Experimental studies on the pulmonary surfactant. Reconstitution of surface-active material

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Abstract The conditions for the reconstitution of surfactant lipoprotein were investigated using pig pulmonary surfactant. Lipids of surface-active material (SAM) were extracted with ethanol–ether and the residue, containing mainly protein, was extracted first with dilute sodium borate buffer (pH 10.0) (fraction A) followed by extraction with deoxycholate solution (fraction B). The former (A) contained mainly protein having a nominal molecular weight of 34,000 daltons, albumin and globulin, and the latter (B) 34,000 and 15,000 daltons. Reconstitution of lipids and proteins using these two fractions revealed a greater affinity of fraction B to lipids than that of fraction A, when lipids and proteins were dialyzed against pH 7.4 Tris–HCl. Observation by freeze-fracture methods revealed that protein particles were incorporated into liposomes. Protein–lipid ratio of this complex (LB) could be regulated by altering the amount of lipids and protein during the dialysis. Fraction A was incorporated into LB (LBA) by dialyzing LB with fraction A at an acidic pH. With this procedure, protein having a nominal molecular weight of 34,000 daltons was selectively incorporated. In negatively stained preparations, LBA had a granular appearance in the area limited by lipid membrane. We suggest that in the lipid–protein complex of SAM, two different proteins are present; a hydrophobic one in lipids and more hydrophilic ones in the water phase that have a close association with the former.—**Suzuki, Y., E. Nakai, and K. Ohkawa.** Experimental studies on the pulmonary surfactant. Reconstitution of surface-active material. *J. Lipid Res.* 1982. **23:** 53–61.

Supplementary key words SDS-polyacrylamide gel-electrophoresis • freeze-fracture method • negative staining

The pulmonary surfactant, which is a lipid–protein complex, is required to maintain the normal mechanical properties of the lung. The major lipid involved was identified as dipalmitoylphosphatidylcholine (DPPC) (1), although other components such as phosphatidylglycerol (2, 3) and cholesterol (4, 5) are also included and the surface properties of the surfactant are thus modified (5–7).

Protein(s) was also reported to be one of the components of surfactant (4, 8, 9) but the functional significance or positional relation to lipids is unknown. One of the apoproteins, which has the nominal molecular weight of 35,000–45,000 daltons was reported to be reassembled with lipids into a lipid-protein complex by dilution of the solution in deoxycholate (10) or by incubation with DPPC in a calcium-containing medium (11). However, another apoprotein with a smaller molecular weight of 10,000–11,000 daltons (11, 12) has not been considered in these reconstitution studies.

In the present work, reconstitution of the surfactant was investigated using two fractions of apoproteins extracted from delipidated pig surface-active material (SAM) with dilute buffer solution and deoxycholate solution, the former containing primarily 34,000-dalton apoprotein and the latter 15,000-dalton apoprotein, corresponding, respectively, to the 35,000-dalton and 10,000–11,000-dalton apoproteins reported by King and MacBeth (11).

MATERIALS AND METHODS

Fresh pig lung was obtained from a slaughterhouse. It was immediately processed for extraction of SAM with 0.145 M NaCl buffered with 0.01 M Tris–HCl (pH 7.4) containing 0.001 M EDTA (hereafter referred to as buffered NaCl) after degassing, or kept frozen at −20°C until analysis.

Isolation of SAM

Alveolar SAM was obtained by washing the lung through the bronchus with cold buffered NaCl. Lung washing was usually performed by introducing 1 liter of buffered NaCl and the fluid was allowed to flow out of the lung by gravity. This same portion of saline was used to wash the same lung again. This procedure was

Abbreviations: SAM, surface-active material; DPPC, dipalmitoylphosphatidylcholine; PL, phospholipid; DOC, sodium deoxycholate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel-electrophoresis.

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repeated twice and a total of 3–3.5 liters of wash solution was obtained from one lung. The pulmonary wash solution was first centrifuged at 1,500 rpm for 5 min to remove cell debris and the supernatant was centrifuged at 20,000 *g* for 1 hr at 4°C. The precipitate was suspended in buffered NaCl and layered over 0.25 M and 0.68 M sucrose solution for centrifugation at 74,800 *g* for 1 hr, according to the method of Frosolono et al. (4). The material concentrated between the two sucrose solutions was removed, washed twice with buffered NaCl, and the washed precipitate was used as purified SAM. For comparison, SAM from both fresh and frozen lungs was used in some experiments and we found that these preparations could be used in the same way as those from alveolar wash; although, in the case of the frozen lung, removal of the contaminating hemoglobin was not feasible by the procedure described above.

Preparation of protein fractions and lipids for reconstitution

Delipidation of SAM was performed according to the method of Scanu (13) and modified as follows. Lipids were extracted from purified SAM by homogenizing in 100 ml of cold ethanol–ether 3:2 (v/v) and then by stirring the homogenates at −10°C for 2 hr. The precipitate was obtained by centrifuging the homogenates at 3,000 rpm for 20 min at −10°C. The procedure was repeated three times and the final precipitate was extracted with ether, overnight at −10°C. Ether was removed by centrifugation as above and the precipitate was washed once with ether, by centrifugation. The residue was then dried under N_2, homogenized in 0.005 M sodium borate buffer (pH 10.0), and incubated at 37°C for 1 hr. The homogenate was centrifuged at 100,000 *g* for 1 hr and the supernatant protein solution was used as fraction A. The precipitate was then homogenized in 30 mM sodium deoxycholate (DOC) and centrifuged at 100,000 *g* for 1 hr. The supernatant was used as fraction B. The precipitate was dissolved in 1.0 N NaOH (fraction C) and used only for protein determination.

The combined ethanol–ether fractions were evaporated to dryness and dissolved in chloroform–methanol 2:1 (v/v) followed by washing with 0.2 vol of 0.1 M KCl solution. After evaporating the chloroform–methanol, the residue was redissolved in chloroform for reconstitution studies.

Procedures for the preparation of proteins and lipids are summarized in **Fig. 1.**

Gel-filtration studies of fractions A and B

Fraction A was dialyzed against 0.01 M borate buffer (pH 10.0) containing 0.145 M NaCl and separated into three subfractions (A-I, A-II, and A-III) by gel-filtration on Sephadex G-200 equilibrated in the same buffer at 4°C. These subfractions were dialyzed against distilled water, lyophilized and then dissolved in the buffer. Fraction B was dialyzed against 0.005 M borate buffer (pH 10.0) containing 0.145 M NaCl and 10 mM DOC, and analyzed by gel-filtration on Sephadex G-75 equilibrated in the same buffer, at room temperature.

Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE)

Lyophilyzed protein fractions or delipidated lipid-protein complexes were dissolved in 0.01 M sodium borate buffer (pH 9.1) containing 2% SDS and 5% mercaptoethanol. After addition of glycerol to a final concentration of 10%, the sample solution was heated at 100°C for 90 sec. Insoluble materials were removed by centrifugation and the sample was analyzed for protein constituents by SDS-PAGE, according to the method described by King et al. (12).

Reconstitution procedure

Lipids in chloroform were evaporated to dryness under N_2 and 100 mM DOC was added, followed by vigorous shaking for 15 sec in a vortex mixer. The mixture was then heated at 40°C for 10 min (this heating is absolutely necessary to clear the solution when the cholesterol content in lipids is high) and diluted with a suitable amount of buffered NaCl to obtain a definite final concentration of materials in the mixture described below. Then, fraction B (or fraction A, in some experiments) was added to the mixture and dialyzed against buffered NaCl for 3 days at 4°C. The final concentration of lipids was arbitrarily changed while the concentrations of DOC, urea, and fraction B were adjusted to 5 mM, 4 M, and 16.7 μg protein/ml of dialyzate, respectively. After completion of the dialysis, the mixture, which turned into a cloudy suspension, was layered over 0.25 M and 0.68 M sucrose solution for centrifugation, as described earlier for the isolation of SAM. The white material at the interface between the two sucrose solutions was removed and washed once with buffered NaCl by centrifugation at 48,000 *g* for 20 min. The washed precipitate was resuspended in buffered NaCl and this was termed LB (meaning a complex of lipids and fraction B). LB containing 50 μg of protein was dialyzed against 0.1 M sodium phosphate buffer with 0.145 M NaCl of various pH values (this buffer was used only for determining optimal pH of the second dialysis), acidified buffered NaCl (pH 5.0), or 0.01 M Tris–HCl (pH 7.4) containing 0.145 M NaCl and 3 mM $CaCl_2$, with various amounts of fraction A or A-I at 4°C for 3 days. This was followed by dialysis against buffered NaCl for 2 days. The dialyzate was again centrifuged by discontin-

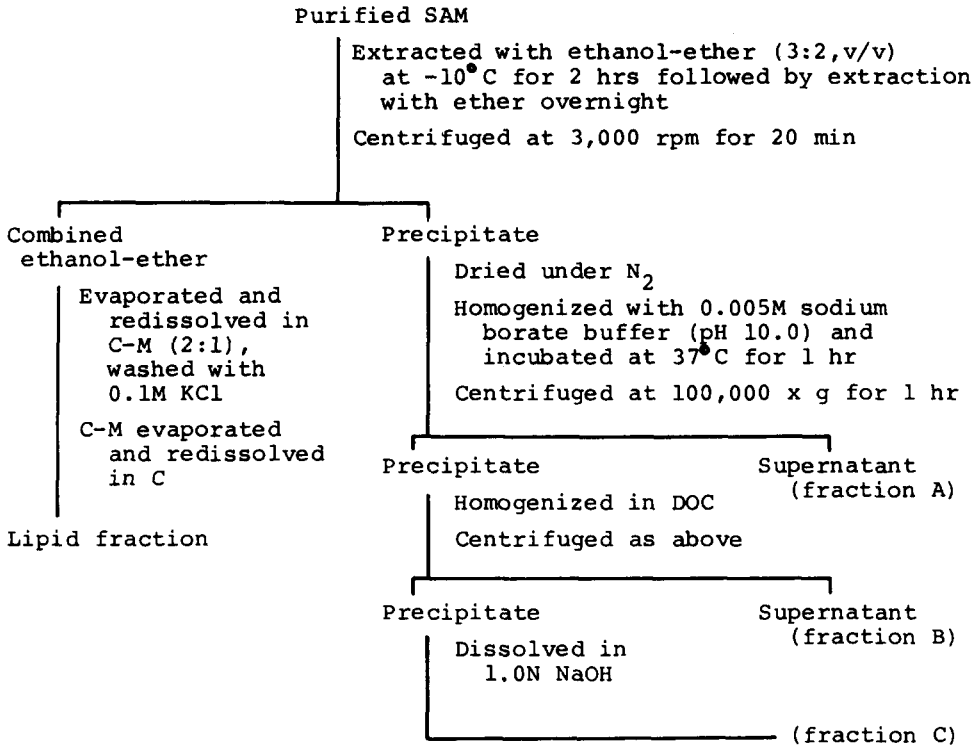

Fig. 1. Procedure of preparation of protein fractions and lipids from pig alveolar surface active material. SAM, surface active material; C, chloroform; M, methanol; DOC, sodium deoxycholate.

uous sucrose density gradient ultracentrifugation as above and the white material was washed once (this final lipid–protein complex was termed LBA).

Electron microscopic study of SAM and the reconstituted lipid–protein complex

Isolated SAM or reconstituted lipid–protein complex in suspension was precipitated by centrifugation at 48,000 *g* for 20 min and the precipitates were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 0.25 M sucrose. Freeze-fracture was carried out on these fixed materials and replicas were examined under a JEOL 100 CX electron microscope (Akishima, Japan). Negative staining of unfixed suspensions was performed with 2% phosphotungstic acid adjusted to pH 7.0 with 1.0 N KOH.

Chemical analysis

Analysis of phospholipid (PL) was carried out as described elsewhere (7) and total phosphorus of PL was determined according to the method of Bartlett (14) after extraction of lipids by the method of Bligh and Dyer (15). Total protein was measured as described by Bensadoun and Weinstein (16).

RESULTS

Differences in the relative content of protein and PL of SAM from various origins

As shown in **Table 1,** the PL–protein ratio of SAM from alveolar wash was more than double that obtained from the homogenates. However, there was only a slight difference in the constituents of PL. In SAM from the alveolar wash, the phosphatidylcholine content was higher and the contents of spingomyelin, phosphatidylethanolamine, and phosphatidylserine + phosphatidylinositol were lower than those of SAM from either fresh or frozen lungs.

Characterization of protein fractions

Protein fraction A accounted for about 72% of the total protein recovered, fraction B about 18%, and fraction C (which was not dissolved even in DOC solution and was not used in this study) was 10% when alveolar SAM was the starting material (Table 1). In the SAM from homogenates, these values were 51–56%, 26–33%, and 11–23%, respectively. Further separation of fraction A with Sephadex G-200 column revealed three peaks of protein (**Fig. 2**). The first peak (A-I) was eluted in the void volume and contained proteins with molecular weight

TABLE 1. Phospholipid–protein ratio, phospholipid composition, and relative amount of protein fractions in pig pulmonary surface active material of various origins

Origin	Alveolar Wash	Homogenates	
		Fresh	Frozen
PL/Protein (w/w)	10.8 ± 1.5[a]	5.2[b]	4.4[b]
Phospholipid (mol%)			
SPH[c]	1.0 ± 0.4	2.9	4.2
PC	82.6 ± 1.8	75.0	72.2
PE	3.0 ± 0.9	6.0	7.8
PS + PI	6.8 ± 3.6	11.6	10.8
PG	6.8 ± 1.8	4.6	5.1
Protein (weight%)			
Fraction A	72.4 ± 5.5	56.2	51.4
Fraction B	18.0 ± 2.3	32.7	26.0
Fraction C	9.6 ± 6.4	11.0	22.6

[a] Mean ± S.D. from five experiments.
[b] Mean value obtained from two to four experiments.
[c] SPH, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol.

over 300,000 daltons, as judged by comparing the elution volume with that of the reference standards. The second peak (A-II) had a molecular weight of 150,000 daltons and the third (A-III), 70,000 daltons.

Protein fraction B, which still had residual PL (1.11 ± 0.89 mg/mg protein) despite the exhaustive extraction of lipids, showed complete dissociation of lipids and proteins when examined by Sephadex G-75 gel-filtration. Proteins were eluted in the void volume and PL was eluted at the position corresponding to molecular weight 10,000 (**Fig. 3**). Attempts to isolate only proteins in fraction B failed since proteins were not redissolved in DOC solution after dialysis against distilled water and lyophilization. For this reason, fraction B was used without further separation from PL.

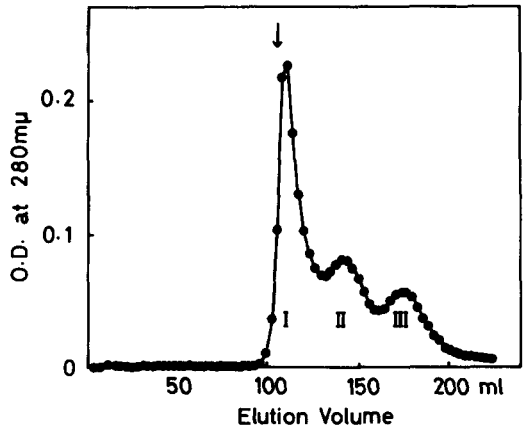

Fig. 2. Elution pattern of protein fraction A on Sephadex G-200 column (1.6 × 170 cm). Arrow shows the elution volume of blue dextran. Elution and equilibration of the column were carried out with 0.01 M sodium borate buffer (pH 10.0) containing 0.145 M NaCl.

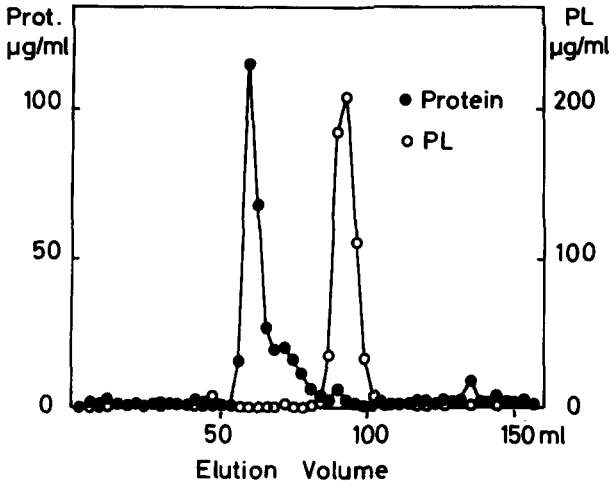

Fig. 3. Elution pattern of protein and phospholipids of protein fraction B on Sephadex G-75 column (1.6 × 70 cm). Elution and equilibration of the column were carried out with 0./005 M sodium borate buffer (pH 10.0) containing 10 mM sodium deoxycholate and 0.145 M NaCl.

Formation of lipid–protein complex by the first dialysis

As shown in **Table 2,** a significantly higher recovery of protein and lipids was obtained by dialysis of lipids with fraction B than with fraction A. The ratio of PL to protein in the starting mixture was 20:1 in both fractions A and B.

Fig. 4 shows the recovery of PL and protein and the ratio of protein to PL in the recovered lipid–protein complex (LB), after dialysis of fraction B with lipids. For this experiment, the amount of protein was fixed and the amount of PL was changed. The recovery of protein was highest when the protein–PL ratio of the initial mixtures was about 0.05 and the protein–PL ratio of recovered LB could be altered by changing the protein–PL ratio of the starting mixtures.

Incorporation of fraction A into LB

LB with a protein–PL ratio of 0.047, containing 50 μg of protein, was dialyzed against buffer solution at various pH's with 575 μg of fraction A. As shown in

TABLE 2. Comparison of the recovery of protein and phospholipids and protein–phospholipid ratio of lipid–protein complex obtained by the first dialysis procedure using protein fractions A and B[a]

Protein Fraction	Recovery		Protein/ Phospholipids (w/w)
	Protein	Phospholipids	
A (4)[b]	14.1 ± 7.0%[c]	42.0 ± 16.0%	0.016 ± 0.005
B (5)	59.5 ± 6.4	68.7 ± 10.6	0.043 ± 0.003

[a] Phospholipid–protein ratio in the dialyzing mixture was 20:1 (w/w).
[b] Number of experiments.
[c] Mean ± S.D.

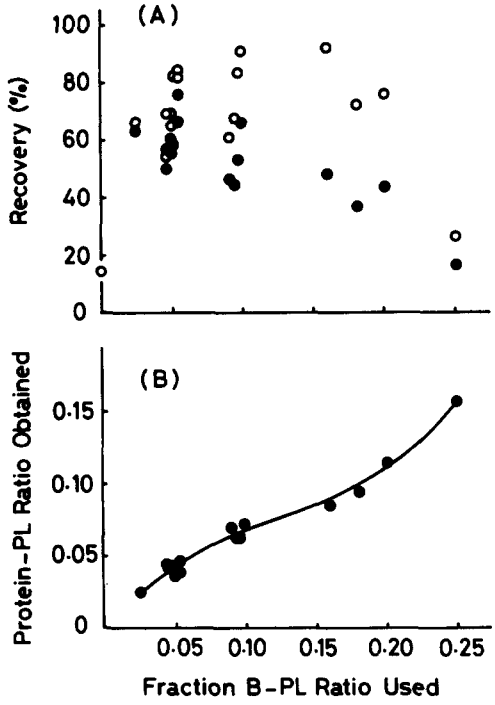

Fig. 4. Recovery of protein and phospholipids (A) and protein–phospholipid ratio (B) of lipid–protein complex obtained by the first dialysis of the mixture of protein fraction B and various amounts of lipids (expressed as weight of phospholipids contained). Open circles and closed circles in A show phospholipids and protein, respectively.

Fig. 5, the protein–PL ratio of the lipid–protein complex (LBA) obtained by this second dialysis was higher in the acidic range of pH than in the neutral or basic range. Recovery of PL by this procedure was 67.6 ± 3.9% of LB used.

Three different LB preparations with mean protein-PL ratios of 0.040, 0.066, and 0.096 (each containing 50 μg of protein) were dialyzed with various amounts of fraction A at pH 5.0 or against 3 mM $CaCl_2$ solution. The results are shown in **Fig. 6.** Increase in the protein–PL ratio of LBA was linear at a low ratio of fraction A to LB protein, but a limit was found where the protein–PL ratio was roughly twice the initial protein–PL ratio of the LBs. A very high protein–PL ratio was, however, obtained when LB having a protein–PL ratio of 0.096 was dialyzed with over 11.5 times of fraction A, and the resulting LBA did not locate at the interface between 0.25 M and 0.68 M sucrose solution. These substances were recovered near the bottom of the centrifuge tubes and showed quite a different specific gravity from others and natural SAM. Dialysis against 3 mM $CaCl_2$ did not increase the protein–PL ratio of LBA, compared to dialysis at acidic pH values. When the subfractions of A were used, a larger increase of protein–PL ratio was obtained in fraction A-I, but not in fractions A-II or III, than in fraction A, even with the same amount of protein.

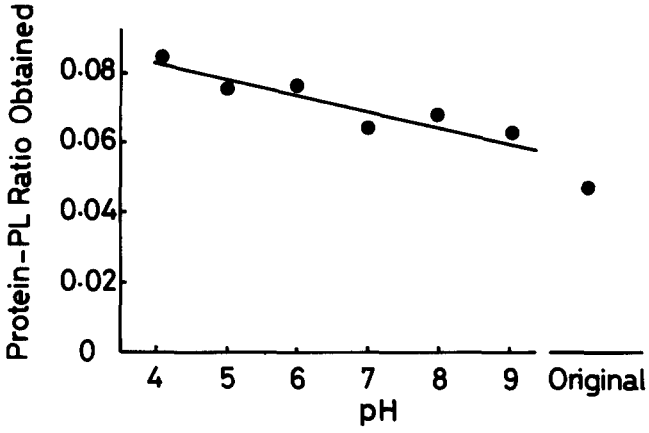

Fig. 5. Effect of pH on the protein–phospholipid ratio of recovered lipid–protein complex by the second dialysis with protein fraction A (575 μg) and LB containing 50 μg of protein (protein–phospholipid ratio, 0.047). LB is the lipid–protein complex obtained by the first dialysis with lipids and protein fraction B.

Analysis of protein by SDS-PAGE

Results of SDS-electrophoresis are shown in **Fig. 7.** The major protein bands in fraction A were found to have nominal molecular weights of 50,000, 45,000,

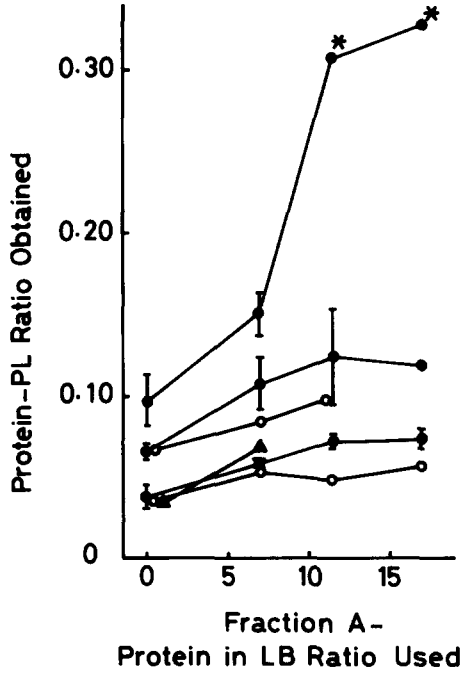

Fig. 6. Protein–phospholipid ratio of recovered lipid–protein complex obtained by the second dialysis of various amounts of protein fraction A and LBs having different protein–phospholipid ratios against acidified (pH 5.0) buffered NaCl (●) or 3 mM $CaCl_2$ at pH 7.4 (○). Triangles indicate the experiment with subfraction A–I of protein fraction A. Asterisks indicate the lipid–protein complex obtained near the bottom of the centrifuge tube instead of at the interface between 0.25 M and 0.68 M sucrose solution (See text). Vertical bars indicate standard deviation of means from three to five experiments.

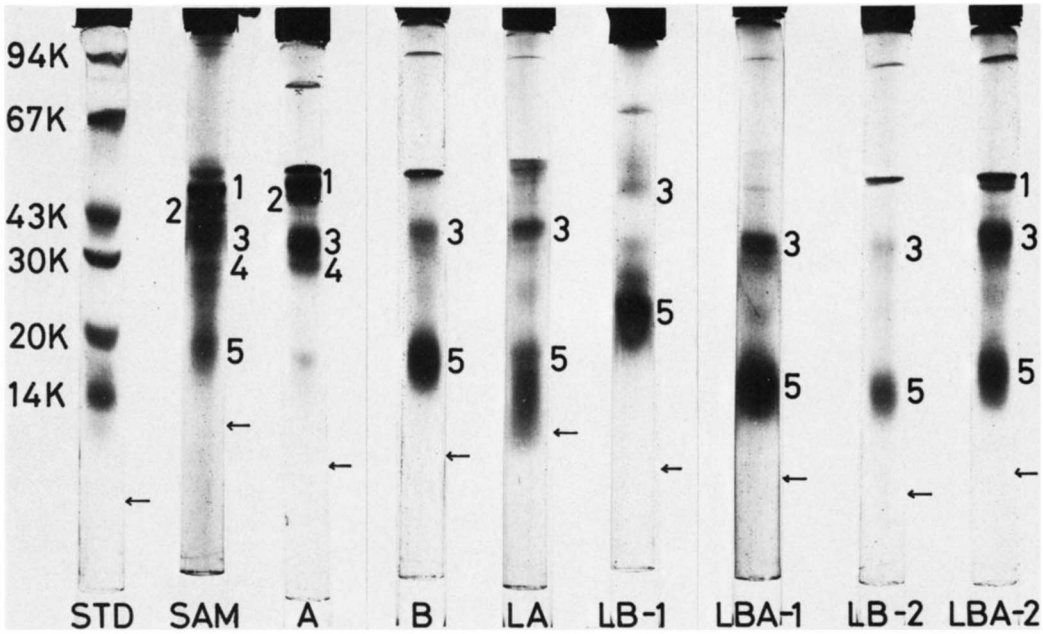

Fig. 7. SDS–polyacrylamide gel–electrophoresis pattern of pig alveolar surface-active material (SAM), protein fraction A (A); protein fraction B (B); lipid–protein complex obtained by the first dialysis with lipids and fraction A (LA); complexes with lipids and fraction B (LB-1 and 2); and complexes obtained in the second dialysis with fraction A and LBs (LBA-1 and 2). Protein–phospholipid ratio of LA, LB-1, LB-2, LBA-1 (formed from LB-1), and LBA-2 (formed from LB-2) were 0.011, 0.047, 0.088, 0.077, and 0.106, respectively. Nominal molecular weights of bands 1, 2, 3, 4, and 5 were 50,000, 45,000, 34,000, 27,000, and 15,000, respectively. Reference protein standards (STD) were phosphorylase b (94,000 daltons), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20, 100), and α-lactalbumin (14, 400). Arrows show migrating positions of bromophenol blue.

34,000 and 27,000 daltons, while in fraction B only two bands having molecular weights of 34,000 and 15,000 daltons appeared. The protein incorporated into LB was 34,000 daltons and 15,000 daltons. The ratio of LB-2 was 1:2.5 compared to the original ratio of 1:1.7 of fraction B, thereby reflecting a selective incorporation of the latter protein into LB. The ratio was reversed to 1:0.76 in LBA-2 with little addition of other protein bands, reflecting a selective incorporation of 34,000-dalton apoprotein into LBA from fraction A by the second dialysis.

Ultrastructure of reconstituted lipid-protein complex

A large number of particles with a diameter of 91 ± 9 Å was observed on the hydrophobic surface of liposomes from SAM (**Fig. 8A**). In the reconstituted LB and LBA, those particles were 109 ± 17 Å and 110 ± 14 Å, respectively (Fig. 8B and C). Although the distribution was somewhat different from sample to sample, there were no fundamental differences between LB and LBA. In negatively stained preparations, there was a fine particulate appearance in the areas limited by the lipid membranes in SAM and LBA, but such was seldom found in the LB (**Fig. 9A, B** and **C**).

DISCUSSION

It has been clearly shown that a lipid fraction and two protein fractions could be reconstituted into a lipid–protein complex with a similar appearance to the native isolated SAM. Although there are reports in which one of the protein components, the 35,000-dalton apoprotein, was reconstituted into a lipid–protein complex with DPPC (11) or with lipid extracts of SAM (10), our findings differ at the following two points. The first is that two major protein components, 15,000-dalton and 34,000-dalton apoproteins, were used and the second is that morphological observations of the reconstituted lipid-protein complex were made.

Although there is one report in which the protein was not a true constituent of SAM (17), a large number of investigators did find evidence for protein(s) in the isolated SAM from various origins (4, 8, 9, 12, 18, 19). King and MacBeth (11) reported two protein components with nominal molecular weights of 10,000–11,000 and 35,000–45,000 daltons. The 15,000-dalton apoprotein in fraction B in the present experiment would correspond to the smaller apoprotein and the 34,000-dalton apoprotein to the larger one in their report. If the smaller apoprotein is indeed the proteolytic fragment of the larger one, as suggested by King et al. (20) and Gikas et al. (21), the same affinity to lipids should be retained in both apoproteins, because the hydrophobic portion of the two apoproteins that reacts with lipids is the same. Appearance of an 11,000-dalton apoprotein was reported (21) in accord with a rapid increase of PL in the tracheal

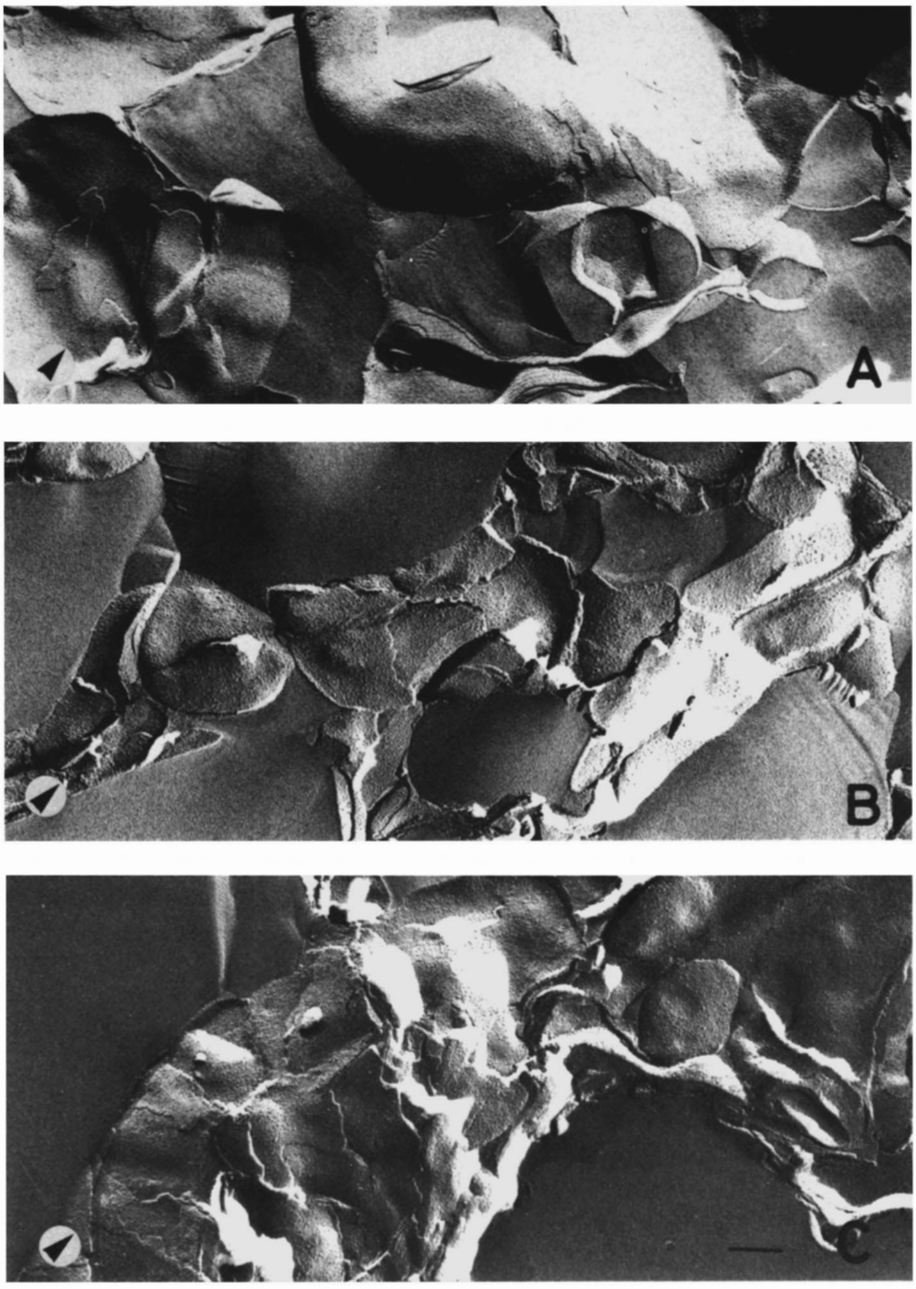

Fig. 8. Freeze-fracture electron micrographs of pig alveolar surface-active material (SAM) (A); reconstituted lipid–protein complex from lipids and protein fraction B (LB) (B); and reconstituted lipid–protein complex from LB and protein fraction A (LBA) (C). Arrow head at the lower left position of each photograph indicates the direction of shadowing and the bar indicates a length of 200 nm. Intramembranous particles were seen in all of these materials having a mean diameter of 9.1 nm in SAM and 10.9–11.0 nm in LB and LBA.

fluid of the fetus in the alveolar stage and, in the canalicular stage, only the 34,000-dalton apoprotein was found without PL. If the 34,000 dalton apoprotein is actually metabolized into the 11,000-dalton apoprotein, it seems reasonable that some stage of lung development is in process when only the 34,000-dalton apoprotein and PL are produced together without the accompanying 11,000-dalton apoprotein. Since we found the lipid affinity quite different between these two apoproteins, they may be independent of each other.

Sawada, Yamabayashi, and Okajima (9) proposed that the molecular weight of native apoprotein of bovine SAM was 20×10^4 daltons. Considering the size of the protein particles in natural SAM found in the freeze-fracture study, it is conceivable that these apoproteins would be present in a combined form of several components. As

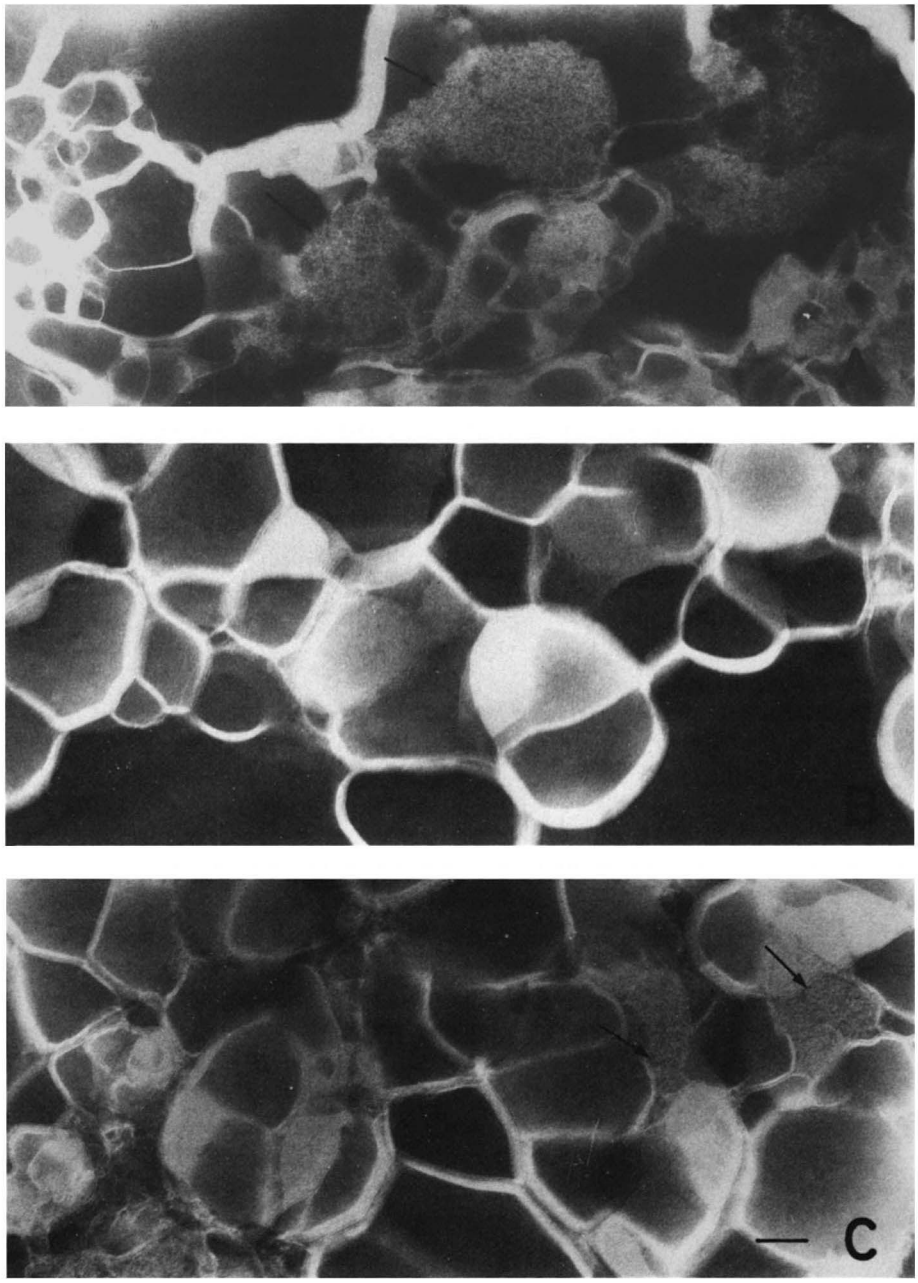

Fig. 9. Electron micrographs of negatively stained preparations from SAM (A), LB (B), and LBA (C). Characteristics particulate appearance in areas limited by lipid membranes (→) were observed in SAM and LBA but not in LB. Abbreviations are the same as in Fig. 8 and the bar indicates a length of 100 nm.

found here, the 34,000-dalton apoprotein was incorporated into the lipid–protein complex in relation to the protein content but not to the lipid content of LB. Therefore, the 34,000-dalton apoprotein is probably closely associated with the 15,000-dalton apoprotein, possibly by ionic interaction. However, the possibility cannot be excluded that this 34,000-dalton apoprotein may interact with the lipids, especially with some boundary lipids of the 15,000-dalton apoprotein. Further investigation is required to assess the relationship between these apoproteins and lipids. From the finding that only the 34,000-dalton apoprotein was selectively associated with LB, simple enclosure of proteins of fraction A by liposomes can be excluded.

Other proteins in fraction A that were relatively abundant in pig SAM were eluted as two peaks having molecular weights of 150,000 and 70,000 daltons in Sephadex G-200 gel-filtration and in SDS-PAGE the former fraction contained mainly 34,000 and 27,000 dalton bands and the latter a 50,000 dalton band in addition

to faint 34,000 and 27,000 dalton bands, which seemed to be contaminants from the former peak. Albumin and globulin were reportedly contained in isolated SAM (12, 17, 22). Part of the 150,000 dalton peak (fraction A-II) was globulin and the 70,000 dalton peak (fraction A-III) was albumin. In this system of SDS-PAGE, the pig serum albumin gave a single 50,000 dalton band. The 34,000-dalton apoprotein was distributed into two peaks with molecular weights of over 300,000 and 150,000 daltons, respectively, thereby suggesting the presence of different aggregates in fraction A. These additional proteins, albumin and globulin, were not reconstituted into the lipid–protein complex by the method described here and they may simply be contamination from serum proteins.

Studies on the surface activity and the effect of changes in the lipid constituents of the reconstituted lipid–protein complexes are published in an accompanying paper (23).

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