CONVERSION OF LAMELLAR BODY MEMBRANES INTO TUBULAR MYELIN IN ALVEOLI OF FETAL RAT LUNGS

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ABSTRACT

Fluid-filled lumina of fetal rat lungs contain lamellar bodies (LBs) as well as tubular myelin (TM), both of which are thought to be stores of phospholipid-rich pulmonary surfactant. The alveolar epithelium is believed to secrete LBs, but neither the origin nor the mechanism of TM formation is entirely certain. The main objective of this study was to determine the relationship between secreted LBs and TM and to define membrane phenomena which occur during TM formation. I examined lung tissues of 20-21 day-old fetuses (day 22 = term) using transmission and high voltage transmission electron microscopy and cytochemistry. My findings indicate that secreted LBs, identified by the presence of an acid-phosphatase reactive core, are the precursor of TM. Secreted LBs are highly organized structures which contain structurally specialized areas, one of which is a "mini-lattice" structure similar to TM. During TM formation, fuzzes or 8.0-nm diameter particles appear on transition membranes, although LB membranes appear to lack both structures. Similar particles are present on TM membranes and are generally associated with membrane intersections. My results provide evidence that TM is formed from LBs within the alveolar lumen by mechanisms which may be complex.

Abundant evidence suggests that pulmonary surfactant (surface-active material), a phospholipid-rich material (5, 32, 33), forms an extracellular layer covering the alveolar epithelium. Numerous studies support the hypothesis that the surfactant prevents alveolar collapse at low lung volumes by decreasing alveolar surface tension; thus, the presence of surfactant is important in maintaining normal respiratory functions (10, 40). After their synthesis by type II epithelial cells (7, 9, 29, 31), lamellar bodies, the intracellular form of surfactant (2, 6, 18), are secreted into the alveolar lumen as layers of membranes arranged in a spherical granule (43). To perform its proposed function, however, the lamellar body material must be arranged upon lung surfaces to form a phospholipid monolayer which is probably a continuous sheet-like film.

Although the sequence of events which takes place in the rearrangement from lamellar form to monolayer has not been directly visualized, morphological, chemical, and physical observations (18, 43, 52, 53) suggest that a second form of surfactant, tubular myelin, is generated within the alveolar lumen. Tubular myelin formation may therefore be an intermediate step in monolayer formation.

As described by Weibel et al. (55), tubular myelin is composed of numerous tightly packed tubules whose walls have a trilaminate structure

similar to that of cell membranes. The walls of individual tubules are formed by shared membrane-like layers about 5–8.5 nm in thickness. In cross section each tubule is a square with sides ~45–52 nm in length (18, 22, 45, 55); groups of tubules cut in cross section therefore appear to be a lattice composed of square subunits. In some preparations (55) but not in others (18), the center of each tubule contains a single electron-opaque particle or filament. The structure of tubular myelin has only a superficial similarity to the hexagonal phase assumed by highly concentrated (~80%; references 38, 50) phospholipids in water; the dimensions of the tubular myelin lattice and the aqueous nature of fetal lung fluid both argue against the possibility that the state of hydration is solely responsible for the unusual lattice-like array. At present the tubular myelin lattice is a unique structural arrangement of biological membranes; thus, details elucidating the mechanism of its formation are of considerable interest.

I have used both conventional and high voltage electron microscopy to examine the formation of tubular myelin in the fetal rat lung. The use of nonbreathing, fluid-filled fetal lungs facilitates the study of possible intermediate stages in formation of surface films because such films are not believed to be formed in the absence of an air-fluid interface. The observations reported here demonstrate (a) continuities between lamellar body and tubular myelin membranes, (b) the appearance of particles 8.0 nm in diameter on transition membranes, and (c) the presence in secreted lamellar bodies of two specialized membrane arrangements, one of which is structurally similar to that of tubular myelin. Taken together, the observations support the hypothesis that lamellar bodies are transformed into tubular myelin within the alveolar lumen. Some of the observations reported here have been previously published in abstract form (58).

MATERIALS AND METHODS

Animals

Fetuses of known gestational age were obtained from Long-Evans rats bred for this study. Successful breeding was detected by the presence of a vaginal plug on day 0. Four 20-day-old fetuses and eight 21-day-old fetuses were used; the observations were the same in the two groups of animals and thus are not reported separately. Pregnant females were maintained on a diet of Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) and water.

Fixatives

For morphologic studies tissues were fixed in 2% redistilled glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.), 1% freshly prepared paraformaldehyde in a 0.1 M Na^+-K^+ phosphate buffer, pH 7.4, at 37°C, as modified from Karnovsky (27). For histochemical procedures, 0.1 M sodium cacodylate was used instead of the phosphate buffer.

Experimental Procedures

FETUSES: Pregnant rats were anesthetized by an intraperitoneal injection of sodium methohexital at a dosage of 6 mg/100 g body weight. The uterus was exposed through a midline incision in the ventral abdominal wall. The umbilical vessels of one fetus were clamped with a hemostat, and the fetus was removed from the uterus. To protect the remaining fetuses the incision in the body wall of the dam was covered with gauze moistened with warm isotonic saline. The cervical spinal cord of the removed fetus was severed and, under a dissecting microscope, the thoracic cavity was carefully opened to expose the right lung which was flooded with fixative. The lung was fixed in situ for 5 min. Additional fixative was added to keep the lung immersed. The entire right lung was carefully removed and placed in fresh fixative for 4 h at room temperature. The upper right lobe, from which all observations were made, was cut into small blocks which were processed for electron microscopy.

Tissue Processing

Tissue blocks were postfixed overnight (16 h) in 1½% OsO_4 in Veronal acetate buffer, pH 7.4, at 4°C, and then rinsed in three 10-min changes of 50 mM Na maleate buffer. Blocks were then stained for 90 min in 1½% uranyl acetate in the same buffer at pH 5.2 (28). Rinses and staining were both carried out at 4°C. Tissue was then rapidly dehydrated in cold, graded acetone solutions, infiltrated with propylene oxide-Epon mixtures, and embedded in Epon 812 (37). 0.5-μm thick sections were stained with 0.5% alkaline toluidine blue for light microscopy. Thin silver sections were mounted on parlodion-filmed, carbon-coated copper grids, stained with 5% aqueous uranyl acetate for 30 min at 37°C, followed by alkaline lead citrate solutions for 10 min.

Histochemistry

For demonstration of β-glycerophosphatase in fetal tissues, lungs were fixed ½–4 h as above. 70-μm thick unfrozen sections were cut on a Sorvall TC-2 Tissue Sectioner (Dupont Instruments, Sorvall Operations, Newtown, Conn.), washed overnight in cold 0.1 M sodium cacodylate with 5% sucrose, pH 7.4, followed by a 3-h incubation at 37°C in a modified Gomori medium with 10 mM β-glycerophosphate (disodium salt, grade 1, Sigma Chemical Co., St. Louis, Mo.) as substrate, pH

5.0, with 5% sucrose (4). Control specimens were incubated in medium lacking substrate or in medium containing substrate and 10 mM NaF. After incubation, slices were washed briefly in cacodylate buffer, postfixed in OsO_4 for 30 min, block stained for 30 min, dehydrated, and embedded as above. Thin sections were examined unstained.

Microscopy

Thin sections were examined in a Zeiss EM 10 or Siemens Elmiskop 1 electron microscope. Measurements were made of specimens photographed at an original magnification of 50,000, and subsequently enlarged to 250,000.

HIGH VOLTAGE ELECTRON MICROSCOPY: $1/2$- or 1-μm thick sections were stained for 60 min at 5% uranyl acetate at 60°C followed by lead citrate for 30 min, and examined in the JEM-1,000 high voltage electron microscope (Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colo., through the courtesy of Dr. Keith R. Porter). Specimens were photographed at 30–50,000 magnification at an accelerating voltage of 1,000 kV.

RESULTS

General Observations

As reported by others (Figs. 4, 5; reference 18) and confirmed in this study, when mature intracellular lamellar bodies are sectioned in a frontal plane, they are ovoid or bell-shaped structures of variable size. They are composed of numerous lamellae which are similar in structure to other cytomembranes as well as to the limiting membrane which surrounds the whole lamellar body (12, 51). Also contained within the limiting membrane is a dense, homogeneous nonlamellar material which forms a central core around which the lamellae fold. The core in turn is continuous with a thin layer of similar nonlamellar material located between the lamellae and the limiting membrane. If the plane of section does not pass through the center of the lamellar body containing the core material, only the outer rim of dense material surrounding the lamellae can be observed. Occasionally, in even large, presumably mature lamellar bodies, a few small membrane-bounded vesicles containing a second dense material are present within the core matrix. The second population of small vesicles is reminiscent of the proposed origin of lamellar bodies from multivesicular bodies (Figs. 16–23, references 47, 9). Cytochemical studies have demonstrated that lysosomal enzymes make up at least part of the homogeneous

nonlamellar material within lamellar bodies (3, 21, 34, 42, 47). The dense matrix, much less abundant than the membrane layers, is therefore presumed to be largely protein.

The presence of an enzymatically active protein core containing occasional small vesicles provides a structural marker which permits identification of a secreted lamellar body. On the basis of these structures, lamellar bodies can be readily distinguished from artifactitious myelin figures, should both be present within the alveolar lumen. Positive identification of a lamellar body following its secretion into the alveolar lumen is essential for interpretation of the observations described in this study.

Light Microscope Observations

In late gestation the rat lung is composed of respiratory tubes lined by columnar epithelial cells embedded in the developing stroma. The cells of the terminal portions of the tubes synthesize, store, and secrete numerous surfactant-containing lamellar bodies beginning on day 19 of gestation (3, 41). If the fetal lung is fixed by immersion in situ, the fluid-filled presumptive air spaces have broad distended lumina within which are trapped groups of spherical particles varying in number from few to dozens (Fig. 1; reference 3). In plastic sections, the particles stain dark purple with toluidine blue as do the lamellar bodies within the adjacent epithelial cells. A pale purple amorphous material intermingles with the spherical particles, but stains less intensely. Some aggregates of particles and amorphous material appear to be lodged in crevices in the tubular lumen, while others appear to float in the alveolar fluid in the center of the tube. The numbers of particles trapped in the lumina increase from day 19 to 22 (term) when air breathing commences.

Electron Microscope Observations

As determined by electron microscopy the aggregates of particles are composed of secreted lamellar bodies (the spheres) and associated tubular myelin (the amorphous material) which extends from, connects, and twines within the lamellar bodies (Figs. 1, 2).

Lamellar Bodies

The structure of secreted lamellar bodies is generally similar to that of those within adjacent cells (Figs. 1–4). Under the present conditions of tissue
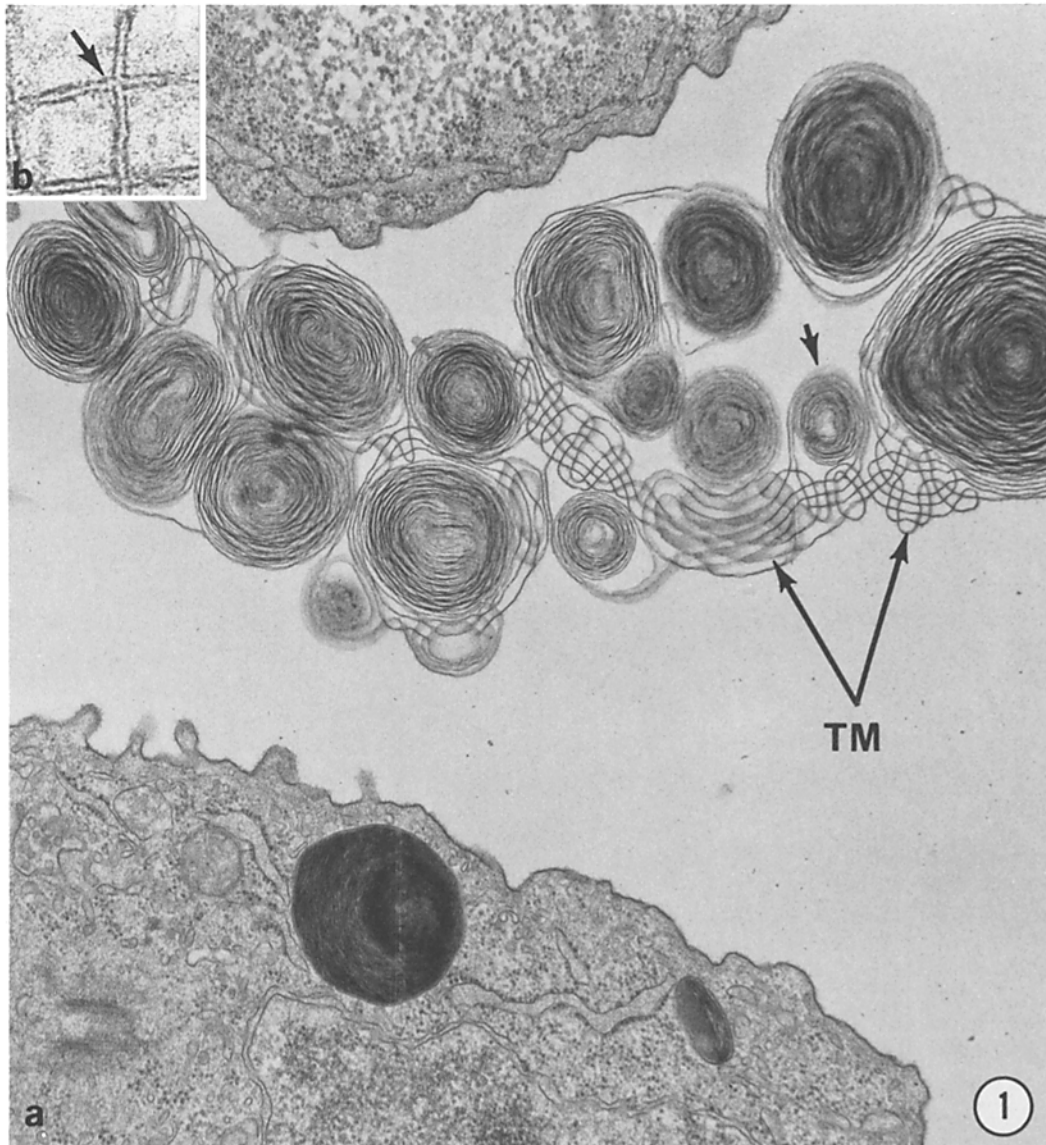
FIGURE 1 Low-power electron micrograph of a portion of a respiratory tubule in a 21-day-old fetal rat lung (Fig. 1a). The presumptive alveolar space contains both secreted lamellar bodies (*LB*) and forming tubular myelin (*TM*). TM is seen in both lattice-like cross section and tube-like longitudinal section (double arrow). One LB (small arrow) is sectioned through the site at which the membrane layers fold back upon themselves to surround the central dense core. The apexes of two alveolar epithelial cells (top and bottom), one cell containing two lamellar bodies, extend into the alveolar lumen. As seen at higher magnification (inset), the electron-transparent portion of TM membranes is continuous in both directions at membrane intersections. (*a*) × 26,000; (*b*) × 244,000.

preparation, the membranes of secreted lamellar bodies are about 8 nm in thickness. The apparent number of lamellae in a single lamellar body, dependent on both lamellar body size and the plane of section, ranges from 3 or 4 to about 40, but accurate counts of layers are difficult because of the tight packing of membranes. Diameters of secreted lamellar bodies have not been directly
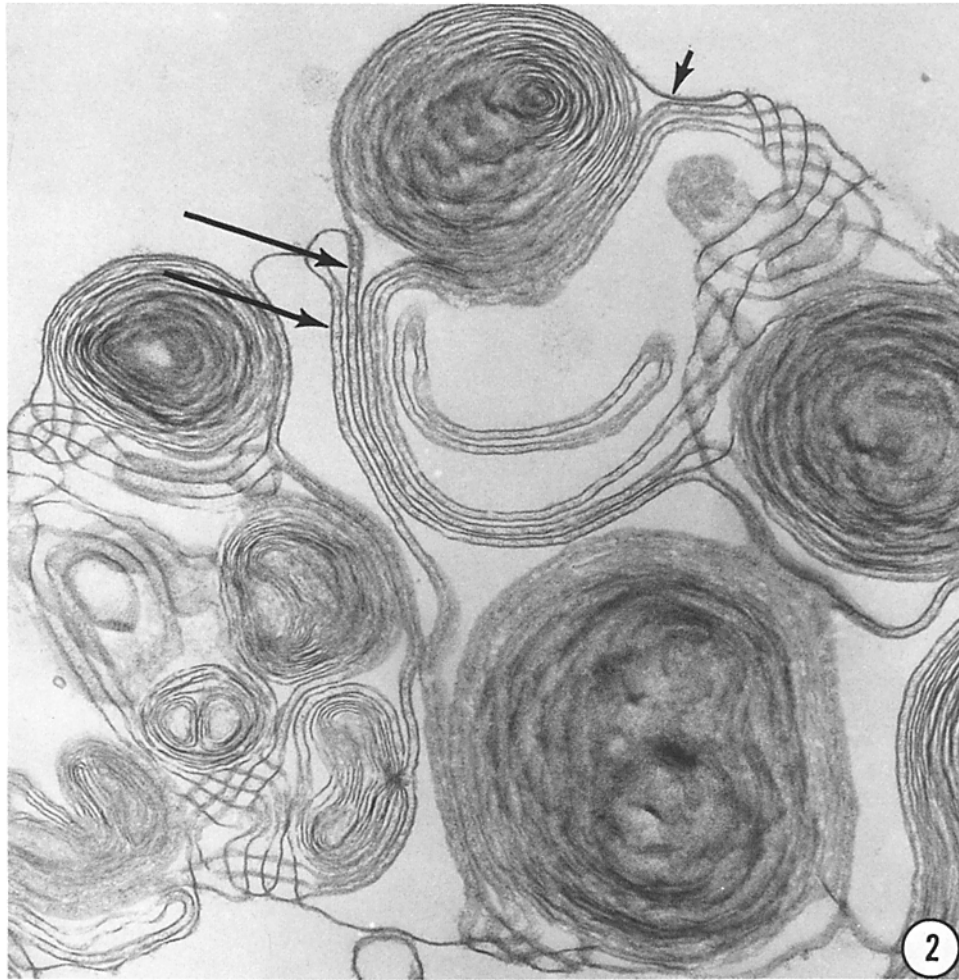
FIGURE 2 Several LBs and associated TM form an aggregate within the alveolar lumen. Pairs of fuzzy-surfaced membranes (double arrows) and occasional single fuzzy membranes (single arrow) extend into the forming TM. × 40,000.

measured. In contrast to the bell-like shape of intracellular lamellar bodies, secreted lamellar bodies tend to be circular in outline, suggesting that they assume a spherical configuration upon release from the limiting membrane (Figs. 1–4). Although the homogeneous core material is present in many secreted lamellar bodies (Figs. 3a, 4a, 5), the rim of protein is absent and presumably has been dissolved in the alveolar fluid.

The three-dimensional arrangement of the membrane layers cannot be determined with certainty, but, in most planes of section, secreted lamellar bodies have the appearance of radially symmetric structures composed of concentrically arranged layers similar to those of an onion. The core material would therefore be enclosed by the innermost spherical layer (Figs. 1, 2, 5). However, other images suggest that it is more likely that lamellar bodies are composed of numerous closed, flattened sacs which fold as a group around a central compartment (Figs. 1, 7).

Two specialized arrangements of lamellae, extending from the outermost layer to the innermost layer, are present in some secreted lamellar bodies. The first of these is a plaque ~255 × 135-nm of closely interdigitated membrane layers which are continuous with the bulk of the lamellar body membranes (Fig. 3a, b). The more regularly and tightly packed membranes at this site appear to be "knit" together, but further structural details cannot be resolved. The lamellar body outline is somewhat indented as a consequence of the tight

packing. The second specialization appears structurally similar to tubular myelin (Fig. 4a, b). Lamellar body layers are arranged into a lattice composed of squares with sides ~15 nm in length when measured between the electron-transparent centers of the lateral membranes. This structure
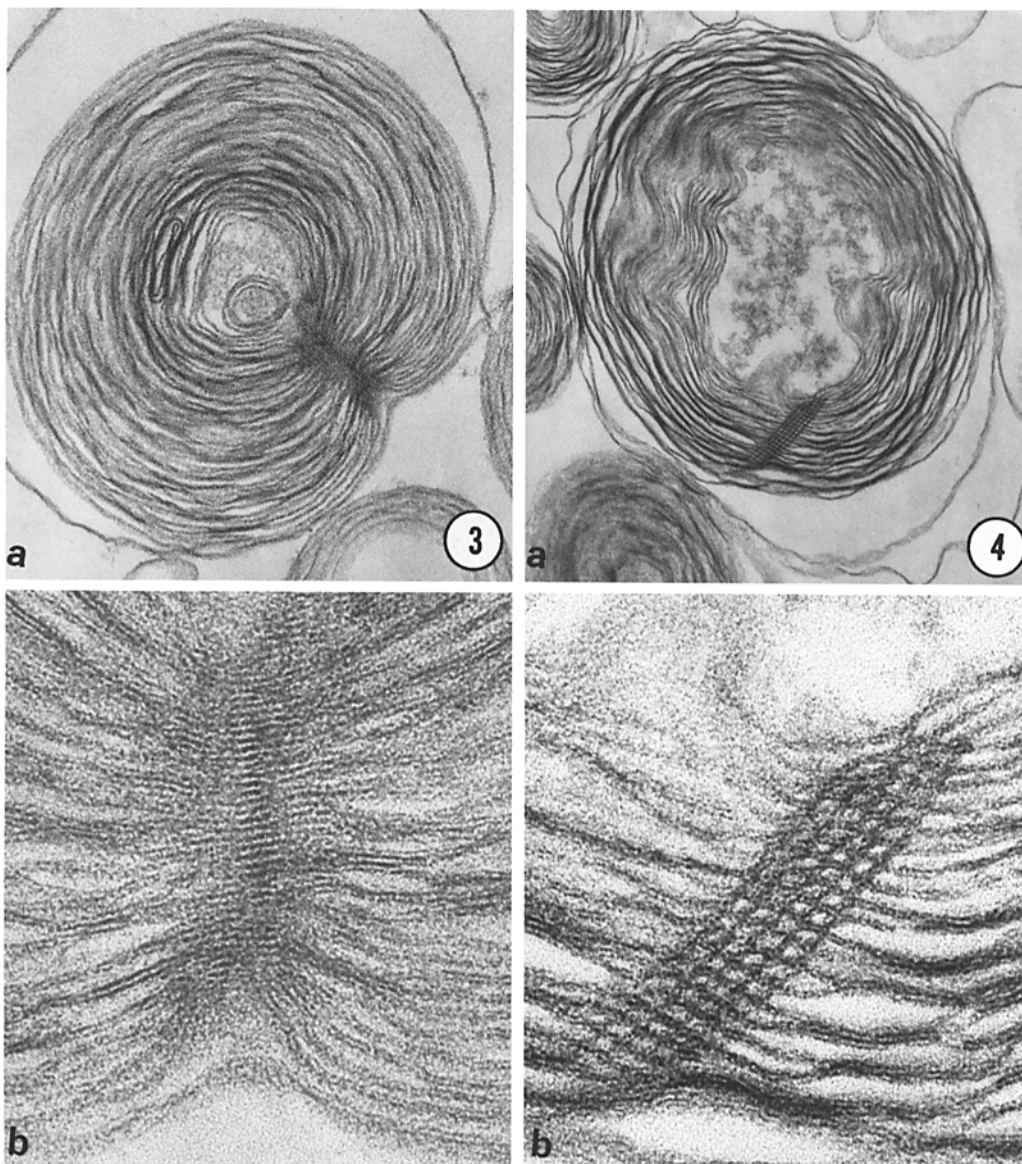
FIGURE 3 In Fig. 3a, a specialized plaque of tightly packed membranes extends across the width of the LB layers. The LB outline is somewhat indented as a consequence of the dense layering. The LB center contains nonlamellar, homogeneous material as well as a small vesicle surrounded by several membrane layers. This small vesicle may be connected to adjacent LB layers in another plane of section, rather than being a vesicle similar to those in Fig. 6. Fig. 3b—enlargement of plaque seen in Fig. 3a. (a) × 57,000; (b) × 208,000.

FIGURE 4 In this secreted LB, a lattice-like specialization is located in a position similar to that of the plaque in Fig. 3. The flocculent material in the LB center is somewht less dense than that of most secreted LBs. As seen in Fig. 4b, each LB layer extends into and is continuous with a single horizontal layer of the lattice. (a) × 38,000; (b) × 192,000.

has approximately one-third the periodicity of the tubular myelin lattice. At this site, there is a one-to-one relationship between the lamellar body layers proper and the membranes forming the lattice. It seems possible that these specializations are actually the same structure sectioned in 90° planes with respect to one another; attempts to answer this question by tilting the section ±45° have so far been uninformative. Neither specialization has been observed in intracellular lamellar bodies.

The flocculent material, which appears to be temporarily sealed within the core of secreted lamellar bodies, is somewhat less electron opaque than that of intracellular lamellar bodies, and occasionally contains a few small vesicles (Figs. 5 b, 6). Additional core-like material is occasionally deposited between adjacent lamellae. When the cytochemical staining for acid phosphatase activity is applied to tissues containing secreted lamellar bodies, the core material reacts strongly (Fig. 5 a) for such enzymatic content but associated tubular myelin is negative. In addition, finely particulate reaction product is often present between membrane layers (Fig. 5 a) in an amount that exceeds that in unreactive sites, such as the nuclei of adjacent cells. These latter sites correspond to the small deposits of core-like material between lamellae mentioned earlier.

Tubular Myelin

The aggregates of material within the alveolar lumen contain varying amounts of lattice-like tubular myelin in addition to lamellar bodies. In cross section, each tubule shares its walls with its four neighboring tubules (Figs. 8, 9). Some tubules, especially those near the periphery of a bundle, have irregular shapes, and may be triangular or dome-shaped in cross section (Fig. 8 a). If the structure of intersecting membranes within the lattice is clearly visualized (Fig. 1 a), it is clear that the membranes are continuous at their perpendicular intersections, rather than being interwoven layers with one membrane passing above or below the other. At the edges of a bundle of tubular myelin, the membranes turn in smooth curves to reenter the lattice; no unsealed cut ends have been observed. The membranes which form the tubular myelin are ~8 nm in thickness. The squares in the most regularly arranged portion of the lattice are 52.4 nm across if measured between the electron-lucent portions of the bounding membranes. In most of the more regularly arranged tubules, and in many tubules at the periphery of a bundle, a 8-nm diameter particle is located near each of the four corners (Figs. 8 b, 9 b). It has not been possible to demonstrate a structure attaching the parti-
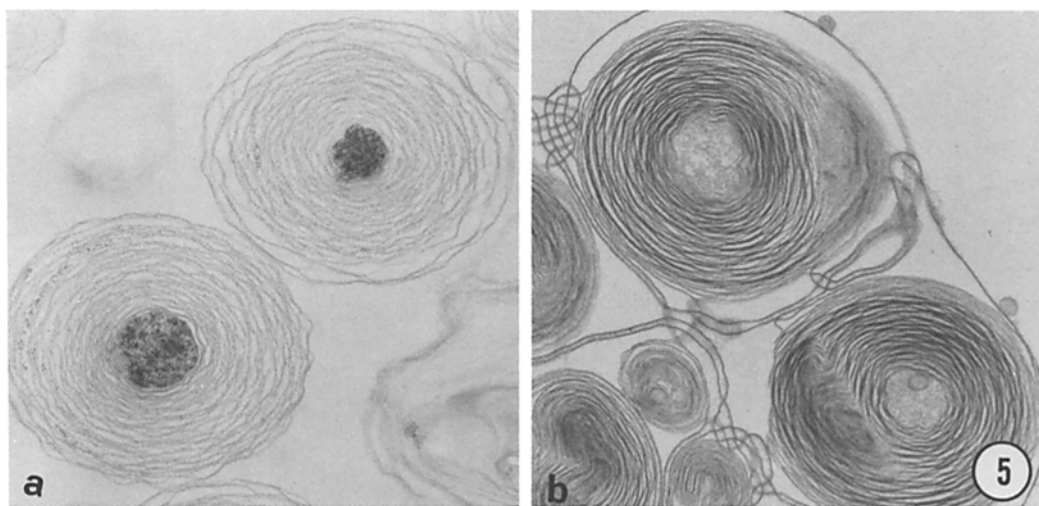

Figure 5 Secreted LBs in lung tissue which has been reacted for β-glycerophosphatase are seen in Fig. 5 a. Dense reaction product fills the LB center and is also seen in lower concentration between some LB layers. The section is unstained. Reaction product is localized to the same part of the LB as the homogeneous core material in tissues prepared for morphologic study (Fig. 5 b). (a) × 28,000; (b) × 27,000.
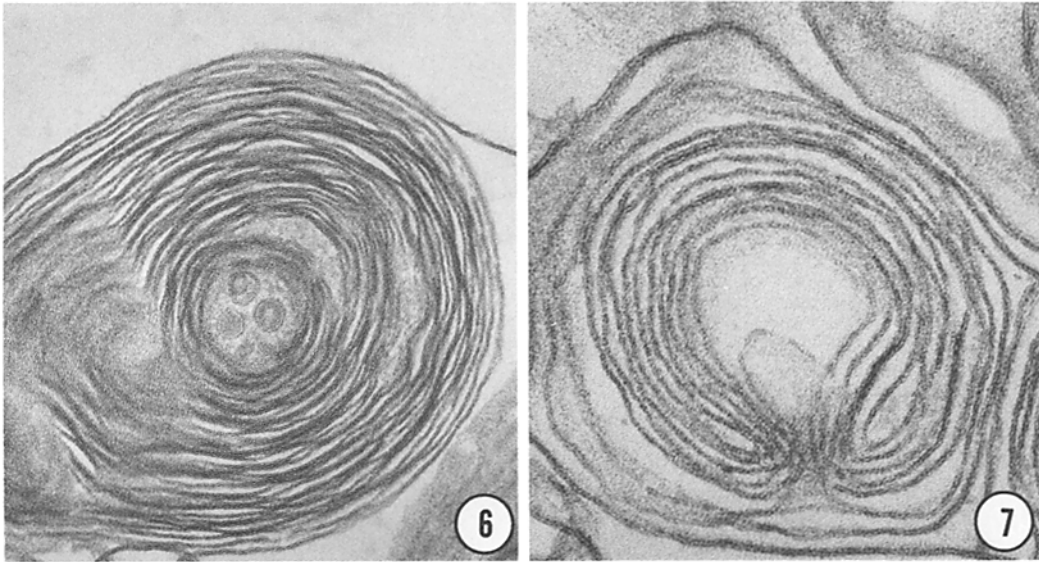
FIGURE 6   The central compartment of this secreted LB contains both homogeneous material and three small vesicles which contain a second dense material which does not react for β-glycerophosphatase. Additional core-like material is present between more peripheral lamellae. × 69,000.

FIGURE 7   One of few images of secreted LBs in which the innermost lamella appears to be in the process of being extruded, leaving behind the more peripheral layers. Alternatively, this image may result from "rupture" of the most peripheral lamellae at the time of fixation. In most instances the remaining LB layers which fold around the now empty central compartment do not appear to be attached to each other. × 120,000.
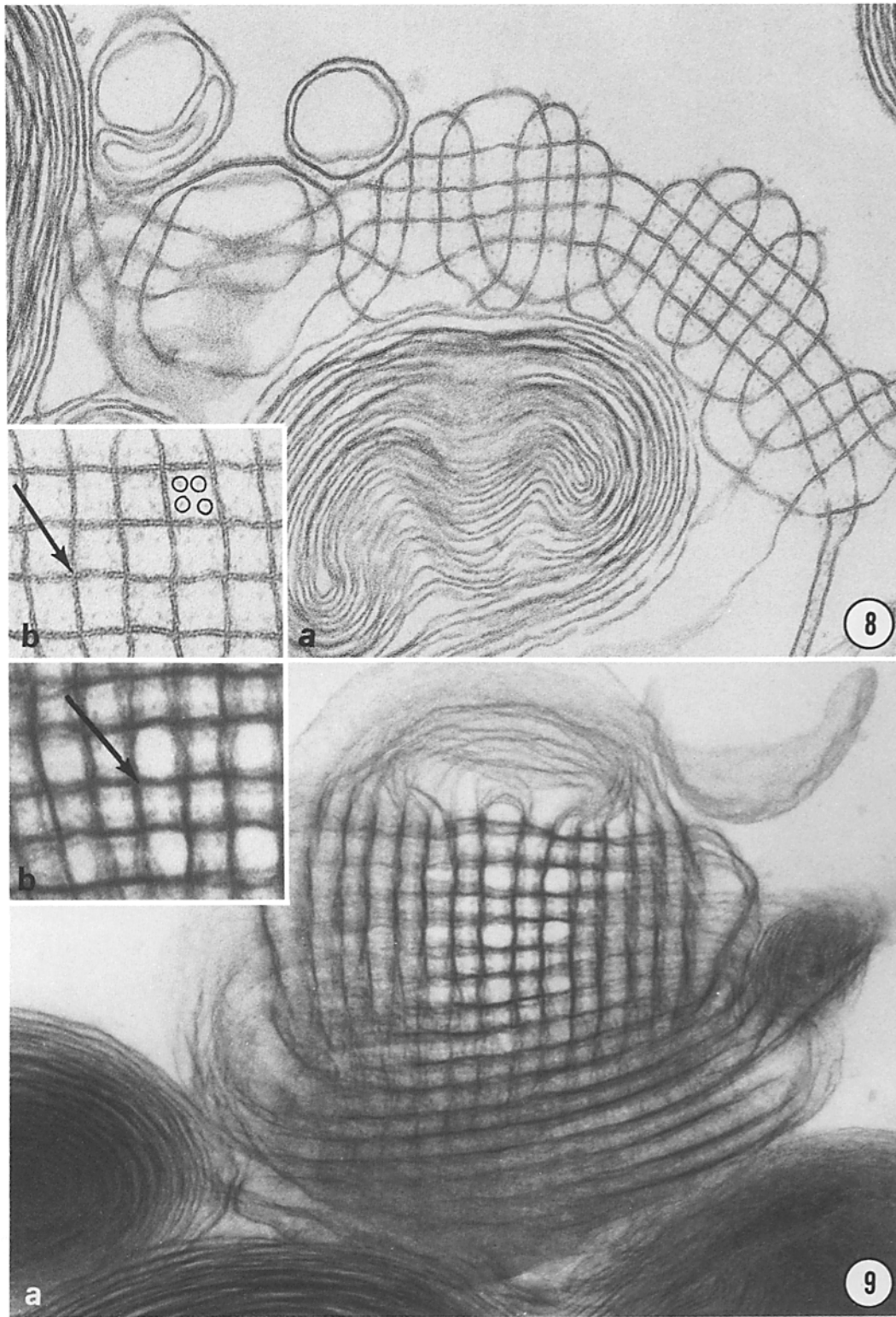
cles to the adjacent membrane, but it seems likely that a slender filamentous connection, if present, might not have sufficient electron-opacity to be resolved with these techniques. The presence of particles on tubular myelin membranes can be readily demonstrated by high voltage electron microscopy where "stacking" of particles within the thickness of the section imparts increased electron opacity (Fig. 9b). Less frequently, particles of similar size and density are present at membrane intersections on the outside of the most peripheral tubules, and consequently extend into the alveolar lumen.[1]

As a bundle of tubules turns within the plane of the section and the images change from cross section (squares) to longitudinal sections of tubes,

the bounding membranes, now seen en face, become gray, blurred images without apparent substructure (Fig. 1). The change in appearance from cross-sectioned to longitudinally sectioned tubular myelin is demonstrated especially well by high voltage electron microscopy where analysis of stereo pairs of micrographs of a single bundle of tubular myelin clearly confirms earlier conclusions of the tubular nature of this material (Fig. 10). As one would predict, the membranes seen en face in longitudinally sectioned tubular myelin are intersected by perpendicularly arranged membranes which have a distinctly trilaminate structure. The spacing between the latter layers is 52.4 nm, comparable to one side of the squares. A row of regularly spaced particles, identical to those seen in cross section, extends along the trilaminate intersecting membrane. Particles are usually less distinct in longitudinally sectioned tubules perhaps due to the presence within the section of a membrane en face in front of or behind the row of particles. The distance between centers of adjacent particles is 15–17 nm in those instances where it has been possible to make measurements.
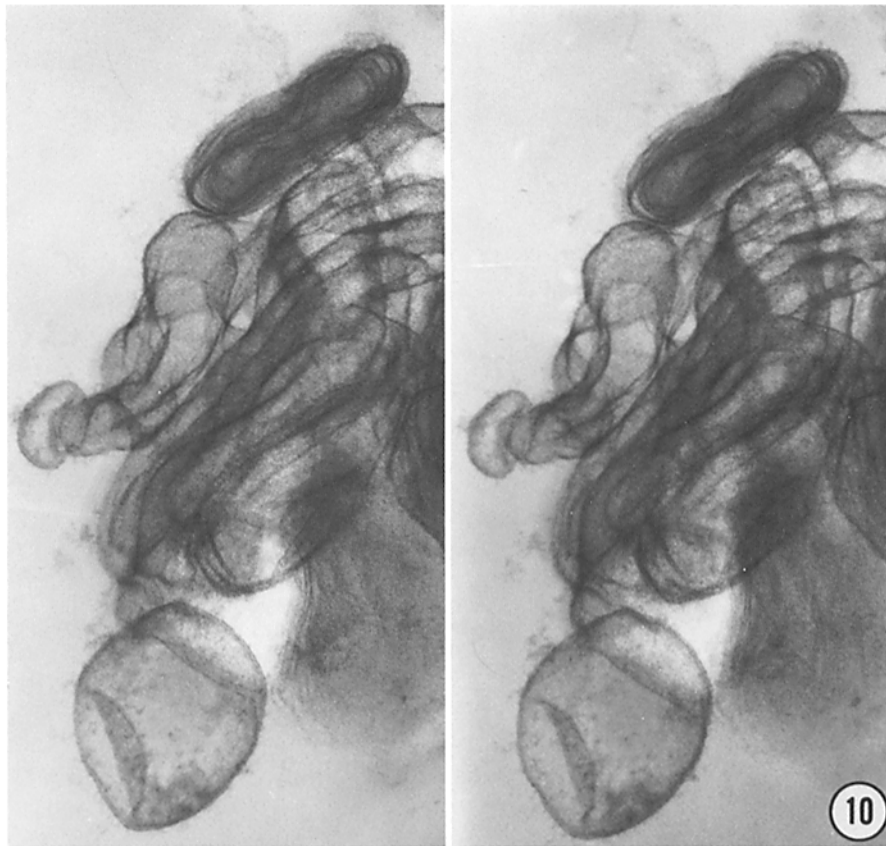
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[1] Particles of similar dimensions are also present associated with tubular myelin membranes in adult rat lungs. In adult tissues demonstration of the particles is less consistent, perhaps because of the presence within the bundles of tubular myelin of amorphous materials which tend to obscure the membranes (unpublished observations).

Figure 10   High voltage electron micrographs of TM and associated structures in a stereo pair (6° tilt) from a 0.5-μm thick section. An intact vesicle, a hemisected vesicle, and a portion of a secreted LB are seen at the lower left, middle left, and upper right, respectively. Several tubules of TM run in a twisted pathway across the field. Labels have been omitted in order to avoid confusion when viewing this plate through a stereo viewer. × 74,000. (This pair of micrographs was photographed by Dr. Douglas Mohr, High Voltage Electron Miroscope Facility, Boulder, Colo.)

Figure 8   In this thin section through an aggregate of LBs and TM, small particles are associated with the more regularly arranged areas of the TM. Prominent fuzzes are present on membrane layers extending into the regular lattice. As seen enlarged in Fig. 8b, small particles (lower right of arrow) of low electron density are present within many corners of the TM lattice, but possible structures connecting them to membranes cannot be resolved. (a) × 81,000; (b) × 138,000.

Figure 9   A large bundle of TM is seen in this high voltage electron micrograph of a 0.75-μm thick plastic section. Small densities, probably comparable to particles seen in thin section in Fig. 8, are present in the corners of much of the lattice. Membrane layers from a small LB at the right of the TM appear to extend into the lattice horizontally, while other layers at the top, perhaps from an LB remnant, extend into the lattice vertically. At higher magnification (Fig. 9b), four small densities protrude from the corners of a cross-sectioned tubule into the center of the tubule. These are best seen in the tubule at the lower right of the arrow. (a) × 72,000; (b) × 121,000.

Formation of Tubular Myelin from
Lamellar Bodies

In all lung tissues examined membranes frequently extend from the outermost layers of a lamellar body and continue without interruption into the lattice structure of tubular myelin, suggestive of conversion of lamellar bodies into tubular myelin. (Figs. 1, 2, 11, 12). Certain structural details related to this conversion are apparent from thin-sectioned material, and confirmed by high voltage electron microscopy of thick sections, but three-dimensional relationships are not yet entirely clear. In general, the observations suggest that membrane used for the assembly of a single bundle of tubular myelin may be provided by more than one, i.e., up to at least three, lamellar bodies (Figs. 11, 12). Conversely, a single lamellar body may contribute membrane for at least two discrete bundles of tubular myelin simultaneously. In some instances one lamellar body appears to provide membrane for tubular myelin layers in one plane while an adjacent lamellar body provides membrane for layers at right angles to the first (Fig. 12). Most frequently, outer layers peel off the lamellar body surface in pairs, but single membrane layers extending into tubular myelin are also present (Fig. 2). Occasional images suggest that lamellar bodies may extrude their innermost layers first, leaving hollow shells of larger lamellae behind them (Fig. 7); however, such an image may be due to breaking or damage of the outermost lamellae during fixation and processing.

Detailed examination of the structure of transition membranes, i.e. those membranes extending between lamellar bodies and tubular myelin, reveals that many such membranes are coated either with closely packed particles (Fig. 13) or with less discrete fuzzes (Figs. 2, 8a), although membranes within the bulk of the unfolded lamellar body proper appear to lack both structures. In one type of modification, arrays of cuboidal particles ~8 nm in diameter coat, and appear to be attached to, the transition membranes on one surface only (Fig. 13a, b). The particles are approximately the same size as those associated with tubular myelin, but are somewhat more electron dense and are packed more closely together. The spacing between particles is ~8.0 nm. Other transition membranes are coated with linear extramembranous densities, or, alternatively, rows of closely packed particles (Fig. 13c, d). It is uncertain whether the linear arrays are in all instances the first type of

membrane particles viewed in a slightly oblique plane of section, but this frequently appears to be the case. Many other transitional membranes are covered on one side by a fuzzy coat ~8.0-12.0 nm in thickness, that extends from the membrane into the alveolar space. The fuzzy coat is most readily seen when the fuzzy surfaces of two membranes are apposed (Figs. 2, 8a). Fuzzes and particles do not both appear to be present on the same transition membrane, and thus the two types of membranes may represent either two different populations of membranes or two phases of a single membrane modification.

DISCUSSION

Although abundant physiologic evidence suggests that mammalian lung surfaces are covered by an extracellular layer of lipid, or lipid-protein complexes (32), which facilitates a decrease in surface tension when the lungs deflate, the morphology of this layer is uncertain (26, 30, 35, 56, 57). Surface layers of mono- or bimolecular dimensions have been demonstrated with such poor reproducibility that it is uncertain that they represent complete layers adsorbed onto all alveolar surfaces. However, several studies have demonstrated the presence of multilayered tubular myelin flattened upon the alveolar surface (8, 16, 17, 26, 53). Although the thickness of the multiple layers of tubular myelin is several orders of magnitude greater than that of the hypothesized monolayer, some observations suggest that tubular myelin, or its outermost layer, may function as the surface-active layer (8) or, alternatively, may be an intermediate form between lamellar bodies and a monolayer (18, 19). Recently, Gil and Reiss (18) demonstrated that tubular myelin, identified unequivocally by its morphology, is capable of forming surface films which can greatly decrease surface tension at an air-water interface in a surface balance. Such behavior is consistent with its high content of disaturated lecithin (86% of total lecithin) as determined chemically in the same study. Tubular myelin, therefore, has both suitable chemical composition and surface-active properties to be considered part of the surfactant system.

The conversion of lamellar bodies to tubular myelin has been frequently suggested (18, 20, 43, 47) but continuities between the two have not been demonstrated with certainty before these observations, principally because of the difficulty in identifying lamellar bodies once they are re-
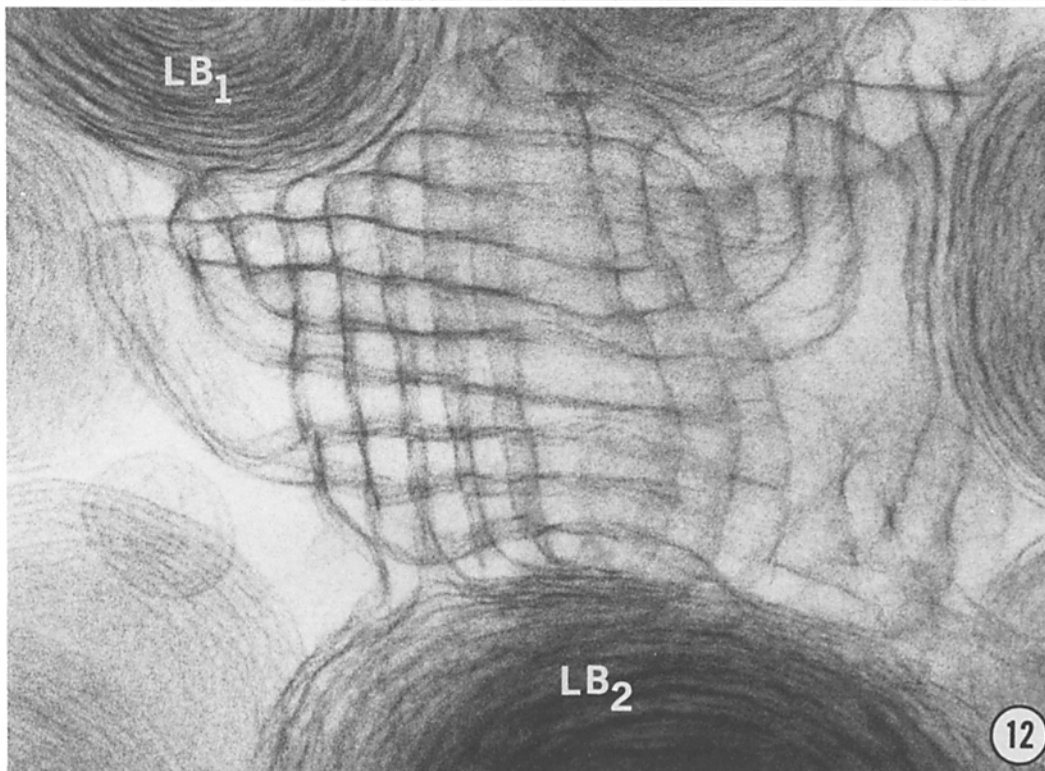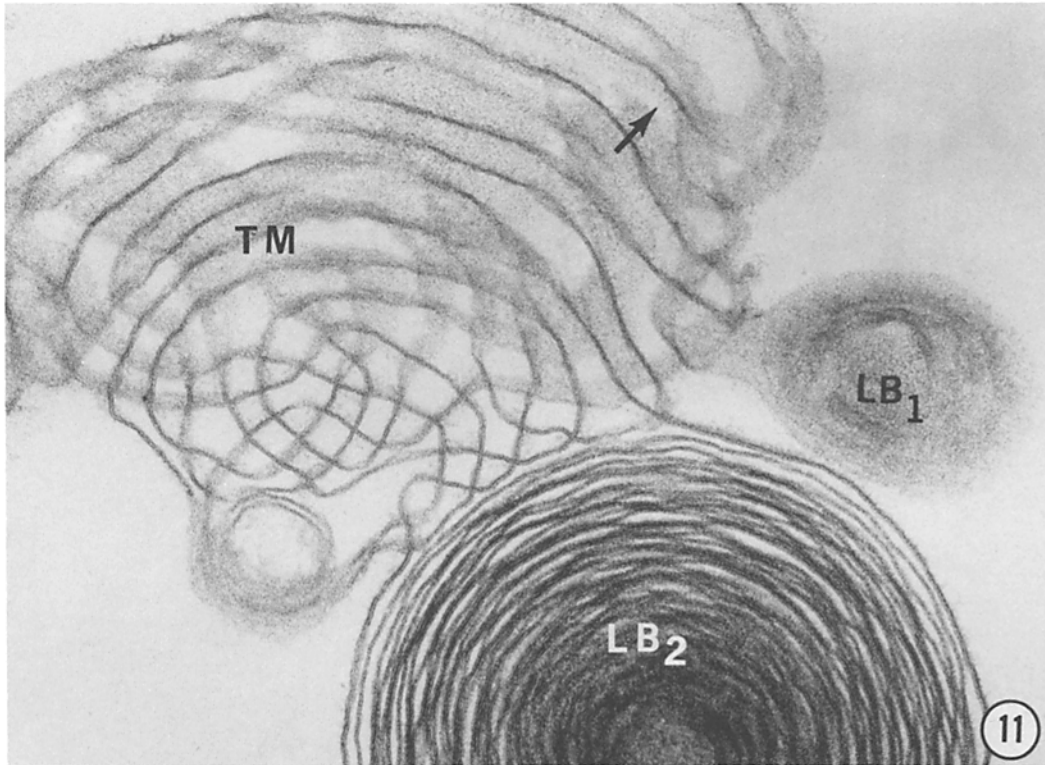
FIGURE 11   The outer membrane layer of LB_2 is continuous with adjacent TM, while other layers, apparently originating from LB_1 also extend into the lattice. The section passes through both cross-sectioned TM (lower) and longitudinally sectioned TM (upper). × 86,000.

FIGURE 12   High voltage electron micrograph of several LBs and TM in a 0.75-μm thick plastic section. Membrane layers from LB_2 extend into the lattice vertically while layers from LB_1 appear to be continuous with both vertical and horizontal TM layers. × 120,000.
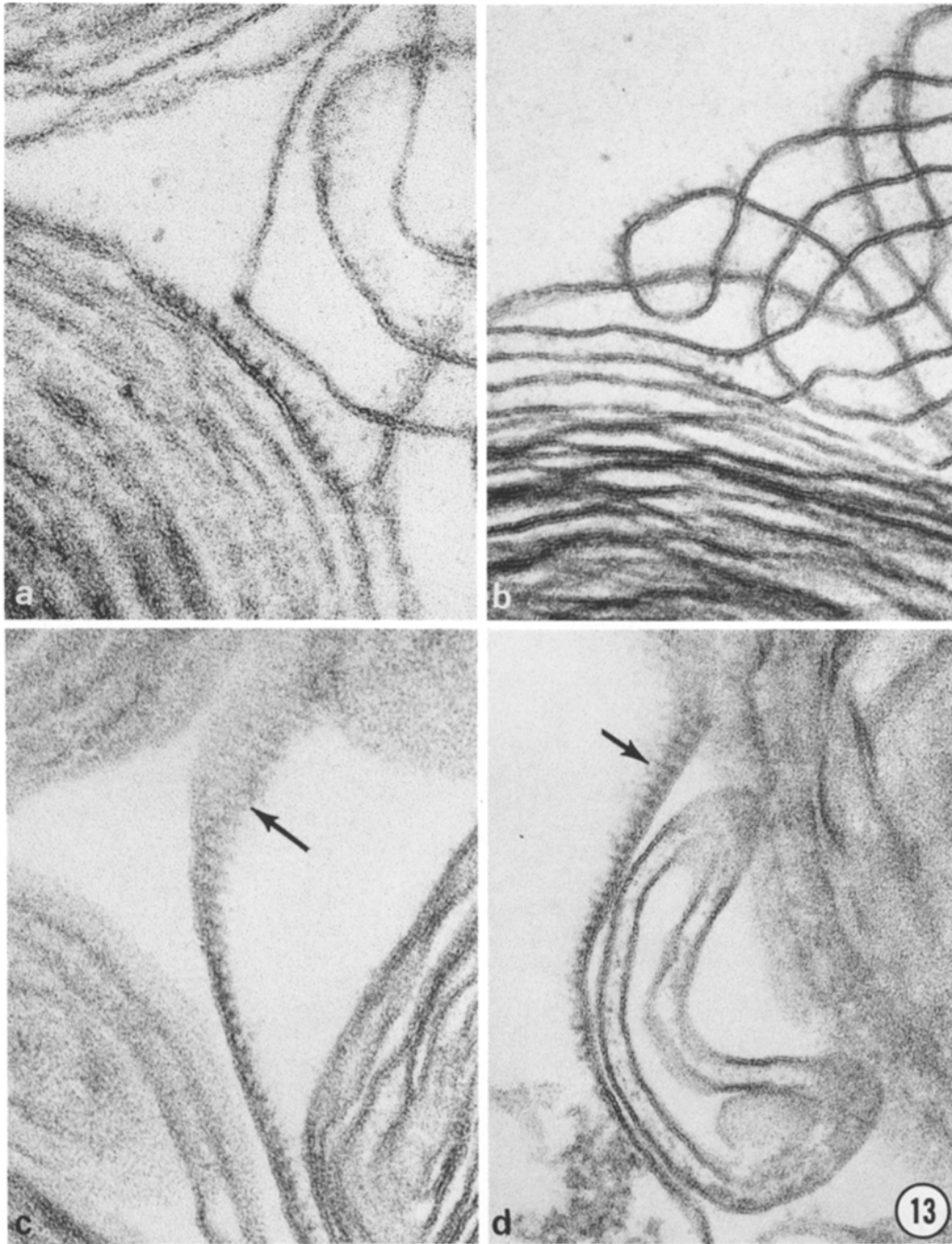
FIGURE 13 Four examples of particulate membranes which extend from secreted LBs into TM, although continuity from LB to TM is not shown in all instances. In (a) and (b), only punctate particles are present, while in (c) and (d) the coated membranes rotate sufficiently within the section to reveal that the particles appear to be arranged in rows when viewed en face (arrows). (a) × 205,000; (b) × 142,000; (c) × 168,000; (d) × 130,000.

leased into alveoli. Because of the propensity of phospholipids to form artifactitious myelin figures during tissue preparation, lamellar bodies must be identified by structural characteristics other than layers of membrane. In this study, I have identified secreted lamellar bodies by demonstrating within lamellae the presence of nonlamellar, homogeneous, acid-phosphatase-reactive material which is similar to that packaged within intracellular lamellar bodies. The presence of this material supports the notion that intra-alveolar lamellar bodies are derived from intracellular lamellar bodies. On the basis of the continuity of membrane layers between secreted lamellar bodies and tubular myelin, I conclude that lamellar bodies are, at least in part, the precursor of tubular myelin.

There are at least two possible mechanisms by which lamellar bodies could be transformed into tubular myelin. First, tubular myelin may be entirely preassembled by the cell as a lattice structure during the process of synthesis. Once the lamellar body is secreted and freed from its limiting membrane, it may expand into tubular myelin without further rearrangement of its molecular components. This phenomenon could be similar to the expansion and compression of a concertina in which, when closed and tightly packed, the components are collapsed on themselves and appear layered, but when expanded are revealed to be arranged into a more complex lattice. Second, it is possible that the components within the lamellar body undergo considerable reorganization, whether catalyzed by enzymatic or physical factors, after secretion into the alveolar lumen. In this case, the lamellae could serve as a pool of partially organized phospholipid. Although the first mechanism is less consistent than the second with the observations reported in this study, there is some morphologic evidence which supports each possibility. Therefore, both will be considered further.

Arguments for Intracellular Preassembly

The best evidence for preassembly of a lattice structure is provided by images of freeze-fractured lamellar bodies within intact type II cells of adult rat lung as reported by Smith et al. (45, 46). In those studies parallel ribs or ridges spaced about 45 nm from one another are present on fracture faces of lamellae, while 10-nm diameter particles are randomly scattered between neighboring

ridges. The particles are similar to those exposed by the fracture of many biological membranes but at present the extremely straight ribs have rarely been reported in other biological membranes. The ribs are unlike the irregular, meandering ridges or linear arrays of particles in freeze-fractured epithelial tight junctions (14), but are somewhat similar to tight junctions reported in choroidal endothelium (49). The spacing between ridges (45 nm) is similar to that between intersecting membrane layers in the tubular myelin lattice (52 nm), and is probably within experimental error when comparing two different techniques.

The structural components or molecular arrangements which are the counterparts of ribs seen by freeze-fracture are not known. One possibility is that ribs represent a cross-fracture of the membrane arranged in a plane perpendicular to the membrane layer cleaved by the fracture; this arrangement would be consistent with the presence of a lattice structure. This possibility is supported by the observation that the 8 nm width of the ridge is identical to the thickness of tubular myelin membranes in thin sections. It is also possible that the ribs, rather than being broken intersecting membranes, represent an unusual arrangement of lipid, lipoprotein complexes, or proteins packed into a linear array. Ordered arrangements of particles have been observed in other fractured membranes such as linear or band-like arrays in developing sperm (15), rosettes in *Tetrahymena* (44), linear arrays in motor end plates (24), etc., but in none of these instances are the particles packed so tightly as to produce continuous ridges. A third possibility is that the ribs may correspond to the "anchoring" portions of the tubular myelin particles, if they do indeed extend into the membrane to any extent. The observations, whether from freeze-fracture replicas or thin sections, do not favor any one of these possibilities over the others.

Arguments for Intra-Alveolar Reorganization

As an alternative mechanism of formation supported by this study, tubular myelin may be assembled by an active rearrangement of phospholipid and protein from a pool of building materials supplied by the lamellar bodies. Five observations favor this possibility, albeit indirectly. These are: (a) the dimensions of the lattice structure demon-

strated in secreted lamellar bodies are smaller (one-third) than those in tubular myelin; (b) no lattice-type structure has been reported in thin sections of intracellular lamellar bodies; (c) no particles have been observed on lamellar body membranes when seen in thin sections, although particles appear to be intimately associated with transition membranes and with tubular myelin; (d) some secreted lamellar bodies appear to be composed of closed sacs of membrane, with adjacent sacs unattached to each other and therefore could not form a lattice; and (e) multiple lamellar bodies appear to participate in the formation of a single bundle of tubular myelin.

The factors which might stimulate rearrangements are varied. For example, molecular rearrangements of considerable magnitude could be initiated by physical or chemical properties of the fetal alveolar fluid such as pH, ions, etc., after secretion of a lamellar body into the alveolar space. This possibility is suggested indirectly by Gil and Reiss (18) who reported that the addition of EDTA to homogenization media prevented the appearance of tubular myelin in lung homogenates. Therefore, Ca^{++} or other divalent cations may be one, but perhaps not the only, requirement for the transformation of lamellar bodies into tubular myelin[2]. Both Ca^{++} (2.5 mg/dl) and Mg^{++} (0.4 mg/dl) are present in lung liquid of fetal lambs (1), and thus are available to participate in tubular myelin formation in that species.

The presence of cytochemically demonstrable enzymes within secreted lamellar bodies (42) suggests the possibility that lamellar body conversion may be mediated by an enzyme which is secreted from the cell as part of a package containing both enzyme and substrate. Lamellar bodies have been reported to contain many enzymes including typical lysosomal enzymes (11, 25), as well as phospholipid-synthesizing enzymes such as phosphatidic acid phosphohydrolase (39, 48), acyl transferases (13) and others (54) which have a wide variety of substrate requirements. A number of arguments can be raised against the importance of enzyme function in tubular myelin formation. First, it seems unlikely that the wide variety of enzymes reported to be present would be required for tubular myelin formation. Second, because

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[2] In my study, tubular myelin was present in all fetal lungs including those processed in fixatives containing either EDTA or Ca^{++}, and in tissues fixed in osmium tetroxide at 4°C.

enzyme and potential substrate (lamellae) are packaged together in the cell, enzymatic activity would have to be blocked intracellularly, followed by activation upon exocytosis. Third, the difference in physical properties between fluid-filled fetal lung and air-filled adult lung makes it doubtful that both circumstances would be equally suited to enzymatic activity. Whether the enzymes in secreted lamellar bodies play a further role in spreading of surface-active material, have a function entirely unrelated to surface tension adjustments, or reflect secretion of now inactive synthetic enzymes is unknown. Studies of the formation of tubular myelin in controlled in vitro conditions may answer these questions.

The presence of particles associated with both transition membranes and tubular myelin, and their apparent absence on lamellar body membranes, strongly suggests that particles either are added to membranes during tubular myelin formation or are formed from precursors, not visible in thin sections, associated with lamellar body membranes. The addition or "unfolding" of particles may subsequently stimulate a dramatic rearrangement of lipid components in a manner comparable to other membrane phenomena. In two such cases, reorganization of presumably nonlipid membrane particles in Tetrahymena membranes or the addition of specific proteins to phospholipid layers has been suggested to result in membrane fusion (44) or membrane splitting (23), respectively. In erythrocyte membranes containing spin-labeled fatty acids, Kury and McConnell (36) have recently suggested that membrane lipid fluidity is altered by the biochemical state of membrane-associated proteins, mediated by the presence of cyclic nucleotides. Whether phenomena similar to one of these occur in tubular myelin formation is unknown. The significance of transition membrane particles cannot be readily evaluated in studies of intact tissue, but remains to be determined by examining preparations of isolated lamellar bodies from which tubular myelin can be generated under controlled conditions.

Finally, the strongest argument against a simple unfolding mechanism of tubular myelin formation is the presence within secreted lamellar bodies of a "miniature" tubular myelin lattice which has a unit size only one-third that of mature tubular myelin. It is tempting to suggest that the "mini-lattice" serves as a template for rearrangement of the remainder of the relatively unorganized phospholipid, but the static nature of my observations does

not provide such evidence. Because the "mini-lattice" is observed infrequently, it is probably not present in all intraalveolar lamellar bodies. It has not been observed in intracellular lamellar bodies. These two factors suggest that "mini-lattice" formation may occur after secretion or, alternatively, may be stimulated by procedures used in preparing tissues for plastic embedding. I am presently examining similar tissues by freeze-fracture techniques to verify the observations reported here.

In summary, although the observations demonstrate that lamellar bodies can be converted into tubular myelin in the lung, that particles appear on transition and tubular myelin membranes, and that a cytochemically demonstrable phosphatase is secreted within lamellar bodies, the interaction of these materials and the mechanism of tubular myelin formation remain uncertain.

REFERENCES

1. ADAMSON, T. M., V. BRODECKY, T. F. LAMBERT, J. E. MALONEY, B. C. RITCHIE, and A. WALKER. 1973. In Foetal and Neonatal Physiology. Cambridge University Press, London. 208-212.
2. ASKIN, F. B., and C. KUHN. 1971. The cellular origin of pulmonary surfactant. Lab. Invest. 25:260-268.
3. BALIS, J. U., P. E. CONEN. 1964. The role of alveolar inclusion bodies in the developing lung. Lab. Invest. 13:1215-1229.
4. BARKA, T., and P. J. ANDERSON. 1962. Histochemical methods for acid phosphatase using hexazonium pararosanilin as a coupler. J. Histochem. Cytochem. 10:741-753.
5. BROWN, E. S. 1964. Isolation and assay of dipalmityl lecithin in lung extracts. Am. J. Physiol. 207:402-406.

6. BUCKINGHAM, S., and M. E. AVERY. 1962. Time of appearance of lung surfactant in the foetal mouse. Nature (Lond.). 193:688-689.
7. BUCKINGHAM, S., H. O. HEINEMANN, S. C. SOMMERS, and W. F. McNARY. 1966. Phospholipid synthesis in the large pulmonary alveolar cell: its relation to lung surfactants. Am. J. Pathol. 48:1027-1041.
8. CALLAS, G. 1974. A new fixative technique for the electron microscopic study of pulmonary surfactant. Anat. Rec. 180:457-463.
9. CHEVALIER, G., and A. J. COLLET. 1972. In vivo incorporation of choline-^{3}H, leucine-^{3}H and galactose-^{3}H in alveolar Type II pneumonocytes in relation to surfactant synthesis. A quantitative radioautographic study in mouse by electron microscopy. Anat. Rec. 174:289-310.
10. CLEMENTS, J. A. 1962. Surface phenomena in relation to pulmonary function. Physiologist. 5:11-28.
11. DIAUGUSTINE, R. P. 1974. Lung concentric laminar organelle. Hydrolase activity and compositional analysis. J. Biol. Chem. 249:548-593.
12. DOUGLAS, W. H. J., R. A. REDDING, and M. STEIN. 1975. The lamellar substructure of osmiophilic inclusion bodies present in rat Type II alveolar pneumonocytes. Tissue Cell. 17:137-142.
13. ENGLE, M. J., and W. J. LONGMORE. 1975. Phospholipid composition and acyltransferase activity of lamellar bodies isolated from rat lung. Fed. Proc. 34:633a.
14. FRIEND, D. S., and N. B. GILULA. 1972. Variations in tight and gap junctions in mammalian tissues. J. Cell Biol. 53:758-776.
15. FRIEND, D. S., and I. RUDOLF. 1974. Acrosomal disruption in sperm. Freeze-fracture of altered membranes. J. Cell Biol. 63:466-479.
16. GIL, J. 1971. Ultrastructure of lung fixed under physiologically defined conditions. Arch. Intern. Med. 127:896-902.
17. GIL, J., and E. R. WEIBEL. 1972. Morphological study of pressure-volume hysteresis in rat lungs fixed by vascular perfusion. Respir. Physiol. 15:190-213.
18. GIL, J., and O. K. REISS. 1973. Isolation and characterization of lamellar bodies and tubular myelin from rat lung homogenates. J. Cell Biol. 58:152-171.
19. GOERKE, J. 1974. Lung surfactant. Biochim. Biophys. Acta. 344:241-261.
20. GOLDENBERG, V. E., S. BUCKINGHAM, S. C. SOMMERS. 1969. Pilocarpine stimulation of granular pneumonocyte secretion. Lab. Invest. 20:147-158.
21. GOLDFISCHER, S., Y. KIKKAWA, and L. HOFFMAN. 1968. The demonstration of acid hydrolase activities in the inclusion bodies of type II alveolar cells and other lysosomes in the rabbit lung. J. Histochem. Cytochem. 16:102-109.
22. HARRISON, G. A., and J. WEIBEL. 1968. The mem-

branous component of alveolar exudate. *J. Ultra-struct. Res.* **24:**334–342.

23. HAUSER, H., R. HENRY, R. B. LESLIE, and J. M. STUBBS. 1974. The interaction of apoprotein from porcine high-density lipoprotein with dimyristoyl phosphatidylcholine. *Eur. J. Biochem.* **48:**583–594.

24. HEUSER, J. E., T. S. REESE, and D. M. D. LANDIS. 1974. Functional changes in frog neuromuscular junctions studied with freeze-fracture. *J. Neurocytol.* **3:**109–131.

25. HOFFMAN, L. 1972. Isolation of inclusion bodies from rabbit lung parenchyma. *J. Cell. Physiol.* **79:**65–72.

26. KAIBARA, M., and Y. KIKKAWA. 1971. 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.

27. KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* **27**(2, Pt. 2):137 a–138 a. (Abstr.)

28. KARNOVSKY, M. J. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* **35:**213–236.

29. KIKKAWA, Y., E. K. MOTOYAMA, and C. D. COOK. 1965. The ultrastructure of the lungs of lambs. *Am. J. Pathol.* **47:**877–903.

30. KIKKAWA, Y. 1970. Morphology of the alveolar lining layer. *Anat. Rec.* **167:**389–400.

31. KIKKAWA, Y., K. YONEDA, F. SMITH, B. PACKARD, and K. SUZUKI. 1975. The type II epithelial cells of the lung. II. Chemical composition and phospholipid synthesis. *Lab. Invest.* **32:**295–302.

32. KING, R. J., and J. A. CLEMENTS. 1972. Surface active materials from dog lung. II. Composition and physiological correlations. *Am. J. Physiol.* **223:**715–726.

33. KLAUS, M. H., J. A. CLEMENTS, and R. J. HAVEL. 1961. Composition of surface-active material isolated from beef lung. *Proc. Natl. Acad. Sci. U. S. A.* **47:**1858–1859.

34. KUHN, C. 1968. Cytochemistry of pulmonary alveolar epithelial cells. *Am. J. Pathol.* **53:**809–833.

35. KUHN, C. 1972. A comparison of freeze-substitution with other methods for preservation of the pulmonary alveolar lining layer. *Am. J. Anat.* **133:**495–508.

36. KURY, P. G., and H. M. McCONNELL. 1975. Regulation of membrane flexibility in human erythrocytes. *Biochemistry* **14:**2798–2803.

37. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9:**409–414.

38. LUZZATI, V., and F. HUSSON. 1962. The structure of the liquid-crystalline phases of lipid-water systems. *J. Cell Biol.* **12:**207–219.

39. MEBAN, C. 1972. Localization of phosphatidic acid phosphatase activity in granular pneumonocytes. *J.*

Cell Biol. **53:**249–252.

40. MORGAN, T. E. 1971. Pulmonary surfactant. *New Engl. J. Med.* **284:**1185–1193.

41. O'HARE, K. H., and M. N. SHERIDAN. 1970. Electron microscopic observations on the morphogenesis of the albino rat lung, with special reference to pulmonary epithelial cells. *Am. J. Anat.* **127:**181–206.

42. O'HARE, K. H., O. K. REISS, and A. E. VATTER. 1971. Esterases in developing and adult rat lung. I. Biochemical and morphological observations. *J. Histochem. Cytochem.* **19:**97–115.

43. RYAN, U. S., J. W. RYAN, and D. S. SMITH. 1975. Alveolar type II cells: studies on mode of release of lamellar bodies. *Tissue Cell.* **7:**587–599.

44. SATIR, B., C. SCHOOLEY, and P. SATIR. 1973. Membrane fusion in a model system. Mucocyst Secretion in Tetrahymena. *J. Cell Biol.* **56:**153–176.

45. SMITH, D. S., U. SMITH, and J. W. RYAN. 1972. Freeze-fractured lamellar body membranes of the rat lung great alveolar cell. *Tissue Cell.* **4:**457–468.

46. SMITH, U., D. S. SMITH, and J. W. RYAN. 1973. Tubular myelin assembly in Type II alveolar cells: freeze-fracture studies. *Anat. Rec.* **176:**125–127.

47. SOROKIN, S. P. 1967. A morphologic and cytochemical study on the great alveolar cell. *J. Histochem. Cytochem.* **14:**884–897.

48. SPITZER, H. L., J. M. RICE, P. C. MACDONALD, and J. M. JOHNSTON. 1975. Phospholipid biosynthesis in lung lamellar bodies. *Biochem. Biophys. Res. Commun.* **66:**17–23.

49. SPITZNAS, M., and E. REALE. 1975. Fracture faces of fenestrations and junctions of endothelial cells in human choroidal vessels. *Invest. Ophthamol.* **14:**98–107.

50. STOECKENIUS, W. 1962. Some electron microscopical observations on liquid-crystalline phases in lipid-water systems. *J. Cell Biol.* **12:**221–229.

51. STRATTON, C. J. 1975. Multilamellar body formation in mammalian lung: An ultrastructural study utilizing three lipid-retention procedures. *J. Ultrastruct. Res.* **52:**309–320.

52. SUN, C. N. 1966. Lattice structures and osmiophilic bodies in the developing respiratory tissue of rats. *J. Ultrastruct. Res.* **15:**380–388.

53. UNTERSEE, P., J. GIL, and E. R. WEIBEL. 1971. Visualization of extracellular lining layer of lung alveoli by freeze-etching. *Respir. Physiol.* **13:**171–185.

54. VATTER, A. E., O. K. REISS, J. K. NEWMAN, K. LINDQUIST, and E. GROENEBOER. 1968. Enzymes of the lung. I. Detection of esterase with a new cytochemical method. *J. Cell Biol.* **38:**80–98.

55. WEIBEL, E. R., G. S. KISTLER, and G. TONDURY. 1966. A sterologic electron microscopic study of "tubular myelin figures" in alveolar fluids of rat lungs. *Z. Zellforsch. Mikrosk. Anat.* **69:**418–427.

56. WEIBEL, E. R., and J. GIL. 1968. Electron micro-

scopic demonstration of an extracellular duplex lining layer of alveoli. *Respir. Physiol.* **4**:42–57.

57. WEIBEL, E. R. 1973. Morphological basis of alveolar capillary gas exchange. *Physiol. Rev.* **53**:419–495.

58. WILLIAMS, M. C. 1975. Conversion of lamellar body membranes into tubular myelin in the alveolar spaces of the late fetal rat lung: TEM and HVEM Observations. *J. Cell Biol.* **67**(2, Pt. 2):456 a. (Abstr.)