Cytochemistry of Pulmonary Alveolar Epithelial Cells

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THE RESPIRATORY AIR PASSAGES of mammalian lungs are lined by a continuous epithelium [1] composed of two different cell types.[2-4] The smaller cell resembles endothelium in structure and in function, serving as a selective barrier to diffusion. This cell Macklin called a "membranous pneumocyte." [5] The cell that he called a "granular pneumocyte" [5] is larger and has characteristic osmiophilic inclusions. Recently, the granular pneumocyte has been considered to be either a phagocyte [6-8] or a secretory cell.[5,9,10] Whereas Macklin believed that this cell secreted a mucinous fluid involved in the removal of particulate matter from the alveoli, recent studies have noted an association between the inclusions of the granular pneumocyte and the surface-active lipoprotein (surfactant) of the alveolar lining.[9-16]

The present report concerns a cytochemical study of the alveolar epithelium which has provided evidence that the inclusions are indeed secretory granules, and that they arise from multivesicular bodies.

Materials and Methods

Electron Microscopy

Samples of human lung from 10 patients were obtained in the operating room from biopsy or pneumonectomy specimens. Blocks selected from grossly normal areas were fixed for 3 hr. at 4° C. by immersion in 2.5% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.2–7.4.[17] Lungs from 8 male Wistar rats weighing 150–200 gm. were briefly fixed in situ by perfusion of 40 ml. buffered glutaraldehyde through the pulmonary artery or by inflation through the trachea at a pressure of 20 cm. of fixative. Blocks no more than 2 mm. thick were then excised and fixed by immersion in the same fixative for an additional 2 hr. After fixation, the blocks were washed overnight in cacodylate buffer. For general morphology 1-mm. blocks were postfixed in 1% osmium tetroxide in Millonig's buffer [18] and embedded in either Epon or Maraglas. In addition, 1-mm. blocks from some of the human biopsy specimens were fixed directly in 1% buffered osmium tetroxide without preliminary glutaraldehyde fixation.

For light microscopy, 0.5- to 2.0-μ sections were mounted on glass slides, stained with paraphenylene diamine,[19] and examined with a phase contrast microscope.

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For electron microscopy, silver to gold sections were mounted on uncoated copper grids, stained successively with uranyl acetate and lead citrate, and examined in a Philips EM 200 electron microscope.

Histochemistry

Histochemical procedures were performed on glutaraldehyde-fixed blocks which had been stored in buffer in the refrigerator for up to 10 days. Frozen sections were cut on a freezing microtome set at 40 μ for rat lung or 80 μ for human lung. To demonstrate acid phosphatase, the sections were floated for 30 min. to 3 hr. at 37° C. in freshly prepared Gomori's medium [20] buffered with 0.1 M Tris maleate, rather than with acetate as in the original procedure. Control sections were incubated in the same medium with the addition of 0.01 M NaF. Incubations for alkaline phosphatase were carried out for 7–15 min. at 37° C. in the medium described by Hugon and Borgers [21] with 0.025% β-glycerophosphate as substrate. Control sections were incubated in the same medium with β-glycerophosphate omitted. Controls for both phosphatases were always negative. Extracellular acid mucopolysaccharide was stained by immersion of frozen sections in the dialyzed iron solution of Rinehart and Abul Haj [22] at pH 1.6 for 20–30 min. Before staining with dialyzed iron, sections of two of the rat lungs were incubated for 2 hr. or overnight at 35° C. in either a solution containing 1 mg. neuraminidase (Sigma Chemicals, Type V, from *C. perfringens*) per milliliter in 0.1 M phosphate buffer, pH 5.4, or in buffer alone.

After histochemical staining, the sections were washed in four changes of 5% sucrose, fixed in the cold for 20 min. in 1% osmium tetroxide in cacodylate buffer, and embedded in Epon. Thin sections were examined either without additional staining or after staining with uranyl acetate.

Blocks from the lungs of 3 additional rats were fixed in 3% glutaraldehyde followed by 1% osmium tetroxide, and embedded in 4:1 butyl-methyl methacrylate mixtures and in glycol methacrylate (GMA).[23] Thin sections were stained with colloidal thorium (Thorotrast) in 30% acetic acid,[24] picked up on grids, and coated with carbon before examination.

Observations

The major findings were identical in rat and human lungs; the two species will be described together. As observed by many authors,[1-4] the membranous pneumocyte has a scanty perinuclear cytoplasm containing a few mitochondria, free ribosomes, cisterns of endoplasmic reticulum, and occasional dense membrane-bound bodies which contain acid phosphatase. The cell has a thin expanse of cytoplasm which extends over most of the alveolar surface and even partially covers the granular pneumocyte.

Granular pneumocytes are larger and tend to be round or cuboidal. Mitochondria are numerous and the rough endoplasmic reticulum is well developed. Characteristic features are the presence of microvilli on the free apical surface of the cell and the presence of dense osmiophilic cytoplasmic inclusions. The microvilli have fine axial filaments (Fig. 1) similar to those in microvilli of other organs.[25,26] These fibrils form part of a fine web of fibrils in the apical cytoplasm. This web

is confined to a zone 0.1 μ beneath the plasma membrane (Fig. 2). The fibrils are preserved by glutaraldehyde but are rarely seen in tissue fixed in osmium tetroxide alone.

The outer or alveolar surface of the apical plasma membrane is covered by a very delicate layer of "fuzz" which is best seen over the microvilli, although it is also present between microvilli (Fig. 3). It is well preserved by osmium tetroxide but is not clearly seen after glutaraldehyde fixation without special staining. At high magnification, the fuzz consists of fine filaments 200–300 Å in length which seem to be continuous with the outer layer of the plasma membrane (Fig. 4). Only rarely and in optimally preserved tissue fixed with osmium tetroxide can a similar layer of fuzz be seen covering the plasma membrane of the membranous pneumocyte. When detectable, the layer is perceptibly thinner than that of the granular pneumocyte.

Typical osmiophilic inclusions are 0.6–1.6 μ in length and are composed of whorls, stacks, or complex tangles of dense lamellae. They are surrounded by a limiting membrane approximately 100 Å in thickness.

Multivesicular bodies (MVB) are also numerous in the cytoplasm of granular pneumocytes. Like the inclusions, their limiting membrane is approximately 100 Å thick. This is perceptibly thicker than the 60- to 70-Å membranes of mitochondria, endoplasmic reticulum, and of the other vacuoles present in the cytoplasm of these cells. Enclosed within the limiting membrane of the MVB there are numerous 0.05-μ vesicles. Between the vesicles, the matrix of the MVB may be either clear or relatively dense and finely granular. A relationship between MVB and osmiophilic inclusions is suggested not only by the similarity of the thickness of their limiting membranes, but also by the presence of structures suggesting transitions (Fig. 5–8). Occasionally lamellae of extremely osmiophilic material can be seen within a typical MVB (Fig. 5 and 6). Inclusions can be found which have a typical osmiophilic lamellar body and an eccentric cluster of 0.05-μ vesicles enclosed within a common membrane (Fig. 7). There are also intermediates between these extremes (Fig. 8).

In a majority of inclusions in well-fixed human lung, the dense material fills the limiting membrane quite completely. However, in the apical cytoplasm near the plasma membrane, a clear zone often separates the limiting membrane from the dense substance of the inclusion (Fig. 9). Fuzz is absent from the inner surface of the limiting membrane. Occasionally a coated vesicle can be seen fusing with an inclusion membrane at this stage (Fig. 10 and 11). Although the great majority of the dense lamellar bodies are in intracytoplasmic inclusions,

occasional bodies of similar material are present in the air spaces. In addition, sometimes the limiting membrane of an inclusion is continuous with the plasma membrane, so that the inclusion vacuole communicates with the air space (Fig. 11–13). When this is seen, the layer of fuzz over the plasma membrane stops at the cell surface and is not continuous over the invagination surrounding the lamellar material (Fig. 11 and 13).

Histochemistry

In sections incubated for acid phosphatase for 30 min., lead deposits are seen in the Golgi apparatus and in small cytoplasmic vesicles. With incubations of 90 min. or longer, lead is also present in the matrix of multivesicular bodies and in many of the osmiophilic inclusions (Fig. 14–16). In rat lungs, small inclusions usually contain acid phosphatase, whereas larger ones do not (Fig. 15). In human lung, too, only some of the inclusions contain reaction product, but there is no obvious correlation with the size of the inclusion.

Alkaline phosphatase activity is mainly demonstrable in the apical plasma membrane of the granular pneumocyte (Fig. 17 and 18). The plasma membrane of membranous pneumocytes does not contain reaction product. Within the cytoplasm of the granular pneumocyte, small vacuoles with alkaline phosphatase activity are seen, but only infrequently. Also, very few of the osmiophilic inclusions show deposits of lead in their limiting membrane. The inclusions with reaction product are always in the apical cytoplasm, very close to the plasma membrane. In several instances, serial sections show that the inclusion membrane showing reaction product is, in fact, continuous with the plasma membrane (Fig. 17).

Dialyzed iron was bound by the alveolar surface of both the membranous and granular pneumocyte (Fig. 19). Over the membranous pneumocyte, it was bound as a narrow line 100 Å thick. Over the granular pneumocyte, the iron was bound in a fuzzy layer 200–300 Å thick in rats and up to 400 Å in man. No intracellular structures were stained in granular pneumocytes. Incubation in neuraminidase solution for 2 hr. eliminated iron staining of the membranous pneumocytes; it seemed to decrease the staining of granular pneumocytes but did not abolish it (Fig. 20). No further changes occurred with overnight incubation. Incubation of sections overnight in phosphate buffer did not alter the iron staining of either cell.

In a few instances in colloidal iron preparations, an inclusion vacuole in a granular pneumocyte was seen in communication with the alveolar

lumen. The iron was bound to the surface plasma membrane of the cell but was not continuous around the vacuole (Fig. 21 and 22).

In contrast, pinocytotic vacuoles at the surface of membranous pneumocytes and phagocytic vacuoles in macrophages were lined by material which bound iron (Fig. 23 and 24).

Penetration of colloidal iron into cells is poor under the conditions used.[27] When successful, this limitation can be overcome by the use of thin sections of tissue embedded in methacrylate for staining with either colloidal iron or thorium.[24] We were unable to obtain staining of tissue embedded in butyl-methyl methacrylate, but did get some staining with GMA. A considerable amount of nonspecific precipitate was unavoidable, however. Thorotrast was bound to the apical plasma membrane of the granular pneumocytes, but not to the limiting membranes of the inclusions (Fig. 25).

Discussion

Early studies favored an origin of the osmiophilic lamellar inclusions from mitochondria,[4,28] but recent workers have suggested a lysosomal origin.[10,29-32] Two reports have shown transition from multivesicular bodies,[29,31] an observation confirmed here. Sjostrand has emphasized that all the membranes within a cell are not of identical structure.[33] The limiting membranes of the osmiophilic inclusions and of multivesicular bodies are of identical thickness, 100 Å, and are thicker than those of mitochondria, which are approximately 70 Å.

Reported studies of acid phosphatase activity in granular pneumocytes have had divergent results. Three studies have shown acid phosphatase, at least in small inclusions,[20,29,32] while others have demonstrated none.[9,13] Neither of the negative reports gave experimental details. In our material, incubation of 90–120 min. was required to show activity in the inclusions, whereas 15 min. will heavily stain alveolar macrophage granules. It seems plausible, therefore, that the negative results could have been the result of too brief an incubation, if staining of macrophages was used as the criterion to time the reaction.

Other lysosomal enzymes have been recently demonstrated in both MVB and inclusions.[32] In addition, parallel reduction in the number of MVB's and inclusions follows vagectomy in rats.[34]

In agreement with the present report, studies of alkaline phosphatase by light microscopy have suggested localization of the enzyme to the plasma membrane of granular pneumocytes.[35-37] However, the single other ultrastructural study only mentioned localization in the inclu-

sions.[13] This was seen in our material only when the inclusions were closely related to the plasma membrane.

The presence of an acid mucopolysaccharide layer on the alveolar lining has been described, as seen by light microscopy.[5,35,38,39] Groniowski and Biczyskowa demonstrated it by electron microscopy.[40] Since the colloidal iron-stained layer over the granular pneumocyte was not completely digested by neuraminidase, it must differ chemically from the polysaccharide layer of the membranous pneumocyte which was removed. The polysaccharide layer is presumably a surface coat similar to that found on intestinal epithelium and many other cells.[41] Features supporting this interpretation are the pattern of radiating filaments continuous with the outer leaflet of the plasma membrane,[42] and the observation that there is a morphologic and chemical difference in the layer associated with cells of differing function—i.e., granular and membranous pneumocytes. A layer of free intraluminal mucus would not show abrupt and reproducible variation.

At present, the surface coat is thought to be an integral part of the cell membrane. This casts doubt on the ability of the colloidal iron-stained layer of the respiratory portion of the lung to function as an extension of the bronchial mucous blanket as suggested by Macklin.[5]

The function of a surface coat is unknown. Pease, however, has pointed out that the one probable result of such a coat is the binding of water.[41] Thus the coat in alveoli may in some way influence the aqueous alveolar lining. Although carbohydrate has been recently emphasized as a component of surfactant,[43] there is general agreement that phospholipid is its major active component.[43-45] Surface-active lung extracts have the "myelin figure" structure expected of lipoproteins with a high phospholipid content.[46] By the use of intratracheal fixation with gelatin-osmium tetroxide mixtures[47] or by freeze cleaving,[48] an extra membrane-like layer has been demonstrated overlying the membranous pneumocyte. A similar layer has been seen in lamb lungs fixed by more conventional methods;[49] its morphology is similar to that predicted for the surfactant. Furthermore, surfactant is relatively easily separated from cell membranes.[50] Thus the iron-stained polysaccharide layer is probably not the surfactant itself.

Many studies have pointed to an association between the surfactant and the dense inclusions of the granular pneumocyte.[10-16] Both the morphology of the inclusions and their solubility in chloroform-methanol suggest a high lipid content.[31] Furthermore, the granular pneumocyte is the alveolar cell most active in the uptake of labeled palmitate, an important component of surfactant.[14]

Since 1964 many illustrations have been published showing lamellar bodies in transit between intracellular vacuoles and the alveolar lumen. It has been difficult to establish from static micrographs whether this represents secretion or phagocytosis. The presence of lysosomal enzymes in certain stages of the evolution of the inclusions is not necessarily evidence in favor of phagocytosis, since lysosomal enzymes have been identified in several secretory granules.[51-54] It has often been assumed that the inclusions are secretory granules because the lamellar inclusions are numerous in granular pneumocytes, whereas inclusions with similar morphology are much less numerous in active phagocytes, the alveolar macrophages. This argument is not conclusive because macrophages are continuously removed via the bronchial mucous blanket, while granular pneumocytes are probably a relatively long-lived population and, thus, could have a much longer time in which to accumulate the lamellar inclusions. If granular pneumocytes phagocytize the inclusions, however, their phagocytic activity shows an extraordinary specificity for the one type of material.

Bensch, Schaefer, and Avery have provided evidence in favor of secretion by a careful analysis of the relationship of the inclusions to edema fluid in experimental CO_2 breathing.[9] The present study also provides evidence in favor of secretion. Phagocytic and pinocytotic vacuoles, being derived from the plasma membrane, are lined by the polysaccharide of the surface coat (Fig. 23 and 24). If the membrane surrounding the lamellar inclusions lacks the carbohydrate surface layer, it cannot be derived from the plasma membrane, although it may still be a precursor of it. The absence of both fuzz and iron staining indicate that the surface coat was absent from the limiting membrane. The failure of penetration of the colloidal iron to intracellular sites could explain the failure to demonstrate a polysaccharide layer associated with inclusion membranes deep within the cytoplasm, but it could not explain the absence of polysaccharide when the inclusion membrane was continuous with the plasma membrane (Fig. 21 and 22). As an additional check, however, a limited number of observations were made by Thorotrast staining[24] of thin sections. These observations confirmed the absence of mucopolysaccharide lining the inclusion vacuoles.

Our observations indicate that the characteristic osmiophilic lamellar inclusions of granular pneumocytes arise from multivesicular bodies and are secreted into the alveolar lumen. During the life history of the inclusion, the limiting membrane undergoes sequential changes. Early in its evolution it has acid phosphatase, but later this is lost and, as

the inclusion approaches the apical plasma membrane, it acquires alkaline phosphatase activity similar to that of the plasma membrane itself. Only after release of the dense contents of the inclusion vacuole, does the limiting membrane develop a surface coat, coincident with its incorporation into the plasma membrane.

Summary

Electron microscopic observations of lungs from rats and from human surgical specimens were supplemented by the ultrastructural localization of acid and alkaline phosphatase and acid mucopolysaccharide. The apical plasma membrane of the granular pneumocyte differs from that of the membranous pneumocyte in that it forms microvilli, has alkaline phosphatase activity, and has a thicker mucopolysaccharide surface coat. The characteristic dense lamellar inclusions of the granular pneumocyte appear to develop from multivesicular bodies, since both contain acid phosphatase activity, and since organelles of intermediate structure can be seen. Lamellar inclusion material is found in the alveolar lumen, within intracellular vacuoles in granular pneumocytes, and within vacuoles which communicate with the alveolar lumen by membrane fusion. The membranes of these vacuoles lack a mucopolysaccharide coat. Since the membrane of a phagocytic vacuole is derived from the plasma membrane, it is lined by surface polysaccharide. The absence of a carbohydrate coat lining the membranes surrounding the lamellar inclusions of granular pneumocytes indicates that the inclusions are secretory granules, rather than products of phagocytosis.

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[*Illustrations follow*]

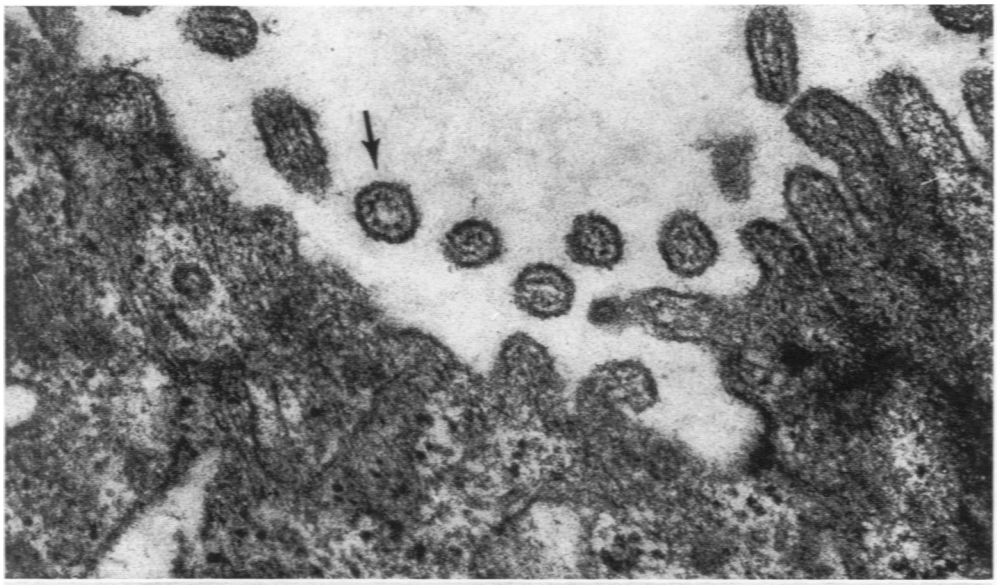
Legends for Figures

Fig. 1. Human granular pneumocyte fixed with glutaraldehyde. Microvilli are cut in cross section showing axial filaments. Arrangement of filaments is inconstant, but they frequently have a circumferential distribution *(arrow)*. Uranyl acetate-lead citrate. × 78,000.

Fig. 2. Apical cytoplasm of human granular pneumocyte shows web of filaments after glutaraldehyde fixation. Filaments are limited to a narrow zone approximately 0.1 μ thick. Uranyl acetate-lead citrate. × 66,000.

Fig. 3. Human granular pneumocyte fixed in 1% osmium tetroxide. Microvilli lack discernible axial structure, but are covered by a layer of fine fuzz. Osmiophilic substance of an inclusion *(I)* is separated from its limiting membrane. No fuzz lines limiting membrane of inclusion. Uranyl acetate-lead citrate. × 66,000.

Fig. 4. At high magnification, fuzz on microvillus consists of filaments which seem to be attached to outer dense leaflet of plasma membrane. Uranyl acetate-lead citrate. × 120,000.



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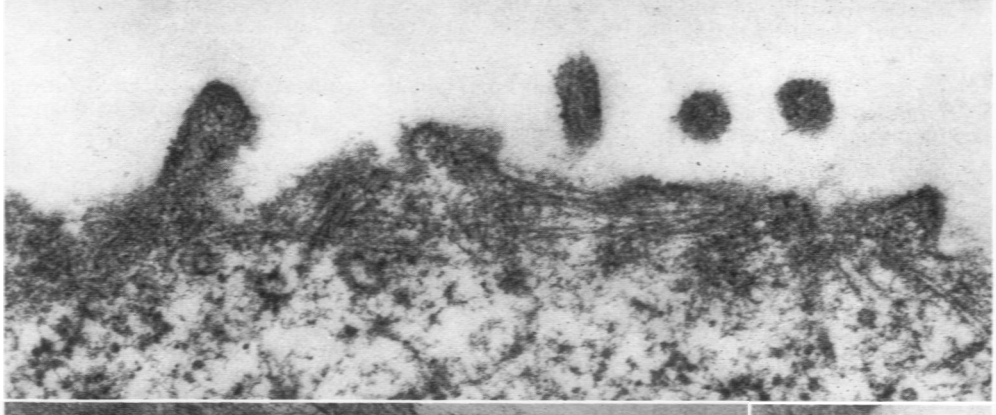

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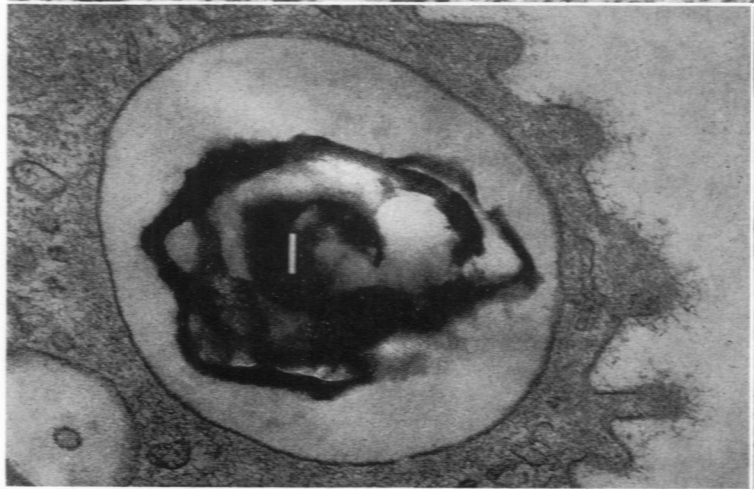

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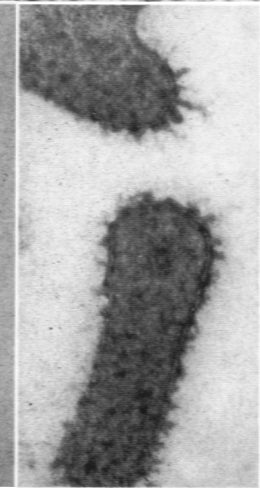

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Fig. 5. Granular pneumocyte of rat lung in which there are two multivesicular bodies. Osmiophilic lamellae are present within one. Lead citrate. × 60,000.

Fig. 6. Lamellas of dense material within multivesicular body in granular pneumocyte of rat lung. Uranyl acetate-lead citrate. × 82,000.

Fig. 7. Inclusion in granular pneumocyte of human lung has eccentric cluster of vesicles within its membrane. Osmium tetroxide fixation; uranyl acetate-lead citrate. × 71,000.

Fig. 8. Two structures *(arrows)* in human granular pneumocyte which can be interpreted as transitions between multivesicular bodies and inclusions. Osmium tetroxide fixation; uranyl acetate-lead citrate. × 50,000.

Fig. 9. Dense material of inclusions deep in cytoplasm of human granular pneumocyte completely fills the limiting membranes. The two inclusions nearest plasma membrane *(top)* are separated from limiting membrane by a space which appears empty. Osmium tetroxide fixation; uranyl acetate-lead citrate. × 21,000.

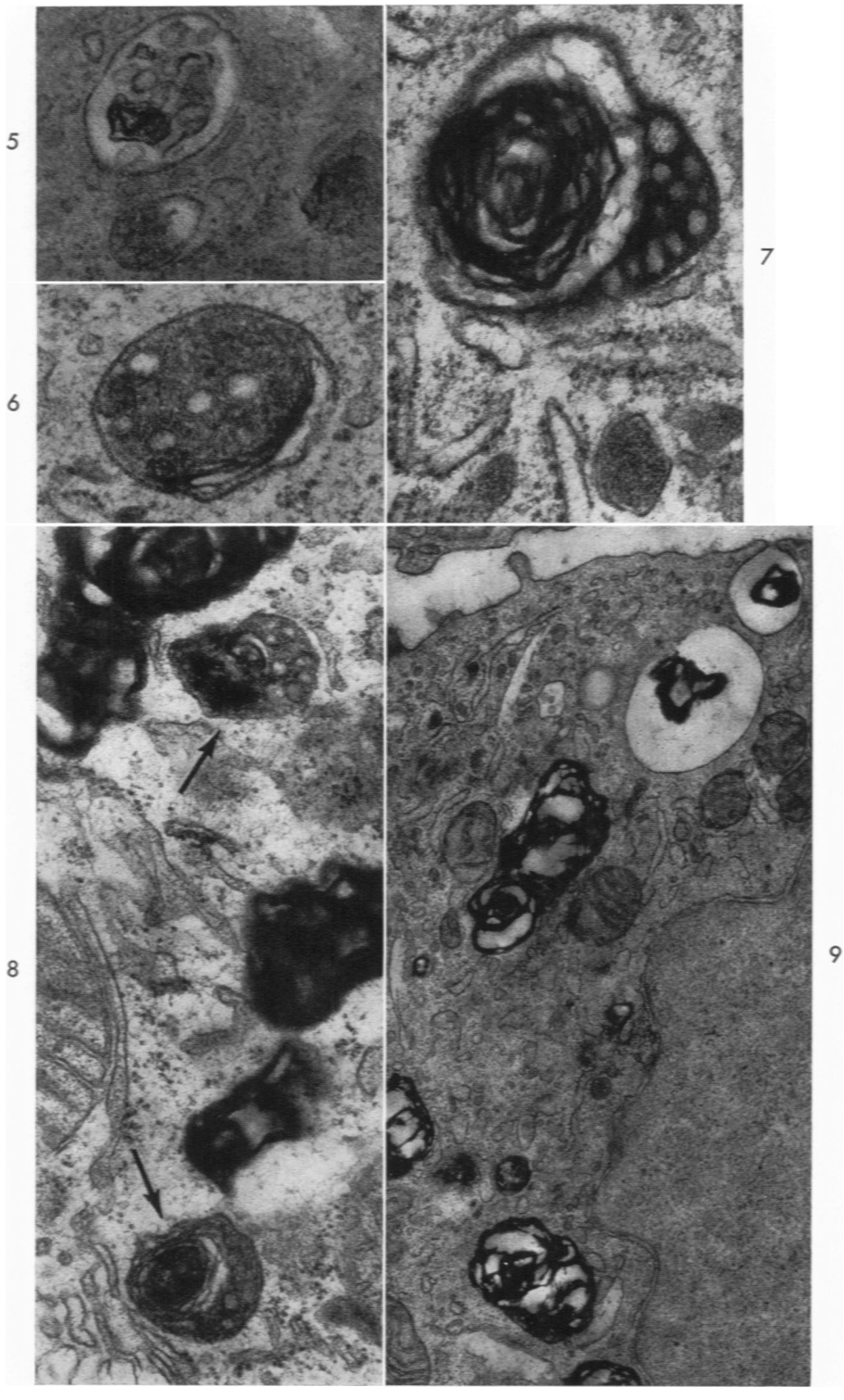
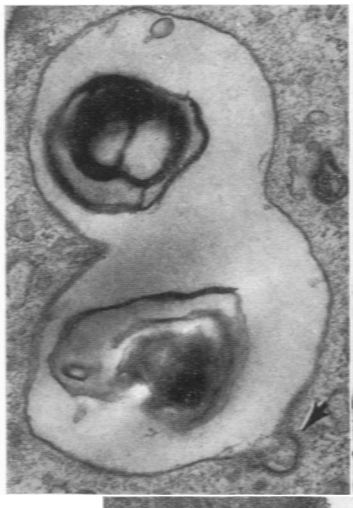


Fig. 10. Coated vesicle *(arrow)* is in communication with inclusion in human lung. Osmium tetroxide fixation; uranyl acetate-lead citrate. × 46,000.

Fig. 11. Human lung. Membrane surrounding inclusion is continuous with plasma membrane. Fuzz of plasma membrane is not evident in inclusion membrane. Coated vesicle communicates with the inclusion *(arrow)*. Osmium tetroxide fixation; uranyl acetate-lead citrate. × 51,000.

Fig. 12. Human lung. Material composed of osmiophilic lamellae is present in alveolar lumen *(arrow)*, in cytoplasmic inclusions, and in transit between lumen and cytoplasm (upper right). Osmium tetroxide fixation; uranyl acetate-lead citrate. × 23,000.



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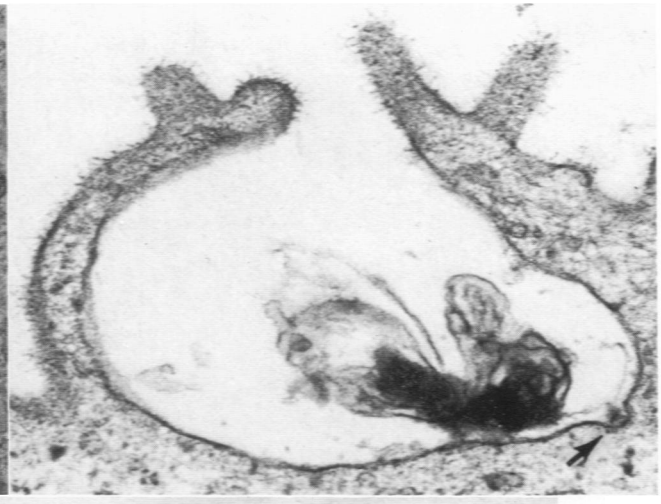

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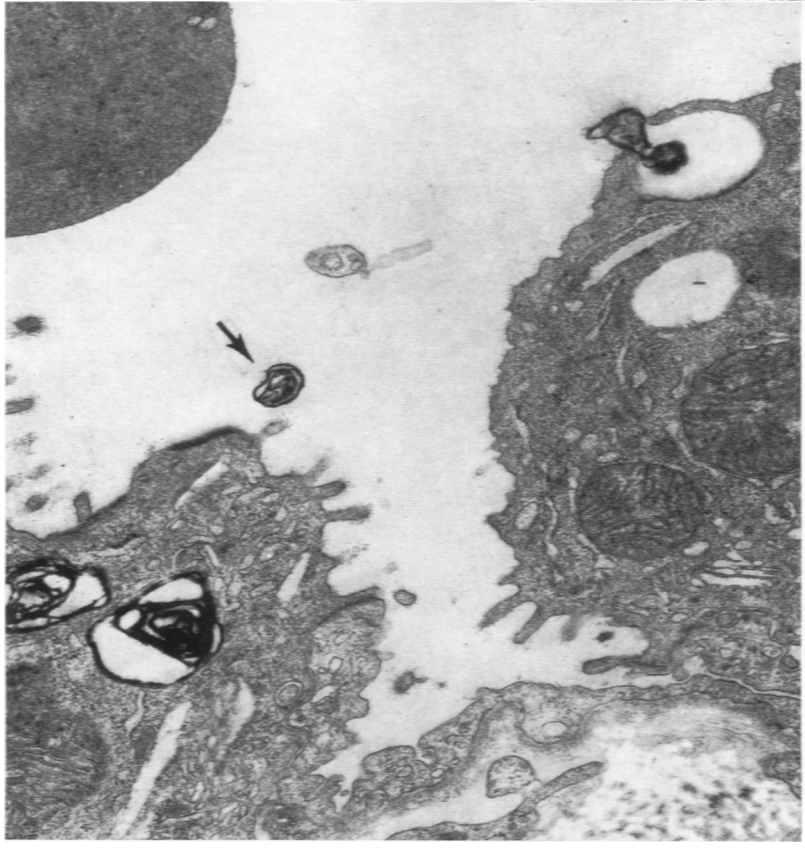

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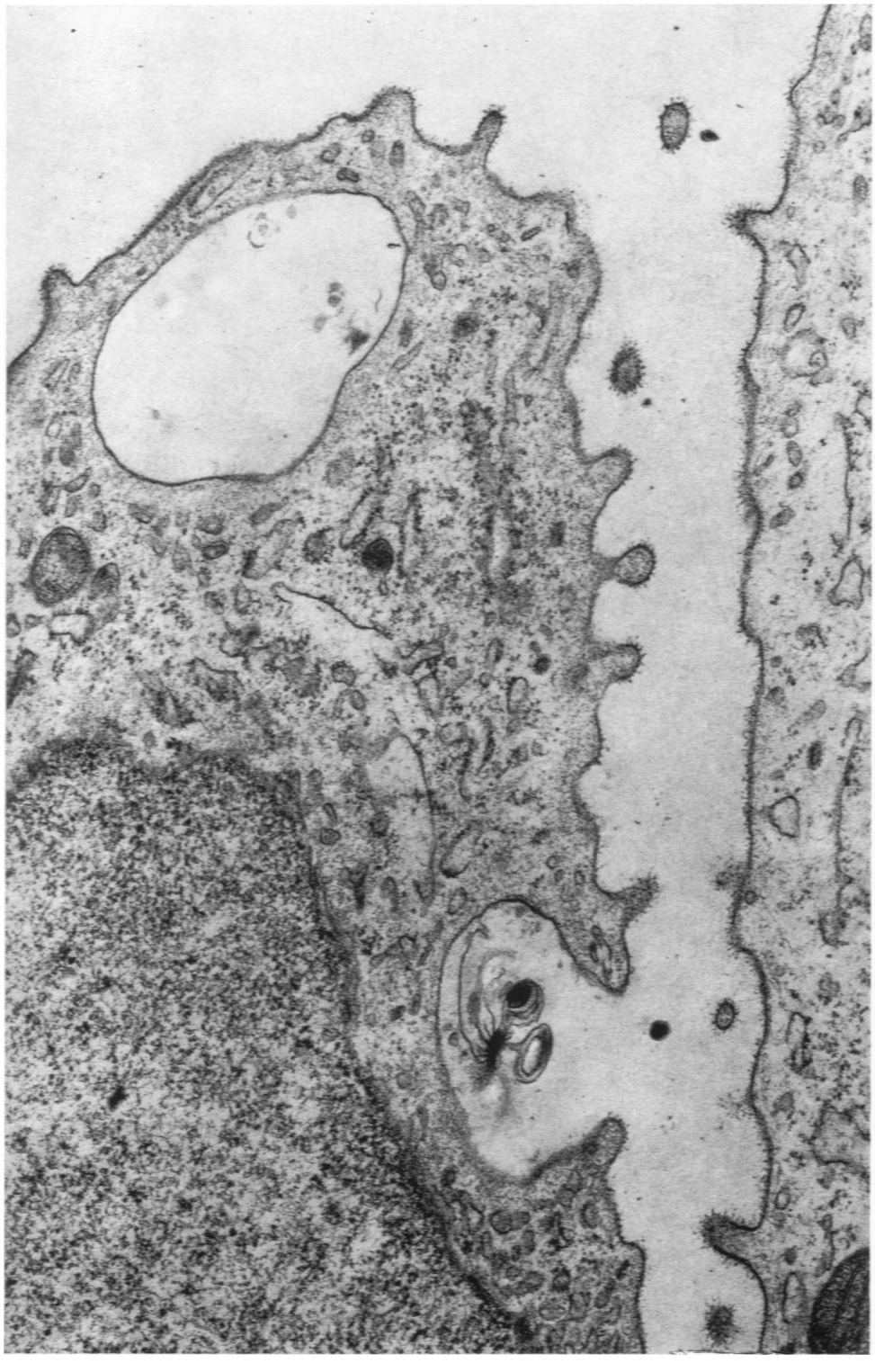
Fig. 13. Human lung. Fuzz is present over plasma membrane of two granular pneumocytes, but is not seen in association with membranes of two inclusion vacuoles. Osmium tetroxide fixation; uranyl acetate-lead citrate. × 32,500.



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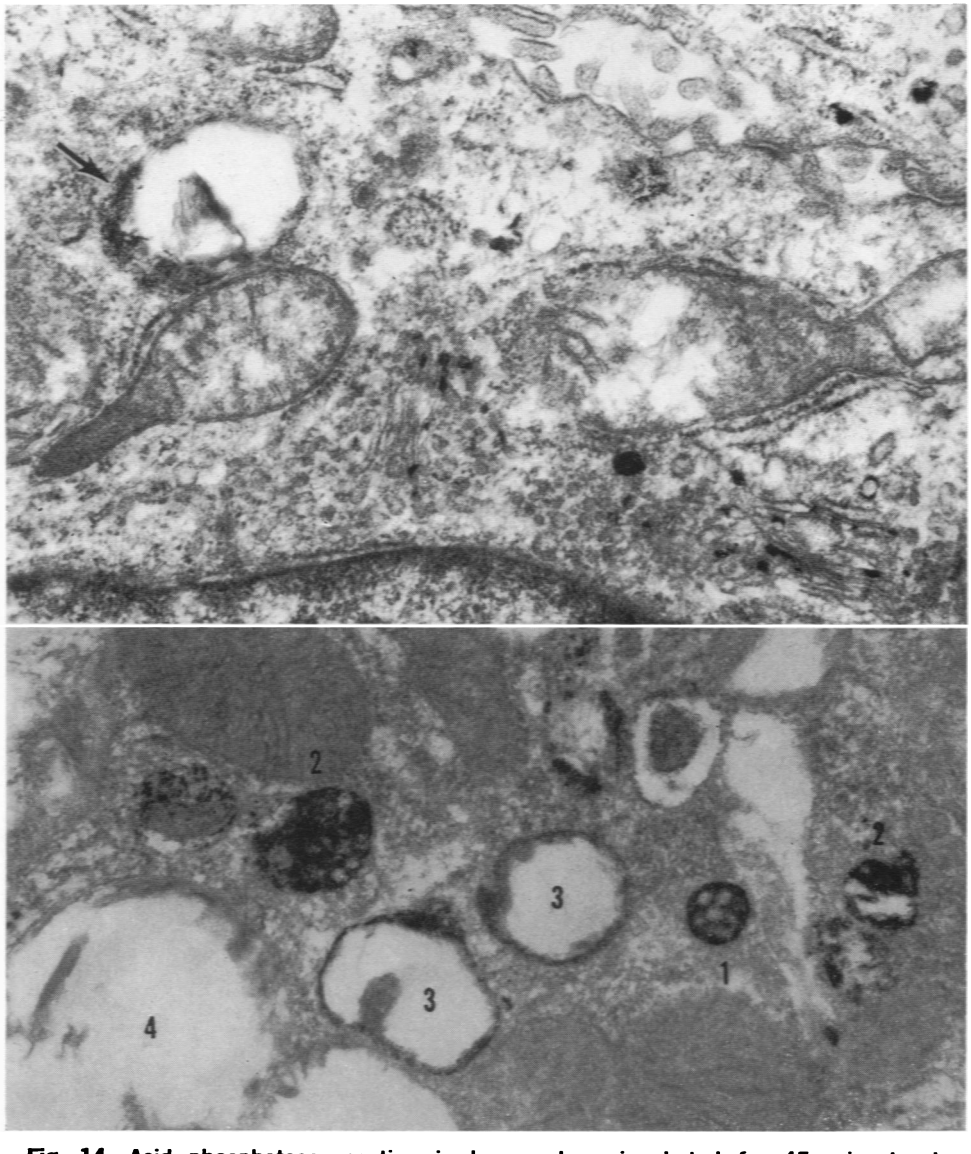


Fig. 14. Acid phosphatase reaction in human lung incubated for 45 min. Lead phosphate is deposited in Golgi cisterns, cytoplasmic vesicles, and an inclusion *(arrow)*. Uranyl acetate. × 31,000.

Fig. 15. Acid phosphatase reaction in rat lung incubated for 150 min. Reaction product is seen in multivesicular body *(1)*, small inclusions *(3)*, and possible transitional structures *(2)*. A large inclusion *(4)* lacks reaction product. Uranyl acetate. × 36,000.

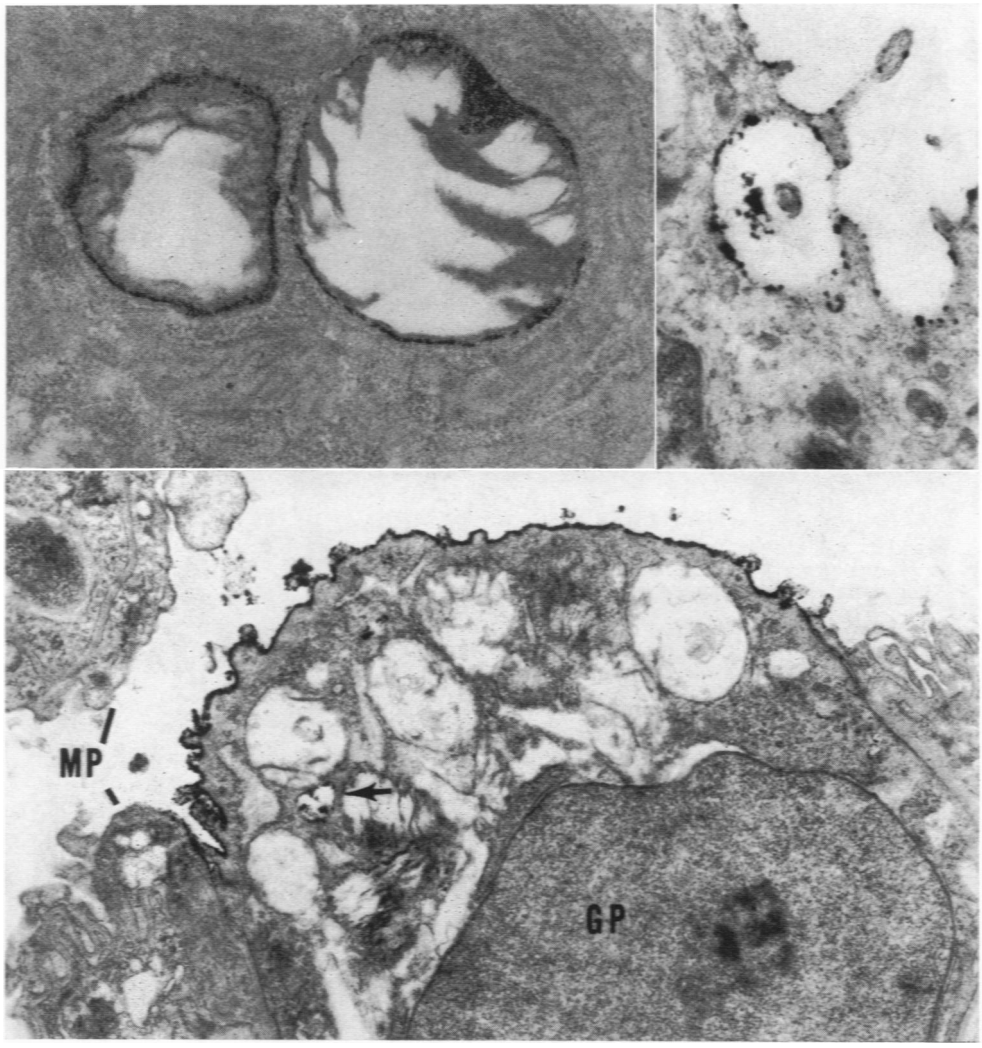


Fig. 16. Acid phosphatase reaction in inclusions in rat lung incubated for 120 min. Reaction product is deposited in or near the limiting membrane. Uranyl acetate. × 41,000.

Fig. 17. Alkaline phosphatase reaction in human lung incubated for 7 min. Lead phosphate is in plasma membrane and membrane of inclusion which is continuous with plasma membrane. Uranyl acetate. × 25,000.

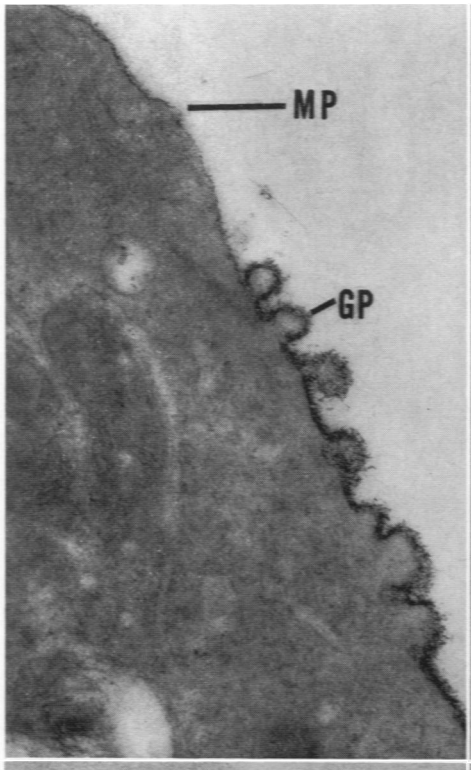
Fig. 18. Alkaline phosphatase reaction in rat lung incubated for 12 min. Reaction product is present in apical plasma membrane of granular pneumocyte *(GP)* and in a small cytoplasmic vacuole *(arrow)*. Plasma membranes of membranous pneumocytes are devoid of lead deposits. Uranyl acetate. × 21,000.

Fig. 19. Rat lung stained with colloidal iron. A delicate layer less than 100 Å thick is stained on membranous pneumocyte *(MP)*. Layer over the granular pneumocyte *(GP)* is much thicker. No counterstain. × 32,000.

Fig. 20. Rat lung stained with colloidal iron after 2 hr. digestion with neuraminidase: no staining of membranous pneumocyte. Staining of granular pneumocyte appears to have diminished. No counterstain. × 51,000.

Fig. 21. Human granular pneumocyte stained with colloidal iron. There is a thick layer of stained material over plasma membrane. Inclusion vacuole communicates with alveolar lumen. There is no staining of limiting membrane of inclusion. No counterstain. × 41,000.

Fig. 22. Rat lung stained with colloidal iron. Vacuole in granular pneumocyte communicates with alveolar lumen. Although no inclusion material is present within the vacuole, osmiophilic membranous material (not shown), which was close by in the alveolar lumen, may have been released from this vacuole. There is heavy staining of plasma membrane in contrast with very faint staining of lining of vacuole. No counterstain. × 51,000.



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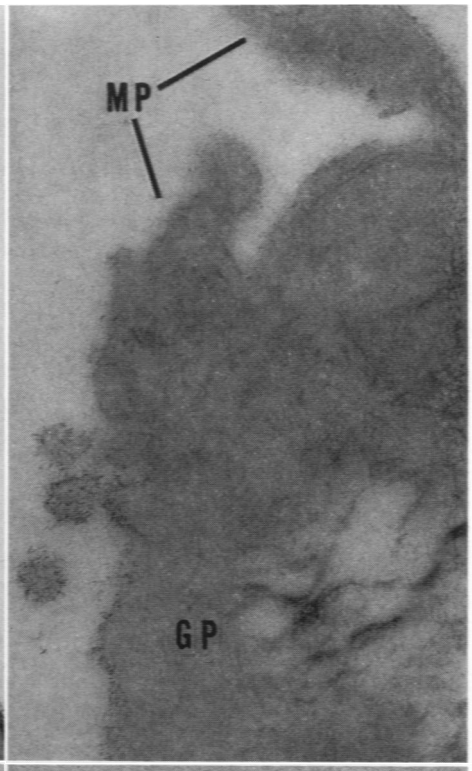

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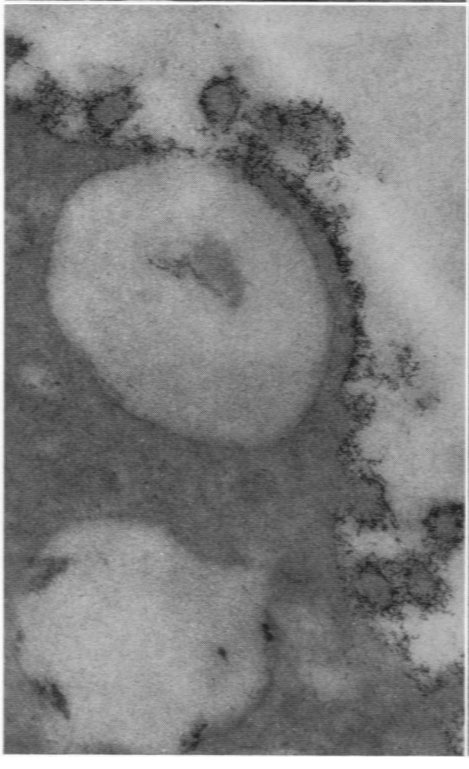

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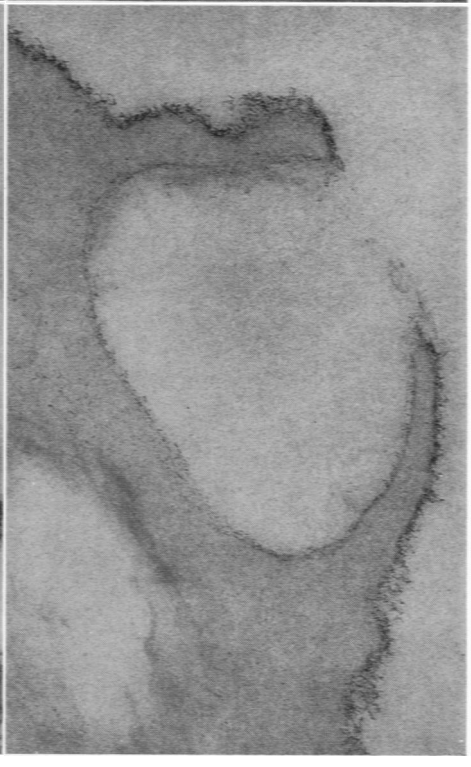

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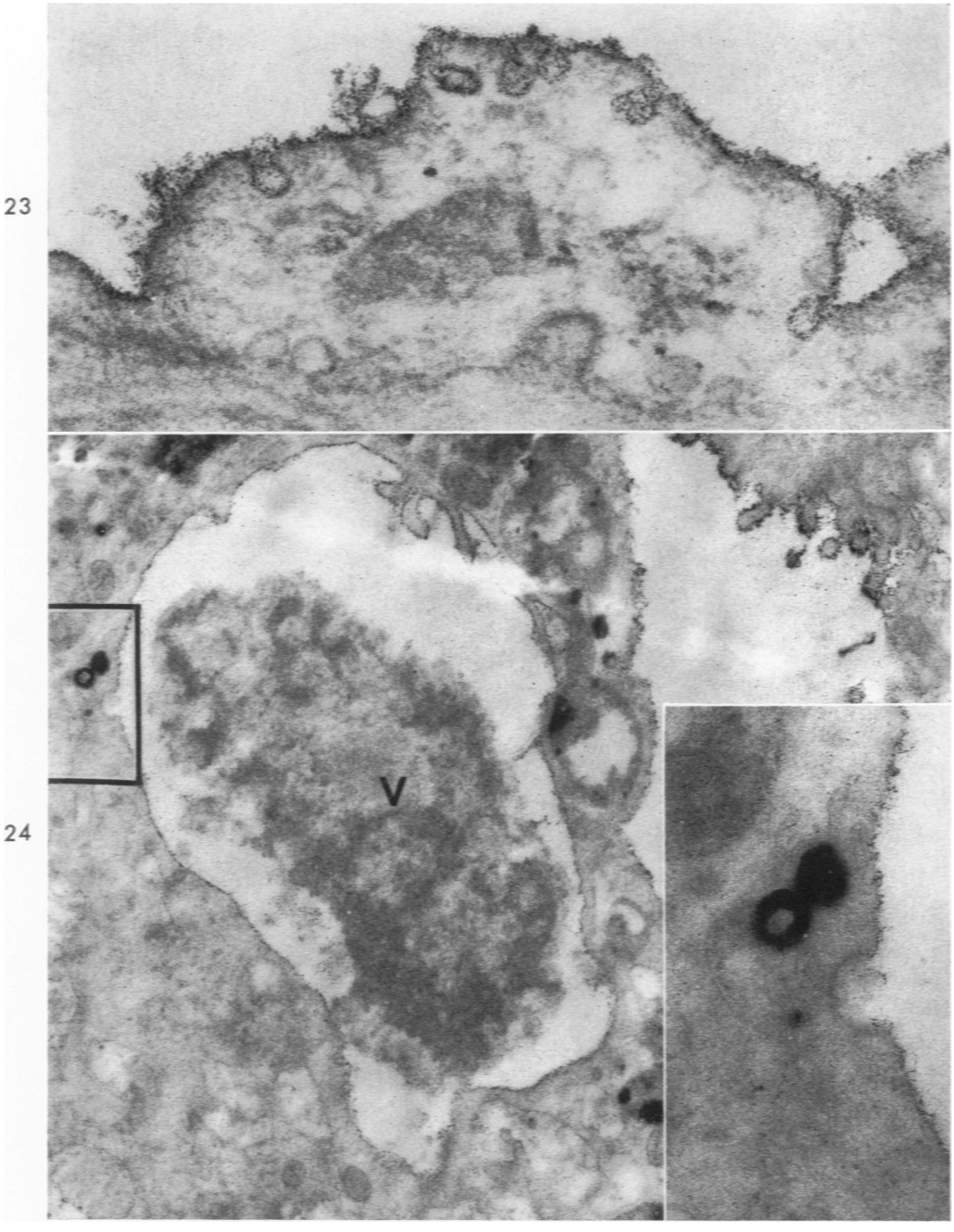

Fig. 23. Membranous pneumocyte from human lung stained with colloidal iron. There is staining of lining of those pinocytotic vesicles which communicate with alveolar lumen. No counterstain. × 93,000.

Fig. 24. Alveolar macrophage in human lung stained with colloidal iron. This micrograph is believed to show a phagocytic vacuole (*V*) with a thin lining stained by colloidal iron. Small vesicle fusing with large vacuole lacks colloidal iron-stained lining: it is probably a primary lysosome fixed at time of discharge into phagocytic vacuole. No counterstain. × 18,200. **Inset:** Higher magnification of enclosed area to show absence of colloidal iron staining of presumed primary lysosome. × 54,000.

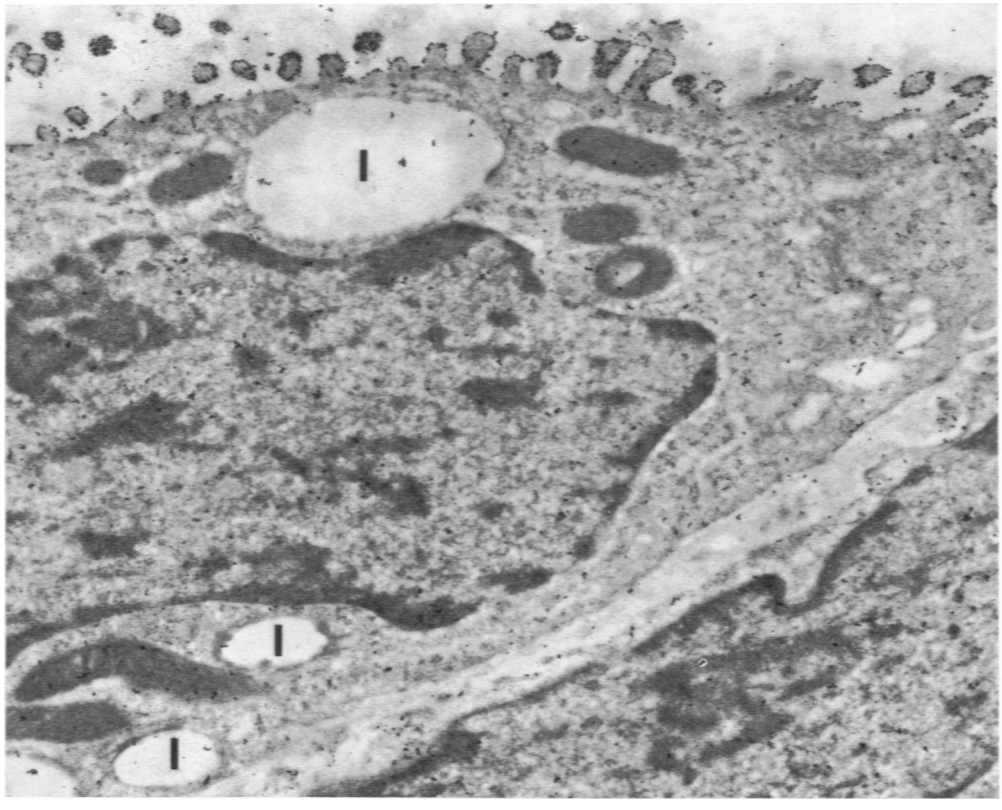

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Fig. 25. Granular pneumocyte of rat; stained with Thorotrast. Apical plasma membrane is stained. There is no staining of inclusion *(I)* membranes. No counterstain. × 17,500.