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# An improved procedure for the isolation of lamellar bodies from human lung. Lamellar bodies free of lysosomes contain a spectrum of lysosomal-type hydrolases

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**We have recently shown that lamellar body fractions purified from human lung contain a distinct acid α-glucosidase distinguishable from lysosomal acid α-glucosidase in that it does not cross-react with antibodies raised against the lysosomal enzyme and does not bind to concanavalin A (De Vries, A.C.J., Schram, A.W., Tager, J.M., Batenburg, J.J. and Van Golde, L.M.G. (1985) Biochim. Biophys. Acta 837, 230–238). In order to study the relationship between the non-concanavalin A-binding α-glucosidase and lamellar bodies more closely a method was developed for the further purification of the organelles. A purified lamellar body preparation isolated from human lung homogenate by discontinuous sucrose density centrifugation was subjected to gel filtration with Sepharose 4B followed by Percoll density gradient centrifugation, which yielded a lamellar body preparation with a phospholipid phosphorus/protein ratio of $12.57 \pm 0.38$ (μmol/mg) ($n = 3$) as compared to a ratio of $3.34 \pm 0.16$ (μmol/mg) ($n = 3$) in the sucrose density gradient preparation. Concomitantly there was a $3.3 \pm 0.1$ ($n = 3$) -fold enrichment in the content of total acid α-glucosidase and a $3.2 \pm 0.1$ ($n = 3$) -fold enrichment of non-concanavalin A-binding acid α-glucosidase. The new purification method removes adhering proteins without changing the phospholipid composition. During the successive purification steps the concanavalin A-sensitive and -insensitive α-glucosidases remained fully lamellar body fraction associated. Differences between a lysosome-enriched fraction and a lamellar body preparation at varying stages of purification with respect to the ratio between soluble acid hydrolases and the membrane-associated lysosomal enzyme glucocerebrosidase indicate that the purified lamellar bodies were not contaminated with lysosomes. The absence of lysosomes in the purified lamellar body fraction was confirmed by experiments with the weak base glycyl-L-phenylalanine-β-naphthylamide, which is an artificial substrate for the lysosomal enzyme cathepsin C and brings about lysis of lysosomes. Morphological examination by electron microscopy endorses the absence of contaminating vesicles and organelles and showed a structural integrity of the lamellar bodies in the final preparation. The**

Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

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**improved isolation procedure strongly suggests that the concanavalin A-insensitive acid α-glucosidase is endogenous to lamellar bodies and supports our earlier idea that it can be used as a lamellar body-specific marker enzyme. In addition, the experiments show that lamellar bodies free of lysosomes contain a spectrum of lysosomal-type enzymes.**

## Introduction

Lung surfactant, a lipid-protein complex that lines the alveoli, reduces the surface tension at air-liquid interface during expiration, thereby reducing the work of breathing and protecting the lung against alveolar collapse [1]. In addition, it prevents the exudation of fluid into the alveoli. Alveolar type II cells are generally accepted to be the sole site of synthesis of surfactant phospholipids (for review see Refs. 1, 2). These surfactant components are stored in the type II pneumocytes in characteristic multilaminar organelles known as lamellar bodies [3,4]. The type II cells secrete these lamellar bodies into the liquid layer covering the alveolar surface. Little information is at present available about the biogenesis of lamellar bodies. It is unlikely that these organelles are involved in the biosynthesis of surfactant lipids, as they are not fully equipped with all the required enzymes (for review see Ref. 5).

In a recent study we presented evidence for the occurrence of lysosomal-type hydrolases in human lung lamellar bodies [6]. Special attention was paid to two acid α-glucosidases found in preparations of human lung lamellar bodies. One of these isoenzymes was similar to the acid α-glucosidase found in the lysosome-enriched fraction. The other acid isoenzyme had no affinity to concanavalin A and seemed to be lamellar body specific. In a subsequent study we provided evidence indicating that these two acid α-glucosidases are products of different genes [7]. We proposed that the concanavalin A-insensitive acid α-glucosidase should prove useful as a lamellar body-specific marker enzyme. So far, metabolic studies on lamellar bodies have been seriously handicapped by the lack of such a marker enzyme. In order to corroborate our idea that this putative lamellar body marker enzyme is indeed localized in the lamellar bodies we developed a procedure to further purify these organelles from human lung. In addition to the usually employed sucrose-gradient centrifugation steps [8,9], this new method involves gel permeation and Percoll density gradient centrifugation. We also characterized the distribution pattern of several other membrane-associated and soluble acid hydrolases in these highly purified lamellar body preparations and in lysosome-enriched fractions. The results of the study are presented in this paper.

## Materials and Methods

*Materials.* 4-Methylumbelliferyl-α-D-glucopyranoside, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside, 4-methylumbelliferyl-α-D-mannopyranoside, 4-methylumbelliferyl-β-D-galactopyranoside and 4-methylumbelliferyl-β-D-glucopyranoside were obtained from Sigma Chemical Co., St. Louis, MO. Sepharose 4B, concanavalin A-Sepharose and Percoll were purchased from Pharmacia, Uppsala, Sweden. Bio-Rad protein reagent, goat anti-rabbit IgG and goat anti-mouse IgG horseradish peroxidase conjugates were bought from Bio-Rad Laboratories, Richmond, CA. Spurr was purchased from Polaron Equipment Ltd., Watford, U.K.

*Isolation of subcellular lung fractions.* Fresh human lung parenchyma was obtained after surgical intervention for a neoplastic lesion. Tumor-free lung parenchyma was kept cool in 0.9% NaCl and the purification procedure was begun within 1 h after lobectomy or pneumectomy. Lamellar bodies, a lysosome-enriched fraction and a microsomal fraction were isolated from the homogenate as described earlier [6,9]. The isolated fractions were suspended in 0.3 M sucrose/1 mM EDTA/10 mM Tris-HCl (pH 7.0). Subsequently, the crude lamellar body preparation was further purified on a Sepharose 4B column (60 × 1 cm). The column was eluted with the above-mentioned buffer solution at a flow rate of 5 ml/h and 1-ml fractions were collected. The elution profile was monitored by measuring the absorbance at 280 nm. The fractions of the void volume which showed a

strong absorbance at 280 nm were pooled and centrifuged at 100 000 × *g* for 1 h. The pellet was suspended in 250 mM mannitol/1 mM EGTA/5 mM Mops-Tris (pH 7.0). Percoll was mixed with this buffer to a final density of 1.070 g/ml. The Percoll-containing buffer (7 ml) was layered on a 1 ml 2 M sucrose cushion and 0.5 ml of the post-Sepharose lamellar body preparation was then layered on top of the gradient. After centrifugation at 30 000 × *g* for 60 min (Beckman R40 rotor) 300 μl fractions were collected from the gradient. In each fraction enzyme activities, protein content, the phospholipid composition and immunochemical characteristics were determined. The fractions forming the main band of the Percoll gradient were combined. In some experiments these combined fractions were subjected to a second gel filtration on the Sepharose 4B column under the conditions described above. The lamellar bodies were found in the void volume, while the Percoll particles were retarded on the column.

*Enzyme assays.* The activities of acid hydrolases were measured fluorimetrically in a Perkin-Elmer fluorimeter with a 366 nm filter for excitation and a 445 nm secondary filter. The following substrates and buffers were used: 0.5 mM 4-methylumbelliferyl-α-D-glucopyranoside in 0.5 mM sodium acetate (pH 4.0) for α-glucosidase; 1.6 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 100 mM sodium acetate (pH 4.0) for β-hexosaminidase; 0.5 mM 4-methylumbelliferyl-β-D-galactopyranoside in 100 mM sodium acetate (pH 4.3) for β-galactosidase. Glucocerebrosidase activity was measured as the conduritol B-epoxide-inhibitable hydrolysis of 4-methylumbelliferyl-β-D-glucopyranoside, exactly as described before [10,11].

This conduritol B-epoxide-sensitive activity represents more than 80% of the total acid β-glucosidase activity found in human lung homogenate. After incubation at 37 °C for 1 h the acid hydrolase reactions were stopped by adding 2 ml 0.3 M glycine/NaOH buffer (pH 10.6). Prior to these enzyme assays all subcellular fractions were sonicated six times for 30 s at 0 °C with an MSE ultrasonic disintegrator at 21 kcycles/s and an amplitude of 6 μm peak-to-peak.

*Incubation with glycyl-L-phenylalanine-β-naphthylamide (Gly-Phe-naphthylamide).* Aliquots of a stock solution of 0.1 M Gly-Phe-naphthylamide in dimethylsulphoxide were added to Percoll gradient lamellar body preparations freed of Percoll by a second gel filtration as described above and to lysosome-enriched fractions at a final concentration of 0.5 mM. In control incubations identical volumes of dimethylsulphoxide were added. After incubation at 37 °C for 20 min the fractions were centrifuged at 40 000 × *g* for 30 min and the non-sedimentable α-glucosidase and β-hexosaminidase activities at pH 4.0 were assayed as described before. The sedimentable activities were calculated by the difference between the total and non-sedimentable enzymic activities.

*Concanavalin A binding assays.* Varying amounts of concanavalin A-Sepharose 4B or uncoated Sepharose 4B were mixed with sonicated isolated lamellar bodies and phosphate-buffered saline to a final volume of 250 μl. The suspension was stirred gently at room temperature for 1 h. Subsequently, the reaction mixture was centrifuged at 2000 × *g* for 2 min and the α-glucosidase activity in the supernatant was assayed as described above.

*Electrophoretic blotting procedure.* Electrophoresis on a 10% polyacrylamide slab gel in the presence of sodium dodecyl sulphate was performed using the discontinuous system described by Laemmli [12]. The proteins were transferred electrophoretically from the gels to nitrocellulose filters according to Towbin et al. [13]. α-Glucosidase was detected on the filters with polyclonal rabbit anti-α-glucosidase antiserum or monoclonal mouse anti-α-glucosidase antibodies as described earlier [6].

*Lipid analysis.* Lipids of the subcellular fractions were extracted according to the method of Bligh and Dyer [14]. Phospholipids were separated by two-dimensional thin-layer chromatography on silica gel G plates impregnated with boric acid [15] using chloroform/methanol/water/15 M $NH_3$ (70 : 37 : 4 : 6, v/v) and chloroform/methanol/water (65 : 35 : 5, v/v) as developing solvents. After detection by brief exposure to iodine vapor, phospholipids were extracted from the silica gel [14]. Phospholipid phosphorus was estimated according to the method of Bartlett [16].

*Protein determination.* Medium (500 μl) containing 0.020% (w/v) Triton X-100, 0.2 M NaOH and lamellar bodies in Percoll suspension was

vortexed and incubated for 15 min at room temperature. Subsequently, the medium was centrifuged for 30 min in a microcentrifuge. To 200 μl supernatant 600 μl water and 200 μl Bio-Rad Protein Reagent were added. After 20 min the absorbance at 595 nm was measured. It was shown in control experiments that no substances interfering with the protein assay were present. In Percoll-free samples protein was determined by the method of Lowry et al. [17]. In both cases bovine serum albumin was used as a standard.

*Electron microscopy.* Pellets of Percoll-free lamellar bodies isolated by the purification method described above were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). The lamellar bodies were concentrated on a 0.2 μm Millipore filter and postfixed with buffer containing 0.67% (w/v) osmium tetroxide and 0.83% (w/v) glutaraldehyde. The preparations were stained with 2% (w/v) uranyl acetate in 0.005% (v/v) acetic acid. After rinsing with cacodylate buffer, the preparations were dehydrated with graded concentrations of ethanol. The pellets were rinsed with 100% propylene oxide, infiltrated with Spurr/propylene oxide mixture (1 : 1, v/v) and subsequently embedded in Spurr.

## Results

In an earlier paper we described a method for the isolation of human lung lamellar bodies in which discontinuous gradient centrifugation was used [9]. The lamellar body preparations obtained in that way were characterized by a relatively high phospholipid phosphorus-to-protein ratio when compared to other subcellular fractions. In the present study we isolated human lamellar bodies by the discontinuous sucrose density gradient centrifugation procedure described earlier [9]. A further purification of this crude lamellar body preparation was achieved by gel filtration on Sepharose 4B.

Fig. 1 shows the elution profile of the phospholipid/protein ratios in the various fractions. It can be seen that there was only one peak (void volume) with a high phospholipid phosphorus/

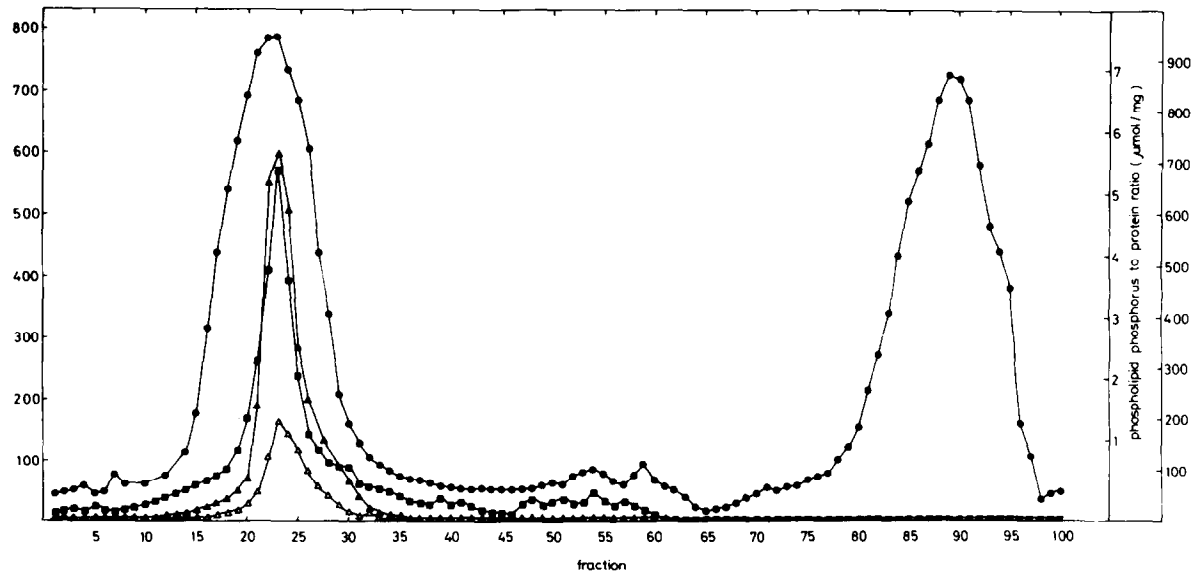


Fig. 1. The elution pattern of human lung lamellar bodies chromatographed on a Sepharose 4B column. The lamellar bodies isolated by sucrose density centrifugations were applied to a Sepharose 4B column and eluted as described in Materials and Methods. All column fractions were sonicated immediately before the enzyme assays. The α-glucosidase activities at pH 4.0 (▲) and pH 7.0 (△) were determined fluorimetrically using 4-methyl-umbelliferyl-α-D-glucopyranoside as a substrate. The phospholipid phosphorus/protein ratio (■) and protein content per fraction (●) were also measured in each fraction. The data are representative for results obtained in three experiments, each carried out with a different preparation.

TABLE I

PHOSPHOLIPID PHOSPHORUS/PROTEIN RATIOS, TOTAL α-GLUCOSIDASE ACTIVITIES AND α-GLUCOSIDASE ACTIVITIES WITHOUT BINDING AFFINITY TO IMMOBILIZED CONCANAVALIN A IN HUMAN LAMELLAR BODY PREPARATIONS SUCCESSIVELY PURIFIED BY SUCROSE DENSITY CENTRIFUGATION, SEPHAROSE 4B GEL FILTRATION AND PERCOLL DENSITY GRADIENT CENTRIFUGATION

| Purification step | Phospholipid/protein | | Total α-glucosidase | | α-Glucosidase non-precipitable with concanavalin A | |
|---|---|---|---|---|---|---|
| | ratio (μmol/mg) | purification (-fold) | activity (pmol/min per mg protein) | purification (-fold) | activity (pmol/min per mg protein) | purification (-fold) |
| Sucrose density centrifugation | 3.34±0.16 | 1 | 354±25 | 1 | 125±5 | 1 |
| Sepharose 4B gel filtration | 5.82±0.06 | 1.7±0.1 | 604±11 | 1.7±0.1 | 193±7 | 1.5±0.1 |
| Percoll density | 12.57±0.38 | 3.8±0.1 | 1183± 5 | 3.3±0.1 | 398±8 | 3.2±0.1 |

protein ratio. Not all the proteins found in the crude lamellar body preparation were found in the phospholipid-associated protein peak. A second peak (fractions 80–100) contained a variable amount of proteins that were not associated with phospholipid. The latter peak may represent contaminating proteins adhering to the lamellar bodies in the crude preparation that were retarded on the Sepharose 4B column. Table I shows clearly that after the Sepharose 4B chromatography the phospholipid/protein ratio in the preparation increases by a factor of about two when compared with the crude preparation. Table II shows that the gel filtration has no influence on the phospholipid composition. Phosphatidylcholine is the most abundant component of the phospholipids followed by phosphatidylglycerol. The phospholipid composition of the isolated organelles is very similar to that of lamellar bodies isolated from rat [8,18–20] and rabbit [21] lung.

Biochemical [22,23] and histochemical [24] studies have demonstrated that lysosomal-type hydrolases are present in lamellar bodies. However, except in one study on phospholipase $A_1$ and $A_2$ in lung lamellar bodies from rabbit [25], the pH optima of the hydrolase activities were not investigated. Fig. 1 clearly shows that all α-glucosidase activity remains associated with the lamel-

TABLE II

PHOSPHOLIPID COMPOSITION OF HUMAN LUNG LAMELLAR BODY PREPARATIONS SUCCESSIVELY PURIFIED BY SUCROSE DENSITY CENTRIFUGATION, SEPHAROSE 4B GEL CHROMATOGRAPHY AND PERCOLL DENSITY GRADIENT CENTRIFUGATION

Data represent means ± S.E. derived from three lung preparations.

| Phospholipid | Relative abundance (%) | | |
|---|---|---|---|
| | Sucrose density centrifugation preparation | Sepharose 4B gel chromatography preparation | Percoll density gradient preparation |
| Phosphatidylcholine | 71.0±0.9 | 72.1±0.8 | 73.2±0.5 |
| Phosphatidylglycerol | 8.6±0.7 | 8.1±0.2 | 8.9±0.2 |
| Phosphatidylethanolamine | 5.6±0.5 | 5.6±0.2 | 5.5±0.2 |
| Phosphatidylserine | 3.5±0.6 | 3.0±0.0 | 2.8±0.1 |
| Phosphatidylinositol | 3.0±0.1 | 3.4±0.5 | 3.1±0.2 |
| Sphingomyelin | 2.8±0.3 | 2.3±0.3 | 2.3±0.2 |
| Lysophosphatidylcholine | 3.0±0.6 | 2.9±0.1 | 3.1±0.2 |
| Cardiolipin | 2.1±0.4 | 2.3±0.2 | 1.2±0.6 |

lar body peak (high phospholipid phosphorus/protein ratio) after Sepharose 4B chromatography of a crude lamellar body preparation and has an acid pH optimum, like lysosomal enzymes. We have recently demonstrated that at least two acid α-glucosidases are present in a preparation of human lamellar bodies: one with a high affinity to the lectin concanavalin A, like the enzyme in lysosomes, and another, absent from lysosomes, which is without affinity for concanavalin A. Table I shows an enhancement of the specific activities of the total and concanavalin A-insensitive acid α-glucosidase activities in the lamellar body preparation obtained by Sepharose 4B chromatography. The phospholipid phosphorus/protein ratio, and the specific activities of the total acid α-glucosidase and the concanavalin A-insensitive acid α-glucosidase increased to about the same extent by the gel filtration process (purification factors of $1.75 \pm 0.07$, $1.74 \pm 0.10$ and $1.55 \pm 0.12$ (mean ± S.E.; $n = 3$), respectively. These observations strongly suggest that both the concanavalin A-sensitive and the concanavalin A-insensitive acid α-glucosidase are endogenous to lamellar bodies.

A further purification was achieved by a Percoll gradient centrifugation step. Fig. 2 shows that the peak with a high phospholipid phosphorus/protein ratio (lamellar body peak) contains all the acid α-glucosidase activity. Tables I and II demonstrate that the Percoll step raises the phospholipid phosphorus/protein ratio by another factor of two, without changing the phospholipid composition. All fractions from the Percoll gradient were analyzed by immunoblotting techniques. The nitrocellulose filters were treated with monoclonal mouse or polyclonal rabbit anti-α-glucosidase antibodies. Only the lamellar body band of the Percoll gradient exhibited immunoreactive bands (one main band of $M_r = 110000$, a second band of about 76000 and a doublet band with $M_r$

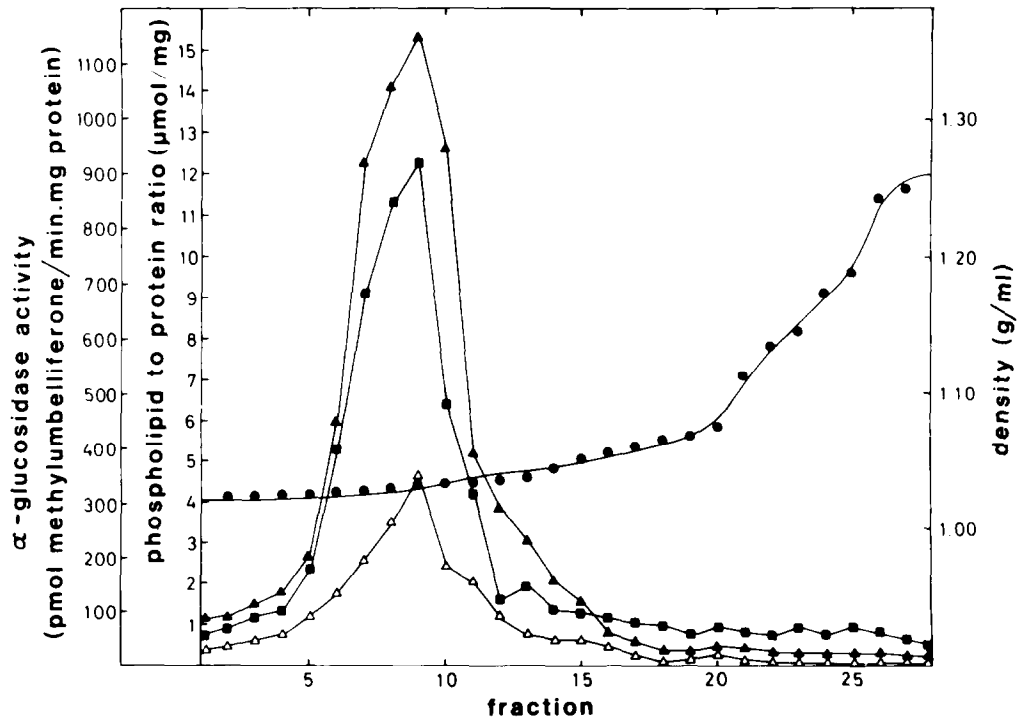


Fig. 2. Separation of human lung lamellar bodies on a Percoll density gradient. The lamellar bodies were first purified by sucrose density gradient centrifugations and, subsequently, by Sepharose 4B gel chromatography (Fig. 1). The preparation (fractions 18–26) obtained in this way was fractionated on a Percoll density gradient (starting density 1.075 g/ml). (●), density of the gradient. The phospholipid phosphorus/protein ratio (■) and the α-glucosidase activities at pH 4.0 (▲) and pH 7.0 (△) were determined in each fraction. The results are representative for three experiments carried out with three different preparations.

of about 70 000) (data not shown). The molecular weights of the protein bands are in accordance with those reported for lysosomal α-glucosidase from human lung [6]. To measure the concanavalin A-insensitive α-glucosidase activity in the lamellar body fraction after the Percoll gradient centrifugation it was necessary to remove the Percoll particles by a second gel filtration step. The effects of titration of this Percoll-free lamellar body preparation with immobilized concanavalin A are shown in Fig. 3. About 30% of the total acid α-glucosidase activity is concanavalin A insensitive (Fig. 3). This concanavalin A-insensitive α-glucosidase has a low pH optimum (data not shown). In the less purified lamellar body preparations the same percentages were found (see Table I). These data demonstrate again that both α-glucosidase isoenzymes are endogenous to lamellar bodies. The lamellar bodies obtained after the Percoll gradient centrifugation have a density of $1.027 \pm 0.001$ g/ml (mean ± S.E.; $n = 3$). The phospholipid phosphorus/protein ratio, and the total and concanavalin A-insensitive α-glucosidase activities are raised to the same extent by the Percoll purification step (see Table I).

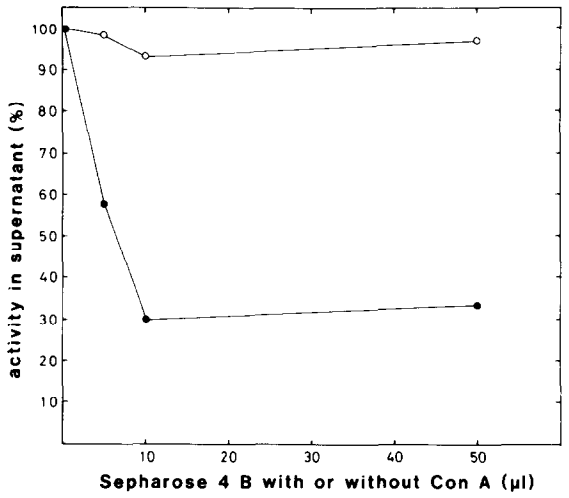


Fig. 3. Effect of titration with concanavalin A-Sepharose 4B on the acid α-glucosidase activity in a lamellar body preparation successively purified by sucrose density centrifugations, Sepharose 4B gel filtration and Percoll gradient centrifugation. Percoll particles were removed by a second gel filtration step on a Sepharose 4B column. Lamellar body preparations obtained in this way were sonicated and preincubated with concanavalin A-Sepharose 4B or uncoated beads as described in Materials and Methods. The figures on the abscissa indicate the volume of Sepharose bead suspension (1 : 1 v/v in buffer) added per 250 µl incubation. After centrifugation, the α-glucosidase activity at pH 4.0 was determined. (●), α-glucosidase activity not precipitated with concanavalin A-Sepharose 4B beads; (○) control values obtained with uncoated Sepharose 4B beads. These data are representative for results obtained in three experiments, each carried out with a different preparation.

For optimal purification the Sepharose gel filtration step cannot be omitted. When the lamellar body fraction obtained by the sucrose density gradient procedure was subjected immediately to Percoll density centrifugation a lamellar body fraction with a phospholipid/protein ratio of about half of the maximal ratio was obtained (data not shown).

In order to establish whether lysosomal contamination contributes to the α-glucosidase activity found in the lamellar body fraction, we have measured the activity of acid α-glucosidase, β-hexosaminidase and β-galactosidase in human lung homogenate, a lysosome-enriched fraction and the most purified lamellar body fraction and related these activities, which are due to soluble enzymes, to that of glucocerebrosidase, a membrane-associated lysosomal enzyme [11]. The results are shown in Table III. The ratios α-glucosidase/glucocerebrosidase, β-hexosaminidase/glucocerebrosidase, and β-galactosidase/glucocerebrosidase are similar in the lysosome-enriched fraction and in the homogenate. In contrast the ratios are 7.5–12-fold higher in the lamellar body fraction than in the homogenate or the lysosome-enriched fraction. This enrichment of the soluble acid hydrolase activities in the lamellar body fraction relative to that of the membrane-associated lysosomal enzyme indicates that the acid hydrolases in the lamellar body fraction are not due to lysosomal contamination.

The absence of lysosomes in the lamellar body fraction was confirmed by an experiment with the weak base, glycyl-L-phenylalanine-β-naphthylamide (Gly-Phe-naphthylamide), an artificial substrate for the lysosomal enzyme cathepsin C (dipeptidylpeptidase I). Oude Elferink et al. [26] have shown that human fibroblast lysosomes rapidly lose their latency upon incubation with Gly-Phe-naphthylamide. Table IV shows the influence of Gly-Phe-naphthylamide on the lamellar body frac-

TABLE III

DISTRIBUTION PATTERN OF VARIOUS ACID HYDROLASES IN THE HOMOGENATE, THE LYSOSOME-ENRICHED FRACTION AND A PURIFIED LAMELLAR BODY PREPARATION FROM HUMAN LUNG

The relative distribution of soluble acid hydrolases to the activity of the membrane-associated lysosomal glucocerebrosidase was measured as described in Materials and Methods. The lamellar bodies were purified by sucrose density centrifugation, Sepharose 4B gel filtration and a Percoll density gradient. Data represent mean ± S.E. derived from three lung preparations.

| Preparation | β-Hexosaminidase/ glucocerebrosidase | | α-Glucosidase/ glucocerebrosidase | | β-Galactosidase/ glucocerebrosidase | |
|---|---|---|---|---|---|---|
| | ratio | enrichment | ratio | enrichment | ratio | enrichment |
| Homogenate | 13.89 ± 0.58 | 1 | 0.82 ± 0.01 | 1 | 1.15 ± 0.001 | 1 |
| Lysosomes | 12.66 ± 0.48 | 0.9 ± 0.1 | 0.72 ± 0.01 | 0.9 ± 0.0 | 0.70 ± 0.01 | 0.6 ± 0.1 |
| Lamellar bodies | 166.67 ± 27.48 | 12.0 ± 2.1 | 8.20 ± 0.74 | 10.0 ± 0.9 | 8.70 ± 0.61 | 7.6 ± 0.5 |

tion and the lysosome-enriched fraction. In the absence of Gly-Phe-naphthylamide both fractions demonstrate a high level of integrity. More than 85% of the acid α-glucosidase and β-hexosaminidase is sedimentable under these conditions. Table IV shows that upon incubation of the lysosome-enriched fraction with 0.5 mM Gly-Phe-naphthylamide, a dramatic decrease in sedimentability of lysosomal α-glucosidase and β-hexosaminidase was seen. However, Gly-Phe-naphthylamide had no significant influence on the sedimentability of these enzymes in the lamellar body fraction. In combination with the results of the experiment examining the distribution pattern of various acid hydrolases in the lamellar body and lysosome-enriched fraction, the incubation with Gly-Phe-naphthylamide strongly suggests that the purified lamellar body preparation is not contaminated with lysosomes.

TABLE IV

EFFECT OF GLYCYL-L-PHENYLALANINE-β-NAPHTHYLAMIDE ON THE INTEGRITY OF LAMELLAR BODIES AND LYSOSOMES

Lamellar body preparations (post-Percoll) and lysosome-enriched fractions from human lung were preincubated with Gly-Phe-naphthylamide or dimethylsulphoxide (controls). After this preincubation the fractions were centrifuged and the sedimentable acid α-glucosidase and β-hexosaminidase activities were determined. For details see Materials and Methods. Data represent mean ± S.E. derived from three lung preparations

| Preparation | Gly-Phe-Naphthylamide in preincubation medium (mM) | Sedimentable activity (%) | |
|---|---|---|---|
| | | α-glucosidase | β-hexosaminidase |
| Lamellar bodies | 0 | 84.2 ± 1.1 | 87.3 ± 2.1 |
| Lysosomes | 0 | 91.0 ± 3.2 | 93.0 ± 4.7 |
| Lamellar bodies | 0.5 | 81.8 ± 4.0 | 84.1 ± 5.6 |
| Lysosomes | 0.5 | 27.2 ± 5.9 | 40.3 ± 4.9 |

Fig. 4 shows electron micrographs of human lamellar bodies isolated successively by sucrose density centrifugation, Sepharose 4B gel chromatography and Percoll density gradient centrifugation. The structures shown are representative for the whole lamellar body preparation. The purified preparation consists primarily of concentric multilamellated structures. These structures resemble very closely lamellar bodies found in the cytoplasm of alveolar type II pneumocytes. No contaminations of the preparation by other subcellular organelles could be detected.

## Discussion

In order to isolate non-disrupted lamellar bodies some precautions should be taken. Gentle homogenization of lung tissue using Teflon and glass homogenizers is necessary and homogenizers with metal cutting blades should be avoided. Another important consideration is that there must be no calcium in the solutions used, because calcium causes lysis of lamellar bodies and the formation of tubular myelin [27]. Most of the isolation procedures are based on those first published by Page-Roberts [28] and Hassett et al. [27]. Similar procedures have been described for the isolation

of lamellar bodies from rabbit [29,30], rat [8,27] and human lung tissue [9]. Apart from some studies on fetal [31–33] and neonatal lung lamellar bodies [34] only a limited amount of information is available on lamellar bodies of adult human lung [6,7,9,34]. When isolating human lamellar bodies, it is essential to use fresh tissue. The isolation procedure needs to start as quickly as possible, preferably within about 1 h after surgical intervention. Especially when the initial purification steps take too much time, a significant part of the concanavalin A-insensitive acid α-glucosidase is inactivated. The concanavalin A-sensitive acid α-glucosidase seems to be more stable (data not shown). A partially purified lamellar body preparation was obtained by the method of Post et al. [9]. Preparations isolated by this procedure contain no mitochondria and less than 5% microsomal contamination [9]. As described by Nijssen et al. for rat lamellar bodies [35], the additional gel chromatography purification step in the present study removes adhering proteins and raises the phospholipid phosphorus/protein ratio. Importantly, the concanavalin A-insensitive acid α-glucosidase remains associated with the organelle.

When the same gel permeation procedure was carried out with rat lung tissue, the phospholipid/protein ratio also doubled. However, we did not achieve the high ratio of 10.0 that was reported by Nijssen et al. [35] for rat lamellar bodies. In contrast to human lamellar bodies, rat lamellar bodies contain an α-glucosidase activity at pH 7.0 which is at least an order of magnitude higher than the activity at pH 4.0 (data not shown). About 60% of this lamellar body associated α-glucosidase activity is concanavalin A insensitive but shows no acid pH optimum. This makes it difficult to ascertain whether rat lamellar bodies also contain a concanavalin A-insensitive acid α-glucosidase like human lamellar bodies.

In order to examine more closely the question whether the non-concanavalin A-binding acid α-glucosidase is, indeed, a lamellar body-specific enzyme we paid particular attention to the purification of lamellar bodies from human lung. Introduction of an additional Percoll gradient purification step after the gel filtration step raises the phospholipid phosphorus/protein ratio of human lung lamellar bodies to the high value of 12.6. This value is higher than that reported earlier for lamellar bodies of rabbit [36–38], rat [19,39], sheep [40], or man [9]. The only report of a higher ratio concerns human lamellar bodies isolated from amniotic fluid which had a phospholipid/protein ratio of 19.4 [33].

The differences between the lysosome-enriched fraction on the one hand and the lamellar body preparation on the other with respect to the ratio between soluble acid hydrolases and the membrane-associated lysosomal enzyme gluco-

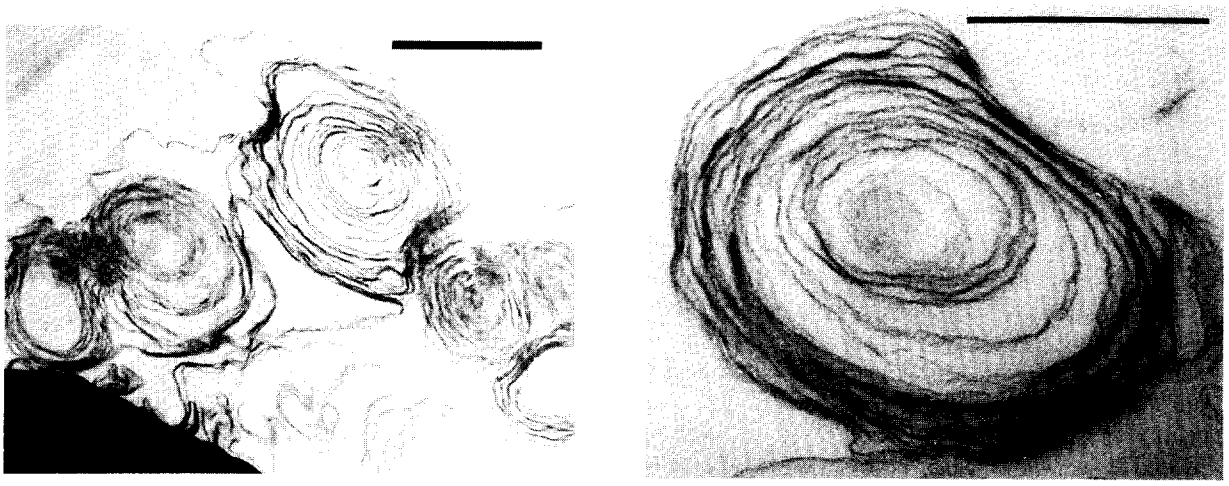

Fig. 4. Electron micrographs of lamellar body preparations successively purified by sucrose density centrifugations, Sepharose 4B gel filtration and Percoll gradient centrifugation. Percoll particles were removed by a second gel filtration step on a Sepharose 4B column. The bars are 0.5 μm.

cerebrosidase (Table III) indicate that the purified lamellar bodies are not contaminated with lysosomes. This is also indicated by the observation that the acid α-glucosidase and β-hexosaminidase activities in a purified lamellar body preparation remained organelle associated after incubation with Gly-Phe-naphthylamide, whereas the activities in the lysosome-enriched fractions lost their sedimentability after this treatment (Table IV). The presence of the acid α-glucosidase activity in the lamellar body preparations is therefore not due to lysosomal contamination. The observations that during the successive purification steps the specific activity of the concanavalin A-insensitive acid α-glucosidase increases by the same factor as the phospholipid/protein ratio (Table I) and remains fully associated with fractions with a high phospholipid/protein ratio (Figs. 1 and 2) indicate that this enzyme is endogenous to lamellar bodies. It can therefore be used as a lamellar body marker enzyme as we suggested earlier [6]. The concanavalin A-insensitive α-glucosidase is not a marker for the neoplastic predisposition, as this enzyme was also found in lung tissue of a Pompe patient [7] suffering from a lysosomal α-glucosidase deficiency.

Finally, the method we have introduced for the isolation of human lamellar bodies yields a preparation which retains many of the morphological features that characterize lamellar bodies in situ. The absence of contaminating organelles or vesicles in the final lamellar body preparation and the structural integrity of the lamellar bodies as shown by electronmicroscopic examination are in accordance with the biochemical data.

Bourbon et al. [41,42] suggested that in the fetal lung, lysosomal α-glucosidase is involved in glycogen catabolism and that lamellar bodies may play a role in this process. It is worth noting that Chi [43] recently discovered the presence of glycogen in lamellar bodies. Lamellar body-specific α-glucosidase could also be involved in the trimming of the oligosaccharide moiety of surfactant apoproteins. Determination of the substrate specificity of this enzyme should be of importance. The activity of lamellar body-specific α-glucosidase and other acid hydrolases may not be restricted to the acidic interior of the organelle itself [44], but the enzyme could also function in the alveolar acidic subphase [45]. Indeed, hydrolytic enzymes have been found in the extracellular lining of the terminal airways and alveoli [46].

The improved isolation method shows that human lamellar bodies contain a spectrum of lysosomal-type hydrolases and endorses our earlier suggestion that the concanavalin A-insensitive acid α-glucosidase could serve as a marker enzyme for lamellar bodies of the human lung.

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