Morphological and lipid analysis of the alveolar lining material in dog lung

T. N. FINLEY, S. A. PRATT, A. J. LADMAN, L. BREWER, and M. B. McKAY

Departments of Medicine and Anatomy, The University of New Mexico School of Medicine, Albuquerque New Mexico 87106

ABSTRACT Endobronchial saline lavage was used to obtain acellular material and cells from the dog lung. The centrifuged lavage fluid yielded a sediment consisting of an upper white layer and a lower brown layer. The white layer was strongly surface-active. It consisted of a mixture of lipids and proteins; the composition of the lipid portion was the same in three dogs. The predominant lipids were phosphatidyl choline, cholesterol, and cholesteryl esters; 75–88% of the fatty acids in each phospholipid fraction were saturated.

Electron microscopy showed a strong morphological resemblance between the white layer and alveolar lining material in situ.

KEY WORDS endobronchial lavage · pulmonary surfactant · alveolar lining material · lung lipids · composition · fatty acids · phosphatidyl dimethyl ethanolamine · dog

THE NATURE OF THE INTERFACE between air and alveolar tissue has intrigued researchers for over 40 yr. In 1926, Terry (1) observed a free fluid on the alveolar walls of several mammalian species in vivo. However, it was not until the observations of Macklin (2) were published in the early 1950's that the existence of this fluid lining of the lung was generally recognized. Macklin studied frozen sections of fresh mouse lung with the light and phase microscopes and saw a thin line (approximate thickness 0.2 μ) at the interface between air and alveolar tissue. Since the material in this line had an affinity for Prussian blue, a characteristic of mucopolysaccharides, he designated it the alveolar mucoid film. Clements (3, 4) demonstrated marked surface activity of saline extracts of lung, and Pattle (5, 6) calculated extremely low surface tension values from stability indices of bubbles squeezed from cut alveolar surfaces. These authors suggested that normal alveoli are lined by a highly surface-active material. Subsequently, surface activity was found to be absent from the lungs of infants dying of hyaline membrane disease (7), a condition characterized by collapse of the alveoli. At present, the theory of alveolar stability assumes the presence of an alveolar lining film which tends to prevent alveolar collapse by markedly lowering surface tension as the alveolar surface area decreases. This lining film, which may be likened to an emulsifying agent that stabilizes bubbles of air in an aqueous phase, has been designated *pulmonary surfactant*.

Alveolar lining material has been demonstrated in situ by various methods of microscopy. A fluorescent material lining normal alveoli was observed by Bolande and Klaus (8) in guinea pigs. Craig (9) demonstrated lining material in normal human lungs by using fluorescent antibodies; this fluorescence was absent from lungs of infants dying of hyaline membrane disease. Alveolar lining material has been seen with the electron microscope, but not characterized, in the lungs of lambs (10), and in the lungs of rats (11–14). To date, this material has not been examined extensively after it has been recovered from the lung. In the present investigation samples of acellular material were collected by endobronchial lavage (15–17) from the lungs of dogs. This material was examined by means of the electron microscope and was compared with the alveolar lining material seen in lung biopsies. The acellular material obtained from such endobronchial lavage is morphologically similar to the alveolar lining material observed in situ; the material contains large amounts of highly saturated phosphatidyl choline and exhibits marked surface activity.

Abbreviation: TLC, thin-layer chromatography.

MATERIALS AND METHODS

Saline Lavage

Mongrel dogs were anesthetized with pentobarbital (30 mg/kg), and a 19F Metras retention catheter (No. 4250 from the American Rusch Corporation, New York 10011) with a cuffed tip was passed into an individual lower lobe. The cuff was inflated and three 100 ml aliquots of isotonic saline were gently instilled and withdrawn through the catheter. The returned fluid was immediately centrifuged at 27,000 *g* for 20 min at 0°C. After this procedure, the centrifuge tube contained a clear supernatant solution and a sediment consisting of an upper white layer and a lower brown layer (Fig. 1, left).

Surface Activity

The surface activity of the white layer of the spun lavage sediment from three dogs was measured by means of a modified Wilhelmy balance (4). Samples consisting of 0.1 ml of white layer material dispersed in 30 ml of saline were used. The trough of the balance was made of Teflon and lined by a continuous polyethylene film. The maximal surface area of the trough was 60 cm^2; the surface area could be reduced to a minimum of 10 cm^2 by a movable barrier driven linearly by a Harvard withdrawal and infusion pump. The cycle of compression and expansion lasted 20 min. Surface tension was measured by a platinum float suspended from a transducer; surface area was measured by means of a potentiometer attached to the drive mechanism. Both surface tension and area were recorded simultaneously and continuously by an *x–y* recorder.

Lung Biopsy

Dogs on which biopsies were performed were ventilated on a respirator while their chests were opened. An inflated peripheral portion of a lobe was clamped with a serrated sponge forceps and a tissue sample approximately 1 cm in diameter was removed. The tissue held in the forceps was plunged immediately in fixative (see below).

Preparation for Electron Microscopy

Biopsies. Lung biopsies from five dogs were fixed for 17–43 hr at 0–5°C. The fixative was prepared by the addition of 5 ml of stock 50% glutaraldehyde to 100 ml of 0.075 M sodium cacodylate–HCl buffer. The pH of the fixative was 7.2 and the osmolality, determined by freezing point depression, was approximately 300 milliosmols. After 2 hr fixation the biopsy specimens were removed from the sponge forceps, diced into 1–1.5-mm^3 pieces, and fixed for the remaining period of time. The samples were washed for 1–2 hr in 0.075 M sodium cacodylate–HCl buffer and then treated for 2 hr in 1% osmium tetroxide in 0.15 M Sörensen's phosphate buffer, pH 7.4. The material was then rapidly passed through 60%, 80%, 95%, 100% ethanol, for dehydration, and then through propylene oxide; the total time was about 20 min. The tissue was embedded in Epon 812 according to Luft (18). Sections were cut on a Porter–Blum MT-2 ultramicrotome and doubly stained with uranyl acetate and lead citrate.

Spun Lavage Sediments. Immediately after the lavage fluid had been centrifuged and the supernatant solution discarded, the white layer was pipetted off and placed in

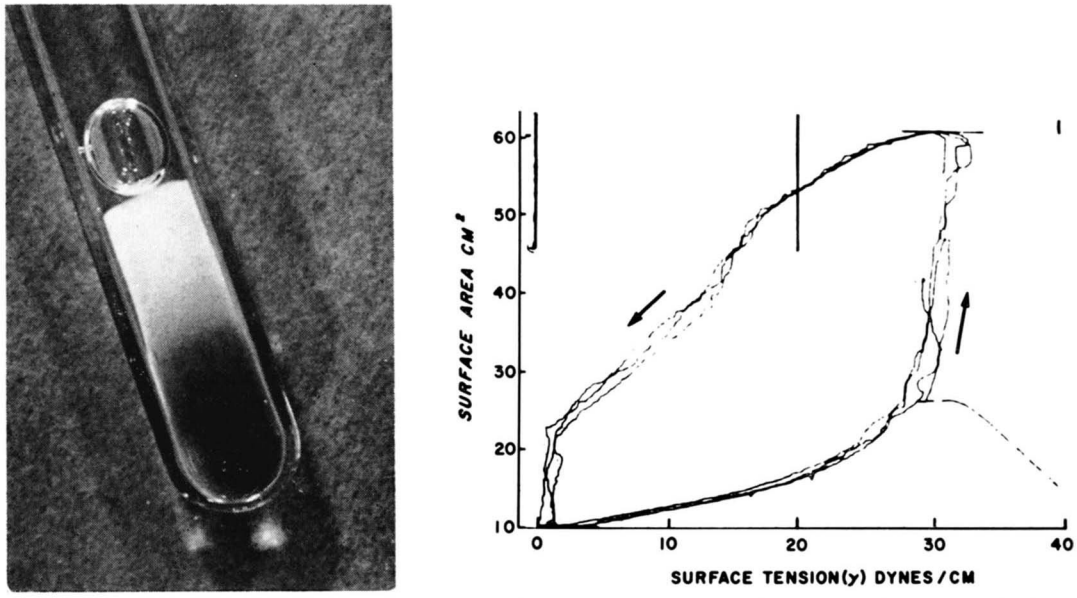


FIG. 1. Left, sediment obtained from a lavaged lung: it consists of a lower brown layer and an upper white layer. Right, typical surface tension–area diagram obtained with the white layer.

2-ml centrifuge tubes. It was prepared for electron microscopy with the same reagents and, so far as possible, by the same procedures as for the lung biopsy specimens. As each reagent was added, the white layer was disrupted by gentle agitation to insure the penetration of reagents and the tubes were then centrifuged for 20 min at 27,000 *g* to sediment the contents again. Because of the time involved in centrifugation, complete dehydration of the white layer took about 2 hr. Upon completion of both the fixation and dehydration procedures, the height of the white layer in the centrifuge tube was measured as an estimate of the amount lost during these manipulations.

Lipid Analysis

Each white layer was placed in a 500 ml separatory funnel and the lipids were extracted by three Folch washes (19) with chloroform–methanol–water 8:4:3. The chloroform portions of the washes were combined in a 500 ml flask, taken to dryness under vacuum, and stored in chloroform–methanol 5:1 at 0°C. Phosphorus and total lipid were determined on an aliquot of the chloroform portion of the Folch wash.

Thin-Layer Chromatography. Thin layers (250 μ) were made with a slurry of 30 g of Silica Gel H (20) in 58 ml of distilled water and washed by development with the solvent mixture (see below), usually overnight. They were activated at 100°C for at least 1 hr. Samples of the unknown lipid mixtures containing 10–20 μg of phosphorus were applied with a microsyringe. Standard solutions of phospholipids (Applied Science Laboratories Inc., State College, Pa.) containing 10–20 μg of phosphorus were also applied. Phospholipids were separated in chloroform–methanol–water–acetic acid 95:37:4:3; the neutral lipids moved with the solvent front. Neutral lipids were separated in petroleum ether-diethyl ether–acetic acid 90:10:1; the phospholipids remained at the origin.

Lipid fractions were detected by spraying with H_2SO_4 and subsequent charring at 400°C. Lipid fractions for fatty acid analysis were detected under UV light after the plates had been sprayed with 2,7-dichlorofluorescein. The identified spots were scraped off for further analysis (see below).

Analytical Procedures. Total lipid was determined by weighing a dried aliquot of the chloroform portion of the Folch washes. Total phospholipid was determined by quantitative analysis of phosphorus, using Bartlett's procedure of perchloric acid digestion (21). The weight of the total neutral lipids was calculated as the difference between the total lipid and phospholipid values. Phosphorus was also analyzed after charring and scraping of each phospholipid fraction obtained by TLC. Neutral lipid fractions separated by TLC were charred and measured on a Photovolt densitometer (22).

Lipid fractions from TLC were methanolyzed with methanolic H_2SO_4 refluxing at 100°C for 1.5 hr (23). Methyl esters, extracted in petroleum ether, were washed with distilled water, taken to dryness under pure nitrogen, and redissolved in 10 μl of hexane for chromatography at 210°C on a Perkin–Elmer gas chromatograph 800 with columns of 8% butanediol succinate polyester on Chrom W HMDS and a hydrogen flame detector.

RESULTS

Saline Lavage

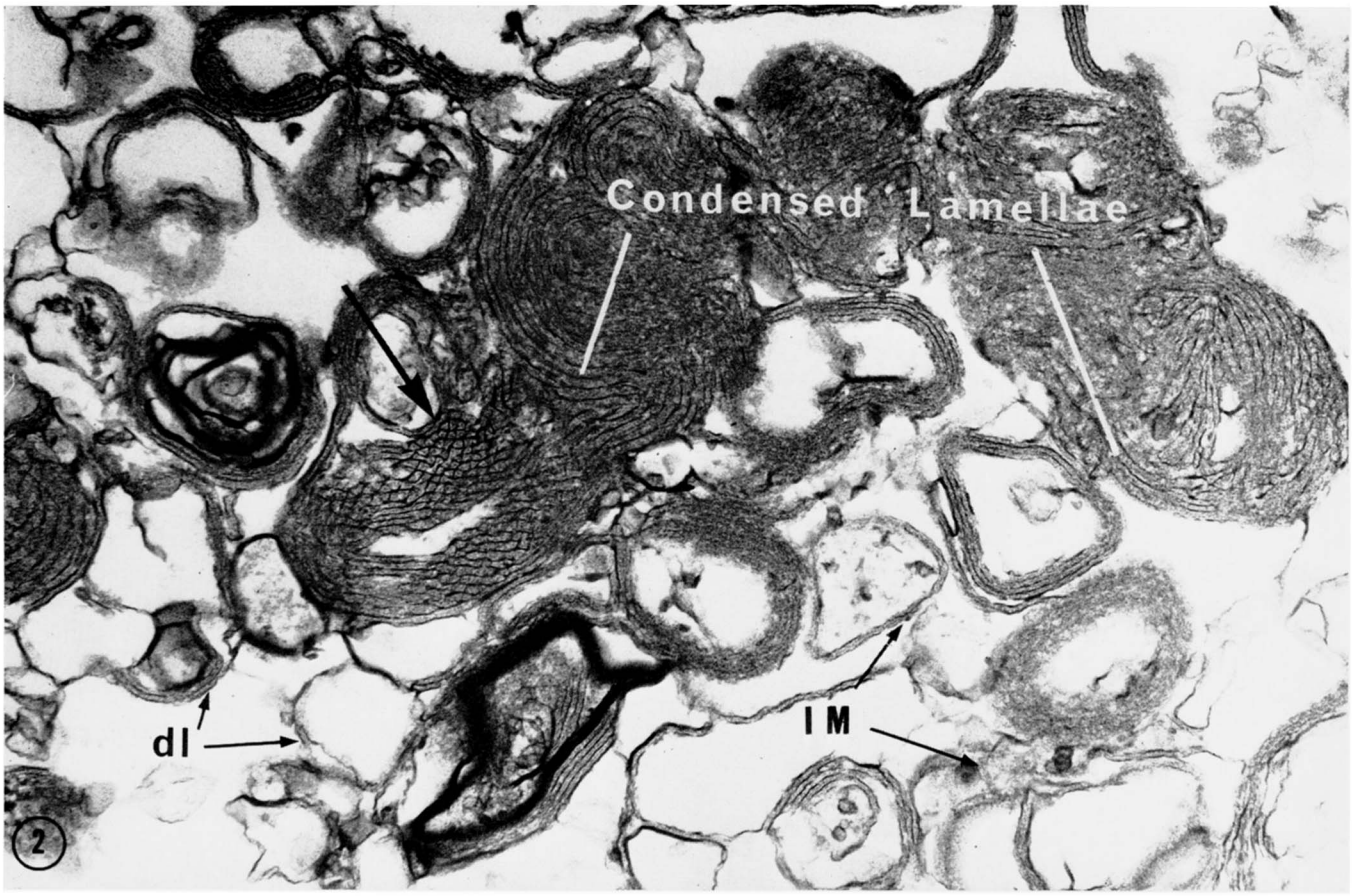
The average recovery of saline from lavaged lungs was approximately 90%. The average volume of the sediment, after centrifugation, was 0.3 ml, varying between 0.1 and 0.5 ml (Fig. 1, left). The white layer represented about half of the volume of the sediment and was flocculent and easily dispersed; the brown layer formed a densely packed pellet.

Surface Activity

The white layer showed remarkable surface activity. Surface tension reached a minimal value of 0–3 dynes/cm in each of the three white layers tested (surface tension of saline is 70 dynes/cm). Fig. 1 (right) shows a typical surface tension–area diagram in which surface tension decreased rapidly to 0 dyne/cm as the surface area of a film of white layer was reduced from 60 to 10 cm^2. As the surface area expanded the surface tension increased rapidly to 30 dynes/cm; the system exhibited marked surface tension–area hysteresis.

Electron Microscopy

The acellular material in the white layer shows at least two morphological forms (Fig. 2). One is represented in single sections by thin dense lines about 70–100 A thick. Many of the lines are roughly parallel to each other and form regularly spaced arrays. In one array, the distance from the center of one dense line to the center of the next is about 470 A (Fig. 3). Occasionally, two less dense and more irregular lines may be distinguished between the dense lines. Another array has a periodicity of 260–280 A and consists of alternating dense and less dense lines separated by light areas (Fig. 3). Often the regularity of the spacings is disrupted, or the lines of one array are continuous with those of the other (arrows with asterisk, Fig. 7). In some regions, lines of two arrays intersect and form irregular rectangles or squares containing material of low electron density (Fig. 4; arrow in Fig. 2). Arrays of the dense lines often occur as large whorls (Figs. 2 and 7). The presence of amorphous struc-



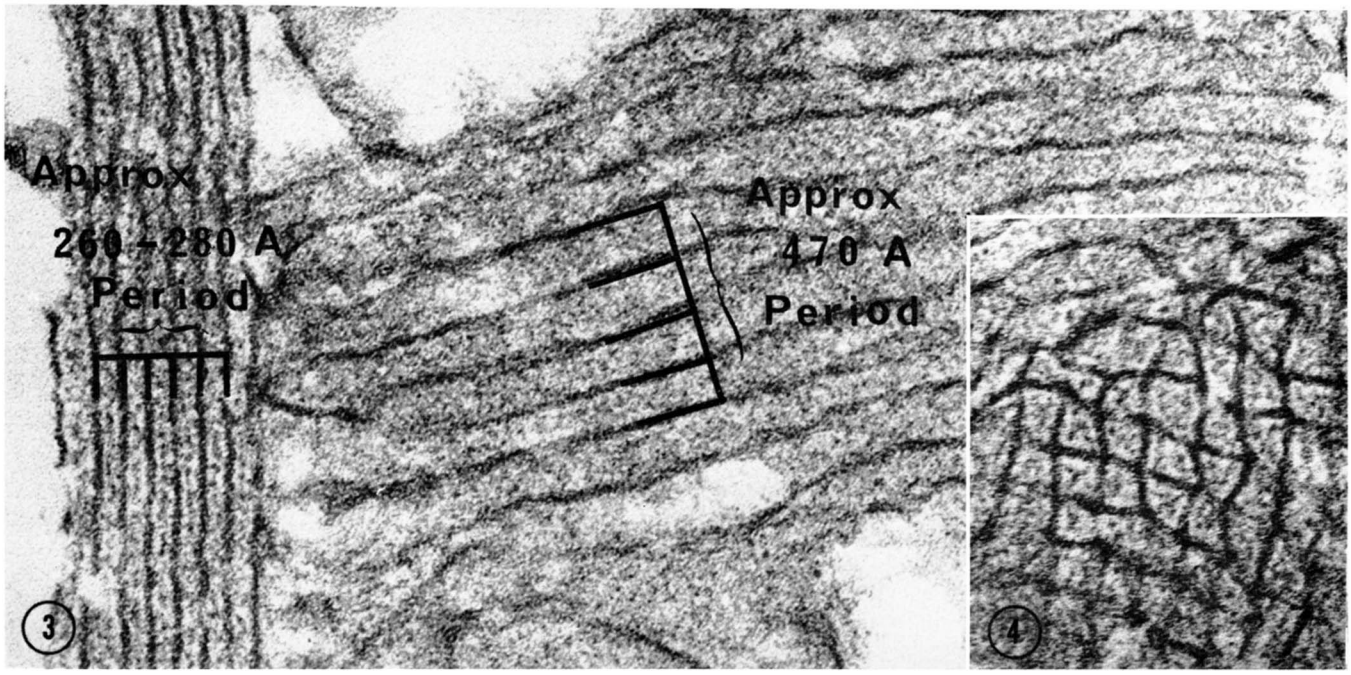


FIG. 2. Electron micrograph of a portion of the white layer. Thin lines, interpreted as the cut edges of lamellae, occur as large whorls Within these whorls, the lines are roughly parallel to each other or form irregular squares and rectangles (arrow). Outside of the whorls, irregularly oriented material (IM) resembling membranes occurs as dense lines of various thicknesses (dl) which often run into broader amorphous areas. × 27,000.

FIG. 3. Two different spacings observed in the white layer where thin lines are roughly parallel to each other. The lines are 70–100 A thick and the spacings are about 470 A and 260–280 A, respectively. Less dense material occurs between the thin lines. × 132,000.

FIG. 4. A region of the white layer in which thin lines form irregular rectangles and squares containing material of low electron opacity. × 130,000.

tures in some regions of the whorls and the absence of images which could be interpreted as transverse sections of filaments suggest that the lines represent lamellae cut on edge.

The second form observed in the white layer is an electron-opaque, somewhat irregular material (IM in Figs. 2 and 7). It is probably membranous since in some regions it appears as well-defined dense lines of various thicknesses (dl in Figs. 2 and 7). In other regions these lines blend into broader, less dense areas suggestive of obliquely cut membranes. Continuity between the thin dense lines of regular arrays (first morphological form above) and the irregular membranous material is sometimes observed (Figs. 2 and 7).

60–65% of the volume of the white layer was lost during preparation for electron microscopy; most of the loss occurred during dehydration in ethanol. The dehydration time could not be shorter than 2 hr since the white layer had to be resedimented by centrifugation between steps. The lung biopsy specimens were dehydrated as rapidly as possible (20 min) in an effort to preserve all of the white layer material in situ. Holes were observed adjacent to alveolar septa in sections of rapidly dehydrated lung tissue and may have been due to insufficient dehydration. However, their presence did not hinder observation of alveolar cytology.

The acellular material in biopsy samples usually lies next to and outside the alveolar epithelium (Figs. 5, 8–10). It is most frequently observed in regions where several alveolar septa meet or in depressions of thicker portions of the alveolar wall, and it is usually absent where the alveolar walls are thin and straight in the inflated lung.

The acellular material found on the alveolar surfaces is strikingly similar to the white layer morphologically (Figs. 5 and 7). Thin lines are arranged in regular arrays with spacings similar to those observed in the white layer. Irregular squares and rectangles are also commonly seen (arrow, Figs. 5 and 10). In addition, membranous material similar to that found in the white layer is closely associated with the regular arrays of thin lines (see Figs. 5 and 9). A third component, absent from the white layer, can be identified in the alveolar lining material. It is either a granular or fibrillar substance of low electron opacity which contains small aggregates of a more electron-opaque nature, possibly fragments of the membranous material (Figs. 5 and 8, and at higher magnification in Fig. 6).

Lipid Analysis

Lipids were determined in the white layers from each of three dogs. Table 1 shows that the predominant lipid was phosphatidyl choline. The fraction labeled "phosphatidic acid (?)" moved more slowly than the standard phosphatidic acid on TLC (Fig. 11). We were unable to isolate enough of this fraction for complete identification; however, it was found to be ninhydrin-negative.

TABLE 1 LIPID ANALYSES OF THE WHITE LAYER

Constituent	% of Total Lipids ± SD
Cholesteryl esters	9.4 ± 1.4
Triglyceride	5.4 ± 2.3
Free fatty acids	1.1 ± 1.1
Cholesterol	11.9 ± 5.7
Diglycerides	1.7 ± 1.9
Phosphatidic acid (?)	1.8
Phosphatidyl ethanolamine	9.7 ± 2.3
Phosphatidyl dimethyl ethanolamine	4.0 ± 1.0
Phosphatidyl choline	51.2 ± 3.0
Lysophosphatidyl choline, sphingomyelin	2.5 ± 0.6

Values are averages from three dogs except for that of phosphatidic acid, which is an average for two dogs.

Table 2 gives the fatty acid composition of the phospholipid classes from the white layer. The predominant fatty acid in each class was palmitic (16:0). The SD values for phosphatidyl choline show that the content of oleic acid was the most variable.

DISCUSSION

Recent studies (24) have shown that endobronchial lavage effectively removes pulmonary surfactant from the lung; the washed lung is devoid of surface activity. Until now there has been no proof that material recovered by the lavage actually originates from alveolar wall surfaces. The present study demonstrates that the material in the white layer of the lavage sediment is morphologically similar to alveolar lining material observed in lung biopsies.

TABLE 2 FATTY ACID COMPOSITION OF PHOSPHOLIPIDS FROM THE WHITE LAYER

	PA	PE	PDME	PC	LPC–SPH
			moles %		
14:0	2.4	1.7	1.0	4.7 ± 0.8	1.5
16:0	40.5	59.5	52.9	75.7 ± 2.1	49.2
17:0	5.4	—	tr.	tr.	1.0
18:0	34.0	20.4	24.3	7.4 ± 1.3	20.5
18:1	16.8	17.3	21.5	12.4 ± 4.1	16.6
18:2	—	—	tr.	tr.	1.0
20:0	—	—	—	—	1.8
20:4	tr.	0.5	tr.	—	6.7
Saturated	83	82	78	88 ± 3.7	75
Unsaturated	17	18	22	12 ± 3.7	25

Values are averages from three dogs except for PA and PDME which are averages from two dogs. SD values are given for phosphatidyl choline. Fatty acids are designated by chain length: no. of double bonds. PA, phosphatidic acid (?); PE, phosphatidyl ethanolamine; PDME, phosphatidyl dimethyl ethanolamine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline; SPH, sphingomyelin.

The most striking similarity of the white layer and alveolar lining material in situ is the presence of regular arrays of thin lines. The spacings observed in regions of the white layer appear to be the same as those observed in similar regions of the lining material in situ. Squares and rectangles occurred in both the white layer and the lining material in situ. Other workers (11, 13, 14) have examined in detail similar material *in* lung, but the only comparable investigation of the material extracted *from* lung is that of Mendenhall and Sun (25). They observed parallel lines with a repeating period of 95 A in bubbles squeezed from lungs; the difference in periodicity from ours may be due to differences in preparation techniques.

Another similarity of the white layer and alveolar lining material in situ is the presence in both of membrane-like, irregularly organized structures. In some regions, this material resembles parts of "lamellated bodies" present in granular pneumonocytes, which some workers consider to be the cellular source of pulmonary surfactant (2, 26, 27). However, our evidence does not permit an unequivocal identification of these irregularly organized structures as "lamellated bodies." The continuity between the lines of regular arrays and the membranous material, observed both in situ and in the white layer, suggests that the material that gives rise to these images can occur in several forms. This is consistent with the experience of others who have worked with lipid systems in vitro (28).

Although many reports on the fine structure of lung have appeared, only a few papers have demonstrated substances which might be regarded as the alveolar lining material in situ. Only a small amount is usually found in normal lungs (11). The fixation process cannot be expected to precipitate the material irreversibly, for 60–65% by volume of the white layer was lost in our dehydration procedure with ethanol. Moreover, Morgan and Huber (29) reported a loss of lipid material after fixation and dehydration of lung biopsy specimens. We therefore dehydrated the biopsy material rapidly, and this may have preserved more of the lining material that would usually be extracted by solvent action over longer periods of time. Permitting the biopsy to remain in the clamp during fixation might also have been a factor in preserving the lining material in situ.

Alveolar lining material was usually observed in depressions of the alveolar wall. It was not seen along the segments of the alveolar wall that were thin and attenuated in the inflated lung, possibly because of loss during dehydration, or else because the material forms a discontinuous layer when the lung is inflated and spreads over the alveolar walls as the lung deflates.

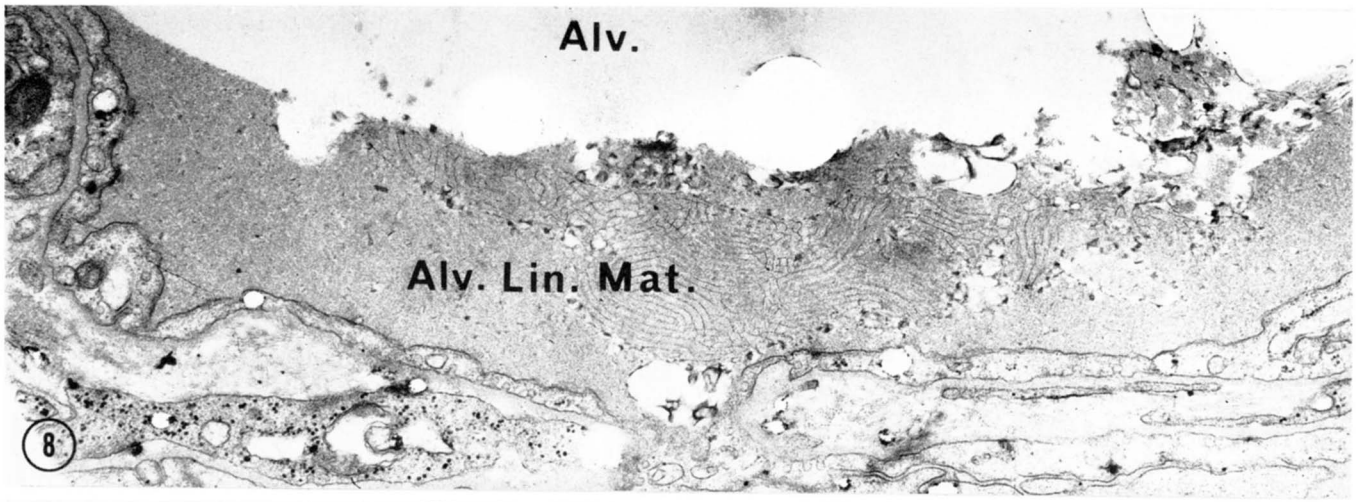
The lipid analyses of white layers from three different dogs (Table 1) are remarkably similar with respect to the distribution of the various lipid components. This suggests that there is (are) present one or more specific substances whose production and removal is (are) well regulated. There have been no previous analyses of "white layers," but our results resemble those reported (16) for a supernatant fraction obtained by centrifugation of lavage fluid at 2000 *g* for 30 min. This procedure sedimented a brown layer but presumably left the white layer material in the supernatant fraction. The percentages reported for phosphatidyl choline, ethanolamine, and dimethyl ethanolamine were 54.8, 6.5, and 5.4% of total lipids; cf our 51.2, 9.7, and 4.0%, respectively. The fatty acids of the white layer phospholipids (Table 2) are more saturated than those of supernatant phospholipids, but this may be due to methodological differences since, in the previous study, phospholipids were separated by column chromatography rather than TLC, and in TLC up to 10% of the unsaturated phospholipids may remain on the silica gel (30).

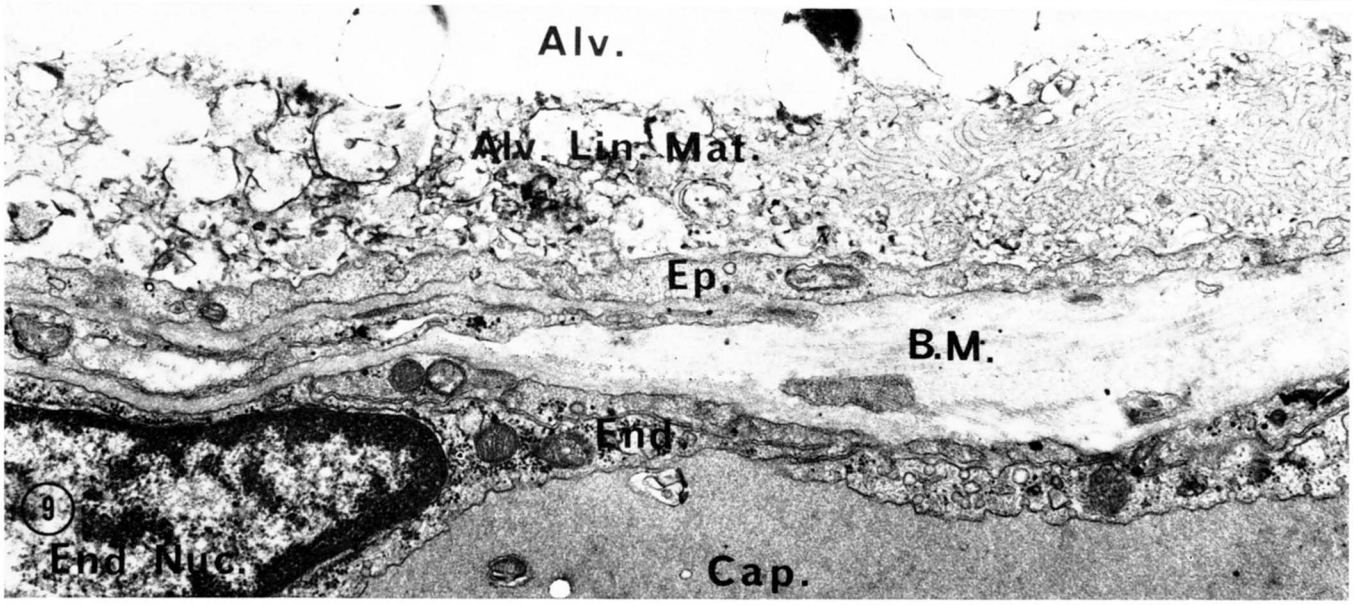
Granular or fibrillar material constituted part of the alveolar lining observed in lung biopsies, but was apparently absent from the white layer. Possibly it was lost during the lavage procedure or during dehydration with ethanol, or else it was too finely dispersed in the white layer pellets to be recognized. Its presence in lung biopsies may support the suggestion of Groniowski and Biczyskowa (31) that the lining material is a composite of substances. These authors, using a Hale technique modified for electron microscopy to localize mucopolysaccharides, found a precipitate over granular material in lung biopsies. Although they observed only the granular material in their study, they suggested that the alveolar lining material is a mixture of lipids and mucopolysac-

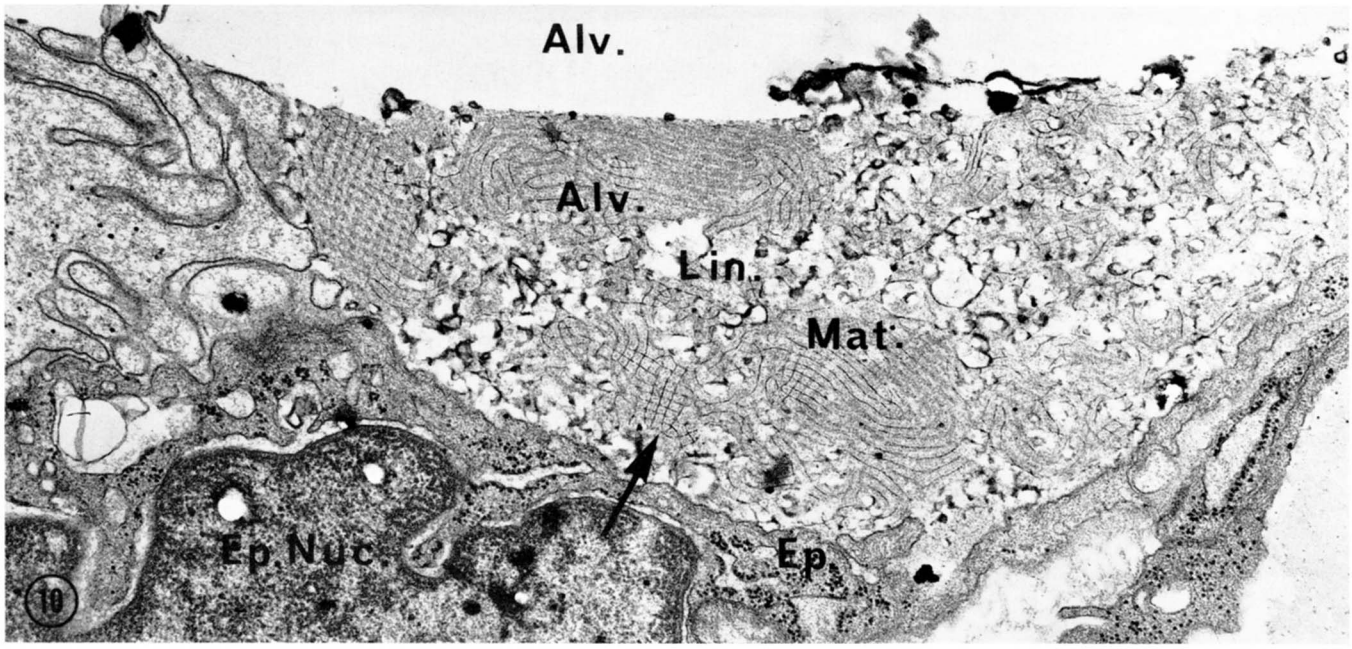
FIG. 5. A section of a biopsy sample containing part of an alveolar septum with acellular material at its surface. The epithelium of the septum is formed by the attenuated cytoplasm (Ep.) of squamous cells whose nuclei were not included in this section, and by a granular pneumonocyte. The epithelium rests on a basement membrane (B.M.). An endothelial cell (End.) containing a large nucleus (End. Nuc.) outlines the lumen of a capillary (Cap.). The acellular material (Alv. Lin. Mat.) shows three forms: a granular substance containing small aggregates of dense material, irregularly oriented membranous material, and thin lines (arrow), interpreted as lamellae, arranged in parallel and in rectangular and square arrays. × 17,000.

FIG. 6. A high magnification view of the irregular membranous material and the granular or fibrillar substance at the surface of an alveolar wall. Small aggregates of dense material are dispersed in the granular substance. The granular substance was apparently absent from the white layer of the lung lavage fluid. × 35,000.

FIG. 7. A micrograph of the white layer at a similar magnification as for the biopsy specimens shown in Figs. 5, 8, and 9. The spacings of the thin parallel lines changes abruptly in one region (arrows with asterisks), where two different arrays meet. Irregularly oriented membranes are also present (IM) and have been cut on edge in some regions (dl). × 16,500.







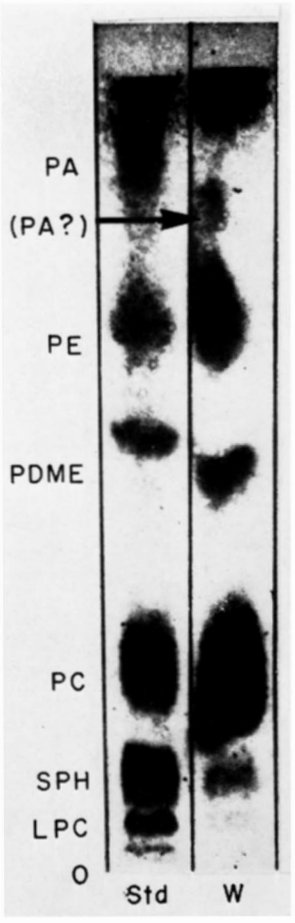


FIG. 11. Thin-layer chromatogram of the white layer (W) phospholipids with the appropriate reference compounds (Std). Silica Gel H, chloroform–methanol–water–acetic acid 95:37:4:3. PA, phosphatidic acid (General Biochemicals, Chagrin Falls, Ohio); PE, phosphatidyl ethanolamine; PDME, phosphatidyl dimethylethanolamine (Mann Research Labs. Inc., New York); PC, phosphatidyl choline; SPH, sphingomyelin; LPC, lysophosphatidyl choline; O, origin.

charides. Macklin (2) originally named the alveolar lining material "alveolar mucoid film" on the basis of staining with Prussian blue, a stain specific for mucopolysaccharides. The nature of the granular or fibrillar material observed in the present study remains unknown, but studies are now in progress to identify it by means of electron microscopic histochemistry.

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FIG. 8. Acellular material lining the alveolar epithelium (Alv.) in a lung biopsy sample. To the left and far right, the material consists of a granular substance. In the center, thin lines or lamellae occur in regular arrays, either as parallel lines or, in a few places, as squares and rectangles. × 17,000.

FIG. 9. Alveolar lining material (Alv. Lin. Mat.) in an alveolar space closely associated with the epithelium (Ep.). The lining material at the left consists mostly of irregularly oriented membranes. At the right, more regularly arranged thin lines or lamellae are present. End., endothelium; End. Nuc., endothelial nucleus; Cap., capillary; BM, basement membrane. × 17,000.

FIG. 10. A depression in an alveolar wall (Alv.) filled with alveolar lining material (Alv. Lin. Mat.). Regular arrays of parallel lines are mixed with less regularly arranged membranous structures. In some regions, the thin lines form squares or rectangles (arrow). Ep., epithelium; Ep. Nuc., epithelial nucleus. × 25,000.