Separation of subfractions of the hydrophobic components of calf lung surfactant

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Abstract This study reports the biochemical separation of the hydrophobic constituents of calf lung surfactant into separate fractions from which specific components are excluded. Gel permeation chromatography on LH-20 with acidified chloroform–methanol separated the constituents of calf lung surfactant extract (CLSE) into fractions containing purified phospholipids (PPL), the neutral lipids and phospholipids (N&PL), or the hydrophobic surfactant proteins (SP)-B and -C together with the phospholipids (SP&PL). Extraction of acid to prevent phospholipid degradation after separation reduced recovery of the apoproteins in SP&PL. This fraction was therefore supplemented with protein purified separately to attain the initial levels present in CLSE. Biochemical analyses confirmed that the resulting preparations had the expected composition not only of protein, neutral lipids and phospholipids, but also of the phospholipid head groups. In addition to these fractions obtained with acidified solvent, elution of CLSE with chloroform–methanol without acid yielded the zwitterionic phospholipids substantially depleted of anionic phosphatidylglycerol and phosphatidylinositol. Limited interfacial measurements also demonstrated that the process of separation did not alter the fundamental surface characteristics of the surfactant constituents. Recombined CLSE (rCLSE) reconstituted from all of the separated components had surface activity indistinguishable from the original CLSE. The individual fractions of surfactant components also had average molecular areas at the air–liquid interface which agreed with predictions based on their biochemical composition. These well defined preparations of the hydrophobic constituents of pulmonary surfactant provide the basis for future studies to establish the role of individual components in the function of this complex surface active material.—**Hall, S. B., Z. Wang, and R. H. Notter.** Separation of subfractions of the hydrophobic components of calf lung surfactant. *J. Lipid Res.* 1994. **35:** 1386–1394.

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The ability of pulmonary surfactant to achieve the extraordinarily low surface tensions observed in the lung requires interaction among its multiple constituents. Pulmonary surfactant consists of a complex mixture of phospholipids combined with smaller amounts of apoprotein and neutral lipid. No single constituent can by itself approach the surface activity of the complete mixture. The most prevalent component, dipalmitoyl phosphatidylcholine (DPPC), does lower surface tension quite effectively when artificially spread films of this compound are compressed at an air–liquid interface. DPPC alone, however, adsorbs extremely slowly from the subphase to form surface films, and by itself functions poorly as a pulmonary surfactant (1). Interactions of DPPC with other constituents are essential for the full activity of the complete surfactant.

Prior studies have generally investigated these interactions in simplified model systems of lipids. For instance, the role of the hydrophobic surfactant apoproteins SP-B and SP-C in the biophysical activity of surfactant has been studied in mixtures with DPPC alone or in combination with a small number of other components, most commonly the anionic phospholipid, phosphatidylglycerol (PG). The use of model systems, however, risks oversimplification of the complex mixture of surfactant components. For instance, the phosphatidylcholines (PC) reported to be present in pulmonary surfactant include compounds with both alkyl (2) and alkenyl (3) ether linkages to the fatty acids, numerous monounsaturated compounds (4), and significant amounts of at least one other disaturated compound (5) in addition to DPPC. These minor constituents may have important roles in surfactant function. Before recognition of the presence of the hydrophobic apoproteins in surfactant extracts, these lesser lipids were thought responsible for the more rapid adsorption compared to DPPC (6). Several of the unsatu-

Abbreviations: CLSE, calf lung surfactant extract; PPL, purified phospholipid; DPPC, dipalmitoyl phosphatidylcholine; PC, phosphatidylcholine; SP, surfactant protein; PG, phosphatidylglycerol; PI, phosphatidylinositol; TLC, thin-layer chromatography; SP&PL, surfactant protein and phospholipid; rCLSE, recombined CLSE; N&PL, neutral lipid and phospholipid; mPPL, modified PPL.

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rated phospholipids in pulmonary surfactant improve the respreading of surface films (7, 8), and may contribute to this interfacial property of pulmonary surfactant. Because the complete mix of surfactant lipids has not been examined, the role of these constituents in adsorption and respreading has never been directly tested.

An alternate approach to simplified model systems for investigating the role of components in surfactant activity is the study of surfactant fractions from which specific constituents have been excluded. The difficulty of separating the hydrophobic proteins from the surfactant lipids has previously prevented this approach. The development of biochemical methods for isolating the hydrophobic surfactant apoproteins, however, has also made feasible the separation of the neutral lipids and phospholipids in purified form.

This paper reports chromatographic methods for the isolation of specific hydrophobic subfractions of calf surfactant for future studies on the role of different constituents in surfactant activity. These studies focus on the hydrophobic components of surfactant isolated by extraction of the intact material into organic solvents. The subfractions are generated from extracts of intact surfactant that lack the apoprotein SP-A (9) but contain all the hydrophobic constituents. Because extracts of this kind are only slightly less active than the native material (9, 10), the hydrophobic constituents of surfactant account for most of its ability to lower surface tension to extraordinarily low levels in dynamically compressed films. The subfractions described in this paper include preparations from which the hydrophobic apoproteins and/or neutral lipids are excluded, as well as a mixture containing zwitterionic phospholipids from which anionic compounds have been substantially removed. Limited interfacial measurements with these subfractions demonstrate that their simple surface characteristics agree with expectation based on their biochemical constituents. The surface behavior of reconstituted surfactant, prepared by recombination of all of the separated components, is also indistinguishable from the initial material, proving that the process of isolation did not alter the interfacial characteristics of the individual constituents.

MATERIALS AND METHODS

Surfactants

Calf lung surfactant extract (CLSE) was obtained by extraction of cell-free bronchoalveolar lavage fluid using protocols described previously (6, 9). Lungs were obtained from freshly slaughtered calves (Conti Packing Co., Henrietta, NY) and lavaged thoroughly with cold saline. Lavage fluid from multiple calves was combined, cells were removed by centrifugation at 250 *g* for 10 min, and surfactant particles were pelleted from the resulting supernatant at 12,500 *g* for 30 min. CLSE was obtained by extraction (11) of the pelleted material, and stored in chloroform at −20°C. Comparisons of characteristics of preparations of surfactant components were only made between materials derived from the same batch of CLSE. DPPC (99% pure), egg PC, and egg PG were obtained from Sigma (St. Louis, MO) and used without further purification.

Column chromatography

The different components of CLSE were separated by gel permeation chromatography using a modification of methods originally developed for the purification of proteolipids from brain tissue (12) and subsequently applied to pulmonary surfactant (13, 14). Samples of CLSE containing 20–30 μmol phospholipid in 200 μl were loaded onto a 50 × 1.5 cm column containing hydroxypropyl dextran (LH-20, Pharmacia-LKB, Pistcataway, NJ) at 4°C and eluted with chloroform–methanol–0.1 N HCl 95:95:10 (v/v/v) by upward flow at 4 ml/h. Fractions of column eluent were collected every 7.5 min. The phospholipid fractions were localized by phosphate assay of 5-μl aliquots. Appropriate fractions were then pooled and extracted into chloroform (11) to remove acid. Samples from which protein was to be removed were then concentrated to approximately 200–500 μl and reloaded for a second pass through the column.

In addition to the acidified solvent system, some separations also used solvents containing no acid. Non-acidified solvents included chloroform–methanol 2:1 (v/v), originally reported by Takahashi and Fujiwara (15), as well as the same solvents in a 1:1 ratio. Fractions eluted by neutral solvent immediately prior to the phospholipids contained the purified protein used in subsequent experiments for supplementation of the apoproteins.

Biochemical determinations

Phospholipid was assayed by determination of phosphorus content (16) of measured aliquots from column fractions. The composition of phospholipid head groups was determined from the phosphorus content of compounds separated by one-dimensional thin-layer chromatography (TLC) on 250-μm thick silica gel G (Analtech, Newark, DE) using solvent system C of Touchstone, Chen, and Beaver (17). Total cholesterol (both free and esterified) was assayed by reduction with ferrous sulfate (18). Other neutral lipids were absent on thin-layer chromatography, with the exception of minor amounts of a band with the approximate mobility of diglycerides. Protein was measured by staining of trichloroacetic acid-precipitable material with amido black relative to known amounts of bovine serum albumin (19). Results with this assay remained constant for a fixed amount of the hydrophobic surfactant proteins over the range of 0–20 mg added phospholipid.

Measurements on interfacial films

The interfacial characteristics of spread films were determined on a Wilhelmy balance designed specifically to maximize the confinement of films at the high surface pressures appropriate for the study of pulmonary surfactant (20). Known amounts of samples dissolved in hexane–ethanol 9:1 were added slowly to the surface of the hypophase (150 mM NaCl, 1.5 mM $CaCl_2$, 10 mM HEPES pH 7.0) enclosed by a Teflon ribbon barrier. Molecular areas of these films were expressed in terms of phospholipid spread in Å²/molecule. A 10-min pause between spreading of the sample and initiation of film compression allowed for evaporation of the solvent. Ambient and hypophase temperatures were monitored continuously throughout each experiment, and maintained at either 23 ± 2°C or 37 ± 0.2°C. Ambient humidity was maintained fully saturated by several open dishes of water in the balance chamber, along with dampened blotting paper on the chamber walls. The surface area was compressed and expanded between 448.6 cm² (100% area) and 103.2 cm² (23.0%) at constant rates of 10 or 1.5 min per complete cycle. Surface pressure was measured continuously during cycling by a sand-blasted Wilhelmy plate within the area enclosed by the Teflon ribbon, and by a second plate outside the ribbon to monitor for film leakage. Experiments in which leakage was detected at any measurable surface pressure were discarded. Analysis of data included tabulations of maximum surface pressure, and of molecular areas at the point of lift-off and at 1 mN/m surface pressure. Lift-off was defined as the point at which surface pressure was first detectably greater than zero during compression of a dilute film.

Oscillating bubble experiments

Measurements of surface activity were also made on dispersions of surfactant during cycling at rates (20 cycles/min) comparable to normal rates of respiration on a pulsating bubble surfactometer (Electronetics, Amherst, NY) (21). Experiments were performed at 37°C with samples dispersed by probe sonication in 150 mM NaCl, 1.5 mM $CaCl_2$, and 10 mM HEPES (pH 7.0). Surface tensions were calculated from the pressure drop across the interface at known minimum bubble radius using the law of Young and Laplace for a spherical surface.

RESULTS

Separation of components of CLSE using acidified solvents

Column chromatography of calf lung surfactant extract (CLSE) separated the protein, phospholipid and neutral lipid into discrete peaks (**Fig. 1A**), shown previously by Hawgood and coworkers (14). This separation formed the basis of the preparation of our subfractions. The protein and phospholipid fractions, however, overlapped significantly. The pooled phospholipid fractions therefore contained small amounts of protein, which were removed by a second pass through the column (Fig. 1B). The neutral lipids formed a separate peak after a single pass through the

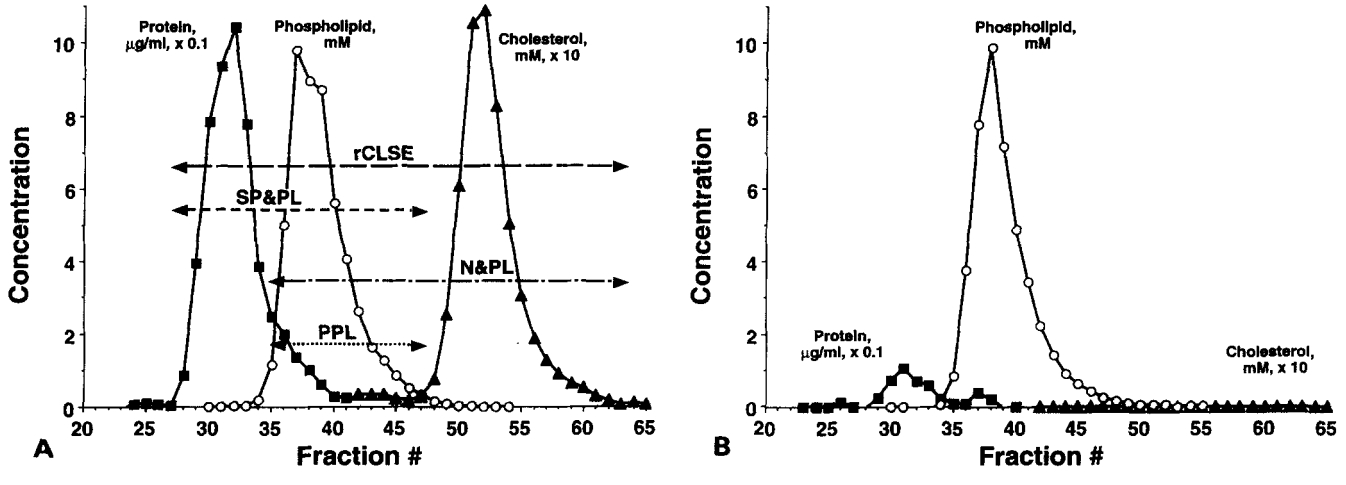

Fig. 1. Separation of purified phospholipids from CLSE with LH-20 column chromatography using acidified chloroform–methanol. Samples were applied in approximately 200 µl chloroform and eluted with chloroform–methanol–0.1N HCl 95:95:10 (v/v/v) at 4 ml/h into fractions collected every 7.5 min. Fractions were assayed for protein (filled squares), phospholipid (open circles), and total cholesterol (filled triangles). A: Components of CLSE (approximately 20 µmol phospholipid) separated by a single pass through the column. Horizontal lines indicate the fractions that were collected to obtain the different preparations of surfactant components: rCLSE, recombined calf lung surfactant extract; N&PL, neutral and phospholipid; SP&PL, surfactant protein and phospholipid; PPL, purified phospholipid. B: Components of PPL separated during a second pass through the column. PPL from the first pass, designated in Fig. 1A, were pooled, extracted into chloroform (11), concentrated to approximately 250 µl, and passed through the column a second time.

column with no overlap with the phospholipids (Fig. 1A).

Only preparations that contained no protein required a second pass through the column. The mix of surfactant protein and phospholipid (SP&PL), for instance, needed no further purification and was obtained simply by pooling the fractions indicated in Fig. 1A. Similarly, recombined CLSE (rCLSE) consisted of all fractions containing any component of surfactant, and was obtained from the fractions from the first pass (Fig. 1A). However, both the purified phospholipids (PPL) and neutral and phospholipids (N&PL) required removal of the protein. Therefore, the relevant fractions from the first pass (Fig. 1A) were pooled and eluted from the column a second time (shown in Fig. 1B for PPL).

Biochemical analysis demonstrated that the lipids in the different preparations included the intended constituents. The head group composition of the phospholipids in PPL was essentially unchanged from that of the CLSE from which they were obtained (**Fig. 2**). Neutral lipids were maintained in N&PL and rCLSE (**Fig. 3A**) at the same ratio of cholesterol to phospholipid (85.1 ± 3.8 and 83.5 ± 4.1 nmol/μmol, respectively) found in CLSE (83.6 ± 3.4 nmol/μmol). Although these neutral lipids were not completely absent from the PPL and SP&PL fractions, the cholesterol-to-phospholipid ratios (6.5 ± 3.6 and 6.0 ± 2.0 nmol/μmol, respectively) were more than an order of magnitude less than found in CLSE (83.6 ± 3.4 nmol/μmol).

This protocol also rigorously excluded protein from preparations containing only pooled lipid fractions (Fig. 3B). The protein content was below the limits of detection for both PPL and N&PL. The limits of the assay suggest that the maximum protein-to-phospholipid ratio possible in these samples would be 0.5 μg/μmol, and that the protein content decreased by at least 16-fold relative to CLSE (9.80 ± 0.29 μg/μmol). However, the preparations that included the protein fractions did not maintain the protein levels found in CLSE. The initial rCLSE and SP&PL both contained significantly less protein than the initial CLSE (Fig. 3B). Recovery of protein varied considerably for these preparations, ranging from 60 to 90% of the protein in CLSE. Fractions obtained directly from the column did contain the expected amount of protein, but significant losses occurred during extraction of the samples to remove acid. Repeated extractions improved the yield of protein, but did not reliably achieve full recovery. Consequently, the initial preparations of SP&PL and rCLSE were supplemented with protein purified separately to restore the protein/phospholipid ratio to the value established for the batch of CLSE from which the components were obtained. This ratio varied from 9.10 to 9.80 μg/μmol for three different preparations of CLSE.

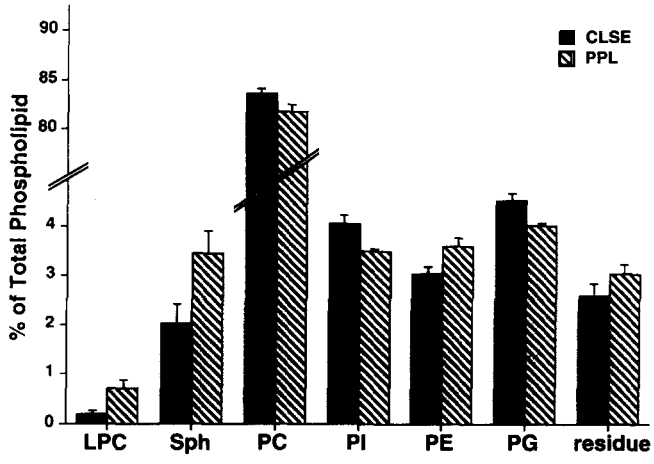

Fig. 2. Composition of phospholipid head groups in CLSE and PPL. Phospholipids containing the different head groups were separated by thin-layer chromatography (17), and quantified by phosphate assay (16). Data are means ± SEM; n = 4.

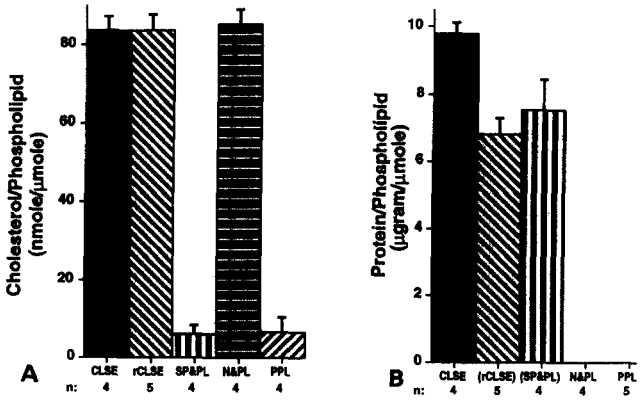

Fig. 3. Relative amounts of total cholesterol and protein in component mixtures derived from CLSE. Data are expressed as protein and total cholesterol relative to the phospholipid content to facilitate comparison between samples containing different amounts of phospholipid. Data are means ± SEM (n as indicated). A: Total cholesterol. B: Protein. (rCLSE) and (SP&PL) indicate these preparations prior to supplementation with protein to levels found in CLSE.

Separation of components using neutral solvents

Solvent systems that contained no acid produced an apparently similar separation of components, but the phospholipids isolated with these systems had greatly reduced amounts of the acidic compounds PG and PI. Takahashi and Fujiwara (15) have previously reported the use of chloroform–methanol 2:1 (v/v) to separate the components of bovine surfactant on LH-20. This solvent system, or chloroform–methanol 1:1, both separated the three components in a pattern similar to that achieved with the acidified solvent (Fig. 1) but with a slightly greater separation between the neutral lipid and phospholipid peaks. However, the modified PPL (mPPL) obtained with either the chloroform–methanol 1:1 or with the 2:1 solvent con-

tained much less PI and PG than CLSE (**Fig. 4**). The 2:1 solvent was particularly effective at eliminating these compounds. PI and PG fell from 3.4 ± 0.1% and 5.4 ± 0.4% in CLSE to 0.2 ± 0.1% and 0.1 ± 0.1% in that solvent (Fig. 4).

Experiments with mixtures of commercially prepared phospholipids suggested that the PG and PI in CLSE elute in neutral solvent after the main phospholipid peak over a broad range of fractions in amounts that were too small to detect. The fate of these anionic compounds was not apparent from the elution pattern of the phospholipids as a whole, which showed a single peak similar to that produced by the original acidified solvent, with no evidence of a distinct population of phospholipids migrating separately. However, a sample containing a large amount of both egg PC and PG (12 mg total; PC:PG 7:5, w/w) produced not only the single sharp peak seen with CLSE, but also a prolonged tail in subsequent fractions in which the phospholipid concentration did not return to baseline (**Fig. 5**). The main peak contained only PC on thin-layer chromatograms, and later fractions had only PG. A similar distribution of the relatively small amount of anionic phospholipid in CLSE would result in quantities per fraction below the limits of our phosphate assay, which explains the absence of a discrete peak containing these compounds.

These non-acidified solvents were also used to produce the protein samples for supplementation of the SP&PL and rCLSE preparations to achieve the correct ratio of protein to phospholipid. Pooled fractions obtained with these neutral solvent systems did not require extraction to avoid acid-catalyzed hydrolysis, and preparation of purified protein was therefore simpler. Fractions eluted with chloroform–methanol 1:1 immediately prior to the phospholipid peak provided the supplements used in the preparations of SP&PL and rCLSE.

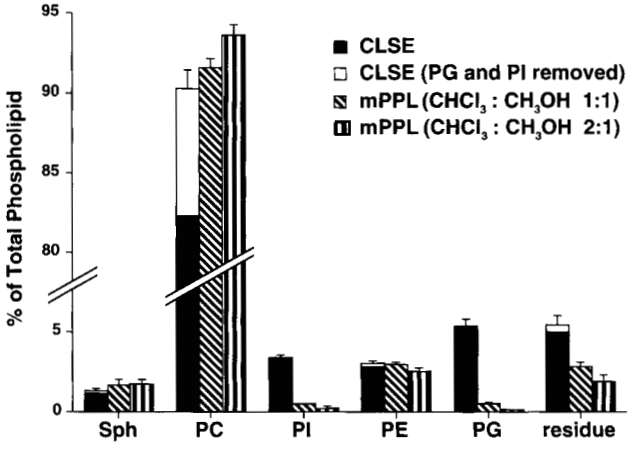

Fig. 4. Composition of purified phospholipids eluted from LH-20 using chloroform–methanol without acid. Solid bars indicate the actual composition of CLSE loaded onto the column. For comparison with the purified phospholipids, the bars with horizontal lines indicate the composition of CLSE recalculated to show the effect of removal of PG and PI. Data are means ± SEM; n = 6.

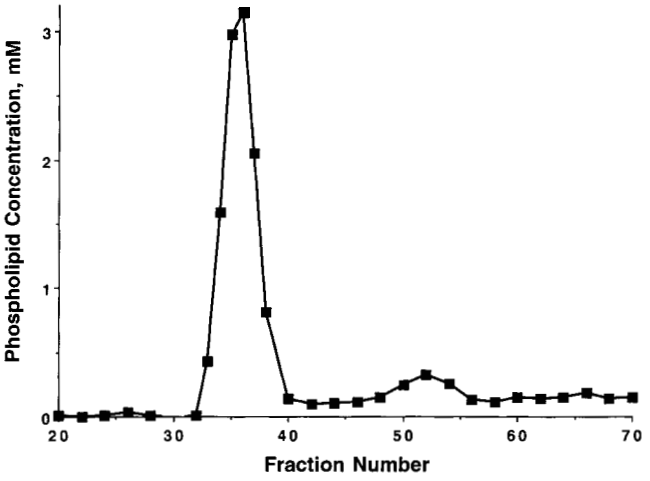

Fig. 5. Elution of phospholipid with chloroform–methanol from column of LH-20 loaded with PC–PG (both from egg yolk) 7:5 (w/w).

Fundamental interfacial characteristics of component mixtures

Limited interfacial measurements showed that the fundamental attributes of the different preparations in surface films agreed with expectations based on the biochemical analyses. The average surface area per molecule of phospholipid for the different preparations fit with measurements of their composition. Compression of films spread at low surface concentrations under quasi-equilibrium conditions allowed determination of highly reproducible molecular areas at specific surface pressures (**Table 1**). At lift-off, at 1 mN/m (Table 1), and at other surface pressures less than 40 mN/m, the order of increase in these areas was: DPPC < PPL < SP&PL, N&PL < CLSE. This order agreed with expected composition of the interfacial films. DPPC, which should pack the most tightly because of its electrostatically neutral head group and fully saturated fatty acids, occupied the least interfacial area. PPL, which contains the complete mixture of surfactant phospholipids including anionic phospholipids and compounds with unsaturated aliphatic chains in addition to DPPC, produced more expanded films. The additional presence of either neutral lipids in N&PL, or proteins in SP&PL, increased the average area further because these areas were expressed per molecule of phospholipid. Finally, the presence of both neutral lipid and proteins in CLSE produced the largest average area per phospholipid.

TABLE 1. Molecular areas for interfacial films of surfactant components during compression on a Wilhelmy balance

Films	Molecular area[a] At Lift-Off	At π^b = 1 mN/m	n
CLSE	118.9 ± 0.2	113.2 ± 0.1	4
N&PL	117.1 ± 0.4	111.5 ± 0.7	6
SP&PL	116.5 ± 0.3	111.0 ± 0.5	5
PPL	114.8 ± 0.1	109.1 ± 0.3	5
DPPC	107.8 ± 0.5	102.9 ± 0.5	6

[a] Molecular areas are expressed as Å²/molecule of phospholipid.
[b] π = Surface pressure. All data are means ± SE. The subphase used in all cases was 150 mM NaCl, 1.5 mM $CaCl_2$, 10 mM HEPES (pH 7.0). Compressions were conducted at 23°C from an initial molecular area of 120 Å² per molecule of phospholipid at 5 min per compression (18.5 Å²/molecule phospholipid/min).

Experiments with recombined CLSE (rCLSE) also showed that the protocol for separating the different preparations did not alter their interfacial characteristics. The surface activity of rCLSE was indistinguishable from that of the CLSE from which it was obtained under multiple conditions designed to probe different aspects of its behavior. At low surface pressures and slow compression, the molecular areas at lift-off (CLSE, 118.9 ± 0.2; rCLSE, 118.5 ± 0.1 Å²/molecule phospholipid) and at 1 mN/m surface pressure (CLSE, 113.2 ± 0.1; rCLSE, 113.4 ± 0.2 Å²/molecule phospholipid), were indistinguishable (**Fig. 6A**). Results were also extremely similar at the higher surface pressures which are particularly important in the function of pulmonary surfactant. These higher pressures were achieved by using a higher initial surface concentration and multiple cycles of compression and expansion (Fig. 6B). Films spread at 60 Å² per phospholipid molecule reached the same pressure at end-compression of approximately 70 mN/m not only on the first compression but on the subsequent six compressions as well (Fig. 6B). Results for the two preparations were also virtually identical under a variety of different conditions, including different temperature (37°C), initial surface concentration (15 Å²/molecule phospholipid), and more rapid compression (1.5 min per complete compression-expansion cycle) (data not shown).

In addition to these experiments with spread films on the Wilhelmy balance, the performance of dispersions of CLSE and rCLSE on the oscillating bubble apparatus was also equivalent (**Fig. 7**). The two samples reached the same minimum surface tension of less than 1 mN/m over virtually the same time course when compared at a phospholipid concentration of 0.75 mM, which was chosen to maximize differences between different surfactants. This assay tests surfactant capabilities beyond those of the interfacial films characterized by the Wilhelmy balance. To lower surface tension effectively on the oscillating bubble, dispersions of surfactant must also adsorb rapidly from the bulk phase to form these interfacial films, as well as lowering surface tension effectively when compressed at the interface. The equivalent performance of CLSE and rCLSE on the oscillating bubble provides further evidence that our method of separation had no effect on the surface activity of surfactant components.

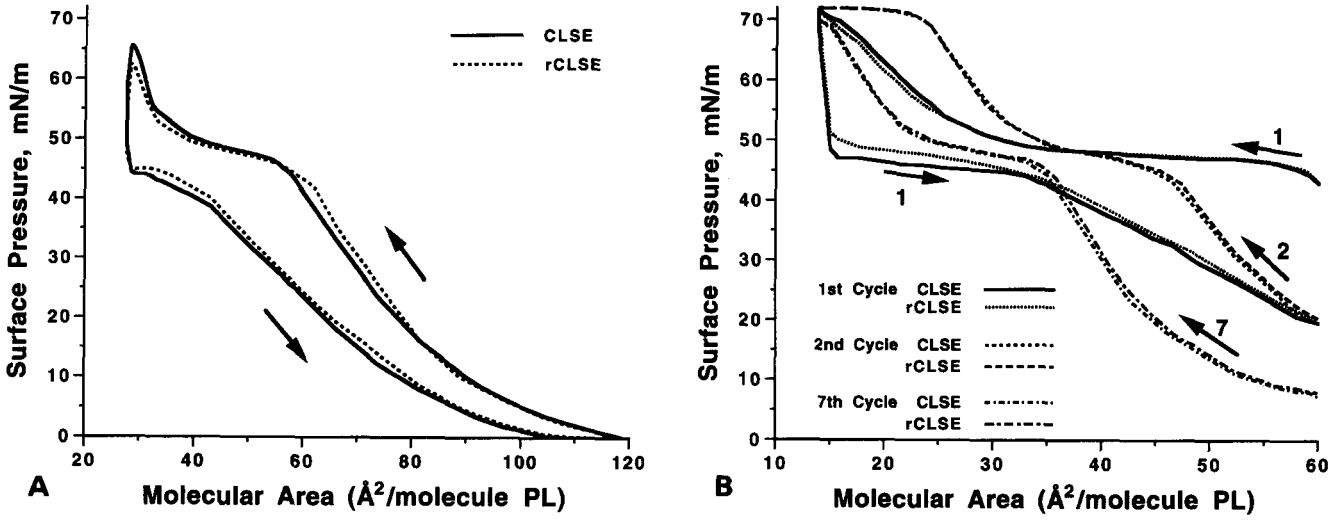

Fig. 6. Comparison of surface pressure for CLSE and rCLSE on a Wilhelmy balance. Samples were spread at the interface in hexane-ethanol 9:1 (v/v), and compressed to 23% of the initial surface area. Individual curves are representative isotherms from a minimum of four experiments. Arrows indicate the direction of area change for each curve. A: First compression-expansion cycle from an initial area of 120 Å² per phospholipid molecule at 23°C and 10 min per complete compression-expansion cycle (18.5 Å²/phospholipid molecule per min). B: Multiple cycles from an initial area of 60 Å² per phospholipid molecule at 23°C and 10 min per complete cycle (9.2 Å²/phospholipid molecule per min). Labels indicate the first compression and expansion, and the second and seventh compressions.

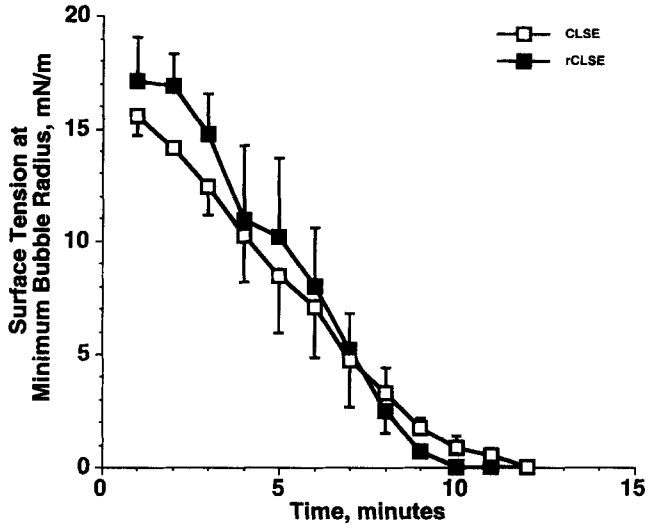

Fig. 7. Reduction of surface tension on an oscillating bubble for CLSE and rCLSE. Surfactant concentration was 0.75 mM phospholipid for both CLSE and rCLSE. Data are means ± SEM for n = 5.

DISCUSSION

These studies document a protocol for separating the hydrophobic components of pulmonary surfactant into subfractions containing the complete set of phospholipids, neutral lipids, and proteins present in the original material. Adjustment of previously published methods using column chromatography with acidified solvent provided a series of preparations from which individual components were excluded. Chromatography using neutral solvent also yielded a modified preparation of surfactant phospholipids which was substantially depleted of the anionic phospholipids PG and PI. Each of these preparations had the expected biochemical composition (Figs. 2, 3, and 4) relative to the CLSE from which they were obtained. They also demonstrated expected interfacial behavior in terms of molecular area at low surface pressures (Table 1).

The chromatographic methods used here represent relatively minor modifications of procedures developed by others previously for the separation of proteolipids (12–14). For our purposes, however, these modifications proved crucial. The acidified solvent produced significant hydrolysis of the phospholipids when not removed from the eluted samples (data not shown). Extraction of the samples to remove the acid, however, resulted in loss of surfactant-associated protein (Fig. 3B), and consequently reduced the ability to lower surface tension relative to CLSE. Supplementation of extracted fractions with protein purified separately restored protein to levels present originally in CLSE. The rCLSE preparation of recombined fractions produced by this method had surface activity indistinguishable from CLSE under several different sets of conditions (Table 1, Figs. 6 and 7, and data not shown), demonstrating that this method of preparation did not alter the interfacial characteristics of the surfactant components.

Modification of this protocol to use neutral rather than acidified solvent yielded preparations of the zwitterionic phospholipids in surfactant, substantially depleted of the acidic phospholipids PI and PG (Fig. 4). Anionic phospholipids generally tend to associate with proteolipids (22), and without added acid, separation of these acidic lipids from hydrophobic proteins can be difficult (23). In our system, however, the acidic phospholipids appeared to bind more tightly to the column than the proteins, similar to findings reported previously by Smith and coworkers (24). Anionic PG eluted from the column over a broad range of fractions following the sharp peak of zwitterionic PC when commercially prepared lipids were applied in neutral solvent (Fig. 5). This pattern suggested that, in the absence of acid, the anionic phospholipids tend to stick to the column, perhaps because of a greater electrostatic charge. Conversely, the overlap between the protein and phospholipid fractions was equivalent for the neutral and acidified solvents, suggesting no greater interaction of the two components in the absence of acid. The loss of these acidic phospholipids suggests that preparations of surfactant phospholipids obtained in prior investigations using neutral solvent systems (15, 25) also lack these compounds. This issue remains unresolved, however, because these prior studies did not report compositional analyses.

The assessment of surface activity in this paper focussed exclusively on confirming that the separated and recombined preparations had reasonable and internally consistent interfacial characteristics. Measurements of the area per molecule of a sample in dilute films provides a sensitive indicator of the presence of unexpected compounds (26). The preparations obtained here all had molecular areas that agreed with expectations based on biochemical composition, and that fit appropriately with data obtained for DPPC and CLSE (Table 1). Molecular areas for rCLSE also agreed well with data for CLSE (Fig. 6A). More extensive measurements comparing the surface activity of the initial and recombined preparations demonstrated their equivalent behavior under several sets of experimental conditions (Figs. 6 and 7, and data not shown). This preservation of activity suggests that any biochemical differences that may result from our protocol are unimportant in surfactant function. For instance, the ratio of SP-B to SP-C in our preparations could differ, because we have assayed only the total amount of protein and not each individual species. The equivalent activity of CLSE and rCLSE, however, argues that any such differences are functionally insignificant.

The preparations of surfactant constituents separated by these methods will be useful in defining the roles of

different components in the surface activity of pulmonary surfactant. A series of mixtures of varying complexity is now possible that can be used to establish the influence of each class of compounds on surface activity. This approach should be particularly valuable in determining the contribution of the phospholipids as opposed to other constituents such as the hydrophobic apoproteins to surfactant function. Prior investigations of the biophysical role of surfactant components have largely relied on simplified model systems of commercially available compounds to replicate the complex mixture of surfactant phospholipids. Comparison of the surface properties of PPL with DPPC can address the functional importance of the many other phospholipids which have long been known to be present in pulmonary surfactant. Similar comparisons with the other preparations obtained here should also define more clearly the role of the apoproteins, neutral lipids, and the anionic phospholipids. The tight regulation of these acidic phospholipids during development, in which the total amount of these anionic compounds remains relatively constant despite substitution of PG for PI around the time of birth in most species, suggests that they have a crucial role in some aspects of surfactant function. Surface studies of mPPL and PPL, which differ only in the absence of these compounds in mPPL, therefore provide the basis for addressing the function of these constituents.

This approach of fractionating extracts of pulmonary surfactant deals only with the hydrophobic constituents of surfactant. Studies with these preparations cannot address the role in surface activity of the major apoprotein SP-A, or of surfactant structures that require SP-A, such as tubular myelin. Extracted surfactants such as CLSE lack SP-A and do not form tubular myelin (27). The activity of these extracts, however, is generally only slightly less than that of the native material (9, 28). This observation suggests that most of the activity of pulmonary surfactant is determined simply by the composition of its hydrophobic constituents, and that the role of specific microstructures and of SP-A is less important.

The protocol presented here is certainly not the only possible approach for preparing subfractions of surfactant constituents. Other possibilities include extraction systems to separate proteolipids from proteins (29, 30), high performance liquid chromatography (31), or modifications of our current system to obtain improved results. A longer column in particular might succeed in separating the phospholipids and proteins on a single pass. Other investigators have used a different column matrix to separate the two hydrophobic apoproteins SP-B and -C into individual fractions (13). The methods of this study do, however, provide preparations with the desired characteristics established by both biochemical and biophysical measurements.

In summary, this paper describes methods of separating the hydrophobic constituents of pulmonary surfactant into preparations from which specific components have been excluded. These preparations provide the basis for determining the role of these components in surfactant activity.

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