Lung Concentric Laminar Organelle

HYDROLASE ACTIVITY AND COMPOSITIONAL ANALYSIS

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SUMMARY

An analytical study was made of the concentric lamellar organelles (CLO) isolated from rabbit lung. These subcellular structures, which are normally located in the type II cells of mammalian lungs, were purified by flotation using a discontinuous sucrose density gradient. Electron microscopy of the isolated CLO revealed many intact structures about 1 \( \mu \)m in diameter with densely packed trilaminar membranes having, also, a granular core and an outer limiting membrane.

The CLO contained a preponderance of polar lipids, mostly as phosphatidylcholine and phosphatidylethanolamine, with trace amounts of neutral lipid. A phospholipid to protein ratio of approximately 12:1, was repeatedly obtained and served as a criterion for organelle purification. Acrylamide gel electrophoresis of the detergent-solubilized CLO revealed a prominent staining band migrating with albumin.

These structures had numerous hydrolases similar to those observed in lysosomes. High specific activities were observed for such enzymes as acid phosphatase, aryl sulfatase and \( \beta \)-N-acetylglucosaminidase. Multiple forms of the latter enzyme were observed in zonal electrophoresis studies. Purification of the CLO resulted in a 20- to 40-fold increase in specific activity over homogenates for most of the hydrolases. Cathepsin D, acid DNase, acid RNase and neuraminidase, other lysosome-associated hydrolases, were not detected in the CLO fraction.

It is suggested from these and other studies that CLO are secreted to the acellular lining of lung where they contribute surfactant phospholipids, and possibly, functional hydrolases. The lung CLO also appear to have many properties in common with those subcellular structures associated with certain human genetic lipidoses.

Two epithelial cell types of the lung, the type I and type II cells are prominent in establishing the thin blood-air barrier for pulmonary gas exchange in the terminal airways. Morphologically, the type II cell (granular pneumocyte, septal cell, dust cell, corner cell, or great alveolar cell) may be readily distinguished from the type I cell, and perhaps all other healthy cell types, by the presence of osmiophilic concentric laminar organelles (CLO) (lamellar bodies, osmiophilic inclusion bodies, cytosomes) which are generally 1 to 2 \( \mu \)m in diameter and occur freely in the cytoplasm. With the discovery of a substance lining the air sacs (alveoli) which reduces alveolar surface forces and maintains lung mechanical stability, the CLO were postulated to be a storage or secretory form of this material (surfactant) (1-4). It has also been shown that the CLO and the surfactant appear simultaneously in the prenatal lung (5). Subsequent studies showed that an active component of this surfactant system was the saturated phospholipid dipalmitylphosphatidylcholine (6, 7). These findings coincided well with investigations which showed that the lung is very active in phospholipid biosynthesis (8, 9). More recently, structures similar in appearance to the lung CLO were shown in brain and other tissues of patients having familial lipid storage disorders where various lipid hydrolases were deficient (10).

The present communication describes some metabolic and compositional properties of a high purity preparation of the rabbit lung CLO. Among other findings is the demonstration of a number of lysosomal acid hydrolases in a subcellular organelle predominantly composed of phospholipids.

EXPERIMENTAL PROCEDURE

Preparation of Tissue Fractions

For most experiments two to four New Zealand White rabbits (1.2 to 1.8 kg) were killed by a sharp blow on the head and the lungs excised, rinsed in 0.15 M KCl, and then cut into small pieces with scissors. The following operations were carried out at about 4°C. The lung pieces were compressed through an Arbor Tissue Press (model 142, Harvard Apparatus Co., Dover, Mass.) and homogenized (four slow passes) in 2 volumes of 0.25 M sucrose-0.05 M Tris-HCl (pH 7.2 to 7.3) containing 0.1 mM ethylene glycol bis(\( \beta \)-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) using a Potter-Elvehjem homogenizer equipped with a loose fitting pestle (clearance 0.004 to 0.006 inch). The homogenate was centrifuged in a Sorvall RC-2B at 1100 \( \times \) g for 20 min. The supernatant was withdrawn and centrifuged at 9000 \( \times \) g for 15 min. The supernatant (Fraction I) was discarded or used for enzyme marker studies and the pellet was overlayed with 1 ml of the sucrose-EGTA solution, which was gently rocked with albumin.

The abbreviations used are: CLO, concentric lamellar organelle; EGTA, ethylene glycol bis(\( \beta \)-aminoethyl ether)-N,N'-tetraacetic acid; benzphetamine, (\( \pm \))-N-benzyl-N,\( \alpha \)-dimethylphenethylamine hydrochloride.
the pellet so as to remove the loosely sedimented material without fragmenting the whole pellet. This was repeated at least four times, or until the washings became clear. The pooled washings were centrifuged at 23,000 \( \times g \) in a Beckman L3-50 ultracentrifuge for 30 min using a 50 Ti rotor. The supernatant was discarded and the pellet overlayed and washed as described above. The residual pellet, or pellet remaining after the wash steps, is shown as Fraction IIa in the flow diagram (Fig. 1). The pooled washings were then centrifuged again at 23,000 \( \times g \). The resulting pellet also was overlayed and washed as described above, leaving a residual pellet which is shown as Fraction IIb in the flow diagram. The washings were layered over 20 ml of 0.9 \( \text{M} \) sucrose-0.1 \( \text{mM} \) EGTA and centrifuged at 160,500 \( \times g \) for 65 min. The fluffy white band that formed at the interface of the sucrose gradient was diluted with 0.15 \( \text{M} \) KCl and centrifuged in an RC2-B centrifuge at 20,000 \( \times g \) for 30 min using an SM-24 rotor. The small compacted white pellet, the concentric lamellar organelles, was occasionally co-sedimented with an unidentified red brown material. The lamellar organelles could readily be removed by covering the pellet with 1 to 2 ml of the KCl solution and gently swirling the tube in a vortex-genie (Scientific Industries). The resuspended lamellar bodies could now be centrifuged again freed from the colored sediment. For best results this procedure was repeated twice, despite lower yields.

Rabbit alveolar macrophages were harvested from the lung according to the method of Myrvick (11). Only preparations free of erythrocytes were used. Cells were sonicated in a tube immersed in ice-cold water with a Bronson Cell Disruptor (model W185) at 80 watts output using two, 10-s bursts. The 250 \( \times g \) supernatant above the macrophage pellet was further centrifuged at 100,000 \( \times g \) for 50 min. The clear supernatant was then concentrated with an Amicon 202 ultrafiltration cell equipped with a Diaflo UM-10 membrane. The concentrate was used to represent the soluble acellular lining fraction of the lung. The preparation of this extracellular fraction for polyacrylamide gel electrophoresis is described below.

**Lipid Analysis**—The lamellar organelle fraction suspended in a few milliliters of 0.15 \( \text{M} \) KCl was added to 100 ml of chloroform-methanol (2:1, \( v/v \)) and extracted and washed under \( N_2 \) as described by Folch et al. (12). The extract was evaporated to dryness in vacuo at 35° and stored under \( N_2 \) at \(-20^\circ C\) in several ml of the chloroform-methanol solution. Separation of the lipid classes was made on columns of silicic acid (1 \( \times \) 20 cm Bio Sil A, 100 to 200 mesh, Bio-Rad Laboratories). The chloroform extract was evaporated to dryness under a stream of \( N_2 \) and then suspended in 2 ml of hexane-benzaene (17:3 \( v/v \)). The suspension was loaded on to the column and chromatographed for the different lipid classes by using the elution series of Barron and Hanahan (13). Forty milliliters were used for each eluting solvent and 10-ml fractions were collected at a flow rate of 1.0 ml per min. Lipid classes were identified in the eluate by thin layer chromatography of the samples on silica gel plates and subsequently measured as previously described (14). Separation of the phospolid classes by two-dimensional thin layer chromatography was made on 20 \( \times \) 20 cm Adsorbosil-5 (250 \( \mu \text{m} \) thickness) thin layer plates (Applied Science Co.) in a manner similar to that of Rouser et al. (15). The first dimension was developed 13 cm from the origin with chloroform-methanol-20.5% aqueous \( \text{NH}_4 \) \( (0.8:38:15, v/v) \). The plate was left at room temperature in the hood for 5 min and then oven-dried at 45° for 20 min before immersion in the second solvent system of chloroform-acetone-methanol-acetic acid-water (25:10:5:5:1, \( v/v \)). Migration was allowed for 16 cm and the plate was again air-dried in the hood. Spots were detected by spraying with 0.05% (\( w/v \)) rhodamine 6 G (Allied Chemical Co.) followed by brief exposure to \( I_2 \) vapors and viewing under ultraviolet light. Phospholipid phosphorus was determined in column eluates and in the silica scrapings in the manner of Shin (16) and Rouser et al. (17), respectively. In the latter method the concentration of the ascorbic acid solution was reduced to 1% (\( w/v \)). Appropriate blank areas of the silica gel plate were also analyzed.

For fatty acid analysis of phospholipids the appropriate rhodamine-stained spot on the silica gel plate was scraped into Fyrex culture tubes (9-ml volume) with Teflon-lined caps and reacted with 1 ml of BF\(_3\)-methanol (14%, \( w/v \)) under \( N_2 \) in the manner described by Morrison and Smith (18). The pentane-extracted fatty acid methyl esters were analyzed on a Packard 904 gas-liquid chromatograph with a flame-ionization detector. The glass coil column (1.52 m \( \times \) 4 mm inner diameter) contained 10% ethylene glycol succinate on Chromosorb W (AW), 80 to 100 mesh. Inlet, oven and detector temperatures were 188°, 181°, and 205°, respectively. \( N_2 \) was used as a carrier gas at a flow rate of 60 ml per min. Fatty acid methyl esters were identified by co-chromatography (relative retention time) with standards.

**Acrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis was done according to the Allen and Moore (19) modification of the original method of Orstem and Davis (20). The gel casting procedure and power settings were adapted from those used for lipoproteins (Orteco Application Note 32, page 1, Orteco Inc., Oak Ridge, Tenn.). Final concentration of polyoxyethylene lauryl ether (Brij 35, Fisher Scientific Co.) was 0.005% (\( w/v \)). Electrophoresis was carried out in an Orteco model 4200 cell using an Orteco 4100 pulsed constant power supply. The lung concentric lamellar organelles were prepared for electrophoretic analysis by lyophilizing an aqueous suspension of the organelles and solubilizing the resulting powder in 0.5 ml of 30% sucrose containing 10% (\( w/v \)) Triton X-100 (New England Nuclear). The same solution was used to apply bovine serum albumin and the lyophilized soluble acellular lining fraction to the gel. Gels to be stained were placed in 0.01% Amido black in 7.5% acetic acid, stained for 18 hours, then destained and stored in the acetic acid solution. Determination of enzyme activity was made by slicing serially 2-mm sections of the gel slab with a razor and placing the sections in the reaction media for 15 hours at room temperature, or in the case of alkaline phosphatase, at 37°. The sections were finely divided with a glass rod in the reaction vessel.

**Electron Microscopy**—To prepare samples for electron microscopy a 0.5-ml suspension of the concentric laminar organelles in 0.15 KCl was mixed with 3 ml of 0.05 \( \text{M} \) cacodylate buffer, \( p\text{H} \) 7.4, containing 0.25 \( \text{M} \) glutaraldehyde and 0.20 \( \text{M} \) sucrose and centrifuged at 500 \( \times g \) for 30 min. The pellet was broken into small pieces, postfixed with 1% OsO\(_4\) (\( w/v \)) in 0.25 \( \text{M} \) sucrose-0.05 \( \text{M} \) cacodylate, \( p\text{H} \) 7.4, for 30 min at 4°. The specimens were washed with the sucrose-cacodylate buffer, stained with uranyl acetate and lead citrate, dehydrated in a graded series of ethanol, dry propylene oxide, and embedded in Epon. The samples were then sectioned with a Porter-Blum MT-1 ultramicrotome and examined with a Phillips EM 300 electron microscope.

**Enzyme Assays**—The low amount of protein in the CL0 gel electrophoresis copy a 0.5-ml suspension of the concentric laminar organelles in 0.15 KCl was mixed with 3 ml of 0.05 \( \text{M} \) cacodylate buffer, \( p\text{H} \) 7.4, containing 0.25 \( \text{M} \) glutaraldehyde and 0.20 \( \text{M} \) sucrose and centrifuged at 500 \( \times g \) for 30 min. The pellet was broken into small pieces, postfixed with 1% OsO\(_4\) (\( w/v \)) in 0.25 \( \text{M} \) sucrose-0.05 \( \text{M} \) cacodylate, \( p\text{H} \) 7.4, for 30 min at 4°. The specimens were washed with the sucrose-cacodylate buffer, stained with uranyl acetate and lead citrate, dehydrated in a graded series of ethanol, dry propylene oxide, and embedded in Epon. The samples were then sectioned with a Porter-Blum MT-1 ultramicrotome and examined with a Phillips EM 300 electron microscope.
change was measured with a Unicam SP1800 spectrophotometer using a temperature-regulated cell block. The close cell position was used for turbid reaction medias. Potentiometric determination of serine protease activity (21) was measured with a Radiometer pH-stat equipped with a model TTT2 titrator, an ABU13 autoburette, and a thermostatted TTA31 microtitration assembly. NaOH, 0.02 to 0.1 M, was used as the titrant. Other enzyme activities, unless otherwise indicated, were assayed in the manner described in the references: acid phosphatase (22), alkaline phosphatase (23), glucose 6-phosphatase (24), 5'-nucleotidase (25), aryl sulfatase (26), β-N-acetylglucosaminidase (27), β-glucuronidase (28), α-amanitinase (29), β-n-galactosidase (27), β-nitrophenylacetate esterase (30), neuraminidase (31), acid ribonuclease (32), acid deoxyribonuclease (33), cathepsin D (34), neutral protease (35), succinate cytochrome c reductase (36), lactate dehydrogenase (37), and benzphetamine-N-deethylase (38).

Protein was measured by the method of Lowry et al. (39) using crystalline bovine serum albumin as the standard. To remove the turbidity caused by the insoluble lipid residue from the laminar organelles, one drop (about 25 mg) of Triton X-100 was mixed with the contents of each tube prior to measuring absorbance. This procedure clarified the experimental samples without affecting standards.

Materials

All chemicals were generally of commercial origin. All solvents were reagent grade and were redistilled when used for column chromatography. Cholesterol was purchased from a local supplier and recrystallized three times from hot ethanol. Bovine heart cardiolipin and bovine cerebrosides were purchased from General Biochemicals and Supelco, Inc., respectively. All other lipid standards were purchased from the Hormel Institute, Austin, Minnesota or Applied Science Laboratories. Neuraminidase (Clostridium perfringens, type VI) and bovine pancreatic trypsin were obtained from Sigma; α-chymotrypsin (bovine pancreas), bovine submaxillary mucin, calf thymus histone, ribonuclease B (beef pancreas), and deoxyribonuclease II (hog spleen) were purchased from Worthington Biochemical Corp. N-Acetylneuramin-lactose was purchased from General Biochemicals. (+)-N-benzyl-N,α-dimethylphenethylamine HCl (benzphetamine) was a gift from Upjohn Co., Kalamazoo, Michigan.

RESULTS

Initial attempts in this laboratory to isolate the lung CLO were based on the assumption that this structure is predominantly composed of phospholipids which, in turn, would confer low density in density-gradient centrifugation studies. High speed centrifugation of a crude mitochondrial preparation in a 0.25 m/0.9 M sucrose density gradient left a narrow fluffy band at the gradient interface. Further studies revealed that this fraction contained numerous intact CLO and was rich in the palmitic acid species of phosphatidylcholine. Fig. 1 summarizes the steps finally developed in purifying the lung CLO. By carefully washing the intact 23,000 g pellet, a preparation could be obtained which when subjected to the density-gradient step was essentially free of mitochondrial contamination. Later in this study it appeared that EGTA, as used by Williams et al. (40), improved the purity of the CLO at the gradient interface as compared with EDTA or the absence of a chelating agent. Density-gradient centrifugation of Fraction II pellet, which was shown to contain mostly mitochondria, gave a greater total yield of acid hydrolases, but further purification of the CLO was difficult to achieve because of the extent of contamination.

When the same work-up as outlined in Fig. 1 was applied to rabbit liver or brain tissue only a faint deposit of material at the density-gradient interface was observed. More vigorous procedures to homogenize lung tissue, such as the use of a Waring Blender, a sonicator, or a small clearance Potter-Elvehjem homogenizer reduced the yield of intact CLO. Disrupted structures bore a strong resemblance to isolated myelin membranes. In order to obtain preparations free of other organelles and of the highest enzyme specific activity, only homogeneous, white pellets were used. Purification steps to obtain this appearance (see "Experimental Procedure") were always associated with an increase in acid hydrolase activity and the ratio of phospholipid to protein. Electron photomicrographs of the red-brown pigment which co-sedimented with the CLO showed only masses of elongated fibers.

Fig. 1. Flow diagram for the preparation of the rabbit lung concentric laminar organelles. Further details are given under "Experimental Procedure."

Fig. 2. Low magnification electron micrograph of the lung concentric laminar organelles (Fraction II) were isolated by discontinuous sucrose-gradient density centrifugation. The enclosed areas have been enlarged and appear as Fig. 3, A and B.
FIG. 3. High magnification electron micrographs of the lung concentric laminar organelle. The structures appeared as densely packed transverse laminations (A), or as concentrically arranged membranes (B). The enlarged inset of a taken from the (Fig. 3A, inset). Occasionally, multivesicular bodies were found contiguous with intact CLO (Fig. 3C).

Composition—The relatively large lipid content of the CLO was evident from the observation that this tissue fraction was virtually solubilized by admixture with chloroform-methanol (2:1, v/v). Silica gel two-dimensional thin layer chromatography revealed, after rhodamine-I staining, materials that co-chromatographed with samples of phosphatidylethanolamine. Phosphorous analysis of the silica scrapings showed that phosphatidylethanolamine was present at one-fifth the amount of phosphatidylcholine. The presence of sphingomyelin or acidic phospholipids was barely discernible on thin layer chromatography plates even when highly concentrated samples were analyzed. A faintly stained unknown material which migrated farther than phosphatidylethanolamine in both solvent systems (see “Experimental Procedure”) was detected in all experimental samples. This material did not co-chromatograph with cerebrosides, mono- and digalactosyl diglycerides, phosphatidyl N,N-dimethylethanolamine or phosphatidyglycerol. Diposphatidylglycerol (cardiolipin) had retention properties on silica gel similar to that of the unknown, but no significant amount of phosphorous could be detected in thin layer chromatography scrapings of the unknown. The fatty acid composition of CLO phosphatidylcholine and phosphatidylethanolamine is given in Table I. Results are expressed as molar per cent of total and represent the averages obtained from four different experiments. All assays were done in triplicate. Details are given under “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>C14:0</th>
<th>C16:0</th>
<th>C18:1</th>
<th>C18:2</th>
<th>C18:3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>5.2</td>
<td>1.4</td>
<td>64.8</td>
<td>8.6</td>
<td>1.7</td>
</tr>
<tr>
<td>PE</td>
<td>5.4</td>
<td>3.6</td>
<td>50.9</td>
<td>10.1</td>
<td>6.1</td>
</tr>
</tbody>
</table>

This represents the sum of fatty acids (methyl esters) eluting from the gas-liquid chromatograph between myristic acid (C14:0) and palmitic acid (C16:0). Lung tissue fractions. It was noted early in this study that the specific activity of a number of acid hydrolases increased with increasing purification of the CLO. The increase in specific activity of one enzyme, e.g. acid phosphatase, usually gave a concomitant increase in the activity of other acid hydrolases, e.g. arylsulfatase. Little variation was observed in hydrolase activity among the different CLO preparations. Measurement of all four monophosphohydrolases (EC 3.1.3) (acid phosphatase, alkaline phosphatase, glucose 6-phosphatase, and 5’-nucleotidase) gave apparent maximum localization in the CLO fraction. Acid phosphatase activity was generally 10-fold greater than that of the Fraction IIa residue.
Table II

Specific activities of hydrolases and marker enzymes in lung tissue fractions

Activity is expressed as nanomoles of substrate metabolized per min per mg of protein. See "Experimental Procedure" and flow diagram (Fig. 1) for details. Results represent the averages obtained from 5 to 10 experiments using at least two pooled lung homogenates per experiment. All assays were made in duplicate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction I</th>
<th>Fraction IIa</th>
<th>Fraction III purified concentric lamellar organelles (CLO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>8</td>
<td>226</td>
<td>590</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>10</td>
<td>11</td>
<td>84</td>
</tr>
<tr>
<td>Glucose 6-phosphatase</td>
<td>0.4</td>
<td>1.1</td>
<td>10</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>4</td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td>Aryl sulfatase</td>
<td>0.3</td>
<td>4</td>
<td>34</td>
</tr>
<tr>
<td>β-N-Acetylglucosaminidase</td>
<td>30</td>
<td>254</td>
<td>1070</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>0.2</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td>6</td>
<td>24</td>
<td>95</td>
</tr>
<tr>
<td>β-d-Galactosidase</td>
<td>4</td>
<td>213</td>
<td>118</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td></td>
<td>0.1</td>
<td>Not detected</td>
</tr>
<tr>
<td>Esterase</td>
<td></td>
<td>35</td>
<td>76</td>
</tr>
<tr>
<td>Succinate cytochrome c reductase</td>
<td>5</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>66</td>
<td>7</td>
<td>Not detected</td>
</tr>
<tr>
<td>Benzphetamine N-demethylase</td>
<td>0.5</td>
<td>2</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

* The specific activity for several lysosomal hydrolases was slightly lower in this fraction when contrasted to Fraction IIIb.
* Expressed as tyrosine released when hemoglobin was used as a substrate.

Thus, washings of the 23,000 X g pellet were selectively removing structures rich in hydrolases. The specific activity of the hydrolysis of p-nitrophenyl-β-d-galactopyranoside failed to increase with purification of the CLO. However, it was previously shown (41) that β-d-galactosidase may not be exclusively localized in lysosomes in some organs. Hydrolysis of p-nitrophenylacetate at pH 7.4 was greater in Fraction IIb than in the CLO. These results are contrary to earlier cytochemical studies where the rat and rabbit lung CLO were shown to be the most prominent ultrastructural site for the hydrolysis of p-nitrophenylacetate (43). Purification of the CLO generally resulted in a 20- to 40-fold increase in specific activity over homogenates for most of the other hydrolases. This is of the same order of magnitude of purification shown for acid hydrolases concentrated in liver lysosomes (44). Attempts to further disrupt the CLO (prior to incubation) by sonication or repeated cycles of freezing-thawing did not increase enzyme specific activity. Preparations stored at −20°C for 2 to 3 days showed no loss of glucosaminidase or acid phosphatase activities.

Unlike liver lysosomes (45), no cathepsin D activity was found in the CLO using albumin or denatured hemoglobin as a substrate, despite the use of up to 0.1 mg of protein per reaction vessel. Similar studies using sonicates of rabbit alveolar macrophages gave positive activity at comparable protein concentrations. No neutral proteolytic activity was detected in CLO fractions when albumin or denatured calf thymus histone was employed as a substrate.

Potentiometric determination of the hydrolysis of acetyl tyrosine ethyl ester or benzoyl arginine ethyl ester by CLO also gave negative results; conversely, no inhibition of the hydrolysis of these synthetic substrates by chymotrypsin or trypsin, respectively, was observed when incubated with the CLO fraction.

Lysozyme (muramidase) activity against suspensions of Micrococcus lysodeikticus (46) also could not be detected in the CLO fraction, whereas activity was observed in sonicated suspensions of the isolated macrophages. This would make the CLO like liver lysosomes, which also cannot attack the β-1,4-glucosidic linkages in cell wall mucopolysaccharides (44). The CLO failed to have digestive activity on the terminal N-acetylmuramyl acid groups of bovine submaxillary mucin or synthetic N-acetylmuramyl-lactose, even for incubation periods up to 15 hours at pH values between 4.0 and 7.0 or in the presence of Ca²⁺ up to 10⁻⁴ m. These substrates, however, were readily degraded by Cl. perfringens neuraminidase. The lung CLO gave no inhibition of the hydrolysis of mucin or N-acetylmuramyl-lactose by the Cl. perfringens enzyme. The CLO also lacked acid nuclease activity against ribonucleic (yeast) or deoxyribonucleic (calf thymus) acids.

Acrylamide Electrophoresis—Penetration of the vertical polyacrylamide gel slabs by CLO proteins was facilitated by solubilizing this tissue fraction with the nonionic detergent Triton X-100 and adapting the gels for lipoprotein separation by using 0.005% Brij. Fig. 4 compares the gel staining of the CLO, the acellular lung lining fraction (see "Experimental Procedure"), and bovine serum Fraction V albumin. In all experiments the most dense band was that which migrated anodically with albumin. A number of less dense bands were observed closer to the cathode; those which were discernible are depicted at the top of Fig. 5. The staining characteristics of the lung acellular lining fraction were not unlike those of the CLO, with most of the dye uptake occurring in the region of albumin. In order to verify that the gel was penetrated by CLO proteins, serial sections were made of the Brij-polyacrylamide gel after electrophoresis and examined for zonal distribution of hydrolases. As
sents tracings of discernible bands taken after electrophoresis of separation of detergent-solubilized concentric laminar organelles about 50 pg of organelles measured as protein. The type II cell of the lung. Other lysosomal structures of greater organelle is a specialized form of lysosome characteristic of the substrates or reaction conditions are yet to be found, or that this density would account for those acid hydrolases absent or of WR-1339 or other materials used to decrease their sedimentation constant (49).

shown in Fig. 5, β-N-acetylglucosaminidase, aryl sulfatase, and alkaline phosphatase penetrated the gel slab to give major activity peaks closer to the cathode. Multiple forms of the glucosaminidase were apparent, one of which remained in the sample well; this diffusion pattern is similar to that shown for the human kidney enzyme (47).

Fig. 5. Hydrolase zonal distribution following electrophoretic separation of detergent-solubilized concentric laminar organelles on Brij-polyacrylamide gels at pH 9.0 (Tris-citrate). A represents tracings of bands taken after electrophoresis of about 50 µg of organelles measured as protein. The dashed lines are representative of bands weakly stained. In B the corre-
sponding distribution of β-N-acetylglucosaminidase (●), alkaline phosphatase (○), and aryl sulfatase (△) is given.

shown in Fig. 5, β-N-acetylglucosaminidase, aryl sulfatase, and alkaline phosphatase penetrated the gel slab to give major activity peaks closer to the cathode. Multiple forms of the glucosaminidase were apparent, one of which remained in the sample well; this diffusion pattern is similar to that shown for the human kidney enzyme (47).

DISCUSSION

It is apparent from these enzyme profile studies that the lung concentric laminar organelle belongs to that heterogeneous group of cell structures referred to as lysosomes. As a general feature, lysosomes are membrane enclosed and contain numerous hydro-
lases with acidic pH optima that serve, usually, as an intracellu-
lar digestive system. Throughout the past decade, lysosomes have been found to degrade such substrates as phosphoric esters, aryl sulfato, lipido, polyaccharides, glycoproteins, proteins, peptides, and nucleic acids (48). The enzyme complement of the lung CLO appears also to have the capacity to degrade many different substrates. The fact that no cathepsin D or nuclease activity was detected in lung CLO would indicate that suitable substrates or reaction conditions are yet to be found, or that this organelle is a specialized form of lysosome characteristic of the type II cell of the lung. Other lysosomal structures of greater density would account for those acid hydrolases absent or of low specific activity in the CLO. Undoubtedly, the high phospholipid content of the CLO rendered it more buoyant than untreated liver lysosomes. The isolation procedure used in this investigation is analogous to the flotation method used for separating liver lysosomes after they have endocytosed Triton WR-1339 or other materials used to decrease their sedimentation constant (49).

The adaption of mammalian lysosomal enyzmes to a variety of functions includes defense (50), absorption (51), cell involution, and differentiation (52). A more recent consideration is the regulation of secretory processes by lysosomes providing a catabolic system for the overproduction of secretory products (53). Such a function might be fulfilled by the lung CLO in the regulation of type II intracellular biosynthesis of phosphatidylycholine. The recent cytochemical demonstration of phosphatidic acid phosphatase (54) in the CLO gives evidence that the catabolic activity of this organel can extend to phospholipid substrates. If the CLO hydrolases are secreted by a process of exocytosis, then a regulatory function for CLO could include those chemicals composing the extracellular lining of the lung. Further studies in this laboratory show that the hydrolases found in the CLO (Table II) do occur in the lung acellular lining fraction (55). At present, there is little evidence for the secretion of lysosome-associated enzymes in mammals under physiological conditions (55).

The results of the analyses in this study make the CLO unique among other mammalian subcellular structures, such as mitochondria and endoplasmic reticulum. The proliﬁc membranal character and phospholipid content of the CLO set it apart from liver lysosomes, which have a phospholipid to protein ratio of about 0.1 (56). In certain diseases in humans, such as the genetic lipidoses or mucopolysaccharidoses, affected tissues can develop abnormal multilamellated membrane structures. In Niemann-Pick disease, for instance, there is an inherited deﬁciency of sphingomyelinase. This deﬁciency leads to the accumu-
lution of sphingomyelin in the form of organelles that strongly resemble the CLO (57, 58). A positive acid-phosphatase stain is given by these lipidoses-associated structures. Thus, these morphological variants of lysosomes, including the CLO, appear to be categorically related to secondary lysosomes, of the residual type, which owe their appearance to undigested accumulated material (59). The concentric membranal arrangement observed in normal or disease states could be characteristic of the excessive intracellular accumulation of phospholipids. In accord with this, biopsy specimens from patients treated with 4,4'-diethyl-
aminoethoxyhexestrol, which was shown to produce cases of unusual phospholipid fatty liver, revealed numerous concentric lamellar structures throughout the cytoplasm of hepatic cells (60). The concentric lamellar sheath of some nerves, or myelin, also might be considered a structural analog of the lung CLO, but studies of purified myelin reveal little qualitative similarities to CLO in composition or enzyme activity (61, 62).

Although the actual biochemical and physiological functions of the CLO are controversial, a number of studies give evidence for the origin or fate of these structures. Sorokin (63), for instance, has reported a sequence of electron photomicrographs which apparently reveal a transformation of the type II cell multi-
vesicular bodies into type II cell CLO. The continual presence of multivesicular bodies among the CLO in the present fractiona-
tion experiments lends support to such a transformation or fusion. The mature organelle is purported to migrate to the apical membrane of the type II cell, where it is secreted to the lung extracellular lining.

The electrophoresis pattern of the CLO and the acellular lining fraction of the lung both had their major stained proteins migrating anodically with albumin. This might be additional support for a transport role for the CLO. Previous studies give evidence that lung cells are capable of rapid synthesis of protein in vitro that some of this newly formed protein is actively secreted into the incubation media (64). More recent pulse-label experi-

ments using electron microscopic radioautography support the concept that the CLO functions also in type II cell intracellular protein transport (65). The concern for protein secreted by the lung is drawn from the fact that the functional properties of the surfactant system might rely on a phospholipid-protein complex (66).

While this work was in progress, other methods of preparation of the lung CLO from various mammals were published. Because of possible species differences and the limited analyses made on these other preparations, it is difficult to make accurate comparisons, however, some salient similarities and differences are noted. Williams et al. (40) were able to obtain intact CLO in a low density sucrose fraction from protease-digested beef lung. Page-Roberts (67) avoided the initial proteolytic digestion step and used also a swing-bucket flotation method in isolating rat lung CLO. This preparation had low cytochrome c oxidase activity and phospholipid to protein ratio about one-third that of mine. Hoffman (68) used a continuous sucrose density gradient in a fixed-angled rotor without any prior proteolytic digestion to isolate rabbit CLO, but the final preparation had a low phospholipid to protein ratio, and no intact CLO as observed in photomicrographs. However, his preparation was similar to mine in the surfactant system might rely on a phospholipid-protein complex (66).

The concern for protein secreted by the lung is drawn from the fact that the functional properties of the surfactant system might rely on a phospholipid-protein complex (66).

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