Intra- and extracellular compartmentalization of the surface-active fraction in dog lung

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ABSTRACT Adult mongrel dogs were killed at various times after injection of ^{3}H-labeled palmitate. The lungs were removed and subjected to an extensive saline lavage. The surface-active fraction was isolated from the lavage and from homogenized residual lung by a procedure based upon differential centrifugation in sucrose solutions. The material isolated from the lavage was designated extracellular surfactant; material from the residual lung was designated intracellular surfactant. Both had similar chemical composition and surface activity. The results of the isotopic labeling studies demonstrate that the two fractions have distinctly different specific activity curves. Label was incorporated into the intracellular surfactant rapidly and reached a peak at 1 hr. No radioactivity was found in the extracellular surfactant for the first 15 min, and the specific activity increased much more slowly than in the intracellular surfactant. These results demonstrate at least two anatomically distinct metabolic "pools" of pulmonary surfactant in the lung. While our data are not conclusive, one possible interpretation is that the biosynthesis of pulmonary surfactant takes place intracellularly with a subsequent secretion onto the alveolar surface.

SUPPLEMENTARY KEY WORDS pulmonary surfactant · alveolar lining · lung lavage

PULMONARY SURFACTANT has been implicated as an important factor in the maintenance of alveolar stability (1). Pulmonary physiologists have generally used a lung lavage to remove the extracellular alveolar lining to yield relatively crude preparations containing pulmonary surfactant (1, 2). Implicit in these early studies is the concept of pulmonary surfactant compartmentalization. Macklin (3) demonstrated silver-staining material, which he assumed was mucopolysaccharide, on the alveolar surface and, intracellularly, in type II cells. He was the first to suggest that a surface tension factor might exist in two compartments in the lung. Buckingham and Avery (4) correlated the onset of surface activity in lung extracts of fetal mice with the appearance of osmiophilic bodies in type II cells (5). Bensch, Schaefer, and Avery (6) submitted electron micrographs as evidence that the extrusion of osmiophilic bodies into the alveolus by type II cells occurs during the recovery-compensated phase of respiratory acidosis. Further morphological data interpreted to show the extrusion of the contents of osmiophilic bodies onto the alveolar surface was provided by Kikkawa, Motoyama, and Cook (7). These findings were further detailed and expanded recently by Goldenberg, Buckingham, and Sommers (8), who showed that pilocarpine stimulated this secretory process.

Gluck, Motoyama, and Smits (9) confirmed and extended the above morphological observations by showing that an acetone-precipitable PC fraction (considered to be a major component of pulmonary surfactant) was not available to a lung lavage until after birth; there was also a concomitant decrease in the number of osmiophilic bodies at birth. Tierney, Clements, and Trahan (10) investigated the half-lives of various molecular species of PC in whole lung tissue and in the surface-active fraction obtained from whole lung by the procedure of Abrams (11). They were unable to detect more than one compartment for disaturated PC. No attempt was made to separate the PC into extra- and intracellular compartments through the use of a lavage procedure. Scarpelli (12) has studied the incorporation of radioactive precursors into the phospholipids of material obtained from a

Abbreviations: PC, phosphatidylcholine; IB, surface-active fraction derived from dog lung.

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lung lavage and from the total residual lung. Young and Tierney (13, 14) have also used a lavage to study the relationship between extra- and intracellular PC. None of these previous studies have, however, combined the two prerequisites needed for a precise understanding of this problem: (*a*) the use of an isolation method which is selective for a definable fraction containing pulmonary surfactant from which the constituent PC may in turn be isolated, and (*b*) the use of a lavage procedure to operationally segregate extracellular and intracellular pulmonary surfactant.

We have recently described the isolation and characterization of a surface-active fraction, containing pulmonary surfactant, from dog lung homogenates (15). The purpose of this communication is to present evidence that our whole-lung surface-active fraction can be separated into intra- and extracellular compartments by the use of a lung lavage. Although these two compartments are chemically and physically similar, radioactive palmitate is incorporated into their constituent PC at markedly different rates. The two fractions do, however, exhibit dissimilar morphology.

MATERIALS AND METHODS

Mongrel dogs were anesthetized with pentobarbital (26.4 mg/kg). Palmitic-9,10-^{3}H acid (specific activity, 200 mCi/mmole, New England Nuclear Corp., Boston, Mass.), 60 μCi/kg, in approximately 0.5 ml of absolute ethanol was injected through a femoral vein catheter. The animals were killed at various times by cutting the abdominal aorta. The thoracic organs were removed *en bloc* and placed on ice. All subsequent procedures were performed at 4°C. The trachea was transected at its bifurcation, and the lungs were cleaned of other tissues and weighed. The bronchus was intubated and the lung was washed six times (12, 16) with 0.15 M NaCl–0.001 M Tris, pH 7.45. (This medium was also used for tissue homogenization and to prepare sucrose solutions for centrifugation procedures.) The lung was inflated with solution until all the alveoli adjacent to the visceral pleura were filled, and the opalescent solution was then withdrawn. An unrecoverable 18% fluid loss occurred from the first lung lavage volume. The subsequent wash volumes, each with fresh solution, were constant and were equal to the volume which was recovered from the first wash. The solution obtained after the fifth lavage was clear. After six lavages, the residual lung was reweighed to determine the amount of retained lavage solution. Enough solution was added to make a ratio of total homogenization medium to tissue of 3.5:1. The lung was minced with scissors, homogenized at low speed in a Waring Blendor, and filtered through nylon gauze.

A surface-active fraction, designated IB, was isolated from the lung wash and the residual lung by the procedure described previously (15). Since the lung wash had a very large volume, 1500–2000 ml, it was first centrifuged for 40 min at 27,300 *g*. The sediment was gently resuspended in homogenization medium, layered over 0.75 M sucrose, and then centrifuged as outlined before (15). Occasionally, the whole lung was also homogenized in order to demonstrate that the surface-active fraction obtained from the whole lung was similar in quantity to the sum of that obtained from the lung wash and homogenized residual lung.

Procedures for the isolation of lipids, analytical methods, surface tension determinations, electron microscopy, and scintillation counting were as described previously (15). The chemical composition and surface properties of the IB fraction do not vary detectably with the method of tissue homogenization, but the ultrastructural details of the fraction are more interpretable when a less vigorous homogenization technique is used (15). Accordingly, electron microscopic characterization was carried out only on IB fractions derived from lungs processed in a Potter-Elvehjem homogenizer (15).

RESULTS

The chemical characterizations of the surface-active fractions, IB, derived from the lung wash and from homogenized residual lung are shown in Tables 1 and 2. The yields of the respective IB fractions are based on the original wet weight of the lung prior to the lavage procedure. In terms of protein and PC, 54% and 57%, respectively, of the total IB is found in the lavage compartment. Control experiments in which an IB fraction was isolated from a total homogenate of one lung and compared with the IB isolated from the wash and residual tissue of the other lung indicated that the sum of the wash and residual IB's was equal in quantity to the IB obtained from the total homogenate. The total lipid to protein and PC to protein ratios were calculated per unit of protein present in each IB fraction. The relative percentage compositions of the fatty acid esters in the α-

TABLE 1 COMPOSITION* OF INTRA- AND EXTRACELLULAR SURFACE-ACTIVE FRACTIONS (IB) FROM DOG LUNG

Fraction	Protein†	Lipid†	PC†	PC‡
	mg/g lung	*mg/mg protein*	*μmoles/mg protein*	*% of total lung PC*
Intracellular	0.105 (0.022)	4.8 (0.7)	4.4 (0.9)	3.2
Extracellular	0.124 (0.028)	6.1 (0.7)	4.9 (0.7)	4.2

* Analytical procedures carried out as previously described (15)
† Values are averages of 12 dogs (± SD).
‡ Based on phosphorus analysis.

TABLE 2 PHOSPHATIDYLCHOLINE FATTY ACID DISTRIBUTION IN INTRA- AND EXTRACELLULAR SURFACE-ACTIVE FRACTIONS (IB) FROM DOG LUNG

Fatty Acid*	Intracellular α†	Intracellular β†	Extracellular α†	Extracellular β†
	% of total‡			
14:0	2.8	4.6	3.2	6.0
16:0	66.7	51.2	73.2	54.2
16:1	5.7	12.4	5.0	13.6
18.0	12.0	1.6	7.8	1.7
18:X§	4.0	7.9	4.8	9.8
18:1	6.2	12.5	3.8	8.5
19:0	0.8	7.8	0.6	3.6

* Fatty acids are designated by chain length:number of double bonds.

† Refers to position on PC molecule. Determined after phospholipase A hydrolysis (15).

‡ Values are averages of duplicate determinations from three different dogs; the agreement was within 5%.

§ Unidentified fatty acid (15).

and β-positions of the constituent PC of each IB fraction are given in Table 2. These criteria clearly establish the chemical similarity of the extra- and intracellular IB fractions. The minimum surface tensions attained by the IB fractions (Fig. 1) indicate that these fractions were similar in this physical respect.

Analysis of whole lung samples from six different dogs gave an average of 14.6 μmoles of PC per g of lung. Using this result and the data given in columns 2 and 4 of Table 1, we can estimate that intracellular and extracellular IB-PC account for 3.2% and 4.2%, respectively, of the total PC found in the lung. Application of the methodology previously described (15) to the determination of the fatty acid ester distribution in whole lung PC indicates that a maximum of 45.6% of this PC can be dipalmitoyl glycerophosphorylcholine. The data given in Table 2 indicate that a maximum of 51.2% of the intracellular IB-PC and 54.2% of the extracellular IB-PC is dipalmitoyl glycerophosphorylcholine. Application of these values for the maximum dipalmitoyl glycerophosphorylcholine content allows us to estimate that intracellular IB and extracellular IB account for 3.6% and 5.0% of the whole lung dipalmitoyl glycerophosphorylcholine.

The incorporation of the radioactive label into the lung wash and residual lung IB fractions at various times is shown in Fig. 2. At the times studied, PC accounted for over 67% of the label incorporated into residual and wash IB. A peak of tritium incorporation into the residual lung IB fraction occurred at approximately 60 min. No significant amount of label was found in the wash IB until after 15 min. After this time, the incorporation into the wash IB slowly increased over the time period studied.

The morphological characterization of the residual and wash IB fractions by electron microscopy is shown

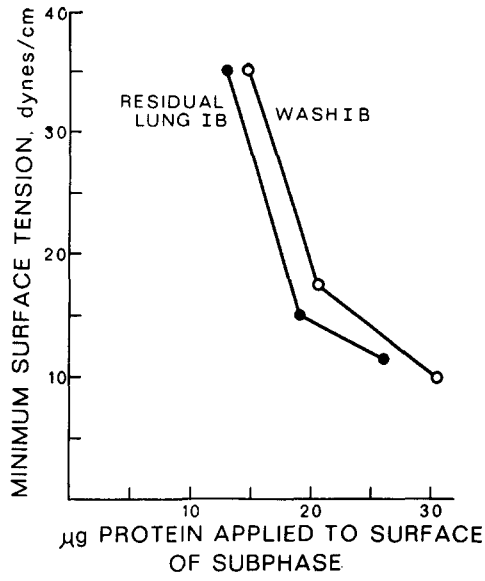

FIG. 1. Surface activity of residual lung and wash IB fractions determined by dynamic compression–expansion of surface area (3). The maximum trough area was 66.0 cm², which was then reduced to 13.2 cm². A 3-min cycling time was used. The subphase was 0.145 M NaCl at room temperature. The minimum surface tension is plotted vs. the quantity of IB (μg of protein) applied to the surface at 100% area. The curves shown are from one dog which was entirely representative of all dogs studied. Each value was determined in duplicate and agreement was within 5%.

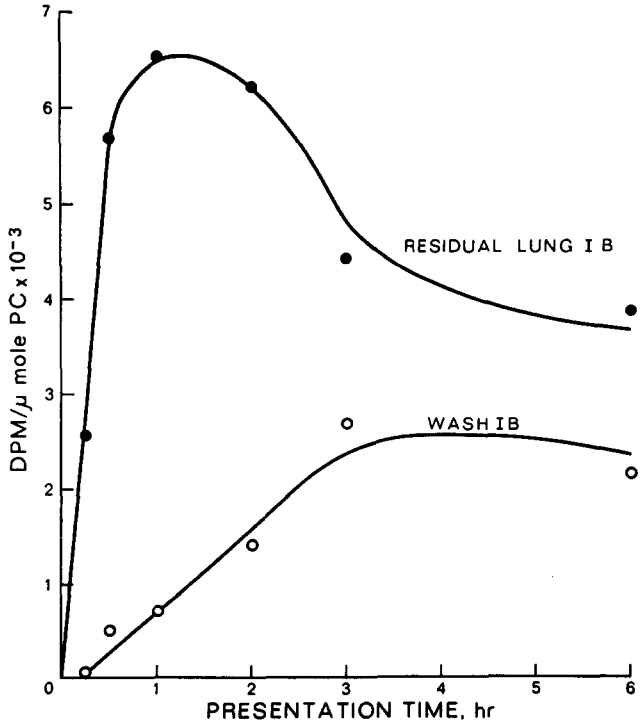

FIG. 2. Incorporation of radioactive label into residual and wash IB fractions after injection of ^{3}H-labeled palmitate. The specific activities at the times shown are averages of at least two different dogs; the results agreed within 5–10%.

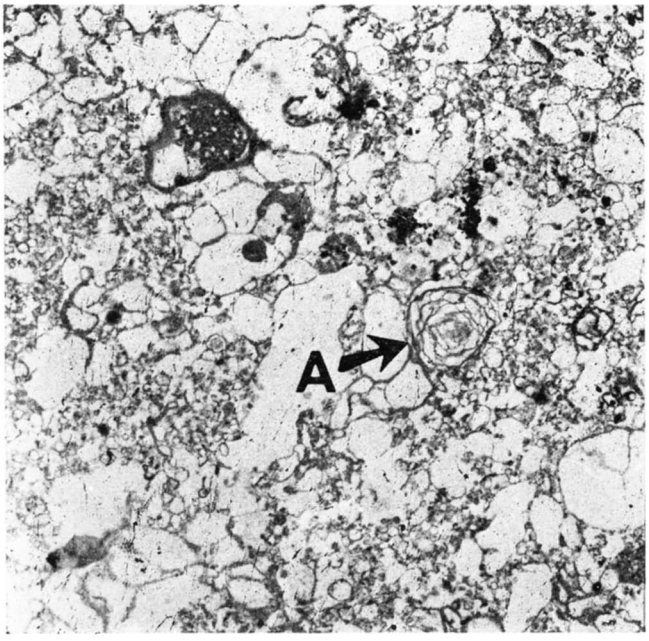

FIG. 3. Residual IB fraction containing laminated bodies, arrow *A*, similar to osmiophilic bodies seen in type II cells (15). The fraction is membranous and vesicular in character. ×6,880.

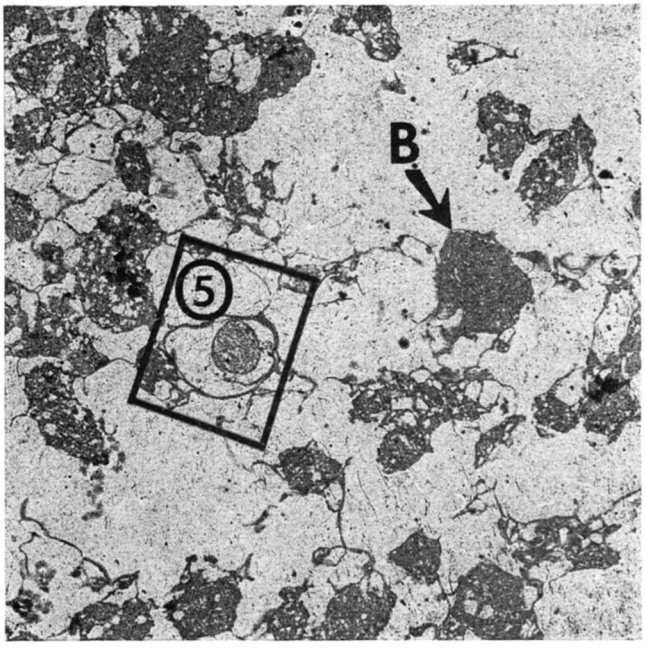

FIG. 4. Wash IB fraction containing osmiophilic masses (as opposed to osmiophilic bodies), arrow *B*, and membranous components which are closely associated, ×6,800. The area outlined is shown in Fig. 5 at higher magnification.

in Figs. 3–6. The membranous and vesicular character of the residual IB is seen in Fig. 3, which is a typical cross section of this fraction. At higher magnifications, these membranous elements for the most part appear to be associated with a fine amorphous or "fuzzy" material. Laminated bodies, arrow *A* in Fig. 3, were seen much more frequently in this fraction than in the wash IB. Fig. 4 is a representative cross section of the wash IB fraction, which consists almost entirely of large osmiophilic masses interspersed with a membranous component. The very close relationship between the membranous component and the osmiophilic masses is emphasized clearly in Fig. 5. Higher magnification of the osmiophilic masses, Fig. 6, indicates a very definite organized structure.

Routine phase microscopy of 1-μ sections of the residual and wash IB fractions indicated that the whole cell contamination was much less than 1%.

DISCUSSION

Pulmonary physiologists have often used a lung lavage to obtain a solution which is very surface-active and which removes extracellular material found in the alveolar lining layer. This procedure does not disrupt cellular integrity (17, 18). After our lavage procedures, routine histological examination of 1-μ Epon sections of whole residual lung indicated that epithelial cell morphology was not appreciably altered. This fact is further supported by the data in Fig. 2 which show that 36 times more label is available in the residual lung IB compared with the lung wash IB after 15 min presentation time; the latter had a specific activity of 70 dpm/μmole of PC. This lag time of incorporation of palmitate into the lung wash IB, in conjunction with the morphological results, is evidence that the lung lavage does distinguish two compartments of surface-active material. If the lung wash IB and the residual lung IB were parts of a single anatomical compartment, then one would expect the form, if not the extent, of their incorporation curves to be similar. Clearly this is not the case.

The wash IB is predominantly composed of osmiophilic masses with associated membranous forms. The ultrastructure of these osmiophilic masses (Fig. 6) appears to be similar to the ordered structures seen extracellularly at the tissue–air interface found *in situ* by others (7, 8, 19–21). Despite extensive modifications of their perfusion procedure to reduce fixation artifacts, Gil and Weibel (21) still found these ordered structures extracellularly.

The residual IB, in terms of surface properties, centrifugational behavior, and chemical composition, should also contain pulmonary surfactant; but, electron microscopy reveals that the wash and residual IB's are not morphologically identical. The latter is much more structurally heterogeneous and apparently does not contain large amounts of the ordered substructure characteristic of the wash IB. To the best of our knowledge, this

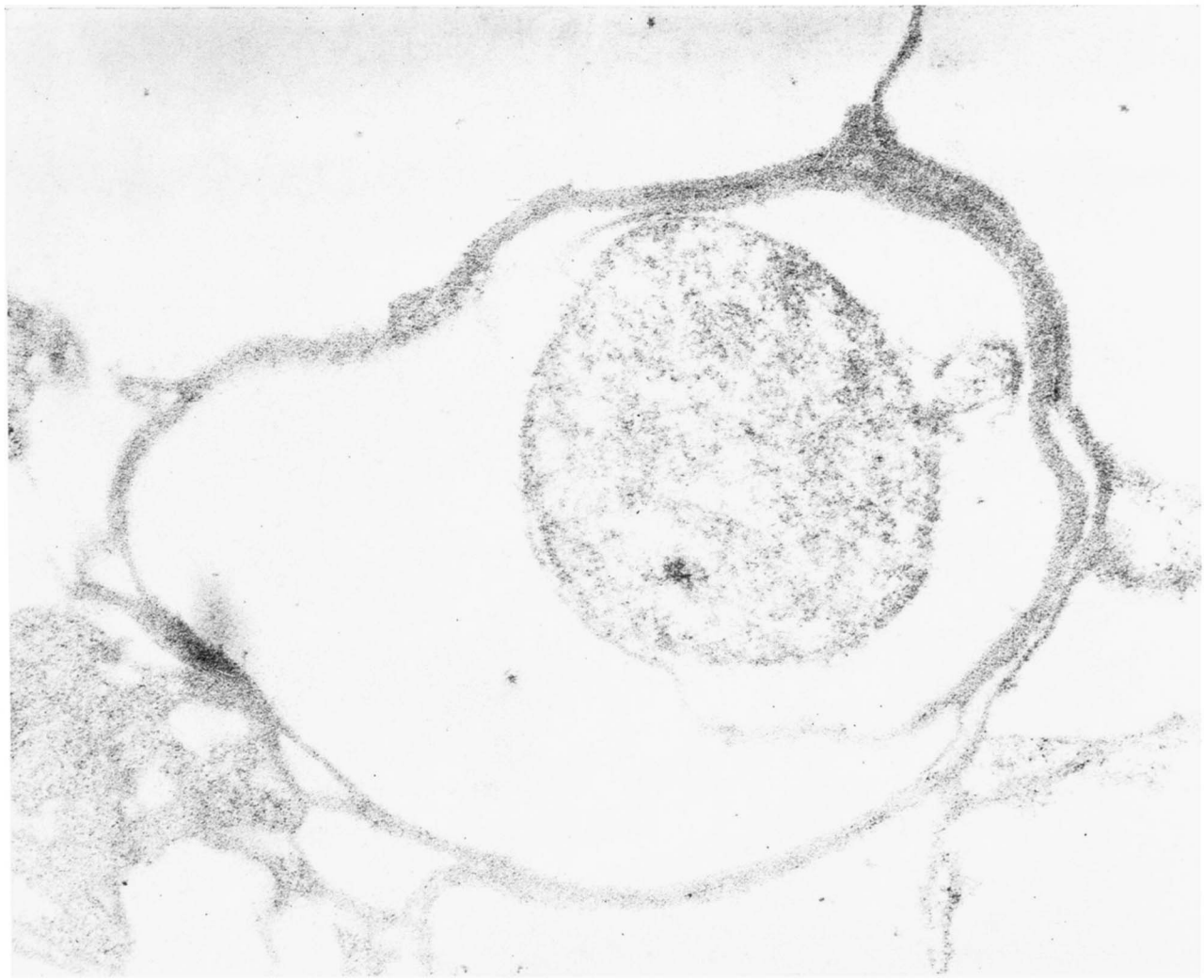

FIG. 5. Wash IB, higher magnification of area shown in Fig. 4, demonstrating close association of osmiophilic masses and membranous forms. ×58,700.

type of ordered structure has not been demonstrated intracellularly in alveolar epithelium, although many lines of evidence indicate that type II cell osmiophilic bodies and multivesicular bodies contain intracellular pulmonary surfactant (4, 5, 8, 22). The ultrastructure of these inclusions (8, 15, 22) more closely resembles structures seen in our residual IB (Fig. 3) rather than the wash IB. As shown previously (15) and in the present study, wash and residual IB's contain large amounts of PC and would be expected to exhibit a marked degree of structural pleomorphism dependent upon the state of hydration and interaction with other cellular components (23). An example of structural pleomorphism may be shown in Fig. 5. This figure can be interpreted to indicate that the osmiophilic mass is in the process of undergoing a phase transformation into the membranous-type structure closely associated with it. The characteristic ultrastructure of the wash IB osmiophilic masses may indeed be a result of the juxtaposition of this material between the air and tissue phases in the alveolar lining, a process which presumably had not yet occurred with the residual IB *in situ*. Consequently, we feel that the morphological dissimilarity between the residual and wash IB's does not necessarily mean that the two fractions do not contain considerable amounts of the same macromolecular entities, a conclusion certainly compatible with the chemical and surface properties of the two fractions.

The data presented in this study demonstrate the existence of two different anatomical compartments of surface-active material in the adult lung. A definite precursor–product relationship between intracellular and extracellular IB is not supported by the data given in Fig. 2, since the two curves do not actually intersect. The interpretation of this relationship is not as clear as that concerned with a simple metabolic pathway, since we are presumably dealing with several phases, i.e., the

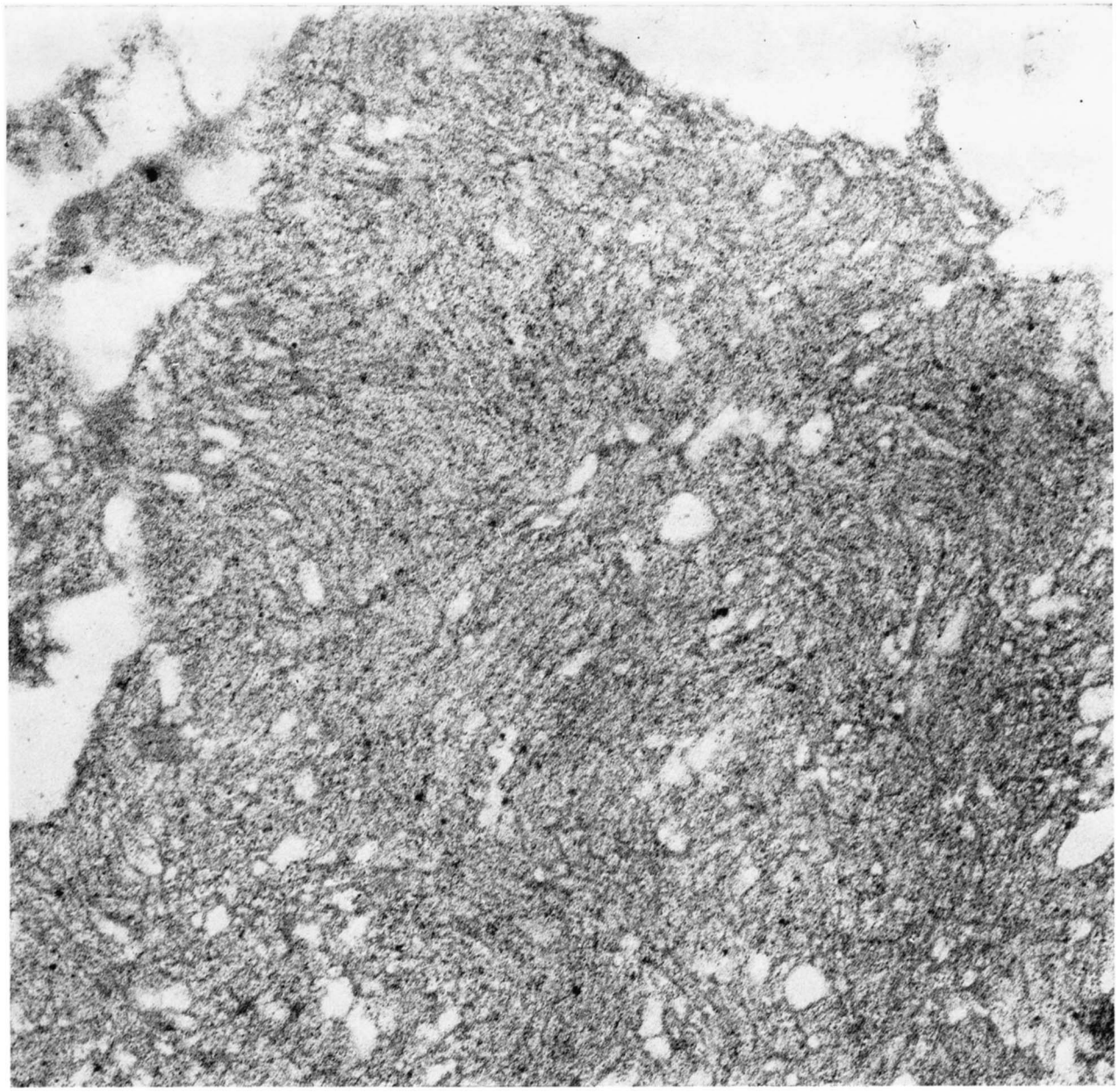

FIG. 6. Wash IB, higher magnification of area similar to that indicated by arrow *B* in Fig. 4. The osmiophilic masses are seen to have a definite ordered structure similar to that seen in the alveolar lining by others (19–21). ×59,500.

biosynthesis of a complex macromolecular particle, concentration into cytoplasmic particles, transport from the basal to the apical portion of the type II cell, and secretion onto the alveolar surface (22). Goldenberg et al. (8) found, after pilocarpine stimulation of the above events, that some osmiophilic bodies were retained by the type II cell. This implies a possible storage mechanism for intracellular surfactant which would also have an effect upon the relationship of the two curves shown in Fig. 2. If a precursor–product relationship does exist, the data in Fig. 2 suggest that the minimum elaboration time for this secretion product, wash IB, is about 15 min.

We have previously (15) discussed the criteria which have led us to consider that our IB fraction contains pulmonary surfactant: (*a*) the isolation procedure yields only one definable fraction, IB, which is capable of reducing surface tension to extremely low values, and (*b*) localization, in part, in the alveolar lining. No other fraction obtained in the procedure is as surface-active as IB. Additionally, IB apparently is a lipoprotein which contains a large amount of dipalmitoyl glycerophospho-

rylcholine, usually associated with the surface activity present in the lung (1). Since, as pointed out earlier in this communication, extra- and intracellular IB's account for only 8.6% of the total lung dipalmitoyl glycerophosphorylcholine, we cannot conclusively state that IB is the only component of the surfactant system in the lung. We do consider, however, that IB is entirely representative of the surfactant system.

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