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FREEZE-FRACTURE STUDY OF THE RAT LUNG I. GENERAL DESCRIPTION OF RAT ALVEOLAR CELLS IN THE GLUTARALDEHYDE-FIXED AND GLYCERINIZED LUNG TISSUE

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Abstract

There is no established standard procedure in processing tissue for freeze-etching or freeze-fracture replication. A review of the literature reveals that specimens suitable and convenient for such study may be obtained from glutaraldehyde-fixed and glycerinized tissue. In this experiment we have undertaken to describe completely the morphological appearance of alveolar type II cells as well as a few other cells in rat lung, after fixation in glutaraldehyde for three hours and glycerinization for one hour before freeze-fracture replication. It is expected that this will provide a standard of reference when structural features of rat lung cells in tissues processed by different procedures are compared.

INTRODUCTION

The freeze-etching or freeze-fracture replication technique has been introduced into ultrastructural investigations of animal tissues. This technique may provide the following advantages: (1) the artefactual changes resulting from chemical fixation or dehydration which is used in conventional electron microscopic preparations can be avoided, and more true-to-life preparations can be obtained; (2) a tridimensional investigation of the tissue structure is possible; and (3) the internal and external faces of cell membrane leaflets are observed *en face*.

No standard procedures for freeze-etching or freeze-fracture replication have been established. A review of the literature[1] reveals that the great majority of workers investigating animal tissue almost routinely employ chemical fixation prior to the application of cryoprotective agents such as glycerol.

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This seems to be done with the intention of reducing tissue necrosis, and of eliminating artefacts allegedly induced by cryoprotective agents. Breathnach *et al.*[2] confirmed the alteration of the cleaving behaviour of plasma membrane at desmosomes and tight junctions after fixation with glutaraldehyde. In their study, however, a comparison of fracture faces of general plasma membranes, nuclear membranes, mitochondrial membranes and membranes of rough endoplasmic reticulum revealed no significant differences between fixed and unfixed material. It was even a great surprise that with the exception of some membranes of liver endoplasmic reticulum, no evidence of aggregation or redistribution of membrane-associated particles in the unfixed material was obtained when processed at 4°C. Stolinski and Breathnach[3] further demonstrated that the general picture of the tissues processed without chemical fixation or cryoprotection was essentially similar to that of material after those processes. Therefore, it may be concluded that specimens suitable and convenient for freeze-fracture study may be obtained from glutaraldehyde-fixed tissue. As they pointed out, however, there may be cell, tissue, and species differences in susceptibility to the effects of fixatives or cryoprotectants.

Freeze-etching technique was also applied in examination of lung tissue[4-14]. Various types of cells and organelles were the objects of investigation. The alveolar type II cell was one of the cells most frequently studied[4,6,8,9,11 and 12]. Procedures and animal species varied among these studies. In early studies [4,6,11.13 and 14], intra-and extracellular ice crystal formation was not completely avoided, which interfered with proper interpretation.

The purpose of this communication is to describe completely the morphological appearance of alveolar type II cells as well as a few other cells in rat lung studied by freeze-fracture replication. Here, glutaraldehyde fixation followed by glycerol immersion was used as a standard procedure. It is expected that this will provide a standard of reference when structural features of rat lung cells in tissue processed by different procedures are compared.

MATERIALS AND METHODS

400 to 500 gm. Wister strain rats were anesthetized by intra-peritoneal injection of Nembutal. The lung was either perfused in situ with 2.5 % glutaraldehyde in 0.1 M cacodylate (pH 7.4) or removed *en bloc* to be immersed in 2.5 % cacodylate-buffered glutaraldehyde after slicing into one to two mm^3 tissue blocks. Fixation was completed in 3 hours and tissues were put in 30 % glycerol in cacodylate buffer (0.1 M, pH 7.4) for one hour. These processes were performed at 4°C. In total, ten rats were used for this study and eight blocks of lung tissue were processed from each animal. Then, tissues were rapidly frozen in liquid freon 22 and were stored in liquid nitrogen. Frozen

specimens were placed on the cold stage of a Balzers' freeze-etching device (BAF - 301). Thereafter, the temperature of the specimen was readjusted and maintained at -110°C. At 10^{-6} Torr, fracture and replication were performed. Platinum-carbon was casted at a 45° angle, followed by carbon coating. Replicas were cleaned with sodium hypochlorite solution and three changes of distilled water, mounted on 200 mesh coated copper grids, and were examined in a Hitachi HU - 12 electron microscope.

RESULTS

I. Alveolar epithelium

The alveolar epithelium consisted of type I and type II alveolar epithelial cells (membranous and granular pneumocytes), (Fig. 1 & 6). Cells with characteristics of the third pneumocyte or alveolar brush cell as reported by Meyrick and Reid[15] were not observed in this study. The type II cells were usually cuboidal or rounded in shape and their luminal plasmalemmas were specialized into several microvilli less than 4000 Å, projecting into the alveolar lumen (Fig. 1 & 3a). No pinocytotic vesicles were apparent. Complicated networks of tight junctions were seen between adjacent cells (Fig. 2). Nuclei were situated in the center or basal portion of the cytoplasm. The karyoplasm was granulated diffusely and surrounded by a double nuclear envelope, which was studded with numerous membrane-associated particles of 150 Å diameter, and contained evenly distributed 1000 Å diameter nuclear pores (Fig. 1 & 3a). Structure of the annulus consisting of eight structural subunits was not clear. Some mitochondria, vacuoles, vesicles, multivesicular bodies and lamellar bodies appeared as either concave or convex spherical bodies (Fig. 1 & 4). Cross-fractured mitochondria showed short and parallel granular steps, representing cristae (Fig. 3c). The Golgi apparatus consisted of several stacks of membrane, each being separated by cisternae and cytoplasm, one after another (Fig. 3b). Small vesicles were surrounded by a single limiting membrane to form multivesicular bodies (Fig. 4).

Though the structure of lamellar inclusions after freeze-fracture preparation seemed to be better preserved than in thin sections, their basic configuration was similar. The lamellae were enveloped by a limiting membrane, which was studded with membrane-associated particles (MAPs) of 150 Å diameter (Fig. 5a). The lamellae showed variability in their configuration (Fig. 5a-g). Some were composed of a variable number of concentric or parallel lamellae, while others were composed of a combination of these. Fusion of two lamellar bodies were also observed. In some lamellar bodies, small vesicles existed between lamellae or between a superficial lamella and the enveloping limiting

membrane. Large concentric lamellar arrangements could be observed between the parallel lamellae or hair-pin-shaped lamellae (Fig. 5f & g). Usually parallel lamellae terminated abruptly on the limiting membrane, but some curved back near the limiting membrane, running parallel to end at the limiting membrane of the opposite side, forming hair-pin image. A few small curved lamellae sat just on the limiting membrane and did not extend up to the other side of the lamellar body (Fig. 5f). The periodicity of lamellae varied from a periodcity of 45 Å in tightly packed parallel areas. In addition, there were large spaces between opposing lamellae in some inclusions. The surface of lamellae was finely granular and such granularity (70 Å) was distinct from particles in the limiting membrane (150 Å). Aspects of the secretory process could be observed in some cases. The continuity of plasmalemma and limiting membrane supports the merocrine secretion of lamellar bodies observed by thin sectioning technique (Fig. 4). The appearance of the type I alveolar epithelial cells did not provide any new information beyond that obtained from observations of thin sections, except for the presence of slight interdigitation with type II cells. Some mitochondria, vacuoles, pinocytotic vesicles were observed.

II. Alveolar space and macrophages

The alveolar space was empty and no alveolar lining layer was observed. This is probably due to dispersion of such layer during chemical fixation and glycerol immersion. Macrophages could be readily identified near alveolar epithelium (Fig. 6). Their plasmalemma was irregularly protruded to form pseudopods which were longer and wider than microvilli of type II alveolar cells. The fracture faces of macrophage plasmalemma revealed a few membrane-associated particles. In general, MAPs of the internal face of plasmalemma were fewer than those of the external face. In addition to mitochondria and endoplasmic reticulum, the cytoplasm contained small round and elliptical vesicles which may represent lysosomes. Although multivesicular and partially lamellar forms of lysosome could be seen, complete lamellar forms in cross fractured faces were not encountered. The nuclear membrane was partially indented. Its surface appearance and nuclear pores were similar to those of other cells described previously.

III. Interstitium and blood vessels.

In freeze-fracture preparations no basement membrane could be distinguished within the ground substance of the alveolar interstitium. Occasionally, grouped collagen fibers of approximately 550 Å diameter showed a helical substructure[16]. The endothelium of the alveolar capillaries had abundant pinocytotic vesicles in both luminal and basal side of plasmalemma (Fig. 1 &

7). When plasma was trapped within the capillary lumen, it showed some granularity. Erythrocytes appeared as granular areas enveloped by linaer membranes in cross fracture and as concave or convex granular cups in *en face* view. Cytoplasmic particles measured about 200 Å. The surface membrane was studded with fine uneven granules. Granularity was more prominent in the external face than the internal face as was the case with other cells. Neutrophilic leukocytes were also occasionally seen in blood vessels, intersitium and alveolar space (Fig. 8). Except for their characteristic lobulation, the nuclear appearance was similar to that of other cells. Their cytoplasm was filled with a heterogeneous population of granules with variable shape and size. Round to oval and oval to elliptical granules could be identified.

IV. Bronchiolar epithelium.

The ciliated cells (Fig. 9a, b, & c) were columnar in shape and were equipped with a large number of cilia whose external and internal surfaces were studded with occasional particles of about 150 Å diameter. The particles tended to gather in the necks of the cilia, and lined circularly to have 6 layers. In cross-cut cilia, the nine outer and one inner tubuli were seen. Most of the cilia were fractured tangentially or transversely to reveal cross view or fissure face, but unfortunately longitudinal cleavage of the microtubuli was not observed. The nucleus was situated basally with the same appearance as in other cells. Mitochondria, vesicles, and smooth endoplasmic reticulum were seen in the cytoplasm. Cells were attached to each other by tight junctions and desmosomes. A few Clara cells were observed. They contained abundant vesicular and tubular structures representing endoplasmic reticulum as well as numerous mitochondria. No lamellar structure was present.

DISCUSSION

A few freeze-fracture studies of the lung, particularly of alveolar type II epithelial cells, have appeared to date. As their procedures and animals used differed to some degree, so did their observations in some respects. Belton *et al.*[4] studied rat lung. Without chemical fixation, tissues were put in 20 % glycerol for two hours before freezing. Roth *et al.*[6,7] examined mouse lung in the same manner. They did not find any particles on the lamellar faces. Smith *et al.*[8] reported the presence of linear sculpturing with a 450 Å spacing on the lamellae of rat type II cells, processed with glutaraldehyde fixation and glycerinization. Lauweryns and Gombeer-Desmecht[11] studied mouse lung frozen after 25 % glycerinization for one hour without fixation, and described small plaques of 170 Å diameter on the lamellar surface. Descriptions of rat lung by Untersee *et al.*[5] using several different procedures concentrated on the

alveolar lining layer and lack details regarding type II cells. Such differences in the appearance of lamellar bodies among these reports led us to study them under different conditions during preparation and to compare them. There is no established standard procedure in freeze-fracture replication or freeze-etching technique, now. Freezing of fresh tissue without chemical fixation and cryoprotectant may yield annoying ice crystal formation introducing misinterpretation. On the other hand, fixatives and cryoprotectants may produce artefactual changes. Breathnach, *et al.*[2], and Stolinski and Breathnach[3] mentioned that although the cleaving behaviour of plasmalemma at desmosomes and tight junctions may be altered after fixation with glutaraldehyde, no significant differences may be noticed in the other cellular structures among fixed, unfixed and cryoprotected materials. In addition MacIntyre[17] mentioned that glutaraldehyde compensates the disadvantage of glycerol when they are used together. These, therefore, indicate that glutaraldehyde-fixed and glycerol-immersed tissue is suitable for freeze-fracture replication and does not have significant artefactual changes.

Contradictory to the published observation of some investigators was the absence of distinct membrane-associated particles within the lamellae of the inclusions of type II cells. No linear sculpturing with a 450 Å spacing was present on the lamellar membrane of the inclusions as described by Smith *et al.*[8]. We have shown the absence of MAPs of the lamellae in fetal rabbits[18]. As we pointed out there, the autoradiographic and histochemical identification of protein in inclusion bodies does not necessarily imply the presence of protein as lipoprotein. We suggested that the proteins may exist not in the form of lipoprotein but in some form outside of the purely lipidic lamellae of the inclusions, and that some portion of the protein constituent of lamellar inclusions may act as an enzyme rather than as an integral part of surfactant system in the form of lipoprotein. The present study using rat also supports the idea that at least the major constituent of lamellar bodies of type II cells is lipid not bound to protein. In contrast to the lamellae, the limiting membrane of lamellar inclusion bodies does have membrane-associated particles of about 150 Å. These particles may well be protein enzymes associated with membranes. Meban[19] has histochemically demonstrated phosphatidic acid phosphatase activity along the lining membrane of lamellar body as well as in multivesicular bodies.

The fine granularity of about 50-80 Å on the lamellar membrane as observed here was also described in the previous reports. The membrane-associated particles they reported are, however, distinct from this granularity. The particles these authors reported may be artefactual or due to the irregular

fissuring of phospholipid bilayers. According to Staehelin[20] and Buchingham and Staehelin[21], formation of 50-200 Å " plaques " can be seen on artificial lecithin membranes, and it may be due to addition of glycerol molecules which focally strengthens the cohesive forces within the surface layers of the bilayer membranes. The process of replication itself can also produce granularity[22]. It is said that prolongation of shadowing causes excessive surface heating which results in local melting and recrystallization on the specimen surface.

The periodicity of 45 Å in this study may differ from that of the other investigators. The variability in periodicity in part depends on the angle of running lamellae and shadowing. This periodicity does not correspond to that studied by thin section technique, because this measurement in freeze-fracture study represents a width from fatty acid tail cleavage to the next fatty tail cleavage of phospholipid bilayer lamellae, and essentially differs from that in thin section, in which a distance from the middle of osmiophilic band to the middle of the next osmiophilic band, namely, a distance from the outside of each phospholipid head to the outside of the other phospholipid head, is considered to be measured.

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LEGEND

Fig. 1. Type II alveolar epithelial cell sitting on the alveolar wall.
The luminal plasmalemma is occasionally specialized into microvilli (mv). The nucleus (N) is in the center of the cell. The external face of the outer nuclear membrane is seen with numerous MAPs and nuclear pores. Through a central defect in the outer membrane, the external face of inner nuclear membrane is revealed. Lamellar bodies (L), vesicles, and mitochondria are seen in the cytoplasm. The lamellar body here is composed of parallel lamellae, concentric lamellae and a combination of multivesicles and lamellae. The external face of the basal plasmalemma is studded with numerous MAPs, but lacks pinocytotic vesicles. In the interstitium, no basement membrane is apparent below the epithelium and endothelium. Bundles of collagen fibers are seen (C). The luminal and basal plasmalemmas of endothelium (End) are rich in pinocytotic vesicles (P, arrows). The linear steps in the lower middle and upper left are artefactual. Type I alveolar epithelium (I).

The encircled arrow of this and succeediug photographs indicates the direction of shadowing.
Magnification: ×13,600

Fig. 2. *En face* view of the external face of type II plasmalemma as well as cross-fractured face of its cytoplasm is shown. A complicated network of tight junction is indicated by arrows. Fissure- and fracture-faces of microvilli (mv) are also seen. Concentric lamellae (L) are shown in the bottom of this picture.
Magnification: ×28,000

Fig. 3. Various intracytoplasmic organelles of type II alveolar cells are shown here.
a. Microvilli Mag.: ×13,000
b. Golgi apparatus Mag.: ×36,000
c. Mitrochondria Mag.: ×21,400

Fig. 4. Several forms of lamellar inclusion bodies are shown in this micrograph. Note multivesicular body (arrowhead). In the left center field, lamellar body is in the process of secretion. Note the the continuity between plasmalemma and the limiting membrane of inclusion body (arrows).
Magnification: ×14,000

Fig. 5. Various types of lamellar architecture are shown.
a. Three lamellar bodies are shown. External (Ext) and internal (Int) faces of the limiting membrane are studded with 150 Å diameter MAPs (arrows). The surfaces of lamellae, either parallel or concentric, lacks MAPs although surfaces are somewhat granular.
Mag.: ×28,000
b. Parallel form of lamellae.
Here two halves of phospholipid bilayer lamellae are shadowed. Periodicity measures about 45 Å at thinnest. The lamellae and abruptly when they come in contact with the limiting membrane. The vesiculolamellar body is seen in the left upper corner.
Mag.: ×28,000
c. Another form of parallel lamellae.
Two stacks of parallel lamellae are enveloped within the same limiting membrane. They run in different directions, and that on the right seems to terminate without continuation when its lamellae come in contact with those of the other stack. An internal face of the outer nuclear membrane is seen in the right lower corner.
Mag.: ×32,600
d. Multiple parallel and concentric lamellae may exist within the same limiting membrane.
Mag.: ×30,700
e. Some forms of the lamellae are not completely concentric and the ends of curved lamellae terminate abruptly at the limiting membrane (arrowheads).
Mag.: ×28,000

f. As seen in this photograph, small vesicles in lamellar inclusion bodies are usually polarized at the periphery. The spaces between lamellae vary in the lower inclusion body. In both inclusion bodies in this photograph, small curved and hairpin-shaped lamellae are seen close to the limiting membrane. They do not extend even up to one quarter of the diameter of the inclusion body.
Mag.: ×28,000

g. The concentric lamellae here are hugged by large hairpin-curved parallel lamellae.
Mag.: ×33,500

Fig. 6. A macrophage (Mac) contains numerous vesicles of varying size, which may correspond to the lysosomes. No lamellar inclusions are seen in its cytoplasm. Pseudopods are larger and wider than the microvilli of type II alveolar cells. Internal faces of the plasmalemma of the outer nuclear membrane of a type I alveolar cell (I) are seen just above the macrophage. The cytoplasm of the type I cell is scanty in organelles. Endothelium (End).
Magnification: ×12,100

Fig. 7. Alveolar wall.
This photograph clearly demonstrates three of the four anatomic barriers between the alveolar space and capillaries (arrows); the alveolar lining layer is not shown in this procedure. No basement membranes are discernible in the interstitial compartment in the freeze-fractnre preparation. White areas represent fissure faces of plasmalemmas. Both epithelial cell (I) and capillary endothelium (End) have pinocytotic vesicles, but they are more prominent in capillary endothelium.
Magnification: ×7,200

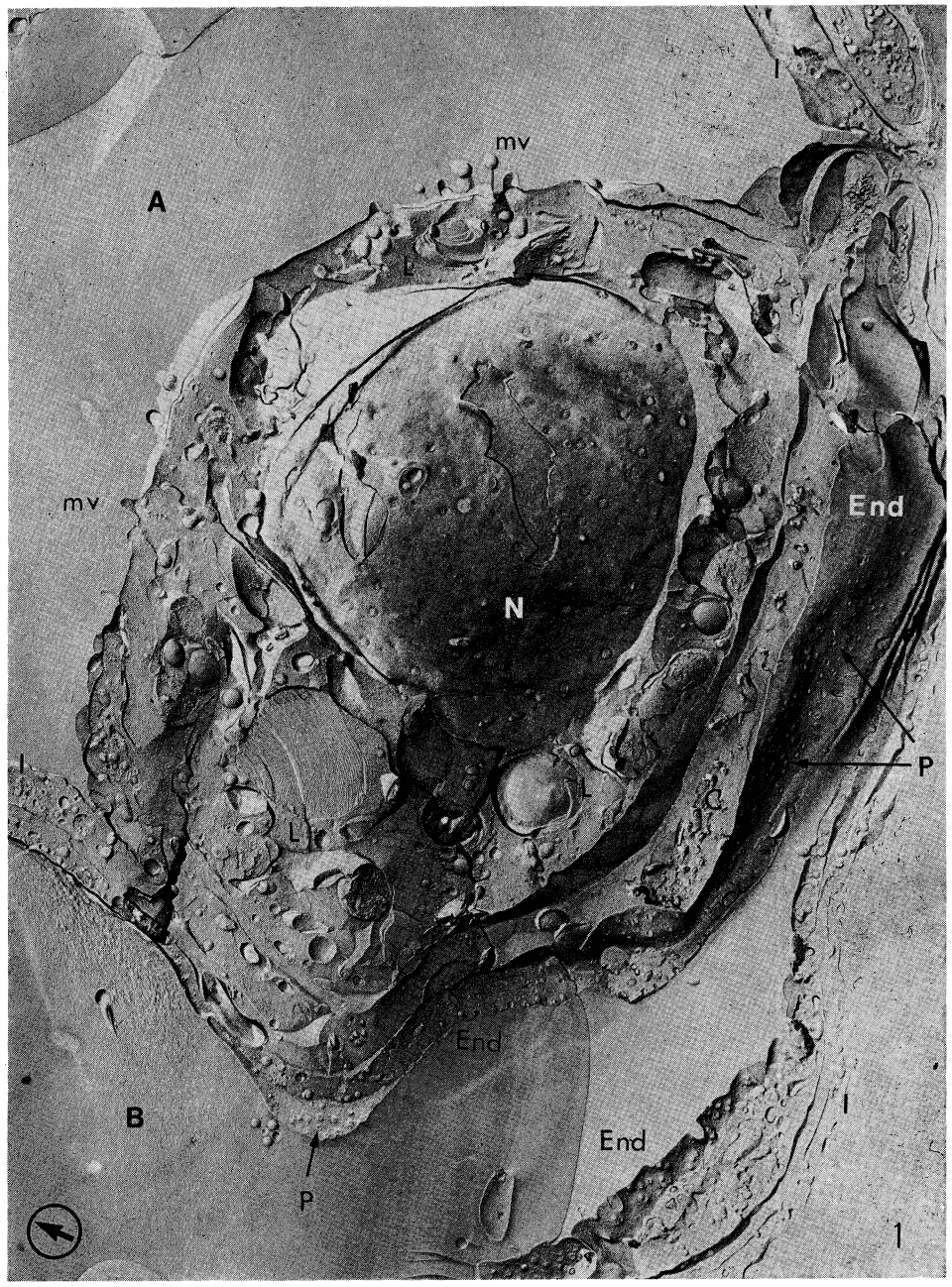
Fig. 8. Neutrophilic leukocytes may be seen in capillary lumens, intersitium or even in the alveolar space. They are characterized by nuclear lobulation and numerous round to elliptical granules.
Magnication: ×20,500

Fig. 9. Ciliated bronchiolar epithelium.

a. Ciliated bronchiolar epithelium at lower magnification. The nuclei are situated basally. Cytoplasmic organelles are scanty. The luminal surface is frilled by cilia.
(9+2) complexes of microtubues are identified in cross sections.
Mag.: ×7,800

b. Cells are attached to each other with tight junctional networks (arrow) and with desmosomes (arrowheads).
Mag.: ×21,500

c. Transition zone of cilia on fissure face shows MAPs which appear in a circular arrangement to form six layers *in toto*. Basal bodies are not seen in the cytoplasm.
Mag.: ×15,000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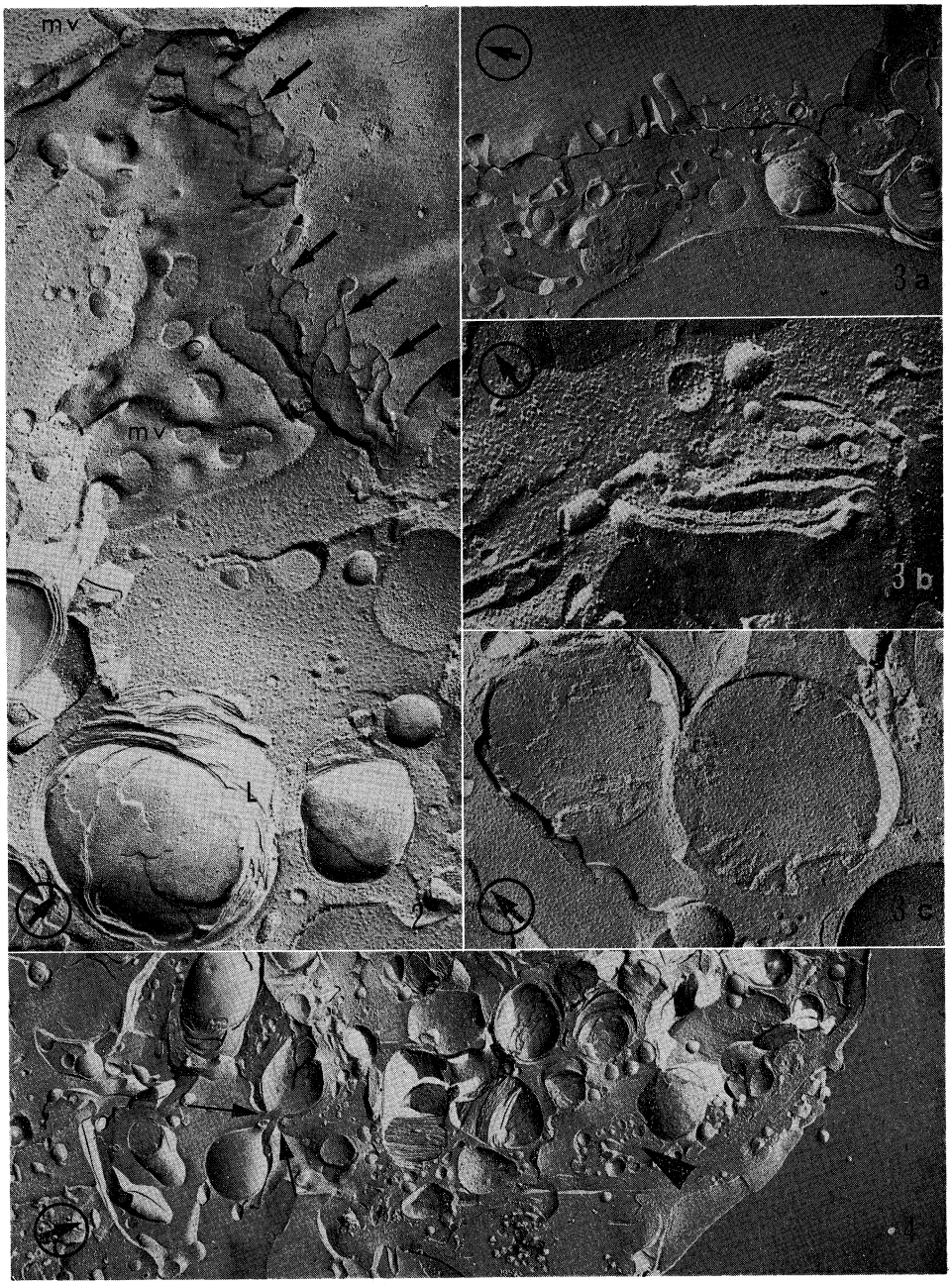
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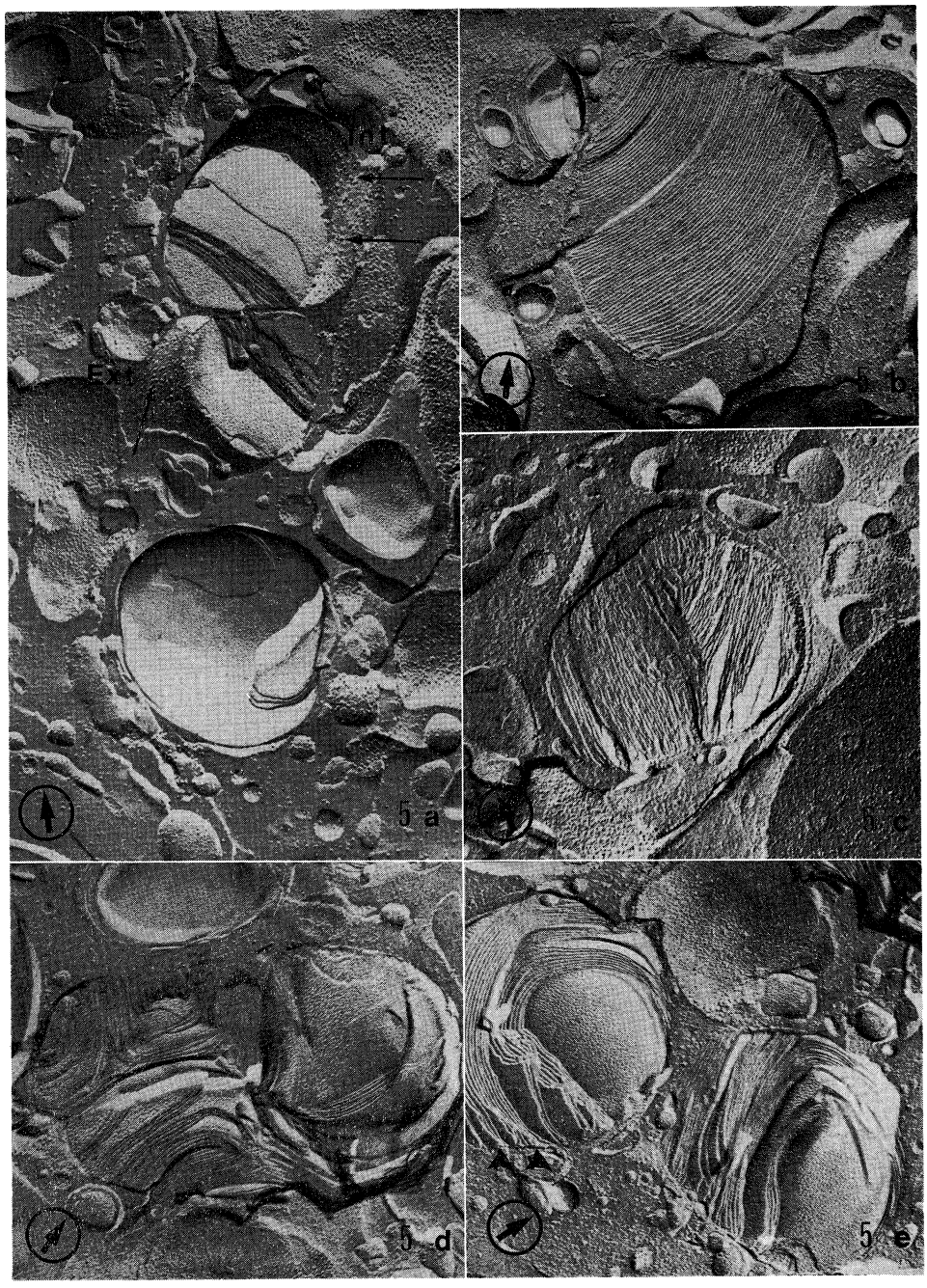
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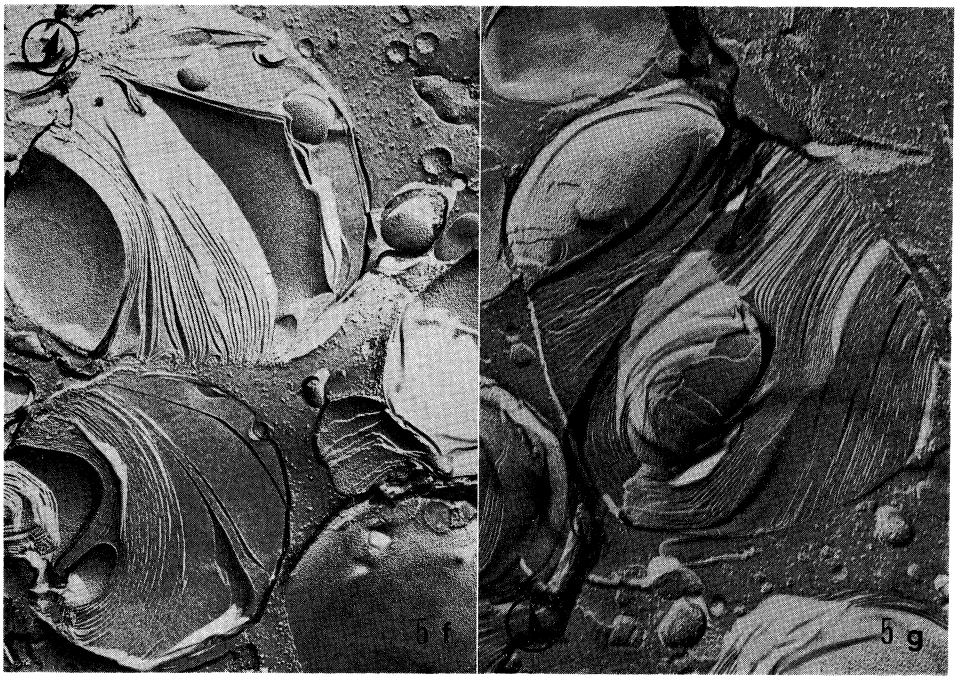
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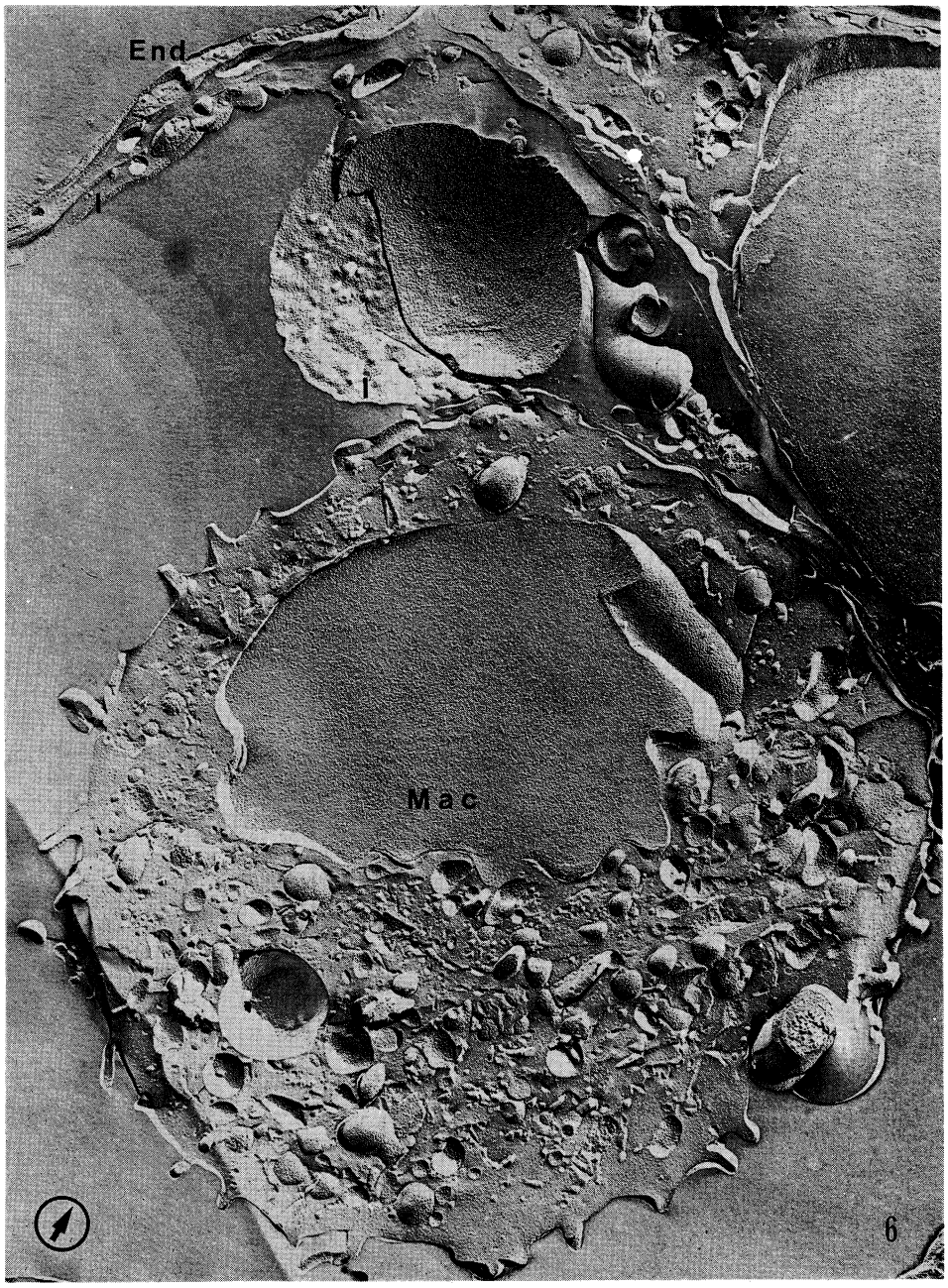
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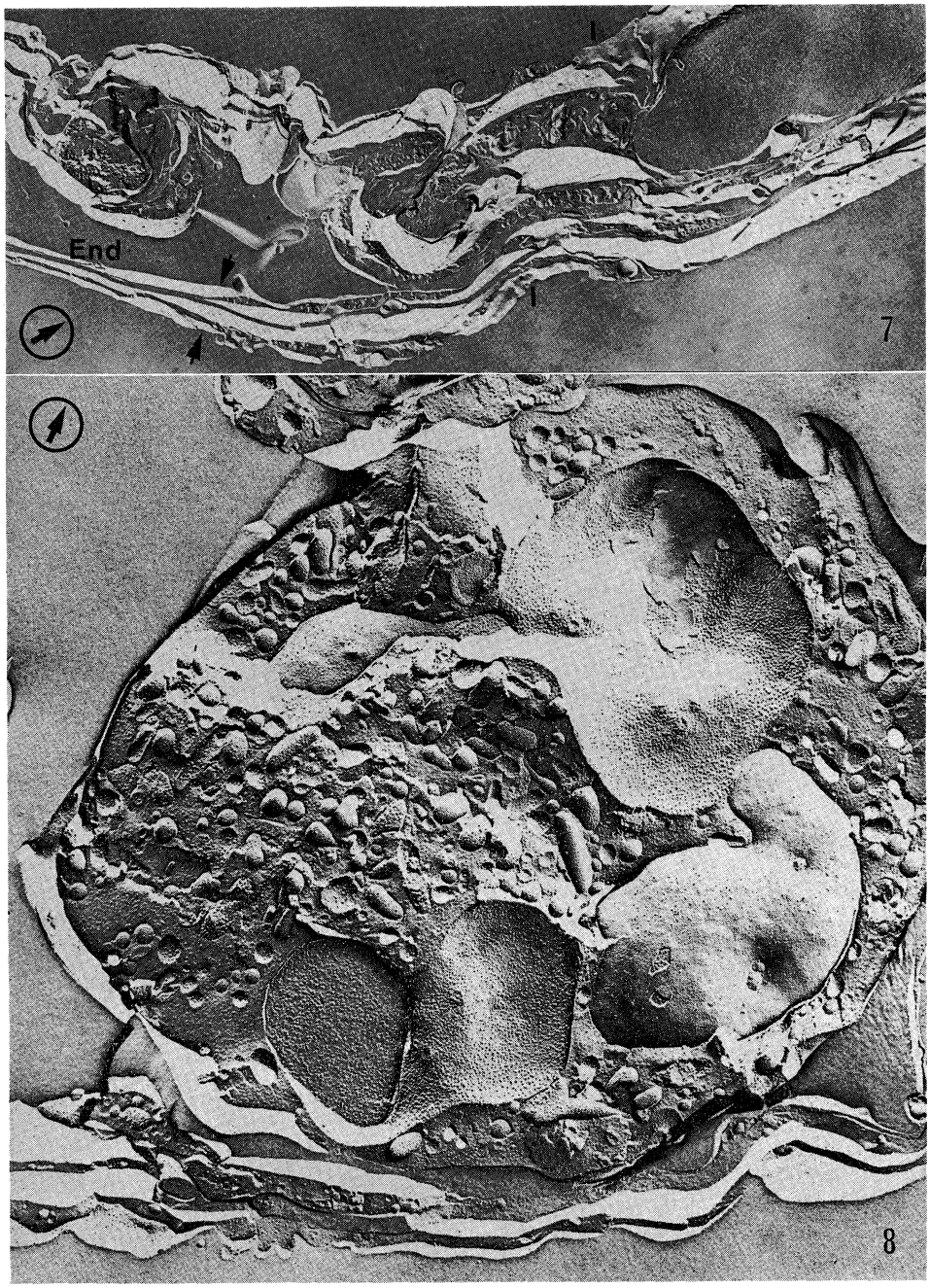
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