Isolation, characterization, and surface chemistry of a surface-active fraction from dog lung

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ABSTRACT A procedure is described for the isolation of a surface-active fraction from dog lung. This material meets the established criteria for pulmonary surfactant. The fraction was shown to contain lipid, protein, and carbohydrate. The predominant lipid present was dipalmitoyl phosphatidylcholine. Surface chemistry studies indicated the surface properties of the fraction could not be explained solely from a consideration of the properties of dipalmitoyl phosphatidylcholine. Electron microscopic studies demonstrated the presence of intact osmiophilic bodies as well as other myelin forms in the surface-active fraction. It is speculated that, *in situ*, the alveolar lining layer is similar to a structured gel.

SUPPLEMENTARY KEY WORDS pulmonary surfactant · dipalmitoyl phosphatidylcholine · osmiophilic bodies

Numerous theoretical and experimental studies have established that the alveoli of mammalian lung are lined with a highly surface-active substance, designated pulmonary surfactant, which presumably reduces alveolar surface forces at low lung volumes during respiration, thereby preventing alveolar collapse (1–6). The preponderance of evidence indicates that this material is a lipoprotein with a high content of dipalmitoyl phosphatidylcholine (7, 8). The absence of demonstrable surface activity in the lung is associated with several pathological conditions, notably hyaline membrane disease (2).

The development of normal surface activity in fetal lung extracts of most mammalian species is associated with the appearance of osmiophilic inclusion bodies in the type II cells (great alveolar cell) of the alveolar epithelium (8–11). The exact physical and temporal relationship between the alveolar lining layer, which contains pulmonary surfactant, and the osmiophilic inclusion bodies is not yet clearly defined (10, 12). Numerous studies, however, indicate that the type II cell inclusion bodies are associated with pulmonary surfactant (8, 10, 13–15). Goldenberg, Buckingham, and Sommers (16) have recently presented micrographs purporting to show the contents of osmiophilic bodies being discharged on to the alveolar surface.

Crude preparations of pulmonary surfactant have been isolated from saline lung lavages or from the filtrate of scissor-minced lung (17). Abrams was the first to isolate a surface-active fraction from lung using an homogenization-centrifugation scheme (6). In our experience, neither this procedure nor subsequent modifications have been reproducible in terms of protein yields or capability to isolate a fraction which lowers surface tension.

The purposes of this paper are to present the following: (*a*) a convenient method for the large-scale isolation of a fraction from whole lung which is very surface active and contains osmiophilic inclusion bodies, as demonstrated by electron microscopy; (*b*) a detailed chemical characterization of this material; (*c*) a modified method for the assay of pulmonary surfactant surface activity; and (*d*) certain aspects of the surface chemistry of pulmonary surfactant.

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Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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METHODS

Isolation of Pulmonary Surfactant

Mongrel dogs were killed by exsanguination under phenobarbital anesthesia, and the lungs were removed. All subsequent operations were carried out at 0°–5°C. The homogenization medium was 0.145 M NaCl in 0.01 M tris buffer containing 0.001 M EDTA, final pH 7.4. All subsequent sucrose solutions were prepared in this medium. The lungs were homogenized in a Waring Blendor at low speed for 1 min and then for 10 sec at high speed, with 2 ml of homogenization medium per g of lung. The homogenate was filtered through nylon gauze. The residue was stirred with homogenization medium, 2 ml/g of original lung, for 5 min and then refiltered. The combined filtrates were made up to a volume of 4 ml/g of lung, and 35-ml portions were carefully layered over 10 ml of 0.75 M sucrose. After centrifugation in the Sorvall SS-34 rotor at 48,000 *g* for 40 min, the interfacial material, I, between the supernatant and 0.75 M sucrose was removed and resedimented from the homogenization medium at 48,000 *g* for 20 min. The washed I was suspended in homogenization medium, 0.3 ml/g of lung, and layered over a discontinuous density gradient consisting of 10–12 ml each of 0.68 M and 0.25 M sucrose. Centrifugation was then carried out in a Spinco SW 25.1 rotor at 25,000 rpm for 60 min. Material concentrating at each interface was removed by aspiration, suspended in 30–35 ml of homogenization medium, and resedimented at 48,000 *g* for 20 min. The resedimentation was repeated. The washed fractions were resuspended in water or homogenization medium and stored at 4°C. By this procedure, the material which collected at the interface between the homogenization medium and 0.25 M sucrose has been designated IA; that between 0.25 M and 0.68 M sucrose, IB; and the precipitate, IC. The isolation procedure is outlined in Fig. 1. As shown in the Results section, only fraction IB was significantly surface active.

From experiments to be reported in detail elsewhere,[1] saline lung lavages subjected to the above procedure yielded an IB fraction that was identical with that obtained when whole lung was homogenized. No IC was obtained from saline lavages.

In studies designed to visualize intact osmiophilic bodies in IB preparations by electron microscopy, whole lung was homogenized with a Potter–Elvehjem homogenizer using the same procedure as above.

In some experiments the surface-active fraction, IB, was isolated at different intervals after dogs were injected intravenously (femoral vein) with the following:

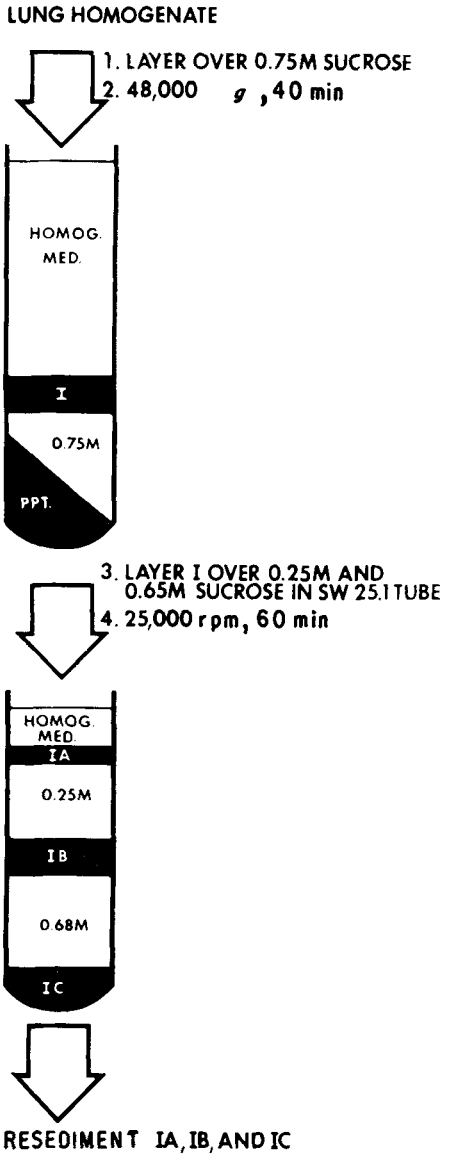

FIG. 1. Outline of isolation procedure. All sucrose solutions were prepared in homogenization medium (see text). As shown in the Results section, only fraction IB was significantly surface active.

(*a*) palmitic-1-^{14}C acid (specific activity of 10 mCi/mM), 10 μCi/kg; (*b*) palmitic-9,10-^{3}H acid (specific activity of 200 mCi/mM), 60 μCi/kg; (*c*) L-alanine-^{3}H, general label (specific activity of 2.1 Ci/mM), 300 μCi/kg; (*d*) L-alanine-^{14}C, uniform label (specific activity of 123 mCi/mM), 100 μCi/kg; or (*e*) combinations of the above isotopes. Palmitic acid was injected in not more than 1 ml of absolute ethanol; alanine, which was obtained in 0.01 N HCl, was diluted with 2 ml of isotonic saline before injection. All radioactive isotopes were obtained from New England Nuclear Corp. (Boston, Mass.).

Enzyme Markers

Assays for cytochrome C oxidase (18) and acid phos-

[1] Submitted by R. Pawlowski in partial fulfillment of the requirements for the Ph.D. degree, Department of Anatomy, Case Western Reserve University, and manuscript in preparation.

phatase (19) were carried out to ascertain the contamination of IB preparations with mitochondria and lysosomes, respectively.

Centrifugation of IB on Continuous Sucrose Gradients

Continuous sucrose density gradients were prepared by placing a 5 ml cushion of 1.75 M sucrose in the bottom of a SW 25.1 tube and then layering on top of this, a 20 ml continuous linear gradient, ranging from 1.00 M to 0.20 M sucrose. The IB, suspended in homogenization medium, was placed on top of the gradient, and centrifugation was carried out for 2 hr at 25,000 rpm. At the end of the run, 0.5-ml fractions were collected from the bottom of the tube for determination of lipid and protein distribution.

Recovery of IB from Lung Homogenates

In order to estimate the recovery of the surface-active fraction, IB, a dog was injected intravenously with palmitic-9,10-^{3}H acid and was killed after 24 hr. The IB fraction was obtained by the above procedure and resuspended in homogenization medium. Equal portions of this ^{3}H-labeled IB were added, in duplicate, to the following: (*a*) the homogenate of the lung of a normal unlabeled dog, and (*b*) a volume of homogenization medium equivalent to that used in (*a*). Both mixtures were carried through the complete centrifugation procedure, and aliquots were removed at each step for the determination of radioactivity.

Gel Filtration Studies

A Sepharose 2B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) column, 2.5 × 75 cm, equilibrated with the homogenization medium described earlier, was used in these studies. Distribution of substances in the eluate was then determined spectroscopically or by scintillation counting. The void volume was shown to be 90 ml with the use of Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.), and the column volume, 360 ml, was determined with glycylglycine. Preliminary experiments indicated that considerable binding of IB to the gel occurred, confirming the earlier observations of Huang (20) with egg phosphatidylcholine. Consequently, we presaturated the gel with a sonicated dispersion of egg phosphatidylcholine in homogenization medium until no additional binding was observed (20). The column parameters were determined after the saturation process.

For gel filtration studies with the IB apoprotein, a 1.5 × 25 cm Sepharose 2B column was equilibrated with 0.005 N sodium borate, pH 11.5. The void volume was 18 ml; the column volume was 64 ml.

IB Apoprotein

An apoprotein preparation was obtained from IB by the third method described by Scanu, Pollard, Hirz, and Kothary (21). The entire procedure was carried out in a 50 ml glass stoppered centrifuge tube. IB, 25 mg of protein, was suspended in 2.0 ml of homogenization medium and added to 20 ml of ethanol–diethyl ether 3:1 at −10°C. The mixture was stirred slowly for 3 hr at −10°C and then centrifuged at 3000 rpm for 10 min. The supernatant was discarded, and 40 ml of ether was added. The precipitate was dispersed with a glass rod, and the mixture was centrifuged at 12 rpm for 12 hr at −10°C. The ether was discarded. The precipitate was dried under N_2 at 0°C and broken up into fine particles with a glass rod before the addition of 10 ml of 0.005 M sodium borate buffer, pH 11.5. After incubation at 40°C for 3 hr, a small amount of insoluble residue (less than 10% of the total protein) was removed by centrifugation. The recovery of protein as soluble apoprotein ranged from 70 to 85%.

Electron Microscopy

Specimens for fixation were taken immediately and placed in 2% glutaraldehyde in 0.2 M Sörensen's buffer, pH 7.3, for 1 hr at 5°C. Tissue was rinsed in buffer two times and remained overnight in buffer solution. Postfixation in 1% osmic acid in similar buffer for 30 min was done at room temperature. Dehydration and embedding in Epon 812 were carried out as described by Luft (22). Thin sections were counterstained with uranyl acetate and lead citrate. Negative staining was carried out with 1% potassium phosphotungstate, pH 6.8 (20). Specimens were examined under the electron microscope (Phillips-200, RCA-3F, or Zeiss 9A).

Isolation of Lipids

Total lipids were extracted from whole lung and tissue fractions by the method of Folch, Lees, and Sloane Stanley (23). Phosphatidylcholine was isolated and purified by the following procedures:

(*a*) *Egg Phosphatidylcholine.* This was isolated from eggs by the procedure of Lea, Rhodes, and Stoll (24). It was purified by column chromatography on alumina (25) and then by column chromatography on Unisil. (The correct fraction was eluted with chloroform–methanol 2:3.) After recrystallization from chloroform–acetone, the material gave the following analysis: 3.62 %*P*, molar ratio ester:*P* = 1.99.

(*b*) *IB Phosphatidylcholine.* This was isolated from IB total lipid by preparative TLC on Silica Gel H plates in chloroform–methanol–water 70:30:4. The analytical characteristics were: 3.72 %*P*, molar ratio ester:*P* = 1.90, and molar ratio aldehydogenic linkages:*P* < 0.05.

(*c*) *Dipalmitoyl Phosphatidylcholine.* A synthetic preparation of L-α,β-dipalmitoyl phosphatidylcholine was obtained from General Biochemicals (Chagrin Falls, Ohio), purified by preparative TLC, and recrystallized from chloroform–acetone. Analytical values were: 4.12 %*P* and molar ratio ester:*P* = 2.00.

Each of the phosphatidylcholine species was shown to be homogenous by TLC when 100 μg were chromatographed.

Analytical Methods and Procedures

Lipid weights were determined gravimetrically using a Cahn M-10 electrobalance. Phosphorus was determined by a modification of the method of Beveridge and Johnson (26) after digestion with perchloric acid. Ester groups were determined according to the procedure of Rapport and Alonzo (27). Aldehydogenic groups were measured by the *p*-nitrophenyl hydrazone method (28). Protein was determined by the procedure of Lowry, Rosebrough, Farr and Randall (29) using crystallized bovine serum albumin, fraction V, as the standard. Carbohydrate was estimated, as glucose, by the anthrone procedure (30). Sialic acid was assayed by the method of Aminoff (31). The analyses for anthrone and sialic acid were carried out on the protein residue of IB preparations after exhaustive dialysis against water and delipidation with diethyl ether–ethanol 3:1.

Individual phospholipid classes were quantitated by phosphorus analysis after separation by TLC on Silica Gel H plates in chloroform–methanol–water 75:27.5:5. Neutral lipid classes, after separation by TLC in hexane–diethyl ether–acetic acid 80:20:1, were estimated by the procedure of Amenta (32). In both instances, areas corresponding to the separated lipid classes were visualized by brief exposure of the plates to I_2 and scraped directly into hydrolysis tubes for analysis. Appropriate blank areas of the silica layers were also analyzed.

Gas-Liquid Chromatography

The composition of the ester residues of the various phosphatidylcholine species was determined by GLC according to the procedure of Morrison and Smith (33) using the reaction tube described by Kishimoto and Radin (34). Distribution of ester residues on the α- and β-positions of the molecule was determined after hydrolysis of phosphatidylcholine with phospholipase A (35) and subsequent separation of fatty acids and 1-acyl-2-lysophosphatidylcholines by TLC. Unsaturated fatty acid residues were catalytically hydrogenated in ethanol at atmospheric pressure using 5% palladium on charcoal. GLC analysis was carried out with a Hewlett-Packard F & M gas chromatograph, model 402, utilizing 10 ft glass columns packed with 6% diethylene glycol succinate on 80–100 Diatoport S. The column temperature was 160°C, isothermal; the carrier gas was helium at a flow rate of 100 ml/min. Peak areas were determined by triangulation.

Disc Electrophoresis

The IB apoprotein was analyzed by polyacrylamide gel electrophoresis according to the procedure described by Nerenberg (36).

Radioactivity Measurements

Incorporation of radioactive precursors was estimated by liquid scintillation counting using a Packard Tri-Carb 3003 liquid scintillation counter, model 526, equipped with automatic external standardization. A diotol scintillation medium was used (37).

Surface Pressure Measurements

A modification of the circular trough of Dawson (38) was used. The procedure has been explained in detail and has been shown to give results identical with those obtained by intermittent compression in a rectangular trough (39, 40). The surface area was 22.42 cm^2. The subphase consisted of 50 ml of 0.145 M saline at room temperature unless otherwise specified. Surface tension was measured with a Wilhelmy plate (41), 5 cm in perimeter, connected to the arm of a torsion balance (Federal Pacific Electric Company, Newark, N.J.). The Wilhelmy plate was constructed from 25 gauge platinum roughened with emery paper. The monolayer was formed by touching the surface of the subphase with the tip of a Hamilton syringe needle and adding 0.5-μl portions of the material under study. After an equilibration period, the surface pressure, π, was determined from the following relationship:

$$\gamma = (m)\ (g)/l$$
$$\pi = \gamma \text{ subphase} - \gamma \text{ film}$$

where

m = mass in grams
g = 980 cm sec^{-2}
l = perimeter of blade in cm
γ = surface tension

The actual results are expressed as plots (surface isotherms) of the surface pressure, π, against the area per μg of material applied to the surface or, if the molecular weight is known, π is plotted against the area per molecule. Successive aliquots of the material under study were added to the surface, at a minimum of 5-min intervals to allow for equilibration, until there was no further decrease in surface tension, namely, until the point of monolayer collapse was reached.

Alternately, surface activity was estimated by the dynamic compression-expansion procedure in a surface

balance as designed by Brown, Johnson, and Clements (17). The maximum trough area was 66.0 cm^2 which, upon maximum compression, was reduced to 13.2 cm^2. Cycling times of 3 and 28 min were used. Surface tension was determined by means of a Wilhelmy plate attached to a strain gauge. Maximum surface pressures (minimum surface tension) obtained at minimum surface area were plotted against the amount of material applied to the subphase at *maximum* surface area. The π_{max} was usually obtained on the third complete cycle.

In both procedures, fractions obtained during the isolation procedure were suspended in distilled water by gentle mixing and agitation to a concentration of 0.20–2.0 μg of protein per μl and added to the surface of the subphase. Lipids were dissolved in chloroform–methanol 85:15 to give a concentration of 1.0 $\mu g/\mu l$.

RESULTS

Compositional Studies

As shown later, only one significantly surface-active fraction, IB, was obtained by the isolation procedure described in the Methods section. This fraction, for reasons discussed in the Discussion section, is thought to contain pulmonary surfactant.

Homogeneity of IB from Continuous Gradient Centrifugation. An IB preparation isolated from a dog given an injection of palmitic-9,10,-^{3}H acid and L-alanine-*U*-^{14}C was centrifuged on a continuous sucrose gradient. The results shown in Fig. 2 demonstrate that protein and ^{14}C- and ^{3}H-labeled material reached the same position on the gradient and, furthermore, that only one band of material is seen.

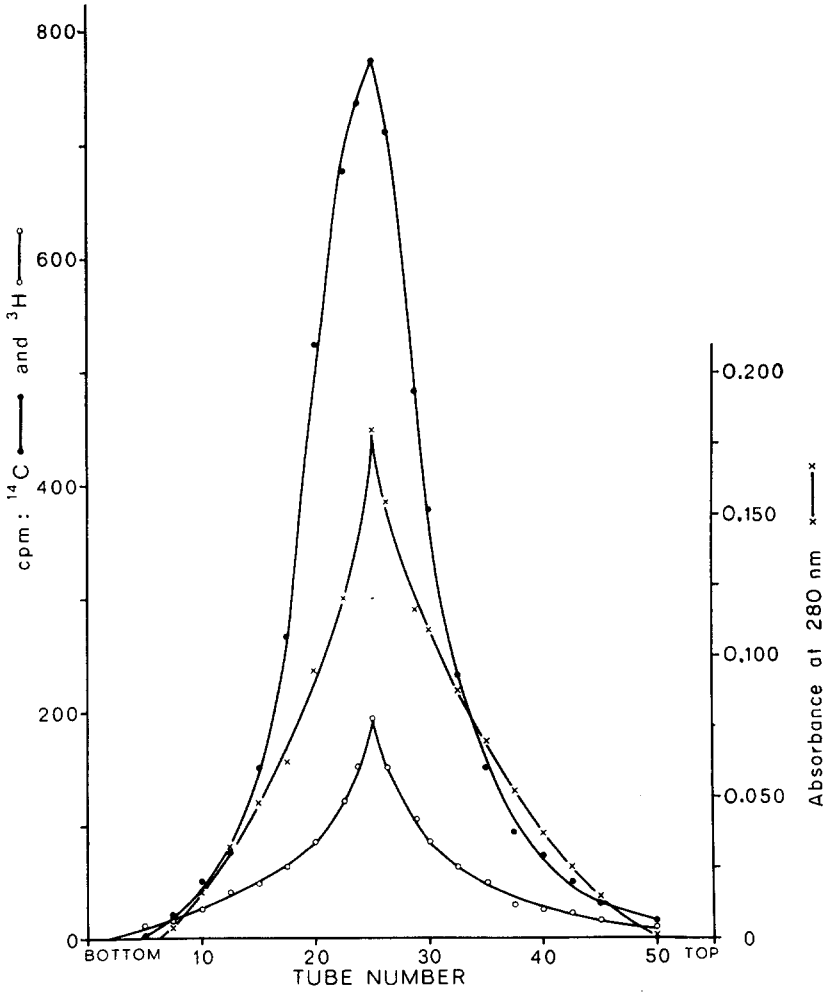

FIG. 2. Centrifugation of IB on continuous sucrose gradient. A 20 ml continuous linear gradient, ranging from 1.00 to 0.20 M sucrose, was layered over a 5 ml cushion of 1.75 M sucrose. Centrifugation was carried out in a Spinco SW 25.1 rotor at 25,000 rpm for 120 min. 0.50-ml fractions were collected from the bottom of the tube for analysis. The dog was given an injection of palmitic-9,10-^{3}H acid and L-alanine-*U*-^{14}C 24 hr prior to isolation of the IB.

Composition of Surface-Active Fraction (IB). Table 1 presents analytical values for the major components found in selected lung fractions obtained by the isolation procedure. Our IB preparations always contained significant amounts of protein. The lipid:protein ratio (w/w) was 3.86. The major lipid present was phosphatidylcholine which accounted for 76% of the total lipid phosphorus. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidyldimethylethanolamine together accounted for 90% of the total phospholipid phosphorus. Total cholesterol was found to be approximately 11% of the total lipid. Although whole lung total lipid contains appreciable quantities of plasmalogens as determined by the assay for aldehydogenic groups, IB preparations contain very little of these compounds. In whole lung, 80% of the total aldehydogenic groups are found in the ethanolamine phosphatide fraction. After delipidation of the IB, carbohydrate, as determined by the anthrone analysis, and sialic acid were found only in the protein component.

The distribution of phosphatidylcholine fatty acid residues from IB preparation is presented in Table 2. Saturated fatty acids comprise 85% of the total α-linked residues and 58% of the total β-linked residues. Despite the high concentration of total saturated fatty acid residues, the β-position does contain more unsaturated residues than the α-position. Palmitic acid is, however, the most abundant fatty acid at each position, and the major phosphatidylcholine species is dipalmitoyl phosphatidylcholine.

The fatty acid designated 18:n is at present unidentified. On the basis of hydrogenation studies, it appears to have at least one point of unsaturation. After hydrogenation, only one peak which had the same relative retention time as 18:0 was found in the 18-carbon region, and it accounted for 22.2% of the total fatty acids. This is what one would expect from the sum of 18:0, 18:1, and 18:n prior to hydrogenation. From a comparison of relative retention times, 18:n is not one of the following: *trans*-9-octadecenoic; *trans*, *trans*-9,12-octadecadienoic; *cis*,*cis*-9,12-octadecadienoic; or *cis*,*cis*,*cis*-9,12,15-octadecatrienoic acids. From the data presented in Tables 1 and 2, it can be seen that while the compositions of IA, IB, and IC are rather similar in some respects, the three fractions are not identical in chemical constitution.

Isotopic Incorporation Studies. The data presented in Table 3 demonstrate that the radioactive label from palmitic-1-^{14}C acid is incorporated into the α- and β-linked ester residues of IB phosphatidylcholine, presumably as palmitate. After 120 min, the specific activity

TABLE 1 COMPOSITION OF PULMONARY SURFACTANT (IB) AND OTHER LUNG FRACTIONS FROM DOGS

Fraction	Protein			Lipid	Lipid							
	Protein	Glucose	Sialic Acid	Lipid	*P*	PC*	PE	PDME	Lyso-PC and Sph	Total Cholesterol	TG	Plasmalogens
	mg/g lung	*mg/mg*		*mg/mg protein*	*%*		*% of lipid phosphorus*			*% of total lipid*		*μmoles/mg lipid*
IB	0.185†	0.19	0.04	3.86	3.06	76.22	7.27	7.77	4.42	10.56	1.73	0.03
	(0.022)	(0.01)	(0.02)	(0.83)	(0.11)	(2.80)	(0.60)	(1.47)	(0.86)	(0.74)	(0.26)	(0.01)
IA	0.032			2.16	2.83	73.67						
	(0.022)			(0.02)	(0.27)	(1.88)						
IC	0.150			0.76	2.62	57.83						
	(0.032)			(0.02)	(0.16)	(3.54)						
Whole Lung					2.64					18.46	6.88	0.13
					(0.16)					(0.40)	(0.34)	(0.01)

* Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PDME, phosphatidyldimethylethanolamine; Sph, sphingomyelin; and TG, triglycerides.

† Values represent the averages of 6–12 animals (±SD).

TABLE 2 PHOSPHATIDYLCHOLINE FATTY ACID DISTRIBUTION IN PULMONARY SURFACTANT (IB) AND LUNG FRACTIONS

Lung Fraction	14:0	14:1	15:0	15:1	16:0	16:1	17:0	17:1	18:0	18:n*	18:1	19:0
	% of total											
IB Total†	4.7	tr.	0.8	0.3	63.3	7.1	0.8	0.2	6.9	6.8	8.1	2.2
α	2.2	0.1	0.5	0.2	67.0	5.2	1.1	0.2	13.7	3.8	4.7	0.7
β	5.4	0.4	1.2	0.6	53.8	9.6	0.5	0.3	3.7	7.7	11.8	3.6
Total† after reduction	5.0				69.0				22.2			2.5
IA: Total†	4.7	tr.	0.8	0.4	69.6	4.4	1.2	0.1	7.7	4.1	4.9	1.0
IC: Total†	3.9	tr.	0.4	0.3	73.8	1.2	0.9	tr.	13.0	tr.	13.0	0.5

Fatty acids designated by chain length: number of double bonds. Values represent means from duplicate determinations from at least six different preparations.

* Unidentified; see text for details.

† Determination performed on complete PC without phospholipase A treatment.

TABLE 3 INCORPORATION OF RADIOACTIVE LABEL FROM PALMITIC-1-^{14}C ACID INTO IB PHOSPHATIDYLCHOLINE

Time	α-Linked Fatty Acids*	β-Linked Fatty Acids*
min	*dpm/μmole PC*	*dpm/μmole PC*
30	30	60
60	120	240
120	280†	360†

* Determined after phospholipase A hydrolysis, see text for exact details.

† Accounts for 77% of label incorporated into total lipids of IB.

TABLE 4 RECOVERY OF RADIOACTIVE SURFACE-ACTIVE FRACTION ADDED TO LUNG HOMOGENATE

Sample	Recovery of ^{3}H-Labeled IB in IB Fraction	
	dpm × 10^{-4}	%
1. ^{3}H-labeled IB* added to lung homogenate	7.04†	78.5
	7.10	79.3
2. ^{3}H-labeled IB* added to homogenization medium	7.92	88.3

* Total dpm added = 8.96×10^4. ^{3}H-labeled IB was obtained from a dog previously injected with palmitic-9,10-^{3}H acid.

† Each value represents the average of duplicate samples carried through the complete procedure.

of the β-linked residues was somewhat higher than that for the α-linked. This indicates that the incorporation into the two positions is independent, to some extent. The incorporation of the label into phosphatidylcholine at 120 min accounts for 77% of the total label incorporated into the lipid component of IB.

Recovery of IB from Homogenates. Since there is no absolute method for the quantitative estimation of pulmonary surfactant *in situ* or in crude homogenates, an "internal standard" procedure was utilized to assess the recovery of surface-active material by the centrifugation techniques. The data are presented in Table 4. Approximately 79% of the ^{3}H-labeled IB (from palmitate-9, 10-^{3}H) was recovered in the IB fraction from the lung homogenate. The greatest single loss of radioactive material occurred in the initial centrifugation step with about 10% of the original radioactive label found in the first precipitate at the bottom of the 0.75 M sucrose. As expected, the recovery of added ^{3}H-labeled IB from an equivalent volume of homogenization medium was approximately 10% higher, 88%. There was no precipitate obtained with these control samples. The data for the recovery experiments indicate that approximately 20% of the surface-active material, IB, is lost during the centrifugation procedure.

Gel Filtration Studies. When samples of IB preparation were chromatographed on a Sepharose 2B column, recovery experiments indicated considerable binding of the IB to the gel. After prior saturation of the gel with a sonicated egg phosphatidylcholine preparation, samples of IB were eluted in the void volume. No other protein or lipid peaks were found in the eluate. Furthermore, when a sample of IB labeled with palmitic-1-^{14}C acid in the lipid moiety and alanine-G-^{3}H in the protein component, was applied to the column, material which was eluted in the void volume had the same ratio of ^{14}C to ^{3}H radioactivity as did the original IB. These results indicate the molecular weight of IB to be greater than 25×10^6 and that protein is an integral component of IB.

Gel filtration of the IB apoprotein on Sepharose 2B in 0.005 N sodium borate, however, gives rise to two protein-containing peaks as seen in Fig. 3. Only after delipidation of IB does protein-containing material, as determined by absorbance at 280 nm, enter into the gel.

Disc Electrophoresis. The results of disc electrophoresis of the IB apoprotein, Fig. 4, demonstrate that at least two bands of material are seen. Although there is considerable material remaining at the origin, the majority of the IB apoprotein does enter into the polyacrylamide gel. Under these conditions, native IB did not enter into the gel.

Surface Chemistry Studies

Isolation of Pulmonary Surfactant. Isotherms for the appropriate fractions obtained during the isolation of pulmonary surfactant are given in Fig. 5. While the isotherms for the fractions shown are for a single preparation, they are entirely typical and representative of results obtained with 12 different preparations. Depending upon the individual preparation, an isotherm for a given fraction may be displaced somewhat along the abscissa to the right or left of the position it occupies in Fig. 5. The relationships exhibited are, however, always in the same direction, that is, IB is always the most surface-active material present. Since, in this procedure, the surface area remains constant (22.42 cm^2), it can be seen that large areas per μg of protein are obtained with small amounts of protein. As an example, a π_{max} of 40 dynes cm^{-1} is obtained with 2 μg IB protein. This was typical of 12 preparations where the amount of IB protein required to reach monolayer collapse ranged from 1 to 6 μg.

When surface activity of the fractions obtained in the isolation procedure was assessed by the dynamic compression-expansion procedure commonly used by lung physiologists (17), only IB preparations were able to reach surface pressures of 57–62 dynes cm^{-1} (γ = 15–10 dynes cm^{-1}) at minimum surface area. The maximum surface pressure values were usually reached after the third or fourth cycle. No other fraction, regardless of the amount of material applied to the

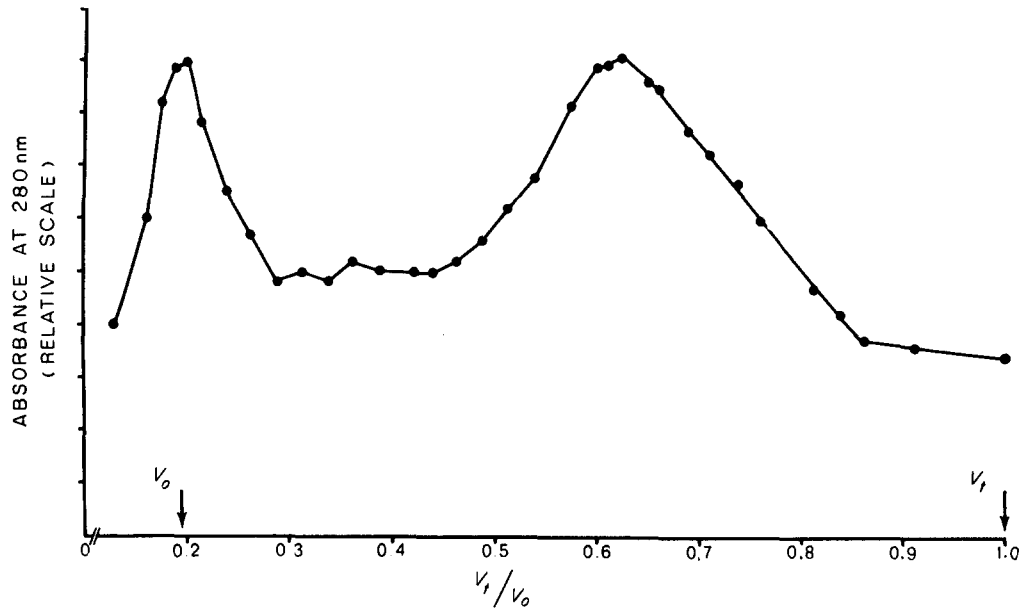

FIG. 3. Gel filtration of IB apoprotein. A Sepharose 2B column, 1.5 × 25 cm, was equilibrated with 0.005 N sodium borate, pH 11.5. V_o, void volume, 18 ml; V_t, total column volume, 64 ml.

surface, was able to reach a surface pressure above 47–52 dynes cm^{-1} (γ = 25–20 dynes cm^{-1}) at minimum surface area. A representation of IB surface activity obtained by plotting the *maximum* surface pressure obtained at minimum surface area as a function of the amount of IB protein initially applied to the surface at 100% surface area is given in Fig. 6. If one arbitrarily defines "significant surface activity" as an increase of surface pressure to 60 dynes cm^{-1} (6), then five times less material is required to reach this point with a 3 min cycle time than with a 28 min cycling period. As shown in Fig. 7, surface layers of dipalmitoyl phosphatidylcholine are not greatly affected by differences in cycling periods. This is in marked contrast to the results obtained with the surface-active fraction isolated from the lung (IB).

Further evidence of the specificity of the IB material in lung was obtained when it was shown that samples of dog brain, liver, whole blood, and serum which were subjected to the above homogenization-centrifugation procedure yielded no surface-active material when assessed by either procedure.

Effect of Subphase pH. Fig. 8 presents the effect of subphase pH and ionic composition on surface isotherms of IB fractions. As expected (42), very little surface activity was obtained with a distilled water subphase. Unbuffered 0.145 M NaCl at pH 5.48 and 0.145 M NaCl prepared in 0.01 M phosphate buffer at pH 5.20 as subphases gave similar surface isotherms. As the pH of the subphase was raised, the surface activity, as judged by π_{max} and the displacement of the isotherm on the abscissa, was decreased. At pH 5.5, no effect upon the isotherm of IB was observed when the NaCl concentration in the subphase was varied between 0.05 and 0.5 M. Similar results were obtained with the dynamic expansion-compression procedure (Fig. 9). The maximum surface pressure (at minimum surface area) that a given amount of IB attained was always lower at an alkaline pH.

Phosphatidylcholine Isotherms. Isotherms for dipalmitoyl, egg, and IB phosphatidylcholines are compared in Fig. 10. Approximately the same π_{max}, 41 dynes cm^{-1}, was obtained by each species. Dipalmitoyl phosphatidylcholine is less compressible than either egg or IB phosphatidylcholine. The fatty acid ester distribution of this egg phosphatidylcholine preparation was the following: 16:0, 37.0%; 18:0, 13.9%; 18:1, 34.4%; and 20:0, 14.6%; 66% of the total fatty acids were saturated. From Table 2, it can be seen that 79% of the total fatty acids from IB phosphatidylcholine are saturated.

Fig. 10 also shows an isotherm for an IB preparation expressed on the basis of the number of PC molecules present *prior* to lipid extraction. The isotherm was determined in the same manner as in Fig. 5. The ratio of μmoles of phosphatidylcholine to μg of protein was subsequently determined by the procedures described in the Methods section. Knowledge of this ratio then allowed expression of the isotherm for the intact unextracted IB as in Fig. 10, that is, on the basis of its phosphatidylcholine content. The isotherm thus obtained for intact IB is more expanded and compressible than those obtained for either dipalmitoyl or extracted IB phosphatidylcholine. Although not shown in Fig. 10, the isotherm for IB total lipid was similar in shape to that of extracted IB phosphatidylcholine.

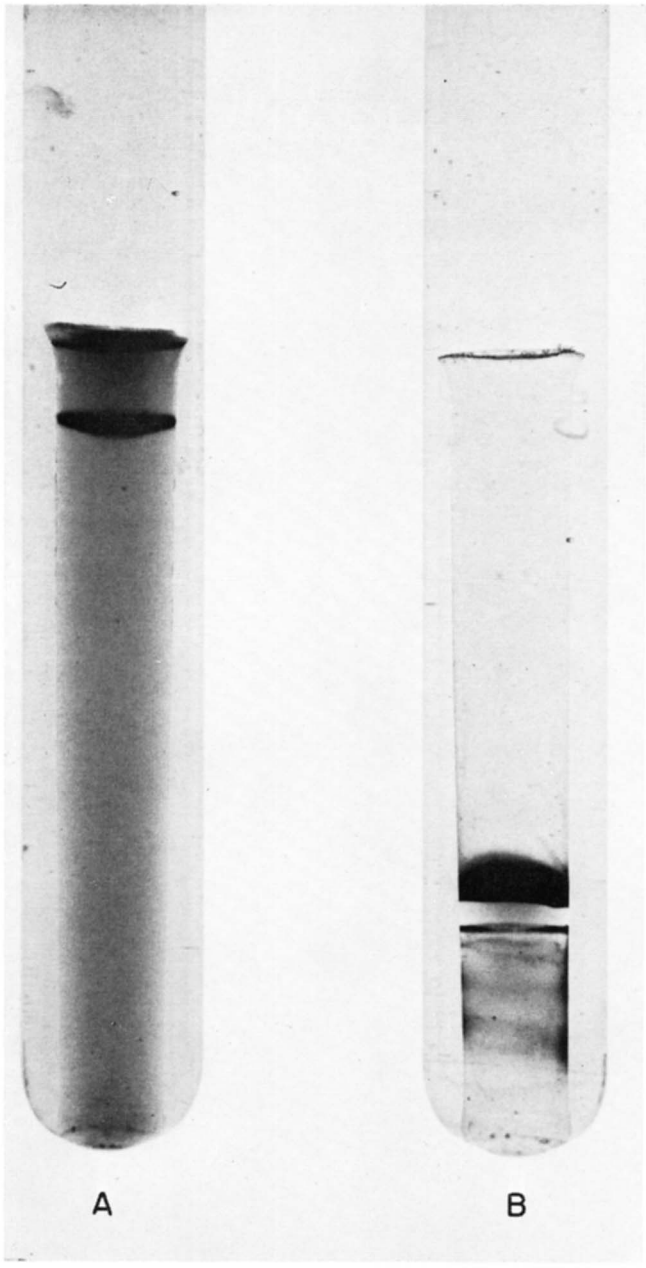

FIG. 4. Polyacrylamide disc electrophoresis of IB apoprotein on 5% *bis*-acrylamide gel. *A*, IB apoprotein; *B*, bovine serum albumin, fraction V. The gel and chamber buffers were 0.05 M glycine-Tris, pH 7.4. Electrophoresis was carried out at 250 volts until the marker dye reached the bottom of the gel column.

Morphological Characterization

Electron Microscopy. Numerous structures morphologically similar to osmiophilic inclusion bodies are found in IB fractions obtained from lung homogenates prepared with a Potter–Elvehjem homogenizer (Fig. 11). This is evident when one compares these structures with those seen in whole lung samples (Fig. 12). Few intact osmiophilic inclusion bodies were found in IB fractions from lungs homogenized in the Waring Blendor; however, as shown in Fig. 13, many "membranous" or extended lamellar structures can be seen. Additionally, a "fuzzy" or amorphous material is seemingly associated with the membranous forms. Fig. 14 is an electron micrograph of a negatively stained IB fraction (Waring Blendor). The structures bear a striking morphological resemblance to the phospholipid spherules or liposomes described by Bangham (43), Sessa and Weissman (44), and Huang (20). Again an amorphous material is seen to be associated with or enclosed by lamellar forms.

DISCUSSION

A surface-active material is present in the alveolar lining layer of mammalian lung, and presumably functions to reduce alveolar surface forces at minimal lung volumes, thereby maintaining alveolar integrity (1–6). The presence of surface-active forces in the lung has often been correlated with a high concentration of dipalmitoyl phosphatidylcholine (3), a phospholipid apparently unique to the mammalian lung (45).

These studies were initiated to develop a reasonably rapid procedure for the isolation of the surface-active fraction from lung in high yield. Our experience with previously published methods (5, 6, 46–48) demonstrated several difficulties. Isolation of surface-active material from saline lavages is time-consuming when large animals are used and is difficult to quantitate since one seldom recovers all of the wash fluid. Only that material accessible to the washing medium can be extracted. This is of particular importance in those pathologic states where airway obstruction or atelectasis are present (49, 50) and in fetal lungs before alveoli are mature. The homogenization techique overcomes these diffiiculties. Our data indicate that centrifugation on sucrose density gradients affords an excellent method for the isolation of surface-active material present in lung homogenates. The use of sucrose partially overcomes the objection applicable to isolation procedures using high salt concentrations with subsequent dialysis, namely denaturation. Morphology of subcellular components would be more likely preserved in isotonic salt and sucrose solutions than in highly concentrated salt solutions (51).

Our procedure yields a definable fraction, IB, from dog lung which meets the following major criteria proposed for pulmonary surfactant: (*a*) reduction of surface tension to extremely low values, and (*b*) localization in the alveolar lining. Pulmonary physiologists generally agree that lavage of the lung removes material at the alveolar lining. We obtained an IB fraction from saline lavages of dog lung that appears to be identical in chemical composition and surface activity with that obtained

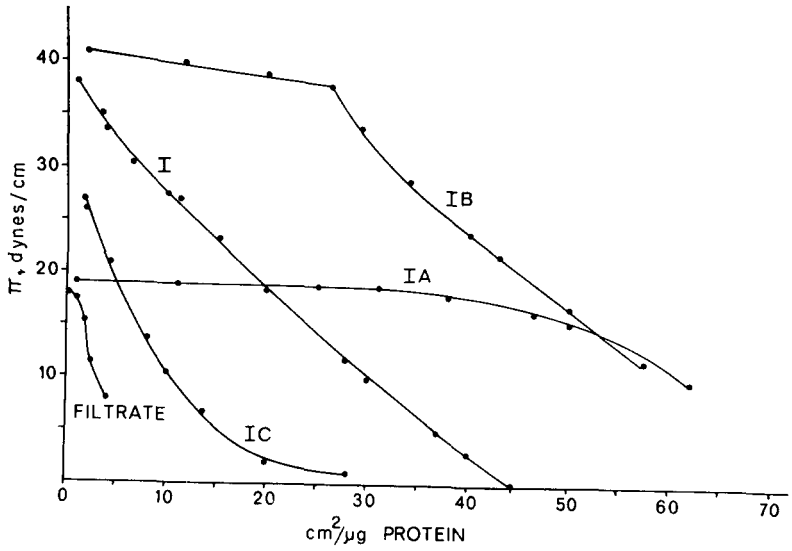

FIG. 5. Surface isotherms of fractions obtained in the isolation procedure. The subphase was 0.145 M NaCl. The surface area was 22.42 cm². Surface tension was measured with a 25 gauge platinum plate, 5 cm in perimeter. Determinations were performed at room temperature, 25°C (±2°C). The fractions, dispersed in water, were added to the surface of the subphase. See Fig. 1.

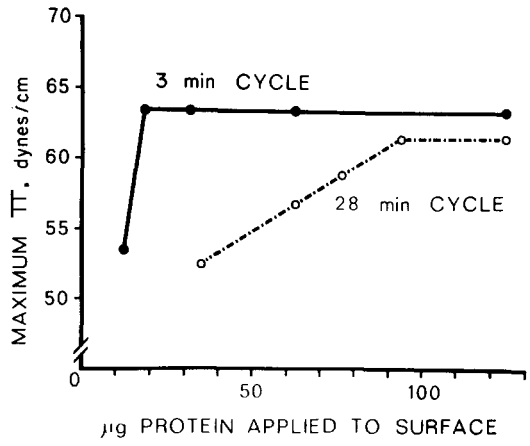

FIG. 6. Surface activity of IB fraction as determined by dynamic compression-expansion of surface area (17). The maximum trough area was 66.0 cm², which was then reduced to 13.2 cm². Cycling times of 3 and 28 min were used. The subphase was 0.145 M NaCl at room temperature. The maximum surface pressure is plotted vs. the quantity of IB, (μg of protein) applied to the surface at 100% area.

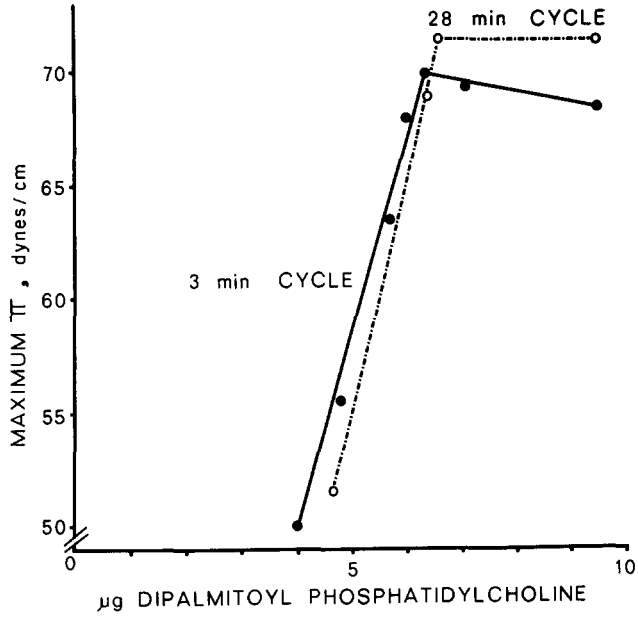

FIG. 7. Surface activity of dipalmitoyl phosphatidylcholine as determined by dynamic compression-expansion. Conditions as in Fig. 6. The maximum surface pressure was plotted vs. the μg dipalmitoyl phosphatidylcholine added to the surface at 100% area.

from lung homogenates.[1] No other fraction obtained in our isolation procedure was as surface active as IB.

The chemical composition of pulmonary surfactant has not previously been rigorously defined. Macklin (52) originally postulated that the alveolar lining layer was a glycoprotein (mucoid). Recently Brooks (53), has concluded that the air-facing side of alveolar cells is coated by a layer containing glycoprotein or polysaccharide molecules with acidic groups. The majority opinion has been that pulmonary surfactant is a lipoprotein (5) with a high content of dipalmitoyl phosphatidylcholine. Scarpelli, Clutario, and Taylor (54) have speculated that their surface-active fraction is a mixture of phospholipid, polysaccharide, and protein. Protein was thought possibly to be due to a contamination from blood. Blood or red blood cells subjected to our isolation procedure did not yield any significant surface active material.

Our IB preparations contained protein. Radioactively labeled alanine was incorporated into the IB, presumably into the protein component. An IB preparation,

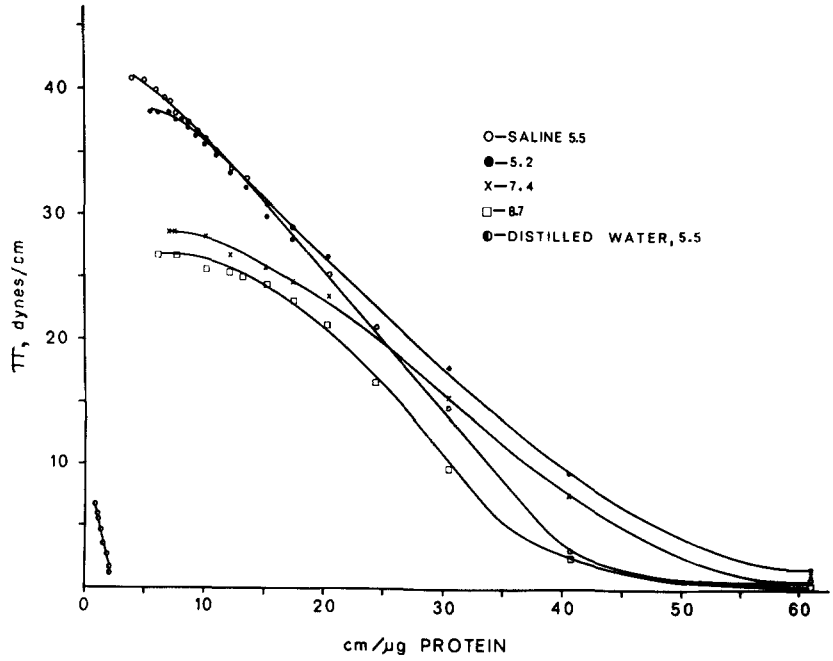

FIG. 8. Effect of subphase pH on surface isotherm of IB. The subphase consisted of 0.145 M NaCl–0.01 M phosphate buffer at the pH indicated except for saline which was unbuffered and distilled water. Other conditions as in Fig. 5.

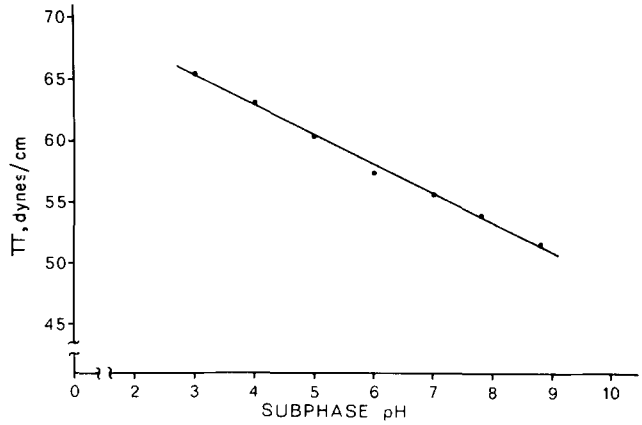

FIG. 9. Effect of subphase pH on surface activity of IB fraction as determined by dynamic compression-expansion of surface area. Subphase as in Fig. 8. The same amount of IB (56 μg of protein) was added at each pH indicated at 100% surface area. Conditions as in Fig. 6.

labeled with both lipid and protein precursors, was centrifuged on a continuous sucrose gradient and gave only one peak which contained both lipid and protein. This indicates a degree of homogeneity and also supports the close association of lipid and protein in IB. Gel filtration of similarly labeled IB yielded a material in the void volume with the same ratio of ^{3}H to ^{14}C as that of the material applied to the column. These results indicate that protein is an integral component of the IB particle and that it does not occur as an artifact due to nonspecific binding.

Anthrone-positive material and sialic acid remain with the protein after delipidation with alcohol–ether; this indicates that the protein is a glycoprotein. These studies, however, do not prove the presence of a covalent linkage between the carbohydrate and protein moieties.

Phospholipid is the major component of IB. Multiplication of the percentage lipid phosphorus by the standard factor of 25 suggests that 76% of the total lipid is phospholipid. Phosphatidylcholine is the major lipid species present. Table 2 indicates that dipalmitoyl phosphatidylcholine comprises, as a maximum, 54% of the phosphatidylcholine class (55). IA and IC, neither of which is as surface active as IB, presumably contain large amounts of dipalmitoyl phosphatidylcholine. The data in Table 3 indicate that the α- and β-residues of IB phosphatidylcholine undergo somewhat independent metabolic pathways as the specific activities of the residues at each position are different. This suggests the operation of a phospholipase–acyl transferase cycle in the lung as has been proposed by Lands (56) for the liver.

Structures morphologically resembling osmiophilic inclusion bodies have been found in our IB preparations. These structures are present in large numbers in IB fractions obtained from lung homogenates prepared with the Potter–Elvehjem homogenizer, but are less frequent in IB fractions derived from homogenates prepared with the Waring Blendor. The Waring Blendor is a more vigorous homogenizer and disrupts delicate biological structures. The lamellar structures (Fig. 13) from lung homogenized with the Waring Blendor could represent

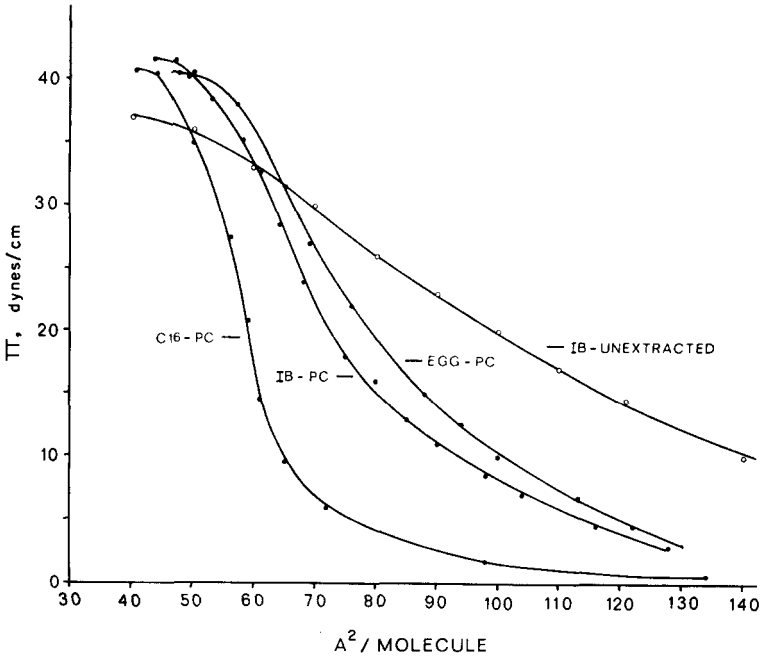

FIG. 10. Phosphatidylcholine surface isotherms. Conditions as in Fig. 5. The isotherm designated, IB-unextracted, is for an IB preparation expressed on the basis of the number of PC molecules present prior to lipid extraction. See text for details.

partially disrupted osmiophilic inclusion bodies. Figs. 11, 13, and 14 do indicate IB is not morphologically homogenous since more than one type of structure can be distinguished. An amorphous material is associated with lamellar forms. These morphological studies do not conclusively prove that the lamellar forms seen in IB are in fact osmiophilic bodies; however, the resemblance is striking.

Fig. 14, obtained from negatively stained material, indicates that IB fractions (from Waring Blendor) resemble lipid spherules or liposomes (20, 43, 44). This is consistent with the high lipid content of the fractions. Using a different isolation procedure, Klein and Margolis (46) isolated a surface-active fraction from lung with similar morphology, but no micrographs were shown.

Both the equilibrium (constant surface area) method (39, 40) and the dynamic compression-expansion procedure (17), Figs. 5 and 6, demonstrate that IB is the only significantly surface-active fraction obtained. Maximum surface pressures (minimum surface tensions) were achieved in the range predicted for pulmonary surfactant in vivo (3). Fraction I (0.75 M sucrose interfacial material) also reached a π_{max} of approximately 40 dynes cm^{-1} although much more material, in terms of protein, was required to reached this value of π than in the case of IB. Our isolation procedure demonstrates that I is composed of the following three fractions: IA, IB, and IC. IA and IC were not surface active.

The cyclic compression time is of great importance when attempting quantitation of surface-active material with the balance designed by Brown et al. (17). Approximately five times as much material must be applied to the surface of the subphase with a 28 min cycle than with the 3 min cycle to reach the arbitrary surface pressure of 60 dynes cm^{-1}. This effect of cycle time may be due to the properties of protein and (or) lipid components other than dipalmitoyl phosphatidylcholine.

Our experience has been that crude homogenates have occasionally been unable to produce a surface pressure above 52 dynes cm^{-1} (dynamic balance), yet yielded completely active IB fractions. Consequently, plots such as those in Fig. 6 are not, in our opinion, suitable for estimating the recovery of surface-active material throughout an isolation procedure. To estimate the amount of surface-active material in a crude homogenate, one would have to rule out the presence of inhibitory substances and other surface-active materials, namely, phosphatidylcholine. We, therefore, estimated the efficiency of our homogenization-centrifugation procedure by the approach exhibited in Table 4. The use of the "internal standard" indicates the recovery of IB from the crude homogenate is approximately 80% and suggests that the values concerning yields of IB shown in Table I might be increased appropriately.

Scarpelli, Gabbay, and Kochen (42) have demonstrated that subphase ions are necessary for the proper expression of pulmonary surfactant surface activity. The

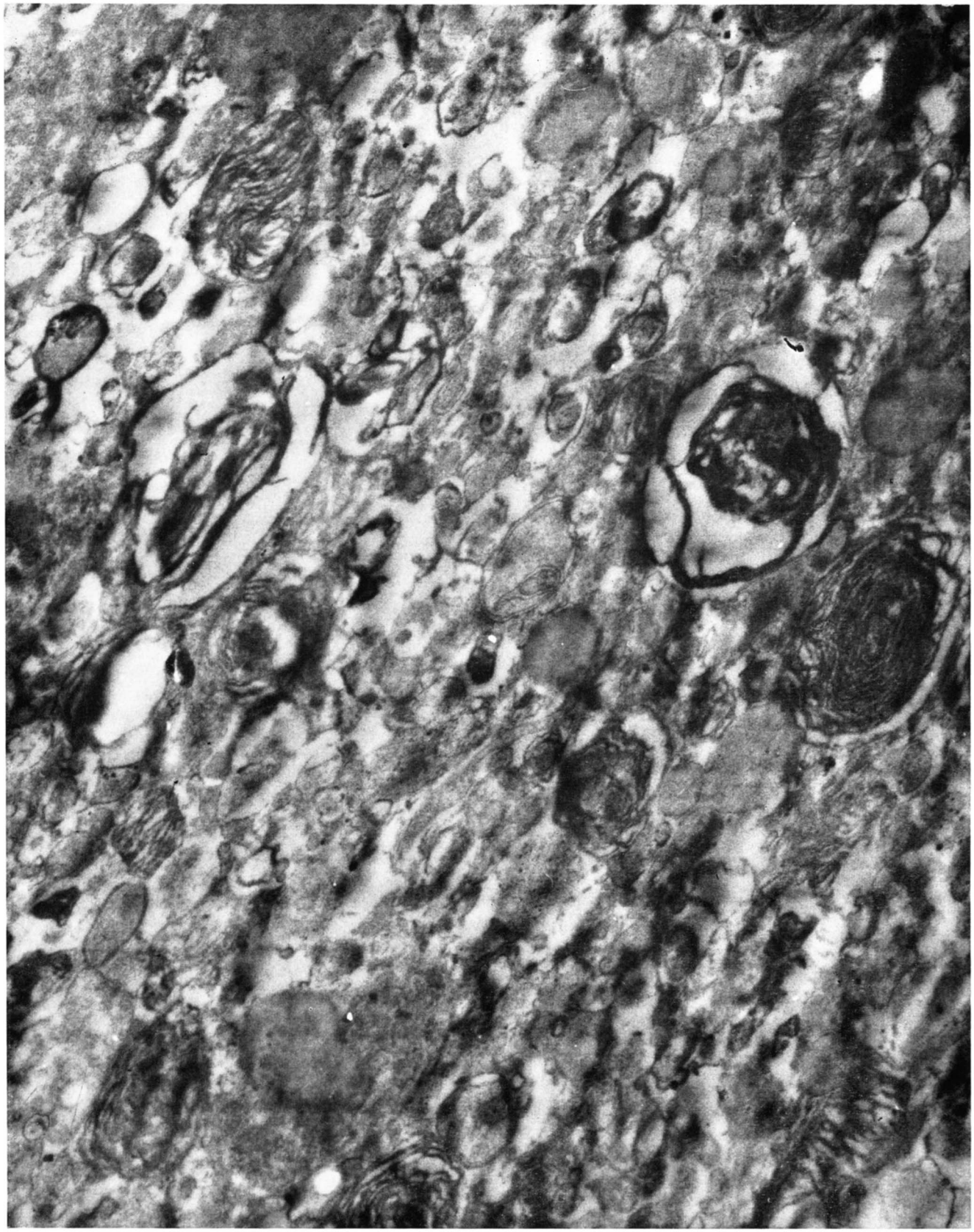

FIG. 11. IB fraction containing morphologically intact osmiophilic bodies from Potter–Elvehjem homogenization. See text for details of the fixation procedure. ×25,960.

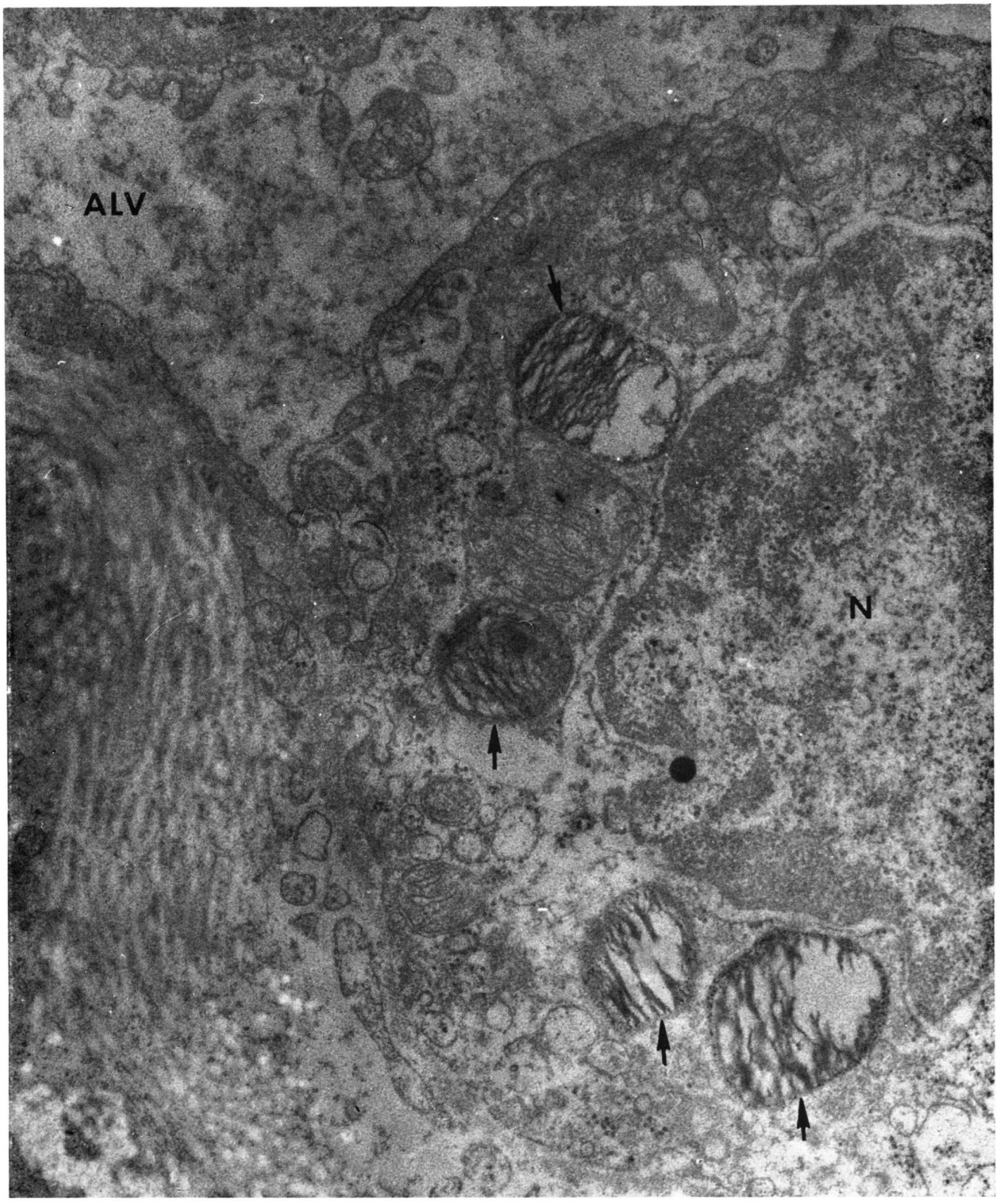

FIG. 12. Type II cell from fixed whole dog lung. Arrows denote osmiophilic bodies. *N*, nucleus of type II cell; *ALV*, alveolus. See text for details of fixation procedure. ×33,000.

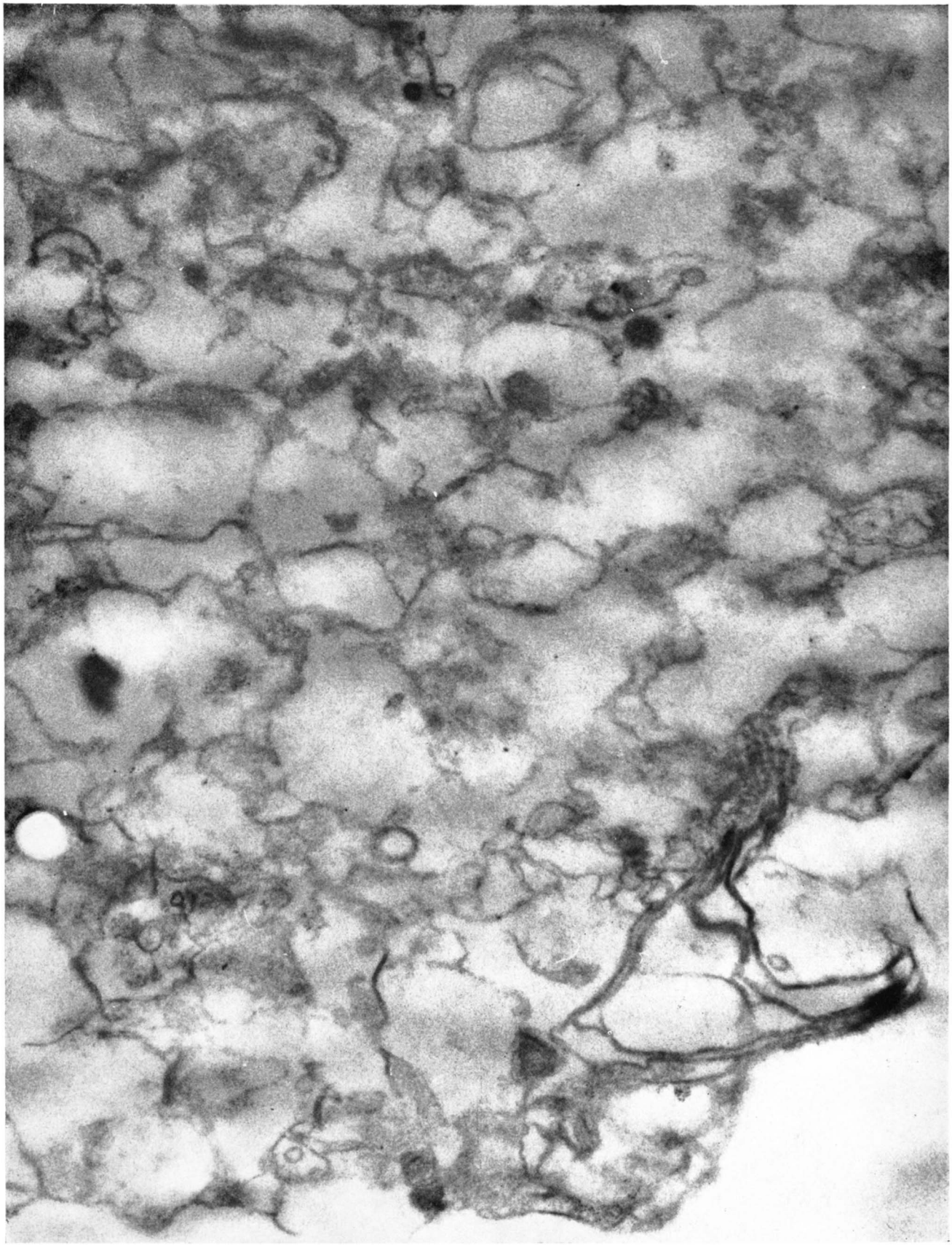

FIG. 13. IB fraction containing myelin forms from Waring Blendor homogenization. See text for details of fixation procedure. ×59,400.

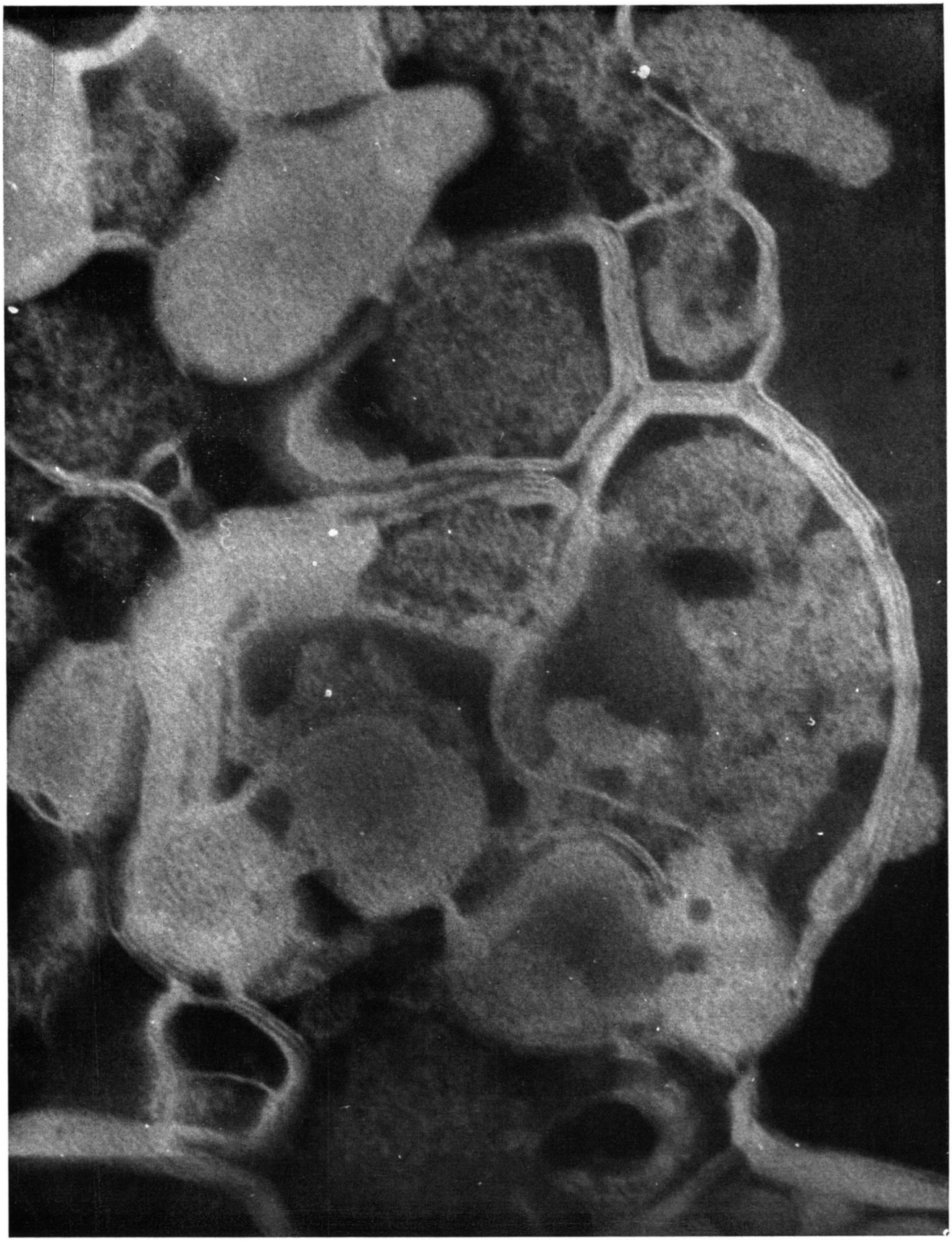

FIG. 14. Negatively stained IB from Waring Blendor homogenization. Negative staining was carried out with 1% potassium phosphotungstate, pH 6.8. ×216,000.

data shown in Figs. 8 and 9 confirm that finding. Furthermore, a definite relationship between subphase pH and maximum obtainable surface pressure was demonstrated. The more alkaline the subphase, the lower the π_{max}, and the more expanded the isotherm. Similar pH effects have been demonstrated for high density lipoproteins of blood by Camejo (57).

Two important observations are presented in Fig. 10. The surface isotherm of phosphatidylcholine isolated from IB resembles more closely that of egg phosphatidylcholine than that of dipalmitoyl phosphatidylcholine. The presence of olefinic unsaturation in phosphatidylcholine species renders the molecule more compressible (58). Secondly, the surface isotherm of an intact (not lipid extracted) IB expressed on the basis of its phosphatidylcholine content is not superimposable on the isotherm obtained for phosphatidylcholine extracted from IB. The isotherm for the intact IB is more expanded and compressible than that for phosphatidylcholine.

On the basis of viscometric studies, Blank, Goldstein, and Lee (59) postulated that a protein network must be present as a component of pulmonary surfactant. This network could function to impart the proper viscosity to pulmonary surfactant *in situ*. Our results concerning the effect of cycling time on IB and dipalmitoyl phosphatidylcholine (Figs. 6 and 7), the effect of pH on IB (Fig. 8), and the fact that phosphatidylcholine surface activity is modified when the intact IB fraction is applied to the surface (Fig. 10) suggest that the expression of pulmonary surface activity is dependent upon the interaction of phosphatidylcholine and other components of the fraction.

The high concentration of dipalmitoyl phosphatidylcholine in IB may be necessary for *in situ* surface activity. As pointed out recently (60), dipalmitoyl phosphatidylcholine is a solid at body temperature. The role of the other components of IB may be to keep dipalmitoyl phosphatidylcholine in the proper physical state in the alveolar lining. Phospholipids ordinarily do not exist free in body tissues but are bound by noncovalent linkages to protein. Similar interactions would be expected in the alveolar lining layer with a subsequent modification of surface properties of phospholipids.

Respiratory physiologists have used the dynamic compression-expansion of a surface film (17) because surface pressures corresponding to those predicted by Clements (3) for the alveolar surface *in situ* are attained. This procedure relates the change in surface pressure to the change in surface area. Clements' predictions (3) assume a change in alveolar surface forces due to a change in alveolar surface area as a function of alveolar volume. Recent reports (61, 62) suggest the change in lung volume is not primarily due to a change in alveolar volume and (or) surface area, although these studies are as yet not conclusive. Thus, the alveolus could be conceived of as a rather inelastic bag whose surface area remains relatively constant while the volume varies with inflation and deflation. This would alleviate the necessity for large cyclical variations in alveolar surface pressure. Pattle has previously postulated that alveolar surface pressure does not change greatly (5). Our results concerning the effect of pH on the surface isotherm of IB (Figs. 8 and 9) indicate that the surface pressures originally postulated by Clements (3) may not be reached *in situ* since, at pH 7.4, the maximum surface pressure reached was much lower than those he predicted.

The maximum surface pressure, 41 dynes cm^{-1}, reached by IB preparations at acid pH when assayed by the static equilibrium procedure (Fig. 4) was much lower than that attained by the dynamic compression-expansion method, 62 dynes cm^{-1} (Fig. 6). This is to be expected on the basis of previous studies in which films of dipalmitoyl phosphatidylcholine and relatively crude surface-active fractions from lung were utilized (63, 64). The static equilibrium surface pressure for these substances is 42–48 dynes cm^{-1}. To maintain a surface pressure greater than this requires that the surface be reduced continuously, and, if compression is halted at pressures above 48 dynes cm^{-1}, the pressure then spontaneously falls to the equilibrium maximum surface pressure, that is, the true point of monolayer collapse.

The fact that marked hysteresis occurs during cyclic compression of lung surface-active fractions indicates that during compression beyond the equilibrium point of monolayer collapse (42–48 dynes cm^{-1}) film components are displaced from the surface. It has been postulated that these displaced components do not return to the surface (63). Mendenhall (65), utilizing a balance designed to permit rapid compression and expansion of surface films at a rate approximating normal breathing, found that maximum surface pressures could not be maintained unless surfactants were continually added to the surface.

Respiratory physiologists have considered the behavior of lung surface-active fractions on cyclical surface balances to be analogous to the behavior of pulmonary surfactant *in situ*. This may be unwarranted. It is highly unlikely that a true liquid subphase exists in the alveolar lining.

We postulate that the alveolar lining may resemble a structured gel. This is a somewhat different view from that in which the surfactant film is interposed between air and a liquid subphase. The lining layer would have a configuration due to lipid-protein-carbohydrate-ion interactions such that the fatty acid chains of phosphatidylcholine (and other lipids) would be oriented toward the air phase. The protein network of the alveolar lining (59) would be attached to the plasma membranes of type I and type II alveolar cells through interactions with car-

bohydrate residues. Carbohydrate could be considered as glycocalyx (66), an interpretation certainly consistent with the results of Brooks (53) who demonstrated ruthenium red stainable material in the alveolar lining. Phosphatidylcholine (and other lipids) would be interspersed in the protein network because of noncovalent bonding. Between the protein network and the air phase, the lipid components could possibly be in the liquid crystalline state. Water, an integral component of the structure, would be less concentrated in the ultimate molecular layer at the air interface. Thermodynamically, this should give rise to an increased surface pressure in the alveolar lining relative to other surrounding tissue components. Additionally, if cyclic compression-expansion of the alveolar surface does occur, the juxtaposition of fatty acid residues would force water out of the ultimate molecular layer either into the air or into the underlying strata of the structure, thus giving rise to variations in surface pressure. The ability of gels and certain liquid crystalline states to tolerate wide variations in water content is well known (67).

As Fergason and Brown have pointed out (67), if a smectic liquid crystalline film contacts a region which lowers its surface energy, it will tend to expand its area of contact, thereby engulfing other materials that tend to lower its surface energy. This would obviously be compatible with the postulated clearance mechanism which may be a physiological function of the alveolar lining layer (68, 69).

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