Roles of different hydrophobic constituents in the adsorption of pulmonary surfactant

Zhengdong Wang,* Stephen B. Hall,[1,§] and Robert H. Notter[1,*,†]

Departments of Pediatrics* and Environmental Medicine,† University of Rochester, Rochester, NY 14642, and Department of Medicine,§ Oregon Health Sciences University, Portland, OR 97201-3098

Abstract Surface tension–time adsorption isotherms were measured at 37°C for calf lung surfactant extract (CLSE) and subfractions of its constituents: the complete mix of surfactant phospholipids (PPL), phospholipids depleted in anionic phospholipids (mPPL), hydrophobic surfactant proteins plus phospholipids (SP&PL, SP&mPL), and neutral lipids plus phospholipids (N&PL). Adsorption experiments were done using a static bubble surfactometer where diffusion resistance was present, and in a Teflon dish where diffusion was minimized by subphase stirring. The contribution of diffusion to bubble adsorption measurements decreased as phospholipid concentration increased, and was small at 0.25 mM phospholipid. At this phospholipid concentration, PPL, mPPL, and N&PL all adsorbed more rapidly and to lower final surface tensions than dipalmitoyl phosphatidylcholine (DPPC) on the bubble. However, none of these phospholipid mixtures adsorbed to surface tensions below 46 mN/m after 20 min, behavior that was significantly worse than CLSE, SP&PL, and SP&mPL which additionally contained hydrophobic SP. Both CLSE and SP&PL rapidly adsorbed to surface tensions below 25 mN/m at 0.25 mM phospholipid concentration on the bubble, as did SP&mPL at a somewhat reduced rate. Further experiments defining the influence of hydrophobic apoprotein content showed that addition of even 0.13% SP (by wt) to PPL improved adsorption substantially, and that mixtures of PPL combined with 1% SP had adsorption very similar to CLSE. Mixtures of SP combined with mPPL had faster adsorption than corresponding mixtures of SP:DPPC, and neither fully matched the adsorption rates of CLSE and SP&PL even at high SP levels (4% in SP:mPPL and 5.2% in SP:DPPC). These results demonstrate that although the secondary zwitterionic and anionic phospholipids and neutral lipids in lung surfactant enhance adsorption relative to DPPC, the hydrophobic SP have a much more pronounced effect in promoting the rapid entry of pulmonary surfactant into the air–water interface.–**Wang, Z., S. B. Hall, and R. H. Notter.** Roles of different hydrophobic constituents in the adsorption of pulmonary surfactant. *J. Lipid Res.* 1996. **37:** 790–798.

Supplementary key words lung surfactant • apoproteins • surface activity • surface tension • adsorption • phospholipids

A primary requirement for functional pulmonary surfactant is its ability to lower surface tension in an interfacial film in the alveoli. To accomplish this, lung surfactant must have the ability to adsorb rapidly at the air–water interface after secretion into the alveolar hypophase (1–4). The rapid adsorption of lung surfactant requires interactions among its different constituents. DPPC, the single most abundant component of pulmonary surfactant, does not by itself adsorb and spread rapidly at the air–water interface at 37°C (5). DPPC films spread directly at the interface lower surface tension to extraordinarily low values <1 mN/m during dynamic compression (6–8), identifying the disaturated DPPC molecule as a crucial contributor to this aspect of lung surfactant surface activity. The adsorption of DPPC is known to be facilitated by other lung surfactant components (5, 9–13), but the relative contributions of each have not yet been fully delineated. The present study investigates the adsorption of subfractions of the hydrophobic constituents of calf lung surfactant to help identify more fully the relative importance of different molecular classes in the adsorption process.

Pulmonary surfactant is comprised of a diverse set of phospholipids in addition to DPPC, plus at least three surfactant proteins (SP)-A, B, and C, and neutral lipids that are predominantly cholesterol and cholesterol ester (3, 14, 15). Prior studies of lung surfactant adsorption have focused primarily on the surfactant proteins, and their ability to enhance the adsorption of DPPC in

Abbreviations: CLSE, calf lung surfactant extract; DPPC, dipalmitoyl phosphatidylcholine; mPPL, purified lung surfactant phospholipids depleted in anionic components; N&PL, CLSE subfraction containing surfactant neutral lipids plus phospholipids; PG, phosphatidylglycerol; PPL, purified lung surfactant phospholipids; SP-A, surfactant protein A; SP-B/C, hydrophobic surfactant proteins or hydrophobic SP; SP&PL, CLSE subfraction containing hydrophobic SP plus PPL; SP&mPPL, CLSE subfraction containing hydrophobic SP plus mPPL; SP:DPPC, hydrophobic SP and DPPC combined in vitro; SP:mPPL, hydrophobic SP and mPPL combined in vitro; SP:PPL, hydrophobic SP and PPL combined in vitro.

[1]To whom correspondence should be addressed.

model mixtures (2–4, 16 for review). The composition of most model systems, however, has differed considerably from that of native lung surfactant. In order to ensure that contributions to surfactant function from minor phospholipids or neutral lipids are fully characterized, an alternative approach is to work with specific subfractions of hydrophobic lung surfactant constituents separated by column chromatography (17, 18). Such subfractions are not subject to the potential for oversimplification inherent in model lipid mixtures, and are directly applicable for defining the effects of all major lung surfactant constituents with the exception of the hydrophilic surfactant protein, SP-A.

In this paper, we measure surface tension–time adsorption isotherms for calf lung surfactant extract (CLSE) and a series of its subfractions including the complete mix of purified phospholipids (PPL), purified phospholipids depleted in anionic phospholipids (mPPL), neutral lipids and phospholipids (N&PL), and hydrophobic surfactant proteins and phospholipids (SP&PL and SP&mPL). Our prior work has shown that these chromatographically generated subfractions contain the appropriate surfactant constituents, and that their surface behavior is unaffected by the separation methodology (17). Recent surface studies with these subfractions by Wang, Hall, and Notter (18) have shown that the secondary surfactant phospholipids (phospholipids other than DPPC) have striking effects in enhancing respreading within the surface film itself during dynamic cycling, and that this film behavior is also facilitated by the hydrophobic surfactant proteins. The current study examines the relative importance of the hydrophobic surfactant proteins, the secondary surfactant phospholipids, and the neutral lipids in the adsorption process by measuring and comparing adsorption isotherms for different CLSE subfractions and for selected model mixtures.

MATERIALS AND METHODS

Synthetic phospholipids

L-α-Dipalmitoyl phosphatidylcholine (DPPC) and egg phosphatidylglycerol (egg-PG) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). DPPC and egg-PG were >99% pure as supplied, and gave single spots on thin-layer chromatography with solvent system C of Touchstone, Chen, and Beaver (19). Model mixtures containing hydrophobic surfactant proteins (SP) combined with DPPC, 9:1 (mole ratio) DPPC:egg-PG, or surfactant phospholipid subfractions, were formulated by adding aliquots of SP in chloroform–methanol 1:1 (v:v) to phospholipids in chloroform at the desired final composition ratio. The organic solvents were evaporated under nitrogen, and the resultant dry mixtures were dispersed in the aqueous phase by sonication (see below).

Lung surfactant extract and surfactant subfractions

CLSE was prepared by extraction of cell-free bronchoalveolar lavage fluid as described by Notter and Shapiro (20), Whitsett et al. (21), and Notter et al. (22). Lungs from freshly slaughtered calves (Conti Packing Co., Henrietta, NY) were lavaged thoroughly with cold 0.15 M NaCl, followed by centrifugation at 250 *g* for 10 min to remove cellular debris. The supernatant was then centrifuged at 12500 *g* for 30 min to pellet surfactant aggregates, which were resuspended and extracted with chloroform–methanol (23) to give CLSE. A series of subfractions was separated from CLSE by gel permeation column chromatography (17). A single pass through an 1.5 × 50 cm column of LH-20 (Pharmacia-LKB Biotechnology, Piscataway, NJ) in chloroform–methanol–0.1 N HCl 47.5:47.5:5 (by volume) separated surfactant proteins and phospholipids (SP&PL) from neutral lipids. Two column passes were used to obtain purified phospholipids (PPL), and neutral lipids and phospholipids (N&PL), having a protein content below the limits of detection by the amido black assay of Kaplan and Pedersen (24). Extraction of samples to remove acid limited recovery of proteins, and SP&PL subfractions were supplemented with SP purified separately to restore the protein content to its original level of 1.3% by weight relative to phospholipid found in CLSE (17). Surfactant phospholipids depleted in anionic components (mPPL) were obtained from CLSE by a related protocol using elution with non-acidified chloroform–methanol 2:1 (v:v) rather than acidified solvent. mPPL had a total content of anionic components PG and phosphatidylinositol (PI) reduced by more than an order of magnitude relative to PPL (17). Total phospholipid concentrations for all surfactant subfractions and synthetic lipids were determined by phosphate assay (25).

Adsorption studies

The majority of adsorption measurements were made on surfactant dispersions in a bubble surfactometer (Electronics, Inc., Amherst, NY) (26) with the bubble fixed at minimum radius (0.4 mm). Surfactants were dispersed in HSC buffer (10 mM HEPES, pH 7.0, 150 mM NaCl, 1.5 mM $CaCl_2$) using three 10-sec bursts of 50 watts power on a Heat Systems Sonicator (Model W-220F). Dispersions were added to the bubble sample chamber, and a static air bubble was created at time zero. Interfacial pressure drop was then measured as a function of time for 20 or 60 min at 37°C with bubble radius held constant. Surface tension was calculated

from the law of Young and Laplace for a sphere. Because these static bubble measurements incorporated a diffusion resistance, comparative adsorption experiments on CLSE were done in a Teflon dish with a stirred subphase to minimize diffusion (9, 22, 27). Experiments in this system were initiated by injection of known amounts of CLSE, dispersed in 10 ml of HSC buffer as above, beneath the surface of a 70 ml adsorption subphase that was continuously stirred by a Teflon-coated bar and magnetic stirrer (9). Surface tension was determined from the force on a sandblasted platinum slide dipped into the interface.

RESULTS

The adsorption of DPPC, CLSE, and subfractions of CLSE (PPL, mPPL, N&PL, SP&PL) measured with the static bubble method is shown in **Fig. 1** for a fixed phospholipid concentration of 0.25 mM. PPL, which contained the complete mix of phospholipids in natural surfactant, had significantly better adsorption than pure DPPC (surface tension 52.9 ± 0.7 vs. 63.0 ± 0.6 mN/m, respectively, after 20 min in Fig. 1). The adsorption of mPPL was almost equivalent to PPL, while the adsorption of N&PL was slightly better (46.7 ± 1.1 mN/m, Fig. 1). The most striking effects on adsorption were generated by the hydrophobic surfactant proteins. Their presence in the SP&PL subfraction gave overall adsorption characteristics very similar to CLSE, both in terms of time-dependent behavior and in the final surface tension reached after 20 min (24.4 ± 0.3 for SP&PL vs. 23.6 ± 0.8 mN/m for CLSE, Fig. 1). The adsorption of CLSE and SP&PL was more rapid and to a much lower final surface tension than found for PPL, mPPL, or N&PL (Fig. 1).

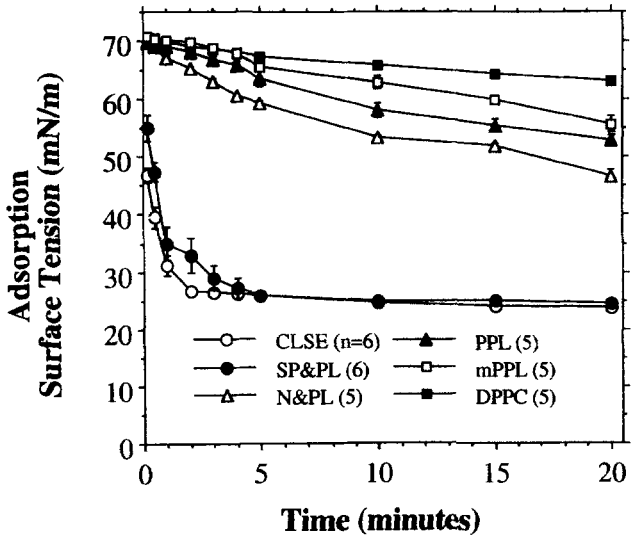

Fig. 1. Adsorption of DPPC, CLSE, and subfractions of CLSE. Surface tension as a function of time was measured for surfactant dispersions adsorbing at the interface of a stationary air bubble at 37°C (Methods). Phospholipid concentration was 0.25 mM. Data are plotted as means ± SEM for n ≥ 5.

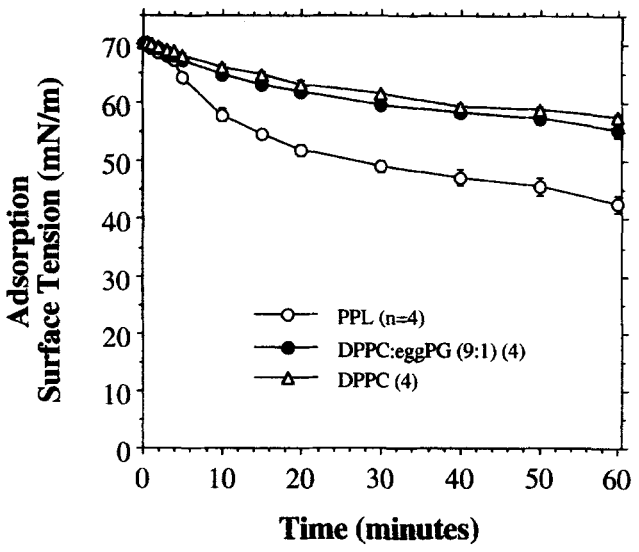

Fig. 2. Adsorption of DPPC, DPPC:egg PG (9:1 by mole), and PPL in the bubble apparatus over longer times. Adsorption experiments with phospholipids were done for 60 min to further assess their potential to lower surface tension during adsorption. Phospholipid concentration (0.25 mM) and temperature (37°C) were as in Fig. 1. Data are means ± SEM for n = 4.

Additional static bubble experiments over a longer timescale of 60 min were done with PPL to assess more completely its ability to lower surface tension during adsorption. Even after 60 min, dispersions of PPL did not approach the equilibrium surface tension values measured for CLSE and SP&PL at much shorter times (**Fig. 2**). PPL dispersions adsorbed to surface tensions of 42.6 ± 1.6 mN/m after 60 min on the static bubble apparatus at a phospholipid concentration of 0.25 mM (Fig. 2). The adsorption of DPPC and 9:1 DPPC:egg PG was even slower, giving surface tensions of 57.6 ± 0.4 and 55.3 ± 1.2 mN/m, respectively, after 60 min (Fig. 2). In contrast, CLSE dispersions generated adsorption surface tensions <30 mN/m after only 2 min, and reached low final equilibrium surface tension within about 5 min (Fig. 1).

To help define the importance of diffusion in static bubble data, the adsorption of CLSE at different concentrations was measured in a Teflon dish with a stirred subphase and compared to corresponding measurements on the bubble apparatus (**Table 1**). For a fixed concentration and time, surface tensions measured for CLSE dispersions on the bubble were always higher than in the Teflon dish apparatus (Table 1), indicating that a diffusion resistance was present in bubble data. As

TABLE 1. Comparison of adsorption of CLSE in a stationary bubble apparatus (A) and in a dish with a stirred subphase (B)

Concentration		Adsorption surface tension (mN/m) at time (minutes)				
		2.0	5.0	10.0	15.0	20.0
mM				mN/m		
0.0625	A	62.6 ± 1.2	51.1 ± 3.3	44.6 ± 2.0	38.9 ± 1.2	37.3 ± 0.7
0.0625	B	38.7 ± 0.9	27.8 ± 0.9	22.9 ± 0.5	22.1 ± 0.1	22.0 ± 0.0
0.125	A	60.8 ± 1.9	45.2 ± 2.5	34.7 ± 1.5	28.5 ± 0.6	23.8 ± 0.3
0.125	B	23.3 ± 0.9	22.1 ± 0.1	22.0 ± 0.0	22.0 ± 0.0	22.0 ± 0.0
0.25	A	26.7 ± 0.5	25.9 ± 0.7	24.6 ± 0.9	23.9 ± 0.8	23.6 ± 0.8
0.25	B	21.3 ± 0.3	21.0 ± 0.0	21.0 ± 0.0	21.0 ± 0.0	21.0 ± 0.0
0.5	A	23.6 ± 0.5	22.6 ± 0.2	22.6 ± 0.2	22.6 ± 0.2	22.6 ± 0.2
0.5	B	21.0 ± 0.0	21.0 ± 0.0	21.0 ± 0.0	21.0 ± 0.0	21.0 ± 0.0

CLSE was dispersed in HSC buffer by sonication (Methods). A: adsorption with diffusion in bubble surfactometer. B: adsorption with minimal diffusion in dish. Data are means ± SEM for n = 4–6.

expected, the magnitude of this diffusion resistance decreased as subphase concentration increased. There was relatively little difference between the adsorption of CLSE dispersions studied in the bubble apparatus and Teflon dish at concentrations of 0.25 and 0.5 mM phospholipid (Table 1). The remainder of reported adsorption results utilized the static bubble method at a subphase phospholipid concentration of 0.25 mM where diffusion effects were small.

Additional experiments examined how phospholipid composition and apoprotein content affected the ability of the hydrophobic apoproteins to facilitate adsorption (Figs. 3–5). The adsorption of SP&PL, SP&mPL, and SP:DPPC mixtures, each having the same 1.3% SP content, is shown in **Fig. 3.** In phospholipid content, SP&PL contained the complete mix of lung surfactant phospholipids, SP&mPL contained surfactant phospholipids depleted in anionic components, and SP:DPPC contained no secondary surfactant phospholipids. The adsorption of all three of these SP-containing preparations was substantially greater than the corresponding phospholipids alone, i.e., PPL, mPPL, or DPPC (compare Figs. 1 and 3). The complete mix of phospholipids in SP&PL gave rise to more rapid adsorption than SP&mPL, although both reached the same final equilibrium surface tension (Fig. 3). There was little difference between the adsorption of SP&mPL and SP:DPPC at short times, but the final surface tension reached was lower for SP&mPL (25.6 ± 1.0 mN/m for SP&mPL at 15 min vs. 30.3 ± 0.5 mN/m for SP:DPPC at 20 min, Fig. 3).

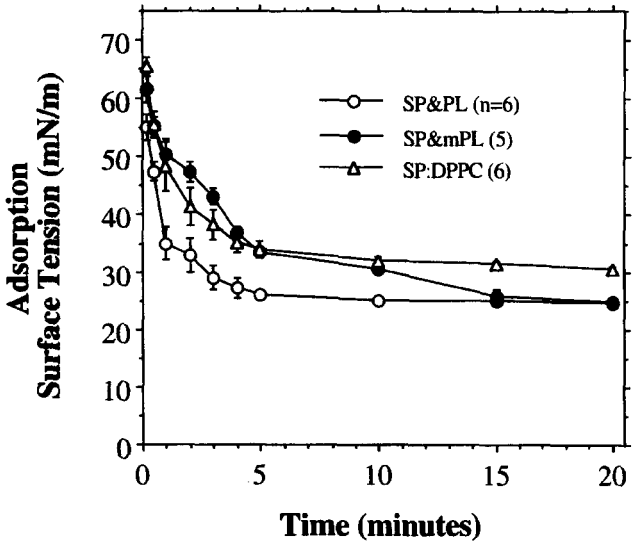

Fig. 3. Adsorption isotherms for SP&PL, SP&mPL, and SP:DPPC (1.3% by weight SP). The three mixtures contained different phospholipids, but had an equal content of hydrophobic SP at the level of 1.3% by weight present in CLSE. Phospholipid concentration 0.25 mM; temperature 37°C. Data are means ± SEM for n = 5–6.

The influence of apoprotein content on adsorption is shown for PPL, mPPL, or DPPC combined in vitro with varying amounts of hydrophobic SP in **Fig. 4(A–C).** The adsorption of all phospholipid:SP mixtures increased in both rate and magnitude as SP content was increased (Fig. 4A–4C). Even small amounts of SP, significantly less than the 1.3% by weight found in CLSE (17), significantly enhanced the adsorption of PPL (Fig. 4A). An SP content of only 0.13% gave a surface tension of 31.7 ± 1.6 mN/m after 20 min, substantially reduced from the 52.9 ± 0.7 mN/m value for PPL alone. Added SP in a weight percent of 0.33% gave a 20-min surface tension similar to CLSE (24.5 ± 0.5 mN/m, Fig. 4A), and the rate of adsorption increased as SP content was increased further. At fixed SP content, the adsorption of SP:mPPL mixtures was reduced compared to SP:PPL (Fig. 4B vs. Fig. 4A), and the adsorption of SP:DPPC was reduced even further (Fig. 4C vs. Fig. 4B).

The surface tension after 20 min of adsorption for SP:PPL, SP:mPPL, and SP:DPPC is plotted as a function of hydrophobic SP content in **Fig. 5.** SP:PPL reached the lowest final surface tension at 20 min for the smallest content of SP relative to phospholipid (Fig. 5). For an

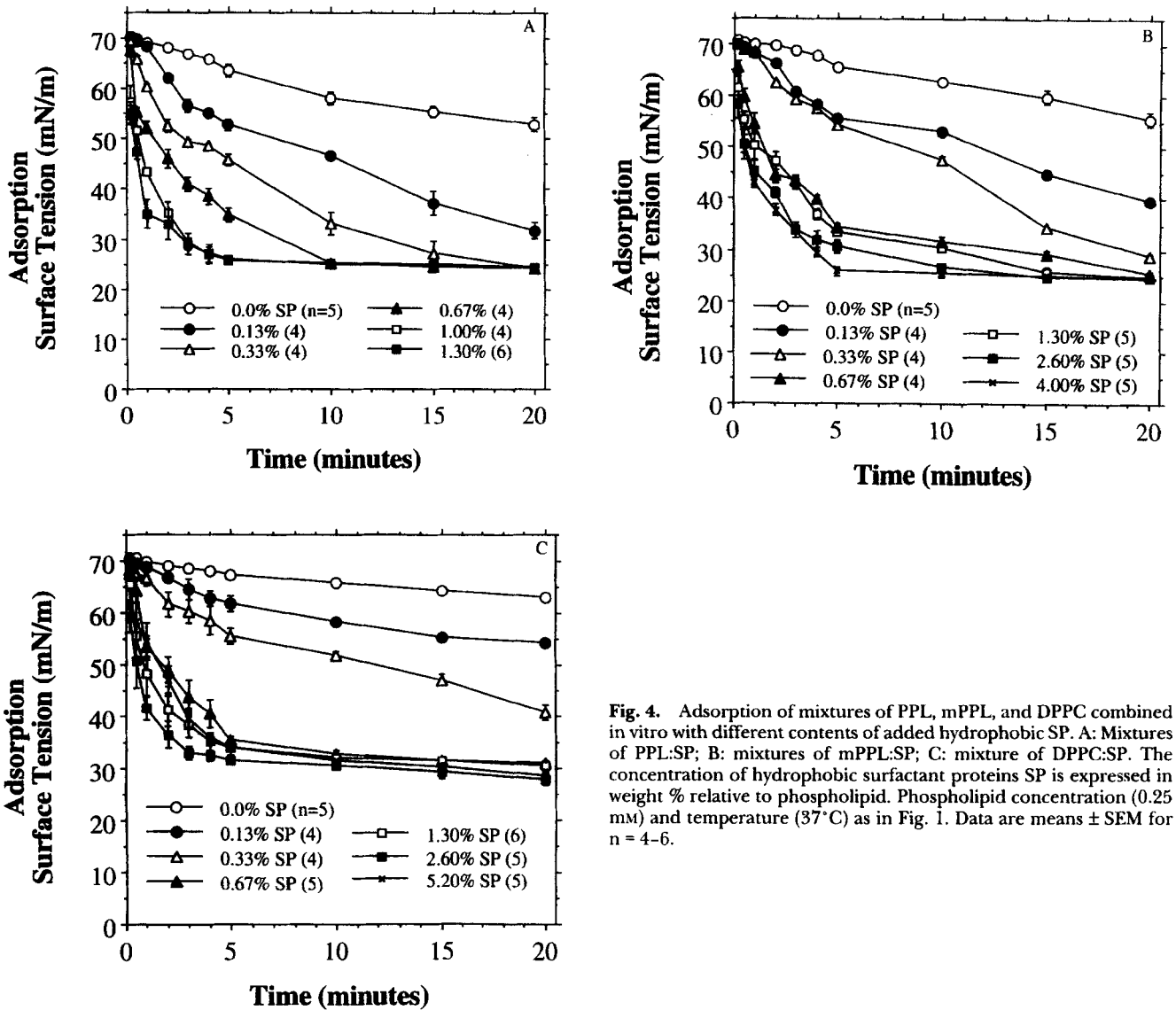

Fig. 4. Adsorption of mixtures of PPL, mPPL, and DPPC combined in vitro with different contents of added hydrophobic SP. A: Mixtures of PPL:SP; B: mixtures of mPPL:SP; C: mixture of DPPC:SP. The concentration of hydrophobic surfactant proteins SP is expressed in weight % relative to phospholipid. Phospholipid concentration (0.25 mM) and temperature (37°C) as in Fig. 1. Data are means ± SEM for n = 4–6.

SP content of 0.33% by weight, SP:PPL had a final surface tension of 24.2 ± 0.5 mN/m compared to 28.9 ± 0.6 mN/m for SP:mPPL and 40.8 ± 1.5 mN/m for SP:DPPC. The final surface tension of SP:mPPL at 0.67% SP (25.3 ± 0.5 mN/m) was almost equivalent to that of SP:PPL (Fig. 5), but the time necessary to reach this value was increased (Fig. 4B vs. 4A). This pattern was also followed by mixtures of SP:mPPL vs. SP:PPL at higher protein contents (Figs. 4B, 4A). Mixtures of SP:DPPC consistently had higher final surface tensions at 20 min than SP:PPL and SP:mPPL (Fig. 5). At 0.67% SP, the surface tension of SP:DPPC after 20 min of adsorption was 30.9 ± 0.3 mN/m, and this value did not fall below 28.9 ± 0.9 mN/m even when SP content was raised to the artificially high value of 5.2 weight % (Fig. 5).

DISCUSSION

This study addresses the importance of different hydrophobic components of lung surfactant in enhancing its ability to adsorb to the air–water interface. The results show that the hydrophobic surfactant proteins have a much more pronounced effect than the secondary surfactant phospholipids and neutral lipids on this functionally important interfacial behavior. The complete mix of lung surfactant phospholipids in PPL adsorbed better than pure DPPC, the single most abundant molecular constituent of the surfactant system (Figs. 1, 2), and the neutral lipids gave a small additional improvement in adsorption (compare results for N&PL vs. PPL and CLSE vs. SP&PL, Fig. 1). However, the most striking facilitation of adsorption was generated by the

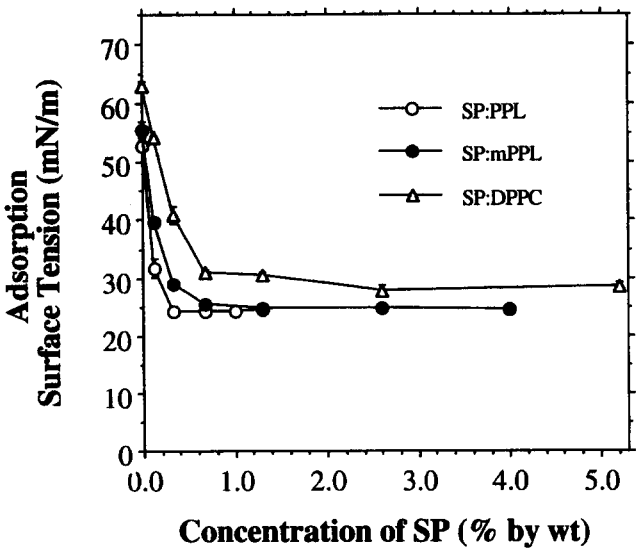

Fig. 5. Dependence of phospholipid adsorption at 20 min on SP concentration. Results are shown for mixtures of PPL, mPPL, or DPPC combined with hydrophobic SP in different weight % concentrations relative to phospholipid. Data are expressed as the surface tension reached after 20 min of adsorption at 37°C on a static bubble surfactometer. Data are means ± SEM for n = 4–6.

hydrophobic surfactant apoproteins. Addition of SP to PPL in increasing amounts from 0.13% to 1.3% by weight gave a pronounced, concentration-dependent increase in adsorption that approached CLSE appropriately (Fig. 4A). Although there were some differences in adsorption magnitude and/or rate when SP were combined with phospholipids of differing composition in PPL vs. mPPL vs. DPPC (Figs. 3, 4), these were small compared to the substantial overall adsorption improvements found for all SP-containing mixtures relative to phospholipids alone (Figs. 1, 2). This is in contrast to actions within the dynamically compressed interfacial film itself, where the complete mix of lung surfactant phospholipids in PPL has a dramatic effect in enhancing film respreading during continuous cycling (18).

Our studies involved only the hydrophobic constituents of lung surfactant, and effects from the more polar surfactant protein SP-A were not defined. Because of extraction into organic solvents like chloroform, preparations such as CLSE do not contain SP-A (21), although all other recognized biochemical components of lung surfactant are present. Surfactant extracts do not adsorb with a tubular myelin microstructure as observed for natural surfactant (22), a process now known to require both SP-A and SP-B (28). However, a large body of prior work has shown that CLSE and related surfactant extracts can be mechanically dispersed in the aqueous phase to give adsorption and overall surface and physiological activity very close to that of natural surfactant (2, 20, 29–31). This implies that the hydrophobic components of lung surfactant incorporate the majority of functionally important surface behaviors and interactions, and it is the roles and importance of specific subsets of these components that are considered here.

The majority of our experiments utilized adsorption measurements at the interface of a static air bubble, a design incorporating measurable bulk phase diffusion. An alternate Wilhelmy plate method with a stirred subphase was used to indicate the contribution of diffusion in CLSE adsorption measurements at different phospholipid concentrations. Although stagnant layer effects are never completely removed at finite stirring rate, this method has been used extensively to characterize the adsorption facility of lung surfactants (1, 2, 9, 27). Comparisons of CLSE adsorption from these two methods at several bulk phase concentrations demonstrated that although diffusion effects were present in the bubble measurements, they decreased as concentration increased (Table 1). The majority of bubble experiments utilized a concentration of 0.25 mM phospholipid where the contribution of diffusion was relatively low. In addition, selected surface tension measurements were carried out for extended periods up to 1 h to demonstrate that phospholipid adsorption data were not diffusion-limited (Fig. 2).

Previous studies investigating the relative contributions of lung surfactant lipids and hydrophobic apolipoproteins to surface activity have been complicated by an inability to define the behavior of the phospholipids and neutral lipids in the absence of protein. Surfactant apolipoproteins are known to enhance the adsorption and/or dynamic surface activity of a variety of model lipid mixtures (2, 4, 16 for review), and also to improve the activity of clinical exogenous surfactants (30–32). The importance of apolipoproteins in biophysical activity has also been suggested by studies with subpopulations of surfactant aggregates separated from animal lungs by differential sedimentation (33) or density gradient centrifugation (34), which form interfacial films at different rates and contain varying amounts of apolipoproteins but the same phospholipids. However, prior studies have not ruled out the possibility that the complete mix of lung surfactant lipids, separate from apolipoproteins, might also have pronounced effects on adsorption. The present work demonstrates that although adsorption relative to DPPC is improved by the secondary surfactant phospholipids and neutral lipids, the hydrophobic SP have a much more important influence on this surface behavior (Fig. 1).

Our experiments address in some detail the dependence of adsorption on the content of hydrophobic apolipoproteins in mixtures with different phospholipids (Figs. 3–5). When added to the complete mix

of surfactant phospholipids in PPL, SP improved adsorption even at a low content of 0.13% SP by weight (Fig. 4A). The amount of hydrophobic SP in typical surfactant extracts is generally 1–2% by weight based on a modified Lowry protein assay (9, 20–22, 30), and Hall, Wang, and Notter (17) report a value of 1.3% for CLSE using amido black staining of trichloroacetic acid-precipated material (24). Yu and Possmayer (13) measured the effect of SP-B or SP-C on the adsorption of DPPC:PG, and found that the minimum protein concentration for optimal adsorption was 1% for SP-B and 1.5% for SP-C. This is consistent with our results that an SP content of about 1% added to PPL gave adsorption very similar to CLSE (Fig. 4A).

The exact mechanism(s) by which the hydrophobic apolipoproteins enhance the adsorption of surfactant phospholipids are currently unknown. In the adsorption process, phospholipids must insert into the interface from the underlying subphase, moving as monomers and aggregates, and interactions with surfactant apolipoproteins clearly facilitate this process. The movement of lung surfactant components into a tensioactive monolayer involves changes in enthalpy, entropy, and free energy. All phospholipids are amphipathic and arrange themselves to minimize contact between their hydrophobic portions and water (35). However, the spectrum of phospholipids in lung surfactant incorporates multiple variables that can affect adsorption and film behavior, including chain saturation and length, and headgroup charge, hydration, and hydrogen bonding (35–37). It is well-known that the hydrophobic SP interact with phospholipids and affect their packing, but specific interactions vary among published studies and on whether SP-B and SP-C are present alone or together (16 for review). Molecular biophysical studies show that SP-B can increase order in the headgroup region of lipid bilayers through interactions involving its positively charged residues (38, 39). SP-C has been found consistently to disorder lipid chains and decrease gel to liquid crystal transition temperature (40–42). FTIR spectroscopic studies have suggested that the combined presence of hydrophobic surfactant apoproteins disturbs the packing and order of phospholipids in lung surfactant (43, 44), potentially facilitating monolayer formation. The relationship of specific molecular interactions between lung surfactant phospholipids and proteins to the generation of rapid interfacial adsorption needs further study.

Secondary zwitterionic and anionic surfactant phospholipids, as well as neutral lipids, had measurable but much less pronounced effects on adsorption compared to the hydrophobic SP. Fluid zwitterionic and anionic phospholipids are known to enhance the adsorption of DPPC (5, 9), and our results show that PPL, mPPL, and N&PL all adsorbed better than DPPC (Fig. 1). In the presence of hydrophobic SP, mixtures of SP:mPPL had better adsorption than SP:DPPC at equal SP contents (Fig. 4B vs. 4C), indicating a contribution from the secondary zwitterionic phospholipids. Adsorption was also improved in mixtures of SP:PPL vs. SP:mPPL at the same SP content (Fig. 4A vs. 4B), indicating that the anionic phospholipids contributed somewhat to the maximal facilitation of adsorption by the hydrophobic apoproteins.

Several previous studies (30, 45, 46) have reported that mixtures of SP-B or SP-C with DPPC do not adsorb to the low equilibrium surface tensions of about 25 mN/m reported here for mixtures of SP:mPPL (Figs. 4B, 5). Lung surfactant contains a variety of secondary zwitterionic constituents other than DPPC, including many saturated and unsaturated PCs, plus smaller amounts of other phospholipids such as phosphatidylethanolamine (4, 14, 15, 47, 48). Anionic phospholipids, also represented in lung surfactant composition, have been shown to interact with the hydrophobic apoproteins (39, 41, 42). Mixtures of SP-C combined with DPPC plus unsaturated PG and PI have been found to adsorb better than similar mixtures of SP-C with DPPC alone (45), although anionic lipids were not essential for protein-mediated lipid insertion into phospholipid films (49). The majority of secondary zwitterionic and anionic phospholipids in lung surfactant have gel to liquid crystal transition temperatures below 37°C (2, 47). This likely contributes to their ability to adsorb more readily than DPPC (Fig. 1), although even complex mixtures of DPPC and fluid phospholipids do not adsorb nearly as well as when surfactant proteins are added (9, 11, 12). The relative fluidity of secondary surfactant phospholipids, in combination with the actions of the hydrophobic SP, may result in more optimal penetration and insertion of DPPC and other poorly adsorbing surfactant components into the interfacial film.

Surfactant neutral lipids as well as secondary phospholipids gave a small improvement in adsorption facility (N&PL vs. PPL, Fig. 1). This is in agreement with the results of Yu and Possmayer (50) showing that depletion of cholesterol in extracted calf lung surfactant decreased adsorption facility. The small difference in adsorption rate found here between CLSE and SP&PL (Fig. 1) most likely reflects the lack of neutral lipid in the latter. However, the small improvement in surfactant adsorption contributed by neutral lipids can be contrasted with their detrimental actions on the stability and surface tension lowering ability of cycled pulmonary surfactant films (18, 50).

In summary, these studies on subfractions of the hydrophobic components of calf lung surfactant show that the mixture of hydrophobic apoproteins SP-B and

SP-C had much more pronounced effects than secondary surfactant phospholipids or neutral lipids in facilitating adsorption relative to DPPC. This is in contrast to recently demonstrated behavior within dynamically compressed surface films (18), where the secondary phospholipids enhance respreading on successive cycles at least as much as do the hydrophobic SP. All surfactant subfractions containing mixed lipids without SP (PPL, mPPL, and N&PL) adsorbed better than DPPC, the single most abundant constituent of lung surfactant. None of these lipid mixtures, however, approached the adsorption rate or magnitude found when hydrophobic SP were present. All mixtures containing hydrophobic SP and lipids (SP&PL, SP&mPL, CLSE) rapidly adsorbed to low equilibrium surface tensions, although the complete mix of zwitterionic and anionic secondary surfactant phospholipids, and surfactant neutral lipids, was associated with maximal adsorption facility in combination with apolipoproteins.

This research was supported by SCOR HL-36543 (RHN, ZW), HL-54209 (SBH), and by funds from the Whitaker Foundation and the American Lung and Heart Associations of Oregon. The technical assistance of Anita Gordon in CLSE preparation, and of Miranda Kahn, Joan Rainey, and Walter Anyan in subfraction isolation, is gratefully acknowledged.

Manuscript received 25 September 1995 and in revised form 10 January 1996.

REFERENCES

1. Goerke, J., and J. A. Clements. 1986. Alveolar surface tension and lung surfactant. *In* Handbook of Physiology. Section 3: The Respiratory System. S. R. Geiger, editor. American Physiological Society, Bethesda, MD. 247–261.
2. Notter, R. H. 1989. Physical chemistry and physiological activity of pulmonary surfactant. *In* Surfactant Replacement Therapy. D. L. Shapiro and R. H. Notter, editors. A. R. Liss, Inc., New York. 19–70.
3. Notter, R. H., and J. N. Finkelstein. 1984. Pulmonary surfactant: an interdisciplinary approach. *J. Appl. Physiol.* **57:** 1613–1624.
4. van Golde, L. M. G., J. J. Batenburg, and B. Robertson. 1988. The pulmonary surfactant system: biochemical aspects and functional significance. *Physiol. Rev.* **68:** 374–455.
5. Notter, R. H., S. Smith, R. D. Taubold, and J. N. Finkelstein. 1982. Path dependence of adsorption behavior of mixtures containing dipalmitoyl phosphatidylcholine. *Pediatr. Res.* **16:** 515–519.
6. Watkins, J. C. 1967. The surface properties of pure phospholipids in relation to those of lung extracts. *Biochim. Biophys. Acta.* **152:** 293–306.
7. Turcotte, J. G., A. M. Sacco, J. M. Steim, S. A. Tabak, and R. H. Notter. 1977. Chemical synthesis and surface properties of an analog of the pulmonary surfactant dipalmitoyl phosphatidylcholine. *Biochim. Biophys. Acta.* **488:** 235–248.
8. Notter, R. H., S. A. Tabak, and R. D. Mavis. 1980. Surface properties of lung surfactant components. *J. Lipid Res.* **21:** 10–22.
9. Notter, R. H., R. D. Taubold, and J. N. Finkelstein. 1983. Comparative adsorption of natural lung surfactant, extracted phospholipids, and artificial phospholipid mixtures to the air–water interface. *Chem. Phys. Lipids.* **33:** 67–80.
10. Ross, G. F., R. H. Notter, J. Meuth, and J. A. Whitsett. 1985. Phospholipid binding and biophysical activity of pulmonary surfactant-associated protein SAP-35 and its non-collagenous C-terminal domains. *J. Biol. Chem.* **261:** 14283–14291.
11. Yu, S-H., and F. Possmayer. 1986. Reconstitution of surfactant activity by using the 6 kDa apoprotein associated pulmonary surfactant. *Biochem. J.* **236:** 85–89.
12. Notter, R. H., D. L. Shapiro, B. Ohning, and J. A. Whitsett. 1987. Biophysical activity of synthetic phospholipids combined with purified lung surfactant 6000 dalton apoproteins. *Chem. Phys. Lipids.* **44:** 1–17.
13. Yu, S-H., and F. Possmayer. 1990. Role of bovine pulmonary surfactant-associated proteins in the surface-active property of phospholipid mixtures. *Biochim. Biophys. Acta.* **1046:** 233–241.
14. Sanders, R. L. 1982. The composition of pulmonary surfactant. *In* Lung Development: Biological and Clinical Perspectives. P. M. Farrell, editor. Academic Press, New York. 193–219.
15. Cockshutt, A. M., and F. Possmayer. 1992. Metabolism of surfactant lipids and proteins in the developing lung. *In* Pulmonary Surfactant: from Molecular Biology to Clinical Practice. B. Robertson, L. M. G. van Golde, and J. J. Batenburg, editors. Elsevier, Amsterdam. 339–378.
16. Keough, K. M. W. 1992. Physical chemistry of pulmonary surfactant in the terminal air spaces. *In* Pulmonary Surfactant: from Molecular Biology to Clinical Practice. B. Robertson, L. M. G. van Golde, and J. J. Batenburg, editors. Elsevier, Amsterdam. 109–164.
17. Hall, S. B., Z. Wang, and R. H. Notter. 1994. Separation of subfractions of the hydrophobic components of calf lung surfactant. *J. Lipid Res.* **35:** 1386–1394.
18. Wang, Z., S. B. Hall, and R. H. Notter. 1995. Dynamic surface activity of films of lung surfactant phospholipids, hydrophobic proteins, and neutral lipids. *J. Lipid Res.* **36:** 1283–1293.
19. Touchstone, J. C., J. C. Chen, and K. M. Beaver. 1980. Improved separation of phospholipids by thin-layer chromatography. *Lipids.* **15:** 61–62.
20. Notter, R. H., and D. L. Shapiro. 1987. Lung surfactants for replacement therapy: biochemical, biophysical, and clinical aspects. *Clin. Perinatol.* **14:** 433–479.
21. Whitsett, J. A., B. L. Ohning, G. Ross, J. Meuth, T. Weaver, B. A. Holm, D. L. Shapiro, and R. H. Notter. 1986. Hydrophobic surfactant-associated protein in whole lung surfactant and its importance for biophysical activity in lung surfactant extracts used for replacement therapy. *Pediatr. Res.* **20:** 460–467.
22. Notter, R. H., D. P. Penney, J. N. Finkelstein, and D. L. Shapiro. 1986. Adsorption of natural lung surfactant and phospholipid extracts related to tubular myelin formation. *Pediatr. Res.* **20:** 97–101.
23. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
24. Kaplan, R. S., and P. L. Pedersen. 1989. Sensitive protein assay in the presence of high levels of lipid. *Anal. Biochem.*

150: 97–104.
25. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate, and phosphatases. *Methods Enzymol.* **8:** 115–118.
26. Enhorning, G. 1977. Pulsating bubble technique for evaluation of pulmonary surfactant. *J. Appl. Physiol.* **43:** 198–203.
27. King, R. J., and J. A. Clements. 1972. Surface active materials from dog lung. II. Composition and physiological correlations. *Am. J. Physiol.* **223:** 715–726.
28. Williams, M. C., S. Hawgood, and R. L. Hamilton. 1991. Changes in lipid structure produced by surfactant proteins SP-A, SP-B, and SP-C. *Am. J. Respir. Cell Mol. Biol.* **5:** 41–50.
29. Venkitaraman, A. R., S. B. Hall, J. A. Whitsett, and R. H. Notter. 1990. Enhancement of biophysical activity of lung surfactant extracts and phospholipid-apoprotein mixtures by surfactant protein A. *Chem. Phys. Lipids.* **56:** 185–194.
30. Hall, S. B., A. R. Venkitaraman, J. A. Whitsett, B. A. Holm, and R. H. Notter. 1992. Importance of hydrophobic apoproteins as constituents of clinical exogenous surfactants. *Am. Rev. Respir. Dis.* **145:** 24–30.
31. Cummings, J. J., B. A. Holm, M. L. Hudak, B. B. Hudak, W. H. Ferguson, and E. A. Egan. 1992. A controlled clinical comparison of four different surfactant preparations in surfactant-deficient preterm lambs. *Am. Rev. Resp. Dis.* **145:** 999–1004.
32. Mizuno, K., M. Ikegami, C-M. Chen, T. Ueda, and A. H. Jobe. 1995. Surfactant protein-B supplementation improves in vivo function of a modified natural surfactant. *Pediatr. Res.* **37:** 271–276.
33. Magoon, M. W., J. R. Wright, A. Baritussio, M. C. Williams, J. Goerke, B. J. Benson, R. L. Hamilton, and J. A. Clements. 1987. Subfraction of lung surfactant: implications for metabolism and surface activity. *Biochim. Biophys. Acta.* **750:** 18–31.
34. Gross, N. J., and K. R. Narine. 1989. Surfactant subtype in mice: characterization and quantitation. *J. Appl. Physiol.* **66:** 342–349.
35. Dervichian, D. G. 1964. The physical chemistry of phospholipids. *Prog. Biophys. Mol. Biol.* **14:** 263–342.
36. Liu, H., Z. Lu, J. G. Turcotte, and R. H. Notter. 1994. Dynamic interfacial properties of surface-excess films of phospholipid and phosphonolipid analogs: I. Effects of pH. *J. Colloid Interface Sci.* **167:** 378–390.
37. Liu, H., J. G. Turcotte, and R. H. Notter. 1994. Dynamic interfacial properties of surface-excess films of phospholipid and phosphonolipid analogs: II. Effects of chain linkage and headgroup structure. *J. Colloid Interface Sci.* **167:** 391–400.
38. Baatz, J. E., V. Sarin, D. R. Absolom, C. Baxter, and J. A. Whitsett. 1991. Effects of surfactant-associated protein SP-B synthetic analogs on the structure and surface activity of model membrane bilayers. *Chem. Phys. Lipids.* **60:** 163–178.
39. Cochrane, C. G., and S. D. Revak. 1991. Pulmonary surfactant protein B (SP-B): structure–function relationships. *Science.* **254:** 556–568.
40. Simatos, G. A., K. B. Forward, M. R. Morrow, and K. M. W. Keough. 1990. Interaction between perdeuterated dimyristoylphosphatidylcholine and low molecular weight pulmonary surfactant protein SP-C. *Biochemistry.* **29:** 5807–5814.
41. Horowitz, A. D., B. W. Elledge, J. A. Whitsett, and J. E. Baatz. 1992. Effects of lung surfactant proteolipid SP-C on the organization of model membrane lipids: a fluorescence study. *Biochim. Biophys. Acta.* **1107:** 44–54.
42. Horowitz, A. D. 1995. Exclusion of SP-C, but not SP-B, by gel phase palmitoyl lipids. *Chem. Phys. Lipids.* **75:** 27–39.
43. Reilly, K. E., A. J. Mautone, and R. Mendelsohn. 1989. Fourier transform infrared spectroscopy studies of lipid/protein interactions in pulmonary surfactant. *Biochemistry.* **28:** 7368–7373.
44. Ge, Z., C. W. Brown, J. G. Turcotte, Z. Wang, and R. H. Notter. 1995. FTIR studies of calcium-dependent molecular order in lung surfactant and surfactant extract dispersions. *J. Colloid Interface Sci.* **173:** 471–477.
45. Pérez-Gil, J., G. A. Simatos, J. Tucker, and K. M. W. Keough. 1992. Interfacial adsorption of simple lipid mixtures combined with hydrophobic surfactant protein from pig lung. *Biochem. Cell Biol.* **70:** 332–338.
46. Morrow, M. R., J. Pérez-Gil, G. Simatos, C. Boland, J. Stewart, D. Absolom, V. Sarin, and K. M. W. Keough. 1993. Pulmonary surfactant-associated protein SP-B has little effect on acyl chains in dipalmitoyl phosphatidylcholine dispersions. *Biochemistry.* **32:** 4397–4402.
47. Kahn, M. C., G. J. Anderson, W. R. Anyan, and S. B. Hall. 1995. Phosphatidylcholine molecular species of calf lung surfactant. *Am. J. Physiol.* **13:** L567–573.
48. Schlame, M., C. Casals, B. Restow, H. Rabe, and D. Kunze. 1988. Molecular species of phosphatidylcholine and phosphatidylglycerol in rat lung surfactant and different pools of pneumocytes type II. *Biochem. J.* **253:** 209–215.
49. Oosterlaken-Dijksterhuis, M. A., H. P. Haagsman, L. M. G. van Golde, and R. A. Demel. 1991. Characterization of lipid insertion into monomolecular layers mediated by lung surfactant proteins SP-B and SP-C. *Biochemistry.* **30:** 10965–10971.
50. Yu, S. H., and F. Possmayer. 1993. Adsorption, compression, and stability of surface films of natural, lipid extract, and reconstituted pulmonary surfactants. *Biochim. Biophys. Acta.* **1167:** 264–271.