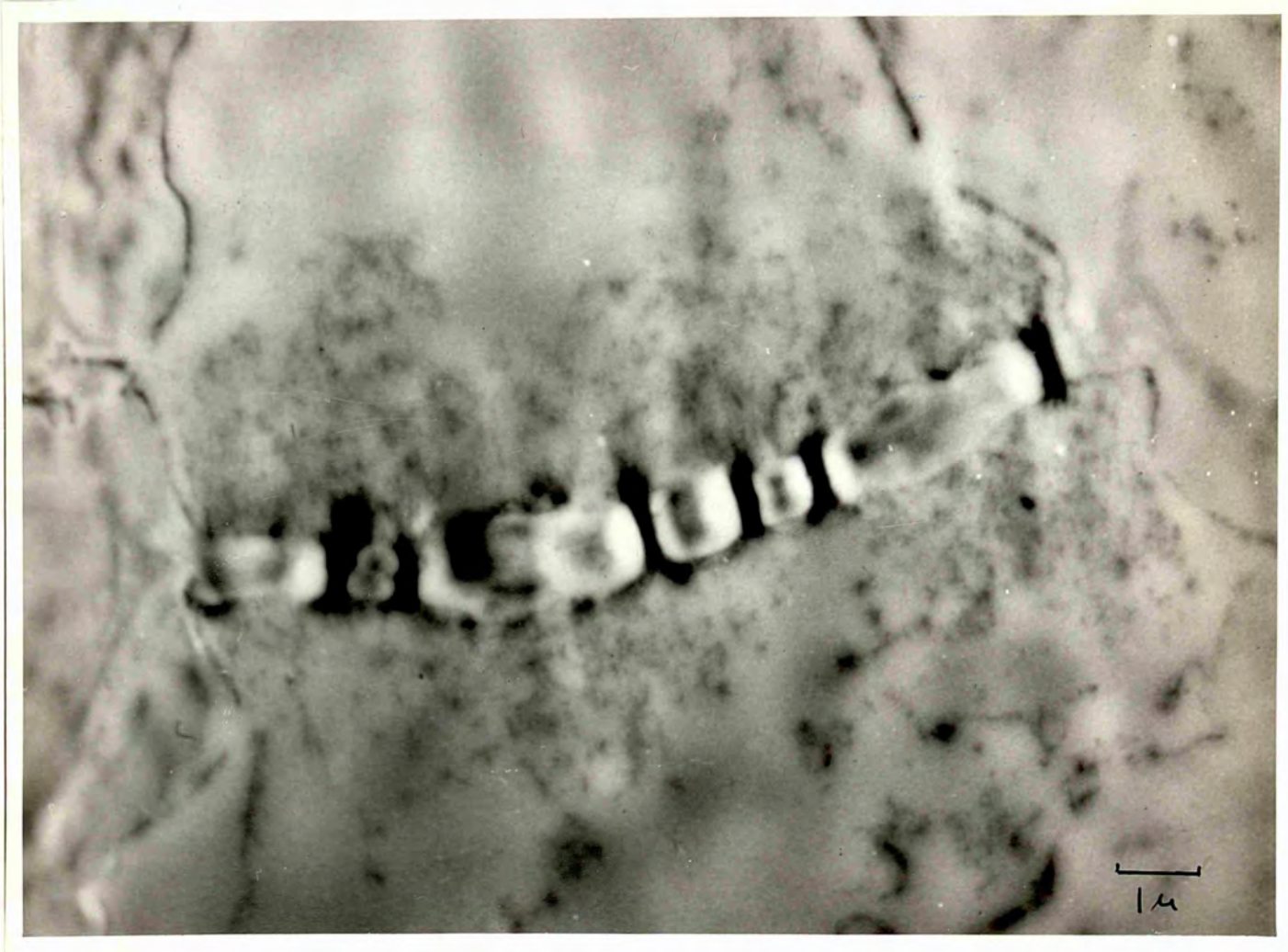

PLATE 29. Electron micrograph of sieve plate fixed in osmic acid containing 1M sucrose. The electron dense material of the pore is continuous from one side of the sieve plate to the other without any break in the middle.

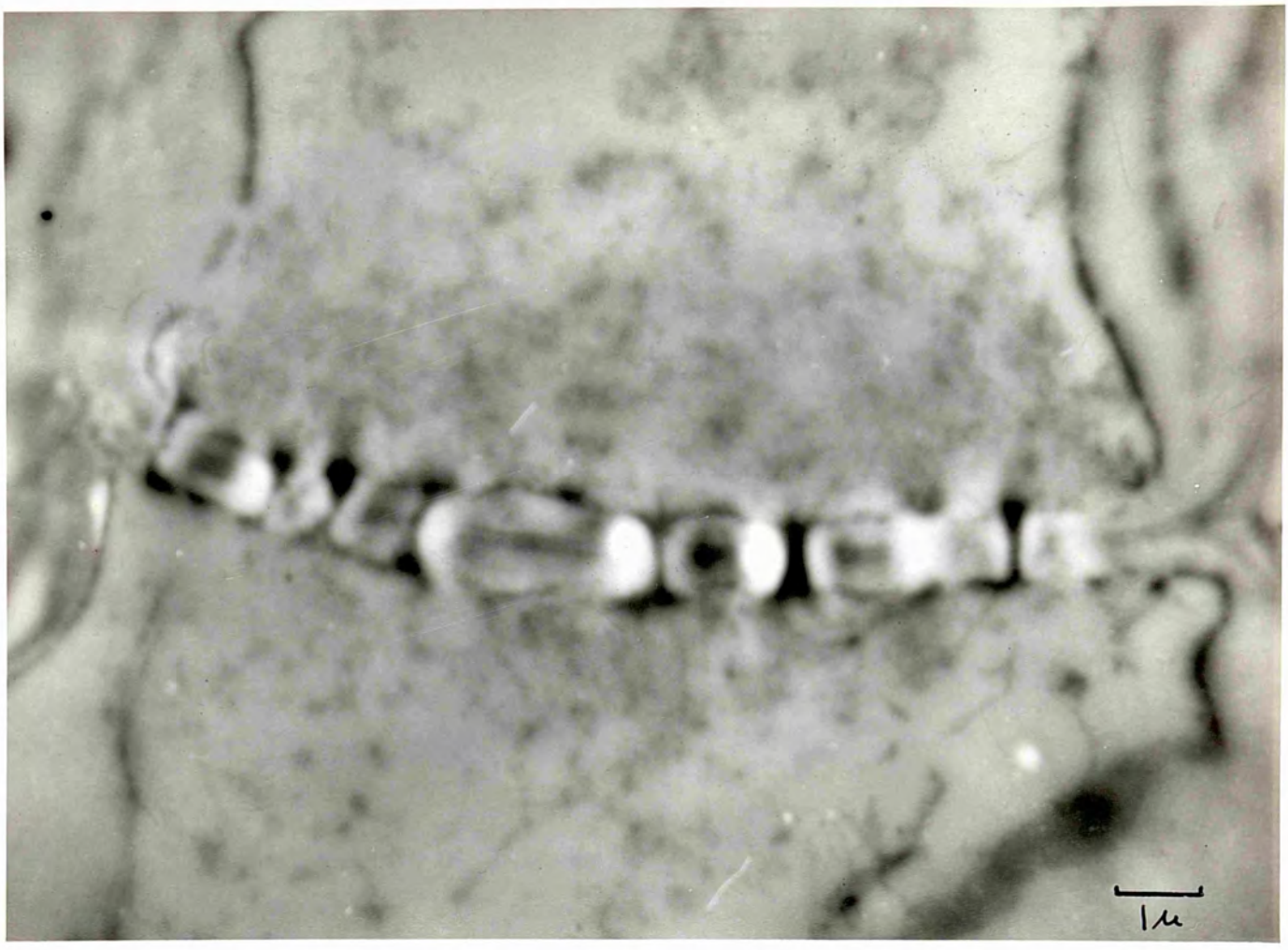

PLATE 30. L.S. Sieve tube showing detailed structure of the sieve plate. Fixed in osmic acid with 1M sucrose.

usual; and so far as one can judge, it shows little evidence of having been in streaming. All these plates show in longtitudinal section mature and presumably functional sieve elements. Since it is difficult to get a sieve-plate in a transverse section by ultramicrotomy, sectioning in longtitudinal direction was used throughout. The results obtained here on the general nature of the sieve-plate and connecting strands confirm the findings of Hepton, Preston and Ripley (1955), Hepton (1957), Preston (1958) and Schumacher and Kollmann (1959) using different plant materials and different preparative techniques.

Passing on now to sieve plates from young sieve tubes, the first one is presented in Plate 31. It shows the same general features. Unfortunately not a single pore is sectional. This is somewhat surprising, though it may be possible that the element is too immature to have developed them. It seems the section has passed rather close to the wall, a part of which appears near the axis on both sides of the plate. An interesting feature of this section is the presence of a large number of inclusions. The larger ones are the plastids each showing several enclosed starch grains. Some of the smaller bodies may be mitochondria, but that is not very clear owing to indifferent fixation and the limitations of the microscope. As is well known, the

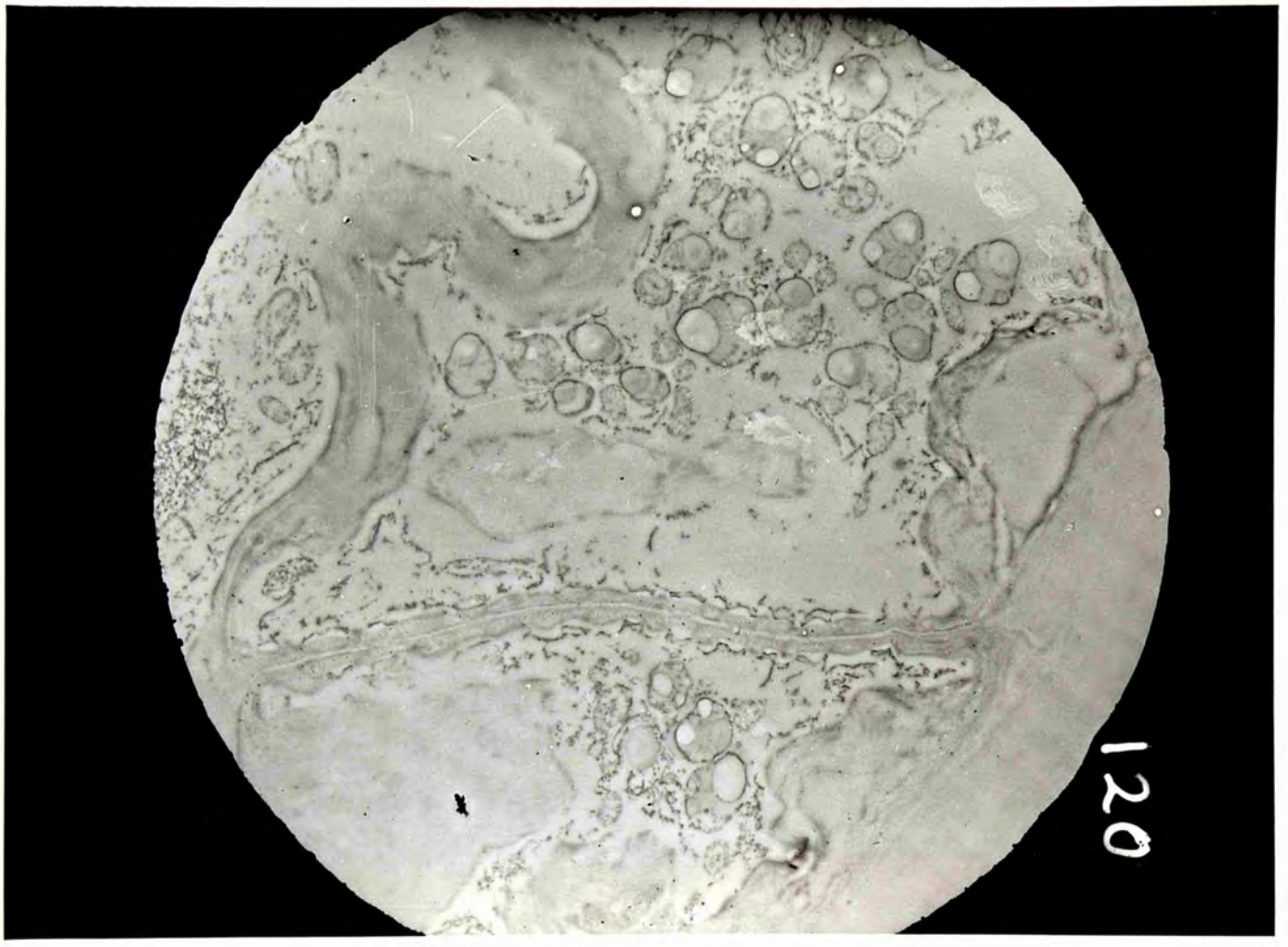

PLATE 31. Young sieve tube showing thick lateral wall, sieve plate, and a number of plastids which show several enclosed starch grains. Note the absence of pores.

X 2

inclusions of the sieve-tube are generally located in the narrow layer of the peripheral cytoplasm, and their presence in such large numbers therefore may be correlated with the fact that the section has passed close to the wall. The next four plates (Plates 32-35) show in serial sections (not consecutive) the features of a sieve plate. The connecting strands show rather different features from what has been presented so far. They are granular rather than fibrillar and show their continuity with the sieve tube cytoplasm. The reason for this difference is not clear, but it may be associated with immaturity or senescence of the element. The conspicuous dark lines passing across the sieve plate in the first three plates seem to be artifacts in spite of the fact that they appear almost in the same situations. They do not resemble the granular connecting strands, do not appear to be encased in callose cylinders, and do not show real continuity with the cytoplasm. Moreover, one of the dark lines in the first plate appears in the region where the lateral wall shows cracks. These dark lines are orientated parallel to the knife edge in the plane of sectioning and may have been formed due to "rucking" of the methacrylate. The last three plates of this series also show a large "reticulate" inclusion on one side of the sieve plate rather similar in appearance to many chloroplasts. The nature of this body will be discussed

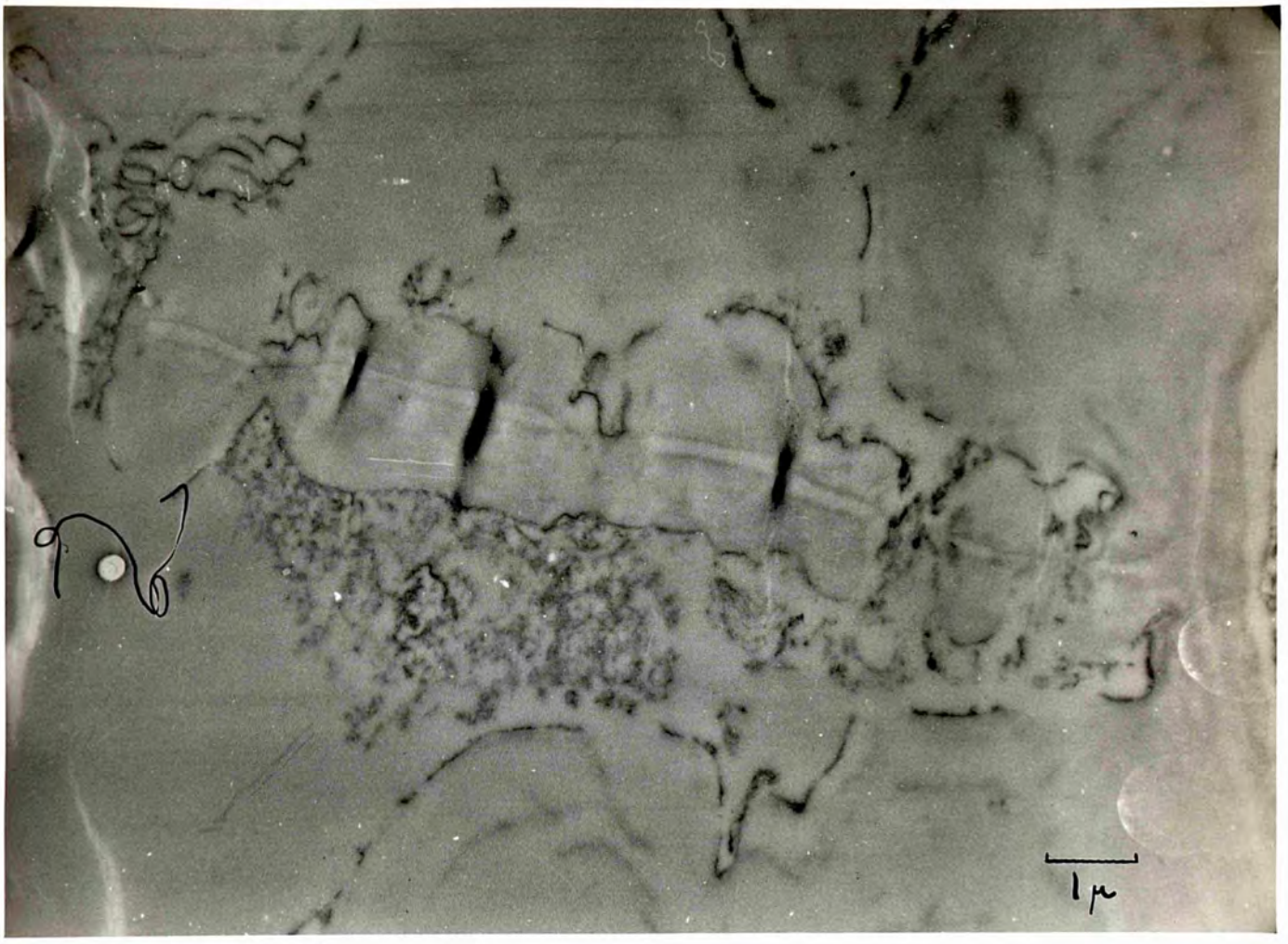

PLATE 32. First of the four serial sections (not consecutive) showing sieve tube from a young petiole. Fixed in osmic acid with 1M sucrose.

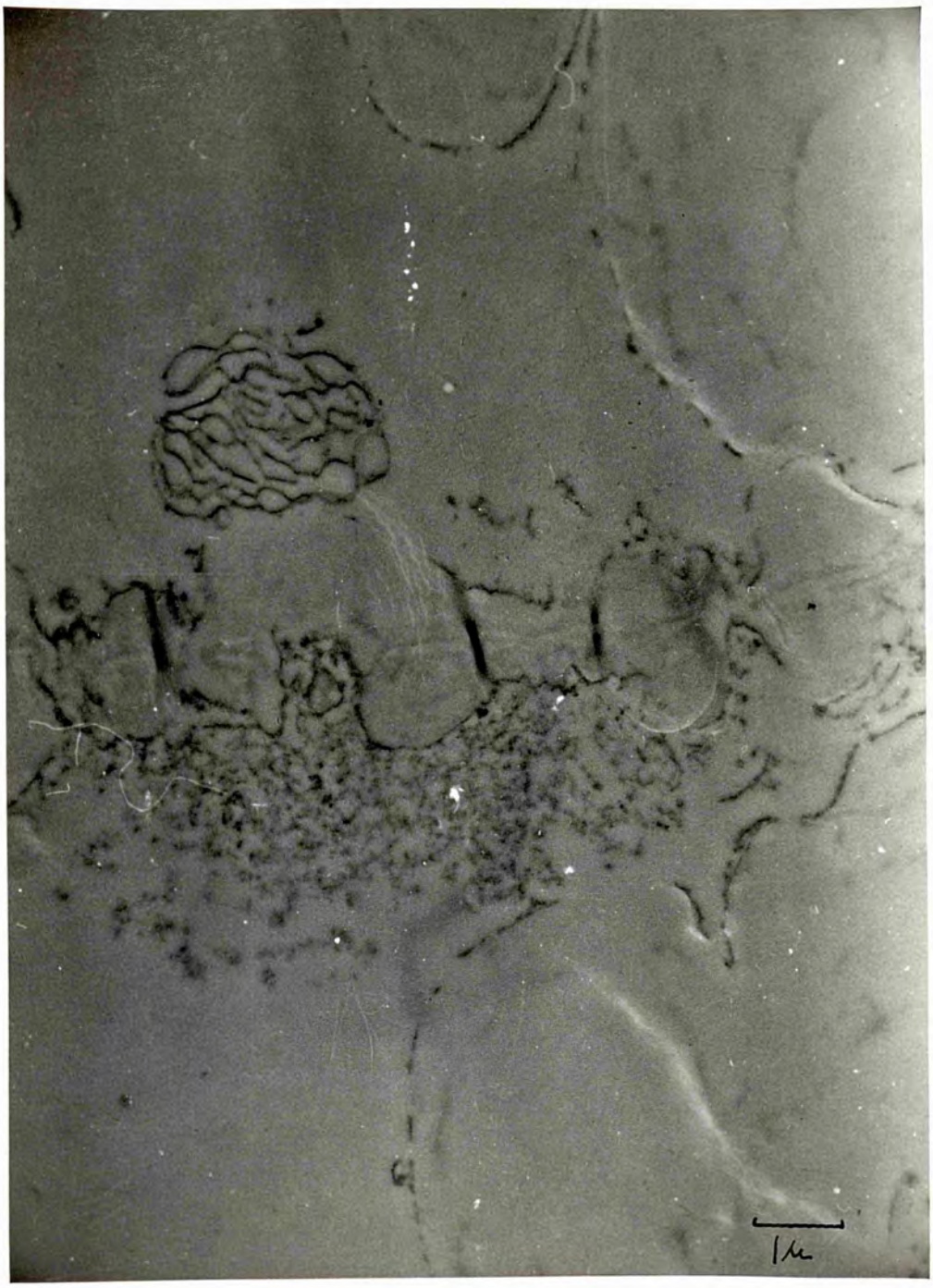

PLATE 33. Second serial section. Note a large "inclusion-like" structure.

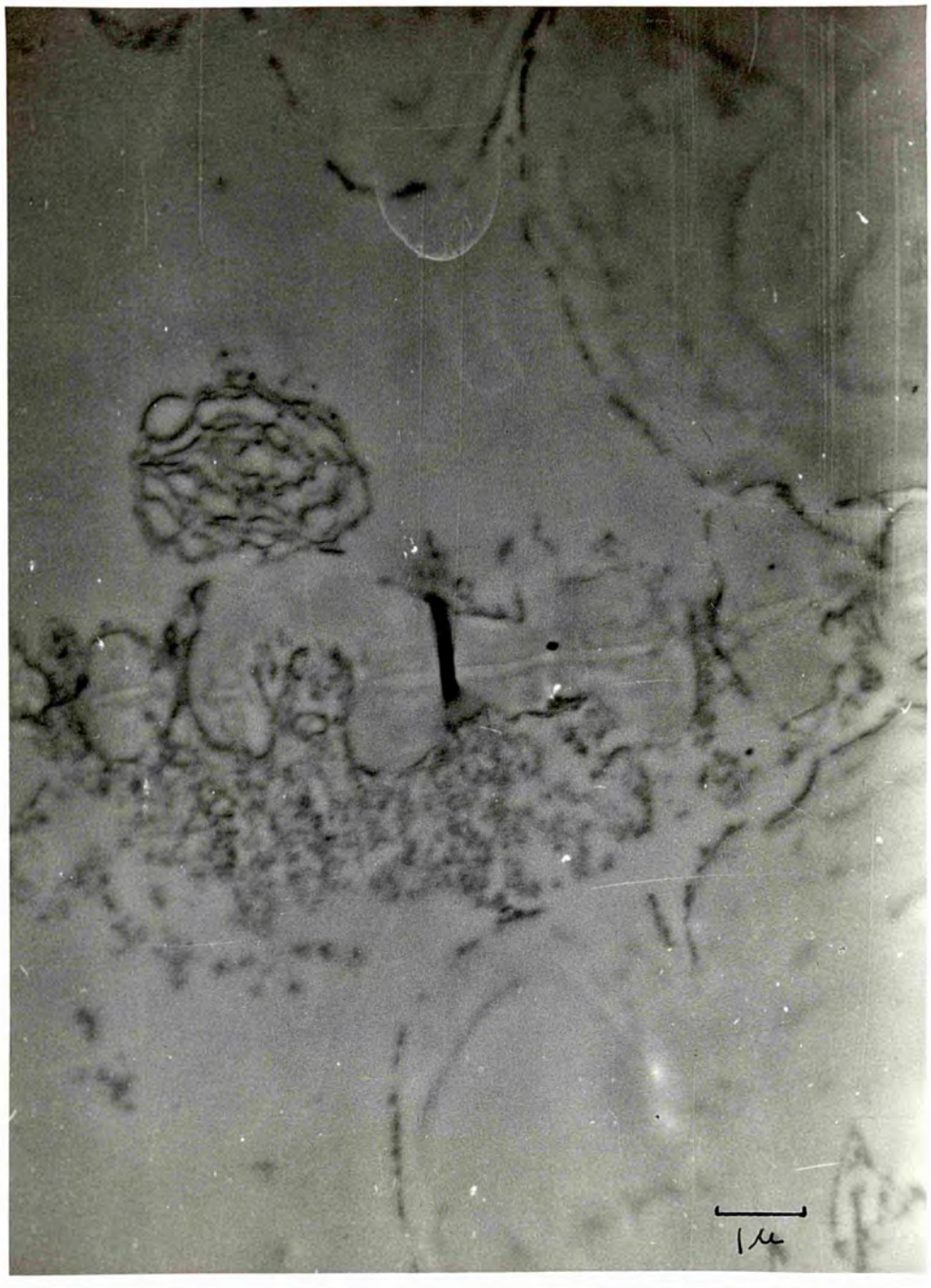

PLATE 34. Third serial section. Note the appearance of the cytoplasm and the connecting strands.

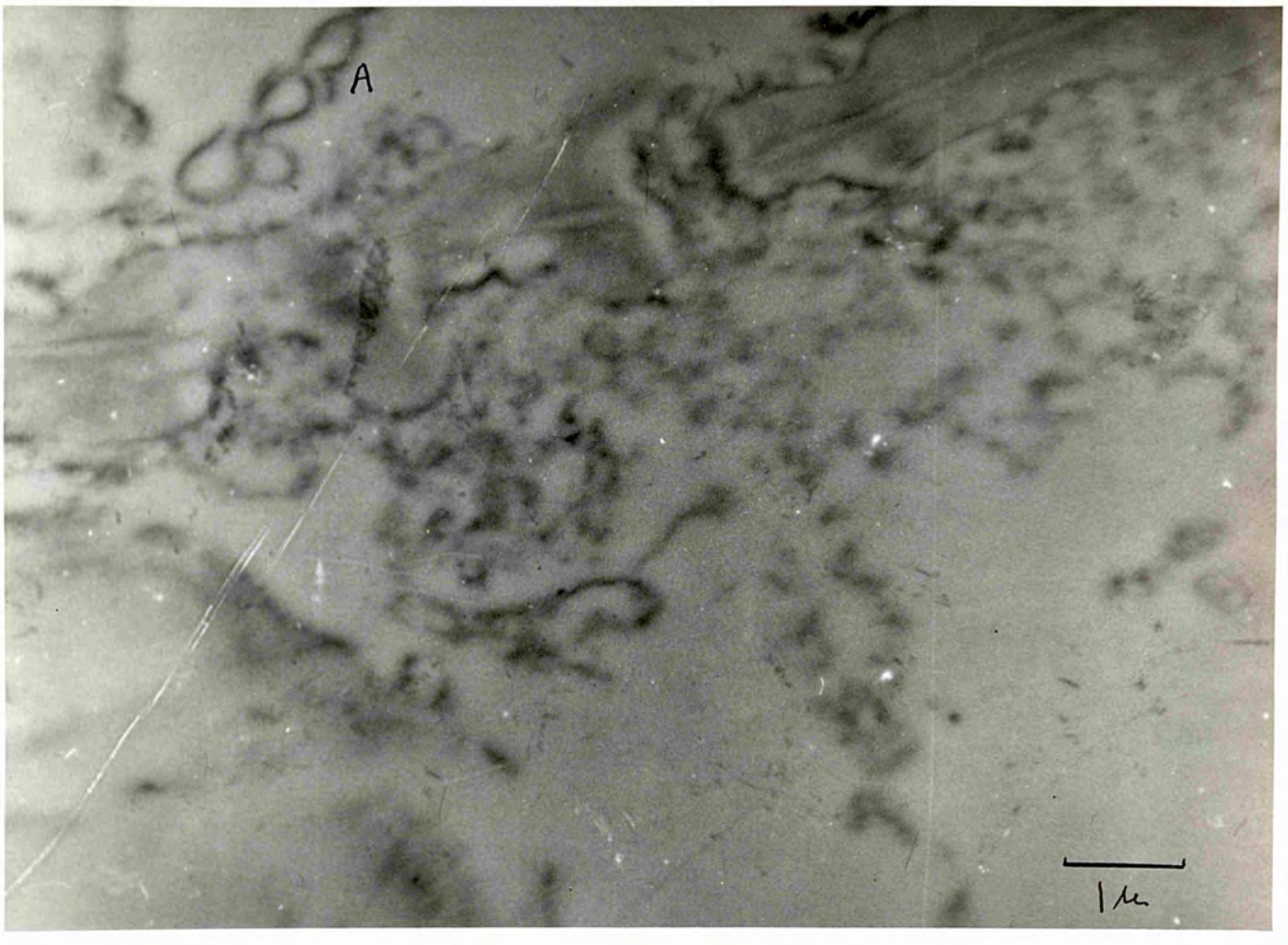

PLATE 35. Last serial section. Note the structure marked A.

in connection with some later photographs taken at much higher resolution with an EM6 microscope. The materials of all these plates were obtained from specimens fixed in 1% OsO_4 with 1M sucrose.

Materials prepared as above, but from sieve-tubes from "senescent" petioles are presented in the next two serial sections (Plates 36 and 37). They show the double dark line in the sieve plate very prominently, the nacreous lateral wall and the granular connecting strands being continuous with the granular fibrillar cytoplasm. There does not seem to be an excessive deposit of callose to indicate cessation of activity by the sieve-tube, but this is hardly to be expected in such a short-lived organ as the petiole of the present subject. These features seem to indicate, as stated earlier, that the sieve-tube is still potentially functional in spite of the fact that it was obtained from the petiole of a senescent leaf. One large body on either side of the sieve-plate can be seen. One of them appears to be of the same nature as that presented in the Plates 33-35. The other might possibly be a large plastid, or alternatively a process from the nacreous wall. Near to it there seem to be several mitochondria.

Before passing on to the results of the specimen preparation according to Schedule 2, a few more micrographs

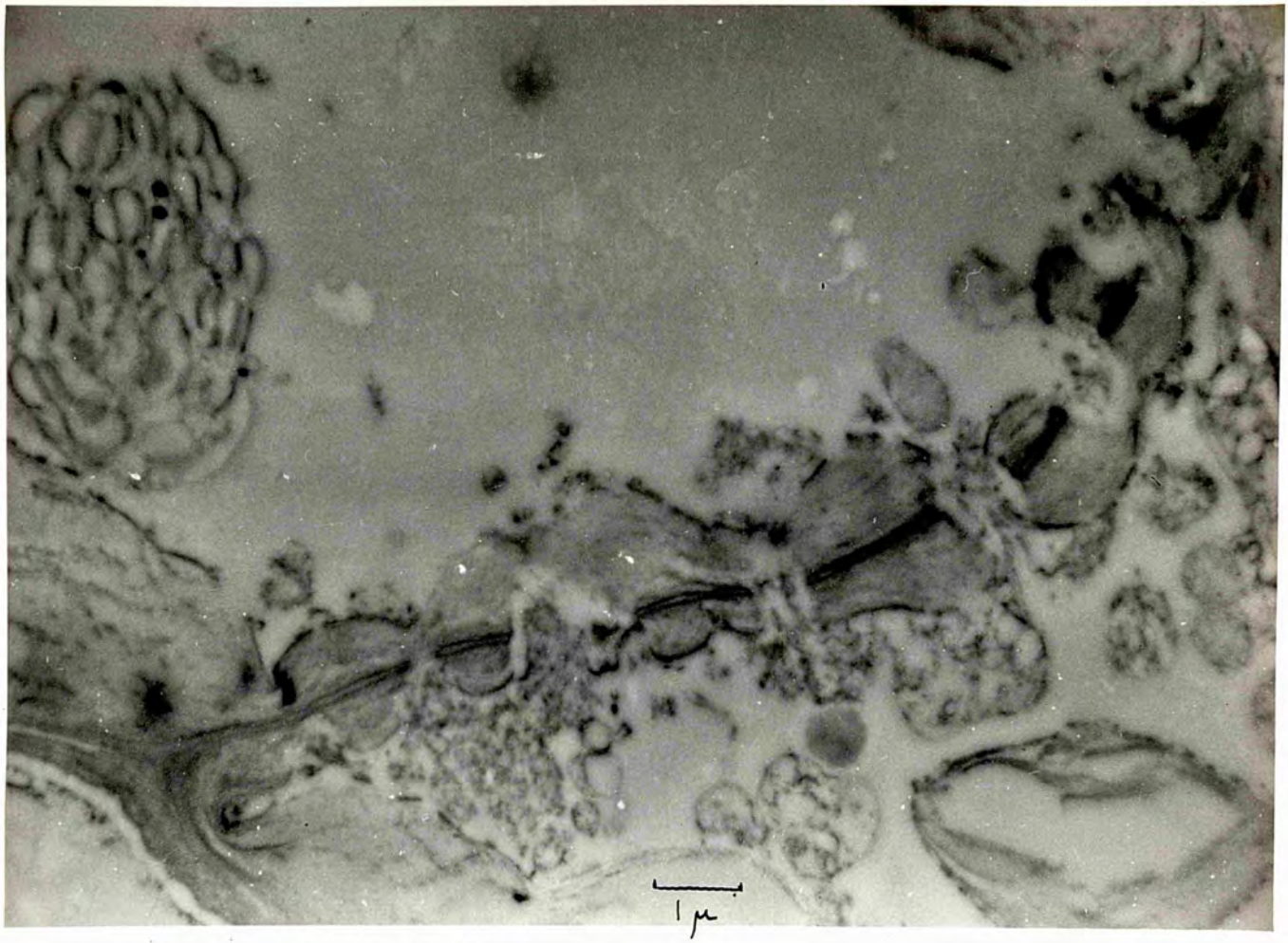

PLATE 36. First of the two serial sections showing a sieve tube from a senescent petiole. Note one large inclusion on either side of the sieve plate. Fixed in osmic acid with 1M sucrose.

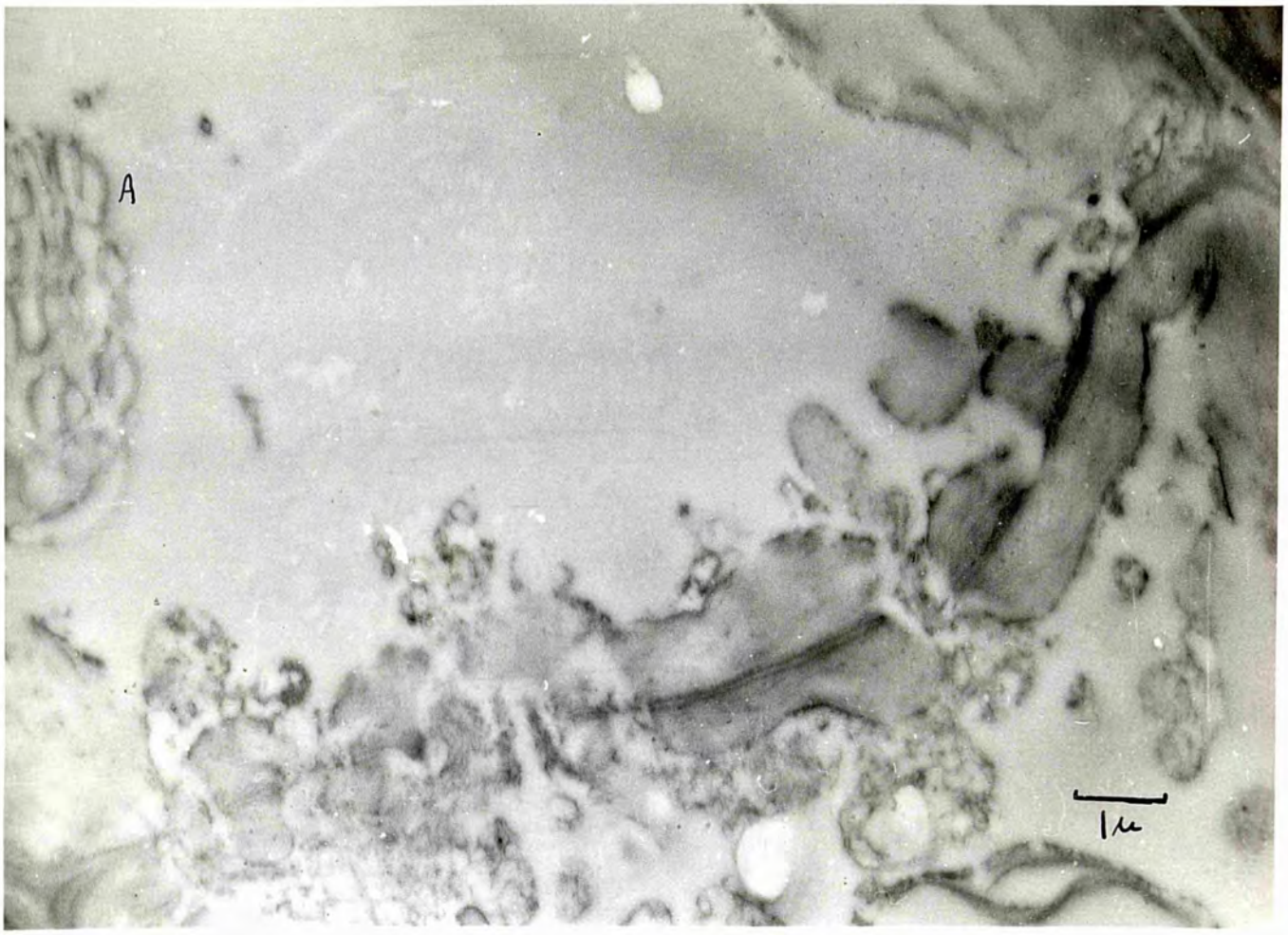

PLATE 37. Second serial section. Note the double membrane nature of the structure marked A.

of mature sieve-tubes are presented to complete the overall picture of the specimen preparation according to Schedule 1. So far only selected micrographs have been described. Plate 38 shows a sieve-tube with sieve plate. The section appears to be very off centre. The sieve plate shows the same features except for the connecting strands, thus giving the impression of vacuolar continuity across the sieve-plate. It seems this sieve-plate was situated on one of the extreme ends of the 0.5-1mm. thick petiole piece used in fixation, and the double cutting method could not neutralise the sudden release of the turgor pressure. This might have blown away the connecting strands, or alternatively osmotic disequilibrium may have forced the cytoplasm towards the cell axis. This micrograph shows the three types of cells found in the meta-phloem as mentioned in connection with the optical Plate 3, namely the sieve-tube, companion cell and parenchyma on its right and left respectively. The preservation of the cytoplasm is certainly unsatisfactory. The material of this micrograph was obtained from the specimen fixed in the fixative without sucrose. The next plate (Plate 39) shows a sieve-tube fixed in 1% OsO_4 with 0.5M sucrose. It resembles very closely the micrograph of the Plate 38 in its absence of the connecting strands. The reason for the absence of the connecting strands could be explained on the basis of the

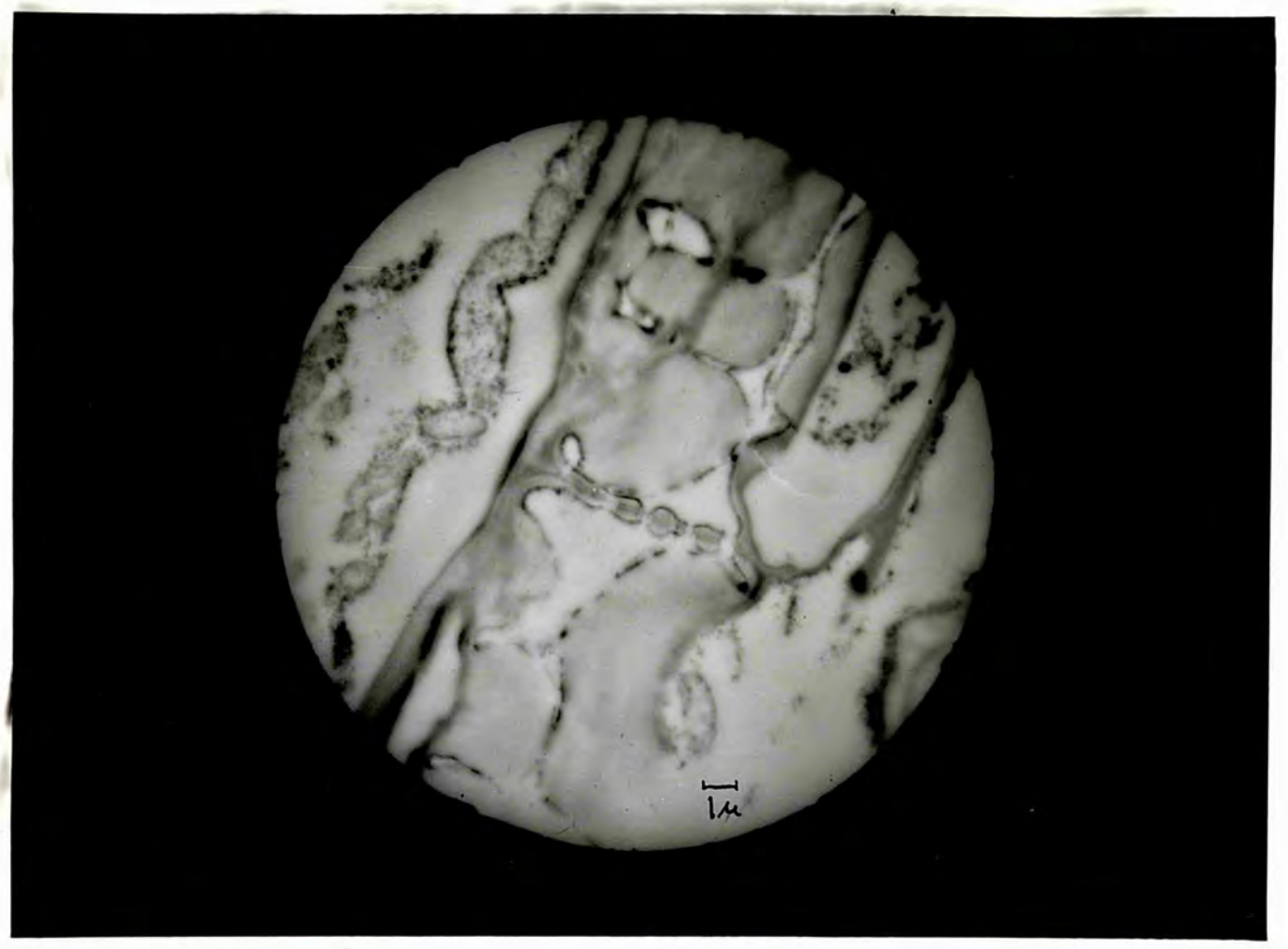

PLATE 38. L.S. Sieve tube with a phloem parenchyma and a companion cell on its left and right respectively. Pores are devoid of contents. Section seems to be very off centre. Fixed in osmic acid without sucrose.

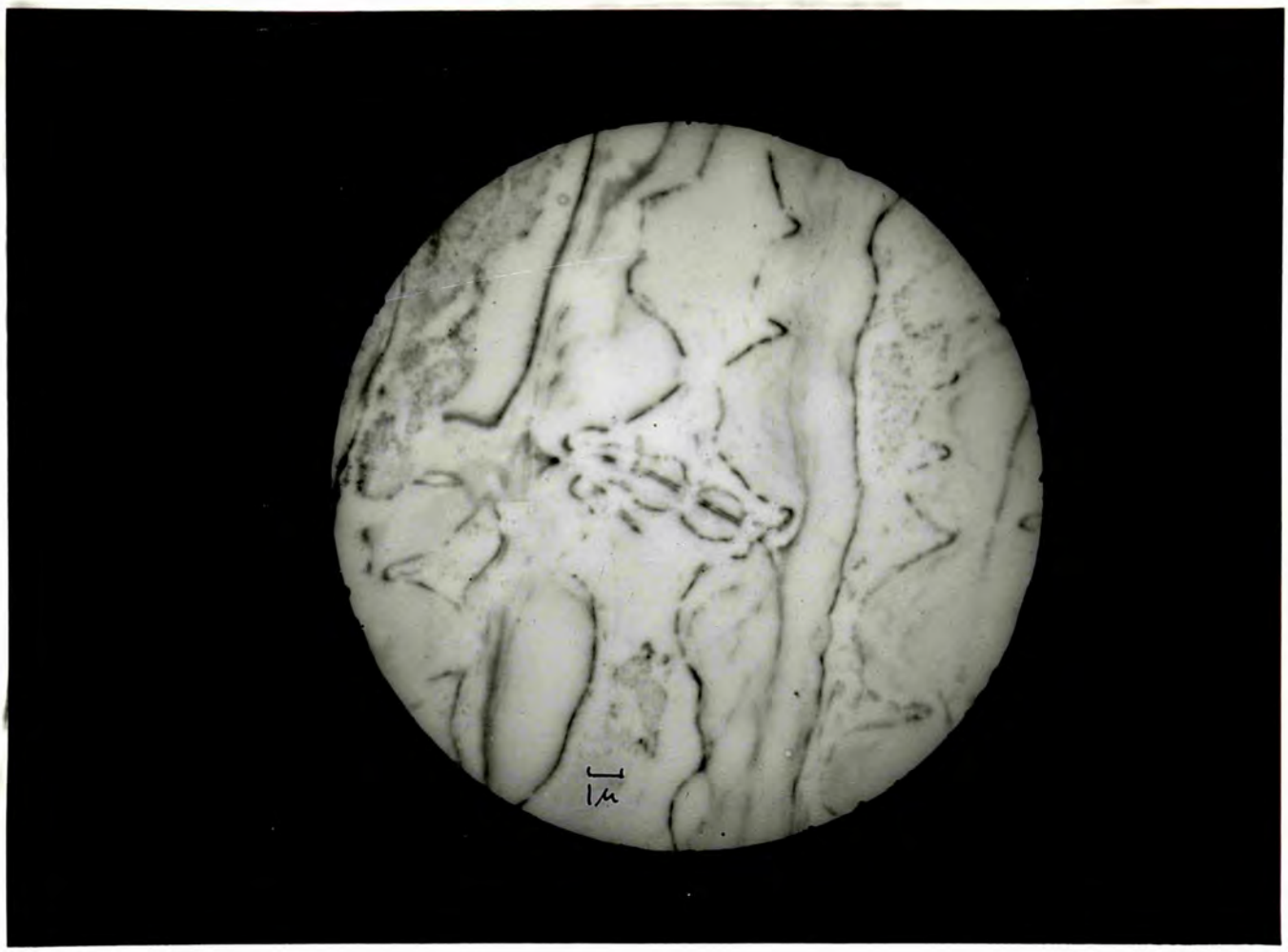

PLATE 39. L.S. of sieve tube with a sieve plate. The sieve pores are devoid of their contents. Fixed in osmic acid with 0.5M sucrose.

arguments advanced for the Plate 38. Alternatively, the section might have passed through the callose cylinder only. That this might also be a possibility is suggested from the Plate 40, which is a higher magnification of the previous plate. In the regions of the pores surface cytoplasm seems continuous and not broken. The dotted appearance of the surface cytoplasm is very regular, and in the light of some of the higher resolution micrographs to be shown later this would seem to be not altogether an artifact. In the last micrograph of this series, of a sieve-tube fixed in the fixative containing 1M sucrose, shown in Plate 41, some of the pores show the dark connecting strands across the sieve plate while others are devoid of it. It would seem that this sieve plate was further inside from the end of the 0.5-1mm. thick petiole piece used in fixation. From these it would appear that so far the double cutting method of Schedule 1 is concerned only the central-most sieve plates of 0.5-1mm. thick petiole pieces would be suitable for electron microscopic investigation. This might account for good preservation of the sieve plates presented in Plates 26 and 30, and bad preservation of the sieve plate of the micrograph 28. Addition of sucrose to the fixative does not seem to improve fixation much in the present study.

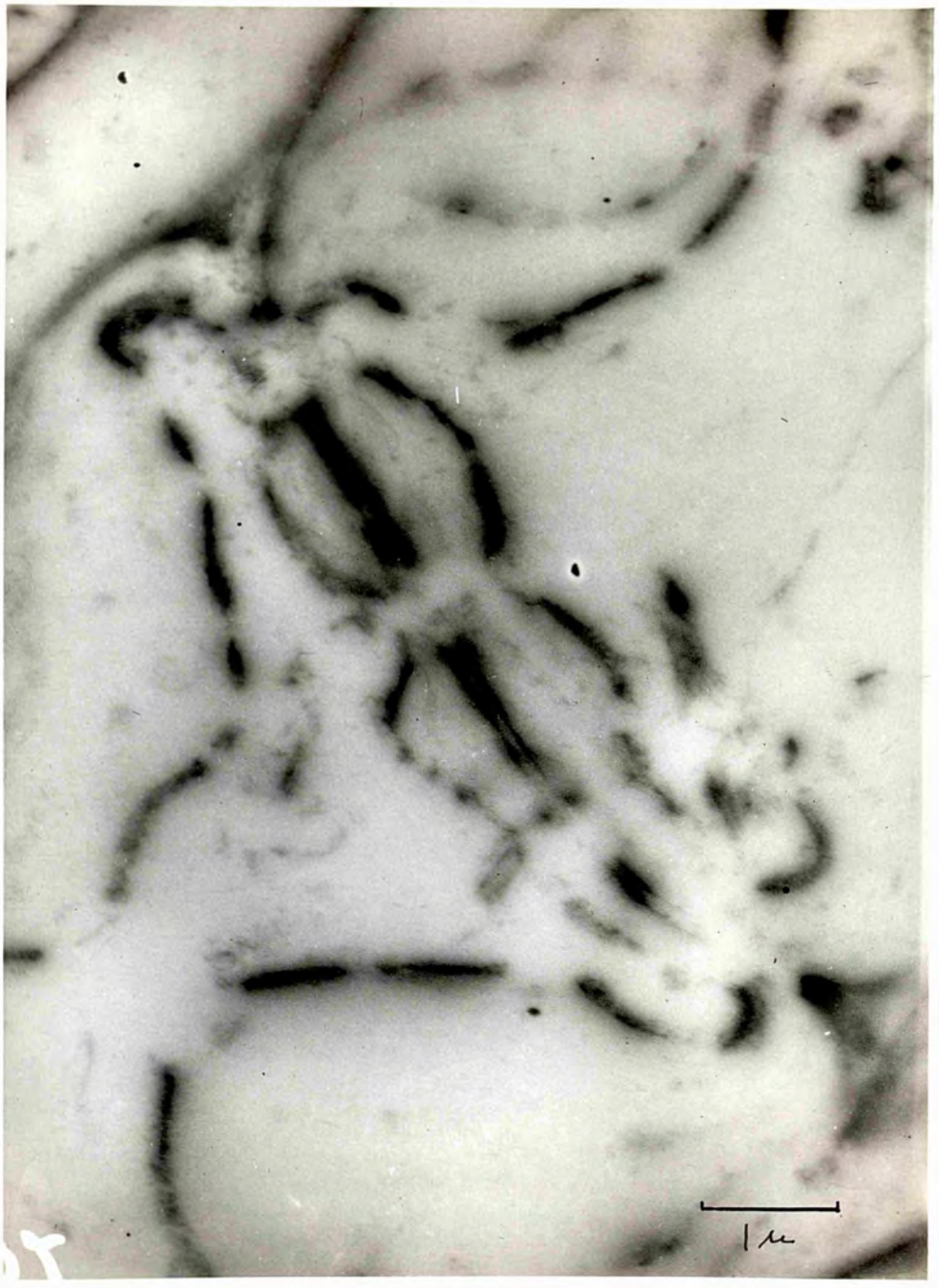

PLATE 40. Higher magnification of Plate 39. The pores seem to be covered over by the surface cytoplasm. Note the "dotted-line" appearance of the inner limit of the nacreous wall.

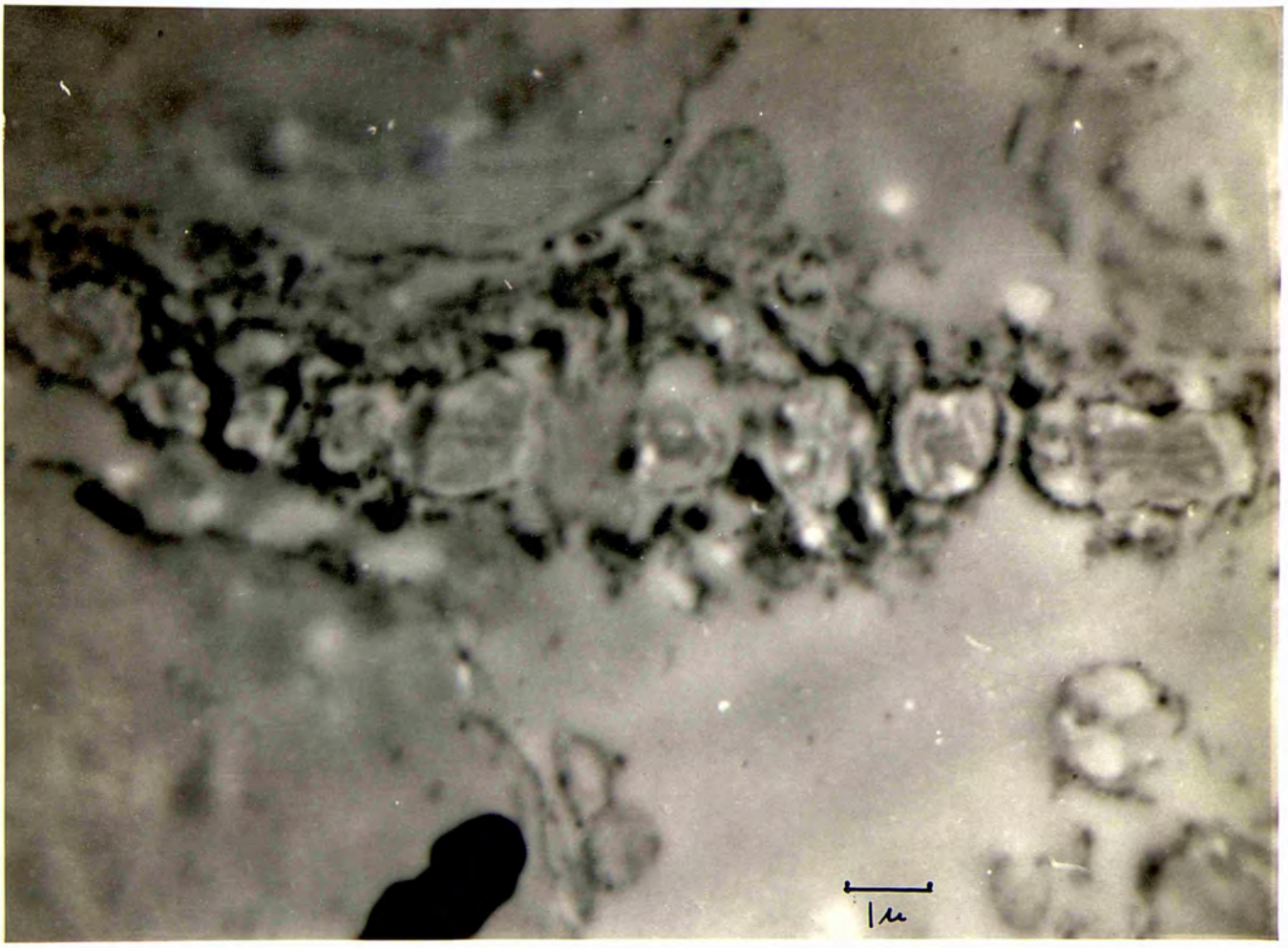

PLATE 41. L.S. Sieve tube with sieve plate.
Preparation is not very good. Fixed in osmic acid with 1M sucrose.

Now the result of the preparative technique according to Schedule 2 will be described. It can be mentioned, however, at once that this method yielded the best result of all the three techniques tried in the present investigation. This is reflected not only in the better fixation of the sieve-tubes themselves but also in the general preservation of almost all the cell components. Further, this method also gave the largest number of sieve plates with connecting strands; in fact a sieve plate without connecting strands could not be found. Use of 1% phosphotungstic acid and 0.5% uranyl acetate as electron stains increased the overall contrast which was not only pleasing to the eye but also brought out plasmodesmata more distinctly. It may, however, be pointed out that slightly less staining would have given still better results. The usefulness of this technique can be emphasised by stating that it was used at a time when the plant was not growing really satisfactorily.

Since the structure of the sieve plate has been fairly fully described earlier, only special features of the micrographs to be presented now will be described. A large number of micrographs will now be described so that a more complete picture of the sieve-tube could be obtained. Plate 42 shows three connecting strands, two of which extend from one side of the sieve plate to the other. The branching nature of

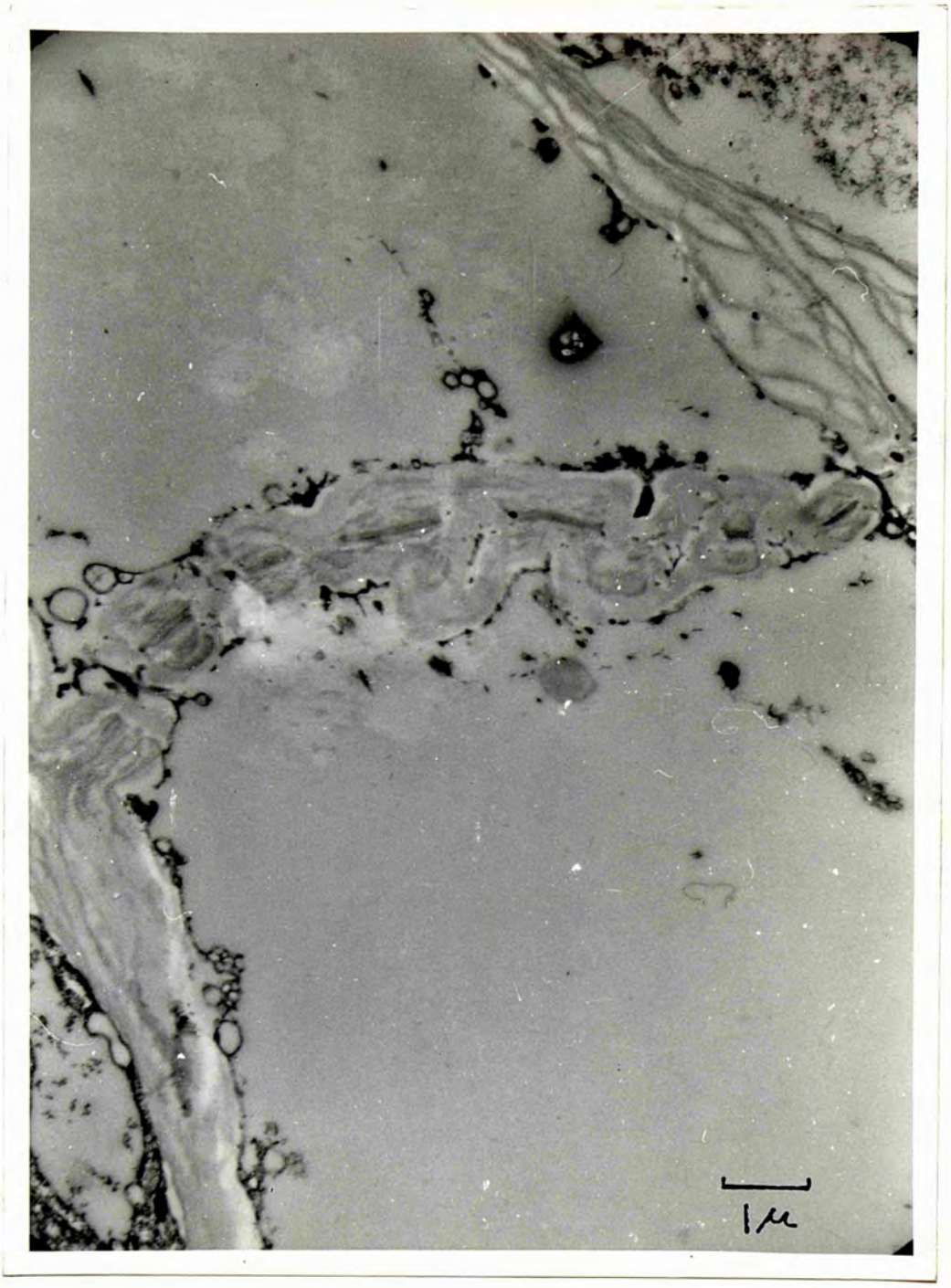

PLATE 42. Sieve plate showing three connecting strands. The two strands towards the lateral walls are continuous from one side of the sieve plate to the other and show branching tendency. Fixed according to Schedule 2.

these two strands is quite distinct. In the next three plates (Plates 43, 44 and 45) are presented the serial sections (not consecutive) of the two adjacent sieve-tubes with sieve plates and a companion cell, a part of which can be seen in Plate 44. The nacreous wall shows good preservation of its structure. The last two micrographs also show a structure which could possibly be interpreted as a tonoplast. A somewhat similar structure has been considered as a tonoplast by Schumacher and Kollmann (1959). It may, nevertheless, be pointed out that small circular bodies in Plate 45 seem to lie without a "cover" on the side walls, a feature which would hardly be expected if a tonoplast were present. In this last plate, the sieve tube with the so-called tonoplast shows a number of released starch grains on one side of the sieve plate. They are circular in outline and show a clear centre. The section of one of the sieve tubes in these plates is very off centre and in this one of the sieve-element shows the "overhang" of the nacreous wall.

Another sieve tube with sieve plate in serial sections (not consecutive) is presented in the Plates 46-49. The sieve plate shows many strands which reveal their continuity with the cytoplasm in one of the sieve-tube elements. If it is assumed that the section is close to the wall, the

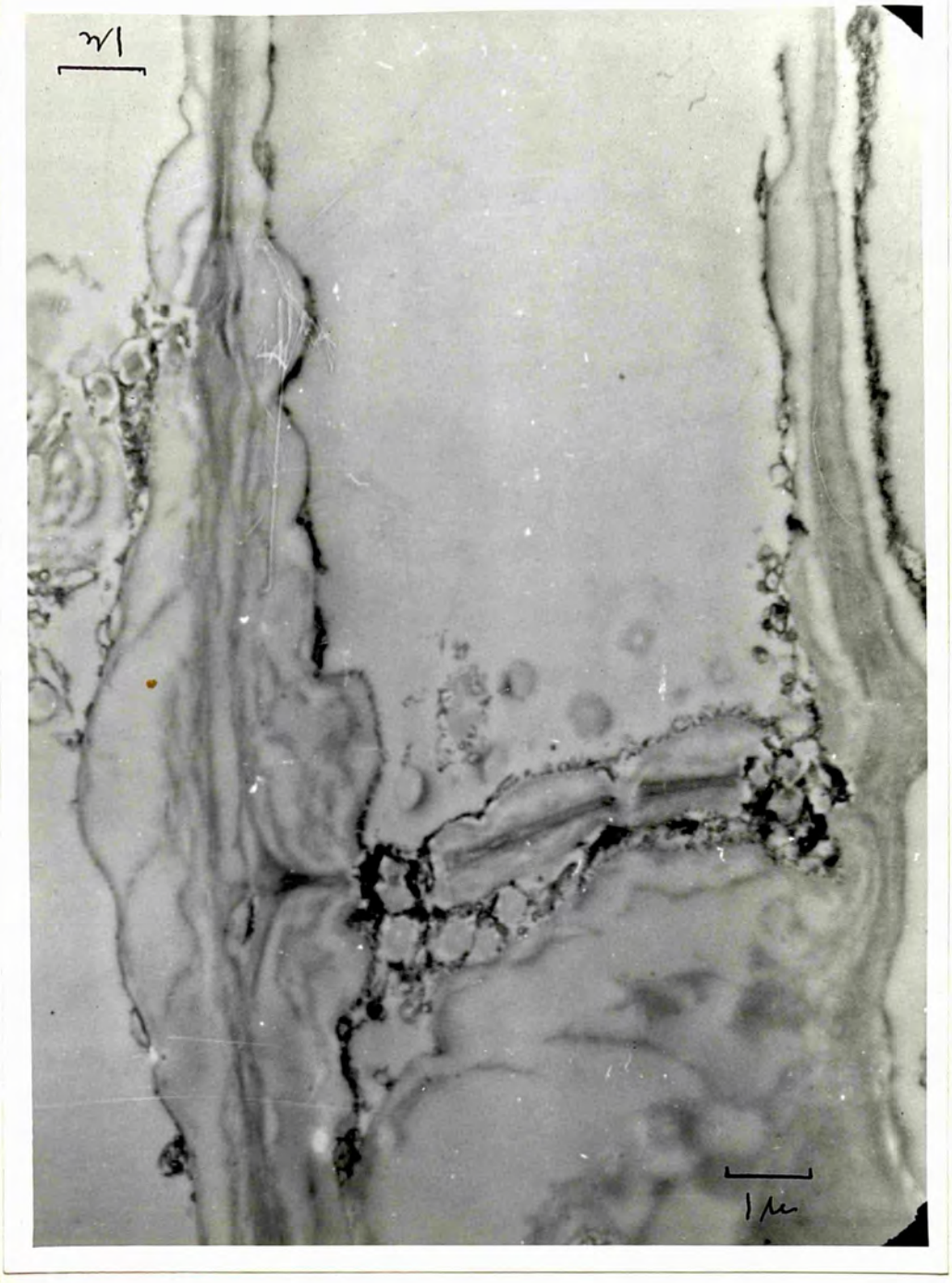

PLATE 43. First serial section. It shows two sieve tubes with their sieve plates and one companion cell. Note the wall structure.

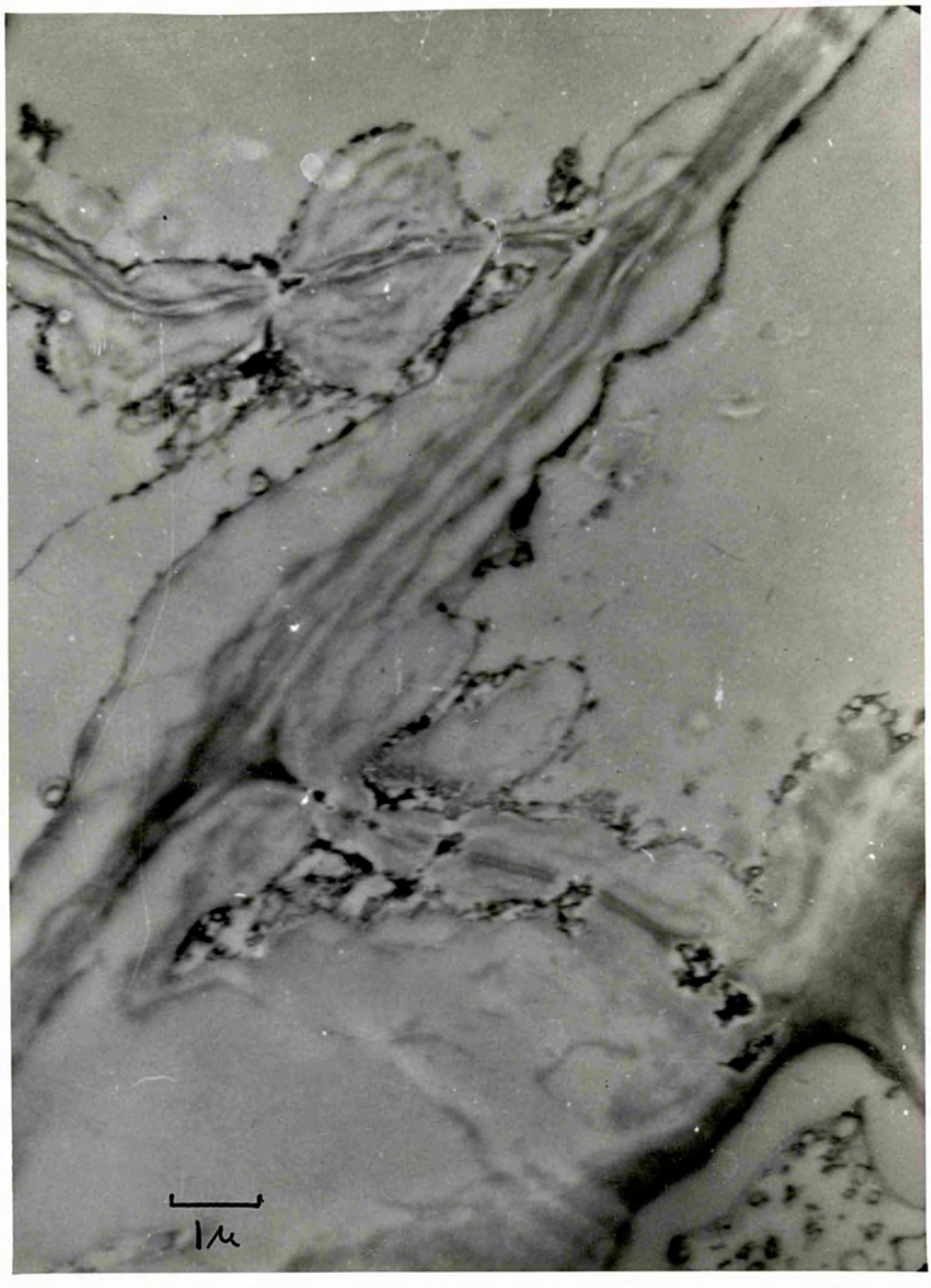

PLATE 44. Second serial section. Note a part of the companion cell and the "overhang" of the nacreous wall in one of the sieve elements.

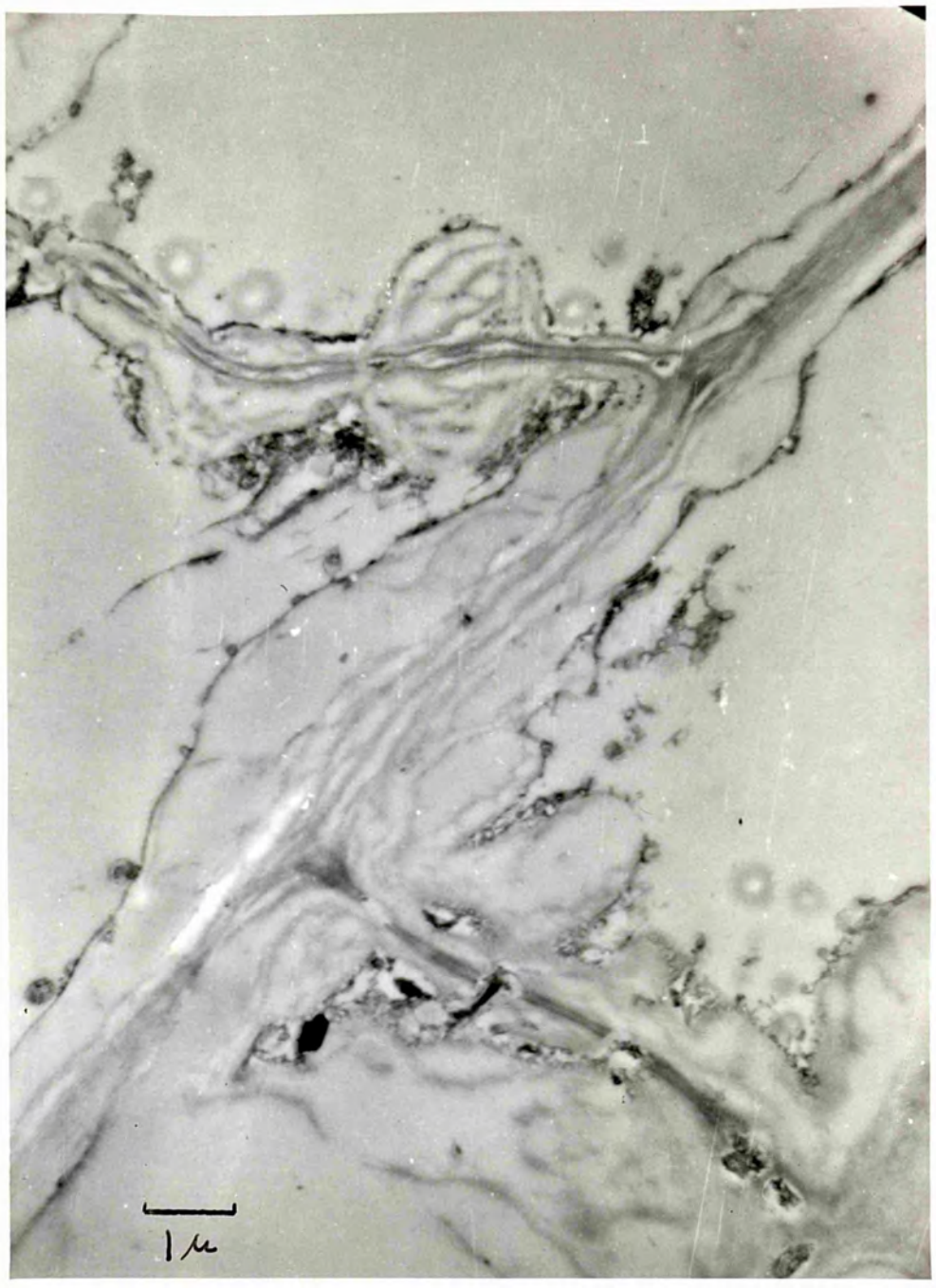

PLATE 45. Third and the last serial section. One of the sieve elements shows a "tonoplast-like" structure.

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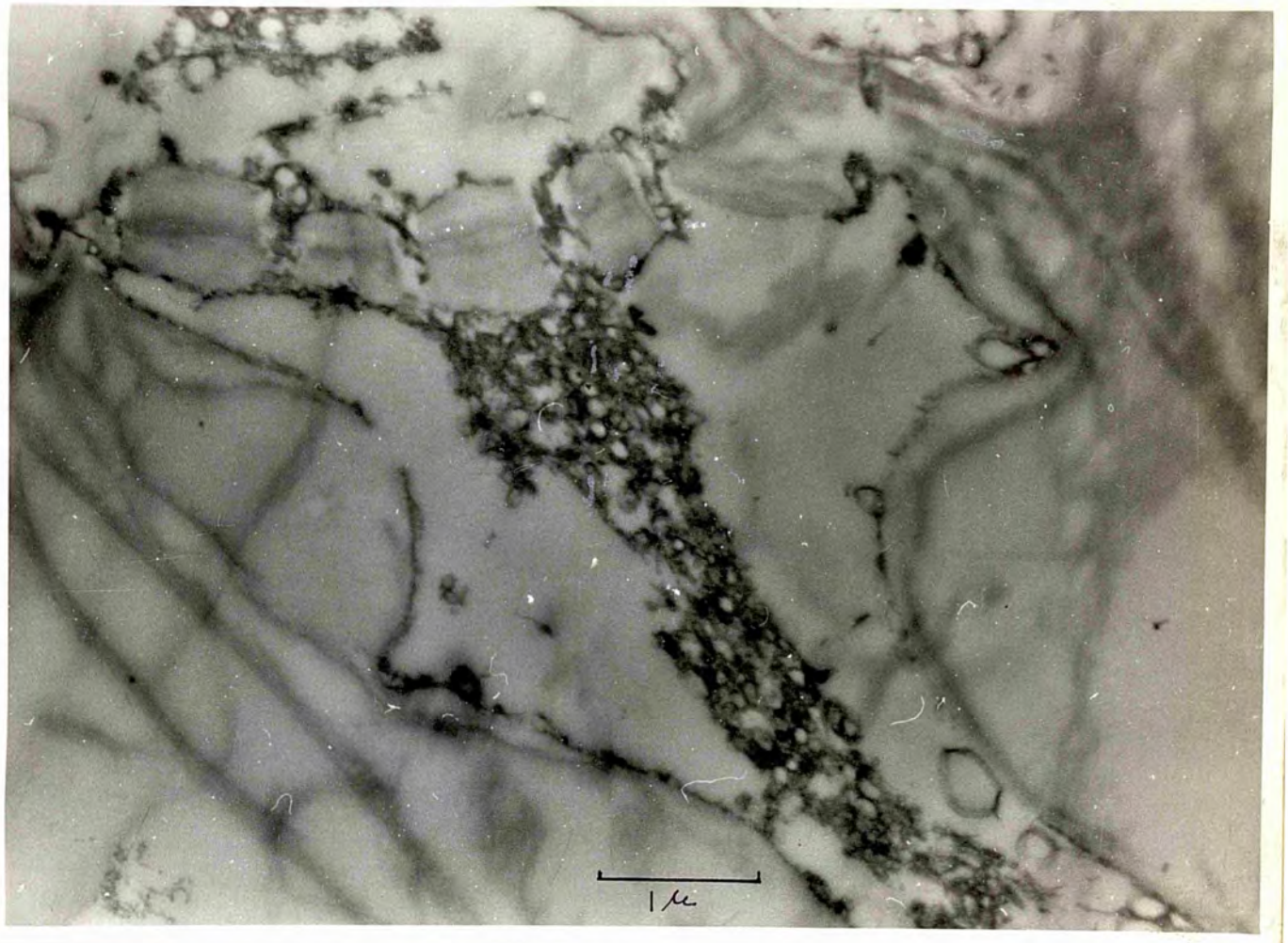

PLATE 46. First of the four serial sections (not consecutive) of the sieve tube with a part of its companion cell. A connecting strand between the sieve tube and the companion cell can be seen.

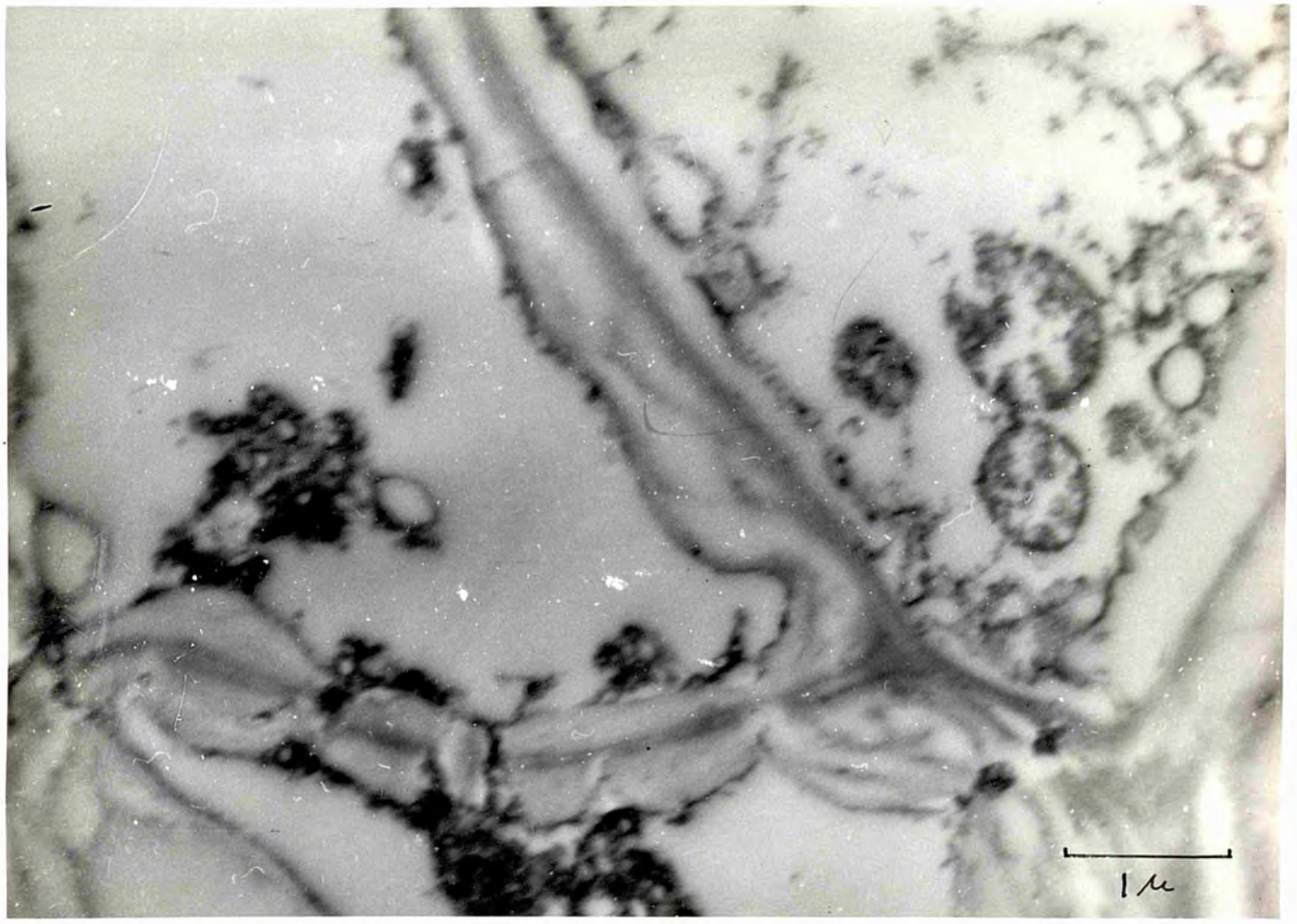

PLATE 47. Second of the four serial sections. Note the connecting strand between the sieve tube and its companion cell which shows two mitochondria.

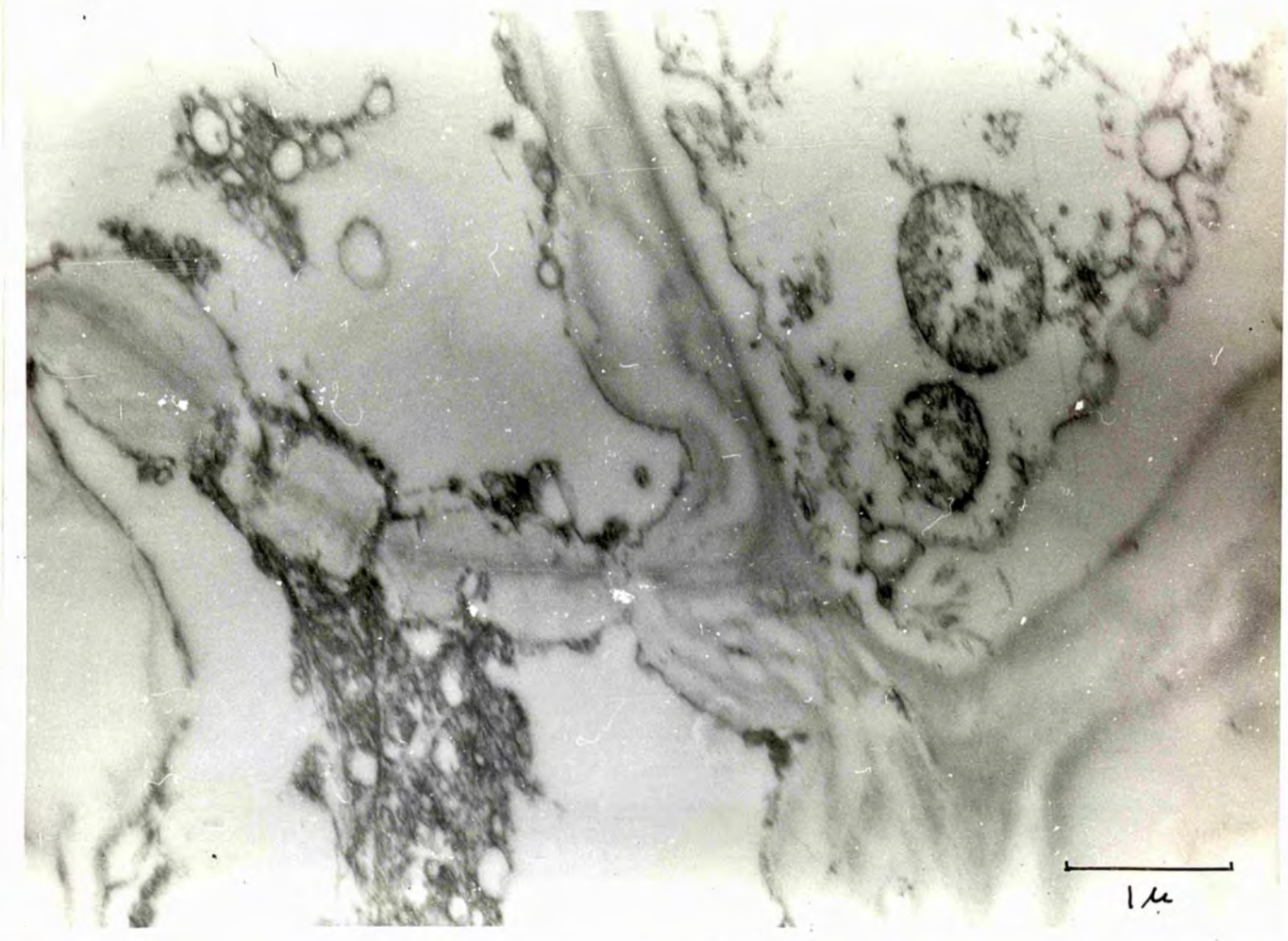

PLATE 48. Third of the four serial sections. Note the appearance of the cytoplasm which is continuous with the connecting strands of the sieve plate. The mitochondria of the companion cell shows the double-membrane nature its cristae.

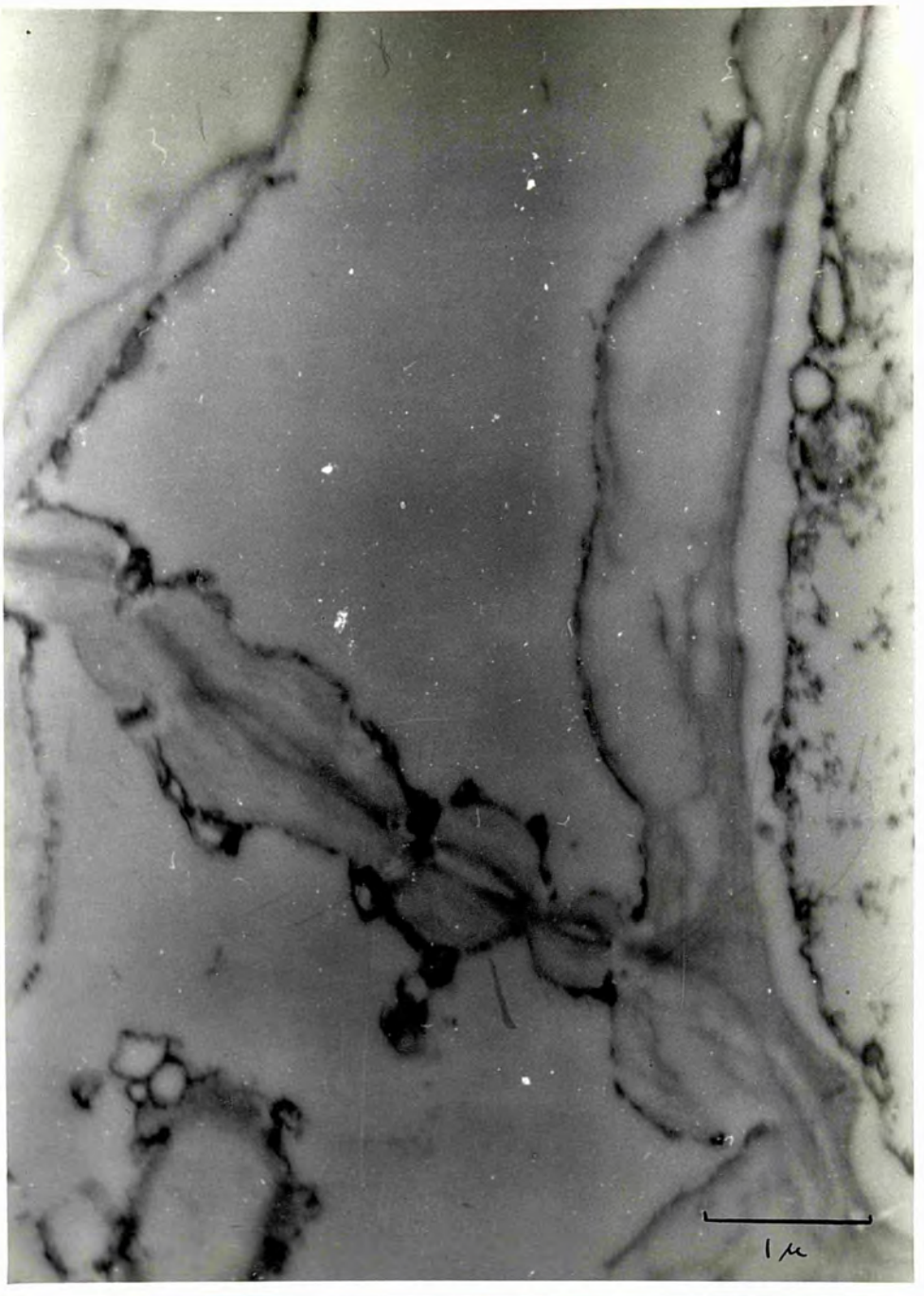

PLATE 49. Last of the four serial sections. Note a part of the nacreous wall in the lumen of the sieve element in the region occupied by cytoplasm in the first three micrographs. This shows the sections were closed to the wall.

presence of this "peripheral" cytoplasm could be explained. The cytoplasm appears to be granular-reticulate in nature and appears similar to the cytoplasm of a parenchyma cell shown in Plate 50. The reticulate nature of the cytoplasm seems also to be reflected in some of the connecting strands. If a longtitudinal section through this cytoplasm is cut a "dotted line" appearance as mentioned earlier might conceivably be obtained. The companion cell of this sieve tube can also be seen and shows the mitochondria which appear better preserved than those fixed at pH5.8 shown in Plate 14. There seems to be a cytoplasmic connection between thesieve tube and its companion cell near the junction of the sieve plate and lateral wall. The peripheral nature of the sieve-tube cytoplasm is rather more clearly seen in the Plates 51 and 52 showing two sections out of a series of five of the sieve tube lumen. The plates show the disposition of the reticulate cytoplasm around the periphery of a hump of the nacreous wall projecting into the sieve tube lumen.

A sieve plate from the sieve tube shown in Plates 51 and 52 is presented in five serial sections (Plates 53-57). These plates have been included to show, besides the general nature of the sieve plate, the definite tendency of the connecting strands to branch. The connecting strands appear

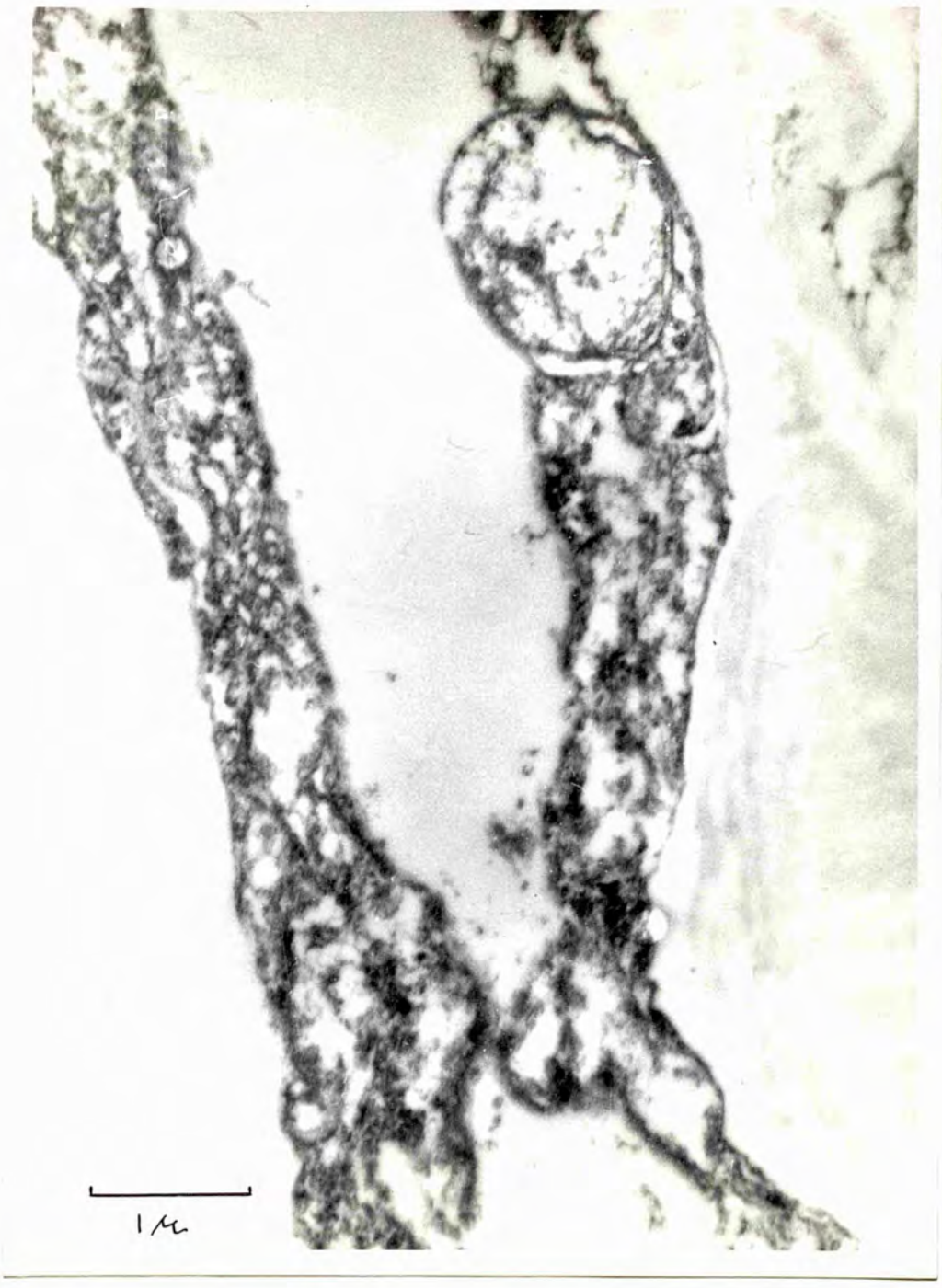

PLATE 50. Cytoplasm in a parenchyma cell for comparison with that of the sieve tube presented in Plates 46-49.

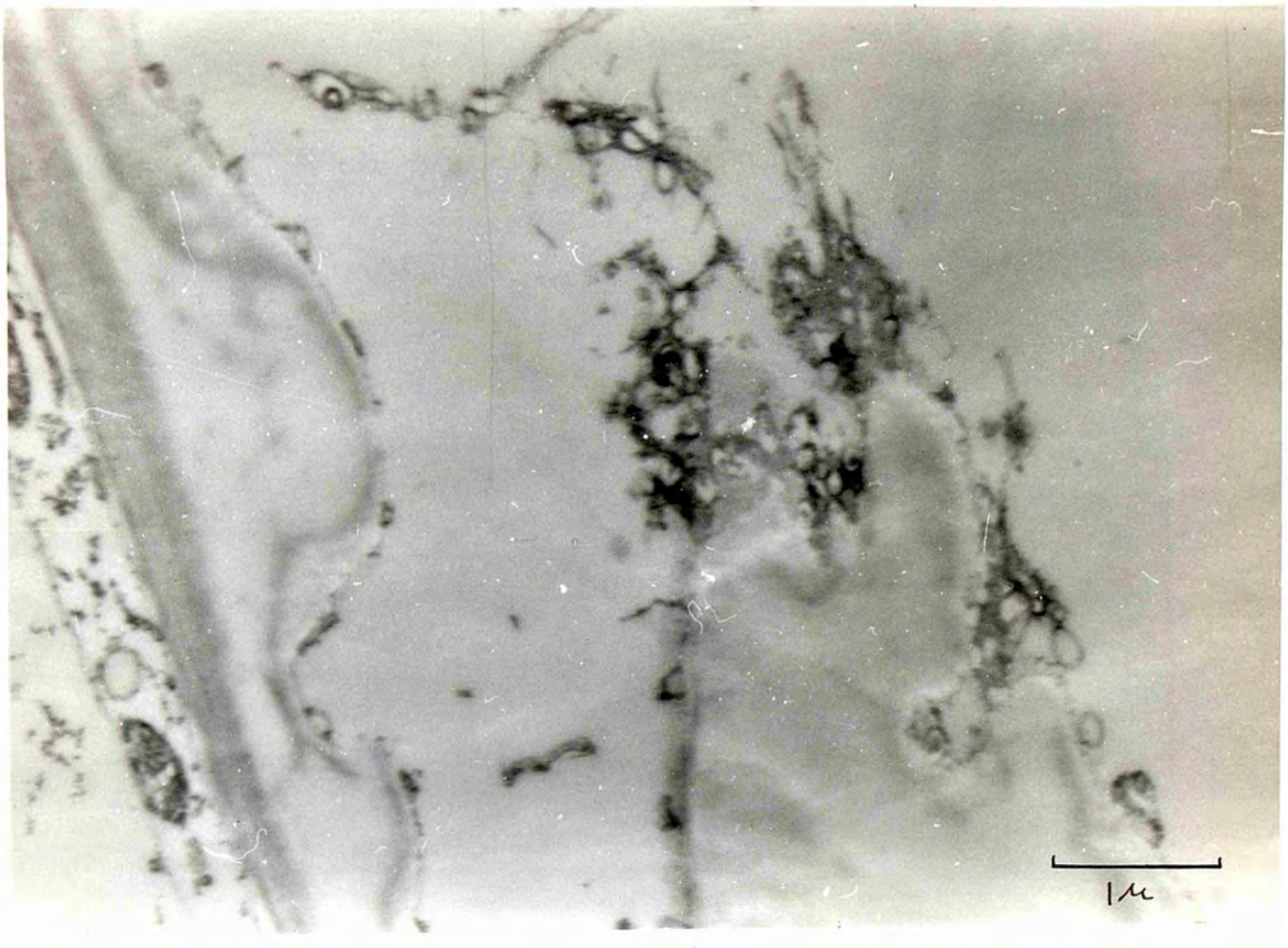

PLATE 51. Sieve tube with a hump of the nacreous wall projecting into the lumen. Peripheral cytoplasm appears around the hump.

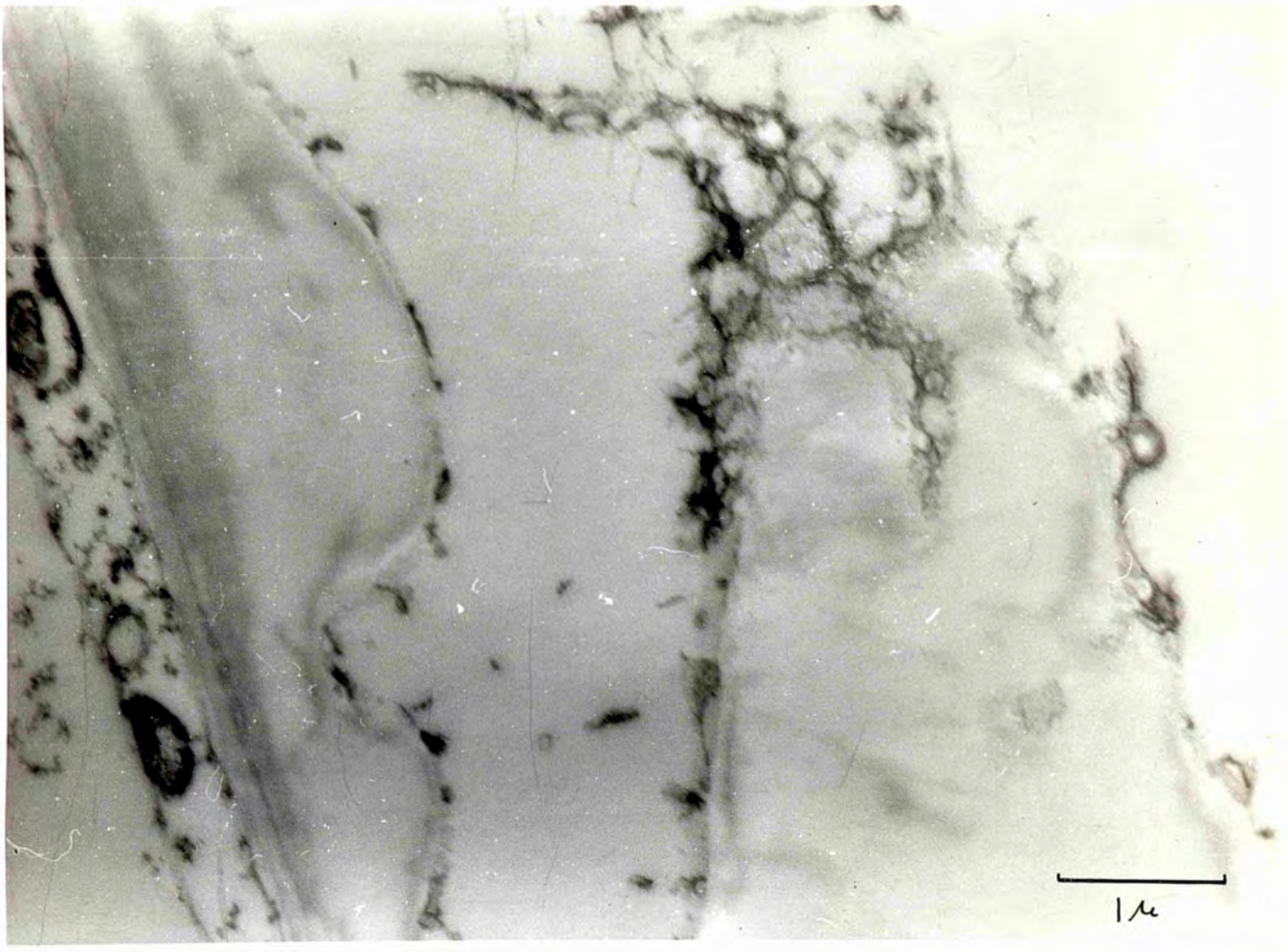

PLATE 52. Second serial section of the sieve tube presented in Plate 51.

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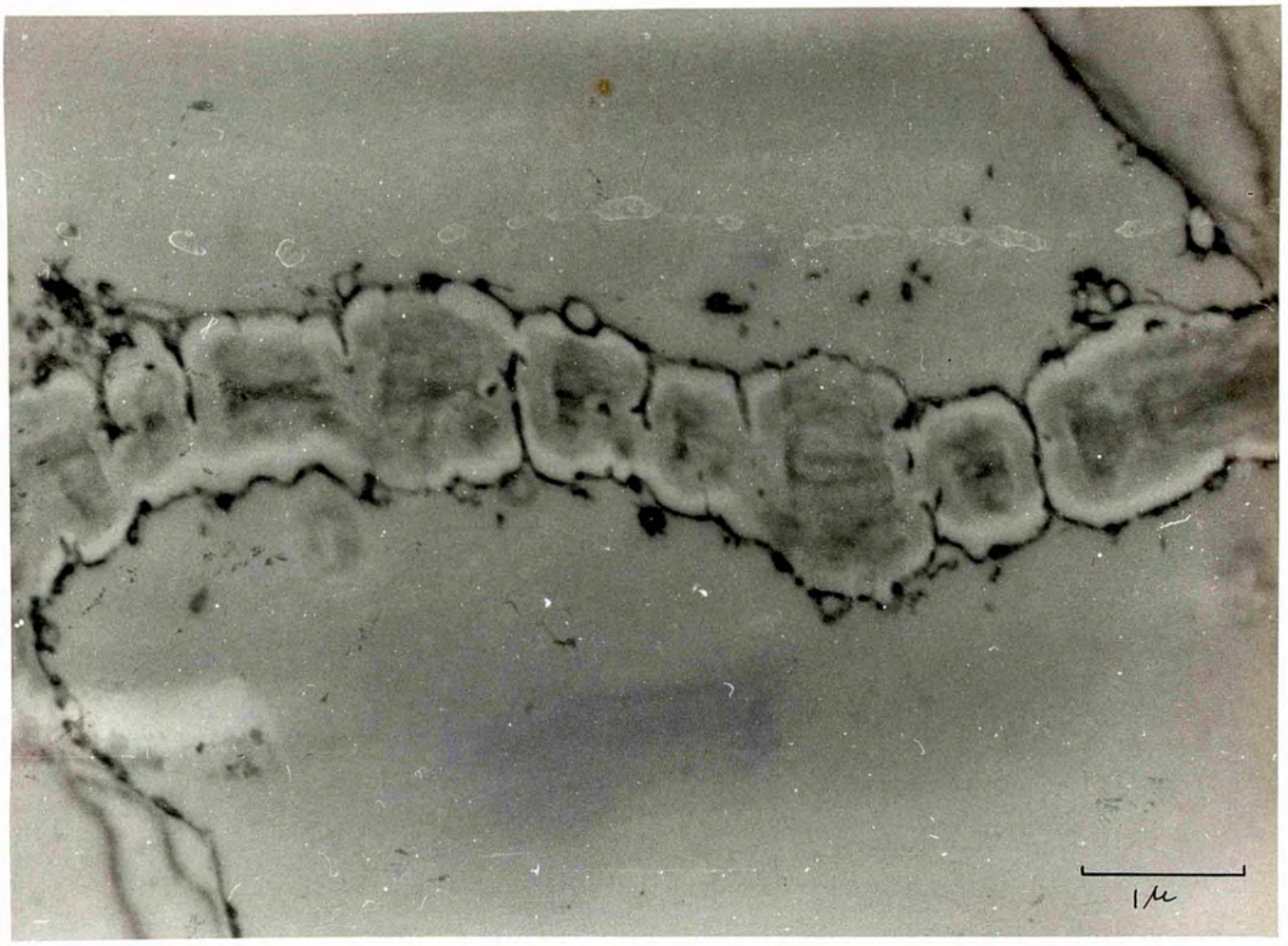

PLATE 53. First of the five serial sections. Note the tendency of the narrow connecting strands to branch. The sieve plate shows heavy callosing.

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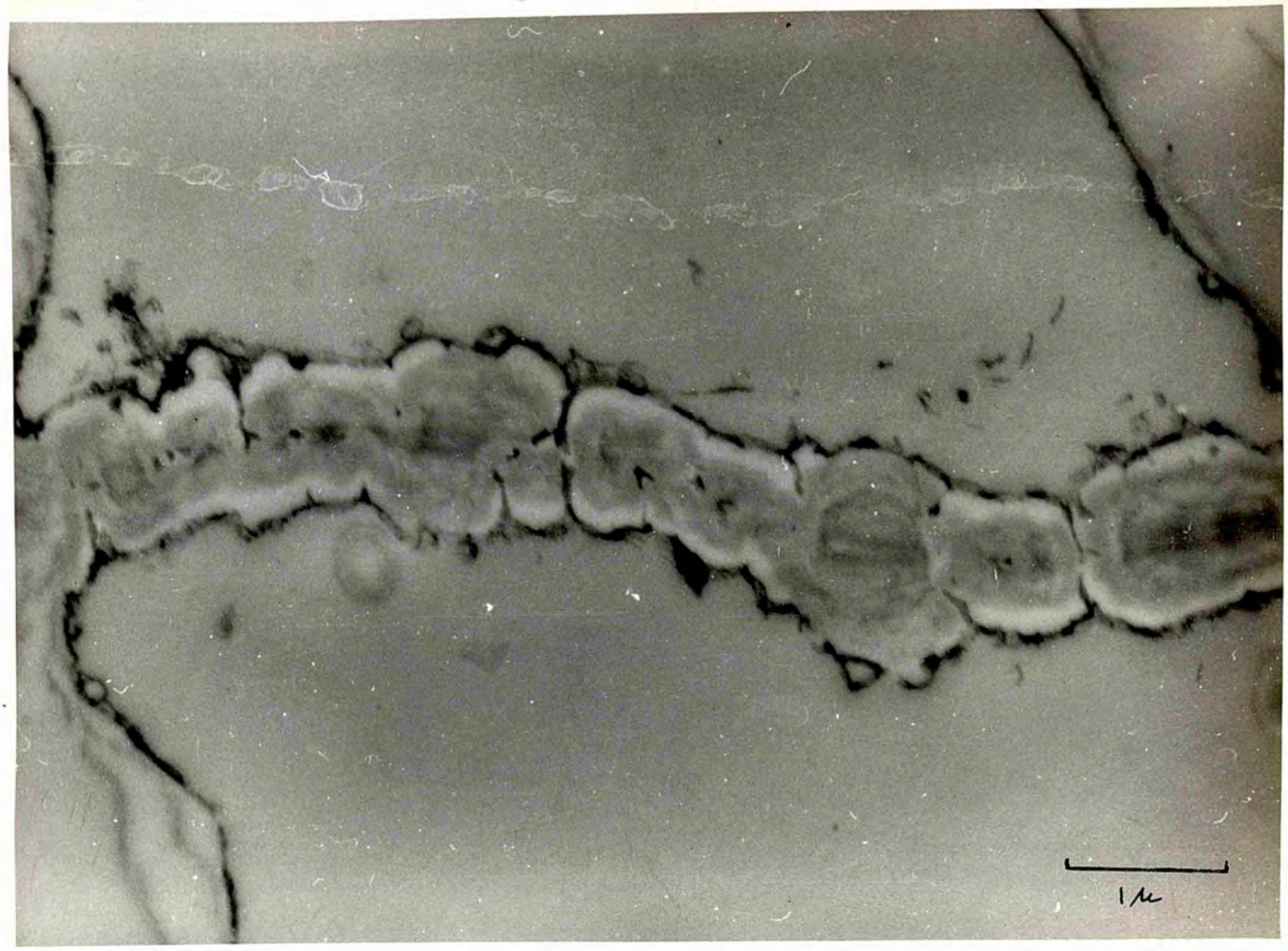

PLATE 54. Second of the five serial sections. Note the continuity of the strands with the peripheral cytoplasm.

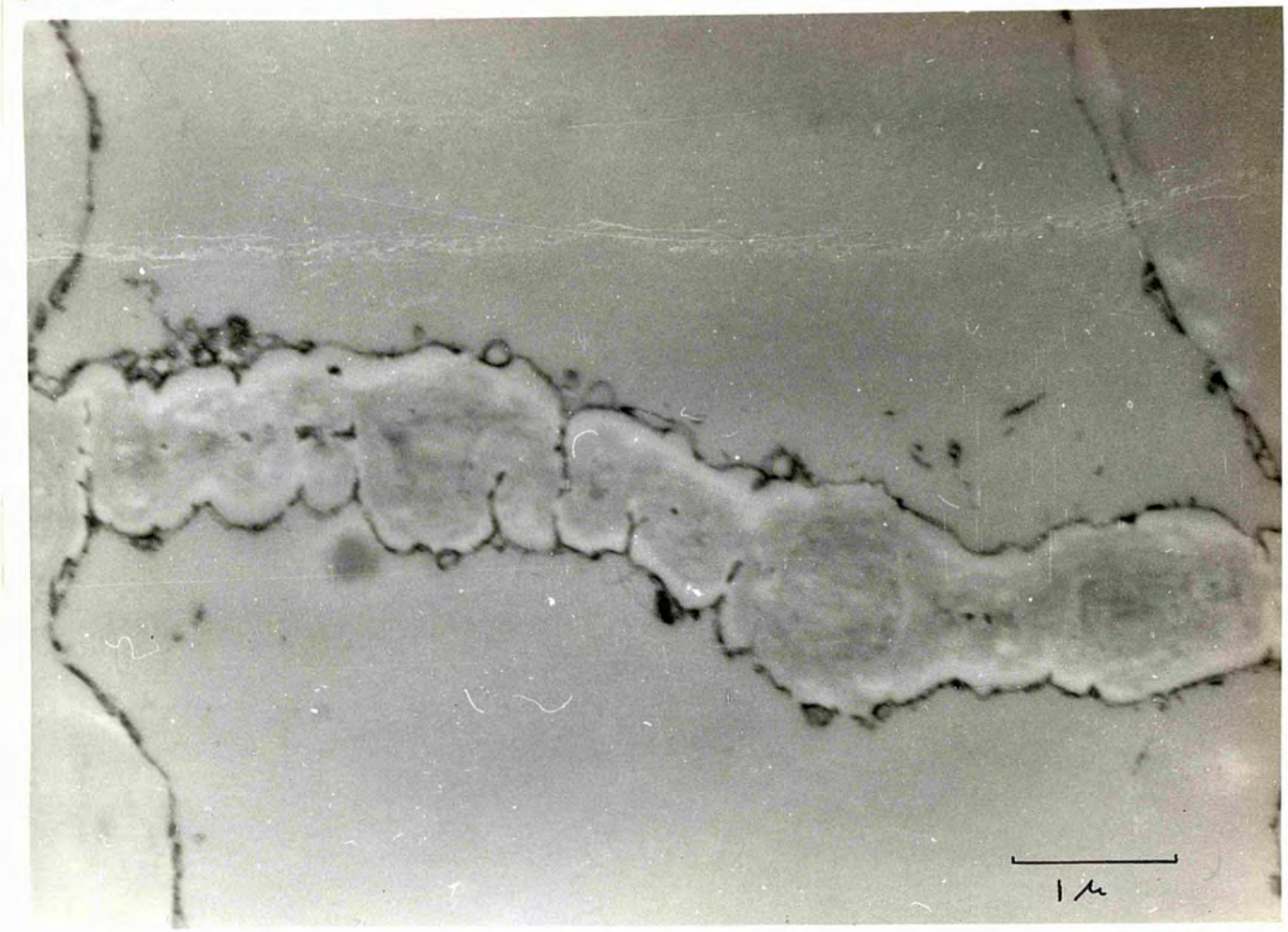

PLATE 55. Third of the five serial sections.

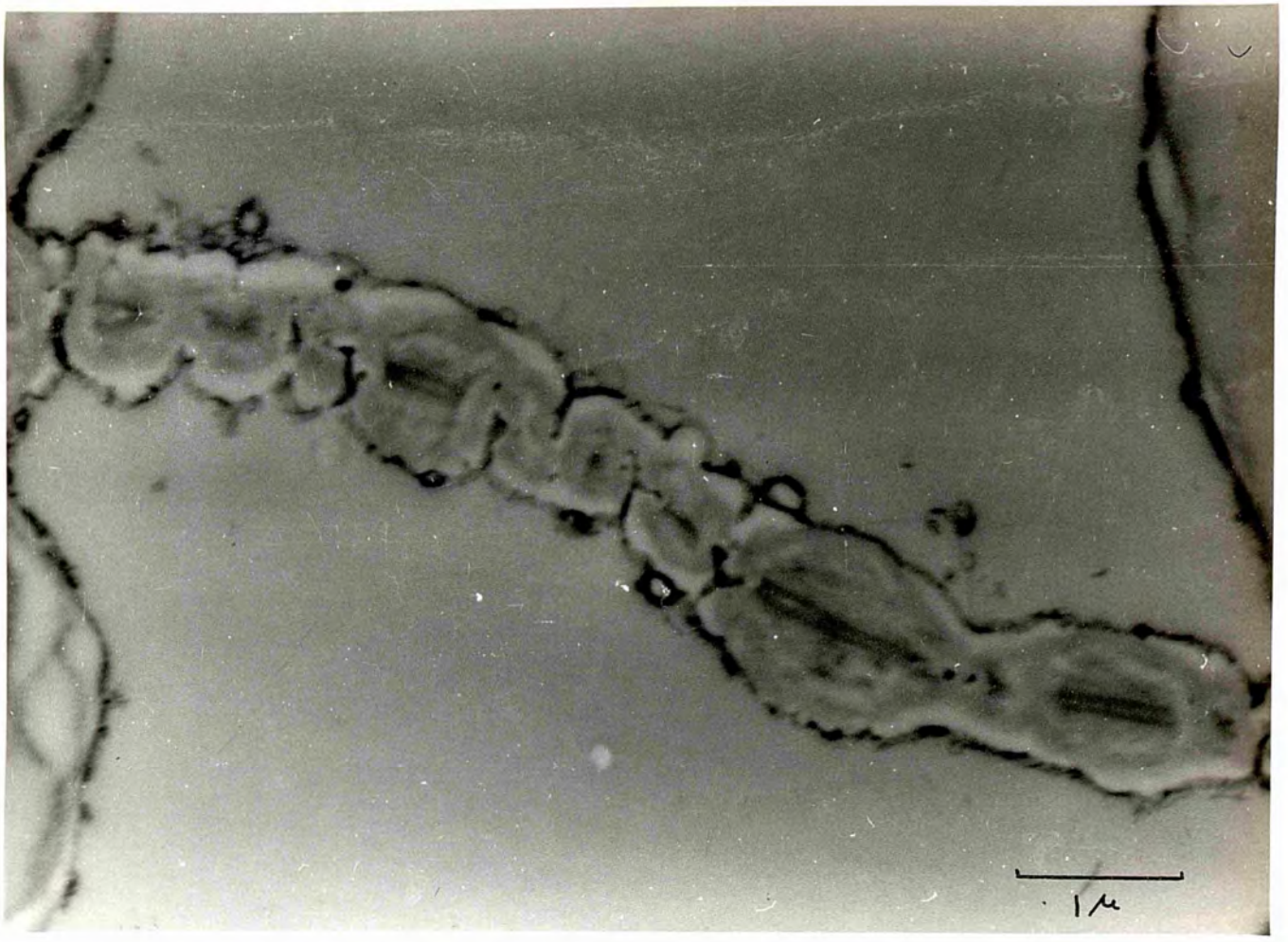

PLATE 56. Fourth of the five serial sections.

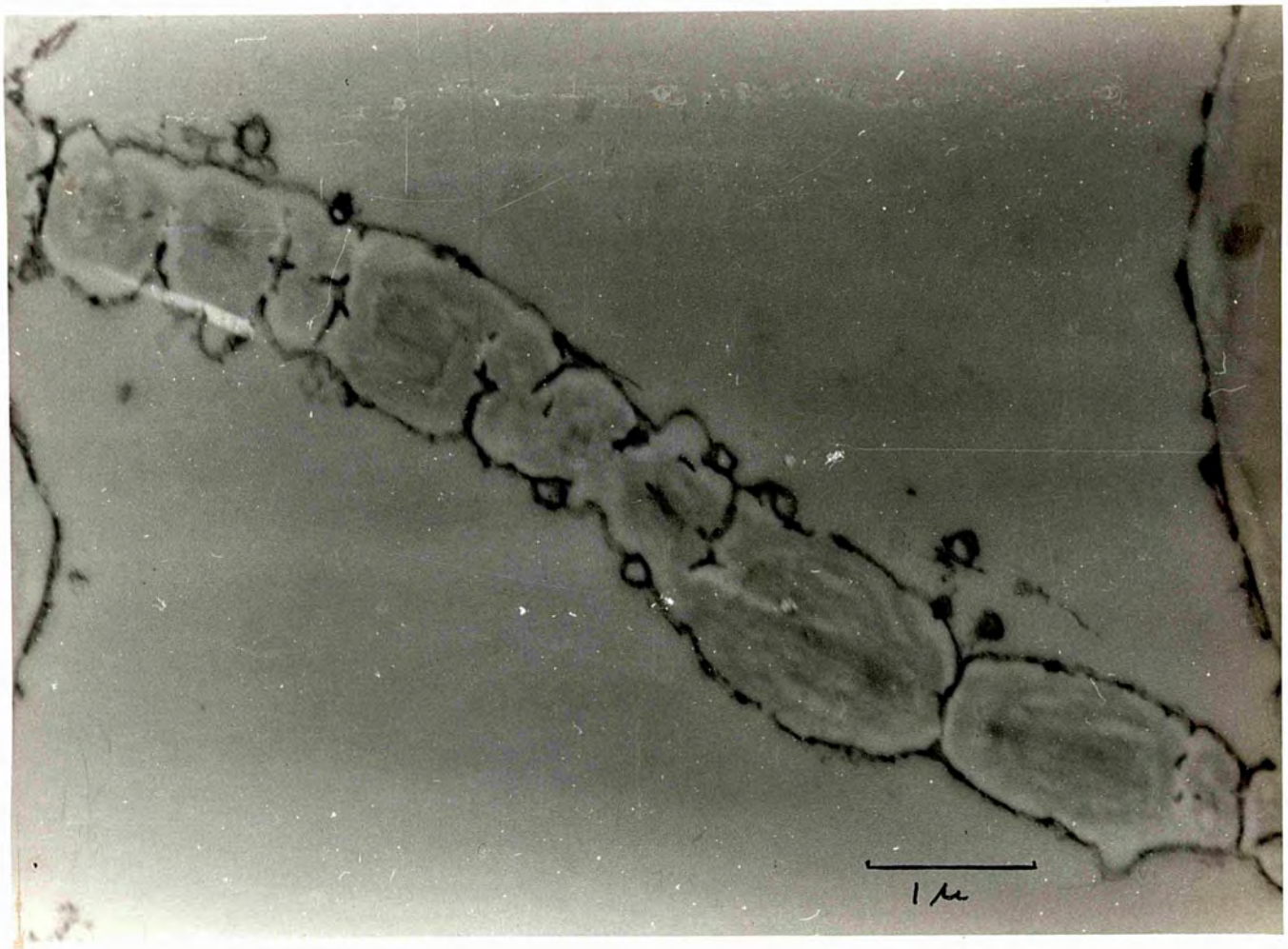

PLATE 57. Last of the five serial sections. Note the branching tendency of the connecting strands which, even though they are narrow, are continuous from one side of the sieve plate to the other.

very narrow. This may be correlated with the fact that there seems to be heavy callosing due to unfavourable growth conditions in winter. The callose is not limited to sieve pores but extends also on either side of the sieve plate.

The next plate (Plate 58) of a rather thick section has been included for comparison with the micrographs presented in Plates 59-62, taken on a new Metropolitan Vickers EM6 electron microscope. In the latter the cytoplasmic structure has been resolved much better than in the former. Only the latter set of micrographs will be described. The cytoplasm on either side of the sieve plate appears to be in a loose reticulum, the fibrils having little definite arrangement except near the plate. In some of the higher magnification micrographs the thread of the "reticulum" appears as a double membrane separated by a space which might perhaps be 15mμ. wide. The total thickness of a thread including the space measures about 29mμ. These threads extend into the sieve pores where they are arranged longtitudinally and compactly. The double membrane nature of these longitudinal "fibres" in the sieve pore can also be conjectured. More will be said about these in the Discussion. A starch grain within the cytoplasm can be seen one side of the sieve plate. We now pass on to the results obtained by preparation according to Schedule 3.

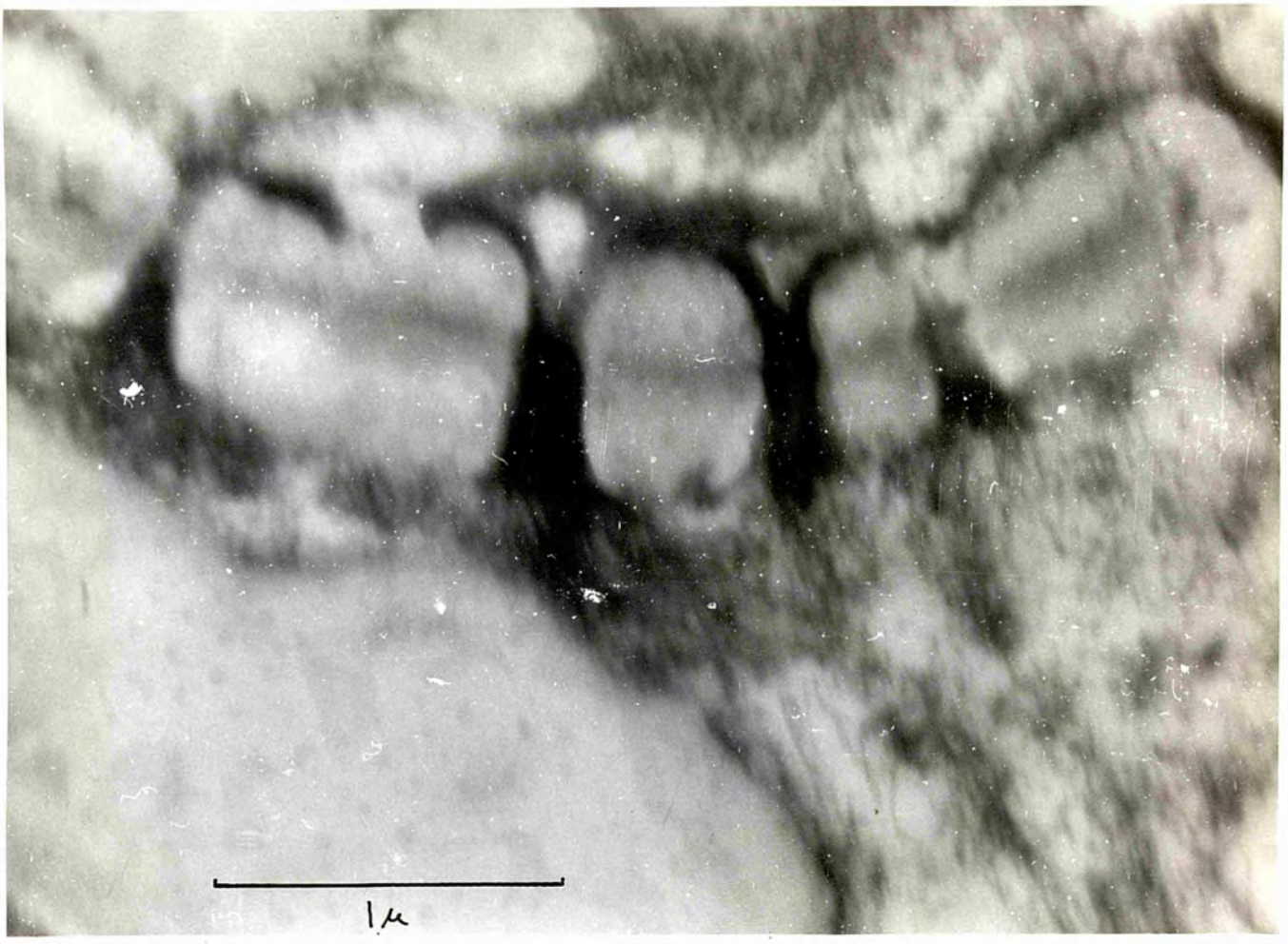

PLATE 58. Sieve plate with connecting strands. The cytoplasm in the sieve elements has an appearance of loose "reticulum".

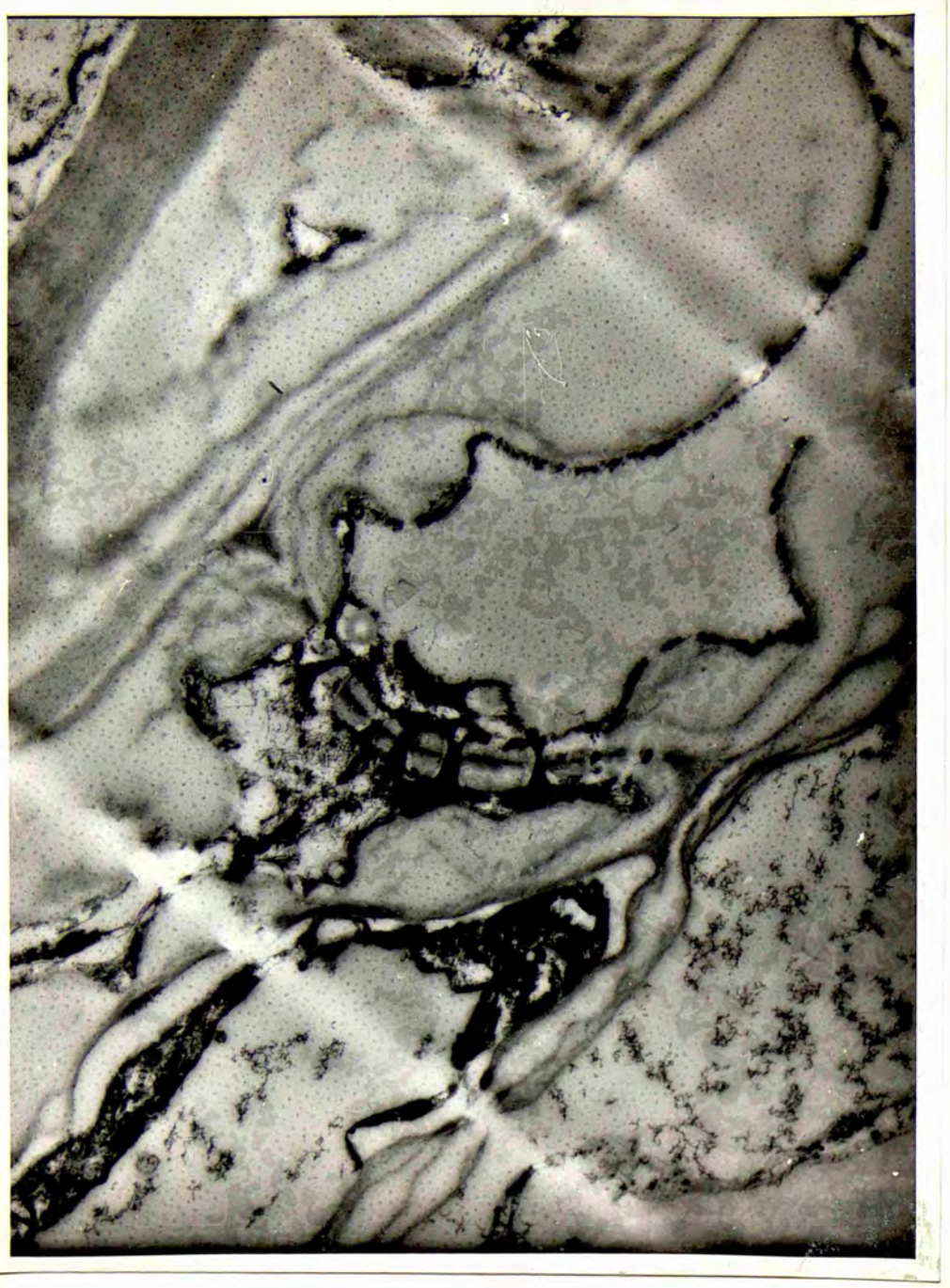

PLATE 59. Same sieve plate which is shown in the previous micrograph. Taken on EM6 electron microscope. Note the difference in resolution.

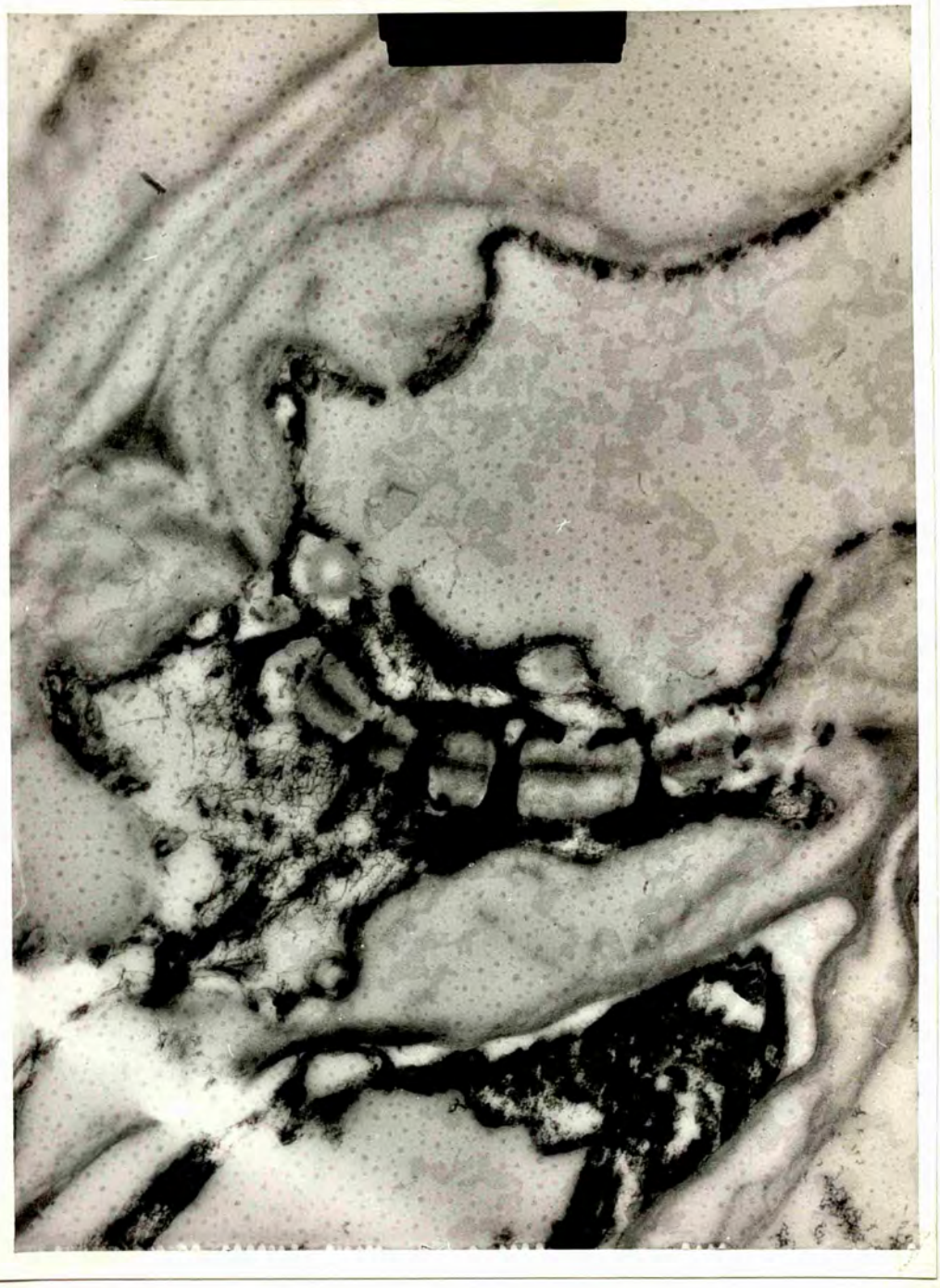

PLATE 60. Higher magnification of the previous micrograph. The threads of the loose "reticulum" show the double-membrane nature. Taken on EM6 electron microscope.

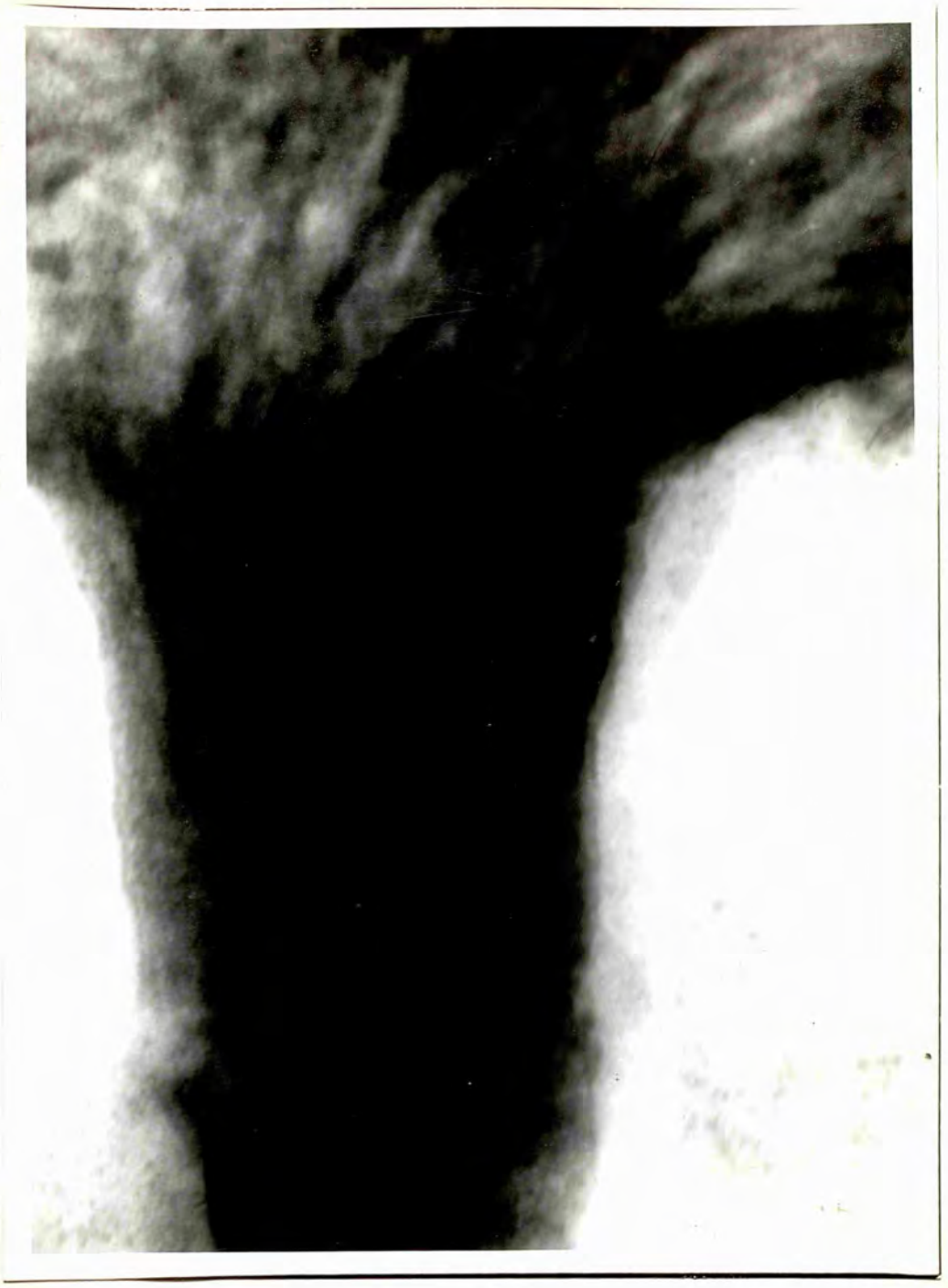

PLATE 61. One of the connecting strands from Plate 60 at a higher magnification. The double-membrane nature of the longitudinal "fibres" in the sieve pore can be seen. Taken on EM6 electron microscope.

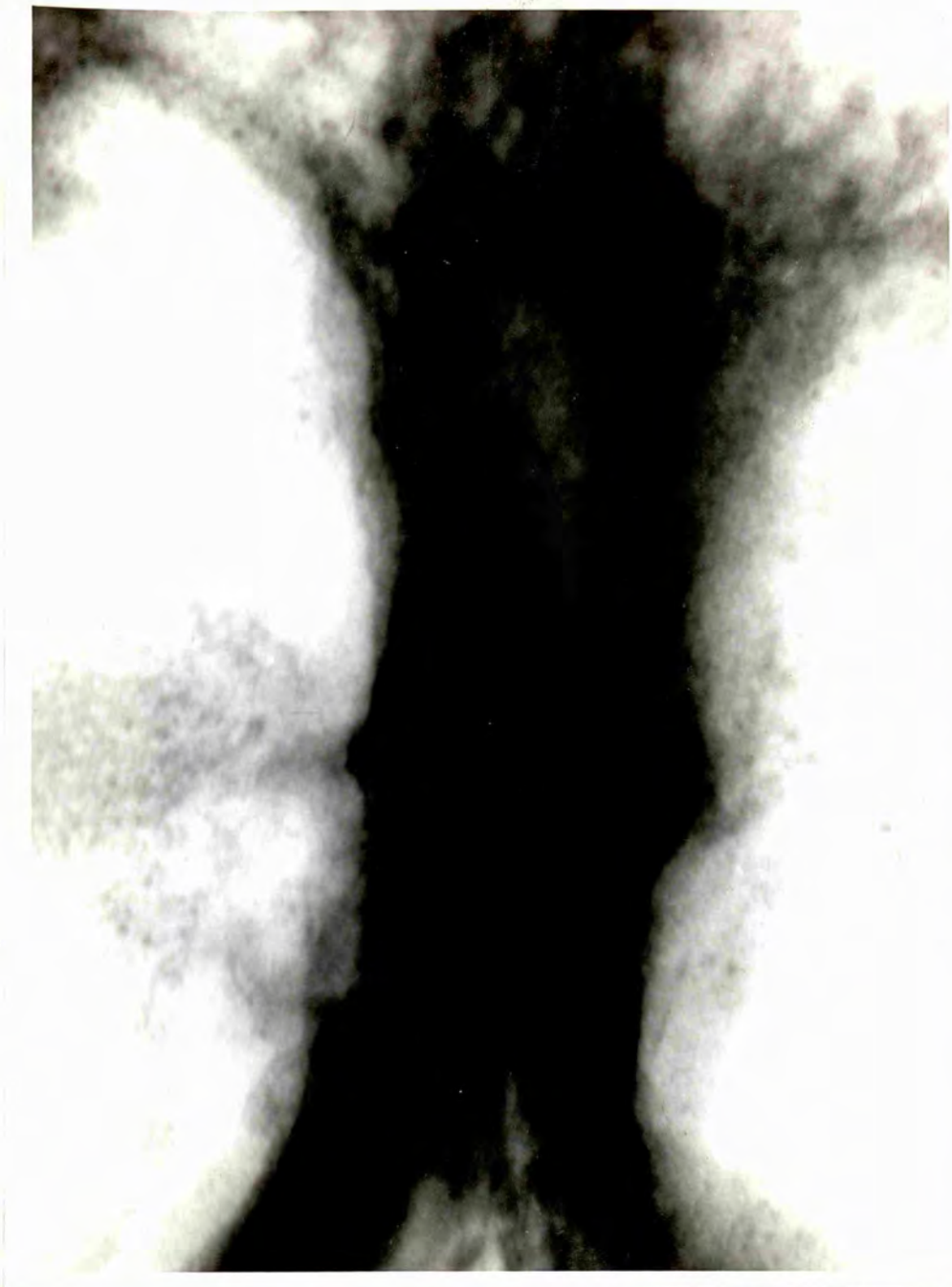

PLATE 62. Another connecting strand from Plate 60 at a higher magnification. Taken on EM6 electron microscope.

Since the results of fixation for 5, 10 and 15 minutes were not noticeably different from each other, the micrographs presented here will be discussed only in terms of $KMnO_4$ fixation and not of the time of fixation. The two Plates 63 and 64 are typical of many that were obtained and the sieve plates appear to be callosed. None of the sieve plates obtained showed the connecting strands even when the section passed through a pore. This can be seen from Plate 64. This is to be regretted since $KMnO_4$ is reported to bring out the membranous structure clearly. Since the initial trial with $KMnO_4$ did not prove satisfactory, and time did not permit carrying on more experimentation, it is difficult to comment further on the absence of the connecting strands in the pores. A possible swelling effect on the middle lamella can be seen, the middle lamella appearing wider than in all the previous micrographs. One very important feature of $KMnO_4$ fixed materials was the presence of a large number of round bodies sometimes with a clear centre and not only on either side of the sieve plate but in the lumen of the sieve tube itself. This was puzzling in the beginning, but later they proved to be released "starch" grains. More will be said about these later on. It may finally be added that $KMnO_4$ fixed material did not show much contrast and sometimes it was difficult to cut ribbons of good serial sections with materials embedded in methacrylate.

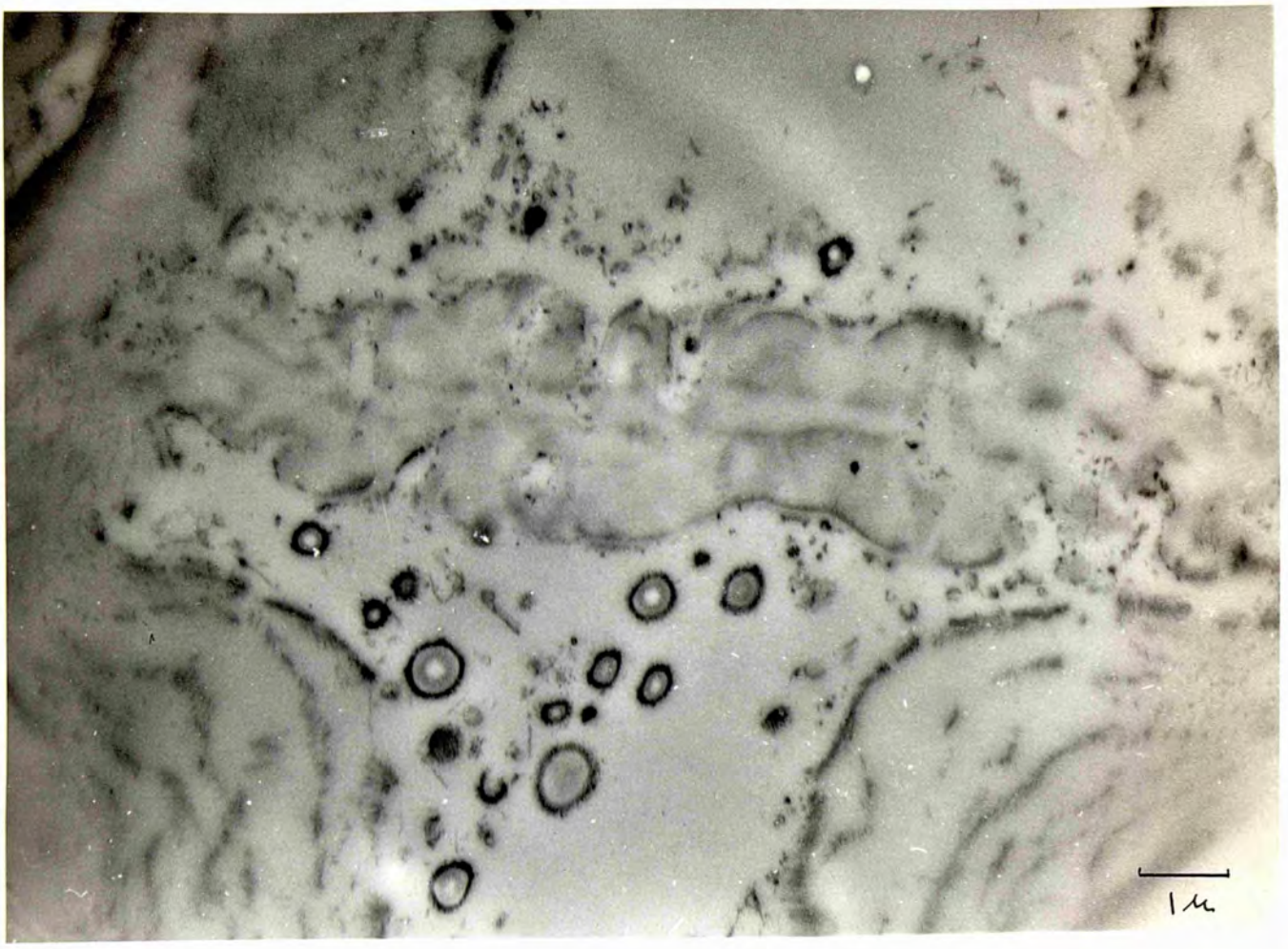

PLATE 63. Sieve tube obtained from petiole fixed in $KMnO_4$. Note the absence of pores and the presence of released starch grains near the sieve plate.

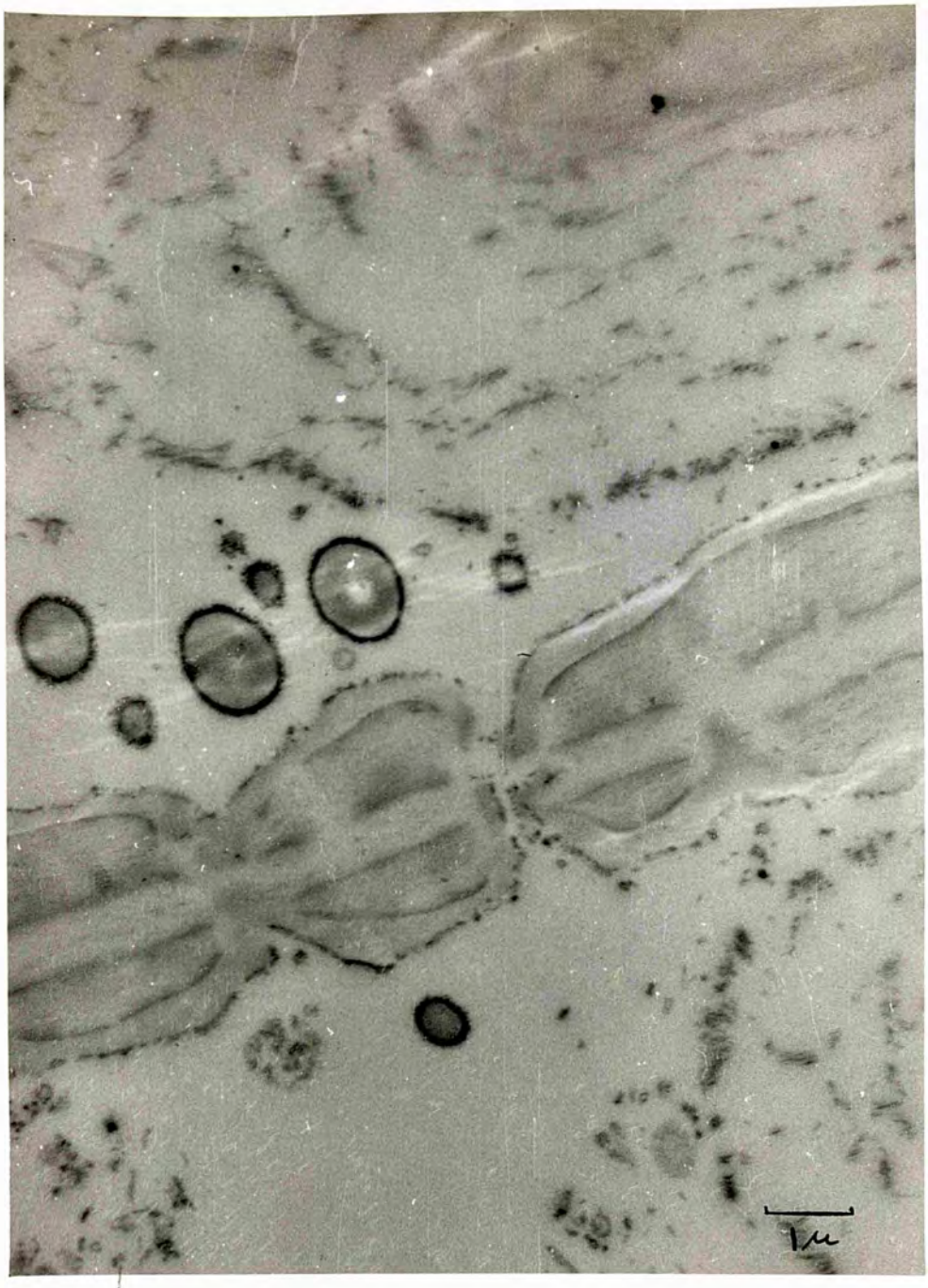

PLATE 64. Another sieve tube from petiole fixed in $KMnO_4$. Middle lamella appears wider. The pore is devoid of its content. Note the released starch grains near the sieve plate.

4. Sieve area

The term sieve area has been used here in the sense of a depression in the longtitudinal wall with clusters of pores through which the cytoplasm of the adjacent sieve tubes or the sieve tube and its companion cell are connected by strands.

In some of the earlier micrographs dealing with the nacreous wall, adjacent sieve tubes are shown. The common longtitudinal wall of these elements frequently shows corresponding depressions which are considered to be the region of sieve area. Evidence for this view is presented in Plate 65. This micrograph shows in longtitudinal section two sieve tubes lying side by side, their common longtitudinal wall showing corresponding thin areas. In the region of this can be seen two pores cut in an approximately median longtitudinal plane, and filled with apparently dense cytoplasm continuous with that lining the sieve element. The connecting strands appear intermediate in size between those of the sieve plate and plasmodesmata of the primary pit fields discussed later.

A connecting strand between the sieve-tube and its companion cell has already been shown in Plate 47. The strand is not shown continuous, but this is probably due to its being inclined to the plane of the section.

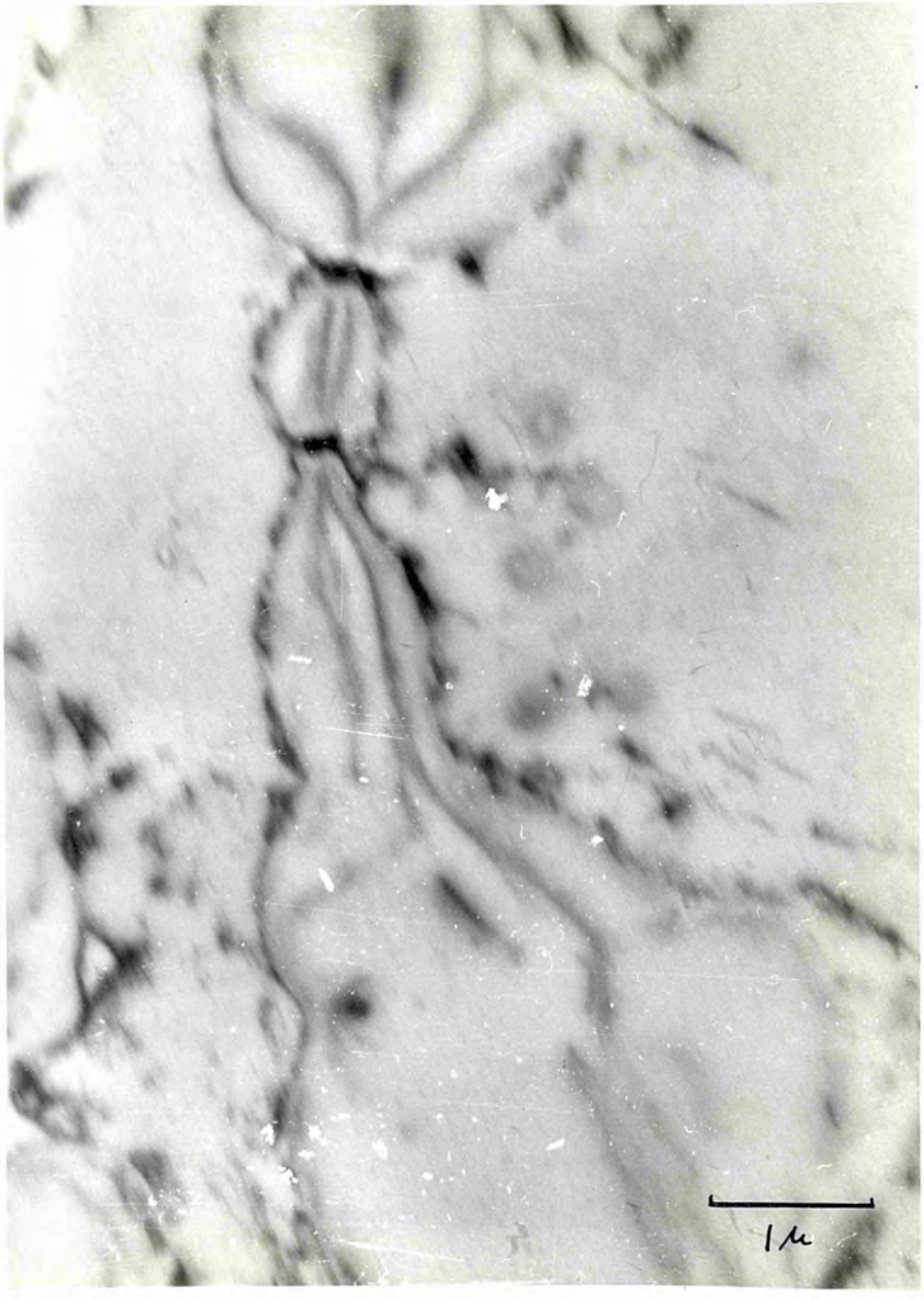

PLATE 65. L.S. of two adjacent sieve tubes. The two pores of the sieve area on the common lateral wall show the connecting strands continuous with that lining the sieve elements.

5. Primary pit fields in the phloem parenchyma

Primary pit fields have been defined to be depressions in the primary wall where plasmodesmata are concentrated. The latter, due to their minute size, are difficult to demonstrate optically. Therefore earlier studies of these structures were carried out by swelling the wall and using precipitation reactions.

In preparations according to Schedules 1 and 3 for electron microscopy it was rather difficult to demonstrate plasmodesmata in the phloem parenchyma cells. This may partly be due to the bad preparative technique and partly due to lack of contrast. Hepton (1957), who used pretreated phloem material, also found difficulty in demonstrating them. This suggests that lack of contrast is a principle obstacle. When the overall contrast of the sections obtained from pretreated phloem was increased by using phosphotungstic acid and uranyl acetate as in Schedule 2, it was fairly easy to demonstrate plasmodesmata. The result from such a preparation is shown in Plate 66. That this is not rare is further shown by four serial sections (not consecutive) presented in Plates 67-70. These micrographs can be compared with those showing plasmodesmata published by Whaley, Mollenhauer and Kephart (1959), Mollenhauer (1959), Marinos (1960) and Porter and Machado (1960). All these workers used $KMnO_4$

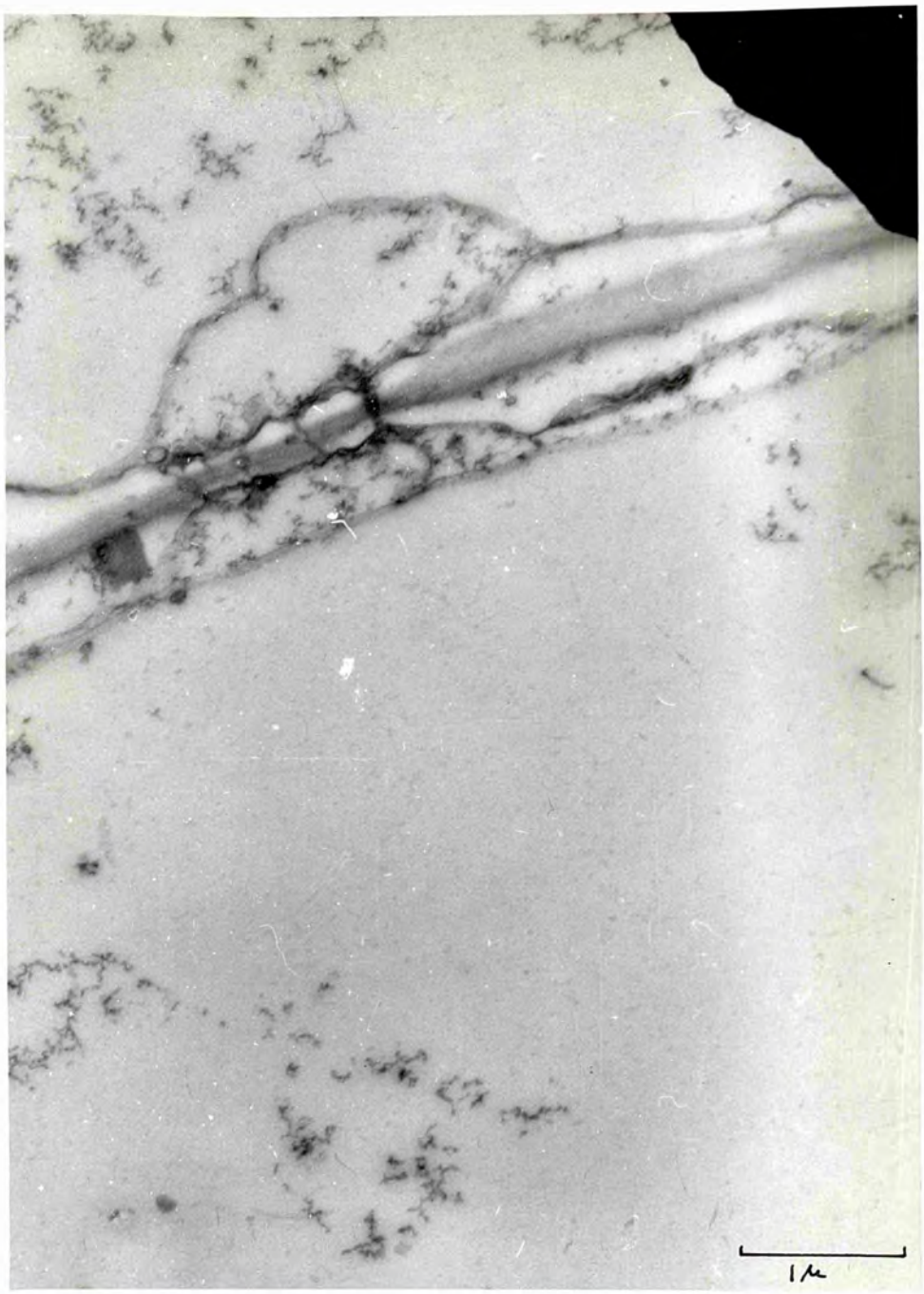

PLATE 66. Plasmodesmata on the common lateral wall of two phloem parenchyma cells. Note the continuation of the plasmodesmata with what is apparently endoplasmic reticulum.

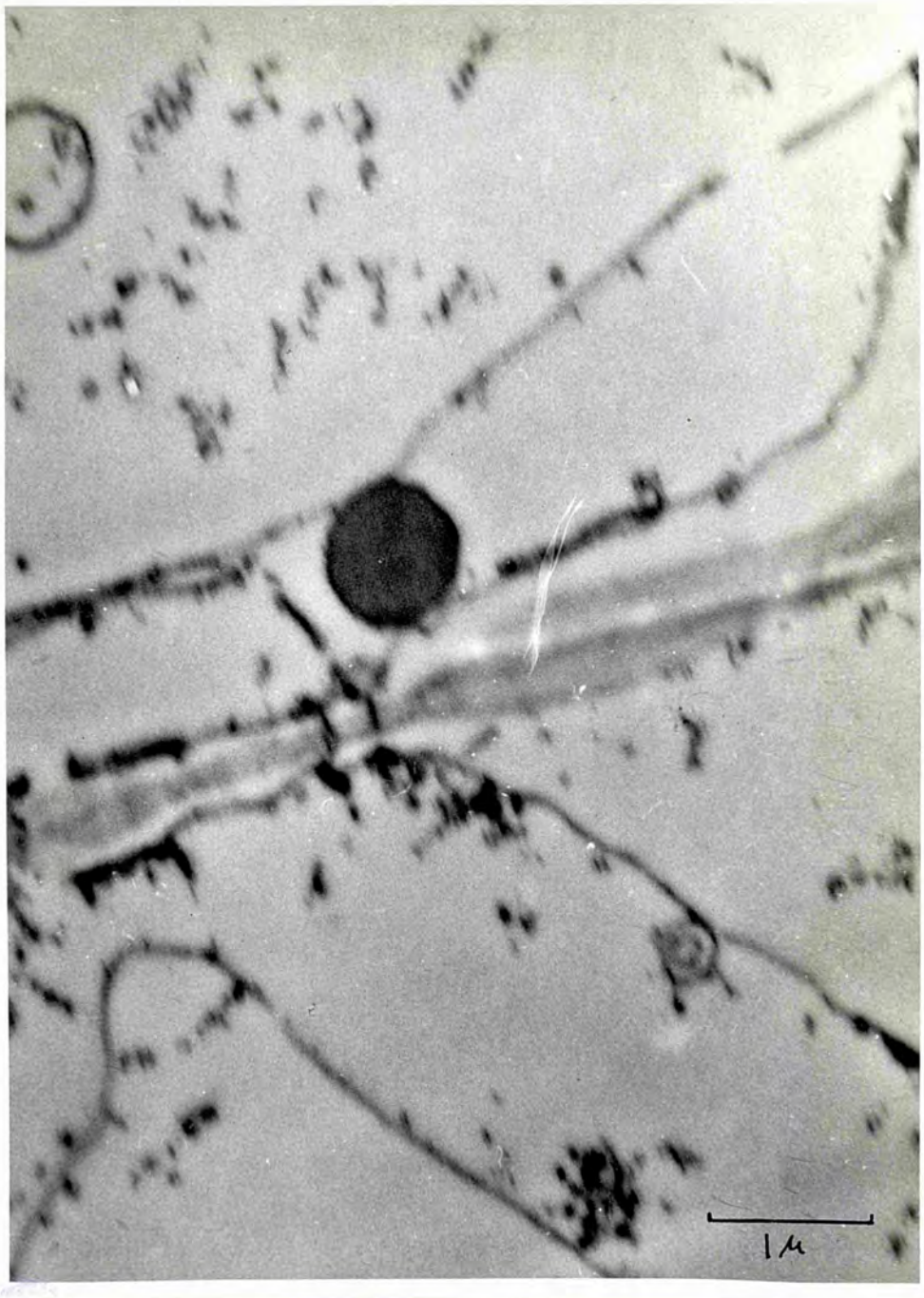

PLATE 67. First of the four serial (not consecutive) sections showing plasmodesmata on the common transverse wall of two phloem parenchyma cells.

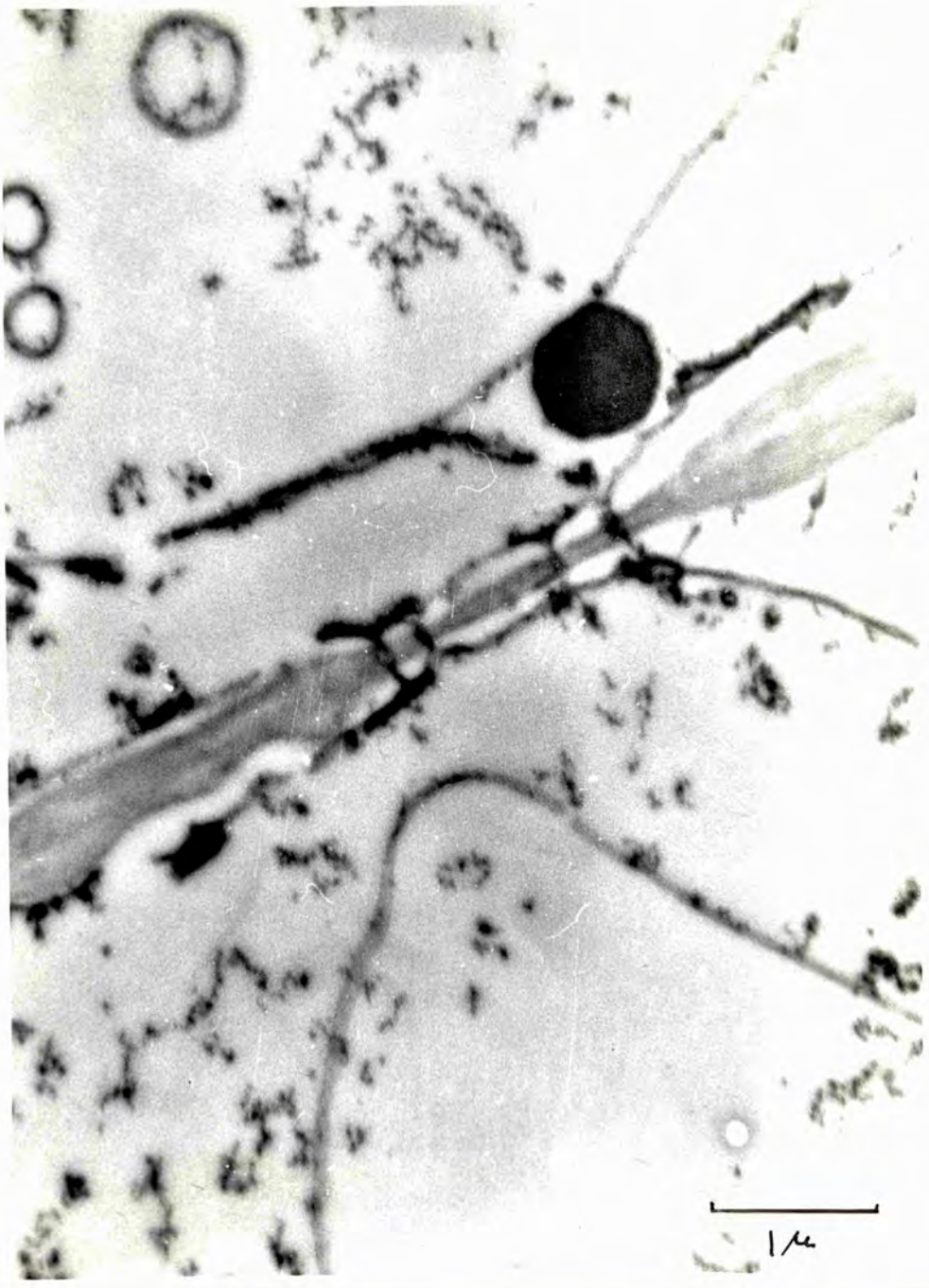

PLATE 68. Second of the four serial sections. Note a median cavity in one of the plasmodesmata.

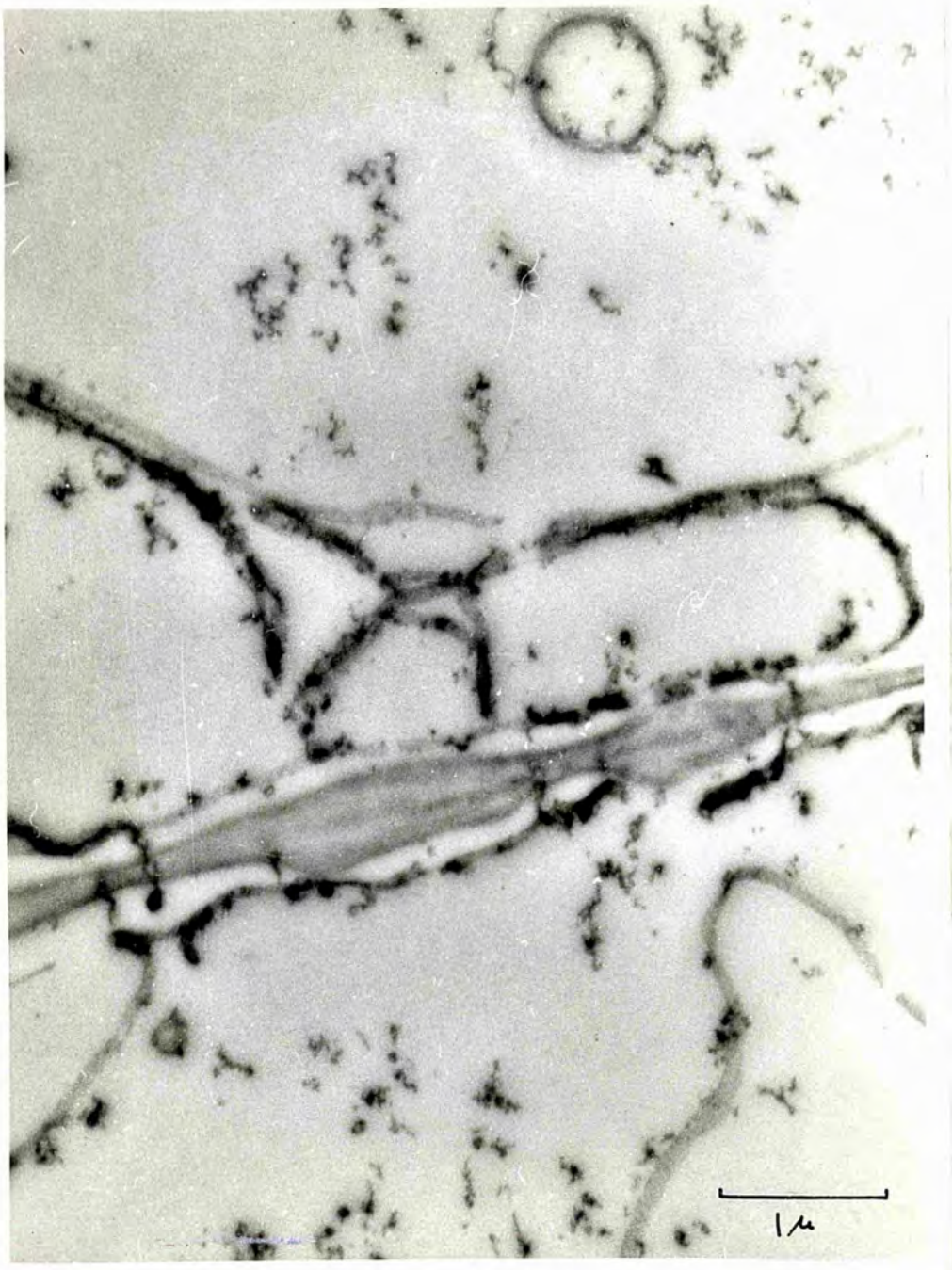

PLATE 69. Third of the four serial sections. One of the plasmodesmata shows a median cavity and another similar cavity outside the wall in the region where it joins the endplasmic reticulum.

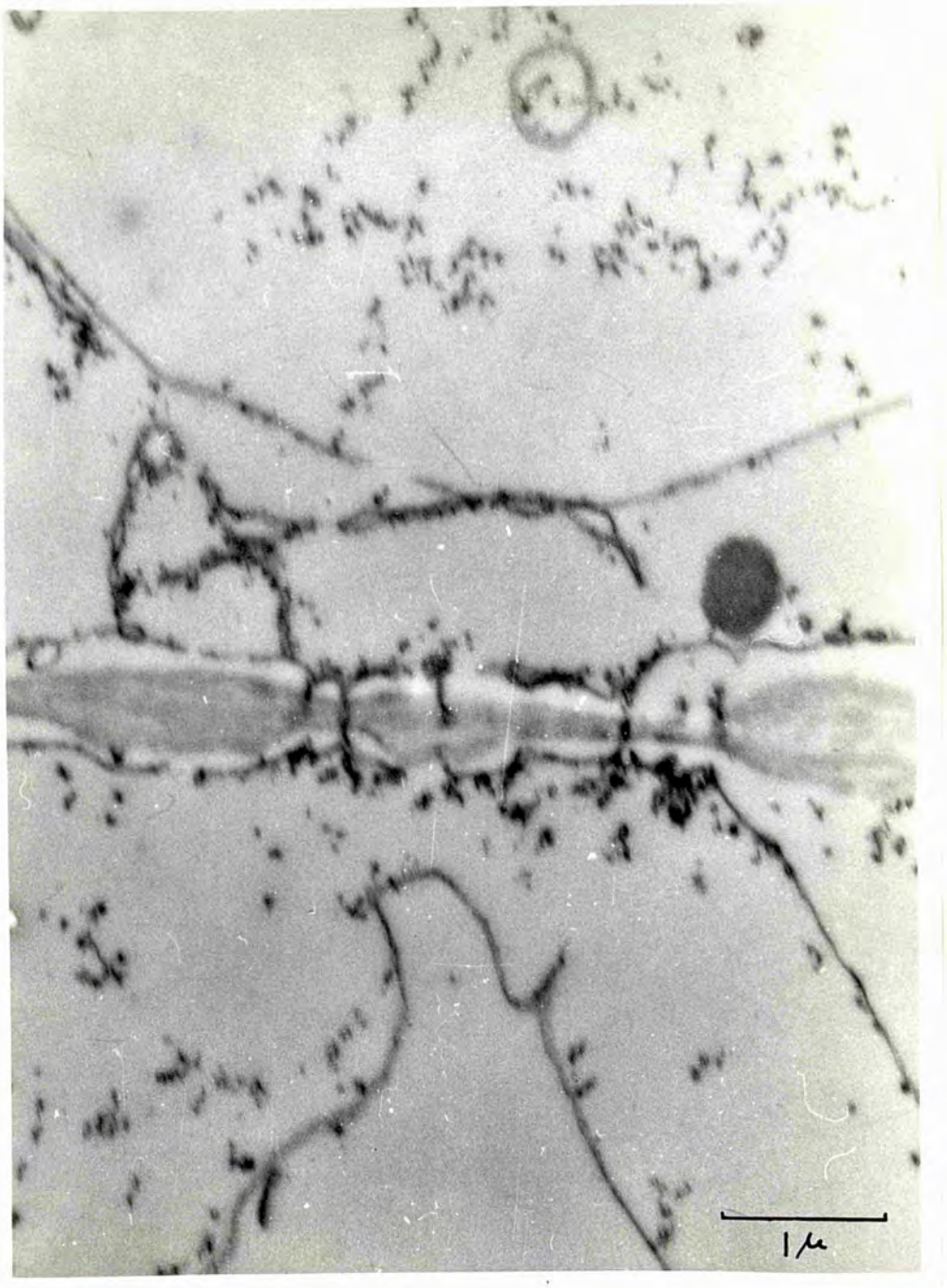

PLATE 70. Last of the four serial sections.

as a fixative. The tissues they worked with, however, were embryonic; in the present case the cytoplasm is vacuoled and not nearly so dense.

The plasmodesmata in Plate 66 are located on the common longtitudinal wall of the phloem parenchyma cells whereas those presented in other micrographs are on the common end walls. In all these cases they extend without natural interruption from one cell to the other. This goes against the findings of Volz (1952), who considered plasmodesmata to be made up of two halves, each being invested by its own membrane. Further they seem in some cases to have a connection with what would appear to be elements of the endoplasmic reticulum. Rarely a plasmodesma, as in Plate 69, shows a median cavity. This feature was also noted by Hepton (1957). It does not appear regularly. If the plasmodesmata are associated with elements of the endoplasmic reticulum, local swelling of the central zone of the latter might result in the appearance of a median cavity. Whaley, Mollenhauer and Kephart (1959) have also reported that the distance between the two membranes of the endoplasmic reticulum is variable, though Porter and Machado (1960) in a recent paper state that it is very constant in plants at least in the root tip cells of onion. This apparent

association of the plasmodesmata with the endoplasmic reticulum goes against the view expressed by Jungers (1930) that they are specialised wall material and not protoplasmic in nature. Another feature to be noted from these micrographs is that the primary pit field is shallow and not very deep, that is the thickness of the wall in the region of the primary pit field and beyond it does vary very much. This is in marked contrast to the sieve area discussed earlier.

6. Tonoplast

In some of the earlier micrographs of sieve tubes a structure has been reported which could possibly be interpreted as a tonoplast. A similar structure is shown in Plates 71 and 72, which are the longtitudinal sections of sieve elements which had typical sieve plates. Sometimes round bodies (mitochondria, starch grains, etc.) appear in the sieve tube without this feature; at others they lie in the space between it and the wall. In these circumstances it is really difficult to interpret it unequivocally as a tonoplast, and the evidence of the present study on this point must be regarded as inconclusive.

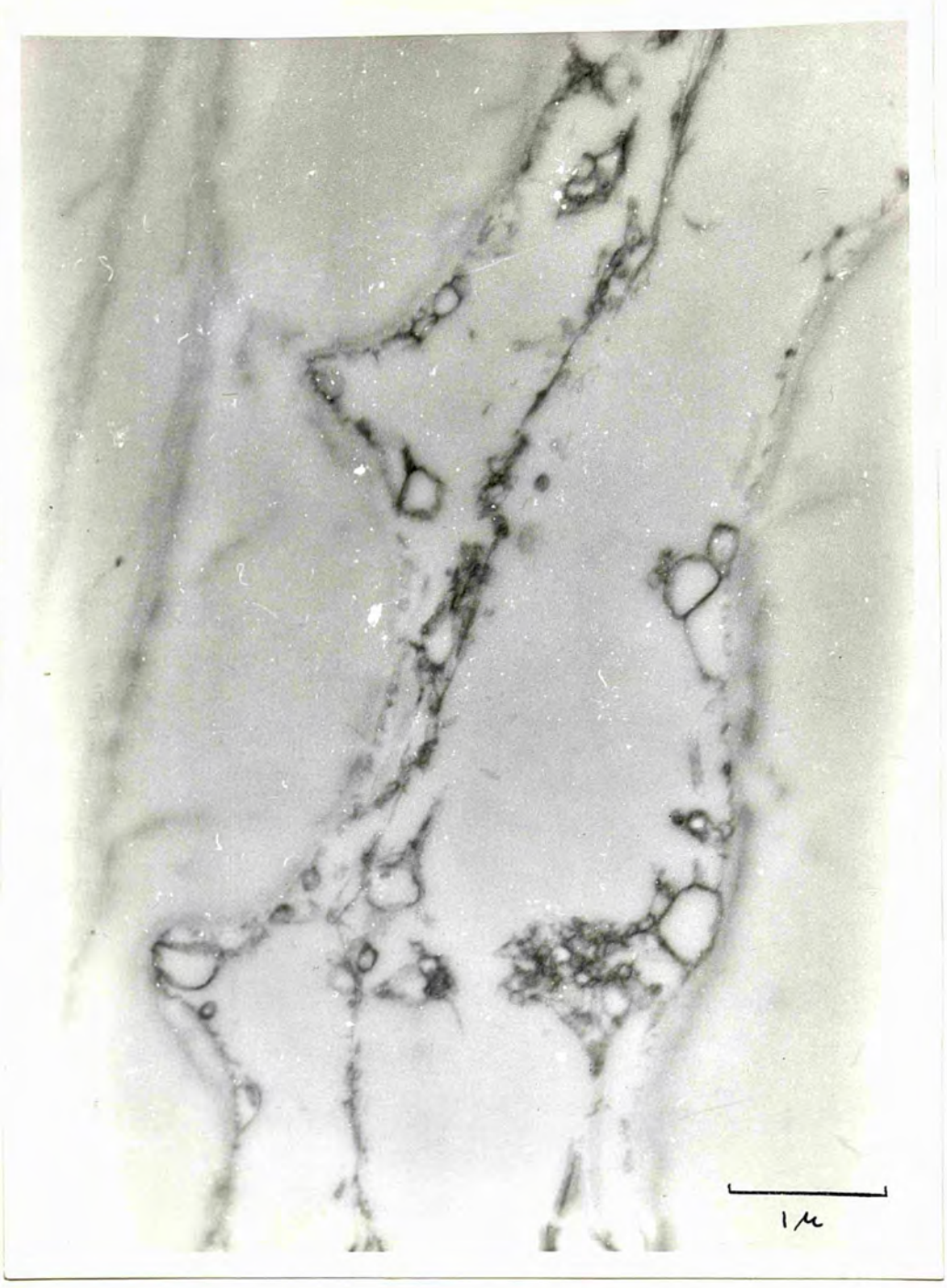

PLATE 71. L.S. of sieve element showing a structure which could possibly be interpreted as a tonoplast.

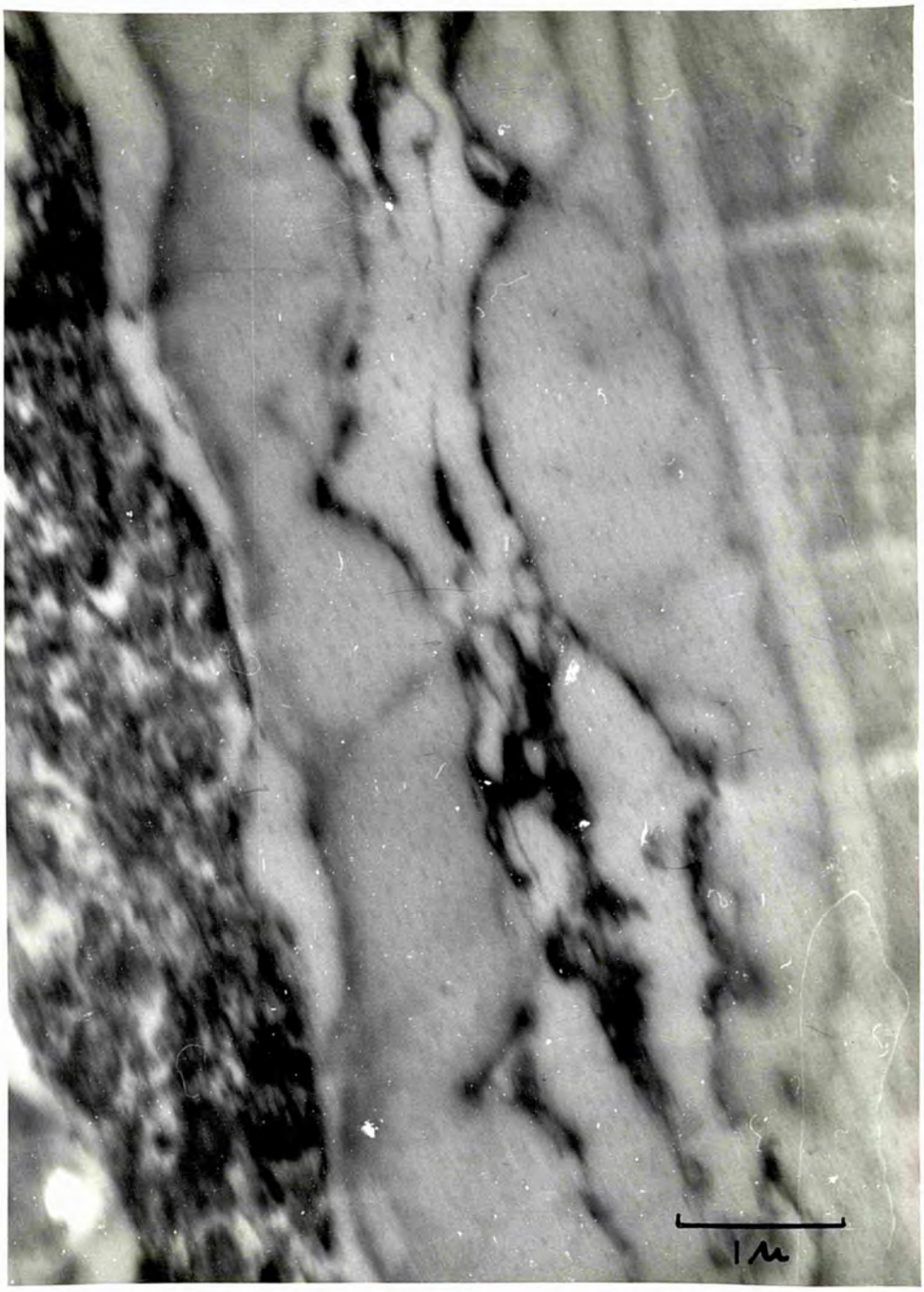

PLATE 72. Another sieve element in L.S. showing scanty cytoplasm and a structure which could be interpreted as a tonoplast. Note the abundance of the cytoplasm in the adjacent parenchyma cell.

7. Inclusions in the sieve tubes

The number of different types of inclusions reported in the sieve tubes is not very large. The earliest ones to be recognised were starch grains which according to Brissi (1873) stain red violet with iodine. Strasburger (1891) found that in Vitis several starch grains are commonly initially present in each plastid from which they are eventually released. The presence of plastids with starch grains as well as "released" starch grains in the sieve tube has subsequently been confirmed for a large number of plants. A commonly reported and very characteristic feature of the sieve tube starch is that it stains brick red with iodine whereas typical starch grains in other types of cells stain blue-black.

Inclusions of a different nature in the sieve tube were described by Salmon (1946) and identified by her as mitochondria. The presence of mitochondria in the living sieve tube has also been shown by McGivern (1957) with the help of vital staining using Janus green B. She has also described some smaller inclusions and has suggested that they might be spherosomes. In a more recent paper Schumacher and Kollmann (1959) have given electron microscopic evidence of mitochondria in the sieve tube of Passiflora.

Inclusions, reported to be extruded from the sieve tube nucleus, have also been reported in the literature on phloem. These were considered to be "albuminous globules" or slime bodies. Their true nature was identified by Esau (1947), who confirmed that these are the extruded nucleolus (or nucleoli) and thus different from the so-called "albuminous globules" or slime bodies. The extruded nucleolus is reported to persist in the sieve tube during its functional period in many plants.

Slime bodies, apparently proteinaceous in nature, have also been reported in the cytoplasm of young sieve tubes of many dicotyledonous plants. They are supposed to spread and disperse in mature sieve tubes and thus lose their discrete identity as inclusions.

In conformity with these observations, inclusions were also found in the sieve tubes of Nymphoides petiole. Already mention has been made (Plate 31) of the plastids containing starch grains. In many of the sections fixed with osmic acid, however, these were rare or absent; but for some reason they appeared much easier to find in material fixed with $KMnO_4$. Thus in Plates 63 and 64, which are from $KMnO_4$ fixed materials, numerous round bodies are seen on either side of the sieve plate. They are also abundant in the sieve tube

further away from the sieve plate as shown in the Plate 73. They have a dark boundary and in many cases a clear centre. Later on they proved to be "released" starch grains. The next plate (Plate 74) shows one such grain and two others still within the plastid, together with fragments of disintegrated plastids. More evidence for this view is presented in Plates 75 and 76, which show "released" starch grains as well as grains still enclosed within the plastids, the latter however showing incipient disintegration of their apparently complex limiting membrane. They have a maximum diameter of about 5μ, whereas the starch grains measure about 1.4μ. That these latter are typical sieve tube "starch" grains was confirmed by staining them in iodine both in the case of fixed and living materials. They stain pink with a clear centre. An iodine stained preparation from fixed material is shown in Plate 77, which shows distribution of "released" grains on the sieve plate as well as in the lumen of the tube itself. These grains are again circular in outline and some of them show a clear centre. Here they measure about 1.6μ in diameter. It was mentioned earlier that starch grains are easier to find in the electron microscopic examination of permanganate-fixed than in osmium-fixed material; an optical investigation of osmium and

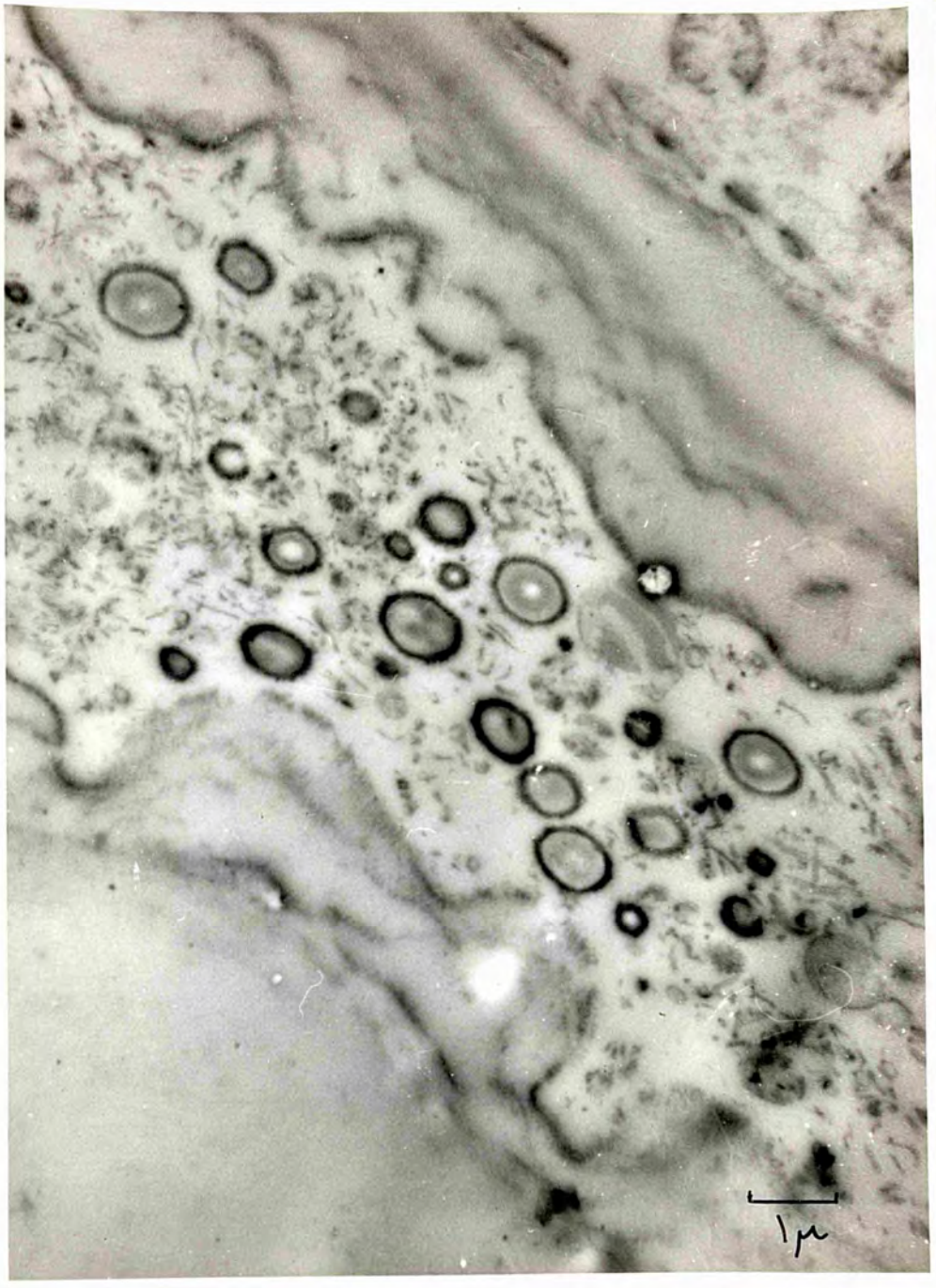

PLATE 73. Released starch grains further away from the sieve plate. Some of these show clear centre. Fixed in $KMnO_4$.

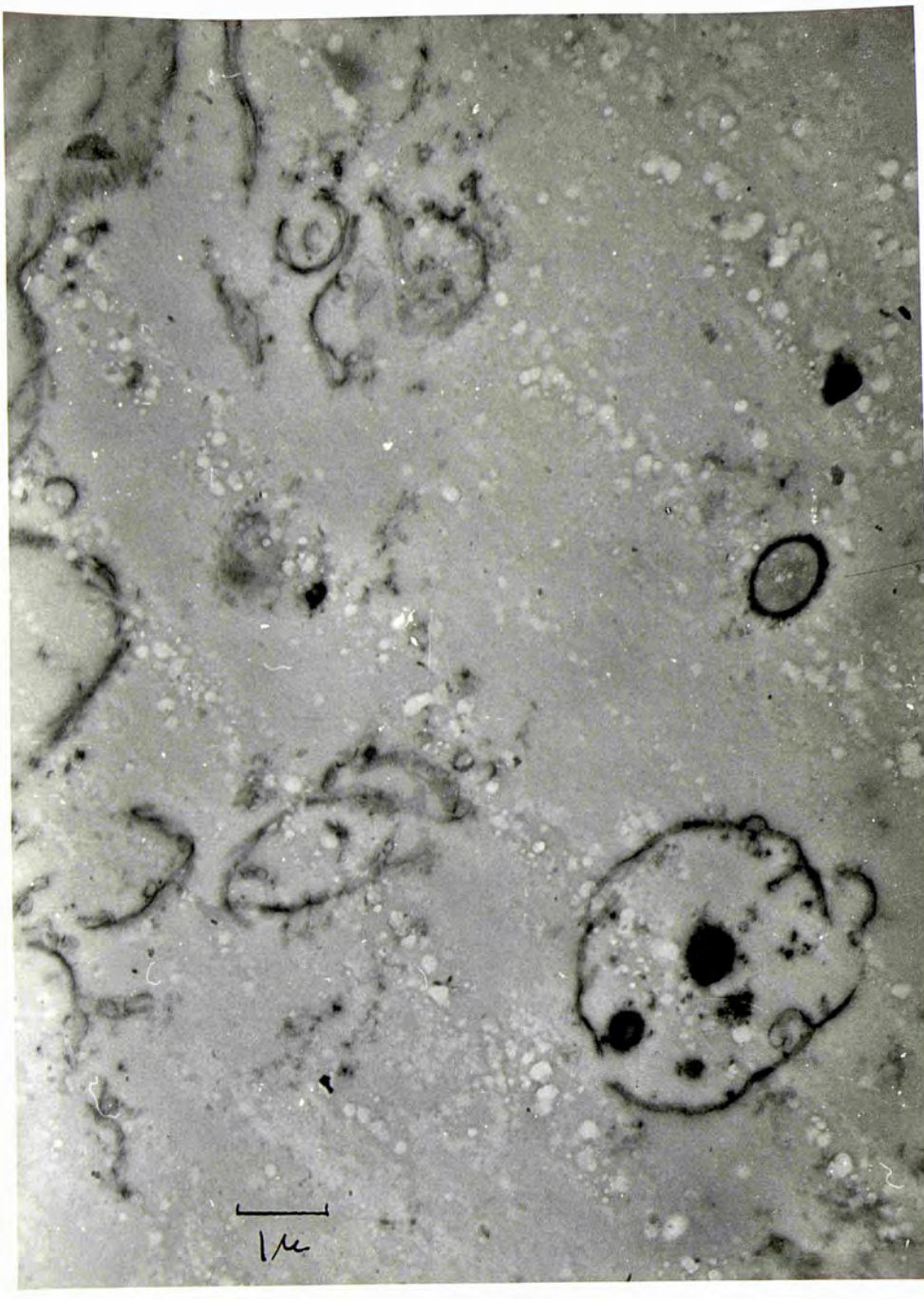

PLATE 74. L.S. Sieve tube showing plastid with starch grains, released starch grains and fragments of disintegrated plastids.

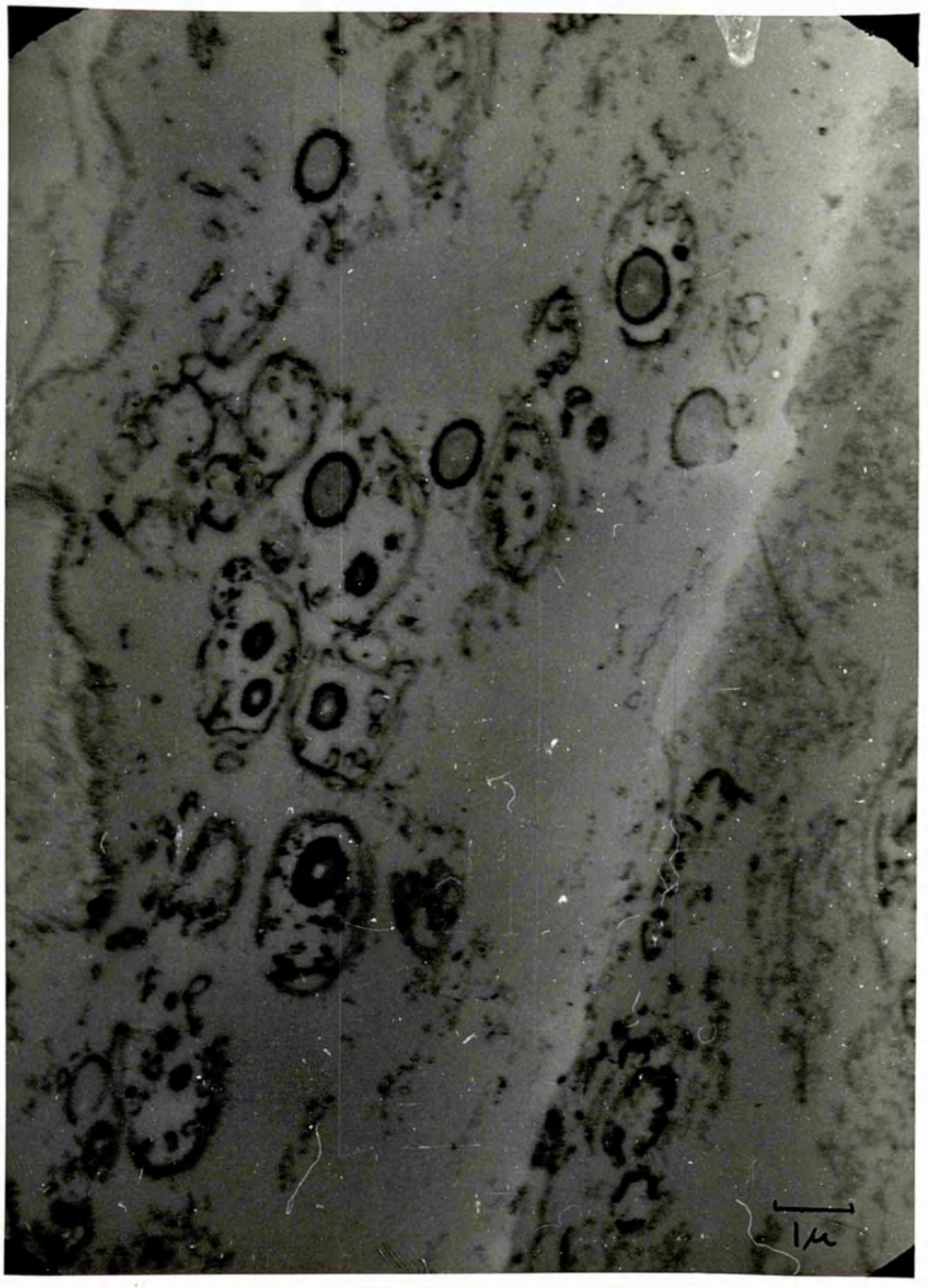

PLATE 75. Another sieve tube showing released starch grains as well as grains still within the plastids.

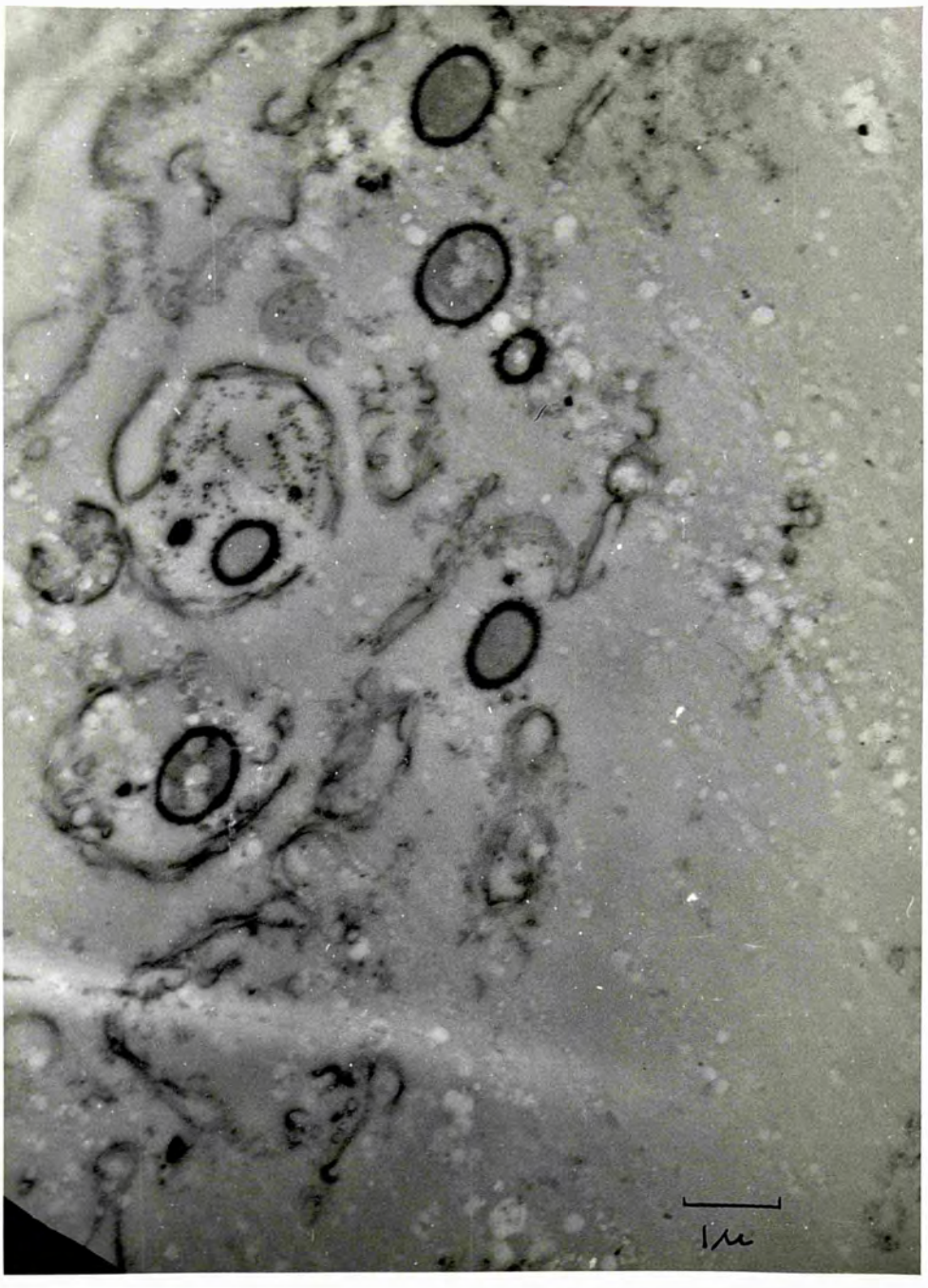

PLATE 76. Sieve tube showing incipient disintegration of the complex limiting membrane of the plastids.

PLATE 77. Optical micrograph of iodine stained preparation from fixed material to show the sieve tube starch which stains pink. Note the presence of starch on the sieve plate as well as away from it. X970.

permanganate fixed sections however did not show any difference in ease of demonstrating starch grains in the sieve tube. It was felt that if the plastid disintegrates releasing the starch grains, they will be difficult to show in electron micrographs in the absence of a stainable background, because starch commonly does not stain in osmic acid. This seems probable in view of the fact that when osmic acid fixed materials were subsequently stained in phosphotungstic acid and uranyl acetate they did show up starch grains more frequently, as can be seen from the Plate 78. Here the starch grains are still within the cytoplasm and they show the same general features, the largest one measuring about 0.86μ. Plate 79 shows another micrograph in which a large number of "released" grains are seen lying near the sieve plate apparently without any cytoplasm. In another plate (Plate 80) an intact plastid having many grains is shown. The plastid is still associated with the boundary cytoplasm; the black line on either side of the plastid could possibly be interpreted as a tonoplast.

The pink colour of the sieve tube starch contrasts sharply with the blue-black of the starch grains of the starch sheath. These features are shown in the coloured micrograph presented in Plate 81.

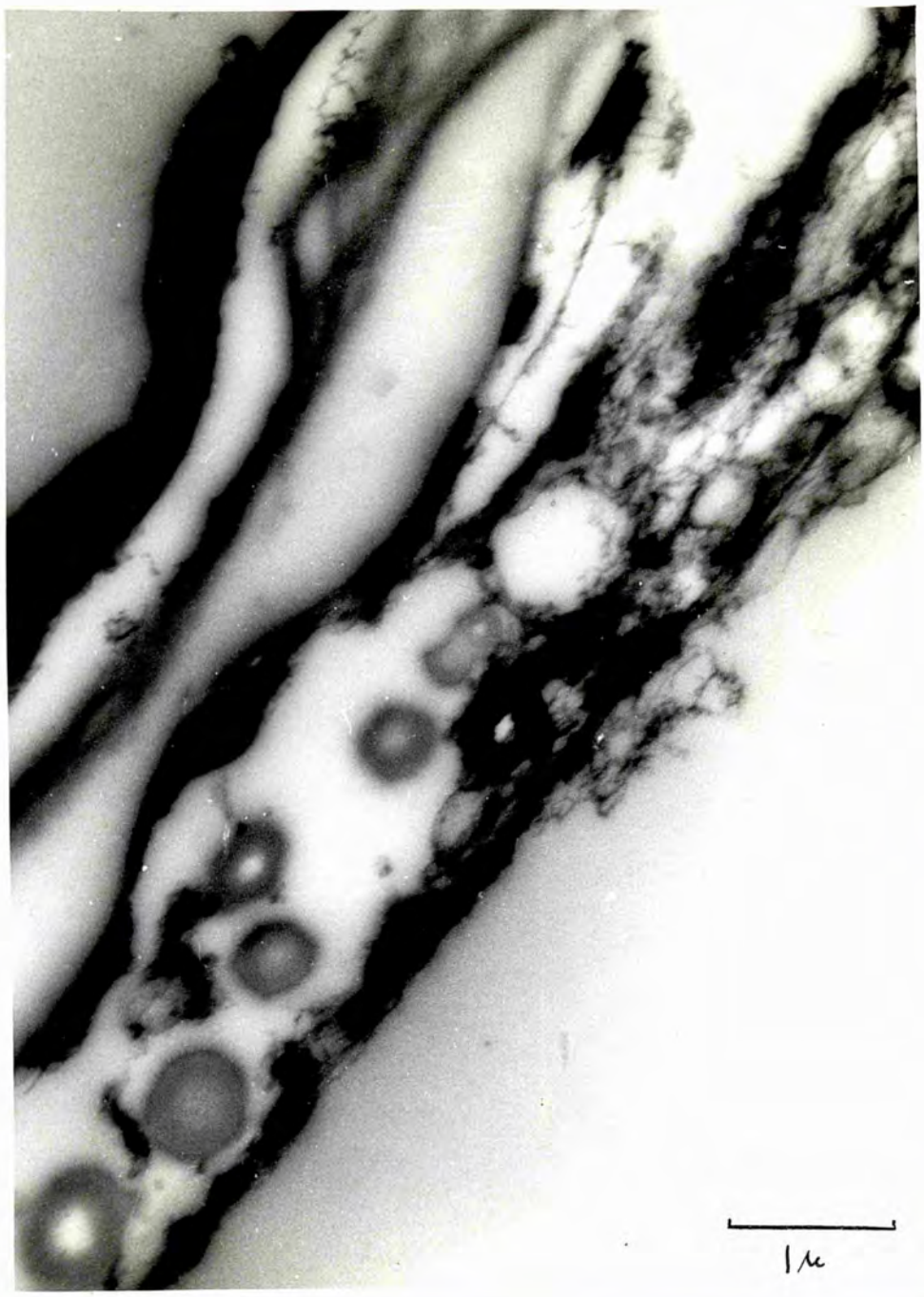

PLATE 78. Sieve tube showing "released" starch grains still within the cytoplasm. Some of them show clear centre. Note the structure of the cytoplasm. Fixed according to Schedule 2.

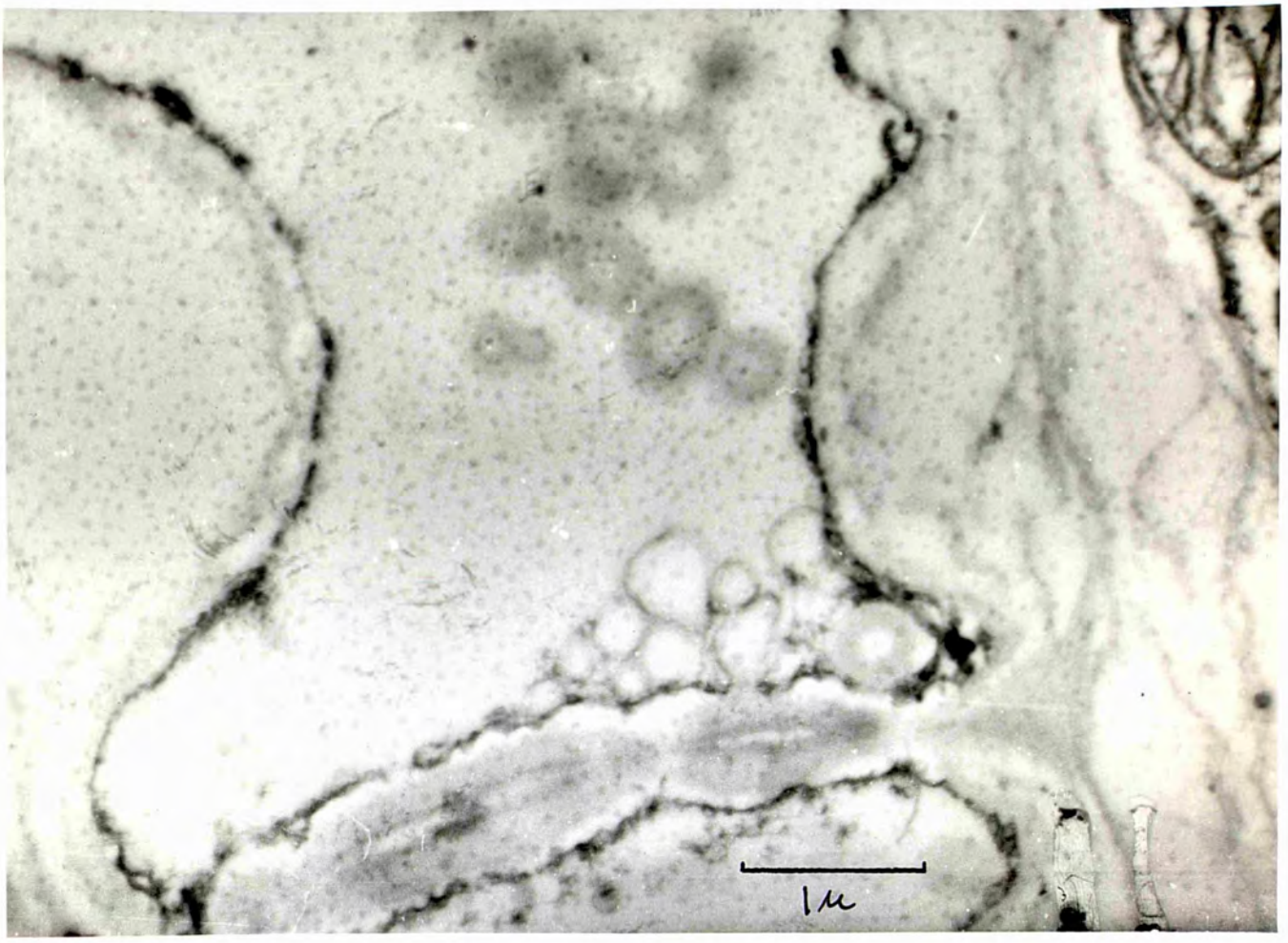

PLATE 79. "Released" starch grains near the sieve plate. They show clear centre.

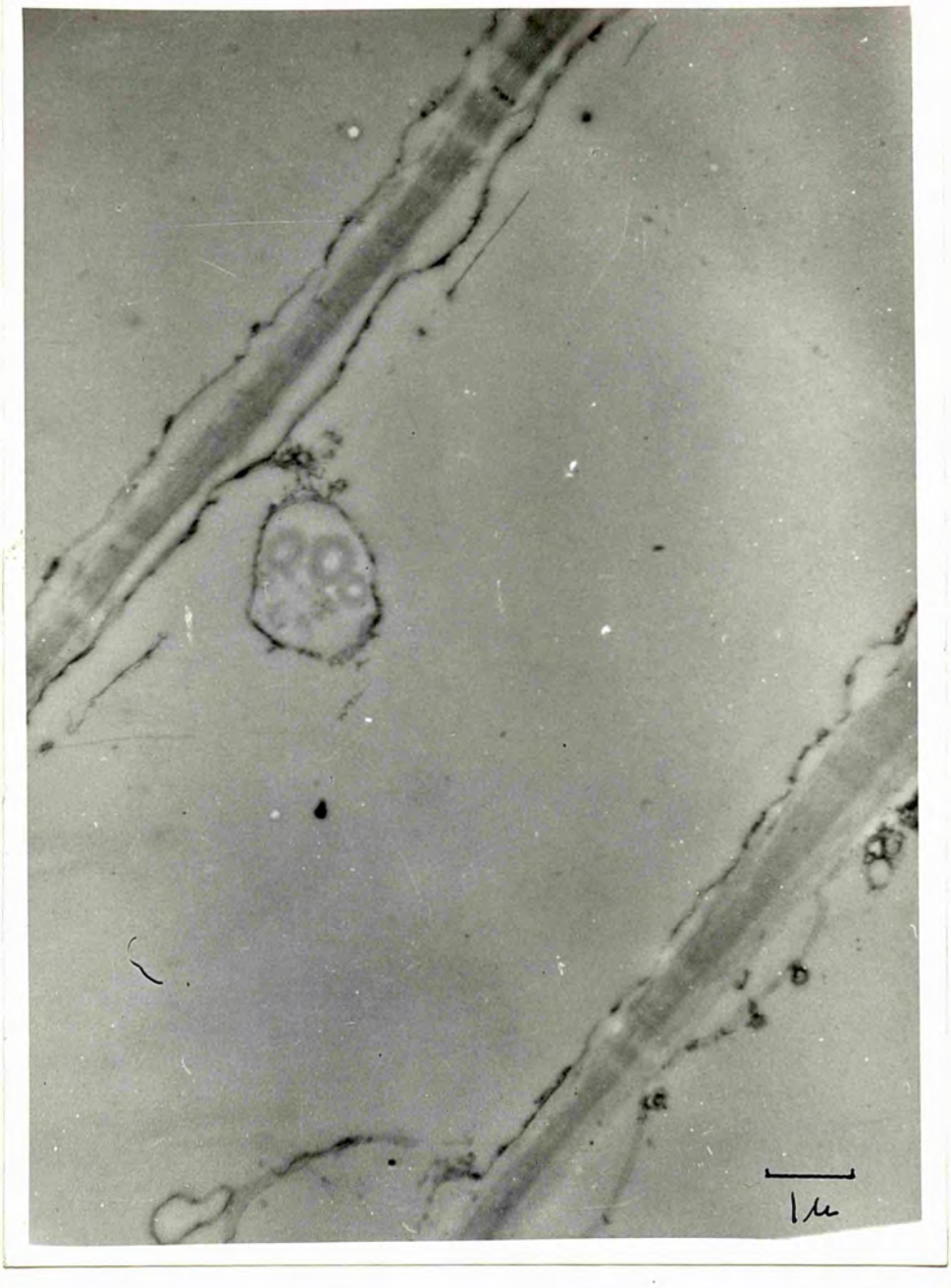

PLATE 80. Sieve tube showing intact plastid with starch grains which show clear centre. The plastid is associated with the boundary cytoplasm. Note a "tonoplast-like" structure on either side of the plastid.

PLATE 81. Optical coloured micrograph of iodine stained preparation from fixed material to show the pink colours of the sieve tube starch and blue black colour of the starch in the starch sheath. Some of the sieve tube starch show clear centre.

Passing on to the smaller inclusions found in the sieve tubes of Nymphoides, a micrograph of a sieve tube with a part of the sieve plate is presented in Plate 82. This shows a round body which measures about 0.7µ in diameter and resembles the mitochondria in the sieve tube of Passiflora as reported by Schumacher and Kollmann (1959). The "mitochondrion" here seems to be partly covered with what has been considered tonoplast by them. Similar inclusions in the sieve tube prepared according to Schedule 2 are presented in the next three plates. The first two plates (Plates 83 and 84) show these bodies away from the sieve plate whereas in Plate 85 it occurs just near it. They measure from 0.4 to 0.6µ in diameter. The fine structure of these bodies is not very clear. This is partly due to the microscope limitation and partly due to the fact that cristae of plant mitochondria are reported to be not so well-defined as those of animal mitochondria.

Besides the fairly definite and obvious inclusions mentioned, one often finds features in electron micrographs whose nature it is difficult to fix with certainty. Some of these are shown in Plates 86, 87, 88, 89 and 90. The structures in Plates 86 and 87 and in the centre of Plate 88 may be plastids, but the "lamellated" ones in Plates 89 and 90

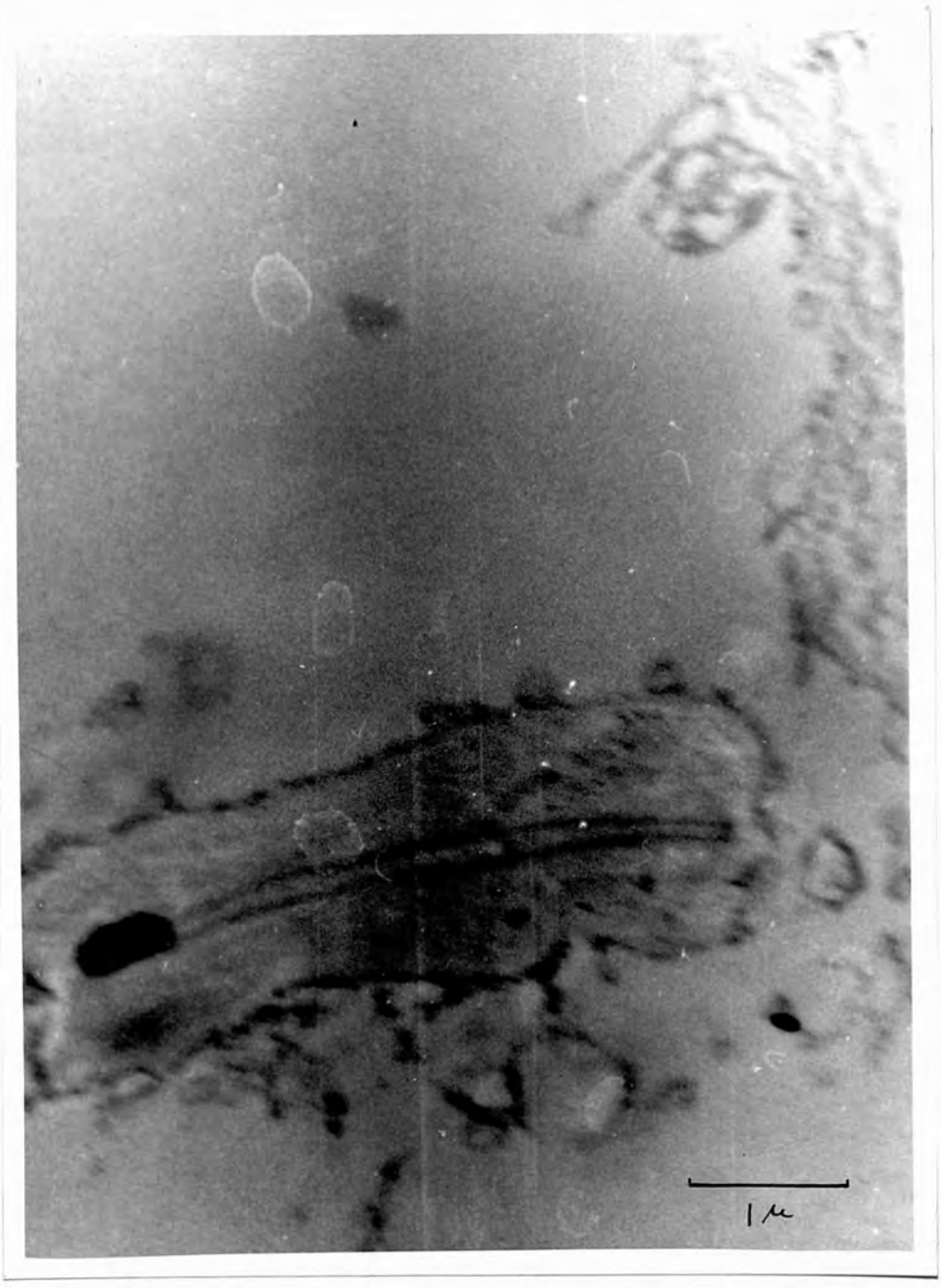

PLATE 82. Sieve tube, with a part of the sieve plate, showing mitochondrion-like inclusion. This is partly covered with a "tonoplast-like" structure.

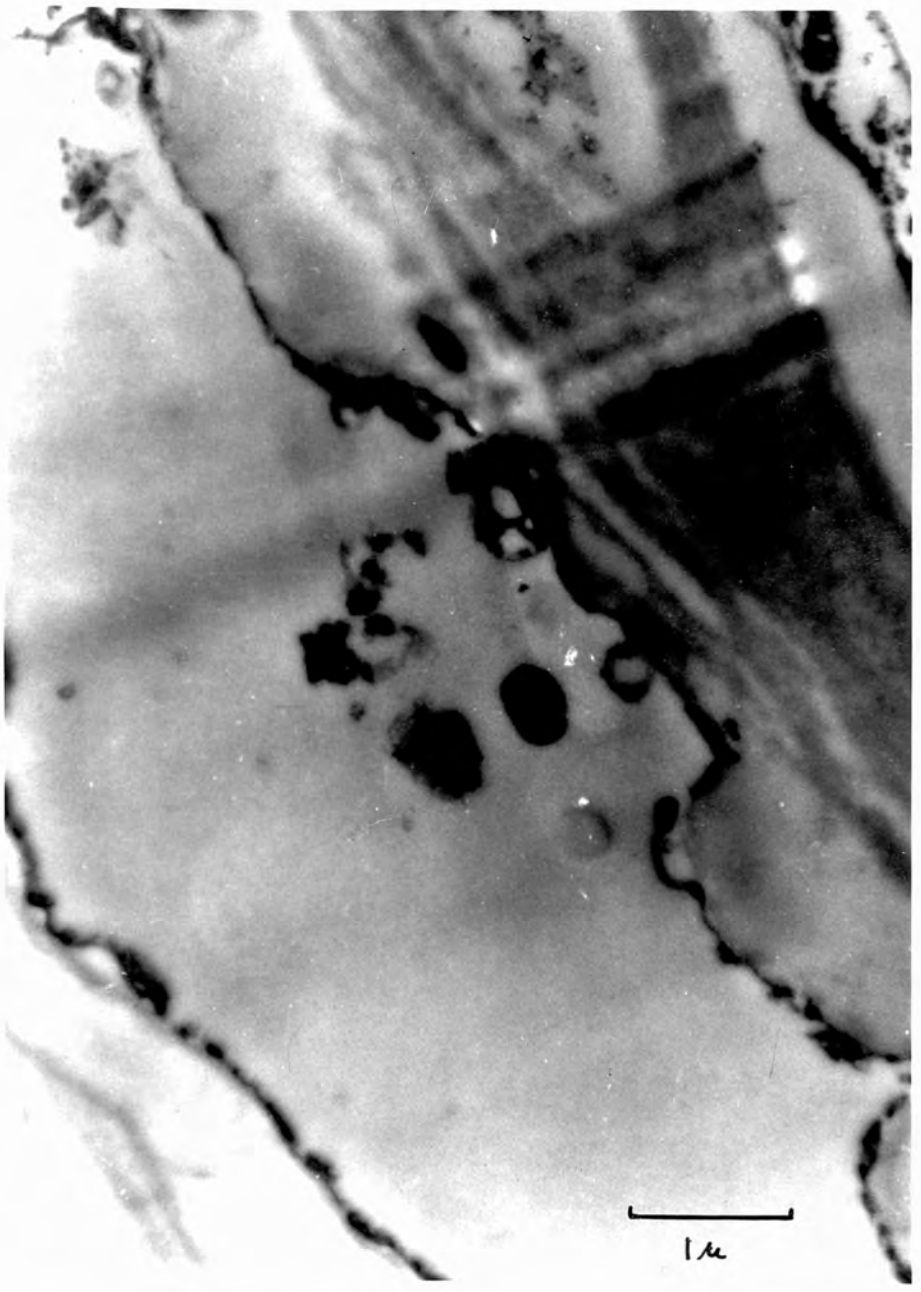

PLATE 83. L.S. sieve tube showing mitochondria-like inclusion.

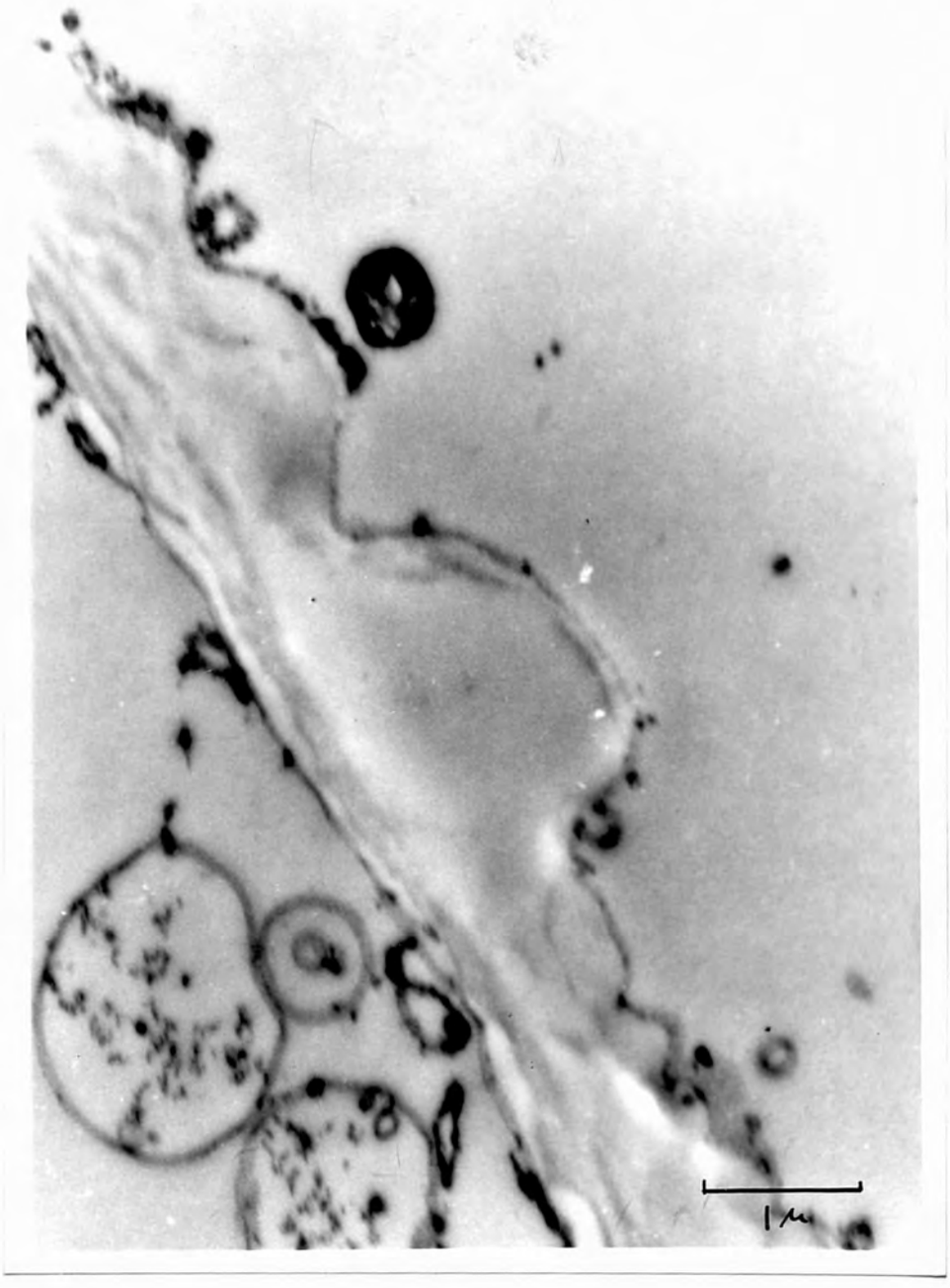

PLATE 84. Another sieve tube showing mitochondria-like inclusion.

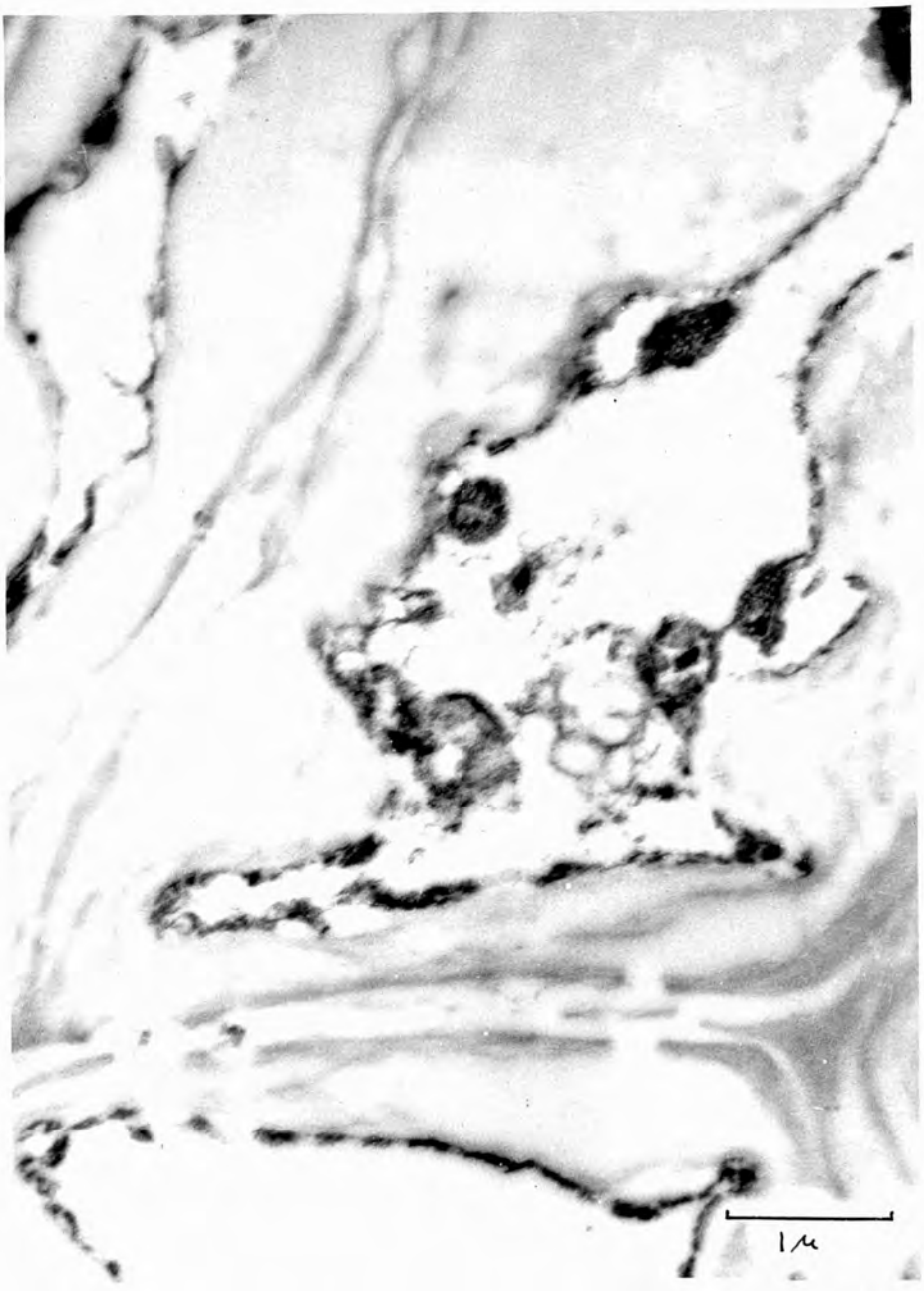

PLATE 85. Two mitochondria-like inclusions near the sieve plate without pores being sectioned.

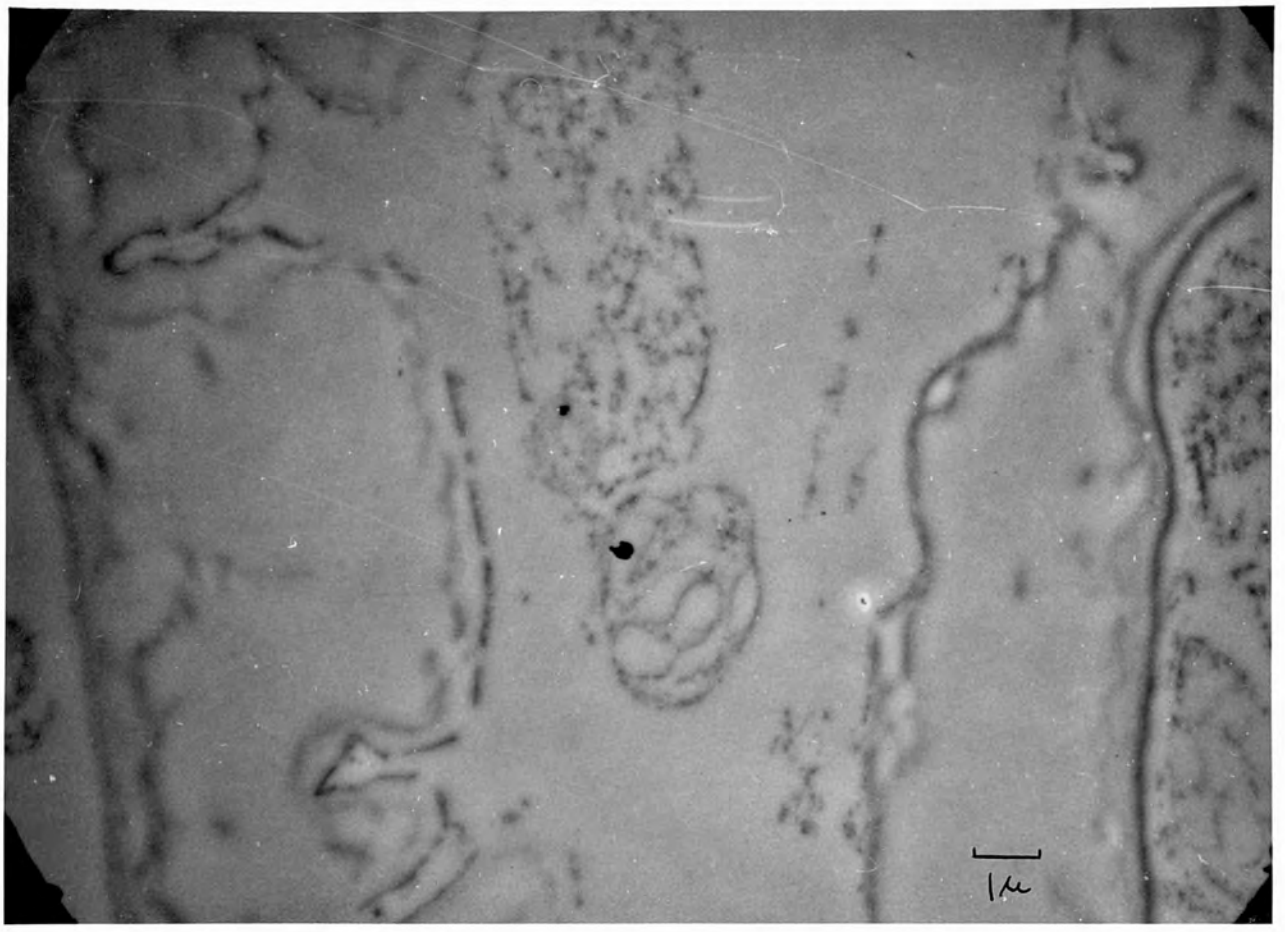

PLATE 86. Sieve tube showing "plastid-like" structure.

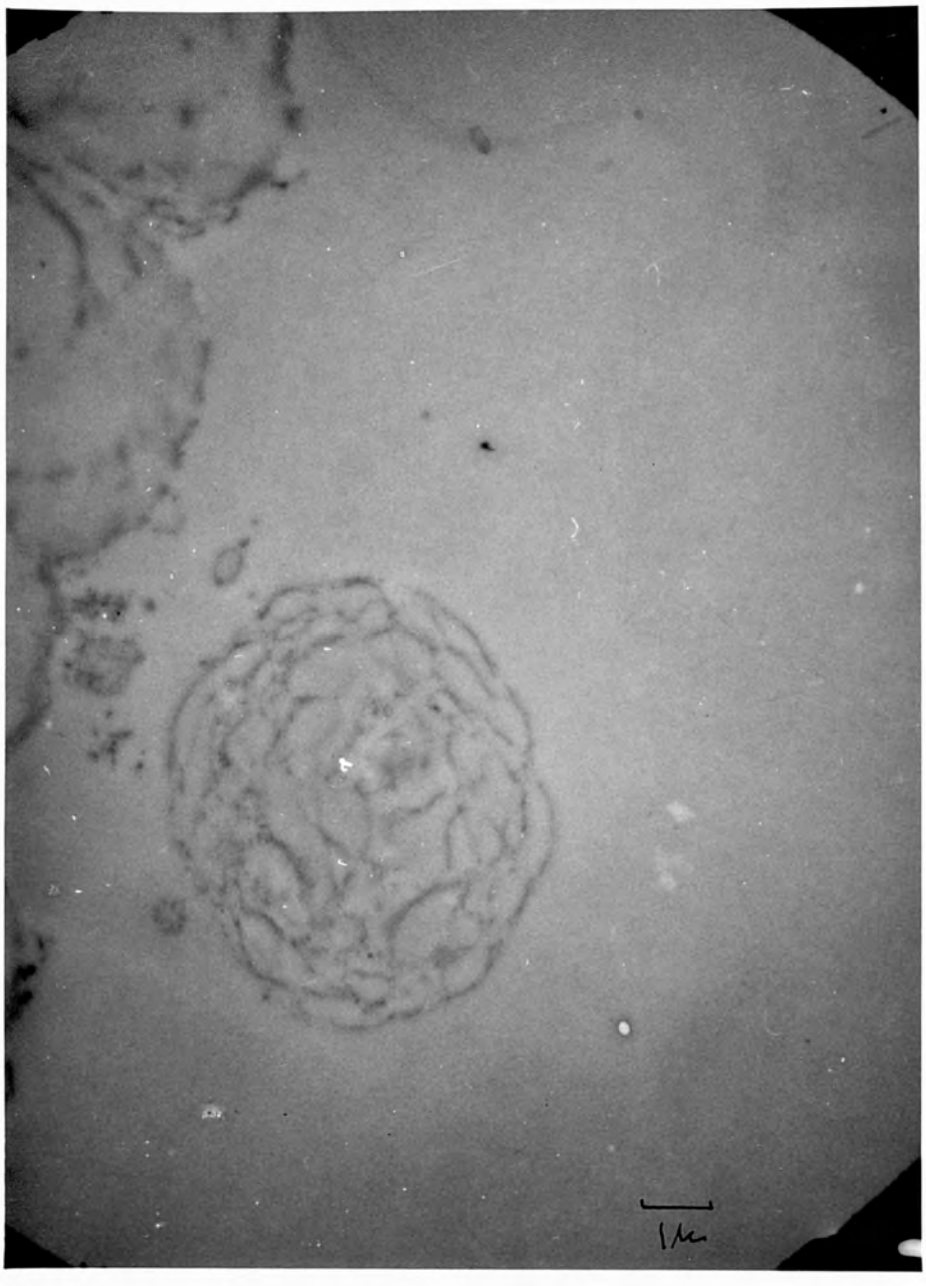

PLATE 87. Another sieve tube with "plastid-like" structure.

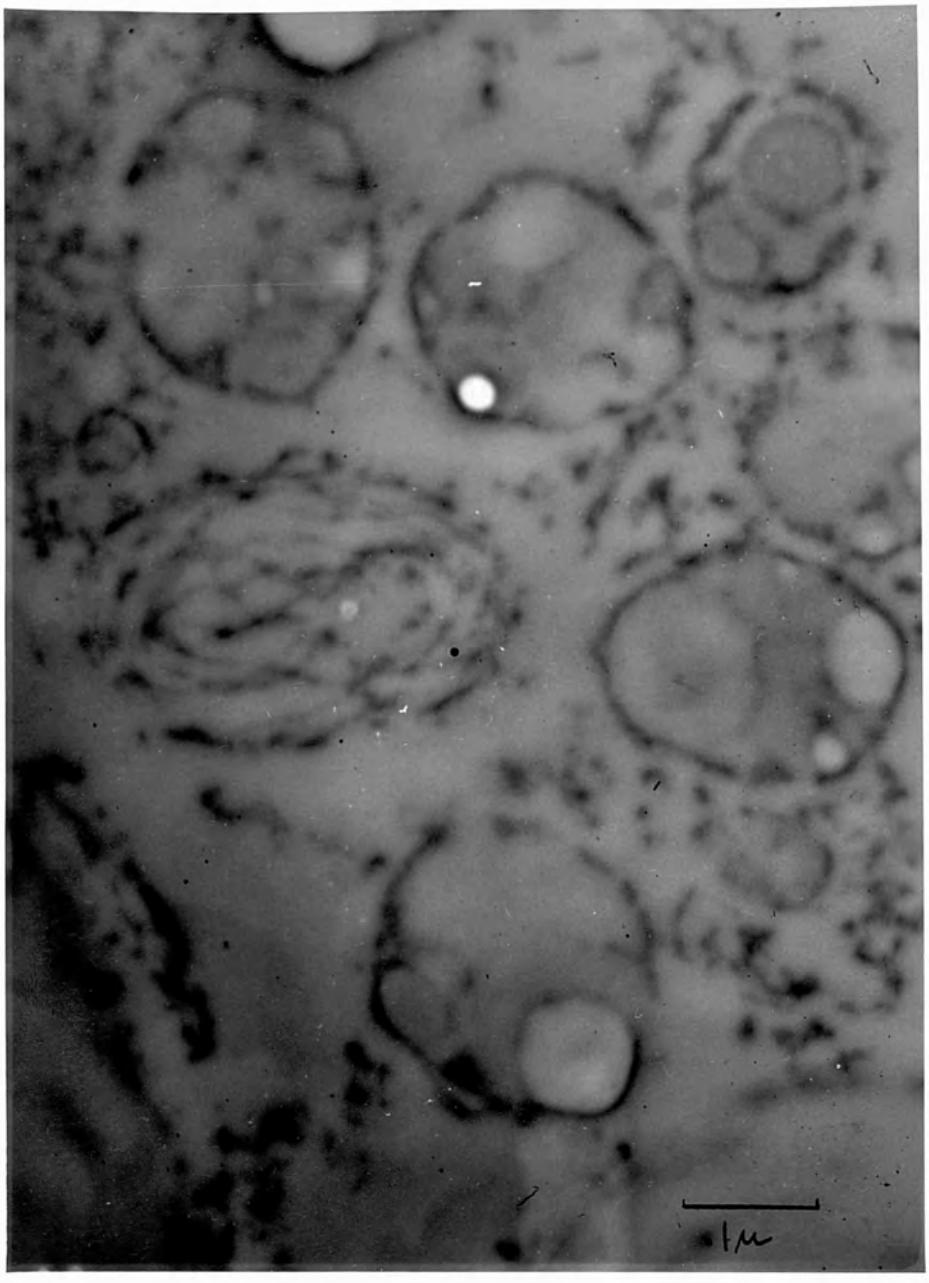

PLATE 88. A sieve tube with plastids and a "plastid-like" structure.

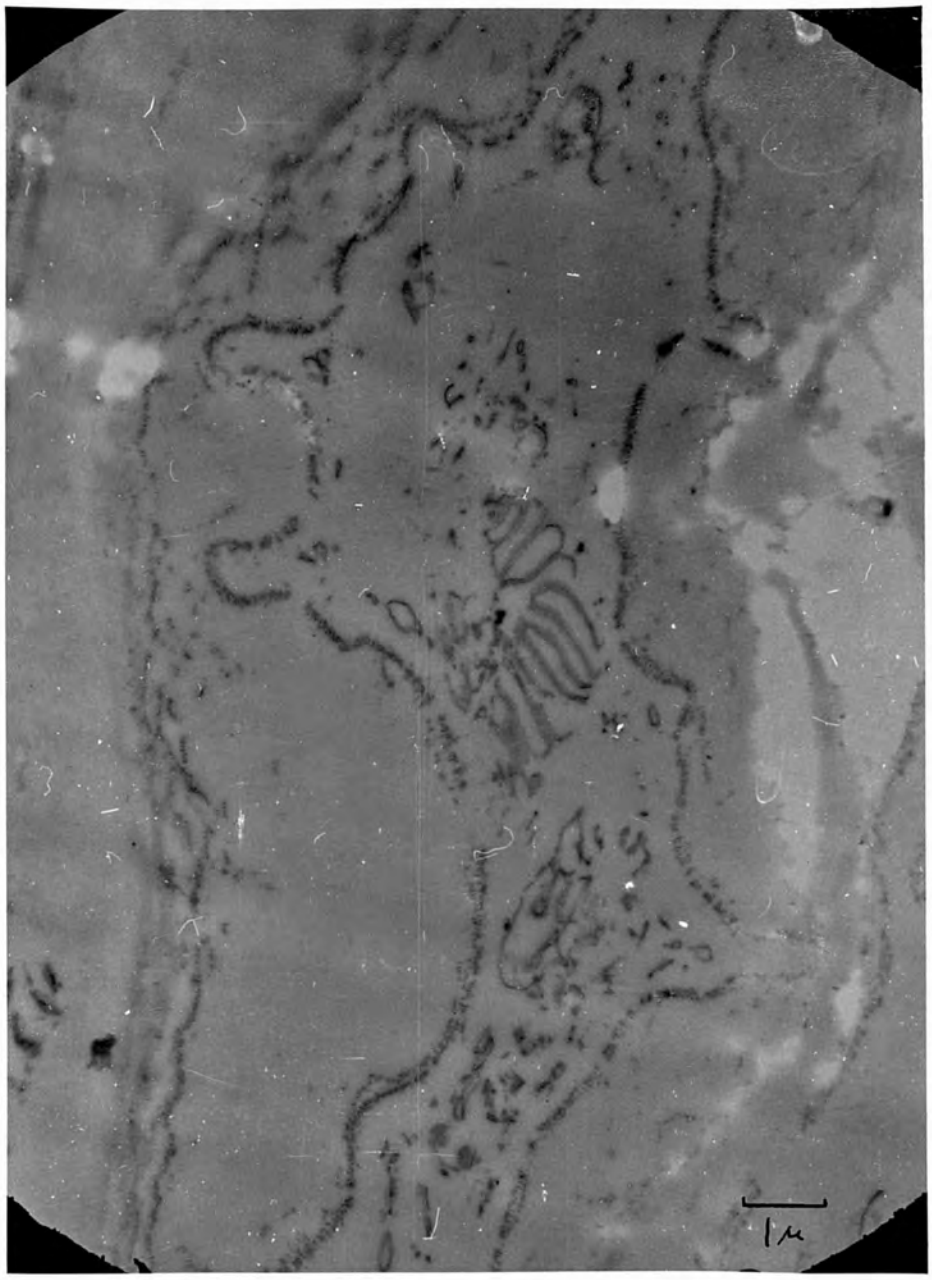

PLATE 89. Sieve tube showing lamellated structure. Fixed in $KmnO_4$.

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PLATE 90. Sieve tube showing sieve plate at one end and a lamellated structure at the other. Note the resemblance of the latter to the endoplasmic reticulum. A thin longitudinal section at right angles to the plane of the paper passing through the lamellated structure will give "dotted-line" appearance. Taken on Metropolitan Vickers EM6 electron microscope.

would seem to be features of the cytoplasm in the narrower sense. The last plate, taken on a Metropolitan Vickers EM6 microscope, shows a sieve plate at one end and the "lamellar" structure on the other. The latter shows an arrangement of the familiar trilaminar type, measuring about 90-140 mμ in thickness. The significance of the "lamellar" structure would appear in Discussion.

DISCUSSION

The first part of the discussion is devoted to the preparative technique for electron microscopy followed by cytological findings and their bearing on the mechanism of translocation.

The variable factors controlling the quality of fixation with osmic acid has been discussed by Palade (1954). His study was concerned with its suitability as a general purpose fixative. In the present study the effect of varying only two factors in the fixation method of Schedule 1 has been considered. These are duration of fixation and the tonicity of the fixative. As far as fixation of 0.5-1mm. thick petiole pieces is concerned, an hour's fixation does not seem to offer any advantage over half an hour's fixation. Since there is a danger of extraction of the specimen during long fixation, a short fixation, wherever possible, is to be preferred. Having this in mind, fixation of the petiole pieces was carried out mostly for half an hour in Schedule 1 as well as in Schedule 2. Regarding the tonicity it can be said that it did not appear within the compass of the present study and using the double cutting method to be a very important factor in the fixation of sieve tubes of Nymphoides. This seems surprising in view of the fact that the sieve tubes are reported to have a high sugar content.

Besides a high sugar content, sieve tubes have also a high turgor pressure which, if released suddenly, displaces their contents. It was to avoid this displacement of the contents that the double cutting method of obtaining petiole pieces was used. It was presumed that by this method longtitudinal surges of the contents due to sudden release of turgor would be avoided. It would seem that this method was only partly successful, the sieve plates in the central region of 0.5-1 mm. thick petiole pieces showing typical pore content, those towards the ends having apparently empty pores.

At this stage it was decided to follow the method of Currier, Esau and Cheadle (1955) to reduce the turgor pressure of the sieve tubes prior to fixation. Hepton (1957) and later Schumacher and Kollmann (1959) also used this method for reducing the turgor pressure. Details of this technique have already been reported in Schedule 2. Further, the contrast of the fixed material was increased by incorporating phosphotungstic acid and uranyl acetate. This method of preparation was far superior to the double cutting method of Schedule 1. Almost all the sieve plates showed pores with normal content. Preservation of the cytoplasm was much better. Incorporation of electron stain in the pretreated material was very successful in demonstrating

plasmodesmata and the sieve tube inclusions. It may however be mentioned that fixation according to this method fave good results at pH7, while fixation at pH5.8 was not very satisfactory. Fixation at higher pH, according to Hepton (1957), is also unsatisfactory. From all these it would seem that fixation of sieve tubes in osmic acid should be carried at a near neutral pH.

Recent reports have demonstrated the usefulness of $KMnO_4$ as a fixative for electron microscopy. It has proved specific for membranous structures. It has proved satisfactory in the case of root tips. Therefore following the method of Mollenhauer (1959) 0.5-1 mm. thick petiole pieces were fixed in $KMnO_4$ for 5, 10, 15 minutes. The petiole pieces were obtained by the double cutting method. The details are described in Schedule 3. There does not seem to be any difference in the quality of fixation following the different times of fixation. As far as the sieve tubes are concerned, fixation in $KMnO_4$ proved disappointing. It was difficult to demonstrate connecting strands in the pores which appear empty. This may be partly due to the double cutting method of obtaining material for fixation, and partly due to the swelling effect of the fixative, the middle lamella appearing wider. Nothing more can be said at the moment without further experimentation. It may however be

mentioned that the contrast was very poor. This is due to the lack of material stainable with $KMnO_4$ in the sieve tube. This difficulty can be overcome by increasing the overall contrast with use of other electron strains. Marinos (1960) has used successfully phosphotungstic acid and uranyl acetate for increasing the contrast of $KMnO_4$ fixed material in his study of the nuclear envelope of plant cells. $KMnO_4$ fixed material, when embedded in methacrylate, is reported by Luft (1956) to be difficult to cut with a glass knife. Some difficulty in cutting sections was also experienced in the present study. It is difficult to advance any explanation for this without further study. It may however be mentioned that Porter and Machado (1960) were successful in cutting sections of $KMnO_4$ fixed, methacrylate embedded materials with a diamond knife.

In spite of all these disadvantages, the use of $KMnO_4$ as a fixative served its purpose in demonstrating "released" starch grains and the presence of membranous structures in the sieve tube. The former were difficult to demonstrate in osmium fixed material according to Schedule 1. More will be said about the membranous structure later. Suffice it to say that the evidence for the presence of the membranous structures and their possible concentration in the sieve pores would seem to pay an attempt to develop a successful technique

of fixing in $KMnO_4$; specially in view of its specificity for the endoplasmic reticulum and other systems.

Now the results of the cytological investigation will be discussed. Since the structure of the sieve plate has already been described in detail, attention will be drawn here only to some of the important features. First of all evidence has been provided that the pores in the sieve plate are filled with non-vacuolated connecting strands joining the cytoplasm of the superposed sieve elements. The connecting strands show dense staining in osmic acid and do not show any indication of a break in the middle. Thus the view of Hill (1908) regarding the hollow nature of the connecting strands is considered untenable, there being no vacuolar continuity through them. Absence of any break in the middle of the connecting strands makes for the cytoplasmic continuity from one sieve element to the other. This finding goes against the views expressed by Mangenot (1926) and Salmon (1946) and later by Velz (1952) on the basis of the electron microscopic studies. Further the continuity of the cytoplasm through the pores of the sieve area on the lateral wall as well as continuity of plasmodesmata across the wall has also been demonstrated in the present study. The finding of Hepton (1957) regarding the continuity of the plasmodesmata is thus confirmed. In fact their continuity across the wall has been in recent times unmistakably demonstrated by the electron microscopic studies of Whaley,

Mollenhauer and Kephart (1959), Marinos (1960) and Porter and Machado (1960). These studies do not support the possibility, suggested by Esau, Currier and Cheadle (1957) that the continuity of sieve-area strands is not a consistent feature and depends on the relative thickness of the strands.

Having discarded the views of Hill (1908) and Mangenot (1926) regarding the nature of the connecting strands, attention is naturally drawn to the views of Crafts (1939) and Schumacher (1939). Both of them consider the connecting strands to be solid cytoplasm; but while Crafts (1939) denies the presence of a tonoplast, Schumacher (1939) thinks that sieve tube elements retain it. As remarked earlier, the evidence of the present study on the existence of a tonoplast is inconclusive. Esau (1950) does not agree with the purely cytoplasmic nature of these strands and thus calls them by a non-committal term "connecting strands". She considers that slime is in some way associated with the cytoplasm in the connecting strands. This might account for their increased avidity for stains. Esau (1948) claims that slime is unmistakably present in the connecting strands in Vitis. Using this material in his electron microscopic studies Hepton (1957) finds no evidence for the presence of the slime in the connecting strands. Crafts (1939) is also of the opinion that the presence of slime is an artifact. The present study also does not seem to give any evidence for the association of slime with the connecting strands.

To explain the osmiophily of the strands Hepton (1957) made the assumption that these are cytoplasmic structures associated in some unknown way with a material having strong affinity for osmium and whose nature remains to be determined. In our opinion connecting strands are truly cytoplasmic in nature. We would try to explain their osmiophily a little later, after the nature of the sieve tube cytoplasm is discussed.

Crafts (1939a, b, 1948, 1951) has repeatedly advanced the view that the sieve tube cytoplasm is "denatured". This view is essentially based on the absence of a nucleus and the tonoplast in mature functional sieve tube and their loss of semi-permeability. Sieve elements are also reported to be of low metabolic activity. This view of Crafts and its implications is not accepted by others. Though the absence of nucleus is confirmed for a large number of species, Schumacher (1939) maintains that a tonoplast is present and the mature sieve tubes can be plasmolysed. A recent study of this problem by Currier, Esau and Cheadle (1955) has confirmed, it seems beyond doubt, the semi-permeability of the sieve elements, though they were unable to detect a tonoplast. Hepton (1957) is of the opinion that the cytoplasm of Vitis has a fine reticulate appearance and does not correspond to Crafts' picture of the denatural protoplast of the functional sieve tube. We are also inclined to this view.

At this stage attention may be drawn to the Plates 46-49 and 50 showing cytoplasm of the sieve tube and a parenchyma cell respectively. Though the cytoplasm in both cases seem to be somewhat coagulated, their similar appearance would suggest that initially they might also have been similar. Since cytoplasm of a parenchyma cell is not considered to be denatured, it seems from this that neither is the cytoplasm in a living and functional sieve tube. More evidence for this conclusion will now be presented. Plates 58 and 59 show the fibrils of the sieve tube cytoplasm arranged in a reticulate manner. These "fibrils" seem to be packed compactly in the sieve pores in longitudinal direction. This view does not seem to be incompatible with what has been shown by Hepton (1957) in shadowed material and by Schumacher and Kollmam (1959, private communication) in an unshadowed preparation. The higher magnification micrographs of Plate 59 are shown in Plates 60, 61 and 62. It would tentatively appear that each "fibre" of the cytoplasm, as remarked earlier, consist of a double membrane separated by a clear space. The double membrane nature of the longitudinal "fibres" in the sieve pore can also be conjectured. From the double membrane nature of the threads of the network it would seem that they belong possibly to the system now well known as the "endoplasmic reticulum" after Palade (1956). If this very tentative view is accepted, then the usefulness of $KMnO_4$, which is specific for membraneous structures, as a fixative can well be visualised. The results of $KMnO_4$ fixation, as mentioned before, are shown in Plates 89 and 90. The last

plate shows very clearly a "lamellar" structure of the familiar trilaminar type, each profile measuring about 90 - 140 mμ in thickness. The size of the profiles, their composite structure, consisting of a relatively light centre flanked by a dense thin membrane, and their apparent lack of internal structure would seem to suggest for them a place within the endoplasmic reticulum. If the presence of the endoplasmic reticulum in sieve tubes could be well established it would seem to mean that the sieve tube cytoplasm is anything but denatured. If the views presented here are substantiated by further studies they might explain the osmiophily of the connecting strands which seem to be revealed here as consisting of numerous tubular threads of the endoplasmic reticulum arranged compactly in the longitudinal direction. In any case the connecting strands would seem to belong to the cytoplasm proper.

So far as inclusions are concerned, the present study, as reported earlier, confirms their presence in the sieve tube. These inclusions are plastids with starch and mitochondrion-like structures. Whether the latter are mitochondria or spherosomes as reported by McGivern (1957) is difficult to decide. On the basis of structural similarity with the mitochondria described by Schumacher and Kollmann (1959) they could be interpreted as mitochondria. The latter workers have also reported lamellar structures comparable to Golgi-bodies - further evidence of the presence of the endoplasmic reticulum.

Before discussing the nature of the undermentioned inclusions in Plates 33-37 attention may be drawn to the "lamellar" structure shown in Plate 90. The latter, as mentioned before, seems to belong to the endoplasmic reticulum. It is in this light that the undetermined inclusions, whose identity in the beginning was a difficult problem, can be viewed. It would seem that they could best be interpreted as surface sections of the cytoplasm located on one of the "humps" of the nacreous wall protruding into the lumen of the sieve tube. If this is so, then the peripheral cytoplasm would seem to contain a very interesting development of the endoplasmic reticulum consisting of more or less parallel and longitudinally oriented tubules. This interpretation would also explain a feature which has been frequently noted in longitudinal sections, the "dotted-line" appearance of the inner limit of the nacreous wall. It can easily be seen how this would arise if the lamellar structure seen in face view in Plate 90 were cut thinly in longitudinal sections at right angles to the plane of the paper (see Fig. 5a) This possibility is

Figure 5a

further noted in the legend to Plate 90. At the moment, however, the suggestion - that the boundary layer of the cytoplasm in the sieve tubes is the seat of a rather unusual

development of the endoplasmic reticulum, and that the tubules of this reticulum run rather densely into and through the pores in the sieve plate - must remain a very tentative one.

The last part of this discussion deals with the implications of the present cytological findings for the prevailing theories of the transport mechanism. These theories have been outlined in the earlier part of this thesis; here they will be considered briefly in order.

The cytological findings of electron microscopic studies on the phloem are potentially of great importance in connection with the mechanism of transport. One finding stands out very clearly from the present as well as from a number of earlier studies; that the pores in the functional sieve plates are occupied, almost certainly, by dense cytoplasm. It is true, of course, that when the material is pre-conditioned for cutting by several hours' treatment with solutions which reduce its turgor, the process of conduction ceases and the sieve tubes may have time to readjust themselves to some extent. But it seems unlikely though not impossible that this readjustment would result in vacuolated strands becoming solid; and in any case there is the evidence from the material processed according to Schedule 1 (when the phloem actually in the process of conducting was severed by two close cuts and fixed within a few seconds), which it will be remembered revealed the same dense cytoplasm in the pores.

Granted, however, that the pores in the functioning state are filled with cytoplasm, is this to be regarded as in a process of rapid longitudinal streaming or is it stationary? There are more-purely-physiological reasons for favouring the latter; but so far as the electron microscopic evidence goes, the arguments which may be put forward are as follows:-

1. As Hepton (1957) has suggested, the material filling the pores seems to be chemically different from that constituting the cytoplasm in the nearby lumens. It would seem unlikely that a rapid chemical change should take place as the latter streamed into, and out of, the pores; hence the inference is that the cytoplasm is stationary. This suggestion is strenthened by the evidence of the present study in which material was not subjected to the lengthy preconditioning, but was actually fixed while conducting.

2. The flow in the lumens and the pores is characterised by a very low Reynold's number (see footnote).

Footnote. Calculation of Reynold's number.

The Reynold's number for a flow through a circular tube is defined as :

$$R = \frac{V.D}{\gamma}$$

where V = Velocity

D = Diameter

γ = Kinematic viscosity = $\frac{\text{Viscosity}}{\text{Density}}$

Assuming that viscosity and density of the translocate are

Thus it will undoubtedly be streamlined apart from the exceptional properties of the flowing substance. The later micrographs of the pore contents may indicate either cytoplasmic flow or merely a cytoplasmic structure correlated with a rapid streaming of a watery solution through it; but the structure of the cytoplasm adjacent to the pores but just within the lumens of the sieve tubes hardly suggests that the living matter is streaming. Unfortunately the

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approximately of the same order as that of water at 20°C we get a value for

$$\gamma = \frac{0.01}{1} = 0.01 \text{ poise/gm per cm}^3.$$

Taking an average velocity of transport of 60 cm/hr and an average diameter of the sieve tube of 20 μ and substituting these values

$$R = \frac{\left(\frac{60}{3600}\right) \times (20 \times 10^{-4})}{0.01}$$

$$= \frac{1}{300}$$

Since a value for Reynold's number of less than 2000 is considered to be characteristic of laminar flow, a value as low as $\frac{1}{300}$ obviously indicates a streamline motion. This clearly applies also to the pores.

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micrograph was from material which was not fixed while in actual process of conduction; and hence the evidence is not unequivocal. Further, cytoplasm which is streaming does not necessarily show evidence of it by streamline contours (see for instance, the work of Shatkin and Tatum (1959) on Neurospora). However, since the parallel orientation within the pores persists after several hours' pretreatment with its enforced quiescence, it hardly seems likely that it represents cytoplasmic streamlines. It would seem to be much more likely that it is evidence of a more or less static configuration.

3. If the cytoplasm is streaming into, and out of, the pores one would expect accumulation of plastids, released starch grains and mitochondria at the sieve plates since these would not be able to, due to their large size, pass through the sieve pores. Presence of these inclusions within the cytoplasm away from the sieve plates shown in the micrographs hardly suggests any streaming movement of the cytoplasm through the sieve pores.

The implications of these considerations is obvious. The outstanding problem facing the pressure flow theory of Münch lies in the fact that it seems to require large pressure gradient to be operative in the sieve tubes. There would appear to be only two possible ways out of this difficulty; either the sieve plate pores are open, or the cytoplasm itself streams rapidly through them. To both these possibilities the evidence of the present study is

decidedly negative, confirming in the case of quite a different subject plant the findings of others.

The next theory, that of protoplasmic streaming, again finds little support from the present study. It was stated in the introduction that two features of this mechanism might be expected to impress themselves on the cytoplasmic morphology; there should be evidence either of a "cyclosis pattern" in the cytoplasm, or of the cytoplasm streaming through the pores - perhaps both together. The last point has been dealt with above, and so far as the former is concerned the general orientation of the "fibrils" of the cytoplasm in the vicinity of the sieve plate is hardly suggestive of cyclosis.

The two remaining theories, that of "activated diffusion" and the electroosmotic theory, can be considered together. The general impression given by a good micrograph of the sieve plate region, and sometimes of the general lumen - see for instance Plate 90 - is that a bulk flow of the contents is taking place down the column. This is suggested by the longitudinal orientation of what has been considered the endoplasmic reticulum both in the tube itself and within the pores; and this is in general agreement with theory. It is difficult to say whether the sieve plate structure favours or otherwise the "activated diffusion" hypothesis; but it would seem at least to be the sort of thing which might be required by the electroosmotic theory.

At least, it seems quite agreeable with the suggestions of the latter.

SUMMARY

The present thesis starts with a short review of the problem of the channel concerned in translocation followed by a short discussion of the prevailing theories of transport. The structural characteristics of the sieve tubes, the main tissue concerned in translocation, are considered in relation to the various theories. In this connection the structure of the sieve plate as described by various workers has been reviewed and the need for more study to elucidate it has been considered.

The reasons for choosing Nymphoides petiole as the subject material have been enumerated. A short description of the fixation techniques suitable for electron microscopy in general and sieve tubes in particular follows. In the light of this three different approaches for the preparation of the present material for electron microscopy have been tried. Two of these involved osmic acid as the fixative. In one the plant material was fixed while in the process of actually conducting by using a "double-cutting" method; in the other, the material for fixation was given several hours of pretreatment to reduce its turgor. The merits of these two techniques have been considered. A third method tried involved $KmnO_4$ as fixative. The results of this approach have been considered in relation to those of the first two.

The sieve tube structure of mature elements is described in detail and compared with that of the sieve tubes obtained from the petioles of young and senescent leaves.

It has been shown that the cytoplasm of the sieve elements, though scanty and peripheral in position is not denatured. Evidence has been brought forward to suggest that the sieve tube cytoplasm contains a remarkable development of the endoplasmic reticulum. The threads of this reticulum appear to run longitudinally in the pore where they seem to be packed compactly. Evidences for and against the stationary nature of the sieve tube cytoplasm have been considered and it is concluded that the cytoplasm probably does not stream through the pores. No conclusive evidence for the absence of a tonoplast in the sieve element has been obtained from the present study.

A number of inclusions of definite identity have been found in the sieve tubes both near the sieve plates and further away from them, and these are briefly described. They include mitochondria, plastids and starch grains.

The sive tubes of Nymphoides show markedly thick and crenulated nacreous walls both in the electron microscope, and with the rather greater difficulty with optical examination. These walls do not seem to be highly hydrated. The matrix of the wall, in electron micrographs, shows black "inclusions", which in many cases resemble the inner limit of the wall.

The detailed study of the wall structure could not however be carried out with the apparatus available. It would appear that the nacreous wall grows so fast that it envelops the peripheral cytoplasm. The inner limit of the

nacreous wall quite frequently shows a dotted-line appearance. The meaning of this is considered.

Lateral connecting strands between sieve elements, companion cells and parenchyma cells have also been demonstrated. The nature of these is briefly discussed.

Lastly the structural characteristics of the sieve plate as demonstrated in this study have been considered in relation to the prevailing theories of translocation. It is concluded that they raise serious difficulties for both the pressure-flow hypothesis and also for the proroplasmic streaming theory. They would seem to fit reasonably well into an activated diffusion hypothesis or into the electroosmotic theory.

PHYSIOLOGICAL INVESTIGATION

INTRODUCTION

Translocation, like any other physiological process, is governed by a set of factors, both internal and external. Some of these control the direction of movement whereas others affect its rate. Among the latter group, temperature is probably one of the most important and most controversial; in fact, reports of the effect of temperature on the velocity of the process (i.e. on its Q10) are extremely conflicting.

There are two main schools of thought on this subject, one led originally by Curtis and the other by Went. According to the former the Q10 of translocation is of the order of 1.5, whereas according to the second its value is less than 1. Thus Curtis and Herty (1936), as a result of an investigation to determine the effect of temperature on translocation from leaves, reported a Q10 of more than 1. This was subsequently confirmed by Hewitt and Curtis (1948). Contrary to this view, Went (1944) adduced evidence that the Q10 of translocation was less than 1 in tomato plants; and further support for a low Q10 of sugar translocation in tomato plants was obtained by Went (1945), Went and Engelsberg (1946), Went and Carter (1948) and Went and Hull (1949). Hull (1952), feeding C^{14} labelled sucrose to one leaf of sugar beet and tomato, also found increased translocation at

a lower temperature (1-3°C). A low temperature coefficient has also been reported for chili pepper by Dorland and Went (1947). However, in recent years another group of workers led by Swanson has joined in this controversy. Swanson and Bohning (1951) have studied the effect of petiole temperature on the translocation of carbohydrates from the bean leaves and have reported a Q10 value of more than 1. A similar result was obtained by Bohning, Swanson and Linck (1952) by varying the hypocotyl temperature. A year later Bohning, Kendall and Linck (1953) found that the Q10 for carbohydrate translocation in tomato in the range of 12-24°C was approximately 1.5. Using radio-active $C^{14}O_2$ Vernon and Aronoff (1952) also report that increase in temperature brings about increased rate of translocation. Swanson and Whitney (1953) have found marked inhibition of translocation of radio-active P^{32}, K^{42}, Ca^{45} and Cs^{137} when the petiole temperature was lowered to about 5°C.

In most of these studies the rate of translocation has been measured only very indirectly in terms of such measurements as the loss of dry weight, the rate of bleeding of decapitated shoots, or stem elongation. Thus, the temperature coefficient obtained has been in general a value relative to an overall process involving perhaps mobilisation of starch, movement into sieve-tubes, transport

proper, growth responses, as well as the complex phenomenon of root pressure. This may perhaps be the clue to the conflicting reports of the value of the Q10. The matter obviously needs further investigation, and the first prerequisite for this is the development of a method giving a more direct measurement of the translocation velocity proper. This is by no means an easy task; more especially in view of the fact that, the precise mechanism and pattern of the translocatory mechanism being still unknown, it is therefore not possible as yet to specify quite unambiguously what is, in fact, being measured. However, it should be possible to get considerably nearer to the essence of the problem than has so far been done.

Prior to the availability of tracers, the rate of translocation was obtained fairly directly either by measuring the mass of material transported per unit of time by assuming a concentration through known cross-sectional areas, or by measuring the increase in the concentration of the translocate at measured distances from the assimilating tissues after definite time intervals. Less directly, it was measured by noting the morphological responses of tissues at measured distances from the point of application of the substance producing the response. As has been remarked earlier, all these methods are complicated by the

intervention of other processes. Immediate determination of the rate was hampered by the inability to distinguish translocating material from the material already present in the sieve tube. Within fairly recent years the use of tracers has offered a more direct means of measuring the rate of translocation, but hitherto no fully satisfactory method of using them has been evolved. Vernon and Aronoff (1952), using $C^{14}O_2$, reported a translocation velocity of 84cm/hr for the photosynthate. This was calculated by extrapolating the activity curves for a 5- and a 20-minute translocation involving two different plants and the assumption that the front of activity is at the intercept of the curve. Rather similar objections are valid for the method of introducing radio-active sugars by foliar application as pointed out by Nelson and Gorham (1957). Later on Nelson and Gorham (1957) introduced labelled sugars through the petiole stump, but here in addition there is the objection that there is the obvious possibility of xylem transport.

One of the best methods from the theoretical point of view of estimating the velocity of the phloem stream itself is probably to measure the wavelength of the natural 24-hour period in sugar concentration down the stem. This method

was first used by Huber, Schmidt and Jahnel (1937) and recently in a modified way by Zimmermann (1958). The result of earlier workers is not very satisfactory as the concentration "crests" are not very well marked. This is not very surprising in the light of Zimmermann's (1958) observations: according to him certain conditions must be satisfied before this method can yield convincing results. Some of these conditions are: (1) a long straight tree trunk without branches, (2) symmetrical live crown and (3) uniformity of light falling on the leaves. It had earlier been felt that use might be made of the method employed many years ago for the somewhat analogous problem of determining the thermometric conductivity of metal bars: applying a periodically varying source of radio-activity to the leaf of Nymphoides and measuring the periodicity of the activity in its long uniform petiole. It can be seen that such an object would fulfil rather well the conditions later suggested by Zimmermann (1958). Further it was felt that the longitudinal pattern of radio-activity might distinguish decisively between a diffusive mechanism and a true mass flow, since Fick's Law implies an extremely rapid falling off in amplitude from one crest to the next. It was therefore felt that this method, using radio-active Cl^{40}_2

and the fairly short period of half an hour or so, might yield more precise results from which to obtain the velocity of translocation. Studies at different temperatures could then be carried out to determine the Q10 of the transport process itself, the petiole being readily maintained at different temperatures while the leaf and rootstock remained at that of the laboratory. This method further held out the promise of yielding a measure of the velocity itself unaffected by such complicating variables as the rate of photosynthesis, mobilisation or growth response. These advantages seemed great enough to warrant embarking on some preliminary trials in spite of the obviously problematical chance of success.

MATERIALS AND METHODS

1. Growing the plant

As mentioned earlier, growth of *N. peltatum* tended to be poor during winter. This was probably due to poor light and low temperature, though air contamination in the laboratory and natural dormancy were also possible factors. Arrangements for a more favourable light period and temperature were however made, and these enabled the plant to continue its growth somewhat more healthily but still not entirely satisfactorily. The plant was grown in seven-inch pots kept in a tank about eighteen inches deep in the laboratory window under a continuous supplementary tungsten light intensity of 150 ft. candles from a 100 watt spot bulb. The bulb was immersed in water in a glass jar to filter off the heat rays. Arrangement was also made for the continuous cooling of the water in the glass jar by a simple convection system. The water in the tank was kept at a thermostatically controlled temperature of 20-23°C.

Some trouble with a bacterial scum which formed on the water was kept in check by a simple method of flushing the surface, fresh tap water being run in below and the surface layers drained off into a submerged funnel. Under these conditions growth was moderately good throughout the winter.

2. Plan of the experiment

It was planned to begin with to supply ordinary CO_2 to the leaf in a perspex leaf chamber for several hours at a steady rate. After this a small dose of radioactive $C^{14}O_2$ (about 6 μc) was to be given and as soon as more or less complete uptake was confirmed by the Infra Red Gas Analyser (IRGA) ordinary CO_2 was to be supplied again to maintain a steady rate of photosynthesis. All this was to be carried out under constant light intensity. After the lapse of a suitable time, the dose of radioactive $C^{14}O_2$ was to be repeated and followed by ordinary CO_2. After a further lapse of time the petiole was to be harvested and freeze-dried in vacuo. When dry, the petiole was to be assayed longtitudinally under an end window counter to get the distribution of C^{14}. It was hoped to get a "wave" as shown in Figure 6 by plotting counts per minute per cm. against distance along the petiole.

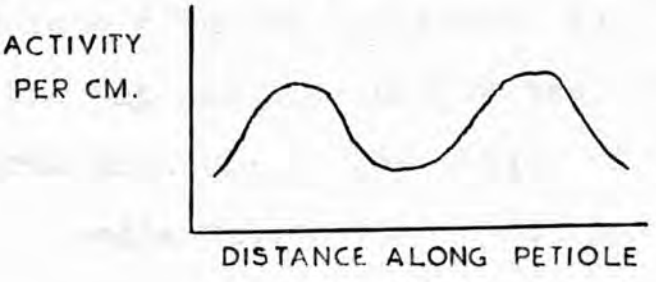


Figure 6.

Then knowing the "period" of the wave the velocity of the phloem stream could be at once found from the wavelength. It

was hoped to repeat the experiment at other temperatures by confining the petiole between two straight copper tubes about $^{3}/_{16}$" in diameter and running warm water through them.

3. Details of the experimental set up

In order to make the changes in the radioactivity of the aerial CO_2 supplied as striking and as abrupt as possible it was decided to use a closed circuit with the smallest possible volume and with an arrangement for the continuous circulation of the enclosed gas. Details of the experimental set up are given diagrammatically in Figure 7. The lamina was enclosed in the air-tight perspex leaf-chamber, having two outlets. One of the outlets was connected to the IRGA while the other was connected through a glass commutating tap 1 to the circulating pump. A very minute air-leak in the form of a fine hypodermic needle inserted into a rubber connecting tube was introduced between the tap and the pump to keep the enclosed gas at atmospheric pressure. The outlet of the pump was joined through a needle valve (to control the rate of flow) to a capillary resistance manometer of fine polythene tubing functioning as a flow meter. The latter was used to determine the rate of flow of the gas and contained water with a little methylene blue and teepol as the manometric

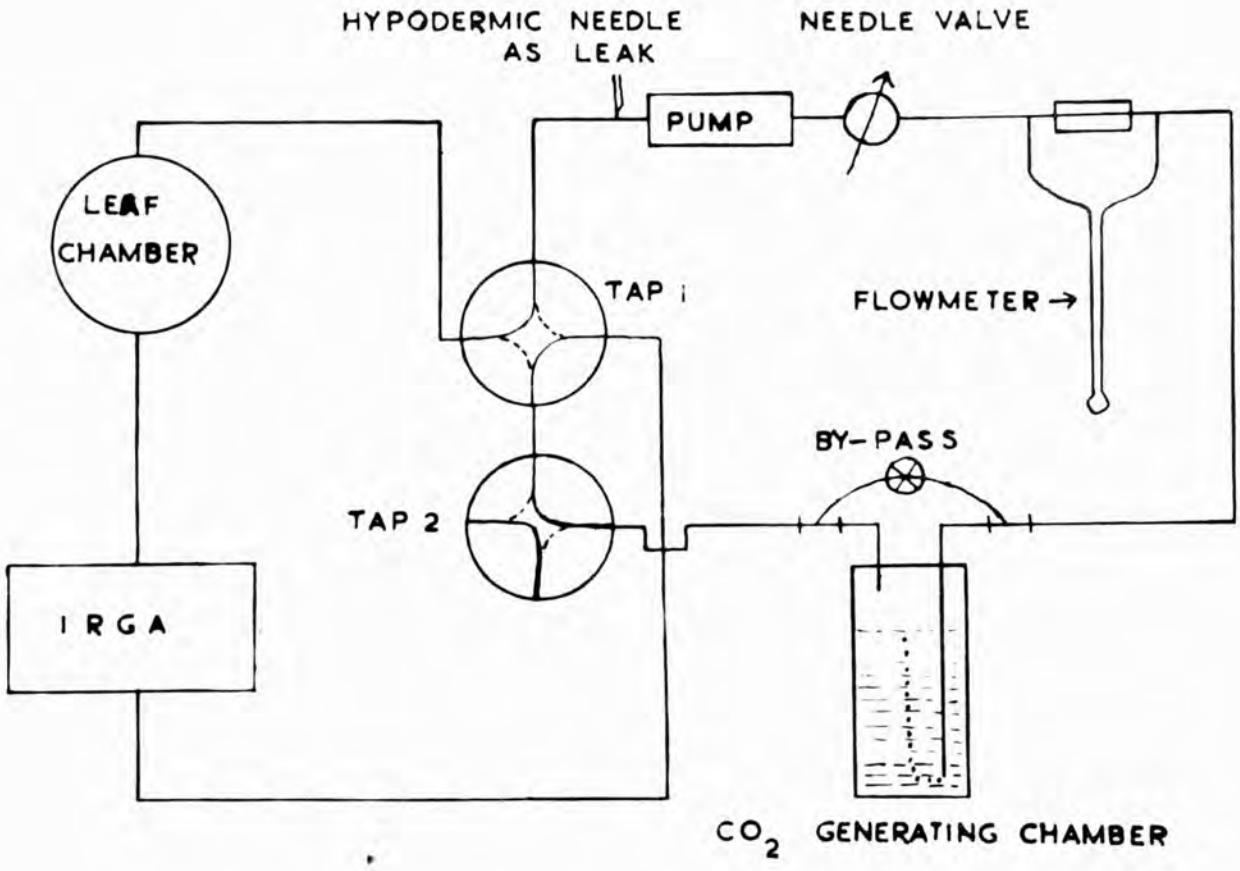


FIGURE 7. DETAILS OF THE CIRCUIT (DIAGRAMMATIC)

fluid. The other end of the flow meter was connected to a CO_2 generating chamber, the details of which are described later, which in its turn was connected through two commutating taps 1 & 2 to the IRGA. The latter as remarked earlier was connected to the leaf chamber, completing the circuit. The second commutating tap was used for flushing out the system.

By the use of the commutating tap 1 the direction of the flow of the gas could be reversed. In one position of the tap the gas would flow from the generating chamber through the IRGA to the leaf. In this case the IRGA would indicate the concentration of CO_2 being supplied to the lamina. In the other position of the tap the direction of the gas flow would be from the generator through the leaf chamber to the IRGA, and in this case the IRGA would give the concentration of the gas leaving the lamina. The difference between the two concentrations would give the CO_2 used in photosynthesis. Thus the commutating tap 1 in conjunction with the flow meter could be used to find the net assimilation rate during a steady state, and this could be compared with the translocation, assuming only a value for the concentration of the sieve tube sap.

4. Details of the leaf chamber

The details of the leaf chamber are shown in Figure 8. It was made of perspex and consisted mainly of a smaller

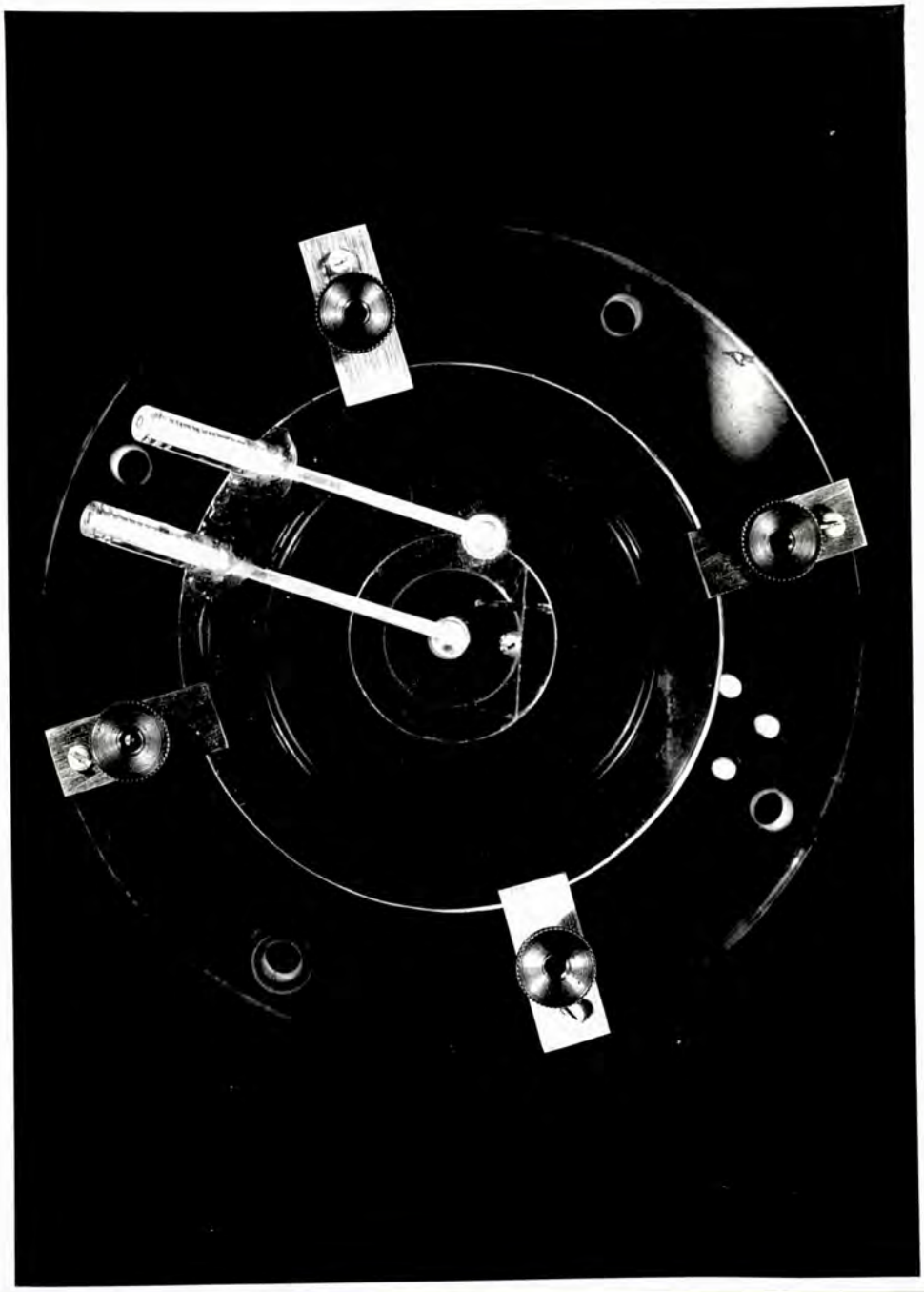


FIG. 8 LEAF CHAMBER

upper disc and larger lower one. The two discs were separated by an O-ring. This arrangement made an air-tight leaf chamber with a small internal volume; the latter ensuring a rapid turnover of CO_2 in the system. Further by using different sizes of O-rings one could have different sizes of leaf chamber (that is, of varying internal diameter) to suit different sizes of lamina. The lamina was introduced into the leaf chamber through a hole in the lower perspex disc, the latter being closed with a circular stepped disc of perspex split along a chord on which was drilled a small hole of diameter just large enough to accommodate the petiole. All the joints were greased with vaseline to make the leaf chamber air-tight, and the petiole was likewise luted with vaseline where it passed through the perspex.

5. Details of the CO_2 generating chamber

This chamber consisted of a small glass jar (Figure 9) ground flat above and covered by a brass disc, through which passed two copper tubes A and B as shown. The former acted as an outlet, the latter as an inlet. The internal volume of the glass jar was reduced by inserting a solid perspex rod so that only a small volume of acid was required to fill the jar to about 1" of the top. A brass rod was soldered onto the top of the brass cap and had two clips attached to it to

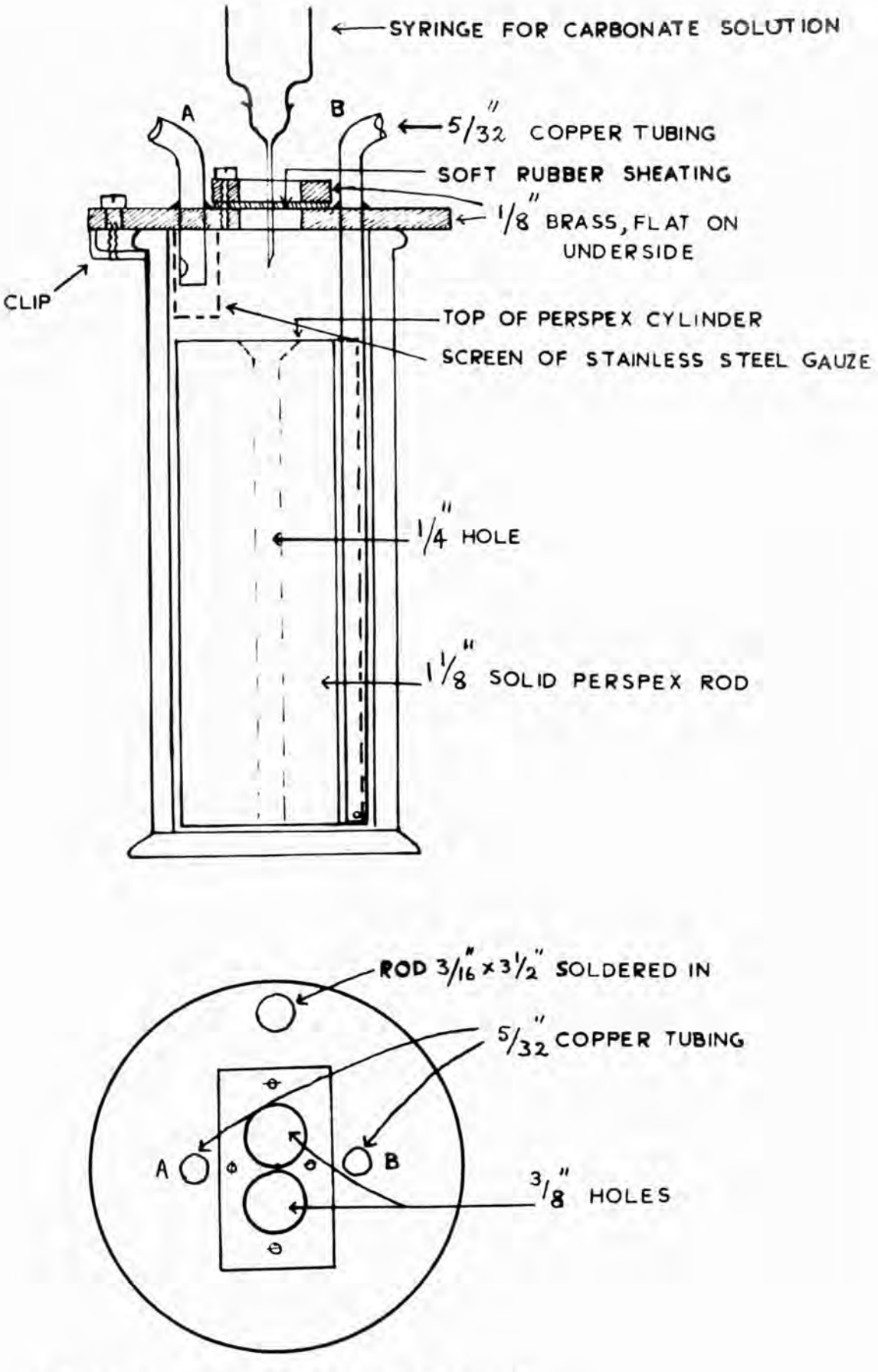


FIGURE 9. CO_2 GENERATING CHAMBER

hold two hypodermic syringes, one for a solution of ordinary Na_2CO_3 and the other for radioactive ($Na_2C^{14}O_3$) solution. The carbonate solutions were run into the acid in the jar at the required rate by applying air pressure from the air-main to the syringes, connection being made from the air-main to the latter with fine polythene tubing ending in hypodermic needle tubing; and from the syringes into the generator with fine needles through which were run close-fitting brass wires. By this means it was possible to secure a very slow and closely-regulated movement of carbonate into the acid; and to change rapidly from one source of carbonate to the other without alteration in rate. As acid, the non-volatile sulphuric acid was chosen, and glycerine added originally to secure a slight dry action before the air traversed the flow meter. The design of the lower part of the chamber was such that the bubbles produced a rapid circulation of the liquid, and the CO_2 produced by the reaction was, as judged by the behaviour of the IRGA, very rapidly removed into the gaseous phase. A variable by-pass was fitted to the generator to control the region of the scrubbing, and an arrangement for periodically withdrawing fluid with a large syringe to prevent the level rising too high.

The strength of the various solutions used was as follows:-

Inactive Na_2CO_3	.. ..	0.06 M
$Na_2C^{14}O_3$	.. .. ..	0.01 M
H_2SO_4	.. .. ..	2½% in 50% glycerine.

The radioactive carbonate, as obtained from The Radiochemical Centre, Amersham, England, was in the form of $BaC^{14}O_3$. It was converted to 0.01 M $Na_2C^{14}O_3$ whose activity was then diluted with ordinary Na_2CO_3 to give a total molarity of 0.06 M with 60μc/ml.

6. The Infra Red Gas Analyser

A factor which hindered the present work somewhat arose from the necessity of modifying the IRGA in the early stages. This was a new instrument by Howard Grubb Parsons, and as delivered the internal junctions to the gas circuit were made with copper-asbestos washers and were far from leak-proof. These were all remade according to high vacuum practice with O-rings and the air-tightness of the four-way metal stopcock was likewise improved. The balancing shutter was found to have far too coarse an adjustment, and a refinement was carried out by cutting down the vertical leading edge to a fairly acute angle. At the same time the backlash on the movement was reduced by fitting a tension spring. The last modifications made were to replace the AC solenoid operating the calibrating wire (which was extremely noisy in operation and very staccato) with a gentle cord-operated device; and

to replace the inlet Porvic filter disc (the mounting for which was very leaky) with a cotton wool filter plug. These modifications made the IRGA much more satisfactory to use with low concentrations of CO_2 and it subsequently performed very well.

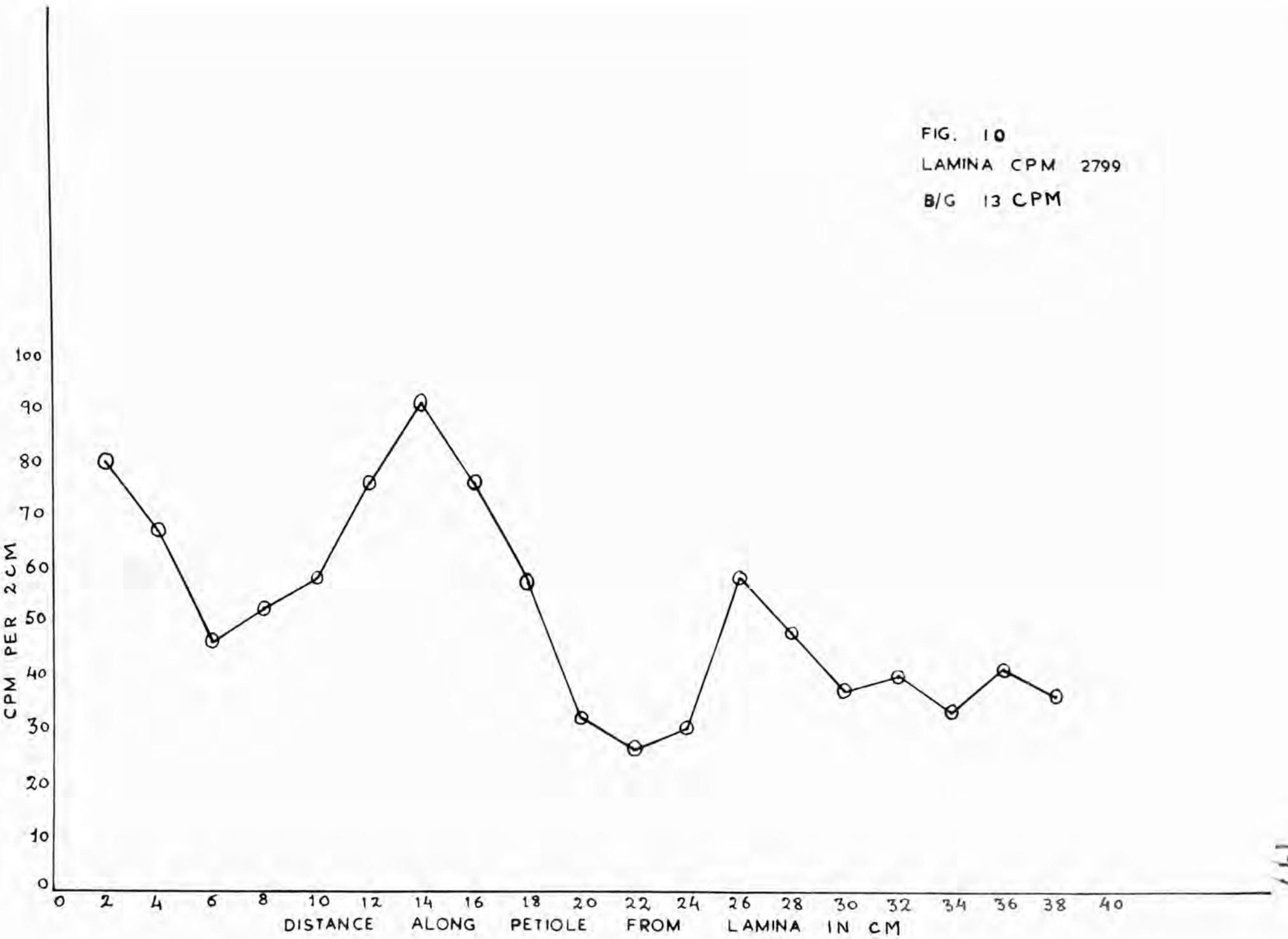
RESULTS

The first experiment was carried out under the following conditions, with the plant growing naturally in the tank and the lamina only under confinement.

Temperature - 16°C, light intensity at the surface of the leaf chamber - 750 ft. candles, three doses of radioactive $Na_2C^{14}O_3$ solution at intervals of one hour each, activity per dose being 6 μc; between these the lamina was given a steady low concentration of inactive CO_2. The leaf had been kept illuminated in inactive CO_2 for about 5 hours before the treatment began; it had been in light (150 ft. candle) overnight. The total length of the petiole harvested was 38 cm. and 2 cm. segments of the intact petiole were assayed for radioactivity after freeze-drying, and using an end window counter. Correction was made for the background count (13 c.p.m.)

The results of this experiment are shown in Figure 10 which appears to show the form of a wave as was hoped. It was at first thought that the three "crests" might represent the three doses of the radioactive $C^{14}O_2$, the first dose corresponding to the far right "crest" and so on. On the basis of this assumption the velocity of the translocate stream would be about 12 cm/hr. which does not seem impossible considering the rather low temperature of 16°C at which the translocation was going on and the general lack of vigour of the plant consequent on the time of the year. It might also be added that the lamina showed the relatively high count of 2799 per minute. However, this interpretation had to be

FIG. 10
LAMINA CPM 2799
B/G 13 CPM



abandoned for several reasons. Drying of the petiole does not result in an uniformly flattened section, but in one with twists and corrugations of varying form. Coupled with the low energy of C^{14} radiations this results probably in a very variable degree of self absorption, quite enough to simulate crests and troughs. The count rates recorded are in any case low; further the crests and troughs are too sharp to appear genuine effects of the type looked for.

At this stage it was decided to attempt to detect a crest by confining the experiment to a single dose of radioactive $Na_2C^{14}O_3$ solution of about 6 μc. For this the plant was removed from the tank and the petiole was laid horizontally in a long groove made in a perspex platform. The fresh petiole was counted in situ every 15 minutes at a fixed point about 6 in. away from the base of the lamina. To decrease the self absorption this part of the petiole was stripped for about 2 cm. to expose the phloem to the counter (see Figure 11a). Counting was started 15 minutes after.

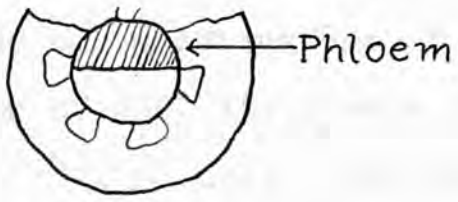


Figure 11a.

the addition of $Na_2C^{14}O_3$ solution. During the period of counting ordinary CO_2 was being supplied to the lamina - at a low concentration. The other conditions of the experiment were - temperature 24°C and light intensity 2500

ft. candles from a 100 watt bulb filtered through a water screen.

The results of this experiment are shown in Figure 11 which shows a curve rising apparently to a maximum and then flattening out with little sign of going down. The maximum count is obtained after about 3 hours and clearly shows not very precise crest. This experiment confirms the doubts as to the early interpretation of the result of the Figure 10 - that the crests correspond to the doses of radioactive $Na_2C^{14}O_3$. The lamina count was again very high - 9075 CPM - it being assayed fresh under the counter at the conclusion.

The attempt to get a sharp crest was continued using a rather different approach. This time the experiment was carried out on a plant which had been kept in darkness for 26 hours, so that the lamina would empty its carbohydrate reserves prior to the commencement. It was hoped thus to follow the translocation of undiluted radioactive carbohydrate. It was with this view that no ordinary CO_2 was supplied to the lamina during the run of the experiment. The experiment was carried out exactly like the previous one at a temperature of 25°C and light intensity of 2500 ft. candle with a single dose of radioactive $Na_2C^{14}O_3$ (6 μc). The counting was started 10 minutes after the addition of $Na_2C^{14}O_3$ and carried out every 15 minutes. The results of this experiment are shown in Figure 12. The curve here has an appearance similar to that in Figure 11. The "crest" occurs after about $3\frac{1}{2}$ hours and then flattens out. The lamina showed a

FIG. II

LAMINA CPM 9075

B/G 26 CPM

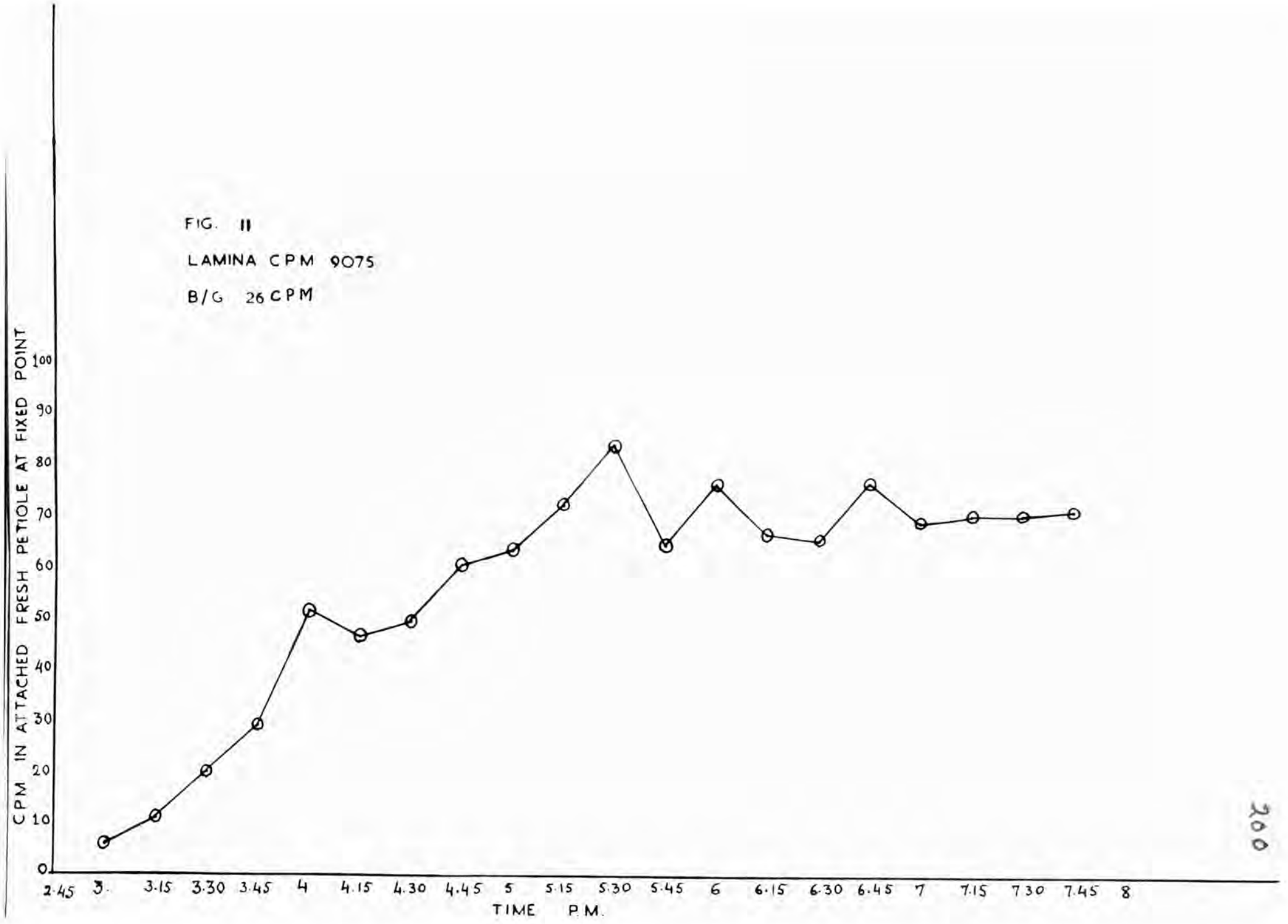
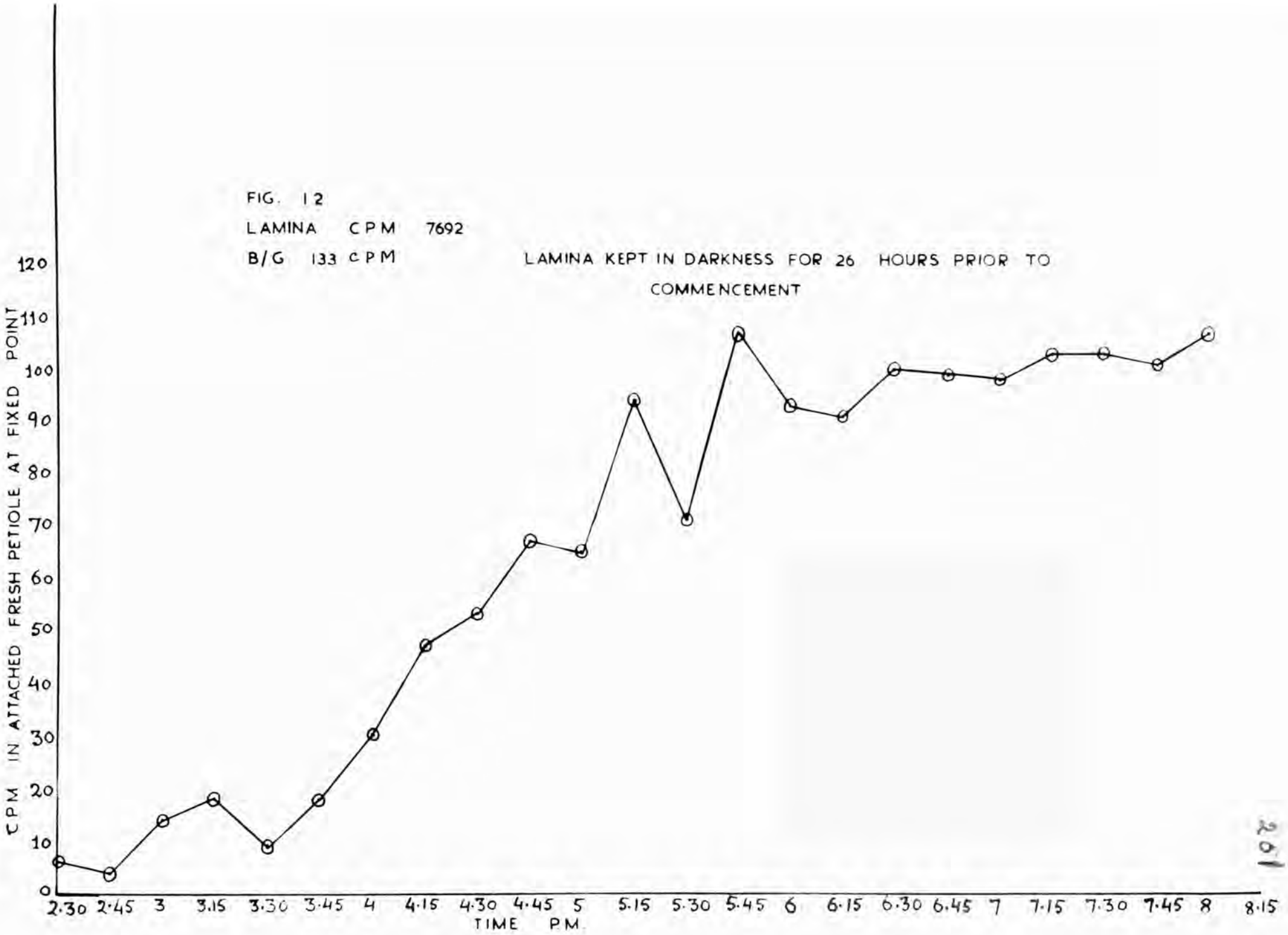


FIG. 12

LAMINA CPM 7692

B/G 133 CPM

LAMINA KEPT IN DARKNESS FOR 26 HOURS PRIOR TO COMMENCEMENT



high count of 7692 per minute at the conclusion.

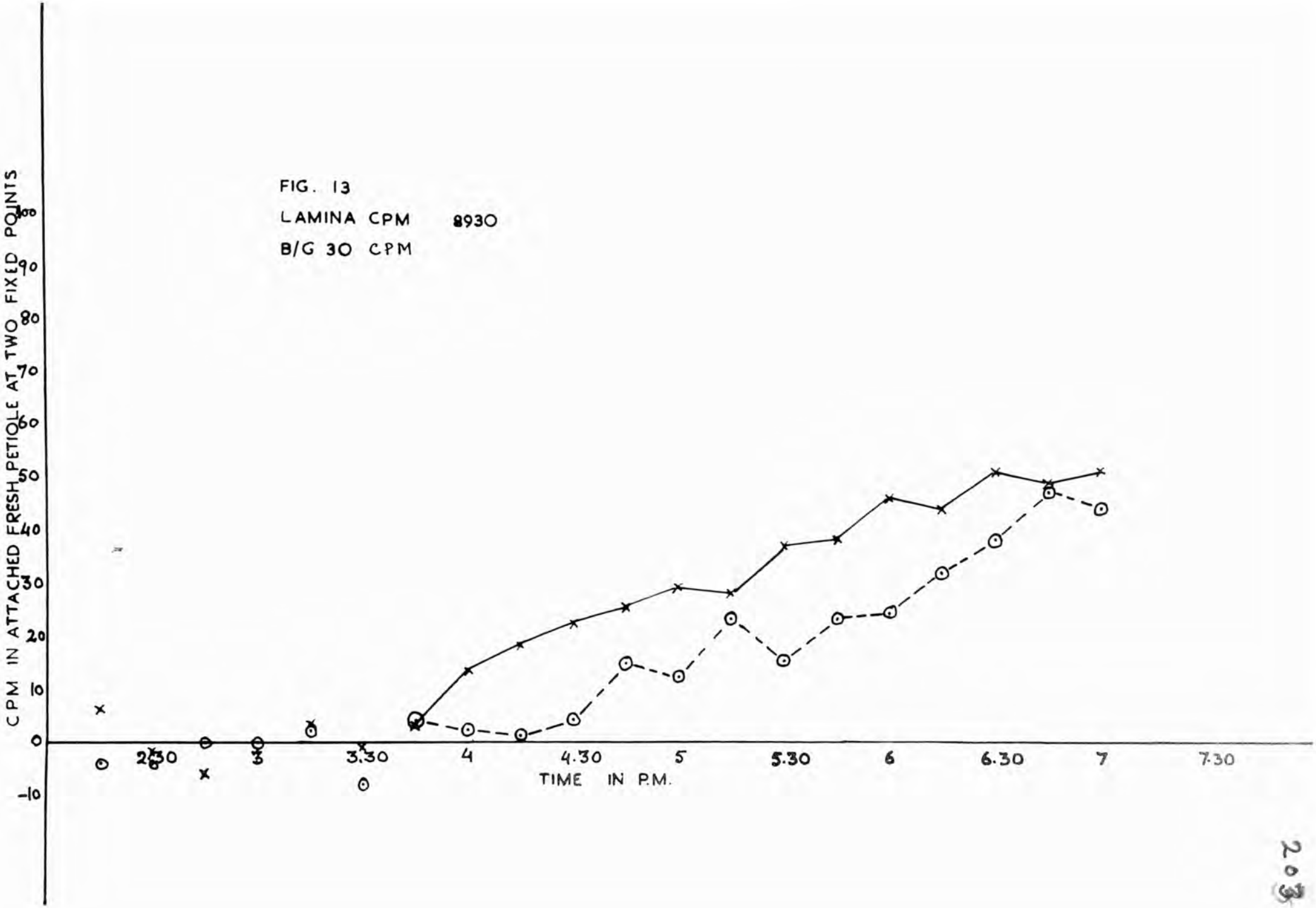
Having failed again to get a precise crest, another approach towards determining the velocity of the translocate stream was tried. This essentially consisted of counting the petiole in situ as in the previous experiment at two different places about 8 cm apart after shaving off the "cortex" for 1 cm. on the upper side facing the counter. The counting was started immediately after the addition of radioactive $Na_2C^{14}O_3$ of about 6 μc to the CO_2 generating chamber and repeated every 15 minutes. The two points at which the petiole was assayed for radioactivity were 19 and 27 cm away from the lamina base. The experiment was carried out at a temperature of 23°C and light intensity of 1600 ft. candles. The results are shown in Figure 13. Though the counts are low the curves resemble in form the first part of those shown in Figures 11 and 12. The lamina as usual had a high count of 8930 per minute at the conclusion.

This exploratory result can be used in an approximate way to calculate the velocity. The horizontal distance between the curves represents about 1.2 hrs. and this may be related to the distance between the stations (8 cm) to give a velocity of 8/1.2 = 7 cm/hr. This method of calculation, in the light of mathematical analysis (see Horwitz, 1958) is, however, open to serious objection.

FIG. 13

LAMINA CPM 8930

B/G 30 CPM



DISCUSSION

Although the physiological work reported here failed in the time available to achieve what was hoped it has produced one or two interesting results. It was thought at the beginning that if a sudden dose of radioactive carbon dioxide was given under conditions in which it was very rapidly assimilated, then a wave of activity with a reasonably well-marked crest might be expected to travel down the petiole; and that this phenomenon could be used to measure the velocity. In the event it proved that the pattern of movement from the leaf was far different. The "crest" is drawn out to such an extent that in the length of petiole available, about 40 cm or so, no precise measurements on the lines intended are possible. It proved practicable to measure the activity in the living petiole at a fixed point during the process of translocation; though the penetrating power of the B particles from C^{14} is such that it was the surface layers, rather than the phloem, which was assayed (see Footnote).

Footnote:- The maximum range of Beta particles from C^{14} having an energy of 0.155 MeV is about 28 mg/cm^2. The mass of the "cortex" outside the aerenchyma of the petiole is perhaps 30 mg/cm^2. If the mass of the mica window of the counter (given by the makers as 1.5 - 2.5 mg/cm^2) is added to this the total mass becomes 32 mg/cm^2. Thus Beta particles from the inner cylinder would not be expected to register in the counter. By shaving off the outer "cortex", only the

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But when this was done it appeared that the activity rose only very slowly and in the length of time allowed (up to 6 hours) showed no sign of falling, in spite of the fact that actual assimilation of the radioactive carbon took only about ten minutes. Thus both in time and space the changes are too gradual to allow the method to succeed with the present plan.

There are two possible causes for this. In the first place the mathematical analysis of Horwitz (1958) suggests that the phloem conduits are "leaky", and allow tracer to move out sideways fairly rapidly into the parenchyma tissue. Since this is so much more bulky than the phloem this will have the effect of reducing the sharpness of the wheel effect; and this will be allthe more so if it is the outer layers of the petiole which contribute most to the count rate.

In the second place it is known from the results of several workers (see for instance Vernon and Aronoff (1952) and Porter, Martin and Bird (1959) that when radioactive CO_2 is assimilated some of it is incorporated fairly rapidly into

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inner "cortex" is left over the phloem with a thickness of about 18 mg/cm^2. With the mica window the thickness above the phloem is then 20 mg/cm^2, giving a transmission for C^{14} of a few percent only. This is naturally low, though in fact lateral equilibration of radioactive sugars into the parenchyma seems to take place rather quickly, and this of course brings the source of radio-activity nearer the surface, though it complicates the problem of analysis.

large molecules, such as starch and other polysaccharides; and that it may only slowly be released from these, if at all. Particularly striking are some very recent results of Jones Martin and Porter (1959) who reported that when $C^{14}O_2$ was assimilated by a mature tobacco leaf about 30% of the activity remained permanently in the leaf, even when it had yellowed. Of the remaining 70%, about 50% was lost during the first six hours and 20% over a period of about 4 days. When a leaf had assimilated $C^{14}O_2$ for two hours and then spent a further two hours in ordinary air and light the activity in the ethanol-insoluble fraction considerably exceeded (40 : 28) that in the soluble. Thus it is evident that newly formed sucrose does not rapidly make its exit entire from the leaf; it undergoes rapid dilution with sucrose subsequently formed from assimilation or from starch reserves, and becomes concurrently in part involved in starch formation itself. The system may in fact, be tentatively likened to a bucket containing contaminated water which is being washed out by an entering stream which is mixing all the time with the contents. The process is a lengthy one.

If the present method is to be used to measure directly the velocity of transport, and so the Q_{10}, it would be necessary first to discover under what conditions the radio-active assimilate can be made to leave the leaf most rapidly. So far as the work reported here is concerned neither the presence of starch in the leaf beforehand, nor its absence

seems to make much difference. A possible alternative approach to the problem is indicated in the last experiment reported, in which the living petiole was assayed periodically at two stations; but the theory of this method needs to be more fully worked out first. It is possible also that traces other than C^{14} might be employed, and if these were of the kind not subject to metabolism (like CO^{137}) this might simplify matters; but the results would then not be directly applicable to the transport of sugars. Time unfortunately did not allow any of these interesting possibilities to be followed up.

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REFERENCES

1. Biddulph, O. & Cory, R. 1957. An analysis of translocation in the phloem of the bean plant using THO, P^{32} and $C^{14}O_2$. Plant Physiol. 32, 608-619.

2. Biddulph, O. & Markle, J. 1944. Translocation of radiophosphorus in the phloem of cotton plant. Amer. Jour. Bot. 31, 65-70.

3. Biddulph, S.F. 1956. Visual indications of S^{35} & P^{32} translocation in the phloem. Amer. Jour. Bot. 43, 143-148.

4. Biddulph, S., Biddulph, O. & Cory, R. 1958. Visual indications of upward movement of foliar applied P^{32} & C^{14} in the phloem of the bean stem. Amer. Jour. Bot. 45, 648-652.

5. Birch - Hirschfeld, L. 1919-1920. Untersuchungen uber die Ausbreitungsgeschwindigkeit geloster stoffe in der Pflanze. Jahrb. Wiss. Bot. 59, 171-262.

6. Bohning, R.H., Kendall, W.A. & Linck, A.J. 1953. Effect of temperature and sucrose on growth and translocation in tomato. Amer. Jour. Bot. 40, 150-153.

7. Bohning, R.H., Swanson, C.A., & Linck, A.J. 1952. The effect of hypocotyl temperature on translocation of carbohydrates from bean leaves. Plant Physiol. 27, 417-421.

8. Borysko, Emil 1956. Recent developments in methacrylate embedding. I. A study of the polymerization damage phenomenon by phase contrast microscopy. J. of Biophysic. & Biochem. Cytol. 2 (Suppl.) 3-14.

9. Bretschneider, L.H. and Elbers, P.F. 1952. As cited by Palade, 1954.

10. Brissi, G. 1873. Uber allgemeines vorkommen von starke in dem siebrohren. Bot. Zeitung 31, 306-351.

11. Caulfield, J.B. 1957. Effects of varying the vehicle for OsO_4 in tissue fixation. J. Biophysic. & Biochem. Cytol. 3, 827-829.

12. Chang, C.Y. 1935. Differentiation of protophloem in the angiosperm shoot apex. New Phytol. 34, 21-29.

13. Cheadle, V.I. 1948. Observations on the phloem in the monocotyledoneae. II. Additional data on the occurrence and phylogenetic specialization in structure of sieve tubes in the metaphloem. Amer. Jour. Bot. 35, 129-131.

14. Cheadle, V.I. & Esau, K. 1958. Secondary phloem of Calycanthaceae. Univ. Calif. Publ. in Botany, 29, 397-510.

15. Cheadle, V.I., Gifford, E.M., Esau, K. 1953. A staining combination for phloem and contiguous tissues. Stain Tech. 28, 49-53.

16. Cheadle, V.I. & Whitford, N.B. 1941. Observations on the phloem in the monocotyledoneae. I. The occurrence and phylogenetic specialization in structure of the sieve tubes in the metaphloem. Amer. Jour. Bot. 28, 623-627.

17. Clapham, A.R., Tutin, T.G. and Warburg, E.F. 1952. Flora of the British Isles, Cambridge.

18. Conn, H.J. 1940. Biological stains. Biotech. Publications, Geneva, N.Y., U.S.A.

19. Crafts, A.S. 1931. Movement of organic materials in plants. Plant Physiol. 6, 1-41.

20. Crafts, A.S. 1939 a. The relation between structure and function of the phloem. Amer. Jour. Bot. 26, 172-177.

21. Crafts, A.S. 1939 B. The protoplasmic properties of sieve tubes. Protoplasma. 33, 389-398.

22. Crafts, A.S. 1948. Movement of materials in phloem as influenced by the porous nature of the tissue. In: Interaction of water and porous materials. Faraday Soc. Disc. No.3, 153-159.

23. Crafts, A.S. 1951. Movements of assimilates, viruses, growth regulators, and chemical indicators in plants. Bot. Rev. 17, 203-284.

24. Currier, H.B., Esau, K., & Cheadle, V.I. 1955. Plasmolytic studies of phloem. Amer. Jour. Bot. 42, 68-81.

25. Curtis, O.F. 1920. The upward translocation of foods in woody plants. I. Tissues concerned in translocation. Amer. Jour. Bot. 7, 101-124.

26. Curtis, O.F. 1935. The translocation of solutes in plants. McGraw - Hill Book Co., New York, N.Y.

27. Curtis, O.F. & Herty, S.D. 1936. The effect of temperature on translocation from leaves. Amer. Jour. Bot. 23, 528-532.

28. De Vries, H. 1885. Uber die Bedeutung der Circulation und der Rotation des Protoplasmas fur den Stofftransport in der Pflanze. Bot. Zeit. 43, 1-6, 16-26.

29. Dixon, H.H. 1922. Transport of organic substances in plants. Pres. Address, Sec.K., Br. Assn. Adv. Sci. 193-203.

30. Dixon, H.H., & Ball, H.G. 1922. Transport of organic substances in plants. Nature Lond. 109, 236-7.

31. Dixon, H.H. & Gibbon 1932. Bast-sap in plants. Nature, 130, 661.

32. Dorland, R.E. & Went, F.W. 1947. Plant growth under controlled conditions. VIII. Growth and fruiting of the chili pepper (Capsicum annuum). Amer. Jour. Bot. 34, 393-401.

33. Esau, K. 1939. Development and structure of the phloem tissue. Bot. Rev. 5, 373-432.

34. Esau, K. 1947. A study of some sieve tube inclusions. Amer. Jour. Bot. 34, 224-233.

35. Esau, K. 1948. Phloem structure in the grape-vine, and its seasonal changes. Hilgardia, 18, 217-296.

36. Esau, K. 1950. Development and structure of the phloem tissue. II. Bot. Rev. 16, 67-114.

37. Esau, K. & Cheadle, V.I. 1955. Significance of cell divisions in differentiating secondary phloem. Acta Bot. Neerl. 4, 348-357.

38. Esau, K. & Cheadle, V.I. 1958. Wall thickening in sieve elements. Proc. Nat. Acad. Sc. 44, 546-553.

39. Esau, K. & Cheadle, V.I. 1959. Size of pores and their contents in sieve elements of Dicotyledons. Proc. Nat. Acad. Sc. 45, 156-162.

40. Esau, K., Currier, H.B., & Cheadle, V.I. 1957. Physiology of phloem. Ann. Rev. Plant Physiol. 8, 349-374.

41. Glanert, A.M., & Glanert, R.H. 1958. Araldite as an embedding medium for electron microscopy. J. Biophysic. & Biochem. Cytol. 4, 191.

42. Hartig, T. 1837. Vergleichende Untersuchungen uber die Organisation des Stammes der einheimischen Waldbaume. Jahresber uber die Fortschritte der Forstwissensch. und forstl. Naturkunde, 1, 125-168.

43. Hepton, C.E.L. 1957. The structure of phloem in relation to its function. Thesis. Leeds.

44. Hepton, C.E.L., Preston, R.D. & Ripley, G.W. 1955. Electron microscopic observations on the structure of the sieve plates in Cucurbita. Nature, 176, 868-870.

45. Hewitt, S.P. & Curtis, O.F. 1948. The effect of temperature on loss of dry matter and carbohydrate from leaves by respiration and translocation. Amer. Jour. Bot. 35, 746-755.

46. Hill, A.W. 1908. The histology of the sieve tubes of Angiosperms. Ann. Bot. 22, 245-290.

47. Horwitz, L. 1958. Some simplified mathematical treatments of translocation in plants. Plant Physiol. 33, 81-93.

48. Huber, Bruno 1958. Anatomical and physiological investigations on food translocation in trees. The Physiology of Forest Trees, 367-379.

49. Huber, B., Schmidt, E., & Jahnel, H. 1937. Untersuchungen uber den assimilatstrom. Tharandt. Forstl. Jahrb, 88, 1017-1048.

50. Hull, H.M. 1952. Carbohydrate translocation in tomato and sugar beet with particular reference to temperature effect. Amer. Jour. Bot. 39, 661-669.

51. Jones, H., Martin, R.V., and Porter, H.K. 1959. Translocation of ^{14}carbon in tobacco following assimilation of ^{14}carbon dioxide by a single leaf. Ann. Bot. 23, 493-508.

52. Jungers, V. 1930. Recherches sur les plasmodesmes chez les végétaux. La Cellule 40, 7-82.

53. Kursanov, A.L. 1952. as cited by Esau, Currier and Cheadle 1957.

54. Latta, H. and Hartman, J.F. 1950. Use of glass edge in thin sectioning for EM. Proc. Soc. Exp. Biol. Med. 74, 436-39.

55. Low, F.N. and Freeman, J.A. 1956. Some experiments with Chromium compounds as fixers for electron microscopy. J. Biophysic and Biochem. Cytol. 2, 629-631.

56. Luft, J.H. 1956. Permanganate - A new fixative for Electron microscopy. J. Biophysic and Biochem. Cytol. 2, 799-801.

57. Mangenot, G. 1926. Communications intercellulaires et synapses. Notes d'histologie végétale. Bul. d'Histol. Appl. à la Physiol. et Path. et de Tech. Micros. 3, 142-160.

58. Maugham, S. 1910-1911. The translocation of carbohydrates in plants (Part I and II). Sci. Prog. 5, 256-285 and 457-479.

59. Marinos, N.G. 1960. The Nuclear Envelope of Plant Cells. J. Ultrastructure Research. 3, 328-333.

60. Mason, T.G. and Maskell, E.J. 1928. Studies on the transport of carbohydrates in the cotton plant. I. A study of diurnal variation in the carbohydrates of leaf, bark and wood, and of the effects of ringing. Ann. Bot. 42, 189-253.

61. Mason, T.G. and Phillis, E. 1934. A tentative account of the movement of food materials during the development of the cotton plant. Empire cotton Growing Review 11, 121- 124.

62. McGivern, M.J. Sister, 1957. Mitochondria and plastids in sieve-tube cells. Amer. Journ. Bot. 44, 37-48.

63. Mollenhauer, H.H. 1959. Permanganate fixation of plant cells. J. Biophysic. Biochem. Cytol. 6, 431-436.

64. Münch, E. 1930. Die Stoffbewegungen in der Pflanze. 1-1234. Gustav Fischer, Jena.

65. Münch, E. 1943. Durchlässigkeit der Siebröhren für Druckströmungen. Flora 36, 223-262.

66. Nelson, C.D. and Gorham, P.R. 1957. Uptake and translocation of C^{14}labelled sugars applied to primary leaves of soybean seedlings. Can. Jour. Bot. 35, 339-347.

67. Nelson, C.D. and Gorham, P.R. 1957. Translocation of radioactive sugars in the stems of soybean seedlings. Can. Jour. Bot. 35, 703-713.

68. Newman, S.B., Borysko, E. and Swerdlow, M. 1949. New sectioning technique for light and E.M. Science, 110, 66-68.

69. Palade, G.E. 1952. A study of fixation for electron microscopy. J. Exp. Med. 95, 285-298.

70. Palade, G.E. 1954. The Fixation of Tissues for Electron Microscopy. Proc. Inter. Conf. Electron Microscopy. 129-142.

71. Palade, G.E. 1956. The endoplasmic reticulum. J. Biophysic. and Biochem. Cytol. 2, 85-98.

72. Porter, H.K., Martin, R.V. and Bird, J.F. 1959. Synthesis and dissolution of starch labelled with ^{14}carbon in tobacco leaf tissue. Journ. Expt. Bot. 10, 264-276.

73. Porter, K.R. and Machado, R.D. 1960. Studies on the endoplasmic reticulum. IV. Its form and distribution during mitosis in cells of onion root tips. J. Biophysic. Biochem. Cytol. 7, 167-180.

74. Preston, R.D. 1958a. The ascent of sap and the movement of soluble carbohydrates in stems of higher plants. Proc. of the tenth Symp. of the Colston Res. Soc. in the University of Bristol, 3, 24-27.

75. Preston, R.D. 1958b. The physiological significance of electron microscopic investigations of plant cell walls. Science Progress, 46, 593-605.

76. Rabideau, G.S., and G.O. Burr. 1945. The use of the C^{13} isotope as a tracer for transport studies in plants. Amer. Journ. Bot. 32: 349-356.

77. Rhodin, J. 1954. In "Correlation of Ultrastructural organisation and function in normal and experimentally changed proximal convoluted tubule cells of the mouse kidney." Aktiebolaget Godwil, Stockholm.

78. Rouschal, E. 1941. Untersuchungen über die Protoplasmatik und Funktion der Siebröhren. Flora (Jena) 135, 135-200.

79. Sachs, J. 1863. Ueber die Leitung der plastischen Stoffe durch verschiedene gewebeformen. Flora 46, 33-42; 49-58; 65-74.

80. Salmon, J. 1946. (Miss). Différenciation des tubes crilites chez les angiospermes; recherches cytologiques. 235 p.

81. Sass, J.E. 1940. Elements of Botanical Microtechnique. McGraw-Hill Book Co.

82. Satir, P.S. and Peachey, L.D. 1958. Thin Sections II. A simple method for reducing compression artifacts. J. Biophysic. and Biochem. Cytol. 4, 345-347.

83. Schumacher, W. 1930. Untersuchungen über die lokalisation der Stoffwanderung in den leitbündeln hoherer Pflanzen. Jahrb. Wiss. Botan. 73, 770-823.

84. Schumacher, W. 1933. Untersuchungen über die Wanderung des fluoreszeins in den Siebröhren. Jahrb. Wiss. Bot. 77, 685-732.

85. Schumacher, W. 1939. Ueber die Plasmolysierbarkeit der Siebröhren. Jahrb. Wiss. Bot. 88, 545-553.

86. Schumacher, Walter und Kollmann, R. 1959. Zur Anatomie des Siebröhrenplasmas bei Passiflora caerulea. Sonderabdruck aus den Berichten der Deutschen Botanischen Gesellschaft, 4, 176-179.

87. Sharman, B.C. 1943. Tannic acid and iron alum with safranin and orange G in studies of the shoot apex. Stain Technology 18, 105-111.

88. Shatkin, A.J. and Tatum, E.L. 1959. Electron microscopy of Neurospora crassa mycelia. J. Biophysic Biochem. Cytol. 6, 423-426.

89. Spanner, D.C. 1958. The translocation of sugars in sieve-tubes. Journ. Expt. Bot. 9, 332-42.

90. Strasburger, E. 1891. Ueber den Bau und die Verrichtungen der Leitungsbahnen in den Pflanzen. Histologische Beiträge, Vol.3, Fischer, Jena.

91. Swanson, C.A. and Böhning. 1951. The effect of petiole temperature on the translocation of carbohydrates from bean leaves. Plant Physiol. 26, 557-564.

92. Swanson, C.A. and Whitney, J.B., Jr. 1953. Studies on the translocation of foliar applied p32 and other radio-isotopes in bean plants. Amer. Jour. Bot. 40-816-823.

93. Vernon, L.P. and Aronoff, S. 1952. Metabolism of Soybean leaves. IV. Translocation from soybean leaves. Arch Biochem. and Biophys. 36, 383-398.

94. Volz, G. 1952. Elektronmicroskopische Untersuchungen über die porengrössen pflanzlicher Zellwände. Mikroskopie 7, 251-266.

95. Ward, R.T. Prevention of polymerisation damage in methacrylate embedding media. J. Hisotchem. and Cytochem. 6, 398.

96. Went, F.W. 1944. Plant growth under controlled conditions. III. Correlation between various physiological processes and growth in the tomato plant. Amer. Jour. Bot. 31, 597-618.

97. Went, F.W. 1945. Plant growth under controlled conditions. V. The relation between age, light, variety and thermoperiodicity of tomatoes. Amer. Journ. Bot. 32, 469-479.

98. Went, F.W. and Carter, M. 1948. Growth response of tomato plants to applied sucrose. Amer. Jour. Bot. 35, 95-106.

99. Went F.W. and Engelsberg, R. 1946. Plant growth under controlled conditions. VIII. Sucrose content of the tomato plant. Arch. Biochem. 9, 187-200.

100. Went, F.W. and Hull, H.M. 1949. The effect of temperature upon translocation of carbohydrates in the tomato plant. Plant Physiol. 24, 505-526.

101. Whaley, W.G., Mollenhauer, H. and Kephart, J.E. 1959. The endoplasmic reticulum and the Golgi structures in maize root cells. J. Biophysic. Biochem. Cytol. 5, 501-506.

102. Zimmermann, M.H. 1958. Translocation of organic substances in the phloem of trees. The Physiology of Forest Trees. 381-400.