Effect of Reconstituted Pulmonary Surfactant Containing the 6000-Dalton Hydrophobic Protein on Lung Compliance of Prematurely Delivered Rabbit Fetuses

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ABSTRACT. Chloroform:methanol extracts of bovine pulmonary surfactant contain small hydrophobic proteins, designated surfactant-associated apoproteins 6,000 (SAP-6), but do not contain the major surfactant-associated 35,000-dalton glycoprotein, designated SAP-35. Examination of lipid extract surfactant on sodium dodecylsulfate-polyacrylamide gel electrophoresis revealed hydrophobic proteins with apparent molecular masses of 15,000, 7,000, and 3,500 daltons prior to reduction. After reduction, the 15,000-dalton species largely disappeared and was replaced by a 5,000-dalton species. In addition, the 7000- and 3500-dalton species exhibited a slightly enhanced mobility. Amino acid analysis demonstrated that SAP-6 possesses a more highly hydrophobic profile than SAP-35. Combining the protein-containing fractions from silicic acid chromatography of lipid extract with synthetic dipalmitoylphosphatidylcholine produced a reconstituted surfactant preparation which was just as active as lipid extract surfactant on a pulsating bubble surfactometer. The reconstituted surfactant contained SAP-6 but not SAP-35. Pressure-volume studies revealed that, at the optimal dose, reconstituted surfactant containing half the SAP-6 concentration of lipid extract exhibited similar effectiveness to lipid extract surfactant in promoting lung expansion with prematurely delivered rabbit fetuses of 27 days gestation. Reconstituted surfactant with an identical SAP-6 protein concentration as lipid extract possessed the same biological properties as the preparation with 1% SAP-6 protein. These studies support the view that an artificial surfactant composed of synthetic or semisynthetic lipids plus human SAP-6 produced via biotechnology could be useful for prevention and/or treatment of the neonatal respiratory distress syndrome. (*Pediatr Res* 23: 23-30, 1988)

Abbreviations

DPPC, dipalmitoylphosphatidylcholine
DPPG, dipalmitoylphosphatidylglycerol
PAGE, polyacrylamide gel electrophoresis
PE, phosphatidylethanolamine
PG, phosphatidylglycerol
SAP, surfactant-associated protein

SDS, sodium dodecylsulfate

R_{max} , maximum radius

R_{min} , minimum radius

Pulmonary surfactant is synthesized in the type II cells of the alveolus, assembled into lamellar bodies, and secreted into the alveolar space by exocytosis (1-4). To reduce the surface tension and stabilize the alveoli, the lipid components of pulmonary surfactant must adsorb and spread at the air-liquid interface as a monolayer with their polar headgroups in the aqueous phase and their fatty acid moieties extending into the air (3-5). During expansion and contraction, the surface film becomes enriched with DPPC (3-7). This phospholipid lowers the surface tension to the low value required to prevent alveolar collapse during expiration (3-7). Phospholipids that are not present in the surface monolayer have a negligible effect on surface and tension. The realization that DPPC, with or without other lipids, does not readily adsorb and spread at the air-liquid interface focused attention on the role of surfactant-associated proteins. Initial studies involved the major surfactant protein, which has been detected in all species examined as minor species with nominal molecular masses of approximately 28,000 daltons and as one or more major glycosylated and sialated species of approximately 35,000 daltons (2, 8). Evidence, primarily obtained with the Wilhelmy plate surface balance, indicates that this 35,000 dalton SAP facilitates the adsorption and spreading of DPPC (9-11). However, it has become apparent that lipid extracts of pulmonary surfactant, which are largely depleted of protein, exhibit the essential characteristics of natural surfactant (4-6) with the pulsating bubble surfactometer (12) and were also effective in promoting lung expansion and survival with prematurely delivered fetal rabbits and lambs (13-17). The biological effectiveness of such protein-poor surfactant preparations has been confirmed through clinical trials involving both the alleviation of the effects of the respiratory distress syndrome and by the prevention of respiratory distress through prophylactic treatment at birth (18-22).

It has recently become apparent that lipid extract surfactant is devoid of SAP-35 but contains a number of small hydrophobic proteins collectively known as SAP-6 (23-29). Previous studies by this laboratory have revealed that the hydrophobic apoproteins can be separated from the PC fraction by column chromatography on silicic acid (23). Combining the protein fraction, which also contains PG and PE, with the PC fraction (23) or

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with synthetic DPPC (30) resulted in active reconstitutes as evaluated by the pulsating bubble surfactometer. The present studies were undertaken to further clarify the relationship between the role of SAP-35 and SAP-6 in surfactant activity and to determine whether reconstituted surfactant containing only SAP-6 possesses biological as well as biophysical activity.

METHODS

Surfactant preparation. Natural bovine surfactant was prepared by a modification of methods previously published by this laboratory (31, 32). Lipid extract surfactant was produced by extraction with chloroform:methanol (33). The surfactant was further purified by precipitation of phospholipid fraction with 20 vol of cold acetone (32). Lipid extract surfactant was normally stored in chloroform:methanol (9:1) at -20°C . The concentration of lipid extract was determined by measuring the phospholipid phosphorous content and multiplying this value by 25 (34).

Lipid extract surfactant was prepared for biophysical and biological assays as follows: the chloroform extracts were evaporated to dryness on the sides of glass tubes with a stream of N_2 . Saline containing 1.0 mM CaCl_2 was added and the samples were dispersed, first by gentle vortexing to avoid foaming, and then by brief sonication with a bath type sonicator (Bransonic 12). The temperature of the sonicator bath was approximately 40°C during this process. The samples used for the lung compliance studies were dispersed at a concentration of 25 mg/ml. After diluting to 10 mg/ml with saline-1 mM CaCl_2 , the biophysical activity of these preparations was assessed on the pulsating bubble surfactometer, as indicated below. For some experiments in which only biophysical parameters were evaluated, the lipid extract surfactant was directly dispersed at 10 mg/ml. Samples were stored at -20°C . Although this has not been studied in detail, in our experience samples prepared as indicated above do not lose appreciable biophysical activity during storage for over 1 yr at -20°C . Normally, the samples were thawed only once prior to testing for biological activity and diluted with saline-1 mM CaCl_2 as required.

Preparation of reconstituted surfactant. Lipid extract surfactant was fractionated by chromatography on silicic acid columns using chloroform:methanol as previously described (23, 35). This procedure produces a minor lipid peak containing PG, PE plus the hydrophobic proteins, and a major lipid peak containing PC. The minor lipid peak, which corresponds to fraction I plus fraction II in our former studies, was taken to dryness and redispersed in chloroform:methanol (9:1). For reconstitution, the protein-containing samples were mixed with an appropriate amount of DPPC in chloroform:methanol (9:1) and taken to dryness under N_2 . Saline-1 mM CaCl_2 solution and fine glass beads were added and the suspension was shaken at room temperature for 1 h. (The term "reconstitution" is used to mean addition of SAP-6 protein back to lipid; it does not imply that the DPPC was obtained from surfactant). Reconstituted surfactant was prepared to yield a relative concentration of either 1 or 2% protein as a percentage of phospholipid. The approximate relative concentrations of the lipids in the reconstituted surfactant preparations were, for 1% protein: DPPC, 90%; PG, 7.5%; PE, 2.5%; and for 2% protein: DPPC, 80%; PG, 15%; PE, 5.0%.

Protein analysis. The protein composition of bovine pulmonary surfactant was examined by SDS-PAGE using a modification (30) of the method of Laemmli (36). The high proportion of lipid (approximately 98%) necessitated the following modifications. The 15% polyacrylamide separation gels were cast with 6 M urea. The concentration of SDS in the running buffer was increased to 0.18%. The sample buffer contained 5% SDS, 0.05 M Tris-HCl, pH 8.5, and where indicated, 2-mercaptoethanol. The 20-cm gels were subjected to electrophoresis at 100 V for 18 h followed by several hours at 200 V until the dye front had emerged from the gel for at least 1 h. The gels were stained with Coomassie-blue as previously described (23) followed by silver

stain from NEN (DuPont Co., Wilmington, DE) as indicated by the manufacturer. Amino acid analyses were conducted using a Beckman model 119CL single column amino acid analyser. The protein samples were hydrolyzed in 6 N HCl for 24 h at 115°C in evacuated sealed tubes.

Biophysical assay. The biophysical activity of natural surfactant, lipid extract surfactant and reconstituted surfactant was determined using the pulsating bubble surfactometer introduced by Enhorning (12). With this apparatus, a bubble communicating with ambient air is created in a suspension of the surfactant preparation. The bubble is pulsated between a R_{max} of 0.55 mm and a R_{min} of 0.4 mm. The pressure difference across the bubble surface is monitored with a pressure transducer. Surface tension is calculated by the law of Laplace which states that the pressure difference across a sphere is directly proportional to twice the surface tension and indirectly proportional to the radius. The surface tension at R_{max} and R_{min} are expressed. Samples are normally tested at a concentration of 10 mg lipid/ml (1.0%, w/v). Lipid extract surfactant contains 2% protein by weight corresponding to a final protein concentration of 0.02% (w/v) in the test samples.

Biological assay. Biological activity was assayed by conducting pressure-volume loops with fetal rabbits delivered at 27 days gestation (term 31 days) as described previously (13, 14). Briefly, the does were given a lethal dose of sodium pentobarbital and the uterus excised. The fetuses were killed *in utero*, delivered, and weighed. After tracheotomy, polyethylene tubes were inserted and the fetuses were placed in a water bath of 37°C . Either saline or surfactant sample was inserted. The tracheal catheters from the fetuses being tested were connected to glass tubes containing 2 ml of air which in turn were connected to a central reservoir of colored water. Pressure volume loops were produced by raising the reservoir so that its surface attained set levels above the fetuses. Pressure was maintained for 15 s at each of two breathing cycles. Changes in the displacement of air into and out of the lung were determined photographically. Values were corrected for body weight.

The lipid extract and reconstituted surfactant samples used in these studies were at a concentration of 25 mg/ml (w/v) based on lipid. In order to facilitate volume measurements and to maintain the volumes of fluid within reasonable limits, sufficient saline-1 mM CaCl_2 was added to adjust the volume of the lower doses to 50 μl . Saline controls received 50 μl of saline-1 mM CaCl_2 .

Twenty-five fetuses from four does were used to determine the pressure-volume loops with different amounts of lipid extract surfactant as follows: 0.25 mg, three fetuses; 0.5 mg, three fetuses; 1.0 mg, five fetuses; 1.25 mg, five fetuses; 1.5 mg, four fetuses; 1.875 mg, five fetuses. Eleven fetuses from two different does were used in the studies with different amounts of reconstituted surfactant containing 1% protein relative to lipid: 0.25 mg, two fetuses; 0.5 mg, one fetus; 0.75 mg, three fetuses; 1.0 mg, two fetuses; 1.25 mg, three fetuses. In addition, five fetuses were treated with 50 μl of saline-1 mM CaCl_2 and 10 fetuses were studied as untreated controls. The studies comparing the effectiveness of reconstituted surfactant containing 2% protein relative to phospholipid were conducted using litter mates for the two different doses of phospholipid. At the 1.25-mg dose, four fetuses were used for lipid extract surfactant and seven for the reconstituted preparation containing 2% protein. For the 1.5-mg dose, six fetuses were treated with lipid extract surfactant and the same number with reconstituted surfactant.

Other methods. Total protein could be approximated by the method of Lowry *et al.* (37) using SDS to disperse the lipid. As has been alluded to previously (31), different responses were obtained for surfactant protein and bovine serum albumin compared to amino acid analyses. In addition, during the course of this study it became apparent that different color yields were obtained by Lowry for the hydrophobic proteins separated by column chromatography. As a consequence, protein concentra-

tion was calculated from the amino acid analyses measured as indicated above. Statistical significance was determined using Student's *t* test for independent variables.

Materials. Standard chemical reagents were obtained from Fisher Scientific Co., Pittsburgh, PA; DPPC was obtained from Sigma Chemical Company, St. Louis, MO; pregnant rabbits of known gestation were purchased from Don Rieman's Fur Ranch, Petersburg, Ontario, Canada.

RESULTS

The protein composition of natural bovine surfactant and its lipid extract was examined by SDS-PAGE using the modified method described in the text. Electrophoresis of natural surfactant in the absence of 2-mercaptoethanol revealed a high molecular weight complex at the top of the separation gel, and a sharp band with an apparent molecular mass of 70,000 daltons. These bands showed positive staining with both Coomassie-blue and silver (*Lane 2*, Fig. 1 *A* and *B*). In addition, broad diffuse bands, which did not photograph well with Coomassie-blue but displayed clear reactivity with silver stain, could be observed with apparent molecular masses of approximately 15,000, 7,000, and 3,500 daltons. Phospholipid could be observed as a grey smudge below the 3500-dalton band. Electrophoresis of natural surfactant in the presence of sulfhydryl reagent, revealed a strong prominent band with an apparent molecular mass of 35,000 daltons and a sharp band with an apparent molecular mass of 28,000 daltons which were stained with both Coomassie-blue and silver stain (*lane 2*, Fig. 1 *A* and *B*). In addition, a faint silver-positive band corresponding to 7000 daltons and darkly stained bands of 5000 and 3500 daltons were observed with the reduced sample of natural surfactant. Electrophoresis of the hydrophobic protein fraction from silicic acid revealed the same

pattern as for lipid extract in Figure 1 but the 7,000 dalton and 3,500 dalton may be slightly reduced relative to the 15,000 dalton species (not shown).

Electrophoresis of lipid extract surfactant failed to reveal any indication of the large molecular weight apoproteins at the top of the gel or the 70,000, 35,000, or 28,000 dalton surfactant apoproteins either in the presence or absence of mercaptoethanol (*lanes 4* and *5*, Fig. 1 *A* and *B*). The low molecular weight proteins were more evident than with whole surfactant. In the absence of mercaptoethanol prominent silver-staining bands were observed with apparent molecular masses of 15,000, 7,000, and 3,500 daltons (*lane 4*, Fig. 1*B*). After reduction, the 15,000-dalton band almost completely disappeared and was replaced by a band at 5,000 daltons (*lane 5*, Fig. 1*B*). The 7000- and 3500-dalton bands stained somewhat more strongly and migrated slightly farther in the presence of sulfhydryl reagent. Although it is difficult to observe a clear separation between the 5000- and 3500-dalton bands in the photograph (Fig. 1), distinct separate bands could be observed with this and with other gels, especially during development. Since the samples electrophoresed in Figure 1 represent complete samples of natural and lipid extract surfactant, the complete protein profile should be represented.

In keeping with the chloroform solubility of the small apoproteins present in lipid extract surfactant, amino acid analysis of these preparations revealed a highly hydrophobic amino acid pattern containing large quantities of leucine and valine and relatively small amounts of highly charged amino acids (Table 1). In contrast, the 35-K apoprotein contained a high proportion of glycine and glutamic acid. The amino acid composition of SAP-6 contained 69% nonpolar and 80% uncharged amino acids compared to the 52% nonpolar and 68% uncharged amino acids present in SAP-35. These calculations have considered the proportions of glutamine present in amino acid sequence data from

Fig. 1. SDS-PAGE of bovine pulmonary surfactant and lipid extract surfactant. The gel was subjected to electrophoresis as described in the text. The gel was stained with Coomassie blue (*A*) and subsequently with silver stain (*B*). *Lane 1*, high molecular weight standards; *lane 2*, bovine pulmonary surfactant, 25 μ g protein; *lane 3*, bovine pulmonary surfactant with mercaptoethanol, 25 μ g protein; *lane 4*, lipid extract surfactant, 4 μ g protein; *lane 5*, lipid extract surfactant, with mercaptoethanol, 4 μ g protein; *lane 6*, low molecular weight standards.

Table 1. Amino acid composition of SAP-6 and SAP-35* from bovine pulmonary surfactant

Amino acid	% Composition	
	SAP-6	SAP-35
Asp	6.7	10.7
Thr	3.3	3.3
Ser	3.0	2.3
Glx	7.8	14.3
Pro	6.0	5.0
Gly	9.0	18.3
Ala	5.4	5.7
Cys	3.6	2.6
Val	13.2	6.5
Met	2.4	2.2
Ile	7.8	3.8
Leu	19.3	8.5
Tyr	1.1	3.0
Phe	1.4	2.4
His	0.8	1.8
Lys	3.2	4.5
Arg	5.9	5.5

* SAP-6 was isolated as indicated in the text. SAP-35 was prepared by high-performance liquid chromatography. A sample of natural surfactant was dispersed in 8 M urea-0.05% trifluoroacetic acid, pH 2.5, applied to a C18 reverse phase column and the SAP-35 eluted with a gradient containing 0.05% trifluoroacetic acid and 2-propanol.

bovine SAP-6 and the cDNA sequences of canine SAP-35 (38) and SAP-6 (39).

Biophysical activity was assessed with the pulsating bubble surfactometer (12). No difference could be observed between the ability of natural pulmonary surfactant or its lipid extract to reduce the surface tension of a pulsating bubble to approximately 27 mN.m⁻¹ at maximum bubble size (0.55 mm) and near 0 mN.m⁻¹ at minimum bubble size (0.4 mm) (Table 2). Reconstituted surfactant was prepared by mixing the apoprotein fraction from silicic acid column chromatography mentioned above with appropriate amounts of DPPC. Because initial recombinations were based on protein values determined using the Lowry procedure, the protein concentration, as a proportion of lipid, was adjusted to 1% (w/w) which is half the protein concentration of lipid extract surfactant as determined by amino acid analysis (see "Methods"). Nevertheless, samples prepared with either 1% protein or 2% protein relative to lipid displayed similar biophysical characteristics to natural surfactant or lipid extract surfactant (Table 2). In contrast, lipid mixtures without protein cannot lower the surface tension of the pulsating bubble to these low values (23, 30).

Previous investigations have demonstrated that lipid extract surfactant is just as effective as natural surfactant in facilitating lung expansion and in promoting survival with prematurely delivered rabbit fetuses of 27 days gestation (13, 14). These former studies revealed an optimal dose for lung expansion of 1.25 mg phospholipid and no effect of volume over a considerable range. In keeping with these earlier findings, increasing the amount of lipid extract surfactant or reconstituted surfactant from 0.25 to 1.25 mg resulted in a progressive increase in lung compliance (Fig. 2A). Increasing the amount of lipid extract phospholipid to 1.5 and 1.875 mg resulted in a small decrease in lung volume, particularly during the second loop but this was not significantly different from the values obtained with 1.25 mg phospholipid.

In order to economize on material and because the biophysical activity of reconstituted surfactant with 1% protein was similar to that with 2% protein, the dose-response curve study was conducted using the lower amount of protein (Fig. 2B). In contrast to lipid extract surfactant, there was little effect at 0.25 and 0.5 mg phospholipid and maximal lung volume was observed

Table 2. Comparison of biophysical activity of Natural surfactant, lipid extract surfactant, and reconstituted surfactant on pulsating bubble surfactometer

Sample	Surface tension (mN/m)*	
	(n = 3)	
	R _{max}	R _{min}
Natural surfactant	25.0 ± 0.4	0 ± 0.0
Lipid extract surfactant	27.0 ± 1.2	0 ± 0.0
Reconstituted surfactant (1% protein)	28.0 ± 2.0	1.5 ± 1.2
Reconstituted surfactant (2% protein)	28.0 ± 2.5	1.8 ± 1.0

* Surfactant activity was assessed by monitoring the pressure difference across the pulsating bubble and determining the surface tension at a maximum bubble radius of 0.55 mm (R_{max}) and at minimum bubble radius of 0.4 mm (R_{min}) after 1 min pulsation at 37° C, using the Laplace equation which states that the pressure difference across a sphere is equal to twice the surface tension divided by the radius. Samples of lipid extract surfactant and reconstituted surfactant were incubated at 37° C for 1–2 h before being tested. All samples were tested at a concentration of 10 mg lipid per ml in saline-1 mM CaCl₂.

with 1.0 mg phospholipid. The pressure-volume loops observed with 1.0 mg of reconstituted surfactant (1% protein) were virtually indistinguishable from the pressure volume loops obtained with 1.25 mg, the optimal amount, of lipid extract surfactant.

Statistical evaluation of the pressure-volume curves obtained with either lipid extract or reconstituted surfactant (1% protein) versus saline-1 mM CaCl₂ and air controls revealed a highly significant increase for all measurements with 1.0 mg or more phospholipid which were beyond the 20-cm pressure point of the first ascending loop. Comparison of the pressure-volume loops obtained with 1.0, 1.25, and 1.5 mg of lipid extract surfactant with those with 1.0 mg reconstituted surfactant (1.0% protein) revealed no significant differences in either loop. However, comparison of the pressure-volume loops obtained with 1.25 mg lipid extract and 1.25 mg reconstituted surfactant (1% protein) revealed that lipid extract was more effective (0.25 > p > 0.025) for the 30-, 35-, and 30-cm H₂O pressure measurements during the initial pressure-volume loops and for the 20-cm values during the descending cycle of the second pressure-volume loop.

In separate experiments, the effectiveness of 1.25 and 1.5 mg of reconstituted surfactant containing 2% protein relative to lipid were compared with the effectiveness of similar amounts of lipid extract surfactant. In order to maximize the possibility of distinguishing between these preparations, litter mates were used for the experiments with each concentration of surfactant. However, the pressure-volume loops obtained with 1.25 and 1.5 mg reconstituted surfactant (2% protein) were statistically indistinguishable from each other and from the pressure loops obtained with either concentration of lipid extract surfactant. In addition, no significant differences were observed between the results obtained with reconstituted surfactant containing 2% protein and the corresponding curves with 1% protein depicted in Figure 2B. Consequently, these pressure-volume curves are not shown. Rather, the data from these experiments are summarized with those from the experiments in Figure 2 by illustrating the relationship between the maximal lung volumes obtained during the first and second loops and the amounts of reconstituted and lipid extract applied (Fig. 3 A and B). Increasing amounts of lipid extract surfactant resulted in a steady increase in maximal lung volume up to 1.25 mg phospholipid where the effect plateaued. In contrast, no increase in maximal lung volume was observed until 0.75 mg of reconstituted surfactant (1% protein) was applied. Maximal lung volumes were observed with 1.0 mg reconstituted surfactant, followed by a definite although statistically insignificant decline. The values obtained with reconstituted surfactant containing 2% protein, depicted with *closed symbols*,

were virtually identical to those obtained with reconstituted surfactant containing 1% protein. It should be noted that neither the pressure volume loops obtained with 0.25 and 0.5 mg of lipid extract surfactant or those obtained with equivalent amounts of reconstituted surfactant were significantly different from the controls. Taken together, the results obtained in these experiments indicate that reconstituted surfactant containing half the SAP-6 apoprotein content of lipid extract surfactant possessed similar biological activity to lipid extract but may not be as effective at low concentrations.

DISCUSSION

The presence of a large molecular weight protein designated glycoprotein A has been documented in pulmonary surfactant from a number of mammalian species (2, 8, 11). When bovine surfactant was subjected to SDS-PAGE in the absence of mer-

captoethanol, a large proportion of this protein remained at the top of the gel and the remainder appeared as a relatively sharp band at 70,000 daltons (Fig. 1, lane 2). Upon reduction bovine glycoprotein A migrated as a minor band of approximately 28,000 daltons and a major band with an apparent molecular mass of approximately 35,000 daltons. Similar staining characteristics were observed with Coomassie blue and silver staining. This protein is both glycosylated and sialylated (40-42). In some species, other higher molecular weight reduced forms can be observed, presumably due to a second carbohydrate chain (11, 42, 43). The cDNA for canine (38) and the genomic DNA (44) for human surfactant SAP-35 have been reported. On the basis of these sequences and on enzymatic and chemical studies, it is apparent that SAP-35 contains both globular and collagen-like sequences (11, 38, 39, 45). Present evidence suggests that it interacts with lipid and this interaction is modified by calcium

Fig. 2. Pressure-volume curves for fetal rabbit lungs of 27 days gestation with various amounts of lipid extract surfactant (A) and reconstituted surfactant containing 1% protein relative to lipid (B). The volumes recorded during the first and second loops as indicated in the text are depicted.

Fig. 3. Comparison of the maximal air volumes achieved during the first and second pressure-volume loops with lungs of prematurely delivered rabbit fetuses. *A*, the effect of increasing amounts of lipid extract on the maximal air volume during the first (□) and second (○) pressure volume loops. *B*, the effects of various amounts of reconstituted surfactant containing 1% protein (open symbols) and reconstituted surfactant containing 2% protein relative to lipid during the first (□, ■) and second (○, ●) pressure-volume loops are plotted.

(9, 10, 4, 47). Circumstantial evidence suggests that SAP-35 is associated with tubular myelin (48, 49).

It has been reported that pulmonary surfactant also contains a low molecular mass protein of approximately 14,000 daltons which is a catabolic fragment of SAP-35 (50). This peptide, which has been referred to as glycoprotein B, shares immunological and physical properties with SAP-35 (2, 8, 45). Glycoprotein B can be distinguished from the small hydrophobic proteins discussed below on the basis of its glycoprotein nature, its method of preparation, and the lack of immunological cross-reactivity (23, 29, 45). For reasons that are not clear, this peptide was not detected in bovine pulmonary surfactant (Fig. 1). Little information is available on its biophysical activity.

In addition to SAP-35 and glycoprotein B, pulmonary surfactant contains a number of small molecular mass proteins which can be distinguished from SAP-35 and glycoprotein B by their physical properties, and by the lack of immunological cross-

reactivity (23, 30, 45). The relation between the small molecular mass proteins reported by various workers is complicated by differences in isolation and analytical techniques, but it is clear that they are very tightly associated with lipid and can be extracted with lipid by organic solvents (23–30). SDS-PAGE electrophoresis of bovine lipid extract surfactant revealed hydrophobic proteins with nominal molecular masses of 15,000, 7,000, and 3,500 daltons. Upon reduction, the 15,000-dalton band disappeared and was replaced by a band at 5,000 daltons (Fig. 1). The hydrophobic proteins were more evident after silver staining, particularly in the presence of mercaptoethanol. The possibility that the unreduced 15,000 and reduced 5,000 bands represent the same protein has been corroborated by dansylation studies demonstrating the presence of N-terminal Leu, Phe, and Ile in these two bands (30). Edman degradation and N-terminal analysis of lipid extracts have provided evidence for three hydrophobic peptides which have been designated SAP-6 (Phe), SAP-6 (Leu), and SAP-6 (Ile) on the basis of the N-terminal amino acids (30). Limited sequence analysis suggests that SAP-6 (Ile) may be the same as SAP-6 (Leu) with the N-terminal Leu deleted (30). These results are consistent with the view that the 15,000-dalton band is due to an oligomer containing SAP-6 (Phe) and SAP-6 (Leu) or Ile bound by sulfhydryl and other interactions. The 7000- and 3500-dalton bands migrate slightly farther after reduction. Dansylation studies detect only N-terminal Leu and Ile in these bands. It has been suggested that the 7000-dalton band represents dimerization of the 3500-dalton band due to sulfhydryl, hydrophobic, and perhaps other interactions (26, 27, 29, 30). However, the possibility of Leu and Ile peptides of variable molecular mass cannot be excluded.

The observations reported herein are in qualitative agreement with other studies on the small hydrophobic proteins associated with pulmonary surfactant from a number of species including dog, cow, pig, and man (25–29, 35, 39, 51, 52). Possibly because of the large amounts of SDS used in the sample preparation and in the running buffer (53–55), the apparent molecular masses observed in the present study are slightly lower than those reported by other workers. It is important to note that, in contrast to previous investigation (23, 25–29, 39, 51, 52), the gel depicted in Figure 1 contains complete lipid extract which has not been subjected to delipidation and/or chromatography. Studies conducted by a number of groups are consistent with the view that pulmonary surfactant contains at least two distinct hydrophobic proteins, SAP-6 (Phe) and SAP-6 (Leu or Ile). SAP-6 (Phe), which migrates with a nominal molecular mass of 15–18,000 daltons has been cloned and sequenced from canine and human lung (39, 56). SAP-6 (Leu) migrates primarily with a nominal molecular mass of 3500–6000 or as a dimer of this species (29, 30). This latter protein contains a polyvaline region containing at least six valines (29, 30, 56). Further studies are required to evaluate the contribution of these two proteins to surfactant activity.

Perhaps not surprisingly, the amino acid composition of the proteins present in lipid extract surfactant possessed a predominance of nonpolar amino acids when compared to SAP-35 (Table 1). The amino acid analyses presented are similar to those reported for SAP-6 and SAP-35 from cow and from other species (25, 26, 29, 52). The differences in these patterns support the view that it is exceedingly unlikely that the small hydrophobic peptides in lipid extract could represent fragments from the high molecular weight proteins present in pulmonary surfactant.

Recombination of the protein-containing fractions from silicic acid chromatography with pure synthetic DPPC gave rise to a reconstituted surfactant that possessed surfactant properties slightly inferior but statistically indistinguishable from lipid extract surfactant as assessed by the pulsating bubble surfactometer (Table 2). The same results were obtained with the protein content adjusted to either half or to the same relative concentration as that of lipid extract surfactant. The ability of reconstituted surfactants, similar to that reported herein, to mimic the essential

characteristics of lipid extract surfactant has been reported for the Wilhelmy surface balance (24, 25, 39) and for a modified Langmuir-Wilhelmy surface tension balance (24, 52) as well as with the pulsating bubble surfactometer (23, 30, 52). The protein fractions used for these reconstitution studies were obtained through chromatography on Sephadex LH-20 (24, 25, 39), silicic acid (23, 30), and by detergent extraction (51, 52). Examination of the purification schemes and the gel patterns in these publications suggest the presence of variable amounts of the 15,000- and 3,500-dalton (unreduced) SAP-6 apoproteins. These preparations did not contain detectable amounts of SAP-35. However, Hawgood *et al.* (39) suggested that the effectiveness of SAP-6 may be enhanced by the presence of SAP-35. Extensive studies have demonstrated that mixtures of pure lipids rarely duplicate the physicochemical properties of natural pulmonary surfactant or its lipid extract (4, 6). Exceptions to this overall rule include mixtures containing hexadecanol as a carrier oil (57) and the hexagonal II phase promoter (58). Whether the biological effectiveness of these preparations will prove adequate for clinical application is still being evaluated.

Considerable evidence has accumulated supporting the biological effectiveness of lipid extract surfactant in compliance and survival studies with prematurely delivered rabbit and lamb fetuses (13–17). Possibly because it is prepared from bovine lung minces rather than bronchoalveolar lavage, the TA-surfactant preparation developed by Fujiwara and coworkers (18, 24, 25) exhibits superior surfactant properties on a modified Langmuir-Wilhelmy surface tension balance after being enriched with DPPC, PG, and free fatty acids. Recent studies have indicated that these additions have little effect on the degree to which TA surfactant improved lung expansion and stability in pressure-volume curves, increased tidal volumes during artificial ventilation, or enhanced alveolar volume density in histological sections (57). On the other hand, recent studies by Fujiwara's group indicate that addition of saturated fatty acids increased both the biophysical and the biological effectiveness of a reconstituted surfactant, similar to that reported herein, so that it became indistinguishable from the modified lipid extract preparation (24). The effect of adding free fatty acids to the reconstituted surfactant preparation reported herein must still be examined. Suzuki *et al.* (52) have demonstrated that reconstituted surfactant 4–5% low molecular weight proteins from porcine surfactant plus DPPC and dipalmitoyl PG exhibited biophysical activity approaching that of natural surfactant with the pulsating bubble surfactometer and with a modified Langmuir trough-Wilhelmy balance. In addition, these authors observed that administration of their apoprotein-based reconstituted surfactant at 5 mg/ml produced a 5-fold increase in tidal volumes of premature rabbit fetuses at an insufflation pressure of 25 cm H₂O. The effect was similar to that observed with natural surfactant. Possibly because only disaturated lipids were present, the apoprotein-based artificial surfactant was viscous and could not be used at higher concentrations. The reconstituted surfactant described herein, which contains unsaturated PG and PE, possessed similar physical properties to lipid extract surfactant. In contrast to the results observed by Suzuki *et al.* (52), the latter preparation had little effect at low doses but at the optimal level was indistinguishable from lipid extract surfactant. No difference was observed in the biological effectiveness of reconstitutes containing either half or the same relative protein content as lipid extract surfactant (2% w/w). These investigations, when taken together with the studies of Tanaka *et al.* (24) and Suzuki *et al.* (51, 52) support the view that an artificial surfactant containing pure lipids plus the SAP-6 apoproteins, possibly produced by molecular biology, could be utilized in the prophylactic prevention of the neonatal respiratory distress syndrome.

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