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PHYSICOCHEMICAL PROPERTIES OF DIPALMITOYL PHOSPHATIDYLCHOLINE AFTER INTERACTION WITH AN APOLIPOPROTEIN OF PULMONARY SURFACTANT

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Summary

We studied the interaction between an apolipoprotein of pulmonary surfactant and the principal lipid found in this material, dipalmitoyl phosphatidylcholine. The apolipoprotein was extracted from canine surfactant and purified to greater than 90% homogeneity. The apolipoprotein was mixed for 16 h at room temperature with dipalmitoyl phosphatidylcholine dispersed in a buffer containing 0.1 M NaCl and 3 mM $CaCl_2$. Unbound lipid, unbound protein, and recombinants of lipid and protein were separated by density gradient centrifugation. 71% of the apolipoprotein was found associated with dipalmitoyl phosphatidylcholine. In comparable experiments using bovine plasma albumin about 13% of the albumin was recovered with the lipid. The physicochemical state of the lipid in the apolipoprotein-lipid complex was modified after binding of the protein. A distinct phase transition at 42°C could no longer be detected, and the rate of adsorption to an air-liquid interface of the apolipoprotein-lipid complex was greater than that of the lipid alone. Surface tension vs. surface area isotherms of the dipalmitoyl phosphatidylcholine-apolipoprotein materials, however, were similar to those exhibited by pure dipalmitoyl phosphatidylcholine.

The results suggest a physiological role for this apolipoprotein. It may bind to dipalmitoyl phosphatidylcholine under conditions expected in vivo, and may modify the physical properties of the aggregated dipalmitoyl phosphatidylcholine to form domains of lipid in a liquid-crystalline array. The complex of dipalmitoyl phosphatidylcholine and apolipoprotein would have the physical properties necessary for its physiological function, allowing it to adsorb to the

Abbreviation: DPPC, dipalmitoyl phosphatidylcholine.

alveolar interface and reduce its surface tension to less than 10 dynes/cm. Dipalmitoyl phosphatidylcholine, by itself, is in a gel-crystalline array below its phase transition temperature (42°C) and would be incapable of effecting these actions.

Introduction

The forces developed across the alveolar surfaces of the lung present a continuing stress to its mechanical stability. The interfacial free energy at the surface of alveolar gas and liquid induces a force tending to collapse alveolar units. Alveoli must either be stabilized by a large distending pressure, or the interfacial tension must be reduced by the adsorption to the surface of a specialized material, pulmonary surfactant [1]. The physiological functions of this material have been studied for several years and are now generally understood [2], and clinical studies have shown that this substance is absolutely vital for life [3]. However, many of the basic physical and chemical properties of pulmonary surfactant, which will affect its actions at the alveolar air-liquid interface, are still unsettled [4]. Pulmonary surfactant is isolated as a complex comprised of neutral lipids, phospholipids, and (usually) protein [5]. During various aspects of its metabolic life cycle it undergoes structural rearrangements [6] including its transition in the alveolar lumen to a configuration showing unique long-range order [7]. All forms of this material probably represent the packing of phospholipids and neutral lipids in extended lamellar arrays. Whether the composition of the lipids changes during these structural transformations is not known with certainty.

There are at least two proteins found in canine pulmonary surfactant [8]. One has a nominal molecular weight of 35 000—45 000; the other is a small peptide (or group of small peptides) of molecular weight equal to or less than 10 000—12 000. Both proteins contain carbohydrate and the nominal molecular weights are provided principally for identification. The larger protein will be referred to in this manuscript as 'apolipoprotein A'; the smaller the 'apolipoprotein B'. The results of metabolic experiments show that the larger protein (apolipoprotein A) is secreted into the alveolar lumen with the same time course as the lipids of surfactant, and suggest that the 10 000 peptide (apolipoprotein B) may be a proteolytic fragment [9]. It is probable that apolipoprotein A is an integral part of the surfactant complex during at least some of the steps in its metabolic cycle [10]. Whether this apolipoprotein interacts with the lipids to help regulate their stoichiometry, induces or stabilizes structural transformations, or in some other way modulates the physiological activities of surfactant, is unknown.

A study of the effects of recombining the apolipoproteins recovered from surfactant with its lipids has not been reported, conceivably because of technical difficulties. Ordinary methods of lipid extraction [11,12] result in irreversibly aggregated proteins which cannot be dissolved in aqueous solutions. We have developed other methods for separating apolipoprotein A from its associated lipids, and for solubilizing and purifying it [13]. The ability to deal with this troublesome technical problem together with the increasing evidence

which indicates that apolipoprotein A is specific to surfactant [8] have encouraged us to undertake experiments investigating the interaction between this protein and quantitatively important lipids found in pulmonary surfactant. The results of experiments investigating the binding between the apolipoprotein and the principal lipid in surfactant, dipalmitoyl phosphatidylcholine (DPPC), are reported here. The apolipoprotein has been found to interact with multilayer lamellar arrays of lipid under gentle test conditions. The physical state of the lamellar arrays changes upon binding the protein, and this is reflected in modified surface properties exhibited by the protein-lipid complex. The results suggest that this apolipoprotein can interact with lipids under conditions expected to be found in a physiological environment, and that the consequences of this interaction may be important to normal respiratory function.

Materials and Methods

Isolation and characterization of the apolipoprotein

The isolation of the apoproteins of pulmonary surfactant was carried out using a modification of a method reported previously [13]. Pulmonary surfactant was purified from canine lavage fluid by density centrifugation [14] and stored at −15°C in aliquots suitable for individual experiments. Just before use, the frozen surfactant was thawed at room temperature and pelleted by centrifugation at 40 000 rev./min for 60 min in a Beckman No. 40 rotor. The pellet was dispersed by hand homogenization in 2 ml of 0.3 M lithium diidosalicylate made up in 0.05 M pyridine, pH 8.4. Hand homogenization of the pellet was continued for 30 min in an ice bath. 2 ml of water and 4 ml of cold butanol were added and the suspension was mixed vigorously by vortexing, and separated into two phases by centrifugation. The upper alcohol-rich phase was removed and a volume of butanol/ethanol (6 : 1, v/v) equal to the remaining lower phase was added to the lower phase. The latter step was repeated twice. The lower phase was made up to 4 ml with water, and the butanol/ethanol (6 : 1, v/v) extractions were conducted. A total of nine butanol/ethanol (6 : 1) extractions were carried out on the surfactant. The aqueous phase material was lyophilized to remove the pyridine, dissolved in 1 ml of water, and centrifuged at 10 000 rev./min for 30 min (Beckman J-21) to remove any debris. The aqueous solution contained the higher molecular weight apolipoprotein together with small amounts of contaminating serum albumin. The alcohol-rich upper phase contained lipid, lithium diiodosalicylate and apolipoprotein B.

To separate the serum albumin from the apolipoprotein we passed the aqueous solution of proteins over a column of Cibacron Blue F3GA coupled to agarose gel beads (Bio-Rad Laboratories, Richmond, CA, Affi-Gel Blue) and eluted the column with a 0.01 M sodium borate buffer, pH 7.1. The apolipoprotein was eluted in the first 3 ml, while albumin was retained. The purity of the protein was characterized by immunological reactions with antisera developed against canine serum proteins and against canine surfactant, and by polyacrylamide gel electrophoresis using the systems of Weber and Osborn [15], and Neville [16]. The concentration of protein was determined from its absorbance

at 277 nm, after determining the weight extinction coefficient by quantitative amino acid analyses.

We tested for the presence of residual lipid bound to the purified apolipoprotein using quantitative gas-liquid chromatography. 350 μg of apolipoprotein, together with 7 μg of diheptadecanoic phosphatidylcholine added as an internal standard, were dried under N_2. To the mixture of apolipoprotein and lipid standard were added 0.6 ml of 5% hydrochloric acid in methanol, and the solution was heated in a closed vial for 24 h at 70°C. The hydrolysate was cooled to room temperature and mixed with 1 ml of water and 2 ml of hexane to form a two-phase system. The fatty acid methyl esters were recovered in the hexane-rich phase. The hexane phase was dried under nitrogen and injected into a gas-liquid chromatograph (Hewlett-Packard Model 5830A). Fatty acid methyl esters were separated on a 6 ft column of 10% SP 2340 on 100/120 mesh Supelcoport (Supelco, Belleforte, PA) run isothermally at 178°C. Quantification of bound fatty acids found in the apolipoprotein was made by comparison with the amount of heptadecanoic methyl esters obtained from the internal standard.

Characterization of lipids used for interaction studies

Dipalmitoyl phosphatidylcholine was purchased from Research Products, Inc. or from Sigma Chemical Company. The lipid obtained from both suppliers migrated as a single spot in silicic acid chromatography even when the silicic acid plate was purposely overloaded. The phosphatidylcholine consisted of greater than 98% palmitic acid residues. The lipid contained 4.2% phosphorus by weight.

Interaction studies

The interaction of purified apolipoprotein with dispersions of dipalmitoyl phosphatidylcholine was carried out by mixing a fixed amount of apoprotein with varying concentrations of lipid for 16 h at room temperature. Sonication was not used to either prepare the dispersions of lipid or to effect the interaction. An appropriate amount of lipid, taken from a stock solution made up in methanol and chloroform, was dried in a Dounce homogenizer under nitrogen. The lipid was dispersed in a buffer of 0.05 M boric acid (pH 7.4) containing 0.10 M sodium chloride and 3 mM calcium chloride and 16—19% sucrose, by gentle homogenization at room temperature. 3 mM Ca^{2+} was included because of the invariable presence of divalent cations in physiological fluids. Lipid concentrations varied from 0.1 to 2.5 μM of lipid/ml. Protein was added to the lipid suspensions to give a final concentration of protein of 11 μg/ml, or $0.31 \cdot 10^{-3}$ μmol/ml calculated by assuming a monomeric molecular weight of 35 000. Thus the molar ratio of lipid to protein in the mixtures ranged from 320 to 8000. The mixtures of lipid and protein were placed in Teflon tubes and allowed to mix overnight at room temperature using a rotary mixing device operated at 10 rotations/min. After 16—18 h the dispersions were poured into a centrifuge tube in a continuous gradient which varied linearly from 16% to 19% from the top to bottom of the gradient. In later experiments we decreased the density difference between the extremes of the gradient by setting up the experiment using 16% and 17% sucrose, respectively. The density gradients

were centrifuged at either 65 000 rev./min for 48 h or 50 000 rev./min for 48 h at 20°C, using a Beckman L5-65 centrifuge. At the completion of the centrifugation we removed bands which had formed within the gradient tubes, and regions where dispersed lipid was not visible but where soluble proteins may have sedimented. For each experiment conducted with mixtures of lipid and apolipoprotein we also set up, in different centrifuge tubes, lipid mixed with bovine serum albumin (Sigma Chemical, crystallized), and lipid without added protein.

We quantified the protein found in the fractions eluted from the density gradients by titration of these fractions with fluorescamine [17]. Since fluorescamine will only react with the terminal primary amine on the protein and with primary amines found in side chains, we increased the sensitivity of the assay by partially hydrolyzing the protein with pronase [18] prior to assay. To aliquots estimated to contain 1—6 μg of protein, we added 0.1 ml of solution of pronase in a concentration of 5 μg/ml. We incubated the samples at 37°C for 24 h in 0.05 M boric acid, 0.10 M NaCl and 3 mM $CaCl_2$, pH 7.4. We then added 0.7 ml of 0.05 M sodium borate buffer, pH 9.0, to bring the pH to about 9, and reacted the proteins with 0.2 ml fluorescamine dissolved in acetone at a concentration of 1.5 mg/ml. All samples were brought to 3.7 ml with 0.5 M sodium borate. Fluorescence yield was measured with a filter instrument (Turner, Model 111) using a 405 primary filter and a 65A secondary filter. Similar procedures were followed with the dispersions containing albumin and lipid, and with the lipid alone. For each experiment separate standard curves were obtained by taking serially increasing volumes of the dispersions prior to centrifugation. Background provided by the lipid and other reagents varied with the batch of fluorescamine that was used, but was generally about 10% of the fluorescence yield obtained with the protein samples and was subtracted from each sample appropriate for the volume of the aliquot.

We obtained standard curves using albumin and apolipoprotein A. As little as 0.5 μg of protein could be quantified, and the relative fluorescence increased linearly with increasing amounts of protein. The fluorescence yields obtained from the two proteins were similar when expressed per unit weight, suggesting that extensive enzymic hydrolysis had occurred. Without hydrolysis the fluorescence yields were barely detectable.

Lipids were partitioned out of the aqueous phase by the method of Bligh and Dyer [12] and were quantified by phosphorus assay [19].

We investigated the extent to which DPPC was degraded to lysophosphatidylcholine during the time interval of mixing and centrifugation. We added radioactively labeled DPPC to mixtures of apolipoprotein-DPPC, or to pure DPPC and carried out the procedures described above. L-α-[*palmitoyl*-1-^{14}C]Dipalmitoyl phosphatidylcholine was obtained from New England Nuclear. The labeled DPPC was mixed with the unlabeled DPPC, and the mixture was dried under N_2 prior to suspending the lipid in buffer. Apolipoprotein was added to the dispersions in a lipid to protein stoichiometry of 500 to 1, the dispersions were mixed for 74 h at room temperature, and the lipids were extracted. Carrier lysophosphatidylcholine and palmitic acid were added to the extracted lipids, and the lipids were separated by silica gel thin-layer chromatography. The lysophosphatidylcholine, phosphatidylcholine and fatty acid spots were

visualized with iodine vapor, and directly scraped into counting vials to quantify the extent of the degradation of label. In four experiments we found that the DPPC, when mixed with the apolipoprotein, was degraded by an average of 2.4%. DPPC, by itself, was degraded to a slightly greater extent. We conclude, therefore, that the apolipoprotein solution did not contain a contaminating phospholipase which catalyzed the hydrolysis of the lipid.

Experiments on physical properties

Thermal phase transitions. We investigated changes in physical state associated with thermal phase transitions by measuring the change in light scattering at 600 nm concomitant with a change in temperature. Dispersions of DPPC or of DPPC and protein were suspended in the centrifugation buffer and their absorbance was measured as temperature was varied from 25°C to 48°C. We used a Gilford Model 2530 spectrophotometer whose cuvette chamber was thermally regulated with an external water bath. The dispersions were heated at a rate of approximately 0.4°C/min with intermittent stirring, and temperature was monitored with a Yellow Springs Model TGB thermistor inserted directly into the cuvette. Measurements were made at one degree intervals, and the curvettes were stirred vigorously with the thermistor probe just prior to each measurement. Similar experiments were carried with egg phosphatidylcholine. In a few experiments we investigated the effects of monovalent and divalent cations on the phase transition by carrying out experiments in a buffer of 0.01 M EDTA titrated to a pH of 7.0 with HCl. The water used for all solutions was purified with a Millipore water system.

Surface balance studies. Our experiments were of two types, both conducted at 25°C: (1) measurement of the rate of adsorption to an air-liquid interface, and (2) recording of the surface tension vs. surface area isotherm of the adsorbed constituents as the surface area was compressed from a surface area of 125 cm^2 to a surface area of 22 cm^2. All experiments were carried out in a carefully defined manner so that differences in the surface properties of the lipid-protein recombined materials could be distinguished from those of the lipids alone. The materials recovered from the density gradients were suspended in 0.05 M boric acid buffer, pH 7.4, containing 0.10 M NaCl and 3 mM $CaCl_2$, and added to the Teflon trough of a surface balance. The surface balance was constructed to specifications similar to those used in previous studies [5] and has been described in some detail. The present equipment was modified from that used in prior studies to allow stirring by Teflon stirring bars extending through the surface into the subphase. Changes in the surface tension of the surface phase were recorded with time.

Dispersions of lipid or lipid and protein were gently mixed with the boric acid buffer to give a concentration of about 10 μg/ml phospholipid in a volume of 170 ml. This was poured into the Teflon trough and mixed with the stirring bars at a mixing rate of 85 rev./min for 15 min. This ensured uniform mixing of the lipid dispersion within the buffered subphase. At the end of this equilibration period the stirring was stopped and the surface was compressed to minimum area with a tight-fitting Teflon barrier. The surface was aspirated to give a clean surface (73 dynes/cm at 25°C), and the surface was reexpanded to maximum area. The platinum paddle was cleaned by flaming and was reinserted

into the surface. Upon recommencement of stirring the measurement of adsorption rate was begun by recording surface tension on a strip-chart recorder. After 60 min the stirring was stopped, the stirring paddles were carefully removed from the subphase, and a surface tension vs. surface area curve was recorded by compressing the surface at a rate of 93 cm^2/min and simultaneously recording surface tension and surface area on an x-y recorder. In separate experiments we compared the curves of the material entering the surface by adsorption with those films obtained by spreading these same materials directly on the surface from a isopropanol/buffer (3 : 1) suspension. Spreading was done at a surface area of 233 cm^2, and the surface was compressed at 93 cm^2/min to a minimum area of 22 cm^2. All experiments were done at room temperature.

Results

Characterization of apolipoprotein

The isolated apolipoprotein migrates in polyacrylamide gel electrophoresis with a nominal molecular weight of 35 000—40 000 (Fig. 1). The protein runs in electrophoresis with a molecular weight equal to or greater than 70 000 if sulfhydryl-reducing agents are omitted from the electrophoresis procedure. It reacts with antiserum developed against canine surfactant, but not against anti-

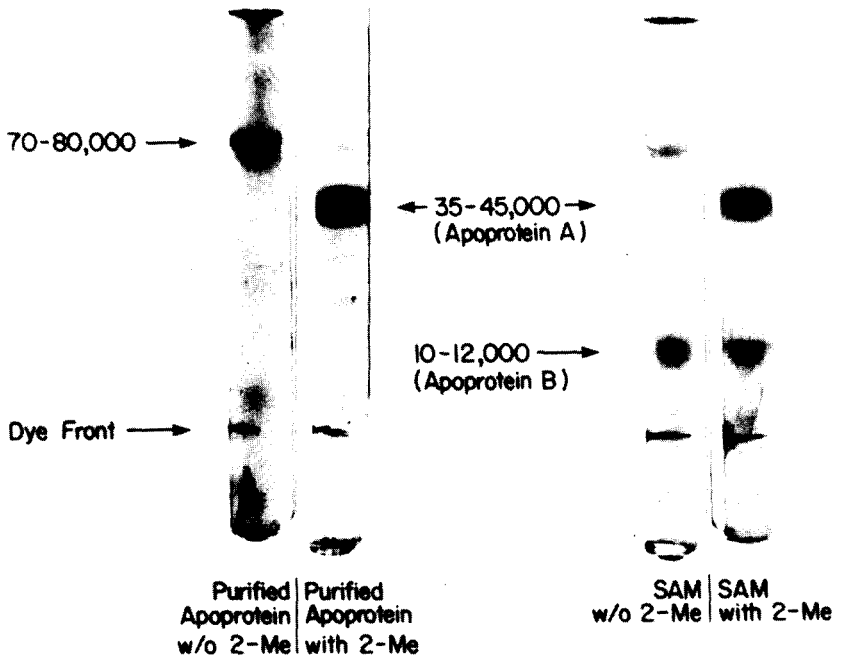

Fig. 1. Polyacrylamide gel electrophoresis of the purified surfactant (SAM) and of apolipoprotein A isolated from surfactant. Electrophoresis was conducted using the method of Weber and Osborn [15] in 6% acrylamide gels, with samples run with and without 2-mercaptoethanol. Greater than 60 μg of apolipoprotein were placed on each gel in order to detect small amounts of impurities.

serum to canine serum proteins. A solution of 1 mg/ml gives an absorbance of 1.50 at 277 nm, as determined by quantitative amino acid analyses. There is an absence of an absorbance peak at 320 nm, which is an absorbance band characteristic of lithium diiodosalicylate [20]. Based upon the sensitivity of our absorbance measurements, and the extinction coefficient of lithium diiodosalicylate [20], we calculate that the apolipoprotein contained less than 0.04 mol of residually bound lithium diiodosalicylate/mol of protein. Amino acid analyses show that the apolipoprotein contains hydroxyproline [21,22]. There was less than 0.2 μmol of fatty acid is associated with each μmol of apolipoprotein. We assume that this very small amount of fatty acid may represent contaminant. These results suggest that fatty acids are not covalently bound to amino acid residues of the apolipoprotein.

Quantitative scanning of the polyacrylamide gels indicates that our apolipoprotein preparation is about 90% pure. The gel shown in Fig. 1 was deliberately overloaded to view the presence of small amounts of impurities. We have sought chromatographic methods which would enable us to further purify the preparation with yields adequate to conduct these studies, but we have, so far, been unsuccessful.

Interaction of proteins with DPPC

We carried out 14 experiments investigating the interaction of the apolipoprotein with dispersions of DPPC. The density gradients used to separate the lipid-protein recombinant materials from the unbound lipid and unbound protein were sufficiently shallow so that materials containing molar ratios of lipid to protein of less than 8000 could be resolved from the unbound lipid and protein reactants. We used a SW 65 rotor which contained three buckets. In all experiments one bucket held a gradient of dipalmitoyl phosphatidylcholine mixed with apolipoprotein, the second dipalmitoyl phosphatidylcholine and bovine plasma albumin, and the third dipalmitoyl phosphatidylcholine by itself. This experimental protocol, therefore, allowed us to measure on every experiment the positions in the gradient of the lipid and lipid-protein bands, and the binding ratios of the lipid-protein recombinants.

The resolution of the constituents in the density gradient is illustrated in Fig. 2. Tube A contained only lipid; tube B lipid and apolipoprotein A; and tube C lipid and albumin. Two bands from tube B were easily resolved. The top band was comprised of material with a DPPC to apoprotein ratio of $8.8 \cdot 10^3$ and contained 25% of the apolipoprotein associated with lipid. The bottom band had material with a lipid to protein ratio of $1.35 \cdot 10^3$ and comprised 75% of the bound apolipoprotein. The DPPC to albumin ratio of the band in tube C was 14 800. 69% of the albumin was unassociated with lipid and was recovered at the bottom of the tube.

Apolipoprotein of surfactant. An average of 71% of the recovered apolipoprotein was associated with lipid. 24% of the protein was recovered at the bottom of the density gradient and was not bound to lipid. About 5% of the protein was unresolved in the gradient. All recovered lipid was resolved into bands.

82% of the bound apolipoprotein (calculated as an average from all experiments) was associated with lipid in a molar stoichiometry nearly identical to

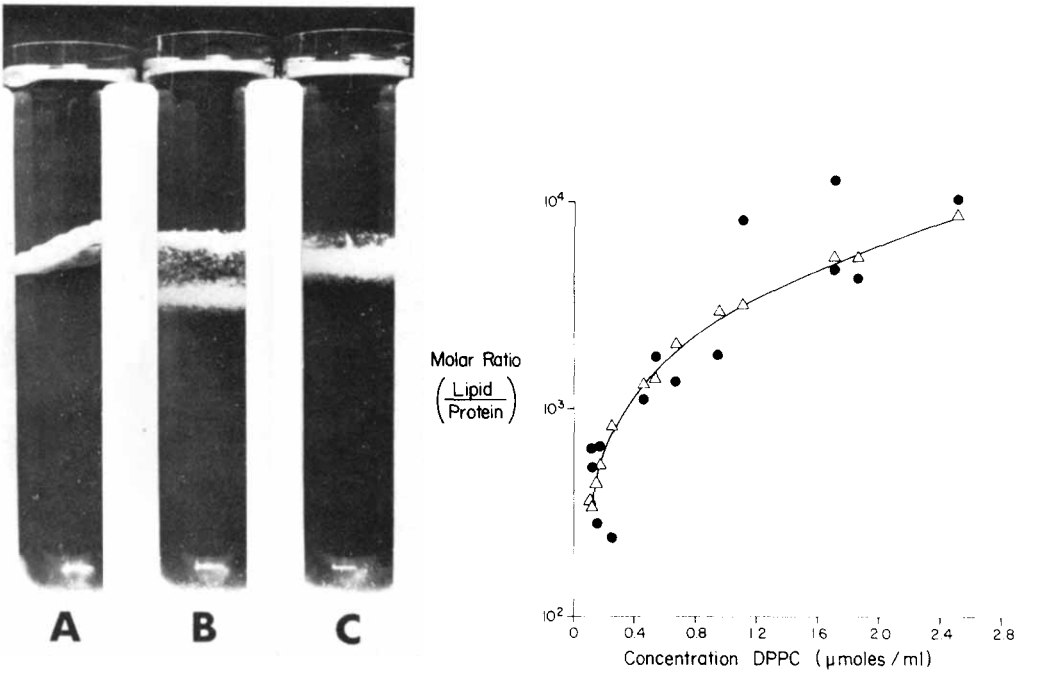

Fig. 2. Separation of recombinant materials by isopycnic density gradient centrifugation. Gradients were poured as continuous density gradients ranging from 1.064 to 1.068 g/ml. Centrifugation was carried out for 48 h at 65 000 rev./min in a Beckman SW-65 rotor at 20° C. (A) DPPC. (B) DPPC and apolipoprotein A. (C) DPPC and bovine plasma albumin. The concentration of DPPC in (A) was 1.1 μmol/ml; in (B) 0.66 μmol/ml, and in (C) 0.89 μmol/ml. The concentration of protein in (B) was 11 μg/ml, in (C) 25 μg/ml.

Fig. 3. The recombination of DPPC with apolipoprotein. Shown are the molar ratios of DPPC to apolipoprotein of all products which constituted greater than 40% of recovered apolipoprotein. △, the molar ratio of lipid to protein of the total starting constituents. •, the molar ratios of lipid to protein in the fractions recovered by density gradient centrifugation. The data are the results of 13 experiments.

that of the starting mixture, irrespective of the concentration of lipid. The mean lipid to protein stoichiometry of the major band recovered from the gradient was 1.2 (S.D. 0.7) times that of the starting constituents. 17% of the bound apolipoprotein was found in stoichiometries greater than three times that of the starting constituents, but these comprised sizable amounts of the recovered apolipoprotein (greater than 40%) in only two experiments. The data from 13 experiments are shown in Fig. 3.

Albumin. In 10 out of 11 experiments we were able to resolve only one band which migrated in the density gradient in about the same position as that found for the free lipid. The stoichiometry of lipid to protein was greater than ten times that of the original ratio of lipid to protein, at all concentrations. In one experiment no protein was found associated with the lipid. Overall, 13% of the recovered protein was associated with lipid, while 86% of the protein was unbound.

Phase transition experiments

The change in the absorbance of light at 600 nm which occurred with a

change in temperature was measured in the lipid and the lipid-protein dispersions in order to detect thermal phase transitions in these materials [23]. The curve obtained for DPPC suspended in a 0.05 M boric acid buffer, pH 7.4, containing 0.1 M NaCl, 3 mM $CaCl_2$, and 17% sucrose (the composition of the density gradient solution) is shown in Fig. 4. DPPC exhibits a reversible phase transition at 41—42°C associated with its well-described transition from a gel-crystalline to liquid-crystalline array. A lower transition associated with a structural transformation of the lattice is present at about 34°C [23], but it is less distinct than that reported by others [23]. As heating is continued through 45°C there is a reversal of the decrease in light scattering, probably associated with an irreversible aggregation of the lipid. The aggregation does not occur when the DPPC is suspended in 0.05 M EDTA, pH 7.0, suggesting that it is dependent upon the ionic composition of the solvent. Abramson [24] has reported that similar effects can be induced by divalent cations on the thermal phase transition curve. Egg phosphatidylcholine, suspended in the buffer containing 0.1 M sodium chloride, 3 mM calcium chloride, and 16% sucrose, did

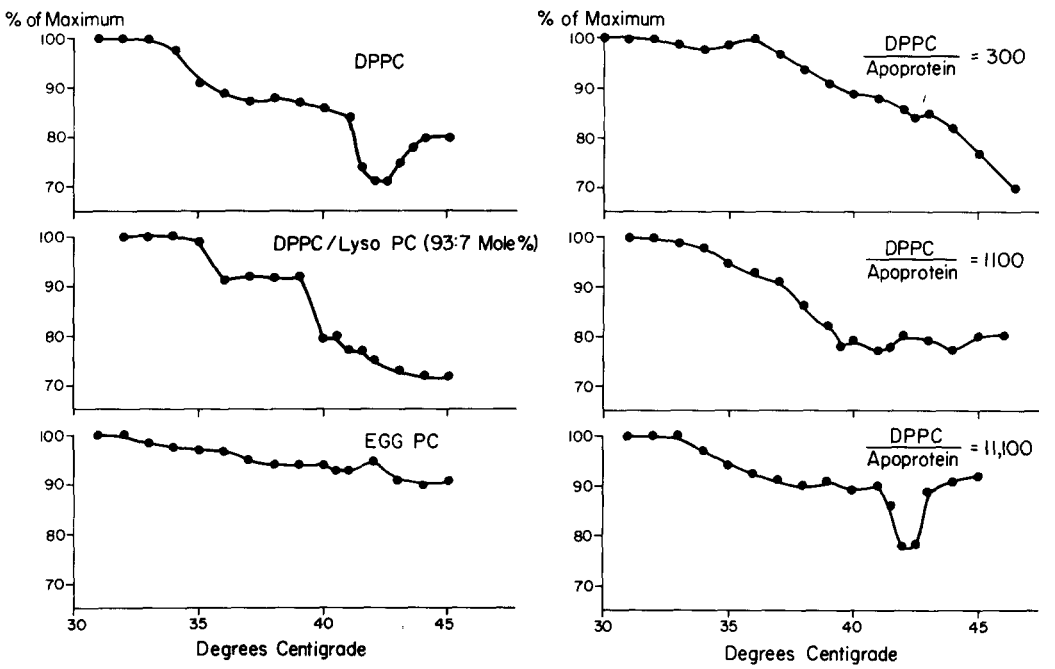

Fig. 4. Thermally induced changes in suspensions of lipid measured by their absorbance at different temperatures. The buffer was 0.05 M boric acid, pH 7.4, containing 0.1 M NaCl, 3 mM $CaCl_2$ and 16% sucrose. The concentration of all lipids was about 0.5 mg/ml. Light scattering was measured by the absorbance of the suspension at a wavelength of 600 nm. The data are expressed as percent of maximum absorbance.

Fig. 5. Thermally induced changes in absorbance measured on three apolipoprotein recombinants with different molar ratios of lipid to protein. The buffer was 0.05 M boric acid, pH 7.4, containing 0.1 M NaCl, 3 mM $CaCl_2$, and 16—17% sucrose. The light-scattering curves were done on recombinants recovered from density gradients. Radioactively labeled DPPC was included in the lipid used in the experiments to detect the extent of formation of lysophosphatidylcholine. The recombinants in the top two figures contained 1.9 and 1.4 mol% of lysophosphatidylcholine, respectively. The content of lysophosphatidylcholine was not measured in the material in the bottom figure.

not exhibit a distinct phase transition associated with a change in turbidity, and the light scattering was reversible with cooling and reheating (Fig. 4). A mixture of 93/7 (mol%) DPPC/lysophosphatidylcholine produced the middle curve shown in Fig. 4. A decrease in light absorbance was evident (presumably associated with a phase transition of the DPPC), but the change in absorbance began at about 39° C and extended through 43° C. Aggregation of the DPPC in the mixture as heating progressed through 45° C was not evident.

The thermally dependent light scattering of three apolipoprotein-DPPC recombinants are shown in Fig. 5. When the ratio of DPPC to apolipoprotein was 300 there was no abrupt change in light scattering occurring over a limited temperature range, and light absorbance decreased monotonically with temperature from 36° C to 46° C. Thermally induced aggregation was absent. As the molar ratio of DPPC to apolipoprotein increased the light-scattering curve of the recombinant began to resemble that of pure DPPC but with a marked broadening of the temperature range of the transition. When the molar ratio of DPPC to apolipoprotein exceeded 11 000 the curve was nearly identical to that of pure DPPC. The molar ratio of DPPC to apolipoprotein in natural surfactant is about 500 [4]. Thus, it appears that DPPC-apolipoprotein recombinants, in stoichiometries approximating those found in vivo, do not show abrupt phase transitions.

Surface tension

DPPC, spread on the surface from a chloroform solution, gives the surface

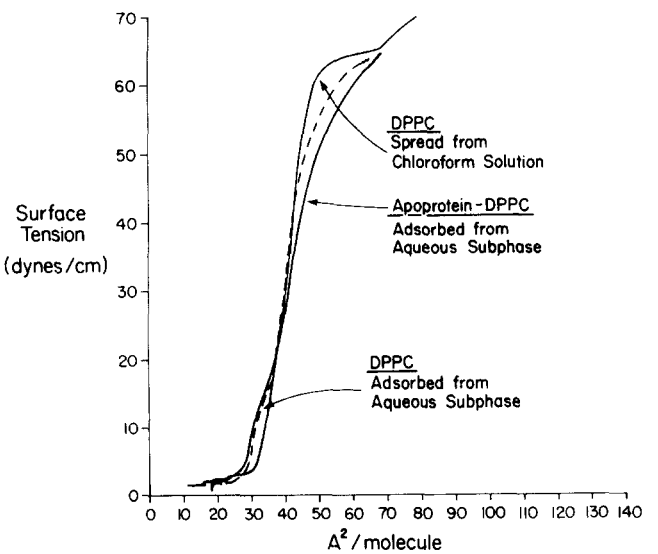

Fig. 6. Surface tension vs. surface area curves of DPPC at 25° C. The films were formed either by the spreading of DPPC from a chloroform/methanol solution onto a clean surface of 0.05 M boric acid, pH 7.4, with 0.1 M NaCl and 3 mM $CaCl_2$, by the adsorption of a dispersion of DPPC suspended in the same buffer at a concentration of 41 μg/ml, or by the adsorption of an apolipoprotein-DPPC recombinant with a lipid-apolipoprotein ratio of 790. The curves of the adsorbed materials were matched to that of the spread DPPC by assuming that the molecular surface areas of all three species were identical at the approximate equilibrium surface tension of DPPC (about 24 dynes/cm).

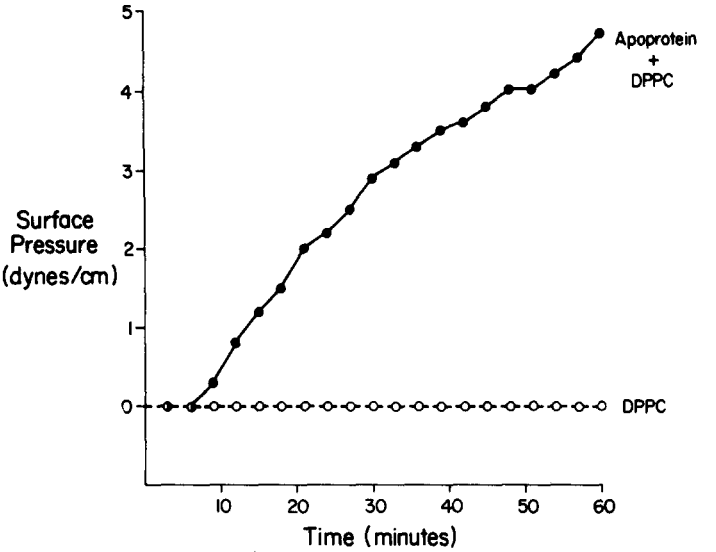

Fig. 7. The adsorption of suspensions of DPPC (20 μg/ml) or recombinants of DPPC and apolipoprotein (8.6 μg/ml of lipid) in the 0.05 M boric acid buffer described in the legend to Fig. 6. The molar ratio of DPPC to apolipoprotein was 2060. The suspensions were stirred with Teflon-coated mixing bars rotating in the subphase at 85 rev./min. The temperature was 25° C.

tension vs. surface area curve shown in Fig. 6. A phase transition is evident at about 65 dynes/cm where the surface concentration is 0.18 μg/cm^2 (66 Å^2/molecule). The continuous compression of the surface results in a reduction of surface tension to below 10 dynes/cm.

The adsorption kinetics of DPPC dispersions isolated from the density gradients are shown in Fig. 7. At room temperature (23—25°C) there is no measurable adsorption in 60 min of DPPC suspended in the subphase at concentrations of 5—20 μg phospholipid/ml. DPPC, suspended at a concentration of 40 μg/ml, formed a measurable film slowly. In contrast, apolipoprotein-DPPC recombinants, in a subphase concentration of 8.6 μg/ml, demonstrated measurable adsorption of material within 1—3 min. The apolipoprotein-DPPC materials with higher ratios of apolipoprotein to lipid had faster rates of adsorption. The surface tension vs. surface area curve of the adsorbed apolipoprotein-DPPC recombinant was similar to that obtained with the adsorbed DPPC, except that the film filled more surface at higher surface tensions (Fig. 6). The interaction with apolipoprotein did not alter the ability of the DPPC to lower surface tension to less than 5 dynes/cm.

Discussion

Binding of apolipoprotein to lipid

The preponderant observation obtained from our experiments was that most of the apolipoprotein interacted with multivesicular suspensions of DPPC in a manner which was indiscriminant of lipid concentration. Over 70% of the apolipoprotein was bound at room temperature, a temperature well below the phase transition temperature of this lipid [23]. One possible mechanism of this

interaction, consistent with the results of the physical studies (see below) is by the insertion of the apolipoprotein into the lamellae of the lipid. We elected to carry out our experiments with multilamellar vesicles [25], and much of the lipid may have been inaccessible to the apolipoprotein. A large ratio of lipid to apolipoprotein was present in all experiments, however, and it does not appear that the concentration of lipid ever limited the interaction. The state of the dispersion might become more important if the ratio of lipid to protein were further decreased. We did not pursue this possibility, as such stoichiometries would be considerably less than those found in natural surfactant.

We have not determined why 26% of the apolipoprotein did not interact with the DPPC. Several explanations can be offered, but none have been confirmed. Our apolipoprotein preparations were usually about 90% homogenous, and part of the unbound protein may include the minor peptides. Some of the apolipoprotein may have been 'denatured' by the procedures used to isolate it from surfactant lipid and to dissolve it in buffer, and the denatured apolipoprotein may have different binding properties. Conceivably the results may reflect small differences between the free energy of self-association of the lipid and of the apolipoprotein, and that of the free energy for the interaction between the lipid and apolipoprotein [26]. Thus, under a strong centrifugal field, self-associated apolipoprotein interacting weakly with lipid may be segregated.

We did not find evidence that the system had ever reached a point of saturation of lipid to protein; that is, that the ratio of lipid to protein remained constant as the lipid concentration increased. The molar ratios of lipid to protein used in these experiments include the ratio found in highly purified pulmonary surfactant. We assume that the apolipoprotein represents 5% by weight of surfactant and has a nominal molecular weight of 35 000 [4], and that the dipalmitoyl phosphatidylcholine represents 50% by weight of the pulmonary surfactant [4] with a molecular weight of 734. The ratio of saturated phosphatidylcholine to apolipoprotein in purified surfactant is about 500 to 1. Similar calculations show that the ratio of total phospholipid to apolipoprotein is about 800 to 1. Our experiments were conducted with mixtures of lipid and protein that ranged from 300 to 1 to 8000 to 1. It seems likely that if the protein were able to bind a fixed amount of DPPC at approximately the ratio found in pulmonary surfactant that this would have been evident over the range of experiments reported here. The results do not lend themselves to this interpretation and do not indicate that the apolipoprotein has a fixed number of discrete sites for DPPC, at least at physiological stoichiometries. All of the experiments reported here were carried out with concentrations of DPPC which were at least six orders of magnitude greater than the critical micellar concentration of this lipid [27,28]. Experiments are not technically feasible using DPPC at a concentration of 10^{-10} M, and we could not determine whether binding to discrete sites would occur at concentrations of lipid below the critical micellar concentration.

In contrast to the results obtained with the apolipoprotein, there was little evidence of an interaction between bovine plasma albumin and dipalmitoyl phosphatidylcholine. 87% of the recovered albumin was not associated with lipid. The albumin that was associated was found with binding ratios that were

all greater than ten times that of the starting ratio of lipid to protein. This may have been protein which was 'trapped' by the macromolecular aggregates of lipid, and which therefore resisted separation by the density gradient centrifugation. These results are in accord with previous studies that show that albumin interacts with dipalmitoyl phosphatidylcholine only under specialized conditions [29].

Physical studies

Interaction of the apolipoprotein with dipalmitoyl phosphatidylcholine results in a material whose rate of adsorption at 25°C to an air-liquid interface is greater than that of the lipid alone. These results are consistent with a change in the structure of the dipalmitoyl phosphatidylcholine after interaction with the protein, resulting in a lipid-protein particle having domains of lipid in a disordered array. The results of the light-scattering experiments support this conclusion. As the amount of apolipoprotein increased in the DPPC-apolipoprotein recombinant the temperature range of the phase transition broadened. A distinct phase transition was not detectable if the ratio of DPPC to apolipoprotein was similar to physiological values. The effect was not due to lysophosphatidylcholine which may have been generated by the experimental procedures. The preparations studied in Fig. 5 contained about 2% lysophosphatidylcholine. The light-scattering curves are clearly different from that obtained in a lipid mixture containing 7% lysophosphatidylcholine (Fig. 4). We conclude, therefore, that the character of the curves seen with apolipoprotein-DPPC recombinants of relatively low lipid to protein ratios cannot be due solely to contamination by lysophosphatidylcholine. We cannot rule out, however, the possibility that the more fluid character of the recombinant may result from the combination of apolipoprotein and lysophosphatidylcholine interacting with the DPPC bilayers, rather than from the apolipoprotein alone.

We were surprised to find that this apolipoprotein interacts with DPPC at room temperature (23—25°C). Most apolipoproteins obtained from either circulating lipoproteins or membranes do not bind phospholipid when the lipid is in an ordered gel-crystalline array [30,31]. In fact, the experiments reported here were begun with this expectation, and we anticipated that a few experiments characterizing the lack of interaction between the apolipoprotein and DPPC at 25°C would provide a useful negative control. Clearly the results show otherwise, and indicate relatively unique binding properties by this protein. The direct interaction between the apolipoprotein of surfactant and dipalmitoyl phosphatidylcholine in vivo would necessarily have to occur at a temperature below the phase transition of the dipalmitoyl phosphatidylcholine (42°C). In contrast, most other apolipoproteins found in circulating or membrane lipoproteins are associated with lipids which are in fluid arrays at 37°C. Thus, the differences in binding characteristics observed for the apolipoprotein of surfactant compared with those from circulating lipoproteins or membranes may reflect the physiological environments of the respective lipoproteins. Pulmonary surfactant, however, contains about 10% by weight of neutral lipid, 50% DPPC, 40% other phospholipids, and probably a small amount of carbohydrate [4]. These experiments do not address the importance of these other constituents in interactions between the lipid and protein. Conceivably the satu-

rated phosphatidylcholine could form mixed myelinic structures with the other phosphatidylcholines or other phospholipids in surfactant, and these might appreciably modify the binding properties of the protein. Additional studies, probing the interaction of this apolipoprotein with defined mixtures of lipids, are necessary in order to ascertain the relative importance of these additional constituents.

The physiological function of this apolipoprotein is unknown. A search for enzymic activity associated with this protein has, so far, been unfruitful [32] *. The results found in these studies do not suggest that this protein could bind a fixed amount of lipid and thereby set the stoichiometry of lipid to protein in a reproducible, definable ratio. They do indicate, however, that the physical properties of the lipid may be modulated when the protein interacts with it, and that the DPPC-apolipoprotein complex exhibits properties similar to some of those observed for surfactant purified from natural sources [5]. Most membrane-derived proteins which bind to lipid do so with a modification of the properties of the protein, and there are now reports of a large number of membrane-bound enzymes whose activities are regulated through the binding of an appropriate lipid [33]. The situation found in pulmonary surfactant may represent an interesting difference. The lipid moiety of pulmonary surfactant is purportedly that part of the material which affects the surface tension properties of the alveolar air-liquid interface, and which is, therefore, the physiologically important part of the lipid-protein complex. The protein may be required to bind to this lipid, and thereby modify its properties so that it can effect its function. We have obtained evidence to support this possibility. DPPC adsorbs to an air-liquid interface very slowly, an observation reported previously [5] and reproduced here. The DPPC-apolipoprotein recombinants, in contrast, absorb and spread at the surface within a few minutes of starting the experiment. Because of restrictions on the amounts of material which we have available for adsorption experiments, however, they were conducted with very low concentrations of materials in the subphase. The changes in surface tension with time observed in these studies are modest compared with those expected in vivo. Thus, the results must be extrapolated in order to demonstrate a trend in the modulation by the apolipoprotein of physiologically important properties of surfactant. They do not address the importance of subphase concentration, or the effects contributed to the physical properties of the entire complex by the other lipid constituents.

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