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INTERACTION OF THE LIPID AND PROTEIN COMPONENTS OF PULMONARY SURFACTANT

ROLE OF PHOSPHATIDYLGLYCEROL AND CALCIUM

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We investigated the interaction of a major apolipoprotein of pulmonary surfactant with mixtures of lipids analogous to those found in natural surfactant. The apolipoprotein was extracted from canine surfactant and was purified to about 90% homogeneity. The apolipoprotein was mixed with liposomes of lipids in buffers containing 0.1 M sodium chloride and 3 mM calcium chloride at 22°C for 2 h or 37°C for 30 min. Two fractions were separated by centrifugation in sucrose density gradients at 15 000 rev./min. One was comprised of an aggregated, relatively high density recombinant lipoprotein which sedimented to a position toward the bottom of the centrifuge tube; the other remained at the top of the centrifuge tube and was mainly comprised of unbound lipid. The amount of lipid recovered as a sedimenting lipoprotein was dependent upon its composition. Those mixtures of lipids which contained dipalmitoyl phosphatidylglycerol formed sedimenting complexes which comprised 14% to 53% of the recovered lipid; those without phosphatidylglycerol formed such aggregates with less than 13% of the available lipid. Moreover, the lipid-to-protein stoichiometry of the recombinant was also dependent upon phosphatidylglycerol, and lipids containing this phospholipid displayed enhanced binding at a critical concentration of lipid which varied with temperature and composition. Calcium was required to form the sedimenting complex at 37°C. These results suggest that phosphatidylglycerol may be involved in the formation of a micelle-like complex, the stoichiometry of which is regulated over a narrow range of lipid concentration, and the structure of which involves calcium. The physiological advantage of forming this complex has not been determined. We found, however, that lipids containing phosphatidylglycerol absorbed more rapidly to an air/liquid interface than did those without. This rate of adsorption was further increased after interaction with the apolipoprotein.

Introduction

The mechanical stability of the gas exchanging units of the lung is dependent upon the physical properties of the material adsorbed at their air/liquid interfaces [1]. A relatively specialized material, pulmonary surfactant, is synthesized by alveolar epithelial cells and secreted into the alveolar extracellular fluid to provide these properties. It adsorbs to the alveolar air/liquid interface and lowers the interfacial tension, thereby reducing the force tending to collapse the alveolar units. Normal alveolar interfacial tension has been measured and found to be less than 15 dyn/cm during much of the respiratory maneuver [2,3]. Increasing alveolar surface tension to 30 dyn/cm either experimentally [4], or in certain clinical conditions [5], results in demonstrable alveolar atelectasis.

We might expect that a material exerting its phys-

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-1-glycerol.

iological function through its physical properties would have a well-defined chemical composition, and this has been confirmed by numerous studies (summarized in Ref. 6). Pulmonary surfactant, as isolated from the lung fluids of several animals in different laboratories, has been found to be a substance comprised of about 50–60% (by weight) dipalmitoyl phosphatidylcholine, 20–25% monoenoic phosphatidylcholine, 5–10% phosphatidylglycerol, 5–10% cholesterol, 5–6% protein, and less than 10% other constituents. The large proportion of saturated phosphatidylcholine allows this material to achieve very low surface tension [7]. It has been postulated that the monoenoic phosphatidylcholine, and perhaps the cholesterol, impart the appropriate physical properties to the complex so that it can readily adsorb to the alveolar interface [1]. The physiological functions of the phosphatidylglycerol and apolipoprotein are unknown. The phosphatidylglycerol is in relatively high proportion compared with that found in other mammalian lipoprotein systems. It is absent in the lung fluids of children with respiratory distress syndrome [8], and it may affect the surface properties of the surfactant complex [9] or help stabilize its physical structure in secretion [10]. At least one of the protein constituents appears to be uniquely associated with this material [6]. This apolipoprotein interacts readily with dipalmitoyl phosphatidylcholine at room temperature, and alters the physical properties of the dispersed lipid [11]. We refer to this protein as apolipoprotein A. What is known of its physical and chemical properties and its specificity has been described in previous publications [6,11, 12].

The work described in this paper was undertaken to describe further the physical properties of the surfactant complex, and to investigate how the individual constituents define these properties. Particular interest has been given to the apolipoprotein and to the phosphatidylglycerol found in this material, as the physiological functions of these constituents have not been thoroughly investigated. Our results suggest that both the apolipoprotein and the phosphatidylglycerol are required to form a complex having the properties expected for natural surfactant, and that the binding of the constituents is mediated by the ionic composition of the buffer and by the physical state of the lipid. Divalent cations are essential for the integration of the lipid and protein in an aggregated complex when the lipids are presented in a fluid state.

Materials and Methods

Interaction studies

Pulmonary surfactant was isolated from the alveolar lavage fluid of canine lungs [13]. Apolipoprotein A was purified from the extracellular surfactant using the procedure described previously [11], which is summarized in the legend to Fig. 1 and in the results. The apolipoprotein was labeled with ^{125}I, using a slight modification [14] of the method of Hunter and Greenwood [15]. DPPC was purchased from Sigma Chemical Co. (St. Louis, MO); POPC from Serdary (London, Ontario); and cholesterol from Sigma. All lipids were the highest purity available, and migrated as single spots in silicic acid thin-layer chromatography. Lyso derivatives could not be detected even with deliberate overloading of the thin-layer plates. GLC indicated that the DPPC and the DPPG contained greater than 98% palmitic acid residues. The molar distribution of the palmitoyl and oleoyl residues in the POPC was 55.3% and 44.7%, respectively. [*palmitoyl*-1-^{14}C]DPPC was obtained from New England Nuclear (Boston, MA). Greater than 98% of the radioactivity migrated with phosphatidylcholine in silicic acid TLC.

The lipids used for the interaction studies were dispersed in an aqueous buffer by the method of Batzri and Korn [16] and were sonicated briefly. Details of this procedure are as follows: Lipid mixtures of desired composition, containing tracer amounts of [^{14}C]DPPC, were dissolved in ethanol at a concentration of about 25 mg/ml. Liposomes were formed by the rapid injection with a Hamilton syringe of the dissolved lipids into a buffer warmed to 48°C, followed by a 30 s sonication using a probe sonicator (Braunsonic Model 1510) set at 100 W. In most experiments, the buffer solution was 0.1 M NaCl/3 mM $CaCl_2$/5 mM sodium borate, pH 7.4, and its volume was greater than 10-times the volume of injected ethanol. Calcium-free buffer was prepared with 0.1 M NaCl/5 mM sodium borate/0.01 M EDTA, pH 7.4. The suspensions of lipids were water-clear immediately after sonication with a slight bluish cast. We observed the morphology of the suspension formed from lipid mixture 1 (see Table I) with

negative staining [16]. Most of the lipid at 37°C was in the form of intact single bilayer vesicles of about 30–40 nm diameter. A small amount appeared as collapsed remnants of these vesicles and formed short bilayer segments with lengths of 1–2 liposome diameters. There was apparent aggregation of the vesicles after 2 h at room temperature, and the suspensions assumed a milky appearance. Electron microscopy indicated an aggregation and fusion into larger multi-layered vesicles.

The interaction of the apolipoprotein with the liposomes was effected by mixing 26 μg of labeled apolipoprotein, dissolved in 0.01 M sodium borate, pH 7.1, with the ^{14}C-labeled liposomes dispersed in buffer. The reactants were mixed in a Teflon tube using a reciprocating arm shaker set at 86 rev./min. Sonication was not used during the interaction. In most experiments done at room temperature the mixing proceeded for 2 h. In a few later experiments we found that the recombination at room temperature could be completed within 30 min. All experiments conducted at 37°C, therefore, were mixed for 30 min.

Bound lipid-protein recombinants which were aggregated and of relatively high density were separated from the unbound lipid and low-density recombinants by density gradient centrifugation. Two systems were used and were equally effective. In the first system the lipid-apolipoprotein mixture, in a total volume of less than 0.6 ml, was layered over a 5 ml continuous density gradient made up of sucrose dissolved in the buffer used for the interaction. The limits of the gradient were defined by 4% sucrose (density = 1.016 g/ml at 22°C) and 40% sucrose (1.165 g/ml). Centrifugation was carried out at 10000 rev./min for 60 min in a Beckman L5-65 centrifuge fitted with a high-temperature control accessory, set at the temperature of interaction. In the second system the lipid-protein mixture was layered over a continuous sucrose gradient ranging from 4 to 17% (density = 1.064 g/ml), poured over a 1 ml cushion of 40% sucrose. Centrifugation was conducted at 15000 rev./min for 15 min. The separation of the low-density recombinant from unbound lipid was carried out in separate experiments using continuous density gradients with a narrow range in density, and centrifuging at forces high enough to effect a separation on the basis of isopycnic density. When buffers containing calcium were used, this density varied from 1.035 to 1.069 g/ml; without calcium in 10 mM EDTA the density ranged from 1.029 to 1.060 g/ml. The samples were centrifuged for 40 h at 50000 rev./min in a Beckman SW-50.1 swinging bucket rotor. Temperature was maintained at 37°C throughout the period of centrifugation.

All gradients were eluted in 0.2 ml samples transferred directly to scintillation counting vials and β-particle scintillation counting was done in 10 ml of Omni-Fluor (New England Nuclear) in toluene supplemented with 0.2 ml Protosol (New England Nuclear). The windows of the scintillation counter were set so that there was no spillover of ^{125}I counts into the ^{14}C channel, and less than 15% spillover of ^{14}C counts into the lower energy window. Quenching was nearly constant for all vials. The specific activity of the lipid was quantified in a separate sample of labeled liposomes by phosphorous assay or by quantitative GLC, using an internal standard of 1,2-dinonodecanoyl-*sn*-glycero-3-phosphocholine (Supelco). The specific activity of the apolipoprotein was qualified by its ultraviolet absorbance at 277 nm [11].

The compositions of the lipid mixtures used in our studies are given in Table I. For simplicity we refer to most results with this nomenclature.

Surface balance studies

We conducted two types of experiment, both at 37°C: (1) the measurement of the rate of adsorption to an air/liquid interface, and (2) the recording of the surface tension versus surface area isotherm of the constituents adsorbed from the subphase. The temperature of the subphase and of the surface balance chamber was controlled to within 0.2°C using heating tapes strategically placed within the enclosure and underneath the Teflon trough. All temperature controls were keyed to the interfacial temperature, mea-

TABLE I

COMPOSITION OF LIPIDS USED FOR INTERACTION STUDIES

Values are expressed as wt%.

Mixture	DPPC	DPPG	POPC	Cholesterol
1	63.0	8.8	19.4	8.7
2	76.6	12.8	–	10.4
3	85.7	14.3	–	–
4	64.4	–	26.8	8.7

sured with a Yellow Springs Model 74 temperature controller using a micro temperature probe placed in the interface. The procedures used to carry out these studies have already been described [11].

Results

Purification of apolipoprotein A

The protein composition of the constituents found after each step in the isolation of apolipoprotein A are shown in Fig. 1. The purified fractions are about 90% homogeneous in apolipoprotein A, as judged by polyacrylamide gel electrophoresis, and they show no immunological reactivity to antiserum developed against canine serum. Quantitative GLC indicates that less than 0.2 μmol of phospholipid fatty acid is associated with each μmol of apolipoprotein [11]. Electrophoresis of the ^{125}I-labeled apolipoprotein shows that most of the protein migrates with the unlabeled apolipoprotein as one band. The preparation, as judged by electrophoresis and isotopic counting, is stable for better than 3 months when stored at −15°C. Greater than 90% of the counts are precipitated by 10% cold trichloroacetic acid. ^{125}I-labeled bovine plasma albumin, prepared and stored in the same manner, appears homogeneous when freshly prepared but tends to aggregate with storage.

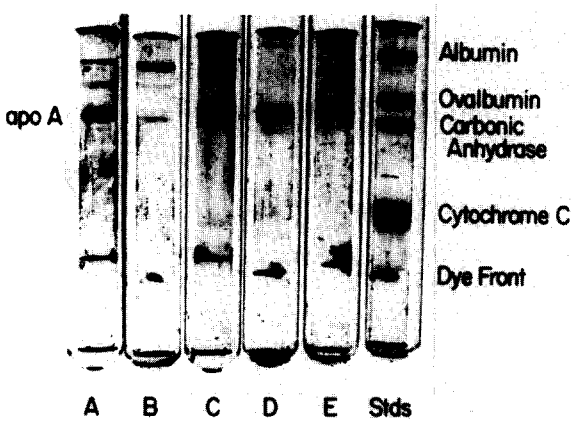

Fig. 1. The isolation of apolipoprotein A (apo A) from canine pulmonary surfactant. Pulmonary surfactant, frozen as a suspension in water, was thawed and centrifuged at greater than 40 000 × *g* for 1 h. The pellet (gel A) was principally comprised of the apolipoprotein, while the supernatant (gel B) contained higher molecular weight proteins which do not bind strongly to the surfactant complex. The pellet is suspended in 0.3 M lithium diiodosalicylate in 0.05 M pyridine, pH 8.4, and extracted with butanol/ethanol (6 : 1, v/v) to partition the lipid into the organic phase and the protein into the aqueous phase. The protein solution was applied to a 7 ml column of Cibracron blue F4GA coupled to agarose gel beads (Affigel Blue, Bio-Rad Laboratories, Berkeley, CA) which has been equilibrated with 0.01 M sodium borate, pH 7.1, and was eluted in 1 ml fractions with the same buffer. The apolipoprotein was highly enriched in fractions 3–6 (gels C, D and E). The electrophoresis was conducted in sodium dodecyl sulfate under conditions which reduce sulfhydryl groups. Stds, standard compounds.

Interaction experiments

Conditions of interaction. Free lipid and free protein, centrifuged separately with low centrifugation

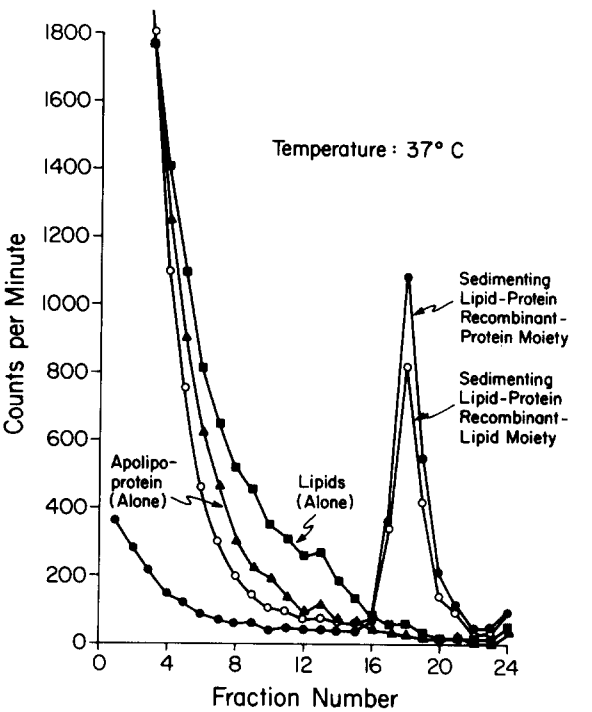

Fig. 2. The distribution of the lipids of mixture 1, the apolipoprotein, and the lipid-protein recombinant in sucrose density gradients. Density gradients were constructed with a 1 ml cushion of 40% sucrose over which were poured 3.6 ml continuous gradients ranging from 4% sucrose to 17% sucrose. The samples in 0.5 ml buffer were carefully layered over the gradients and centrifuged at 15 000 rev./min for 15 min in a Beckman SW 50 rotor. The results of three separate experiments are shown in the figure. ■———■, lipids of mixture 1; ▲———▲, apolipoprotein (alone); ●———●, the protein portion of the recombinant of apolipoprotein and lipid; ○———○, the lipid portion of the recombinant. Temperature: 37°C.

forces, are found at the top of the density gradients, as shown in Fig. 2. The mixture of lipid and protein, centrifuged together after lipid-protein interaction, can be separated into an aggregated fraction, which readily sediments to a position low in the gradient, and a supernatant fraction which migrates in these gradients in the same position as unbound lipid. We investigated by isopycnic density gradient centrifugation whether this supernatant fraction might also contain a small amount of a recombinant lipoprotein, the physical properties of which were similar to those of the unbound lipid. Three fractions could be partially resolved in some lipid-protein mixtures: a lipoprotein, the stoichiometry of which was similar to that of the fraction sedimenting at the lower centrifugation speeds described in Fig. 2; a lipoprotein of lower density which comprised a small amount of the recovered lipid, and unbound lipid. The lipid-to-protein stoichiometry of the lower density recombinant was difficult to quantify because of its incomplete resolution from unbound lipid, and we have not attempted to isolate it and describe its properties. We have, however, estimated the maximum of lipid which might be comprised of this low density recombinant using the distribution of the lipid and protein components in the gradient. We assumed that wherever both lipid and protein were found in the same fraction they were bound as a lipoprotein. This calculation maximizes the estimate of the lower density recombinant since it ignores band spreading and the incomplete resolution of this fraction from the larger amount of unbound lipid. Nevertheless, we still found that this lower density recombinant made up less than 9% of the recovered lipid. We have ignored this small fraction in our calculations, and our discussion is confined to the aggregated, relatively high density fraction, which readily sediments in 15 min at 15 000 rev./min. For preciseness, however, we generally refer to this fraction as the 'sedimenting' lipoprotein recombinant.

Most of our interaction experiments done at 22°C were carried out using 2 h of mixing, while those at 37°C were done with 30 min of agitation. Control experiments showed that the interaction was completed within these times, and the results do not reflect kinetic limitations. These times of mixing did not lead to significant hydrolysis of the phosphatidylcholine. [*Palmitoyl*-1-^{14}C]DPPC was added to a reaction mixture and mixed for 60 min at 37°C. The lipids were extracted [17] and the lysophosphatidylcholine and phosphatidylcholine were separated by silicic acid TLC. We found that less than 0.5 mol% of our lipid was hydrolyzed in 60 min at 37°C. In a previous study we found that less than 2.5 mol% of a suspension of DPPC was degraded to lysophosphatidylcholine in 74 h at room temperature [11]. We conclude from these findings, therefore, that the mixing of apolipoprotein with lipids for 30 min at 37°C, or for 2 h at 22°C, does not result in the formation of appreciable lysophosphatidylcholine.

Interaction of apolipoprotein with lipids

Effects of calcium. Lipid mixture 1 (Table I) was constituted to resemble the composition of the principal lipids in surfactant. Our most extensive experimentation was done with these lipids, and included studies at room temperature (about 22°C) and 37°C, with and without calcium in the suspending buffer.

The results obtained at 22°C are shown in Fig. 3, where the amount of lipid bound per mole of apo-

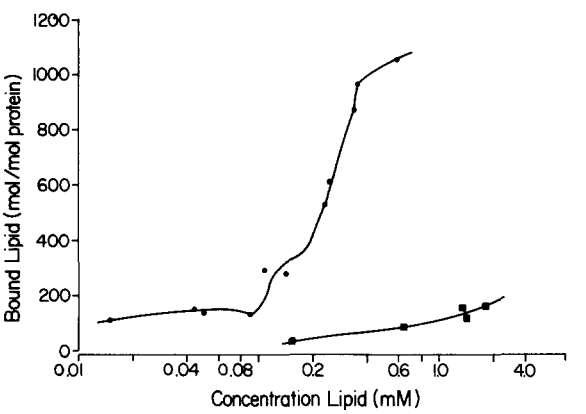

Fig. 3. The binding of the lipids in mixture 1 (DPPC/DPPG/POPC/cholesterol, 63 : 9 : 19 : 9 wt%) by the apolipoprotein. Lipids were mixed for 2 h at room temperature with 0.74 nmol of apolipoprotein, and the bound and free constituents were separated by density gradient centrifugation carried out at 20°C under the conditions described in Fig. 2. The amount of bound lipid, expressed as mol/mol total protein in the system, is plotted against the logarithm of the concentration of lipid in the supernatant fraction, which approximates the amount of unbound lipid (see text). The buffers differ in the presence (●——●) or absence (■——■) of 3 mM calcium chloride.

lipoprotein in the sedimenting fraction is plotted against the logarithm of the molar concentration of lipid. The amount of bound lipid was about 150 mol per mol apolipoprotein when the concentration of lipid was 0.015 mM (the lowest concentration of lipid which we tested), and increased to about 240 as lipid concentration changed by 10-fold to 0.15 mM. Between 0.25 and 0.4 mM of lipid the binding increased rather abruptly to over 1000 mol lipid bound per mol apolipoprotein, suggesting that a highly cooperative interaction had occurred over a relatively small concentration range. Maximum binding of 1200 mol lipid per mol apolipoprotein occurred at 0.6 mM lipid, and the slight inflection in the curves suggests that this may be approaching the level of saturation. This was not confirmed, however, as we did not carry out experiments with higher concentrations of lipid at this temperature.

The pattern of interaction between the apolipoprotein and the lipid at 37°C (Fig. 4) was qualitatively similar to that obtained at 22°C. Over a wide span of lower concentrations of lipid the amount of bound lipid increased from about 50 to 120 mol per mol protein, but increased rather abruptly to about 250 mol bound lipid per mol protein when the lipid concentration increased from 1.8 to 2 mM. Saturation at 250 mol lipid per mol protein apparently occurred when the concentration of lipid reached 2–2.5 mM.

At both 22°C and 37°C Ca^{2+} markedly affected the stoichiometry of the sedimenting recombinant lipoprotein. At 22°C, without Ca^{2+}, the molar ratio of lipid to apolipoprotein at saturation was about 150, about 10% of that when calcium was present, and the amount of lipid involved in this complex was about one-fifth of that with calcium. At 37°C there was no detectable formation of a sedimenting lipoprotein without calcium, even when the concentration of total lipid was 1.8 mM.

Effects of phosphatidylglycerol. We investigated the importance of DPPG for the interaction between lipids and the apolipoprotein by carrying out experiments with mixture 4 and with 100% DPPC in calcium-containing buffers. The results are shown in Fig. 5. The lipids of mixture 4 were bound by the apolipoprotein in the sedimenting complex at about the same stoichiometry of binding as that found with mixture 1 when the concentration of lipid was less than 0.8 mM. Differences between the interaction of the apolipoprotein with these two mixtures of lipids became manifest, however, at higher concentrations of lipid. Enhanced binding occurred with mixture 1

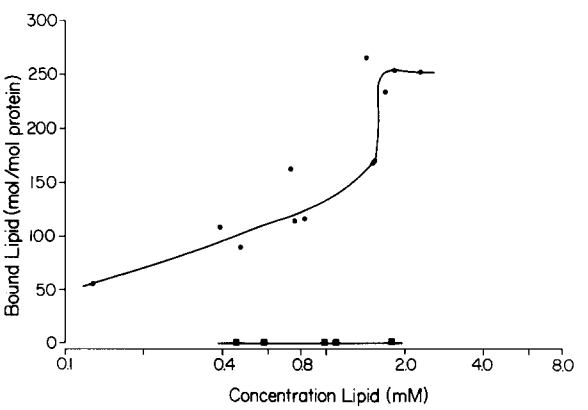

Fig. 4. The binding of the lipids in mixture 1 (DPPC/DPPG/POPC/cholesterol, 63 : 9 : 19 : 9 wt%) by the apolipoprotein. The interaction proceeded for 30 min at 37°C, and the density gradient centrifugation was conducted at the same temperature. The other conditions of the experiments are described in the text of the legend to Fig. 2 and 3. No lipid was present as a sedimenting complex when calcium was absent, and we have indicated a stoichiometry of zero.

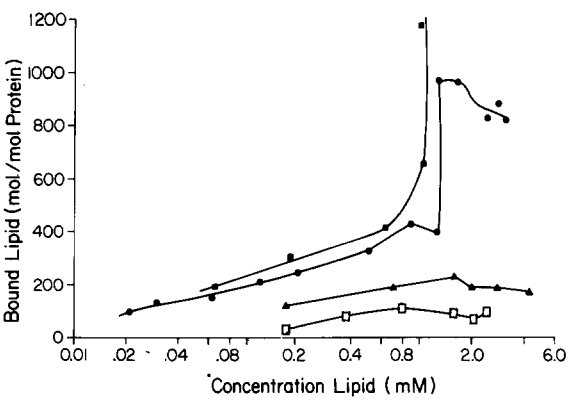

Fig. 5. The binding of the lipids in mixtures 2 (DPPC/DPPG/cholesterol, 77 : 13 : 10 wt%), 3 (DPPC/DPPG, 86 : 14 wt%) and 4 (DPPC/POPC/cholesterol, 64 : 27 : 9 wt%), and DPPC, by the apolipoprotein. The interactions occurred at 37°C in buffers with 3 mM Ca^{2+}. Lipids of mixtures 2 and 3, which contained phosphatidylglycerol, were bound with affinities 5- to 10-times greater than those without phosphatidylglycerol (mixtures 4 and 5). ■———■, mixture 2; ●———●, mixture 3; □———□, mixture 4 and ▲———▲, 100% DPPC.

(containing DPPG) between 1.5 and 1.8 mM, and the amount of bound lipid increased by about 2-fold over that range of lipid concentration. In contrast, the lipids of mixture 4 (no DPPG) were bound to a maximum extent at 0.8 mM lipid, and there is no cooperative interaction. Similar results were obtained for the interaction between the apolipoprotein and 100% DPPC. The apolipoprotein bound a maximum of about 250 mol of DPPC when the concentration of DPPC was about 1.5 mM. The amount of bound lipid decreased slightly at higher concentrations.

Lipid mixtures containing phosphatidylglycerol consistently involved a greater percentage of the lipid in a sedimenting lipoprotein than did those without. The mean percentages of lipid recovered at 37°C in this type of complex were 14% for mixture 1, 53% for mixture 2, and 41% for mixture 3. In comparison, only 7% of the lipids in mixture 4 and 13% of the pure DPPC were found in this form.

Effects of physical state and of cholesterol. The results of most of our experiments indicated that the amount of lipid bound by the apolipoprotein as a sedimenting complex increased as the proportion of lipid in a fluid state was decreased. 77% of the lipid of mixture 1 was bound in this fraction at 22°C while only 14% was bound at 37°C (Figs. 3 and 4). In addition, the stoichiometry of bound lipid per mol protein was markedly increased at 22°C over that at 37°C (Fig. 3 and 4). Similarly, when comparing the lipids without DPPG, more of the 100% DPPC was associated with protein than was the lipid comprising mixture 4 (Fig. 5), which can be expected to have a greater proportion of its mass in a liquid-crystalline (fluid) state. The largest stoichiometries of bound lipid occurred with mixtures not containing unsaturated phospholipids (Fig. 5). 86 : 14 wt% DPPC : DPPG (mixture 3) was bound to a saturation stoichiometry of about 900 mol/mol protein. This interaction was slightly enhanced by 10 wt% cholesterol. We observed by light scattering a clearly demarcated phase transition in mixture 3 which was spread over a temperature range of 41–43°C; this was not detectable in mixture 2 containing 10% cholesterol. This augmented binding may reflect special properties imparted by the cholesterol (or the cholesterol in conjunction with the DPPG) to the apolipoprotein-lipid system which may be distinct from the effect of cholesterol on lipid fluidity [18]. We did not further investigate the effects of cholesterol, but additional studies may be warranted. Pulmonary surfactant contains over 20 mol% cholesterol, the physiological function of which is unknown.

Interaction of albumin with lipids

We investigated whether albumin would form a sedimenting complex with the lipids of mixtures 1 and 3 at 37°C. With mixture 1, at lipid concentrations of 0.5 mM and 0.6 mM, 0 and 10 mol lipid were bound per mol albumin, respectively. This represents less than 1% of the lipid bound by the apolipoprotein at these concentrations of lipid. The lipids of mixture 3, at concentrations of 0.4, 1.5 and 3.0 mM, were bound by albumin to an extent of 1, 4 and 12% of that bound by the apolipoprotein. Similar results were found for all other mixtures of lipid. Over 90% of the apolipoprotein was found in combination with lipid in calcium-containing solutions. In comparison less than 10% of the albumin was recovered bound in any form to lipid. We conclude, therefore, from the results of the present experiments as well as those of the previous ones [8], that albumin interacts with the lipids of surfactant with far less affinity than does the apolipoprotein.

Selective binding of lipids

Molar binding ratios in all experiments were estimated from the ratio of [^{14}C]DPPC bound to ^{125}I-labeled apolipoprotein. We wished to be certain that the radiolabeled DPPC was a suitable marker for the lipids in the lipid mixtures; i.e., that the amount of [^{14}C]DPPC was always proportional to the amount of total lipid. In two experiments, therefore, we investigated whether the composition of bound lipid in the sedimenting complex differed from that of the original lipid mixture. In the first experiment, using mixture 1 at 22°C, we found that the bound lipid containing 64.4% DPPC, 6.8% DPPG, 20.4% POPC, and 8.4% cholesterol, which compares closely with the composition of the original lipid. The bound lipid contained a slightly smaller proportion of DPPG, but in general the binding of the lipids was not selective and followed that of the labeled DPPC. In the second experiment we investigated whether the cholesterol in mixture 2 migrated throughout the density gradient with the DPPC. We used [^{3}H]cholesterol and [^{14}C]DPPC (the ^{125}I-labeled apolipoprotein was

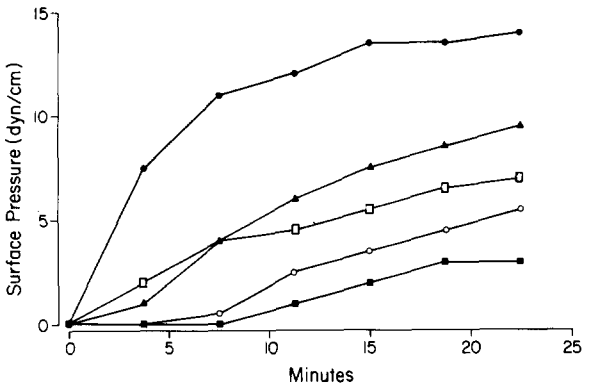

Fig. 6. The adsorption of the principal lipids used in these studies, and that of mixture 1 mixed with apolipoprotein. The lipids were suspended in 125 ml of the subphase buffer containing 3 mM Ca^{2+}. Surface tension was recorded after mixing the subphase and aspirating the surface to obtain a surface tension characteristic of a clean surface of buffer at 37°C. The initial time was taken as the time when mixing in the subphase was restarted. The data are presented as surface pressure ($\pi = \gamma_0 - \gamma_t$) versus time. ●———●, mixture 1 (44 μg/ml) apoprotein (4.4 μg/ml); ▲———▲, mixture 3 (50 μg/ml); □———□, egg phosphatidylcholine (63 μg/ml); ○———○, mixture 1 (44 μg/ml) and ■———■, DPPC (50 μg/ml).

omitted), and measured the cholesterol and DPPC distribution at 37°C in a density gradient separating unbound lipid and protein from recombinant lipoproteins. The distribution of cholesterol was almost identical to that of DPPC, and the ratios of cholesterol to DPPC were similar in bound and unbound lipid.

Surface properties

We investigated the rate of adsorption of the lipids of mixture 1 at concentrations in the subphase of 10, 20, 35, and 50 μg/ml. As expected, the rate of adsorption increased as the concentration of lipid in the subphase was increased from 10 to 35 μg/ml. Adsorption rates at concentrations of 35 and 50 μg/ml were not markedly different, and we chose the latter concentration with which to do comparative studies.

The rates at which the major lipids lowered surface tension are shown in Fig. 6. Egg phosphatidylcholine (used to approximate the properties of POPC), mixture 1, and DPPC had rates which decreased in that order, confirming predictions made earlier [1] on the basis of the reported physical properties of these types of lipids. We found that 14% DPPG in mixture 3 accelerated the adsorption of DPPC. The most rapid adsorption was obtained with suspensions of the apolipoprotein and the lipids of mixture 1, as shown in Fig. 6, and these results are consistent with earlier findings [11]. Mixing apolipoprotein with the lipids of mixture 3 resulted in a slight increase in the rate of adsorption of these lipids, but it was still less than the rate obtained with the apolipoprotein and mixture 1 (not shown). Only the adsorbed lipids of mixture 3, or these lipids mixed with apolipoprotein, lowered the surface tension to less than 10 dyn/cm at 37°C. However, these results may have reflected the physical conditions of our surface balance rather than physicochemical limitations of the lipids in mixtures. In order to conserve material we conducted the adsorption at a maximum area of 100 cm^2 and then compressed the film to a minimum area of 22 cm^2. This compression ratio may have been insufficient to squeeze the unsaturated lipids from the film and leave it enriched with enough DPPC to reduce the interfacial tension to characteristic values. When we spread the lipids of mixture 1 at an area of 266 cm^2 and then compressed to 22 cm^2 we obtained a minimum surface tension of 6 dyn/cm. The calculated surface packing of this film at 24 dyn/cm (the surface tension of the close-packed film prior to surface collapse) was 55.7 $Å^2$/molecule. The nominal surface packing at 12 dyn/cm, calculated as if DPPC were the only lipid left in the film at this surface tension, was 22.9 $Å^2$/molecule. This area is about half of that required to accommodate DPPC at this surface tension and temperature [1], and indicates that much of the DPPC in the lipid mixture which was originally spread in the film had been lost during compression and film collapse.

Discussion

These experiments were designed to investigate how the major lipid constituents of pulmonary surfactant affect the overall interaction between the apolipoprotein and the complex of lipids. Our approach has been to study a mixture of lipids similar to those found in natural surfactant, and systematically to eliminate one or more of the lipid constituents and ascertain how the particular constituent might affect the binding between the lipid and apolipoprotein, or the physical properties of the complex. Permutations in the ionic composition of the buffer solutions have

not been done extensively, but the effects of calcium have been investigated at 37°C and at room temperature.

The results of the interaction between the apolipoprotein and the lipids in the most complex mixture which we studied, the one which approximates the composition of the lipids found in surfactant [6], indicate that the lipid is not bound as a monomer to discrete sites acting independently (Figs. 3 and 4). At lower concentrations of lipid the binding of the constituents in the sedimenting complex proceeds with a relationship which is linearly dependent upon the logarithm of lipid concentration. At a concentration of lipid of about 2 mM (at 37°C), the interaction appears to occur in a highly cooperative manner. Ca^{2+} is obligatory for formation of this sedimenting lipoprotein at 37°C. The results obtained at room temperature are similar, except that the amount of lipid bound per mole protein is about 4-fold higher than that seen at 37°C. Without Ca^{2+} the binding is markedly reduced, but is not completely abolished as it is at 37°C.

The 'cooperative' interaction seen at about 1.5–2 mM lipid is dependent upon having DPPG in the lipid complex. Neither pure DPPC nor a mixture of 64% DPPC, 27% POPC and 9% cholesterol (Fig. 5) displays enhancing effects. With these lipids the amount of bound lipid increased linearly with the logarithm of lipid concentration until about 2 mM lipid, where bound lipid, instead of increasing abruptly over a small concentration range of lipid, appears to reach a saturation value. The physical state of the 100% DPPC at 37°C is gel-crystalline, while the other lipid mixture was principally in a liquid-crystalline state. Neither, however, contains DPPG, and neither interacts cooperatively with the apolipoprotein.

We are uncertain how Ca^{2+} acts with phosphatidylglycerol to effect a maximum binding of lipid over a limited concentration range. Three possibilities exist:

1. Ca^{2+} may interact with the apolipoprotein to induce a change in the secondary or tertiary structure of the protein. We do not yet have information to evaluate the possibility of such a process.

2. Ca^{2+} may serve as an ionic bridge between negatively charged groups on the phosphatidylglycerol and on the protein. This type of interaction has been observed in other systems [19]. Recent studies, however, show that phosphatidylcholines in methanol can also bind calcium [20], and conceivably this calcium could also form bridges to the apolipoprotein. If so, the cooperative effects seen only with lipid containing phosphatidylglycerol would involve other mechanisms. These, perhaps, may be augmented by electrostatic interactions [21].

3. Ca^{2+}, with phosphatidylglycerol, may induce perturbations in the bilayer structure of the intact liposomes. These effects have been followed in detail by Papahadjopoulos and coworkers [22], and have been shown to result in the aggregation and fusion of lipid bilayers. They are apparently induced by the generation of a high energy hydrocarbon-aqueous interface at the positions of disruption in the liposomal bilayer. Apolipoprotein A may have the ability to interact with these hydrophobic surfaces [11], thereby lowering the free energy of the system. Interactions of this type should result in the binding of lipid to protein with large stoichiometries, and in the formation of structured aggregates of surfactant.

We cannot be certain that the results of these in vitro studies are applicable to the natural processes which form and stabilize the surfactant complex, in vivo. We note, however, that tubular myelin [23], an extracellular form of surfactant which exhibits remarkable long-range order, may be dependent upon calcium for its formation [24]. It has not been shown that specific proteins are required to maintain this structure, but it is difficult to imagine that surfactant lipids by themselves, being subject to rapid lateral diffusion [25], would be capable of interacting into a form of such morphological regularity. Thus, we think it possible that surfactant apolipoprotein may be involved in the formation of this complex structure and in the regulation of the stoichiometry of the bound lipid. These effects, together with those which are known to be exerted by the protein and by the phosphatidylglycerol on the interfacial properties of the complex [11], present important reasons to view calcium, phosphatidylglycerol and apolipoprotein as integral and necessary constituents of pulmonary surfactant. The regulation of their intracellular metabolism and secretion, therefore, may be an important aspect of respiratory physiology.

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