

# Quantification of surfactant phospholipids in the dog lung

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**Abstract** We quantified total phospholipid (PL), total and disaturated phosphatidylcholine (PC and DSPC), phosphatidylglycerol (PG), and total protein in alveolar washings and lung tissue in 22 dog lungs. Quantitative recovery of alveolar material and assessment of its possible contamination by blood lipids were important determinants of methodology. To remove blood, the vessels of half the lungs were perfused with a fluorocarbon emulsion before lavage. The volume of blood removed by perfusion and the quantity and fatty acid patterns of its whole blood and plasma PL and PC were determined. Washings of unperfused lungs contained means of 21% more PL and 24% more PC than those of perfused lungs. Although this excess could be accounted for by the PL and PC in pulmonary blood, the hemoglobin and total protein content of washings and their PC fatty acid patterns indicated that blood lipids were not a major source of the excess lipid in washings of unperfused lungs. Using more recent morphometric estimates rather than the indirect ones previously used by others, the quantity of alveolar DSPC (1 mg/g lung) is calculated to be 1.8 times the amount necessary to form a packed monolayer on the internal surface of the lung at functional residual capacity.—Ryan, S. F., S. A. Hashim, G. Cernansky, C. R. Barrett, Jr., A. L. L. Bell, Jr., and D. F. Liau. Quantification of surfactant phospholipids in the dog lung. *J. Lipid Res.* 1980. 21: 1004–1014.

**Supplementary key words** pulmonary surfactant • disaturated phosphatidylcholine • phosphatidylglycerol • lung internal surface area

Many studies have established that the major surface-active lipids of the lung surfactant system are dipalmitoyl phosphatidylcholine and phosphatidylglycerol (PG) (1–4). These lipids are believed to be synthesized and stored by the Type II alveolar epithelial cells and secreted into the alveoli as part of a complex mixture of lipids and proteins (5–8).

Surfactant lipids from the alveoli and from the cells can be separately isolated, the former by lavaging alveoli and the latter by analyzing lung tissue post-lavage (9) or by studying purified Type II cells (10).

Most previous studies of surfactant isolated by these methods have been qualitative and few attempts have been made to quantify the key surfactant lipids or to relate the quantity of the alveolar fraction to the internal surface area of the lung (11). Such data, together with available electron microscopic evidence (12), should allow deductions about the distribution of surfactant within individual alveoli, information that is critical to a clear understanding of surfactant function.

Contamination by pulmonary blood presents a problem in quantifying lipids and proteins in lung tissue (13) and there is evidence that it may occur in alveolar washings as well (14, 15). Quantitative aspects of this contamination have not been studied.

This study presents results of quantitative analysis of lipids and proteins from alveolar washings and post-lavage lung tissue in the dog, evaluates the contribution of blood lipids and proteins to those in the washings, and relates the quantity of disaturated phosphatidylcholine (DSPC) to the internal surface area of the lung.

## MATERIALS AND METHODS

These studies were performed in healthy adult male mongrel dogs weighing 11.6 to 23.9 (mean 20.25) kg. In six dogs, one lung was lavaged without first perfusing the blood vessels. In six other dogs, one lung was lavaged following perfusion of the blood vessels with a stabilized fluorocarbon emulsion to remove blood. In a third group of five dogs, referred to as two-lung experiments, both lungs were lavaged,

Abbreviations: PL, phospholipid; PC, phosphatidylcholine; PG, phosphatidylglycerol; DSPC, disaturated phosphatidylcholine; TLC, total lung capacity; FRC, functional residual capacity; ISA, internal surface area.

one without perfusion and one after perfusion of the vessels with fluorocarbon emulsion. In each dog, total phospholipids (PL), phosphatidylcholine (PC), PG, and total protein were quantified in washings and (except PG) in post-lavage lung tissue. DSPC was quantified in washings from six of the unperfused lungs. PL, PC, and total protein were quantified in whole venous blood of seven of these animals and in the plasma of eight.

The blood phospholipids removed from the lung by perfusion were quantified by multiplying the concentration of phospholipids in peripheral blood by the blood volume in the collected perfusate. This volume was determined by measuring the hemoglobin in the perfusate collected from the pulmonary veins and dividing by the peripheral hematocrit.

### Isolation procedures

Following anesthesia with intravenous pentobarbital, the thorax was opened widely, the trachea was clamped, and the heart and lungs were removed en bloc. The lungs were then carefully separated and weighed.

### Perfusion

The lung to be perfused prior to lavage was degassed in a vacuum and the bronchus, pulmonary artery, and pulmonary veins were cannulated. The pulmonary artery was then perfused at a pressure of 30 cm H<sub>2</sub>O with an emulsion of the fluorocarbon FC-80, stabilized with the non-ionic detergent F-68. The emulsion was prepared by dissolving 4.8 g F-68 (BASF-Wyandotte Corp., Wyandotte, MI) per 100 ml Tris buffer, adding 40.0 g of FC-80 (Minnesota Mining and Mfg. Co., St. Paul, MN) and sonicating for 3 min in 25-ml volumes. The emulsion was then filtered through a 2-micron millipore filter.

Perfusion was begun with the lung degassed and during perfusion it was inflated with air in a stepwise fashion using a Hamilton syringe until fully inflated. One and one-half liters of emulsion were used and the venous effluent of blood and perfusate were collected for hemoglobin measurement. Perfusion by this method consistently produced uniform blanching of the entire lung. Hemoglobin concentration in the collected perfusate and the cell-free alveolar washings were determined spectrophotometrically following conversion to cyanmethemoglobin with Drabkin's solution.

### Lavage

After degassing, the lung was lavaged six times via the cannulated bronchus, each time with 10 ml/g lung of cold 0.005 M Tris-HCl buffer (Tris buffer)

containing 0.15 M sodium chloride and 0.003 M each of calcium chloride and magnesium chloride (pH 7.35; 300 milliosmoles/liter) using a 1500 ml Hamilton syringe. During each of the six successive lavages, the Tris buffer was alternately washed in and out of the lung six times. Any experiment in which less than 90% of the total washing was recovered, was discarded. After vascular perfusion lungs were again degassed and lavaged in the same way. In the two-lung experiments, the perfused and unperfused lungs were lavaged simultaneously. In all experiments, the six washings per lung were kept separate and analyzed individually. Final lung weight was obtained by carefully trimming away all extrapleural vessels and bronchi and subtracting their weight from the pre-lavage lung weight.

### Lipid studies

The six washings from each lung were immediately centrifuged at 160 g at 4°C for 10 min to remove cells. The sediments were resuspended in Tris buffer, centrifuged again, and the supernatants combined. The phospholipid content of the second sediment was determined before and after passing it through a 5-micron millipore filter. The individual supernatants were then lyophilized, weighed, and stored in a freezer at -20°C under nitrogen.

The lung tissue was finely minced, ground, and lyophilized in Tris buffer. The dry tissue was further ground, pulverized, weighed, and also stored as powder in the freezer at -20°C until further analysis.

Phospholipid standards, DL- $\alpha$ -phosphatidylcholine (dipalmitoyl), L- $\alpha$ -phosphatidyl-DL-glycerol, DL- $\alpha$ -phosphatidylethanolamine (dipalmitoyl), and L-3-phosphatidyl-N,N-dimethylethanolamine (dipalmitoyl) were purchased from Sigma Chemical Co., St. Louis, MO. Fatty acid methyl esters were supplied by Nu Chek Prep, Elysian, MN. All other chemicals were reagent grade. The purity of the lipid standards was checked by thin-layer chromatography.

### Extraction of Lipids

Total lipids of lung washings, lung tissue, and plasma were extracted from aliquots of the lyophilized powders by the method of Folch, Lees, and Sloane Stanley (16) with several modifications. To assure complete removal of all lipids, we increased the volume of chloroform-methanol 2:1 (v/v) tenfold and added no salts to the upper and lower phase solvents (because the lavage Tris buffer already contained salts). The washings were extracted twice and the lung tissues three times. The whole blood was extracted similarly without increasing the chloroform-methanol 2:1 volume except that it was added

dropwise first to methanol with chloroform added later to prevent congealing. The washed lipid extracts were concentrated to a small volume on a flash evaporator, transferred into screw-top tubes, made to a volume of 20 ml with chloroform-methanol 2:1 and stored at  $-20^{\circ}\text{C}$ . All subsequent lipid analyses were done from these stock solutions.

### Quantification of phospholipid phosphorus

Phospholipid phosphorus was determined on each of the six washings, tissues, whole blood, and plasma from their stock solutions according to the modified method of Beveridge and Johnson (17). We assumed that phospholipids contained an average of 4% phosphorus.

### Quantification of phosphatidylcholine (PC)

Aliquots of stock solutions of the six washings, tissues, and whole blood were spotted on chromatoplates ( $20 \times 20$  cm, pre-coated with a  $250\text{-}\mu\text{m}$  layer of silica gel 60-Kieselgel, E. Merck). At least two spots of the reference standard PC were also spotted on each plate. Both the standard and the unknown PC contained similar concentrations. The plates were developed in tanks lined with filter paper and saturated with the solvent chloroform-methanol-conc. ammonium hydroxide 85:30:6(v/v/v),  $1\frac{1}{2}$  hr before use. No attempt was made to separate all the other phospholipids. After air-drying, the plates were sprayed to complete saturation with Rhodamine 6G 0.05% in 50% ethanol and dried again. A Model 111 Turner fluorometer equipped with a Camag-Turner Model 2 automatic scanner was used for sensing the fluorescence produced by the PC spots. The ultraviolet light source was a 4-watt UV lamp with a maximum wave length emission at 360 nm (Turner #110-850). Two primary filters (Turner #110-811 and #110-822) and two secondary filters (Turner #110-824 and #110-823B) were employed. The emitted fluorescence was detected as peak areas on a Corning Model 841 chart recorder at a chart speed of 4 cm per minute. Areas under the peaks were measured with a compensating polar planimeter. Quantification of PC was achieved by comparing the areas of the unknown with those of the standard on the same plate. Four determinations were carried out on each unknown, two on each of two plates.

### Quantification of phosphatidylglycerol (PG)

PG, the second most abundant phospholipid in lung washings, was also quantified by fluorometry. The same chromatoplates were used for spotting as for PC. We were able to separate PG from other phos-

pholipids by one-dimensional thin-layer chromatography in chloroform-methanol-distilled water 65:25:4 (v/v/v) (18). Both the standard PG and the unknown PG migrated above PC, but below the standard phosphatidyl-*N,N*-dimethylethanolamine and phosphatidylethanolamine. The chromatoplates after developing were treated exactly the same way as described for PC, and the method for quantification was identical.

### Fatty acid composition of PC

Fatty acid composition of PC was determined on all six washings, lung tissue, plasma, and whole blood. PC was separated from other phospholipids by one-dimensional thin-layer chromatography on Analtech plates (silica gel G,  $250\text{ }\mu\text{m}$ ). The plates were developed in the same solvent used for PC quantification. PC spots were visualized in iodine, marked, and scraped into tubes. After methylation with sodium methoxide, the samples were cooled, neutralized with 5% HCl, washed, and extracted overnight in heptane. The upper layer was removed and dried down to  $20\text{-}\mu\text{l}$  aliquots. Gas-liquid chromatography was done in a Hewlett-Packard research chromatograph using a  $6' \times \frac{3}{16}"$  column packed with 12% DEGS on Anakrom ABS 70/80 mesh. Column temperature of  $180^{\circ}\text{C}$  was maintained. Fatty acid methyl esters were determined by comparison of retention time to known standards. Peak areas were calculated by multiplying peak height  $\times$  width at half-height.

### Isolation and quantification of disaturated phosphatidylcholine (DSPC)

Aliquots of stock solutions were reacted with osmium tetroxide in carbon tetrachloride according to the method of Mason, Nellenbogen, and Clements (19) except that the isolation of DSPC from the reaction mixture was accomplished by thin-layer chromatography rather than by a column. After evaporation of the mixture under  $\text{N}_2$ , the residue was redissolved in chloroform-methanol 2:1 and spotted quantitatively on thin-layer chromatography plates (Kieselgel-250 microns). On the same plate, a known amount of the unreacted stock solution and a DPPC standard were also applied. The plates were developed in chloroform-methanol-distilled water 65:25:4 (v/v/v). After air-drying the plates were sprayed to complete saturation with Rhodamine 6G 0.05% in 50% ethanol and dried again. The scanning and quantification were done as described above. The percentage of DSPC was obtained by comparing the quantity of DSPC of the reacted stock solution with that of the PC of the unreacted stock solution spotted on the same plate.

## Determination of protein

Protein content of cell-free washing and of tissue powder digested with 1N sodium hydroxide was determined by the method of Lowry et al. (20) with the addition of 1% sodium dodecyl sulfate (SDS) to the reagent. The addition of SDS was found necessary to prevent the turbidity caused by lipids.

## Statistics

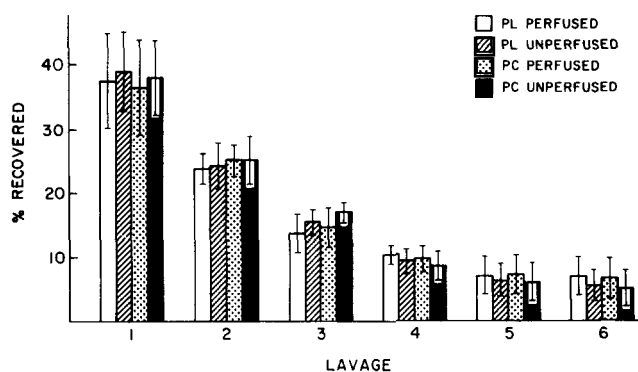
Differences between mean quantities of phospholipids and protein in washings and tissue in perfused and unperfused lungs were evaluated using a conventional unpaired *t* test.

## RESULTS

**Fig. 1** shows the mean recovery of total PL and PC in each of the six serial washings of perfused and unperfused lungs expressed as the percent of the total recovery. An average of 88% of both total PL and PC was recovered in the first four lavages. The quantity of PC in the residual washing in the lung after the sixth lavage was calculated to average less than 2% of the total, assuming equal concentration in recovered and in residual washing (post-lavage lung weight minus pre-lavage lung weight  $\times$  concentration of PC in the sixth washing). By comparing total phospholipid content of the washed precipitate before and after millipore filtration, PC loss during preliminary centrifugation of the washings was calculated to average less than 10%. These amounts were neglected in subsequent calculations of total lavage phospholipids.

Electron microscopic examination of the unperfused lungs postlavage showed focal edema of connective tissue around larger vessels and bronchioles and occasional swelling of Type I cells. In the lungs lavaged after perfusion with FC-80 emulsion, striking interstitial edema and destruction of Type I cells were widespread (**Fig. 2**). In both the perfused and unperfused lungs, Type II cells were intact with well preserved lamellar bodies (**Fig. 3**).

**Table 1** presents quantitative data for phospholipids and total protein in washings and post-lavage tissue in the two groups of lungs, one group lavaged without perfusion of the blood vessels and one group lavaged after perfusion of the blood vessels. The washings of the unperfused lungs contained more total PL (21%), PC (24%), and PG (25%) than those of the perfused lungs. The differences in PL and PC were highly significant. In the two-lung experiments wherein both lungs of each of five dogs were lavaged (one with and one without perfusion), the sum of the PC content



**Fig. 1.** Recovery of total phospholipid (PL) and phosphatidylcholine (PC) in each of six serial washings of the same lung. Bars represent the mean percent of total recovery for 11 perfused and 11 unperfused lungs. Bracketed lines represent  $\pm$  one S.D.

of perfusate and washings of the perfused lung was comparable to that of the washings from the unperfused lung. DSPC made up a mean of  $51 \pm 2.7\%$  of the total PC in washings of unperfused lungs.

Differences in tissue PL and PC between perfused and unperfused lungs were not significant, because blood lipid constituted only about 4% of total lung tissue lipid and its removal did not cause significant reduction in the total.

The post-lavage tissue of the unperfused lung contained a mean of 52 mg/g more total protein than that of the perfused lungs (Table 1). No more than a trace of hemoglobin was found in the cell-free washings of both perfused and unperfused lungs.

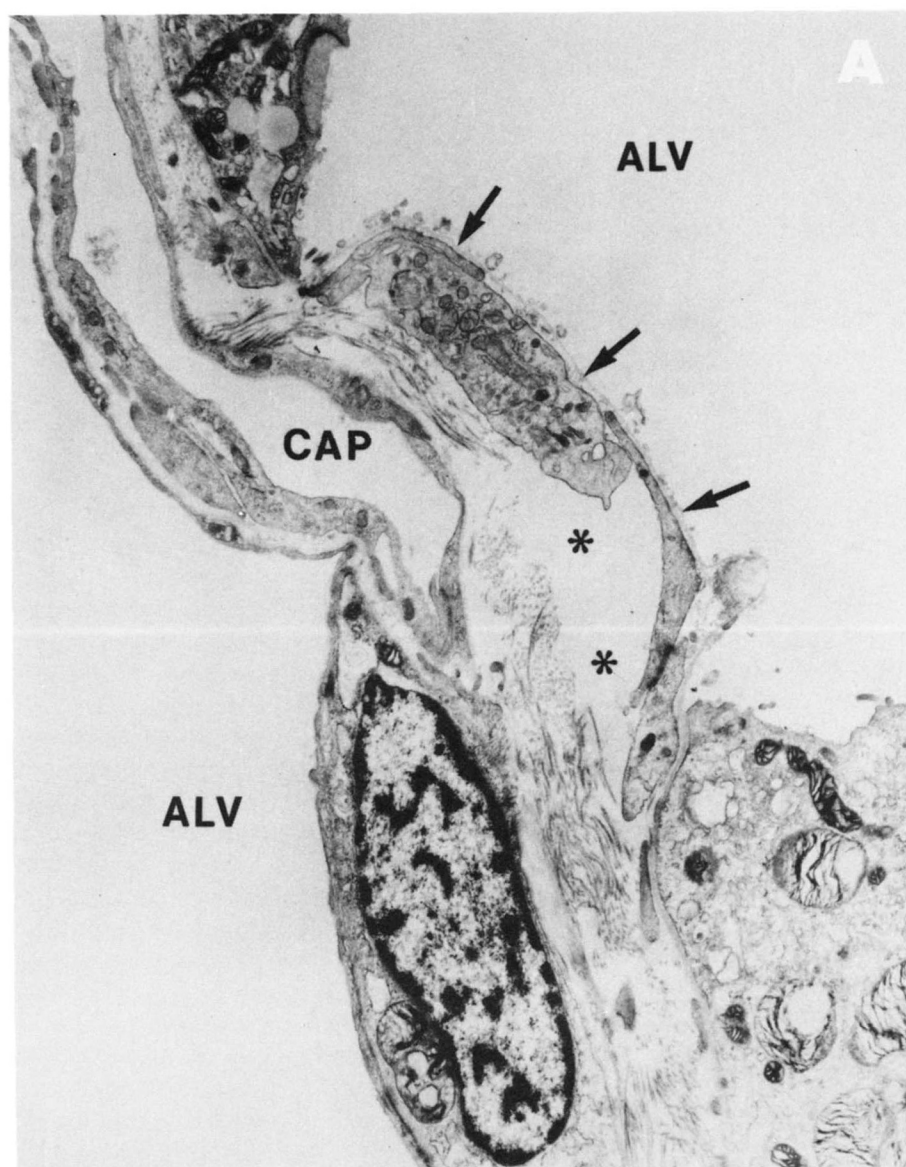
**Table 2** presents the composition of fatty acids of PC from washings, whole blood, and serum. Eighty percent of the fatty acids of the PC of the washings was saturated and 68% was palmitate. Although fatty acid patterns of whole blood and plasma were quite different from those of washings, no significant difference in fatty acid pattern was detected between washings from perfused and nonperfused lungs. When as little as 5 ml of whole blood was added to washings, the fatty acid pattern was quite different from that of blood-free washings (Table 2).

## DISCUSSION

Accurate quantitation of alveolar surfactant is important to a clear understanding of its function, for it is still uncertain whether sufficient material is present in the alveoli both to form the deep pools seen in alveolar recesses by electron microscopy and to provide the remainder of the alveolar surface with at least a monolayer (21).

The qualitative characteristics of the phospholipids in our crude washings were similar to those reported





**Fig. 2A.** Electron micrograph of transversely sectioned interalveolar septum from lung following vascular perfusion and alveolar lavage. Endothelium of capillaries (CAP) is intact and there is focal edema of the septal connective tissue (asterisks). An intact Type II cell lies adjacent to a zone in which Type I cells are disrupted and the basal lamina (arrows) is denuded. (Lead citrate, uranyl acetate  $\times 2900$ .)

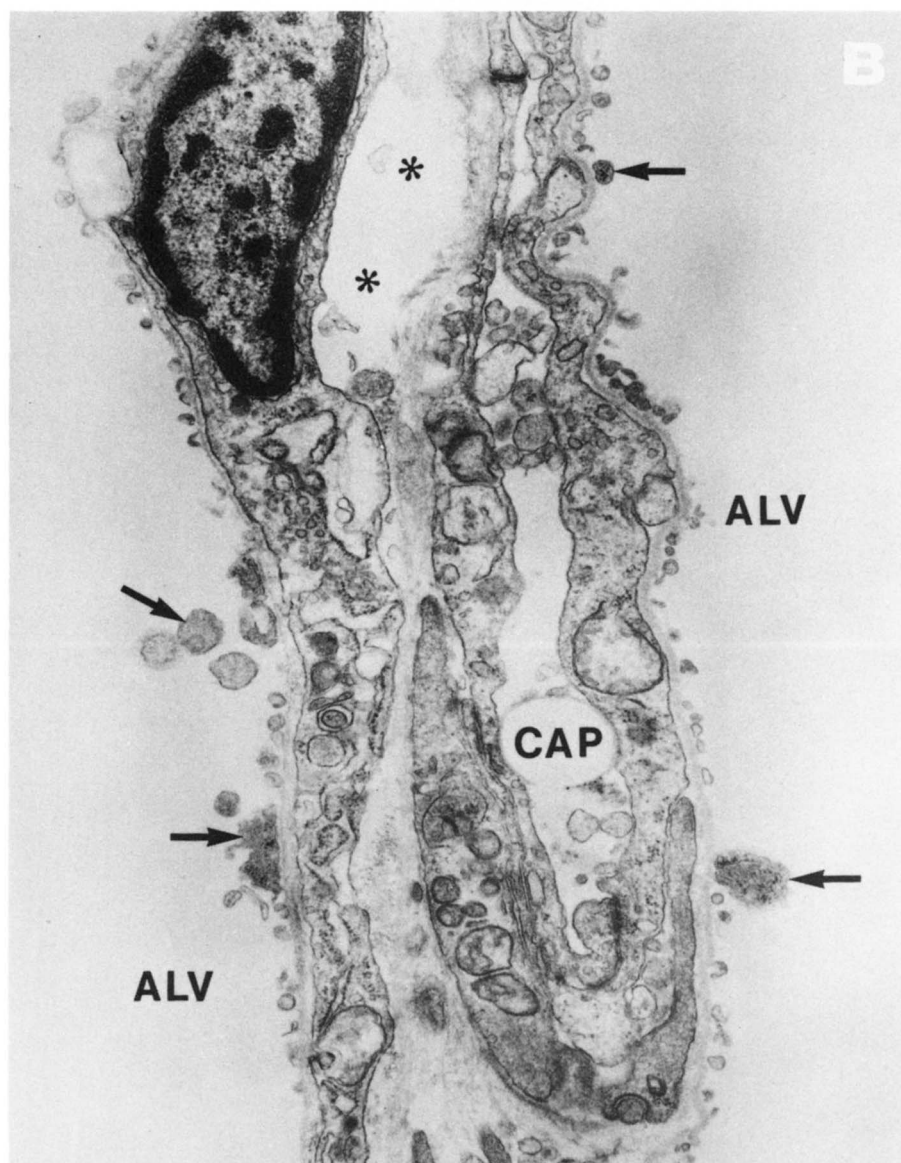
by others (22, 23). In both perfused and unperfused lungs, PC composes 75–78% of the total PL. About 80% of the fatty acids of the PC is saturated and 68% of them is palmitate. Of the total PC, 51% is DSPC. PG composes about 9% of the total phospholipids.

Aside from the obvious importance of rigorous analytical procedures, we considered three prerequisites to be critical to accurate quantitation of surface-active lipids per unit internal surface area of lung: 1) quantitative recovery of alveolar material;

2) possible contamination of alveolar washings by lipids from the pulmonary capillary blood; and 3) accurate estimation of the internal surface area of the lung.

#### Quantitative recovery of alveolar material

We applied the compartment concept proposed by Pawlowski et al (9) in which lipids recovered by alveolar lavage are considered to be those already secreted into the alveoli by the Type II cells and lipids extracted from lung tissue following lavage to include



**Fig. 2B.** Electron micrograph of transversely sectioned interalveolar septum from lung following vascular perfusion and alveolar lavage. Endothelium of capillaries (CAP) is intact and there is focal edema of the septal connective tissue (asterisks). Type I cells are extensively disrupted and their rounded fragments (arrows) lie on the basal lamina or free in the alveolus. (Lead citrate, uranyl acetate  $\times 7500$ .)

those synthesized, but not yet secreted by the Type II cells, plus lipids of the other lung cells.

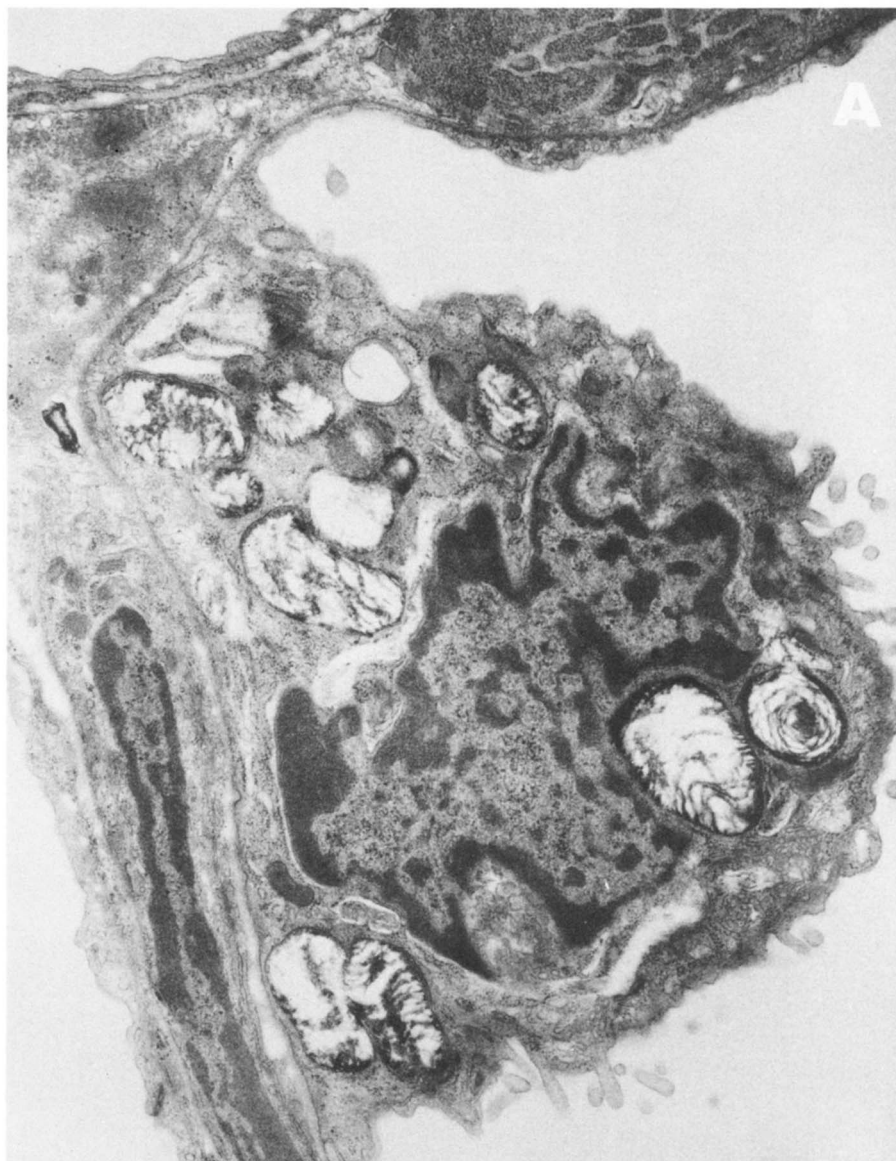
We used very large volumes of washings and repeated lavage in order to recover a high proportion of alveolar lipids. In spite of this vigorous lavage, electron microscopic examination of the lung following lavage showed no injury to the Type II cells. Washout curves (Fig. 1) suggest that a large proportion of alveolar lipid was removed by this method. Ice-cold lavage was used because it is believed to minimize the release of surfactant lipids from the cells into

the alveoli caused by distention of the lung, as demonstrated by Faridy (24).

#### **Possible contamination of alveolar washings by lipids from the pulmonary capillary blood**

Several investigators have suggested that constituents of the pulmonary capillary blood contaminate alveolar washings (14, 15, 25). Albumin, presumably from the blood, is found in crude alveolar washings (14) and in small amounts even in the surfactant material isolated by density gradient centrifugation (25).



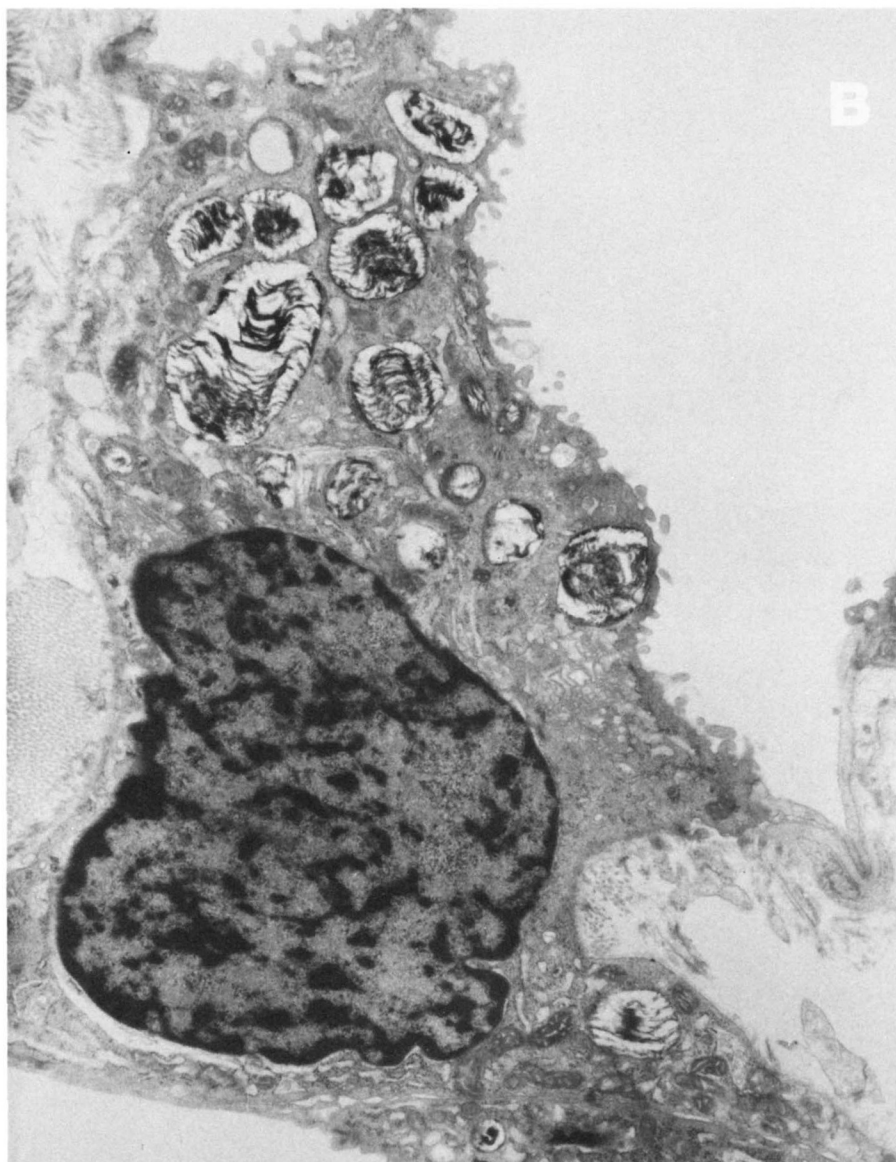


**Fig. 3A.** Electron micrograph of representative Type II epithelial cells in non-perfused lung following alveolar lavage. The cells and their lamellar bodies are intact. (Lead citrate, uranyl acetate  $\times 6100$ .)

Hurst, Kilburn, and Lynn (15) found that FC-80 perfusion of the pulmonary artery prior to lavage greatly reduced the amount of lipid and protein recovered in the washings. We used a stabilized emulsion of the fluorocarbon FC-80 because it approximates the viscosity and osmotic-oncotic properties of whole blood and has been used in vivo as a blood substitute (26).

The data clearly show that perfusion of the lung with the FC-80 emulsion reduces the amount of PL and PC recovered in washings by 20–25%. Although data from the two-lung experiments indicate that sufficient quantities of PC are present in the blood of the

unperfused lung to account for the excess of PC in its washings, this excess cannot be entirely attributed to the presence of blood lipids in the washings. In fact, the evidence strongly suggests that very little of the blood lipids appears in the washings. 1) The PL from greater than 10 ml of whole blood or 20 ml of plasma would be required to account for the difference in lavage PL from perfused versus unperfused lung. The finding of no more than a trace of hemoglobin in any washing indicates that significant numbers of blood cells do not leak into the washings. 2) If plasma lipids were the principal component of the excess lipid in unperfused over perfused washings,



**Fig. 3B.** Electron micrograph of representative Type II epithelial cells in perfused lung following alveolar lavage. The cells and their lamellar bodies are intact. (Lead citrate, uranyl acetate  $\times 3300$ .)

a greater quantity of protein would be expected in the washings from unperfused lung. This is because the plasma lipids exist almost entirely as lipoproteins, and their proteins as well as their lipid moieties would be expected to appear in the washings. In fact, the opposite is true. Much more protein is found in the washings of perfused than of unperfused lungs. Furthermore, the amount of protein in the washings from unperfused lung would account for only 1 ml of blood, whereas the excess protein in the tissue of the unperfused lung would account for almost 20 ml of blood left behind after lavage. These data differ from those of Hurst and Kilburn (15) who found almost no pro-

tein in washings from the rabbit lung perfused with fluorocarbon, and much more (about 90 times more) in washings from unperfused lungs. The discrepancy in our findings may be due to differences in method of perfusion. These investigators used FC-80 alone to perfuse the pulmonary blood vessels rather than the stabilized emulsion which we employed. Our attempts to perfuse with pure FC-80 were not successful. The source of the excess protein in the washings from perfused lung is uncertain, but it may be the extensively disrupted Type I cells, demonstrated by electron microscopy. 3) Fatty acid composition of PC from washings of perfused and unperfused lungs are virtu-



TABLE 1. Phospholipids and proteins in washings and post-lavage tissue of perfused and unperfused lungs<sup>a</sup>

	Washings				Post-Lavage Lung Tissue			
	PL	PC	PG	Total Protein	PL	PC	Total Protein	Mean Final Lung Weight
	(mg/g lung)				(mg/g lung)			
Unperfused (n = 11)								
Mean	2.72	2.12	0.24	2.41 <sup>b</sup>	16.12	7.11	99.15	Lav. lung 68.6 g
S.D.	± 0.68	± 0.46	± 0.09	± 0.31	± 2.62	± 1.68	± 1.9	Right & left 142.5 g
Perfused (n = 11)								
Mean	2.16	1.61	0.18	7.60 <sup>b</sup>	15.40	7.30	47.4	Lav. lung 64.2 g
S.D.	± 0.22	± 0.19	± 0.08	± 3.13	± 2.65	± 1.25	± 8.48	Right & left 153.5 g
Difference	0.56	0.51	0.06	5.19	0.72	0.2	51.75	
	(21%)	(24%)	(25%)	(68%)				
P	<0.025	<0.005	<0.1	<0.001	NS	NS	<0.001	

<sup>a</sup> Final lung weight was the wet weight of the lung after trimming away all extrapleural bronchi and vessels. This weight was used to calculate quantity of lipid per unit lung weight.

<sup>b</sup> These data were obtained in two-lung experiments wherein perfused and unperfused lungs of the same animal were compared (n = 5). NS, not significant.

ally identical and quite different from those of either whole blood or plasma (Table 2). When as little as 5 ml of whole blood is added to a whole (6 liter) lavage, a marked change in fatty acid composition is seen (Table 2).

These observations indicate that the excess lipid recovered in the washings of unperfused lungs over that from perfused lungs is not attributable in any significant degree to leakage of blood lipids into the unperfused washings. An alternate possibility is that perfusion removes or binds a fraction of the alveolar phospholipid. Regardless of the mechanism involved,

it seems clear that the quantity of lipid in washings from the unperfused lung more accurately reflects the true alveolar lipid than that from perfused lung whose structure is extensively altered by perfusion.

#### Accurate estimation of the internal surface area of the lung

According to current concepts, sufficient quantities of surfactant should be present in the alveoli to form at least one tightly-packed lipid monolayer over the alveolar surface at functional residual capacity (FRC) (2). We calculated the area which would be covered

TABLE 2. Fatty acid patterns (chain length:degree of unsaturation) of phosphatidylcholine<sup>a</sup> from washings of perfused and unperfused lungs from whole blood, from plasma, and from washings to which whole blood was added

	14:0	15:0	16:0	16:1	Other	18:0	18:1	18:2	20:4	Sat.	Unsat.
<i>Lavage</i>											
Perfused (N = 15)											
Mean	6.29	1.02	67.68	9.30	1.96	5.17	8.42	1.23	0.64	80.20	19.84
S.D.	± 1.58	± 0.29	± 5.50	± 2.10	± 0.78	± 1.06	± 2.25	± 0.43	± 0.04	± 4.80	± 4.80
Unperfused (N = 14)											
Mean	6.43	1.14	67.84	9.20	2.10	5.27	6.94	1.63	1.42	80.68	19.31
S.D.	± 1.16	± 0.41	± 5.93	± 2.46	± 0.63	± 1.11	± 1.97	± 1.15	± 0.00	± 4.84	± 4.84
<i>Whole blood</i> (N = 7)											
Mean	0.74	0.48	29.28	2.17	1.51	26.54	12.45	16.28	12.06	56.67	43.32
S.D.	± 0.28	± 0.15	± 1.61	± 0.31	± 0.61	± 5.77	± 2.11	± 2.51	± 4.94	± 5.94	± 5.91
<i>Plasma</i> (N = 8)											
Mean	0.87	0.28	22.67	2.19	1.85	30.62	13.36	22.14	9.00	54.05	45.91
S.D.	± 0.47	± 0.21	± 2.47	± 0.83	± 0.43	± 4.68	± 3.96	± 4.34	± 4.00	± 6.11	± 6.00
Lavage	6.32	0.80	70.69	7.33	1.83	5.22	6.14	1.54	T	83.03	16.97
Lavage + 5 ml WB	6.87	0.91	62.67	6.96	1.73	9.27	8.39	3.70	T	79.72	20.28
Lavage + 10 ml WB	6.04	0.99	52.22	6.55	1.03	9.94	10.29	7.24	5.63	69.19	30.81

<sup>a</sup> The phosphatidylcholine was isolated by thin-layer chromatography of lipid extracts and the fatty acid patterns (percent molar ratios) were determined by gas-liquid chromatography.


Sat., saturated; Unsat., unsaturated, including mono- and polyunsaturated fatty acids; WB, whole blood.

by the mean quantity of DSPC isolated in lavage from 1 gram of lung tissue (1.08 mg or  $1.47 \times 10^{-3}$  mM DSPC/g lung) using an area of  $42 \text{ \AA}^2/\text{molecule}$  of DSPC (27). The number of DSPC molecules per g lung was calculated to be  $8.85 \times 10^{17}$  (molecules  $(6.02 \times 10^{20})$  per mmol DSPC  $\times$  mmol  $(1.47 \times 10^{-3})$  DSPC per g lung). The area covered by this number of molecules as a packed monolayer would be  $3.72 \times 10^{19} \text{ \AA}^2$  (molecules  $(8.85 \times 10^{17})$  of DSPC per g lung  $\times$  area  $(42 \text{ \AA}^2)$  per molecule). We then estimated the internal surface area (ISA) per gram lung in the dog at FRC using data from the literature as follows. 1) The data of Siegwart et al. (28) for internal surface area/kg body weight obtained in excised dog lungs inflated to 30 cm transpulmonary pressure were taken as pertaining to total lung capacity (TLC). For our mean dog weight of 20.25 kg, that internal surface area (ISA) is approximately  $71 \text{ m}^2$ . 2) The in vivo measurements of functional residual capacity and total lung capacity made by Robinson et al. (29) were used to estimate FRC. The FRC/TLC ratio of 0.4 was used to estimate lung volume at FRC. 3) Using the finding of Gil and Weibel (30) and of Forrest (31), that ISA varies almost linearly with lung volume, an ISA of  $28.4 \text{ m}^2$  was calculated at the derived mean FRC for our dogs. Dividing by observed mean total lung weight (right plus left) gave  $0.208 \text{ m}^2/\text{g lung}$  or  $2.08 \times 10^{19} \text{ \AA}^2/\text{g lung}$ . In summary, we found that the surface that would be covered by a packed monolayer of DSPC in washings from 1 gram of lung was  $3.72 \times 10^{19} \text{ \AA}^2$ , and we estimated the ISA/g lung at FRC to be  $2.08 \times 10^{19} \text{ \AA}^2$ . Thus, 180% as much DSPC was recovered as would cover the internal surface area with a packed monolayer at FRC.

The value for FRC which we used in these calculations is among the highest in literature. Had a lower figure been used, the excess of DSPC would have been higher. While the finding supports those of others, that an excess of surfactant or of DSPC exists in the alveoli, these previous estimates have been based on data which are much different from ours.

King and Clements (2) used not the alveolar surfactant but the sum of that isolated from washings and lung homogenate to arrive at the conclusion that more than twice the amount of surfactant necessary to make a single lipid protein duplex film was present in the lung. Furthermore, they used an internal surface area of  $1,000 \text{ cm}^2/\text{g lung}$ , a figure less than half that derived here. Using our figure for ISA at FRC, they recovered only slightly more lipoprotein than would be necessary to make one duplex layer and had they used only the alveolar lipoprotein, they would have found much less than the amount necessary to make a single duplex layer.

Gail, Steinkamp, and Massaro (11) also concluded that an excess of DSPC is present in alveoli. The quantities of DSPC in washings per gram lung reported by these investigators is only about 50–65% as much as we found (depending upon whether they used total or parenchymal lung weight), but the surface area that they used, which was based on estimates of surface-free energy from observations on surfactant films, was 60% lower than our figure for dogs of the same size. The reasons for the large discrepancy in quantity of DSPC in washings found by these investigators and by us are unclear, but that may be due, in part, to the very large wash volumes and repeated lavage which we used.

An 80% excess of DSPC in alveolar washings allows some conclusions about the distribution of surfactant within the alveoli. Electron microscopic observations of excess tubular myelin, presumably containing the DSPC, in alveolar recesses suggest an uneven distribution of surfactant with higher concentration in areas of sharper curvature (12). If amounts sufficient to provide only a single monolayer at FRC were present, it would follow that certain parts of the alveolar surface would be devoid of a surfactant film, because other parts were covered by an excess. The finding of an 80% excess allows the possibility that an excess can occupy areas of high curvature and that sufficient material remains to provide a thinner layer over the remainder of the alveolar surface. 

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