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Characterization of Lung Surfactant Proteins and Lipid-Protein Complexes

Sue Ann Eckhardt Shelley
Loyola University Chicago

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CHARACTERIZATION OF LUNG SURFACTANT PROTEINS
AND LIPID-PROTEIN COMPLEXES

by

SUE ANN SHELLEY

A Dissertation Submitted to the Faculty of the Graduate School of
Loyola University of Chicago in Partial Fulfillment
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LIFE

Sue Ann Eckhardt Shelley was born in Moweaqua, Illinois, on December 30, 1938. She graduated from Moweaqua High School, Moweaqua, Illinois in June, 1956, and received a Bachelor of Arts degree from Millikin University, Decatur, Illinois, in June, 1959. After five years as a Research Assistant in the Plant Virus Laboratory, Department of Botany, University of Illinois, Urbana, Illinois, and a year and one-half in the Department of Pathology at the University of Chicago, Chicago, Illinois, she joined the Department of Pathology, Loyola University Stritch School of Medicine in September, 1966, and at present, she is a Research Associate in that department.

In January, 1969, she began graduate study in the Department of Biochemistry and Biophysics, Loyola University Stritch School of Medicine, under the guidance of Dr. Maurice V. L'Heureux. A Master of Science degree was awarded in June, 1971, following completion of her thesis on the "Effect of Vitamin D on Ribonucleic Acid Synthesis in Bone."

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CHAPTER I

INTRODUCTION

The alveoli of mammalian lungs are lined by a fluid layer which was first observed by Terry in 1926. He found that when a capillary glass tube was inserted into the superficial alveoli of inflated lungs, it could be seen under the microscope to fill with a clear fluid containing bubbles. Terry (1945) continued to call attention to the fluid normally present on respiratory surfaces, and observed its existence in the alveoli of various mammals (cat, dog, rat) as well as in the pulmonary sinuses of reptiles (turtle, lizard) and of amphibians (frog, salamander), but it was not until the 1950's that other investigators began to recognize the importance of this fluid lining layer.

The 25 year delay in understanding its significance was probably due to the fact that investigators were engaged in a controversy as to whether the capillaries of the lung were "naked" or covered by a continuous lining of epithelial cells. Low (1952 and 1953), first published a study of lung tissue sections by electron microscopy and resolved the question by clearly showing the presence of a pulmonary epithelium which forms a complete covering of the alveolar walls. Present knowledge of alveolar structure was developed from a large number of subsequent inquiries which have been published and recently reviewed by Weibel (1973). Research demonstrates that the air-blood barrier is composed of three layers: the capillary

endothelium, the alveolar epithelium, and a very narrow interstitial space. The alveolar epithelium is composed largely of two distinct types of cells. Type I, or squamous alveolar cells, line the major part of the alveolar surface and are characterized by thin cytoplasmic extensions that may extend 50 micrometers or more from the nucleus. Type II, or granular alveolar cells, are cuboidal cells with no lateral extensions. They contain numerous mitochondria, a well-developed endoplasmic reticulum with many ribosomes, and a Golgi complex. Their relative wealth in organelles suggests that these cells play a prominent role in lung metabolism. The main feature of type II cells is the presence of characteristic granules which are membrane-lined and contain an osmiophilic material disposed in the form of lamellae. This material is believed to be a precursor to, or a stored form of surfactant which may be secreted into the alveolar space to replenish the extracellular lining layer.

The fluid layer on the surface of the pulmonary epithelial cells was described by Macklin in 1954 as an "aqueous mucoid film." Examining fresh, frozen, and stained lung tissue by light microscopy, he observed remnants of a film at the interface between air and alveolar tissue which had an affinity for Prussian Blue stain, a characteristic of mucopolysaccharides. He suggested that the alveolar fluid forms a film containing acid mucopolysaccharides, perhaps 0.2 micrometers thick on the alveolar wall, and that it maintains a constant favorable surface tension.

In 1955, Macklin observed, by phase-contrast microscopy of frozen sections of lung mounted in aqueous media, myelin figures emerging "from

the alveolar walls until they fill the spaces with strange forms made of bimolecular lamellae around aqueous cores." He stated that this material, which seemed to come from the aqueous alveolar fluid, is either lecithin or cholesterol. In the same report, Macklin described observation of the formation of bubbles during the collapse of a cut surface of lung and proposed that it was impossible to explain their existence without invoking the theory that the participation of an alveolar fluid of relatively low surface tension was involved.

The importance of surface forces in the elastic behavior of lung was described as early as 1929 by the Swiss physiologist von Neergard. Using excised mammalian lungs, he compared pressure-volume curves recorded during deflation of air-filled lungs with those obtained during deflation of liquid-filled lungs. The difference in his findings between liquid-filled lungs, in which the retractive pressure was described as a function primarily of tissue elasticity, (the alveolar-air interface having been removed by replacement of air with liquid), and air-filled lungs, was then an expression of the retractive force of the lung due to surface forces. From these experiments, von Neergard concluded that approximately two-thirds to three-fourths of the retractive pressure of the lung was due to surface forces, and therefore, the surface tension in the alveoli must be lower than that of physiological fluids owing to the accumulation of a surface active material at the alveolar-air interface.

Between 1929 and 1955, the theoretical importance of the surface properties of the lung was mentioned in several papers dealing with the

significance of interfacial forces in the initial expansion of the lungs after birth (Wilson and Farber, 1933; Gruenwald, 1947). However, it was not until 1955, when the classic paper by Pattle was published, that the nature and importance of the alveolar-lining fluid were generally recognized. Pattle, both in 1955 and in his more detailed report of 1958, described the unusual properties of bubbles from the fluid and foam found in the trachea of rabbits with acute lung edema. These bubbles were observed to be unaffected by chemical anti-foaming agents which rapidly destroyed foam produced by shaking serum with air, or even bubbles formed by detergents. In air-saturated water, the bubbles from the lung foam remained unchanged in size for long periods of time, whereas similar bubbles from serum and other foams dissolved and disappeared within a few minutes. Similar foams were also obtained from healthy lungs by either cutting and squeezing under water, or after the introduction of saline into the trachea. Pattle proposed that the surface tension of lung bubbles approaches zero due to the presence of an insoluble surface layer on the bubbles. The stability of the bubbles from lung was destroyed by pancreatin and by trypsin, leading to the suggestion that the lining material was an insoluble protein. He deduced from this careful study of the properties of the bubbles that lungs contain a powerful surfactant, which reduces the surface tension in the alveoli to nearly zero. Since neither blood nor tracheal mucus showed these unusual surface properties, he concluded that lung surfactant must be a specialized secretion of the lung and not a transudate from blood. He also studied fetal guinea pigs and found that

the lung surfactant develops late in fetal life and suggested that its absence may pose difficulties for premature infants.

Additional understanding of the nature and importance of lung surfactant was provided by Clements (1957) who studied the properties of lung surfactant using the standard techniques of surface physics and chemistry. He examined the surface properties of saline after it had been used to inflate the lungs via the trachea. He also studied minces of whole lung tissue and saline to which slices of lung had been touched. The results of all three methods were similar in that the tension of a lung-derived surface varied from 46 to 10 dynes/cm as the area of the surface was changed. This ability of lung surfactant to produce changes in surface tension when the area changes is believed to be a major force preventing alveolar collapse during expiration.

Avery and Mead (1959) used Clements' method of testing surface properties of lung surfactant to demonstrate the absence of surface active material in infants dying with hyaline membrane disease. This was the first pathological condition, and the only one to date, convincingly to be shown attributable to a lung surfactant deficiency.

In 1961, three reports were published which characterized lung surfactant as a lipoprotein. Pattle and Thomas (1961) examined the dry residue from bubbles of lung foam by infrared absorption spectrophotometry. By comparison of the infrared spectra with that obtained from lecithin-gelatin mixtures, they concluded that the bubbles were lined with a lipoprotein containing approximately 1% protein. Unwilling to accept this

very low value of protein content, they suggested that some of the lecithin present may be adventitious.

Buckingham (1961) harvested a compressed surface film formed from sheep lung extracts. On the basis of staining reactions, solubility, and nitrogen content, she proposed a lipoprotein nature for the lung surfactant.

Klaus, Clements and Havel (1961) made the most careful early measurements of the chemical composition of lung surfactant. Using a method for the preparation of surface active material from lung which was proposed by Bondurant (1960), beef lungs were perfused with saline via the pulmonary artery and the foam which poured out the trachea was collected, washed with distilled water, and dried. The dried powder was highly surface active and was found to contain 50-70% lipids and 5% nitrogen. Analysis of the lipid fraction showed that of the lipids 74% were phospholipids, 8% cholesterol, 10% triglycerides, and 8% free fatty acids. The phospholipid fraction was the only fraction showing surface activity similar to that of the crude extract.

The major phospholipid present in the surface active material from lung was soon identified as phosphatidyl choline (PC). According to Clements (1962), Siakotos separated the phospholipid fraction of Klaus et al. (1961) by paper chromatography and found that it contained about 40% PC. Brown, in 1962, isolated from lung washings, by precipitation with trichloroacetic acid and then from an ethanol solution with cadmium chloride, a highly surface active fraction which was not soluble in acetone. This precipitate was shown to contain choline, indicating that pulmonary surface active

material is PC. In 1964, Brown further characterized this material as dipalmitoyl phosphatidylcholine (DPPC) by melting point, paper chromatography, and gas-liquid chromatography.

Many subsequent reports have confirmed that PC is the major phospholipid of surface active material from mammalian lung, regardless of the method of preparation or species from which it was obtained. Fujiwara and coworkers (Fujiwara and Adams, 1964; Fujiwara et al. 1964) prepared extracts from normal rabbit, guinea pig and human lungs by mincing. All the extracts contained PC, sphingomyelin, and phosphatidyl ethanolamine (PE), with PC present in the highest concentrations. Analysis of lipid composition of dog (Finley et al., 1964) and human (Finley et al., 1965) lung washings, after removal of cells, revealed that PC was the major component, making up 57% of the lipids in dog lung washings and 43% in human washings. Morgan et al. (1965) compared the lipids of washings of dog lungs with the lipids from whole lung homogenates. He found that whole lung contained proportionately more cholesterol and sphingomyelin and less PC than did the lung washings. The PC in the washings was also found to contain a higher percentage of saturated fatty acids than did whole lung lecithin.

The conclusion that the PC in lung surfactant is largely DPPC is generally based upon indirect evidence, namely, an exceedingly high content of palmitic acid in the PC molecules. The presence of DPPC in beef lung tissue had been demonstrated in 1946 by Thannhauser et al. using chemical methods of separation and analysis. Tierney et al. (1967) identified DPPC in rat lung by using argentation thin-layer chromatography. Recent studies

(Toshima and Akino, 1972) of the PC in alveolar washings of rats, also using the method of argentation thin-layer chromatography, showed that 70% of the PC contains two saturated fatty acids, mainly palmitoyl residues. Toshima and Akino (1972) reported, in agreement with Morgan et al. (1965) that the PC of lung tissue is less saturated (47%) than that of lung washings.

Although DPPC is generally recognized to be the most abundant lipid in lung surfactant, the exact lipid composition of surfactant is still under investigation. Various reports differ as to the amounts of other phospholipids and neutral lipids present in preparations of lung surfactant. Perhaps the most important controversy concerns the presence or absence of phosphatidyl dimethylethanolamine (PDME) because of its possible role in the synthesis of PC by methylation of PE, and the proposed importance of this pathway in premature infants (Gluck et al., 1972). Morgan et al. (1965) first reported finding significant amounts of PDME in dog lung washings. Gluck et al. (1967) reported isolating PDME from adult rabbit alveolar washings and Frosilono et al. (1970) identified PDME as a component of their surface active fraction from dog lungs. However, Baxter et al. (1969) failed to find any PDME in a study of lung phospholipids in rat, mouse, frog, human and cow. Likewise, Body (1971) found phosphatidyl glycerol (PG) but no PDME in pig lung surfactant and suggested that, under some conditions of chromatography, PDME and PG would have similar properties. Other workers have also reported the presence of substantial quantities of PG and small quantities, or none at all, of PDME in lung

surfactant lipid extracts from dog lung (Pfleger and Thomas, 1971; Pfleger et al., 1972) and from rat lung (Toshima and Akino, 1972). Whether these discrepancies have resulted because of differences in techniques, or from variations in the species studied, remains to be determined. The importance of phospholipids other than PC and of neutral lipids in lung surfactant also remains to be demonstrated, especially in light of recent experimental evidence suggesting that the alveolus is normally lined with saturated PC, essentially free from other lipids (Hurst et al., 1973). The latter study will be discussed in detail later in this chapter.

The concept that lung surfactant is a lipoprotein has received rather widespread acceptance, largely through repetitious application of the term, lipoprotein, rather than by substantial experimental evidence. The exact molecular nature of lung surfactant is not known. Two main approaches have been used to study the relationship of the various components of the alveolar lining layer: (1) direct observation by techniques of microscopy, and (2) application of the methods of modern biochemistry to surface active fractions from lungs or lung washings.

Macklin (1954 and 1955) gave the first morphological evidence for the existence of an extracellular lining layer when he observed the presence of both mucopolysaccharides and lipids in the alveolar fluid. A continuous layer of mucopolysaccharides closely associated with the plasma membrane of the alveolar epithelial cells was demonstrated by Groniowski and Biczyskova (1964) using histochemical techniques with electron microscopy. Luke and Spicer (1965), using histochemical techniques at the light

microscopy level, showed that the alveolar cells are lined by sialomucin and that no sulfomucins were detectable. The mucinous coat is apparently not a unique feature of the alveolar epithelium, since a similar coating appears to exist on many other mammalian cell surfaces (Pease, 1966; Rambourg et al., 1966). More recent studies (Adamson and Bowden, 1970; Balis et al., 1971) have shown that removal of surface active material by alveolar lavage does not appreciably change the mucinous coat of the alveolar cells. Adamson and Bowden (1970) found that the mucopolysaccharide layer of type II cells is much thicker than the coating on type I cells. These findings suggest that the mucinous coat is an integral part of the epithelial cell membranes rather than of the surfactant layer.

Visualization of the fluid layer on the surface of the alveolar epithelium has been very difficult. Conventional methods of preparation of tissue for electron microscopy, which include cutting the lung tissue into small blocks before fixation, or instillation of a fixative solution into the alveoli by way of the trachea, have failed to demonstrate the presence of a smooth continuous layer on the alveolar surface as would be required to fit a model consistent with the proposed function of surfactant. Occasionally, a patchy osmiophilic layer has been seen on the cell surfaces (Kikkawa et al., 1965). Also, the presence of lipid in the form of "myelin figures," which are lamellar structures with a repeating period of 42 \AA was observed by Weibel et al. (1966) in the alveolar spaces. Thus, it appears that conventional methods of fixation have not been adequate to preserve the surface layer, presumably because instillation of liquid into the alveolar

spaces tends to wash off or disperse the surfactant when it has not been fixed prior to the destruction of the alveolar-air interface.

Direct demonstration of the alveolar lining layer was achieved by Weibel and Gil in 1968. They described a method of "fixation from behind" which employs perfusion of the lungs with a fixative solution through the vascular bed. When the fixative diffused from the capillary to the alveolar surface, it was able to preserve a portion of the alveolar lining layer. Their results, as reported above and in a subsequent paper (Gil and Weibel, 1969/70), clearly demonstrate that the alveolar epithelium is coated by an extracellular lining layer composed of two phases, a base layer and a superficial layer. The structure of the superficial layer, a fine osmophilic film at the surface of the base layer with occasional lamellae, is consistent with the expected presence of polar lipids and water. The base layer, called the aqueous hypophase, appears as a flocculent, and for the most part, homogeneous layer. Alveolar macrophages are present in the hypophase between the surface layer and the alveolar epithelium. The hypophase is believed to contain proteins and possibly mucopolysaccharides in aqueous solution, as well as some lipids which can be seen in the form of osmophilic myelin figures and lamellae. The base layer is of varying thickness, the thickness probably varying with the degree of inflation of the alveolus. In clefts between adjacent and closely proximated capillaries, they observed the hypophase to be several microns deep, while over other parts of the cell membrane, it was barely observable.

Weibel and Gil's findings have been reproduced utilizing other techniques which allow preservation of the air-liquid interface, such as fixation from the pleural side. Kikkawa (1970) rapidly immersed whole air-filled lungs into fixative allowing diffusion from the tissue side. Freeze-substitution (Kuhn, 1972) and a freeze-etching method (Untersee et al., 1971) have also demonstrated the presence of duplex lining layer with a hypophase and a surface film. These methods indicate that the film appears to be continuous, and by variations in the depth of the base layer, to provide a smoothing of the air-alveolar interface relative to the epithelial surface.

Although these elegant morphological methods, in conjunction with the physiological function of surfactant, indicate that the extracellular lining of the alveoli is composed of an aqueous hypophase topped by a monomolecular phospholipid film, the possibility that all or part of the phospholipid in the film is bound in some manner to proteins or mucopolysaccharides of the hypophase cannot be excluded. The nature of the phospholipid monolayer is likewise unknown. The film may be exclusively DPPC or it may be a mixture of this phospholipid with other phospholipids, or could even contain neutral lipids. The relationships of the lipids in the hypophase to other components of the hypophase as well as to the surface film is also unknown. Many attempts to answer these questions have been made in the past nine years, using biochemical techniques, and with varying degrees of success.

Abrams, in 1966, was the first to report isolation of a surface active material from lung using differential centrifugation. In separate

experiments, lung tissue from rabbits and from human infants, obtained at the time of autopsy, were homogenized with 0.9% NaCl. After preliminary centrifugation at 300 x g for 10 minutes, the surface active material was found to sediment from a 0.9% NaCl solution when centrifuged for 60 minutes at 1,000 x g at 4° C, and to float as a pellicle when suspended in 1.15 specific gravity NaCl solutions and centrifuged at 1,500 x g for 25 minutes. After dialysis against 0.9% NaCl, chemical analysis of the surface active fraction revealed that it contained 40% lipid; the remainder being protein. Phospholipids were the major component of the lipid fraction, accounting for 70% of the total lipid or 28% of the total surface active fraction. PC and PE were both identified in the phospholipids with six times as much PC as PE present. Fatty acid analysis of the PC revealed that 75% of the fatty acids was palmitic acid. Other lipids present were triglycerides (14% of total lipids), free fatty acids (10%), cholesterol (6%) and cholesterol esters (0.5%).

The lipid composition compares quite closely with that reported by Klaus et al. (1961) for surface active material obtained from lung foam. However, Abrams' surface active lipoprotein contained only 40% lipid, as compared with 70% reported by Klaus et al. This difference might be explained by the fact that lung homogenates would contain intracellular as well as extracellular surfactant, and intra- and extracellular surfactant might well differ in protein content. Another possibility is, of course, that the surface active fraction might contain non-specifically absorbed proteins from the lung tissue. The recovery of surface active lipoprotein

by Abrams' method from rabbit lung was 5.2 mg per gram wet weight of lung. Similar values were obtained from infant lungs when the infant died from other than lung problems. However, lungs of infants dying of hyaline membrane disease contained less than 0.5 mg of surface active lipoprotein per gram of lung.

That at least a part of the surface active material of lungs is readily sedimentable was shown by Abrams when he obtained a surface active pellet after centrifugation at $1,000 \times g$ for one hour. In fact, surface active material from dog lung washings sedimented so readily that for a short time Wetton et al. (1967) proposed that the surface activity might be due to the cells present in alveolar washings. They found that when the white opalescent, foamy material from dog lung lavages was centrifuged at $2,000 \times g$ for five minutes, the resulting cellular pellet was surface active when dispersed in a small amount of solution, whereas the supernatant, even after concentration to a smaller volume by vacuum distillation, was not. They also reported a correlation between the numbers of cells present and the surface tension values; the more cells that were present, the greater the ability to lower surface tension. The idea that cells themselves are surface active was quickly disproven. Cavagna et al. (1967) found that the surface active material in the cellular pellet could be recovered in solution by repeated washing of the cells and that cells alone, or their membranes and particles obtained by sonification, lacked surface activity. This study suggested, as did Said et al. (1968), that the surface active material is absorbed onto the cells, possibly during sedimentation, and can be desorbed by shaking and

rinsing. Cavagna et al. (1967) also considered the possibility that the surface active material could be particulate with sedimentation properties similar to those of cells or nuclei. This possibility was discounted because such large particles should sediment in the second as well as the first centrifugation.

Sedimentation is still frequently used to recover surface active material from lung homogenates or washings. In some cases, such as the method described by Abrams, it is only one of several steps in a purification procedure. In others, the surface active pellet obtained by centrifugation of lung washings has been characterized. Finley et al. (1968) obtained saline washings from dogs and centrifuged them for 20 minutes at 27,000 x g. The sediment contained an upper white layer over a lower brown layer of cells. The acellular white layer was highly surface active and similar, morphologically, to the material found on the alveolar surface. Lipid analysis of the white layer showed that over 50% of the lipid was PC, containing predominantly palmitic acid. Galdston et al. (1969) obtained rabbit lung washings by lavage with saline. After centrifugation at 1,650 x g for 20 minutes, a white, fluffy, non-cellular layer on top of the cells and the supernatant were removed and centrifuged at 50,000 x g for one hour. The sediment was washed by resuspending in buffer and centrifuging again. This final sediment was surface active and was found to contain about 60% lipid and 40% protein by weight, with 90% of the lipid being phospholipid. In contrast to these results, Balis et al. (1971) found that after the cells had been removed by centrifugation at 450 x g for five minutes, the

sediments from the cell-free lung washings of rabbits obtained by centrifugation for 90 minutes at 100,000 x g, contained a highly surface active sediment composed largely of phospholipid (72%) and with much less protein (8%).

Scarpelli et al. (1967), using gel filtration techniques to study lung surfactant, proposed that surfactant contains polysaccharides but no protein. Three types of lung preparation from both rabbits and dogs were used. Both unperfused lungs and lungs which had been freed from blood by perfusion through the pulmonary vasculature with saline, were minced in saline. After centrifugation for 20 minutes at 39,000 x g, the supernatants were then utilized as a source of surfactant. No explanation was offered for this procedure which appears somewhat unusual in that other workers have found that surfactant sediments during such a procedure. Scarpelli and coworkers, however, reported that these supernatants were surface active. In addition, washings were obtained from some lungs and used without prior centrifugation. These samples were applied to Sephadex G-200 columns and the surface active material appeared in the void volume of the column. This fraction contained lipids, (primarily PC), polysaccharides, and no detectable protein. Indeed, the only protein that could be identified in later fractions from perfused mince or washings was albumin. The appearance of the surface active material in the void volume of the column indicated a molecular weight greater than 200,000. Scarpelli et al. (1967), on the basis of these experiments, proposed that surfactant is a lipopolysaccharide. It should be pointed out, however, that the presence of both micellar lipids and high

molecular weight polysaccharides in the same fraction does not provide any evidence of association as a complex.

Scarpelli and coworkers continued until quite recently to propose that lung surfactant is not a lipoprotein. In 1970, Scarpelli et al. studied lung surfactant preparations by polyacrylamide gel electrophoresis, using the same methods which are commonly applied to the study of serum lipoproteins, and detected no such lipoproteins in surfactant. When a surface active pellet recovered by centrifugation of lung washings was dispersed in saline and applied to gel, electrophoresis revealed that 90-100% of the lipid remained in the spacer gel with trace amounts of albumin, while most of the protein migrated in five or six bands. These results suggested that there are no lipoproteins in surfactant similar to serum-type lipoprotein, and if any sort of lipoprotein is present, it is so fragile that its components can be separated by electrophoresis.

Similar conclusions were reached using ultracentrifugation methods (Colacicco and Scarpelli, 1970). They reported that lipid from pulmonary washings floated as a single fraction in NaCl-KBr solutions of density 1.080 or higher after 48 hours of centrifugation at $115,000 \times g$ and $15^{\circ} C$. The lipid pellicle was surface active and contained less than 2 percent protein which had the electrophoretic mobility of albumin, suggesting that any association of lipid and protein is a rather tenuous one that can be broken by ultracentrifugation as well as standard electrophoretic methods.

In 1971, Scarpelli and coworkers (Colacicco et al., 1971) reported the results of attempts to further characterize the proteins present in pulmonary

washings. Disc gel electrophoresis of rabbit washings showed three major proteins: one which migrated as albumin, another migrating protein which they call "T", and a third protein, "S", which remained in the spacer gel with the lipid. The results of separation of the washings by filtration on Sephadex G-200 columns as reported in the above abstract and a later detailed paper (Colacicco et al. 1973a) are quite different from those of the first report (Scarpelli et al. 1967). The latter paper reports that three fractions with qualitatively reproducible composition were obtained from Sephadex columns using pulmonary washings which had been concentrated 20-fold by vacuum dialysis and eluted with 1.0 M NaCl in 0.1 M Tris buffer containing 1 mM EDTA. Fraction I contained most of the phospholipid (greater than 85%) and proteins T and S. Fraction II was composed of gamma globulin and fraction III contained primarily albumin. When fraction I was centrifuged at 49,000 x g for one hour, a white pellet containing most of the phospholipid and protein S was obtained. The supernatant had less phospholipid and protein T. Protein T was found to be extracted with the lipid into chloroform and behaved as a proteolipid protein, whereas protein S was water soluble and was removed from the lipid by solvent extraction. Immunological studies (Scarpelli et al., 1973) showed that protein T had no counterpart in serum.

The most recent report by this group (Colacicco et al., 1973b) attempts to characterize the lipid-protein complexes of rabbit and sheep pulmonary washings by sucrose density gradient centrifugation. After removing the cells, the supernatant of lung washings was placed on continuous sucrose

gradients made in 0.15 M NaCl solution. Two surface active bands were found close to each other at densities of 1.056 and 1.065. The upper band at 1.056 was reported to contain 3 times as much phospholipid as protein, while the lower band had 9 times as much phospholipid as protein, a finding which is difficult to understand considering the relative densities of phospholipids (1.03) and proteins (1.33-1.37). Over 60% of the phospholipid of the washings was found in the two bands which contained only protein T. At densities less than 1.05, neutral lipids and albumin were found. At densities greater than 1.07, cholesterol and glycolipids were present. Protein T was further characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Two bands were found corresponding to molecular weights of 150,000 and 60,000. When treated with 2-mercaptoethanol, a band was found corresponding to a molecular weight of 25,000. This protein did not react with antiserum to immunoglobulin G and was found to contain 60-65% hydrophobic amino acid residues.

Concurrently, a number of other researchers were using these and other techniques in attempting to understand the nature of the surface active material in the lungs. McClenahan and Ohlsen (1968) lavaged human lungs at autopsy and separated surfactant by differential centrifugation using a method modified from that of Abrams (1966). The surface active material was fractionated using butanol, and the aqueous phase material was used to prepare antiserum in rabbits. Immuno-electrophoretic analysis of the material indicated that albumin was closely associated with surfactant and was the only water soluble protein present in the surface active fraction.

Other studies using immunologic methods have produced conflicting results. Craig (1964) made antibodies to human surface active material (washed foam) and showed that under all conditions studied, the antibodies failed to react with plasma proteins, but they formed a single precipitation band on Ouchterlony slides when tested against the surfactant preparation. Using this antiserum and immunofluorescent techniques, he studied the distribution of surface active material in infant lungs. Marinkovich and Klein (1972) isolated surfactant from both bovine and human lung homogenates and from bovine lung washings by differential centrifugation and starch block electrophoresis. On injection into chicken, these surfactant preparations caused production of an antiserum that neutralized the surface activity of the surfactant and produced a rapid, visible aggregation of the surfactant. This reaction was completely blocked by pre-incubation of the antibodies with homologous serum albumin, but not by solutions of serum gamma globulins or heterologous serum albumin. Chicken antiserum, specific for serum albumin, also caused aggregation of the surfactant preparation. These results suggest that serum albumin or a molecule antigenically related to it, is intimately associated with the surface active lipids. The most recent immunological studies are those reported by Klaus in 1973. He produced antibodies in rabbits by injecting dog surface active material isolated and purified from lung washings using the methods of King and Clements (1972a). Three distinct antigenic determinants were found in the pulmonary surface active material. Two of these were identical with the serum proteins, albumin and immunoglobulin G. The third was identified

as a non-serum protein. He also reported that by immunofluorescence, this non-serum protein could be seen to be located at the alveolar interface and was not demonstrable in organs other than the lung.

A number of attempts were being made to improve methods for isolation and purification of lung surfactant during this period. Klein and Margolis (1968) concentrated surface active material from extracts of rabbit lung minces in saline by centrifugation in KBr solutions with a density of 1.21 g/ml for 18 hours at 100,000 x g at 1-4° C. The surface active material was found in the pellicle and contained about 3% of the total protein and 75% of the total phospholipid of the crude extract and had a phospholipid to protein ratio of 2.7/1. When this pellicle was resuspended in KBr of density 1.21 and centrifuged at 100,000 x g for 2 hours, small amounts of both phospholipids and protein were lost from the pellicle, but the ratio of the two was changed only slightly to 2/1. Other pellicles were adjusted to densities of 1.063 or 1.15 g/ml and recentrifuged for 2 hours at 100,000 x g for further purification of the lipoprotein. The pellicles isolated at both of these densities were surface active but contained less phospholipid and protein. The pellicle isolated at density 1.15 had a phospholipid to protein ratio of about 3/1, while the pellicle obtained at density of 1.063 had a ratio of about 8/1, indicating the removal of a more dense protein or lipoprotein from the original pellicle by centrifugation in media of lower density, and therefore, the heterogeneity of protein-phospholipid complexes present in crude lung extracts.

Stein et al. (1969), using lung washings from dogs, applied both differential and density gradient techniques to the isolation and purification of lung surfactant. After removing the cells, the surfactant was sedimented by centrifugation at 16,000 x g for 1 hour. The resulting pellet was reported to contain 79% of the phosphorus and less than 2% of the protein of the original cell-free washing. The pellet was dispersed by dialysis against 0.02 M Tris-EDTA at pH 8.0 and then against distilled water. The deionized surfactant was layered over a linear sucrose gradient with densities of 1.000 to 1.100 and centrifuged at 4° for 20 hours at 100,000 x g. The band which showed surface activity was centered at a density of 1.035. Based upon this experiment, a purification scheme was devised in which the dialyzed pellet was alternately sedimented in sucrose at a density of 1.010 and floated in a solution with a density of 1.055. The surfactant thus obtained was highly surface active, but its total protein and carbohydrate content was less than 3%. The conclusion made by the authors was that native surfactant is a mixture of lipids and not a lipoprotein.

Frosolono et al. (1970) described a procedure for the isolation of surface active material from dog lung homogenates and lung lavages using discontinuous sucrose gradients containing NaCl, Tris buffer and 0.001 M EDTA at pH 7.4. After several steps of centrifugation in discontinuous sucrose gradients and sedimentation from the homogenization medium without sucrose, the surface active material was found at an interface of 0.25 M (density = 1.03) and 0.68 M (density = 1.09) sucrose. This fraction was found to contain lipid, protein and carbohydrates with the lipid to protein

ratio being 3.86 and with phospholipids constituting the major portion of the lipids. With gel filtration of the fraction on Sepharose 2B columns, the surface active material appeared in the void volume, indicating a molecular weight of greater than 25×10^6 . The lipid to protein ratio was not changed by gel filtration, indicating that the protein is an integral component of the surface active fraction. With disc electrophoresis, the fraction did not enter the gel. Protein was extracted from this fraction using ethanol-ether at -10°C and suspended in sodium borate buffer at pH 11.5. Both gel filtration and disc electrophoresis of the protein fraction revealed the presence of two proteins. After delipidation, the carbohydrate remained with the protein, suggesting that the proteins are glycoproteins.

Rabbit lung washings were studied by Reiss (1971) who reported purification of the particulate material on sucrose gradients. Three bands were found on gradients, two of which at densities of 1.042 and 1.052, were surface active. The band at 1.035 was not surface active. The proteins of the surface active bands were extracted with ethanol-ether at -10°C and the amino acids were analyzed. The amino acid, as well as the lipid composition of the two bands, were reported to be similar. Two times as many acidic as basic residues were found along with a high percentage of hydrophobic amino acids, few aromatic amino acids and no methionine.

Pig lung surfactant was studied by Pruitt et al. (1971). Lungs were lavaged with Tris-NaCl solution, pH 7.4 and after removal of the cells, the surface active material was sedimented at $27,000 \times g$ for 20 minutes. The

pellet was suspended in buffer and subjected to density gradient centrifugation on discontinuous sucrose gradients ranging from 0.25 M to 0.75 M. Two bands were found at 0.50 M (density = 1.065) and 0.55 M (density = 1.072) sucrose. Both of these fractions were found to contain 4 mg of phospholipid per mg of protein and 80% of the phospholipid was DPPC. Repeated density gradient centrifugation of either band produced both bands. Disc-gel electrophoresis of both bands which had been solubilized using 1% octyl phenoxy polyethoxy-ethanol revealed the presence of two components. The slower moving band stained strongly for lipid and weakly for protein. The faster moving band stained strongly for protein and weakly for lipid. This fast moving band had a mobility similar to that of albumin. However, immunoelectrophoresis and immunodiffusion studies of both bands revealed no reaction with antiserum to porcine albumin.

King and Clements (1972a) published an elaborate method for isolation of surface active materials from dog lung homogenates and washings. They used a combination of differential and density gradient centrifugation steps in NaBr solutions buffered to pH 7.35 and with the calcium and magnesium concentrations approximating physiological proportions. Their procedure for isolation of surface active materials from washings involved seven steps of centrifugation, and included four sedimentations of the surface active material, two flotations and one density gradient. Total time of centrifugation was 52.5 hours. The isolation scheme for surface active material from lung homogenates was even more lengthy. After this long series of steps, four surface active fractions were obtained, one from washings and

three from homogenates. Each had well defined and reproducible isopycnic densities, and protein and phosphorus compositions. The phospholipid to protein ratio of the surface active fraction from lung wash was 6.62 and those from the homogenate were 4.00, 5.11 and 6.00. The isopycnic densities were 1.089 for the fraction from washings and ranged from 1.088 to 1.103 for the fractions from homogenates. The recovery of surface active material was estimated by surface titration data, which determined that the recovery from the lavage fluid was about 50% and that from lung homogenates was about 25%.

In their second paper (King and Clements, 1972b), the four fractions isolated as described above were further characterized. All were composed almost entirely of lipid and protein and contained less than 2% hexose and hexosamine. The four fractions differed from each other in protein content, but did not differ significantly in lipid composition. In all of the surface active fractions, the phospholipids constituted more than 80% of the total lipids. All fractions contained over 73% PC. Other lipids present included PE, sphingomyelin, triglycerides, cholesterol, and free fatty acids. Separation of the PC into saturated and unsaturated PC species revealed that more than 60% of the PC was fully saturated and that this fraction was composed of nearly 90% palmitic acid.

Attempts to further separate the surface active material from washings by electrophoresis in agar/agarose gels resulted in almost all of the material remaining at the origin. The material at the origin stained both for protein and lipid. Isoelectric focusing of the surface

active fraction from lung washings was carried out. The largest amount of material migrated to an apparent pI of 3.8-4.1 and had a phospholipid to protein ratio close to that of the starting material and retained its ability to lower surface tension. Even in light of this evidence, these authors remain cautious in defining lung surfactant as a lipoprotein and they state that the "exact molecular definition still remains to be achieved" and "that final conclusions concerning the lipoprotein nature of this material should be postponed."

In a following paper, King et al. (1973) reported further characterization of the proteins of their surface active fraction from lung washings. Most of the lipids were removed by ethanol-ether at -15° C and the protein fraction containing about 12% of the phospholipid present in the original fraction was solubilized by sonication in sodium borate solution, pH 9.0, containing sodium dodecyl sulfate. Following centrifugation, the clear supernatant was used for study of the proteins. SDS-PAGE revealed the presence of three bands: immunoglobulins at a molecular weight of 150,000, albumin at 68,000, and a non-serum protein at 10,000. Addition of 2-mercaptoethanol before electrophoresis revealed bands at 75,000, 50,000, 32,000 and 25,000, most of which were derived from immunoglobulin G. The 10,000 molecular weight band was not affected by 2-mercaptoethanol. From intensity of staining and densitometric scans, it was estimated that the 10,000 dalton component constituted about 50 to 75% of the total protein. This component was separated from the other proteins in larger quantities by gel filtration in the presence of SDS. This non-serum protein contained 16.6 ug organic

phosphorus, 5.6 ug of sialic acid and 13.5 ug hexose per 100 ug of protein. The phosphorus could be extracted with chloroform-methanol and it represented phospholipid phosphorus. Therefore, there were about 400 ug of phospholipid present per 100 ug of protein in this fraction. Studies of the lipid composition revealed that PC made up 56% of the total lipid while another 32% was tentatively identified as PG. Both PC and PG contained 65-75% palmitic acid. Studies of the amino acid composition revealed the presence of twice as many acidic as basic amino acids and a large portion of hydrophobic residues.

Gil and Reiss (1973) used discontinuous sucrose gradients to separate three morphologically distinct, surface active fractions from rat lung homogenates. After homogenization of the rat lung, without prior washing to remove the extracellular lining layer, a mitochondria-rich fraction was isolated and washed by centrifugation. The washed pellets were resuspended in a small volume of the homogenization medium which contained 0.32 M sucrose in Tris buffer, with calcium, pH 7.4, and layered on the top of a discontinuous sucrose gradient. After centrifugation, fractions found at densities of 1.051, 1.059 and 1.089 were determined to be surface active and each was morphologically homogeneous. Common myelin figures, found at the density of 1.051, had a phospholipid to protein ratio of 8.7/1, and the layer contained mostly osmiophilic structures that could be interpreted as damaged lamellar bodies. However, protein electrophoresis indicated the presence of a protein in this fraction which was not present in the lamellar bodies, suggesting that the common myelin figures contained material from other sources as well.

The fraction at the density of 1.059 contained the lamellar bodies, and had a phospholipid to protein ratio of 4.7/1. It was clearly of intracellular origin. The last fraction, described as tubular myelin figures, contained twice as much phospholipid as protein, and was found at a density of 1.089. It was postulated by the authors to be a liquid crystalline state of the extracellular surface active lipoprotein.

The lipids of all three fractions contained approximately 94% polar lipids, of which 73% was PC in the common myelin figures and lamellar bodies. Tubular myelin figures, however, contained only 58% PC. Electrophoresis of the proteins of each fraction revealed that the lamellar bodies and the tubular myelin figures contained proteins with different mobilities, whereas the common myelin figures contained the protein found in both of the other fractions.

A unique approach has been used by Hurst and coworkers (1973) in their attempts to identify the components of lung surfactant. Since a large portion of the protein found in alveolar washings is albumin, they designed experiments to explore the possibility that the proteins in lung washings might be contaminants from the capillary blood. In some rabbits, the pulmonary vasculature was perfused with either 0.25 M sucrose or FC-80 fluorocarbon, an inert, non-aqueous liquid, before obtaining surfactant by alveolar lavage. This was done in order to minimize any transport of blood constituents during the lavage procedure. After removal of cells by low speed centrifugation, the lipid and protein contents of these lavages were compared with those of lavages obtained from non-perfused animals.

Lavages from non-perfused rabbits (those in which pulmonary capillary blood was present) contained substantial quantities of both lipid and protein. Five successive washings from each rabbit yielded an average total of 117 mg of lipid and 23 mg of protein. Only a slightly smaller average total lipid content was found in washings from perfused lungs. Perfusion with sucrose and fluorocarbon yielded lavages containing an average of 94 mg and 96 mg of lipid, respectively. However, the amount of protein in the washings from perfused lungs was drastically altered. Sucrose perfusion yielded lavages containing an average of only 3.8 mg of protein and following perfusion with fluorocarbon, less than 0.3 mg of protein were found in the washings.

Thin-layer chromatography of the lipid fractions revealed that the lipid present in greatest quantities in all cases migrated as PC. Small amounts of neutral lipids and other phospholipids, amounting to 10 to 15% of the total lipids, were also present in the washings from non-fluorocarbon perfused animals. The lipid from lavage of fluorocarbon-perfused animals contained almost exclusively PC and consumed essentially no iodine when titrated using starch as an indicator. This indicated the absence of unsaturated fatty acids in the PC.

The proteins from lavages of non-perfused and sucrose-perfused animals were separated on DEAE cellulose columns and characterized by SDS-PAGE in the presence of mercaptoethanol. A small peak from the DEAE cellulose column contained a heterogenous mixture of large and small molecular weight polypeptides. The second peak, which was the major component, contained essentially

only one protein which could not be separated from pure rabbit serum albumin in the SDS gel electrophoresis system.

On the basis of these studies, the authors have suggested that the alveolus is normally lined with saturated PC, essentially free from other lipids, proteins and carbohydrates.

Protein synthesis and secretion by lung have been studied by Massaro and coworkers with the results reported in a series of papers. In the most recents of these, Dickie et al. (1973) reported that a surface active fraction from lung tissue contains a protein which is synthesized in the lung. They found that in an in vitro system, lung slices incorporated radioactively labeled leucine into the proteins of a surface active fraction which floats at a density of 1.21 and which had been purified from homogenates by the method of Klein and Margolis (1968). Using a combined in vivo and in vitro system, they found that surface active material from both lung homogenates and lung washings contained radioactively labeled protein, and that the specific activity of the protein in the lavage surface active fraction was decreased by incubation at low temperatures.

Polyacrylamide gel electrophoresis of surface active pellicles from both lung homogenates and washings which had been sonicated showed a very rapidly migrating band which moved more rapidly than any band in similarly treated serum. Staining reactions showed that the band contained both lipid and protein. Dickie and coworkers concluded that the surface active material isolated from lung contained a protein-lipid moiety which is not a contaminant from serum. Furthermore, they suggest that their evidence indicated that the protein, a non-serum protein, is synthesized in the lung.

STATEMENT OF THE PROBLEM

Although most investigators agree that DPPC is the major substance lowering surface tension at the alveolar tissue-air interface, several uncertainties remain about the exact chemical composition and physical properties of the pulmonary surface active material. Whether native surfactant is only lipid, as proposed by Steim et al. (1969) and by Hurst et al. (1973), or whether it is some sort of lipid-protein complex, as suggested by most researchers, poses a question which has not yet been resolved. The solution to this problem 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 and physical state of surfactant in various pathological conditions.

Biochemical characterization of lung surfactant is a difficult problem for a number of reasons. The first of these concerns the method of obtaining material for analysis. Lung lavage with Ringer's lactate solution causes little, if any, damage to the epithelial cells or to the alveolar macrophages as observed by electron microscopy (Balis et al., 1971), thus yielding a more pure preparation of material present on the alveolar surface than could be obtained from tissue homogenates, even after extensive purification. However, since 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, and since it is known that plasma components can move across this tissue barrier, as for example, in pulmonary edema, the possibility that lung washings may contain material from plasma cannot be excluded. Indeed, in

a recent paper, Reifenrath and Zimmerman (1973) using a micropuncture technique, concluded that the albumin found in lung washings was not a true constituent of the lining layer, but came from plasma and was an artifact of the lavage procedure.

Hurst and coworkers (1973) attempted to eliminate contamination of lung washings with plasma components by perfusion of the pulmonary circulation with sucrose solutions, or with the non-aqueous liquid, FC-80 fluorocarbon. They assumed that in such treatments, alveolar contents are not lost into the tissue or the vascular space. However, it has been shown by Bensch et al. (1967) that albumin and globulins can migrate from the alveoli into the circulatory system. Although the results of Hurst et al. (1973) could be interpreted as indicating the absence of at least any strongly-bound lipid-protein complexes on the alveolar surface, the possibility that perfusion might cause absorption of some components of the alveolar lining layer from the alveolar surface remains open.

The second problem in the characterization of lung surfactant, as pointed out by Clements (1970) and again by King and Clements (1972a), is that the physical properties of lung surface active materials severely limit the methods which may be utilized for their isolation. In appearance, lung washings are an opalescent, white fluid, containing a considerable amount of material in an insoluble suspension. This insoluble surface active material can be observed to settle on the bottom of the container if the washings are allowed to stand for a few hours, or it can be readily collected as a sediment following centrifugation at fairly low speeds.

The large and possibly variable particle size of surface active material also poses problems in designing methods for their isolation and characterization. King and Clements (1972a) estimated that surfactant is composed of particles which are generally greater than 100 μ in diameter. This large particle size precludes the use of solid chromatographic supports, such as agar, agarose and polyacrylamide gels. Also, these large particles are beyond the effective ranges of separation afforded by molecular sieving gels.

King and Clements (1972a) suggested that their method of purification by differential and density gradient centrifugation in a salt media was based on density only, and eliminated the factor of particle size. This appears to be true for density gradient centrifugations which they carried out; however, the sedimentation and flotation steps which they used prior to density gradient centrifugation do not appear to be independent of particle size. Virtually all of the methods used for the study of lung surfactant begin with the isolation of surface active fractions by sedimentation or flotation. Therefore, the possibility that such centrifugations could cause the loss of some important components because of their smaller particle size, or could cause the production of artifactual lipid-protein complexes, must be considered, particularly since the concept that lung surfactant is lipoprotein in nature comes largely from the fact that lipids and proteins are found together in various fractions obtained upon centrifugation. As can be seen from the preceding literature review, the so-called lung surfactant lipoproteins

vary widely in their composition, especially with respect to the amount of protein present, and the nature of the protein itself is not yet established.

The experimental work to be reported in this investigation was carried out using methods designed to overcome some of these problems. Lung washings were utilized with an understanding that they might contain some plasma components, but this factor did not preclude their use since washings appeared to be the best available source of the alveolar lining material. Specifically, two main approaches were employed. Analytical ultracentrifugation, a technique that has been widely applied in the study of serum lipoproteins, was used in an attempt to discover the possible existence of any discrete classes of lipoprotein particles in the lung washings. Density gradient centrifugation was also used, and in much the same manner as other workers have employed it, but with the important distinction that the entire non-cellular washing was used as the starting material for density gradient centrifugation, thus avoiding any possible formation of artifactual lipid-protein complexes by sedimentation or flotation. The proteins of the non-cellular washings, as well as some centrifugation fractions of the washings, were characterized by SDS-PAGE. Analysis of some of the problems encountered in these experiments resulted in a better understanding of some of the divergent data in previously published reports.

CHAPTER II

MATERIALS AND METHODS

COLLECTION OF LUNG WASHINGS

Adult albino rabbits were anesthetized by intravenous injection of sodium pentobarbital. The animals were exsanguinated by cutting the aorta and inferior vena cava, and before opening the chest cavity, the trachea was clamped to prevent the entry of blood into the tracheobronchial tree. Ringer's lactate solution (purchased from Baxter Laboratories, Morton Grove, Illinois) was introduced into the lungs through a cannula in the trachea. Initially, 35 ml of solution was instilled and slowly removed by gravity drainage and gentle suction. This procedure was repeated with another 35 ml of Ringer's lactate solution (RLS), and from a total of 70 ml of solution introduced into the lungs, about 50 ml of a white, opalescent washing was recovered.

In some experiments, variations of the above procedure were used. In some cases, washings were obtained with RLS chilled to 4° C before use. In other experiments, a Tris buffer solution was used in place of RLS, 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 (1972a), with the following composition:

0.15 <u>M</u> NaCl	(8.77 g/l)
0.003 <u>M</u> MgCl ₂	(0.61 g of MgCl ₂ ·6 H ₂ O/l)
0.003 <u>M</u> CaCl ₂	(0.44 g of CaCl ₂ ·2 H ₂ O/l)
0.005 <u>M</u> Tris/HCl	(0.78 g of Trizma/l, pH 7.0)
	Sigma Chemical Company

In an experiment designed to test the effect of adding protein to lung surfactant, 4 ml of rabbit serum was mixed with 66 ml of RLS to make the 70 ml of solution used for lavage. In other experiments, an attempt was made to obtain a more concentrated solution of washings by using only 35 ml of RLS, instilling it first into one lung, and then into the other, or by instilling only 10 ml of RLS solution via the trachea and perfusing with RLS through the pulmonary artery.

Washings were obtained from 31-day gestation rabbit fetuses using the procedure as first described with the exception that the total amount of RLS in mls used was determined by multiplying the body weight in grams by 0.2.

Lung washings were obtained by lavage with RLS from one human infant. The amount of solution used was determined by multiplying 0.06 times the infant's weight in grams, and then using one-half of this amount in two successive lavages.

PREPARATION OF LUNG WASHINGS FOR ANALYTICAL ULTRACENTRIFUGATION

A number of different methods were applied to lung washings in attempting to find a suitable way of preparing the sample for analytical ultracentrifugation. In all experiments, the washings were centrifuged for 5 minutes at 480 x g for the purpose of removing the cells, which

were mostly alveolar macrophages. This centrifugation was shown by cell counts both before and after centrifugation to remove more than 99% of the cells present in the washings (Balis *et al.*, 1971).

In some experiments, the lung washings were separated into sedimentable and non-sedimentable fractions as described by Balis *et al.* (1971). The non-cellular supernatant was centrifuged at 127,000 x g, max. for 90 minutes to obtain a surface active sediment B and surface inactive supernatant C. 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. The details of such experiments are described in Chapter III along with the results of those particular experiments.

Lung washing sediments obtained by centrifugation were on some occasions subjected to short periods of ultrasonic irradiation, a method which 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 second intervals separated by a 1 minute or longer period in order to prevent overheating. Samples were sonicated for 1, 3, or 10 minutes.

In other experiments, cell-free lung washings or lung washing fractions were concentrated by pressure ultrafiltration in an Amicon L52 stirred cell (Amicon Corp., Lexington, Mass.). Depending on the pore size of the membrane utilized in the ultrafiltration cell, various sized components could be removed from the solution. Three different kinds of membranes

were used in this study. UM05, the smallest pore size, retains all components with a molecular weight greater than 500. PM10 membranes were used to retain molecules larger than 10,000 and XM300 membranes for molecules larger than 300,000. The retention values are only an approximation, since molecular size, shape, and charge also influence the retention of individual components.

ANALYTICAL ULTRACENTRIFUGATION

Sedimentation velocity measurements were used to characterize the components of lung washings. This procedure allows detection of the presence of macromolecular species, calculation of the sedimentation coefficients, detection of molecular heterogeneity and estimation of the relative amounts of the components. Solute molecules, subjected to high centrifugal forces, move outward from the axis of rotation, forming a fairly sharp boundary between the solution and pure solvent. The rate of movement of the boundary is observed by changes in the refractive index by means of a schlieren cylindrical lens system. The boundary appears as a peak in the schlieren pattern, and the maximum ordinate of the peak is a reasonably accurate indication of boundary position. The position of the peak at given time intervals is recorded photographically. Flotation rates are measured in the same manner, except that the sample is dissolved in a solution of higher density and the solute molecules, when subjected to high centrifugal forces, move toward the center of rotation (deLalla and Gofman, 1954).

The rate of sedimentation is expressed in terms of the sedimentation coefficient, S , which is the velocity for unit centrifugal field of force and has the dimensions of time. From observations of the rate of boundary movement, S can be calculated from the following formula.

$$S = \frac{1}{w^2 x} \cdot \frac{dx}{dt}$$

where " x " represents the distance from the center of rotation and " w ," the angular velocity in radians per second. A sedimentation coefficient of 10^{-13} seconds is termed "one Svedberg unit" or " s ." Flotation rates are determined in the same manner, except that the s value is negative since the boundary is moving in the opposite direction. Therefore, the terms " S_f " or " F " which equal $-1 s$ are used to describe flotation under defined conditions.

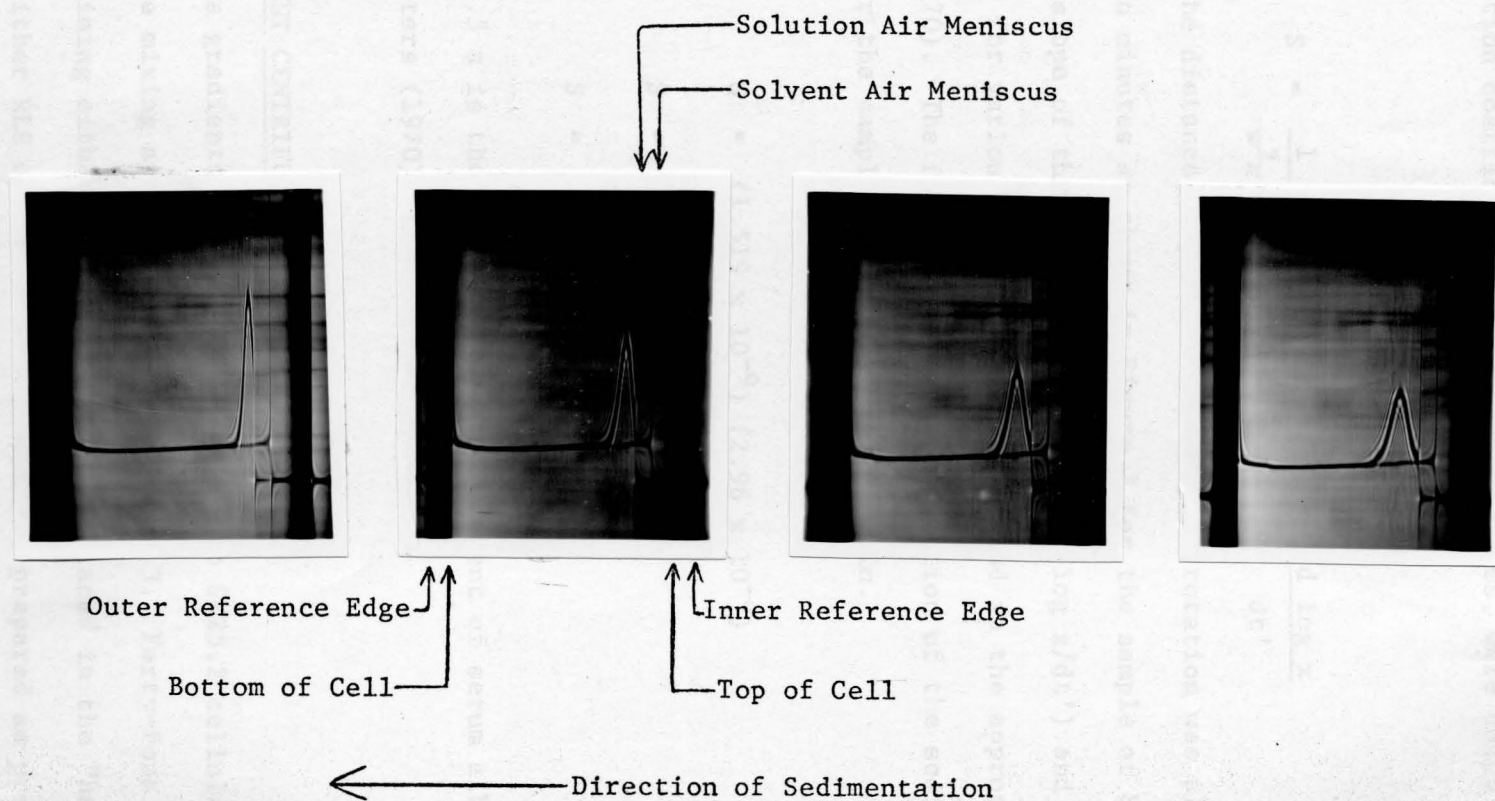
Samples of non-cellular lung washings and various fractions obtained by centrifugation of the washings, were investigated using the above methods. In addition, a sample with a known sedimentation coefficient, bovine serum albumin, was also subjected to the same analysis as a check to determine that the procedures and calculations were being carried out correctly. Each sample was placed in one sector of a 12 mm double-sectored cell, and the other sector was filled with the appropriate reference solution. For example, 24% NaBr in Ringer's lactate solution was used as the reference solution for a sample in a solution of 24% NaBr in RLS. The

cell was placed in an An-D rotor opposite a schlieren counterbalance and centrifugation was carried out at 20° C in a Spinco Model E ultracentrifuge. The speed of centrifugation and intervals at which photographs were taken varied and are noted in the results of the experiments.

A series of photographs taken at 8 minute intervals during the centrifugation of a sample of bovine serum albumin, 5 mg/ml, is shown in Figure 1. This sample was centrifuged at 48,000 rpm, and the first photograph was taken 19 minutes and 35 seconds after zero time, the time when 2/3 of the operating speed had been attained.

Because of the magnification of the camera lens, the dimensions of the photographic plates are larger than the actual dimensions in the cell and counterbalance. The ratio of image size to cell size, the magnification factor, was determined by measuring the distance between the inner reference edge and the outer reference edge on the photographic plate, and dividing this value by the known distance between the reference edges of the counterbalance (Chervenka, 1970).

Measurements of the positions of peaks were made relative to the image of the inner reference edge, which is 5.70 cm. from center of the rotor at low speed and 5.71 at 40,000 rpm (Schachman, 1957). The actual distance of the peak from the axis of rotation was calculated by dividing the measured distance from the inner reference edge to the peak by the magnification factor, 2.1375, and adding 5.70 or 5.71, depending upon the speed of centrifugation.



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Figure 1. Schlieren pattern of bovine serum albumin. The sample was centrifuged at 48,000 rpm and 20° C. Photographs were taken at 8 minute intervals.

Sedimentation coefficients, or flotation rates, were calculated using the equation:

$$S = \frac{1}{w^2 x} \cdot \frac{dx}{dt} = \frac{2.303}{60 w^2} \cdot \frac{d \log x}{dt'}$$

The \log_{10} of the distance of peak from center of rotation was plotted against time in minutes as shown in Figure 2 for the sample of bovine serum albumin. The slope of this line is the value $(d \log x/dt')$ and the value of $2.303/60 w^2$ for various speed settings is found in the appropriate tables (Chervenka, 1970). The following is the calculation of the sedimentation coefficient for the sample of bovine serum albumin.

$$S = (1.519 \times 10^{-9}) (2.96 \times 10^{-4})$$

$$S = 4.496 \times 10^{-13}$$

$$S = 4.5 \text{ s}$$

The value of 4.5 s is the sedimentation coefficient of serum albumin recorded by Peters (1970).

DENSITY GRADIENT CENTRIFUGATION

Continuous gradients were prepared in Spinco SW25.2 cellulose nitrate tubes using the mixing apparatus shown in Figure 3. Forty-four ml of solution containing either NaBr or sucrose was placed in the "heavy" flask and 24 ml of either RLS or Tris buffered saline, prepared as previously

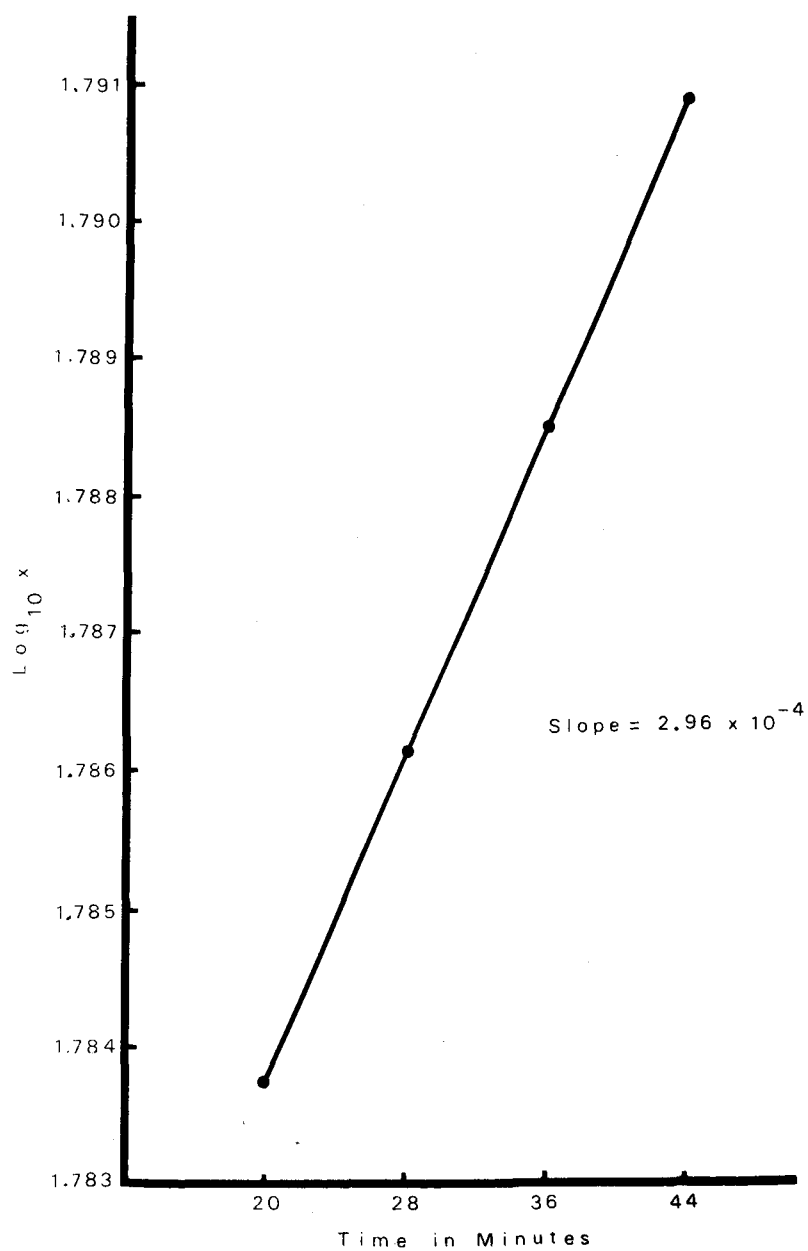
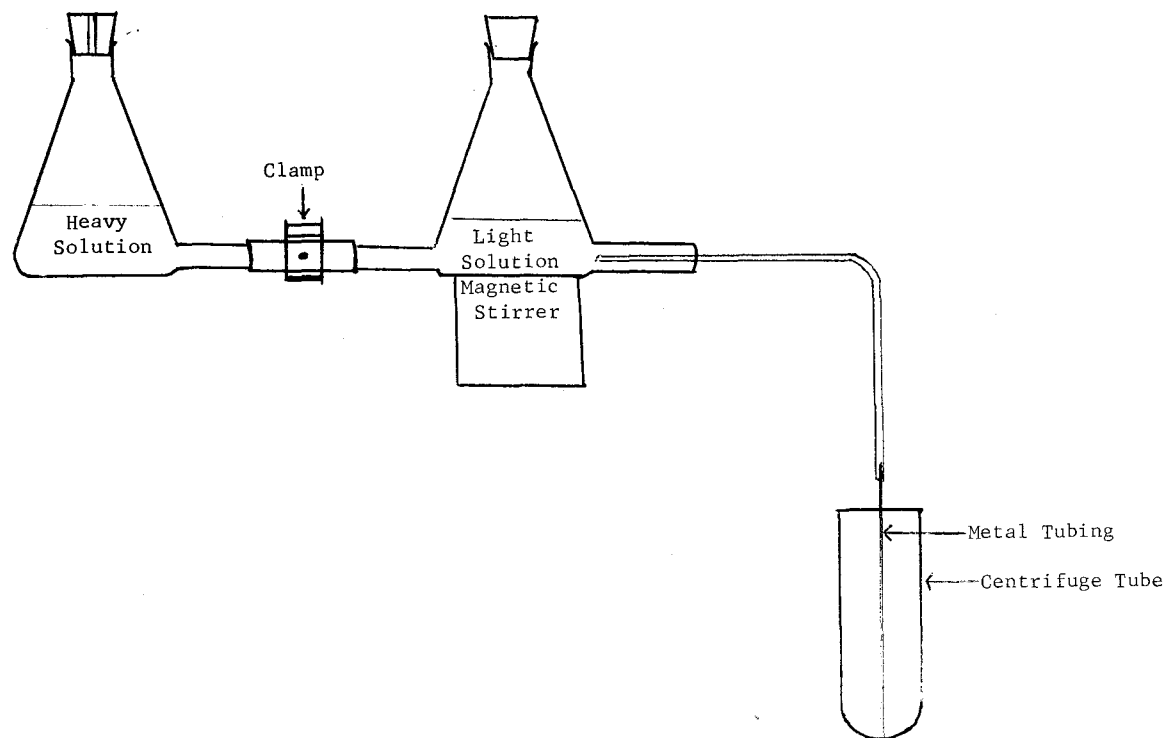


Figure 2. Plot of $\log x$ versus time for calculation of sedimentation coefficient of bovine serum albumin.



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Figure 3. Apparatus for preparation of continuous density gradients.

described, was placed in the "light" flask. After the flow of solution from the light flask into the centrifuge tube was started, the flasks were stoppered and the clamp between the two flasks was released, allowing gradual mixing of the heavy solution into the light solution. The flow was stopped when the meniscus was within 3 mm of the top of the tube, giving a volume of about 58 ml in the gradient tube.

In order to check the gradient-making apparatus, Orange G dye was dissolved in a heavy solution containing 30% NaBr (density = 1.28) in RLS before mixing a gradient using RLS as the light solution. After the gradient was formed, the bottom of the tube was punctured with a needle contained in a plastic cylinder designed to hold the SW 25.2 tubes. The tube was drained through a flow cell in a Beckman DB spectrophotometer and the absorbance at 480 nm was recorded. The results are shown in Figure 4 indicating that a satisfactory gradient had been formed with densities ranging in excess of 82% of the range of the original solutions.

Gradients to be run at 20° C were prepared at room temperature and those to be run at 4° C were prepared at 4°. Cell-free lung washings were used as the heavy solution after adding the appropriate amount of either NaBr or sucrose to them. This resulted in the sample being spread throughout the tube at the beginning of the run although not evenly. The gradients were centrifuged in the SW 25.2 swinging bucket rotor of the Spinco model L2 ultracentrifuge at 23,000 rpm (90,000 x g, max.) at either 20° C or 4° C. The runs were generally 18 hours in duration, but as noted in specific

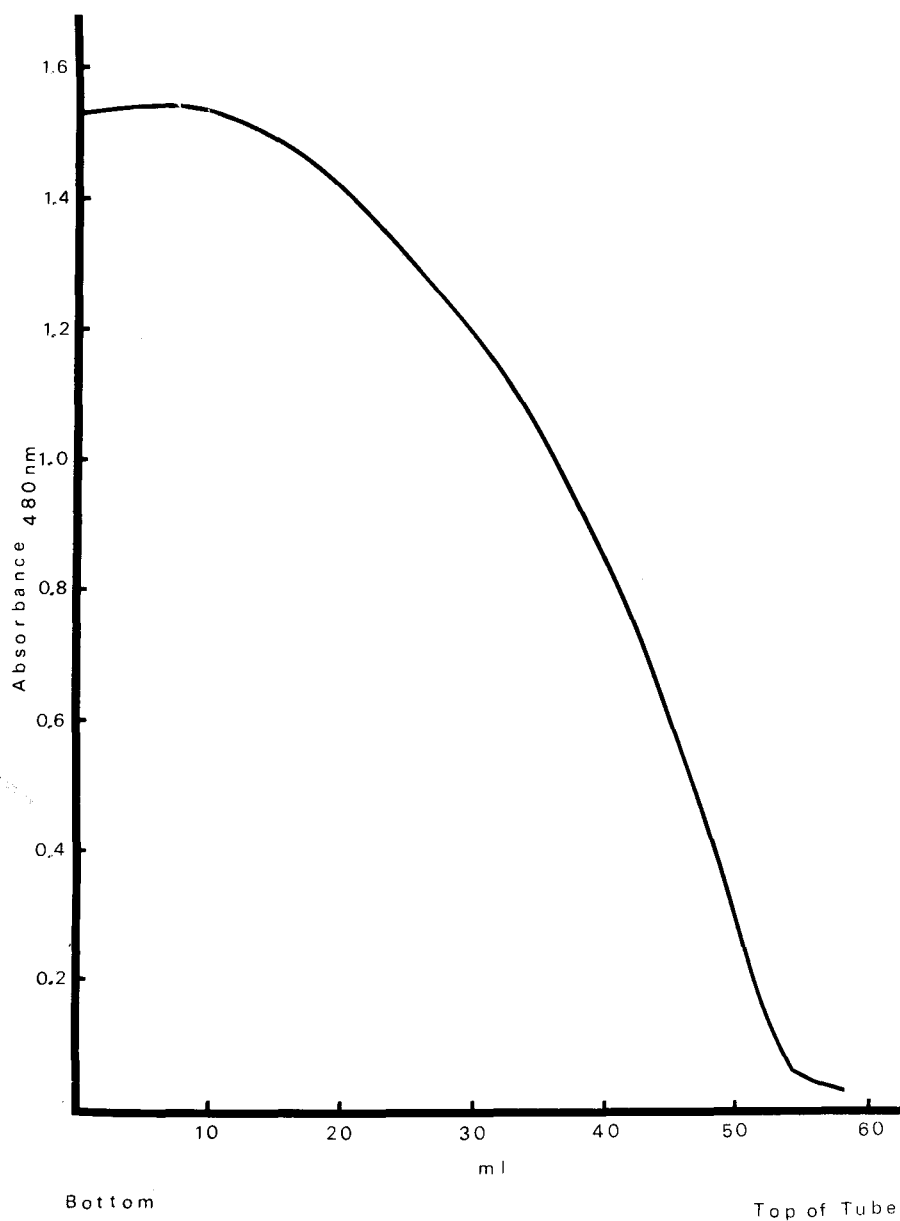


Figure 4. NaBr gradient prepared with Orange G dye. The absorbance of the heavy solution ($d = 1.28$) used to mix the gradient was 1.83 and that of the light solution ($d = 1.00$) was 0.00. Based on these values the range of densities in the gradient was calculated to be from 1.01 to 1.24.

experiments, the length of time of centrifugation varied from 3 to 24 hours and were stopped without use of the brake.

In early experiments, fractions were removed from the visible bands and other areas of the tube using syringe and hypodermic needle bent in such a way that the bellowed opening could be placed at the bottom of the band to be removed. In later experiments, fractions were collected from the bottoms of the tubes after puncturing with a needle held in the plastic cylinder designed to hold SW 25.2 tubes.

The densities of various fractions were estimated by weighing a measured volume of the fractions of interest. It was determined in the course of the experiments, that the most useful gradient at 20° C was made from 10% NaBr, and at 4° C from 14% NaBr. Therefore, careful density measurements were made using a 10 ml pycnometer on fractions from these gradients in which no sample had been placed. Fractions from tubes which had been run at 20° C were weighed at 20° C and the density expressed relative to water at 4° C (d_4^{20}) (Figure 5). Density measurements from tubes which had been centrifuged at 4° C were made at 4° C and related to water at 4° C (d_4^4) (Figure 6).

MEASUREMENT OF SURFACE ACTIVITY

The dynamic surface tension properties of the pulmonary lavage fluids and the various fractions obtained from these fluids were measured in a modified Langmuir-Wilhelmy surface balance. This instrument, described by Greenfield and Kimmell (1967) and purchased from Kimray, Inc., Oklahoma

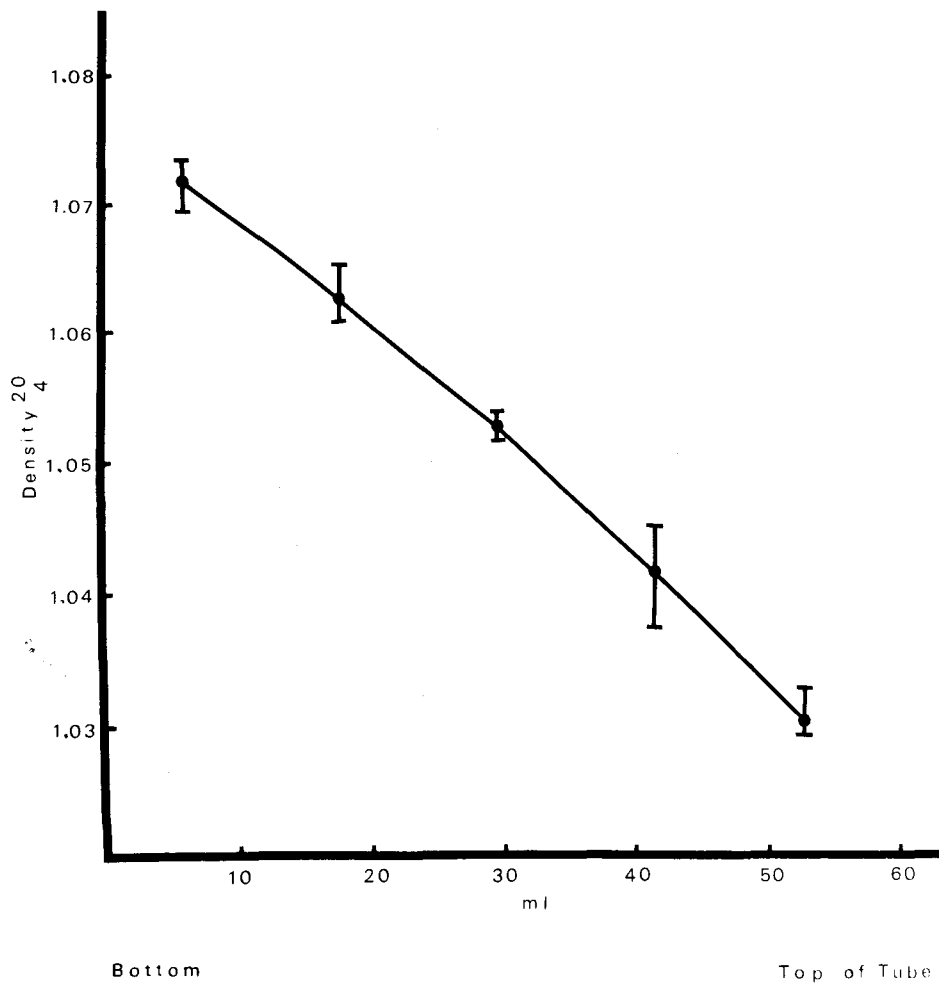


Figure 5. NaBr density gradient for density measurements at 20° C. These gradients were prepared from RLS and 10% NaBr in RLS, and centrifuged for 18 hours. Each point is the average of 3 measurements. The ranges of values are indicated by the vertical lines.

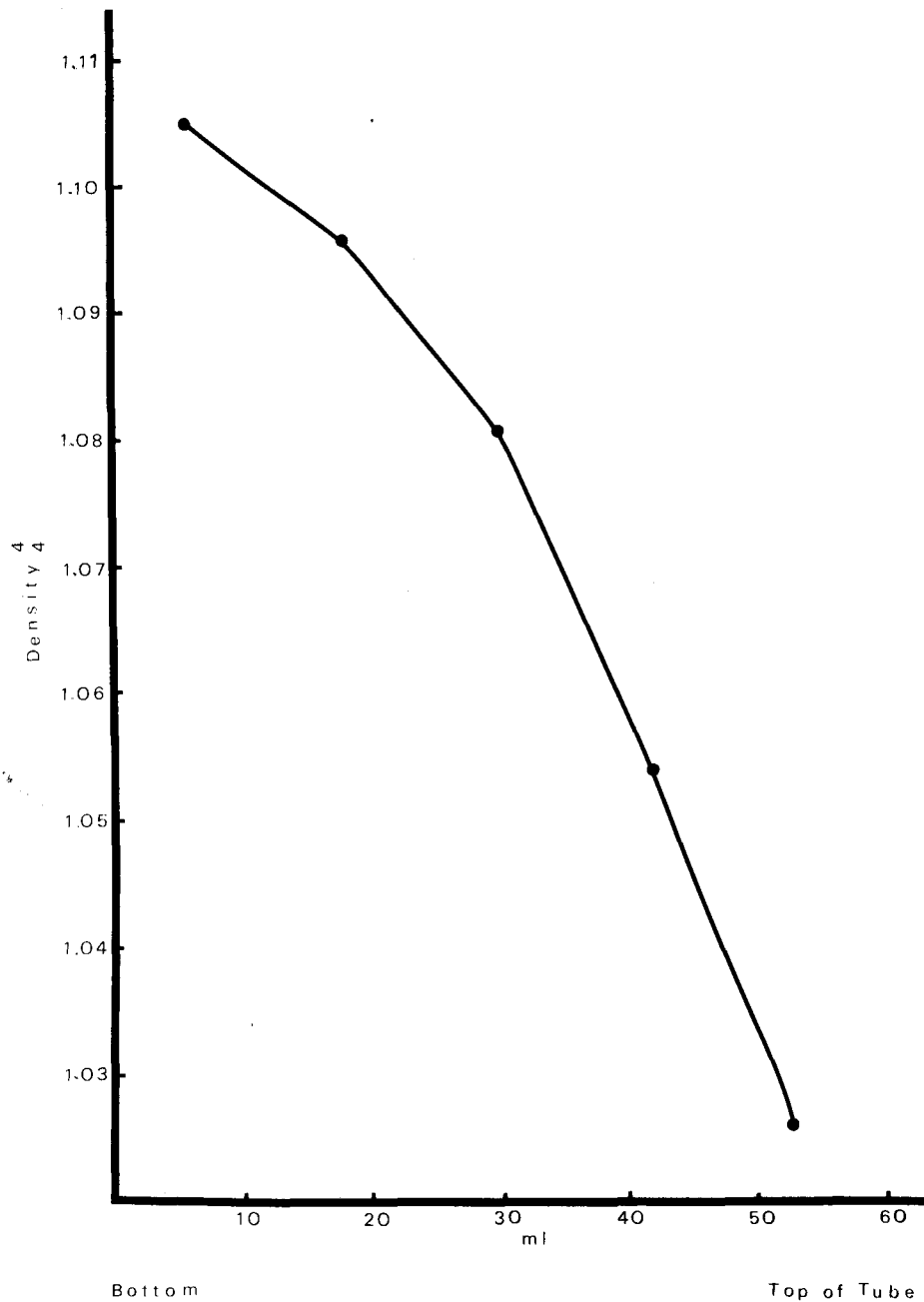


Figure 6. NaBr density gradient for density measurements at 4° C. This gradient was prepared from RLS and 14% NaBr in RLS, and centrifuged for 18 hours. Each point represent a single measurement.

City, Oklahoma, allows direct measurement of surface tension forces on a vertical platinum strip subtending the surface of the sample in a teflon trough, while the surface area is changed by a moving barrier. A pneumatic sensor and amplifier are connected to a stylus which continuously records the surface tension. The maximum surface area of the trough is 57.2 cm^2 and can be reduced to 7.0 cm^2 , representing a 12% residual area when the moving barrier is closest to the platinum strip.

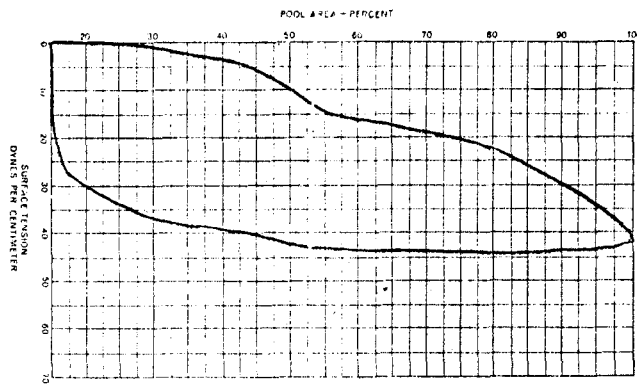
Before a sample was tested, the teflon trough and barrier were cleaned with ethanol and distilled water and the platinum strip was rinsed with ethanol and flamed. Prior to introducing the sample into the teflon trough, the zero position of the platinum strip was adjusted and then 25 ml of RLS was placed in the trough and the instrument allowed to cycle. If a straight line at 70 to 72 dynes/cm was not observed, the cleaning procedure was repeated.

When the instrument was properly cleaned and calibrated, the sample was placed in the trough. If 10 ml or less of sample was to be tested, it was carefully poured onto the RLS already in the trough. If larger volumes of sample were to be tested, the RLS was removed from the trough and replaced by the sample. In all cases, the total volumes of solution in the trough were between 25 and 35 ml and all studies were carried out at room temperature. The surface film was allowed to "age" for 10 minutes before compression and expansion cycles were started. A cycling time of 3 minutes was used. The cycling was continued until no changes were observed in 3 consecutive cycles.

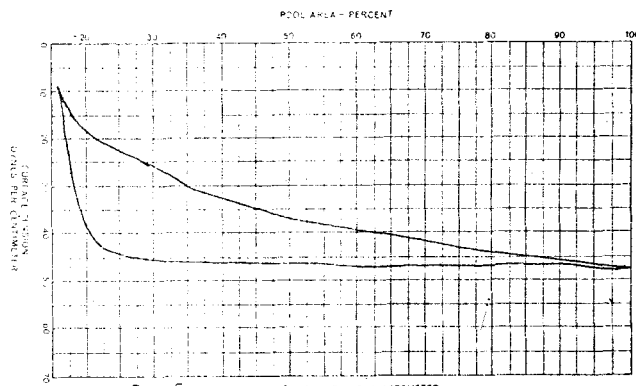
Examples of highly surface active, surface active, and surface inactive samples are shown in Figure 7. Highly surface active samples reduced the surface tension to 5 dynes/cm or less at the minimum surface area. On expansion, a large hysteresis loop was observed indicating that surface pressure changes preceded surface area changes. This phenomenon is also observed during ventilation of the lung. Samples are described as surface active if they reduced the surface tension to between 5 and 15 dynes/cm and showed some hysteresis. Surface inactive samples showed very little hysteresis and generally did not reduce surface tension to below 20 dynes/cm.

EXTRACTION OF LIPIDS

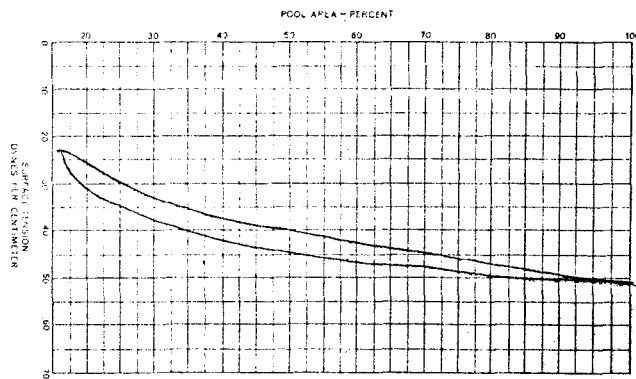
Lipids were extracted from lung washings and lung washing fractions using chloroform-methanol, 2:1 (v/v) following the method of Folch et al. (1957). Lyophilized samples, derived from a sample with a volume of 3 ml or less, were extracted overnight at room temperature with 2 ml of chloroform-methanol. Using an additional 2 ml of chloroform-methanol, the samples were transferred to conical centrifuge tubes. After centrifugation at about $2,000 \times g$ for 5 minutes, the supernatants were transferred to clean tubes and the residues re-extracted for 1 hour with 1 ml aliquots of chloroform-methanol. Following centrifugation, the supernatants were added to the previous supernatants. Larger lyophilized samples were extracted with appropriately larger volumes of solvent. The lipid extracts were then washed by adding 0.2 volumes of 0.37% KCl. After thorough mixing, the



Highly Surface Active



Surface Active



Surface Inactive

Figure 7. Surface tension-area diagrams of 3 different preparations of lung washings. The numbers on the abscissa indicate the percent of total pool area and the numbers on the ordinate are the surface tension in dynes per centimeter.

phases were separated by centrifugation at about 2,000 x g for 10 minutes. The upper phase and any particulate material present at the interface were removed without disturbing the lower phase. The interface was rinsed 3 times with "pure solvent upper phase" (3 ml chloroform, 48 ml methanol, 47 ml distilled water, and containing 0.37% KCl). A few drops of methanol were added to make the entire sample one phase. Each sample was then transferred to a 10 ml volumetric flask and after washing the tube with chloroform-methanol (2:1), diluted to 10 ml.

Lipids were extracted from samples in aqueous solutions by adding one volume of methanol and 2 volumes of chloroform, mixing vigorously and centrifuging to separate the layers. These samples were then handled as above except that when the entire sample was to be used for a phosphorus determination, the addition of methanol and dilution to 10 ml was omitted.

DETERMINATION OF PHOSPHOLIPID CONCENTRATION

The phospholipid content of the lipid extracts was determined by measuring the phosphorus content after digestion of the lipids with perchloric acid. The method used was that described by Wuthier (1966) and is an adaptation of the method employed by Martin and Doty (1949) for inorganic phosphate. The amount of phospholipid was calculated on the assumption that the phospholipids present contained an average of 4% phosphorus.

Preparation of Solutions

1. Perchloric Acid with Ammonium Molybdate

1 gm of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4 \text{H}_2\text{O}$ was dissolved in 1 liter of 70% HClO_4 .

2. 10% Ammonium Molybdate

2 gm of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4 \text{H}_2\text{O}$ were dissolved in distilled water to give a volume of 20 ml. This reagent could be kept for only 3 to 4 days.

3. Benzene-Isobutanol (1:1)

Equal volumes of benzene and isobutanol were mixed.

4. Ethanol-Sulfuric Acid (96.8:3.2)

32 ml of concentrated H_2SO_4 was added to 968 ml of ethanol.

5. Stannous Chloride Reagent

A stock solution of 10% stannous chloride was made by dissolving 0.5 gm of $\text{SnCl}_2 \cdot 2 \text{H}_2\text{O}$ to a volume of 5 ml with concentrated HCl .

This reagent can be used for 3 to 4 days. The working solution was prepared just before use by diluting 1 ml of the stock solution to a volume of 100 ml with 1 N H_2SO_4 .

6. Standard Phosphorus Solution

1.0 gm of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ was dissolved in distilled water and diluted to a volume of 1.0 liter. A working standard solution was prepared by

diluting 1.0 ml of the standard solution to 20.0 ml with distilled water.

Samples for the preparation of a standard curve were prepared by measuring out aliquots of the diluted standard. Samples were prepared either by evaporating a measured volume of the lipid extract or the entire lipid extract, to dryness. Reagent blanks were also run with each determination.

To each blank, standard and sample, 0.5 ml of perchloric acid reagent was added and they were heated at 180° C in a sand bath or heating block for about 1 hour in stoppered test tubes. After cooling, 1.5 ml of distilled water, 0.25 ml of 10% ammonium molybdate, and 1.5 ml of benzene-isobutanol 1:1 were added to each tube and mixed thoroughly using a vortex mixer. After brief centrifugation to insure complete separation of phases, 1.0 ml aliquots of the upper phase were transferred to another set of tubes. If the yellow color of the upper phase of any sample tube was greater than that of the highest phosphorus standard, an appropriately smaller aliquot was taken. The volume of each sample was then made up to 3.0 ml with ethanol-sulfuric acid reagent and mixed. 0.5 ml of stannous chloride reagent was added and the contents of the tubes were mixed thoroughly. After 5 minutes, the absorbance was read at 725 nm. The amount of phospholipid present in each sample was calculated by reference to the standard curve (Table I and Figure 8).

TABLE I

STANDARD CURVE DATA FOR DETERMINATION
OF PHOSPHOLIPID CONCENTRATION

Micrograms Phosphorus	Micrograms Phospholipid	Number of Samples	Absorbance* 725 nm
0.56	14	8	0.070 ± 0.003
1.12	28	8	0.140 ± 0.004
2.24	56	8	0.281 ± 0.007

*Mean \pm Standard Deviation

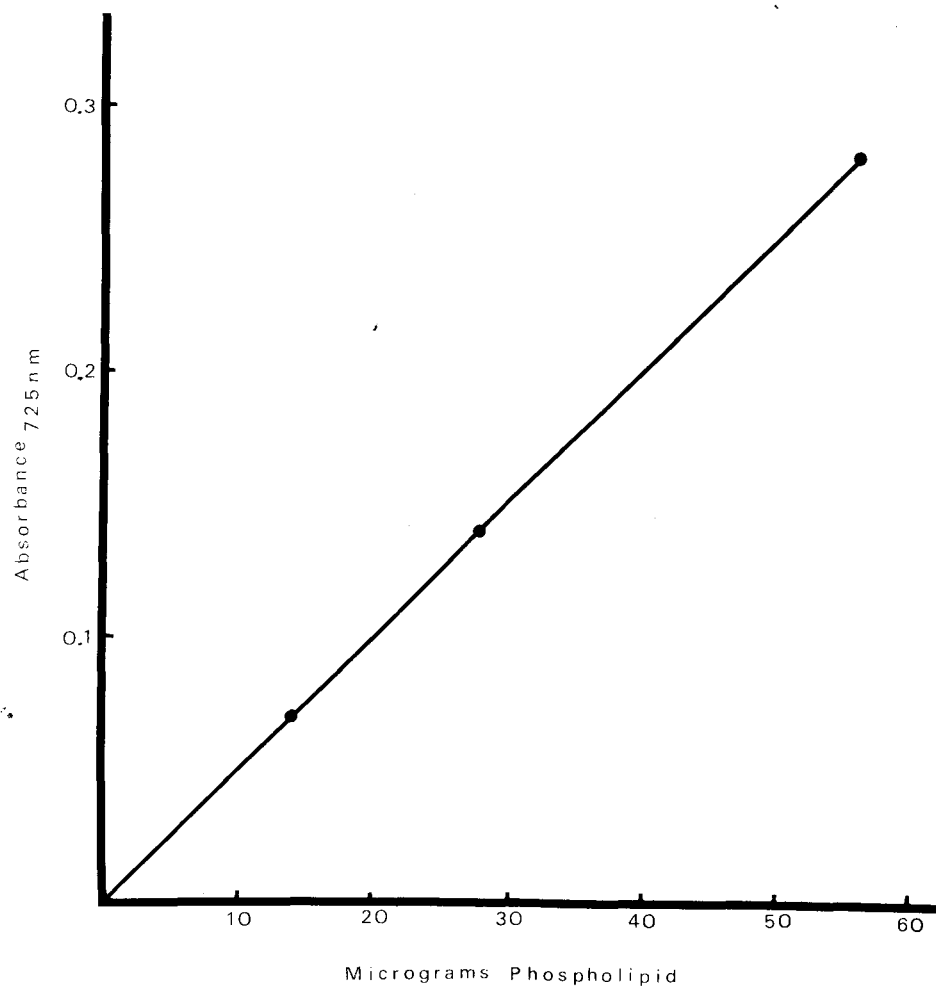


Figure 8. Standard curve for determination of phospholipid concentration.

SEPARATION OF LIPID CLASSES

A qualitative determination of the classes of lipids present was carried out by thin-layer chromatography in some experiments. Silica gel G plates (Analtech, Inc., Newark, New Jersey), 250 microns thick, were washed by development to the top of the plate in a solvent composed of chloroform, methanol and distilled water (75:22:3). After the plates were dried, aliquots of the lipid extract, concentrated to a small volume by evaporation under a stream of nitrogen, were applied with 5 μ l pipettes. The plates were then developed in a solvent composed of petroleum ether, ether and acetic acid (80:20:1) to within 1 cm of the top of the plate. After drying, the lipid spots were visualized by spraying with 50% sulfuric acid solution and heating on a hot plate.

GAS-LIQUID CHROMATOGRAPHY OF FATTY ACIDS

The fatty acid composition of some of the lipid extracts was determined after preparing fatty acid methyl esters by alkaline hydrolysis and treatment with BF_3 -methanol reagent (Metcalf *et al.*, 1966). The fatty acid methyl esters were separated on a 10% EGSS-X column in a Varian 2100 gas chromatograph. Quantitation was achieved using a Vidar digital integrator. Results obtained with National Heart Institute Fatty Acid Standard D (Applied Science, State College, Penn.) agreed with the stated composition data with a relative error less than 3% (Horning *et al.*, 1964).

DETERMINATION OF TOTAL PROTEIN CONCENTRATION

The protein concentrations of the samples were measured using the method of Lowry et al. (1951). This very sensitive method is based on the fact that a deep blue color is formed when protein in alkaline copper solution is treated with the phenol reagent of Folin and Ciocalteu (1927). Two distinct steps are involved in the reaction. First, the protein reacts with copper in alkaline solution, and secondly, a reduction of phosphomolybdic and phosphotungstic acid by the copper-treated protein occurs. This method is not susceptible to interference by most substances found in biological samples, so extraction of the protein from the samples is not necessary.

Preparation of Solutions

1. 1% Sodium Dodecyl Sulfate (SDS)

1 gram of $\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3\text{Na}$ was dissolved in distilled water to a volume of 100 ml.

2. 2% Sodium Carbonate in 0.1 N Sodium Hydroxide

0.1 N NaOH was prepared by adding 0.4 grams of NaOH to enough distilled water to make 100 ml of solution. Two grams of Na_2CO_3 were mixed with a quantity of the 0.1 N NaOH solution sufficient to give 100 ml of solution. This reagent was made fresh for each determination.

3. 2% Sodium Tartrate

2 grams of $\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2 \text{H}_2\text{O}$ were dissolved to a volume of 100 ml with distilled water.

4. 1% Copper Sulfate

1 gram of $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ was dissolved to a volume of 100 ml with distilled water.

5. Phenol Reagent of Folin and Ciocalteu, 1 N

A commercially prepared solution of the phenol reagent, 2 N, was purchased from Harleco, Philadelphia, Penn. A fresh working solution was prepared from this, each day of use, by diluting a small volume of the stock solution with an equal volume of water to give a 1 N solution.

6. Standard Protein Solution

Crystallized human albumin purchased from Dade Division of American Hospital Supply Corporation, Miami, Fla., was used for the preparation of the standard protein solution. When reconstituted with 3.0 ml of distilled water, the standard contains 80 mg of protein/ml. A protein standard solution of 80 ug/ml was prepared by diluting 0.1 ml of the protein standard to 100.0 ml with distilled water.

Samples for the preparation of a standard curve were prepared by diluting various amounts of the working standard solution to 1.0 ml with distilled water. One ml of distilled water was used as blank. Each sample of the lung washings or fraction from lung washings was also diluted to a total volume of 1.0 ml with distilled water, except in some instances where the sample of washings or washing fractions were turbid, 0.5 ml of 1% solution of SDS was added and the volume then adjusted to 1.0 ml with distilled water.

Five ml of a reagent prepared by mixing 1 ml of each 2% sodium tartrate and 1% copper sulfate with 100 ml of 2% sodium carbonate in 0.1 N sodium hydroxide was added to each blank, standard, and sample. After mixing, they were left at room temperature for 10 minutes. One-half ml of 1 N phenol reagent was added to each tube and mixed immediately. After 30 minutes or longer the absorbance was measured at 700 nm in a Beckman DB spectrophotometer. The amount of protein present in the samples was calculated by reference to the standard curve (Table II and Figure 9).

Standard protein solutions were also prepared with SDS, as well as with the various solutions in which some fractions of lung washings were obtained, to determine which of 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 in the Tris buffer used in some gradients, and NaBr in the Tris buffer, did interfere with the protein determination. Therefore, fractions which contained these materials were dialyzed before protein determinations were made.

TABLE II

STANDARD CURVE DATA FOR DETERMINATION
OF PROTEIN CONCENTRATION

Micrograms Protein	Number of Samples	Absorbance* 700 nm
20	8	0.073 ± 0.002
40	8	0.142 ± 0.004
60	8	0.211 ± 0.007
80	8	0.270 ± 0.005

*Mean \pm Standard Deviation

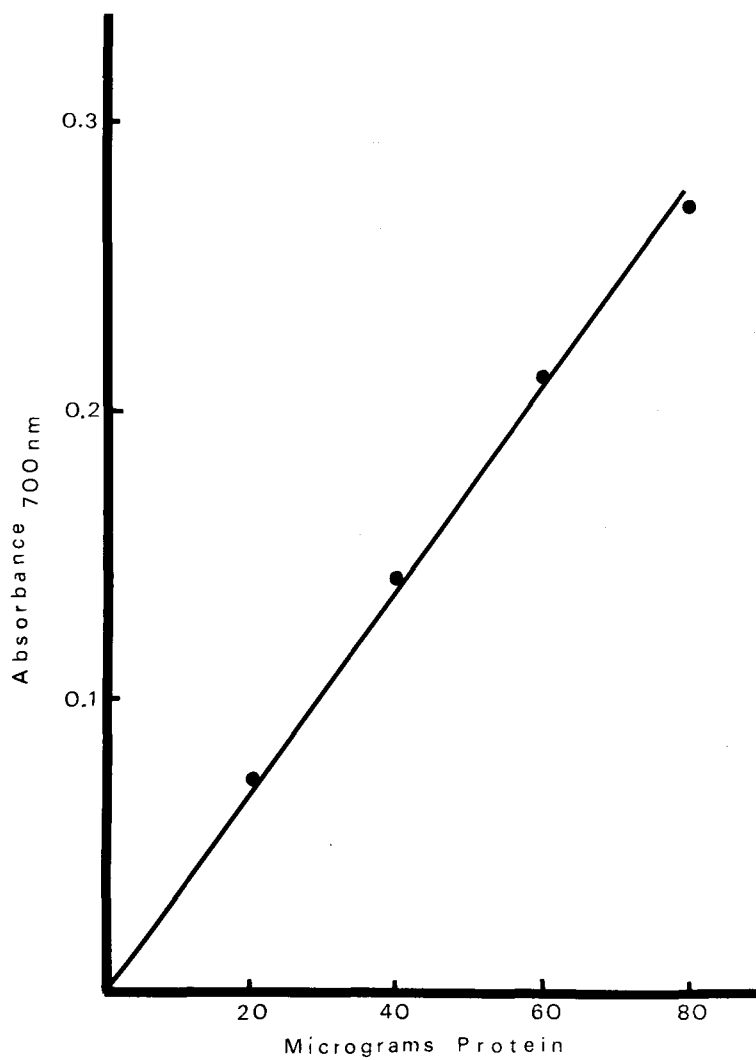


Figure 9. Standard curve for determination of protein concentration.

SEPARATION OF PROTEINS BY SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

The proteins of non-cellular lung washings and of the fractions obtained from the washings by the methods previously described in this chapter, were separated and their molecular weights estimated using the technique of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separation of proteins by gel electrophoresis without the use of SDS is dependent on both charge and molecular size. Shapiro et al. (1967), reported that the separation of proteins by polyacrylamide gel electrophoresis in the presence of the anionic detergent SDS is dependent only on their molecular weights. They studied 11 proteins varying in isoelectric points from 4 to 11, and in molecular weights from 15,500 to 165,000, and found that all of the proteins fell on a straight line when the distance of migration was plotted against the logarithm of the molecular weight. These results suggest that SDS minimizes the native charge differences and that all the proteins are negatively charged as a result of the formation of complexes with SDS.

Weber and Osborn (1969) studied the accuracy of this method by applying it to 40 proteins with well characterized molecular weights. When the electrophoretic mobilities were plotted against the logarithm of the molecular weights, a smooth curve was obtained showing that SDS-PAGE could be used for the separation and estimation of the molecular weight of a variety of proteins.

This method has been widely used for separation of membrane proteins since SDS helps to solubilize these insoluble proteins and helps to dissolve the lipid present in the protein preparations. The solubilized proteins and lipids can then be separated from one another by electrophoresis. The foregoing method was selected for use in the present study for these reasons, rather than for obtaining exact information concerning molecular weights of the proteins. Since lung washings contain much lipid, the possibility was recognized that there might be some lipid-associated proteins which would be insoluble in aqueous solutions and therefore, not amenable to study by conventional electrophoretic methods. Details of the procedure used in this study were essentially as described by Weber and Osborn (1969).

Preparation of Gels

1. Gel Buffer - 0.2 M Sodium Phosphate, pH 7.2, containing 0.2% SDS.

Buffer was prepared by adding 7.8 gm $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 20.44 g Na_2HPO_4 and 2 gm of SDS to enough distilled water to give 1 liter.

2. 10% Acrylamide Solution

22.2 gm acrylamide and 0.6 gm N, N'-methylene bisacrylamide (both obtained from Eastman Organic Chemicals, Rochester, New York) were dissolved in distilled water to a volume of 100 ml. After filtering through Whatman No. 1 filter paper, the solution was stored in dark bottles at 4° C.

The glass tubes used for preparation of the gels were 11 cm long, with an inner diameter of 5 mm. They were cleaned by washing with a hot SDS solution and after thorough rinsing with distilled water, were coated with a tube-rinse solution (Canalco, Inc., Rockville, Maryland) and oven-dried.

Gels were prepared by mixing 15 ml of gel buffer, 13.5 ml of 10% acrylamide solution, 1.5 ml of a freshly made ammonium persulfate solution (15 mg/ml) and 0.045 ml of N, N, N', N'-tetramethylethylenediamine (Eastman Organic Chemicals). Each gel tube was filled to a height of about 8 cm with this solution and before the gels hardened, a few drops of distilled water were layered on top of the gel solution. Just before use, the water was shaken off and the tubes were placed in a Canalco model 1200 gel electrophoresis apparatus.

Proteins Used for Standards

Proteins for use as standards were obtained from Sigma Chemical Co., St. Louis, Missouri. They are listed in Table III with the molecular weights for the intact proteins and for the polypeptide chains found after reduction of the disulfide bonds by 2-mercaptoethanol. The molecular weights for 4 of the 5 proteins were calculated from amino acid compositions listed by Dayhoff (1969). Bovine serum albumin molecular weight is as reported by Peters (1970).

TABLE III

PROTEINS USED AS STANDARDS FOR SODIUM DODECYL SULFATE-
POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein	Source	Molecular Weight	Molecular Weight of Polypeptide Chains
Gamma globulins	Human, Cohn Fraction II	150,000	50,000 23,500
Albumin	Bovine serum	65,500	-----
α -Chymotrypsin	Bovine pancreas	25,250	13,900 10,100
Myoglobin	Equine skeletal muscle	17,000	-----
Cytochrome-c	Horse heart	11,700	-----

Preparation of Samples

Proteins were dissolved in 0.01 M sodium phosphate buffer with a pH of 7.0, and containing 1% SDS, and incubated at 37° C for 2 hours. For each gel, 1 drop of tracking dye (0.01% pyronin Y in distilled water), 1 drop of glycerol, and 50 or 100 ul of the protein solution were mixed in a small test tube and then applied to the top of the gel.

In some experiments, 2-mercaptoethanol was used to disrupt the disulfide bonds for separation of polypeptides of the proteins. In these experiments, the solution in which the proteins were dissolved contained 1% 2-mercaptoethanol, and a small drop of mercaptoethanol was added to each tube in which the samples were mixed prior to application to the tops of gels.

Electrophoresis

Gel buffer, diluted 1:1 with distilled water, was layered on top of each sample to fill the tube. Both compartments of the electrophoresis apparatus were filled with the diluted gel buffer. Electrophoresis was performed at a constant current which varied from 2 to 5 ma per tube in different runs, with the positive electrode in the lower chamber.

Electrophoresis was continued until the tracking dye had moved to the lower 1 or 2 cm of the gel.

After electrophoresis, the gel was removed from the tube by squirting water from a syringe and 22 gauge needle between the gel and wall of the glass tube. The length of the gel and the distance the dye moved were then measured.

Staining and Destaining

1. Staining Solution

1.25 gm of Coomassie Brilliant Blue R250 was dissolved in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid and filtered through Whatman No. 1 filter paper.

2. Destaining Solution

75 ml of acetic acid, 50 ml of methanol and 875 ml of distilled water were mixed.

Each gel was placed in a 12 x 125 mm test tube and stained by filling the tube with the staining solution of Coomassie Brilliant Blue. After 1 to 2 hours in the staining solution, the gels were rinsed with distilled water and placed in destaining solution. In some experiments, after at least 30 minutes in the destaining solution, the gels were further destained electrophoretically by replacing the gels in the Canalco electrophoresis apparatus containing destaining solution in both compartments. In other experiments, the gels were destained over a period of days simply by repeatedly changing the destaining solution in the tubes.

Measurement of Position of Bands and Calculation of Relative Mobilities

After destaining was completed, the lengths of the gels were measured and the gels were stored in 7.5% acetic acid solution. The gels were scanned using a Photovolt densitometer equipped to scan acrylamide gels.

A reproduction of a densitometric trace of a gel containing the five protein standards without mercaptoethanol is shown in Figure 10.

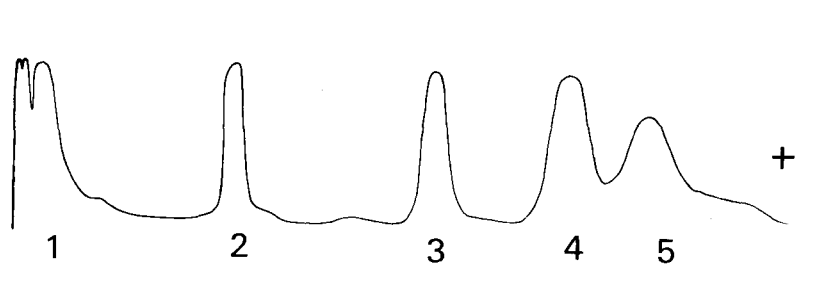
The distance from the top of the gel to the center of each protein band was measured on the densitometer tracing, and the mobility of each protein in relation to the leading edge of the tracking dye was calculated. The gels swell in the staining and destaining solution, and this factor had to be considered in the calculation of migration rates.

$$\text{Relative Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

The mobilities of the standard proteins, as shown in Table IV, were plotted against their known molecular weights on a semi-logarithmic scale (Figure 11). The molecular weights of the proteins in the lung washing samples were estimated by reference to the standard curves.

ELECTROPHORESIS AND IMMUNOELECTROPHORESIS ON CELLULOSE ACETATE MEMBRANES

Electrophoresis on cellulose acetate membranes is a widely used method for separation of serum proteins, and recently, Schwartz (1972) has shown that immunoelectrophoresis can also be performed adequately on cellulose acetate membranes. These methods have been applied to the study of lung washing proteins in this investigation. Also, the presence of lipid components was determined in some samples by use of appropriate staining techniques.



- 1 = Gamma Globulin
- 2 = Albumin
- 3 = α -Chymotrypsin
- 4 = Myoglobin
- 5 = Cytochrome c

Figure 10. Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

TABLE IV

STANDARD CURVE DATA FOR ESTIMATION OF PROTEIN MOLECULAR WEIGHTS BY
SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Without 2-Mercaptoethanol			
Protein	Molecular Weight	Number of Determinations	Relative Mobility*
Gamma globulin	150,000	12	0.026 ± 0.011
Albumin	65,500	12	0.230 ± 0.010
α -Chymotrypsin	25,250	14	0.456 ± 0.011
Myoglobin	17,000	11	0.614 ± 0.019
Cytochrome-c	11,700	12	0.697 ± 0.020
With 2-Mercaptoethanol			
Protein	Molecular Weight	Number of Determinations	Relative Mobility*
Albumin	65,500	6	0.187 ± 0.012
Gamma globulin	50,000	6	0.240 ± 0.014
	23,500	6	0.445 ± 0.018
Myoglobin	17,000	6	0.603 ± 0.011
α -Chymotrypsin	13,900	3	0.673 ± 0.019
	10,100	3	0.753 ± 0.024
Cytochrome-c	11,700	6	0.679 ± 0.013

*Mean \pm Standard Deviation

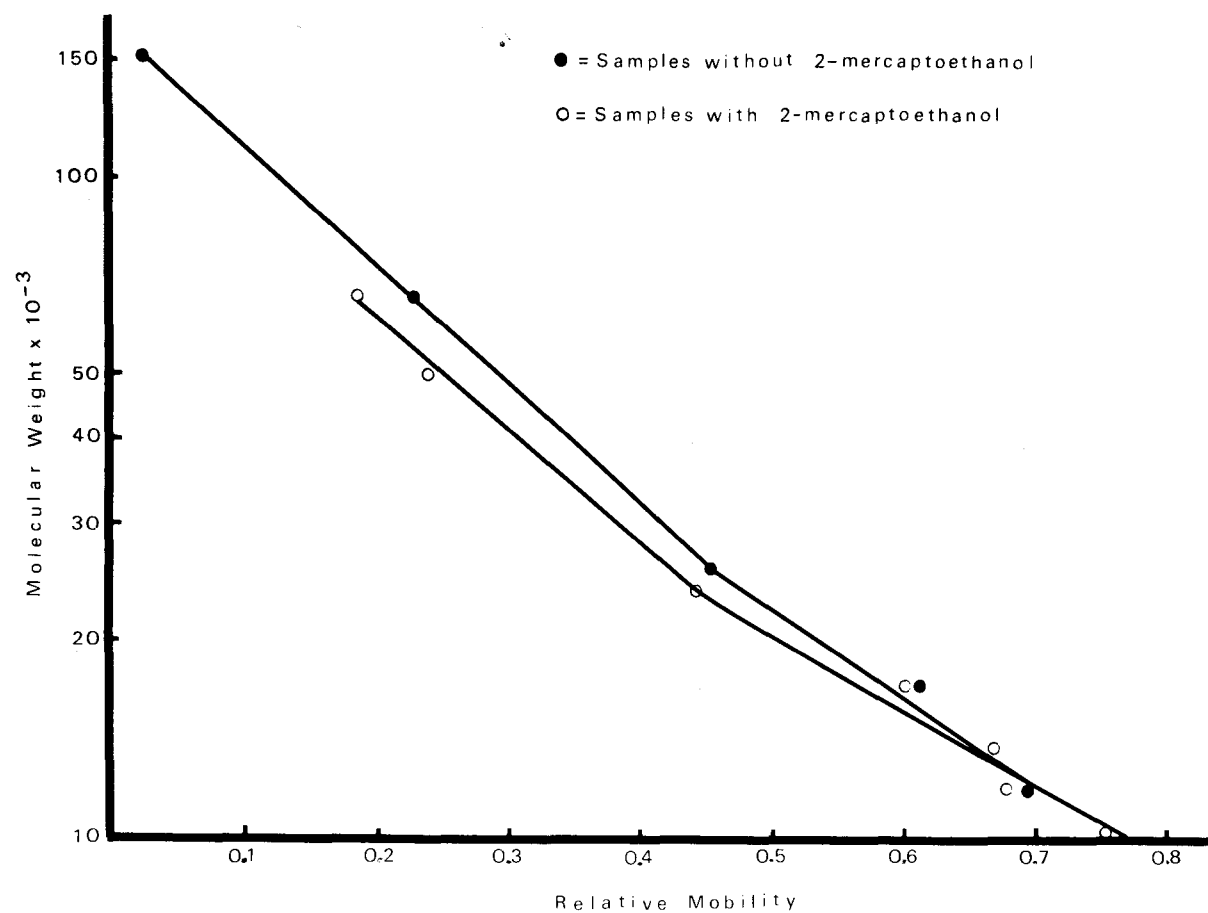


Figure 11. Standard curve for estimation of protein molecular weights by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Preparation of Solutions

1. Barbitol Buffer, pH 8.6

Barbitol-sodium barbitol buffer, with a pH of 8.6 and ionic strength of 0.05 was prepared by diluting the contents of 1 vial of Buffer-Salt-Type B-1 (Harleco, Philadelphia, Penn.) to 1000 ml with distilled water.

2. Ponceau S Stain

The staining solution was prepared by dissolving 500 mg of Ponceau S in 100 ml of 5% trichloroacetic acid.

3. Antisera

Goat antiserum to rabbit serum and antisera to individual proteins of rabbit plasma were obtained in lyophilized form from Cappel Laboratories Inc., Downingtown, Penn. Goat antiserum to human serum was purchased from Hyland Lab., Costa Mesa, Calif.

Protein Electrophoresis

The membranes used in this study were 1" x 6 3/4" Sepraphore III cellulose polyacetate strips (Gelman Instrument Company, Ann Arbor, Michigan). The membranes were first soaked in the buffer solution and then gently blotted to remove excess buffer. They were then positioned in a Gelman Model 51170 Electrophoresis Chamber which had been previously filled

with the barbital buffer solution. Samples were then applied to the membranes in straight narrow bands using a Gelman sample applicator. The electrophoresis of the samples was then carried out at 250 volts for about 45 minutes. Immediately after electrophoresis, the membranes were placed in the Ponceau S stain for a period of 5 minutes. The excess stain was then removed by washing in several changes of 5% acetic acid. The proteins appeared as red bands on a white background.

Immunoelectrophoresis

In the immunoelectrophoretic studies, the samples were applied as a small round dot using a 5 ul pipette. Generally, 2 samples were placed on each strip. After electrophoresis in the manner previously described, the membranes were removed from the chamber and placed on the bottom of shallow plastic trays. The antiserum was applied in a long streak between the two samples using a Cordis 50 ul microapplicator (Cordis Laboratories, Miami, Fla). Each tray was then covered by another tray which fit tightly, but which did not touch the membranes below it. The stacked trays were then wrapped in wet paper towels covered tightly with aluminum foil in order to prevent drying of the membranes. They were then placed in an incubator at 37° C for approximately 16 hours. After incubation, the membranes were washed for 1 hour in NaCl solution in order to remove the non-precipitated protein. They were then placed for 1 hour in distilled water to remove the salt. The membranes were then stained in Ponceau S as previously described.

Detection of Lipids on Cellulose Acetate Membranes

Lipids were detected on the cellulose acetate membranes in one of two ways. In some experiments, samples were prestained with Sudan Black B as described by McDonald and Ribeiro (1959). In other experiments, lipids were detected by the application of Schiff's reagent after ozonization following the procedure described by Kohn (1961).

CHAPTER III

EXPERIMENTAL RESULTS

COMPOSITION OF NON-CELLULAR RABBIT LUNG WASHINGS

As discussed in the Statement of The Problem, Chapter I, lung washings appear to be the best available source of material for the biochemical study of the alveolar lining layer. However, since the lining layer is closely associated with the capillaries of the lung, the possibility exists that the lavage procedure may result in contamination of the washings by components of the blood. For this reason, a systematic study of the phospholipid and protein concentrations in lung washings was undertaken to determine the extent of variation in the composition of the material used by many researchers for the isolation of lung surfactant.

Thirty-two rabbit lung washings, 3 pooled from 2 rabbits and 29 from individual rabbits, obtained in RLS at room temperature were studied. After centrifugation for 5 minutes at 480 x g to remove cells, the phospholipid and protein concentrations of the cell-free lung washings were measured and the phospholipid to protein (PL/Protein) ratios calculated.

The results as shown in Table V demonstrate that there is a wide variation in the amounts of both phospholipid and protein present in the lung washings obtained from rabbits by the standard method of alveolar lavage. The mean value of phospholipid concentration was 346 ± 112 ug/ml with values ranging from 140 to 624. The mean value of protein concentration

TABLE V

PHOSPHOLIPID AND PROTEIN CONTENT OF NON-CELLULAR RABBIT LUNG WASHINGS

Experiment Number	Appearance of Cellular Pellet ^a	ug Phospholipid/ml	ug Protein/ml	PL/Protein
17	+	319	215	1.48
18	0	379	216	1.75
21 ^b	0	205	205	1.00
23	++	177	165	1.07
24	++	140	286	0.49
26 ^b	+	319	165	1.93
28	+	477	242	1.97
31	+	393	190	2.07
32	0	376	146	2.58
35	+	343	169	2.03
37	++	429	346	1.24
38	++	481	424	1.13
39	0	411	364	1.13
40	+	224	282	0.79
42	0	361	234	1.54
43	+	317	196	1.62
44	0	223	181	1.23
45	++	246	297	0.83
46	0	397	295	1.35
47	++	328	196	1.67
48	++	511	270	1.89
49	0	551	161	3.42
50	+	308	315	0.98
51	0	356	183	1.95
52	++	476	346	1.38
56	0	167	119	1.40
57	++	624	209	2.99
59	0	378	222	1.70
60	0	264	166	1.59
61	++	349	203	1.72
65 ^b	0	304	308	0.99
71	0	224	168	1.33
Average of 32 washings		346 ± 112 ^c (140-624) ^d	234 ± 72 (119-424)	1.57 ± 0.61 (0.49-3.42)
Average of 14 washings in which no RBC visible in pellet		328 ± 100 (167-551)	212 ± 66 (119-364)	1.64 ± 0.64 (0.99-3.42)
Average of 8 washings in which few RBC visible in pellet		338 ± 68 (224-477)	222 ± 26 (165-315)	1.61 ± 0.46 (0.79-2.07)
Average of 10 washings in which many RBC visible in pellet		376 ± 148 (140-624)	274 ± 78 (165-424)	1.44 ± 0.57 (0.49-2.99)

^a 0 = no RBC, + = few RBC, ++ = many RBC. ^b Washings from 2 rabbits pooled.

^c Standard deviation. ^d Range of values.

was 234 ± 72 ug/ml with values ranging from 119 to 424. The PL/Protein ratios varied from 0.49 to 3.42 with the average being 1.57 ± 0.61 .

The contents of phospholipid and protein could be 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 changed 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 which appeared grossly red in color were not used in these experiments, some washings which were white in color gave a cellular pellet containing red blood cells. Therefore, the data from these 32 lung washings was divided into 3 groups on the basis of qualitative observation as to whether the cellular pellets contained no, few, or many red blood cells. As is shown in Table V, both the mean phospholipid and protein concentrations were higher in washings containing more red blood cells. Analysis of the data using the Student's "t" test revealed that the differences in phospholipid content were not statistically significant. The mean protein content of the samples yielding a pellet with many red blood cells was significantly greater than samples giving a pellet with no red blood cells ($p < 0.05$). The mean ratio of PL/Protein was slightly, but not significantly, lower in samples with red blood cells. However, even in samples in which no red blood cells were observed in the cellular pellet, the PL/Protein ratio varied from 1.00 to 3.42, or more than a three-fold range.

These results demonstrate that even in normal animals, the components of the acellular lining of the alveoli, as recovered by alveolar lavage,

vary considerably. It should be pointed out that the presence of varying amounts of proteins in alveolar washings does not necessarily mean that they are contaminants from the plasma resulting 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.

Proteins Present in Lung Washings

Many studies of the lipid composition of lung washings have been reported and it is generally agreed that fully saturated PC is the major component. However, only one group of workers (Colacicco et al., 1971) has reported investigation of the protein components of the entire cell-free lung washings. Using disc-gel electrophoresis and immunological methods, they found that albumin, immunoglobulin G and several "minor" serum proteins were present. In addition, they found one protein that had no counterpart in serum, and some protein that was not separated from the lipid, but remained with the lipid in the spacer gel. The non-serum protein they describe is quite different from that found by King et al. (1973) in their surface active fractions.

In this study, the method of SDS-polyacrylamide gel electrophoresis was used in order to study all of the proteins present, including those which were lipid-associated. This method, which has been widely applied to membrane preparations, results in the solubilization of both lipids and proteins which are then readily separated by electrophoresis.

The proteins of the non-cellular washings were concentrated by lyophilization after dialysis against distilled water to remove the salt. The lyophilized material was dissolved in a small amount of buffer containing SDS but without 2-mercaptoethanol, resulting in a clear solution. In order to compare the proteins of the lung washings with plasma proteins, aliquots of rabbit plasma were also lyophilized and resuspended in buffer containing SDS. A densitometric tracing of a gel on which the components of a non-cellular lung washings had been separated is shown in Figure 12. At least 9 distinct protein bands were present, but the relative mobilities of these bands could not be calculated because the tracking dye appeared as a broad, diffuse band which could not be measured. This streaking of the tracking dye was presumably caused by the large amount of phospholipids present in the sample. To confirm that phospholipids were causing this problem, 500 ug of PC were mixed with a protein sample before application to a gel. Following electrophoresis, the dye was streaked and the faster moving protein bands were distorted.

In order to overcome this problem and also to find a way in which the proteins could be separated from the lipid without the use of a detergent, which might alter their immunological properties, other methods of sample preparation were examined. It was found that if the lyophilized washings were resuspended in a small volume of distilled water, a cloudy solution resulted and when this solution was centrifuged at $29,000 \times g$ for 1 hour, about 98% of the phospholipid appeared in the pellet. The clear supernatant contained much of the protein; however, the amount of protein lost in the

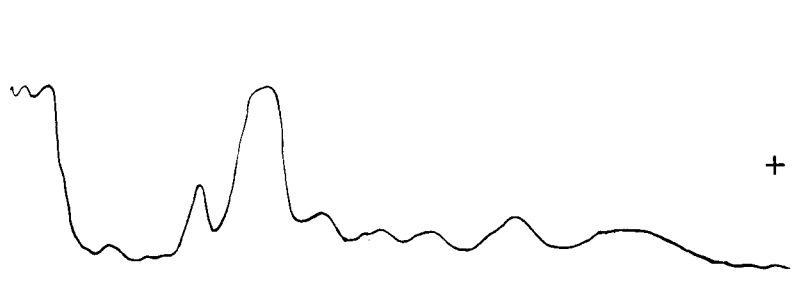


Figure 12. SDS-PAGE of non-cellular lung washings.

pellet varied greatly. In 3 separate experiments, the percentage of protein remaining in the supernatant amounted to 82, 77 and 57, respectively. A portion of the supernatant from the experiment in which only 57% of the protein was found in the supernatant was lyophilized and then examined by SDS-PAGE. As can be seen in Figure 13, all of the protein bands seen in the entire non-cellular washing (Figure 12) were also present in this preparation. Since only a small amount of lipid was present, the relative mobilities of the protein bands could readily be calculated and are shown in Figure 13. The largest protein band had a relative mobility of 0.21 which is close to that of albumin. A band with a relative mobility similar to that of immunoglobulin G, 0.03, was also present. Comparison of this tracing with the tracing of a gel on which rabbit plasma had been separated (Figure 14) clearly showed that in addition to albumin and immunoglobulin G, several non-plasma proteins, with molecular weights ranging from about 20,000 to 50,000, were present in the lung washings.

To confirm that the bands found in the positions of the serum proteins were indeed those proteins, immunoelectrophoretic studies were carried out using the protein supernatant prepared as above. In this experiment, the supernatant contained 82% of the protein of the washings. The concentrated protein solution contained about 30 mg of protein per ml and a control rabbit serum was diluted to the same protein concentration. When the lung washings were tested against antiserum to rabbit albumin and antiserum to rabbit immunoglobulin G, strong reactions were observed. A much weaker reaction was noted with anti-rabbit immunoglobulin M, as was also the case for the

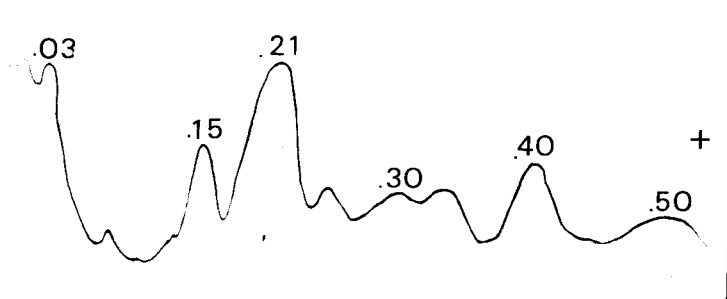


Figure 13. SDS-PAGE of protein supernatant of lyophilized lung washings. The numbers above the major peaks represent the relative mobilities of those protein bands.



Figure 14. SDS-PAGE of rabbit plasma. The numbers above the major peaks represent the relative mobilities of those protein bands.

control serum. These results demonstrate that the rabbit lung washings do indeed contain serum proteins, and supported by the results of the SDS-PAGE experiments, indicate that albumin is the predominant protein present in the lung washings.

In the course of the experiments using SDS-PAGE, it was observed that much larger amounts of the standards cytochrome c and myoglobin were needed to produce bands similar in size to those of the other protein standards. Investigation of this problem revealed that electrophoretic destaining resulted in partial loss of the smaller molecular weight proteins from the gels. Since the previous experiments with non-cellular lung washings had been carried out using electrophoretic destaining, the possibility that some proteins had been lost due to the destaining procedure was investigated. Also, since certain experiments described later required a more complete separation of the lipids and proteins without loss of protein into the lipid fraction, the use of organic solvent extraction was explored.

A cell-free lung washing which had been dialyzed and lyophilized was suspended in cold ethanol:ether (1:3) and, after incubation at -10° C for 4 hours, centrifuged to separate the protein precipitate from the lipid-containing supernatant. The protein precipitate was extracted with another aliquot of ethanol:ether for 2 hours at -10° C. After collection by centrifugation, the precipitate was dissolved in buffer plus SDS. The combined supernatants were evaporated to dryness and redissolved in buffer with SDS. Electrophoresis of the ethanol:ether supernatant revealed only a broad smear of lightly staining material typical of phospholipids, and no protein

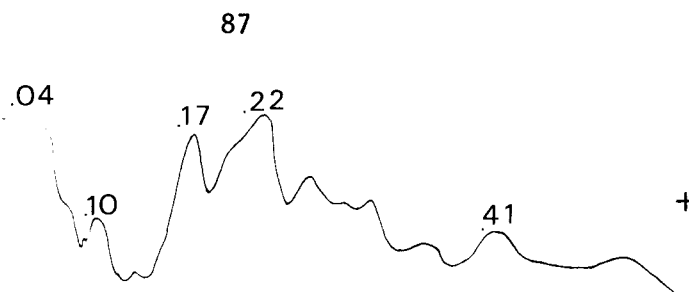
bands. The ethanol:ether precipitate dissolved easily in the SDS solution and a densitometric tracing of a gel containing this sample is shown in Figure 15. Several additional minor protein bands were visible in this gel which had not been seen in gels electrophoretically destained.

. The results of these experiments clearly demonstrate that lung washings contain a heterogeneous mixture of proteins. The serum proteins, albumin and immunoglobulin G, appear to be quantitatively the major proteins. However, the presence of several proteins which do not have counterparts in serum indicates that even though the washings may be contaminated to some extent by proteins from serum, there are also proteins in lung washings that originate from another source or sources.

CHARACTERIZATION OF SEDIMENTATION FRACTIONS FROM LUNG WASHINGS

When lung washings in dilute salt solution are centrifuged, even at relatively low speeds, a surface active pellet and a surface inactive supernatant are obtained. This is the first step used by most investigators in their attempts to isolate a specific surfactant lipoprotein. During the course of this investigation, it became necessary to further characterize both the sediment and the supernatant in order to interpret the data obtained using the analytical ultracentrifuge.

Previous results (Balis et al., 1971) had demonstrated that centrifugation of the non-cellular lung washings for 90 minutes at 100,000 x g yielded a surface active sediment and a non-surface active supernatant. Use of non-cellular washings with an average PL/Protein ratio of 2.7



<u>Relative Mobility</u>	<u>Molecular Weight</u>
0.01	>150,000
0.04	143,000
0.07	125,000
0.10	112,000
0.12	102,000
0.17	83,000
0.20	73,000
0.22	67,000
0.25	60,000
0.28	53,000
0.30	48,000
0.34	41,000
0.41	31,000
0.50	22,000

Figure 15. SDS-PAGE of the ethanol:ether precipitate from non-cellular lung washings. The numbers above the peaks represent the relative mobilities of those protein bands. The molecular weights corresponding to these relative mobilities are shown below the figure.

yielded a sediment which contained an average of 83% of the phospholipids of the cell-free washings and a supernatant which contained an average of 74% of the protein. The mean PL/Protein ratio of the sediment was 8.5. Other investigators have obtained very different results. For example, Galdston et al. (1969) reported that their sedimentable fraction contained only slightly more phospholipid than protein, while Steim et al. (1969) found less than 2% of the protein of the cell-free washing in the sediment.

In order to better understand the factors affecting the sedimentation properties of lung surfactant, several experiments were carried out. In the first of these, the effect of the speed of centrifugation on the phospholipid and protein contents of the sediment was studied. The cell-free washings were centrifuged at 3000 x g for 20 minutes at 4° C, yielding sediment B-1. The supernatant was further centrifuged at 127,000 x g, max. for 60 minutes at 4° C to yield sediment B-2. The phospholipid and protein contents of these fractions, as well as that of the final supernatant C, are shown in Table VI. About one-half of the phospholipid of the washings, and more than one-half of the sedimentable phospholipids were found in sediment B-1. The very slight difference in PL/Protein ratios between sediments B-1 and B-2 suggests that variation in particle size, rather than a difference in densities accounted for their differing sedimentation properties.

In subsequent experiments it became apparent that the phospholipid to protein ratios of sediments obtained from lung washings varied in relation to the relative amounts of phospholipid and protein present in the non-cellular

TABLE VI

PHOSPHOLIPID AND PROTEIN CONTENTS OF SEDIMENTATION FRACTIONS
FROM NON-CELLULAR LUNG WASHINGS

Fraction	mg Phospholipid	mg Protein	PL/Protein
Non-cellular washing	30.84	13.80	2.2
Sediment B-1	15.07	1.97	7.6
Sediment B-2	10.06	1.14	8.8
Supernatant C	5.11	10.09	0.5

washings from which they were obtained. As shown in Table VII and Figure 16, the PL/Protein of the sediment was directly dependent upon the PL/Protein ratio of the non-cellular washing from which it was obtained. In addition, some extreme cases are discussed later in this chapter. A mixture of lung washings and serum having a PL/Protein of 0.07 yielded a sediment with a PL/Protein of 0.17. At the other extreme, a fraction from a density gradient centrifugation of lung washings which had a PL/Protein ratio of 8.7, yielded a sediment with a ratio of 18.4.

Since the major component of lung washings is PC, an investigation of the sedimentation properties of aqueous suspensions of PC, and the effect of protein on these sedimentation properties was undertaken. PC (obtained from egg yolk, Sigma Chemical Co.) was suspended in RLS with various amounts of bovine serum albumin. With pure PC, centrifugation at $127,000 \times g$ for 1 hour produced a pellet which contained 62% of the PC. Mixing of PC and protein in a 1:10 ratio gave a pellet with a PL/Protein ratio of 6 to 1 and containing 98% of the PC of the initial mixture. Mixing PC and protein in a 1:1 ratio yielded a pellet with a PL/Protein ratio of 17 to 1, and this pellet contained 63% of the PC. Although these results are quantitatively unlike those of lung surfactant, as would be expected since lung washings contain other lipids in addition to PC and protein other than albumin, they do demonstrate that the amount of protein present in the solution affects the sedimentation properties of PC and the ratio of PL/Protein in the sediments.

TABLE VII

DEPENDENCE OF PL/PROTEIN RATIO OF SEDIMENTABLE FRACTIONS
ON THE PL/PROTEIN RATIO OF THE CELL-FREE WASHINGS

Experiment Number	PL/Protein Ratio of Cell-Free Lung Washings	PL/Protein ratio of Sediment
LP 65 ^a	0.99	2.35
LP 71 ^a	1.33	4.93
LP 35 ^b	2.03	9.37
LP 31 ^b	2.07	9.59
LP 5 ^a	2.23	8.08
56 ^{a,c}	2.44	8.18
55 ^{a,c}	2.47	7.43
47 ^{a,c}	2.94	8.79
48 ^{a,c}	3.34	11.81
LP 49 ^b	3.42	10.12

^aSediment obtained by centrifugation at 127,000 x g for 60 to 90 minutes.

^bSediment obtained by centrifugation at 37,000 to 48,000 x g for 30 to 60 minutes.

^cThese results are from Balis et al., 1971.

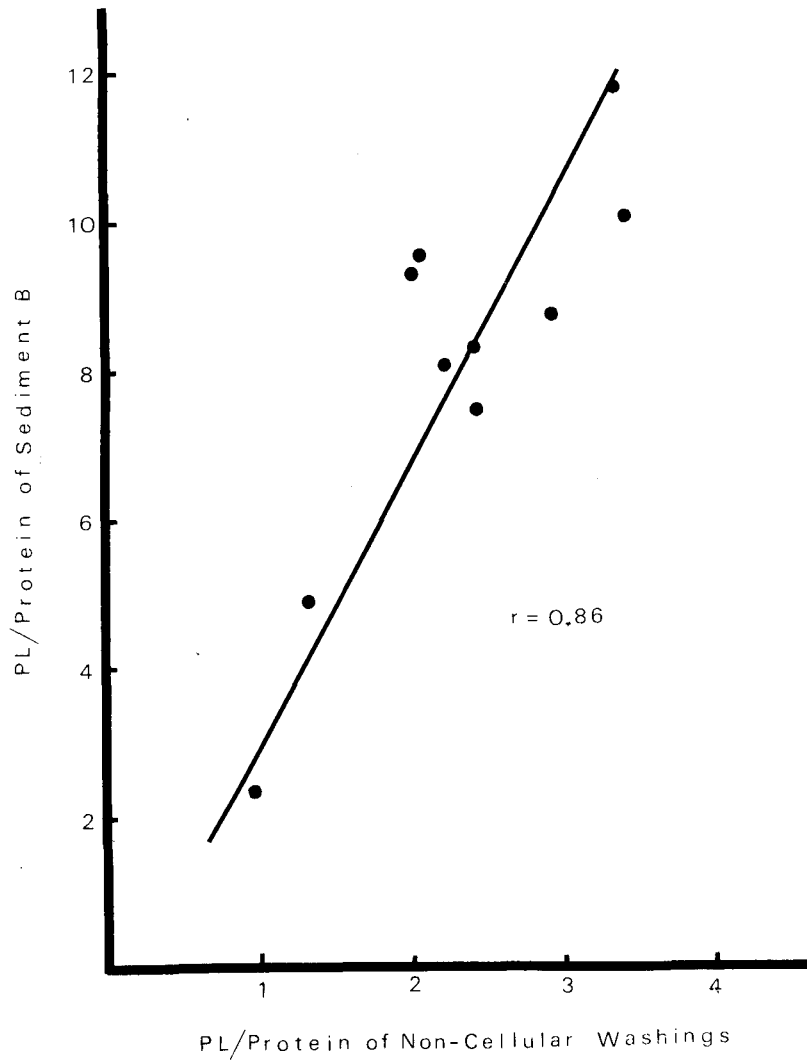


Figure 16. Relationship of the PL/Protein ratio of the sedimentable fraction to the PL/Protein ratio of the cell-free washings. The correlation coefficient (r) for this relationship was 0.86.

The supernatant remaining after removal of the sedimentable fraction from lung washings appeared as a clear solution containing most of the protein of the washings. When examined by cellulose acetate electrophoresis, this fraction showed only one band which could not be distinguished from bovine serum albumin. Analytical ultracentrifugation revealed a major peak with a sedimentation coefficient of 4.4 and two very small, faster moving peaks.

In order to characterize the minor protein components of the supernatant fraction, an attempt was made to concentrate supernatant C by dialyzing against distilled water, lyophilizing and resuspending in a small volume of distilled water. In three experiments, the supernatant remaining after centrifugation of lung washings at 48,000 x g for 1 hour was treated in this way. In each experiment, when distilled water was added to the lyophilized material to give a 1:10 ratio to the original volume, a cloudy suspension was formed. On cellulose acetate electrophoresis, this suspension gave 3 bands when stained for protein. One band remained at the origin, the second was found in about the same position as the β globulins of serum, while the third migrated as albumin. Centrifugation of the concentrated supernatant fraction at 48,000 x g for 1 hour yielded a clear supernatant and a large white pellet. This supernatant gave only 2 bands on cellulose acetate electrophoresis with no observable band at the origin. The pellet, which was found to be highly surface active, showed only a band at the origin on cellulose acetate electrophoresis, which stained with both Ponceau S for protein and with Sudan Black for lipid.

These results demonstrated that the clear, surface inactive supernatant remaining after sedimentation of the largest portion of surface active material from lung washings also contains surface active lipids. These surface active components were readily sedimented only after concentration of supernatant C by lyophilization. Most of the procedures used for the isolation of lung surfactant begin with obtaining a sediment by centrifugation and discarding the supernatant. The above findings suggest that such a step results in an artificial separation of some of the surface active material, from another portion of surface active material, a separation that is probably the result of differences in particle size.

CHARACTERIZATION OF LUNG WASHING LIPID-PROTEIN COMPLEXES BY ANALYTICAL ULTRACENTRIFUGATION

The lipoproteins of serum have been well characterized and 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.

Methods as for Serum Lipoproteins

In the first experiment, the methods commonly used for isolation and characterization of serum lipoproteins (deLalla and Gofman, 1954) were applied to lung washings. This procedure involves the concentration of the lipoproteins by flotation in sodium bromide solutions using the

preparative ultracentrifuge, and then resuspending the lipoproteins in NaBr solutions of appropriate density for flotation in the analytical ultracentrifuge. Solid NaBr was added to the non-cellular lung washings obtained from an adult rabbit in RLS to give a 24% solution of NaBr. The density of a 24% solution of NaBr is 1.21, a density at which all known classes of serum lipoproteins will float (Del Gatto *et al.*, 1959). The lung washings containing 24% NaBr were centrifuged in the R50 rotor in the Spinco Model L2 ultracentrifuge at 40,000 rpm (127,000 x g, max.) for 20.5 hours at a temperature of 18-20° C. At the end of this centrifugation, a thin, white pellicle was found at the top of each tube. These pellicles were removed with a fine-tipped pipette along with a small amount of the solution immediately under the pellicle in a total volume of 2 ml. The combined pellicles were easily dispersed, giving a very cloudy white suspension. The phospholipid and protein contents of the resuspended pellicle were measured and found to be 8.06 mg and 0.93 mg respectively, giving a PL/Protein ratio of 8.7, similar to that of sedimentable surfactant (Balis *et al.*, 1971). A sample of the resuspended pellicle containing 400 ug of phospholipid was tested for surface activity and was found to be highly surface active. However, when an aliquot of the resuspended pellicle was centrifuged at 44,000 rpm and 20° C for about 1 hour, no peaks were seen. When the cell was removed from the rotor, a pellicle of material was seen at the top of a clear solution, indicating that the surfactant lipid-protein complexes had indeed floated. These results showed that although a pellicle of surface active material containing both

phospholipid and protein could be isolated by flotation in the preparative ultracentrifuge, the pellicle did not contain discrete particles of lipoprotein as does fractions isolated from serum in this manner.

A number of other attempts were made to observe surfactant lipoproteins in the analytical ultracentrifuge. The entire non-cellular washings, obtained by washing with a minimal amount of RLS in order to produce a more concentrated solution of washings, as well as resuspended pellicles and sediments, were centrifuged at various speeds and using various concentrations of NaBr. No peaks were seen, but it was found that non-cellular washings gave a pellicle at the top of the sample compartment whenever the concentration of NaBr was 6% or more, whereas with less than 6% NaBr, the surfactant sedimented. However, resuspended sediments, when centrifuged in 6% NaBr, gave a pellet rather than a pellicle.

Two possible explanations for these initial failures to detect lipoproteins in lung washings were considered. First, the non-cellular washing could have contained lipoproteins, but not in sufficient concentrations to be detected in the analytical ultracentrifuge. Secondly, when the surface active "lipoproteins" were concentrated by flotation or sedimentation, they may have formed large aggregates of lipoprotein particles which were not dispersed by merely stirring the pellet in solution. A number of experiments were undertaken to investigate these possibilities.

Sedimented Surfactant

In one experiment, a sediment B (sediment obtained from cell-free lung washings by centrifugation at 127,000 x g for 60 to 90 minutes) was resuspended in RLS by homogenizing in a glass tube with a teflon pestle in order to more thoroughly dispense the pellet. After adjusting the density to 1.21 by addition of NaBr, this sample was run in the analytical ultracentrifuge. As can be seen in Figure 17, a small, rapidly moving lipoprotein peak was observed. Based on these 2 pictures, a $F_{1.21}$ of about 500 was calculated.

Sonication is a method often used in solubilizing membrane preparations and was recently employed by Barclay et al. (1972) in experiments designed to isolate lipoprotein subunits from plasma membranes. Since one of the problems in working with lung surfactant preparations is their lack of solubility in aqueous solutions, it was decided to try sonication in order to further break down these possible large aggregates of material into their "basic lipoprotein subunits."

A sediment B was sonicated for 3 minutes and was examined by analytical ultracentrifugation in 24% NaBr. As can be seen in Figure 18, sonication did allow the observation of 2 typical lipoprotein peaks. These peaks moved very rapidly, with all of these photographs being taken during the first 15 minutes of centrifugation and at speeds up to 24,000 rpm.

A number of similar experiments were then carried out at a speed of 14,000 rpm and with photographs taken at regular intervals in order to determine flotation rates of the 2 components. The results of some of

Direction of Flotation \longrightarrow



Figure 17. Schlieren pattern of Sediment B resuspended by homogenization. This sample was centrifuged at 14,000 rpm and photographs were taken at 30 seconds and 2 minutes and 30 seconds after reaching speed.

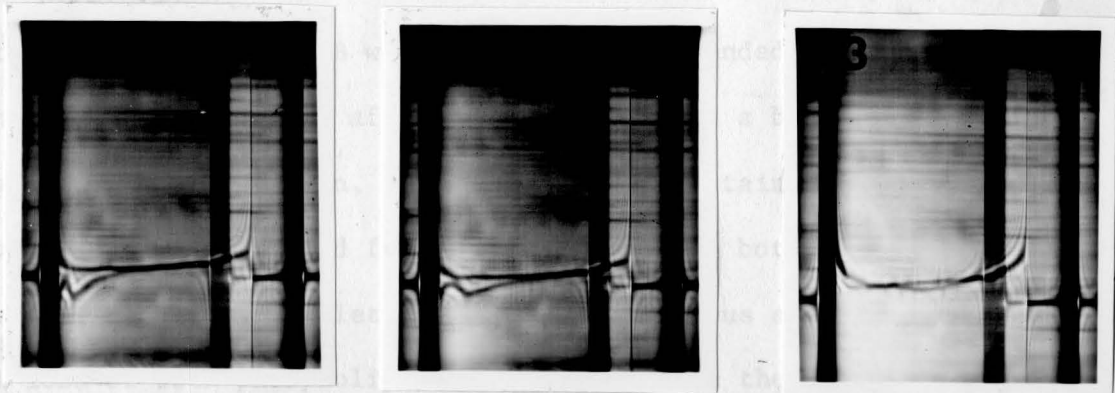


Figure 18. Schlieren pattern of Sediment B resuspended by sonication.

these experiments are shown in Table VIII and in Figures 19 and 20.

The variation in flotation rates from one experiment to another, especially for the faster moving peak, suggests that these peaks do not represent homogeneous classes of lipoproteins, but rather particles of varying sizes. A comparison of Figures 19a and b shows that by increasing the length of time of sonication, the amount of material in the slower moving peak increased at the expense of the faster moving peak. A similar phenomenon can be observed in Figure 20, in which after 3 minutes of sonication, the faster moving peak cannot be clearly seen. In addition, Figure 20b shows the instability of these peaks which suggests that the components are changing even during centrifugation.

Concurrently, several other experiments were carried out in order to determine the effect of sonication on sediment B. Comparison by cellulose acetate electrophoresis of a sediment B which had been resuspended by sonication, and a sediment B which had been resuspended by vigorous stirring revealed that only after sonication, could a band moving with, or slightly ahead of albumin, be seen. This band stained for both lipid and protein, as did the band found at the origin in both samples.

Resedimentation of pellets suspended by vigorous stirring resulted in a small loss of both phospholipids and protein from the pellet, but no change in the PL/Protein ratio (Balis *et al.*, 1971). That result was confirmed in the course of this work and the effect of sonication on the PL/Protein ratio of this fraction was examined. The results shown in Figure 21 demonstrate that sonication does result in the loss of more

TABLE VIII

FLOTATION RATES OF SURFACTANT "LIPOPROTEINS"

PREPARED BY SONICATION

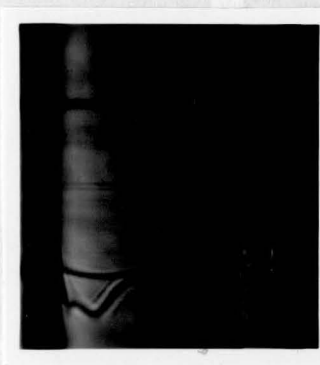
Experiment Number	Length of Time Sonicated	F _{1.21}	
		Faster Moving Peak	Slower Moving Peak
LP-7	3 min.	300	27
LP-8	3 min.	151	30
	10 min.	165	54
LP-13	1 min.	87	39
	3 min.	---	42

Direction of Flotation \longrightarrow

6 minutes



16 minutes

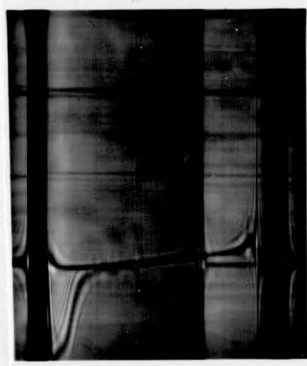


24 minutes

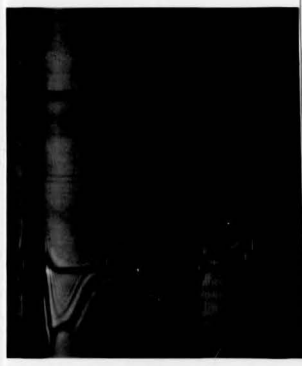


Figure 19a. Schlieren pattern of Sediment B resuspended by sonication for 3 minutes. This sample was centrifuged at 14,000 rpm. Photographs were taken at the times indicated above each frame.

8 minutes



12 minutes



16 minutes



24 minutes

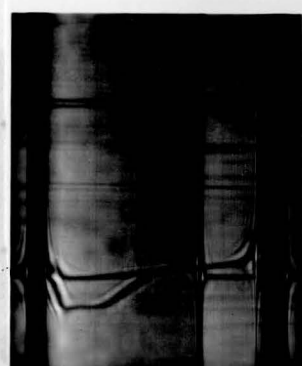


Figure 19b. Schlieren pattern of Sediment B resuspended by sonication for 10 minutes. This sample was centrifuged at 14,000 rpm. Photographs were taken at the times indicated above each frame.

Direction of Flotation \longrightarrow

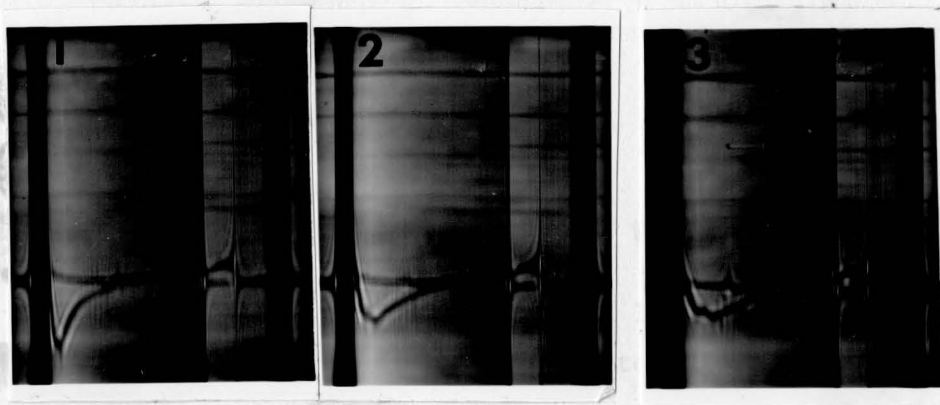


Figure 20a. Schlieren pattern of Sediment B resuspended by sonication for 1 minute. This sample was centrifuged at 14,000 rpm and photographs were taken at 8 minute intervals.

centrifuged 127,000 \times g
for 90 minutes

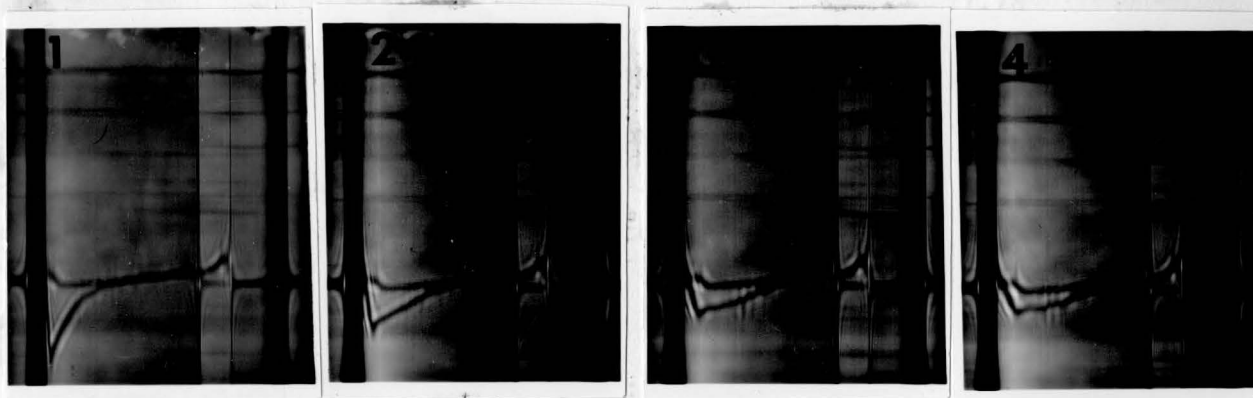


Figure 20b. Schlieren pattern of Sediment B resuspended by sonication for 3 minutes. This sample was centrifuged at 14,000 rpm and photographs were taken at 8 minute intervals.

	<u>PL/Protein</u>
Non-cellular Lung Washings	1.33
↓ Centrifuged 127,000g x g for 90 minutes	
Sediment B-1	4.93
↓ Resuspended in RLS, sonicated for 3 minutes, centrifuged 127,000 x g for 90 minutes	
Sediment B-2	7.78
↓ Resuspended in RLS, sonicated for 3 minutes, centrifuged 127,000 x g for 90 minutes	
Sediment B-3	9.24

Figure 21. Effect of sonication on sedimentable surfactant.

protein than phospholipid from the sedimentable material. After 3 centrifugations and 2 periods of sonication, the pellet contained 8% of protein and 79% of the phospholipid present in the original cell-free washing.

These results suggest that the sediments obtained by centrifugation contained some proteins non-specifically absorbed to the lipids. Whether the small amount of protein remaining with the phospholipid sediment after sonication is part of a specific lipoprotein or whether it, too, is non-specifically absorbed to the lipids is not clear. Although the results of the analytical ultracentrifugation studies of the sonicated sediments do not reveal the presence of unique lipoproteins, the possibility that specific lipoprotein particles are present and obscured by the non-specific associations of protein which occurs during sedimentation must be considered.

Concentrated Non-Cellular Washings

In order to avoid the problem of non-specific aggregation of proteins with the lipoproteins or lipids of lung washings produced by sedimentation, methods which did not include a sedimentation step were explored. Use of the entire non-cellular washing had been tried without success. Possibly the concentrations of "lipoproteins" were too low. The usual range of concentration for detection with the schlieren optical system is 1 to 10 mg/ml, and serum lipoproteins are often studied with concentrations at the upper end of this range (Hully et al., 1971). The average phospholipid

concentrations of lung washings was found to be 346 ug/ml (Table V) and even though attempts were made to obtain more concentrated washings by use of less RLS and by perfusion, these were near the lower limits of usable concentrations.

For these reasons, several experiments were carried out using an Amicon Ultrafiltration system to concentrate the non-cellular washings to a smaller volume. Use of a UM05 or a PM10 filter resulted in production of a clear filtrate and a cloudy white concentrate which was surface active. Filtration using the XM300 filter gave a slightly cloudy filtrate, which following concentration with PM10 filter, gave a solution from which a surface active sediment was obtained. This result indicates that at least some of the surface active components of the lung washing have molecular weights of less than 300,000. The majority of the surfactant, however, appeared to remain in the ultrafiltration cell and would therefore, be composed of higher molecular weight components.

A cell-free lung washing which had been concentrated using a PM10 filter was examined in the analytical ultracentrifuge after adding NaBr to give a density of 1.21. As can be seen in Figure 22, a large slow moving peak and several small faster moving peaks were observed. The $F_{1.21}$ of the large peak was found to be 44. Similar experiments were performed several times but the results have been inconsistent. In one experiment, a similar large peak was seen with an $F_{1.21}$ of 53, while in others, many small peaks with indications of instability were present (Figure 23).

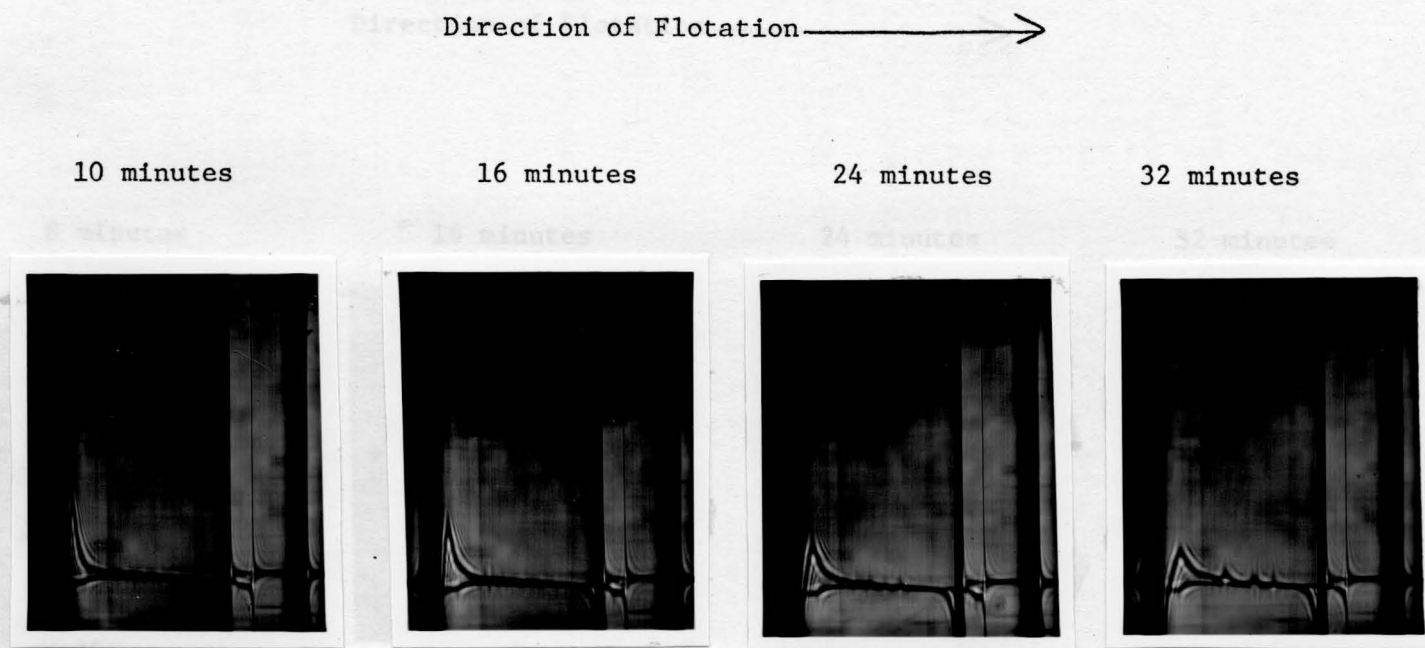


Figure 22. Schlieren pattern of concentrated non-cellular lung washings.
This sample was centrifuged at 14,000 rpm and photographs
were taken as indicated.

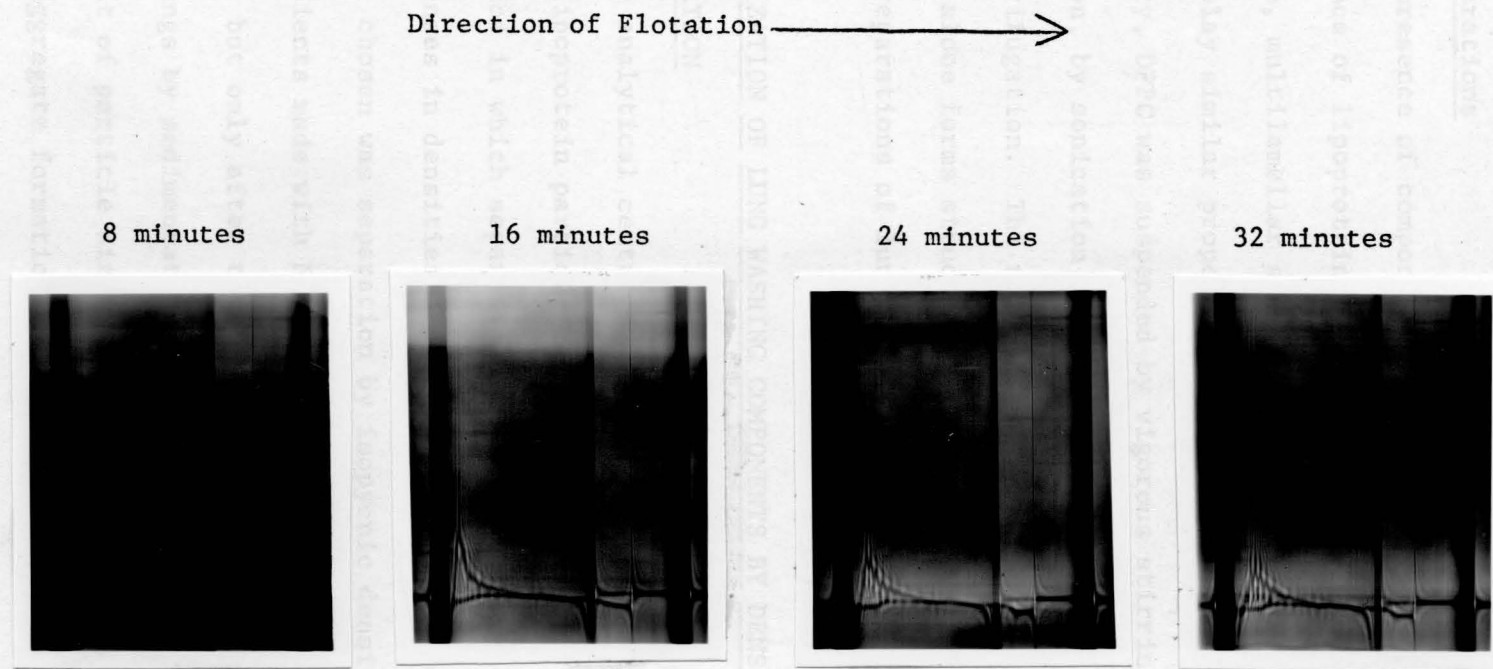


Figure 23. Schlieren pattern of concentrated non-cellular lung washings. This sample was centrifuged at 14,000 rpm and photographs taken as indicated.

DPPC Preparations

The presence of components which float in NaBr solutions could indicate the presence of lipoproteins. However, phospholipid in aqueous suspensions form large, multilamellar structures (Bangham and Horne, 1964) which 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 results are shown in Figure 24 and demonstrate that DPPC alone forms structures with flotation rates comparable to those of various preparations of lung surfactant.

CHARACTERIZATION OF LUNG WASHING COMPONENTS BY DENSITY GRADIENT CENTRIFUGATION

Since analytical centrifugation failed to reveal the presence of specific lipoprotein particles in lung washings, it appeared desirable to use a method in which separation of components would be made on the basis of differences in densities, thus discounting the factor of particle size. The method chosen was separation by isopycnic density gradient centrifugation using gradients made with NaBr. King and Clements (1972a) used this procedure, but only after the preliminary separation of fractions from the lung washings by sedimentation and flotation. Such methods are not independent of particle size. Since it had been found that sedimentation promotes aggregate formation, and results in loss of some surface active material, it seemed appropriate to apply the technique of continuous

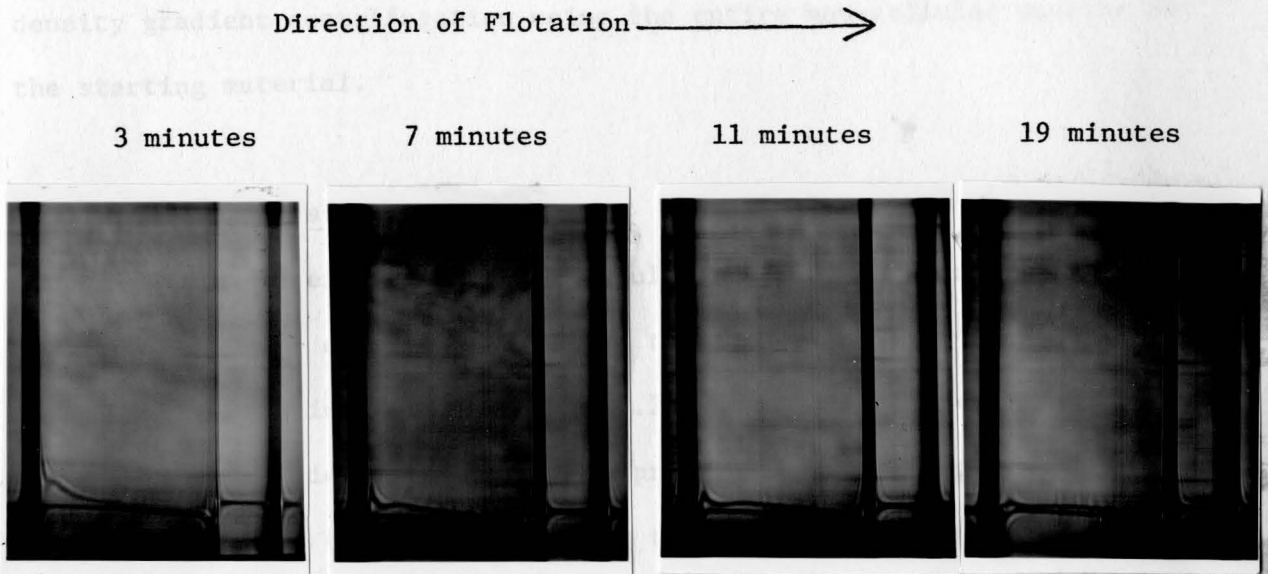


Figure 24a. Schlieren pattern of DPPC suspension. This sample was centrifuged at 14,000 rpm and photographs were taken as indicated.

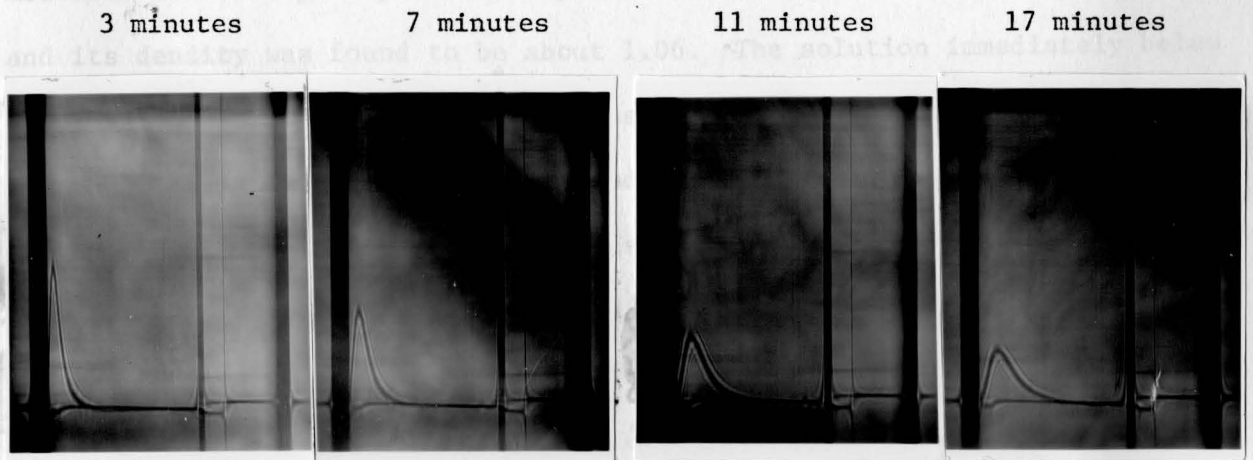


Figure 24b. Schlieren pattern of a sonicated preparation of DPPC. This sample was centrifuged at 14,000 rpm and photographs taken as indicated.

density gradient centrifugation using the entire non-cellular washing as the starting material.

Choice of Gradients

In the first experiment, non-cellular lung washings from rabbit were mixed with 39.6 gm of NaBr and diluted to 132 ml with RLS. This provided enough heavy solution of 30% NaBr ($d=1.28$) to make 3 gradients. Using RLS as the light solution, gradients were prepared at room temperature and centrifuged at 20° C for 18 hours. At the end of the run, a white, opalescent band containing particulate material was observed near the top of each of the three tubes (Figure 25a). About 8 ml of this band was removed from each tube with a syringe and bent needle. The particulate material was evenly dispersed. A portion of this fraction was weighed and its density was found to be about 1.06. The solution immediately below the bands was also removed and its density was found to be about 1.10.

The results of this experiment indicated that lung surfactant lipid-protein complexes were of densities less than 1.10, and that a less steep gradient should afford a better separation. Therefore, in the next experiment, 14% NaBr ($d=1.12$) instead of 30%, was used as the heavy solution and the gradients were prepared as before. Following centrifugation at 20° C for 18 hours, adjoining opalescent and particulate bands could be observed in each of the 3 tubes (Figure 25b). Fractions from the portion above the opalescent band, the opalescent band, the particulate band and immediately below the particulate band, were removed with a syringe and bent needle.

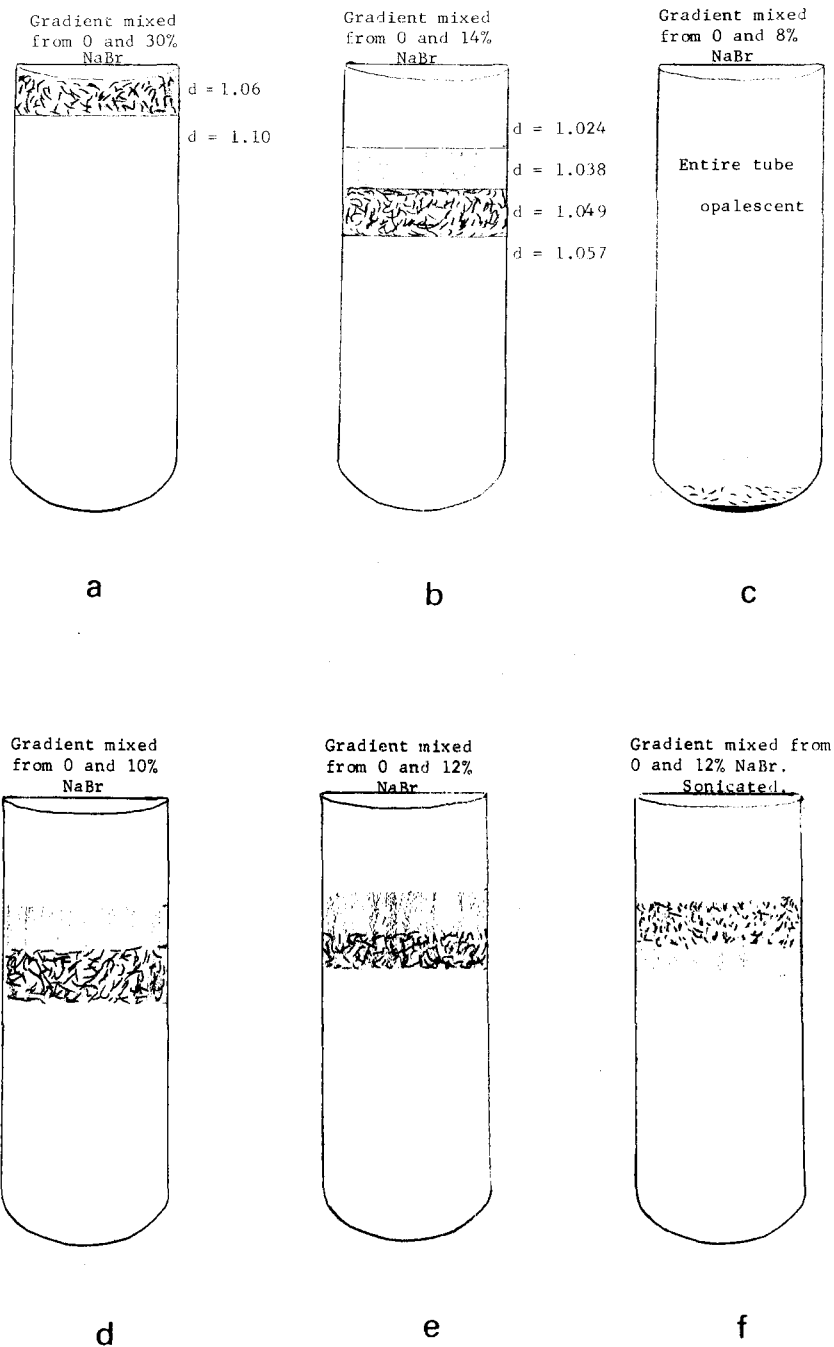


Figure 25. Use of various gradients for separation of lipid-protein complexes from non-cellular washings.

Each fraction was weighed, and their densities are shown in Figure 25b. Both the opalescent and particulate fractions were found to be highly surface active, but each required about 1 hour of cycling to reduce the minimum surface tension to zero.

In subsequent experiments, various concentrations of NaBr, 8, 10, and 12%, were used in the heavy solution in an attempt to make a gradient in which the opalescent and particulate band would be separated from each other. Gradients were prepared as above and centrifuged for 18 hours at 20° C. Use of 8% NaBr ($d=1.06$) resulted in much of the material accumulating as a pellet on the bottom of the tube (Figure 25c). Ten percent NaBr ($d=1.08$) and 12% NaBr ($d=1.10$) both gave patterns similar to that obtained with 14% NaBr with adjacent opalescent and particulate bands (Figures 25d and e).

Effect of Sonication

The effect of sonication on non-cellular lung washings before gradient centrifugation was determined using a heavy solution of 12% NaBr. Non-cellular washings were mixed with 10.56 gm NaBr and enough RLS to yield 88 ml of heavy solution. One-half of the heavy solution was used to mix a gradient as before. The other half was sonicated for 3 minutes and then used as heavy solution to mix another gradient. After centrifugation for 18 hours at 20° C, the bands appeared at the same position in both tubes, but the opalescent band in the sonicated sample was below the particulate band, which was composed of smaller particles than were observed in the non-sonicated sample (Figure 25f). The 2 bands were removed from each of the

tubes with a syringe and bent needle, and the phospholipid and protein contents of the bands were determined. These results are shown in Table IX and show that although sonication of the non-cellular washings changed the appearance of the bands and resulted in some rearrangement of the components within the visible bands, no change in the overall density of the surface active materials occurred, and the total phospholipid and protein contents of the bands and the overall PL/Protein ratio remained constant. The significance of these findings were not apparent until after other experiments had been carried out.

Distribution of Phospholipid and Protein after Centrifugation on NaBr Gradients

A careful investigation of phospholipid and protein distribution in the gradients was then undertaken. For the heavy solution, 10% NaBr (0.98 M) was chosen because in such gradients, which ranged in density from about 1.02 to 1.08, the visible band of surface active material was found near the center of the tube and was fairly wide. Therefore, if differences in the composition of various parts of the visible band did occur, they would be detectable. Also, this was the minimum concentration at which surface active material was kept in the gradient rather than in a pellet, and use of as little salt as possible was desirable to prevent possible ionic disruption of lipid-protein complexes.

Nine gradient tubes, using washings from 5 different rabbits, were prepared in this manner, with 1/3 to 1/2 of the washings from one rabbit in

TABLE IX

DISTRIBUTION OF PHOSPHOLIPID AND PROTEIN IN VISIBLE BANDS
ON NaBr GRADIENTS WITH AND WITHOUT PRIOR SONICATION

Sample	Band	ug Phospholipid in Band	ug Protein in Band	PL/Protein
Non-Sonicated Washings	Opalescent	959	47	20.40
	Particulate	5669	480	11.81
	TOTAL	6628	527	12.58
Sonicated Washings	Particulate	3914	187	20.93
	Opalescent	2008	295	6.81
	TOTAL	5992	482	12.29

RLS adjusted to contain 10% NaBr in a volume of 44 ml. This heavy solution, and 24 ml of RLS for the light solution, were used to prepare each gradient. Gradients were made at room temperature and centrifuged at 20° C for 3, 18 or 24 hours. Following centrifugation, the positions of the visible bands were measured and fractions were collected after puncturing the bottom of the tube. The absorbance of each fraction was measured at 400 nm and the higher readings, indicating turbidity, corresponded to the visible bands. Initially, the flow cell was used, but the particulate nature of the sample made such direct monitoring impractical. However, the particulate matter did resuspend easily, making turbidity readings possible after the collection of fractions. When left standing for several hours, the particulate material settled to the bottom of the tubes.

The phospholipid and protein concentrations, as well as the absorbance, of each fraction from 7 of these tubes were determined and the results are shown in Figures 26 to 32. In experiment LP 48, the four fractions which were collected from near the bottom of the tube and the four fractions from near the top of the tube from each of the 3 gradients were pooled after optical density readings, but before phospholipid and protein determinations.

In the first experiment (LP 47), the total amounts of phospholipid and protein in the gradients were calculated and the percent recovery of the phospholipid and protein present in the washings used to prepare the gradients were determined in order to ascertain that there was no large loss of either component. More than 90% of the phospholipid was found in the gradient. The 10% loss might well have been due to the loss of solution in

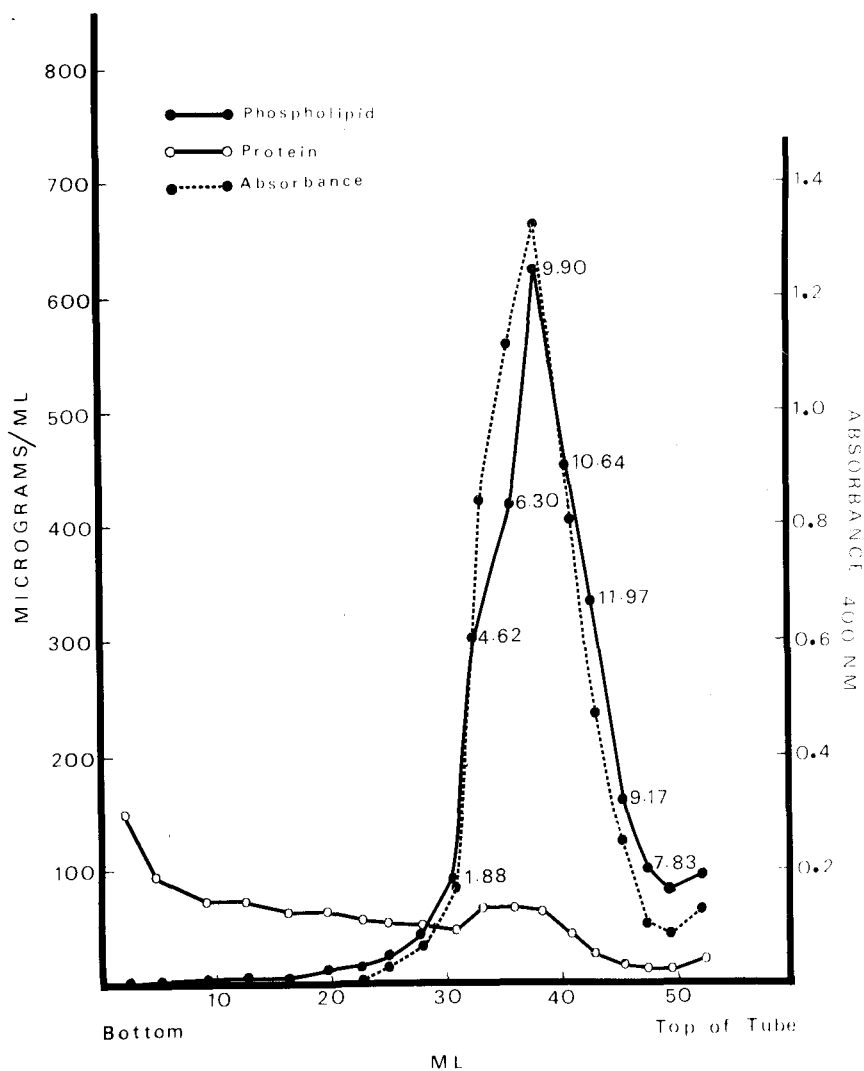


Figure 26. Distribution of phospholipid and protein in NaBr gradient (LP 47, Tube 1). The non-cellular lung washings used to prepare this gradient were obtained with RLS and had a PL/Protein ratio of 1.67. The gradient tube was centrifuged at 20° C for 18 hours. The number by each point represents the PL/Protein ratio of that fraction. The PL/Protein ratio of these fractions combined is 7.21.

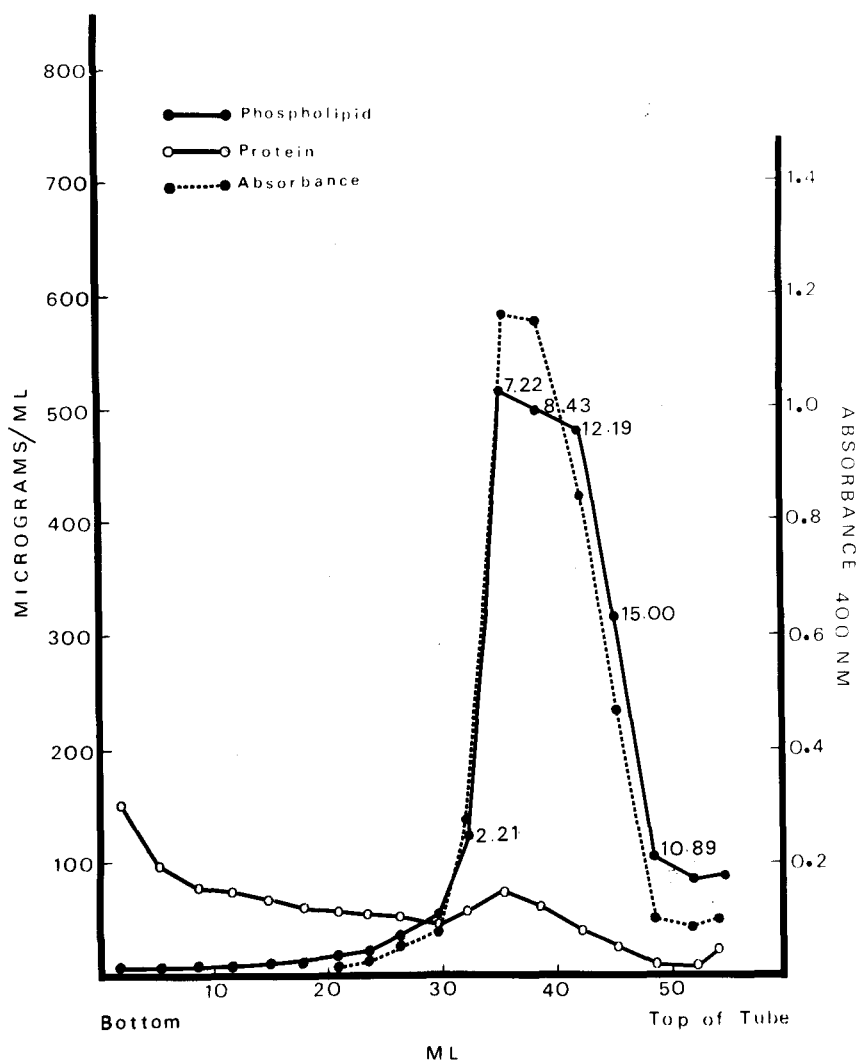


Figure 27. Distribution of phospholipid and protein in NaBr gradient (LP 47, Tube 2). The non-cellular lung washings used to prepare this gradient were obtained with RLS and had a PL/Protein ratio of 1.67. The gradient tube was centrifuged at 20° C for 18 hours. The number by each point represents the PL/Protein ratio of that fraction. The PL/Protein ratio of these fractions combined is 8.00.

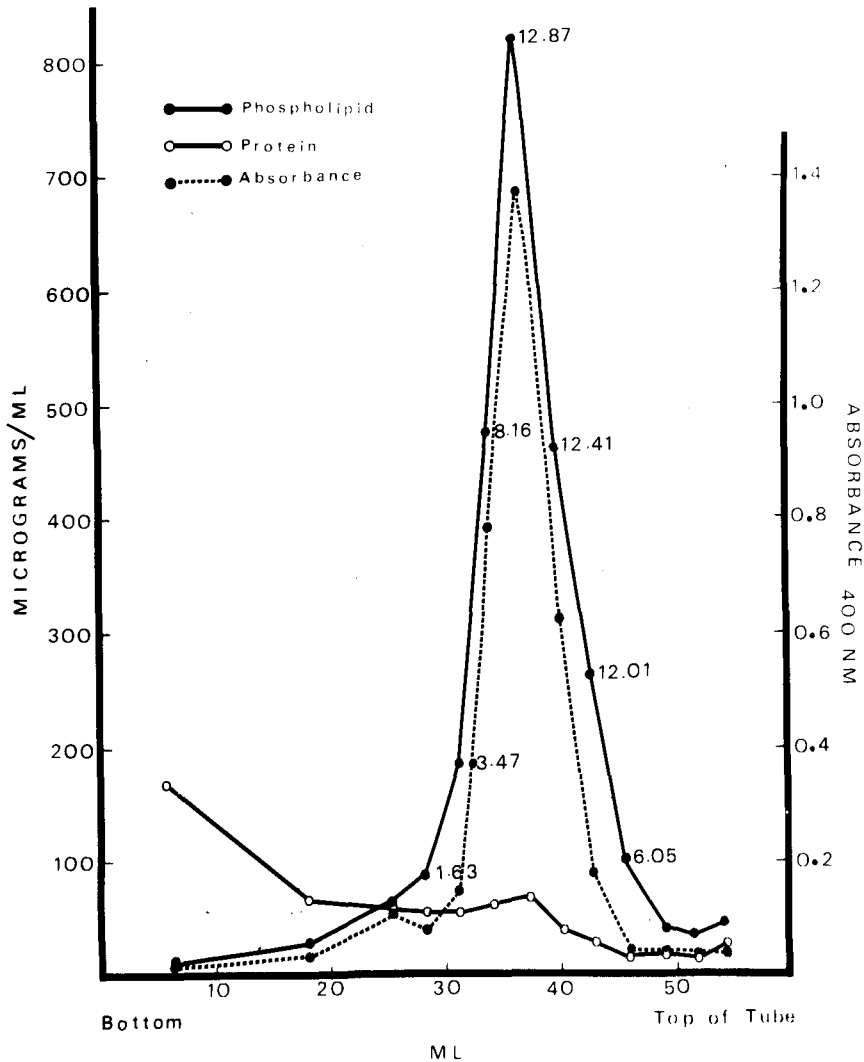


Figure 28. Distribution of phospholipid and protein in NaBr gradient (LP 48, Tube 1). The non-cellular lung washings used to prepare this gradient were obtained with RLS and had a PL/Protein ratio of 1.89. The gradient tube was centrifuged at 20° C for 24 hours. The number by each point represents the PL/Protein ratio of that fraction. The PL/Protein ratio of these fractions combined is 7.87.

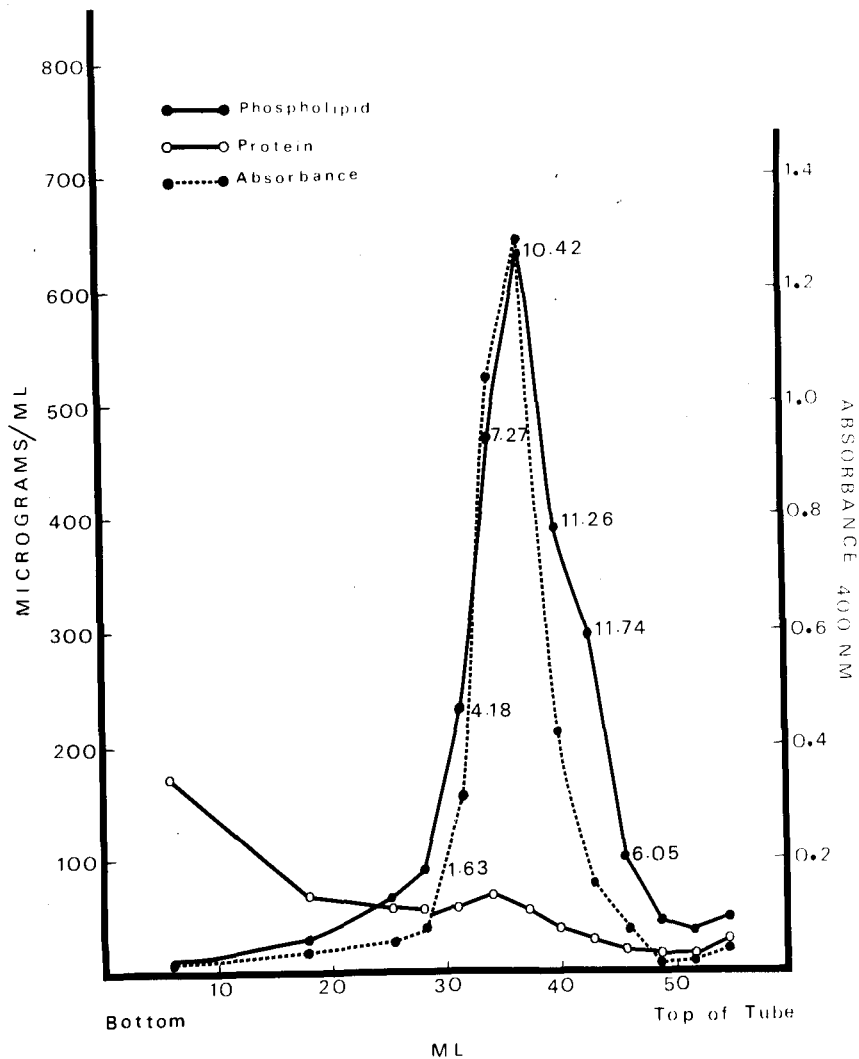


Figure 29. Distribution of phospholipid and protein in NaBr gradient (LP 48, Tube 2). The non-cellular lung washings used to prepare this gradient were obtained with RLS and had a PL/Protein ratio of 1.89. The gradient tube was centrifuged at 20° C for 24 hours. The number by each point represents the PL/Protein ratio of that fraction. The PL/Protein ratio of these fractions combined is 7.10.

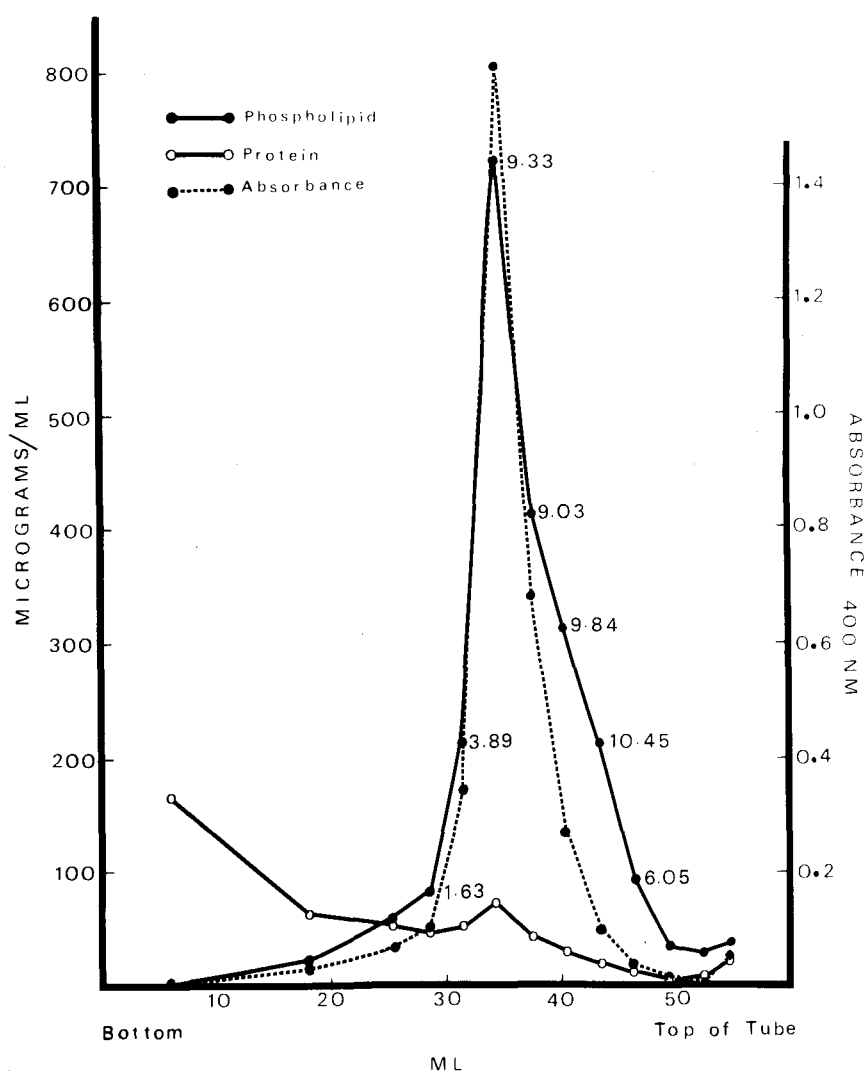


Figure 30. Distribution of phospholipid and protein in NaBr gradient (LP 48, Tube 3). The non-cellular lung washings used to prepare this gradient were obtained with RLS and had a PL/Protein ratio of 1.89. The gradient tube was centrifuged at 20° C for 24 hours. The number by each point represents the PL/Protein ratio of that fraction. The PL/Protein ratio of these fractions combined is 6.88.

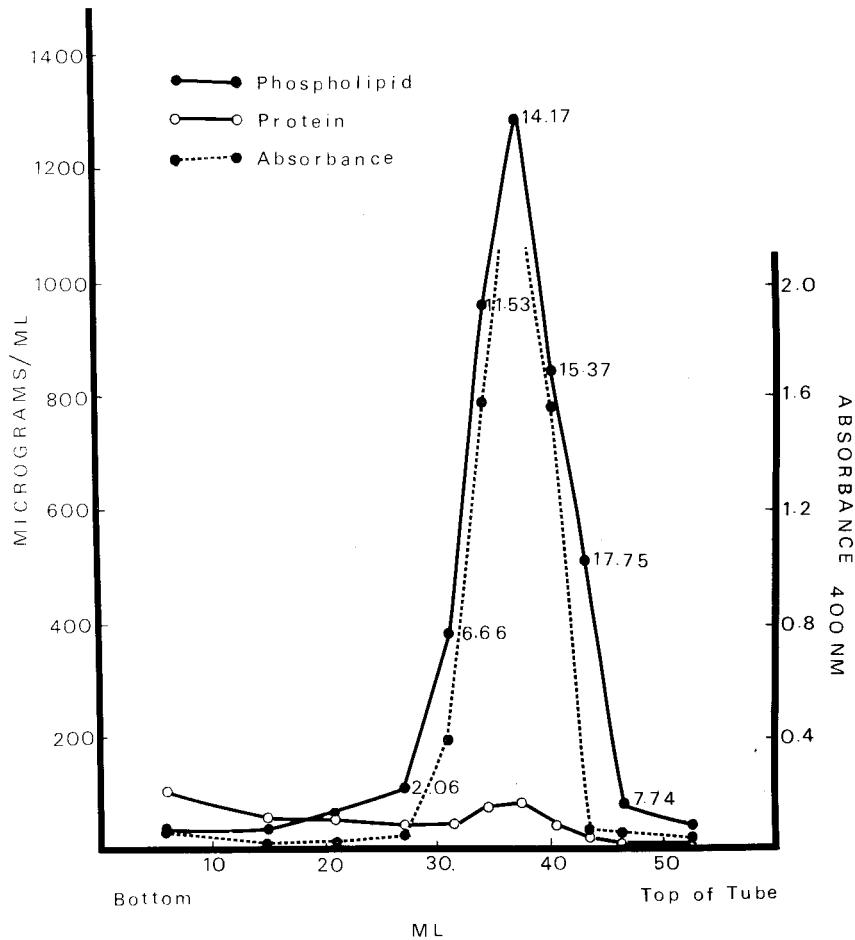


Figure 31. Distribution of phospholipid and protein in NaBr gradient (LP 57, Tube 3). The non-cellular lung washings used to prepare this gradient were obtained with RLS and had a PL/Protein ratio of 2.99. The gradient tube was centrifuged at 20° C for 18 hours. The number by each point represents the PL/Protein ratio of that fraction. The PL/Protein ratio of these fractions combined is 10.88.

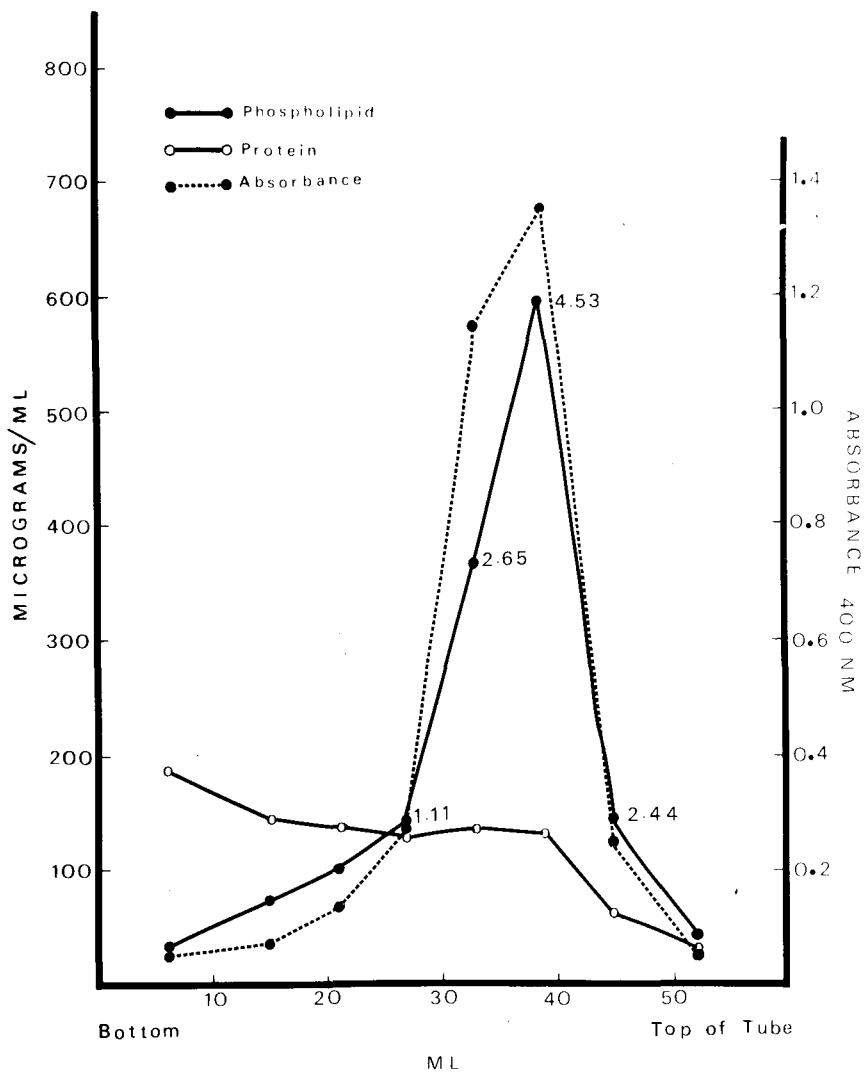


Figure 32. Distribution of phospholipid and protein in NaBr gradient (LP 65, Tube 1). The non-cellular lung washings used to prepare this gradient were obtained with RLS and had a PL/Protein ratio of 0.99. The gradient tube was centrifuged at 20° C for 3 hours. The number by each point represents the PL/Protein ratio of that fraction. The PL/Protein ratio of these fractions combined is 2.73.

the density gradient mixing apparatus. About 80% of the protein was found in the gradient, with this larger loss probably attributable to a higher concentration of protein in the small pellet found on the bottom of the gradient tubes.

As can be seen in Figures 26 to 32, the phospholipids were concentrated in an area of the tube corresponding to the visible band. Since the volumes of the fractions collected were insufficient to make accurate density measurements with the available equipment, the density at which the phospholipid band was located was estimated by reference to the data in Figure 5. The phospholipid bands were centered at an average density of 1.046. In experiment LP 48, after aliquots of each fraction were removed for phospholipid and protein determinations, the samples remaining from the peak were pooled from all three gradient tubes and the density was found to be 1.043.

The proteins were spread throughout the tube with a small peak of protein associated with the visible band of phospholipids in all 7 tubes. The phospholipid to protein ratios of fractions containing the highest concentrations of phospholipid from each of the 7 tubes varied from 4.53 to 14.17 and the PL/Protein ratios of the entire band of phospholipid ranged from 2.73 to 10.88. The method of collecting the fractions might have accounted for some of the variations, but these values also appear to depend in some measure upon the PL/Protein ratio of the non-cellular washings used as starting material in each gradient (Table X). The results of these experiments revealed that the "background protein," that is, protein which is

TABLE X

PL/PROTEIN RATIOS OF PHOSPHOLIPID-CONTAINING FRACTIONS
FROM NaBr DENSITY GRADIENTS

Experiment Number	PL/Protein of cell-free washings	PL/Protein of fraction with highest phospho- lipid concentration	PL/Protein of entire phospho- lipid peak
65	0.99	4.53	2.73
47	1.67	9.90	7.21
		7.22	8.00
48	1.89	12.87	7.87
		10.42	7.10
		9.33	6.88
57	2.99	14.17	10.88
	—	—	—
Average	1.86	9.78	7.24

not associated with the phospholipid, is spread throughout the gradient tube at the end of the centrifugation period in much the same manner as the sample was distributed during preparation of the gradient and may tend to obscure any possible specific lipid-associated protein.

The nature of the protein found in the phospholipid band and the relationship of this protein to the lipids was further investigated using a number of different methods as described in the following sections.

Sedimentation of Surfactant from the Phospholipid Band

In experiment LP 48, the fractions collected from the phospholipid peaks from each of the 3 tubes were pooled. There included fractions #5 through 8, the fractions being numbered beginning at the bottom of the tube. Following measurement of its density, the pooled sample was dialyzed at room temperature against RLS to remove excess NaBr. A portion of this sample which had a PL/Protein ratio of 8.7 was centrifuged at $127,000 \times g$ for 90 minutes in order to produce a sedimentable surfactant fraction B and a supernatant fraction C. The PL/Protein ratio of the pellet B was 18.4 and that of the supernatant was 2.6. These data, along with previous results shown in Figure 16 and the results of washings mixed with serum, which will be discussed later in this chapter, support the contention that the PL/Protein ratio of the sedimentable fraction is dependent upon the PL/Protein ratio of the starting material.

Surface Activity of Gradient and Sedimentation Fractions

The non-lipid containing fractions, (fraction #1) from each of the 3 gradients in experiments LP 48 were also pooled, and after dialysis against RLS at room temperature, they were tested for surface activity. As expected from the low phospholipid content, this fraction, as well as the supernatant C of the centrifugation described above were surface inactive. The material from the phospholipid band and the sediment B obtained from a portion of the phospholipid band were both highly surface active, reducing surface tension to 0 dynes/cm.

SDS-PAGE of Proteins from Gradient Fractions

Even though 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 were compared with the protein composition of gradient fractions without substantial quantities of phospholipids.

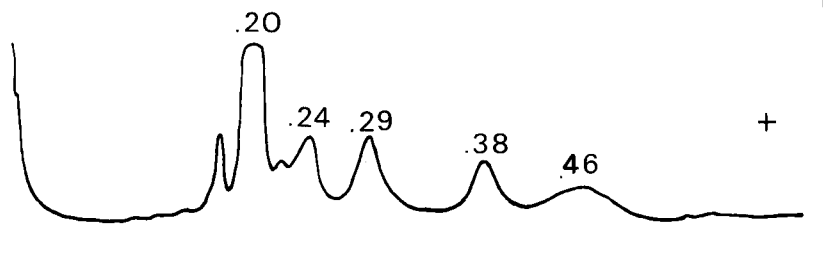
In experiment LP 47, after removing aliquots of each fraction for phospholipid and protein determination, fractions 1 through 5 from both tubes were pooled. This sample was, for convenience, designated Sample I and contained 1.78 mg of protein and 0.12 mg of phospholipid. Fractions 11 through 15 from both tubes were also pooled, and the pooled material was designated Sample II. This sample contained 0.76 mg of protein and 5.54 mg of phospholipid. Samples I and II were dialyzed against several changes of

distilled water to remove the NaBr and then lyophilized. Each sample was dissolved in 1 ml of buffer containing SDS and 2-mercaptoethanol and subjected to electrophoresis as described in Chapter II. The gels were electrophoretically destained.

Densitometric tracings of two such gels are shown in Figure 33. The gel of Sample I contained 36 ug of protein, and the gel of Sample II contained 38 ug of protein. Each gel showed 7 protein bands. Use of 89 or 178 ug of protein from Sample I revealed no additional bands. Doubling the amount of Sample II resulted in distortion of the bands due to overloading the gel with lipid. Even the gel of Sample II shown in Figure 33 could not be used for accurate measurement of relative mobilities of the protein bands since the tracking dye was streaked and the distance of dye migration could not be measured accurately. However, both visual observation and densitometric scanning indicated that Sample II contained the same proteins as Sample I.

The major protein band had a relative mobility of 0.20, close to that of albumin, and bands at 0.24 and 0.46, corresponding to the heavy and light chains of Immunoglobulin G. Comparison with rabbit plasma subjected to electrophoresis in this system (Figure 34), revealed that the bands at 0.29 and 0.38 do not have counterparts in plasma.

The proteins from various parts of the density gradients in experiment LP 48 were also studied by SDS-PAGE. Pooled fractions obtained from near the bottom of the gradient (Sample I) and from the phospholipid-containing peak (Sample II) which had been tested for surface activity were recovered



Sample I



Sample II

Figure 33. SDS-PAGE of fractions of lung washings centrifuged on NaBr density gradients. The numbers above the peaks represent the relative mobilities of those protein bands.

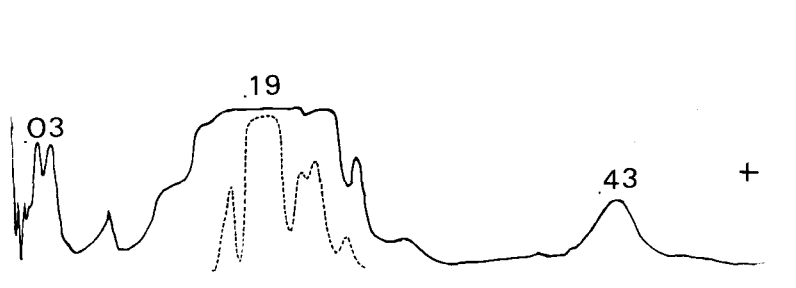
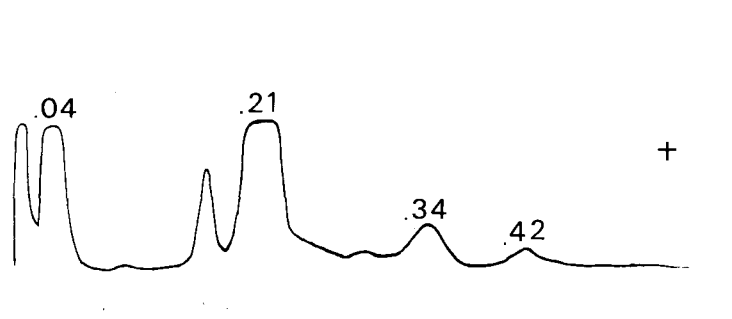


Figure 34. SDS-PAGE of rabbit plasma. The dotted line is a tracing obtained with the zero adjustment of the densitometer set at a lower point in order to resolve closely migrating bands. The numbers above the peaks represent the relative mobilities of those protein bands.

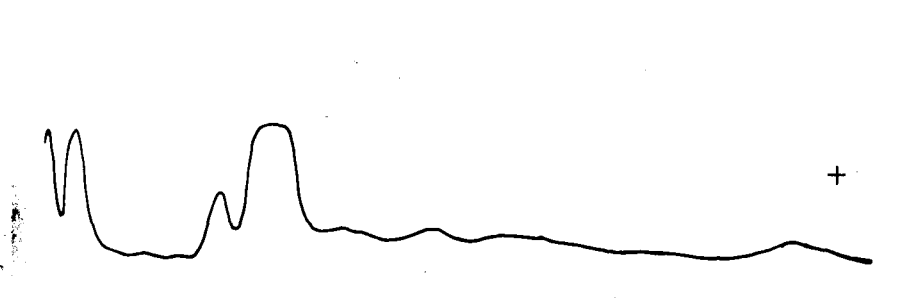
from the surface balance. Following dialysis against distilled water, each sample was equally divided into two portions and lyophilized. One portion of each sample was then dissolved in buffer with SDS and 2-mercaptoethanol, while the other portion was dissolved in solution without mercaptoethanol. Each portion of Sample I contained 1.78 mg of protein and 0.09 mg of phospholipid and was dissolved in 1 ml of solution. Each portion of Sample II contained 0.18 mg of protein and 1.20 mg of phospholipid and was dissolved in 0.5 ml.

Electrophoresis in the presence of 2-mercaptoethanol produced substantially the same pattern as in the previous experiment. Densitometric tracings of samples without 2-mercaptoethanol are shown in Figure 35. Again, no difference was observed between Samples I and II, except that the faster moving bands were distorted by the presence of phospholipid in Sample II and their relative mobilities could not be determined because of the streaking of the tracking dye. A band with the mobility of albumin predominated and a large band corresponding to immunoglobulin G was also present. The bands with relative mobilities of 0.42 and 0.34 correspond reasonably well to the bands of 0.38 and 0.29 in samples containing 2-mercaptoethanol, since as can be seen in Figure 11, the relative mobilities of the standard proteins are less in the presence of 2-mercaptoethanol than in its absence. These bands represent molecular weights of approximately 30,000 and 40,000 and they have no counterpart in serum.

The results of these experiments indicate that there are no differences in the proteins present in various parts of the density gradient tubes and



Sample I



Sample II

Figure 35. SDS-PAGE without 2-mercaptoethanol of fractions of lung washings separated on NaBr density gradients. The numbers above the peaks represent the relative mobilities of those protein bands.

therefore, no unique lipid-associated protein is present. They further show that although quantitatively, plasma proteins are the most abundant proteins in both phospholipid-rich and phospholipid-poor fractions, small amounts of non-serum proteins are also present in both fractions.

Centrifugation of Phospholipid Band on a Second Gradient

Since the same proteins were found in the phospholipid band as in other parts of the gradient, it was decided to centrifuge a portion of a phospholipid band on a second gradient to determine if the protein would remain associated with the phospholipid or be spread throughout the tube. Part of the phospholipid band from Tube 3 in Experiment LP 57 (Figure 31) was diluted to 44 ml with 10% NaBr in RLS and used to prepare another gradient. Following centrifugation at 20° C for 18 hours, the phospholipids and proteins were distributed as shown in Figure 36. The position of the phospholipid peak in the tube was unchanged from the previous run. Only a very small amount of protein remained associated with this peak, and the PL/Protein ratio of the peak was 23.11, considerably higher than the PL/Protein ratio of the material from the previous peak.

Centrifugation of Chloroform-Methanol Extracted Surfactant Lipids on NaBr Density Gradients

The results of the previous experiment indicated that very little protein was specifically associated with the phospholipids of lung surfactant. Therefore, an experiment was carried out to determine whether complete

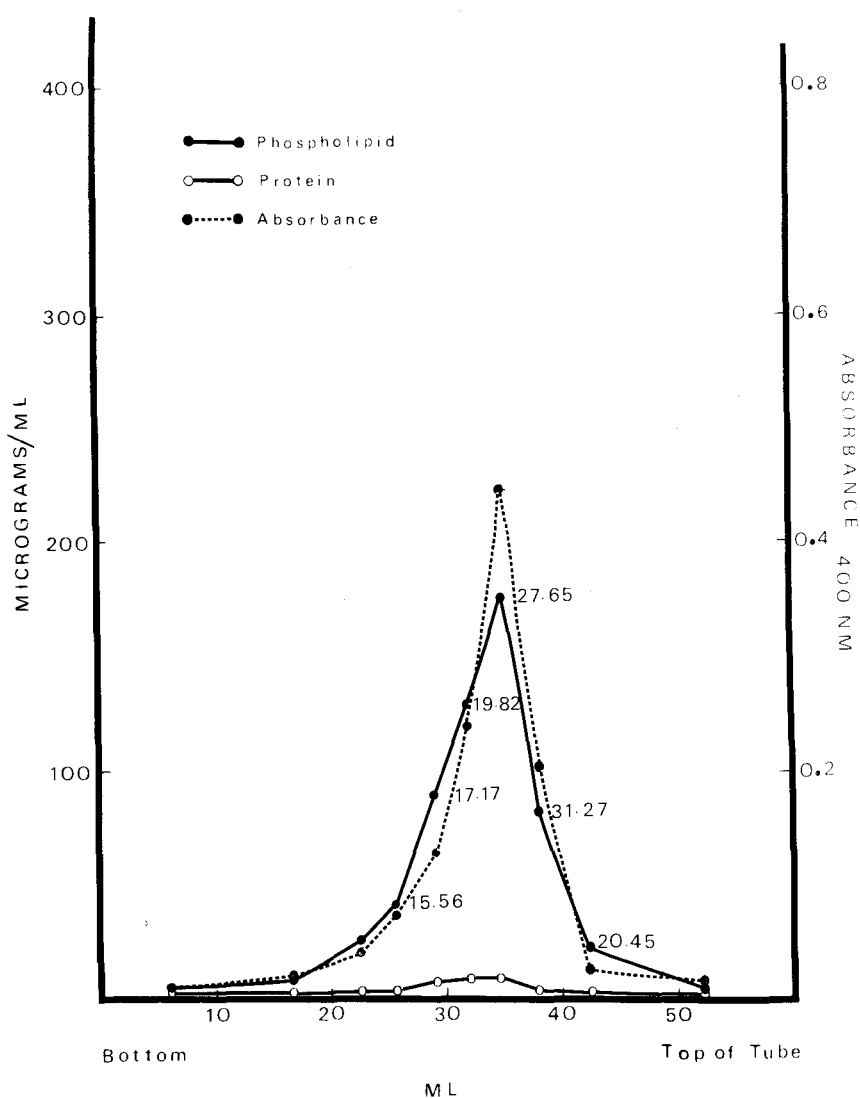


Figure 36. Distribution of phospholipid and protein of the material from the center of a phospholipid band of a previous gradient on a second NaBr gradient. The PL/Protein ratio of the starting material was 13.50. This tube (LP 57, Tube 5) was centrifuged for 18 hours at 20° C. The number by each point represents the PL/Protein ratio of that fraction. The PL/Protein ratio of these fractions combined is 23.11.

separation of the lipid and protein components by organic solvent extraction would alter the density at which the phospholipid was found. Another portion of the phospholipid band from tube 3 in Experiment LP 57 (Figure 31) was treated with chloroform-methanol to extract the lipids. After washing the extract following the method of Folch *et al.* (1957) as outlined in Chapter II, the solvent was evaporated. The dried lipid extract was resuspended in 10% NaBr and used as heavy solution for the preparation of another gradient. This lipid extract suspended quite readily and yielded a cloudy white suspension. After centrifugation for 18 hours at 20° C, a fine, white particulate band was seen at a position slightly higher in the tube than when unextracted washings were used. Fractions were collected and the results of absorbance measurements and phospholipid and protein determinations are shown in Figure 37.

The chloroform-methanol extraction removed essentially all of the protein. The phospholipid band was found centered at a slightly lower density (1.040) than is usual when the entire cell-free lung washings were used (1.046). These results suggest that the association of protein with the phospholipids of lung surfactant only slightly alters their density and, therefore, it can be assumed that only a very small amount of protein is attached to the lipids.

Use of Other Conditions for Density Gradient Centrifugation of Lung Washings

The results of the previous experiments indicate that lung washings contain no specific lipoproteins. The fact that the PL/Protein ratios varied

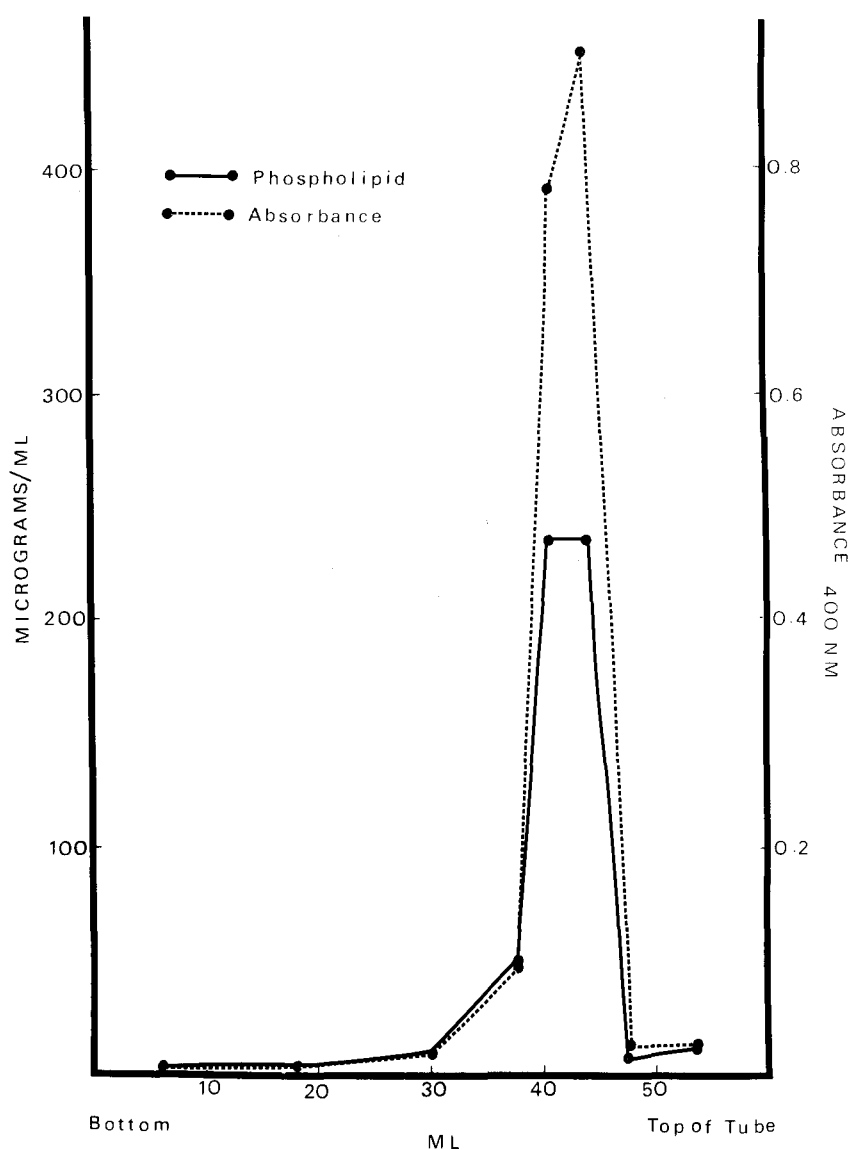


Figure 37. Distribution of phospholipid on a NaBr gradient of a chloroform-methanol extract of a phospholipid band from a previous gradient. This tube was centrifuged for 18 hours at 20° C.

greatly in both sediments and density gradient fractions, and appeared to depend on the relative amounts of phospholipid and protein present in the original washings, as well as the fact that no differences could be observed in the protein composition of various gradient fractions supports this conclusion. However, the possibility that specific lipoprotein complexes which were broken by the high ionic strength of the NaBr (0.98 M) or by long periods of centrifugation at 20° C and at a pH between 6 and 7, could not be excluded. For this reason, centrifugation of the non-cellular washings under a variety of conditions was carried out.

In the first of these experiments, the conditions used by King and Clements (1972a) were duplicated. Lung washings were obtained in Tris-buffered saline, pH 7.35, chilled to 4° C. After removal of the cells, NaBr was added to make a 10% solution, and the calcium and magnesium concentrations adjusted so that the sum of the monovalent cations was 25 times the sum of the divalent cations. This solution was used as the heavy solution and Tris-buffered saline was used as the light solution to prepare the gradients. Following centrifugation at 4° C for 18 hours, much of the visible material was observed as a pellet at the bottom of the tube, with some particulate material and an opalescent band immediately above the pellet. These results suggested the possibility that a more dense lipoprotein could be present in this system.

To further investigate this situation and to determine whether the temperature or the nature of the solution was the important factor in producing these differing results, lung washings were obtained from a rabbit with RLS

chilled to 4° C. These washings were used to prepare two gradients, one with 10% NaBr and the other with 14% NaBr as the heavy solution. The gradients were centrifuged for 18 hours at 4° C. In the gradient made from 10% NaBr, a white pellet was found on the bottom of the tube and the bottom one-half of the tube was opalescent. The gradient made from 14% NaBr revealed the presence of a particulate band near the center of the tube with a narrow, opalescent band above and below it. Fractions were collected from this gradient and the results of the phospholipid and protein determinations are shown in Figure 38. By reference to Figure 6, this band was found to be centered at a density of about 1.075. However, there does not appear to be any more lipid-associated protein in this sample than was found in samples centrifuged at 20° C. Centrifugation of washings in Tris-buffered saline at 20° C or at 4° C yielded results similar to those obtained with washings in RLS at these respective temperatures.

Substitution of sucrose for NaBr was also undertaken. In one experiment, washings obtained in RLS were made 20% with respect to sucrose. The density of 20% sucrose is 1.083; the density of 10% NaBr is 1.082. Gradients were prepared using 20% sucrose as the heavy solution and RLS as the light solution. Following centrifugation at 20° C for 18 hours, a narrow particulate band was found centered in a much wider opalescent band. The results of absorbance measurements and phospholipid and protein determinations are shown in Figure 39. If one can assume that the densities of this gradient are similar to those of a NaBr gradient, the phospholipid peak appears at nearly the same, or at a slightly lower density, as found in NaBr gradients in RLS at 20° C.

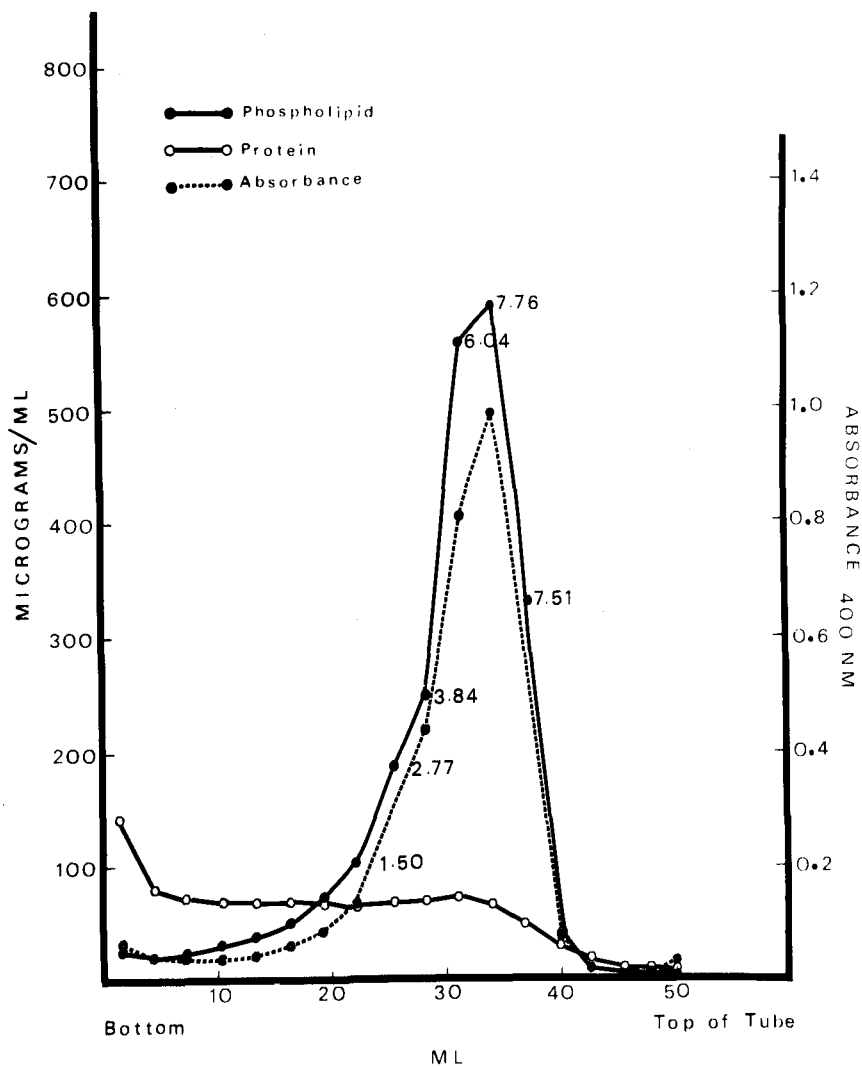


Figure 38. Distribution of phospholipid and protein on a NaBr gradient centrifuged at 4° C. The non-cellular lung washings used to prepare this gradient were obtained with RLS and had a PL/Protein ratio of 1.98. The gradient tube was centrifuged for 18 hours. The number by each point represents the PL/Protein ratio of that fraction. The PL/Protein ratio of these fractions combined is 5.38.

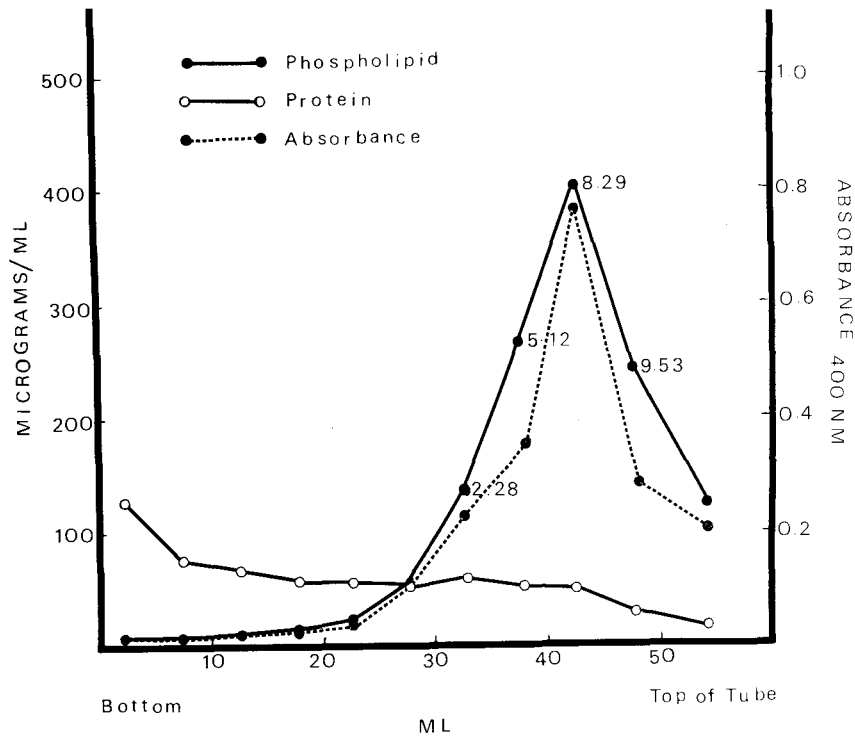


Figure 39. Distribution of phospholipid and protein on a sucrose gradient. The non-cellular lung washings used to prepare this gradient were obtained in RLS and had a PL/Protein ratio of 1.59. The gradient tube was centrifuged at 20° C for 18 hours. The number by each point represent the PL/Protein ratio of that fraction.

Also, the amount of protein found associated with the phospholipid peak is no greater than that found in NaBr gradients. In another experiment, sucrose in Tris-buffered saline at 4° C was used and the results revealed no significant differences from similar gradients in which NaBr had been used.

These experiments indicate that temperature influences the density at which the phospholipid band is found, but does not appear to influence the relative amount of protein found in this band. The use of sucrose in place of NaBr and the use of Tris-buffered saline instead of RLS caused no apparent changes in either the position or the protein content of the phospholipid band.

Centrifugation of DPPC in NaBr Density Gradient

In an attempt to understand the behavior of phospholipids in density gradient centrifugation for the purpose of interpreting the results obtained with lung washings, DPPC (Applied Science, State College, Penn.), was suspended in NaBr solutions and used to prepare gradients. In two such experiments, 2 mg of DPPC was suspended by vigorous stirring in 44 ml of 10% NaBr in RLS and was used as the heavy solution in preparation of gradients. After centrifugation at 20° C for 7 or 18 hours, a fine particulate band was found near the bottom of the gradient tube. Fractions were collected and from measurements of absorbance at 400 nm and reference to Figure 5, the density at which the band was located was estimated to be about 1.065. However, phospholipid determinations revealed that less than 10% of the DPPC of the starting solution was in the gradient. Since no pellet was observed, it

was assumed that the rest of the DPPC must have been lost in the gradient mixing apparatus. This was not unexpected since the DPPC did not disperse well and probably settled out of the suspension in the "heavy flask." The absorbance and phospholipid concentrations of these samples were too low to accurately locate the phospholipid band.

Sonication was used in the next experiments in order to suspend more of the DPPC. Two mg of DPPC were sonicated for 3 minutes in 10 ml of 10% NaBr in RLS. This solution was then diluted to 44 ml with 10% NaBr and used as the heavy solution to prepare a gradient. Following centrifugation at 20° C for 18 hours, a particulate band was found near the bottom of the tube. Fractions were collected from this tube and their absorbances and phospholipid contents determined (Figure 40). By reference to the data in Figure 5, it was found that the band was centered at a density of about 1.07, a density considerably greater than that at which the phospholipid band from lung washings was found after centrifugation under similar conditions.

The effect of temperature on the behavior of DPPC in NaBr gradients was investigated, repeating the above experiment at 4° C. When sonicated DPPC was centrifuged for 18 hours at 4° C in a gradient mixed from 10% NaBr, no bands were visible; rather, the material was found in pellet-form on the bottom of the tube. Use of 14% NaBr as the heavy solution resulted in the DPPC appearing as a particulate band near the bottom of the tube. From absorbance readings and with reference to the data in Figure 6, it was estimated that the band was located at a density of about 1.10.

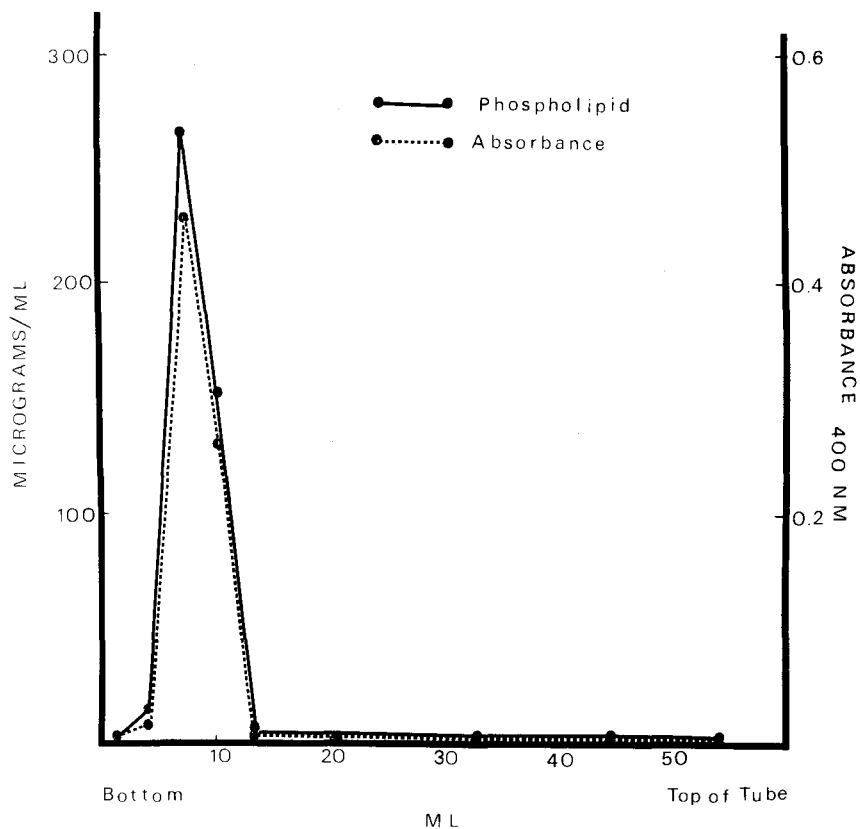


Figure 40. Distribution of phospholipid on a NaBr density gradient containing sonicated DPPC. The DPPC was sonicated in 10% NaBr in RLS and used to prepare this gradient, which was then centrifuged for 18 hours at 20° C.

The results of the above experiments demonstrated that temperature has a significant effect upon the behavior of DPPC in NaBr density gradients. The same effect was also noted with surfactant lipids. When lipids extracted from lung washings were centrifuged on a 0-10% gradient at 4° C, the material was found in the pellet and in a slightly opalescent solution just above the pellet.

Fetal Lung Washings

Since fetal lung washings contain more protein than adult lung washings and since the sedimentation fractions have a lower PL/Protein ratio than do those of adult rabbit lung washings (Pagast, et al., 1972), the possibility that more lipid-associated protein might be found in density gradients was investigated. Washings were obtained from 31-day gestation rabbit fetuses in RLS, and after centrifugation to remove the cells, NaBr was added to give 10% NaBr. A gradient was prepared and centrifuged for 20 hours at 20° C. A fairly narrow band of particulate material was found slightly lower in the gradient tube than is usual in the adult washings. Fractions were collected, and absorbance, phospholipid and protein measurements were made. The results are shown in Figure 41. By reference to the data in Figure 5, the band was estimated to be centered at a density of 1.057; however, again, there was no indication of a specific, lipid-associated protein peak. The remaining material from the phospholipid band (Fractions #8, 9, and 10) were pooled and used to prepare another gradient. The phospholipid band was again found in the same position, at a density of 1.057.

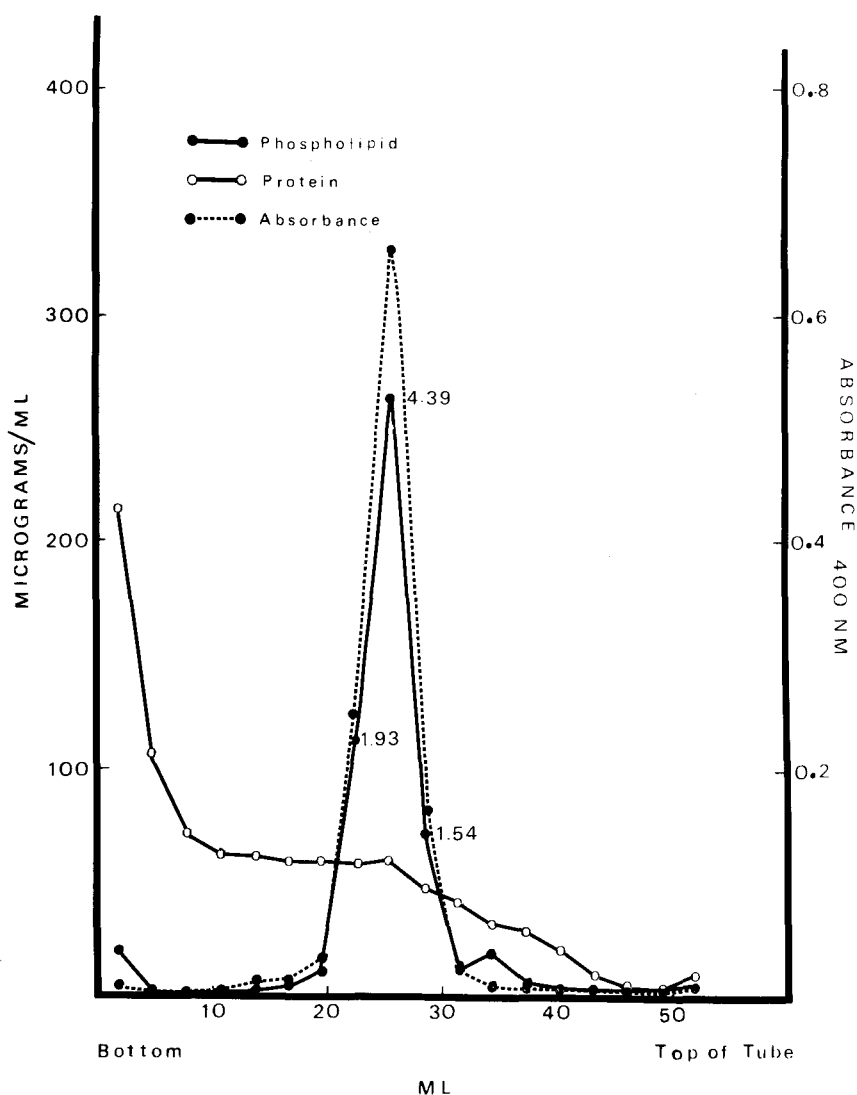


Figure 41. Distribution of phospholipid and protein from fetal lung washings in a NaBr density gradient. The washings were obtained in RLS and the gradient was centrifuged at 20° C for 20 hours. The number by each point represents the PL/Protein ratio of that fraction.

The presence of additional lipid-bound protein could account for the finding of this band at a higher density; however, no obvious peak of protein was observed. The fact that extracted surfactant lipids, a mixture of lipids composed largely of PC with several neutral lipids present in small quantities, appeared in density gradients at a density of about 1.04, while pure DPPC was found at a much higher density, 1.07, suggested that variations in lipid composition might be an important factor in determining the density at which the band from lung washings would be found. For this reason further characterization of the lipid composition was undertaken. Lipids were extracted from the phospholipid band of the re-centrifuged fetal washings and compared with the lipids obtained from the phospholipid after two density gradient centrifugations of adult lung washings. Gas-liquid chromatography of the fatty acids revealed a similar percentage of saturated fatty acids in both samples. Thin layer chromatography showed the presence of the same lipid classes in both samples. However, quantitative total lipid determinations by the method of Marsh and Weinstein (1966) revealed that fetal lipid extract had a slightly higher percentage of phospholipids than did the adult lipid extract. Since the density of phospholipids is greater than that of at least some neutral lipids, this could explain the difference in the densities at which the fetal and adult phospholipid bands were found. It should be pointed out that such a suggestion is based only upon one determination and needs further study before conclusive results can be obtained.

In Vitro Interaction of Lung Washings with Serum

Two experiments using density gradient centrifugation were carried out in an attempt to investigate the nature of interactions of phospholipids of lung washings with serum proteins and to determine whether density gradient centrifugation would be an appropriate method for separation of lung surfactant from the contaminating serum components present in virtually all pathologic conditions of the lung. In the first of these experiments, lung washings were obtained by alveolar lavage with a dilute solution of normal rabbit serum. The cell-free washings were mixed with NaBr and RLS to give a sufficient quantity of 10% NaBr solution for the preparation of two gradient tubes. After centrifugation at 20° C for 18 hours, a broad, opalescent band with some particulate matter was observed near the center of the tubes. Fractions were collected from one tube and the absorbance at 400 nm and phospholipid and protein contents were measured. As can be seen in Figure 42, the phospholipid peak is centered at about the same density as in previous experiments without added protein. However, the phospholipid to protein ratios of these fractions were much lower, due to the amount of added protein spread throughout the gradient tube.

The remaining portions of the fractions from phospholipid band (#9 through 12) and the visible band (24-36 ml) from the other gradient which had been prepared and centrifuged in the same way, were combined and used for further study. After dialysis against RLS to remove the NaBr, the material was diluted with RLS and NaBr added to give a 10% NaBr solution.

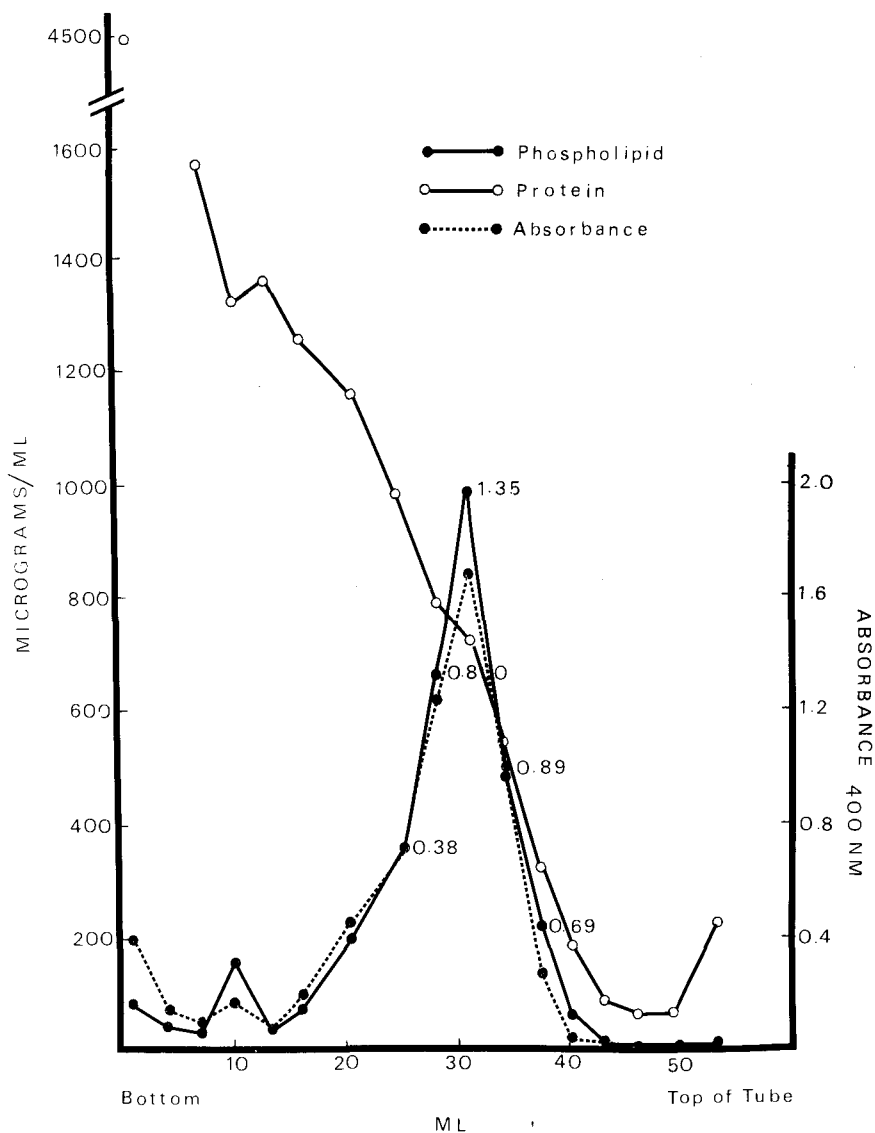


Figure 42. Distribution of phospholipid and protein in a NaBr gradient of non-cellular lung washings obtained in dilute serum. The gradient was prepared using RLS and was centrifuged at 20° C for 18 hours. The number by each point represents the PL/Protein ratio of that fraction.

This solution was used to prepare another gradient. After centrifuging at 20° C for 18 hours, the phospholipid and protein were distributed as shown in Figure 43. The phospholipids were located in the usual position in the tube, while the proteins were again spread throughout the tube. However, since there was less total protein in this gradient than in the previous gradient, the PL/Protein ratio of the fractions containing phospholipid were higher.

In the second experiment, 27 ml of a non-cellular lung washing with a PL/Protein ratio of 0.99 was mixed with 3 ml of normal rabbit serum. A sedimentable fraction obtained from this mixture by centrifugation at 127,000 x g for 90 minutes at 20° C had a PL/Protein ratio of 0.17 and it contained about 50% of the phospholipids of the mixture. Another sample of the non-cellular lung washing was also centrifuged as above, but without addition of serum. The sediment obtained from this sample had a PL/Protein ratio of 2.35 and contained about 75% of the phospholipids of the washing.

Each sediment was then resuspended in 10% NaBr in RLS and used to prepare gradients which were centrifuged at 20° C for three hours. The results obtained from these two gradients are shown in Figures 44 and 45. As is observed when the entire non-cellular washings are used to prepare a gradient, a particulate band below an opalescent band was found in the tube containing the sediment without serum. However, only a broad, opalescent band, and no particulate material was found in the gradient prepared from the sediment of the washing-serum mixture. Phospholipid and protein determinations revealed that both of these components were spread

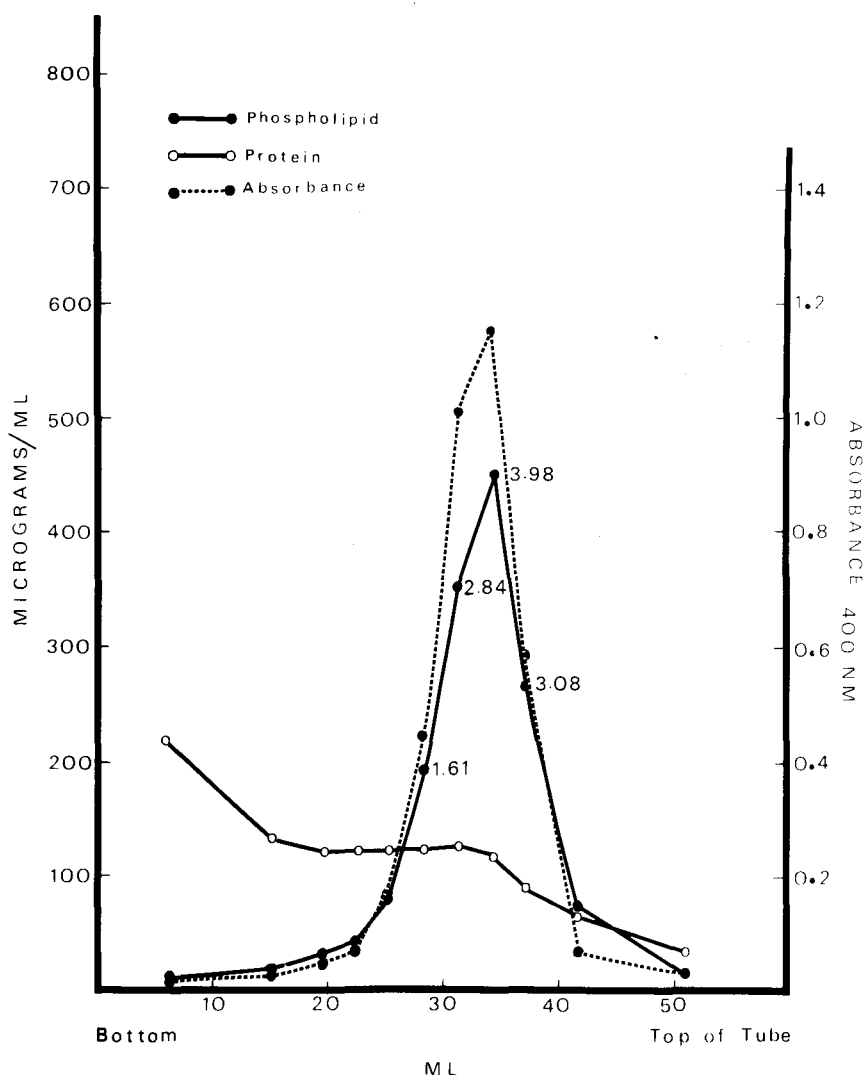


Figure 43. Distribution of phospholipid and protein from lung washings obtained in dilute serum after second centrifugation on NaBr density gradient. The number by each point represents the PL/Protein ratio of that fraction.

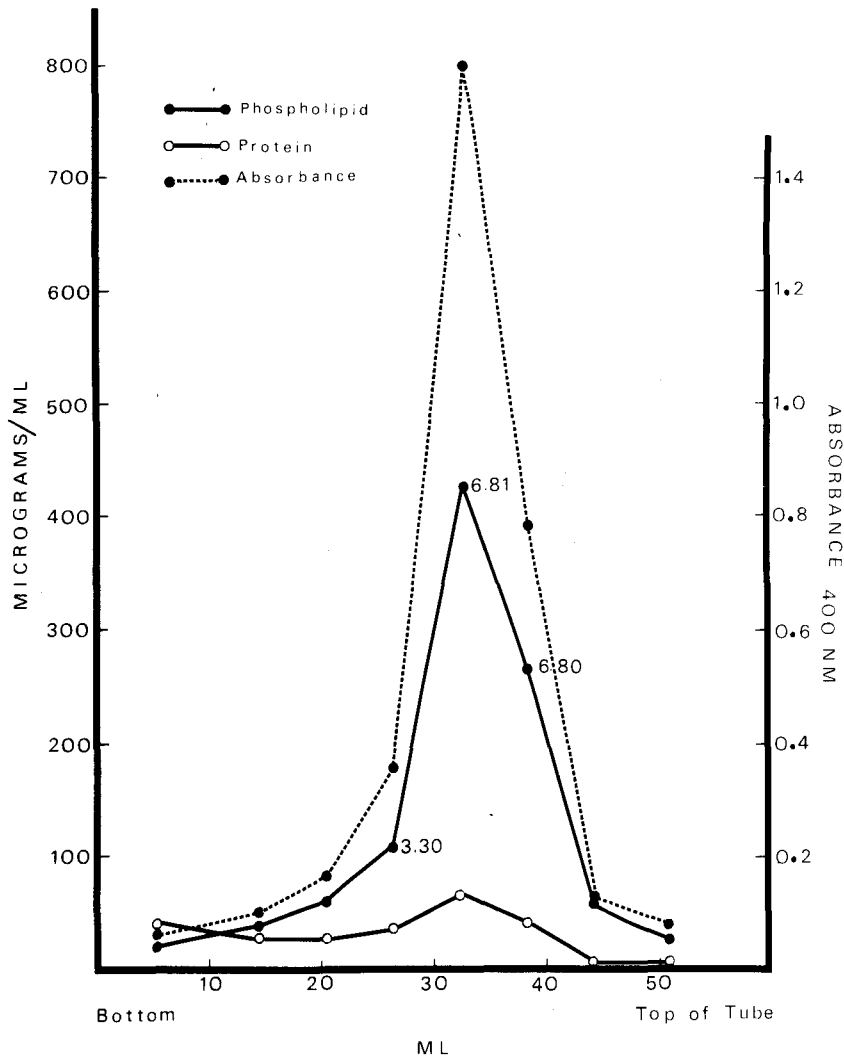


Figure 44. Distribution of phospholipid and protein from resuspended Sediment B on NaBr gradient. The Sediment B was resuspended in RLS and had a PL/Protein ratio of 2.35. The gradient was centrifuged for 3 hours at 20° C (LP 65, Tube 2). The number by each point represents the PL/Protein ratio of that fraction.

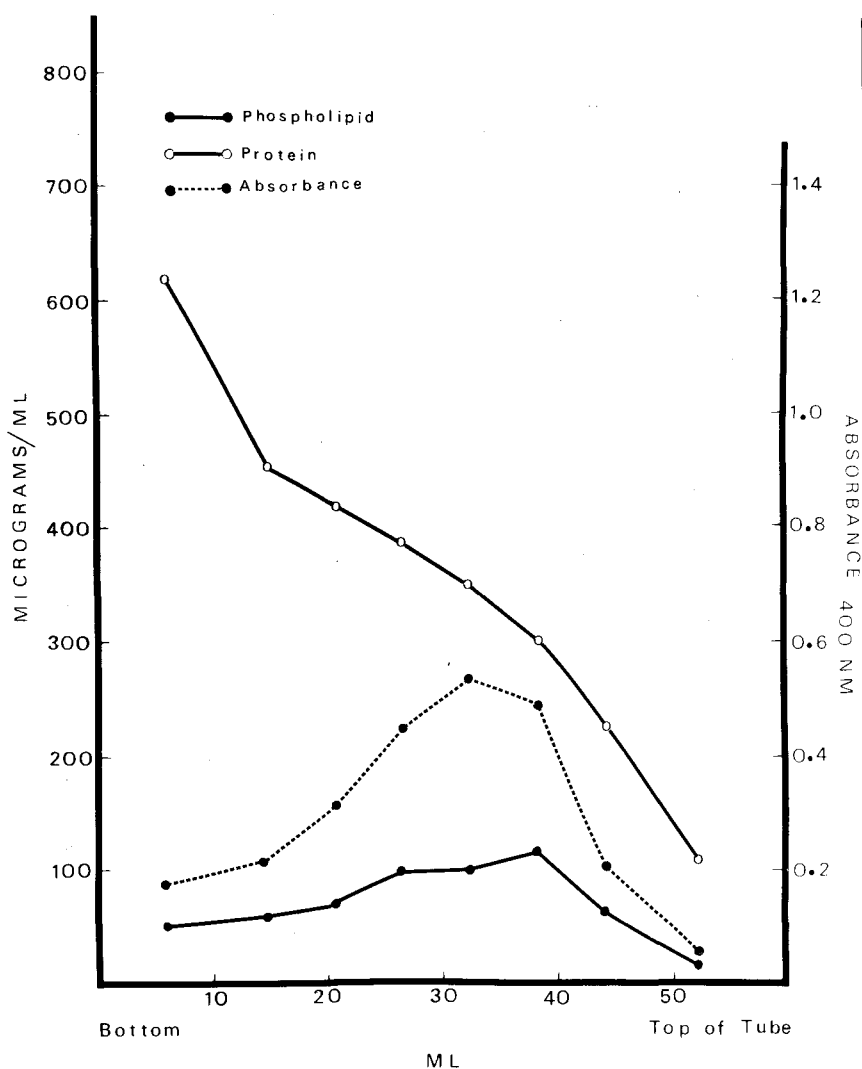


Figure 45. Distribution of phospholipid and protein from resuspended sediment obtained from a mixture of lung washings and serum on NaBr gradient. The gradient was prepared in RLS and centrifuged for 3 hours at 20° C (LP 65, Tube 3).

throughout the tube, whereas in the gradient without serum, a typical phospholipid peak was found, along with a small amount of associated protein.

The results of these two experiments indicate that sedimentation, in the presence of large amounts of protein, results in the formation of lipid-protein aggregates which are not readily dissociated. If sedimentation is avoided, as in the first experiment, there is little tendency for the excess protein and the surfactant phospholipid to associate, suggesting that density gradient centrifugation of the cell-free washings is a useful method for isolation of surfactant.

Human Lung Washings

Human lung washings obtained at autopsy are generally much more contaminated than are rabbit lung washings. Since it is extremely desirable to design techniques which will allow meaningful study of human material, the method of density gradient centrifugation was applied to human lung washings.

Washings were obtained at the time of autopsy from a 2830 gm, 3 day-old infant in which there was no evidence of hyaline membrane disease. After removal of the cells, the washing appeared as a yellowish-colored, slightly cloudy solution. This solution was found to be surface active and to contain 246 ug of phospholipids and 740 ug of protein per ml, giving a PL/Protein ratio of 0.33.

A portion of the washings were mixed with solid NaBr to give a 14% NaBr solution which was used as the "heavy" solution for 2 gradients. From

another portion of the lavage fluid, lipids were extracted with chloroform-methanol. After evaporation to dryness, the lipids were suspended by stirring in 14% NaBr in RLS and used as the "heavy" solution for one gradient. RLS was used as the "light" solution in each tube. The tubes were centrifuged for 18 hours at 4° C. This temperature was chosen because of the greater possibility of contamination of human washings and because the only difference which had been observed with variation in temperatures was the density at which the phospholipid band was found.

The results of this centrifugation are shown in Figure 46. The phospholipid band from the lung washings was found in the same location in the gradient tube and appeared similar to that of rabbit washings previously run under similar conditions (compare with Figure 38). As was expected from a consideration of the PL/Protein ratio of the non-cellular washing, a large quantity of protein was present in all fractions with the distribution similar to that from rabbit lung washings obtained in serum (Figure 42). As was observed with rabbit lung washings, the chloroform-methanol extracted lipids from the human lung washings were found slightly higher in the tube, that is, at a lower density than were the phospholipids of the unextracted washing.

After phospholipid and protein determinations were carried out, the remainder of the fractions with high concentrations of phospholipid (fractions 6, 7, and 8), were combined with the lipid-containing fractions from the other gradient tube which had been prepared and centrifuged in the same way. Only the absorbance at 400 nm was measured on fractions collected

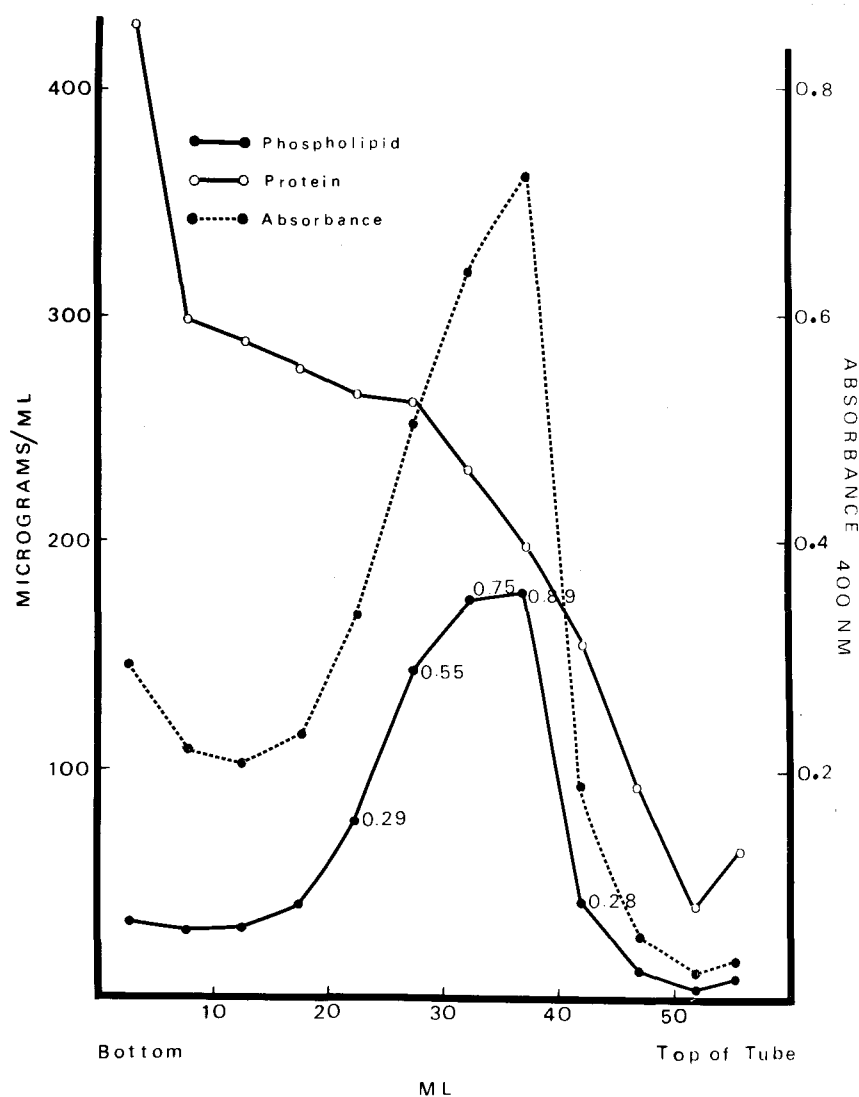


Figure 46. Distribution of phospholipid and protein from non-cellular human lung washings in NaBr gradient. The washings were obtained in RLS and had a PL/Protein ratio of 0.33. The gradient was centrifuged at 4° C for 18 hours. The number by each point represents the PL/Protein ratio of that fraction. The fractions near the bottom of the tube were yellow in color.

from this tube in order to preserve more material for further study. The combined phospholipid-containing fractions were dialyzed against several changes of RLS at 4° C. NaBr and RLS was then added to the sample to give 44 ml of solution containing 14% NaBr and another gradient was prepared. Centrifugation for 18 hours at 4° C resulted in the phospholipids appearing in a narrow, particulate band (Figure 47). The protein was again spread throughout the tube, but with a slight indication of a peak corresponding with the phospholipid peak. The material from the phospholipid peak remaining after phospholipid and protein determinations were carried out, was again dialyzed and used to make yet another gradient. The distributions of phospholipid and protein in this gradient tube are shown in Figure 48. The phospholipid band was found at the same density as before with a more definite protein peak at the same density.

This experiment demonstrates that human lung washings, obtained at the time of autopsy, and rabbit lung washings behave in the same manner in this procedure of density gradient centrifugation. Three successive gradients were needed to remove the majority of the "background" protein.

A New Approach to Density Gradient Centrifugation of Lung Washings

A difficulty in the method of gradient centrifugation used in this investigation is that at the end of a run, protein is spread throughout the tube. This "background" protein obscures characterization of lipid-associated protein. When the gradients were prepared, the samples were spread unevenly throughout the tube, being more concentrated at the bottom than at the top.

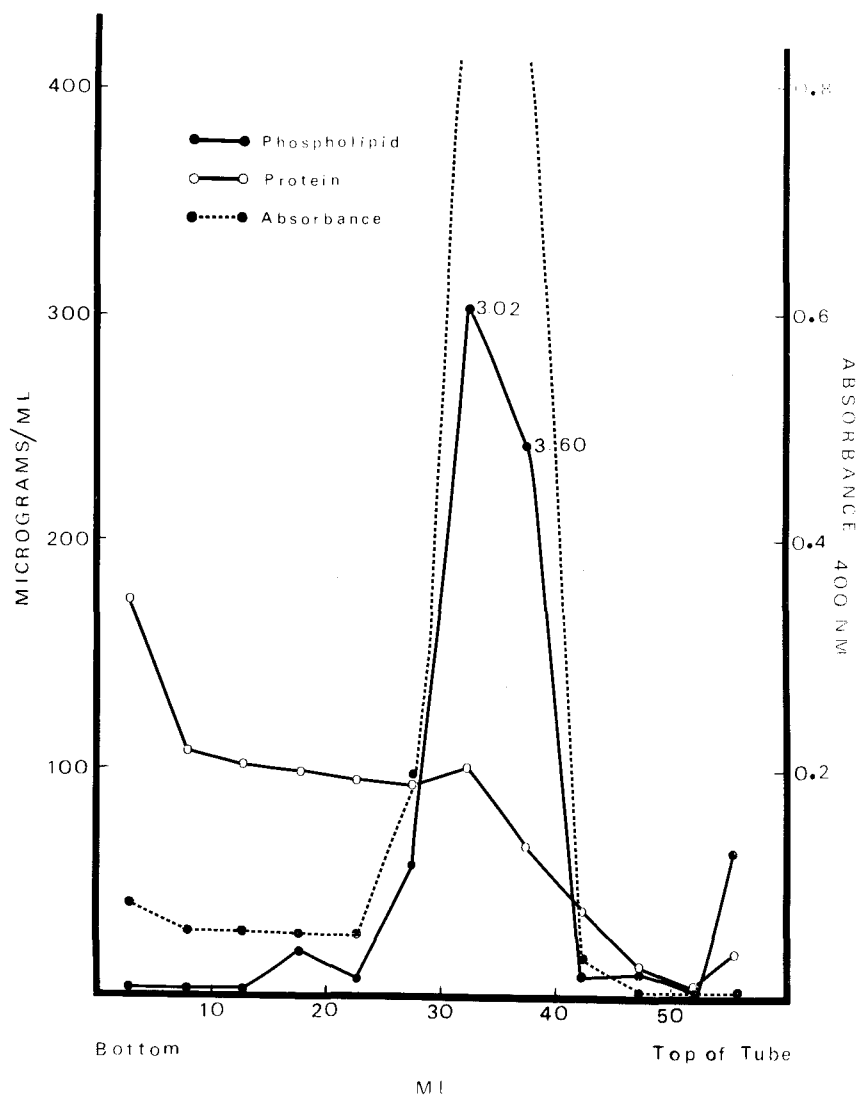


Figure 47. Distribution of phospholipid and protein from human lung washings in a second density gradient. The gradient was prepared using NaBr in RLS and was centrifuged for 18 hours at 4° C. The PL/Protein ratio of the sample used to prepare this gradient was about 0.70. The number by each point represents the PL/Protein ratio of that fraction.

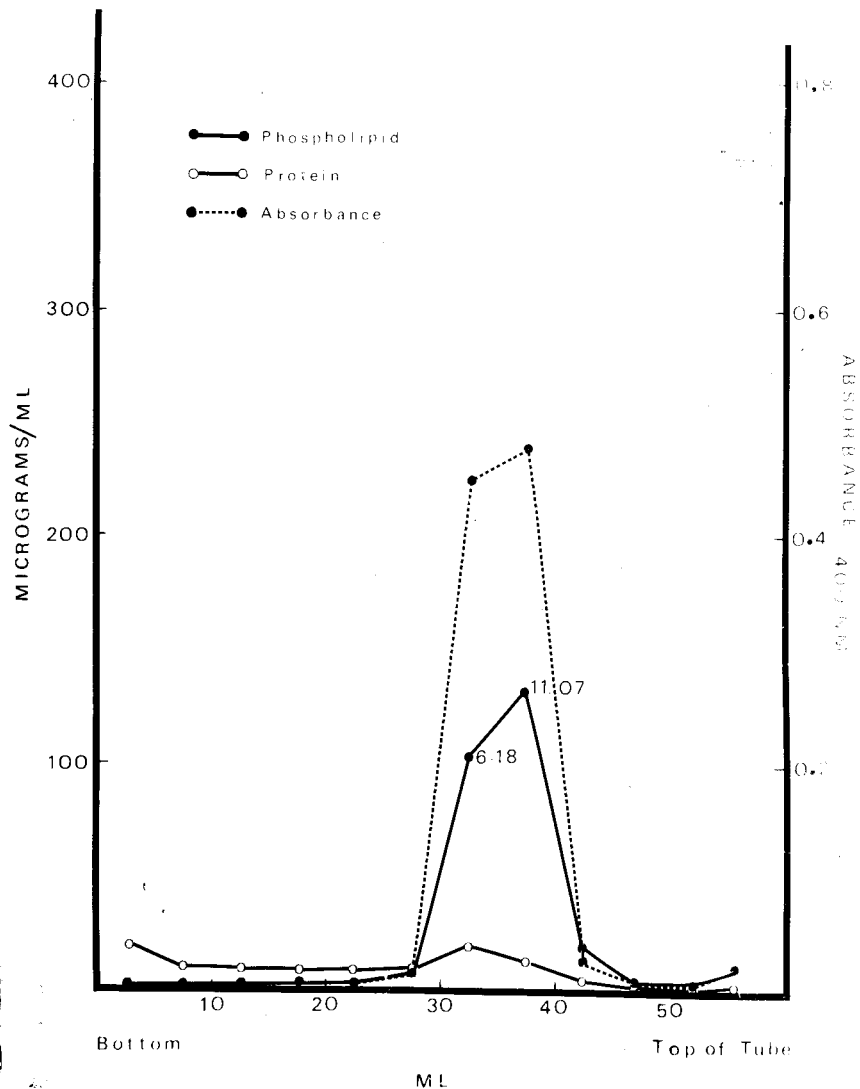


Figure 48. Distribution of phospholipid and protein from human lung washings in a third density gradient. The gradient was prepared using NaBr in RLS and was centrifuged for 18 hours at 4° C. The PL/Protein ratio of the sample used to prepare this gradient was about 3.25. The number by each point represents the PL/Protein ratio of that fraction.

The protein was still distributed in much the same manner at the end of the centrifugation period. The density of protein is generally 1.33-1.37 (Hatch and Lees, 1968) and such a density indicates that the protein should have moved toward the bottom of the tube. However, the relatively small size of the protein molecules apparently tends to limit their downward movement.

In an experiment designed to overcome this situation, gradients were prepared filling the centrifuge tubes to only 60% of their capacity. NaBr was added to lung washings to give a solution of a density greater than that on the bottom of the gradient and the washings were then placed at the bottom of the gradient tubes as shown in Figure 49a. The gradients were prepared at 4° C from RLS and 14% NaBr in RLS. The washings were obtained in RLS at room temperature, made 16% with respect to NaBr and chilled to 4° C. Since there was no protein in the gradient at the beginning of the run, and since the densities were such that phospholipids would move into the gradient during the run, any protein found with the lipid in the gradient at the end of the run could be assumed to be associated with the lipid and not "background" protein. An additional advantage of this design is that a preliminary centrifugation of the washings to remove the cells is unnecessary, thus avoiding a step that might result in the loss of surfactant.

After centrifuging for three hours at 23,000 rpm at 4° C, a white, particulate band was observed at 2.1 to 2.8 cm from the top of the tube (Figure 49b). Five ml fractions were collected from this tube and the phospholipid and protein contents of each fraction were measured. As can be seen in Figure 50, about 65% of the phospholipid and about 8% of the

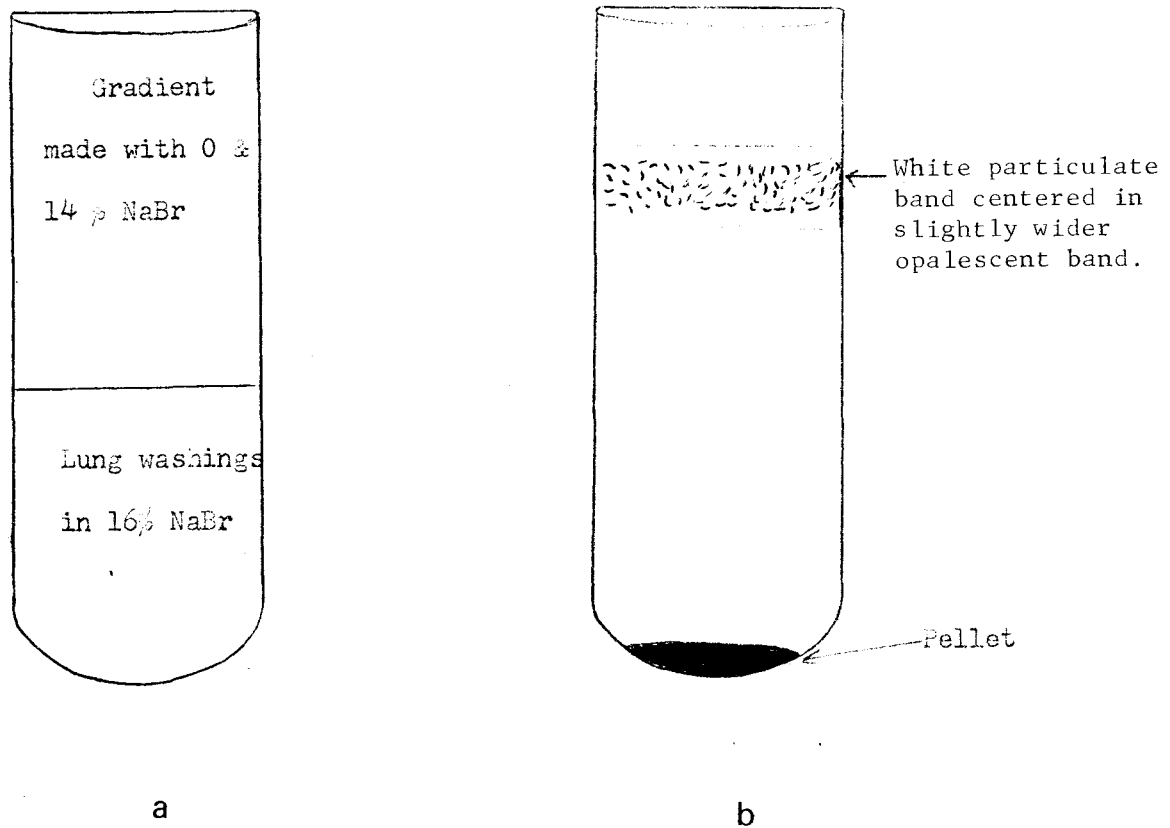


Figure 49. Gradient designed to separate lipid-associated proteins from other proteins of lung washings.

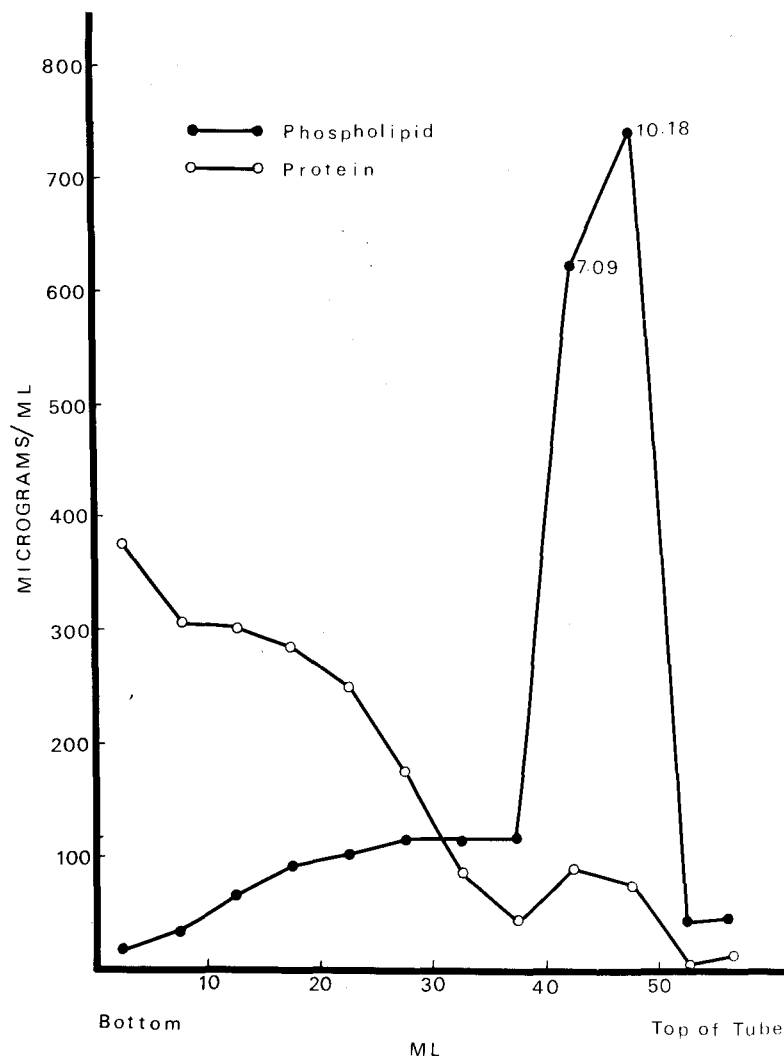


Figure 50. Distribution of phospholipid and protein from rabbit lung washings on gradient designed to separate lipid-associated proteins from other proteins of lung washings. The washings were obtained in RLS and used to prepare a NaBr gradient which was then centrifuged for 3 hours at 4° C. The number by each point represents the PL/Protein ratio of that fraction. The PL/Protein ratio of all the material in the gradient tube (excepting the cellular pellet) was 1.05, whereas the PL/Protein peak of the phospholipid peak was 8.50.

protein of the non-cellular washings were found in the particulate band. Thin layer chromatography revealed that this band also contained neutral lipids. The pellet, presumably cells and cell debris, contained large amounts of triglycerides and cholesterol and had a PL/Protein ratio of 0.53.

The proteins of the lipid-containing peak were compared with the proteins of the fractions found near the bottom of the tube by SDS-PAGE to determine whether any unique protein or proteins were associated with the phospholipids or whether this protein peak represented a non-specific association of the entire spectrum of proteins present in the washings with the lipid. The remaining portions of fractions 2, 3, 4 and 5, from this gradient were combined giving a total of 2.56 mg of protein to represent the non-lipid protein (Sample I). The remainder of fractions 9 and 10 and the particulate band from another gradient which had been prepared and centrifuged in the same way were combined giving a total value of 0.92 mg of phospholipid-associated protein (Sample II). Samples I and II were each dialyzed for two hours at room temperature against several changes of distilled water to remove the NaBr. Each sample was then lyophilized in 2 bottles.

One bottle of each sample was used directly for SDS-PAGE. Sample I was dissolved in 0.6 ml and Sample II in 0.2 ml of phosphate buffer with SDS, but without 2-mercaptoethanol, giving a concentration of approximately 2 mg of protein/ml. After incubation at 37° C for 2 hours, Sample II was still cloudy, presumably due to the presence of a high concentration of lipid. Enough solid SDS was added to this sample to achieve a clear solution. The

results of separation of the proteins of these 2 samples by SDS-PAGE are shown in Figures 51 and 52. Sample I, the non-lipid associated protein contained at least 18 bands, with the largest having the same relative mobility as albumin. However, the protein pattern was not the same as that of rabbit plasma as can be seen by comparison with Figure 53. These gels were not electrophoretically destained, which may explain why more faster moving bands were visible than in previous gradient fractions.

Sample II, the lipid-associated protein, revealed the presence of only 5 bands. Interference by the large amount of lipid present resulted in the tracking dye being streaked and the distance of migration unmeasurable. Therefore, although the major band appeared to be in the position of albumin, the relative mobility could not be calculated. Also the absence of other protein bands which were seen in Sample I might also have been due to the interference of lipid.

In order to more thoroughly investigate the proteins of Sample II, the other lyophilized aliquots of Sample I and of Sample II were treated with ethanol:ether to extract the lipid, prior to separation by SDS-PAGE. Five ml of ethanol:ether (1:3) was added to each sample at -10° C and after transfer to centrifuge tubes, the samples were kept overnight at -10° C. Some of the material of Sample I was lost because it did not resuspend well. Following centrifugation, the lipid-containing supernatant was separated from the protein-containing precipitate. The precipitates from both Samples I and II were prepared as before for SDS-PAGE, except that no additional SDS was needed to solubilize Sample II. Sample I appeared similar to the non-extracted

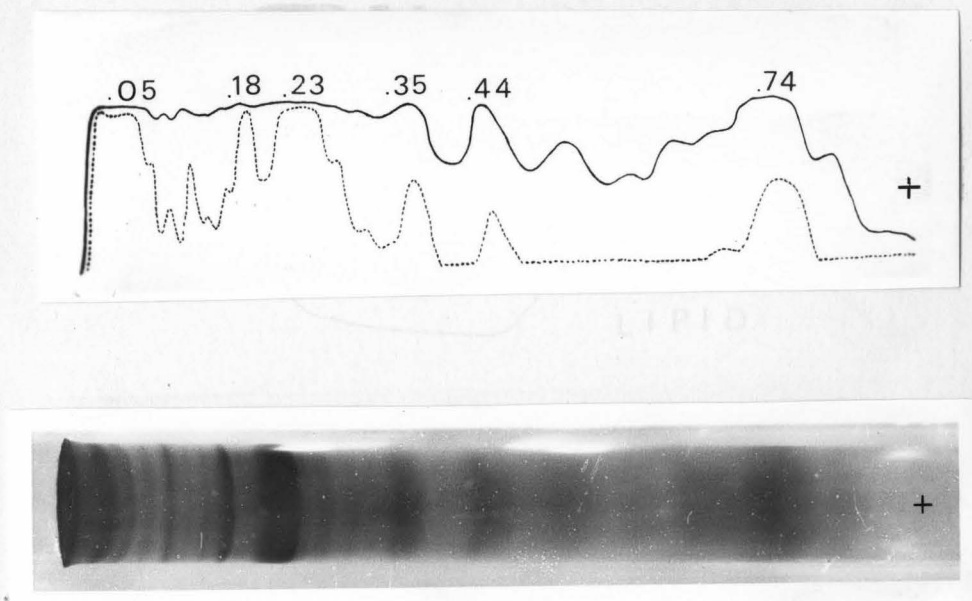


Figure 51. SDS-PAGE of Sample I from gradient designed to separate lipid-associated proteins from other proteins of the lung washings. The dotted line is a tracing obtained with the zero adjustment of the densitometer set at a lower point in order to resolve closely migrating bands. The numbers above the peaks represent the relative mobilities of those protein bands.

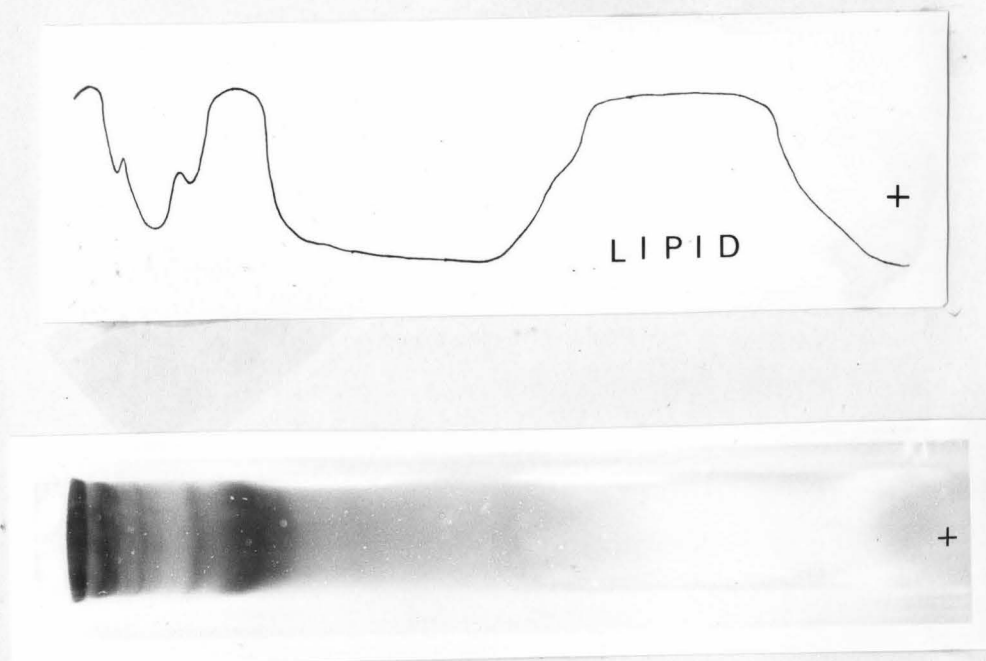


Figure 52. SDS-PAGE of Sample II from gradient designed to separate lipid-associated proteins from other proteins of the lung washings.

material except that some of the smaller bands were lost due to a loss of material during the extraction procedure. The bands of sample II, following ether-ether extraction, are shown in Figure 53. The extraction of lipids was not complete and although several bands could be seen that in the non-extracted sample, the migration could not be seen.

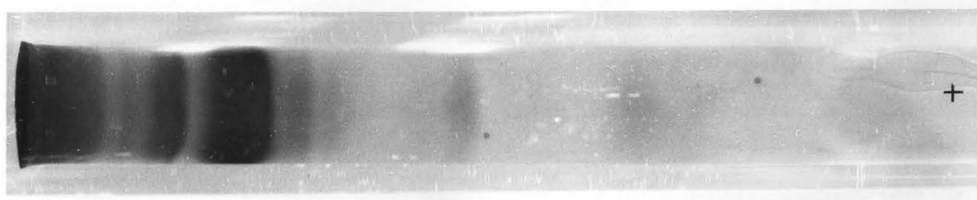
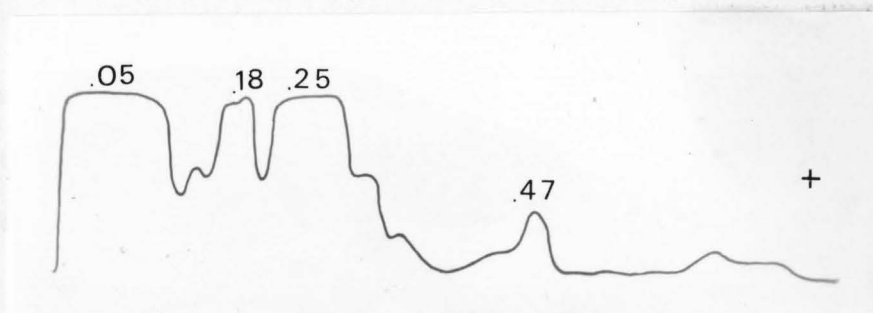


Figure 53. SDS-PAGE of rabbit plasma. The numbers above the peaks represent the relative mobilities of those protein bands.

material except that some of the smaller bands were not present, presumably due to a loss of material during the extraction procedure. The proteins of Sample II, following ethanol:ether extraction, are shown in Figure 54. The extraction of lipids was not complete and although several more protein bands could be seen than in the non-extracted sample, the migration rates could not be accurately measured. However, a comparison of Figures 51 and 54 indicates that the major components of the non-lipid associated protein and the lipid-associated protein are the same, and that the particulate band contains no unique protein detectible by SDS-PAGE.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

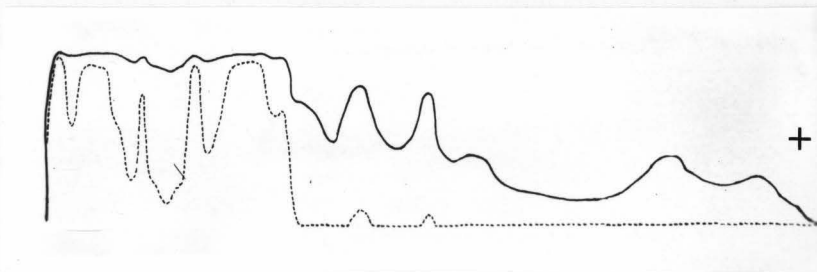
A lipoprotein is generally defined as a complex of lipid and protein

soluble in aqueous

resist separation

lung surfactant

nevertheless,



the basis of previously published results, it could be proposed that lung

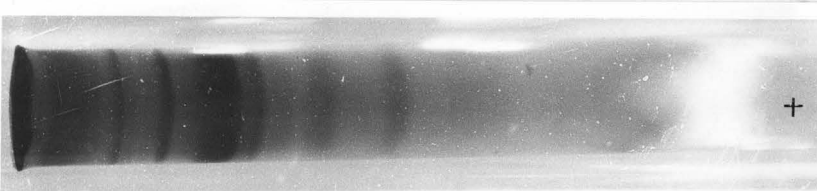
surfactant is a lipid-protein complex of some nature. The evidence for the

presence of lipid-protein complexes in lung washings comes largely from

the fact that

obtained from

the presence



mean that they are part of a specific lipid-protein complex which exists at

the alveolar surface. The results of this investigation clearly indicate

that many of the methods of centrifugation used for isolation of surfactant

"lipoproteins" are unsatisfactory in that they result in the formation

Figure 54. SDS-PAGE of ethanol:ether precipitate of Sample II from gradient designed to separate lipid-associated proteins from other proteins of the lung washings. The dotted line is a tracing obtained with the zero adjustment of the densitrometer set at a lower point in order to resolve closely migrating bands.

washings in such a way as to obtain a surface active sediment. The various

CHAPTER IV

DISCUSSION AND CONCLUSIONS

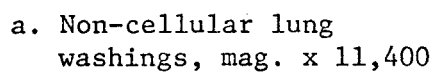
A lipoprotein is generally defined as a complex of lipid and protein, soluble in aqueous media, whose particles migrate as discrete units and resist separation into lipid and protein by physical methods (Burley, 1971). Lung surfactant is not a lipoprotein in terms of the above definition; nevertheless, lung surfactant is frequently called a lipoprotein and on the basis of previously published results, it could be proposed that lung surfactant is a lipid-protein complex of some nature. The evidence 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 several 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 which exists at the alveolar surface. The results of this investigation clearly indicate that many of the methods of centrifugation used for isolation of surfactant "lipoproteins" are unsatisfactory in that they result in the formation of artifactual lipid-protein complexes, and are thus clearly unsuitable for the separation of lung surfactant from other components of lung washings.

The first step used by most investigators in the isolation of lung surfactant "lipoproteins" is centrifugation of the non-cellular lung washings in such a way as to obtain a surface active sediment. The present

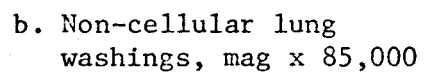
study reveals that the PL/Protein ratio of such a sediment is directly dependent upon the PL/Protein ratio of the lung washings from which it was obtained. Using healthy rabbits, a standardized technique of lung lavage and evaluation of only those washings which showed no evidence of contamination with blood, the PL/Protein ratio of the non-cellular washings varied from 1 to 3.4. Only one previously published report (Colacicco et al., 1973a) includes any data on the phospholipid and protein contents of lung washings. They found, in accordance with our results, that the phospholipid and protein contents of washings from individual rabbits varied over a wide range. The phospholipid concentration ranged from 75 to 354 mg/ml, and from about one to three times those amounts of protein were present. Most workers have not reported any characterization of the material from which the surface active sediment was obtained. The data in this investigation indicates that even under carefully controlled conditions, sediments obtained from rabbit lung washings contain phospholipid and protein in ratios varying from 3:1 to 12:1. Lung washings obtained with various lavage solutions or contaminated with blood would be expected to show even more variation. These data suggest that much of the protein found in surface active sediments is the result of a non-specific aggregation of lipid and protein.

The presence of an extracellular, alveolar lining layer with a monomolecular film of phospholipid at the air-liquid interface has been demonstrated by electron microscopy (Gil and Weibel, 1969/70) and this is in accordance with the function of lung surfactant, that is, to lower the

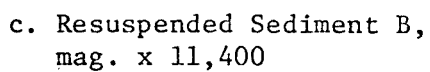
surface tension upon expiration, thereby preventing collapse of the alveoli. One might even theorize that if the phospholipid were bound in some sort of stable lipoprotein molecule, its compressibility would be hindered, and it would not be able to perform its function as efficiently. When the surface film is disrupted by lavaging the lungs with an aqueous solution, insoluble phospholipids would be expected to aggregate and form the same sort of multilamellar, bilayered structures, called liposomes, that are observed when solid phospholipid is dispersed in aqueous solutions (Bangham and Horne, 1964). The results of several experiments in this study suggest that such surfactant liposomes do exist in lung washings, and as particles of varying sizes. Ultrafiltration revealed the presence of some surface active material with a molecular weight of less than 300,000, although most of the surface activity resided in particles of larger molecular weight. The fact that more than one-half of the phospholipid of lung washings was sedimented at very low speeds also suggests that much of the material consists of large particles. However, the fact that some surface active material is sedimented only after concentration by lyophilization indicated that much smaller particles are also present. Direct confirmation of the presence of structures resembling aqueous dispersions of PC in lung washings has been accomplished by electron microscopy using negative staining techniques (unpublished data, collaborative work with Dr. J.U. Balis). As can be seen in Figure 55a, these liposomes are of varying sizes, measuring from about 100 \AA to 1000 \AA in diameter. Figure 55b shows the multilamellar, bilayered nature of these liposomes.



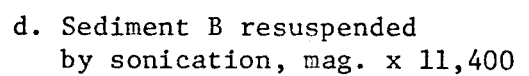
a. Non-cellular lung washings, mag. x 11,400



b. Non-cellular lung washings, mag x 85,000



c. Resuspended Sediment B, mag. x 11,400

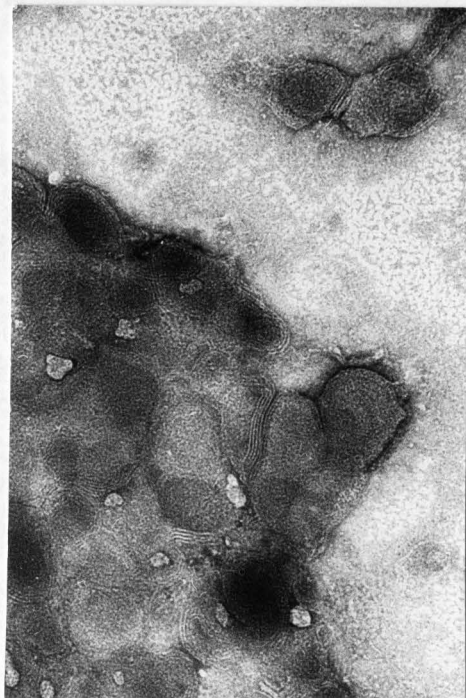
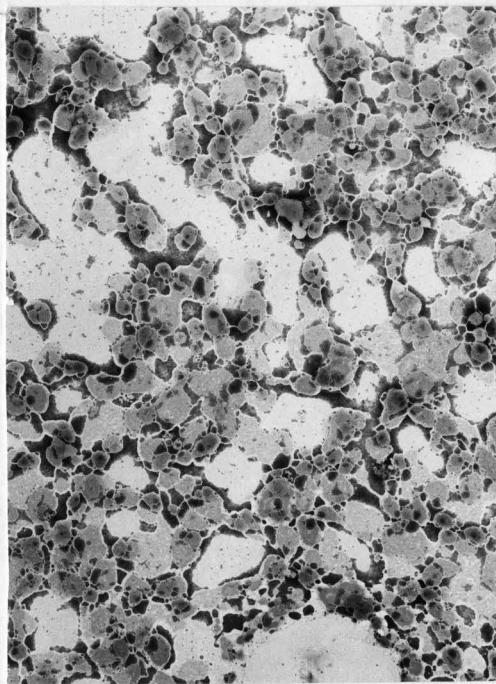
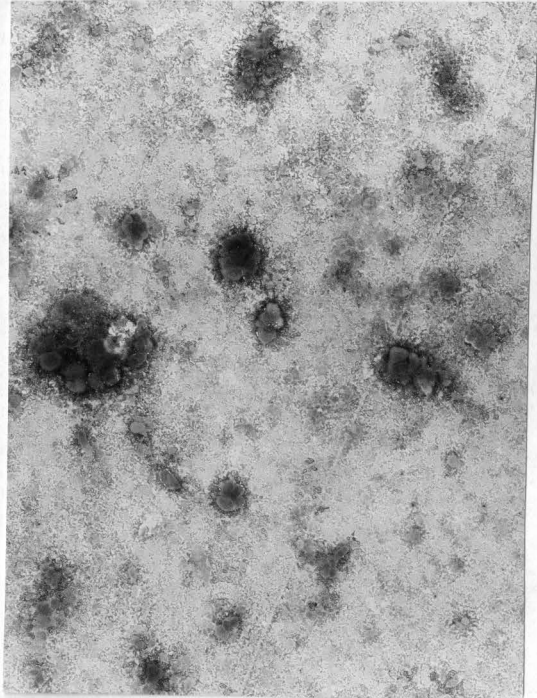
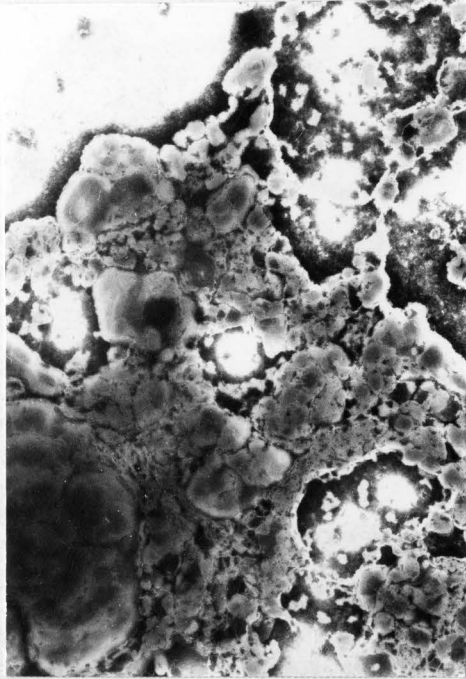


d. Sediment B resuspended by sonication, mag. x 11,400

Figure 55. Negatively stained preparations of lung surfactant.

Images for page 171

(photos are detached and out of order)



Negative staining also revealed that following sedimentation and resuspension by vigorous stirring, extremely large aggregates of these liposomes, measuring up to $10,000 \text{ \AA}$ in diameter were present (Figure 55c). When the resuspended pellets were subjected to sonication, the average particle size was reduced considerably (Figure 55d). These data agree well with the results of analytical ultracentrifugation studies of resuspended sediments in which it was observed that the flotation rate apparently depended upon the degree to which the sediment was dispersed. A sediment resuspended by vigorous stirring gave a peak with a $F_{1.21}$ of about 500. Following dispersion of the sediment by sonication, peaks with lower flotation rates were observed. Variations in average particle sizes obtained following sonication could account for the wide range of flotation rates observed in the analytical ultracentrifugation studies. The tendency of these particles to reaggregate following sonication could account for the apparent instability of some peaks observed in the analytical ultracentrifugation studies. That the amount of protein associated with the phospholipid was reduced by sonication was shown by repeating sedimentation following sonication of a resuspended pellet. The PL/Protein ratio of the pellet increased indicating that more protein than phospholipid was lost from the pellet.

Sedimentation and flotation, methods of differential centrifugation used in analytical ultracentrifugation and in some preparative methods of centrifugation, 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 sample for these gradients were large aggregates of surfactant liposomes and protein which would not readily disassociate 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. The presence of background protein may also explain the apparently inconsistent finding of Colacicco *et al.* (1973b) that a band at a lower density had a lower PL/Protein ratio than did a band at a higher density.

Density gradient centrifugation of the entire non-cellular 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 non-cellular 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. An experiment designed to eliminate the presence of background protein in the gradient by placing the sample of lung washings below the gradient also resulted in the finding of a small amount of protein in the phospholipid band.

That the protein present in the phospholipid band is not part of a specific lipoprotein molecule is indicated by the fact that the same proteins are present in both the phospholipid containing band and in other parts of the gradient. 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 that is found with the phospholipid in the density gradient is the result of a non-specific aggregation possibly formed during the lavage procedure.

Aggregates formed during the lavage procedure can be broken by sonication as was indicated by the results of density gradient centrifugation of sonicated non-cellular lung washings. As shown in Table IX, sonication caused more of the phospholipid to be found near the top of the band, that is at a lower density, indicating that sonication caused the release of some protein. However, the presence of background protein did not allow detection of changes in the PL/Protein ratios.

The possibility that specific lipoprotein complexes were present in lung washings and that they were disassociated by the methods employed in this study appears unlikely since changing the conditions of density gradient centrifugation did not change substantially the density at which the phospholipid band was located or the amount of protein associated with the band. Use of sucrose in place of NaBr demonstrated that no ionic disruption of a lipoprotein complex was caused by NaBr. The results of centrifugations at a lower temperature, and in the presence of Tris buffer indicated that no denaturation of lung washing components occurred. The only change found was temperature dependence, that is, the phospholipid band of lung washings was found at a higher density at 4° C than at 20° C. That this change was due to the temperature-dependent change in lipid density was shown by the fact that both pure DPPC and extracted surfactant lipid exhibited the same behavior.

The lack of effect of proteins on the density at which surfactant lipid-protein complexes are found may also be inferred by comparison of the densities of the various components. Proteins generally have densities of

greater than 1.3 whereas the densities of lipids are considerable lower. In these experiments, DPPC alone was found at a density of 1.07 and surfactant lipids at a density of about 1.04. The lipid-protein complexes were found at a density only slightly higher than this, 1.046. Since DPPC is the major component of lung surfactant, these results suggest that the other lipids found in lung washings are much more important in determining the density at which the phospholipid band will be located than are the proteins.

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. The finding of several proteins in lung washings that are not plasma components is of interest. The report of Dickie et al. (1973) 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. (1973). They found that in vitro, proteins stabilize PC monolayers against loss of surface activity upon repeated compression. An alternative hypothesis might be that these proteins are of importance in the transport of intracellular surfactant to the phospholipid 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 if the study of abnormal surfactant is to be undertaken. The results of this investigation indicate strongly that the PC 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 dispersion 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 not only unnecessary, but also undesirable because they result in the fractionation of surface active components on the basis of particle size and in the formation of artifactual lipid-protein complexes.

The method of density gradient centrifugation of the entire non-cellular lung washings described in this investigation overcomes shortcomings of previous methods. This method results in separation on the basis of only

the density of the components of the lung washings. The problem of background protein can be alleviated by the application of the sample below the gradient rather than spreading it throughout the gradient. This method allows recovery of both the phospholipid and protein components of lung washings and without denaturation of the proteins as occurs when the lipids are extracted with organic solvents. The phospholipid band still contains a small amount of the protein of the washings. On the basis of the data from this investigation, it is reasonable to suggest that this protein could be released by sonication and could then be separated from the phospholipid by density gradient centrifugation.

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ABSTRACT

Abstract of the dissertation entitled "CHARACTERIZATION OF LUNG SURFACTANT PROTEINS AND LIPID-PROTEIN COMPLEXES" submitted by Sue Ann Shelley in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1974, Loyola University of Chicago.

Although dipalmitoyl phosphatidylcholine (DPPC) is known to be the major surface active component of the alveolar lining layer, uncertainty exists as to whether DPPC is a part of a lipoprotein molecule or whether it is free on the alveolar surface. This 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 obtained by centrifugation were found to contain phospholipid and protein in ratios directly dependent upon the relative amounts of phospholipid and protein in the lung washings from which they were obtained. Comparison of negatively stained lung washings and sediments revealed that sedimentation of lung surfactant caused extensive aggregation of surfactant "liposomes." These aggregates were not readily broken, but following sonication, soluble protein was released and the particle size was reduced.

Analytical ultracentrifugation of lung washing fractions prepared by various techniques revealed the presence of components with flotation rates ($F_{1.21}$) ranging from 25 to 400. However, suspensions of pure phosphatidylcholine were observed to have components with similar flotation rates.

Density gradient centrifugation of the non-cellular lung washings, without prior concentration by sedimentation, revealed the presence of a visible band which contained most of the phospholipid of the washings. The density at which this band was located depended upon the temperature at which centrifugation was carried out. Only a small amount of the proteins of the washings was found in the phospholipid band. Comparison by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins of the phospholipid band with the proteins found in other parts of the gradient revealed the presence of the same proteins in all fractions. Albumin, IgG and IgM, identified by immunoelectrophoresis, were among the proteins found. Small quantities of several non-plasma proteins were also present.

These results indicate that there is little, if any, protein specifically attached to the phospholipids of lung surfactant; rather, the properties of lung washings are very similar to those of DPPC in aqueous suspensions. The presence of both lipid and protein in fractions obtained by centrifugation appears to be the result of a non-specific association caused by the techniques of removing the water-insoluble surfactant from the lung and promoted by sedimentation. With this understanding of the nature of lung surfactant, it was possible to design a relatively simple method of density gradient centrifugation in which the distinct components of the lung surfactant system were separated. This method promises to be useful in the further study of normal surfactant, as well as in the study of surfactant in various pathologic conditions of the lung.

Approval Sheet

The dissertation submitted by Sue Ann Shelley has been read and approved by six members of a committee appointed by the Dean of the Graduate School of Loyola University of Chicago.

The final copies have been examined and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to form, content, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 15, 1974

Date

Maurice V. P. Heeneey

Signature of Advisor