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THE FINE STRUCTURE OF THE PROTOPLAST IN PRIMARY TRACHEARY ELEMENTS OF THE CUCUMBER AFTER PLASMOLYSIS

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SUMMARY

A striped pattern consisting of lighter and darker bands can be seen under the light microscope on the surface of the cell in tracheary elements in the hypocotyl of the cucumber. After plasmolysis, a striped pattern can also be observed both on the surface of the protoplast and on the cell wall.

The nature of the striped pattern on the plasmolysed protoplast was investigated by electron microscopical examination.

In the non-plasmolysed cell, high densities of cisternae of the endoplasmic reticulum and of Golgi vesicles are found in the areas between the developing cell wall thickenings. At the same time, bands of microtubules are present along the plasmalemma around the thickenings.

Comparing these results with the electron microscope picture of the plasmolysed cell, the following observations can be made: 1. the regular distribution of the endoplasmic reticulum and the Golgi vesicles has disappeared; all cell organelles are disorganized. 2. The bands of microtubules are still present at their original sites. 3. The surface of the plasmolysed protoplast does not become smooth after detachment from the cell wall. It shows low, somewhat irregular ridges. These ridges are the areas which were originally located between the thickenings.

Several hypotheses which might explain the pattern observed under the light microscope are discussed.

1. INTRODUCTION

In cells in which cell wall material is deposited against the primary cell wall to form rings, spirals, or a kind of network, a similar ring-, spiral-, or net-shaped pattern is observed with the light microscope on the surface of the protoplast during the formation of this cell wall thickening.

Crüger (1855) and Dippel (1867) described the pattern on the surface of the protoplast as a more densely granular protoplasm which was associated with an intensive streaming of the protoplasm. Crüger made his observations on cells in the developing velamen of aerial roots of *Catasetum tridentatum*; Dippel studied young vessels of *Impatiens noli-tangere*. These authors suggested that the dense bands of protoplasm were responsible for the development of the system of corresponding cell wall thickenings. Barkley (1927) studied vessels in fixed *Trichosanthes anguina* material and described a structure on the protoplast with the same pattern as the cell wall thickening. It consisted of alternating bands with small vacuoles and bands with larger vacuoles. The

author thought that the thickening was formed in the areas with small vacuoles. MAJUMDAR (1940), studying protoxylem in *Heracleum sphondylium*, agreed with the nature of the bands on the protoplast described by Barkley. SINNOTT & BLOCH (1945) observed in fixed material (wound vessel members of *Coleus hybridus*) a granular pattern on the protoplast which was similar in shape to the pattern of the cell wall thickenings.

After the introduction of the electron microscope, many investigators attempted to study the nature of the "pre-pattern" of the cell wall thickening pattern on the surface of the protoplast.

Most of the investigators (WOODING & NORTHCOTE 1964; CRONSHAW 1965; CRONSHAW & BOUCK 1965; ESAU, CHEADLE & GILL 1966 and PICKETT-HEAPS & NORTHCOTE 1966) could not find anything that might represent the densely granular or vacuolar bands by use of the electron microscope. Concentrations or patterning of cell organelles or vacuoles, except for microtubules, were not observed.

However, some authors correlated the densely granular bands with a concentration of some cell organelle or of various cell organelles. PORTER & MACHADO (1960) and PORTER (1961) observed a pattern of the endoplasmic reticulum which corresponded to the cell wall thickening pattern in protoxylem in the root of *Allium*. HEPLER & NEWCOMB (1963, 1964) reported concentrations of mitochondria, plastid-like bodies, endoplasmic reticulum, Golgi bodies, and numerous small vesicles in the cytoplasm corresponding to the thickenings in wound vessel members of *Coleus*. GOOSEN-DE ROO (1973b) could discern a pattern in the distribution of the cisternae of the endoplasmic reticulum and, to some extent, of Golgi vesicles corresponding to the cell wall pattern after density determination of these cell organelles in xylem of the hypocotyl of *Cucumis* 'Sporu-Origineel'. The density of these two organelles is higher between the developing thickenings than in the cytoplasmic areas facing the thickenings.

A patterning in the distribution of microtubules corresponding to the developing cell wall thickening is found in tracheary elements during thickening formation and has been described by many authors after the use of glutaraldehyde as a fixative for electron microscopy (HEPLER & NEWCOMB 1964; PICKETT-HEAPS & NORTHCOTE 1966; HEPLER & FOSKET 1971; MAITRA & DE 1971 and GOOSEN-DE ROO 1973a). The microtubules are present along the plasmalemma surrounding the thickenings. Their localization alternates with the pattern of the cisternae of the endoplasmic reticulum and the Golgi vesicles (GOOSEN-DE ROO 1973b).

Crüger and Dippel used plasmolysis to make sure that the pattern was present on the protoplast and was not confused with the already present cell wall thickening pattern. In cells in which wall thickenings had already been formed, the pattern on the surface of the protoplast which is similar in shape to the wall pattern is clearly visible after plasmolysis. In cells in which the formation of the thickening has not yet begun, a pattern similar to the future cell wall pattern also seems to be present on the protoplast (DIPPEL 1867). We must,

however, take account of the fact that Dippel discussed in detail that he was not sure of this last observation.

Plasmolysis has been employed in light microscopic, but not in electron microscopic investigations, for the study of the "pre-patterning" of the thickenings on the surface of the protoplast. The patterns observed before and after plasmolysis were considered identical when studied by light microscopy.

The aim of this study was to investigate the effect of plasmolysis on the two cell organelle patterns in primary tracheary elements of the hypocotyl of *Cucumis* 'Sporu-Origineel' with the electron microscope and to discuss the results in relation to the light microscopical observations after plasmolysis.

2. MATERIAL AND METHODS

2.1. Culture of the plants

Seeds of the cucumber (*Cucumis* 'Sporu-Origineel' from de Ruyter and Son, Bleiswijk, Holland) were germinated for 48 hours in a controlled culture room. Details concerning the culture of the plants are described in a previous paper (GOOSEN-DE ROO 1973a).

2.2. Plasmolysis

Plasmolysis was accomplished by exposing pieces of the hypocotyl to a 0.6 M sucrose solution at a temperature of 4°C for 10, 20 or 30 minutes. A comparable series without plasmolysis was also prepared for light and electron microscopy. The pieces used for light and electron microscopy were placed in the solution of 0.6 M sucrose before they were killed in the various fixatives. These pieces were prepared as follows: slices of the hypocotyl, with a thickness of about 2 mm, cut from the part just below the insertion of the cotyledons, were divided into four wedges. A large vascular bundle was present in each part. In this part of the hypocotyl, the cell wall thickening formation in the tracheary elements is in full progress and the two alternating patterns of cell organelles are present.

2.3. Light microscopy

After plasmolysis the pieces for light microscopy were placed for 21 hours at room temperature in a solution of glutaraldehyde diluted to a concentration of 6.5% in a 0.2 M sodium cacodylate buffer, pH 7.6. After dehydration by way of an alcohol and tertiary butyl alcohol series, the pieces were embedded in diglycolstearate (Pegosperse 100 S). Longitudinal sections were cut for light microscopical examination. The thickness of the sections was 10 microns and they were stained according to MAÁCZ & VÁGÁS (1963).

2.4. Electron microscopy

The pieces for electron microscopy were placed in the same glutaraldehyde solution as were the pieces for light microscopy for 21 hours at room temperature. This was followed by washing in water for 10 minutes before a post-

fixation in a 1% osmium tetroxide solution in a sodium veronal acetate buffer, pH 7.4, for three hours at 4°C. After dehydration in alcohol and propylene oxide, the pieces were embedded in Epon 812. Longitudinal sections of 1–2 microns thickness were cut with a glass knife on an LKB-ultramicrotome. These sections were stained with a mixture of 1% toluidine blue and 1% borax in a ratio 1:1, pH 8.6–8.8, at a temperature of 50°C for about 45 seconds. In order to ascertain a correct orientation for ultrathin sectioning and to have sections for light microscopical examination from nearly the same area as the ultrathin sections, sections 1–2 microns in thickness were cut before and after ultrathin sectioning. Gold or silver coloured ultrathin sections were cut with a glass knife on the ultramicrotome. The sections were stained for 45 minutes with a saturated uranyl acetate solution in water. This was followed by a lead citrate staining for 8 minutes by REYNOLDS' technique (1963), or by the technique of VENABLE & COGGESHALL (1965) for 7 minutes (referred to in the legends of the figures as (R) and (V)). The stained sections were then examined with a Philips E.M. 300 electron microscope which was operated at 60 or 80 kV.

3. RESULTS

Some accidental observations are interesting for the "pre-patterning" of the wall thickening on the protoplast under investigation here. In preceding experiments it was occasionally observed that the protoplast was set free from the cell wall in electron microscopical sections *(fig. 1)* and also in sections with a thickness of 1–2 microns *(fig. 2)*, which were made before and after ultrathin sectioning for light microscopical examination.

The outline of the outer surface of the protoplast when set free from the cell wall is nearly identical to that of the in situ protoplast *(fig. 3B)*. The parts of protoplasm originally present between the cell wall thickenings appear as free ridges 0.5 to 1.5 microns high. Ridges of this size can be clearly seen with the light microscope.

The plasmolysis experiments could show whether the surface of the protoplast becomes smooth after plasmolysis or whether ridges originally fitting between the cell wall thickenings remain visible. After all of the plasmolysis times employed, the plasmolysed protoplast never became completely smooth. The shape of the ridges varies, depending on the duration of plasmolysis time *(fig. 3)*. The height of the ridges becomes smaller as the duration of plasmolysis increases. The illustrations in this paper are from material plasmolysed for 30 minutes.

Light microscopical examination gave the following results: a more or less regular pattern is visible on the protoplast *(fig. 4)*. It is difficult to decide whether this pattern is really present on the surface of the protoplast or whether it is an optical illusion caused by the already formed cell wall thickening pattern of the tracheary element. The image is rather confusing. However, some of the protoplasts accidentally became dislocated from the cells to which they belonged *(fig. 5)*, probably during sectioning. In these cases, the pattern

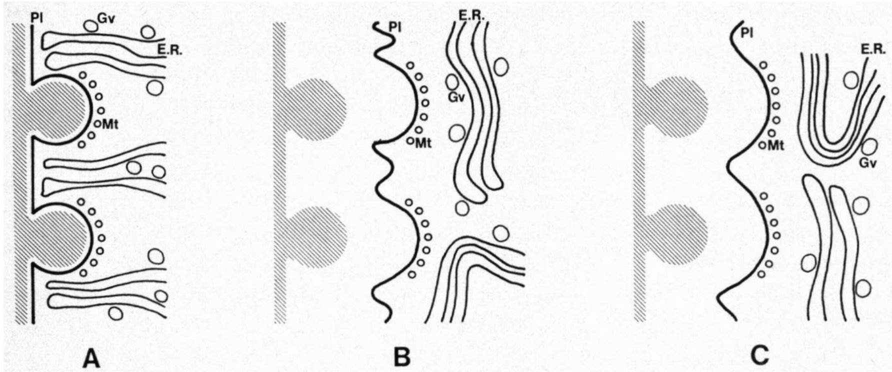

Fig. 3. Three schematic representations of a part of a radial section through a tracheary element.
A. The non-plasmolysed cell with the distribution of the cisternae of the endoplasmic reticulum, Golgi vesicles, and microtubules in the cytoplasm.
B and C. After plasmolysis: two frequently occurring shapes of the ridges on the outer surface of the protoplast. Microtubules are present between the ridges or the remnants of the ridges. Endoplasmic reticulum and Golgi vesicles are randomly distributed in the cytoplasm. In B the plasmolysis time is shorter than in C. The hatched areas represent cell wall material.

on the surface of the protoplast is clearly visible *(fig. 6)*.

Electron microscopical examination of the plasmolysed material also shows ridges on the protoplast, but the regularity in the pattern is not so clear as in the light microscopical photographs *(fig. 7)*. This may be due to the higher magnification of the electron micrographs. Depending on the plasmolysis time, the height of the ridges varies; the sites corresponding with the cell wall thickenings (between or facing) always remain recognizable on the surface of the contracted protoplast *(fig. 3)*. The ridges represent the parts which were located between the cell wall thickenings.

The light microscopical photographs do not give any information about the distribution of the two patterns of cell organelles in the cytoplasm. Only the nucleus and some large vacuoles can be observed *(fig. 4)*.

Electron microscopical examination gave the following characteristics of the morphology of the cell organelles and structures present and of their distribution in the plasmolysed protoplasts. The rough cisternae of the endoplasmic reticulum become arranged parallel to each other. It is a conspicuous arrangement of many curves which are randomly dispersed in the protoplast *(fig. 8)*. Dictyosomes and many electron-transparent Golgi vesicles are clearly visible *(fig. 9)*. The dictyosomes are present in a shape different from that in the normal non-plasmolysed situation. The cisternae of the stack are bent *(figs. 9* and *10)*. Sometimes a group of dispersed ribosomes is visible, more or less surrounded by membranes *(fig. 11)*. This membrane possibly represents one cisterna of a dictyosome. Mitochondria and plastids are present; these cell organelles seem to be stretched out a little *(fig. 12)*. The membrane system in the mito-

chondria sometimes shows a deviated parallel arrangement *(fig. 10)*. Furthermore, myelin-like arrangements of membranes are visible *(fig. 12)* resembling the myelin-like bodies of smooth endoplasmic reticulum as described by ROBARDS & KIDWAI (1969) for differentiating cells of the vascular cambium. A few lipid bodies surrounded by a membrane are present *(fig. 10)*. Some vesicles with electron-opaque contents are observed, but perhaps these vesicles are present in plastids *(fig. 13)*. Many dispersed ribosomes are present which give the impression of a densely packed protoplasm *(fig. 12)*. Many small vacuoles without visible contents *(fig. 14)* and a dark stained nucleus with nucleoli are present *(fig. 7)*. The above-mentioned structures and cell organelles are not distributed in a special way; all structures seem to be disorganized. A pattern in the distribution of the cisternae of the endoplasmic reticulum and the Golgi vesicles is certainly not present.

The microtubules remain situated at their original sites along the plasmalemma, as in the non-plasmolysed situation *(fig. 15)*. They lie between the ridges or the remnants of the ridges.

The plasmolysed protoplast remains surrounded by the plasmalemma. The plasmalemma is smooth as in the non-plasmolysed situation.

As far as could be seen, during plasmolysis no change takes place in the cell wall thickenings.

It is clear from the electron micrographs that plasmolysis did not damage the protoplast surface or the cell wall with the cell wall thickenings.

External to the plasmalemma, various structures are present: various vesicles *(fig. 10)*; elongated structures *(figs. 9* and *10)* (cytoplasmic threads?; HECHT 1912); small membrane bound parts of protoplasm and fibrillar material resembling cellulose microfibrils *(fig. 13)*; and myelin-like membrane systems *(fig. 16)*.

The vesicles are possibly involved in the deposition of cell wall material of the thickenings. The function(s) of the membrane system is not known. Both vesicles and membrane systems can also be observed between the plasmalemma and the cell wall in the non-plasmolysed material. Therefore, it is not likely that these structures are artifacts caused by plasmolysis.

In more tangentially sectioned tracheary elements, the microtubules are sectioned longitudinally. Sometimes it seems that the fibrillar material which is present between the protoplast and the cell wall is connected with the microtubules. The orientation of microtubules and the fibrillar material is the same *(fig. 17)*. A relationship between microtubules and the fibrillar material seems to be evident.

4. DISCUSSION

The situation concerning the non-plasmolysed and the plasmolysed cell will first be considered separately.

Light microscopical examination of the non-plasmolysed cell shows a pattern on the surface of the protoplast in the tracheary elements in the hypocotyl of

Cucumis 'Sporu-Origineel' (*figs. 2* and *18*). Detailed structures such as granules or vacuoles can be observed neither in the lighter bands nor in the darker bands.

The same material when observed with the electron microscope does not show a clear pattern on the surface of the protoplast. At close examination, however, by determining densities, a pattern appears to be present in the distribution of the cisternae of the endoplasmic reticulum and Golgi vesicles corresponding with the developing thickenings (GOOSEN-DE ROO 1973b). A pattern of microtubules alternating with the pattern mentioned above can also be observed (GOOSEN-DE ROO 1973a).

In the plasmolysed cell both the light and the electron microscope show ridges on the surface of the protoplast. The height of the ridges varies with the plasmolysis time. The ridges, however, are more conspicuous in the material prepared for light microscopical examination than in the material prepared for electron microscopy. This might be due to the differences in the fixation and embedding methods for light and electron microscopy.

The patterning of the microtubules corresponding with the developing cell wall thickenings is still present in the material prepared for electron microscopy. However, the pattern of the cisternae of the endoplasmic reticulum and Golgi vesicles has disappeared. The situation in the protoplast is not the same before and after plasmolysis. Two reasons can be mentioned as to why the observed pattern in the non-plasmolysed situation is of a different nature than in the plasmolysed situation. Firstly, the pattern of the cisternae of the endoplasmic reticulum and the Golgi vesicles together has vanished. Secondly, the occurrence of free ridges after plasmolysis may cause differences. It appears that, in the non-plasmolysed situation, the cell wall thickenings are clearer than the areas of protoplasm (non-free ridges) between the cell wall thickenings. The free ridges, although they are lower than the original non-free ridges, can be observed after plasmolysis.

The occurrence of the free ridges observed after plasmolysis can be explained in three different ways:

1. the relief on the outer surface of the protoplast is merely an imprint of the cell wall thickening.
2. The ridges are caused by a differential shrinkage of the protoplast. This could be the result of differences in dehydration of protoplasmic areas with many cell organelles and areas with fewer organelles.
3. The ridges are caused by longitudinal shrinkage of the microtubules resulting in local constrictions of the protoplast.

By using the term imprint as an explanation, one implicitly makes the following assumptions: 1. the protoplast is a structure that can hold an imprint such as wax or clay; 2. the internal structure of the protoplast, although dehydrated, is basically unaltered. Both assumptions must be repudiated. The protoplast is notorious for its high water content. Unless held in place by the cell wall, a protoplast will usually assume a rounded shape. Therefore it seems improbable that a simple imprint will be formed. Besides, the original distribu-

tion of the cisternae of the endoplasmic reticulum and the Golgi vesicles has disappeared; all cell organelles are disorganized.

In case of differential shrinkage one might assume that areas of the protoplast with high densities of cell organelles contain less water than areas with low densities. Under these conditions, it seems likely that the areas with high water contents lose more water and more quickly than the other areas during plasmolysis. The effect of such a differential loss of water would be differential shrinkage of the various areas of the protoplast. It is conceivable that this differential loss of water causes both the disappearance of the density differences in endoplasmic reticulum and Golgi vesicles and the appearance of ridges on the outer surface of the protoplast.

With respect to the third explanation (longitudinal shrinkage of microtubules), it is possible that the microtubules shrink more than does the rest of the protoplast. Therefore, it might be that the microtubules are actually constricting the protoplast after plasmolysis. If that is so, the ridges are formed by the protoplasmic areas bulging between these constrictions. From our electron micrographs it seems likely that the microtubules do shrink; at least, there are no indications that the microtubules have maintained their original length in the plasmolysed protoplast. If they had, one would expect to find turns and loops in the microtubules. As a consequence we should find them in all directions in sections. Since we found only cross sections of microtubules, it is likely that the microtubules did indeed shrink. The electron micrographs of the ridges, however, are not very suggestive of strong constrictions by the microtubules. Although we cannot exclude this possibility, it seems unlikely that constriction is a major factor in the formation of the surface ridges on the plasmolysed protoplast.

Crüger and Dippel observed a pattern of lighter and darker bands on the surface of the plasmolysed protoplast. The darker bands were composed of granules. In our plasmolysed material we also observed a striped pattern of lighter and darker bands under the light microscope. Comparing the light microscope findings with the electron microscope findings, the striped pattern can have different origins in the plasmolysed material. It may be that the pattern is due only to the presence of the surface ridges; i.e., to local differences in the diameter of the protoplast. Another possiblility is that the alternation of bands with microtubules and bands without microtubules is an actuality.

At first sight, it may seem unlikely that microtubules can be observed under the light microscope. In connection with this, however, we have to recall the fact that the nuclear spindle which is also composed of microtubules is clearly visible under the light microscope – especially in fixed plant material. When the bands composed of microtubules are visible on light microscopical examination, it is difficult to understand that circumferentially arranged microtubules have been observed as granules.

Two other points seem to be in agreement with the supposition that the pattern is the result of the alternation of bands with and without microtubules: 1. if the microtubuli dictate the observed pattern and represent the darker

bands, these would correspond with the sites where the cell wall thickenings develop. This is in agreement with the suggestions of the authors cited in the introduction. 2. During nearly the whole period of thickening formation the microtubules are located at sites corresponding with the thickenings. Only at the end of the cell wall thickening formation they are no longer present in bands but are dispersed along the whole plasmalemma between as well as surrounding the thickenings (Goosen-de Roo 1973a). Therefore, in the final stage of thickening formation, light microscopical examination no longer shows the pattern on the surface of the protoplast. Dippel's observations are in complete agreement with these statements.

5. CONCLUSION

After plasmolysis during the formation of cell wall thickenings, the electron microscope reveals two patterns on the surface of the protoplast in tracheary elements which are related to the cell wall thickenings.

These patterns are: 1. ridges caused by differential shrinkage of the protoplast; and 2. bands of microtubules corresponding with the developing cell wall thickenings.

Both of these patterns can be related to the pattern observed on the surface of the protoplast by light microscopy after plasmolysis. There are a few points in favour of the second pattern, but no conclusive evidence can be given at present.

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Legend of photographs

Fig. 1. In the most central tracheary element the protoplast has become detached from the cell wall. The outer shape of the protoplast is largely maintained; (V), × 14,200.
Fig. 2. Section of 1 micron thickness. The protoplast in one of the tracheary elements has become partially detached from the cell wall (arrows). A pattern on the protoplast seems to be present in the cell marked with an asterisk, × 460.
Fig. 4. Section of 10 microns thickness. A striped structure appears to be present on the contracted protoplast and in the cell wall. The nucleus and some large vacuoles are visible, × 580.
Fig. 5. A dislocated protoplast lying on a section following plasmolysis, × 180.
Fig. 6. Detail of *fig. 5*; the striped structure is clearly visible on the surface of the protoplast, × 580.
Fig. 7. Electron microscopic view of the plasmolysed material. Ridges are present on the outer surface of the protoplast. Note the electron-opaque nucleus; (R), × 2,400.
Fig. 8. Tracheary element after plasmolysis; cisternae of the rough endoplasmic reticulum are arranged parallel to each other and in many curves. The cisternae are no longer restricted to certain areas in the cytoplasm; (V), × 6,870.
Fig. 9. Detail of a plasmolysed protoplast showing a dictyosome and many Golgi vesicles. Note the bent cisternae of the dictyosome, also the elongated structure at the surface of the protoplast; (R), × 36,500.
Fig. 10. Protoplasmic area with bent cisternae of a dictyosome, a mitochondrion with disoriented internal membranes, and a lipid body. Various vesicles are visible between the cell wall material and the plasmalemma. Note the elongated structure at the surface of the protoplast; (R), × 36,500.
Fig. 11. Some groups of ribosomes seem to be surrounded by a membrane (arrows). This membrane is probably a bent cisterna of a dictyosome; (R), × 36,500.
Fig. 12. General view of a plasmolysed protoplast which is densely packed with ribosomes. Mitochondria and plastids seem to be stretched out. A myelin-like arrangement of membranes is visible; (V), × 15,500.
Fig. 13. Vesicle with electron-opaque contents (arrow). Fibrillar material is present between the cell wall thickenings and the plasmalemma; (R), × 36,500.
Fig. 14. Area of protoplasm with many small vacuoles; (R), × 36,500.
Fig. 15. Microtubules are situated along the plasmalemma between the remnants of the ridges; (R), × 22,800.
Fig. 16. A myelin-like membrane system is present between cell wall material and plasmalemma; (R), × 36,500.
Fig. 17. A tangentially sectioned area of a plasmolysed protoplast. Microtubules are sectioned longitudinally; fibrillar material seems to be connected with the microtubules. The orientation of the microtubules and the fibrils is identical; (V), × 34,470.
Fig. 18. Non-plasmolysed protoplasm in tracheary elements. A pattern is visible on the protoplast (arrows), × 380.

List of abbreviations used in figure and photographs

D	dictyosome	N	nucleus
ER	endoplasmic reticulum	P	dlastid
Fi	fibrillar material	Pl	plasmalemma
Gv	Golgi vesicle	R	ribosomes
Li	lipid body	Ri	ridge
M	mitochondrion	T	cell wall thickening
Mt	microtubule	V	vesicle
My	myelin-like membranes	Va	vacuole

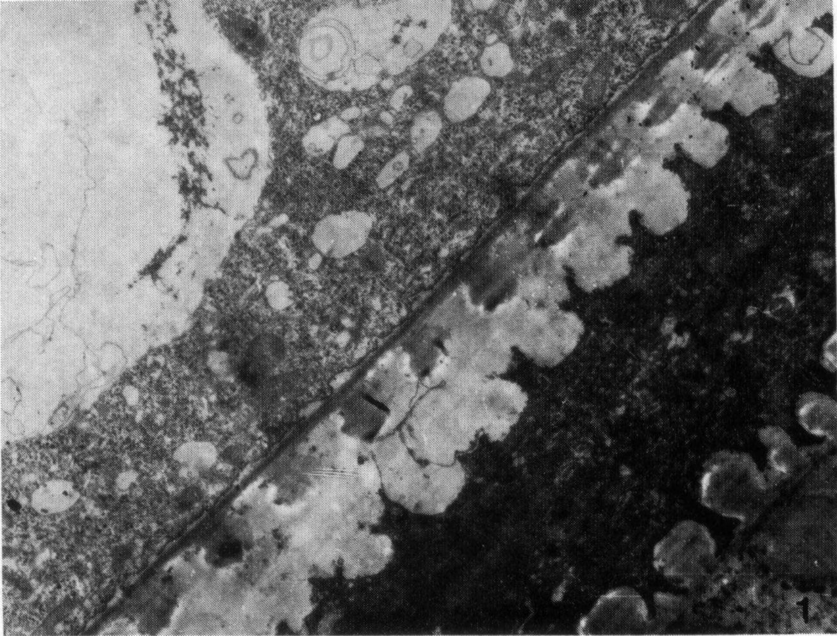


Fig. 1.

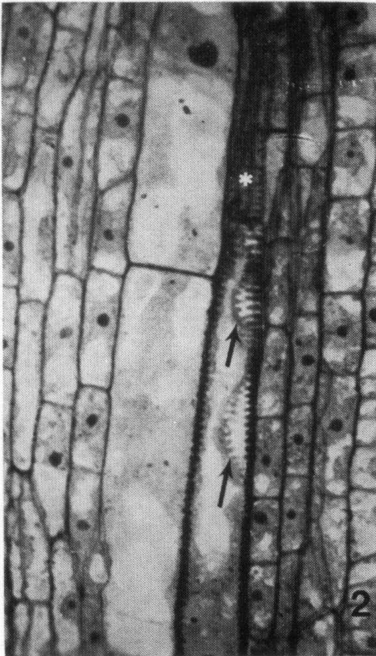


Fig. 2.

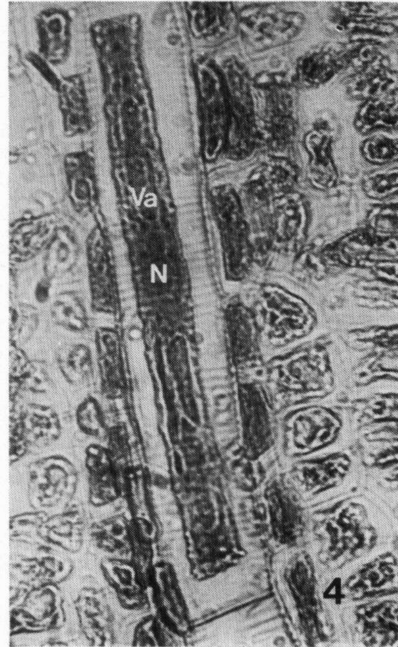


Fig. 4.

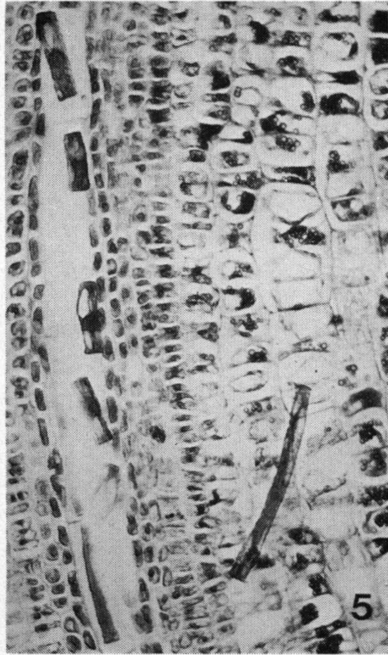
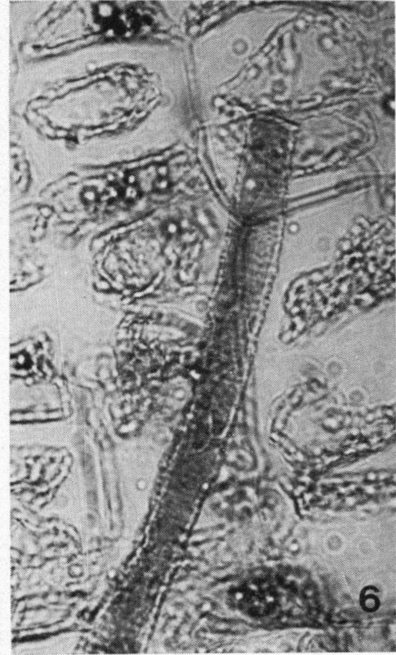


Fig. 5.



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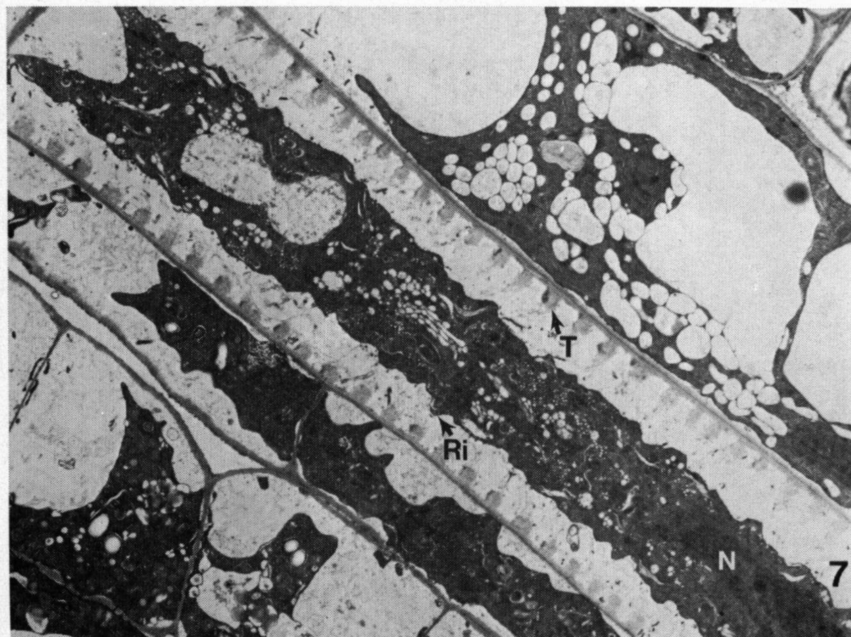

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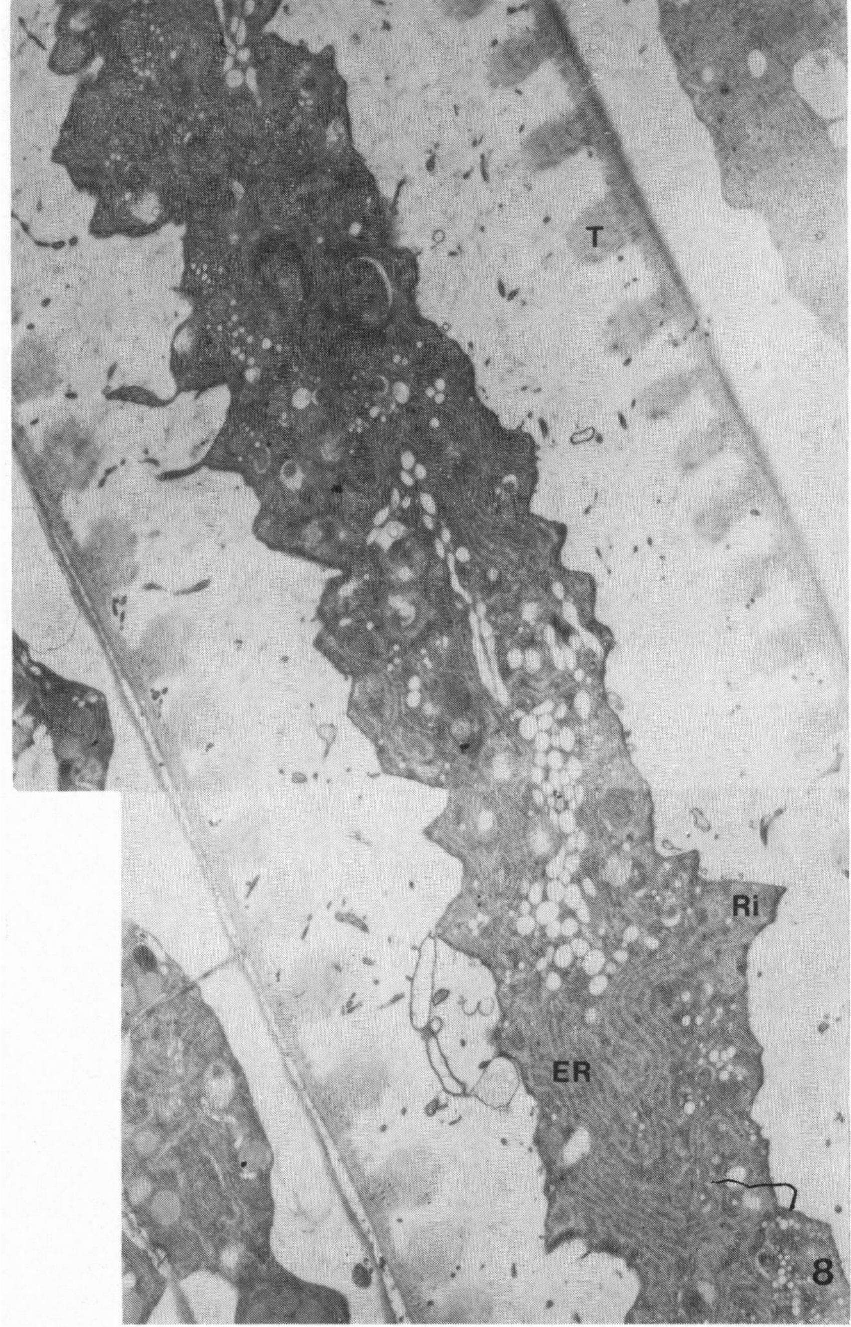

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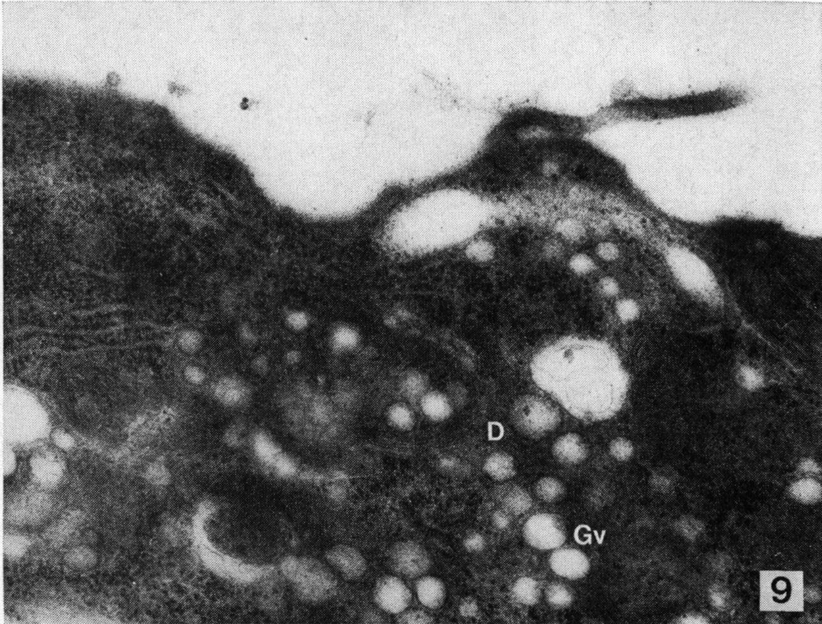

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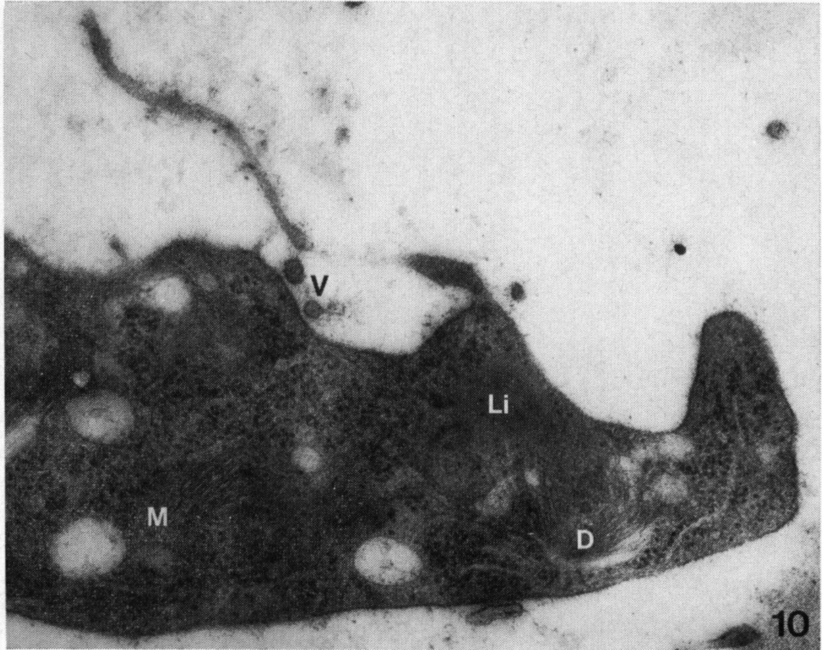

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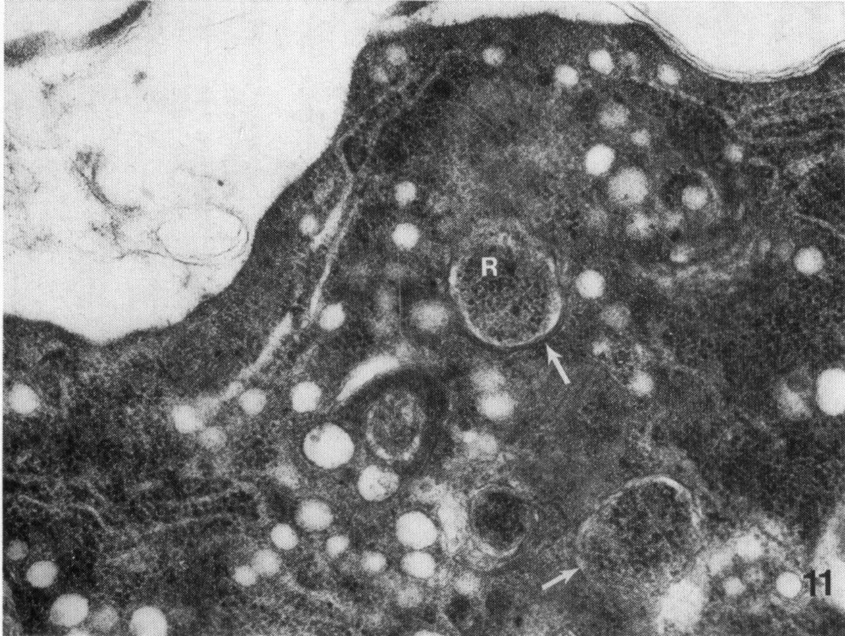

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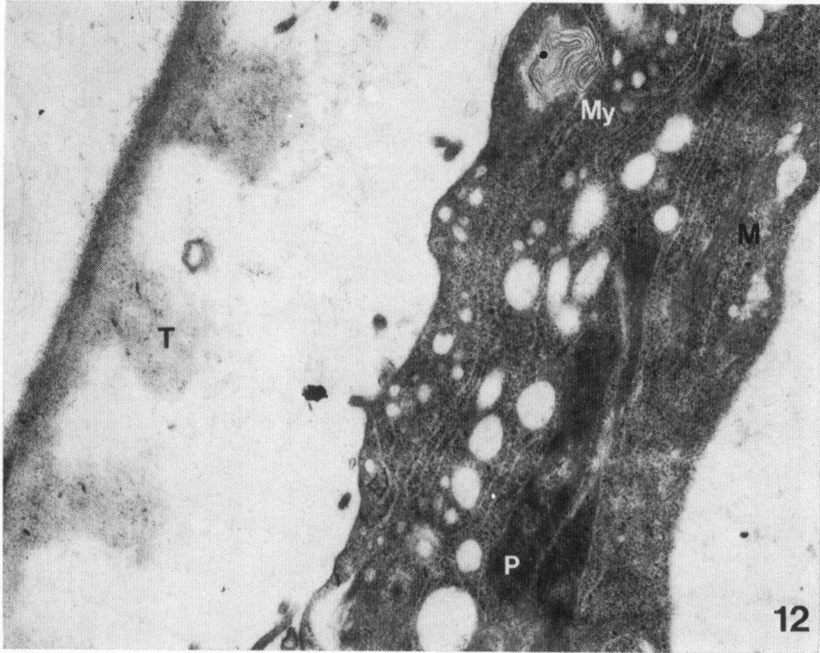

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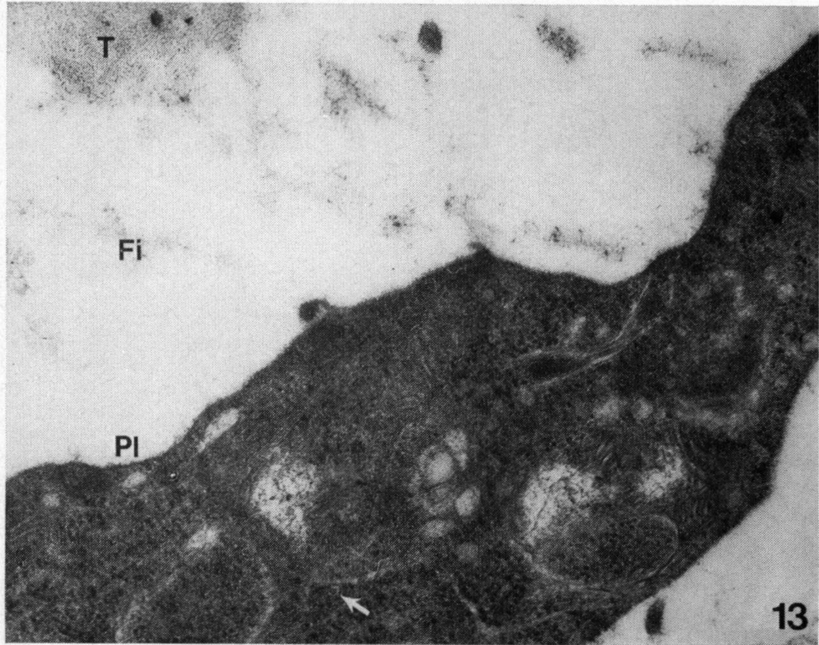

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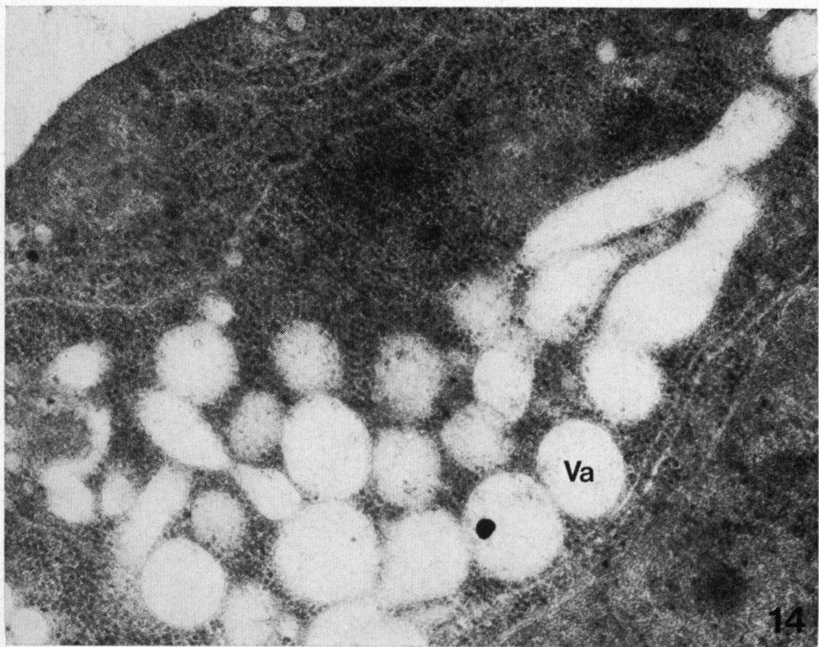

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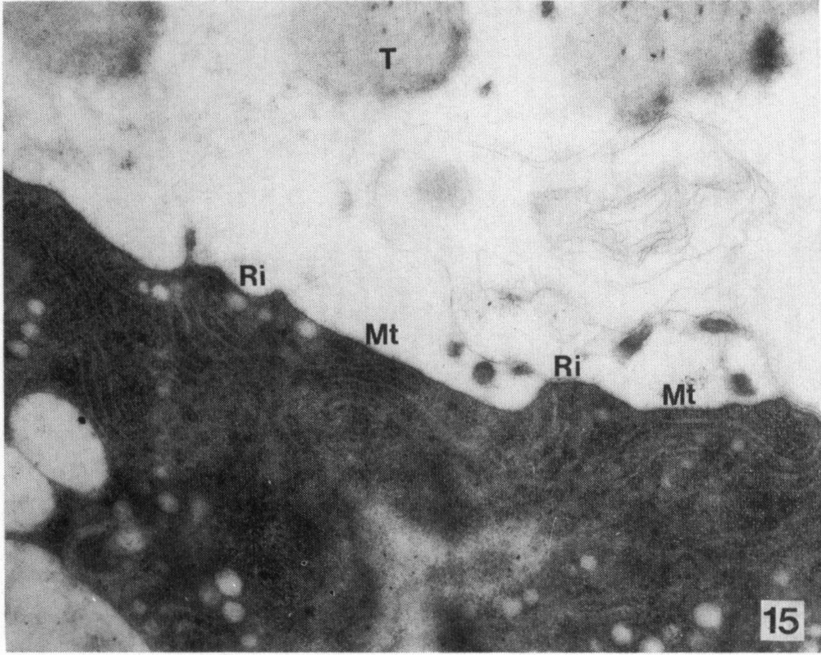

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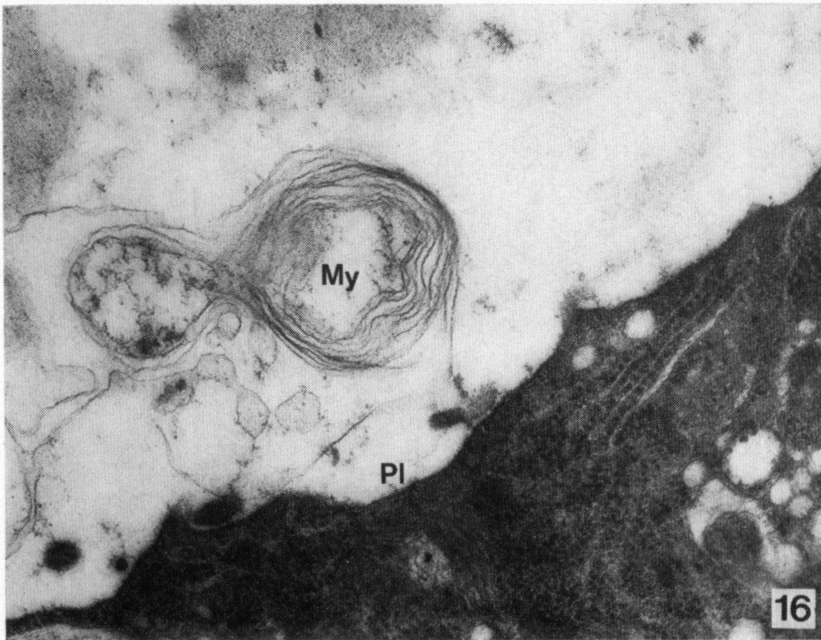

Fig. 16.

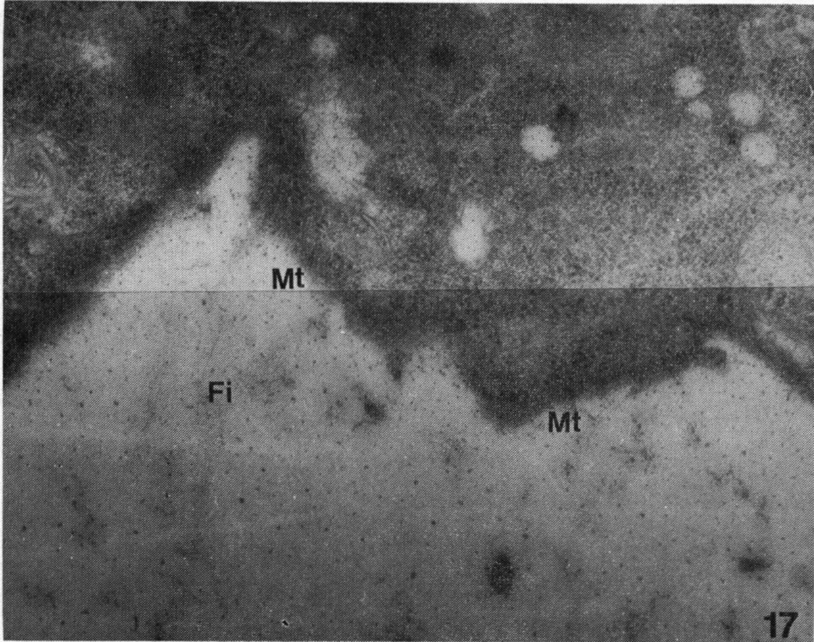

Fig. 17.

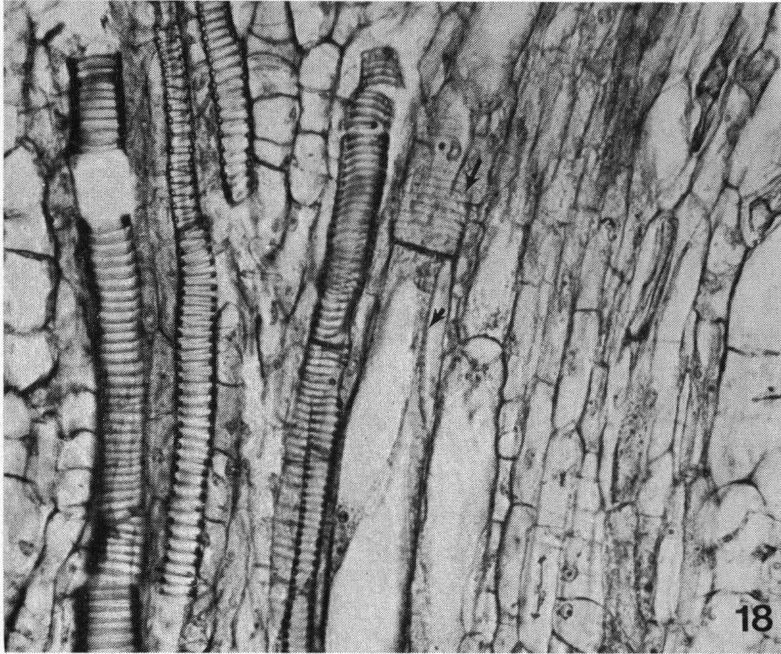

Fig. 18.