Interaction of Lung Surfactant Lipids with Proteins of the Alveolar Space

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INTERACTION OF LUNG SURFACTANT LIPIDS WITH PROTEINS
OF THE ALVEOLAR SPACE

by

PETER PAGAST

A Dissertation Submitted to the Faculty of the Graduate School of
Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

June
1975
ACKNOWLEDGMENTS

The author is indebted to Dr. Richard Schultz for his ideas, guidance and direction during his research work. The author wishes to thank Dr. John U. Balis for the privilege of being part of his research team; for his guidance, his advice, his ideas, his encouragement and especially, his patience. The author wishes also to express his gratitude to other staff members and students of the Departments of Biochemistry and Biophysics and Pathology, with special thanks to Dr. Mary Manteuffel for her helpful guidance in writing this thesis. The author thanks his family for their tolerance and understanding throughout this work.

Finally, the author wishes to dedicate this thesis and any publication derived from it to his father, the late Dr. Felix Pagast.
Peter Pagast was born in Königsberg, Germany, on April 7, 1940. He graduated from Oberrealschule and Gymnasium, Miltenberg, Germany, in June, 1961, and received a "Vordiplom," the equivalent of a Bachelor's degree, in biology from the University of Freiburg, Germany, in February, 1965.

In December, 1965, he came to the U. S. A. and worked for four years for several chemical companies, while attending night school in chemistry at Northwestern University.


In September, 1969, he joined the Department of Pathology, Loyola University Stritch School of Medicine as a Research Assistant. As part of Dr. John U. Balis' research team from the Department of Pathology and in conjunction with Dr. Steven Keresztes Nagy, Peter Pagast began graduate study in the Department of Biochemistry and Biophysics, Loyola University Stritch School of Medicine. A Master of Science degree was awarded in January, 1972, following completion of his thesis on the "Biochemical Maturation Changes in Developing Lung Surfactant in Rabbits."
ABSTRACT

Abstract of the dissertation entitled "INTERACTIONS OF LUNG SURFACTANT LIPIDS WITH PROTEINS OF THE ALVEOLAR SPACE" submitted by Peter Pagast in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1975.

Dipalmitoyl phosphatidyl choline (DPC) is the major component of extracellular pulmonary surfactant. Model studies show it to be the primary constituent of surfactant responsible for the lowering of the surface tension in the alveolar air liquid inter-phase, which is essential for the respiratory process. There is little information as to the biochemical or physiological function of other lipids and the proteins found in the pulmonary surfactant. However, on isolation of pulmonary surfactant by the procedure of Frosolono et al. (J. Lipid Res. 11, 439-457, 1970), it is shown to consist of lipid-protein complexes with a consistent phospholipid to protein ratio. This work studies the composition, the physical, and the spectral properties of native and artificial model phospholipid-protein complexes for pulmonary surfactant.

Dog alveolar surfactant was isolated and separated into its constituent lipid and protein components by partition chromatography over Sephadex LH-20 in 2-chloroethanol-water. A series of
experiments resulting in reconstitution of surfactant lipid-protein complexes were performed to investigate the interaction of surfactant lipids with surfactant proteins. Reconstitution of the lipid and protein of the surfactant fraction of dog resulted in the formation of lipid-protein complexes which compared favorably with the original isolated surfactant complexes. These complexes were characterized by certain well-defined properties including their density, particle size, phospholipid to protein ratio, protein composition and circular dichroism.

Reconstitution experiments were performed substituting delipidated albumin and delipidated dog serum proteins in place of surfactant proteins. Also, reconstitution experiments were performed utilizing commercial phosphatidylcholine in place of surfactant lipids. In all these experiments only a small yield of lipid-protein complexes were found, and the complex that did form had different physical and spectral properties from the reconstituted complex utilizing pulmonary surfactant lipids and proteins. When pulmonary extracellular proteins other than found in the pulmonary surfactant fraction were utilized in place of surfactant proteins in reconstitution experiments, the yield of phospholipid-protein complexes was high. However, the density of the complexes were diffuse and dissimilar from that of native complexes.

The \( \alpha \)-helix content of the reconstituted complexes in SDS was higher than that of the delipidated apoprotein. A similar increase in \( \alpha \)-helix content was not observed following
reconstitution of surfactant lipids with delipidated protein from surface inactive fractions of lung washing, serum, or bovine albumin. Likewise, reconstitution of commercial phosphatidylcholine with surfactant protein was not associated with increased \( \alpha \)-helix content of the formed complex. In a discontinuous gradient, the reconstituted lipid-protein complexes of the surfactant fraction appeared as a single band on a sucrose gradient, while complexes of surfactant lipids with delipidated dog serum proteins or extracellular proteins of the lung other than found in the pulmonary surfactant fraction displayed a wide range of distributions on a sucrose gradient.

Negatively stained preparations examined by electron microscopy demonstrated that the original surfactant fractions (obtained by a procedure involving sedimentation) contained large aggregates of liposomes widely varying in size and more uniformly distributed. The liposomes of reconstituted complexes were similar in size and distribution to those found in lung washing.

SDS-polyacrylamide gel electrophoresis showed that the protein composition of surfactant fraction was similar to that of serum, but two additional bands with approximate molecular weights of 11,000 and 35,000 were only seen in proteins of the surfactant fraction, and comprised 15 per cent of the surfactant proteins. The distribution of the individual proteins comprising the surfactant protein fraction did not change on reconstitution. Analysis of the protein in originally isolated and reconstituted
surfactant showed proteins of molecular weight approximately 11,000, 35,000, 66,000 and 150,000, respectively. It appears that the two low molecular weight proteins were essential for the observed characteristic properties of the surfactant lipid-protein complexes.

The results suggest that the in vitro model employed in this study is suitable to evaluate lipid-protein interactions which may occur following close contact of various proteins and lipid constituents present in the alveolar space in normal and pathologic conditions of the lungs.
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In a classic paper in 1929, the Swiss physiologist, Karl Von Neergard (1), was the first to describe the action of surface forces in the function of the lung. He recorded static volume-pressure curves on excised mammalian lungs during deflation with air and after removal of the air with a liquid solution of gum arabic. When the lung was filled with gum arabic solution, the surface forces at the alveolar-air interface were eliminated. The difference between the two static volume-pressure curves gave Von Neergard the size of the retractive forces which were due only to interfacial forces. The results of these experiments showed that 1/2 to 3/4 of the retractive pressure of mammalian lung is due to surface forces. Neergard claimed that the surface tension of the alveoli was lower than that of other physiological fluids and felt that surface-active material at the alveolar-air interface was responsible for this phenomenon.

In several papers since 1929, the significance of the surface properties of the lung were described. It was not until 1955 that Pattle realized the full significance of the alveolar lining fluid
in mammalian lung (2). In a classic paper he demonstrated that the bubbles present in foam obtained from rabbit lungs are stable because they contain a surface-active material.

Pattle obtained foam by producing acute pulmonary edema in animals and by washing these lungs *in vivo* and *in vitro* with saline solution. The bubbles which made up this foam had certain characteristic properties.

1. They were highly resistant to antifoaming agents.
2. The bubble sizes were extremely stable when placed in an air-saturated medium.

From these properties, Pattle felt that the lining film of the bubbles either were characterized by extremely low surface tension or were highly impermeable to air. By placing this foamy material into air-free water, Pattle further demonstrated that bubbles rapidly dissolved and, therefore, were not impermeable to air. Pattle concluded from these findings that the bubbles were lined by a strong surface-active film which lowered the surface tension of the bubbles to zero. He further suggested that the lining film of the bubbles originated from the lung alveoli which presumably were coated with a surface-active material, similar to that of the bubbles.

In 1957, Clements confirmed Pattle's statements and made further important observations: lung extracts which he obtained by rinsing the lung with 0.9% NaCl were tested for surface tension
with a modified-Wilhelmy surface balance (3). Tension was measured during the cyclic compression and expansion of the surface film. During compression and expansion of the film, a wide separation of the compression isotherms, which he called "surface hysteresis loop," was noted. An analogous situation was known to exist in excised lung specimens which characteristically show a hysteresis loop following lung deflation. The static volume pressure behavior of an air-filled lung is due to tissue elastic and surface tension forces. The findings of Pattle and Clements suggested that during expiration, the surface tension is abolished due to the surface activity of a lung surfactant and, therefore, the normal lung is capable of retaining air at low inflational pressure resulting in a characteristic hysteresis loop.

Hyaline membrane disease of the newborn was the first major disorder which was attributed to an underlying surfactant defect. Using the method of Clements, Avery and Mead, in 1959, demonstrated that lung extracts from newborn infants with hyaline membrane disease lacked significant surfactant activity (4).

The first strong evidence for the existence of an extracellular lining was reported by Macklin in 1955 (15). He claimed that the alveoli are lined by an exterior alveolar mucoid fluid, containing polysaccharide, but he disregarded his observation of specific lipid containing myeloid figures. Early electron microscopic studies demonstrated that the alveolar air-blood barrier
of the lung is composed of three layers: the capillary endothelium, the alveolar epithelium, and enclosed, a narrow interstitial space. From a series of morphological investigations, which have been recently reviewed by Weibel, there is now convincing evidence that a non-cellular layer coats the epithelial lining of the alveoli (6). This non-cellular lining layer is composed of a liquid base layer (hypophase) and a 40 Å-thick monomolecular surface layer. The hypophase is believed to contain proteins as well as lipids which can be seen in form of highly structured osmiophilic myelin figures. These myeloid figures contain osmiophilic lamellae which, like phospholipids, show a repeating distance of 40 Å. There is also evidence that the monomolecular surface film originates from these myelin figures.

The alveolar epithelium is composed of two types of cells: a squamous cell with thin cytoplasmic extension (membranous pneumocyte or type I cell) which lines the largest portion of the alveolar surface and the granular pneumocyte or type II cell which periodically interrupts the continuity of the type I cells. From various lines of evidence, it is generally believed that these type II cells synthesize surfactant or precursors of the surfactant which is stored in the inclusion bodies of these cells and eventually secreted into the air space (hypophase) and forms the surface film of the alveoli.

Pattle and Thomas, in 1961, suggested that the surface-active lining in the alveolar space is composed of lipoprotein
complexes (7). They found that infrared spectra of a mixture of purified egg lecithin with gelatin was qualitatively the same as that with dried lung washing. The conclusion of their investigation was that 1% of the surfactant forming the lining of bubbles is composed of proteins; the rest are lipids.

Buckingham et al., in 1961, did a screening study on surfactant obtained from sheep lung extracts (8). Based on diverse results from staining reactions, nitrogen content and solubility, they suggested the surface-active material is a lipoprotein composed of phospholipids and protein.

The first extensive composition study by Klaus, Clements and Havel, in 1961, indicated that surfactant powder obtained from beef lung washing was composed of 50-70 per cent lipid and 5 per cent nitrogen (9). Separation of the lipid fractions revealed 74 per cent phospholipids, 8 per cent cholesterol, 10 per cent triglycerides and 8 per cent fatty acids. Surface tension study on a modified-Wilhelmy balance suggested that the fractions containing cholesterol triglycerides and fatty acids alone did not show any unusual surface activity. The phospholipids, however, revealed high surface activity.

In 1962, Brown first suggested that lecithin was the main component of surfactant and in 1964, he further characterized the lecithin as dipalmitoyl phosphatidylcholine (10). Numerous investigations on the surfactant composition, especially their
phospholipids, followed and the overall conclusion was that
dipalmitoyl phosphatidylcholine is the most surface-active and
major component of the surfactant.

Fujiwara and Enhorning, in 1964, examined the phospholipids
of the surfactant-rich extracts from total lung tissue and washing
from several species (11, 12, 13). They found that all extracts
contained lecithin, sphingomyelin and phosphatidylethanolamine
and that the lecithin content was two to three times as high as
the other two components combined.

Morgan et al., in 1965, reported a detailed survey on
pulmonary phospholipids obtained from washing of living dogs(14).
He separated the phospholipids from the total lipids and the
individual phospholipids were separated by thin layer chromatog-
raphy. The surface activity and the fatty acids of each phospho-
lipid were determined. His findings showed that the lipids of
extracts obtained by alveolar lavage, which apparently contained
primarily material lining the alveoli, represented a relatively
small fraction of the total lipids of the lung. The composition
of whole lung lipids were similar to those of other body tissues,
whereas the lipids from lung washing were quite different. Thus,
extracts from homogenized lung contained proportionally more
cholesterol and less lecithin than the washings. The fatty acid
residues of the lecithin obtained from washing were 78% saturated
(66% palmitic acid), whereas the per cent of saturated fatty acids
in lecithin from homogenized lung was only 50%. Moreover,
lecithin isolated from the whole lung homogenate showed less surface activity than highly saturated lecithin recovered from washings.

Morgan et al. reported that lung washings and whole lung homogenates contain phosphatidyl-N,N-dimethylethanolamine. He speculated that the presence of this phospholipid indicated that besides the main pathway of lecithin synthesis from the incorporation of cytidine diphosphate choline and diglycerides (Diglyceride + CDP-choline \(\rightarrow\) lecithin + CMP), a conversion of phosphatidylethanolamine to phosphatidylcholine by transmethylation may occur: Phosphatidyl ethanolamine +CH\(_3\) \(\rightarrow\) phosphatidyl monoethyl ethanolamine +CH\(_3\) \(\rightarrow\) phosphatidyl dimethyl ethanolamine +CH\(_3\) \(\rightarrow\) lecithin). Morgan also suggested that under conditions of hypoperfusion or hypoxia, the N-methyl-transferase pathway may become important for the dipalmitoyl phosphatidylcholine. Thus, during fetal development, the low uterine \(P_02\) may give the methyl transferase lecithin synthesis relative preponderance over the CDP-choline pathway.

Gluck investigated the different pathways for the synthesis of lecithin in the developing fetal rabbit in vitro (15). Lung homogenates were incubated with (a) CDP-(1, 2-\(^{14}\)C) choline for CDP choline pathway (CDP-choline + D- -diglyceride \(\rightarrow\) lecithin) and (b) (\(^{14}\)CH\(_3\))-S-adenosyl-L-methionine for the transmethylation pathway (phosphatidylethanolamine + 3 (CH\(_3\)))-S-adenosyl-L-methionine \(\rightarrow\) lecithin). All incubation procedures
were stopped after one hour and the lipids were extracted and chromatographed on DEAE cellulose acetate. The individual phospholipids were separated on thin layer plates. Gluck's data obtained from these studies indicated that the same pathways which are active for the de novo synthesis of lecithin in adult lung are also active in the fetal lung of rabbits. The CDP-choline pathway was overall the most active during fetal development although a strong rate of transmethylation at 28-29 days of gestation (full term 31 days gestation) from phosphatidylethanolamine to lecithin was found. Gluck et al., in 1972, (16) in a study on pulmonary lecithin synthesis in the human fetus and newborn and the etiology of the respiratory distress syndrome came to the following conclusions, which were primarily based on PDME (phosphatidyl-dimethyl-ethanolamine) variations in the surfactant evaluation: In human fetal surfactant synthesis, there are two major pathways for surfactant synthesis.

1. CDP-choline + D,α, β-diglyceride $\rightarrow$ α-palmitic/α-palmitic lecithin. There is very little activity of this pathway until the 36-37th week of gestation

2. Phosphatidyl ethanolamine + 3 CH$_3$ $\rightarrow$ α-palmitic/β-myristic lecithin.

Gluck et al. suggested that the second pathway is the major pathway as early as 22 weeks and can sustain alveolar stability alone until the other pathway matures. They further claimed that PDME
is absent in aspiration of infants with hyaline membrane disease suggesting that the premature newborn is strongly dependent on this pathway. They also claimed that the effect of various therapies could be assessed by this way.

Body et al., in 1971, isolated from pig surfactant phosphatidylglycerol but no PDME (17). Since phosphatidylglycerol and PDME have similar chromatographic properties, Body suggested that when only TLC is used for the identification of the lipids, the investigators who claim to have found PDME in lung could have mistaken phosphatidylglycerol for PDME. Pfleger et al., in 1972, criticized parameters used by many pulmonary surfactant investigators for the identification of phospholipids in surfactant material and suggested that in addition to TLC of the total surfactant lipids, other methods for the analysis of phospholipids should be used (18). Phosphatidyl glycerol was isolated from normal Beagle dogs but no PDME besides the regular surfactant lipids. The techniques for the characterization of the phospholipids isolated from Beagle lung surfactants included selective hydrolysis (enzymatic and mild alkaline), TLC separation and identification of hydrolyzate constituents, quantitative chemical analysis of glycerol and phosphorus, TLC of derivatives of both the lipids and polar head group constituents, and infrared spectral analysis of the deacylated phospholipids. In a recent study, Hallman and Gluck actually retracted their original findings relating to PDME in lung surfactant (19). In this
investigation, in addition to TLC, chemical analysis of the separated compounds were undertaken and in addition to usual surfactant phospholipids, only phosphatidylglycerol but not PDME were identified.

From the above studies it is apparent that there is still no significant information as to the precise role (if any) of methylation pathway in normal and altered surfactant biosynthesis.

Young and Tierney, in 1972, presented an investigation on dipalmitoyl lecithin secretion and metabolism by the rat lung (20). The study was undertaken since there was evidence that phosphatidylcholine (DPL) is an essential component of the surface active material which was based on the following:

1. Of the several phospholipids found in surfactant material, which can reduce surface activity below 10 dynes/cm, only DPL is in sufficient quantity present to form a continuous layer in the pulmonary alveoli.

2. Phospholipase C, which cleaves lecithin, raised the surface tension in surfactant extracts to such an amount that the normal surface-pressure curves could not be obtained. It was also shown that there is a good correlation between the amount of DPL in fetal lamb lung and their surface activity of lung extract from fetal lamb.
In order to study the secretion and metabolism of DPL, Young and Tierney injected rats with 10 µc of palmitate-1-C\textsuperscript{14} into the tail veins. At intervals of 1 to 72 hours after injection, rats were killed. The lungs were thoroughly washed and the washings of each animal were pooled separately. The washed lungs were then homogenized. From both the extracellular washings and the lung tissues the lipids were extracted using the Folch method. The different lipid classes were separated and the specific activity in the DPL from the lung tissues and the extracellular washings was determined.

The above investigations indicated that the rat lung rapidly esterify radio-labeled palmitate into neutral lipids and phospholipids and about one-third of the radioactivity appears in dipalmitoyl-lecithin one hour after injection. This radio-labeled DPL is not secreted immediately, but appears in increasing amounts on the alveolar surface over the next 8 hours. The relative pool size of DPL in the lung and on the surface is in the ratio of 7:1. Of the radioactivity in DPL which is lost from the lung tissue between 1 and 8 hours after the injection of palmitate-C\textsuperscript{14}, at least one-third appears in the lavage fluid. Therefore, a significant fraction of the newly synthesized DPL is secreted onto the surface. The relationship of the specific activities of DPL in the lung and lavage fluid does not follow the precursor-product relationship expected for a single pool of the precursor within the lung. All pulmonary DPL may be in the surface-active material,
but the possibility exists that it may also be in other components of the lung, such as membranes. From these findings it seems clear that metabolic studies of surfactant DPL using precursor of DPL may be misleading for an estimation of the metabolism of surface active material.

Realizing the difficulties of using the total dipalmitoylcholine from lung tissue or washing for the estimation of lung surfactant, King et al., in 1973, explored the possibility whether the intracellular and alveolar surfactant contains surfactant specified lipids (other than DPL) to be used as a marker for surfactant determination (21). Surface active material was obtained by lavage of adult dog lung, newborn puppies and dog fetuses and by homogenization of the whole lungs using procedures developed previously. In order to estimate the loss of surface active material (SAM) during the isolation procedure, labeled dipalmitoyl phosphatidylcholine (DPC-$^{14}$) was added to the unfractionated dog lung lavages and dog lung homogenates. Assuming that the labeled DPC was distributed in the same way as the DPC of SAM, they estimated the loss of surface active material during the isolation procedure from the recovery of the isotope in the isolated surfactant material. The lipids were extracted by standard procedures and the structures of the isolated lipids, especially the phosphatidylcholine, were identified by GLC.

The results indicated that a special phosphatidylcholine containing Cis-5-ostadecenoic acid was isolated in the purified
SAM. King et al. further found in the analysis of the different isolated surfactant materials that Cis-5-18:1 phosphatidylcholine made up 6.6% of the total SAM, but only traces in total lung. Since phosphatidylcholine exists besides in SAM as well as in other components, this special phosphatidylcholine could be used for the estimation of surface active material in lung. King et al. however, pointed out that since this special phosphatidylcholine is found only in such small quantities in the total lung, the measurement of this lipid requires the techniques of micro-lipid chemistry. In addition, up to now, this special phosphatidylcholine was only found in dog lung. Therefore, the use of Cis-5-18:1 phosphatidylcholine as a parameter of SAM is of limiting value in the measurement of surfactant material and can only be used in research experiments but cannot be adapted for the clinical assay of human lung surfactant.

There is a controversy regarding the exact composition of the lung surfactant. One group has suggested that surfactant is mainly a mixture of phospholipid and is free of proteins (22, 23). Other investigators maintain that surfactant is composed of phospholipids and proteins in relatively constant proportions (24, 25, 26, 27, 28, 29).

In 1966, Abrams reported that the surface active material is a lipoprotein (24). Lung tissue from rabbits and from infants who died from hyaline membrane disease was homogenized in 0.9% sodium chloride solution, and subjected to differential
centrifugation. The homogenate was first centrifuged at 300 x g for 10 minutes. The supernatant was centrifuged for 60 minutes at 1000 x g. The resulting precipitate was suspended in 1.15 specific gravity sodium chloride and centrifuged at 1,500 x g for 25 minutes. A floating pellicle was obtained and dialyzed against 0.9% sodium chloride solution. Abrams describes this surface active material as a lipoprotein consisting of 40% lipids and 60% protein. The lipids were comprised of 70% phospholipid, 10% fatty acids, 14% triglycerides, 6% free cholesterol and less than 0.5% cholesterol ester. Six times more phosphatidyl choline than phosphatidylethanolamine were found and gas liquid chromatography showed that about 75% of the fatty acids in the lecithin was palmitic acid. Agar electrophoresis indicated that the lipoprotein had the mobility of \( \gamma \)-globulin. Abrams lipoprotein contained a high percentage of protein (60%) and a low percentage of lipids (40%).

These results led Scarpelli to believe that Abrams' proteins in the lipoprotein was comprised predominantly of serum contaminants (22). Scarpelli stated that lung phospholipids are not a part of a lipoprotein, but may be associated with carbohydrates and other lipids. Scarpelli et al. perfused the pulmonary vasculature with isotonic NaCl until the dog lung lobes appeared white. They minced these lobes and suspended the material in 0.9% NaCl, The suspension was filtered through gauze and centrifuged at 3,000 x g for 10 minutes. The centrifugation was repeated with
the supernatant at 39,000 x g for 20 minutes. Ten milliliters of the last supernatant and aliquots of tracheal dog lung washing were applied on a Sephadex G-200 column. The column effluents were pooled into four fractions and each fraction was dialyzed against distilled water. Lipids were extracted with chloroform/methanol (2:1 v/v) and analyzed. Aliquots of the fractions in distilled water were lyophilized and analyzed for proteins by disc electrophoresis. The fraction which contained most of the phospholipids had a negligible protein content, (phospholipid to protein ratio = 65/1). It should be noted that the method used by Scarpelli is unsatisfactory in that surfactant readily sediments by centrifugation and therefore the supernatant fractions that were used for analysis were actually sharply depleted of surface active material. One has also to consider the possibility that the lipid and protein moieties of the lung surfactant were separated during the passage through Sephadex G-200. Scarpelli et al. felt that this was not the case since there was no significant phospholipid or protein adsorption on to the gel.

In contrast to the results of Scarpelli, the investigation of Wetton et al. suggested that surface-active material is confined to cellular material (30). Dog lung washings obtained from dogs under sedation were centrifuged at 2,000 x g for a period of 10-30 minutes. A white cellular sediment was obtained and a cell-free supernatant. Surface tension measurements seemed to indicate that there had to be a minimum of the sedimental cells
present in order to obtain good surface tension measurements. The supernatant, even after concentration to a small volume by vacuum distillation did not show any surface activity.

Cavagna et al., in 1967, further investigated the work of Wetton et al. and suggested that surface-active material is adsorbed to sedimented cells, possibly during the centrifugation procedure (31). In their investigation, opalescent white dog lung washing was sedimented at 2,000 x g for 10 minutes. The sediment was resuspended by shaking until the sediment was no longer visible. This was done in order to desorb any surface-active material from the surface of the cells. The suspension was centrifuged again at 2,000 x g. After this centrifugation step, the surface-active material remained in the supernatant and the cellular sediment no longer had any surface activity.

Said et al., in 1968, came to a similar conclusion (32). They obtained by sedimentation centrifugation a white cellular surface-active material. The continued washing followed by centrifugation of the cellular material removed adsorbed surfactant. Said et al. speculated on the possibility of a similar phenomenon of surfactant adsorption to the large alveolar cells in situ.

While the sedimentation method first described by Abrams is frequently still used, most investigators apply several steps of centrifugation in buffer media or on continuous, discontinuous salt or sucrose gradient for the recovery of pulmonary surfactant.
Klein, in 1968, points out the need for the development of methods which allow us to isolate surfactant as it is in situ, before pulmonary surfactant can be analyzed and before its significance is understood (25). Results of his study gave evidence for the lipoprotein nature of surfactant. Phospholipids and proteins were present in all fractions which had surface activity. Efforts to remove the proteins by repeated suspension and centrifugation were unsuccessful.

Finely et al., in 1968, used endobronchial isotonic saline washings for the isolation of strong surface-active material (33). The washing fluid was subjected to centrifugation at 27,000 x g for 20 minutes. A clear supernatant solution and a sediment consisting of a lower brown layer of cells and an upper white acellular layer was obtained. Electronmicroscopic studies suggested that there was a striking similarity between the material of the white layer and the alveolar material in situ. Lecithin, containing primarily palmitic acid, comprised more than 50 per cent of the lipids.

In an attempt to isolate lung surfactant, Steim et al., in 1969, used differential and linear sucrose gradient separation techniques in application to extracellular lung washing from dogs (23). The cellular debris was removed by centrifugation of the washing for 15 minutes at 600 x g. The obtained pellet had 79% phosphorus and only 2% protein of the original washing. A rather large amount of this material was necessary to obtain
sufficient surface activity on a surface balance. The pellet was dispersed in saline to a flocculent suspension, dialyzed against 0.02 M Tris-EDTA at pH 8.0 and then against distilled water for 24 hours. The material was layered over a continuous sucrose gradient with a density range of 1.000 - 1.100 and centrifuged at 4° C for 20 hours at 100,000 x g. A white band was visible at the density 1.035 and showed high surface activity.

Steim et al. developed a modified procedure based on this equilibrium centrifugation. A dialyzed pellet, obtained after centrifugation at 16,000 x g was layered on sucrose (density 1.010) and centrifuged at 100,000 x g for one hour. The sedimented material was resuspended in buffer and layered on a sucrose solution of density 1.055 and recentrifuged. The last separation step was repeated several times and after each step a yellowish protein-rich pellet remained at the bottom. The complete surface activity was still unaltered after the last remaining 3% proteins associated with this surfactant was removed by organic solvents. Although Steim suggested that the proteins were contaminants, he points out that the proteins could have a modifying effect in the alveoli, where proteins, mucopolysaccharides and salts are found under normal conditions.

Galdston et al., in 1969, described an isolation procedure of surfactant which includes several centrifugation steps (26). Rabbit lung washing with isotonic saline was centrifuged at 1.650 x g. A supernatant fluid and a fluffy white material on
top of a pelleted, packed cell layer was carefully removed and centrifuged at 50,000 x g for one hour. The sediment was resuspended in citrate-phosphate buffer and centrifuged for another hour at 50,000 x g. A surface active sediment was obtained which was composed of 65% lipid and 36% protein. Ninety per cent of the lipid consisted of phospholipids. Proteins of the surfactant were investigated by disc electrophoresis and immunologic methods and Galdston et al. claimed they were different from serum components. Galdston et al. concluded from these results, the proteins in the surfactant lipoprotein originate in the lung.

Using a similar procedure for the isolation of surfactant from rabbit lung, Balis et al., in 1971, found slightly different results (34). After cells were removed at 450 x g for 5 minutes, the supernatant was subjected to centrifugation for 90 minutes, at 100,000 x g. The obtained sediment was highly surface-active and had a phospholipid-to-protein ratio of 9/1. More than 80% of the phospholipid found in the extracellular lung washings were isolated in the surfactant fraction. Two-dimensional chromatography revealed that phosphatidylcholine comprised 90% of the total phospholipids. Seventy-five per cent of the total lipids present in the bulk solution was isolated in the surfactant fraction. The protein content remained constant with additional centrifugation. Electron microscopic studies showed that the granular material in the surfactant fraction was composed of osmiophilic myeloid figures.
For several years, Scarpelli and co-workers maintained that surfactant was free of protein. Because the concept that surfactant is a lipoprotein or lipid-protein complex was shared by many other investigators, Scarpelli et al., in 1970, undertook a series of experiments to clarify the lipoprotein character of surfactant (35).

Surfactant material was isolated by three different methods: Unperfused pulmonary tissue was minced; unperfused lung airways were washed; pulmonary washings were centrifuged and a white cellular precipitate was recovered. Studies of proteins separated by disc electrophoresis suggested that the proteins in pulmonary extract are not associated with lipid in a form of lipoprotein. A surface-active pellet, recovered by centrifugation of lung washing, was dispersed in saline. Analysis of this material by disc gel electrophoresis showed that between ninety and one-hundred per cent of the lipids remained in the spacer gel. Six bands comprised the majority of the protein. From these results, Scarpelli et al. suggested that if there were lipoproteins present, they must be fragile and thus broken up of their lipid and protein components by electrophoresis.

Clements, in 1970, discussed the semantic difficulty of describing pulmonary surface-active material (27). He referred to complexes of lipids and proteins, which exist under physiologic circumstances, despite the fact that these complexes might dissociate under certain conditions, used during their isolation.
Material obtained by differential centrifugation always contained protein, were rich in dipalmitoyl lecithin and were devoid of polysaccharides. Further purification under more vigorous conditions, such as using chelating agents and ultracentrifugation, seemed to break up any lipid-protein association.

Frosolono et al., in 1970, by differential centrifugation of continuous and discontinuous sucrose gradients, isolated and characterized surface-active material from dog lung (28). Dog lung homogenates in Tris buffer (pH 7.4), containing EDTA and NaCl were filtered through nylon gauze and layered over 0.75 M sucrose. The suspension was centrifuged for 40 minutes at 48,000 x g. The material on top of the sucrose layer was carefully removed. The washed material was layered over a discontinuous gradient consisting of 12 ml of 0.68 M and 12 ml of 0.25 M sucrose. After centrifugation for one hour, two interfaces were visible: One was on top of 0.25 M sucrose; the other floated on 0.68 M sucrose. The interfaces were removed and resedimented in buffer. Most of the material floated on the 0.68 M sucrose layer. This material was highly surface-active. The surfactant had a constant protein content. The lipid-to-protein ratio (w/w) was 3.86. Radioactively labeled alanine was incorporated into this surfactant, presumably in the protein fraction. Labeled surfactant material was then centrifuged on a continuous sucrose gradient. There was only one lipid and protein-containing peak. This gave evidence for the close association of lipid and protein
in the surfactant material. Results from gel filtration of labeled surfactant on a Sepharose 2B column also supported this notion. Material obtained from gel filtration had the same $^{3}$H to $^{14}$C ratio as that of the material applied to the column.

The apoprotein of the surfactant was obtained by adding ethanol-diethylether (3:1, v/v) at $-10^\circ$ C. The mixture was stirred and then centrifuged at a low centrifugal force for 10 minutes. The supernatant was discarded and pure ether was added. The precipitate was suspended in sodium borate buffer (pH 11.5) and incubated at $40^\circ$ C for three hours. Ninety per cent of the precipitate was dissolved by this procedure and was capable of entering a polyacrylamide gel. This was in contrast to the unpenetrability of the lipid-protein complex obtained by gel filtration. Two protein bands were detected. Traces of carbohydrates found in the delipidated protein were thought to be glycoprotein (Frosolono et al., 28).

Colacicco and Scarpelli, in 1971, further investigated the function of pulmonary proteins obtained from rabbit lung washings (37). They found three proteins by polyacrylamide gel electrophoresis: one which migrated like albumin, a second lipid-free protein which appeared identical with that described by Frosolono (38) and a third protein which remained with the lipids in the spacer gel.

Pruit, in 1971, reported on the physical and chemical characters of surfactant obtained from pig lungs (29). The lungs were
washed with Tris buffer, pH 7.4, containing isotonic NaCl. After filtering the lavage through cheesecloth, the material was centrifuged at 1,000 x g for ten minutes to sediment the cellular debris. The supernatant was then further centrifuged at 27,000 x g for a minute. The pellet was suspended in buffer and layered on a gradient ranging from 0.25 M to 0.75 M sucrose. Following centrifugation for 16 hours at 35,000 x g, two bands were found: one at 0.50 M sucrose and a second at 0.55 M sucrose. In both fractions, the ratio of phospholipid-to protein was 4/1. Each of these two surfactant fractions appeared at the same place in the gradient when recentrifuged. Phospholipid analysis revealed that about 80 per cent of the phospholipids was phosphatidylcholine. Analysis of the two surfactant bands by disc electrophoresis revealed the presence of four protein bands, three of which were also containing phospholipids. Both surfactant fractions had a slow moving protein band, which was associated with lipids, and a faster moving protein band having the same mobility as albumin. However, immunoelectrophoresis and immunodiffusion studies could not verify that this band was albumin.

Klass, King and Clements, in 1971, did an immunochemical characterization of proteins in pulmonary surface-active material from dogs (38). Purified surfactant revealed high surface activity. Approximately 11.5% of the material was protein and approximately 88% was lipid. The remaining 1% was comprised of carbohydrates and nucleic acids. Rabbit antisera to pulmonary dog surfactant
was produced by immunizing rabbits with periodical injections of surfactant over a period of four weeks. The rabbit antisera contained antibody against IgG, dog albumin and a third antigen which was not a plasma constituent. Surfactant obtained from homogenized dog lung also showed this specific antigen. Immunofluorescence revealed that this third antigen is also present on the alveolar surface of the lung.

King and Clements, in 1972, developed a complex method for the isolation of surface-active material from dog (39). The bulk of the surface-active material was obtained as an insoluble suspension, with a large particle size (100 nm in diameter). The large particle size eliminates the use of any solid chromatography, such as agar, starch, agarose or polyacrylamide for the purification of the surface-active material. King and Clements felt that ion-exchange resins use, due to their large surface area, may be hazardous. They felt that these resins would irreversibly adsorb the surfactant. Since the pulmonary surfactant might be made of lipid-protein complexes, dispersing agents, such as detergents, chelating agents, bile salts or alcohol should not be used, as they possibly break the lipid to protein complex. The new methods of King and Clements were based on differential and density gradient centrifugation in NaBr solutions. They felt that the separation of the surface-active material was based only on density. In addition, they felt that particle size had been eliminated as an interfering factor.
King and Clements studied composition and physiological properties of four surface-active fractions obtained by their new procedure (40). The four fractions were almost entirely composed of lipids and proteins. The phospholipid content was greater than 80% of the total lipids, of which 73% was phosphatidylcholine. Smaller amounts of phosphatidylethanolamine and sphingomyelin and several neutral lipids could also be identified.

When the surface-active material was placed on agarose gels, the majority of the material remained at the origin, and only small amounts of either lipid or protein migrated into the gel. Finding both protein and lipid at the origin was of prime significance to these experiments and were substantiated by isoelectric focusing. Most of the material migrated to an isoelectric point of 3.8 - 4.1 and had a protein-to-phosphorus ratio almost identical with that of the starting material.

In a subsequent study, King and Clements investigated the surface activity of the four surfactant fractions (one fraction was recovered from extracellular washings while the remaining three were obtained from lung homogenates) by thermal analysis (41). The four surface-active fractions did not differ significantly in terms of their surface properties, as measured by adsorption kinetics, or in terms of their surface tension-surface area dependencies. In view of these results, each of the four fractions could be considered a type of surfactant. In addition,
the surface tension measurements revealed that the protein content had no influence on the surface activity of the isolated materials.

Marinkovich and Klein, in 1972, isolated surfactant from human lung homogenates, bovine lung homogenates and lung washings using the techniques of differential centrifugation and starch block electrophoresis (42). An antiserum to the isolated surfactant was produced by injection of the surfactant preparation into chickens. This antiserum neutralized the surface activity of the surfactant and produced a visible aggregation of the surfactant mixture. A three per cent solution of homologous serum albumin completely stopped the production of visible surfactant aggregation. Solutions containing heterologous serum albumin or concentrated solutions of heterologous serum gamma globulin failed to impede the antiserum-surfactant reaction. The surfactant did not enter polyacrylamide gels; however, after electrophoresis a faint band, presumably corresponding to albumin, could be seen in the gel. These investigators felt that due to albumin's intimate association with surface-active material, the albumin may play a role in the function of pulmonary surfactant.

In 1972, we studied the biochemical maturation changes in developing rabbit lung surfactant (43). Lung washings from 26-31 days gestation fetuses, newborn and adult rabbits were fractionated into cells, sedimentable surfactant and supernatant using sedimentation techniques designed to characterize and quantitate
distinct constituents of the alveolar space. In 26 and 28-day fetuses, more than 70% of the phospholipids (composed almost entirely of phosphatidylcholine) were found in the supernatant fraction of the lung washing. After day 28, the phospholipid content of the sedimentable surfactant fraction per gram lung weight revealed a rapid increase in association with a more gradual rise in the protein content of the same fraction. Since the rate of increase of phospholipids was significantly greater than that of proteins, the amount of protein per mg dry weight of the surfactant fraction decreased with advancing maturation of the fetus. From the above inverse relationship between phospholipid and protein, it was possible to establish a reliable maturation index for the developing surfactant. Thus, the phospholipid to protein ratio was 0.13 and 1.94 on days 28 and 31 fetuses, 3.80 and 8.90 in newborn and adult rabbits, respectively. From this finding, we felt that the composition of surfactant lipoprotein underwent significant changes during late stages of fetal development. We further concluded that the phospholipid to protein ratio is a reliable and convenient parameter to evaluate the state of surfactant maturation.

Scarpelli and Colacicco's study on surfactant preparations from rabbit lungs supports the possibility of membrane-like proteins in association with lipids (44). Aliquots of lung washings were used for antibody production in goat. The rest of the material was subjected to Sephadex G-200 column chromatography.
Three fractions were eluted with 1.0 M NaCl in 0.1 M Tris buffer containing 1 mM EDTA. The first fraction contained most of the phospholipids and proteins S and T. This contradicts the earlier study by Scarpelli et al., which claimed there were no proteins in this fraction (22). The pooled fraction II contained 5% of the total phospholipids. Disc gel electrophoresis demonstrated that the protein was γ-globulin. The third fraction contained 9% of the total phospholipid. The major component of the proteins of this fraction was albumin. After centrifugation of Fraction I at 49,000 x g for one hour, a white pellet was obtained containing protein S and phospholipids. Protein T stayed in the supernatant and could be extracted with chloroform. This characteristic is unusual for proteins and points to its possible similarity with brain proteolipid proteins. Scarpelli et al. immunological studies suggested that there are counterparts of this protein in the serum. Protein S which precipitated with the phospholipids did not produce any antibodies in serum. This protein could be pulmonary surfactant-specific.

In another investigation, these protein-containing pulmonary washings were layered on continuous sucrose gradients ranging from 0.1 M to 1.0 M sucrose (Colacicco et al., 45). The gradient was centrifuged for 16 hours at 80,000 x g and two bands were visible at densities 1.056 and 1.065. The upper band had a phospholipid-to-protein ratio of 3/1 while the lower band (density=1.065) had a ratio of 9/1. At first glance these findings seemed to be hard
to understand since the average protein has a much higher density than the phospholipid. Only protein T was found in the two density bands. Over 60% of the phospholipids from the lung washings was isolated in these two bands. Large quantities of cholesterol and appreciable amounts of glycoproteins were present at a density greater than 1.07. At densities less than 1.05, neutral lipid and albumin were found. When protein T was investigated by disc sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Colacicco saw two bands corresponding to molecular weights of 150,000 and 60,000.

Since over 85% of the phospholipids was recovered together with the two proteins, T and S, one might speculate that similar lipid-to-protein aggregates can form in situ. The association of the phospholipids with protein T seems to resemble a proteolipid while protein S might form a lipoprotein which is somewhat different from the known serum lipoproteins.

King et al., in 1973, extended their study on the characterization of the three protein fractions which they had isolated from dog pulmonary surfactant preparation by addition of ethanol/ether, 1:3 (v/v) at -15°C and extraction of most lipids overnight (46). The proteins and about 12% of the phospholipids in the original surfactant fraction precipitated during this procedure and were solubilized by sonication in sodium borate buffer containing 0.1% sodium dodecyl sulfate (pH 9.0). A water-clear supernatant was obtained after the suspension and was centrifuged for two hours
at 19,000 x g. Polyacrylamide gel electrophoresis with SDS gave
the following results: a band of approximately 150,000 M. W.
which was probably immunoglobulin; a second band coincided with
albumin position, M. W. 68,000 and a third band of approximately
10,000 M. W. which was apparently not a serum protein. Addition of
2-mercaptoethanol prior to electrophoresis resulted in additional
bands of 25,000, 23,000, 50,000 and 75,000 molecular weights.
The components at 25,000, 50,000 and 75,000 seemed to be derived
from immunoglobulin. The intensity of the 10,000 molecular weight
component was not affected by the 2-mercaptoethanol treatment.
By comparison of the densitometric gel scan, it was estimated
that 50-70% of the total protein in adult dog lung had a molecular
weight of 10,000. In fetal or neonatal lung, only 10,000 molecu-
lar protein could be detected. The 10,000 molecular weight pro-
tein was isolated by gel filtration using Sephadex G-150 in the
presence of SDS. In the first pooled fraction eluted from the
column, all of the proteins with larger molecular weights were
separated while the second fraction consisted almost exclusively of
the 10,000 molecular weight material. In this fraction, the non-
serum protein was eluted with the phospholipids, which were pre-
cipitated in the ether/ethanol fractionation. No phospholipids
were found in association with the larger molecular weight proteins.

King et al. speculated that the lipids were either zwitter-
ionic (phosphatidylcholine) or acidic (phosphatidylserine and
phosphatidylglycerol) and may be associated with the proteins by a
divalent cationic linkage, hydrophobic interactions, or both. The amino acid analysis of the protein revealed the presence of twice as many acidic as basic amino acids. The hydrophobic residues of the proteins could interact with the fatty acid varieties of the phospholipids.

Klass, in 1973, points out many obstacles which still prevent a total understanding of surfactant (47). The exact chemical composition of this pulmonary surfactant is not known. Unfortunately, it was not known how surfactant is synthesized and transported to its site of action. Thus, it is somewhat difficult to uncover the defect in various pathological situations of the hyaline membrane-like diseases which could be due to underproduction, faulty production or inactivation of the surfactant. Klass characterized, immunochemically, the proteins which were isolated from dog lung by King et al. Antiserum to surfactant was produced by intramuscular injection of pulmonary surface-active material mixed with Freund's complete adjuvant into rabbits four times in a period of four weeks. In immunoelectrophoresis performed with 0.005% (w/v) SDS in the gel, the antiserum reacted with two components of the surfactant mixture which corresponded to albumin and IgG. A third protein band in the polyacrylamide gel did not react with the antiserum. Information obtained previously by immunofluorescence indicated that this third protein was lung-specific and was found in the alveolar lining.
Hurst maintains that most proteins in pulmonary surfactant are contaminants \(48\). Hurst et al. in 1973, introduced a new approach to the study of pulmonary surfactant. They designed experiments which they felt minimized contamination of the alveolar space by pulmonary capillary content. The pulmonary vasculature of a group of rabbits was gently perfused with either 0.25 molar sucrose or isotonic NaCl until the washing was free of blood. In a second group, the vasculature was gently washed with FC-80 fluorocarbon, an inert non-aqueous liquid. Hurst et al. believed that the isotonic sucrose solution and especially the inert FC-80 fluorocarbon would minimize any capillary transfusion into the alveolar space. Following this procedure, the pulmonary lavage was obtained by five consecutive washings of the lung with a total of 150 ml of 0.25 molar aqueous sucrose solution. A third group of animals was taken as controls. In this group, there was no rinsing of pulmonary vasculature. However, the lungs were washed with five 30 ml fractions of 0.25 molar aqueous sucrose solution. The lavage material was centrifuged for 15 minutes at 250 \(x\) g to eliminate cellular debris. The lipid and protein contents obtained from lavages of vasculature-perfused-lungs were compared with washings obtained from non-perfused animals. The surfactant material obtained from non-perfused rabbits was comprised of 30% proteins and 70% lipids. The total lipid content of the surfactant of the sucrose-and-fluorocarbon-perfused and the nonperfused lung did not differ substantially. However, the
perfusion procedure used prior to the washing seemed to change the protein content drastically. The sucrose-perfused-lung washing contained 3.8 mg protein. Traces of protein could still be detected after the fourth washing. There was 0.25 mg protein in the fluoro-carbon-perfused lung; no detectable traces of protein were seen after the second washing. By thin layer chromatography it was shown that lecithin comprised the majority of the lipids. Neutral lipids and other phospholipids were also detected from the washings of non-fluorocarbon perfused animals. The lipid content from the fluorocarbon washings was made up almost exclusively of phosphatidylcholine. DEAE cellulose columns separated the proteins from nonperfused and sucrose-perfused animals into three peaks, one of which contained 75% of the total protein. This major protein peak could not be differentiated from albumin by SDS gel electrophoresis. It was considered that the albumin might be derived from the capillary blood rather than a normal alveolar component. The other smaller peaks contained a mixture of non-homogeneous, large and small molecular weight polypeptides. Based on these findings, Hurst et al. concluded that the alveolar space is normally lined with diphosphatidylcholine and is practically free of other lipids, proteins or carbohydrates.

In preliminary studies with surfactant obtained by sedimentation techniques, Shelley, in 1974, found that surfactant material formed aggregations of phospholipids and proteins which could not be readily broken; however, following sonication, soluble
protein was released and the particle size of the aggregates was reduced (49). Non-cellular rabbit lung washing, prepared by various techniques, revealed components with flotation rates ranging from 25 to 400. However, pure phosphatidylcholine suspensions had similar flotation rates. Shelley suggested that the isolation of surfactant fractions containing both lipids and proteins seemed to be the result of non-specific associations, which are artifacts promoted by the technique of sedimentation. Based on these results, Shelley developed a relatively simple method for isolation of surfactant. Non-cellular alveolar rabbit lung washings were centrifuged on continuous NaBr-gradient without any prior concentration by sedimentation. A visible band, which contained most of the phospholipids of the washing was obtained. The density at which this material was separated was temperature dependent. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins of the phospholipid band revealed no difference in composition when compared with proteins from all the other fractions of the gradient. Albumin, IgG and IgM were identified by immunoelectrophoresis. In addition, small quantities of several non-plasma proteins were identified in the rabbit lung washings. Shelley concluded from her results that there is little, if any, protein specifically attached to the phospholipids of lung surfactant; rather, the properties of lung washing are very similar to those of phosphatidylcholine in aqueous suspension.
King et al. recently developed a radioimmunoassay for pulmonary surface-active material in sheep lung using solid-phase techniques (50). Pulmonary surfactant from sheep lung washing was isolated by their previously developed standard techniques. The isolated material consisted of 7.7 per cent protein and 72.5 per cent phospholipids. Antibodies to the proteins in pulmonary surfactant were obtained using procedures previously developed. A non-serum apoprotein from the isolated surfactant was obtained by the following procedure:

Eight mg of the surface active material was brought up to a volume of 8 ml with 0.3 M lithium diiodosalicylate buffered to 7.4 with 0.1 M Tris. After mixing the suspension in an ice bath for 15 minutes, the suspension was centrifuged for 90 minutes at 48,000 x g.

The precipitate was discarded and the supernatant diluted with an equal volume of water and mixed with 16 ml of 44 per cent phenol for 60 minutes. A phase system was then obtained by adding 1.6 ml of 80% (v/v) phenol. The two phase system was then separated by centrifugation and the upper phase was removed and lyophilized. The lyophilized material was extracted three times, with 100 per cent alcohol and the precipitating protein recovered by centrifugation. The protein was dissolved in water and the solution centrifuged for two hours.
at 81,000 x g to remove insoluble material. The isolated apoprotein was labeled with NaI$^{125}$. The radioimmunoassay was done the following way. Disposable polystyrene tubes were coated with sheep surfactant. The tubes lined with sheep surfactant were then coated with antiserum (containing antibody against sheep surfactant). For control, some tubes were also coated with non-immune rabbit serum. The assay was then carried out by adding aliquots of sheep surfactant ranging from 0 to 100 µl to a series of coated tubes (antiserum-coated and non-immune sera coated). Following this process, 3,000 CPM of $^{125}$I-labeled apoprotein dissolved in 20 µl of water were added to each tube and after incubation for four hours at 37° C. The tubes were washed with distilled water three times and the bound radioactivity was measured by gamma-particle scintillation counting. Surfactant was also obtained from sheep lung tissue and subjected to the same procedure as with the surfactant obtained from extracellular lung washing.

The lung surfactant apoprotein from 9 sheep varying in age between one month and adult had a range from 0.29 mg per gram of lung (wet) to 0.70 mg per gram lung (wet). The adult lung contained the highest content of SAM. Taking to account that 7.7 per cent of surface-active material comprised of apoprotein, the surfactant material varied from 3.7 mg per g to 9.1 mg per g lung (wet).
King et al. came to the conclusion that radioimmunoassay is a very helpful tool in measuring small amounts of surfactant-specific antigen in the lung and can distinguish fluids where this antigen is apparently absent. The radioimmunoassay study revealed also that the dipalmitoyl phosphatidylcholine is not a specific marker for pulmonary surfactant in whole lung, since the radioimmunoassay study combined with the chemical determination of the amount of dipalmitoyl phosphatidylcholine seemed to indicate that less than 40 per cent of the dipalmitoyl phosphatidylcholine in whole lung tissue belong to pulmonary surfactant.

Dickie et al. studies revealed that lung slices can incorporate leucine $^{14}$C into proteins of surface-active material, which floated on KBr-gradient of density 1.21 g/ml (51). Polyacrylamide gel electrophoresis of the surface-active material showed a single band which stained for lipids and proteins. This band moved much faster than the fastest migrating protein or lipid staining material of the serum. Dickie et al. concluded from their study that rabbit lung can synthesize a lung specific protein, which is part of the surface-active material.

Bensch et al. showed that albumin and globulin can traverse the air-blood barrier under normal conditions (52).

Moreover, transudation of plasma proteins in the alveoli is a common alveolar reaction to injury. Heard and co-workers recently investigated at the functional level in vitro effects of serum proteins on lung surfactant (53). Surface properties of lung
surfactant and phospholipid suspensions were investigated by the
diameter changes of the air bubble method first developed by
Pattle and Burgess (54). The diameter of bubbles composed of
either surfactant or phospholipid material were measured at zero
time and after 20 minutes. A stability ratio of these bubbles
was then calculated as the square of the final diameter divided
by the square of the initial diameter. Heard et al. added separately
albumin, globulin or plasma to air bubbles composed of either
pure phosphatidylcholine, phosphatidylinositol or surfactant
lipids and found that the stability ratio of these bubbles was
raised. On the basis of this in vitro study, Heard et al. sug-
gested that plasma proteins exert a protective mechanism on sur-
factant in lungs with moderate pulmonary edema.

Chapman has discussed the phase transitions and fluidity of
biological membranes (55). Phospholipids possess an endothermic
phase transition from crystalline gel to liquid crystal depending
in part on the length of aliphatic chain and saturation. Many
membranes are in a dynamic condition with considerable flexing,
twisting and lateral movement of the lipids. Experiments with
model systems have shown that the characteristic transition
temperature of the lipids can be shifted up or down dependent upon
the type of interaction with the proteins.

Since the mammalian lung is at constant temperature, such
thermal phase transition cannot play a role. However, lipid
phase transition can also be produced by changes in surface
pressure, and protein-lipid interaction may have an influence in the pressure-area diagram of surfactant films. Thus, in the transition area of a pressure-area diagram, small changes of surface pressure may produce large changes in the surface area.

Trauble et al. investigated the lipid phase transitions in lung surfactant and found that lung alveolar surfactant revealed an ordered to fluid phase transition between 20 and 40° C. (56). Bubbles lined by surfactant material showed critical areas in a volume-pressure diagram where small changes in pressure caused abrupt changes in the bubble volumes. No difference in the volume pressure diagrams of bubbles lined with either alveolar surfactant (containing both phospholipids and proteins) or extracted surfactant lipids were seen which led Trauble et al. to the conclusion that proteins present in the alveolar surfactant had no influence on the volume pressure relationship of the bubbles. Trauble et al. suggested that pulmonary surfactant exhibits, besides the known function of reducing surface tension in the alveolar space, an additional property of spontaneous opening and closing of alveoli at a critical pressure area.

Scanu et al. studied the separation and reconstitution of high density serum lipoprotein and investigated the lipoprotein structure by the technique of circular dichroism (57). Lipids were separated from the serum lipoprotein after treatment with ethanol-ethyl ether mixture. The precipitated proteins were dissolved in aqueous buffer. Relipidation or reconstitution of
the original structure was carried out by incubating a lipid dispersion with the protein solution. The resulting complexes were isolated by ultracentrifugation. The CD studies revealed that the serum high density lipoprotein had a high content of \( \alpha \)-helix. The most significant observation of their study was that the \( \alpha \)-helical spectra of lipid-free apoprotein is not clear. However, Scanu maintained that during the delipidation procedure, the proteins underwent a change in their tightness and length of the helical segments. Another possibility was that there were no true conformational changes of the proteins with or without the lipids, but rather that there was a change in the environment around the active chromophores of the proteins. Lux et al. studied the influence of lipids on the conformation of human plasma high density apolipoproteins (58). The native high density lipoprotein contained about 70% \( \alpha \)-helix. Upon delipidation, the helical content decreased by 20% with the corresponding increase in the disordered structure of the protein. These investigators found that 20 to 30% of the amino acid residue in HDL apoprotein were involved with the helix-disordered transition which occurred when lipid was removed from the serum lipoprotein. The significance of the ability of phospholipids to induce \( \alpha \)-helix structure in proteins was not clear. They further speculated as Scanu et al. had previously that the binding of lipids could decrease the local polarity in the amino acid residues, which are involved in the lipid binding sites. The results of the lipid binding could be
the formation of a weak hydrogen binding environment, which could favor the formation of increased helical structures.

Hammes et al. investigated the structure of macromolecular aggregates comprising of phospholipids and polypeptides (59). The complex formation between poly-L-lysine and phosphatidylserine or phosphatidylcholine liposomes were studied by circular dichroism, optical rotatory dispersion and electron microscopy techniques. In a buffer range of pH 5-9.5, phosphatidylserine had one negative charge per molecule, while phosphatidyl choline had zero negative charge. The results from the circular dichroism study indicated that polypeptides (poly-L-lysine) can be converted from random coil into essential all helix structure induced by the interaction with phosphatidylserine liposomes. Only small and nonreproducible effects were seen when phosphatidylcholine instead of phosphatidylserine was used.

Hammes et al. concluded that although electrostatic interaction is a requirement for the stabilizing effect of phospholipids on polypeptides, a hydrophobic bond effect cannot be excluded since high salt concentrations as high as 1 M NaCl could not disrupt the phosphatidyl-serine-poly-L-lysine complex. While no generalization can be made as yet predicting the effect of phospholipids on protein structure, Hammes et al. felt that in their specific model of water soluble polypeptide and phospholipid interaction the following points can be said:
1. Electrostatic interaction was necessary for the complete formation between the polypeptides and phospholipids.

2. Hydrophobic bonding between the protein and lipids stabilized the interaction.

3. The interaction favored the formation of membrane-like vesicles consisting of phospholipid bilayer and coated by polypeptides.

4. The $\alpha$-helix structure of the polypeptides investigated had the tendency to increase upon complex formation with the phospholipids.

5. The mobility of the fatty acid groups was reduced as a result of the interaction.

Hammes et al. speculated that this was due to a tighter packing of the polar moieties in their crystalline array.
STATEMENT OF THE PROBLEM

The alveolar space of the mammalian lung is lined by surface-active material designated lung surfactant, which stabilizes the alveoli of the lung. Although it is known that phosphatidylcholine is the primary component responsible for this surface activity, the exact composition of the lung surfactant has not been determined. One group of investigators has suggested that the surfactant is composed mainly of phospholipids, while other investigators maintain that the surfactant is a lipid-protein complex. Notwithstanding the controversy about the precise composition of surfactant, proteins, especially plasma proteins as well as lung-specific proteins, are consistently associated with surfactant fraction of lung washing and apparently represent a natural constituent of the alveolar space.

The present study was undertaken to investigate the interactions of proteins, recovered from the alveolar space, with surfactant lipids, since it seemed plausible that such interactions may occur in the hypophase and surface film of the surfactant system, under normal and pathological conditions and that these interactions may relate to the functional state of surfactant. By trying to reassemble surfactant lipid-protein complexes from its previously separated constituents, some information could be obtained about the way these constituents interact and the
influence these proteins and lipids have on each other. Such recombination experiments and circular dichroic studies of the delipidated surfactant proteins as well as proteins complexes with surfactant lipids may provide valuable information relevant to the nature of lipid-protein interactions in the alveolar spaces.

More specifically, this research will attempt to provide information relevant to the following questions:

1. Do proteins from the alveolar space associate with surfactant lipids in a defined stoichiometry or in a random and nonspecific manner?

2. Which of the major plasma proteins, if any, can interact and associate with lung surfactant lipids?

3. Do there exist unique proteins native to alveolar surfactant that are not proteins simply transudated from plasma?

4. Does interaction of alveolar lipids with plasma proteins result in the formation of complexes showing properties similar of those of the alveolar surfactant lipid complexes?

In our initial studies, lipid-protein complexes were isolated from rabbit and dog lung using the method of Frosolono and it was noted that the surfactant lipid-protein complexes and the delipidated surfactant proteins were not water-soluble. Due to the insolubility of our apoprotein mixture in aqueous media, the Scanu method for the reconstitution of surfactant complexes was
unsuccessful. Zahler and Weibel described the separation of the lipids and proteins of erythrocyte membranes using Sephadex LH-20 and 2-chloroethanol-H$_2$O (9/1, v/v) as eluent (60). They found that lipids and proteins dissolved in 2-chloroethanol and dialyzed against aqueous media reaggregate and electron microscopic studies reveal the typical trilaminar membrane structure. This procedure seemed suitable for both the separation of surfactant lipid-protein complexes and for their reconstitution especially since it has been found that after 2-chloroethanol treatment, proteins do not undergo alterations detectable by CD studies (61).
CHAPTER II

MATERIALS AND METHODS

COLLECTION OF DOG LUNG WASHING

Ten mongrel, male dogs, weighing 35-40 kg, were killed in the morning by intravenous injection of sodium pentobarbital. During the 24-hour period prior to sacrifice, the dogs were maintained without food. After opening the chest wall and inspection of the lungs, washing solution consisting of 0.01 M Tris buffer, pH 7.4, and 0.145 M NaCl, was introduced by gravity into the trachea until the lungs were completely expanded (28). The amount of buffer used for each lavage varied from 2.0 to 2.5 liters corresponding to the size of dog used. The lavage was removed by gravity drainage. This procedure was repeated four times and all lavages were pooled; the amount of the total washing varied from 9 to 12 liters.

PREPARATION OF THE LUNG WASHING FOR THE ISOLATION OF SURFACTANT FRACTION

The isolation of lung surfactant was done according to a modification of the procedure of Frosolono (28) (Figure 1).
Alveolar dog washing, 10-12 liters, Sedimented at 48,000 x g, 45 min.

a) Layer of resuspended sediment over 0.75 M sucrose
b) 48,000 x g, 45 min.

Supernatant

Interphase material

0.75M

Cellular material

Washing of interphase material, suspended in buffer, by sedimentation at 48,000 x g, 45 min.

a) Layer of washed interphase material over 0.33 M and 0.68 M sucrose in SW-25.2 tubes.
b) 69,000 x g, 90 min.

Designated surfactant

Figure 1. Outline of isolation procedure.
The bulk washing was concentrated by sedimenting the opalescent material for 40 minutes at 48,000 x g in a Sorvall SS-34 rotor. The combined sediments were resuspended in 1/10 of the original volume (bulk washing). Thirty-five milliliter portions of the suspension were carefully layered over 10 ml of 0.75 M sucrose. After centrifugation in the Sorvall SS-34 rotor at 48,000 x g for 40 min, the interphase material which was isolated between the clear supernatant buffer solution and the 0.75 M sucrose was removed by a 35 ml glass syringe, resuspended in buffer and sedimented again at 48,000 x g for 45 min. The washed 0.75 M cell-free interphase material was then suspended in 0.01 M Tris buffer, pH 7.4 and layered on top of a discontinuous gradient, containing 23 ml of 0.68 M and 23 ml of 0.33 M sucrose. The tubes were centrifuged at 69,000 x g for 2 hours in a Spinco SW-25.2 rotor. Material at the two interphases was collected with a 35 ml glass syringe, resuspended in 35 ml of 0.01 M Tris buffer, pH 7.4 and sedimented at 20,000 rpm (48,000 x g) for 60 min in the Sorvall SS-34 rotor. The 0.33 M and 0.68 M interphase materials were pooled separately and resedimented a second time. Ninety per cent of the cell-free material which originally floated on top of 0.75 M sucrose migrated to the 0.68 M interphase, while the other 10% was found on top of the 0.33 M interphase. Only the material which was isolated from the 0.68 M interphase was significantly surface-active as shown in the results section and was henceforth designated as surfactant. Surfactant was stored at 4°C until evaluation.
SEPARATION OF CELL AND SURFACTANT-FREE LUNG ALVEOLAR FRACTION--

DESIGNATED SUPERNATANT

Lung bulk washing (400 ml) was centrifuged for 15 min at 400 x g. The obtained sediment was discarded and the supernatant centrifuged at 100,000 x g for 60 minutes. The sediment from this centrifugation was also discarded, while the supernatant (designated supernatant) was dialyzed against distilled water and lyophilized (Figure 2).

ISOLATION OF RABBIT SURFACTANT

Basically the isolation procedure of Frosolono was applied (28). It was shown that rabbit surfactant had a lower density than dog surfactant on sucrose gradient; therefore, Frosolono's procedure was modified by adding an additional density of 1.060 (0.45 M sucrose) in the sucrose gradient.

Extracellular Washing (4 rabbits)

<table>
<thead>
<tr>
<th>Centrifuged 70 min</th>
<th>48,000 x g</th>
<th>on 0.75 M sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment +</td>
<td></td>
<td>Interphase PL/Pr = 8.3 (w/w)</td>
</tr>
<tr>
<td>Supernatant discarded</td>
<td></td>
<td>PL amount 106 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The interphase was washed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and layered on 0.33 M, 0.45 M, 0.68 M sucrose. Centrifugation 180 min, 23,000 RPM, SW-25.2 bucket</td>
</tr>
</tbody>
</table>
Figure 2. Scheme of the procedure used for obtaining of designated Supernatant Fraction. The reason for this procedure was to obtain a fraction of the lung washing other than surfactant. Reconstitution experiments of the proteins of this fraction was compared with surfactant proteins.
Three clear bands were collected:

1. 0.33 M interphase (density 1.045)
   PL/Pr = 10.5/1.0 (w/w)
   PL amount 19 mg

2. 0.45 M interphase (density 1.060)
   PL/Pr = 9.7/1.0 (w/w)
   PL amount 42 mg

3. 0.68 M interphase (density 1.090)
   PL/Pr = 5.6/1.0 (w/w)
   PL amount 17 mg

**ISOLATION OF LIPID-FREE SURFACTANT PROTEIN**

Surfactant material was initially delipidated by a procedure of Scanu (Figure 3). The delipidated surfactant proteins were insoluble in water and therefore a procedure of Zahler and Weibel was used (60).

Delipidation of surfactant lipid-protein complex was achieved by gel filtration on Sephadex LH-20. A solution containing 2-chloroethanol-water (9:1, v/v) was used as solvent. Due to the fact that 2-chloroethanol extracted a yellow color from the connections of commercial columns, a special column was made completely out of glass (column 2.5 in. x 65 in.). Sixty-five gm of Sephadex LH-20 was swelled in 200 ml of 2-chloroethanol-H₂O (9:1, v/v) for 16 hours. The column was packed under the hood with the swelled material and washed for 24 hours with solvent (2-chloroethanol;water = 9:1). The column was used immediately
Surfactant 1 ml

EtOH-Et<sub>2</sub>O 3:1 (v/v)
50 ml

3 hr, -10° C
12 rpm

Precipitate

Supernatant
discarded

Et<sub>2</sub>O
50 ml

18 hr, -10° C
12 rpm

Precipitate

Supernatant
discarded

wash 3x with Et<sub>2</sub>O
dry under N<sub>2</sub>

Figure 3. Scheme of delipidation of surfactant by a procedure of Scanu (57).
since it was reported that Sephadex LH-20 is unstable during long contact with 2-chloroethanol. The surfactant material (40 mg phospholipid, 10 mg protein) was resuspended in distilled water and sedimented. The sediment was resuspended in 1.5 ml of distilled water. This was mixed vigorously and dissolved in 7.5 ml of 2-chloroethanol. The surfactant solution was concentrated seven-fold under a stream of nitrogen at room temperature. The concentrated solution (5 mg protein per ml) was applied to the column. Fractions, ranging between 2 ml and 10 ml, were collected automatically. Designated supernatant fraction, albumin and serum were delipidated by the same procedure. Zahler (60) has shown that proteins are washed off the column in the void volume, while the lipids should be retained until they are washed off in the bed volume in this system. In order to determine the distribution of the proteins and lipids, the content of protein (64) and phospholipid (63) was measured. An example of the distribution of the surfactant proteins and lipids in a typical experiment is shown in Figure 4.

EXTRACTION OF LIPIDS BY NON-COLUMN METHOD

Lipids were extracted by the method of Folch et al. (1957) (62). Two ml aliquots of a 2:1 chloroform:methanol mixture (C:M, v/v) were placed in vials containing lyophilized samples of surfactant. The vials were mixed and left for sixteen hours at room
Figure 4. Sephadex LH-20 gel filtration of surfactant with 2-chloroethanol-\( \text{H}_2\text{O} = 9:1 \) (v/v) as eluate. Forty 5 ml fractions were collected.
temperature. The samples were then transferred to centrifuge tubes. The vials were washed with additional 2 ml of the C-M solution and this solution was added to the centrifuge tubes. After centrifugation at about 2,000 x g, the supernatants were removed to test tubes. The C-M insoluble residues were re-extracted with one ml C-M for one hour and centrifuged. The supernatants were combined. The lipid extracts were washed with 0.2 volumes of 0.37% KCl. The mixture was centrifuged to separate layers. The upper layer and any material at the interphase was discarded. The interphase was rinsed carefully three times with pure solvent upper phase, (chloroform-methanol-0.37% KCl in water, 3-48-47). The samples were transferred to volumetric flasks and diluted with C-M to either 10 ml or 25 ml.

SEPARATION OF PHOSPHOLIPID CLASSES

The phospholipid classes were separated by paper chromatography using the method of Wuthier (63). Whatman paper SG-81 was cut to 15.3 x 19 cm. Samples containing five to fifteen g phosphorus were applied in a spot near one corner, 2 cm from the edge, and were dried with an air dryer. Solvent I (chloroform-methanol-diisobutylketone-pyridine-distilled water, 90:30:60:40:8, v/v/v/v/v) were used. After developing the paper in the long direction for two 1/2 hours, the papers were dried for at least two hours, washed with distilled water, dipped into a solution
of .005% (w/v) rhodamine 6G in water, and washed again with distilled water. For the identification of the phospholipid spots, the papers were examined under UV light. The phospholipid spots were cut out and each spot was weighed. Blanks were also cut out for correction of the background. For the quantitative determination of the single spots, phosphorus was determined as described (Figure 5).

**PROTEIN DETERMINATION**

The method of Lowry et al. (64) was used to determine protein content. The method is a combination of two reactions. There is an initial interaction of protein and Cu⁺⁺ in alkali (related to biuret reaction), followed by the reduction of the phosphotungstic and phosphomolybdic acid to molybden blue and tungsten blue initiated by the Cu-protein complex and by the tyrosine and tryptophan of the protein.

**Reagents**

1. 1% Sodium Dodecyl Sulfate (SDS), (K and K Laboratories, Inc.)
   
   One gram \( \text{CH}_3(\text{CH}_2)_{12} \text{OSO}_3\text{N} \) was dissolved in 100 ml distilled water.

2. 1% Copper Sulfate (Solution B)
   
   One gram of \( \text{CuSO}_4 \cdot 5 \text{H}_2\text{O} \) was dissolved in 100 ml distilled water.
Figure 5. The phospholipids (isolated surfactant) were separated by 2-dimensional paper chromatography as described in the text. As identification for the single phospholipids, the findings by Wuthier was used (63). The following phospholipids were identified with the use of standards treated in the same way. 1. lysolecithin, 2. sphingomyelin, 3. lecithin, 4. phosphatidylethanolamine, 5. phosphatidylinositol, 6. unidentified phospholipids.
3. 2% Sodium Tartrate (Solution E)
   Two grams Na₂, C₄H₄O₆ • 2 H₂O were dissolved in 100 ml distilled water.

4. 2% Sodium Carbonate in 0.1 N Sodium Hydroxide (Solution A)
   0.1 N NaOH was prepared by adding 4 gm of NaOH to 1000 ml distilled water. Two grams of Na₂CO₃ were then added to the 0.1 N NaOH to make 1000 ml of solution.

5. Phenol Reagent of Folin and Ciocalteau (Solution D), in a 2 N reagent, was obtained from Harleco, Philadelphia, Penn. A 1 N reagent was obtained by diluting the solution 1:1 with distilled water.

6. Standard Protein Solution
   Crystallized human serum albumin was obtained from Dade Division of American Hospital Supply Corporation, Miami, Fla.
   A standard solution was obtained by adding 3 ml of distilled water to the crystallized albumin. The concentration of this standard solution was 80 mg/ml. A standard solution of 80 mg/ml was prepared by diluting 0.1 ml of this standard to 100 ml with distilled water.

7. 100 ml of solution A were mixed with 0.5 ml of solution B and E to obtain solution C.
The samples of the lung washings were diluted to 1 ml with distilled water. Eight standards were prepared for each protein determination using two samples of 20 \( \mu g \), 40 \( \mu g \), 60 \( \mu g \) and 80 \( \mu g \) of the diluted stock solution. When the lung surfactant samples were turbid, 0.25 ml or 0.5 ml of 1% SDS solution was added and the volume was adjusted with distilled water to 1 ml. To the sample of 1 ml protein in a 10-15 ml test tube, 5 ml of reagent C is added.

The samples were allowed to stand at room temperature for 10 minutes. A 0.5 ml aliquot of the diluted Folin reagent was added and mixed rapidly. After 30 min, the optical densities of the samples and the standards were measured at 700 nm (Table 1, Figure 6).

**PHOSPHOLIPID DETERMINATION**

Phospholipid content was determined by Wuthier's modification (63) of the procedure by Martin and Doty (73).

Standards were prepared as follows:

1. Perchloric acid with ammonium molybdate. \((0.1\% \text{(NH}_4\text{)}\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O in 70\% HClO}_4)\)

   One gram \( \text{(NH}_4\text{)}\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O} \) was dissolved in one liter of 70\% HClO\(_4\).
TABLE I

Standard Curve Data for Determination of Protein Concentration

<table>
<thead>
<tr>
<th>Micrograms Albumin</th>
<th>Number of Samples</th>
<th>Absorbance at *700 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>5</td>
<td>0.069 ± 0.001</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>0.137 ± 0.003</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>0.197 ± 0.005</td>
</tr>
<tr>
<td>80</td>
<td>5</td>
<td>0.258 ± 0.008</td>
</tr>
</tbody>
</table>

*Values are expressed as the mean ± standard deviation
Figure 6. Standard curve for the determination of protein concentration.
2. 10% Ammonium molybdate.
   Ten grams (NH₄)Mo₇O₂₄ • 4 H₂O were dissolved in 100 ml of distilled water.

3. 10% Stannous chloride reagent.
   Two and one-half grams of stannous chloride (SnCl₂ • 2 H₂O) were dissolved in 25 ml of distilled water. The working solution was prepared just by diluting the stock solution with H₂SO₄.

4. Benzene:isobutanol (1:1)
   Equal volumes of benzene and isobutanol were mixed.

5. Ethanol:sulfuric acid (96.8:3.2)
   3.2 ml of concentrated H₂SO₄ was mixed with 96.8 ml of absolute ethanol.

6. Standard phosphorus solution
   One gram of NaH₂PO₄ • H₂O was added to one liter H₂O, the solution having a concentration of 224.5 mg P/ml. This stock solution was then diluted 1 to 20.

Six standards were prepared for each phosphorus determination using two 0.05 ml, two 0.1 ml and two 0.2 ml of the diluted stock solution. To each sample (unknown, standard and blank), 0.5 ml of 70% perchloric acid containing one gram of ammonium molybdate/liter was added and heated in a sand bath at 180° C for about one
hour or until the solutions were colorless. After cooling, 1.5 ml water, 0.25 ml 10% ammonium molybdate and 1.5 ml benzene-isobutanol (1:1) were added to each test tube. The contents of the tubes were then mixed and centrifuged to separate phases. An aliquot of the upper phases (1 ml) was transferred to other tubes and the volumes adjusted to 3.0 ml with absolute ethanol-conc. sulfuric acid (96.8:3.2, v/v).

Samples were then mixed with 0.5 ml of stannous chloride reagent (10% in conc. HCl and diluted 1:100 with 1 N sulfuric acid just before using) was added to each tube and mixed again. After 5 min, the absorbance was read at 725 nm with the Beckman DB spectrophotometer. The phospholipid content was calculated assuming that phospholipids contain 4% phosphorus by weight (Table II, Figure 7).

RECONSTITUTION

Reconstitution experiments were performed according to a modification of the procedure of Zahler and Weibel (65). Protein samples included albumin, dog surfactant apoprotein and dog supernatant protein. Lipid samples contained dog surfactant lipids or commercial diphosphatidylcholine. The protein and lipid components were dissolved separately in 2-chloroethanol. The concentration of phospholipids and proteins varied between 1 and 2 mg/ml. The
<table>
<thead>
<tr>
<th>Microgram Phosphorus</th>
<th>Microgram Phospholipid</th>
<th>Number of Samples</th>
<th>Absorbance* at 725 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.56</td>
<td>14</td>
<td>5</td>
<td>0.067 ± 0.003</td>
</tr>
<tr>
<td>1.12</td>
<td>28</td>
<td>5</td>
<td>0.133 ± 0.009</td>
</tr>
<tr>
<td>2.24</td>
<td>56</td>
<td>5</td>
<td>0.275 ± 0.004</td>
</tr>
</tbody>
</table>

*Values are expressed as the mean ± standard deviation
Figure 7. Standard curve for determination of phospholipid concentration.
two solutions were combined in a ratio of protein/phospholipids - 1/1 (w/w). These clear mixtures were transferred to Visking dialysis bags, which had previously been boiled in distilled water for one hour and dialyzed for 14 hours at 4°C against 1000 vol. of 0.01 M Tris, pH 7.6, containing 0.01 M CaCl₂ and 0.001 M MgCl₂. The solutions started to precipitate after one hour. After 7 hours, the buffer solution was changed. The resulting suspension was placed on a discontinuous gradient which contained 1.4 ml of 0.33 M sucrose, 1.4 ml 0.68 M sucrose and 1.4 ml of 0.90 M sucrose. In some cases, the gradient was made up of 1.4 ml of 0.33 M sucrose, 1.4 ml 0.45 M sucrose and 1.4 ml of 0.68 M sucrose. The discontinuous gradient was centrifuged in a Spinco SW-50L swinging bucket rotor for 2 and 1/2 hours at 25,500 rpm at 4°C. The reconstituted material was carefully collected at the interfaces with a glass syringe. The different fractions were dialyzed against 0.01 M Tris, pH 7.6. The amount of material reconstituted was determined by evaluating the percent of total proteins incorporated into the reconstituted material. For all fractions, the phospholipid-to-protein ratio was determined and the circular dichroism was analyzed.

PROTEIN SEPARATION BY SODIUM DODECYL SULFATE (SDS) - POLYACRYLAMIDE GEL ELECTROPHORESIS

The delipidated proteins of the surfactant, designated supernatant fraction and dog serum were separated by polyacrylamide...
gels containing sodium dodecyl sulfate, according to the procedure of Druse et al. (66).

Preparation of the Gels for Electrophoresis Separation

1. Preparation of reagents.

Solution A_1 - 10% acrylamide
Forty grams of acrylamide and 1.04 gm of N,N'-methylene bis-acrylamide (both obtained from Eastman Organic Chemicals, Rochester, New York) were dissolved in distilled water to a volume of 180 ml.

Solution A_2 - 1% SDS, 0.1 M Phosphate Buffer pH 7.2
40 ml of 1% SDS, 40 ml of 1 M phosphate buffer pH 7.2 were added to 100 ml of distilled H_2O.

Solution B
Immediately prior to use, 37.5 mg ammonium persulfate was dissolved in 5 ml of a 0.5% TEMED (N,N,N',N'-tetramethylene-diamine) (TEMED obtained from Eastman).

12 glass tubes (12 cm x 0.6 cm) were boiled in nitric acid, washed in distilled water, and dried.
2. Preparation of 12 gels

Paraﬁlm was tightly wrapped around the bottom of the 12 glass tubes. The tubes were placed in a stand. The gel solution was prepared by mixing together 22.5 ml of A₁, 22.5 ml of A₂ and 5 ml of B. The solution was then deaerated for 1/2 - 1 minute. Approximately 2.5 ml of the deaerated gel mixture was pipetted into each tube to a height of 5 cm. The gels were gently overlayered with distilled water and allowed to polymerize. The polymerization took about 30 minutes. Immediately before use, the water on the upper surface of the gels was emptied and the tubes were placed in a Buchler Polyanalyst gel electrophoresis apparatus.

Preparation of Samples

The proteins were dissolved in 0.1% SDS containing 0.01 M phosphate buffer pH 7.2 and 1.6 M urea and stabilized in this solution for 12 hours at room temperature. Standard proteins were prepared the same way. The amount of protein placed on the gels varied between 40 and 150 g. In a few cases, we put up to 1 mg on the gels. The following standards were used:
<table>
<thead>
<tr>
<th>Source</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma globulins</td>
<td>Human Cohn</td>
</tr>
<tr>
<td>Bovine Serum</td>
<td>65,500</td>
</tr>
<tr>
<td>Bovine Pancreas</td>
<td>25,250</td>
</tr>
<tr>
<td>Horse Heart</td>
<td>11,700</td>
</tr>
</tbody>
</table>

**Electrophoresis**

A buffer, containing 0.1% SDS and 0.1 M phosphate pH 7.2 was placed into the lower portion of the electrophoresis apparatus. Samples containing a trace of bromphenol blue tracking dye were carefully layered on top of the tubes. The 0.1% SDS, 0.1 M phosphate buffer pH 7.2 was layered carefully on top of each sample and the upper chamber was filled with the buffer. Electrophoresis was performed at a constant current (2 mA per tube). The electrophoresis was continued until the tracking dye moved to about 1 cm from the bottom end of the gel. After the electrophoresis, the gels were removed with a spinal puncture needle attached to a syringe by squirting ice cold water between the gels and the walls of the glass tubes. The gels were placed in test tubes and fixed in a solution of methyl alcohol:acetic acid: distilled water, 45/10/45 (v/v/v) for at least 10 hours.
Staining and Destaining

One gram of Fast green (Sigma Chemical Co.) was dissolved in 100 ml fixing solution. The gels were stained for four hours in staining solution. Repeated washing over a period of several days with the destaining solution (methyl alcohol:acetic acid: distilled water, 45/10/45, v/v/v) destained the gels and the protein bands became visible.

The stained gels were scanned with a Densicord Recording Electrophoresis Densitometer. The relative areas of densitometric peaks were determined by weighing (Figure 8).

CIRCULAR DICHOISM SPECTRA

The circular dichroism spectra were obtained with a Durrum-Jasco OR/CD/UV-5 Recording Spectropolarimeter modified to a maximum sensitivity of $1 \times 10^{-3}$ deg/cm. The instrument was standardized with a solution of L-10 camphorsulfonic acid, each day prior to use. Measurements of the circular dichroism in the spectral region between 260 and 200 nm were carried out at room temperature using quartz cells of 0.1 cm path length, and a scan speed less than 0.5 nm/mm. The protein concentrations ranged from 50 to 300 g/ml. The values of molar ellipticity $\Theta_{MRW}$ (deg. cm$^2$/decimole) were calculated from the relationship
Figure 8. Densitometric graph of dog surfactant, separated by SDS-polyacrylamide gel electrophoresis as described in the text. The per cent of protein composition was calculated by weighing the densitometric peaks.
\[
\Theta_{\text{MRW}} = \frac{\Theta}{10} \frac{\text{MRW}}{L \times C}
\]

where \( \Theta \) is the observed ellipticity in degrees/cm

\( L \) = the path length (0.1 cm)

\( \text{MRW} \) = the mean relative weight of 112 was utilized

In the wavelength of interest, the lipids were found to contain insignificant circular dichroism. For the calculation of the \( \alpha \)-helix content, the negative maxima at 222 nm was used and compared to the circular dichroism of the \( \alpha \)-helical configuration of \( \gamma \)-polyglutamic acid at 222 nm, i.e.,

\[
\Theta_{222} = 3.1 \times 10^{-4} \text{ deg cm}^2/\text{decimole}
\]

(100% \( \alpha \)-helix)

**DETERMINATION OF SURFACE ACTIVITY**

The surface tension properties of lung washings and fractions obtained from these washings were measured with a modified Langmuir-Wilhelmy surface balance (Figure 9). This instrument was purchased from Kimray, Inc., Oklahoma City, Oklahoma and first described by Greenfield and Kimmell in 1967 (67). This modified Wilhelmy
Figure 9. Surface tension - area diagrams of two preparations of lung washing. The numbers at the ordinate indicate the surface tension in dynes per centimeter. The numbers on the abscissa represent the per cent of total pool area.
surface balance allows direct measurement of surface tension forces on a vertical platinum strip subtending the surface of the prepared sample placed in a teflon trough, while the surface area is changed by a moving barrier. With the help of a pneumatic sensor and an amplifier connected to a stylus, a record of the varying surface tension is obtained. The surface area change caused by the movement of the teflon dam is between a maximum of 57.5 cm$^2$ and a minimum of 7.0 cm$^2$.

Before the use of the instrument, the teflon trough and barrier were cleaned with ethanol and distilled water. The platinum strip was rinsed with ethanol and flamed. The platinum strip was then connected to a beam and the zero position of the instrument was adjusted. Following this, 25 ml of Ringer's lactate was placed in the trough and the instrument was allowed to cycle. If a straight line of 70 dynes/cm was not obtained, the instrument was turned off and the washing procedure repeated. Between one and five ml of the solution to be checked was then carefully layered on top of the Ringer's lactate solution and the Ringer's lactate with the top layer composed of surfactant material was allowed to "age" for 20 minutes at room temperature. The instrument was then turned on and the compression and expansion cycles were recorded by the stylus on special graph paper. A cycling time of three minutes was used and the cycling was continued until no changes were observed in consecutive cycles.
CHAPTER III

RESULTS

ISOLATION AND PROPERTIES OF SURFACTANT FRACTION RECOVERED
FROM MONGREL DOG LUNG

A lipid-protein complex with phospholipid-to-protein ratio of 3.6 to 4.0 was isolated on a discontinuous sucrose gradient of density 1.090 (Table III). This complex was shown to be surface-active as demonstrated by the fact that this material lowered surface tension dramatically (Figure 10). Repeated ultracentrifugation of this material, designated as surfactant, on discontinuous sucrose gradients, resulted in no significant change in the phospholipid to protein ratio (Table IV). The originally isolated surfactant and that isolated after repeated ultracentrifugation had the same protein composition (Figure 11).

The surfactant fraction was delipidated as described in material and methods. No trace of phospholipid was detected in the pooled protein fraction. SDS-polyacrylamide gel electrophoresis (PAGE) of this delipidated surfactant (Figures 11, 12 and 13) revealed several major protein bands which appeared to be identical with those observed in dog serum protein delipidated in the same
TABLE III

Phospholipid/Protein Ratio of the Isolated and Reconstituted Surfactant Fraction

<table>
<thead>
<tr>
<th>Phospholipid/Protein Ratio of Original Isolated Dog Surfactant</th>
<th>Phospholipid/Protein Ratio of Reconstituted Dog Surfactant (Dens. 1.090)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6</td>
<td>1.2</td>
</tr>
<tr>
<td>3.8</td>
<td>2.2</td>
</tr>
<tr>
<td>3.8</td>
<td>1.5</td>
</tr>
<tr>
<td>4.0</td>
<td>2.3</td>
</tr>
<tr>
<td>3.9</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Figure 10. Surface tension - area diagrams of two preparations of lung washing. For each measurement, 150 μg of phospholipids were used. The numbers of the ordinate indicate the surface tension in dynes per centimeter. The number on the abscissa represents the per cent of total pool area. Dog Surfactant Fraction (Density 1.090) was very surface-active since it decreased the surface tension to zero dynes/cm. The fraction at density 1.045 was less surface-active. It decreased the surface tension to 13 dynes/cm.
### TABLE IV

<table>
<thead>
<tr>
<th>Description</th>
<th>Phospholipid/Protein Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original Surfactant</td>
<td>3.8/1</td>
</tr>
<tr>
<td>One Centrifugation Step</td>
<td>3.7/1</td>
</tr>
<tr>
<td>Two Centrifugation Steps</td>
<td>3.75/1</td>
</tr>
<tr>
<td>Three Centrifugation Steps</td>
<td>3.8/1</td>
</tr>
<tr>
<td>Four Centrifugation Steps</td>
<td>3.7/1</td>
</tr>
</tbody>
</table>
Figure 11. Protein distribution of dog serum (A) and dog surfactant (B) after four repeated centrifugation steps of the original surfactant material.
Fig. 12 Percent Protein Distribution of Serum, Supernatant and Surfactant Proteins.

One hundred micrograms of each protein sample was applied to a 10% (W/V) polyacrylamide gel containing SDS. Proteins were separated, stained with Fast Green and quantitated as described in the text.
Figure 13. Densitometric graph of dog surfactant (case 4) proteins, separated by SDS-polyacrylamide gel electrophoresis. The percent of protein composition was calculated by weighing the densitometric peaks.
manner as the surfactant material. However, in the surfactant, the bands were not as clearly separated as in the serum (Figures 12; 15). The major band, comprising about 45% of the total proteins had the same electrophoretic mobility as albumin. Two bands, having molecular weights of approximately 30-40,000 and 10-11,000 were not found in plasma proteins, even when the PAGE gels overloaded with 500 µg of plasma proteins (Figure 16). These two proteins were only detected in the pulmonary surfactant fraction (Figures 12-15).

Evaluation of the isolated surfactant suspended in 0.01 M Tris buffer pH 7.6 by circular dichroism revealed a double trough at 222 and 208 nm, typical of helical structures (Figure 17). An exact determination of the helical content of the surfactant material was not obtained because of interference of light-scattering artifacts by lipid and protein particles in the suspension; however, estimates suggested that surfactant has approximately 26 per cent α-helix content.

The circular dichroism study of the surfactant lipid-protein complex dissolved in 1% SDS and 0.01 M Tris buffer pH 7.6, also showed the double trough at 222 and 208 nm for helical structures (Figure 18). The calculated α-helix content of the isolated surfactant in SDS was 31.8. The trough at 222 nm seemed to be flattened by the interaction with SDS in comparison with spectra from serum lipoprotein in buffer (Figures 17, 18).
Figure 14. Densitometric graph of the proteins from supernatant fraction (case 4) separated by SDS-polyacrylamide gel electrophoresis. The per cent of protein composition was calculated by weighing the densitometric peaks.
Figure 15. Densitometric graph of dog serum proteins (case 4) separated by SDS-polyacrylamide gel electrophoresis. The per cent of protein composition was calculated by weighing the densitometric peaks.
Figure 16. Delipidated samples, containing 500 ug of each protein were applied to 10% (w/v) polyacrylamide gel, containing SDS.
Figure 17. Circular Dichroism Spectrum of isolated suspension in 0.01 M Tris buffer pH 7.6.
Figure 18. Circular dichroism spectra of A) surfactant protein and isolated surfactant, B) supernatant protein, C) serum protein and D) bovine albumin protein the reconstituted lipid-protein complexes of A) surfactant lipid-protein, B) surfactant lipid supernatant protein, C) surfactant lipid-serum protein and D) surfactant lipid-bovine albumin, dissolved in 0.01 M Tris, 1% SDS, pH 7.6. The numbers on the ordinate indicate the molar ellipticity expressed in $10^{-4}$ x deg cm$^2$/decimole.
While the study of these lipid-protein complexes in SDS introduced perturbation from the native spectrum of these proteins, the use of SDS is necessary to dissolve the protein and lipid-protein complexes for spectrometric studies. The differences in the spectrum observed for the various apoproteins and lipid-protein complex fractions described, led to a better characterization of these complexes and thus, justify the use of this procedure.

Reconstitution Experiments

Surfactant was initially delipidated by a procedure of Scanu using ethanol/ethyl ether mixtures (Figure 3). However, since proteins delipidated by this method were found to be insoluble in aqueous buffer, the partition chromatography procedure of Zahler and Weibel was utilized (60). This latter procedure resulted in the complete separation of lipid and protein components of the surfactant complex (Figure 4). Both the proteins and the lipids were soluble in the eluting solvent (2-chloroethanol-water, 9/1, v/v).

Proteins and lipids were dialyzed separately against buffer and isolated after centrifugation on discontinuous sucrose gradient on density greater than 1.12 and at density of 1.060, respectively.

Reconstitution is then effected by mixing in predetermined ratios, lipids and proteins dissolved in 2-chloroethanol. In most
reconstitution experiments, the reconstitution mixture prior to dialysis had a phospholipid/protein ratio of 1/1 (w/w). In some experiments, a starting mixture of phospholipid to protein of 4/1 (w/w) was used, in order to evaluate the preferential stoichiometry and stability of the reconstituted material. Reconstitution experiments were carried out on apoprotein and lipid mixture derived from lung surfactant. Also experiments were performed with bovine albumin, delipidated dog serum proteins and dog supernatant apoprotein in place of surfactant apoprotein. Substitution of these latter proteins for the native surfactant apoprotein in reconstitution experiments were carried out in order to compare the reconstitution properties of the surfactant proteins to other proteins found in serum and the lung. It is evident that surfactant proteins from lung contain albumin and other proteins which apparently originate from plasma proteins. However, it is not clear from the literature whether the surfactant proteins from the lung are simply a coincidental mixture of plasma proteins transudated into the lung or a group of proteins with a unique composition and properties. In addition, a series of reconstitution experiments were carried out using delipidated surfactant proteins substituting commercial egg dipalmitoyl phosphatidylcholine for surfactant lipids. The purpose of these experiments was to determine the influence of lipids on complex formation. Since surfactant contained large amounts of albumin and lecithin,
reconstitution experiments using bovine albumin and egg lecithin were also performed in order to study the most optimum conditions under which albumin and lecithin dissolved in 2-chloroethanol will form complexes after dialyzation against buffer.

In all reconstitution experiments with surfactant material or with the reconstitution control models, the complexes material appeared at a density which was different from that of the pure lipids or proteins, indicating a change in their properties induced by complex formation.

a. Reconstitution Experiments with Apoprotein and Lipids of the Surfactant Fraction

Reconstituted dog surfactant was placed on a discontinuous sucrose gradient by procedures similar to that utilized for the isolation of native surfactant. On such gradients, reconstituted material went either to a density of 1.090 or 1.12 (Table V). In a particular experiment, all the reconstituted material appeared to go to one or the other of these density gradients. This differed from the properties of native surfactant which always went to a density of 1.090.

The phospholipid-to-protein ratio (w/w) of reconstituted surfactant of 1.090 and 1.12 density varied from 1.2/1.0 to 3.3/1.0 (Table III). This is a low PL/Pr ratio compared to the native surfactant with PL/Pr ratio between 3.6 and 4.0 (Table III). In recombination experiments, 75% of the total available surfactant
TABLE V

Distribution of Proteins in Reconstitution Experiments with Surfactant Dog Lipids

<table>
<thead>
<tr>
<th></th>
<th>Albumin 1/1 + Surfactant Lipid</th>
<th>Albumin 10/1 + Surfactant Lipid</th>
<th>Surfactant* Apoprotein + Surfactant Lipid</th>
<th>Supernatant Apoprotein + Surfactant Lipid</th>
<th>Serum Apoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top Layer Dens 1.045</td>
<td>84.3%</td>
<td>25.8%</td>
<td>70.2%</td>
<td>17.6% ± 2.0</td>
<td>4.2% ± 1.2</td>
</tr>
<tr>
<td>Dens 1.090</td>
<td>88.3%</td>
<td>74.9%</td>
<td>825.8%</td>
<td>847.4% ± 5.1</td>
<td>829.3% ± 4.5</td>
</tr>
<tr>
<td>Dens 1.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>875.6% ± 1.7</td>
<td>837.3% ± 12.5</td>
</tr>
<tr>
<td>Below Dens 1.12</td>
<td>5.5%</td>
<td>-</td>
<td>4.0%</td>
<td>7.6% ± 4.0</td>
<td>829.2% ± 11.0</td>
</tr>
</tbody>
</table>

\[ ^b \text{standard deviation (from either three or four different cases)} \]
\[ ^a \text{indicates the per cent of protein which was incorporated into the reconstituted material (over 80\% of the lipids incorporated in this fraction).} \]
\[ ^* \text{recombination with Dog Apopr. and Dog Lipids resulted in two species.} \]
proteins were found to complex with the lipid and migrate to high densities in the sucrose gradient. Uncomplexed protein remained above in the lower density and below in the higher density of the sucrose gradient.

The protein of the reconstituted surfactant was delipidated over LH-20 in 2-chloroethanol-H_2O (9:1, v/v) and the apoprotein of reconstituted surfactant was compared with that of the native surfactant apoprotein by polyacrylamide gel electrophoresis. The proteins contained seven bands with identical mobilities and similar relative amounts as that of the native surfactant apoproteins. They included the two bands of molecular weight 30-40,000 and 10-11,000 not found in electrophoresis pattern of dog serum proteins (Figure 12).

Delipidated surfactant protein has a α-helix content in SDS of 28.1% (Figure 20, Table VI). Recombination of surfactant apoprotein with the dog lipid mixture increased the α-helical content to 31.8%, identical to the original amount of α-helix structure in SDS found for the native lipid-protein complex (Figure 20, Table VI).

b. Reconstitution Experiments with Supernatant Apoproteins and Surfactant Lipids

Reconstitution experiments were carried out with delipidated proteins isolated from the designated supernatant fraction of the
Fig. 20 The α-helix content of delipidated proteins and their lipid-protein complexes were determined as described in the text. In the samples with bars—-the percent α-helix represents the average of either four or five determinations. The bars indicate the range of the single determinations. In the case of samples without bars the α-helix content comparison was done only on one case.
### TABLE VI

Evaluation of Surfactant Dog Lipid and Protein in Complex Formation (obtained by reconstitution experiments)

<table>
<thead>
<tr>
<th>Per cent of Protein Incorporated into Complex</th>
<th>α-Helix Content in Per Cent Apoprotein</th>
<th>Lipid-Apoprotein Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog Surfactant Lipid and Dog Surfactant Apoprotein</td>
<td>75.2 ± 3.4</td>
<td>28.1 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dog Surfactant Lipid and Supernatant Dog Apoprotein</td>
<td>96.0*</td>
<td>28.3 ± 1.5</td>
</tr>
<tr>
<td>Standard Lecithin and Dog Surfactant Apoprotein</td>
<td>86.0</td>
<td>28.1 ± 1.4</td>
</tr>
<tr>
<td>Dog Surfactant Lipid and Bovine Albumin</td>
<td>8.3</td>
<td>40.0 ± 1.4</td>
</tr>
<tr>
<td>Dog Surfactant Lipid and Serum Apoprotein</td>
<td>22.8</td>
<td>34.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>indicates standard deviation (of at least four measurements).

*protein incorporation into three different species of reconstituted material.
centrifugation procedure (Figure 1) in place of the surfactant apoproteins. The delipidation procedures for these proteins were identical with those for delipidation of surfactant apoprotein. Reconstitution with surfactant lipids in a ratio of 1:1 phospholipids/protein (w/w) led to the formation of lipid-protein complexes which had different densities than the lipids and proteins by themselves when isolated by sucrose density gradient centrifugation. Twenty-nine per cent of the total protein migrated to a density of 1.090, 37% to a density of 1.12 and 29% to a density of more than 1.12 (Table V). Ninety-six per cent of the total protein recombined with lipid and migrated with lipid into the sucrose. Only 4% of the total protein remained free in the supernatant fraction. The phospholipid to protein ratio of the reconstituted material ranged from 1:1 to 4.5:1.0 (Figure 21). The protein of the recombined phospholipid-protein complex was delipidated over LH-20 in 2-chloroethanol-H2O (9-1, v/v) and the proteins of the formed complex were compared to the original supernatant proteins. The proteins contained several bands with the same distribution as that of the original apoprotein and serum (Figure 22). They did not contain the bands of 10,000 and 30-40,000.

The lipid-supernatant protein complexes were pooled and their circular dichroism pattern in SDS was studied at 222 nm. The 222 nm complex had an ellipticity of 0.781 x 10^4 deg cm^2/decimole and an α-helix content of 25.0%. Thus, the lipid-protein
Figure 21. Phospholipid/Protein ratio of reconstituted lipid-protein complexes. The original phospholipid/protein ratio before starting the reconstitution experiment was 1:1. Phospholipids and proteins were determined according to the methods cited in the test. Values represent the mean of at least four different samples. The bars indicate the range of values. Only one bovine albumin-dog surfactant lipid sample was determined.
Figure 22. Per cent protein distribution of delipidated reconstituted supernatant, surfactant protein-lipid complexes. Protein samples, containing 100 micrograms of protein were applied to 10% (w/v) polyacrylamide gels, containing SDS. Proteins were separated by electrophoresis, stained with Fast Green and quantitated as described in the text. The protein distribution in the reconstituted supernatant protein-surfactant lipid and surfactant protein-surfactant lipid complex was the same as in the original apoprotein fractions.
supernatant complex had 11% less ellipticity than that observed for the supernatant apoprotein in SDS (Figure 20, Table VI).

c. Distribution of Phospholipids in Original Isolated Dog Surfactant Reconstituted Material

The composition of the phospholipids of the different lipid-protein complexes was obtained by two dimensional paper-chromatography (63). Table VII shows the average values of the original isolated surfactant obtained from four different cases. Each determination of phospholipid distribution of reconstituted surfactant and supernatant protein-surfactant lipid complex formation was obtained from an individual case. Phosphatidylcholine was the predominant phospholipid (77%) in the surfactant fraction. Other phospholipids found in this fraction were lysolecithin, sphingomyelin, phosphatidylinositol, phosphatidylethanolamine and unidentified phospholipids. No significant changes in the phospholipid distribution between the original isolated surfactant and the reconstituted material was found.

d. Reconstitution Experiments with Surfactant Apoprotein and Com.Dipalmitoyl phosphatidylcholine

Surfactant apoprotein was reconstituted with com.dipalmitoyl lecithin leading to a lipid protein complex that migrated to a sucrose density of 1.12 in centrifugation. The phospholipid/
TABLE VII

Phospholipid Distribution in the Original Dog Surfactant and Reconstituted Material (expressed in per cent of total phospholipids)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LPC</td>
<td>8.5 ± 1.42</td>
<td>9.7</td>
<td>8.9</td>
</tr>
<tr>
<td>Sph</td>
<td>2.6 ± 0.45</td>
<td>2.8</td>
<td>3.4</td>
</tr>
<tr>
<td>PC</td>
<td>76.8 ± 1.00</td>
<td>75.0</td>
<td>75.3</td>
</tr>
<tr>
<td>PI</td>
<td>2.5 ± 0.88</td>
<td>4.0</td>
<td>3.7</td>
</tr>
<tr>
<td>PE</td>
<td>3.7 ± 0.97</td>
<td>3.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Unidentified PL</td>
<td>4.3 ± 0.97</td>
<td>5.1</td>
<td>4.4</td>
</tr>
</tbody>
</table>

LPC - Lyso-phosphatidylcholine  
Sph - Sphingomyelin  
PC - Phosphatidylcholine  
PI - Phosphatidylinositol  
PE - Phosphatidylethanolamine  
a - Standard deviation (four different cases)
protein ratio was found to be 1.6:1.0. Eighty-six per cent of the total surfactant proteins migrated with the lecithin to this density, 20% remained uncomplexed in the supernatant sucrose gradient.

The ellipticity observed for the complexed protein at density 1.12 at 222 nm in SDS was 0.837 deg x cm$^2$/decimole, similar to that observed for surfactant apoprotein in SDS.

e. Reconstitution Experiment with Delipidated Serum Proteins and Surfactant Lipids

Dog serum proteins were isolated and delipidated on LH-20 resin in 2-chloroethanol-$H_2O$ (9:1, v/v). Combination with lipids of the surfactant was carried out leading to complexes of lipid and protein migrating to densities of 1.090 and 1.12 in the sucrose gradient with phospholipid/protein ratios of 1.0/1.0 and 2.2/1.0 (Table V, Figure 21), respectively. Twenty-two per cent of the total delipidated serum protein combined with the dog surfactant lipids, 78% remained in the supernatant sucrose gradient fraction and in the gradient with higher density and did not complex with lipids. The ellipticity obtained for the combined lipid-protein complexes that migrated to density 1.090 and 1.12 was $0.682 \times 10^4$ deg cm$^2$/decimole representing a 37% decrease of $\alpha$-helical content from that calculated for delipidated serum protein in SDS ($\theta_{222} = 1.070 \times 10^4$ deg cm$^2$ decimole) (Figure 20).
f. Reconstitution Experiments with Bovine Albumin/Surfactant Apoprotein Mixtures and Surfactant Lipids

A bovine albumin/surfactant apoprotein mixture of 1/1 (w/w) ratio was reconstituted with surfactant lipids. A lipid-protein complex was formed that migrated to a sucrose density of 1.090 during centrifugation (Table V). The phospholipid/protein ratio of this complex was found to be between 1.0 and 4.5 (w/w). Seventy-five per cent of the total protein mixture migrated with surfactant lipid to a density of 1.090 sucrose on centrifugation, while 25% of the protein remained uncomplexed in the lesser and greater density sucrose gradient (Table V). The ellipticity observed in the complex was $1.24 \times 10^4$ deg cm$^2$/decimole (40% $\alpha$-helix) the same as it is for pure albumin in SDS (Figure 20).

A surfactant apoprotein/bovine albumin in a ratio of 1/10 (w/w) formed a complex with the surfactant lipids which was isolated on sucrose density of 1.090 on centrifugation (Table V). The phospholipid/protein ratio of this complex was found to be between 1.0 and 2.2. However, only 25% of the total protein mixture together with the surfactant lipids was incorporated into the complex. Seventy-five per cent of the total protein remained uncomplexed in the supernatant and higher density sucrose gradient (Table V). The ellipticity obtained for the combined lipid-protein complex was $1.15 \times 10^4$ deg cm$^2$/decimole (37% $\alpha$-helix) (Figure 20).
APPENDIX

This section includes certain pilot experiments, which are, however, not integral parts of the final experimental design.
STABILITY TEST ON ISOLATED RABBIT SURFACTANT

Centrifugation of rabbit pulmonary washing, on a gradient comprised of sucrose solution having densities of 1.045, 1.060 and 1.090, resulted in three bands. Twenty-four per cent of the material was separated on top of 1.045 with a PL/Pr ratio of 10.5/1.0 (w/w), 22% on top of 1.090 with a PL/Pr ratio of 5.6/1.0 (w/w) and 54% on top of 1.060 with a PL/Pr ratio of 9.7/1.0. The material isolated at the 0.45 M sucrose interphase (density = 1.060), designated surfactant, was further centrifuged on a discontinuous sucrose gradient containing 0.33 M, 0.45 M and 0.68 M sucrose, for 180 minutes at 23,000 rpm to investigate the stability of the phospholipid/protein ratio of this fraction. The new 0.45 M interphase material was then centrifuged again for 180 minutes at 23,000 rpm over a discontinuous gradient containing 0.33 M, 0.45 M and 0.68 M sucrose. The original surfactant material (density 1.060) was always found at the 0.45 M interphase (density 1.060). No bands were found on top of either 0.33 M or 0.68 M sucrose. The phospholipid-to-protein ratio of the original isolated surfactant material was 9.7/1.0, 9.5/1.0 after the second centrifugation and 9.2/1.0 after the third centrifugation.

In order to further investigate the composition of surfactant fractions, aliquots of the material obtained in the first, second and third centrifugations were layered on a continuous sucrose gradient ranging in density from 1.090 to 1.010. Gradients were centrifuged for 16 hours at 25,000 rpm in an SW 25.1 rotor.
Eleven fractions, each containing 2 ml, were collected after separation by puncture from the bottom. The fractions were dialyzed against 0.01 M Tris buffer, pH 7.4. The content of protein and phospholipid was determined on each fraction and the PL/Pr ratios calculated (Figure 19). The distributions of the PL/Pr ratios of the first, second and third centrifugations did not differ significantly.

RECONSTITUTION EXPERIMENTS WITH BOVINE SERUM ALBUMIN AND COMMERCIAL DIPHOSPHATIDYLCHOLINE

In pursuit of a better understanding of surfactant complex formation, we also investigated the complex formation of commercial lecithin with bovine albumin. The bovine serum albumin (delipidated) was combined with commercial diphosphatidylcholine by the general procedure utilized in reconstitution experiments. A lipid-protein complex was formed with 8% of the total protein that migrated to a density of 1.090 in the sucrose gradient and contained a phospholipid/protein ratio that varied between 4/1 and 20/1. Ninety per cent of the total protein remained uncomplexed in the supernatant sucrose gradient on centrifugation. The ellipticity found for the complexed lipoprotein SDS at 222 nm was $0.558 \times 10^4$ deg cm$^2$/decimole (10% $\alpha$-helix), representing a 55% decrease in ellipticity content from that of delipidated albumin in SDS of $1.240$ deg x cm$^2$/decimole (40% $\alpha$-helix) (Figure 20).
Figure 19. (----) Isolated rabbit surfactant (density 1.060) centrifuged on discontinuous sucrose gradient containing 0.33 M, 0.45 M and 0.68 M sucrose, for 180 min at 23,000 rpm to investigate stability. (---) The above isolated surfactant material (density 1.060) are centrifuged a third time over discontinuous sucrose gradient. (-.-.) For further investigation of the surfactant, compositions aliquots of the material obtained in the first, second and third centrifugations were separated on continuous sucrose gradient ranging in density from 1.090 to 1.010. Two milliliter fractions of the gradients were obtained and the phospholipid to protein ratios of these fractions calculated.
RECONSTITUTION EXPERIMENTS WITH BOVINE ALBUMIN AND COMMERCIAL
DIPALMITOYLPHOSPHATIDYLCHOLINE, DIALYZED AGAINST THIS BUFFER, pH 7.6, FREE OF CaCl$_2$ and MgCl$_2$

The CaCl$_2$ in the dialyzation buffer appeared to have no effect on the amount of lipid-protein complexes reconstituted nor on the quality of these complexes. Only ten per cent of the total proteins were reconstituted and the reconstituted material had an $\alpha$-helix content of 18%.

RECONSTITUTION EXPERIMENT WITH BOVINE ALBUMIN AND COMMERCIAL
LECITHIN UNDER STANDARD CONDITIONS WITH THE ADDITION OF EITHER
CHOLESTEROL OR PALMITIC ACID

It is generally known that cholesterol is a component of membranes. Thus, an experiment was performed where cholesterol was added to the lecithin/albumin reconstitution mixture.

The reconstituted material was collected at a density of 1.090 after centrifugation as described. Ten per cent of the total protein material formed a complex with the lipids and was isolated at a density of 1.090. The phospholipid to protein ratio was 8/1 (w/w).

Since it is known that albumin has several strong binding sites for free fatty acids (74), we were interested in determining the influence that palmitic acid would have on the complex formation of bovine albumin-lecithin. The obtained data suggested that
there was no influence either on the amount of albumin incorporated into the complex or on the phospholipid/protein ratio.

**RECONSTITUTION EXPERIMENT WITH BOVINE ALBUMIN AND CHARGED COMMERCIAL DIPALMITOYL PHOSPHATIDYLCHOLINE AGAINST 0.01 M ACETIC ACID PHOSPHATE BUFFER-pH 3.5, CONTAINING 0.01 M CaCl₂ and 0.001 M MgCl₂**

C. Sweet and J. E. Zull undertook a study of the binding of bovine serum albumin to lecithin as a function of pH, ionic strength and liposome charge. They were able to bind positively charged serum albumin strongly to negatively charged lecithin cholesterol-dicetyl phosphate liposomes by dialyzing these protein lipid mixtures against a buffer with a pH below that of the isoelectric point of albumin. In a modification of their procedures, we reconstituted a positively charged lecithin liposome albumin mixture in 2-chloroethanol by dialysis against 0.01 M acetic acid phosphate buffer, pH 3.5 containing 0.01 M CaCl₂ and 0.001 M MgCl₂. Ten per cent of the proteins were incorporated into the lipid-protein complex and the α-helix content was 18%, the same as that observed in the standard reconstitution of albumin and lecithin.
STABILITY TEST OF RECONSTITUTED RABBIT SURFACTANT

Isolated rabbit surfactant material was delipidated the same way as the dog material described under materials and methods for the dog surfactant. The reconstituted rabbit surfactant complex in 0.01 M Tris pH 7.6, containing 0.001 M MgCl₂ and 0.01 M CaCl₂ was spun down at 1500 rpm for 10 minutes in a Sorvall SS-34 rotor. The phospholipid to protein ratio was 1.0/1.0.

Half of the rabbit surfactant complex was layered on a discontinuous containing sucrose gradient, solutions having densities of 1.045, 1.060, and 1.090, and centrifuged for three hours at 48,000 x g. No bands were visible in the gradient but a sediment was obtained and suspended, dialyzed against 0.01 M Tris buffer, pH 7.6. The phospholipid to protein ratio of this surfactant complex was 2.1/1.0.
A. APPLICATION OF A PROCEDURE FOR CHARACTERIZATION OF ALVEOLAR LIPID-PROTEIN INTERACTIONS IN THE SURFACTANT SYSTEM

There has been no agreement among different investigators as to whether certain proteins of the alveolar surfactant film which is formed at the air-liquid interface of the alveoli represent an integral component of the surface active films. This dispute is largely due to the fact that until the present time, there has been no widely accepted method for the isolation of surfactant material from mammalian lung. Aside from this controversy, it has been widely accepted that proteins are always present in the alveolar space but it has not been known whether these proteins can interact with surfactant lipids under both normal and pathological conditions.

The present study was performed to investigate alveolar lipid-protein interactions by reconstitution of surfactant lipid-protein complexes from previously separated constituents. Several researchers have studied the reconstituted serum lipoproteins by adding micellar lipids to aqueous protein solutions (57, 58).
Because preliminary studies indicated that delipidated surfactant proteins were insoluble in aqueous media, the method of Zahler and Weibel (65) using 2-chloroethanol was employed for the reconstitution of alveolar surfactant complexes. Zahler and Weibel's method has several advantages. It has been shown that when 2-chloroethanol was used as a solvent for membranes, it did not destroy the lipid binding capacity of the proteins (65). In addition, both the protein and lipid components of surfactant could be totally dissolved in one solvent (2-chloroethanol) and then slowly dialyzed against aqueous buffer. During dialysis, it was possible to partially reconstitute the lipids and proteins. In addition, this procedure does not require the usual sonication step (Scanu), which could potentially result in artificial complex formation. A third advantage is that artificial surfactant lipid-protein complexes produced by sedimentation procedures (49) are completely separated when these complexes are dissolved in 2-chloroethanol. Accordingly, the choice of method for the original isolation of surfactant is not likely to affect the result of the recombination experiments. Finally, the separation of proteins and lipids in 2-chloroethanol is complete; thus, facilitating a more reliable evaluation of proteins using polyacrylamide gel electrophoresis. In previous procedures for analyzing surfactant proteins, traces of lipids were present leading to difficulties of the interpretation of the polyacrylamide gels.
B. PROPERTIES OF ORIGINAL SURFACANT FRACTION AND RECONSTITUTED LIPID-PROTEIN COMPLEXES

The original surfactant complex was found to be stable as indicated by the constant PL/Pr ratio after repeated density gradient centrifugations. In addition, the electrophoretically distinguished protein components of the surfactant material, which had been centrifuged several times, did not differ significantly from the original surfactant material. When the original surfactant lipid-protein complex isolated from dog washing was separated into lipid and protein components, they could be reconstituted into lipid-protein complexes, which retained some of the properties found in the original surfactant fraction. After gradient centrifugation, the reconstituted material had a density of 1.090 or 1.12. The original surfactant had a density of 1.090. There appeared to be no exact stoichiometry of phospholipid/protein. Rather, the original PL/Pr ratio in the recombination mixture, dissolved in 2-chloroethanol appeared to predetermine the PL/Pr ratio in the formed complex after dialyzation against the buffer. Following repeated centrifugation, the PL/Pr ratio of the original surfactant fraction remained essentially the same (Table IV). On the other hand, the PL/Pr ratio of the recombined surfactant complex changed after repeated gradient centrifugation. The apparently higher stability of the original isolated surfactant material could be due to the fact that for the isolation of the original surfactant sedimentation was used, while in the isolation
of the reconstituted lipid-protein complexes this step was omitted. This interpretation is supported by electron microscopic evaluation of original surfactant fractions and reconstituted complexes using the negative staining technique (unpublished data, collaborative work with Dr. J. U. Balis). These studies revealed that liposomes of lung washing were relatively uniform in size measuring from 200 \( \AA \) - 1000 \( \AA \). On the other hand, liposomes of the isolated surfactant fraction (obtained by the Frosolono technique, which involved sedimentation) was composed of large aggregates, which varied widely in size measuring up to 10,000 \( \AA \). By contrast, liposomes of the reconstituted material were smaller in size and more uniform resembling those found in the original lung washing. This finding suggests that large surfactant particles are stable on repeated gradient centrifugation over sucrose while the small surfactant particles are unstable after repeated centrifugation. Therefore, the increase of stability of the original surfactant fraction is the result of the sedimentation procedure involved.

The composition of protein associated with the surfactant fraction was similar to that of dog serum with the exception that two additional bands having molecular weights approximately 30-40,000 and 10-11,000 were present in surfactant protein. These two additional proteins were observed in both the original and reconstituted surfactant complex and represented 7 and 8% of the total surfactant proteins, respectively.
King and Clements isolated dog surfactant using an elaborate procedure which included eight different centrifugation steps (39). Ten per cent of the surfactant material consisted of proteins. About half of the proteins were composed of a 10,000 dalton protein. In testing the immune properties of this specific protein as a basis for quantitative assay of surface active material, King and Clements found that this protein is more concentrated in the purified surface-active material than in the whole homogenate. The enrichment was four-fold based on the content of phosphorus (phospholipids) and fifty-fold relative to the amount of the surfactant specific protein. In concurrence with our results and those of King and Clements, it follows that the surfactant specific proteins have a high affinity to phospholipids. This affinity to phospholipids appears greater than to other proteins which have originated in the serum. The possibility cannot be excluded, however, that the above enrichment is related to centrifugation procedure. The latter possibility is supported by our finding that further enrichment of specific proteins or phospholipids does not occur following reconstitution of surfactant lipids and proteins. Following delipidation of the surfactant complex, there was a 12% decrease of ellipticity in the surfactant proteins. When surfactant proteins were recombined with surfactant lipids, there was a 12% increase in ellipticity at 222 nm. The ellipticity of the recombined surfactant and the original surfactant was identical. The increase in ellipticity was only observed when the
recombination experiment was carried out with delipidated surfactant apoprotein and surfactant lipids. An increase in negative ellipticity at 222 nm was not observed when surfactant lipids were complexed with either designated supernatant proteins, delipidated bovine albumin or delipidated serum proteins. Likewise, the ellipticity was not increased following reconstitution of lecithin with surfactant apoproteins. These findings suggest that the mixture of surfactant lipids interact in a characteristic manner only with surfactant proteins and not with supernatant or serum proteins.

Significant differences between circular dichroism spectra of lipid-free and lipid-bound proteins were also reported for serum lipoproteins. Scanu et al. studied the structure of human serum high-density lipoproteins and found that the delipidated lipoproteins (apoprotein of HDL) had a lower \( \alpha \)-helix content calculated from the ellipticity at 222 nm than the heavy density lipoprotein with the bound lipids (57). Scanu's studies were done in the absence of detergent. Scanu et al. felt that the increase of \( \alpha \)-helix content in the formation of heavy density lipoproteins from its apoprotein and lipid constituents reflected a change in length, tightness, or orientation of the various helical segments of the polypeptide chain on the bindings of lipids. Alternatively, the changes in the CD may not reflect true conformational changes of the protein's secondary structure, but rather a change in the environment around the active chromophores of the proteins on complexing with lipids.
Lux et al. studied the influence of lipids on the conformation of human plasma high density apolipoproteins (58). The native high density lipoprotein contained about 70% α-helix, calculated from its ellipticity at 222 nm. Upon delipidation, the helical content decreased by 20% corresponding to an increase in the disordered structure of the protein. Lux et al. calculated that 20 to 30% of the amino acid residue in HDL apoprotein may be involved with the helix-disordered transition, which appeared to occur when lipid was removed from the serum lipoproteins. Although it was not clear to Lux et al. how lipids can influence protein folding, they felt that bound lipids would decrease the local polarity and produce an environment for the protein of low dielectric strength which would favor an increase in α-helix structure in the proteins.

Morrisett et al. investigated the interaction of apolipoproteins from very low density lipoprotein with phosphatidylcholine (75). When a solution of apolipoprotein was titrated with a sonicated dispersion of phosphatidylcholine, the CD spectrum indicated the α-helical content in the formed complex increased from 22% to 54%. The formed complexes consisted of heterogeneous population of species when investigated on gradients. In agreement with the results obtained by Scanu (57) and Lux (58), the study of Morrisett et al. (75) also suggested that a shift from a disordered to a helical secondary protein structure of the protein is mediated by the interaction with phospholipids and that
disordered regions in the protein structure were changed into helical segments.

In a study on light density serum lipoproteins, Scanu found that when LDL was dissolved in SDS, most of the lipids were preferentially partitioned into the detergent phase, leaving the proteins with some bound SDS (68). Accordingly, he concluded that the spectrum of LDL in SDS is the same as that of a delipidated protein.

Rottem et al. investigated the lipid-protein interactions of Mycoplasma membranes in the presence of SDS by circular dichroism (69). They found that the lipid-protein interactions had little or no effect on secondary protein structure. However, they could not exclude the possibility that the effect of the added lipids on the secondary protein structure was inhibited by the binding of SDS to the hydrophobic sites of the membrane proteins.

It has been suggested that the microenvironment of the amino acid residues of apoproteins which are involved in binding lipids is changed from a polar (aqueous) to a non-polar environment, when lipids bind, producing a weakly hydrogen-bonding environment (58). In such an environment, peptide groups would be able to form helices by forming hydrogen bonds with each other. The well known influence of 2-chloroethanol and anionic detergent such as SDS on increasing helix formation, is believed to act in an analogous way (70, 71). It may be inferred from the above discussion that SDS should be avoided for the solubilization of lipid-protein complexes.
in experiments designed for the study of lipid-protein interactions. However, this assumption is not supported by our CD data on lipid-protein interactions, which were all performed in the presence of SDS to solubilize the lipid-protein complexes. It is known that SDS introduces perturbation in the CD spectra. However, significant differences were observed from our control studies using other proteins (supernatant proteins, albumin and serum proteins) and lipids (surfactant lipid and lecithin). These results indicate that the lipids of the surfactant fraction (but not surfactant lecithin alone) have a supporting effect on the α-helix content of the surfactant proteins in spite of the presence of SDS.

C. EFFECT OF PLASMA PROTEINS ON THE RECONSTITUTION OF SURFACTANT LIPID-PROTEIN COMPLEXES

About 40% of the dog surfactant protein appeared to be composed of albumin. It was felt, therefore, that a binding study of the serum albumin to dipalmitoyl phosphatidylcholine could provide information about the specificity of lipid-protein interaction in the surfactant system.

Sweet et al. had described a binding study of serum albumin to lecithin (72). However, these investigators had to charge lecithin micelles (originally with a zero net charge) to a
negatively charged micelle complex by adding dicetylphosphate to the lecithin mixture in order to obtain significant lipid-protein complexes in aqueous media. The optimal interactions between albumin and lecithin liposomes were obtained at the pH range of 3.5 to 3.7, just below the pI of albumin. At these pH values, albumin is positively charged. Their findings suggested that electrostatic forces may be involved in the interaction. High ionic strength up to 1.0 M NaCl broke up the complexes but the appearance of rebinding at higher salt concentrations (up to 1.5 M) suggested that non-ionic interactions were also involved in the complex formation of albumin and charged lecithin liposomes.

In the reconstitution experiments with albumin, utilizing the standard procedure (solvent-2-chloroethanol-dialyzation against 0.01 M Tris pH 7.6), I was not able to find more than 10% of the albumin complexed to the lecithin or surfactant lipids. The findings were actually not too surprising since no natural phospholipid albumin complexes are known. The addition of either cholesterol or palmitic acid in other experiments had no effect on either the amount of albumin incorporated or the complex formed as far as CD data were concerned. In further investigations of the lipid-protein interactions of surfactant, I incorporated the method of Sweet and coworkers to the 2-chloroethanol method and evaluated the complex formation of albumin and lecithin.

Lecithin dissolved in chloroform was charged with dicetylphosphate, dissolved in 2-chloroethanol and albumin added. After
dialyzation against 0.01 M acetic phosphate buffer of pH 3.5, containing 0.01 M CaCl₂ and 0.001 M MgCl₂ and isolation of the complex on discontinuous sucrose gradient, I found that only ten per cent of the albumin was incorporated into the complex. From this finding and the identical albumin incorporation with out standard recombination procedure, I may conclude that electrostatic charge does not have a significant effect on the binding of albumin to lecithin when reconstituted by our procedure. Rather, albumin is probably either trapped by the large amount of lecithin or bound to the lecithin by non-ionic forces.

While there was only minimal incorporation of albumin as well as proteins from delipidated dog serum into complexes with surfactant lipid, the presence of surfactant protein was found to promote incorporation of albumin into lipid-protein complexes and to increase the negative ellipticity of 222 nm of the complex. Thus, when half of the delipidated albumin was substituted with surfactant proteins, 75% of the protein mixture was incorporated into complexes with surfactant lipid. Control studies showed that supernatant proteins of alveolar washing easily form complexes with surfactant lipids. However, the finding of scattered densities of the lipid-protein complexes may point to a more random process of recombination. In addition, there was no increase in ellipticity following the formation of these lipid-protein complexes. The above finding indicates that the surfactant proteins
interact differently with surfactant lipids than the supernatant, serum or albumin proteins.

Further investigations utilizing, among others, the introduced *in vitro* model employed in this study should be designed to determine (a) the ability of various lipid or protein components of the surfactant system to promote formation of lipid-protein complexes; (b) whether lipid-protein interactions are involved in the transport of lipids from the cells through the alveolar hypophase to the surface film and (c) whether different types of lipid-protein interactions occurring in normal versus edematous conditions of the lung modulate the functional state of the surfactant system.
BIBLIOGRAPHY


APPROVAL SHEET

The dissertation submitted by Peter Pagast has been read and approved by a committee from the faculty of the Graduate School.

The final copies have been examined by the director of the dissertation 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 content, form and mechanical accuracy.

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

May 15, 1975
Date

Signature of Advisor