Characterization of lung surfactant: factors promoting formation of artifactual lipid–protein complexes

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Abstract Because uncertainty exists as to whether dipalmitoyl phosphatidylcholine, the major component of lung surfactant, is part of a lipoprotein molecule, a study was designed to investigate the relationships between the phospholipids and proteins of rabbit lung washings obtained by lavage with aqueous solutions. Surface-active sediments contained phospholipid and protein in ratios directly dependent upon the ratios in the washings from which they were obtained. Comparison of negatively stained lung washings and sediments revealed that sedimentation caused extensive aggregation of surfactant "liposomes." Analytical ultracentrifugation revealed that both cell-free washings from lungs and suspensions of pure phosphatidylcholine contained components with flotation rates ranging from 25 to 400. Density gradient centrifugation of washings, without prior sedimentation, resulted in the appearance of a phospholipid band associated with only a small amount of protein. The density of the band varied depending upon temperature. No qualitative differences in the protein compositions of the phospholipid band and other gradient fractions were found by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Albumin, IgG, IgM, and several nonplasma proteins were present. These results indicate that little, if any, protein is specifically attached to the phospholipids of lung surfactant; rather, the lipid–protein complexes of lung washings are the result of a nonspecific association caused by removal of water-soluble surfactant from the lung and promoted by sedimentation.

Supplementary key words lung surfactant purification · pulmonary surfactant · dipalmitoyl phosphatidylcholine

Although most investigators agree that dipalmitoyl phosphatidylcholine (DPPC) is the major substance lowering surface tension at the alveolar tissue–air interface, several uncertainties remain concerning the exact chemical composition and physical properties of the pulmonary surface-active material. Many researchers have suggested that native surfactant is a lipid–protein complex (1–8). Others were unable to identify distinct lipoproteins in lung washings (9, 10) and proposed that the association of proteins with lipids in surfactant fractions may occur during the preparation of pulmonary washings (11). The results of two other groups of investigators have led them to the conclusion that lung surfactant is a mixture of lipids containing no protein (12, 13).

The concept that lung surfactant is a lipoprotein is based largely on the observation that lipid and protein are found together in fractions obtained by centrifugation of lung washings or lung homogenates. However, the lung surfactant "lipoproteins" isolated by different researchers vary widely in composition, especially with respect to the amount of protein present. Although several recent studies have reported the presence of nonplasma proteins in lung surfactant preparations (14–17), it is not yet clear whether the proteins washed from the lung by alveolar lavage are components of the lining layer or are largely contaminants from the blood and tracheobronchial secretions (18, 19).

The choice of a method for obtaining material for the biochemical characterization of lung surfactant poses a difficulty. Surfactant can be recovered by lavage of the lung with aqueous solutions, and this probably yields a purer preparation of the material from the alveolar surface than can be obtained from tissue homogenates after extensive purification. However, because the alveolar surface is separated from the capillary blood by only a very thin layer of tissue over most of the area of the lung, the possibility that lung washings may contain material from plasma cannot be excluded. A second problem in the characterization of lung surfactant, as pointed out by Clements

Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; PAGE, polyacrylamide gel electrophoresis; PL, phospholipid; RLS, Ringer's lactate solution; SDS, sodium dodecyl sulfate.

[1] The data forming the basis of this paper were taken from a dissertation submitted by Sue A. Shelley to Loyola University of Chicago, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1974.

(20), is that the physical properties of lung surface-active materials severely limit the methods that may be utilized for their isolation.

An understanding of the exact molecular nature of lung surfactant is important in order to determine what procedures are appropriate for the study of the synthesis and degradation of lung surfactant in normal conditions as well as to understand alterations in the turnover, chemical composition, and physical state of surfactant in various pathological states. This report describes the results of an investigation concerning the influence of various factors on the nature of the lipid–protein complexes obtained from lung washings by several methods of centrifugation. A preliminary report of the results has previously appeared (21).

MATERIALS AND METHODS

Lung washings were routinely obtained from male New Zealand white rabbits, weighing 1.4–1.6 kg, by alveolar lavage with Ringer's lactate solution (RLS) following a standardized procedure previously described (22). In some experiments, washings were obtained with RLS chilled to 4°C before usage or by lavage with a Tris buffer solution either at room temperature or at 4°C. Tris-buffered saline, pH 7.35, containing calcium and magnesium, was prepared as described by King and Clements (6).

The washings were centrifuged for 5 min at 480 *g* to remove the cells. In some experiments, the lung washings were then separated into sedimentable and nonsedimentable fractions by centrifugation of the noncellular washing at 127,000 *g* (max) for 90 min (22). Sedimentations for shorter times and at lower speeds were also utilized. Other methods of differential centrifugation, including flotation on NaBr solutions, were used in individual experiments for specific purposes that are described later.

In some instances, lung washing sediments obtained by centrifugation were subjected to short periods of ultrasonic irradiation, a method that has frequently been used to disperse insoluble lipids in aqueous media. Sonication was carried out using a 20-kilocycle Branson model S75 Sonifier at power level 4. Samples were placed in an ice bath and sonicated for 30-sec intervals spaced a minute or longer apart in order to prevent overheating. Samples were sonicated for 1, 3, or 10 min.

The dynamic surface tension properties of the pulmonary lavage fluids and of the various fractions obtained from these fluids were measured in a modified Langmuir-Wilhelmy surface balance as described by Greenfield and Kimmell (23). Aliquots of lung washing fractions containing 200–500 μg of phospholipid were gently layered onto the surface of RLS in the trough. The surface film was allowed to "age" for 10 min at 22°C before starting the compression and expansion cycles. A cycling time of 3 min was used. The cycling was continued until no change was observed in three consecutive cycles. The fractions were considered surface active when the minimum surface tension was reduced to 5 dynes/cm or less.

Lipids were extracted from lung washings and lung washing fractions using chloroform–methanol 2:1 (24). The phospholipid content of the lipid extracts was determined by measuring the phosphorus content after digestion of the lipids with perchloric acid (25). The amount of phospholipid was calculated on the assumption that the phospholipids present contained an average of 4% phosphorus.

The protein concentrations of the samples were measured using the method of Lowry et al. (26). Standard protein solutions were also prepared with sodium dodecyl sulfate (SDS), as well as with the various solutions in which some fractions of lung washings were obtained, to determine whether these substances would interfere with the protein determination. SDS, at 0.5%, did not change the absorbance at 700 nm obtained with the protein standard. Likewise, up to 12% NaBr in RLS with or without 0.5% SDS did not affect color development in the Lowry protein determination. Sucrose, in either RLS or the Tris buffer used in some gradients, and NaBr in the Tris buffer, did interfere with the protein determination. Therefore, fractions containing these materials were dialyzed before protein determinations were made. More than 95% of the protein present in the lung washings used to prepare the gradients was recovered in the gradient fractions after dialysis.

The proteins of lung washings and of lung washing fractions obtained by centrifugation were characterized by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS. Details of the procedure used in this study were essentially those described by Weber and Osborn (27), except that 2-mercaptoethanol was not used. Lyophilized samples of lung washings or fractions obtained from lung washings were dissolved in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% SDS, and incubated at 37°C for 2 hr. For each gel, 1 drop of tracking dye (0.01% pyronin Y in distilled water), 1 drop of glycerol, and 50 or 100 μl of the protein solution were mixed in a small test tube and then applied to the top of the gel. After electrophoresis and staining, a densitometric tracing of each gel was made. Immunoelectrophoresis was carried out on cellulose acetate membranes as described by Schwartz (28).

Analytical ultracentrifugation was carried out in the manner commonly used for serum lipoproteins (29). Fractions prepared from lung washings by flotation or sedimentation were adjusted to a density of 1.21 by addition of NaBr in RLS. Centrifugation was carried out in an An-D rotor at 20°C in a Spinco model E ultracentrifuge.

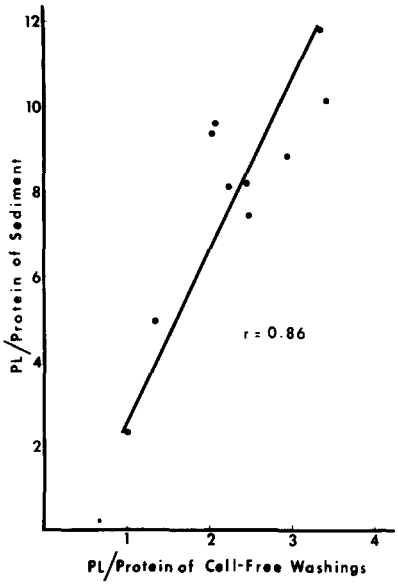

Fig. 1. Relationship of the PL/protein ratio of the sedimentable fraction to the PL/protein ratio of the cell-free washings. Sediments were obtained by centrifugation of the cell-free washings at 127,000 *g* (max) for 60 to 90 min.

Density gradient centrifugation was done using continuous gradients of NaBr in RLS. At the beginning of the run, the sample was spread unevenly throughout the tube, being most concentrated at the bottom. This was done by adding the sample to the "heavy" solution in the gradient mixing apparatus. The gradients were centrifuged in an SW 25.2 rotor at 90,000 *g* (max) in a Spinco model L2 ultracentrifuge at 20°C for 18 hr. In other experiments, sucrose was used in place of NaBr and Tris buffer, pH 7.35 (6), in place of RLS, and centrifugation for 18 hr as well as for shorter periods of time was carried out at 4°C instead of 20°C. Fractions were collected from the bottoms of the tubes after puncturing with a needle held in a plastic cylinder designed to hold SW 25.2 tubes.

For electron microscopy, small aliquots of washings and of washing fractions obtained by centrifugation were mixed with equal volumes of 2% sodium phosphotungstate, pH 7.4. A droplet from each of the above mixtures was placed on a Formvar–carbon-coated grid, and after removal of the excess fluid with filter paper, the negatively stained preparation was immediately examined in an RCA-EMU-3G electron microscope.

RESULTS

Characterization of sedimentation fractions of lung washings

The first step generally used in the isolation of surfactant lipid–protein complexes is the centrifugation of cell-free washings in such a manner as to obtain a surface-active sediment. This sediment contains both lipid and protein, suggesting an interaction between the lipids and proteins of the washings. However, large variations in the relative amounts of phospholipid and protein found in such sediments have been reported (3, 22). For this reason, a systematic study of the phospholipid and protein contents of both cell-free washings and surface-active sediments was undertaken.

TABLE 1. Phospholipid and protein content of cell-free rabbit lung washings

	μg PL/ml	μg Protein/ml	PL/Protein
32 washings	346 ± 112[a]	234 ± 72	1.57 ± 0.61
	(140–624)[b]	(119–424)	(0.49–3.42)
14 washings with no RBC visible in pellet	328 ± 100	212 ± 66	1.64 ± 0.64
	(167–551)	(119–364)	(0.99–3.42)
8 washings with few RBC visible in pellet	338 ± 68	222 ± 26	1.61 ± 0.46
	(224–477)	(165–315)	(0.79–2.07)
10 washings with many RBC visible in pellet	376 ± 148	274 ± 78	1.44 ± 0.57
	(140–624)	(165–424)	(0.49–2.99)

[a] Standard deviation.
[b] Range of values.

The phospholipid and protein contents of 32 cell-free rabbit lung washings were determined, and the phospholipid to protein (PL/protein) ratios were calculated. The results as shown in **Table 1** demonstrate a wide variation in the amounts of both phospholipid and protein present in lung washings obtained from rabbits by the standard method of alveolar lavage. The content of phospholipid and protein could have been influenced to some degree by the size of the rabbits and the care with which the lavage material was recovered. However, neither of these factors should have altered the PL/protein ratio. Normal lung washings often contain some blood, as evidenced by a red color and the presence of red blood cells in the pellet of alveolar macrophages. Although washings that appeared grossly red were not used in these experiments, some washings that were white gave a cellular pellet containing red blood cells. Therefore, the data from these 32 lung washings were divided into three groups on the basis of qualitative observations as to whether the cellular pellets contained no, few, or many red blood cells. The differences in phospholipid content and PL/protein ratios among these groups were not statistically significant. The mean protein content of the samples yielding a pellet with many red blood cells was significantly greater than that of samples giving a pellet with no red blood cells ($P < 0.05$). However, even in samples in which no red blood cells were observed in the cellular pellet, the PL/protein ratio varied from 0.99 to 3.42, or more than a threefold range.

Sediments were obtained by centrifugation of 10 of the cell-free washings for 60–90 min at 127,000 *g* max, and the data shown in **Fig. 1** indicate that the PL/protein ra-

tios of the sediments were dependent upon the relative amounts of phospholipid and protein present in the cell-free washings from which they were obtained. The sediments obtained from cell-free lung washings with PL/protein ratios ranging from about 1/1 to 3.5/1 had PL/protein ratios varying from about 2/1 to 12/1, with the ratio in the sediment increasing in proportion to the increasing ratio in the washings. In addition, in some extreme cases, it was found that a mixture of lung washings and serum having a PL/protein ratio of 0.07 yielded a sediment with a PL/protein ratio of 0.17. At the other extreme, a fraction from a density gradient centrifugation of lung washings that had a PL/protein ratio of 8.7 yielded a sediment with a ratio of 18.4. Other experiments indicated that neither the speed of centrifugation nor the length of time of centrifugation influenced the PL/protein ratio of the resulting sediment. These results suggest that much of the protein found in the sediment is nonspecifically associated with the phospholipid.

Although the supernate remaining after removal of the sedimentable fraction from lung washings is not surface active, it was found to contain surface-active material. The supernates remaining after centrifugation of lung washings at 40,000 *g* for 1 hr were concentrated by dialyzing against distilled water, lyophilizing, and resuspending in 1/10 the original volume of solution. A cloudy suspension was formed that was surface active and that, on further centrifugation, yielded a clear supernate and a pellet of surface-active material. These findings reveal that sedimentation of lung washings results in separation of surface-active particles on the basis of difference in sedimentation rate, resulting in a loss of some of the surfactant in the supernate.

The nature of the protein present in the cell-free lung washings and in the sediment and supernatant fractions was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. A densitometric tracing of a gel containing cell-free washings is shown in **Fig. 2,** *a*. For comparison, a tracing of a gel on which rabbit plasma had been separated is shown in Fig. 2, *b*. Comparison of the cell-free washing proteins and plasma proteins on SDS–PAGE reveals that, in addition to bands corresponding to the plasma proteins, rabbit lung washings contain several bands of protein not seen in the plasma. The largest band of protein present in the cell-free washings had a mobility similar to that of albumin. Immunoelectrophoretic studies revealed reaction of the cell-free lung washing with anti-rabbit albumin, IgG, and IgM. No differences were observed between the SDS–PAGE patterns of sediments and supernates from cell-free lung washings. Albumin appeared to be the major protein of each fraction, with small amounts of other proteins present. The large amount of lipid present in the sediment interfered with the electrophoresis and prevented a more quantitative comparison of the sediment and supernatant proteins.

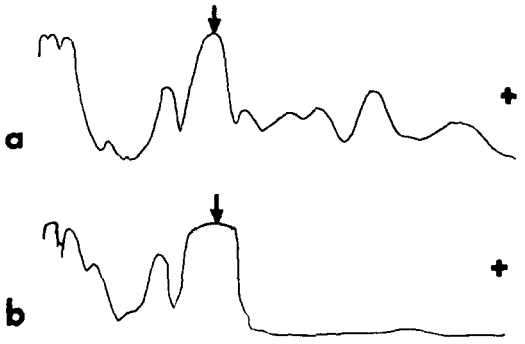

Fig. 2. Densitometric tracings of polyacrylamide gels containing (*a*) cell-free rabbit lung washing proteins and (*b*) rabbit plasma proteins. The position of the albumin band in each tracing is indicated by an arrow.

Characterization of lung washing lipid–protein complexes by analytical ultracentrifugation

The results of the sedimentation experiments suggested that much of the protein found in the sediment is nonspecifically associated with the phospholipid. However, these results did not rule out the possible existence of a specific lipoprotein molecule containing a relatively small proportion of protein. The lipoproteins of serum have been well characterized and are divided into distinct groups on the basis of their flotation rates as determined by analytical ultracentrifugation. Therefore, it appeared reasonable that if lung surfactant is composed of any type of discrete lipoprotein particles, these particles should be observable in the analytical ultracentrifuge.

The method commonly used for isolation and characterization of serum lipoproteins was applied to lung washings. This procedure involves the concentration of the lipoproteins by flotation in NaBr solutions using the preparative ultracentrifuge and then resuspending the resultant pellicle in NaBr solutions of appropriate density for flotation in the analytical ultracentrifuge. Preparative centrifugation of noncellular lung washings in NaBr at a density of 1.21 gave a surface-active pellicle that had a PL/protein ratio of 8.7. When aliquots of the resuspended pellicle were centrifuged in NaBr at a density of 1.21 in the analytical ultracentrifuge, no peaks were observed, although at the end of the centrifugation period a pellicle of material was seen at the top of a clear solution. Resuspended sediments gave similar results, floating to the top of a NaBr solution of 1.21 so rapidly that no schlieren pattern was observable, indicating the presence of large aggregates of the surface-active material. Experiments using NaBr solutions of lower densities revealed that any density greater than 1.05 gave the same results as did NaBr at a density of 1.21, whereas at densities of less than 1.05, the surface-active material sedimented but,

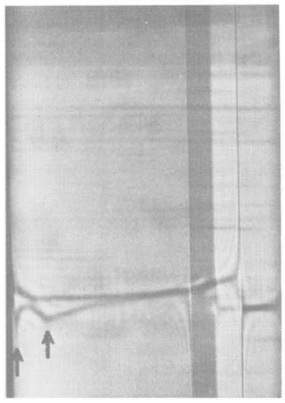

Fig. 3. Schlieren pattern of a sediment from cell-free lung washings resuspended in NaBr, density of 1.21, by sonication for 3 min and centrifuged in the analytical ultracentrifuge at 14,000 rpm at 20°C. This photograph was taken 8 min after reaching full speed. The lipoproteinlike peaks are indicated by the arrows.

again, very rapidly and with no schlieren pattern observable.

In attempts to disperse the particles of surface-active material more thoroughly, sediments from lung washing were suspended by vigorous homogenization. After adjusting the density to 1.21 with NaBr, analytical ultracentrifugation revealed the presence of a very small, rapidly moving peak with a flotation rate of about 500. Sonication of sediments was then tried to break down further the large particles into their possible "basic lipoprotein units." The results of one such experiment are shown in **Fig. 3.** Two typical lipoproteinlike peaks were seen with flotation rates of 300 and 27, respectively. Continued study of sonicated sediments from a number of lung washings gave inconsistent results, with the faster-moving peaks having flotation rates from 87 to 300 and the slower peak having flotation rates from 27 to 54. By increasing the length of time of sonication, the amount of material in the slower-moving peak increased with a concurrent loss of material from the faster-moving peak.

The effect of sonication on the chemical composition of the sediment from a cell-free lung washing was studied by resedimentation of the sonicated suspension of such a sediment. Noncellular lung washings with a PL/protein ratio of 1.33 gave a sediment with a ratio of 4.93 after centrifugation at 127,000 *g* for 90 min. When this pellet was resuspended in RLS and sonicated for 3 min, centrifugation yielded a pellet with a PL/protein ratio of 7.78. After a second sonication and centrifugation, a pellet with a PL/protein ratio of 9.24 was obtained. The results showed that although sonication causes solubilization of more protein than phospholipid, the protein nonspecifically absorbed to the phospholipid during sedimentation is not readily removed. That some protein remains with the phospholipid is not necessarily indicative of a specific association because the short duration of sonication used in these experiments is insufficient to completely disperse phospholipids into particles of uniform size (30).

The flotation rates measured for surfactant lipid–protein complexes were quite high compared with those of serum lipoproteins and were in the range found for the very low density lipoprotein of serum. However, the density of the solution needed to float surfactant lipid–protein complexes (1.05) was in the range of low density lipoprotein. These findings, as well as the inconsistent flotation rates found for various sonicated sediments, indicated that the peaks did not represent homogeneous lipoprotein classes of different densities but, rather, particles of varying size. This interpretation was confirmed by electron microscopic findings of negatively stained preparations of lung washings and lung washing sediments. Numerous small particles of various sizes, measuring from about 20 nm (200 Å) to 100 nm (1000 Å) in diameter were present in cell-free lung washings prior to sedimentation (**Fig. 4,** *a*). At higher magnification (Fig. 4, *b*) the particles, or liposomes, revealed characteristic, often concentrically arranged lamellae. In sediments resuspended by stirring in a small volume of RLS, the small particles that were present in the washings were clumped into very large aggregates, usually measuring 1000 nm (10,000 Å) or more in diameter, which were not dispersed by stirring (Fig. 4, *c*). Sonication of a resuspended sediment caused a reduction in particle size, as shown in Fig. 4, *d*.

The presence of components that float in NaBr solutions could indicate the presence of lipoproteins. However, phospholipid in aqueous suspensions form large, multilamellar structures (31) that also might display similar properties upon centrifugation. To investigate this possibility, DPPC was suspended by vigorous stirring, and, in another preparation, by sonication in 24% NaBr in RLS, and examined by analytical ultracentrifugation. The resulting peaks had flotation rates ranging from 25 to 320. This finding demonstrates that DPPC alone can form structures with flotation rates comparable to those of various preparations of lung surfactant and shows that the method of analytical ultracentrifugation is not adequate to determine the presence or absence of specific lipoprotein components.

Characterization of lung washing components by density gradient centrifugation

The results of sedimentation and analytical ultracentrifugation studies indicated that sedimentation steps should be avoided in the procedure for isolation of lung surfactant lipid–protein complexes, because sedimentation results in the loss of some surface-active material in the supernate and promotes the formation of aggregates containing varying amounts of protein. These aggregates were not readily

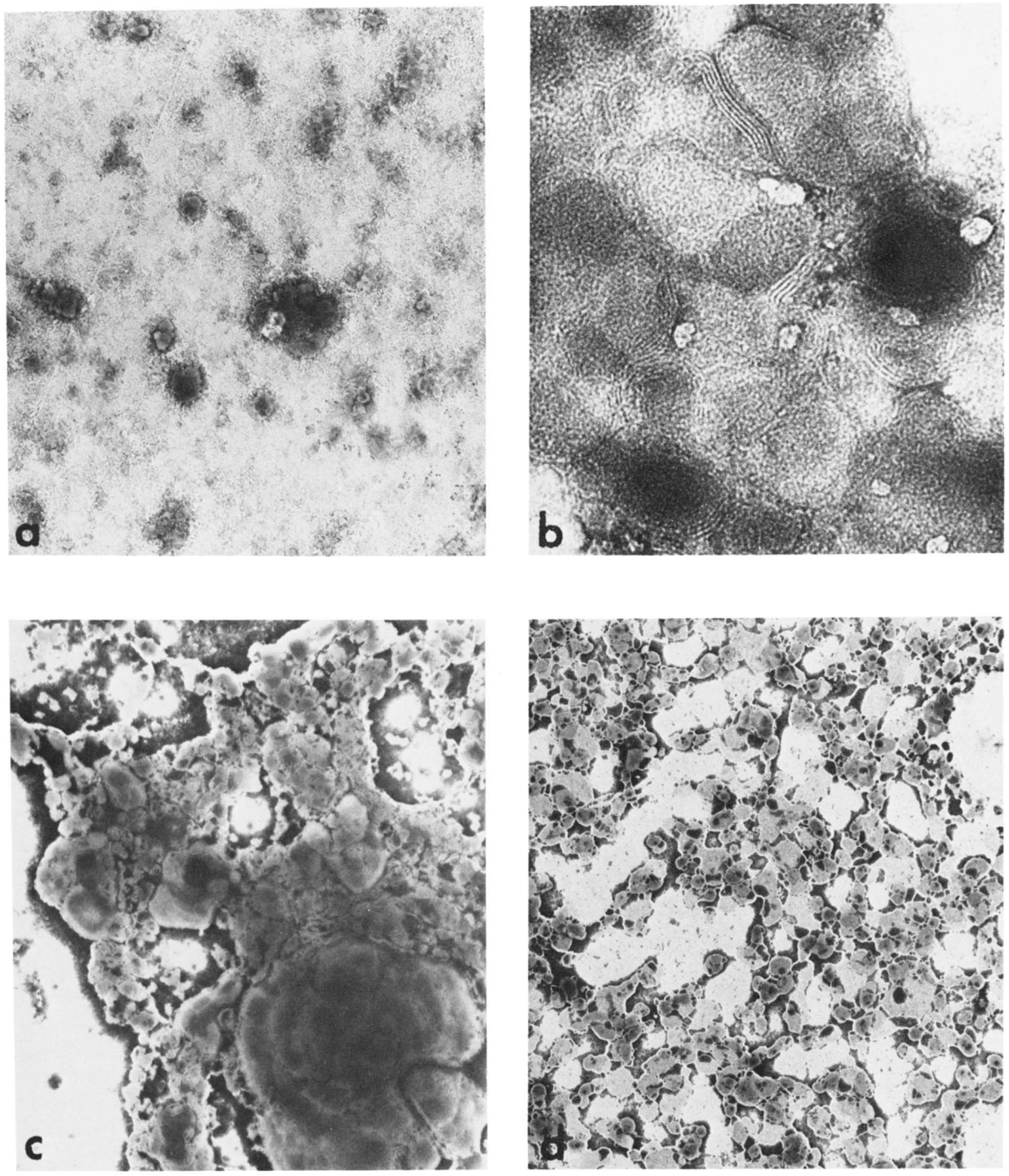

Fig. 4. Negatively stained preparations of lung surfactant. (*a*) Surfactant liposomes of cell-free lung washings; × 11,400. (*b*) Same as *a*, at higher magnification to show characteristic, concentrically arranged lamellae of surfactant liposomes; × 140,500. (*c*) Sediment obtained by centrifugation of a cell-free lung washing and resuspended by stirring in RLS. The surfactant liposomes are present in the form of large aggregates; × 11,400. (*d*) Same as *c*, but sediment was resuspended by sonication, which results in some dispersion of the surfactant liposomes; × 11,400.

dispersed; even sonication did not give particles of uniform size. A better method would also be one in which the components of the lung washings are separated on the basis of density only, because even the cell-free washings were seen to contain particles of varying size. Isopycnic density gradient centrifugation is such a method and has been used for the separation of surfactant lipid–protein complexes, but only after a sedimentation step. In this study,

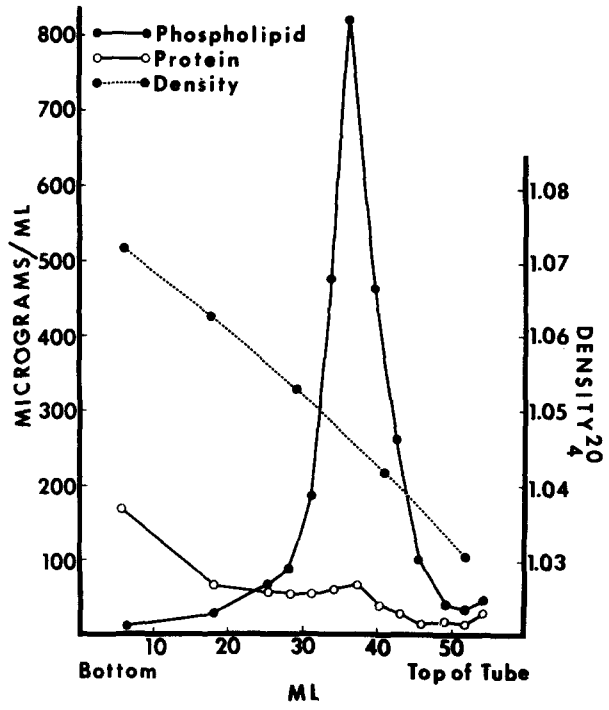

Fig. 5. Distribution of phospholipid and protein in a continuous NaBr gradient after centrifugation at 20°C for 18 hr. The noncellular lung washings used to prepare this gradient were obtained in RLS at 25°C and had a PL/protein ratio of 1.89.

density gradient centrifugation of the entire cell-free washings was carried out on a continuous gradient of NaBr in RLS.

Gradients with different ranges of densities were tried, and a gradient ranging in density from about 1.02 to 1.08 at 20°C was chosen for further study of surfactant lipid–protein complexes. With such a gradient, more than 90% of the phospholipid contained in the noncellular washings used in preparation of the gradient was recovered in fractions collected from the gradient. Centered slightly above the middle of the tube was a visible band of material, the bottom portion of which was composed of a white, particulate material, while the top half was opalescent. The distribution of phospholipid and protein in such a gradient is shown in **Fig. 5.** The phospholipids of the washings were present in a relatively narrow band centered at a density of 1.045. The protein was spread throughout the gradient tubes in much the same manner as it was at the beginning of the run, with a small amount of protein associated with the phospholipid band. However, the PL/protein ratio of the band varied from gradient to gradient, depending upon the PL/protein ratio of the noncellular washing from which the gradient was prepared (**Table 2**). A small amount of protein appeared to be associated with the phospholipid band, but "background protein" made a determination of PL/protein ratio of the phospholipid-containing fractions unreliable as a parameter for measuring the extent of associated protein.

TABLE 2. PL/Protein ratios of phospholipid-containing fractions from NaBr density gradients

	PL/Protein of Cell-free Washings	PL/Protein of Fraction with Highest Phospholipid Concentration	PL/Protein of Entire Phospholipid Peak
	0.99	4.53	2.73
	1.67	9.90	7.21
		7.22	8.00
	1.89	12.87	7.87
		10.42	7.10
		9.33	6.88
	2.99	14.17	10.88
Average	1.86	9.78	7.24

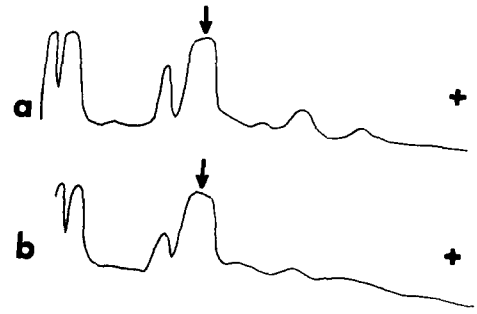

Fig. 6. SDS–PAGE of fractions of lung washings separated on NaBr density gradients: (*a*) fractions from near the bottom of the gradient tube containing very little phospholipid and (*b*) fractions from center of the phospholipid band. The position of the albumin band in each tracing is indicated by an arrow.

Even through the phospholipid peaks from the gradients contained some "background protein," the presence of any unique lipid-associated protein in this fraction should be observable by SDS–PAGE. Therefore, the protein composition of the gradient fractions containing high concentrations of phospholipids was compared with the protein composition of gradient fractions without substantial quantities of phospholipids. The results are shown in **Fig. 6.** In addition to albumin and immunoglobulin, which were also identified by immunoelectrophoresis, the lung washing fractions contained several bands of protein that were not present in plasma. However, no differences could be found in the protein from the various parts of the density gradient tubes. All the protein bands in the phospholipid-containing fractions were also present in other gradient fractions. These results indicate that although several nonplasma proteins are present in lung washings, they are not specifically associated with the phospholipid. In fact, these proteins were observed more readily in non-phospholipid-containing fractions, but this may be due to the interference of the lipids with the methods of SDS–PAGE.

Other studies of the phospholipid band from the gradient revealed that this material was highly surface active,

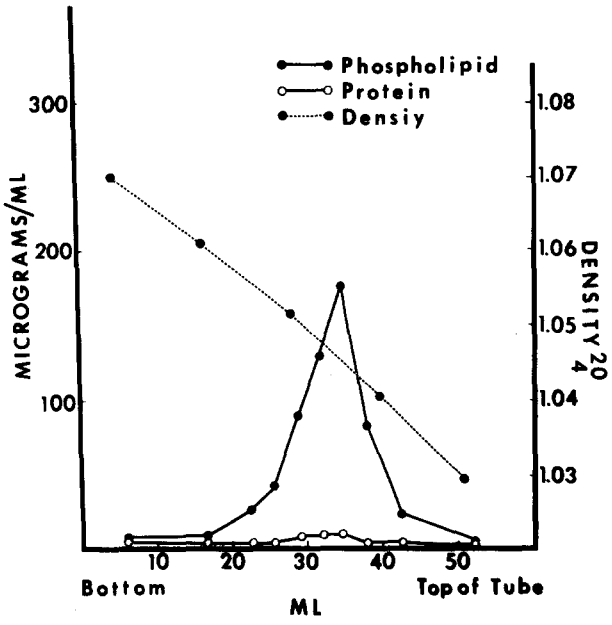

Fig. 7. Distribution of phospholipid and protein of the material from the center of a phospholipid band of a previous gradient on a second continuous NaBr gradient. The gradient was centrifuged for 18 hr at 20°C. The PL/protein ratio of the starting material was 13.5, and the ratio of this phospholipid band was 23.0.

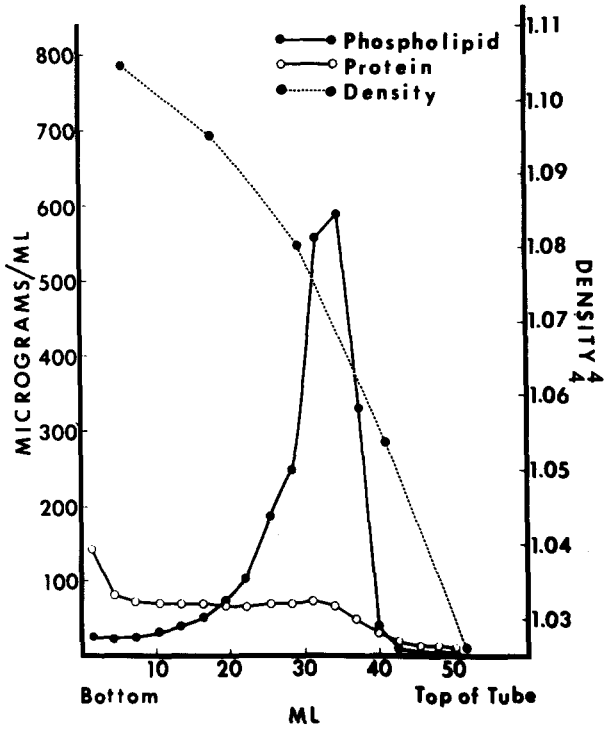

Fig. 8. Distribution of phospholipid and protein on a continuous NaBr gradient after centrifugation at 4°C for 18 hr. The noncellular lung washings used to prepare this gradient were obtained in RLS and had a PL/protein ratio of 1.98.

reducing surface tension to 0 dynes/cm. When a portion of the material from the phospholipid band with a PL/protein ratio of 8.7 to 1 was centrifuged in such a way as to obtain a sedimentable surfactant fraction, the sediment had a PL/protein ratio of 18.4 to 1 and was also highly surface active. When a portion of the phospholipid band was used to prepare a second gradient and centrifuged in the same manner, the pattern in **Fig. 7** was observed. The position of the phospholipid peak in the density gradient tube was unchanged from the previous run. By contrast, only a very small amount of protein remained associated with this peak, the PL/protein ratio of this band being about 23 to 1. Complete separation of the lipid and proteins of the phospholipid band by organic solvent extraction, followed by another density gradient centrifugation, slightly altered the density at which the phospholipid band was found. The band was centered at a density of 1.040, compared with the original 1.045, suggesting that a small amount of protein had been removed from the lipids.

The results of these experiments do not support the concept that lung surfactant phospholipids are part of a specific lipoprotein molecule. However, the possibility that specific lipoprotein complexes were present in lung washings and were fragmented by the methods used in density gradient centrifugation was investigated by carrying out density gradient centrifugation of cell-free washings without prior sedimentation under various conditions. Sucrose was used in place of NaBr to investigate the possibility that specific lipoproteins might have been dissociated by the high ionic strength of NaBr. Samples were also centrifuged at 4°C, and Tris buffer, pH 7.35, was used in place of RLS. These changes, singly and in various combinations, did not reveal the presence of any specific lipid–protein complexes. The only major difference observed was that the phospholipid band was found at a higher density at 4°C than at 20°C (**Fig. 8**). That this density change was not due to the fact that the lipid was associated with more protein at 4°C than at 20°C was shown by the demonstration that lipids extracted from lung washings by organic solvents, as well as pure DPPC, exhibited the same density change as a function of temperature. DPPC sonicated in RLS and used to prepare a gradient was found in a band centered at a density of 1.07 after centrifugation at 20°C. After centrifugation at 4°C, sonicated DPPC was located at a density of 1.10. Surfactant lipids extracted from lung washings by chloroform–methanol were found at a density of 1.065 at 4°C compared with 1.04 at 20°C.

DISCUSSION

The results of this investigation indicate that the lung surfactant phospholipids do not exist as a part of a specific lipoprotein molecule and that the surfactant "lipoproteins," isolated by methods using differential centrifugation, are artifacts produced by those methods. The evi-

dence for the presence of lipid–protein complexes in lung washings comes largely from the fact that lipids and proteins are found together in various fractions obtained from lung washings by methods of centrifugation. However, the presence of lipid and protein in the same fractions does not necessarily mean that they are part of a specific lipid–protein complex that exists at the alveolar surface.

The first step used by most investigators in the isolation of lung surfactant "lipoproteins" is centrifugation of the noncellular lung washings in such a way as to obtain a surface-active sediment. The present study reveals that the PL/protein ratio of such a sediment is directly dependent upon the PL/protein ratio of the lung washings from which it was obtained. Electron microscopic and analytical ultracentrifugation studies revealed that these sediments are composed of very large aggregates of material that are not readily dispersed.

Both sedimentation and flotation result in the separation of components on the basis of both particle size and density. Since lung surfactant does not appear to exist as discrete particles of uniform size, a method based only on density, such as isopycnic density gradient centrifugation, appears to be more appropriate for the isolation of surface-active components of lung washings. Density gradient centrifugation has frequently been used to isolate surfactant "lipoproteins," but virtually all workers have applied a resuspended sediment to the density gradient. The results just discussed suggest that the starting samples for these gradients were large aggregates of surfactant liposomes and protein that would not readily dissociate during density gradient centrifugation. The amount of protein in these aggregates and, therefore, the density at which they would be found upon centrifugation would depend upon the amount of protein in the washings from which the sediment was obtained. In addition, previously published papers have reported measurement of the phospholipid and protein content of only the visible bands and not of fractions from the entire gradient tube. The presence of "background" protein is a possible explanation for some of the variations in the PL/protein ratios reported for lung surfactant purified by density gradient centrifugation.

Density gradient centrifugation of the entire noncellular washings without prior sedimentation was used in this investigation and appears to be a useful technique for the study of the lipid–protein complexes of lung washings. Without prior sedimentation, only a small amount of phospholipid-associated protein is found. In most of the experiments in this study, even this small amount of protein is obscured by the presence of background protein. The presence of this background protein results in the PL/protein ratio of the phospholipid band in the density gradient tubes being directly proportional to the PL/protein ratio of the noncellular washing. However, the fact that the phospholipid band is found at the same density regardless of the PL/protein ratio strongly indicates that most of the protein is background protein. When the proteins and lipids were completely separated by the use of organic solvents, the protein-free phospholipid band was found at a slightly lower density than before the extraction, suggesting that a small amount of the protein is closely associated with the phospholipid. However, SDS–PAGE of various gradient fractions revealed that the same proteins were present in all gradient fractions studied and that the most abundant proteins in all fractions were serum proteins. These results suggest that the protein present in the phospholipid band is not part of a specific lipoprotein molecule but results from a nonspecific aggregation possibly formed during the lavage procedure.

King and Clements (6, 7), using dog lung washings and homogenates, were able to isolate fractions of surface-active material that contained reproducible amounts of phospholipid and protein and that remained intact as lipid–protein complexes under several experimental techniques. However, they did not exclude the possibility that the protein components of these stable fractions are contaminants entrapped in lipid aggregates. Our studies provide evidence that centrifugation procedures promote formation of stable lipid–protein complexes, and, therefore, stability or reproducibility of isolated fractions is not indicative of surfactant purity.

The observation by King and coworkers (17) that a certain protein with a molecular weight of 10,000–11,000 is found in increased concentrations in purified fractions of surfactant cannot readily be reconciled with the results of this study. One possible explanation is species differences, because their work was with dogs whereas the present study utilized rabbits.

The structure of the extracellular alveolar lining layer proposed on the basis of electron microscopic studies is a monomolecular film of phospholipid at the surface of an aqueous layer or hypophase. The hypophase may contain proteins and possibly carbohydrates (32). When such a monomolecular film of phospholipid is disrupted by lavaging with an aqueous solution, the insoluble phospholipid would be expected to aggregate and form the same sort of multilamellar, bilayered liposomes that are observed when solid phosphatidylcholine is dispersed in aqueous solutions (31). The properties of surfactant liposomes are consistent with the expected behavior of dispersions of phospholipids in aqueous solutions. Liposomes can entrap other substances, which may account for the finding of a small amount of protein in the phospholipid fractions. The fact that the same proteins are present in both the phospholipid-containing fractions and other fractions of lung washings is strong evidence suggesting that this association is nonspecific.

The origin of the proteins found in lung washings is not known. Quantitatively, the largest amounts of protein

present in the washings are serum proteins. Whether these proteins are normal components of the alveolar hypophase or whether they are contaminants obtained from the serum during the lavage procedure is a question that remains to be answered. It should be pointed out that the presence of varying amounts of proteins in the alveolar washings does not necessarily mean that they are contaminants from the lavage procedure. The possibility that the protein content of the hypophase of the alveolar lining layer varies from one animal to another, or even from one time to another in the same animal, must be considered.

Hurst, Kilburn, and Lynn (13) attempted to eliminate contamination of lung washings with plasma components by perfusion of the pulmonary circulation with sucrose solutions or with the nonaqueous liquid FC-80 fluorocarbon. They found that material obtained by lavage after such treatment contained very little protein. However, they assumed that in such treatments alveolar contents are not lost into the tissue or the vascular space. It has been shown by Bensch, Dominguez, and Liebow (33) that albumin and globulins can migrate from the alveoli into the circulatory system. Therefore, the possibility that perfusion might cause absorption of some components of the alveolar lining layer from the alveolar surface remains open. The finding of several proteins in lung washings that are not plasma components is of interest. The report of Dickie et al. (34) suggests that lung washings contain some protein that is synthesized in the lung. Although the source and function of these proteins is presently uncertain, one may speculate that these proteins may be of importance in providing a solution on which a stable surface film could be formed. This suggestion is supported by the data of Hurst et al. (13), who found that, in vitro, proteins stabilize phosphatidylcholine monolayers against loss of surface activity upon repeated compression. An alternative hypothesis might be that these proteins are of importance in the transport of DPPC from the cell through the hypophase to form the monomolecular surface film.

This investigation was undertaken for the purpose of characterizing the functional components of the lung surfactant system in normal conditions in order that future experiments could be designed for the meaningful study of lung surfactant in various pathological conditions. Transudation of fluid and proteins from the vascular bed of the lung to the alveolar space occurs in virtually every pathological condition in which surfactant activity is deficient or altered. Therefore, the method used for isolation of lung surfactant must be capable of separating the surfactant from large excesses of protein as well as from nonsurfactant phospholipids if the study of abnormal surfactant is to be undertaken. The results of this investigation indicate strongly that the phosphatidylcholine of the alveolar lining layer is not an integral part of a complex lipoprotein and that the so-called lipoproteins reported by various investigators actually represent lipid–protein complexes artifactually produced by the fractionation procedures. This conclusion is supported by the finding that aqueous dispersions of DPPC behave in much the same way as does lung surfactant in the procedures of analytical, differential, and density gradient centrifugation employed.

These findings are of significance to the field of surfactant research because they suggest a radical departure from the currently used elaborate schemes of surfactant "purification" designed to isolate distinct lipoproteins from lung washings. Lengthy purification schemes appear to be not only unnecessary, but also undesirable, because they result in the fractionation of surface-active components on the basis of sedimentation rate and in the formation of artifactual lipid–protein complexes. The method of density gradient centrifugation of the entire noncellular lung washings described in this investigation overcomes many shortcomings of previous methods and results in separation on the basis of only the densities of the components of the lung washings. Further studies based on the principles outlined in this report, designed to simplify and shorten the gradient centrifugation method and to quantitate yields of various components of lung surfactant, are in progress.

This work was supported in part by U.S. Public Health Service Grant HD-04434 and USAMRDC Contract DADA17-70-C-0041.

Manuscript received 5 August 1974; accepted 31 January 1975.

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