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FREEZE-FRACTURE STUDY OF THE RAT LUNG II. COMPARTMENTALIZATION OF LAMELLAR INCLUSION BODIES IN RAT ALVEOLAR TYPE II CELL

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Abstract

Differences in cleaving behavior of alveolar type II cell lamellar bodies examined by the freeze-fracture technique were observed in specimens processed by various preparatory methods. All methods preserved the general appearance of rat type II cells; however, in tissue frozen after glycerinization at 38°C without chemical fixation, the lamellar bodies were fractured more superficially, so that the outermost layer of the lamellar bodies could be observed. Results of this study suggest that some lamellar bodies are composed of parallel stacks of discoid compartments which consist of multilayered lamellae. I believe that glycerinization at higher temperatures enhances the mutual adhesiveness of lamellae during subsequent freeze-fracturing. Based on these observations, I propose that disc-like compartments represent lamellar aggregates of surfactant phospholipids synthesized by enzymes at the limiting membrane, and that the linear zone of intersection of these discs with the membrane represents the site of active formation for these structures.

INTRODUCTION

Partial extraction and poor preservation of phospholipid components during commonly used methods of chemical fixation and dehydration have hampered visualization of the natural appearance of the type II cell lamellar inclusion body. A number of techniques have been developed in order to improve this situation[1-5]. With these techniques, a regular periodicity of lamella within inclusions has been demonstrated. Freeze-etching (FE) and freeze-fracture replication (FFR) techniques have also proven useful in understanding the morphology of the inclusion body[6-9]. Using this technique, either the processes of fixation and of dehydration, which may result in lipid extraction, can be omitted. FE and FFR have added some new data on the periodicity and

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biochemical nature of lamellae, but none regarding the internal structual configuration of the lamellar inclusion[6-9]. With either technique, the lamellar inclusion bodies appear to be composed of a variable number of concentric or parallel lamellae. Changes in procedures during freeze-fracture preparation may alter the morphological appearance of lamellar bodies. I decided, therefore, to study the effects of various combinations of preparatory procedures, including the use of glycerol and of chemical fixation, and of altering preparation temperature, in order to evoke differences in fracturing behaviour or in the arrangement of phospholipid or protein components in lamellae. As a result of these studies, I report here an unusual feature of lamellar inclusion body, namely, a compartmentalization of lamellar contents, and a possible mechanism for development of these compartments is discussed.

MATERIALS AND METHODS

400 to 500 gm. Wister strain rats were anesthetized by intraperitoneal injection of Nembutal. The lungs were removed *en bloc* and then sliced into one to two mm^3 tissue blocks. Such tissue blocks were grouped as follows.

(1) Group I and II. Tissues were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 hours and then glycerinized in 30 % glycerol in 0.1 M cacodylate buffer (pH 7.4) for one hour. All processes were performed at 4°C (group I) and 38°C (group II), respectively.

(2) Group III and IV. Tissues were fixed in cacodylate-buffered 2.5 % glutaraldehyde for three hours at 4°C and 38°C, respectively. Glycerinization was omitted.

(3) Group V and VI. Tissues were glycerinized in 30 % glycerol in 0.1 M cacodylate for 15 to 30 min. at 4°C and 38°C, respectively. Chemical fixation was not done.

(4) Group VII. Tissues were processed without chemical fixation or glycerinization.

The tissues were rapidly frozen in liquid freon 22 and were stored in liquid nitrogen. Frozen specimens were placed on the cold stage (-150°C) of a Balzers' freeze-etching device (BAF-301). Thereafter, the temperature of the specimens was readjusted and maintained at -110°C. Fracture and replication were performed at 10^{-6} Torr. 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 copper grids covered with Formvar and examined in a Hitachi HU-12 electron microscope.

RESULTS

The general appearance of rat type II cells in all groups was almost identical to that described in a previous report.[12] The basic architecture of lamellar bodies was also identical in groups I, II, III, IV, V and VII. Group VI (glycerinized at 38°C without chemical fixation) showed a somewhat different fracture-pattern of lamellar bodies. For the purpose of this communication, one unusual feature of the lamellar inclusion body, that is, compartmentalization, will be detailed here, and other changes will be described elsewhere.

As shown in figures 1 and 3, type II alveolar cells contained numerous lamellar inclusion bodies. Lamellar bodies were fractured at various depths in group VI. When the lamellar body was fractured most superficially, it revealed an external face of the limiting membrane, which had so-called "membrane-associated particles" (MAPs) of 150 Å diameter (Fig. 2-a). The distribution of MAPs was not so uniform as that seen in glutaraldehyde-fixed specimens, probably because of the tissue change during glycerinization before freezing. Slightly deeper planes of fracture revealed stacks of cigar-shaped, convex lamellar surface which was identical in appearance to that of parallel lamellae (Fig. 2-a). Their widths and lengths varied from 700 Å to 2500 Å, and from 0.7 μm to 1.2 μm, respectively. Fracture faces at deeper planes disclosed elliptically concentric multilayered lamellar structures within the inclusion body (Fig. 2-b). Even inclusion bodies apparently comprised almost entirely of parallel stacks of lamellae may contain elliptically concentric multilayered compartments (Fig. 2-c). Some others, however, clearly demonstrated the abrupt termination of parallel lamellae at the limiting membrane without reflection (Fig. 2-d). In the same inclusions, however, compartmental architecture can be traced in places (Fig. 2-d, arrow). In some cases, lamellae were fractured irregularly to reveal a cross-fracture face as well as fissure-face of compartmentalized lamellae simultaneously (Fig. 2-e). Compartmentalized lamellae were sometimes observed partially surrounding large concentric aggregates of lamellae (Fig. 2-f). When the superficial layer of small lamellar compartments was observed from the internal aspect, elongated concave cups lined up in parallel within the limiting membrane could be seen (Fig. 3, arrows). Such disc-shaped compartments on either fissured or cross-fractured faces were observed in about one out of ten lamellar bodies.

DISCUSSION

Lamellar inclusion bodies of alveolar type II cells are known to vary in configuration.[9,10] The lamellar inclusion body may be composed of stacks of

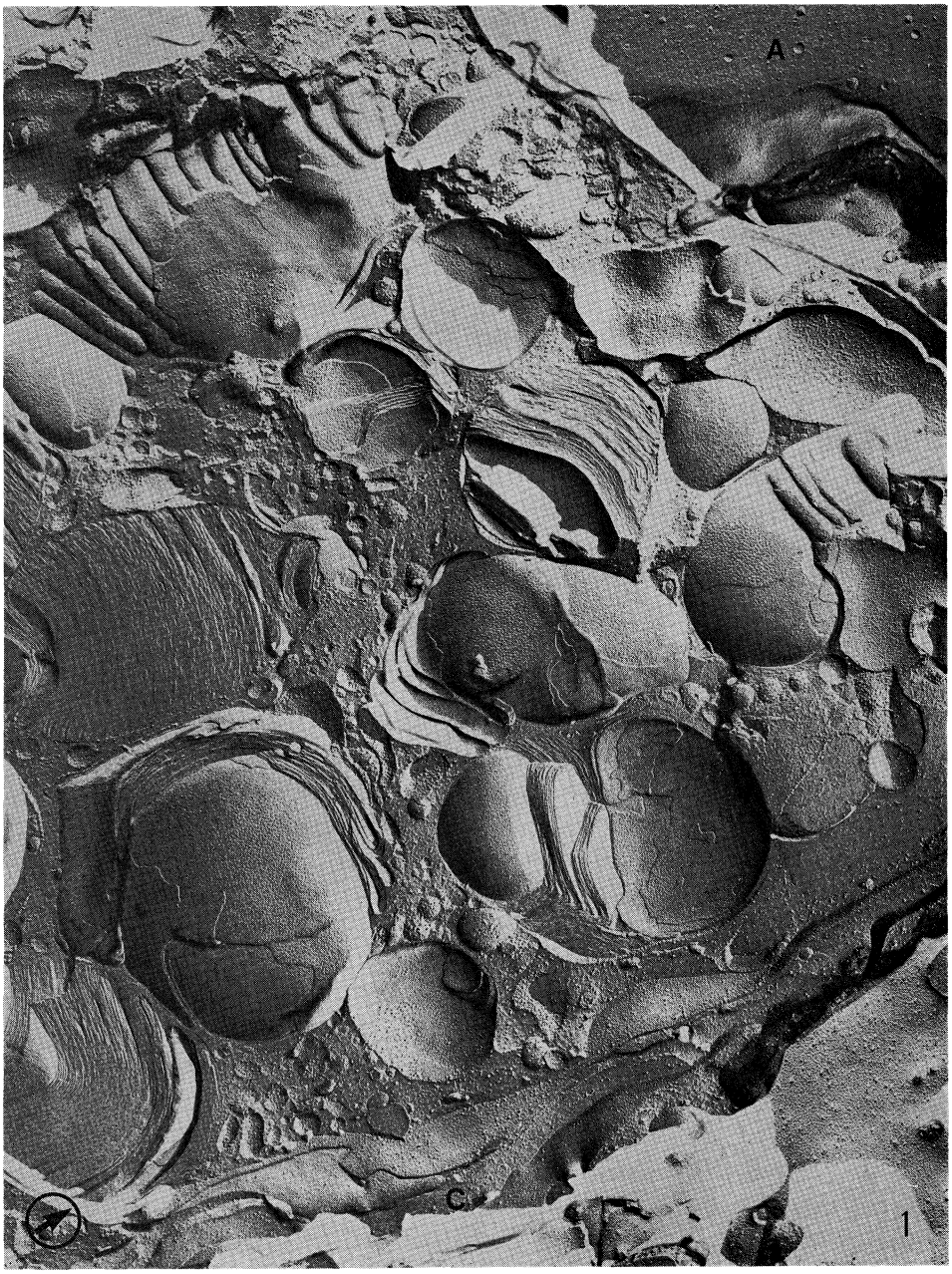


Fig. 1. A type II alveolar epithelial cell showing numerous lamellar inclusion bodies, fractured at various depths. Note fracture faces of the limiting membrane, and various compartmental lamellae. The plasmalemma faces the alveolar space (A) on the right upper corner, and the interstitial connective tissue layer (C) on the lower margin of the field. Mag.: ×28,000

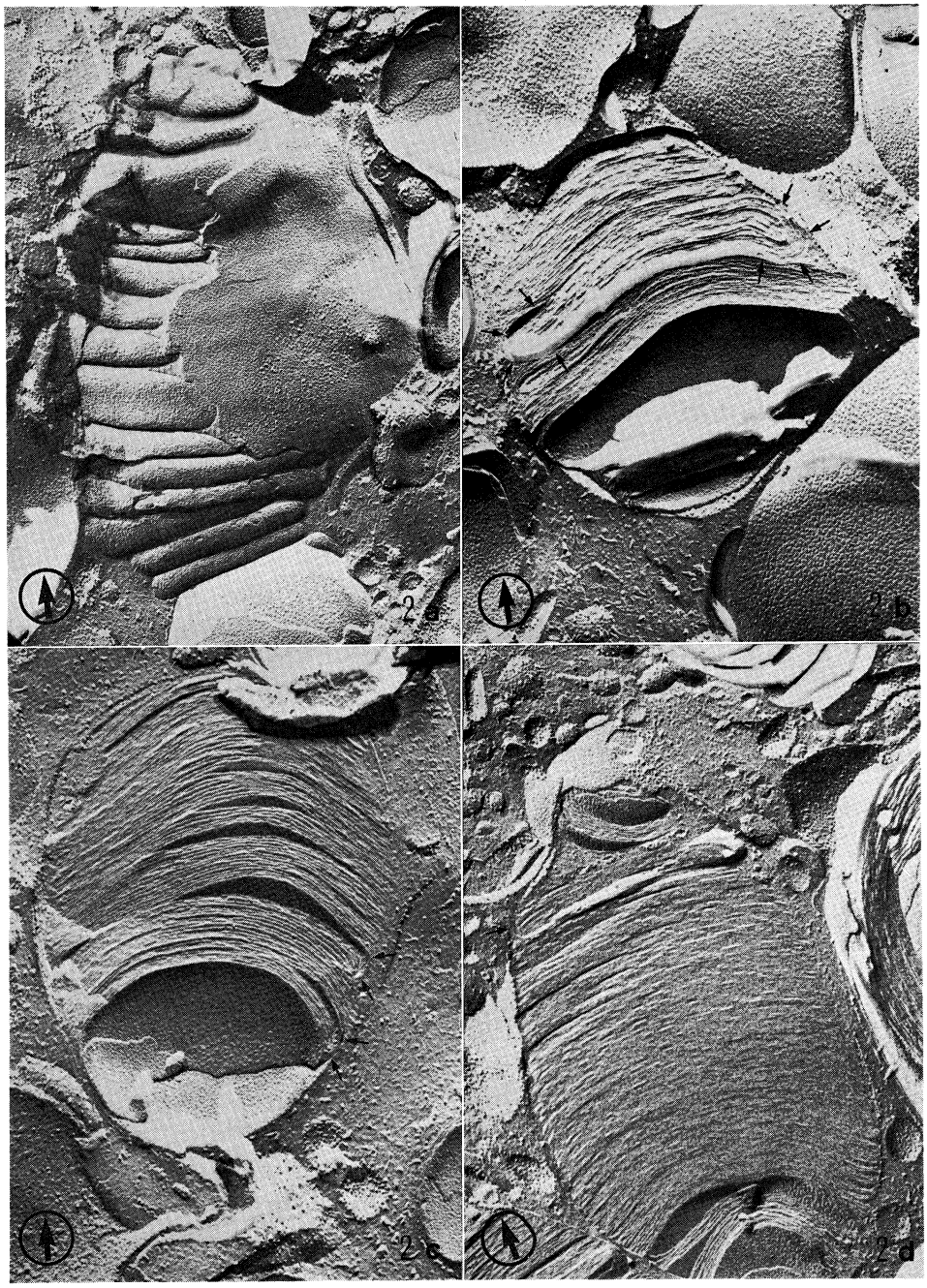

2a
2b
2c
2d

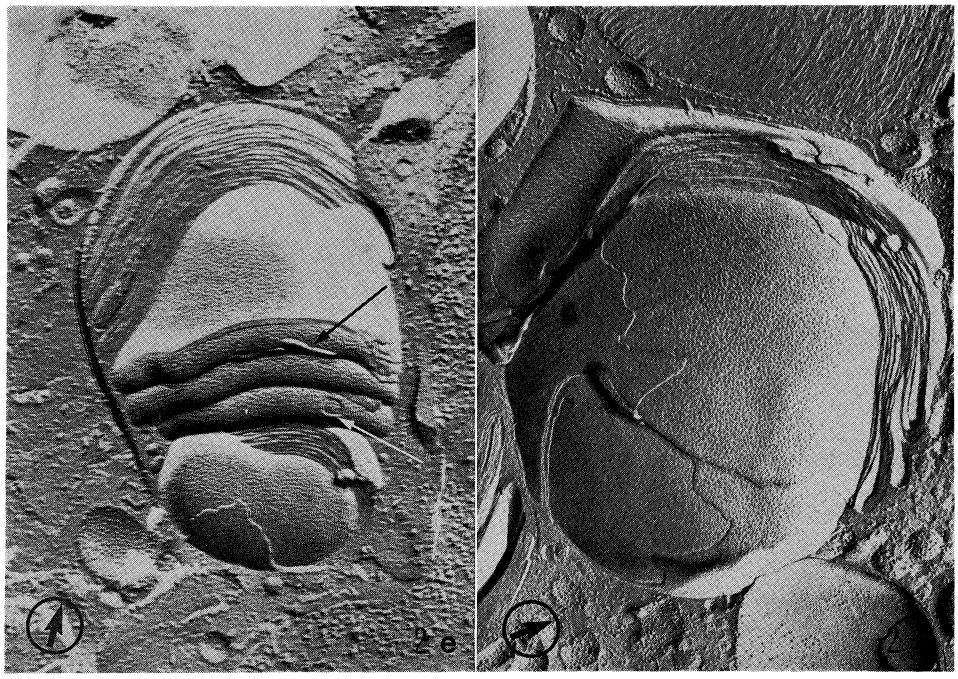
Fig. 2. Lamellar bodies cut at various fracture depths are shown here at high magnification.

a. This lamellar body is fractured at the level of the limiting membrane (right) and outermost layer of the discoid compartment lamellae (left). MAPs of 150 Å diameter are seen mainly in the middle of the limiting membrane in this photograph. This distribution of MAPs may be due to the tissue change during glycerinization before freezing. The background of the limiting membrane is finely granular as are the surfaces of compartment lamellae. Mag.: ×40,000

b. A lamellar body fractured in the midportion showing a disc-shaped lamellar compartmet, which is outlined by arrows. Mag.: ×50,000

c. In this photograph, curved corners (arrows) of compartmentalized lamellae are appreciated on the right, while the left side ends of lamellae are obscure. Mag.: ×50,000

d. All the lamellae appear parallel with sharp ends, except near the top (arrows) where a discoid compartment is apparent. Mag.: ×42,000



e. This photograph more clearly shows that the cigar-shaped compartments like those seen in Fig. 2-a are made of elliptical multilayered lamellae. Note the presence of a small piece of a lamella on such compartment (black arrow) and lamellar surface through a window in the outer surface of the compartment (white arrow). Mag: ×50,000

f. A compartment may partially surround concentric lamellae. Mag.: ×39,000

Fig. 3. A type II alveolar cell
Two of the lamellar bodies here show concave grooves (arrows) on the limiting membrane. These grooves are interpreted as fracture faces of the outermost layer of discoid compartment lamellae. Lamellar patterns are discernible at the curved arrow. In the collapsed alveolar space (A), a tubular myelin figure (tm) is seen.
Nucleus (N), Collagen fibers (C) Mag.: ×18,000

parallel lamellae, single concentric lamellae, multiple concentric lamellae, or of concentric and parallel lamellae. Their substructure and molecular arrangement, particularly in relation to the limiting membrane, remain unclear.

A change in procedure during freeze-fracture preparation may produce a different pattern of cleaving, and thereby reveal previously unfamiliar morphological details when examined under electron microscope. Breathnach *et al.*[11] have demonstrated alteration of the cleaving behaviour of junctional plasma membranes after glutaraldehyde fixation.

In our present experiments, groups I, II, III, IV, V and VII, did not reveal any unusual cleaving of lamellar inclusion bodies. In these groups, as has been described in a previous communication[12], the ends of lamellae, either in parallel or in partially concentric form, were usually abrupt, and only a few lamellae curved back near the limiting membrane, running parallel to end at the limiting membrane of the opposite side. A few small curved lamellae sat just on the limiting membrane and did not extend up to the other side of the lamellar body. Internal lamellae were never seen forming stacks of concentric discs within the inclusion. In the group glycerinized at 38°C without chemical fixation (group VI), on the other hand, lamellae were found further separated into several compartments within a single inclusion body. In preparations of this type, each compartment seemed to consist of whorl of lamellae packed in disc-like configuration; discoid compartments in turn were aligned in parallel fashion and surrounded by limiting membrane, studded with MAPs, to comprise the entire lamellar body (Fig. 4). The presence of the usual parallel form of lamellae, microvilli, and direct contact of the plasmalemma with the underlying connective tissue layer without intervention of cellular components confirmed that cells containing compartmentalized lamellar bodies were in fact alveolar type II cells. Factors which caused this appearance are undetermined. This, however, cannot be considered a random phenomenon. It is also hardly conceivable that the use of glycerinization without chemical fixation alone has affected the cleaving behaviour, because the similarly processed tissue at 4°C did not reveal the same appearance. Rather it seems probable that glycerinization at a higher temperature may have enhanced the tightness or adhesiveness of the lamellae in the compartment. In fact, it is known that glycerol may strengthen the adhesiveness of phospholipid bilayers to cause irregularities in fracture faces[13,14]. Kikkawa and Manabe[10] previously reported some features of lamellar body compartmentalization in fetal rabbit lung studied by freeze-fracture replication, where aggregated elliptical and concave cups were noted in glutaraldehyde-fixed specimens. These were interpreted as superficial fracturing of lamellar bodies composed of compartments, because other lamellae in

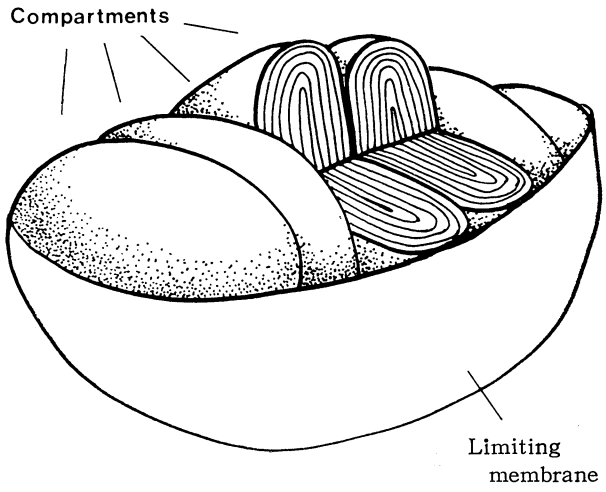


Fig. 4. This drawing helps an interpretation of freeze-fracture images in photographs and enables a better understanding of tridimensional structure of a compartmentalized lamellar inclusion body.

the specimens frozen after glycerinization without chemical fixation revealed disc-like lamellar compartment on the concave cups similar to the present case (unpublished observation). Of course, this feature differs from the freeze-fracture image of mitochondria[15].

The development of inclusion bodies has been studied to some extent. According to Kikkawa[11], inclusion bodies seem to originate from vesicles around the Golgi zone in immature epithelial cells. Either by fusion of two vesicles or the fission of one, they become tubulovesicular bodies, which may attach and fuse with the multivesicular body concurrently present. Such complex bodies which possess tubular loops are the basic units responsible for the production of mature inclusion bodies. Morphologic stages in development of lamellar bodies in mature epithelial cells have not been established, although fusion of multivesicular bodies and lamellar bodies has been occasionally observed. Precise mechanisms of lamellar body formation, in particular, still remain obscure. Histochemical studies have suggested multivesicular bodies and inclusion bodies to be lysosomal derivatives engaged in a secretory process[17,18,19]. Autoradiographic study demonstrated precursor substance of surfactant to have been incorporated into lamellar bodies of type II cells[20]. Also, a certain enzyme responsible for lecithin biosynthesis has been revealed localized to the limiting membrane of the inclusion body[21]. These findings suggest that the lamellar body is a site of lecithin biosynthesis. It would be possible that these vesicles or tubular loops play an important role in the lamellar body

formation, being a core for a concentric or disc-shaped lamellar inclusion to form multiconcentric vesicles. The following facts, however, are against this theory; small vesicles may still be present in the lamellar body despite the presence of mature parallel forms in the same lamellar body. Small multilayered concentric vesicles have never been observed within inclusion bodies. A previous communication[13] demonstrated curved lamellae of various sizes, each end of which sat on the limiting membrane. Together with the results of the present study, this tempts one to speculate on the possible development of discoid lamellar compartments. The lamellae may be synthesized on the limiting membrane, with constituent phospholipid molecules moving toward the center of the inclusion body as newly synthesized phospholipid are generated at the membrane; such evolving lamellar aggregates may appear in two dimension as multilayered hairpin-shaped and tridimensionally as multilayered tubes or discs with blind ends. In view of the fact that some enzymes were found histochemically along the limiting membrane[20], and that the MAPs (protein particles) were found in the limiting membrane by freeze-fracture study[10,13], this seems reasonable, but of course remains speculation. It must be emphasized here that all of the lamellar inclusion bodies were not compartmentalized. Abrupt termination of parallel lamellae was also seen in the lamellar inclusions from group VII.

In summary, the present study demonstrates for the first time that lamellar inclusion bodies are composed of compartments of lamellae. This appearance was seen only in the specimen frozen after glycerinization at 38°C without chemical fixation. It is speculated that surfactant phospholipid molecules may be synthesized at the limiting membrane and arranged in bilayers to form linear or curved lamellae, end of which sits on the limiting membrane. The top of the curved lamellae may extend to the limiting membrane of the opposite side, which may be fractured as a disc.

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REFERENCES

1) Kaibara, M. and Kikkawa, Y.: "Osmiophilia of the saturated phospholipid, dipalmitoyl lecithin, and its relationship to the alveolar lining layer of the mammalian lung" Am. J. Anat. 132: 61-78, 1971
2) Stratton, C. J.: "Multilamellar body formation in mammalian lung: An ultrastrucfual study utilizing three lipid-retention procedures-lung multilamellar body formation" J. Ultrastruct. Res. 52: 309-320. 1975

3) Douglass, N. L., Redding, R. A. and Stein, M.: "The lamellar substructure of osmiophilic inclusion bodies present in rat type II alveolar pneumocytes." Tissue & Cell 7: 137-142, 1975
4) Stratton, C. J.: "The three dimensional aspect of mammalian lung multilamellar bodies." Tissue & Cell 8: 693-712, 1976
5) Stratton, C. J,: "The high resolution ultrastructure of the periodicity and architecture of lipid retained and extracted lung multilamellar body laminations." Tissue & Cell 8: 713-728, 1976
6) Belton, J. C., Branton, D., Thomas, H. V. and Muller, P, K.: "Freeze-etch observations of rat lang" Anat. Rec. 170: 471-486, 1971
7) Roth, J. and Jeyer, H. W.: "Electron microscopic studies in mammalian lung by freeze-etching II. Morphological observations in the metabolism of the surfactant." Exp. Path, Bd. 6: 291-302, 1972
8) Smith, D. S., Smith, U. and Ryan, J. W.; "Freeze-fractured lamellar body membranes of the rat lung great alveolar cell" Tissue & Cell 4: 457-468, 1972
9) Lauweryns, J. M, and Gomeer-Desmecht, M.: "Freeze-etching electron microscopy of the lung" Pathol. Annu. 8: 257-282, 1973
10) Kikkawa, Y. and Manabe, T.: "The freeze-fracture study of alveolar type II cells and alveolar content in fetal rabbit lung" Anat. Rec. 190: 620-638, 1978
11) Breathnach. A. S., Gross. M., Martin, B. and Stolinski, C.: "A comparison of membrane fracture faces of fixed and unfixed glycerinated tissue" J. Cell Sci. 21: 437-448, 1976
12) Manabe, T. and Manabe, R.: "Freeze-fracture study of the rat lung. I. General description of rat alveolar cells in the glutaraldehyde-fixed and glycerinized lung tissue." Kawasaki Med. J. 4: 1-17, 1978
13) Staehelin, L. A.: "The interpretation of freeze-etched artificial and biological membranes" J. Ultrastruct. Res. 22: 326-347, 1968
14) Buckingham, J. H. and Staehelin, L. A.: "The effect of glycerol on the structure of lecithin membrane; a study of freeze-etching and x-ray diffraction J. Microscopy 90: 83-106, 1968
15) Tewari, J. P., Malhotra, S. K. and Tu, J. C.: "A study of the structure of mitochondrial membranes by freeze-etch and freeze-fracture techniques" Cytobios 4: 97-119, 1971
16) Kikkawa, Y. and Spitzer, R.: "Inclusion bodies of type II alveolar cells: Species differences and morphogenesis" Anat. Rec. 163: 525-541, 1969
17) Goldfisher, S., Kikkawa, Y. and Hoffman, L.: "Demonstration of acid hydrolase activities in the inclusion bodies of type II alveolar epithelial cells and other lysosomes in the rabbit lung." J. Histochem. Cytochem. 16: 102-109, 1968
18) Corrin, B., Clark, A. E. and Spencer, H.: "Ultrastructural localization of acid phosphatase in the rat lung" J. Anat. 104: 65-70, 1969
19) Smith, R. E. and Farqueh, M. G.: "Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland" J. Cell Biol. 31: 319-347, 1966
20) Chevalier, G. and Collet, A. J.: "In vivo incorporation of choline-^{3}H, Leucine-^{3}H in alveolar type II pneumocytes in relation to surfactant synthesis. A quantitative radioautographic study in mouse by electron microscopy" Anat. Rec. 174: 289-310, 1972
21) Meban, C.: "Localization of phosphatidic acid phosphatase activity in granular pneumocytes" J. Cell Biol. 53: 249-252, 1972