RAPID COMMUNICATIONS

A MODE OF FORMATION OF TUBULAR MYELIN
FROM LAMELLAR BODIES IN THE LUNG

R. J. SANDERSON and A. E. VATTER. From the Webb-Waring Lung Institute and Department of Microbiology, University of Colorado Medical Center, Denver, Colorado 80262

ABSTRACT

A mechanism is suggested by which the membranes of lamellar bodies are converted to tubular myelin (TM) in the lung. It is argued that a simple corrugation of the membranous sheets can produce the TM formation. Such corrugation would occur in response to simple stresses acting on the lamellar body membranes. The intersections of the tubular figures are formed by fusion of adjacent corners in the corrugations. This results in a more stable hydrophobic bonding of phospholipid molecules. Strong supportive evidence for the mechanism is given by electron micrographs of TM formations.

KEY WORDS
lamellar bodies · lung surfactant · membrane fusion · tubular myelin

The observation of myelin lattices in the alveolar spaces of rat lung was first reported by Campiche (2) in 1960. Weibel et al. (19) established the tubular nature of the structure of the myelin figure through a stereologic electron microscope study, as a consequence of which it was given the name tubular myelin (TM). The membranous nature of both TM and the lamellar bodies (LB) of type II alveolar cells led to the postulation that TM was formed from the contents of extruded LBs. Extrusion images have been observed both in our laboratory and elsewhere (15). While LBs are undoubtedly the intracellular source of pulmonary surfactant (1), it has been suggested that TM represents either an extracellular reservoir (5) or a breakdown product of that material (9). The studies of Gil and Reiss (5) showed LBs and TM to be surfactant forms by virtue of their composition. The morphology of LBs has been described by a number of workers, including most recently Stratton (17) and Williams (20). Williams also noted the continuity between secreted LB material and TM membranes. She presented arguments for both intracellular preassembly and intra-alveolar reorganization of the membranous material of the LBs into TM. Many enzymes have been identified by cytochemical means within LBs (14, 3, 16, 18). These observations have given rise to the speculation that the reorganization might be enzymatically mediated. Arguments against this have been summarized by Williams (20).

Whatever the mechanism causing the LB-TM transformation, no description of its geometric detail has been made up to this time. Assuming that there is no dissolution of the LB membranes followed by a reassembly of their components into TM, for which there appears to be no evidence, we must look for either a folding of the planar LB membranes into TM lattice, or a partial disassembly followed by reassembly. By careful examination of electron micrographs of suitably sectioned TM preparations, we are able to interpret TM formation as the result of a relatively simple folding process. This enables us to make a number of postulations concerning the physical mechanisms which might lead to the organization as well as the stabilization of the tubular form. We believe that
the mechanism suggested may have relevance to the fusion of other phospholipid bilayers as well as to TM.

MATERIALS AND METHODS

Lungs of rats anesthetized intraperitoneally with sodium pentobarbital (5 mg/100 g body wt) were perfused with oxygenated dextran-75 (6% Gentran in 0.9% NaCl, Travenol Laboratories, Deerfield, Ill.) through the pulmonary artery until the lung was cleared of visible erythrocytes. This required 30–60 s of perfusion. The lungs were then lavaged, via the trachea, with 2–4 ml of a solution containing 0.85% NaCl, 6% dextran-75 or 0.5 M sucrose, with or without 3 mM CaCl₂.

The material obtained by lavage was initially prepared in suspension by the addition of 1 vol of fixative to 2 vol of specimen followed by sedimentation in a Sorvall HB4 rotor (Sorvall Operations, DuPont Instruments, Newtown, Conn.) at 25,000 g for 5 min. The fixatives used were 1% glutaraldehyde (Biological Grade, Fisher Scientific, Pittsburgh, Pa.) and 1% tannic acid (Mallinckrodt, Inc., St. Louis, Mo.) in 0.1 M sucrose containing 0.1 M cacodylate buffer (pH 7.4) or 0.5 M sucrose and adjusted to pH 6.8 using 0.05 M cacodylate when 3 mM CaCl₂ was added at room temperature.

After this procedure, the pellet was treated with undiluted fixative for 2–4 h, rinsed several times in a solution of similar composition with the fixative omitted, and postfixed for 30 min in 1% cold osmium tetroxide in 0.1 M cacodylate (pH 7.3). The pellet was dehydrated cold with either glycol methacrylate or acetone, and embedded in Epon.

The structural organization of the figures to be discussed was similar if they were prepared by any of the following three variations: (a) 2% glutaraldehyde with osmium tetroxide postfixation, (b) mixture of cold glutaraldehyde and osmium tetroxide (8), or (c) glutaraldehyde-tannic acid mixture with added Ca²⁺, followed by osmication (11). The last method, however, yielded specimens whose images contained superior fine structural detail. Specimens were sectioned with a diamond knife in a Sorvall Porter-Blum MT2 ultramicrotome in the silver and gold range and observed without poststaining. Stereo-micrographs were prepared on a Philips EM-300 electron microscope.

RESULTS AND DISCUSSION

At the time of secretion of a LB into the alveolar cavity of the lung, a reorganization of its membranous components occurs presumably as a result of the changed environment. Although it is not possible to define the system of forces acting on the lamellae after the surrounding membrane has been disrupted, it is useful to consider the behavior of a material under uniaxial tension. For the simple case of a homogeneous, isotropic material, it is easy to show that the maximum stress acts at an angle of 45° to the direction of the applied tension (see, for example, Fung, page 79 [4]). Although the situation is more complex for a laminated material loaded normal to the planes of the laminations, we can say that, for the case of a dissociating LB, a membrane will begin to flow in the direction of maximum stress. This flow will continue until a configuration is reached for which the energy of distortion, i.e., the strain energy, is a minimum and the membranes are aligned at about 45° to their original planes. If this is so, typical planar sheets in LBs subjected to a suitable stress field might be distorted into corrugated sheets, i.e., into a "zig-zag" pattern, in the TM, as shown schematically in Fig. 1. If so, these sheets should be observable in microscope sections.

We have observed such sheets and found them to be most readily visible in lattice aggregates which are sectioned slightly obliquely. A stereo pair of images so obtained is shown in Fig. 2. Although the zig-zag pattern of membrane folding is best visualized stereoscopically, it can be readily deduced from the separate photographs. For this purpose the detail on Fig. 3a, which is a portion of Fig. 2 enlarged, has been sketched (Fig. 3b) to assist interpretation. In particular, the gap between adjacent ribbons (arrow) has been exaggerated.

We must next examine the detail of the "intersections" in the tubular myelin structure at a magnification sufficiently high to allow the membrane bilayers to be observed. If the zig-zag mode of formation suggested by the photographs of Fig. 2 occurs, the pattern made by the bilayers in the vicinity of the intersections should be as shown in Fig. 4a. Also shown is a simplified arrangement of the membrane lipid molecules (Fig. 4b). That this configuration is not often observed is probably a reflection of its inherent metastability in the alveolar environment. Such instability would lead to transition to the pattern shown in Fig. 4c by translation and rotation of the polar molecules of divalent cations, particularly calcium (6), the constrained bilayer system. 

![Figure 1](image-url) (a) An array of planar sheets under uniaxial load normal to the laminations. (b) A mode of distortion of the system under such a load.
centration of which is higher in the extracellular fluid of the lung alveoli (20) than in the intracellular environment of the type II lung cells from which the lamellar bodies originate. Gil and Reiss (5) noted that TM is never seen in homogenates when an EDTA-containing buffer is used. This led them to suggest that either Ca$^{++}$ or some other cation strongly bound by EDTA is required for TM formation. In fact, Hassett et al. (6) have recently shown that incubation of isolated LBs with 5 mM Ca$^{++}$ or Mg$^{++}$ at 37°C leads to the formation of TM. That calcium enables certain disrupted membranes to fuse has been known for many years. For example, Heilbrunn (7) showed in 1926 that protoplasm emerging from a broken sea urchin egg would seal off to form a droplet only in the presence of calcium ion. More recently, LeNeveu et al. (10) measured the forces between electrically neutral zwitterionic phospholipid bilayers, and showed that the energy required to fuse them is significant compared with energies available from thermal motion. When fusion of bilayers does occur, therefore, it is likely that it results from some destabilization of the
polar head groups such as we suggest is brought about by divalent ions. Such destabilization would allow the necessary rotation of the phospholipid molecules, and hence the establishment of a strong hydrophobic association in the interior of the junctions. It should be also noted that the scale of the tubular myelin grid, which has a repeating dimension of about 500 Å, is well within that required for London dispersion forces to be significant. These forces, which are attractive, may be subdivided into unretarded and retarded components which are effective at distances of less than 150 Å and greater than about 300 Å, respectively, depending upon the actual geometry of the surfaces. At intermediate distances, Born repulsive forces of electrostatic origin dominate (12). An alternation of attractive and repulsive forces is probably important in producing a stable grid and in fact may even provide the basic stresses causing the grid to be formed.

If the transient configuration for TM shown in Fig. 4a does exist, it is more likely to be found in the near vicinity of a dissociating LB than in a completely formed grid of “old” TM. Fig. 5 shows the formation of the lattice in such a region. In several locations, some of which are marked with arrows, the transient configuration is clearly visible. In other places, the cross configurations have already been formed. Both forms can also be clearly seen in Fig. 5 of a recent paper of Nichols (13).

**Figure 4** Diagrammatic representation of the tubular myelin membrane in the vicinity of intersections. (a) The metastable configuration arising from the zig-zag formation of TM. (b) The arrangement of phospholipid molecules corresponding to Fig. 4a. (c) The final stable configuration. (d) The strong hydrophobic association of the nonpolar ends of the phospholipid molecules corresponding to Fig. 4c.

**Figure 5** Tubular myelin formation near a dissociating lamellar body. The transient configuration which is the precursor to the final stable lattice intersections is clearly visible (arrows). × 165,000.
Up to this point, no attempt has been made to explain the regularity of the TM. Many photographs, taken both in our laboratory and elsewhere (13, 20), show fine projections extending from the four corners of the tubules toward the center. It is possible that these projections stabilize the tubules, and it is conceivable that through their association with the precursor membranes of the LBs they may dictate the repeating dimension of the TM. On the other hand, the grid structure represents the configuration of minimum free energy, and the distance between corners may allow the optimal distribution of hydrophobic and hydrophilic bonds. This could account for the observation made by Williams (20) of a much tighter lattice within a LB where the ionic environment is presumably different.

Finally, it should be made clear that the zig-zag mode of TM formation applies to the idealized case where an array of parallel planar membranes is transformed into a lattice. An LB, however, has a more complex structure than such an array, being a set of parallel closed membranes whose ends have been doubled back upon themselves (see Stratton [17] and Williams [20]). In addition, more than one LB can feed membrane material into a TM grid. Such nonideal geometry leads to the frequent formation of more complex and less regular figures than the ideal square grid discussed here. These are always seen toward the periphery of a TM grid. In these regions, three-, five- and occasionally, six-sided tubules may be observed with the usual four-sided figures.

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