papers and notes on methodology

Isolation and characterization of lamellar body material from rat lung homogenates by continuous linear sucrose gradients

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Abstract A technique is described for isolating lamellar body material from rat lung. Membranes with relative densities ranging between 1.050 and 1.074 g/ml were isolated by centrifugation of crude lung homogenates upward through continuous linear sucrose gradients at 40,000 rpm (199,000 *g*) for 3 hr. Their protein and lipid content was characteristic of that of lamellar bodies. They were free of contaminating microsomal and mitochondrial marker enzymes but contained enzyme activities associated with lysosomes and Golgi complex. Longer or repeated centrifugation resulted in a reduced yield and an apparent transformation of some of the material to lower densities. Electron microscopy revealed that most of the images represent disrupted rather than intact lamellar bodies. Other methods for preparation of lamellar bodies entail either sedimentation or pelleting at interfaces between sucrose solutions. Such preparations are often contaminated with endoplasmic reticulum membranes and have apparently lost the more fragile bodies. The present technique reveals the heterogeneous nature of lamellar body material and should be useful in a search for lamellar body precursors and in the investigation of the mechanisms by which surfactant is synthesized or assembled.—**Gilder, H., R. H. Haschemeyer, G. F. Fairclough, Jr., and D. C. Mynarcik.** Isolation and characterization of lamellar body material from rat lung homogenates by continuous linear sucrose gradients. *J. Lipid Res.* 1981. **22:** 1277–1285.

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About one-tenth of the volume of lung parenchyma consists of Type II cells or granular pneumocytes. These are the cells that synthesize lung surfactant, a specific complex of lipid and protein which is essential for maintaining the stability of the small air spaces in the lungs (1, 2). Type II cells contain distinctive inclusions, the lamellar bodies which are the source of the surfactant. These bodies appear to originate from the endoplasmic reticulum, with the Golgi complex an intermediate step in the formation (3–6). Multivesicular forms, some containing lysosomal enzymes, are early manifestations of the lamellar bodies (1, 3, 6–9). The mature lamellar body migrates to the apex of the cell and is extruded into the alveolar lumen forming a surface film (3, 4, 6, 10). The surfactant produced or assembled within the lamellar body is characterized by a high content of phosphatidylcholine, 50 to 70% of which is the dipalmitoyl species (11–14).

The high lipid content of the lamellar bodies facilitates their isolation from other organelles by density gradient techniques, and several methods for doing this have been described (15–23). In these reports, the amounts recovered, as well as the compositions of the lamellar bodies, differ considerably depending on details of the isolation technique. For example, the phospholipid-to-protein (mg/mg) ratios varied between 1.2 and 12 (11, 15–19). In addition, the enzymatic makeup of these isolated lamellar

Abbreviations: CLH, crude lung homogenate; AF, alveolar fluid; TLC, thin-layer chromatography.

bodies indicates contamination by other organelles, especially by endoplasmic reticulum membranes.

The present work describes a new centrifugation method which isolates membranous material derived from lamellar bodies on a continuous sucrose density gradient. The study characterizes fractions at different densities in terms of yield, composition, enzymatic activity, stability to further centrifugation, and freedom from contamination by other organelles. Since the different fractions may be associated with different stages in lamellar body maturation, the method has a potential for a more detailed investigation of the synthesis and secretion of lung surfactant.

METHODS

Male rats weighing 250–300 g were anesthetized with intraperitoneal sodium pentobarbital (0.2 ml of a 6-mg/ml solution per 30 g of rat). The abdominal cavity and thorax were opened. The descending aorta at the level of the kidney was severed and the perfusion of the pulmonary vessels was immediately begun by the slow injection of 20 ml of ice-cold saline into the right auricle via a 22-gauge needle in the inferior cava. Complete blanching of the lung usually occurred after 15 ml had been injected. A plastic catheter was tied into the trachea, the lungs were filled to capacity with air (about 10 ml), deflated, and then lavaged with four portions of 5 ml each of cold saline. From this time, except as noted, samples and reagents used in the preparation were kept on crushed ice. All sucrose solutions mentioned hereafter were prepared in 0.01 M Tris–HCl buffer (pH 7.4).

The lungs were removed, rinsed with ice-cold saline, blotted, trimmed of all visible airways, weighed, minced finely with scissors and homogenized with 1.5 parts of 0.51 M sucrose, using a loosely fitting Teflon plunger (clearance of about 0.09 mm) with a Pyrex Potter homogenizer. Because of the excess moisture in the lung due to the perfusion and lavage, the final concentration in the homogenate was approximately 0.33 M sucrose. Debris and intact cells were spun down at 2200 rpm (600 *g*) for 10 min in a Sorvall RC2-B ultracentrifuge. The sediment was re-extracted with 1/4 volume of 0.33 M sucrose and the two supernatants were pooled and strained through four layers of gauze.

The crude lung homogenate was brought to 0.75 M sucrose by the addition of 2.5 M sucrose. Three-ml aliquots were placed in 13.4-ml polyallomer tubes and overlaid with a continuous linear density gradient prepared with equal volumes of 0.66 M and 0.30 M sucrose (densities relative to water at 4°C of 1.088 g/ml and 1.040 g/ml, respectively). The tubes were centrifuged for 3 hr at 40,000 rpm (199,000 *g*) at 4°C in a Beckman L2-65B preparative ultracentrifuge with an SW40 swinging bucket Ti rotor.

The gradients were separated into 0.75-ml increments with a density gradient fractionator, fraction collector, and absorbance monitor (Instrumentation Specialties Company, Lincoln, NE). This step was carried out at room temperature and the fractions were refrigerated as they came off the gradient. The relative densities (referred to hereafter simply as "densities") of sucrose gradient fractions were calculated from refractometric measurements at room temperature, and are reported here as values at 4°C. A spectrophotometer continuously monitored absorbance at 254 nm of fractions removed from the gradient. At this wavelength, absorbance is due to both protein content of the particles and the scatter of light due to their size. Because of the semiquantitative nature of this measurement, no attempt to calculate absorbance was made. Rather, the areas under the UV absorption curve tracings were measured in cm^2, and these areas were summed for the figures to indicate the total derived from the number of rats used in the experiment.

Fractions of identical gradients were pooled, Tris buffer was added to bring the sucrose concentration to 0.25 M, and the samples were spun at 10,000 rpm (20,000 *g*) for 1 hr. The sedimented material was suspended in Tris buffer and frozen at −20°C until used (within 7 days) for protein, lipid, and marker enzyme analysis.

The lamellar body material prepared as above was compared with preparations isolated by the technique of Page-Roberts (16) as modified by Engle (11), which utilizes two centrifugations. In the first, particles are collected at the interface between 0.33 M and 0.75 M sucrose; in the second, they are resuspended in 0.58 M sucrose (density of 1.08 g/ml), layered with 0.33 M sucrose, and centrifuged. The lamellar body film found between the 0.33 and 0.58 M sucrose layers is collected. Some of these bodies, prepared by the two-step pelleting technique, were resuspended in 0.75 M sucrose and centrifuged through the continuous gradient as above.

The alveolar lavage fluid was spun at 2200 rpm (600 *g*) for 10 min to remove cellular elements. The supernatant was brought to 0.01 M Tris buffer and centrifuged at 10,000 rpm (20,000 *g*) for 1 hr in the Sorvall centrifuge. The sediment was suspended in 0.75 M sucrose, overlaid with the continuous sucrose gradient, and centrifuged as above.

Mitochondria and light and heavy microsomes were isolated by the method of Page-Roberts (16).

Lipids were extracted from the suspensions by the technique of Folch, Lees, and Sloane Stanley (24),

and were quantitated by the charring method of Marsh (25). The standard curves for phospholipids and phosphatidylcholine by the charring method were prepared by pipetting dipalmitoylphosphatidylcholine dissolved in chloroform (in amounts from 0.01 to 0.12 mg) into test tubes. These were dried under a stream of nitrogen at 38°C. About 25 mg of silica was added to each tube as its presence affected the total amount of charring and it was present in the unknown samples separated by TLC. We found that total lipids of rat lung, when charred as above, gave a curve identical to that of dipalmitoylphosphatidylcholine, so that one standard curve was used for analyses of total lipid, phospholipid, and phosphatidylcholine.

Aliquots of the extracted lipids were applied to silica gel G on glass plates. For total lipid, the silica was scraped into 15 × 150 mm test tubes without thin-layer chromatography (TLC). For total phospholipids, the plate was developed first in isopropylether–acetic acid 96:4 (v/v) to move apolar lipids about 10 cm from the point of application. The plate was dried and developed in the same direction in the second solvent system, chloroform–methanol–water 65:25:4 (v/v/v) until the front had moved about 3 cm. For phosphatidylcholine, the plates were developed in the second solvent system only and the front was allowed to go to the top of the plate. Phospholipid and phosphatidylcholine spots were visualized with iodine vapor and identified by known markers applied to each plate.

Acid phosphatase, as a marker for lysosomes, was measured by a modification of the method of Bessey, Lowry, and Brock (26), wherein 7.6 mM p-nitrophenylphosphate in sodium citrate buffer at pH 4.85 was the substrate. Activity was expressed in μmol/min of p-nitrophenol released.

Golgi material was quantitated by the method of Morré (27) in which UDP-galactose: N-acetylglucosamine galactosyltransferase (UDP-galactose transferase) was measured. Radioactive galactose is transferred from UDP-[^{14}C]galactose to N-acetylglucosamine to form [^{14}C]-N-acetylaminolactose which is isolated by passage through an ion-exchange column. The hydrolysis of UDP-galactose in this procedure was calculated and used as an additional assay for lysosomal activity.

The NADPH-cytochrome c reductase procedure of Sottocasa et al. (28) was utilized as a marker enzyme for microsomes, and the succinate-INT (2-(p-iodophenyl)-3-3 (p-nitrophenyl)-5-5 phenyltetrazolium) reductase method of Pennington (29) was utilized as a marker enzyme for mitochondria. In all enzyme assays, results are proportional to the amount of protein present and to the time of incubation.

Protein was determined by the method of Lowry et al. (30). Amounts of protein, lipids, and enzymes in the lamellar body material are referred to in terms of the amount recovered from 100 mg of crude lung homogenate (CLH) protein (see Tables). Approximately 50 mg of protein are present in the crude lung homogenate prepared from a 250-g rat. Enzymatic data are also presented as specific activity, that is, in terms of activity per minute per mg of protein.

For electron microscopy, material from the gradients was fixed in a mixture of osmium tetraoxide and glutaraldehyde in cacodylate buffer, stained with uranyl acetate and dehydrated using the technique of Gil and Reiss (15). Additional preparations were studied using the freeze fracture technique of Miller and Torreyson (31).

RESULTS

When crude lung homogenate is centrifuged on a continuous sucrose gradient between densities of 1.040 and 1.088 g/ml for 3 hr at 40,000 rpm (199,000 *g*), material accumulates in a cloudy band about one-third of the way down from the top of the gradient, with clear areas above and below the band and a thin lipid film at the top. Results from a typical experiment are shown in **Fig. 1-A** which records the area under the tracing of the absorption at 254 nm of the fractions collected from the gradient. A broad band between densities of 1.050 and 1.074 g/ml suggests that the material isolated from crude lung homogenates on this gradient is heterogeneous in lipid content and size. For comparison, the same Fig. 1-A shows the corresponding curve for lamellar bodies prepared by a commonly employed pelleting method (16) and then resuspended and centrifuged through the continuous sucrose density gradient. The latter differs in that the position of the curve is shifted somewhat toward a heavier density, and the yield is reduced. The densities at which the material was isolated by the present method were the same as those observed by other workers using other centrifugal procedures to isolate lamellar bodies. Hoffman (17) and Gil and Reiss (15) placed resuspended pellets from lung homogenates on top of discontinuous sucrose gradients and found lamellar bodies to band between densities of 1.059 and 1.070 g/ml. These are the same densities between which Page-Roberts (16) and others (12, 21, 22) isolated lamellar bodies by pelleting in two sequential steps.

Material from alveolar fluid sediments, centrifuged through the same sucrose gradients, peaked at a density of 1.054 g/ml and frequently showed a secondary smaller peak at 1.064 g/ml (Fig. 1-A).

Prolonged centrifugation on the gradient alters the

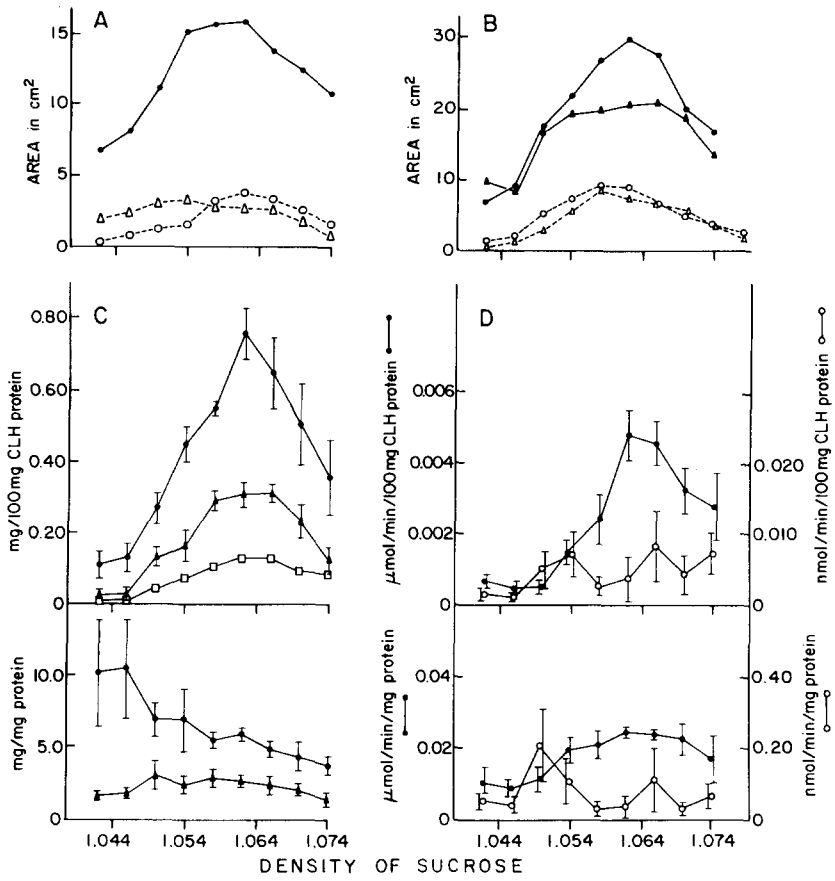

Fig. 1. Lamellar body material recovered on continuous linear sucrose density gradients. A: Recovery of particles absorbing UV at 254 nm from the gradient of rat lung homogenate ● —— ●; from a suspension of lamellar bodies prepared by pelleting O-----O; and from a suspension of alveolar fluid sediment Δ-----Δ, in a single experiment. Lungs from seven rats totaling 1,720 g body weight yielded 288 mg of crude lung homogenate protein. One-half of this homogenate was used for centrifugation through continuous sucrose gradients. Lamellar bodies were prepared by the technique of Page-Roberts (16) from the other half and a suspension of alveolar fluid sediment was used under an additional gradient. All gradients were centrifuged for 3 hr. The areas in square cm under the recorded UV absorption curves for the density of each 0.75 ml increment were summed. The curves represent the total areas derived from the number of rats used. B: Effect of 3 and 17-hour centrifugation on the recovery of lamellar body material as measured by absorbance of UV at 254 nm of the gradient. Areas represent the amounts recovered from 475 mg of crude lung homogenate protein from eight rats, totaling 2,780 g body weight. Procedure was similar to that described for A. Gradient of crude lung homogenate, 3 hr ● —— ●; gradient of crude lung homogenate, 17 hr ▲ —— ▲; gradient of suspension of pelleted lamellar bodies (16), 3 hr O-----O; and gradient of suspension of pelleted lamellar bodies, 17 hr Δ-----Δ. C: Lipid, phospholipid, and protein content of lamellar body material isolated on gradients from crude lung homogenates. Fractions from each gradient with the same density were pooled, diluted with buffer, and sedimented. Protein and lipid contents are in terms of mg per 100 mg of crude lung homogenate (CLH) protein (above) and mg/ml protein or specific activity (below). The figure represents the mean of four experiments. Total lipid ● —— ●; phospholipid ▲ —— ▲; protein □ —— □. Vertical lines show standard errors. D: UDP-galactose:acetylglucosamine galactosyltransferase and acid phosphatase activity in fractions from particles isolated on gradients from crude lung homogenates. The curve of the galactosyltransferase O —— O represents the mean of five experiments; that of acid phosphatase ● —— ●, the mean of six experiments. Vertical lines show standard errors.

distribution of lamellar body material (Fig. 1-B). The amount of material present at a density of 1.060 g/ml at 3 hr is reduced markedly at 17 hr of centrifugation. Protein and lipid analyses also show a loss of material from the fraction due to the longer centrifugation (not illustrated). In contrast, the figure shows that particles from a gradient prepared from pelleted lamellar bodies do not show this difference between the 3- and 17-hr centrifugations. Similarly, the UV absorption of gradient fractions of alveolar fluid material, having been pelleted in their preparation, were not depressed further by centrifugation (not shown).

The mean lipid, phospholipid, and protein contents of lamellar body material from gradients in four experiments are shown in Fig. 1-C. The specific activity in terms of mg of phospholipid per mg of protein shows an increase as the gradient fractions decrease in densities from 1.074 to 1.050 g/ml. The slope of the specific activity curve of total lipid is about twice that of the phospholipid suggesting that both phospholipid and non-phospholipid lipid contribute

to the decreasing density of the particles resulting in their distribution along the gradient. The small amount of lipid at the top of the gradient with the very high lipid specific activity probably consists in part of free lipid.

The composition of lamellar body material isolated as described above is compared to that for lamellar bodies prepared by the two-step pelleting method in **Table 1**. For the table, the protein and lipid content of material isolated from fractions with densities between 1.050 and 1.074 g/ml were summed. Although the total amount of protein in preparations isolated by each of the two methods is about the same, the total lipid, phospholipid, and phosphatidylcholine in the material isolated from crude lung homogenates by our one-step continuous gradient procedure is higher, with a resulting higher ratio of lipid to protein in this procedure than in the pelleting procedure.

When the lamellar bodies prepared by the pelleting method are resuspended in 0.75 M sucrose by gentle homogenization and recentrifuged through a continuous sucrose gradient, a substantial loss occurs, especially of the protein. This results in an increase of the specific activities of all three lipids to levels that approach those of the one-step procedure. The additional manipulation eliminated some of the lamellar bodies, but those remaining are qualitatively similar to material prepared by the one-step procedure.

The purity of the lamellar body material isolated from crude lung homogenates in high yield by our method was assessed in terms of several important criteria. **Table 2** shows that this material is devoid of enzyme activities commonly employed as markers for microsomal and mitochondrial material. On the other hand, lamellar bodies prepared by the two-step pelleting technique contained NADPH-cytochrome c reductase activity (for microsomes) at 0.5% of the total amount present in crude lung homogenates. However, this enzyme activity is lost from the preparation after recentrifugation through the continuous gradient (not shown in Table). Succinate-INT reductase, the mitochondrial marker, is absent from both preparations.

Since the lamellar bodies are presumably derived from the Golgi complex (5, 6), one might expect enzymatic evidence of this in lamellar body preparations. The activity of UDP-galactose transferase, a marker enzyme for Golgi membranes, is shown in **Table 3.** The enzyme is present in both preparations to the extent of about 0.7% of that in crude lung homogenates. Most of the activity of the material prepared by pelleting is lost when it is centrifuged through the gradient.

Two marker enzymes used for detecting lysosomes are present in the preparations as well (Table 3). Acid phosphatase is present in both preparations at levels of less than 2% of that in the crude lung homogenate. As with the Golgi marker enzyme, the total activity in the particles prepared by the one-step method is comparable to that of the two-step pelleted preparation; however most of the latter is lost upon recentrifugation through the continuous sucrose gradient. Acid phosphatase activity in the alveolar fluid material also is lost during centrifugation through the continuous gradient. The UDP-galactose hydrolase activities of these preparations parallel those of the acid phosphatase (Table 3). The mean UDP-galactose transferase and acid phosphatase activities of lamellar body material isolated at different sucrose densities using the one-step centrifugation technique are plotted in Fig. 1-D. UDP-galactose transferase activity is present along the entire gradient but a small increase in the specific activity at a density of 1.054 g/mol is apparent. On the other hand, acid phosphatase content is low at low densities but increases to a peak in material having a density of 1.060 g/ml in parallel with the protein peak. This results in a constant specific activity in the fractions having densities between 1.054 and 1.074 g/ml.

TABLE 1. Protein, total lipid, phospholipid, and phosphatidylcholine content of lamellar body preparations

Method of Isolation	No. of Experiments	Protein	Total Lipid		Phospholipid		Phosphatidylcholine	
		per 100 mg CLH Protein[c]	per 100 mg CLH Protein	per mg Protein	per 100 mg CLH Protein	per mg Protein	per 100 mg CLH Protein	per mg Protein
One-step gradient[a]	6	0.70 (0.08)	4.07 (0.58)	4.97 (0.72)	2.24 (0.37)	2.73 (0.49)	1.53 (0.30)	2.00 (0.31)
Two-step pelleting[b]	6	0.66 (0.12)	1.51 (0.32)	2.06 (0.27)	1.04 (0.21)	1.52 (0.13)	0.90 (0.20)	1.14 (0.21)
Two-step pelleting, recentrifuged through gradient[a]	3	0.19 (0.04)	1.07 (0.14)	3.38 (1.01)	0.46 (0.12)	2.42 (0.65)	0.41 (0.10)	2.36 (0.73)

[a] The content of protein and lipid in the gradient fractions at densities from 1.046 to 1.074 g/ml were summed.
[b] Method of Page-Roberts (16).
[c] To facilitate comparison between experiments with different numbers of rats, data are in terms of mg (SE) derived from that amount of crude lung homogenate that contains 100 mg of protein (CLH protein).

TABLE 2. Microsomal and mitochondrial enzyme activity of subcellular fractions of lung

	NADPH-Cytochrome Reductase			Succinate-INT Reductase		
	No. of Experiments	Total Activity μmol/min per 100 mg CLH Protein	Specific Activity μmol/min per mg Protein	No. of Experiments	Total Activity nmol/min per 100 mg CLH Protein	Specific Activity nmol/min per mg Protein
Lamellar body material (one-step gradient)	5	<0.001	<0.001	3	<0.1	<0.1
Lamellar body material (two-step pelleting)	7	0.016 (0.006)	0.017 (0.004)	5	nd[a]	
Light microsomes	5	0.037 (0.010)	0.76 (0.20)	3	nd	
Heavy microsomes	4	0.023 (0.008)	0.032 (0.008)	3	nd	
Mitochondria	4	0.023 (0.008)	0.010 (0.004)	3	4.27 (1.86)	3.19 (1.31)
CLH	7		0.018 (0.005)	6		2.36 (0.30)
AF	4	<0.002		5	0	

[a] nd, Not detected.

Total activities are in terms of the mean (SE) in crude lung homogenate (CLH) containing 100 mg of protein and, for alveolar fluid (AF) sediment, the sediment collected from rats yielding that amount of CLH protein.

An experiment was designed to determine the extent of deterioration or alteration of lamellar body material during the centrifugation process and the feasibility of purifying fractions by recentrifugation. For these purposes, a crude lung homogenate was initially centrifuged for 3 hr through a continuous gradient prepared with equal volumes of 1.167 M and 0.20 M sucrose (densities of 1.156 and 1.027 g/ml, respectively). The top six 0.75-ml fractions having mean densities from 1.032 to 1.087 g/ml were collected, brought to 0.75 M sucrose without sedimentation, and recentrifuged through the standard continuous 0.66 M to 0.30 M sucrose density gradient for 3 hr.

The data from one of the above described experiments is given in **Fig. 2.** The peak of each fraction is true to its original flotation characteristics. Thus, the densities of fractions from the first gradient are 1.032, 1.043, 1.054, 1.065, 1.076, and 1.087 g/ml, while the corresponding densities at the peaks of the secondary gradients are 1.042, 1.051, 1.053, 1.064, 1.074, and 1.080 g/ml, respectively (Fig. 2). It is apparent that a portion of the material in each fraction has become heavier or lighter than its original density and some of it has sedimented. Protein, phospholipid, and acid phosphatase levels were determined for the initial fractions and in pools of the secondary fractions. Although nearly 100% of the protein and phospholipid in the fractions with densities between 1.043 and 1.076 g/ml can be accounted for in the sum of all of the fractions of the secondary gradients, including the sedimented material, only 34 to 49% of the protein at the densities of the initial fractions of the first gradient is re-

TABLE 3. Golgi and lysosomal enzymes in lamellar body preparations, crude lung homogenate, and alveolar fluid sediments

	UDP-Galactose:N-Acetylglucosamine Galactosyltransferase			Acid Phosphatase			UDP-Galactose Hydrolase		
	No. of Experiments	Total Activity nmol/min per 100 mg CLH Protein	Specific Activity nmol/min per mg Protein	No. of Experiments	Total Activity μmol/min per 100 mg CLH Protein	Specific Activity μmol/min per mg Protein	No. of Experiments	Total Activity μmol/min per 100 mg CLH Protein	Specific Activity μmol/min per mg Protein
Lamellar body material (one-step gradient)	10	0.067 (0.015)	0.070 (0.014)	6	0.048 (0.004)	0.062 (0.010)	9	0.24 (0.06)	0.27 (0.06)
Lamellar body material (two-step, pelleting)	3	0.073 (0.064)	0.028 (0.019)	4	0.059 (0.008)	0.091 (0.019)	3	0.11 (0.04)	0.12 (0.05)
Lamellar body material (two-step, pelleting, recentrifuged through gradient)	3	0.005 (0.002)	0.043 (0.009)	2	0.012 (0.004)	0.016 (0.005)	4	0.14 (0.08)	0.19 (0.07)
CLH	10	9.42 (1.29)	0.095 (0.013)	6	3.20 (0.38)	0.032 (0.004)	9	13.0 (1.27)	0.13 (0.01)
AF	5	0		5	0.012 (0.002)	0.017 (0.003)	3	0.38 (0.08)	0.99 (0.34)

Total activities are in terms of the mean (SE) in crude lung homogenate (CLH) containing 100 mg of protein, and for alveolar fluid (AF), the sediment collected from rats yielding that amount of crude lung homogenate protein. Specific activities are in terms of mean (SE) per mg protein.

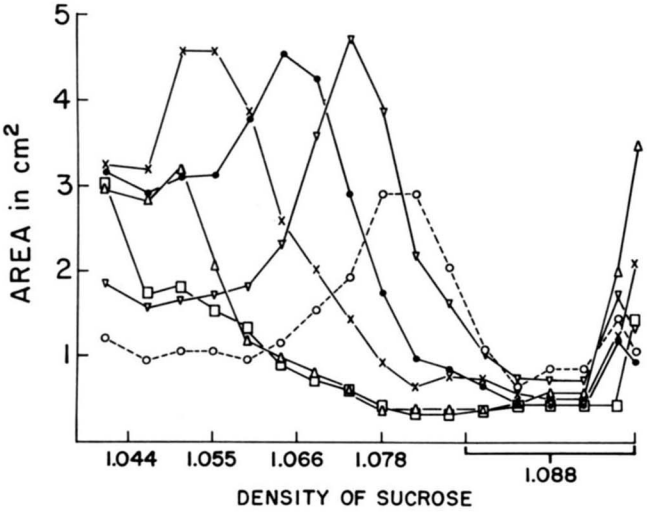

Fig. 2. Recovery of material monitored by absorbance at 254 nm from fractions of a preparation of lamellar bodies re-applied to a second gradient. Areas represent the amount recovered from 358 mg of crude lung homogenate protein from six rats, totaling 2,070 g body weight. Fractions in 0.75-ml increments from the first gradient centrifugation were pooled, brought to 0.75 M sucrose, and 4-ml aliquots were centrifuged through the second gradient ranging in concentration from 0.66 M to 0.30 M sucrose. Densities of the first fractions and their symbols are, as follows: □ —— □, 1.032; △ —— △, 1.043; × —— ×, 1.054; ● —— ●, 1.065; ▽ —— ▽, 1.076; and ○ – – – ○, 1.087.

covered at that density peak in the secondary gradient. Similarly, 39 to 69% of phospholipid in the initial fractions of the first gradient is recovered at the corresponding density of the secondary gradient. In contrast to the high total recovery of protein and phospholipid, acid phosphatase activity markedly decreases in the secondary gradients presumably because of inactivation during the extra manipulation.

An electron micrograph of a typical section of the gradient material at the peak density of 1.06 g/ml, sedimented, fixed, and stained according to Gil and Reiss (15) is shown in **Fig. 3-A.** An occasional lamellar structure is seen but most of the section consists of characteristic images that are atypical for other cellular subfractions. Samples taken above and below the peak give similar pictures. On freeze fracture (Fig. 3-B), most of the material is in the form of elongated sacs. Typically these are joined together in one place. Particles that are presumed to be proteins are associated with the regions where the sacs are joined, while the rest of the sacs appear devoid of proteins. Besides the joined sacs there are numerous other sac-like membranous structures, but no membranes typical of plasma membranes, mitochondria, nor rough endoplasmic reticulum.

DISCUSSION

The objective of this work was to develop a method of isolating biologically active lamellar body material from lung homogenates in high yield and free of contaminating organelles in order to permit the study of surfactant synthesis or assembly and the maturation of lamellar bodies. The method we

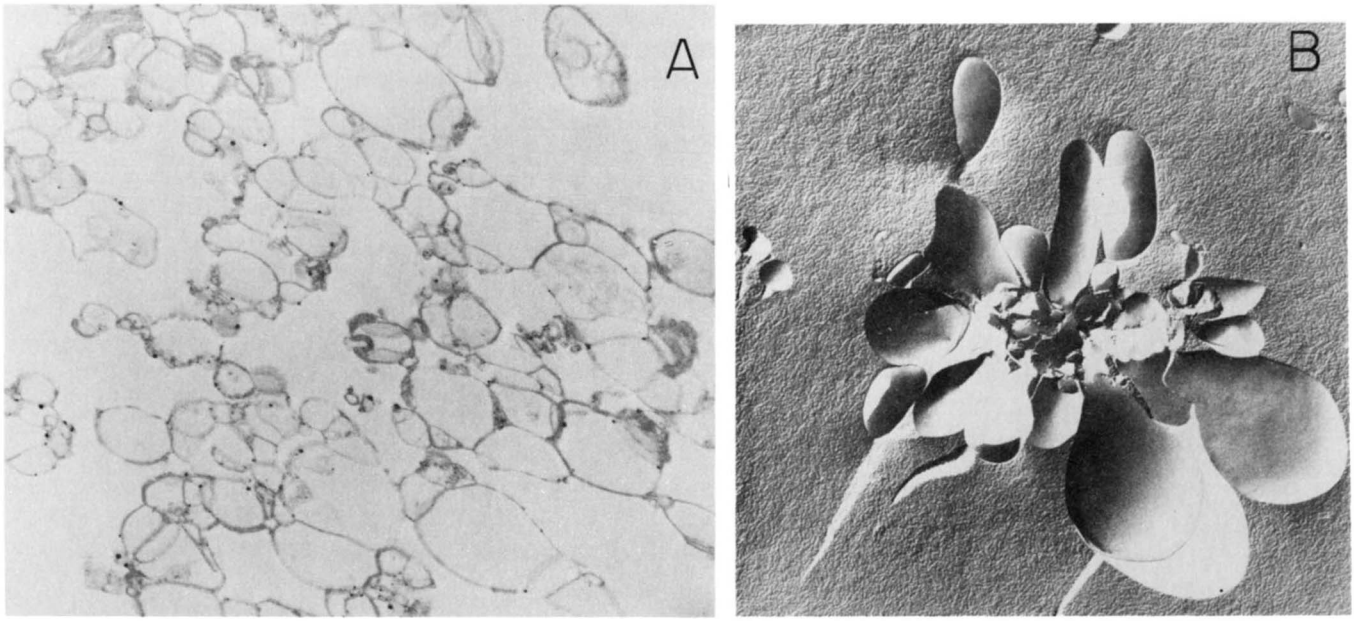

Fig. 3. Electron micrographs of the lamellar body material isolated at the peak of the density gradient and sedimented before processing for electron microscopy. A, Pellet fixed and stained according to Gil (15); loose membranous material showing an occasional fragment which appears lamellated. 10,200×. B, Pellet freeze fractured; multiple sacs joined in one place; scattered particles dot a few surfaces in the area where the sacs are joined. 15,300×.

report avoids pelleting until after separation of the particles on density gradients. The result is a high yield of material which ranges in densities from 1.050 to 1.074 g/ml.

Techniques which require pelleting or packing between sucrose solutions risk absorption of microsomal particles to clumped lamellar body material at the interfaces (15–17). This appears to be the case here in the preparation using the two-step procedure which was found to contain microsomal marker enzyme activity (Table 2). Such techniques also result in lower yield of lamellar body material and may produce artificially homogeneous preparations because of incomplete collections and the elimination of fragile portions. This is illustrated in the present study in which the lipid yield of our preparations was three times that of the pelleting method although the protein yield was about the same. It is probable that the extra protein arises from contamination by other organelles.

Electron microscopy of our material indicated that it consisted largely of fragmented rather than intact lamellar bodies. The fragmentation may result from the experimental protocol, for example, a more vigorous homogenization, and the exposure to higher sucrose concentrations than employed in other procedures. It could also be due, at least in part, to the presence in the preparations of some lamellar bodies with greater *a priori* fragility.

Several considerations support our conclusion that the material we isolate are fragments of authentic lamellar bodies even though we do not see many typical lamellar structures. First, the density at which the material is separated is typical of the range at which lamellar bodies are isolated by others (12, 16, 21, 23) and is reproducible from one experiment to another. Second, the phospholipid to protein ratio is typical of these organelles. This ratio is frequently used to judge the purity of lamellar body preparations, but in fact the present study reveals that a high ratio does not guarantee that the preparation is free of contamination from other organelles. Finally, acid phosphatase and UDP-galactose hydrolase, enzyme markers for lysosomes, are present not only in our preparations but also in those using the pelleting technique. Lysosomes are known to be closely associated with lamellar bodies and have been detected in them both chemically (17) and cytochemically (1, 3, 6–9). They are believed to facilitate the secretion of lamellar bodies.

The slopes of the specific activity curves of total lipid and phospholipid between densities of 1.050 and 1.074 g/ml suggest that phospholipid and nonphospholipid lipid contribute equally to the decreasing densities of the particles over the range, thus providing a basis for the separation achieved on the gradient. However, since we avoided the long time centrifugation required to assure isopycnic equilibrium, some variability in lipid levels at these densities may be the result of size variation.

Some lamellar body fragments in the above experiments can be altered during in vitro preparation as is evident in the recentrifugation study described in Fig. 2. The experiment is consistent with the work of Sanders et al. (32) and Petty et al. (33) who showed that the material in lamellar bodies within granular pneumocytes was transformed into less dense forms upon secretion or under other deleterious conditions. Williams (34) has presented morphological evidence of the conversion of lamellar bodies to tubular myelin in the alveoli of the fetal lung.

The presence of the Golgi assay marker enzyme may be interpreted in several ways. The present technique may have resulted in isolation of Golgi bodies along with lamellar bodies. As precursors of the lamellar bodies they would tend to form vesicles containing lipid mixtures typical of them and having the same densities. The other possibility is that the lamellar bodies do indeed contain enzyme activity typical of the Golgi body as an integral component. The Golgi marker enzyme seems to be concentrated somewhat near a density of 1.050 g/ml where the specific activity peaks and is twice that of crude lung homogenate (Fig. 1-D, Table 3).

In this study, lamellar body fragments are fractionated with respect to their densities. We believe the procedure described will prove useful in investigating the maturation of lamellar bodies and the synthesis of lung surfactant. We have already demonstrated this in studies described in another article showing different patterns of distribution along the gradient of three enzymes in the biosynthesis of phospholipid (35).

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