# Role of lamellar inclusions in surfactant production: studies on phospholipid composition and biosynthesis in rat and rabbit lung subcellular fractions

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**Abstract** Lamellar inclusion bodies in the type II alveolar epithelial cell are believed to be involved in pulmonary surfactant production. However, it is not clear whether their role is that of synthesis, storage, or secretion. We have examined the phospholipid composition and fatty acid content of rabbit lung wash, lamellar bodies, mitochondria, and microsomes. Phosphatidylcholine and phosphatidylglycerol, the surface-active components of pulmonary surfactant, accounted for over 80% of the total phospholipid in lung wash and lamellar bodies but for only about 50% in mitochondria and microsomes. Phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyelin accounted for over 40% of the total in mitochondria and microsomes but for only 6% in lung wash and 15% in lamellar bodies. The fatty acid composition of lamellar body phosphatidylcholine was similar to that of lung wash, but different from that of mitochondria and microsomes, in containing palmitic acid as a major component with little stearic acid and few fatty acids of chain length greater than 18 carbon atoms. The biosynthesis of phosphatidylcholine and phosphatidylglycerol was examined in the mitochondrial, microsomal, and lamellar body fractions from rat lung. Cholinephosphotransferase was largely microsomal. The activity in the lamellar body fraction could be attributed to microsomal contamination. The activity of glycerolphosphate phosphatidyltransferase, however, was high in the lamellar body fraction, although it was highest in the mitochondria and was also active in the microsomes. These data suggest that the lamellar bodies are involved both in the storage of the lipid components of surfactant and in the synthesis of at least one of those components, phosphatidylglycerol.

**Supplementary key words** osmiophilic inclusions · phosphatidylcholine · phosphatidylglycerol · cholinephosphotransferase · glycerolphosphate phosphatidyltransferase.

Pulmonary surfactant, material that lines the alveoli and stabilizes air spaces at small lung volumes, is believed to be synthesized in the type II alveolar epithelial cell (1–6). A characteristic feature of the type II cell is the presence of osmiophilic lamellar inclusion bodies (7). Indirect biochemical and morphological evidence suggested that this organelle is intimately involved in surfactant biochemistry (7–11), but there was little direct evidence to support the idea, nor was it clear whether the role of the lamellar body was that of biosynthesis, storage, or secretion of surfactant.

If the lamellar body is indeed the source of pulmonary surfactant, it would be expected that its lipid composition would resemble that of the alveolar lining layer rather than that of whole lung tissue or other lung subcellular fractions. If the lamellar body is active in the synthesis of surfactant, it would be expected that it would be active in the synthesis of phosphatidylcholine and phosphatidylglycerol, the major surface-active components of pulmonary surfactant (12, 13). In this study we have examined these hypotheses in rats and rabbits by comparing the phospholipid content and fatty acid composition of lamellar bodies with those of whole lung, lung wash, mitochondria, and microsomes and by comparing the activities of cholinephosphotransferase and glycerolphosphate phosphatidyltransferase in lamellar bodies with those of whole lung, mitochondria, and microsomes.

Part of this work has been published in abstract form (14).

## EXPERIMENTAL PROCEDURE

### Rabbit lung

Adult, female, albino New Zealand rabbits, weighing 2.5–3 kg, were used. The animals were anesthetized and heparinized by intravenous administration of sodium pen-

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tobarbital (50 mg/kg) followed by sodium heparin (500 units/kg). The chest was opened, and the lungs were perfused in situ under gravity via the pulmonary artery with chilled Hanks' balanced salt solution (15). During perfusion the lungs were alternately inflated and deflated with air via the trachea (16). The blood-free lungs were lavaged with 30–50-ml aliquots of cold 0.9% NaCl four or five times. The parenchymal tissue was scraped off the bronchial tree, which was discarded. Any bloody unperfused areas were also discarded. The remaining tissue was homogenized in 0.33 M sucrose in 0.01 M Tris–HCl, pH 7.4, (0.33 M sucrose–Tris) in a Potter-Elvehjem homogenizer. A 40% homogenate in 0.33 M sucrose–Tris was filtered through four layers of cheesecloth. Aliquots of 6 ml were layered onto 5 ml of 0.75 M sucrose–Tris and centrifuged at 40,000 *g* for 40 min, when a pellet, as well as interfacial material, was obtained. The pellet was further fractionated into cell debris–nuclear, mitochondrial, and microsomal fractions by differential centrifugation as described previously (17). The material at the interface, 1 ml of 0.33 M sucrose–Tris and 2 ml of 0.75 M sucrose–Tris, was collected and diluted to 0.58 M sucrose–Tris. Aliquots of this were layered onto 0.75 M sucrose–Tris in a ratio of 2.33:1 and centrifuged at 60,000 *g* for 1 hr. The floating lamellar bodies were removed, diluted to 0.33 M sucrose–Tris, and collected as a pellet by centrifugation at 56,000 *g* for 20 min.

### Rat lung

Adult, Sprague-Dawley rats, weighing 200–250 g, were killed by concussion and bled by decapitation. The lungs were finely minced, homogenized, and filtered as described above. Lamellar bodies, mitochondria, and microsomes were prepared from 40% homogenates as described for rabbit lung.

A 20% homogenate was also centrifuged sequentially at 800 *g* for 15 min, 12,500 *g* for 10 min, 16,500 *g* for 10 min, and 105,000 *g* for 1 hr to yield pellets designated cell debris–nuclear, mitochondrial, intermediate, and microsomal, respectively, as well as a final supernate. The microsomal fraction was further fractionated into rough and smooth microsomes by the procedure of Dallner, Siekevitz, and Palade (18). All supernates were removed to within 0.5 cm of the pellets, and the final supernate and a portion of the original 20% homogenate were retained for assay. Pellets were washed once by resuspension and resedimentation and were finally suspended in 0.33 M sucrose–Tris to give a protein concentration of 4–10 mg/ml.

### Lipid extraction and fractionation

Lipid was extracted from lung wash, after centrifugation at 1,000 *g* for 15 min to remove cellular material, as well as from the homogenate and suspensions of subcellular fractions by the procedure of Bligh and Dyer (19) and from whole lung by the procedure of Folch, Lees, and Sloane Stanley (20) as described previously (13). Crude lipid extracts were washed with sodium chloride (20). Chloroform solutions of the total lipid were fractionated on columns of silicic acid into neutral lipid, glycolipid, and phospholipid (13).

Thin-layer chromatography of the phospholipids was carried out on layers of silica gel G, 0.33 mm thick (analytical) or 0.5 mm thick (preparative), in chloroform–methanol–7 M ammonia 60:35:5 (v/v/v). Lipids were located on the chromatograms with potassium dichromate (21), rhodamine 6G (22), or water (22) and were eluted from the gel with chloroform–methanol–water–formic acid 97:97:4:2 (by vol). The phospholipids were identified as described previously (13) by comparison of their retention times with those of standards in several thin-layer chromatographic systems, by their staining characteristics, and by paper chromatography of their deacylated products.

### Quantitative determinations

Phosphate was measured by the procedure of Bartlett (23), and protein was measured by the procedure of Lowry et al. (24).

### Fatty acid analysis

After saponification (25) of the phospholipids, the fatty acids were methylated with diazomethane, and the methyl esters were analyzed by gas–liquid chromatography on a column (1/8 inch × 8 ft) of 10% EGSS-X on Gas-Chrom P (100–120 Mesh; Applied Science Laboratories, State College, Pa.) at 190°C. Fatty acid methyl esters were identified by comparing their retention times with those of standards. Quantitation was by peak height multiplied by peak width at half the height.

Fatty acid methyl esters were hydrogenated in methanol over 5% palladium on charcoal. Hydrogen was bubbled through for 1 hr at room temperature. The methyl esters were recovered by washing with chloroform–methanol 2:1 (v/v).

### Enzyme assays

Cytochrome *c* oxidase (EC 1.9.3.1) was measured polarographically (26). NADPH–cytochrome reductase (EC 1.6.2.4) was measured as described by Sottocasa et al. (27) using an extinction coefficient of 27,700 $M^{-1}$ $cm^{-1}$ for cytochrome *c* (28).

Cholinephosphotransferase (EC 2.7.8.2) was assayed by measuring the rate of incorporation of radioactivity from CDP [*methyl*-$^{3}$H]choline into lipid in the presence of 1,2-dipalmitin. The reaction mixture was similar to that of Mudd, van Golde, and van Deenen (29) and contained 50 mM Tris–HCl, pH 7.2, 10 mM $MgCl_2$, 5 mM glutathione, 0.006% Triton X-100, 0.1 mM CDP [*methyl*-$^{3}$H]choline (1 $\mu$Ci/$\mu$mole), 0.88 mM 1,2-dipalmitin (pre-



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TABLE 1. Enzyme activities in subcellular fractions of rabbit lung

| Fraction | Cytochrome *c* Oxidase | NADPH–Cytochrome Reductase |
|---|---|---|
| | *nmoles/min/mg of protein* | |
| Mitochrondria | 379 | 20 |
| Microsomes | 125 | 104 |
| Lamellar body | Not detectable | 21 |

Subcellular fractions were prepared from the pooled lungs from three rabbits.

pared by sonication in 0.01% Triton X-100), and 2–5 mg of protein in a total of 1 ml. Incubation was carried out with shaking at 37°C. The reaction was linear with time for up to 20 min. Duplicate aliquots (0.1 ml) were removed at intervals and put on 2.4-cm disks of Whatman 3MM filter paper, which were immediately plunged into ice-cold trichloroacetic acid and washed as described by Goldfine (30). After drying, the disks were counted in toluene containing 0.5% PPO and 0.03% POPOP.

Glycerolphosphate phosphatidyltransferase (EC 2.7.8.5) was similarly assayed by measuring the rate of incorporation of radioactivity from [$^{14}$C]glycerol-3-phosphate into lipid in the presence of CDPdipalmitin. The reaction mixture was similar to that of Kiyasu et al. (31) and contained 50 mM Tris–HCl, pH 7.6, 5 mM glutathione, 0.2 mM L-[$^{14}$C]glycerol-3-phosphate (2 $\mu$Ci/$\mu$mole), 0.1 mM CDPdipalmitin, and 2–5 mg of protein in a total of 1 ml. The reaction was carried out with shaking at 37°C. The reaction was linear with time for at least 15 min. Aliquots (0.1 ml) were removed at intervals and processed as described above.

The radioactive products of the cholinephosphotransferase and glycerolphosphate phosphatidyltransferase reactions were shown to be phosphatidylcholine and phosphatidylglycerol, respectively, as follows. Aliquots (1 ml) of the reaction mixture were extracted with chloroform and methanol by the procedure of Bligh and Dyer (19). Portions of the extracts, together with appropriate phospholipid standards, were chromatographed on thin-layer plates of silica gel G developed in chloroform–methanol–acetic acid–water 80:13:8:0.3 (by vol) and chloroform–methanol–7 M ammonia 65:35:5 (by vol). The lipids were visualized on the plates with an iodine stain (22). After the iodine had sublimed, the lipid-containing areas were scraped into vials and were counted directly. All of the radioactivity from the cholinephosphotransferase reaction cochromatographed with phosphatidylcholine while that from the glycerolphosphate phosphatidyltransferase reaction cochromatographed with phosphatidylglycerol in both systems. Cardiolipin was not labeled in the latter experiment.

In simultaneous incubations, the results obtained by measuring the radioactivity on the disks agreed well with those obtained by measuring the radioactivity of the extracted lipid.

### Electron microscopy

Small pellets of the subcellular fractions were prepared for electron microscopy as described previously (17).

### Chemicals

Radioisotopes were purchased from New England Nuclear, Boston, Mass.; CDPdipalmitin was purchased from Serdary, London, Ontario; fatty acid methyl esters from Supelco, Bellefonte, Pa.; phospholipid standards from Sigma (St. Louis, Mo.), Supelco, and Serdary; and all other biochemicals from Sigma. All other chemicals were reagent grade.

## RESULTS

### Purity of subcellular fractions

The results of marker enzyme studies on the subcellular fractions of rabbit lung are shown in **Table 1.** Similar find-

TABLE 2. Phospholipid compositions of lung wash and lung subcellular fractions from rabbit

| | Lung Wash[a] | Lamellar Body Fraction[b] | Mitochondria[b] | Microsomes[b] |
|---|---|---|---|---|
| | | *% of total phospholipid P*[c] | | |
| Phosphatidylcholine | 86.2 | 73.0 ± 2.36 | 40.4 ± 2.00 | 47.6 ± 1.66 |
| Phosphatidylethanolamine | 3.0 | 6.8 ± 0.45 | 26.5 ± 0.92 | 24.9 ± 2.01 |
| Phosphatidylglycerol | 6.2 | 8.1 ± 0.82 | 8.5 ± 1.23 | 5.8 ± 1.69 |
| Phosphatidylinositol, phosphatidylserine, and sphingomyelin | 3.4 | 7.7 ± 0.69 | 17.9 ± 1.16 | 17.9 ± 1.04 |
| Unknown | 1.2 | 4.5 ± 1.37 | 6.7 ± 0.75 | 3.8 ± 1.18 |

[a] The lung wash data are from 18 animals; 4 pairs and 14 unilateral lungs were pooled.
[b] The data for the subcellular fractions are the means (±SE) from five experiments. In each experiment the lungs from three animals were pooled.
[c] Each value is the mean of three separate determinations.



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ings were obtained with rat lung. The mitochondrial marker, cytochrome *c* oxidase, was not measurable in the lamellar body fraction whereas the microsomal marker, NADPH-cytochrome reductase, indicated a maximum of 20% contamination of the lamellar body fraction with microsomes. These findings were confirmed by electron microscopy when it was seen that the lamellar body fraction contained no mitochondria and few microsomes, which were seen as a small vesicular contaminant.

Marker enzymes showed about 30% contamination of mitochondria with microsomes and about 20% contamination of microsomes with mitochondria.

### Phospholipid composition

Phospholipid accounted for 60% of the total lipid in rabbit lung mitochondria, 78% in microsomes, 90% in the lamellar body fraction, and 86% in lung wash.

The phospholipid composition of rabbit lung lamellar body fraction is compared with that of lung wash, mitochondria, and microsomes in **Table 2.** The lamellar body phospholipid composition resembled that of lung wash but differed from that of mitochondria and microsomes. Phosphatidylcholine accounted for 40-50% of the total phospholipid in mitochondria and microsomes but 86% in lung wash and 73% in lamellar bodies. Phosphatidylethanolamine and combined phosphatidylinositol, phosphatidylserine, and sphingomyelin each accounted for 18-27% of the total in mitochondria and microsomes but only 3% in lung wash and 7-8% in the lamellar body fraction. The amounts of phosphatidylglycerol and the unknown phospholipid were reasonably constant in the lung wash and subcellular fractions.

The unknown phospholipid (Table 2) was not characterized in the present study. However, it may be lyso-bis-phosphatidic acid, which has been shown to be a pulmonary phospholipid component (13).

The distribution of lipid among the subcellular fractions is shown in **Table 3.** Approximately 40% of the total lipid and phospholipid was recovered in the interface containing the lamellar bodies obtained during the first density gradient centrifugation step (not shown). When this material was further fractionated, 13% of the total lipid and 16% of the phospholipid was recovered in the purified lamellar body fraction. The remainder was recovered in the soluble fraction. Thus a large portion of the lamellar bodies may be disrupted during purification with loss of their contents to the soluble fraction resulting in the rather large recovery of lipid in this fraction. 7-8% of the lipid was recovered in the mitochondria and 10% in the microsomal fraction.

### Fatty acid composition

The fatty acid composition of phosphatidylcholine from lung wash, mitochondria, microsomes, and lamellar bodies is shown in **Table 4.** The fatty acid compositions of the lung wash and lamellar body fractions were similar in that palmitic acid accounted for over 60% of the total and there were few fatty acids with more than 18 carbon atoms. They contained relatively little stearic acid. Saturated fatty acids accounted for over 90% of the total. On the other hand, phosphatidylcholine from the mitochondria and microsomes contained much less palmitic acid, although it was still the most abundant fatty acid, there were more longer-chain fatty acids, and stearic acid was present in greater amount.

The fatty acid compositions of phosphatidylethanolamine and phosphatidylglycerol, phosphatidylserine, and phosphatidylinositol combined are given in **Table 5.** The fatty acids of sphingomyelin are not included in the latter because they are not released during saponification. In contrast to phosphatidylcholine, palmitic acid was not the major fatty acid in these phospholipids. Further, longer-chain fatty acids were more abundant as were those with

TABLE 3. Lipid distribution among subcellular fractions of rabbit lung

| Fraction | Total Lipid | | Phospholipid | |
|---|---|---|---|---|
| | *mg* | *% of total* | *mg of phospholipid P* | *% of total* |
| Homogenate | 127.4 | 100.0 | 4.00 | 100.0 |
| Nuclei and cell debris | 22.0 | 17.3 | 0.68 | 17.0 |
| Mitochondria | 9.4 | 7.4 | 0.33 | 8.3 |
| Microsomes | 12.8 | 10.0 | 0.40 | 10.0 |
| Lamellar body fraction | 16.9 | 13.3 | 0.62 | 15.5 |
| Soluble fraction | 45.1 | 35.4 | 1.38 | 34.5 |

Pooled lungs from three animals were used in this experiment.

TABLE 4. Fatty acid composition of phosphatidylcholine from rabbit lung wash and lung subcellular fractions

| Fatty Acid Methyl Ester | Lung Wash (3) | Lamellar Body Fraction (2) | Mitochondria (4) | Microsomes (3) |
|---|---|---|---|---|
| | | *% of total fatty acids* | | |
| 14:0 | 2.2 ± 0.1 | 2.4 ± 0.6 | 2.1 ± 0.6 | 1.6 ± 0.3 |
| 14:2 | | | 2.8 ± 0.7 | 1.7 ± 0.6 |
| 16:0 | 62.8 ± 0.6 | 61.5 ± 7.4 | 25.7 ± 11.8 | 37.8 ± 2.9 |
| 16:1 | 5.9 ± 0.8 | 5.5 ± 0.7 | 4.0 ± 0.4 | 3.4 ± 0.5 |
| 16:2 | | | 1.9 ± 0.9 | |
| 18:0 | 2.5 ± 0.4 | 2.1 ± 0.3 | 7.2 ± 0.2 | 9.1 ± 0.7 |
| 18:1 | 14.8 ± 0.6 | 11.1 ± 0.5 | 13.5 ± 0.5 | 16.6 ± 0.6 |
| 18:2 | 10.6 ± 1.0 | 15.0 ± 4.2 | 17.8 ± 1.8 | 20.8 ± 0.8 |
| 19:0 | | | 2.2 ± 1.2 | |
| 20:0 | | | 1.3 ± 0.5 | |
| 18:3 | 1.3 ± 0.1 | 1.2 ± 1.2 | | 1.4 ± 0.3 |
| 21:0 | | | 4.0 ± 2.3 | |
| 20:2 | | | | 1.0 ± 0.3 |
| 22:0 | | 1.3 ± 1.3 | 6.4 ± 2.8 | |
| 20:4 | | | | 6.5 ± 0.5 |
| 23:0 | | | 5.0 ± 2.9 | |
| 24:0 | | | 6.1 ± 2.7 | |

Each value is the mean (±SE) of duplicate determinations from the number of experiments indicated in parentheses. Fatty acids that accounted for less than 1% of the total are not included.



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TABLE 5. Fatty acid compositions of phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine + phosphatidylinositol from rabbit lung wash and lung subcellular fractions

| Fatty Acid Methyl Ester | 14:0 | 14:1 | 15:0 | 14:2 | 16:0 | 15:2 | 16:1 | 17:0 | 16:2 | 17:1 | 18:0 | 18:1 | 18:2 | 20:0 | 18:3 | 20:1 | 21:0 | 20:2 | 22:0 | 20:4 | 23:0 | 24:0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phosphatidylethanolamine** | | | | | | | | | | | | | | | | | | | | | | |
| Lung wash | 3.4 | | | 8.4 | 7.4 | | 2.7 | | 5.0 | | 9.9 | 37.9 | 10.6 | | | 9.6 | | 5.3 | | | | |
| Lamellar body fraction | 6.4 | | | 2.2 | 2.3 | | 1.3 | | | | 4.4 | 33.7 | 3.4 | | | | 11.5 | 2.5 | | | 2.4 | 30.0 |
| Mitochondria | 1.8 | | | 12.7 | 3.9 | | 2.6 | | 15.9 | | 20.6 | 20.5 | 11.5 | | | | | | | 10.7 | | |
| Microsomes | | | | 13.4 | 3.6 | 1.2 | 1.9 | | 13.7 | | 15.7 | 21.6 | 8.7 | 3.3 | | | 2.8 | 2.0 | | | | 12.1 |
| **Phosphatidylglycerol** | | | | | | | | | | | | | | | | | | | | | | |
| Lung wash | | 1.2 | | 1.5 | 26.5 | | 3.7 | | | 1.8 | 9.4 | 36.7 | 12.1 | 2.1 | 2.0 | | 1.6 | | | 1.4 | | |
| Lamellar body fraction | 5.6 | 1.5 | | 6.7 | 7.0 | | 5.6 | | 3.9 | 6.3 | 16.7 | 16.0 | 5.1 | 8.7 | | | 5.4 | 7.0 | 2.3 | | | 2.0 |
| Mitochondria | 3.5 | 2.0 | | 6.5 | 10.6 | | 6.9 | | 11.7 | | 19.7 | 12.5 | 14.2 | 3.2 | | | | 6.2 | | 3.2 | | |
| Microsomes | 7.0 | | 16.2 | 3.2 | 14.7 | | 5.0 | 14.2 | 8.0 | | 11.7 | 2.9 | 7.0 | | 5.8 | 2.3 | | 1.8 | | | | |
| **Phosphatidylserine + Phosphatidylinositol** | | | | | | | | | | | | | | | | | | | | | | |
| Mitochondria | 13.1 | 1.9 | | 6.0 | 5.0 | | 6.7 | | | 7.5 | 13.4 | 15.6 | 2.6 | 7.7 | | | 5.9 | 8.3 | 6.2 | | | |
| Microsomes | 8.8 | 1.0 | | 4.2 | 4.7 | | 4.6 | | | 5.6 | 25.7 | 13.3 | 3.0 | 8.4 | | 6.8 | | 5.3 | 3.8 | 3.6 | 1.4 | |

Results are expressed as percentages of the total fatty acid content. Each value is the mean of duplicate determinations from two experiments. Fatty acids that were present in amounts less than 1% of the total are not included.

TABLE 6. Subcellular distribution and specific activity of cholinephosphotransferase and glycerolphosphate phosphatidyltransferase in rat lung

| Fraction[a] | Cholinephos-photransferase | | Glycerol-phosphate Phosphatidyl-transferase | | Protein |
|---|---|---|---|---|---|
| | *pmoles/min/mg of protein* | *% recovery* | *pmoles/min/mg of protein* | *% recovery* | *% recovery* |
| Homogenate | 13 | 100 | 19 | 100 | 100 |
| Lamellar body fraction | 41 | 0.7 | 87 | 0.8 | 0.2 |
| Pellet + supernate[b] | 16 | 109.3 | 23 | 93.2 | 94.0 |
| Cell debris–nuclei | 15 | 9.5 | 30 | 12.4 | 11.4 |
| Mitochondria | 19 | 7.7 | 133 | 31.4 | 7.3 |
| Microsomes | 178 | 42.2 | 74 | 38.8 | 4.2 |
| Supernate[c] | 0 | 0 | 0 | 0 | 65.7 |

[a] Subcellular fractionation was carried out on a 40% homogenate as described in Experimental Procedure.

[b] The pellet + supernatant fraction was further fractionated into cell debris–nuclear, mitochondrial, microsomal, and supernatant fractions.

[c] Supernate data are from a separate experiment.

14 carbon atoms, particularly 14:2, which was a relatively major component of phosphatidylethanolamine. 16:2 was also abundant in phosphatidylethanolamine and phosphatidylglycerol, but it was absent from phosphatidylserine and phosphatidylinositol and was found in mitochondrial phosphatidylcholine in small amounts only. The unusual fatty acids 14:2 and 16:2 were only tentatively identified by comparison of their retention times with those of standards. However, their identity as fatty acids rather than aldehydes is supported by the fact that they were not extracted with the nonsaponifiable lipids after saponification.

In many of the fatty acid analyses an unidentified peak eluted after 18:1 and was often poorly separated from it. On hydrogenation it disappeared but could be accounted for by the increase in the amount of 18:0. It may be *cis*-5-18:1, which was reported by King, Ruch, and Clements (32) to be a specific component of phosphatidylcholine from canine surface-active material. However, in the present study it was not exclusively associated with any phospholipid or subcellular fraction. It is included with 18:1 in Tables 4 and 5.

## Phospholipid synthesis

The highest specific activity of cholinephosphotransferase was found in the microsomal fraction, which also accounted for most of the total activity (**Table 6**). The activity in the cell debris–nuclear, mitochondrial, and lamellar body fractions could be attributed to microsomal contamination.

Glycerolphosphate phosphatidyltransferase activity was distributed over several fractions (Table 6). The highest specific activity was in the mitochondria, but those in the



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microsomal and lamellar body fractions were also quite high. The total activity recovered in the cell debris–nuclear fraction could be attributed to the large amount of mitochondria seen in this fraction on electron microscopy, but the large recovery, as well as high specific activity, in the microsomal fraction was not due to mitochondrial contamination.

As shown in **Table 7,** the relative specific activities of cytochrome *c* oxidase and glycerolphosphate phosphatidyltransferase were markedly different, especially in the intermediate fraction, which was shown by electron microscopy to contain small mitochondria, some lysosomes, and numerous microsomes, which were mainly rough surfaced. The high specific activity of this fraction compared with that of the total microsomes suggested that the extramitochondrial phosphatidylglycerol synthesis might be located primarily in the rough microsomes. Experiments to separate microsomal subfractions confirmed that phosphatidylglycerol synthesis could occur in both the mitochondria and the microsomes, the enzyme system of the latter being localized primarily in the rough subfraction (Table 7).

The specific activity of glycerolphosphate phosphatidyltransferase in the lamellar body fraction exceeded that of the microsomes and was unlikely to be due to mitochondrial or microsomal contamination.

TABLE 7. Relative specific activities of cytochrome *c* oxidase and glycerolphosphate phosphatidyltransferase in subcellular fractions of rat lung

| Fraction[a] | Glycerolphosphate Phosphatidyltransferase | Cytochrome *c* Oxidase |
|---|---|---|
| Homogenate | 1.0 | 1.0 |
| Mitochondria | 5.0 | 4.2 |
| Intermediate | 5.4 (6.3, 4.5) | 1.1 (0.9, 1.3) |
| Microsomes | 1.6 (1.2, 2.0) | 0.8 (0.8, 0.8) |
| Rough microsomes | 6.8 | 2.6 |
| Smooth microsomes | 1.4 | 0.9 |

The relative specific activity of a fraction is the specific activity of the enzyme in that fraction divided by its specific activity in the homogenate.

[a] Data for the homogenate and rough and smooth microsomes are from the same experiment. Data for the intermediate and microsomal fractions are means, with the individual values in parentheses, from two separate experiments. Fractionation was carried out on a 20% homogenate as described in Experimental Procedure.

## DISCUSSION

Previous studies (5–7, 9–11) suggested that the osmiophilic lamellar inclusion bodies in the type II alveolar epithelial cell are intimately involved in the synthesis, storage or secretion of pulmonary surfactant. These findings were derived mainly from physiological and morphological experiments; biochemical evidence was limited (8).

Recently, however, lamellar body preparations have been obtained in varying degrees of structural integrity from rat (17, 33–35), rabbit (36, 37), guinea pig (38), fetal and newborn lamb (39), and bovine (40) lung and they have been shown to exhibit surface activity (17, 33) and to contain large amounts of lipid relative to protein (17, 33, 37). In agreement with the findings in the present study, the lipid has been shown to be largely phospholipid (33, 36, 37), the major component of which is phosphatidylcholine (33, 36, 37, 39) containing palmitic acid as the major fatty acid and with little fatty acid of chain length greater than 18 carbon atoms (33, 36, 37). Phosphatidylethanolamine (33, 36, 37), sphingomyelin (39), and phosphatidylserine and phosphatidylinositol (33) were also minor components of other lamellar body preparations, but phosphatidylglycerol, a second surface-active component of pulmonary surfactant (14, 41), was reported in only one other study (34, 35). There were unidentified lipids, however, in some studies (33, 37).

In the present study we have directly compared the phospholipid composition and fatty acid content of rabbit lung lamellar bodies with that of lung wash, which is rich in surfactant, mitochondria, and microsomes. The phospholipid composition of lamellar bodies is similar to that of lung wash, as well as to that of surface-active material (11, 42–46), but is different from that of whole lung (13) mitochondria and microsomes. The major surface-active lipids of pulmonary surfactant, phosphatidylcholine and phosphatidylglycerol (12, 13, 41), were the most abundant phospholipids in both the lung wash and lamellar bodies. Because dipalmitylphosphatidylcholine is the major highly surface-active species of surfactant phosphatidylcholine (12), we examined the fatty acid composition of that phospholipid. Phosphatidylcholine from lung wash and lamellar bodies was rich in palmitic acid, whereas that from mitochondria and microsomes was not. These findings, therefore, strongly support the morphological evidence (5–7, 10, 11) that the lamellar bodies are involved in surfactant biochemistry and suggest that one of their roles may be that of storage of surfactant. Further evidence in support of this comes from the studies of Frosolono and coworkers (11, 47), whose data indicate that intracellular material, resembling lamellar bodies, is the precursor of extracellular surfactant. Morphological studies with electron microscopy indicate that lamellar body contents are extruded from the type II cell into the alveolar space (10, 48).

Although radioautographic and cytochemical evidence (3, 4, 49) suggested a role for the lamellar bodies in surfactant phospholipid biosynthesis, there was little biochemical evidence other than that of Morgan (8), who reported that lipid *N*-methyltransferase was associated with lamellated structures in dog lung, and Gray et al. (39), who measured choline kinase in lamb lung lamellar bodies during fetal and postnatal development. The data in our study show that the lamellar body fraction from rat lung exhibits a rel-



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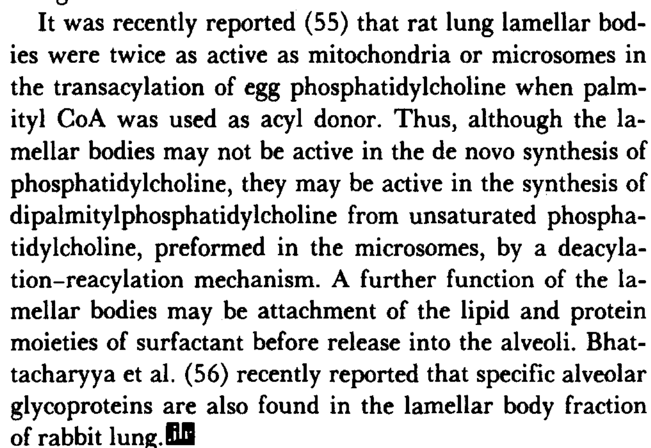
atively high glycerolphosphate phosphatidyltransferase specific activity, although its contribution to the total synthesis of phosphatidylglycerol is small, but its contribution to the synthesis of phosphatidylcholine could be attributed to microsomal contamination. The lamellar body fraction, therefore, in addition to storing surfactant phospholipid, is active in the synthesis of at least one of the major surface-active components of pulmonary surfactant, phosphatidylglycerol.

Phosphatidylglycerol synthesis also takes place in the mitochondria and microsomes. While this study was in progress, the data of Hallman and Gluck were published (34). These authors also reported that phosphatidylglycerol synthesis takes place in both the mitochondria and microsomes, but they attributed lamellar body synthesis to microsomal contamination. These workers, however, demonstrated the transfer of newly synthesized phosphatidylglycerol from the microsomes, but not from the mitochondria, to lamellar bodies and alveolar wash material. Thus, it is possible that surfactant phosphatidylglycerol is synthesized both in the lamellar bodies themselves and in the unique rough microsomal system from which it is transferred to the lamellar bodies. Hallman and Gluck (34) suggested that mitochondrial phosphatidylglycerol synthesis may be for cardiolipin formation, as is the case in brain (50) and liver (31, 51). In the present study, however, there was no evidence of cardiolipin synthesis.

Cholinephosphotransferase activity in rat lung, as in other organs (52), is largely microsomal. This is in agreement with Gluck, Sribney, and Kulovich (53), who found that the incorporation of labeled choline from CDPcholine into phosphatidylcholine in fetal rabbit lung was mainly microsomal. Chevalier and Collet (54), who studied choline incorporation in vivo in the mouse, also concluded that the bulk of phosphatidylcholine biosynthesis took place in the endoplasmic reticulum, the lamellar body contribution being small.



It was recently reported (55) that rat lung lamellar bodies were twice as active as mitochondria or microsomes in the transacylation of egg phosphatidylcholine when palmityl CoA was used as acyl donor. Thus, although the lamellar bodies may not be active in the de novo synthesis of phosphatidylcholine, they may be active in the synthesis of dipalmitylphosphatidylcholine from unsaturated phosphatidylcholine, preformed in the microsomes, by a deacylation–reacylation mechanism. A further function of the lamellar bodies may be attachment of the lipid and protein moieties of surfactant before release into the alveoli. Bhattacharyya et al. (56) recently reported that specific alveolar glycoproteins are also found in the lamellar body fraction of rabbit lung.

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