Fine Structure of Secondary Wall Thickening and a Role of Microtubules in Primary Xylem Cells of Poplar*

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Summary——Secondary wall thickening and organization of the cytoplasm during development of primary xylem cells of poplar were investigated by sectioning and freeze etching technique.

Microtubules usually aggregate only above the helical thickenings or bands throughout the the development of primary xylem cells but not above the areas where no secondary wall deposition occurs, and run parallel to the microfibrils within the bands. In view of their general distribution throughout cell organelles, plasmalemma particles of primary xylem cells visible by freeze etching technique are questionable to be considered as the enzyme complex involved in the synthesis and orientation of cellulose microfibrils of secondary wall. Thus, microtubules may be considered as the only organella to determine the orientation of microfibrils.

A possible association of microtubules and Golgi-derived vesicles during wall thickening is assumed by the fact that the position and alignment of microtubules are closely related to those of Golgi-derived vesicles. It seems that the microtubules play a part for the incorporation of Golgi-derived vesicles into the destined site of cell wall.

Introduction

It was suggested that particles arranged in regular arrays on plasmalemma surface of yeast were involved in the synthesis of glucan fibrils of the cell wall[1]. PRESTON (1964)[2] subsequently postulated that the regular arrays of wall particles were responsible for the orientation of cellulose microfibrils in *Chaetomorpha*. ROBINSON and PRESTON (1972)[3] recently suggested that granule bands at the plasmalemma of *Oocystis apiculata* were involved in not only microfibril synthesis but also orientation of microfibrils. In higher plant, BRANTON and MOOR (1964)[4], MÜHLETHALER (1967)[5], and NORTHCOTE and LEWIS (1968)[6] proposed that plasmalemma particles were involved in the synthesis of cellulose microfibrils, although regular patterns of plasmalemma particles were not observed. However, CHAFE and WARDROP (1970)[7] found no close correlation between microfibril orientation and the particle distribution on the plasmalemma of collenchyma of *Apium graveolens*, differentiating xylem fibers of *Eucalyptus maculata* and cortical parenchyma of *Avena sativa*. They concluded that the microtubules are the structures which are most likely to be involved in determining microfibril orientations in the cell

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wall.

Since the first investigations by LEDBETTER and PORTER (1963)[8] on the role of microtubules involved in microfibril orientation, many investigations[9~21] have demonstrated the role of oriented microtubules in controlling the microfibril orientation in different types of plant cells undergoing secondary wall thickening. On the other hand, NEWCOMB and BONNETT (1965)[22] observed that the oriented microtubules of root hairs of radish extended through the 20 to 25 μ zone near the tip where the wall structure consisted of random microfibrils. ROBARDS and HUMPHERSON (1967)[23] found many cases where microtubules are not parallel to microfibrils through the investigation of developing bordered pit of *Salix fragilis*. BERLYN (1970)[24] observed the sporadic occurrence of microtubules, which does not substantiate a general role in elementary fibrillar orientation. Thus, the involvement of microtubules in microfibril orientation has not been clearly understood.

Furthermore, the function of Golgi bodies is well known as secreting their vesicles to the cell wall[25]. It is the problem how the Golgi-derived vesicles could be successfully transported to the areas where the deposition of cell wall substances occur. Northcote (1968)[26] postulated that microtubules might be concerned with supplying matrix substance from Golgi bodies to the wall. Evidences for this postulation were noted by PICKETT-HEAPS (1968)[27] on the autoradiographic investigation of xylem wall deposition with lignin precursors, ROBARDS (1968)[28] in differentiating xylem cells of *Salix fragilis*, NEWCOMB (1969)[29] in a tracheary element of root tip of *Arabidopsis thaliana* and MAITRA and DE (1971)[30] in differentiating xylem element of *Alfalfa*. The present author could fully substantiate the association of microtubules and Golgi-derived vesicles in primary xylem cells of poplar.

The present paper was undertaken to see whether either microtubules or plasmalemma particles are involved in determining the microfibrillar orientation in the development of primary xylem cells of poplar. The paper also discussed the relationship between microtubules and vesicles.

Material and Methods

The material was obtained from young shoots of poplar tree (*Populus nigra* var. *italica* Koehne), grown in the experimental field of Wood Research Institute, Kyoto University, Uji, Kyoto. The shoots were cut and placed directly into an aqueous solution of 0.5% colchicine. After appropriate exposure, they were washed, cut into sugments and fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) or KARNOVSKY's solution[31] for 6~12 hr at 4°C. The non-treated shoots were cut with a razor blade and immediately fixed in the above fixation solution. Then, the segments

of colchicine-treated and non-treated shoots were washed thoroughly in a solution containing 0.05 M phosphate buffer (pH 7.2) and postfixed in 1 % osmium tetroxide in 0.05 M phosphate buffer (pH 7.2) for 6～12 hr at 4°C. After further washing, they were dehydrated through a graded acetone series. They were then passed through propylene oxide and finally embedded in Epoxy resin.

Gold or silver sections were cut using glass kinves on a Porter-Blum type MT-1 ultramicrotome and were mounted on 150 mesh copper grids coated or non-coated with collodione. The sections were then stained on the grids with uranyl acetate and lead citrate.

Freeze fractured and freeze etched replicas were prepared in an apparatus deviced by NISHIURA[32] (now available as HFZ-1 Hitachi Freeze Replication Apparatus). The specimens were usually immersed in 50 % glycerol solution before freezing. After vacuum pressure below 1×10^{-5} Torr was obtained, specimens were cut with a knife and, when desired, etched by elevating the specimen temperature to −100°C for 2 min. Replication was carried out by Pt/Pd shadowing and carbon coating. Replicas were cleaned with acidified sodium chlorite solution, 72 % Sulfuric acid and commercial bleaching solution before mounting on non-coated 200 mesh grids.

Both thin sections and replicas were examined in a JEM T6S electron microscope using 60 kV.

Results

Primary xylem cells are characterized by their helical wall thickenings. This is important for studing the formation of secondary wall, since, when examined by sectioning technique, both thickening and non-thickening area could be seen in the same field of electron microscope and be compared with them each other.

Figs. 1, 2 and 3 show the secondary wall thickenings at different stages of primary xylem development. For convenience, three stages of helical wall thickening which were supposed to be useful for dynamic observation were distinguished as early, middle and late ones. In the early and middle stages, many microtubules were found only above the thickening, whereas they were sparsely found in the late stage. In a section cut throughout the thickened bands, microtubules ran parallel to the regularly oriented microfibrils within the bands.

Figs. 4, 5 and 6 showed freeze fractured or freeze etched image of primary xylem cells. Microfibrils within the bands clearly ran parallel to the direction taken by the bands (Fig. 4). Many plasmodesmata sometimes aggregated on the plasmalemma surface between the incipient thickenings and thus constructed a broad area of primary pit field. Lomasome like structures were found on the concave plane of plasmalemma

of developing primary xylem cells (Fig. 5). In addition, 110~150 A particles distributed randomly throughout the convex and concave planes of plasmalemma situated not only above the thickenings but also above the areas between the incipient thickenings. The broad grooves on the convex plane of plasmalemma, indicated by arrows in Fig. 6, showed the traces of band thickenings removed by fracturing. There could be seen no regularly arrayed plasmalemma particles on the traces of band thickenings.

Figs. 7 and 8 show the band thickenings of developing primary xylem cells treated with colchicine. Although PICKETT-HEAPS (1967)[17] and HEPLER and FOSKET (1971)[18] reported irregular deposition of secondary wall material, similar evidence was not observed above thickenings of poplar primary xylem cells. This may be because secondary wall materials were not fully incorporated into the poplar tissues during the treatment of colchicine. In the present study, the application of colchicine resulted in the almost total disappearance of cortical microtubules (Figs. 7 and 8). However, the same reagent did not destroy the microfilaments (Fig. 7). Moreover, in colchicine treated cells many vesicles distributed randomly above the thickenings as well as between the incipient thickenings (Fig. 8).

Cell organelles located near the cell wall were mainly ER, Golgi bodies and microtubules. It was not apparent whether ER-derived veiscles occur during development of the bands, but some ER had the inflated appearance, suggesting the possibility that ER-derived vesicles occur (Fig. 10). Similar evidence was noted by the present author (1974)[20] in developing cotton fiber. Golgi bodies excreted vesicles actively in early and middle stages, and many vesicles were located near the plasmalemma. In late stage, however, they were located apart from but not near the plasmalemma. In transverse sections of differentiating primary xylem cell, microtubules were present in the peripheral cytoplasm and cytoplasmic vesicles aligned in a row along a microtubule (Fig. 9). It was also shown that these vesicles were mainly derived from Golgi bodies (Figs. 1, 2, 3 and 9). Vesicles surrounded with microtubules were found and some vesicles seemed to be incorporated in the cell wall (Fig. 11). In grazing longitudinal sections of the differentiating cells, microtubules and vesicles assembled above thickenings which were protruding into the cytoplasm (Fig. 12). In this figure, the close correlation of microtubules and vesicles could be seen all above four thickenings. The relation was shown more clearly in Fig. 13; the bundles of microtubules extended at the thickenings together with vesicles.

Since the finding of microtubules by LEDBETTER and PORTER (1963)[8], the shape of microtubules observed by sectioning method is straightly extending or moderately curving tube. In the present investigation, however, undulated microtubules were sometimes observed (Figs. 14 and 15).

Disussion

Recently, by the development of freeze etching technique, plasmalemma particles have been increasingly noticed as the enzyme complex for the formation of cell wall fibrils both in lower and higher plants[1~6,33,34]. Plasmalemma particles visible by this technique, however, could not be decided as the enzyme complex involved in the synthesis of cell wall substances, since these particles distributed randomly not only above the secondary wall thickenings but also above areas between the incipient thickenings where little or no cell wall materials were synthesized. Although several investigations have suggested that orientation of cell wall fibrils is determined by the regular arrangement of plasmalemma particles[1~3], the particles of poplar primary xylem cells are not regularly arranged at the localized thickenings. Accordingly, plasmalemma particles may not also be concerned with controlling the orientation of cellulose microfibrils.

Microtubules are considered to be concerned with cell development in various ways[5,29]. Although SANGER and JACKSON (1968)[35] have reported a case that microtubules appear to play a cytoskeletal role in controlling the shape of pollen in *Haemanthus* during its development, the fact that microtubules run parallel to microfibrils of the bands and were found only above the thickenings and not above the areas between the incipient thickenings suggests that microtubules mirror the orientation of microfibril deposition, but not function in supporting of plasmalemma or cell form. If the microtubules are regarded as elements supporting the form of immature primary xylem cells, they should also appear in the areas between the incipient thickenings. From the results of both freeze etching and sectioning experiments, it is reasonable to suppose that microtubules are the only organella which determine the orientation of cellulose microfibrils.

In the early and middle stages of band thickening occur many microtubules which might be related to the active incorporation of Golgi-derived vesicles, although they decreases in late stage. Moreover, occurrence of the complex of microtubules and vesicles above four thickenings as shown in Fig. 12 means functionally a close correlation between them. This relation is further evidenced by the appearance of vesicles surrounded with several microtubules. Fig. 13 may further strengthen the correlation. Both microtubules and vesicle arrange like an arc; vesicles seems to be conducted by microtubules. Through these observations, it is indicated that the occurrence of complex of microtubules and vesicles is not a case of chance association of two organelles.

According to van ITERSON (1937)[36], the cellulosic fibrils is produced by the flowing cytoplasm, and subsequently deposited and incorporated into walls in the direction of the stream. The view is well illustrated by close relationship between the microtubules and protoplasmic streaming found by LEDBETTER and PORTER[8,37]. Their finding is strongly supported by the fact that microtubules are numerous in streaming endoplasm[38~41].

However, other investigators[42,43] have concluded that microfilaments are involved in protoplasmic streaming for the reason that microfilaments paralleled the main axis of the cell and the direction of streaming, while microtubules sometimes observed in the same cell were transversly oriented. Similar results obtained in the present investigation suggest that microfilaments are related to a main motive source for protoplasmic streaming. Undulated microtubules, however, were observed above the band thickenings (Figs. 14 and 15). Similar structures were noted by ESAU (1968). The undulated form of microtubules may indicate that localized streaming of more peripheral cytoplasmic layers immediately beneath the thickenings may be produced by the cortical microtubules for the purpose of the active incorporation of Golgi-derived vesicles into the secondary wall.

BURGESS and Northcote (1968)[44] showed that smooth elements of ER were found in close association with the microtubules during the onset of prophase and the development of the mitotic spindle. He thought that the ER could thus be important for the processes of transport and aggregation of the microtubular subunits. In differentiating xylem cells, however, there is no definite evidence demonstrating that any organellae may be involved in the synthesis of microtubules. Some authors[45,46] presented the possibility that the microfilaments might be subunits of microtubules. Since microtubules disintegrated through the experiment of colchicine impregnation but microfilaments did not, microtubules and microfilaments might be different in their chemical composition.

It is well known that the role of Golgi bodies is to transport wall precursors to the walls in the form of vesicles[25]. It is not obvious, however, whether cellulose originates from precursors being secreted by Golgi bodies. Autoradiographic and chemical studies on sycamore seedling stems by WOODING (1968)[47] suggested that cellulosic precursors might not pass through Golgi bodies. Moreover, on the investigation of developing cotton fiber which consists of almost all cellulose, the present author[20] found that vesicle-secreting ability of Golgi bodies seems to be very poor and suggested that Golgi-derived vesicles could not excrete cellulosic precursors to the wall. The results obtained by NOBUCHI and FUJITA (1972)[19] and FUJITA et al. (1974)[21] both in differentiating tension wood fiber (which is also rich in cellulose) of poplar seem to support the above finding. In view of these fact, it is considered that the Golgi-derived vesicles observed in the present investigation may contain the precursors other than cellulose, that is, those of matrix substance and/or lignin.

In addition to the Golgi-derived vesicles, great notice has been taken on lomasome in relation to the formation of cell wall[24,48~51]. The funtion of lomasome could not be clearly understood in this study. Since chemical structure and biosynthetic pathway of cellulose are related to those of matrix substances such as pectin or hemicellulose[52],

both cellulose and matrix substance might be incorporated in the cell wall even in the ultrastractural level.

Further studies are needed to demonstrate clearly the way of incorporation of cellulose, lignin and matrix substances through cytoplasm into secondary wall under ultrastructural level.

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Abbreviations used

G Golgi body	Lo Lomasome	Mf Microfilament
PL Plasmalemma	PPF Primary pit field	T Tonoplast
⌒ Convex plane	⌣ Concave plane	⇔ Direction of shadowing

Fig. 1. Early stage of band thickening. Many microtubules occur at the thickenings, and Golgi bodies (G) are actively engaged in vesicle secretion. ×41,000.

Fig. 2. Middle stage of band thickening. Microtubules occur at the thickenings, and Golgi bodies are actively engaged in vesicle secretion. ×41,000.

Fig. 3. Late stage of band thickening. Microtubules occur spatially at the thickenings, and the Golgi bodies are not so active in vesicle secretion as in the former two stages. ×41,000.

Fig. 4. Freeze etched wall of a primary xylem cell. Microfibrils clearly run parallel to the dirction taken by the bands. Between the bands are seen many plasmodesmata, which construct broad primary pit fields. ×8,000.

Fig. 5. Freeze fractured primary xylem cell. In the right are shown concave plane of plasmalemma which holds much less numerous particles and several lomasome like structures. ×3,500.

Fig. 6. Freeze fractured. Broad grooves on the convex plane of plasmalemma (arrows) indicate the traces which are produced by the splintering away of thickened bands. Plasmalemma particles distributed randomly not only above the band thickenings but also above the areas between the incipient thickenings. ×8,000.

Fig. 7. Colchicine treated. Microtubules are disintegrated by colchicine, while axially oriented microfilaments are not. ×20,000.

Fig. 8. Colchicine treated. No microtubules can be seen above the thickenings. Golgi bodies are active to produce vesicles which are distributed randomly above the thickenings as well as between the thickenings. ×30,500.

Fig. 9. Cross section through the band thickenings of differentiating primary xylem cell. A number of vesicles arrange along a microtubule (arrow) just beneath the plasmalemma. These vesicles are derived from Golgi bodies. ×28,000.

Fig. 10. Some inflated ER (small arrows) and vesicles surrounded with microtubules (large arrows) are shown. ×44,500.

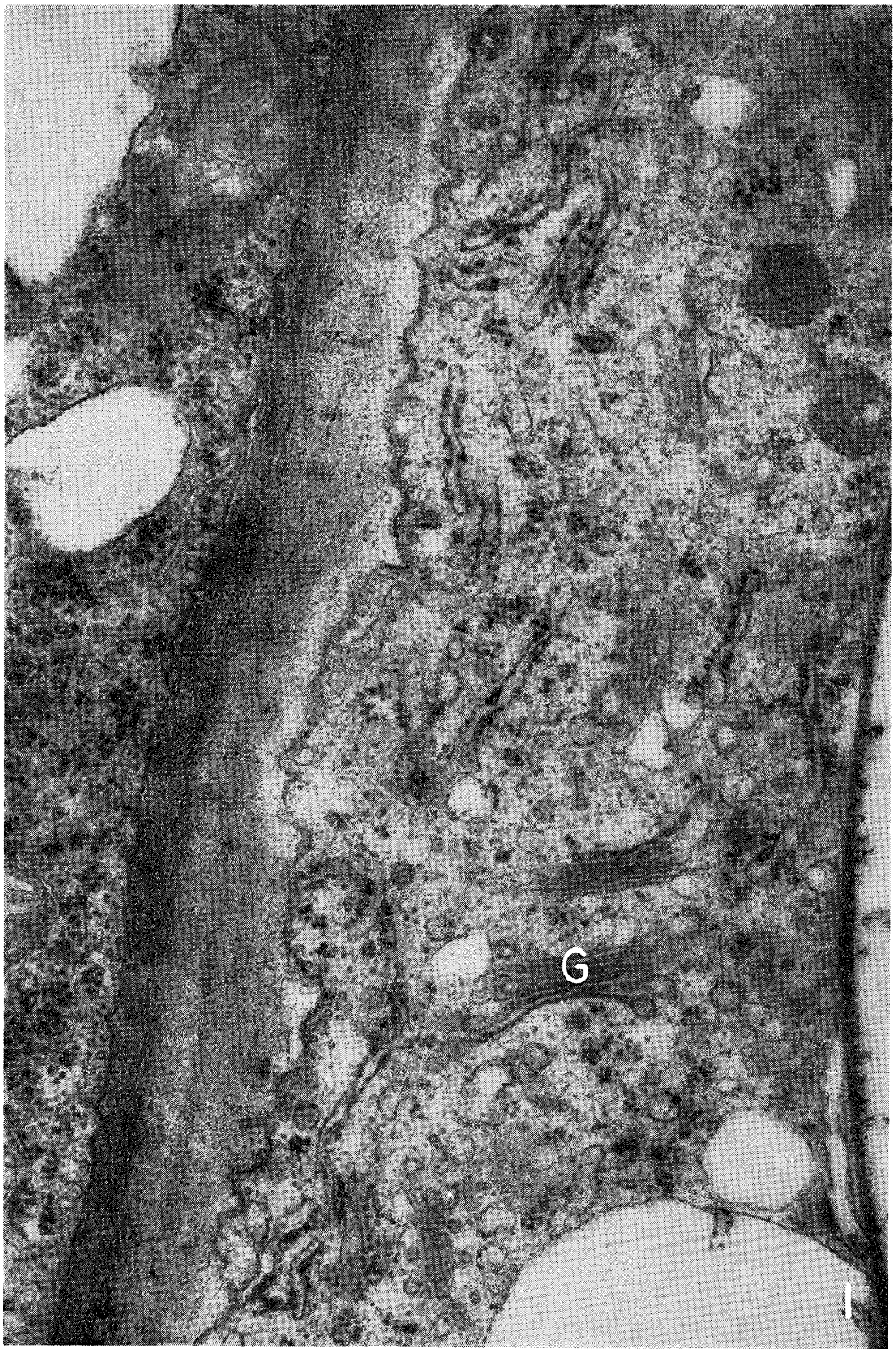
Fig. 11. Some vesicles surrounded with microtubules (small arrows) and those incorporated in the thickenings (large arrow) are shown. ×44,500.

Fig. 12. Close association of microtubules and vesicles are shown at the thickenings (arrows). ×24,000.

Fig. 13. Microtubules extending like an arc together with vesicles are shown. ×44,500.

Fig. 14. Groups of undulated microtubules (arrows). ×44,500.

Fig. 15. Undulated microtubules (arrow) extending from a thickening to the opposite one. ×44,500.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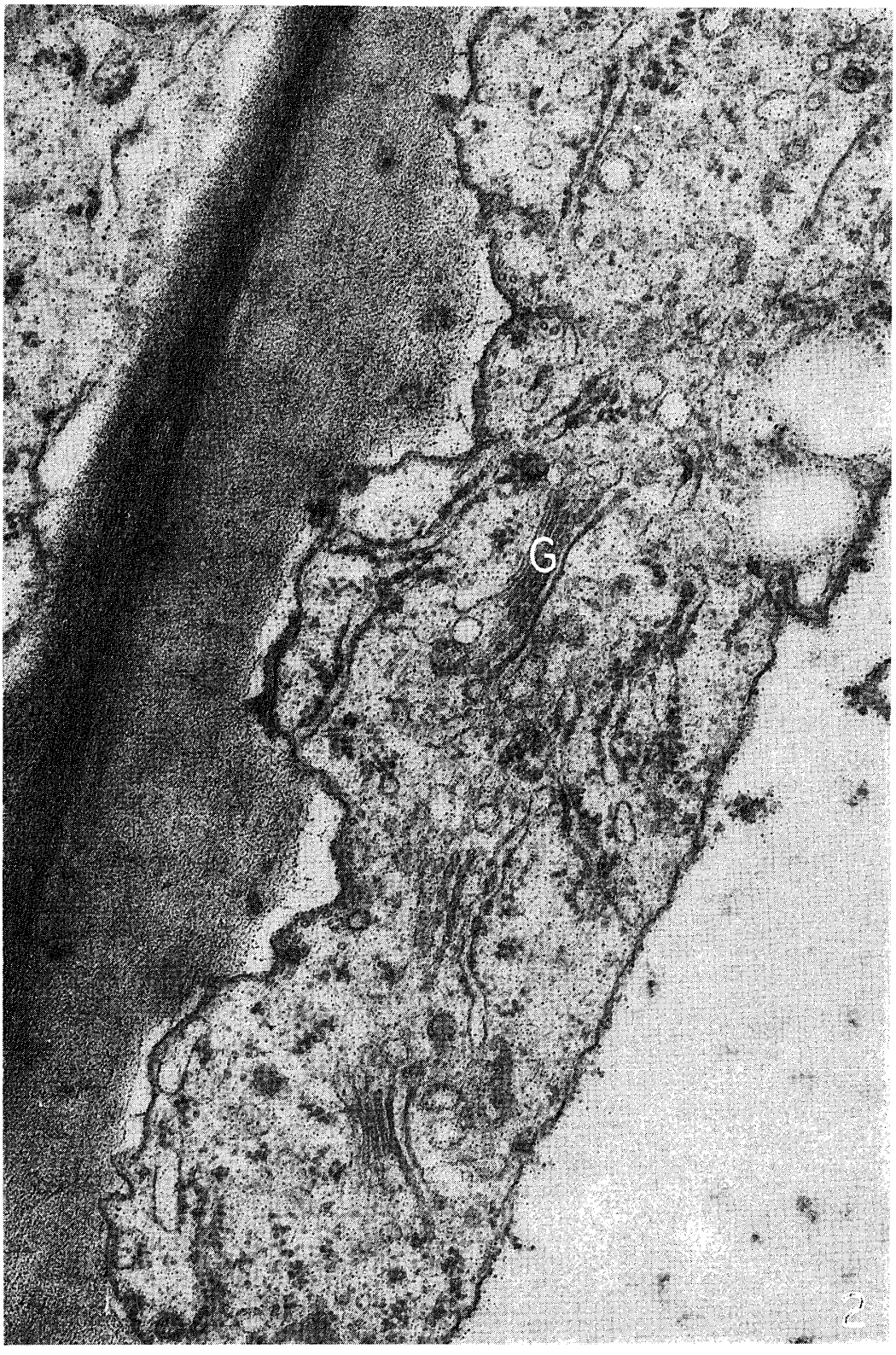
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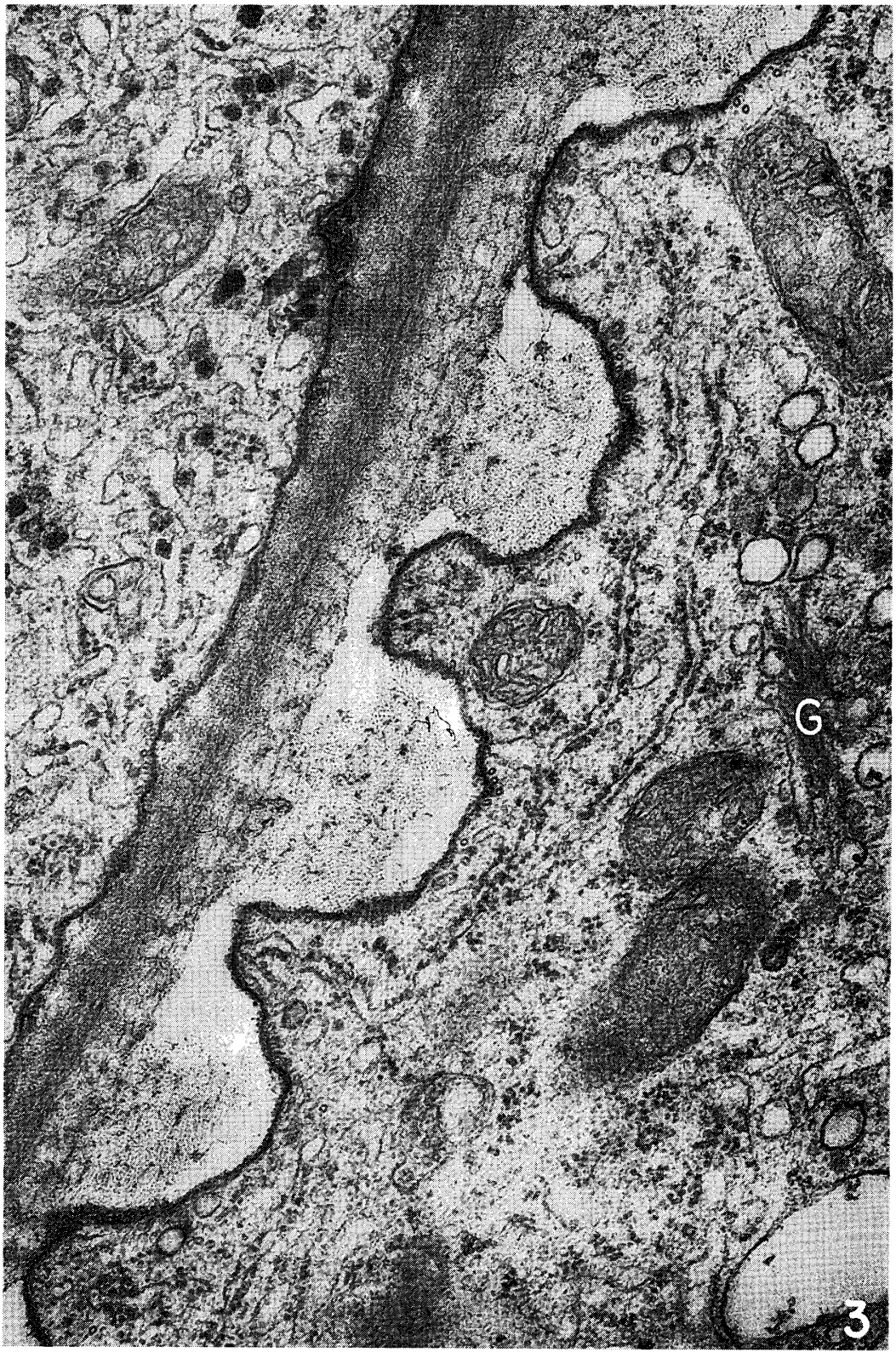
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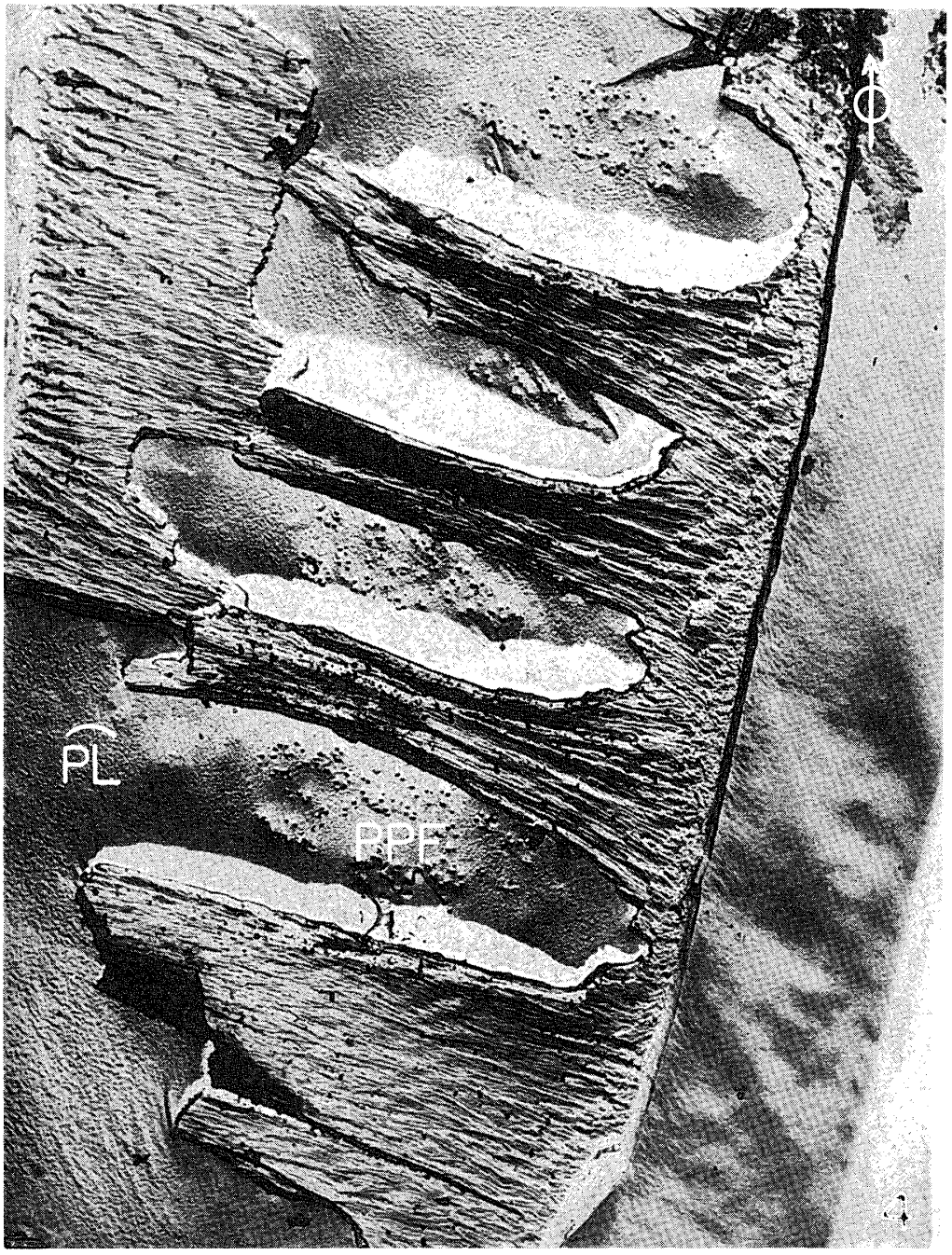
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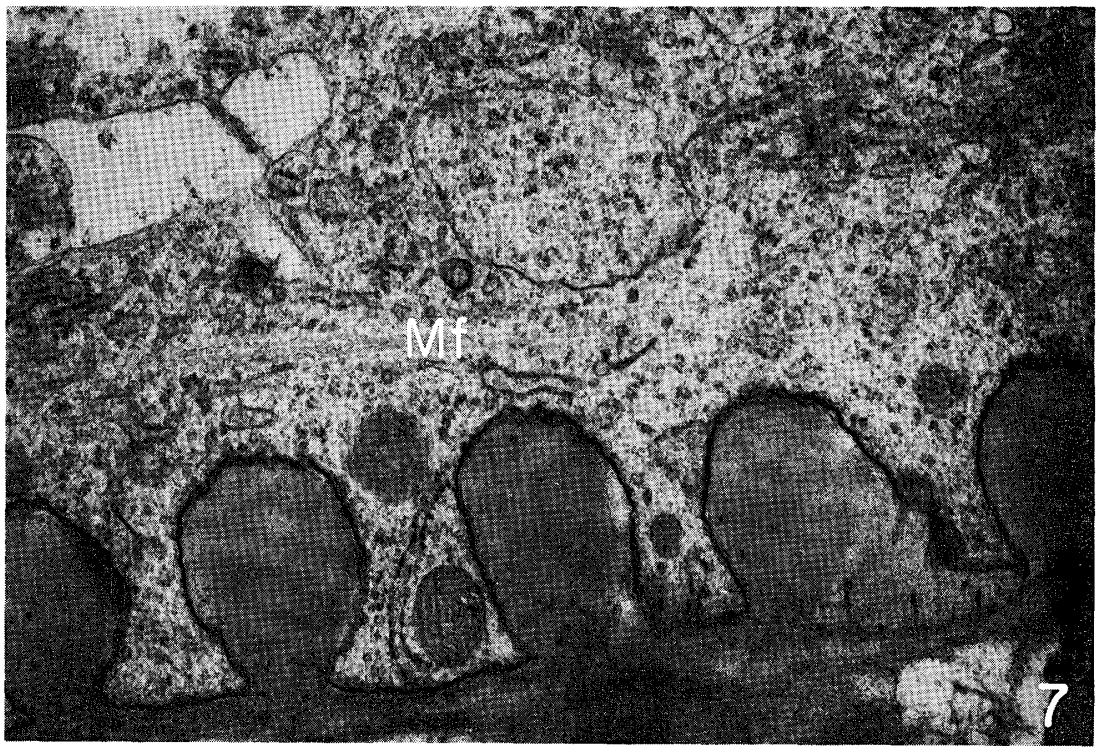
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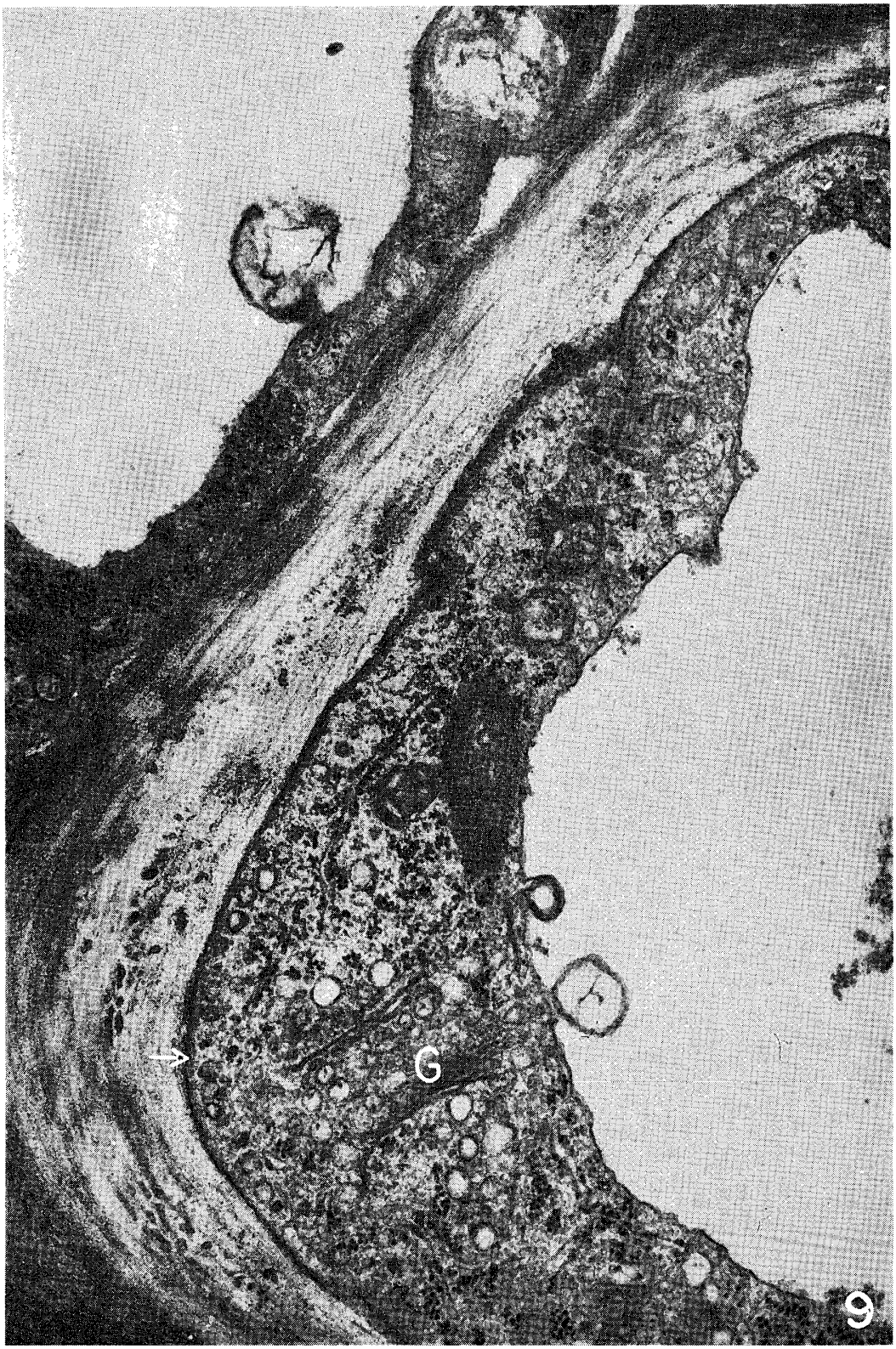
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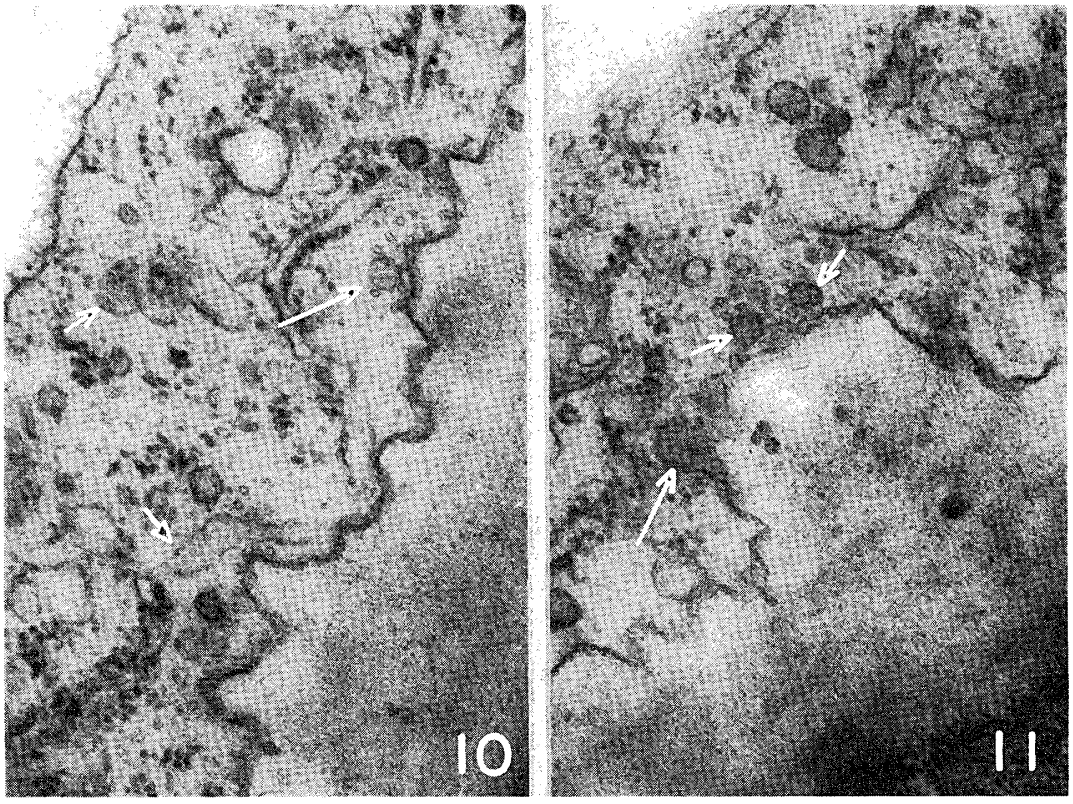
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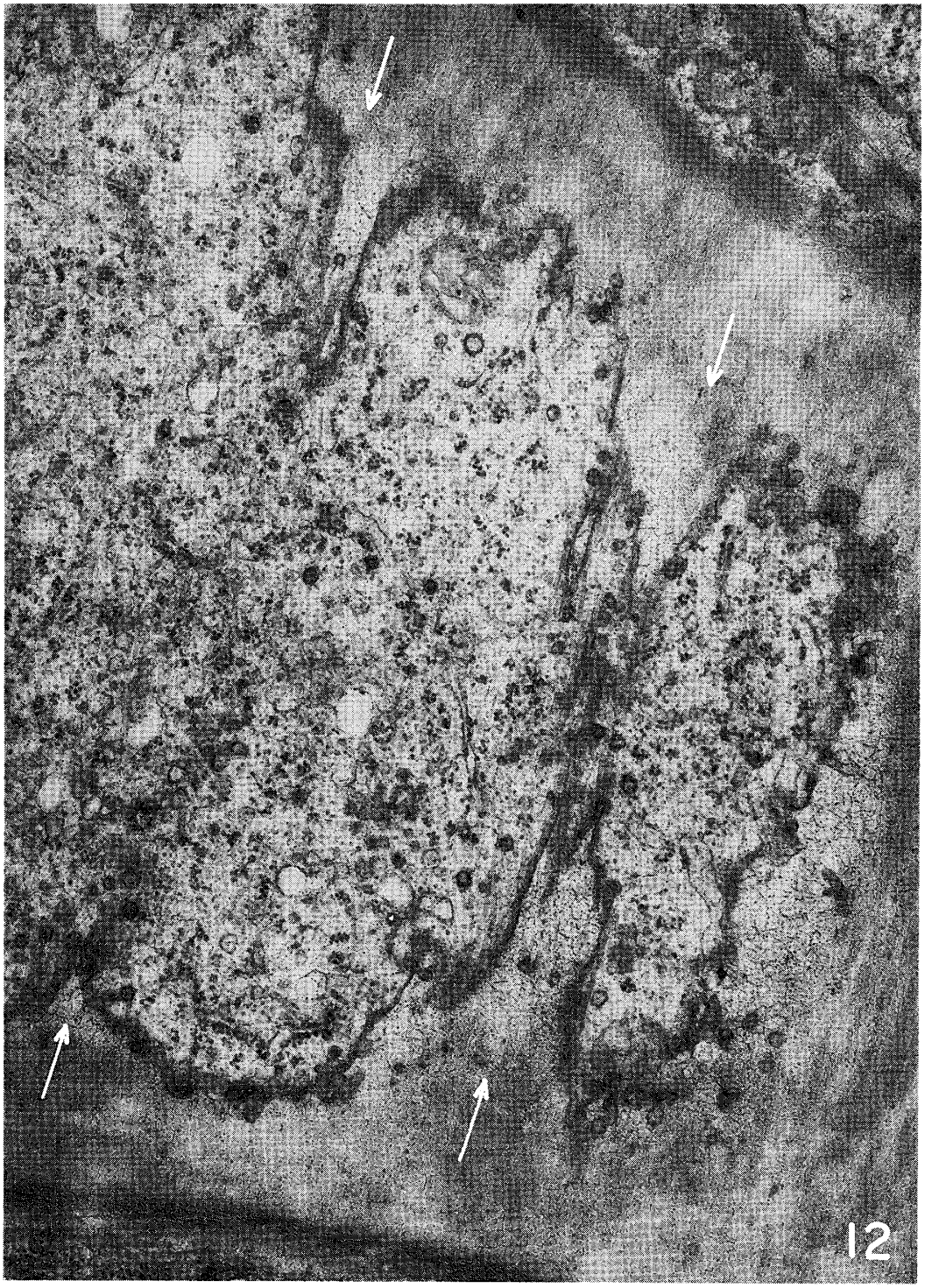
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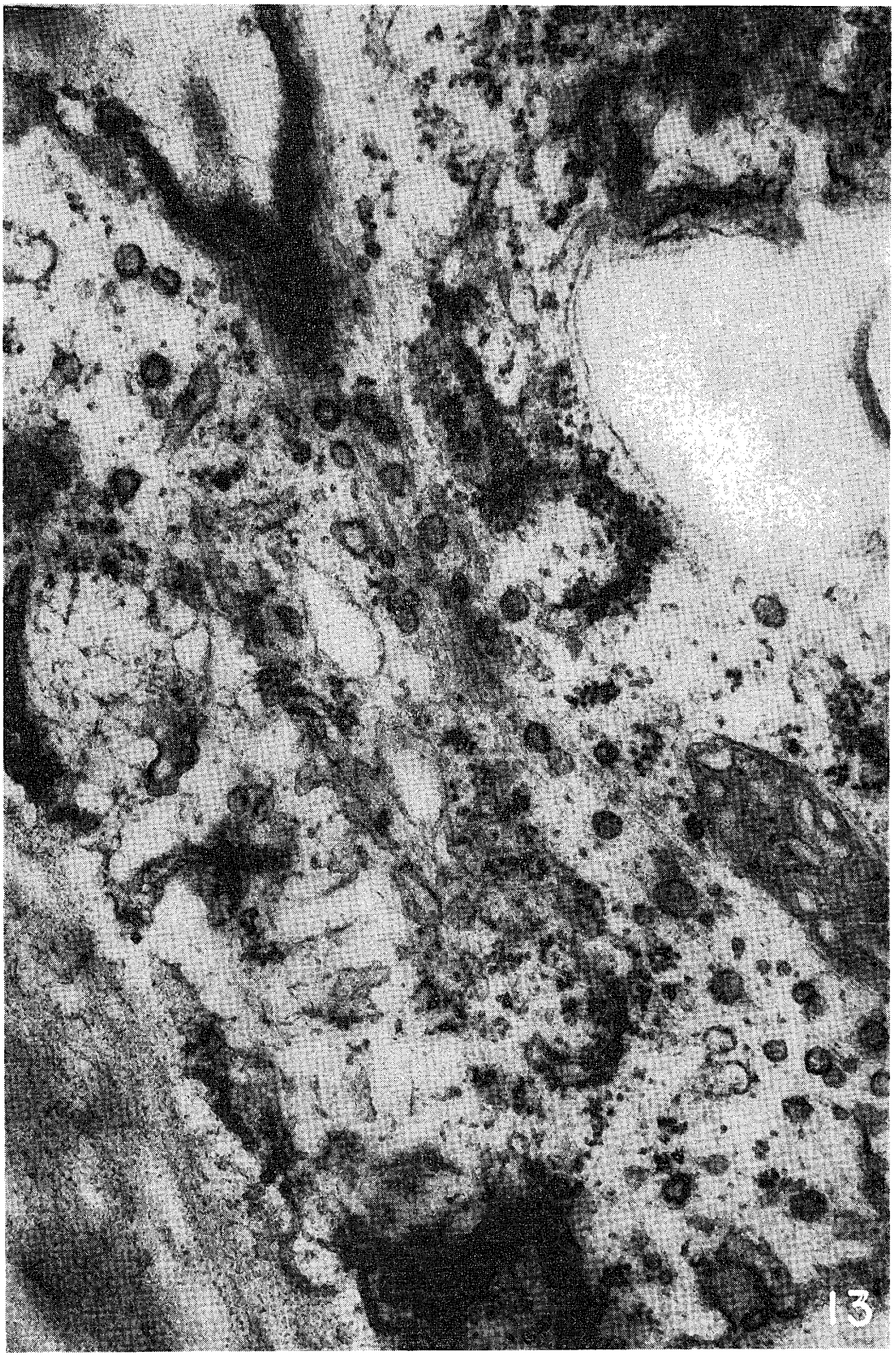
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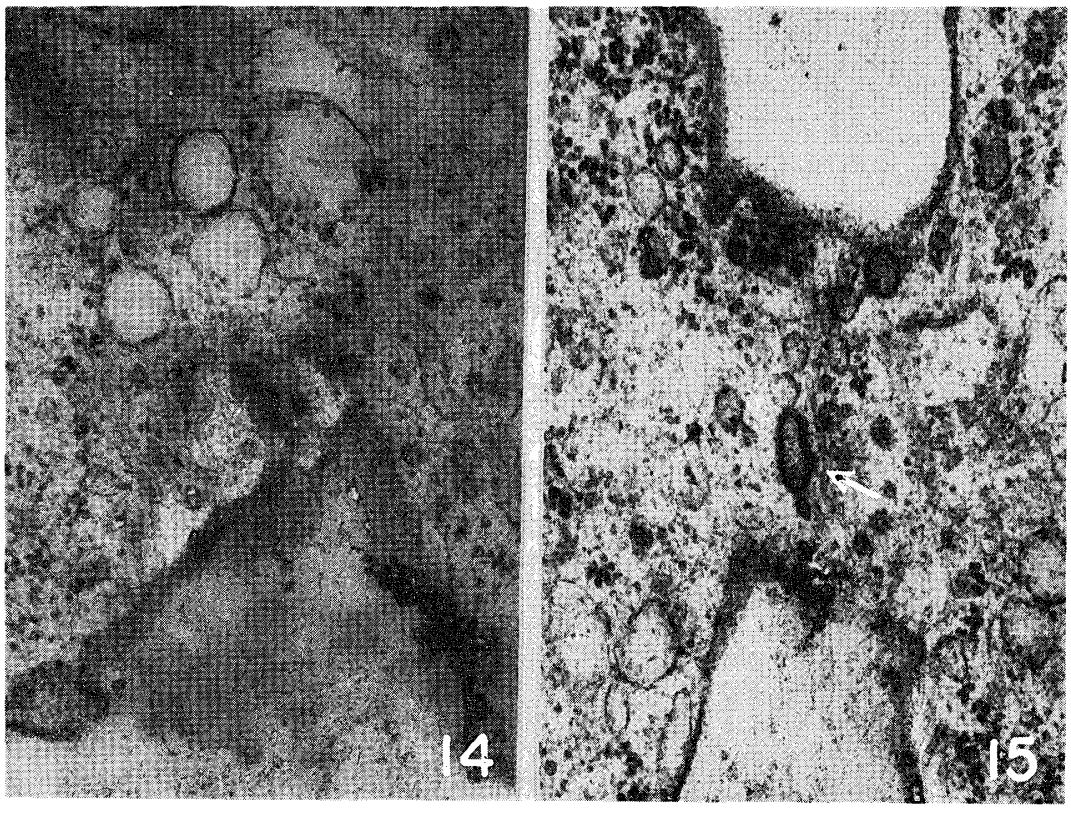
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