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The relationship between intra- and extra-cellular surfactant phospholipids in the lungs of rabbits and the effects of silica-induced lung injury

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Extensive homogenization of lung tissue by nitrogen decompression in a Parr disruption bomb increased by 5-fold the yields of low-density phospholipid ($d = 1.06$) achieved by other methods. This intracellular phospholipid preparation was high in phosphatidylcholines (84.3%), particularly disaturated phosphatidylcholine (51.2%). On the basis of its low density, composition, and morphological appearance, we concluded that this phospholipid was derived from the intracellular compartment of pulmonary surfactant. We examined the relationship between intra- and extra-cellular surfactant pools according to age, gender and silica-induced pulmonary injury. In normal animals the intracellular pool of surfactant phospholipids increased from 1.54 ± 0.14 mg at 1 day after birth to 62.30 ± 4.50 mg per pair of lungs after 31 months, and over the same time period the extracellular pool increased from 1.04 ± 0.15 mg to 27.45 ± 2.30 mg per pair of lungs. The ratio between the extracellular and intracellular pools of surfactant increased from 1.50 ± 0.19 at 1 day after birth to 2.28 ± 0.23 after 31 months of age. The ratio between the two pools was not influenced by gender, but was changed by the intratracheal injection of silica into the lungs. Intratracheal injection of silica dust increased the levels of surfactant in both compartments, but not to the same extent, indicating that the ratio between the pools could be changed by toxic materials. These data suggest the existence of a size relationship between the intra- and the extra-cellular pools of surfactant, a relationship which implies a common regulatory mechanism that can be disturbed during pulmonary injury.

INTRODUCTION

The pulmonary-surfactant system consists of two anatomically distinct pools, one intracellular and the other extracellular (Pawlowski *et al.*, 1971). The intracellular pool is contained in the Type II alveolar epithelial cells, where surfactant is synthesized and stored in the form of cytoplasmic organelles called lamellar bodies. Upon appropriate physiological stimuli, lamellar bodies are secreted into the alveolar, or extracellular, compartment where surfactant phospholipids lower surface tension at the air/cell interface, thereby preventing alveolar collapse. Neither the intra- nor extra-cellular pools of surfactant appear homogeneous, and various stages in the maturation of surfactant have been noted (Magoon *et al.*, 1983). Evidence indicates that these two pools exist within a controlled precursor–product relationship (Jacobs *et al.*, 1982), although the regulatory mechanisms are not known. Investigation of this relationship and the metabolic processes that control it has been hampered by lack of knowledge concerning the sizes of these pools.

Isolation and quantification of the extracellular surfactant pool may be achieved by using bronchoalveolar lavage (Hook, 1978). Quantification of the intracellular pool of surfactant phospholipids has proven much more difficult primarily because of the absence of unique phospholipid markers. Many studies have taken the intracellular pool to be that phospholipid remaining in the lungs after lavage (Ryan *et al.*, 1980), an approach that ignores the fact that the lungs do not consist solely of Type II cells. Type II cells account for only about 20% of the total cells in the lungs (Young *et al.*, 1981). Furthermore, Young *et al.* (1981) have estimated that intracellular surfactant phospholipid comprises only 16% of the total phospholipid present in the lungs of rats.

Few attempts have been reported on the quantification of the intracellular pool of surfactant phospholipids. Abrams (1966) was the first to attempt quantification of the intracellular pool, although he did not estimate recoveries of surfactant phospholipid. Many methods for the isolation of lamellar bodies have since been devised, although yields have generally been too low and variable for use in the estimation of the pool of intracellular surfactant phospholipids. Duck-Chong (1978) adapted the basic procedure of Page-Roberts (1972) for the isolation of lamellar bodies and devised a method that produced relatively high yields of low-density phospholipids from the lungs of rats, although, again, estimates of yields were not made. We have also used the Duck-Chong (1978) method with rat lungs and showed that the procedure recovers as much as 75% of the total

Abbreviations used: LPC, lysophosphatidylcholine; Sph, sphingomyelin; PC; phosphatidylcholine; USPC, unsaturated phosphatidylcholine; DSPC, disaturated phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

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intracellular surfactant phospholipid (Dethloff *et al.*, 1986). The Duck-Chong (1978) method appears to be a reliable procedure with high yields for the estimation of intracellular surfactant phospholipids in the lungs of rats; however, when we applied this method to the lungs of rabbits, we obtained yields of phospholipid that appeared to be unrealistically low.

We wished to know what the size of the intracellular pool of surfactant phospholipids was in the lungs of rabbits and to determine, through its relationship with the extracellular pool of surfactant phospholipids, whether these two pools were in steady-state equilibrium. Because previously published procedures did not appear suitable for the estimation of the size of the intracellular pool of surfactant phospholipids in rabbit lungs, we developed our own method. The present paper describes a new procedure for the quantification of the intracellular pool of surfactant phospholipids and examines the size relationship between this pool and the extracellular pool as a function of age, gender, lung weights and silica-induced pulmonary injury.

MATERIALS AND METHODS

Chemicals

The chemicals used were as follows: sodium cacodylate, uranyl acetate, lead citrate, propylene oxide, sodium potassium tartrate (Fisher Scientific Co., Fairlawn, NJ, USA); sucrose (density-gradient grade, Schwarz/Mann, Orangeburg, NJ, U.S.A.); OsO_4 (Colonial Metals, Elkton, MD, U.S.A.); cytochrome *c*, NADPH, bovine serum albumin, AMP, Tris hydrochloride, paraformaldehyde, Hepes (Sigma Chemical Co., St. Louis, MO, U.S.A.); silica in the form of Berkeley Min-U-Sil (< 5 μm particle size; Pennsylvania Glass Sand Corp., Pittsburgh, PA, U.S.A.); phospholipid standards (Applied Science, State College, PA, U.S.A.); glutaraldehyde (Ladd Research Industries, Burlington, VT, U.S.A.); Epon 812 epoxy resin (Ernest F. Fullam, Schenectady, NY, U.S.A.); [*methyl*-^{3}H]choline chloride (80 Ci/mmol) (New England Nuclear, Boston, MA, U.S.A.). All other chemicals were of analytical grade.

Animals

Adult male albino rabbits of the New Zealand strain weighing 2–2.5 kg (Dutchland Laboratory Animals, Denver, PA, U.S.A.) were used, except where noted otherwise. All animals were allowed free access to food and water.

Isolation of extracellular surfactant phospholipid

Rabbits were killed by injections of 3 ml of sodium pentobarbital solution (50 mg/ml) into the marginal ear vein. Lungs were removed quickly with the trachea intact. Extraneous tissue was removed and the lungs were filled to capacity with ice-cold 0.9% NaCl and then drained via the trachea. Ice-cold saline was used to minimize secretion by the Type II cells and cotton gauze was used to prevent contamination of the effluents by fluid from the exterior surfaces of the excised lungs. The lavage procedure was performed eight times. The combined lavage effluents were centrifuged at 580 ***g*** for 10 min at 4 °C to sediment cells. Lavage fluid was stored frozen at −20 °C until biochemical analyses were performed.

Disruption of tissue and isolation of intracellular surfactant phospholipids

All steps were performed at 4 °C. Lavaged lungs were homogenized in 0.9% NaCl (4 ml/g of tissue) in a Potter–Elvehjem homogenizer with seven passes of a Teflon pestle [0.102–0.152 mm (0.004–0.006 in) clearance]. To compare the yields of low-density phospholipid released by various methods of tissue disruption, portions of the homogenates were treated further by homogenization with a Polytron (9 min) (Brinkman Instruments, Westbury, NY, U.S.A.), by sonication (Branson Sonifier; Branson, Danbury, CT, U.S.A.) (output 6, 30% duty cycle for 20 or 60 pulses), or by N_2 decompression [N_2 pressure 10.5 MPa (105 bar), 10 min] (Parr Disruption Bomb; Fisher Scientific Co., Fair Lawn, NJ, U.S.A.). Because extensive treatment of homogenates resulted in the formation of stable foams which made volume determinations difficult, we measured the homogenates by weight instead of volume. Homogenates (8.5 g) were layered over 20% (w/v) sucrose and centrifuged at 82500 ***g*** for 60 min at 4 °C (Beckman L3-50 ultracentrifuge, SW 27 rotor). The lamellar bodies appeared as an opaque band at the saline/sucrose interface. The portion of the gradients above and including the saline/sucrose interface was collected and stored frozen until biochemical analyses were performed. Lamellar bodies were identified by using electron microscopy and by their phospholid composition.

Lamellar bodies were also isolated by the procedure of Duck-Chong (1978). Briefly, lavaged lungs were homogenized in 1.0 M-sucrose by using a Potter–Elvehjem homogenizer with a Teflon pestle (clearance given above). Seven passes of the pestle were made and the resulting homogenate was filtered through one layer of monofilament nylon cloth (160 μm pore size) (New York Stencil Co., Halesite, NY, U.S.A.). The filtrate was adjusted to 18 ml with 1.0 M-sucrose. A 2 ml portion each of 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, and 0.2 M-sucrose was layered on top of the filtered homogenate and the gradients centrifuged for 15 min at 1000 rev./min (200 ***g***) (Beckman L3-50 ultracentrifuge, SW 27 rotor). Without interruption the gradients were then accelerated to 25000 rev./min (82500 ***g***) for 180 min. The lamellar bodies, which appeared as an opaque band at a relative density of approx. 1.06, were aspirated and stored frozen at −20 °C until biochemical analyses were performed. Lamellar bodies were identified by using electron microscopy and by their phospholipid composition.

Isolation of microsomal fractions

Microsomes were isolated by the method of Hook *et al.* (1972). All steps were conducted at 4 °C. Lungs were homogenized in 0.25 M-sucrose (4 ml/g of tissue) and the homogenates were centrifuged at 10000 ***g*** for 10 min. The supernatant was centrifuged at 143000 ***g*** for 30 min. The resulting pellet was resuspended in 0.25 M-sucrose, layered over 30 ml of 20% (w/v) sucrose and centrifuged at 82500 ***g*** for 60 min. The microsomal pellet was resuspended in 0.25 M-sucrose and stored frozen at −20 °C until biochemical analyses were performed.

Distribution of intact and disrupted lamellar bodies on density gradients

The distribution of highly purified lamellar bodies on continuous density sucrose gradients was determined.

Lamellar bodies were isolated by the method of Spalding *et al.* (1983). Briefly, lungs were homogenized in 0.25 M-sucrose (4 ml/g of lung) by using seven passes of the pestle. The homogenate was centrifuged at 1100 *g* for 10 min at 4 °C (Sorvall RC-2B centrifuge, SS-34 rotor). The supernatant was then centrifuged at 4300 *g* for 10 min at 4 °C. The resulting supernatant was again centrifuged at 21000 *g* for 20 min. The pellet was resuspended in 0.25 M-sucrose and re-centrifuged at 21000 *g* for 20 min. This pellet was resuspended in 0.25 M-sucrose. An 8.5 ml portion of the suspension was layered over 30 ml of 20% sucrose and centrifuged at 82500 *g* for 60 min at 4 °C (Beckman L3-50 ultracentrifuge, SW 27 rotor). The lamellar bodies appeared as an opaque band which was aspirated from the gradient and diluted with water to give a final sucrose concentration of 0.25 M. The lamellar bodies were disrupted further by sonication (Branson Sonifier, output 6, 40% duty cycle, 75 pulses). A portion of the lamellar bodies was left untreated. A 4 ml portion of sonicated and a 4 ml portion of unsonicated lamellar bodies were layered over sucrose gradients composed of 12 ml of 12% (w/v) sucrose, 14 ml of 25% (w/v) sucrose, and 8.5 ml of 50% (w/v) sucrose and were centrifuged at 82500 *g* for 16 h (Beckman L3-50 ultracentrifuge; SW 27 rotor). Fractions (0.7 ml) were collected from the gradients by infusing 50% sucrose into the bottom of the tubes. The fractions were extracted in chloroform/methanol (2:1, v/v) as described by Folch *et al.* (1957). The phospholipid content of each fraction was estimated by measuring phospholipid phosphorus by the method of Shin (1962).

Estimation of the recovery of intracellular surfactant phospholipids

We estimated the recovery of intracellular surfactant phospholipids as follows: [*methyl*-^{3}H]choline in 0.9% NaCl was injected into the marginal ear vein of several rabbits (1 mCi/rabbit). After 16 h the rabbits were killed and highly purified lamellar bodies were isolated by the method of Spalding *et al.* (1983) as described above. The specific radioactivity of the lamellar bodies was determined and portions of the labelled lamellar bodies were used as internal standards upon which estimates of intracellular surfactant recoveries were based. Radiolabelled lamellar bodies were mixed thoroughly with homogenates of lung tissue (lung tissue that had been homogenized by using seven passes of the pestle in a Potter–Elvehjem homogenizer). The mixture was treated by N_2 decompression and the intracellular pool of surfactant phospholipids was isolated as described above. We assumed that the isolated radiolabelled lamellar bodies behave similarly to the unlabelled lamellar bodies during the subsequent isolation procedure and the percentage of radioactivity recovered was used as an indication of the recovery of intracellular surfactant phospholipids.

Electron microscopy

The intracellular surfactant pool was prepared for electron microscopy essentially by the procedure of Williams (1977). The gradient fractions were diluted with 0.9% NaCl and centrifuged at 143000 *g* for 30 min at 4 °C. The pellet was washed once by diluting with 0.9% NaCl and re-centrifuging at 55000 *g* for 30 min. The resulting pellet was fixed with 2% glutaraldehyde/1% formaldehyde in 0.1 M-sodium phosphate buffer, pH 7.4, at 4 °C overnight and then treated with 1.5% OsO_4 in 0.1 M-cacodylate buffer, pH 7.4, for 18 h at 4 °C. The pellets were rinsed in three changes of 50 mM-sodium maleate buffer, pH 6.0, at 4 °C, stained for 90 min in 1.5% uranyl acetate in 25 mM-sodium maleate buffer, pH 5.2, at 4 °C, then rapidly dehydrated in cold graded acetone solutions and infiltrated with Epon 812 (Luft, 1961). Ultrathin sections were cut on a Porter–Blum Ultramicrotome MT-1 with a du Pont diamond knife (E. I. du Pont de Nemours and Co., Wilmington, DE, U.S.A.). The sections were stained with 5% uranyl acetate (Watson, 1958) for 10 min, then with lead citrate (Reynolds, 1963) for 6 min. Sections were examined in a Philips EM-400 electron microscope.

Phospholipid analyses

Extracellular and intracellular surfactant phospholipids were extracted in chloroform/methanol (2:1, v/v) by the method of Folch *et al.* (1957). The phospholipids were separated into their component species by t.l.c. on silica-gel H plates (Applied Science) with choroform/methanol/acetic acid/water (60:32:5:4, by vol.). PS and PI migrated together in this system as did PE and PG. These phospholipids were recovered from the gel, re-plated and run in a solvent system of tetrahydrofuran/methylal/methanol/2 M-NH_3 (50:25:25:5, by vol.). The phospholipids were eluted from the gel with methanol and quantified by analysis of phospholipid phosphorus by the method of Shin (1962). DSPC was separated from USPC by the OsO_4 method of Mason *et al.* (1973).

Enzyme assays

NADPH:cytochrome *c* reductase activity in lamellar body fractions was measured by the method of Williams & Kamin (1962). The reaction was run in 0.2 M-Hepes buffer (pH 7.7), 0.1 M-KCl, 2.1 mM-KCN, 2 mM-NADPH containing cytochrome *c* (1 mg/ml). Succinate:cytochrome *c* reductase activity was determined by the method of Singer & Kearney (1957) in 30 mM-phosphate buffer, pH 7.6, 1.8 mM-KCN, 3.2 mM-sodium succinate, containing 3.6 mg of bovine serum albumin and 0.9 mg of cytochrome *c*/ml. All enzyme assays were run at 37 °C. Protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Intratracheal injection of silica

Rabbits weighing 2–2.5 kg were anaesthetized by slowly injecting sodium pentobarbital (25 mg/kg) into the marginal ear vein. The animals were placed prone on an inclined board and the trachea was located with the aid of a laryngoscope. A cannula attached to a 1 ml syringe was inserted into the trachea and the silica, suspended in 1.0 ml 0.9% NaCl, was injected rapidly into the lungs.

RESULTS

Intracellular surfactant phospholipid and lamellar bodies

In the present paper the term 'lamellar bodies' refers to the highly purified and structurally intact Type II cell organelles isolated from lung homogenates (contamination of the lamellar bodies with microsomal phospholipid was 17.7% and mitochondrial contamination was not detectable by measurements of NADPH:cytochrome *c*

reductase and succinate:cytochrome *c* reductase respectively). 'Intracellular surfactant phospholipid' refers to the low-density phospholipid isolated after extensive disruption of lung tissue. We show below that most of this low-density phospholipid is derived from lamellar bodies and that quantification of this material is appropriate for the quantification of the intracellular pool of pulmonary surfactant.

Isolation of intracellular surfactant phospholipids

Isolation of intracellular surfactant phospholipids from the lungs of rabbits by the method of Duck-Chong (1978) yielded only 0.30±0.01 mg of phospholipid/g of lung (Table 1), a value that did not compare well with that of rat lungs (Young *et al.*, 1981). The low yields appeared to be attributable to two factors. First, examination of lung homogenates under the electron microscope indicated incomplete disruption of the rabbit lungs. The Potter–Elvehjem homogenizer left many Type II cells damaged but not sufficiently for the release of lamellar bodies. Second, the upward migration of low-density phospholipids through the density gradients as required by the Duck-Chong (1978) method appeared to trap lamellar-body-like material within subcellular debris.

Table 1. Release of low-density phospholipid from lung homogenates by various homogenization procedures

Lavaged lungs were homogenized by using seven passes with the pestle of a Potter–Elvehjem homogenizer. Intracellular surfactant and lamellar bodies [Duck-Chong (1978) method] were isolated as described in the Materials and methods section. Before sucrose gradient centrifugation, aliquots of homogenates were treated further (as indicated) with a variety of homogenization procedures. Equivalent amounts of lung tissue were used in all procedures. Results are expressed as means of three determinations±S.D.

	Method	Yield of low-density phospholipid (mg/g of lung)
I.	Duck-Chong (1978) method	0.30±0.01
	Centrifugation of homogenate downward through 20% sucrose	0.51±0.03
II.	Polytron*	1.07±0.15
III.	Sonication†	1.07±0.37
IV.	N_2 decompression‡	1.65±0.04

* Brinkman Polytron, 9 min treatment.
† Branson Sonifier, output 6, 30% duty cycle, 60 pulses.
‡ N_2 pressure, 10.5 MPa (105 bar), 10 min.

We overcame these problems to some extent by sedimenting the homogenates downward through 20% sucrose and by applying a more effective means of homogenization to the lung tissue. As shown in Table 1, sedimentation of lung homogenates (homogenization achieved by seven passes with the pestle in a Potter–Elvehjem homogenizer) downward through the sucrose increased the yield of low-density phospholipids from 0.30±0.01 to 0.51±0.03 mg of phospholipid/g of lung, an increase of 67%. Further treatment with the Potter–Elvehjem homogenizer by using as many as 29 additional passes of the pestle did not improve the yield of low-density phospholipid significantly (results not shown). Further homogenization with a Brinkman Polytron homogenizer or Branson sonicator increased the yield of low-density phospholipid from 0.30±0.01 to 1.07±0.15 and 1.07±0.37 mg/g of lung respectively. The most effective method of homogenizing rabbit lungs appeared to be by N_2 decompression in a Parr disruption bomb. This method increased our yield of low-density phospholipids to 1.65±0.04 mg/g of lung. The yield of low-density phospholipids by using N_2 decompression was five times greater than that obtained with the Duck-Chong (1978) method. The yield of low-density phospholipids was influenced by both the N_2 pressure and the time spent under pressure (Fig. 1). We obtained optimum results by treatment of homogenates with a N_2 pressure of 10.5 MPa (105 bar) for a period of 10 min.

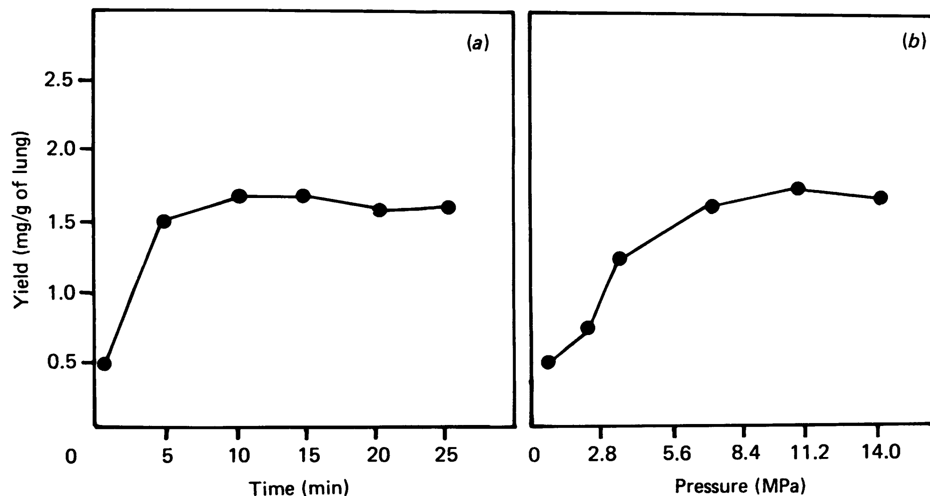

Fig. 1. Effects of N_2 decompression on the release of low-density phospholipid from homogenates of rabbit lung

(*a*) Lavaged lungs were homogenized in 0.9% NaCl and then treated with high-pressure N_2 [10.5 MPa (105 bar)] for various times. Treated homogenates were centrifuged over 20% (w/v) sucrose for 1 h and the yield of low-density phospholipid was determined. (*b*) Homogenates of lavaged lungs were treated for 10 min at various pressures of N_2. Treated homogenates were centrifuged over 20% (w/v) sucrose for 1 h and the yield of low-density phospholipid was determined.

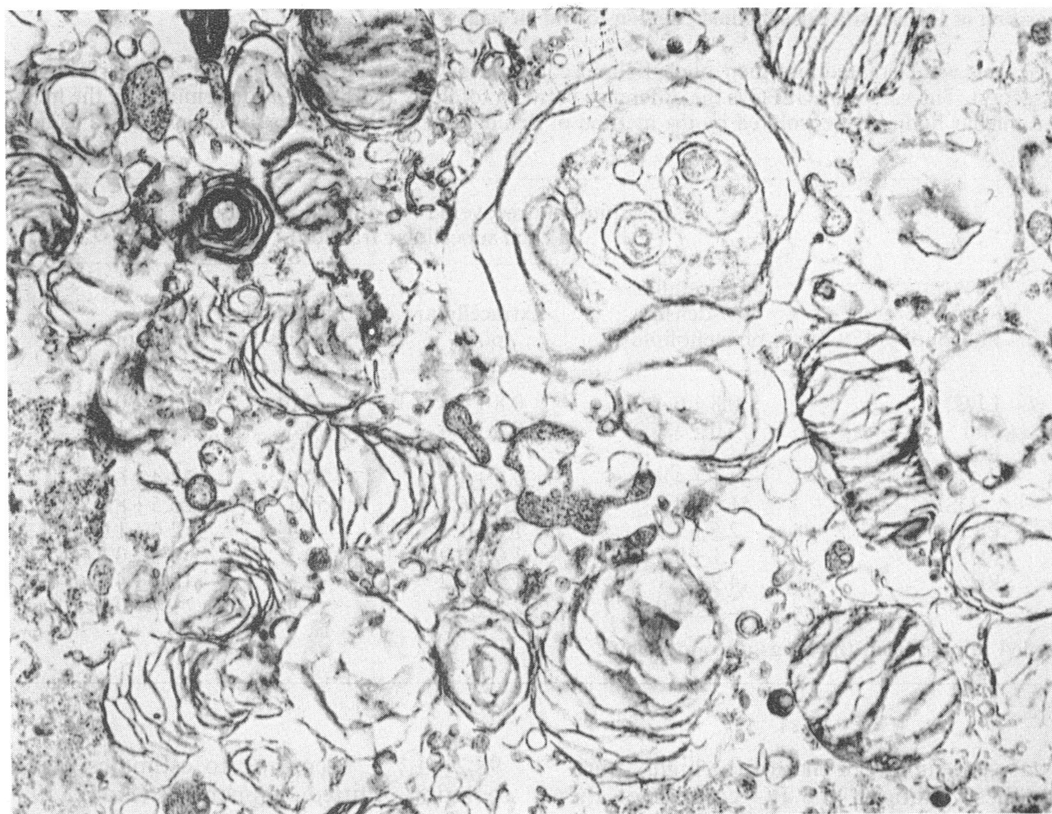

Fig. 2. Electron micrograph of lamellar-body fraction isolated by N_2 depressurization and discontinuous sucrose-gradient centrifugation

The fraction consists of disrupted lamellar bodies and membrane fragments. Magnification ×33000.

Effect of disruption on the density of lamellar bodies

Intracellular surfactant phospholipids exist in the Type II cells primarily in the form of lamellar bodies. Extensive disruption of tissue would be expected to result in the disruption of lamellar bodies, but, because our objectives were to simply recover as much of the intracellular surfactant phospholipids as possible with minimal contamination, structural damage to lamellar bodies was not considered objectionable. However, it was necessary to determine the effects of disruption on the density of the lamellar bodies. It was conceivable that the density of lamellar bodies or lamellar-body fragments might be altered by their disruption in such a manner as to prevent or reduce their recoveries on sucrose density gradients. We isolated structurally intact lamellar bodies from the lungs of rabbits and then disrupted them by sonication. We were unable to use the Parr disruption bomb with the purified lamellar bodies because of the small amounts available and the losses incurred by foam formation upon decompression. Intact and disrupted lamellar bodies were then subjected to isopycnic centrifugation on continuous sucrose density gradients. Intact lamellar bodies banded on the gradient as a single band with a relative density (*d*) of 1.06. Lamellar bodies disrupted by sonication banded in the same region but, in addition, a band of phospholipid was found at lower relative densities. Nothing with a density higher than that of intact lamellar bodies was found. Because all phospholipid of *d* 1.00–1.06 was recovered, and because disruption of lamellar bodies did not result in the formation or release of any higher-density phospholipid structures, we concluded that extensive disruption of the tissue during the homogenization procedure would not lead to unrecoverable intracellular surfactant phospholipid.

We estimated the yield of intracellular surfactant phospholipid by adding radiolabelled lamellar bodies to lung homogenates and treating the homogenates by N_2 decompression followed by centrifugation over 20% (w/v) sucrose. We assumed the recovery of radiolabelled lamellar bodies to be the same as that of the non-labelled structures and estimated our recoveries of the intracellular surfactant phospholipid to be $69.5 \pm 11.6\%$ (mean ± S.D. of five separate determinations). Although it was possible to estimate the recovery of lamellar-body phospholipid, we could not exclude contamination of that lipid with other subcellular organelles or low-density phospholipid from non-surfactant-related sources (if such sources exist).

Composition of intracellular surfactant phospholipid

The phospholipid composition of pulmonary surfactant is characteristic of that material. No other subcellular organelle appears to contain phospholipids in proportions similar to those found in pulmonary surfactant. The composition of the intracellular low-density phospholipid isolated from the lungs of rabbits by using the technique of N_2 decompression is shown in Table 2. PC was the major phospholipid accounting for 84.3% of the total. All of the other phospholipids, including PE and Sph each accounted for less than 5% of the total phospholipid. The composition of the intracellular low-density

Table 2. Composition of low density phospholipid fractions of rabbit lung

Subcellular fractions were isolated and their phospholipid composition was determined by t.l.c. as described in the Materials and methods section. The value for USPC is the difference between total PC and DSPC as determined by the method of Mason *et al.* (1973). Lamellar bodies were isolated by the method of Spalding *et al.* (1983). Results are expressed as means ±S.D. for four individuals.

	Phospholipid recovered (% of total recovered in each subcellular fraction)			
Phospholipid	Intracellular low-density phospholipid	Extracellular pool	Lamellar bodies	Microsomes
LPC	0.8±0.4	0.8±0.4	2.0*	0.5±0.2
Sph	4.0±3.2	0.8±0.4	2.8	11.0±1.6
PC				
USPC	33.1±4.4	29.3±1.9	29.9	38.4±6.5
DSPC	51.2±4.9	62.1±6.5	59.1	26.5±8.3
PS	2.5±1.1	1.3±1.1	1.4	2.6±1.0
PI	1.1±0.5	1.0±0.9	0.3	0.6±0.2
PE	4.9±2.1	1.9±0.2	0.6	18.7±4.7
PG	2.4±1.0	2.8±1.0	3.9	1.7±0.4

* Values for pooled sample from the lungs of four rabbits.

phospholipid was very similar to that of the highly purified lamellar bodies, although the PC content of the lamellar bodies was 5% higher. The DSPC content was lower, and that of PE higher, than that found in the lamellar bodies, suggesting that the intracellular material was contaminated to a small extent with phospholipid from some other source. The most likely contaminants were microsomes, whose phospholipid composition is also shown in Table 2. Rabbit lung microsomes contained considerably more PE, and considerably less DSPC, than lamellar bodies. Thus contamination of the intracellular low-density phospholipid with microsomes would tend to increase PE and decrease DSPC. Microsomal contamination was confirmed by the presence of NADPH:cytochrome *c* reductase (52 nmol of cytochrome *c* reduced/min per mg of phospholipid) in the intracellular surfactant phospholipid. Because the specific activity of NADPH:cytochrome *c* reductase in the microsomes was 294 nmol of cytochrome *c* reduced/min per mg of phospholipid, we estimate that the contamination of the intracellular phospholipid with microsomal phospholipid was 17.7%. Mitochondrial contamination of the intracellular surfactant phospholipid was not detected when succinate:cytochrome *c* reductase was used as a marker.

The appearance of the intracellular surfactant under the electron microscope is shown in Fig. 2. Most of the structures appear to be disrupted lamellar bodies, fragments of lamellar bodies and membranous vesicles.

From its composition, its appearance under the electron microscope and its characteristic low density, we concluded that this intracellular low-density phospholipid isolated by using N_2 decompression arose primarily from the intracellular pool of surfactant. Hereafter this pool will be referred to as 'intracellular surfactant'.

Extracellular-surfactant phospholipids

Extracellular-surfactant phospholipids were recovered by repetitive bronchoalveolar lavage by a previously described (Hook, 1978) procedure. Six lavages recovered 98.2±1.7% according to estimates based upon extrapolation of fitted exponentials (Hook, 1978).

Effects of age, gender, and silica on extracellular and intracellular surfactant phospholipids

The intracellular and extracellular pools of surfactant phospholipid in the lungs of adult male rabbits was 1.54±0.25 and 2.30±0.66 mg of phospholipid/g of tissue respectively. No differences were found in the concentration of intracellular and extracellular surfactant phospholipids in the lungs of male and female rabbits. The intracellular and extracellular pools in the lungs of female rabbits were 1.56±0.52 and 2.04±0.46 mg of phospholipid/g of lung tissue respectively. In the lungs of both male and female rabbits the extracellular pool was about 50% larger than the intracellular pool. Furthermore, the concentration of intracellular and extracellular surfactant phospholipids in the left lung were the same as in the right lung.

Intracellular and extracellular surfactant phospholipids were measured in the lungs of rabbits of increasing ages (Table 3). The sizes of the intracellular and extracellular pools increased from birth up to 3 months and appeared to remain almost constant up to about 9 months. However, both pools almost doubled in size between 9 and 31 months of age. The ratio between the extracellular and intracellular pools was variable, increasing from 1.50±0.19 at 1 day after birth to 1.92±0.63 at 17 days, decreasing to 0.66±0.21 at 35 days, and thereafter increasing to 2.28±0.23 at 31 months of age. The ratio between extracellular- and intracellular-surfactant phospholipids generally appeared to lie between 1.5 and 2.3, excepting for a period around 35 days of age. In adults of 9 months or older the ratio appeared to be remarkably constant.

Both intra- and extra-cellular pools increased linearly with weight of the lungs, although the extracellular pool increased disproportionately in lungs weighing above

10 g (Fig. 3*a*). Both pools increased linearly with body weight (Fig. 3*b*).

To determine whether the relationship between the intra- and extra-cellular pools in the lungs of adult rabbits could be altered, we injected silica into the lungs via the trachea. At a dose of 125 mg of silica/kg body weight, the extracellular pool of surfactant was not changed when measured at either 14 or 28 days after dosing (Table 4). However, the intracellular pool was nearly doubled at 14 days, but had returned to normal by 28 days after dosing. Increasing the dose of silica to 250 mg/kg body weight increased both the intracellular and extracellular pools of surfactant at 14 days and 28 days after dosing.

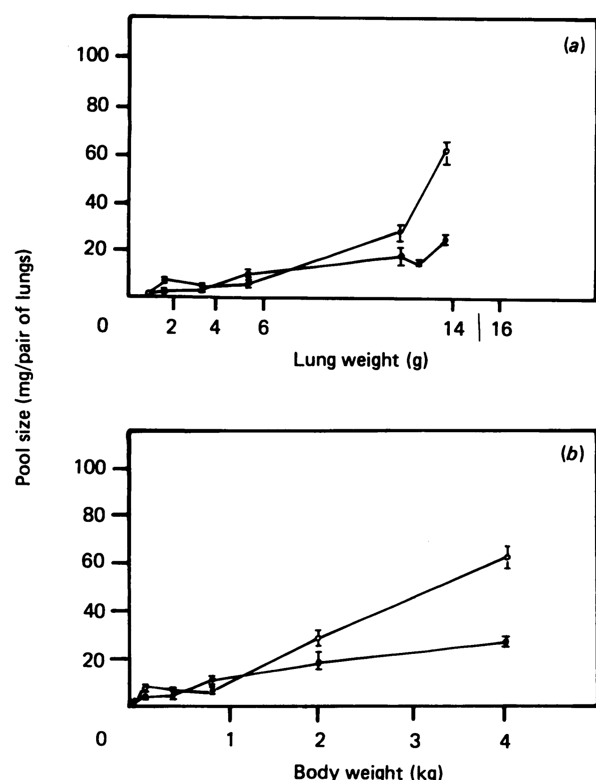

Fig. 3. Changes in (○) intra- and (●) extra-cellular surfactant pool sizes as functions of lung and body weights

The intra- and extra-cellular pools of surfactant phospholipid were isolated from rabbits of various ages as described in the Materials and methods section. Each point represents the mean result (±S.D.) for four animals.

DISCUSSION

Many methods have been devised for the isolation of intracellular surfactant phospholipids in the form of lamellar bodies (Page-Roberts, 1972; Duck-Chong, 1978). Because most of these methods have aimed at isolating lamellar bodies in highly purified form and as structurally intact as possible, the yields of surfactant phospholipid have often been either very low or, as in most cases, not determined. We have devised a method for the isolation, from the lungs of rabbits, of a low-density fraction, containing phospholipids mostly from the intracellular pool of surfactant. This method appears suitable for the routine quantification of the intracellular pool of surfactant. The intracellular surfactant pool of the rabbit has not been quantified previously. In addition, we have shown that a relationship exists between intracellular and extracellular surfactant pools in the lungs of adult rabbits such that the ratio between the two pools remains constant. However, this relationship may be disturbed in the presence of toxic agents such as silica dust.

The intracellular pool of surfactant in the lungs of rats has been quantified by others (Young *et al.*, 1981) using the method of Duck-Chong (1978). This method did not prove satisfactory in our hands for the determination of intracellular-surfactant levels in the lungs of rabbits because of low yields. Considerable improvement was obtained when more efficient methods were used for the disruption of lung tissue. Homogenization with the Potter–Elvehjem homogenizer is not an effective method for the disruption of rabbit lungs, presumably because of the presence of greater amounts of connective tissue than in the rat lungs. Homogenization by N_2 decompression appeared to be highly effective in releasing low-density phospholipids from lung tissues. Although rarely used with animal tissues, this method has been used extensively for the disruption of bacteria (Hughes *et al.*, 1971).

Because intracellular surfactant does not contain known specific markers, estimation of surfactant yield is difficult and at best an approximation. Surfactant

Table 3. Changes in surfactant phospholipid pool sizes in the lungs of rabbits with age

Intra- and extra-cellular surfactant phospholipids were isolated as described in the Materials and methods section. Results are means ± S.D. (*n*), where *n* is the number of individuals

Age of rabbit	Total extracellular surfactant (mg)	Total intracellular surfactant (mg)	Extracellular / Intracellular
1 day	1.54 ± 0.14	1.04 ± 0.15	1.50 ± 0.19 (4)
17 days	7.00 ± 1.06	3.79 ± 0.58	1.92 ± 0.63 (4)
27 days	5.78 ± 1.11	4.41 ± 0.53	1.34 ± 0.41 (3)
35 days	6.54 ± 1.63	10.30 ± 1.87	0.66 ± 0.21 (5)
3 months	30.55 ± 3.31	19.70 ± 3.52	1.58 ± 0.29 (4)
9 months	31.15 ± 3.43	15.05 ± 0.96	2.08 ± 0.25 (4)
31 months	62.30 ± 4.50	27.45 ± 2.30	2.28 ± 0.23 (4)

Table 4. Effect of silica on the intracellular and extracellular pools of pulmonary surfactant in the lungs of rabbits

Rabbits weighing 2–2.5 kg were injected intratracheally with silica suspended in sterile saline (0.9% NaCl). Controls received saline only. Intracellular and extracellular surfactant was isolated as described in the Materials and methods section. Results are expressed as means ± S.D. for four or five individuals. *$P < 0.001$.

	Phospholipid (mg/pair of lungs)			
Time after dosing (days)...	14		28	
Dose of silica (mg/kg)	Intracellular	Extracellular	Intracellular	Extracellular
0	12.8±3.0	24.5±3.9	11.9±2.5	22.0±4.5
125	22.8±2.6*	25.4±4.1	15.3±4.2	24.4±1.4
250	34.6±4.8*	38.4±4.2	42.7±17.1*	54.8±21.8*

apoproteins have been found associated with extracellular surfactant phospholipids (Clements & King, 1976), but their association with intracellular surfactant has not been satisfactorily demonstrated. Immunohistochemical localization of surfactant apoproteins has revealed their presence in the endoplasmic reticulum of Type II cells but not in lamellar bodies (Williams & Benson, 1981). The phospholipid composition of surfactant is highly characteristic, although each individual phospholipid may be found in other cellular membranes. Our identification of intracellular surfactant is dependent upon its compositional similarity to highly purified lamellar bodies and its appearance under the electron microscope. We used radiolabelled lamellar bodies to determine the recovery of lamellar-body phospholipid in our preparations of intracellular surfactant. Unfortunately this method did not recognize surfactant phospholipids that could have arisen from other parts of the Type II cells such as the endoplasmic reticulum or the presence of low-density non-surfactant phospholipids that might have arisen from other cell types. We estimate that approx. 17.7% of the phospholipids in our preparations were derived from endoplasmic reticulum (based upon the presence of the marker enzyme NADPH:cytochrome *c* reductase). This level of contamination is similar to that reported by others for purified lamellar bodies (Tsao & Zackman, 1977; Spalding *et al.*, 1983).

The intracellular and extracellular pools of pulmonary surfactant share a precursor–product relationship (Jacobs *et al.*, 1982). Surfactant phospholipids are synthesized and stored in the form of lamellar bodies within the cytoplasm of the alveolar Type II cells. Subsequently, lamellar bodies are secreted by these cells into the extracellular, or alveolar, pool of surfactant. Extracellular-surfactant phospholipids may be recycled back into the intracellular surfactant (Jacobs *et al.*, 1983), although the mechanism by which this occurs has not been determined. Our findings indicate that these two pools of surfactant may share a commonality that extends beyond a simple precursor–product relationship. In the lungs of adult rabbits the ratio between the intracellular and extracellular pools of surfactant phospholipids appears constant, suggesting the presence of a common regulatory mechanism(s), perhaps mediated through recycling of surfactant via the Type II cells. This mechanism could be of fundamental importance in controlling the pulmonary-surfactant system, especially under conditions where the lung is injured by the presence of toxic agents. Ryan *et al.* (1980) estimated that the amount of surfactant present in the alveoli is approx. 75% greater than that required to cover the entire surface of the alveoli. If this is true, then the intracellular pool of surfactant may be just sufficient to replace the entire extracellular pool in cases of sudden loss.

Silica dust is known to cause increased levels of both intracellular and extracellular surfactant in the lungs of exposed animals (Gabor *et al.*, 1978; Dethloff *et al.*, 1986), a condition that closely resembles pulmonary alveolar proteinosis in humans. The mechanisms by which silica stimulates the pools of surfactant are not known. Our study indicates that intratracheal injections of silica result in the expansion of the intracellular pool of surfactant before any effect is seen in the extracellular compartment. These data support the findings of Heppleston *et al.* (1974), who measured the rates of phospholipid biosynthesis and disappearance in whole lungs and concluded that accumulation of phospholipids in the lungs of silica-treated rats was due to an imbalance between the two rate processes. Biosynthesis was increased 4-fold, but disappearance was increased only 2-fold, thereby leading to accumulation of phospholipid. There are several indications that increased surfactant may be a desirable compensatory response to lung injury. Maintenance of normally low surface tension would minimize transudation from the capillaries and prevent alveolar collapse after injury (Clements *et al.*, 1958; Hildebrandt & Kirk, 1979). Surfactant may also be important in the clearance of fluid and particulates from the lungs, which would be of particular importance in the case of exposure to silica or other dusts (Sorokin & Brain, 1974).

In summary, we have presented a method for the isolation and quantification of the intracellular pool of rabbit lung surfactant. This method is rapid and results in greater yields of low-density phospholipid than previously published procedures. Application of this method to the lungs of normal adult rabbits has revealed a fixed relationship between intracellular and extracellular pools of surfactant, suggesting that both pools may be controlled by a common regulatory mechanism.

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