

ISOLATION AND CHARACTERIZATION OF LAMELLAR BODIES AND TUBULAR MYELIN FROM RAT LUNG HOMOGENATES

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ABSTRACT

Three surface-active fractions which differ in their morphology have been isolated from rat lung homogenates by ultracentrifugation in a discontinuous sucrose density gradient. In order of increasing density, the fractions consisted, as shown by electron microscopy, primarily of common myelin figures, lamellar bodies, and tubular myelin figures. The lipid of all three fractions contained approximately 94% polar lipids and 2% cholesterol. In the case of the common myelin figures and the lamellar bodies, the polar lipids consisted of 73% phosphatidylcholines, 9% phosphatidylserines and inositols, and 8% phosphatidylethanolamines. In the case of the tubular myelin figures, the respective percentages were 58, 19, and 5. Over 90% of the fatty acids of the lecithins of all three fractions were saturated. Electrophoresis of the proteins of the fractions in sodium dodecyl sulfate or Triton X-100 revealed that the lamellar bodies and the tubular myelin figures differed in the mobilities of their proteins. The common myelin figures, however, contained proteins from both of the other fractions. These data indicate that, whereas the lipids of the extracellular, alveolar surfactant(s) originate in the lamellar bodies, the proteins arise from another source. It is further postulated that the tubular myelin figures represent a liquid crystalline state of the alveolar surface-active lipoproteins.

INTRODUCTION

Mitochondrial-rich fractions of the mammalian lung, in contrast to the mitochondrial fractions of other organs, exhibit marked surface activity (1). We will show in the present work that this activity is related mainly to the presence in lung mitochondrial fractions of two discrete components that can be isolated and purified by fractionation in discontinuous sucrose gradients. These components are the lamellar bodies, a cell organelle uniquely encountered in type II alveolar cells, and "tubular myelin figures," a constituent of the extracellular lining layer of alveoli. The lamellar bodies have been the object of much research

because of the essential role that they are supposed to play in the metabolism of the surface-active lipoproteins of the mammalian lung (2, 3). The evidence in favor of the notion that lamellar bodies store and/or synthesize essential components of the surface-active lipoproteins is based on developmental (4-9), cytochemical (8, 10-13), and autoradiographic (14-16) studies. Although the isolation of these organelles has been previously attempted (17, 18), the only reliable criteria for their identification is correlation of fine structure with surface activity. In previous reports the electron micrographs lacked adequate struc-

tural detail and were not accompanied by measurements of surface activity.

Tubular myelin figures were first shown in the alveolar spaces of conventionally fixed mammalian lungs by Policard (19) and Campiche (20). They were designated "tubular" by Weibel et al. (21) because these authors showed by means of three-dimensional reconstructions of electron micrographs that the characteristic square lattice pattern was a section through a system of tightly packed tubules. These structures have been repeatedly observed in the alveoli of normal and diseased lungs by other authors (22-25). By using methods of fixation that permit the preservation of an intact air-liquid interface, it was shown that tubular myelin figures are a constant element of the extracellular lining layer of mammalian alveoli (26-30). They also appear on the bronchiolar surfaces (31), in pictures of freeze-etched alveoli (32), and their presence in lung washings has been reported (33, 34). Their occurrence is not limited to mammalian lungs since it has been seen in intact frog lung.¹

MATERIALS AND METHODS

A. Preparations of the Lung Fractions

Adult Sprague-Dawley rats (6 wk or older) of either sex were sacrificed by cervical dislocation. The lungs from 30 rats were removed immediately, rinsed in an ice-cold solution containing 0.32 M sucrose, 0.01 M Tris hydrochloride, and 0.001 M CaCl₂ final pH 7.40 (STC medium), and stored in the same solution. All solutions and glassware were precooled and kept in ice during the subsequent operations. A lung mitochondrial-rich fraction was isolated and washed by modifying the procedure used for the preparation of rabbit lung mitochondria (35). The lobes of the lung were separated, all major visible bronchi and blood vessels removed, and five lungs were minced with scissors and placed into a homogenizing vessel (Arthur H. Thomas Co., Philadelphia, no. 4288-B, size C). The lungs were first homogenized in 8-10 ml of the STC medium at 150 rpm with a Teflon pestle that had been milled to an outside diameter of 2.45 cm until the vertical motion was uniform. This required 12-24 vertical strokes. Homogenization was continued at 250 rpm with a pestle of 2.50 cm diameter for another 6-12 strokes. The volume of the homogenate was then adjusted to 50 ml with the STC medium, the contents mixed with the pestle, and strained through a

38-mesh nylon screen cloth (Turtex no. 105A6045A). The filtrate was spun first at 500 g for 10 min (International centrifuge model CL head no. 331) and the supernatant fraction recentrifuged in the same centrifuge at 1,600 g for 5 min. The supernatant fraction from the latter centrifugation was spun in the International B-20 centrifuge, head no. 873 at 16,000 rpm (15,000 g) for 10 min, the resulting pellets resuspended in 0.5 of the original volume of the STC medium and recentrifuged under the same conditions. Finally all pellets were resuspended, pooled, and made to 32 ml with the STC medium.

SEPARATION ON A DISCONTINUOUS SUCROSE GRADIENT: The following six-zone gradient was introduced by means of a pump (Watson-Marlow MHRE-200) into tubes of the Beckman Spinco SW-27 rotor: 4.0 ml of 0.90 M, 8.0 ml 0.80 M, 6.0 ml 0.70 M, 6.0 ml 0.55 M, 6.0 ml 0.45 M sucrose all in 0.16 M NaCl, and adjusted with a minimum 0.1 M Tris-hydrochloride to bring the pH to 7.45 (Fig. 1). Each tube was overlaid with 5.0 ml of the lung suspension in the STC medium. The tubes were spun for 120 min at 25,000 rpm (90,000 g) in the Beckman Spinco L-2 65B centrifuge. Five turbid fractions (F I-F V) were removed by micropipette, and the sixth fraction (F VI) was recovered as the pellet. After establishing the sucrose concentration in the five fractions by refractometry, the fractions were then diluted with 0.16 M NaCl to be 0.32 M with respect to sucrose and pellets were formed in the Beckman Spinco no. 30 rotor at 28,000 rpm (68,000 g) for 40 min. Each of the five pellets was resuspended in 2.0 ml of 0.16 M NaCl. The mitochondrial pellet was directly suspended in 0.16 M NaCl. Aliquots of each suspension were used for (a) reapplication on a continuous sucrose density gradient (see below), (b) electron microscopy, (c) protein and lipid-phosphorus determinations. The remainder was dialyzed overnight at 4°C against 0.16 M NaCl in a Hofer microdialyzer (Hofer Scientific Instruments, San Francisco, Calif.), then dialyzed for 30 min against triple distilled water at 4°C, made to volume with water, an aliquot removed for sodium analysis, and the remainder lyophilized in tared tubes.

SEPARATION OF FRACTIONS ON A CONTINUOUS SUCROSE DENSITY GRADIENT: Aliquots of fractions (F I, F II, and F IV) were placed on a linear, continuous sucrose density gradient in order to test their stabilities to the fractionation process. The gradients were constructed with a Buchler Universal density gradient mixer and transferred by a pump into tubes of the Beckman Spinco SW-41 rotor. For examination of F I and F II, 5.0 ml of 0.60 M sucrose were mixed with 5.5 ml of 0.20 M sucrose and placed on a cushion of 1.0 ml 0.60 M sucrose, for F IV 5.0 ml of 0.8 M sucrose and 5.5

¹ Weibel et al. Personal communication.

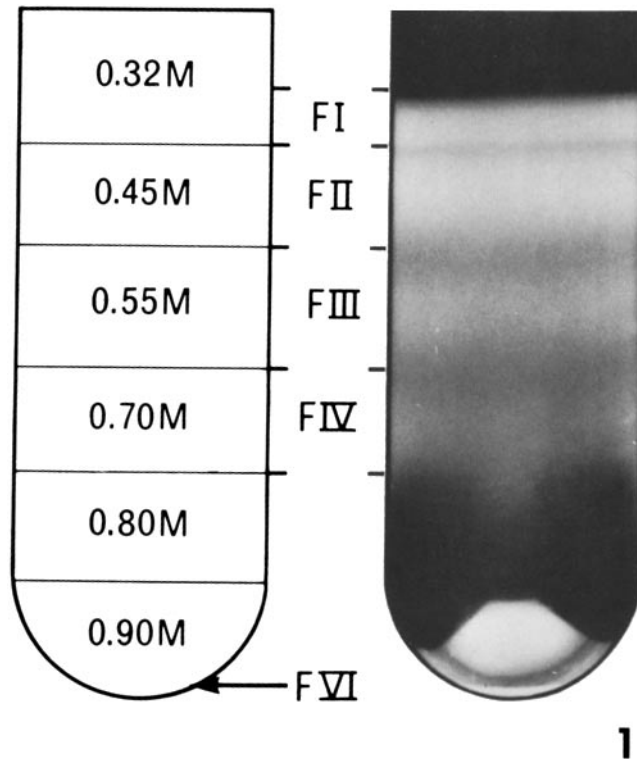


FIGURE 1 Schematic of discontinuous sucrose density gradient and photograph of the preparation after centrifugation. Turbid layers were removed with micropipettes and the nomenclature used is indicated in this figure. For volumes and exact composition of the fractions, see text.

ml of 0.40 M sucrose were placed on 1.0 ml of 0.8 M sucrose. All sucrose solutions were made up in 0.16 M NaCl. The samples were spun in the Beckman Spinco L-2 65B for 120 min at 35,000 rpm (165,000 g). It had been established previously that the sample reaches its isopycnic density under these conditions (36, 37). The fractions were removed with a Buchler Auto-density flow apparatus, 0.2-ml fractions were collected, and the concentrations of sucrose in the fractions containing the samples were determined by refractometry and used to establish the isopycnic densities.

B. Lipid Extraction and Recovery of Protein

The lyophilized pellets were brought to constant weight in a vacuum desiccator, and these weights minus the amount of sodium chloride (calculated from the sodium content in aliquots, see section A) were recorded as the dry weights of the fractions. The lipids were then extracted by the method of Bligh and Dyer (38, 39) with the modification of the procedure to include a refluxing of the water: chloroform:methanol (0.8:2:1)-pellet mixture for 30

min before centrifugation, a step that had been found necessary for the total extraction of lipid from alveolar surface-active lipoproteins (36, 37). After cooling of the mixture, the resulting pellet was used for isolation of "water-insoluble protein" (see below). After addition of the chloroform and water to produce a two-phase system, the chloroform layer was washed two times with water, dried over anhydrous sodium sulfate, and taken to dryness in a rotary evaporator. The lipid was redissolved in redistilled chloroform, filtered through sintered glass (coarse), and collected in a tared 10 ml Erlenmeyer flask. The solvent was removed under nitrogen, and the total lipid weight determined on an M-5 Mettler microbalance (40). The protein pellet was washed with 8.0 ml ice-cold water, centrifuged at 20,000 g for 10 min, and the resulting pellet transferred in a tared tube and lyophilized. The weight of total insoluble protein was established gravimetrically at $\pm 10 \mu\text{g}$.

C. Fractionation of the Lipids

Total lipids were applied to 0.5×6.5 cm columns containing silicic acid (40) (adapted from Barron

and Hanahan [41]). The distributions of the lipids were determined gravimetrically. From 91 to 109% of the weights of the applied materials were recovered. Identities of the lipids in each of the fractions except the polar lipids (see below) were established by thin-layer chromatography of the samples and standards on silicic acid developed in hexane:ethyl ether:acetic acid (70:30:1) (42) followed by charring with dichromate-sulfuric acid. The amounts of cholesterol in the fractions containing both cholesterol and diglyceride were determined according to Abell et al. (43).

The polar lipids were fractionated by applying 500 μ g of the methanol fraction to thin-layer plates (Redi-coats; Supelco, Inc. Bellefonte, Pa.) which were developed in two dimensions with chloroform:methanol:28% ammonia (65:25:5) and chloroform:acetone:methanol:acetic acid:water (3:4:1:1:0.5) (Rouser et al. [44]). Plates were charred by spraying with dichromate-sulfuric acid. The spots were removed by aspiration and digested for phosphorus determination (45).

To determine the fatty acid composition of the lecithins, a similar two-dimensional plate of the methanol fraction was run. The spot containing the lecithins was visualized by spraying with ice-cold water and removed from the plate by aspiration. The lecithins were eluted from the silicic acid with methanol containing 1% formic acid; the solvent was removed in a stream of N_2 , and finally *in vacuo*. The lecithins were then treated with 2.0 ml of 0.5 N NaOH in absolute methanol. The resulting methyl esters and fatty acids were extracted with hexane and, after removal of the hexane, were completely methylated with BF_3 -methanol (46). The yield of fatty acid methyl esters with this procedure is over 95%, assuming that all the lecithin is dipalmitoyl lecithin (see below).

The fatty acid methyl esters were identified and quantitated in a Barber-Coleman model 5000 gas chromatograph equipped with a dual flame ionization detector. The columns (5 ft-by- $\frac{1}{4}$ inch) were packed with 15% HIEFF-IPB on Chromosorb P 80/100 (Applied Science Labs, Inc., State College, Pa.) and were maintained at 149°C. A standard mixture of methyl esters ([GLC-1], The Hormel Institute, Austin, Minn.) was used to establish the retention times of the various methyl esters, and the relative quantities of the various fatty acids were calculated from the integration units (IU) obtained with a Disk Chart Integrator. The fractional composition of a fatty acid was calculated from the IU of a given fatty acid divided by the sum of IU of all the fatty acids.

D. Analyses of the Protein Components

Aliquots of the water-insoluble protein of the fractions were applied to two different polyacrylamide disk gel electrophoretic systems.

Microdisk gels containing Triton X-100 were prepared in 100- μ l pipettes (Corning no. 7099-S; inside diameter 1.5 mm) that had been treated by immersion in a 1% aqueous suspension of Siliclad (Clay-Adams, Inc., Parsippany, N.J.) and dried at 180°C for 30 min before use. The separating gel was 6 cm long and was cast by polymerizing a mixture containing 1.8 g acrylamide, 32 mg *N,N*-methylene bis acrylamide (Bis), 2.0 g sucrose, 6.0 μ l *N,N,N',N'*-tetramethylethylenediamine (TEMED), 7.5 mg ammonium persulfate, and 0.1% Triton X-100 in 20 ml of 0.31 M Tris-hydrochloride pH 8.2. The stacking gel was made from: 175 mg acrylamide, 12.5 mg Bis, 1.5 g sucrose, 3.1 μ l TEMED, 31.5 mg riboflavin, and 0.1% Triton X-100 in 5.0 ml 0.05 M Tris-hydrochloride pH 8.2. The insoluble proteins were dispersed by sonic oscillation in a solution that contained 70 mg acrylamide, 5 mg Bis, 1.2 μ l TEMED, 32 μ g riboflavin, tracking dye, and 1% Triton X-100 in 2.4 ml of 0.05 M Tris-hydrochloride pH 8.2. 50–75 μ l of this solution containing 50–75 μ g of protein were applied on the polymerized separating and stacking gels. The detailed preparation of these gels without the Triton X-100 has been described previously (47). The buffer compartment contained 0.005 M Tris-hydrochloride, 0.04 M glycine, 0.1% Triton X-100, final pH 8.2. The gels were subjected to 2 mA per tube for 45 min, then fixed in 20% sulfosalicylic acid overnight, stained for 4–6 h in a 1% solution Coomassie blue in methanol:acetic acid:water 5:1:5, and destained in 5:1:5 methanol:acetic acid:water. Gels were rehydrated in water and photographed with a Polaroid MP-3 camera. Several other investigators have reported the use of Triton X-100 for the separation of membrane proteins in standard size gels (48, 49).

For electrophoresis in sodium dodecyl sulfate (SDS) containing gels, the procedure of Weber and Osborn (50) was modified. The tubes (85 \times 5 mm), after cleaning in dichromate-sulfuric acid, were coated with a 1% Photoflow solution (Eastman Kodak, Rochester, N. Y.) and dried. The gels were made from: 1.8 g acrylamide, 32 mg Bis, 10 μ l TEMED, 7.5 mg ammonium persulfate, 2 g sucrose, and 0.2 g SDS dissolved in 20 ml 0.1 M sodium phosphate pH 7.4. The samples were incubated in a solution containing 1% SDS, 4 M urea, and 1% 2-mercaptoethanol overnight at 4°C (1 mg sample/ml solution). These conditions had previously been found to give a linear logarithm molecular weight-mobility relationship with the standard proteins listed below and also to yield sharp bands with our

preparations. Undialyzed samples (30–50 μg) were applied to the polymerized gels. The cathodal buffer chamber was filled with a 0.01 M sodium phosphate buffer, pH 7.2, containing 1% 2-mercaptoethanol and 1% SDS, while the anodal chamber was filled with 0.01 M sodium phosphate buffer pH 7.2. The current applied was 7 mA per tube for 105 min. Fixation, staining, destaining, and photographic procedures were identical with those used on microdisk gels except that the time of staining was extended to 16 h. A standard curve was prepared by plotting the relative mobilities against the logarithm of the reported molecular weights of the following proteins: transferrin (mol wt 76,600), ovalbumin (mol wt 46,000), trypsin (mol wt 23,800), myoglobin (mol wt 17,600), chymotrypsin (mol wt 13,900 and 10,200), cytochrome (mol wt 12,400), insulin (mol wt 5,770) (51). Molecular weights of the proteins of our preparations was estimated from this calibration graph.

E. Analytical Procedures

Proteins were determined by a modified Folin procedure (52) with crystalline bovine plasma albumin (Armour Pharmaceutical Co., Chicago, Ill.) as a standard. Total phosphorus was determined according to Bartlett (53) after total digestion with perchloric acid (54). The concentrations of all sucrose solutions used in this investigation were determined by refractometry (Abbe-3L; Bausch & Lomb Inc., Rochester, N.Y.) and compared with values reported in the literature (55). The relationships of refractive index and concentrations of sucrose solutions containing 0.16 M NaCl were established by preparing a calibration curve. Apparent densities of sucrose solutions at 20°C were obtained from tables (55) and corrected to 4°C. Sodium concentrations were determined by flame spectrophotometry.

Surface tension measurements were determined on a modified Wilhelmy balance (56) (Kimray, Inc., Oklahoma City, Okla.). The system was calibrated with triple distilled propanoic acid-water mixtures, using the surface tension values determined by Tamamushi (57). Exact molarities of the propanoic acid were established by titration with standard base. Daily checks of the pneumatic transducer were made with a platinum weight. All components of the balance in contact with liquid were cleaned in a nitric acid and rinsed in triple distilled water. The lung fractions were placed on the subphase containing 0.16 M sodium chloride, 0.01 M sodium phosphate pH 7.4. They were allowed to sit overnight at room temperature (21°–24°C) before cycling. The

area of the surface trough changed from 51.5 to 10.5 cm^2 with a cycle time of 12 min. The force area diagrams obtained with these materials were reproducible beginning with the second cycle and exhibited the usual hysteresis characteristics of these materials (3). For quantitative estimations we applied known amounts of surface-active lipoproteins to the film balance and calculated the lipoprotein needed to cover one square centimeter. This method was based on the following technique previously developed by one of us (O. K. Reiss, unpublished observation): by extrapolating the initial compression slope of the force-area diagram to the axis of the area, it is possible to obtain a relationship of micrograms lipoprotein per square centimeter that stays constant in a small range of weight applied on the subphase. In other words: if the extrapolation of the diagram obtained with 40 μg lipoprotein cuts the area axis at 60%, 20 μg will cut it at 30%. The amounts of lipoprotein where this relationship holds range from 20 to 60 μg and yield minimum tensions between 4 and 18 $\text{dynes}\cdot\text{cm}^{-1}$. According to our previous experience with other pulmonary surface-active lipoproteins, we can produce lower minimal surface tension by applying larger amounts of lipoproteins. We believe that this method is useful for quantitative work.

The chloroform, methanol, acetic acid, and hexane used for chromatography of lipids were redistilled. Triglycerides, fatty acids, diglycerides, dipalmitoyl lecithin, egg lecithin, phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl inositol used as standards for lipid chromatography were obtained from Supelco Inc.

F. Electron Microscopy

150 μl of F I and F II, 100 μl of F III, F IV, and F V, and 25 μl of F VI were placed in small cellulose tubes. The tubes were filled with the following cold fixative: two parts of 1% OsO_4 in 0.1 M cacodylate buffer mixed with one part of 2.5% glutaraldehyde in 0.1 M cacodylate (58, 59). The contents of the tubes were mixed and spun in a refrigerated International centrifuge at low (1,500 g) speed for 30–45 min. The supernatant was decanted and the bottom ends of the tubes containing black pellets were cut and immersed in 1% aqueous uranyl acetate for 20–30 min. The specimens were quickly dehydrated in graded ethanols (5 min each step). The fragments of tubes were dissolved in propylene oxide. The pellets were embedded in Epon 812 according to Luft (60). Thin sections were cut with a diamond knife, mounted on naked grids, stained with lead citrate according to Reynolds (61), and examined in a Philips 200 electron microscope.

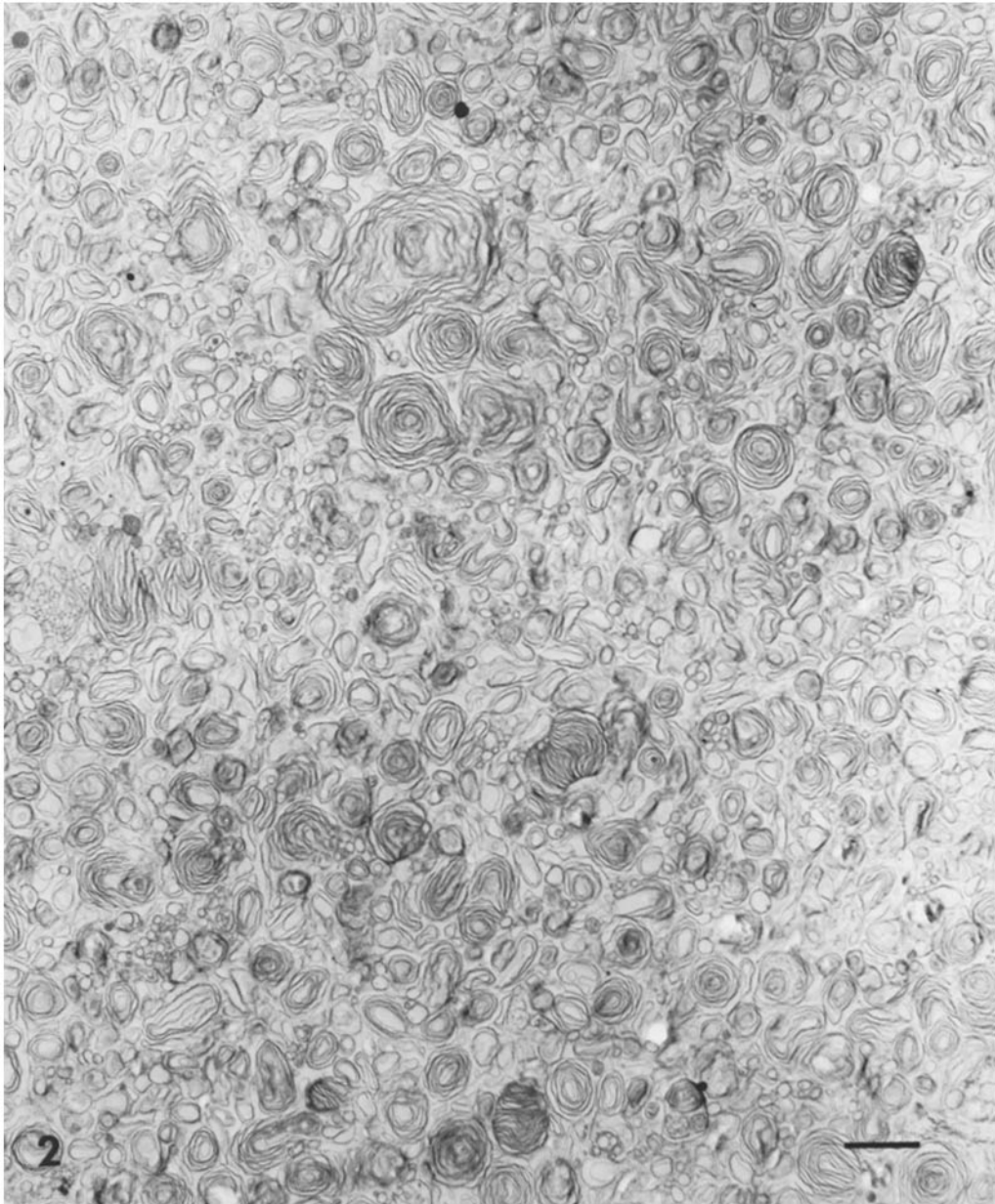


FIGURE 2 Low power micrograph from F I. This fraction mainly contains myelin figures, often resembling the arrangement of the lamellae of the lamellar bodies. Intact lamellar bodies can occasionally be seen. $\times 10,800$. Horizontal bar = $1 \mu\text{m}$.

RESULTS

A. Electron Microscope Characterization of the Fractions

FRACTION I: This fraction (Fig. 2) consisted principally of osmiophilic structures that can be

regarded as common myelin figures.² According to present knowledge (62-64) formations of this

²The term "common myelin figures" is used here solely to contrast the structures found in this fraction from those found in the tubular myelin figures (in the sense of Weibel et al., [21]).

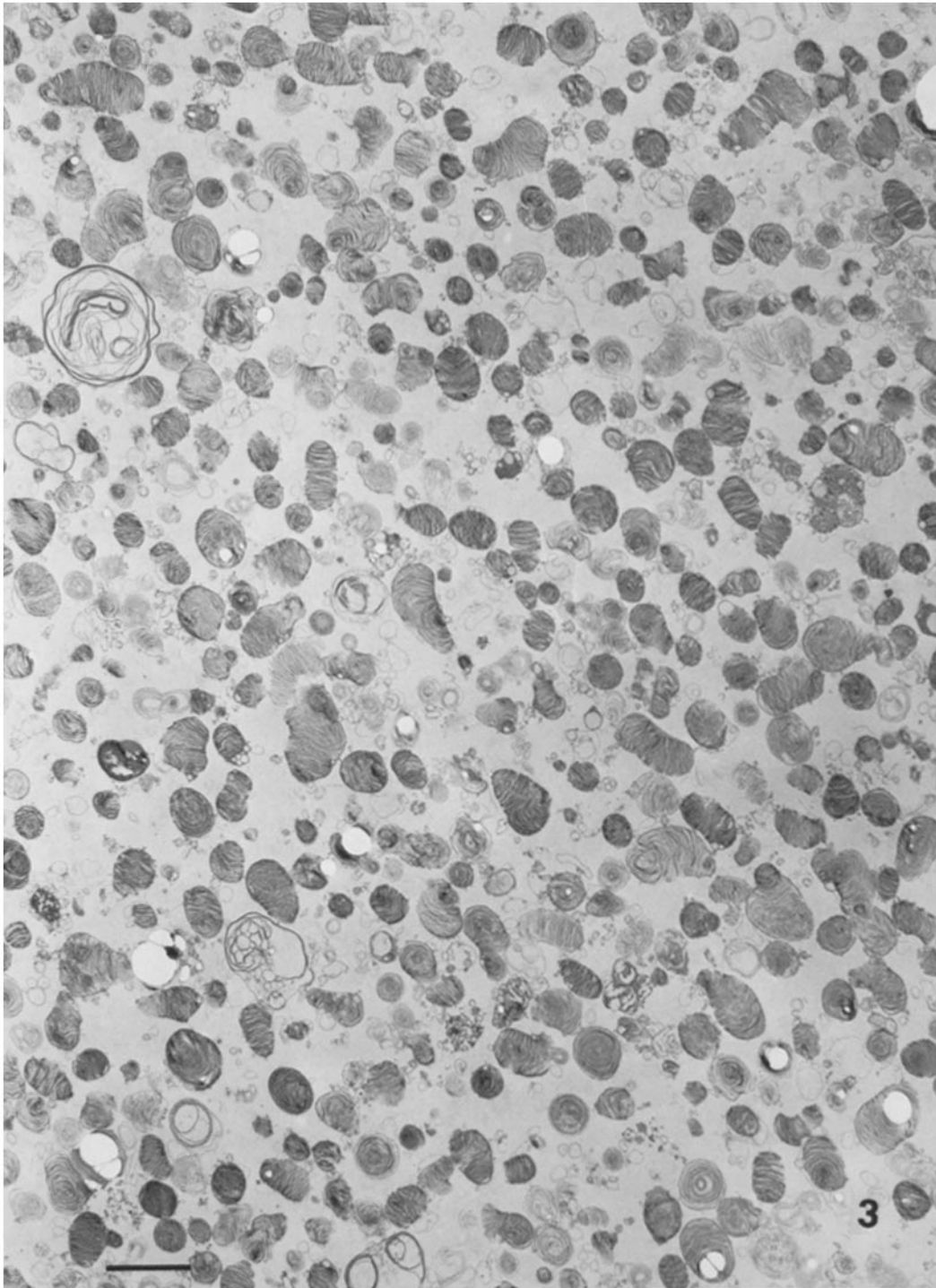


FIGURE 3 Low power electron micrograph from F II showing concentrated lamellar bodies of type II alveolar cells. Infiltration of this fraction with Epon was difficult. $\times 6,230$. Horizontal bar = $2 \mu\text{m}$.



FIGURE 4 Frontal section through lamellar body of F II. Note bell-like arrangements of lamellae. Limiting membrane is clearly shown. In addition to the osmiophilic lamellae, an amorphous material can be distinguished. Outpocketings of the limiting membrane containing amorphous material are not seen in preparations of intact lung and are probably artefactual. $\times 140,000$.

type are supposed to represent aggregates of polar lipids in an aqueous phase. Occasional structures were seen that meet the criteria for lamellar bodies (see below). The structure of many myelin figures often resembled the typical arrangement of the lamellae inside these organelles, thus suggesting that the source of material in this fraction might well be in part lamellar bodies damaged during the preparation.

FRACTION II: Fig. 3 shows a low power micrograph of this fraction. It contains osmiophilic organelles that can be identified as lamellar bodies of type II alveolar cells according to the following criteria:

(a) They exhibit distinct limiting membranes. This membrane consists of a double osmiophilic leaflet (Figs. 4 and 6).

(b) Since many of the lamellae inside the lamellar body are also grouped in bilayers, the limiting membrane can be distinguished if it shows a different orientation and a continuity on the exterior. Furthermore, the lamellae never insert directly in the limiting membrane.

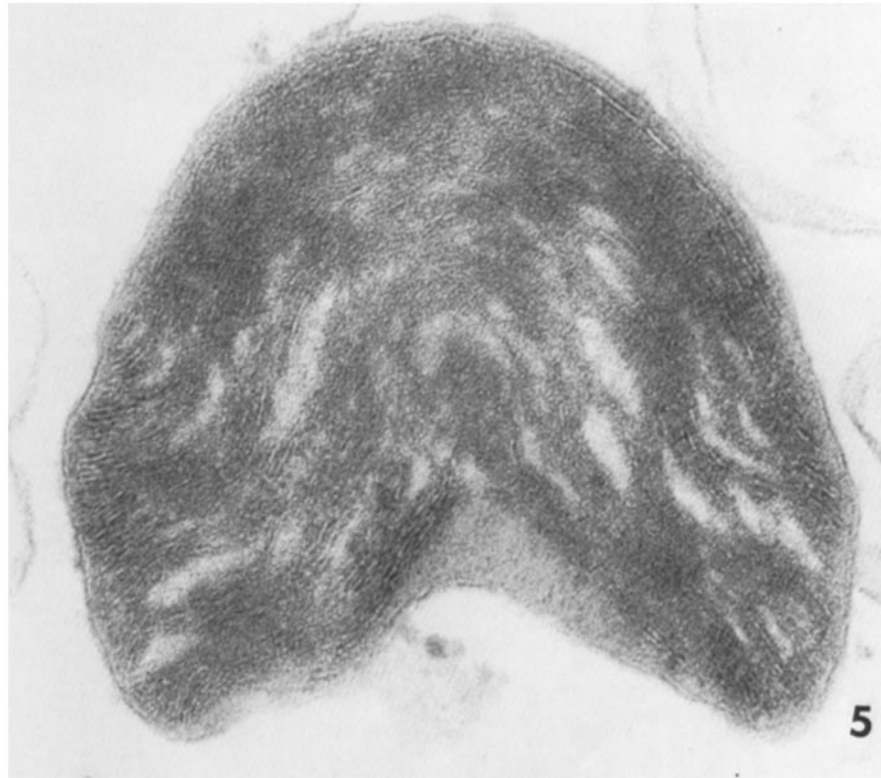


FIGURE 5 F II. Section similar to Fig. 4. Existence of an amorphous material in addition to the osmiophilic lamellae can be recognized. $\times 150,200$.

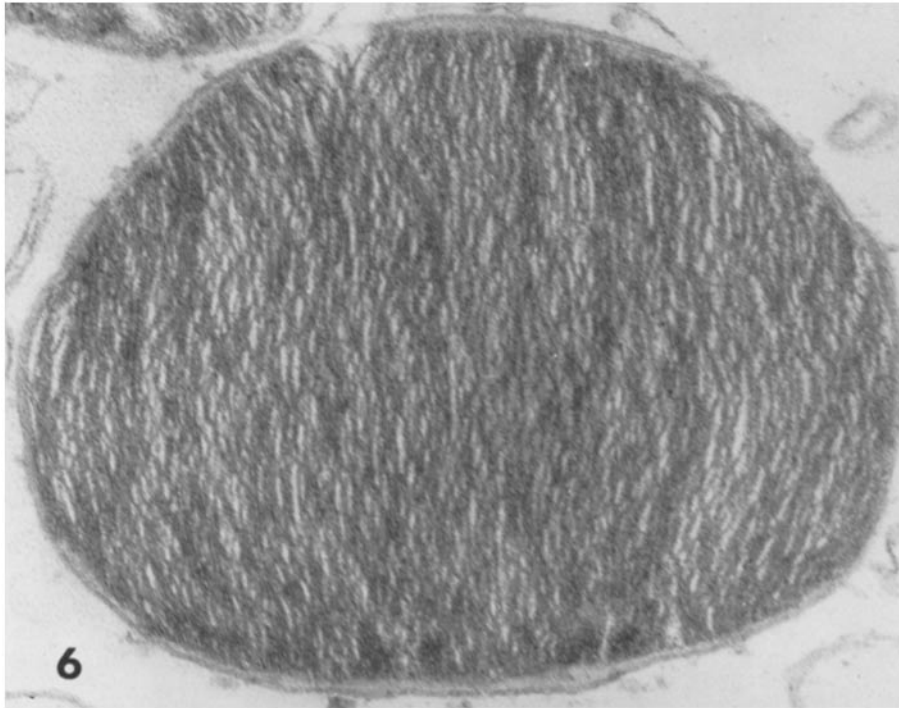


FIGURE 6 F II. Other plane of section through a lamellar body. Limiting membrane and amorphous material between these and the lamellae are shown. The limiting membrane is broken in one area. $\times 97,200$.

(c) Recent studies on lamellar bodies *in situ* in rat lungs³ have demonstrated nonrandom orientation of the lamellae. In a frontal section, they would form a kind of bell-like structure (Figs. 4 and 5). Other planes of section may result in quite different morphological presentations. Hence random sections of lamellar bodies must show different orientations of the lamellae (see Figs. 3-6).

(d) In addition to the dark lamellae, these bodies contain, as they do *in situ*,³ an amorphous material characteristically placed between the lamellae and the limiting membrane (Figs. 4 and 6).

In our material from rat lung homogenates, preservation of the lamellae inside the organelles was variable but usually good. The repeat period of the lamellae was about 4.3 nm. Pictures of freeze-etch preparations of lamellar bodies *in situ*,³ show that the lamellae are originally closely packed

without gaps: those gaps seen between lamellae in our specimens are possibly the result of lipid extraction during processing for electron microscopy. Infiltration of this material with Epon and cutting ultrathin sections proved to be very difficult. Most specimens exhibited numerous holes.

FRACTION III: F III contained mainly a mixture of the materials described in fractions II and IV and were, therefore, not examined chemically.

FRACTION IV: F IV contained the constituent of the intra-alveolar lining layer named by Weibel et al. (21) "tubular myelin figures" (Figs. 7-9). Usually they form aggregates of moderate size. From our pictures, it is evident that the orientation of the structure in relation to the plane of section changes even within very short distances. This is related to the fact that the structures we have isolated are considerably less regular than those described *in situ*. Weibel et al. (21) reported that the transverse dimensions of the tubular units in alveolar tubular myelin figures were about

³ Gil, J., E. R. Weibel, and P. Untersee. 1973. A three-dimensional reconstruction of lamellated bodies of type II alveolar cells. In preparation.

45 nm, whereas in our specimens (Fig. 7) they were about 52 nm. This leads us to the assumption that our isolated tubular myelin is somewhat "swollen."

A further difference between tubular myelin *in situ* and *in vitro* lies in the orientations of tubular elements at the surface of the structure (Fig. 8). In perfused rat lungs (26, 27) it has been shown that the osmiophilic membranes are always parallel or perpendicular to the air space and are

never oriented in a 45° angle as in Fig. 8. The wall of the cross-sectioned tubules consists of two osmiophilic lines whose combined thickness varies from 5 to 6 nm.

It has been claimed (30) that tubular myelin figures are the result of the aggregation of very regular "crosses" (we will not discuss here the three-dimensional implications), each one being shared by four squares. Pictures of isolated crosses

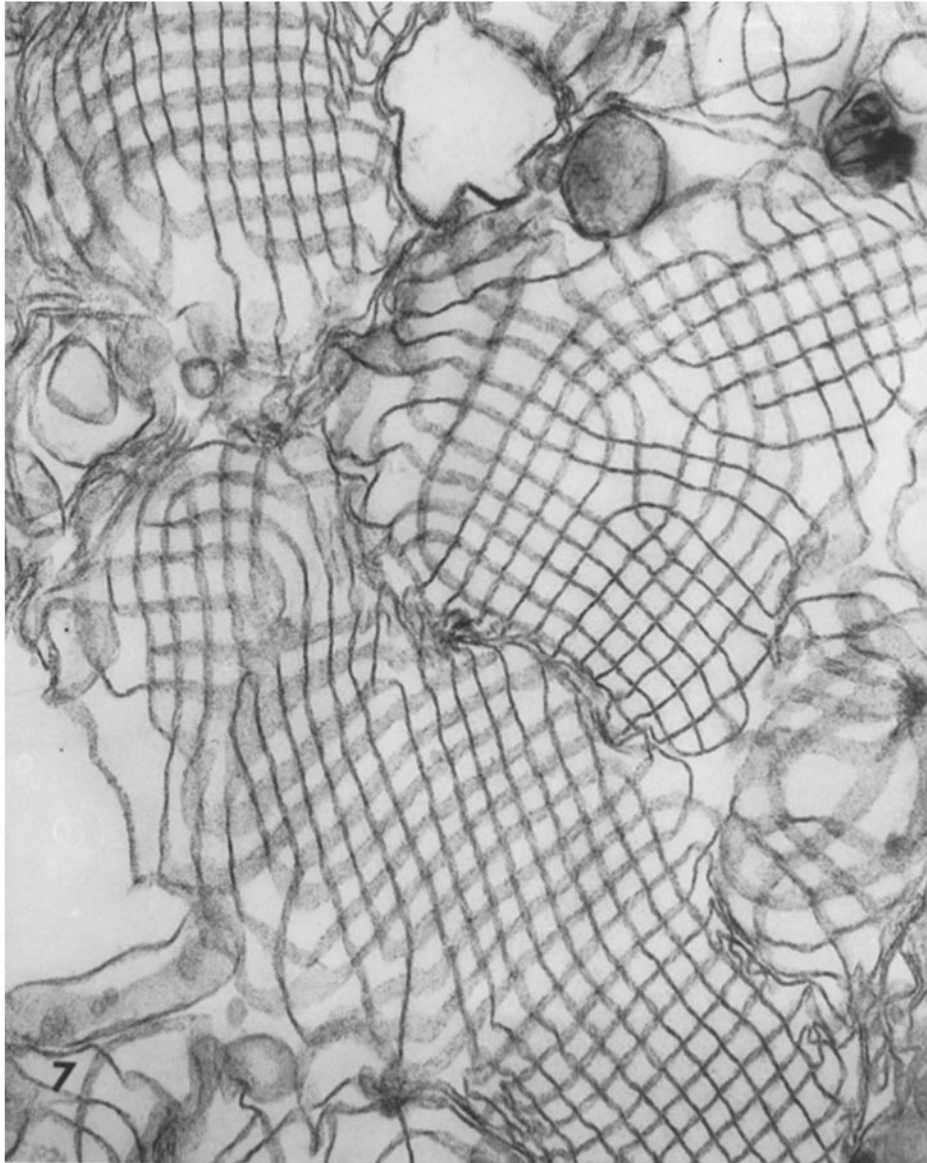


FIGURE 7 F IV (tubular myelin). Square lattice is relatively regular. Observe loss of sharpness due to changes in the orientation of structure. The osmiophilic wall of the square lattice consists of two leaflets. $\times 73,800$.

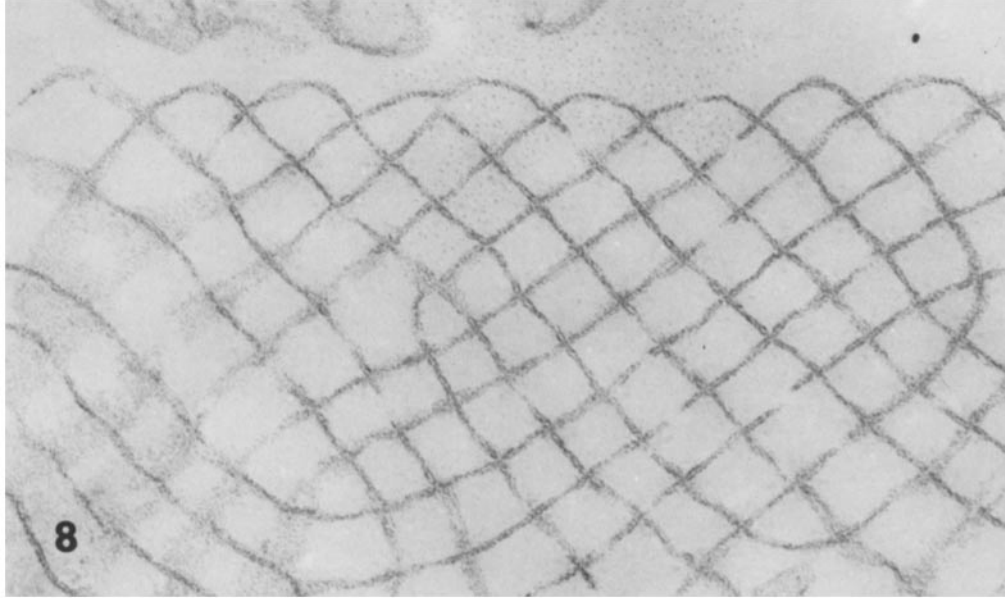


FIGURE 8 F IV (tubular myelin). In this picture, it is evident that many crosses are incompletely fused, resulting in irregularities. Observe the peculiar closing of the structure in the border. $\times 135,000$.

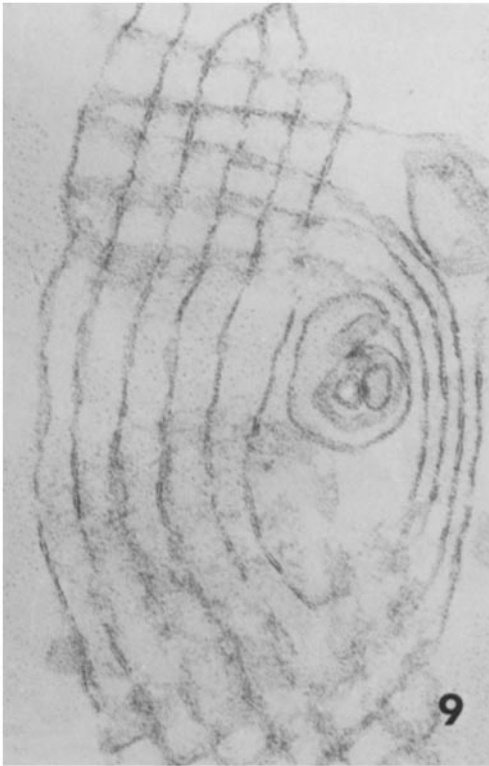


FIGURE 9 F IV. This electron micrograph shows a transition from the regular tubular myelin (left) to a structure of smaller periodicity (right). Transition lines are unsharp, possibly owing to torsion. $\times 108,000$.

in the perfused rat lung have been published (27, 30). The present observations confirm this view. Fig. 8 shows areas where the fusion between neighboring crosses is not complete. Furthermore it is apparent that the lines of a cross itself have distinct bilayer character while the dark lines connecting them usually do not show clearly this feature. Tubular myelin was originally described (21) to contain a filament of moderate electron density running inside the tubules. We have seen this feature several times but it was not frequent in our specimens.

Weibel et al. (21) also noted the existence in the alveolar space of other structures formed by concentric, possibly spherical shells of membranes with 27 nm spacing. A continuity between the tubular myelin and those concentric lamellae was observed. In our preparations (Fig. 9), we have found several times a transition from regular square lattice structures to a parallel system of smaller repeat period, although this was only of 23 nm or less.

FRACTION V: F V showed mainly tubular myelin figures, some dark, heterogeneous membranous materials, and some mitochondria. This fraction was not further studied.

FRACTION VI: F VI contained mitochondria, usually poorly preserved, probably due to the calcium ions present in the homogenizing medium.

The lipid from this fraction was isolated for comparative purposes.

B. Physical and Chemical Properties of the Fractions

Some of the chemical and physical properties of the lamellar bodies (F II), tubular myelin figure (F IV), common myelin figures (F I), and mitochondrial fraction (F VI), are listed in Table I. (Fractions III and V contained mixtures of different structures and were not analyzed.) The values of the phospholipid:protein ratios determined on the resuspended fractions before lyophilization were calculated from results of the phosphate analyses obtained by total digestion (milligrams of $P_i \times 25 =$ milligrams of lipid) and protein analysis with the phenol reagent. The isopycnic densities of the various fractions are shown in Table I. The fraction with the greater density has a smaller phospholipid:protein ratio.

The stability of fractions I, II, and IV was examined by applying aliquots on separate linearly continuous sucrose density gradients. Recentrifugation of each fraction leaves its characteristic isopycnic density unaltered, and no new bands appear. This indicates that each fraction is a stable aggregate of lipid and protein and that these lipoproteins can be isolated reproducibly.

In the foregoing experiments, all sucrose solutions contain 0.16 M NaCl, because we had established previously that in the absence of inorganic ions or in the presence of high concen-

trations of NaCl the alveolar surface-active lipoproteins, isolated by lung lavage, are unstable and tend to lose protein (37). For the same reason prolonged dialysis of the various fractions against water was avoided.

The fractions containing the lamellar bodies (F II), the tubular myelin figures (F IV), and the common myelin figures (F I) are surface active, while the mitochondrial fraction (F VI) is devoid of such activity. The differences in the observed surface tension-lowering properties of the surface-active fractions are not significant. Based on their lipoprotein content, the surface tension-lowering activity of these fractions is similar to that of the partially purified lipoproteins prepared from lung washes (36, 37).

The total dry weight of each fraction determined after lyophilization, the total lipid, and total water-insoluble protein after extraction of the lipids are listed in Table II. The data represent the material from 30 rat lungs. The recovery of total lipid plus total water-insoluble protein from each fraction ranges from 90 to 102% of the total dry weight. The lipid:protein ratio calculated from the isolated total lipid and total water-insoluble protein agrees well with the ratios obtained by colorimetric analyses of the fractions for phosphorus and protein recorded as phospholipid:protein ratio (Table I). Since we recovered over 96% of the P_i of the resuspended fractions before lyophilization in the total lipid after extraction, very little phosphorus was bound to the protein.

These data provide evidence that lipid and pro-

TABLE I
Physical and Chemical Properties of Rat Lung Homogenate Fractions

Fraction no.	Content of fractions	Phospholipid* ‡		Isopycnic densities‡	Surface activity§
		Protein			
			<i>g/g</i>	<i>g/ml at 20°C</i>	
II	Lamellar bodies	4.7 ± 0.40		1.059 ± 0.001	2.2
IV	Tubular myelin figures	2.0 ± 0.14		1.089 ± 0.005	2.1
I	Common myelin figures	8.7 ± 1.1		1.051 ± 0.001	1.6
VI	Mitochondria	0.36 ± 0.06		ND	>20.

* Total protein was determined with the phenol reagent (52). Total lipid was calculated from the inorganic phosphorus found after acid digestion (53, 54).

‡ Values indicated are averages of three experiments with their calculated standard errors.

§ Expressed as micrograms of lipoprotein required per square centimeters of surface area to reduce surface tension to at least 18 dynes·cm⁻¹. (For method of determination, see text.) Total lipoprotein was calculated from total lipid plus protein (see first footnote).

|| This fraction never yielded surface tension values below 21 dynes·cm⁻¹ even when large amounts of this material were tested.

TABLE II
Protein and Lipid Content of Rat Lung Homogenate Fractions

Fraction no.*	Content of fractions	Dry weight†	Total lipid	Protein‡	Lipid‡
					protein
		mg	mg	mg	
II	Lamellar bodies	1.24	0.96	0.19	4.7 ± 0.7
IV	Tubular myelin figures	2.03	1.19	0.66	1.5 ± 0.3
I	Common myelin figures	4.10	3.76	0.41	8.7 ± 1.1
VI	Mitochondria	152	ND	ND	

* For identification of fractions, see Fig. 1.

† All values were determined gravimetrically.

‡ The protein listed here is the water-insoluble protein as defined in the text.

|| Figures represent the average of three isolations with their standard errors.

TABLE III
Separation of Lipids from Rat Lung Homogenate Fractions by Chromatography on Silicic Acid

Fraction no.*	Content of fractions	Hydrocarbons and sterol esters	Triglycerides‡	Fatty acids‡	Cholesterol‡ §	Diglycerides‡	Polar lipids
			II	Lamellar bodies	0.8 ± 0.3	1.8 ± 0.5	2.8 ± 0.8
IV	Tubular myelin figures	<0.1	0.3 ± 0.2	1.6 ± 0.4	2.5 ± 0.5	0	95.6 ± 3.8
I	Common myelin figures	<0.2	2.2 ± 0.4	0.8 ± 0.3	1.7 ± 0.3	0.5 ± 0.3	94.5 ± 0.4
VI	Mitochondria	4.4	26.8	4.4	10.4	0	52.1

* For identification of fractions, see Fig. 1.

‡ Identified by thin-layer chromatography (42).

§ Cholesterol and diglycerides are eluted together from this column. The mixture was analyzed colorimetrically for cholesterol by adapting the procedure of Abell to a microscale (43). Diglycerides were calculated by subtracting the calculated weight of cholesterol from the total weight of the lipid eluted in this fraction (hexane:ether, 80:20). Thin-layer chromatography indicated that cholesterol and diglycerides were the only components of this fraction.

|| All values are the average of two determinations (except the analyses of the mitochondrial fraction) and are expressed as the percentage of the fraction of total weight applied to the column. The recovery of total weight from the column varied from 91 to 109% with an average of 97.1 ± 4.9%.

tein are the major components of these fractions and that they are therefore best described as lipoproteins. Crude surface-active preparations from lung have been shown to contain lipid and protein (65), and more recently similar results have been obtained from highly purified preparations (37, 66, 67).

C. Lipid Composition of the Fractions

The total lipid was separated into neutral lipid and polar lipid fractions by column chromatography on silicic acid. In all surface-active fractions (II, IV, I) over 90% of the lipid consists of polar lipids as compared with the mitochondrial

fraction containing 52% polar lipids (Table III). The cholesterol content of the surface-active fractions varies from 1.5 to 2.5% as contrasted with 10% in the mitochondrial fraction. The remaining traces of neutral lipids in the surface-active fractions (hydrocarbon and sterol esters, triglycerides, fatty acids, and diglycerides) are present in variable amounts in different preparations. It is not certain whether these lipids are components of the surface-active lipoproteins or trace contaminants. Some may be due in part to the action of hydrolytic enzymes. We should also point out that in the mitochondrial fraction the triglyceride and cholesterol contents are higher

than that reported for mitochondria from other organs (68).

The composition of the polar lipids from the various surface-active fractions was determined by two-dimensional thin-layer chromatography on silicic acid. The chromatographic patterns given by the polar lipids from the lamellar bodies, tubular myelin figures and common myelin figures were indistinguishable from each other and each had lecithins as the most prominent class of lipid. For quantitative analyses the spots were aspirated, digested, and analyzed for total P_i . Because of the small quantities available, phosphatidyl serines and phosphatidyl inositols were aspirated as one sample. The composition of the polar lipids of the various fractions is shown in Table IV. Phosphatidyl cholines make up over 70% of the polar lipid of the lamellar bodies (F II) with the sum of phosphatidyl serines and phosphatidyl inositols

accounting for 10% of the lipid P and phosphatidyl ethanolamines for 7%. Traces of other phospholipids are present at the origin and in the region next to the origin. Sphingomyelin is present only in variable trace amounts. The polar lipid fraction from the tubular myelin fraction (F IV) contains less lecithins (58%) and more phosphatidyl serines plus phosphatidyl inositols (19%) than the lamellar bodies. In addition, the recovery of P_i near the origin is greater. The polar lipid composition of the common myelin figures is identical with that of the lamellar bodies.

D. Fatty Acid Compositions of the Lecithins

In order to determine the fatty acid composition of the lecithin fractions, the polar lipids were separated by two-dimensional thin-layer chromatography as described previously, but the spots

TABLE IV
Distribution of Polar Lipids after Separation of Lipids by Two-Dimensional Thin-Layer Chromatography

Fraction no.	Content of fractions	Phosphatidyl cholines	Phosphatidyl serines and phosphatidyl inositols	Phosphatidyl ethanolamines	Origin	Other spots
II	Lamellar bodies	71 ± 5.6*	13 ± 2.0	7 ± 0.8	3 ± 0.9	6 ± 3.7
IV	Tubular myelin figures	58	19	5	4	16‡
I	Common myelin figures	76 ± 3.1	8 ± 0.2	9 ± 0.4	3 ± 2.2	4 ± 3.5

* All values are expressed as percent of total P_i recovered. They are the averages of two determinations except for fraction IV where only one determination was possible.

‡ 12% of this phosphorus was found near the origin but not on the origin. Compounds such as lysolecithins are found in this region.

TABLE V
Fatty Acid Composition of the Lecithins from the Surface-Active Fractions

Origin of lecithin	No. of determinations	C _{14:0}	C _{16:0}	C _{18:0}	Saturated fatty acids total	C _{16:1}	C _{18:1}	C _{18:2}	Unsaturated fatty acids total
<i>percentage of total fatty acids*</i>									
Lamellar bodies	4	6.4 ± 0.6‡	81.1 ± 3.6	4.0 ± 1.1	91.5	4.2 ± 0.3	4.2 ± 1.7	<0.2	8.4
Tubular myelin figures	2	6.6 ± 2.5	75.9 ± 2.8	4.0 ± 0.5	86.5	6.1 ± 0.9	7.3 ± 0.8	<0.2	13.4
Common myelin figures	4	6.2 ± 1.1	82.4 ± 0.4	2.1 ± 0.8	90.7	6.0 ± 0.5	3.4 ± 1.0	<0.2	9.4

* Calculated as follows: $\frac{\text{IU given by methyl esters of fatty acid}}{\text{sum of IU given by the methyl esters of all fatty acids}} \times 100$.

‡ Figures indicate averages and the calculated standard errors.

were visualized by spraying with ice-cold water. After aspiration of the lecithin-containing spots, the lecithins were eluted from the silicic acid with methanol-formic acid, saponified, and the fatty acids converted to the methyl esters. Table V shows the fatty acid compositions of the lecithins in the various fractions. Over 86% of the fatty acids of the lecithins from the three surface-active fractions are saturated. The lecithins from the tubular myelin figures are slightly less saturated (86%) than those of the other two fractions. The results from these analyses indicate that all the surface-active fractions contain large amounts of dipalmitoyl lecithin.

E. The Protein Compositions of the Fractions

In a previous communication it has been established that over 65% of the proteins of the surface-active lipoproteins isolated by tracheal lavage

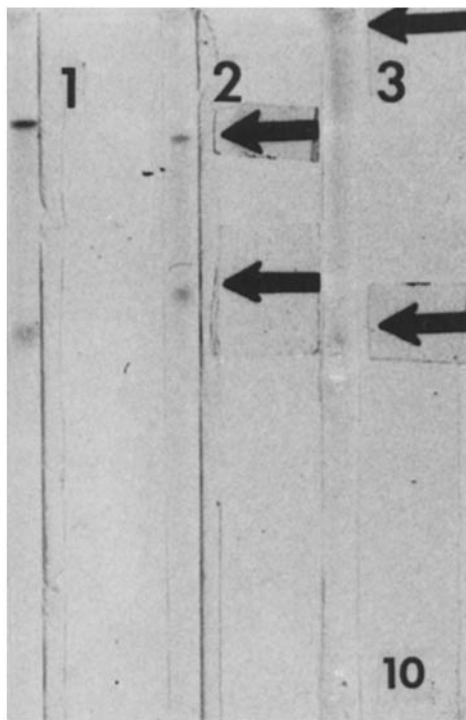


FIGURE 10 Separation of insoluble proteins on polyacrylamide gels containing Triton X-100. Gel no. 1 proteins of common myelin figures; no. 2 proteins of lamellar bodies, no. 3 proteins of tubular myelin figures. Arrows were used to point to bands, which are poorly visible after photography.

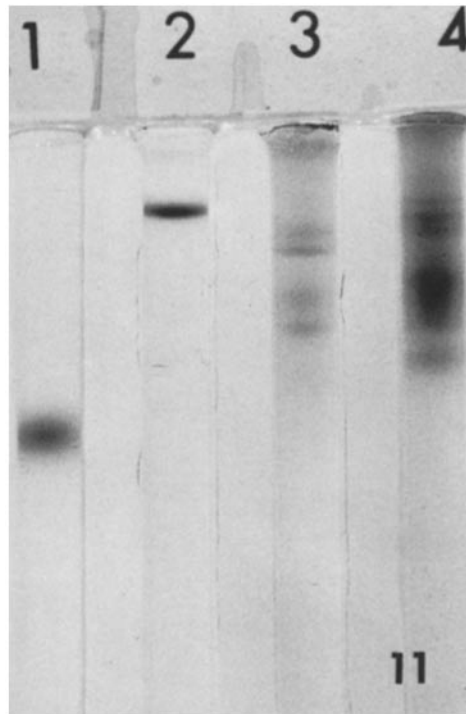


FIGURE 11 Separation of insoluble proteins on polyacrylamide gels containing SDS. Gel no. 1 insulin, no. 2 transferrin; no. 3 common myelin figures; no. 4 tubular myelin figures.

from lungs of rabbits can be recovered as water-insoluble proteins after lipid extraction (37). While it is not possible in the present preparations from rat lung homogenate due to the limited amounts of material available to achieve recoveries of equivalent accuracy, it appears that the sum of the lipid and water-insoluble protein can account for most of the dry weight of the surface-active fractions (Table II).

The proteins of all the surface-active fractions were separated by electrophoresis in polyacrylamide microgels containing Triton X-100 (Fig. 10). The pattern obtained from the lamellar body fraction differs from that of the tubular myelin fraction (gel no. 2 and 3). Neither the slowly (top) nor the rapidly migrating proteins found in the lamellar body fraction are present in the tubular myelin figure fraction. Instead the latter contains proteins migrating both slower and faster than the lamellar body proteins. The common myelin figures (gel no. 1) contain the slowly migrating protein present in the lamellar bodies and the

TABLE VI
*Apparent Molecular Weights of Proteins from the
 Lung Fractions by SDS Electrophoresis*

Band no.	Tubular myelin figures	Common myelin figures
1	196,000	—
2	98,000	—
3	56,500	55,000
4	—	41,000
5	23,000	24,500
6	15,500	15,900
7	9,550	9,200

The molecular weights were estimated by plotting the logarithm of the molecular weight against the observed mobility. The following proteins were used as standards: transferrin (mol wt 76,600), ovalbumin (mol wt 43,000), insulin (mol wt 5,770) (51).

rapidly migrating proteins of the tubular myelin fraction.

The molecular weight of the proteins of tubular myelin figures was determined in polyacrylamide gels containing SDS (Fig. 11, gel no. 4). In these gels the proteins are dissociated into subunits. Molecular weights of the bands can be estimated by simultaneously running proteins of known molecular weights (51) (insulin and transferrin gels are shown in gel no. 1 and 2). Six distinct bands can be seen with molecular weight ranging from 9,550 to 196,000 (Table VI). Some of the common myelin figures proteins (gel no. 3) are similar, while others are different (Table VI). Insufficient proteins from the lamellar bodies precluded their analysis in this system.

DISCUSSION

Only three of the six fractions isolated were studied in detail: the lamellar bodies, the tubular myelin figures, and the common myelin figures. These were the fractions that were morphologically pure and contained surface activity. The fraction containing the common myelin figures (F I) was studied because the physical characteristics and chemical composition of this fraction are similar to the surface-active fractions isolated from lung lavage of rabbit (37) and dog (66, 67). Electron micrographs of this fraction showed mostly osmiophilic structures that by morphological criteria could be interpreted as damaged lamellar bodies that had lost their limiting membrane and amorphous material; on the other hand, protein analyses indicated the presence of protein not

present in the fraction containing the lamellar bodies. This fraction, therefore in addition to damaged lamellar bodies contains other materials, such as the alveolar surface-active lipoproteins or possibly, the phagocytotic vesicles of alveolar macrophages, whose isolation has been recently reported (69). We conclude that material in this fraction was of mixed intra- and extracellular origin.

F II contained the lamellar bodies, which were identified by ultrastructural criteria. This was the only surface-active fraction clearly of intracellular origin. The surface activity exhibited by this fraction on the surface tension balance is probably due to disruption of the limiting membrane with subsequent spreading of the content of the bodies on the subphase. The lamellae of these bodies are heavily osmiophilic although Table V shows that they contained large amounts of fully saturated lecithins. This is surprising since it has been previously shown (70, 71) that pure saturated lipids *in vitro* are not osmiophilic. The preservation of the content of the lamellar bodies may vary considerably from species to species. After initial attempts with rabbit lungs, we decided in favor of rats only because the morphological preservation of this organelle was better in lungs from this species.

F IV contained tubular myelin figures. It is known that this structure is a frequent constituent of the lining layer of mammalian and amphibian lungs (27, 29) fixed by a method that preserves the integrity of the air-liquid interface. It was surface active (see Table I). Its chemical analysis showed basically the same qualitative composition but containing slightly less lecithins than the other preparations of surface-active lipoproteins (37). All these considerations together allow an identification of this material as surface-active lipoprotein. We favor at the present time the following interpretation: tubular myelin represents an aggregation of molecules at the alveolar surface, which may include some or all of the surface-active lipoprotein molecules. Being an obviously anisotropic fluid, tubular myelin may be classified as liquid crystal. This aggregation may be reversible, thus opening the possibility that it may be an intra-alveolar storage form of the surface-active molecules. Here again, the question of the surface activity of this fraction deserves a comment: tubular myelin, a three-dimensional structure by definition can hardly be imagined forming a thin

surface layer on a subphase. Deaggregation upon spreading will be necessary for the expression of a lowered surface tension. We always allowed 16 h between application of the specimen and actual measurements. Our homogenization medium was buffered with Tris, contained Ca^{++} , and was devoid of EDTA. It is noteworthy that tubular myelin never appears in homogenates if an EDTA-containing buffer is used. This would suggest that either Ca^{++} or some other cation strongly bound by EDTA is required for the formation of this structure.

Results of lipid analysis of all the fractions were generally in good agreement with the previously reported composition of alveolar surface-active lipoproteins (3, 36). The most prominent lipids are fully saturated lecithins, characteristic of the pulmonary surfactants, with smaller amounts of lecithins containing other fatty acids, phosphatidyl serines plus phosphatidyl inositols, phosphatidyl ethanolamines, and cholesterol present.

Dipalmitoyl lecithin, first isolated from lung by Thannhauser (72) was subsequently identified as one of the main components of surface-active lung extracts (73). It is believed that it is the principal substance responsible for lowering the surface tension of lung extracts (2, 3). All the lipids from the surface-active fractions contain a higher percentage of myristic ($\text{C}_{14:0}$) and palmitoleic acid ($\text{C}_{16:1}$) than is normally found in other tissues. The dog lung lipoproteins are also high in these fatty acids (66), and this may be a characteristic property of the various surface-active lipoproteins regardless of species. The presence of phosphatidyl glycerol in the pulmonary surfactant of beagle lungs has been reported (74). In the two-dimensional thin-layer chromatography system used in this work, this phospholipid is difficult to resolve from phosphatidyl ethanolamine. The small quantities of material available precluded further analyses.

The lipid composition of the common myelin figure and the lamellar body fraction was identical (Table III), whereas the composition of polar lipids differed slightly in the tubular myelin figures. The patterns of the three were clearly different from the lipid composition of the mitochondria. The proteins of the three fractions differed, however, considerably. The comparison of the composition of lamellar bodies and tubular myelin figures is of special interest, for it may provide some insight into the steps of the synthesis of

the alveolar surface-active lipoproteins. It is clear (Fig. 11) that tubular myelin contains proteins not present in the lamellar bodies. These findings, together with the observed decreased lipid:protein ratio (and increased isopycnic density of the tubular myelin figures compared to the lamellar bodies) suggest that lamellar bodies can be the sole intracellular source of the intra-alveolar lipids, but some proteins present in the tubular myelin (and in the common myelin figures) are added after secretion into the alveolar spaces. The latter proteins may originate in an intracellular site other than the lamellar bodies. Studies on synthesis turnover and transport of proteins in lung have been published (75-77). The most reasonable source for synthesis of export proteins in the lung would be the endoplasmic reticulum of type II alveolar cells. Autoradiographic studies of lung tissue (78) have shown heavy labeling of this organelle with L-[4,5- ^3H]leucine. We suggest that lamellar bodies secrete their content into the alveolar space; there it meets protein synthesized elsewhere, and the final assembly of the surface-active lipoproteins takes place. Whether the molecular components aggregate in an obligatory sequence or in a random fashion needs to be established in the future.

It has been suggested by Young and Tierney (79) on the basis of turnover studies that there are several pools of dipalmitoyl lecithin in the lung. Our studies seem to pinpoint at least in part the nature of the pools because one of the surface-active fractions, the lamellar bodies, is intracellular, whereas the tubular myelin figures were extracellular, and both contain large quantities of dipalmitoyl lecithin.

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