Pulmonary surfactant synthesis. A highly active microsomal phosphatidate phosphohydrolase in the lung

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Abstract Lung cell-free homogenate, which contains about twice the units of phosphatidate phosphohydrolase per mg of protein compared to liver, was fractionated by differential centrifugation and the fractions were assayed for phosphatidate phosphohydrolase and marker enzymes of endoplasmic reticulum, mitochondria, and lysosomes. Over 60% of the lung phosphatidate phosphohydrolase was associated with the endoplasmic reticulum, compared to 50% of the total liver enzyme. Thus a major portion of the more active lung enzyme is potentially involved in lipid biosynthesis by the endoplasmic reticulum. Less than 0.2% of the total lung enzyme was found in a lamellar body fraction, consistent with previous findings. The lung microsomal phosphohydrolase was specific for lipid substrates, showing equal activity towards phosphatidic acid or lysophosphatidic acid and relatively low activities towards glycerophosphates. It had a neutral pH optimum, similar to the liver enzyme, but differed somewhat in its relative activity at extremes of pH. Stability at 65°C was greater for the lung enzyme. Fluoride inhibited lung (or liver) microsomal phosphatidate phosphohydrolase, while tartrate, $MgCl_2$, or EDTA had no effect. The presence of a high activity of phosphatidate phosphohydrolase in lung endoplasmic reticulum is consistent with the rapid synthesis of pulmonary surfactant phosphatidylcholine.

Supplementary key words endoplasmic reticulum · lamellar bodies · lipid synthesis · subcellular localization

Lung function is dependent upon the presence in the alveolar lumen of a unique lipid–protein mixture known as pulmonary surfactant. This surface-active mixture is believed to allow expansion and contraction of the alveolar air–tissue interface with a mininum of energy expenditure (1). The major surface-active component of pulmonary surfactant is dipalmitoyl phosphatidylcholine, a species that is more surface-active than the unsaturated species of phosphatidylcholines which predominate in other tissues. Thus a pathway of phosphatidylcholine synthesis that is specific for incorporation of large amounts of palmitate should distinguish lung lipid metabolism from metabolism in other tissues. Several studies have appeared testing the fatty acid specificity of lung phosphatidylcholine synthesis (2). Available data are consistent with the hypothesis that a large fraction of the palmitate in surfactant phosphatidylcholine is incorporated via a deacylation–reacylation pathway that remodels unsaturated phosphatidylcholine synthesized de novo.

In addition to the high content of palmitate, a second distinguishing characteristic of surfactant phosphatidylcholine is its rapid rate of turnover. A half-life of 14 hr has been reported for the palmitoyl and glycerol moieties of lung dipalmitoyl phosphatidylcholine (3). This is considerably shorter than half-lives of several days which have been generally found for other tissue phospholipids (4). Thus, the synthesis of surfactant dipalmitoyl phosphatidylcholine by lung apparently occurs at a rate several times higher than the rate of synthesis of "normal" tissue phospholipids.

The high specific activity of a lung microsomal phospholipase A_2, which was over 10-fold more active than the same enzyme in liver microsomes (5), is consistent with a high rate of lung dipalmitoyl phosphatidylcholine synthesis by a deacylation–reacylation pathway. Enzymes capable of reacylating lysophospholipids have also been found in lung with high specific activity (6–8). A rapid remodeling pathway, however, is dependent upon a rapid rate of de novo phosphatidylcholine synthesis for a continuous supply of starting material. Enzymological data presently available on de novo phosphatidylcholine synthesis by lung do not reveal any striking differences in possible rates of synthesis compared to other tissues.

Abbreviations: PA, phosphatidic acid; LPA, lysophosphatidic acid; PAPase, phosphatidate phosphohydrolase; dipalmitoyl phosphatidylcholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

Specific activities reported for lung enzymes of this pathway are roughly similar to (9–11) the corresponding enzyme activities reported in studies on liver (12–14).

In this report we present evidence for a highly active phosphatidate phosphohydrolase (PAPase) in lung microsomes, including a direct comparison between lung and liver. The specific activity of the lung enzyme is several-fold higher than the enzyme in liver. Phosphatidate phosphohydrolase has been suggested to be a rate-controlling enzyme of liver glyceride synthesis (15), based on the low activity of this enzyme relative to other enzymes in the pathway and on the response of this enzyme to dietary manipulation. The rate-limiting step in lung glyceride synthesis remains to be determined. The high activity of lung microsomal PAPase reported here is consistent, however, with a rapid de novo synthesis of phosphatidylcholine which could be remodeled to surfactant dipalmitoyl phosphatidylcholine.

EXPERIMENTAL PROCEDURE

Preparation of subcellular fractions

Male, Long-Evans hooded rats, weighing 200–250 g, were injected intraperitoneally with 40 mg of sodium pentobarbital. Chests were opened and the lungs were perfused through the right ventricle with about 40 ml of ice-cold saline–EDTA (0.9% sodium chloride, 3 mM EDTA) until they were free of visible blood. The lungs were excised, lavaged three times with 6 ml of ice-cold saline–EDTA and the lung parenchyma was separated from visible bronchi and blood vessels by scraping with a scalpel. The separated parenchyma was further minced with a razor blade, rinsed with cold saline–EDTA, and homogenized in 4 ml per lung of ice-cold Tris–sucrose buffer (0.33 M sucrose, 0.01 M Tris HCl, pH 7.4, 1 mM EDTA). A Potter Elvehjem Teflon–glass homogenizer was used with a loose-fitting pestle (0.043 cm clearance) for the initial six strokes, followed by six strokes with a tighter-fitting pestle (0.018 cm clearance). The homogenate was filtered through two layers of cheesecloth and centrifuged at 300 *g* for 15 min to remove unbroken cells and large debris. The pellet was discarded. The supernatant was designated the cell-free homogenate and contained about 15 mg of protein per rat lung, which had a wet weight of about 2 g. The cell-free homogenate was subfractionated by the following differential centrifugation procedure. All pellets were resuspended in 1–2 ml of Tris–sucrose buffer. The following fractions were obtained: fraction 1, pellet from 1600 *g* for 10 min; fraction 2, pellet from 8000 *g* for 10 min; fraction 3, pellet from 16,000 *g* for 10 min; fraction 4, pellet from 100,000 *g* for 60 min; fraction 5, supernatant from 100,000 *g* for 60 min.

Rat liver fractions were prepared by the same procedure, starting with a homogenate of 2.5 g (wet weight) of liver in 12 ml of Tris–sucrose buffer. Cell-free homogenate of liver contained about 80 mg of protein per 2.5 g of liver.

Protein content of the cell-free homogenate and of fractions derived from it was determined by the procedure of Lowry et al. (16). Fractions were divided into 0.5-ml portions and frozen in a liquid nitrogen refrigerator until they were assayed for enzyme activity. Fraction 4 is also designated the microsomal fraction.

Enzyme assays

Phosphatidate phosphohydrolase was assayed in a 0.2-ml reaction mixture containing 0.05 M Tris–maleate buffer, pH 7.0, 1 mM PA (Sigma Chemical Co., St. Louis, MO; from egg lecithin) suspended by ultrasonication and 20–100 μg of protein. The assay was initiated by the addition of protein. It was incubated at 37°C for 15 min and terminated by the addition of 0.8 ml of a solution containing 0.13% sodium dodecylsulfate, 1.25% ascorbic acid, 0.32% ammonium molybdate·$4H_2O$, and 0.75 N H_2SO_4. The phosphomolybdate color was developed at 45°C for 20 min (17) and absorbance was determined at 820 nm. An extinction of 0.014 absorbance units per nmol of phosphate was determined with a phosphate standard solution. Incubations without protein and without substrate were used to correct for nonenzymatic phosphate release.

Acid phosphatase was assayed in a 0.2-ml reaction mixture containing 0.05 M sodium acetate buffer pH 5.0, 0.1% Triton X-100, and 0.05 M β-glycerophosphate titrated to pH 5. The reaction was initiated with 10–200 μg of protein, incubated at 37°C for 15 min, and terminated and analyzed for released phosphate in the same way as the PAPase assay.

NADPH cytochrome *c* reductase (18), succinate cytochrome *c* reductase (19), *N*-acetyl-β-glucosaminidase (20), and aryl sulfatase (21) were assayed according to published procedures. All enzyme assays were linearly proportional to time and added protein over the ranges used.

Characterization of lipid products of PAPase

For characterization of the lipid product of PA hydrolysis, 1-acyl-2-[1′-^{14}C]linoleoyl-*sn*-glycero-3-phosphate was used as substrate in the assay. The reaction was terminated by the addition of 0.6 ml

of 0.01 M HCl and the lipids were extracted by the procedure of Bligh and Dyer (22), with 50 μg each of monoglyceride, diglyceride, triglyceride, and fatty acid added as carrier. Neutral lipid composition of the extracted lipids was determined by chromatography on a 250 μm thin-layer plate of silica gel G (Analtech, Newark, Del.). The chromatogram was initially developed with acetone to 30% of its length, dried under N_2, and developed completely in the same direction with petroleum ether–diethyl ether–acetic acid 80:75:1.5. This system resolves monoglyceride, diglyceride, triglyceride, and fatty acids, while phospholipids remain at the origin. Phospholipid composition of the extracted lipids was determined by thin-layer chromatography on a 250 μm silica gel H plate, sprayed with 10 mM Na_2CO_3 before activation, and developed in chloroform–methanol–acetone–acetic acid–water 100:20:40:20:10. This system resolves PA and LPA while neutral lipids run just behind the solvent front.

Appropriate standards were cochromatographed with radioactive samples and visualized with iodine vapor. The chromatograms were divided into bands containing the standards and the gel was scraped into scintillation vials for counting in the presence of toluene–Triton X-100–water 2:1:0.2 containing 5 mg of PPO and 0.5 mg of bis-MSB per liter of toluene.

Preparation of radiolabeled lipids

Preparation of 1-acyl-2-[1′-^{14}C]linoleoyl-*sn*-glycero-3-phosphate was by incubation of 1-acyl-2-[1′-^{14}C]-linoleoyl-*sn*-glycero-3-phosphocholine with cabbage phospholipase D (Sigma) in the presence of $CaCl_2$ and diethyl ether (23). The calcium salt of phosphatidic acid was extracted from the reaction mixture with chloroform and converted to the sodium salt by washing the chloroform solution with an equal volume of 0.1 M sodium citrate pH 5.8. The phosphatidic acid was purified by thin-layer chromatography in the system described above for phospholipid analysis.

The method of Waite and Van Deenen (24) was used for preparation of 1-acyl-2-[1′-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine.

RESULTS

Characterization of lipid product of PA hydrolysis by lung and liver microsomes

Lung microsomes, under normal assay conditions, yielded monoglyceride as the major radioactive product when 1-acyl-2-[1′-^{14}C]linoleoyl-*sn*-glycero-3-phosphate was used as substrate. The monoglyceride to diglyceride ratio was 2.7 and no labeled fatty acid

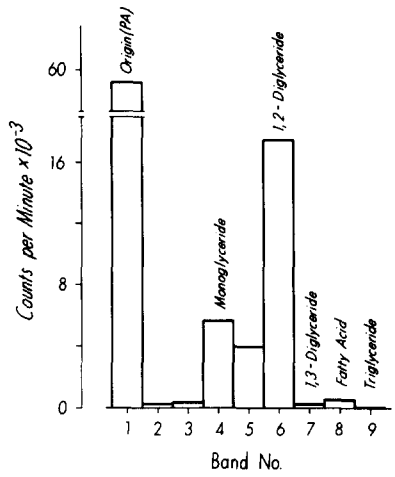

Fig. 1. Characterization of radioactive product of lung microsomal PAPase using 1-acyl-2-[1′-^{14}C]linoleoyl-*sn*-glycero-3-phosphate as substrate. Lung microsomal fraction (60 μg protein) was preincubated at 37°C with 100 nmol of unlabeled PA and 300 nmol of unlabeled diolein (suspended by sonication) for 20 min in a total volume of 150 μl. Radiolabeled PA (100 nmol, 180,000 cpm) and additional unlabeled diolein (300 mmol) were then added in a volume of 50 μl and the incubation was continued for 20 min. The reaction was terminated and analyzed as described in Experimental Procedure.

was released (data not shown). This is consistent with two hypotheses: *1*) a phospholipase, specific for the fatty acyl ester bond at position 1, is hydrolyzing PA to LPA, which is then acted upon by a phosphohydrolase, or *2*) PA is initially hyrolyzed to diglyceride by phosphatidate phosphohydrolase and the diglyceride is further hydrolyzed to monoglyceride by a diglyceride lipase specific for position 1. Hypothesis 1 seems unlikely because no radiolabeled LPA was detectable in the reaction products and PA was the only labeled phospholipid recovered. Hypothesis 2 is consistent with the observation of a diglyceride lipase in lung microsomes reported by Sarzala and Van Golde (25). As a test of hypothesis 2, we attempted to "trap" the radioactive product as diglyceride by adding 600 nmol of unlabeled diolein to the reaction mixture. The trapping was partially successful, reducing the monoglyceride/diglyceride ratio to 0.91 (data not shown). The persistence of radioactive monoglyceride formation in the presence of an excess of unlabeled diolein suggests that the PAPase-generated diglyceride exists as a membrane-bound pool whose exchange with exogenous diglyceride is slow relative to its hydrolysis by diglyceride lipase.

The experiment shown in **Fig. 1** was therefore performed as a further test of hypothesis 2. Lung microsomes were incubated with unlabeled PA and diglyceride prior to addition of radiolabeled PA, to allow formation of a pool of unlabeled PA-derived diglyceride, which should facilitate trapping of sub-

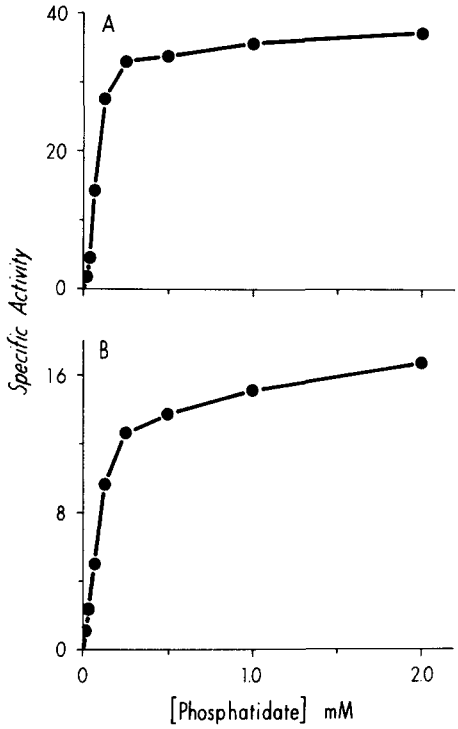

Fig. 2. Effect of phosphatidate concentration on phosphohydrolase activity of microsomal fraction. Specific activity is in nmoles per min per mg of protein. *A*, lung microsomal fraction; *B*, liver microsomal fraction.

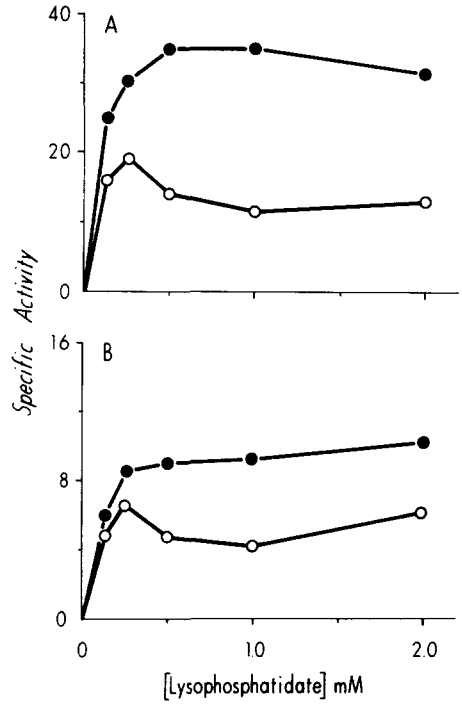

Fig. 3. Effect of lysophosphatidate concentration on phosphohydrolase activity of microsomal fraction. Reaction conditions were those described in Experimental Procedure for phosphatidate phosphohydrolase, substituting lysophosphatidic acid as substrate, with (●) or without (○) addition of 1.8 mg/ml of bovine serum albumin. Specific activity is expressed as in Fig. 2. *A*, lung microsomal fraction; *B*, liver microsomal fraction.

sequently formed labeled diglyceride. The results (Fig. 1) were consistent with hypothesis 2, as the monoglyceride/diglyceride ratio was reduced to 0.32 and diglyceride was the major radioactive product. Results similar to these were obtained by studying hydrolysis of radiolabeled PA by liver microsomes (data not shown). Monoglyceride/diglyceride ratios were 1.4, 0.8, and 0.4, respectively, for untrapped, exogenous diglyceride trapped, and PA-generated diglyceride trapped experiments with liver microsomes.

It is clear from these experiments that hydrolysis of phosphate from PA is most likely the initial reaction of PA catalyzed by lung or liver microsomes and that determination of PA-dependent phosphate release is a valid assay of PAPase activity in these tissues. The amount of radioactive neutral lipid recovered (Fig. 1) was consistent with the specific activity of the lung microsomes as measured by phosphate release. The colorimetric phosphate assay for PAPase was therefore used in all subsequent experiments.

Substrate specificity

Substrate concentration curves for PA and LPA are shown in **Figs. 2 and 3** for both lung and liver microsomes. With PA as substrate, the curve was sigmoidal for both tissues. A rapid increase in activity occurred over the range of 0–0.5 mM PA. Only a slight further increase occurred above 0.5 mM PA.

With LPA as substrate, activity increased over the range of 0–0.3 mM. Above 0.3 mM, increasing LPA caused a decline in activity, suggestive of inhibition of the enzyme by LPA, perhaps due to its detergent-like properties. Adding bovine serum albumin to the assay mix prevented this apparent substrate inhibition.

The specific activities shown in Figs. 2 and 3 are typical of several preparations of lung and liver microsomes. With PA as substrate, preparations of lung microsomes showed specific activities ranging from 28 to 40 nmol per min per mg of protein[1]; for liver microsomes, the values were 7–13 nmol per min per mg. Rates of hydrolysis of LPA by a given prepara-

[1] We have previously reported a specific activity of 16 nmol per min per mg for PAPase in a microsomal fraction of lung (26). That value was the lower limit of a range of 16–28 nmol per min per mg observed in different microsomal preparations in that study. The low value was reported in that study because it was obtained in a preparation in which all of the enzymes of interest were assayed. We attribute the higher range of specific activities in the present study to refinements in our methods of lung fractionation (e.g., perfusion and lavage to remove more extraneous protein), the use of phosphatidic acid substrate from a different source, and possibly the use of a different species of rat.

tion of lung or liver microsomes were nearly identical to the rate of PA hydrolysis.

The hydrolysis of α- and β-glycerophosphate was poorly catalyzed by lung and liver microsomes under conditions of the PAPase assay. At 2 mM, α-glycerophosphate was hydrolyzed at about 10% of the rate of PA hydrolysis. Hydrolysis of 2 mM β-glycerophosphate was barely detectable at about 1% of the activity towards PA. Increasing concentration of α- or β-glycerophosphate resulted in increasing rates of hydrolysis. Saturation was not apparent at 50 mM.

The possibility that PA, LPA, and α-glycerophosphate were hydrolyzed by the same enzyme in microsomes was investigated by combining these substrates in the assay mixture. If separate enzymes catalyze the hydrolysis of two substrates present together in one assay, the total phosphate released should equal the sum of the individual activities. If both substrates are hydrolyzed by the same enzyme and one substrate is present at a concentration that is near saturation, addition of the second substrate should produce little release of additional phosphate. Increasing the concentration of LPA up to 1 mM in the presence of 1 mM PA (bovine serum albumin was also present to prevent inhibition by LPA) showed no increase in phosphate released over that observed with PA alone. Addition of α-glycerophosphate up to 50 mM in the presence of 1 mM PA resulted in a small increase in phosphate released, about 30% of that expected if a separate enzyme catalyzed the hydrolysis of each substrate. Mixing experiments with β-glycerophosphate were not performed because of the low activity towards this substrate relative to PA. While these mixing data, similar for liver or lung, are consistent with a single enzyme catalyzing the hydrolysis of PA, LPA, and α-glycerophosphate, conclusive evidence regarding this point requires purification of the enzyme or enzymes.

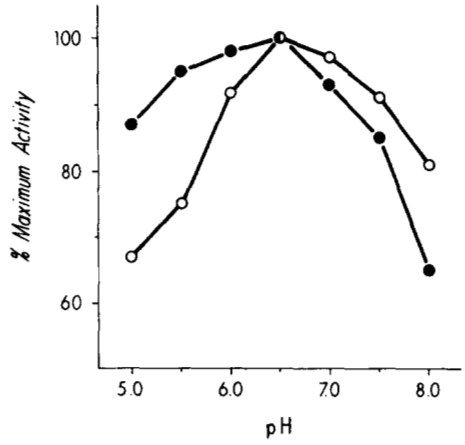

Fig. 4. Effect of pH on PAPase activity of microsomal fraction from lung (○) and liver (●).

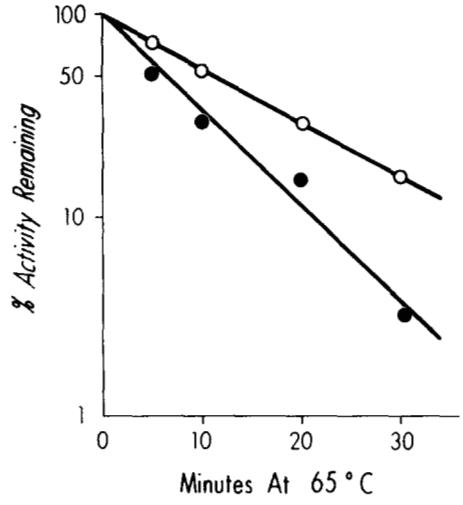

Fig. 5. Thermolability of PAPase activity in microsomal fraction from lung (○) and liver (●).

Effect of EDTA and $MgCl_2$

No significant effect on the activity of lung or liver microsomal PAPase activity was observed when either EDTA or $MgCl_2$ was added to the assay in concentrations from 0.5 to 8.0 mM.

Effect of pH

The pH profiles of lung and liver microsomal PAPase are shown in **Fig. 4**. The enzymes from both tissues showed maximum specific activities between pH 6 and 7. The two enzymes differed, however, at extremes of pH. The liver enzyme at pH 5 retained nearly 90% of its maximum specific activity while the lung enzyme was less than 70% as active at pH 5 compared to pH 6.5. Conversely, at pH 8, the liver enzyme decreased to 65% of its maximum activity while the lung enzyme at pH 8 retained over 80% of its activity at pH 6.5.

Thermolability

Lung microsomes incubated at 65°C lost activity in an apparent first order reaction with 50% inactivation occurring in about 11 min. Liver microsomes lost activity more rapidly at 65°C, with a half-time of about 6 min (**Fig. 5**).

Effect of fluoride and tartrate

The effects of fluoride, a known inhibitor of liver PAPase (27) and tartrate, which is a specific inhibitor of lysosomal acid phosphatase (28), on microsomal hydrolysis of PA, LPA, α- and β-glycerophosphate are shown in **Table 1**. Fluoride at 80 mM produced

TABLE 1. Phosphohydrolase activities in lung and liver microsomes in the presence of fluoride or tartrate

Source of Microsomes	Substrate	Specific Activity nmol phosphate released per min per mg protein		
		Control	80 mM Tartrate	80 mM Fluoride
Lung	1 mM PA	33	28 (15%)[b]	9.9 (70%)
Liver	1 mM PA	11	9.4 (15%)	3.2 (71%)
Lung	1 mM LPA[a]	34	30 (12%)	7.3 (79%)
Liver	1 mM LPA[a]	12	10 (15%)	3.4 (71%)
Lung	100 mM α-glycerophosphate	23	18 (23%)	5.4 (77%)
Liver	100 mM α-glycerophosphate	60	57 (6%)	24 (60%)
Lung	50 mM β-glycerophosphate	7.8	2.3 (71%)	1.0 (87%)
Liver	50 mM β-glycerophosphate	3.2	1.6 (50%)	1.3 (60%)

[a] Bovine serum albumin (1.8 mg/ml) was included to prevent substrate inhibition by LPA.
[b] Percentage inhibition.
Assay conditions were the standard PAPase assay with substrates as indicated.

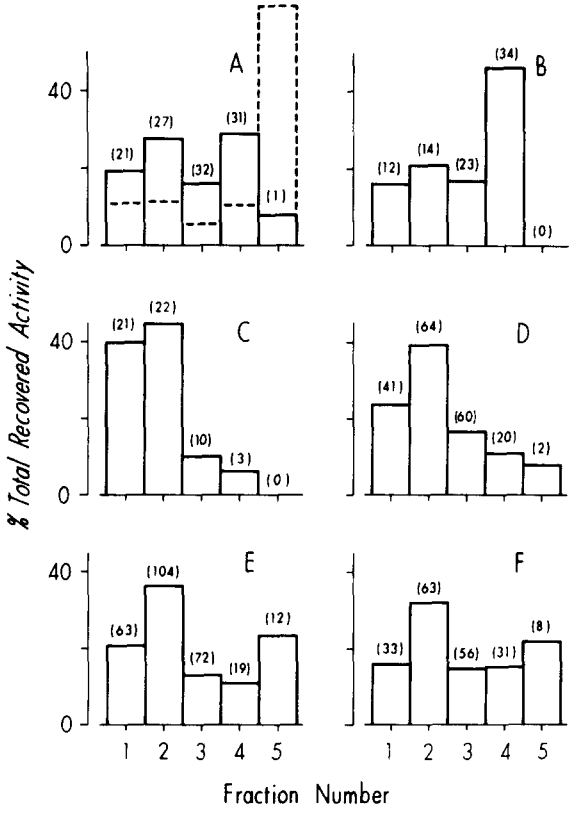

Fig. 6. Distribution of PAPase, protein, and marker enzymes in subcellular fractions of rat lung. Fractions 1–5 were prepared from rat lung cell-free homogenate as described in Experimental Procedure. Specific activity of each fraction (nmol per min per mg protein) is shown in parentheses. *A*, phosphatidate phosphohydrolase, solid line; protein, broken line; *B*, NADPH cytochrome *c* reductase; *C*, succinate cytochrome *c* reductase; *D*, *N*-acetyl-β-glucosaminidase; *E*, aryl sulfatase; *F*, acid phosphatase. Specific activity of each enzyme in the cell-free homogenate was: *A*, 10; *B*, 8; *C*, 7; *D*, 20; *E*, 28; *F*, 20. Total recovery of succinate cytochrome *c* reductase in the five fractions was 68% of the activity in the cell-free homogenate. Recovery of all other enzyme activities was 82% or more. Protein recovery was 87%.

60–87% inhibition of the hydrolase activity towards all four substrates in both lung and liver. Tartrate at the same concentration showed little inhibition towards hydrolysis of PA, LPA, or α-glycerophosphate, while inhibiting β-glycerophosphatase 50–70%.

Subcellular distribution

The distribution of PAPase in subcellular fractions of lung and liver is shown in **Figs. 6 and 7**, along with the content of a number of enzymes commonly used as biochemical markers for mitochondria, lysosomes, and endoplasmic reticulum. The distribution of the marker enzymes studied was similar in lung and liver. Mitochondria appeared to be nearly exclusively localized in fractions 1 and 2, which contained 85–100% of the recovered succinate cytochrome *c* reductase activity. Fractions 1 and 2 also contained major amounts of the lysosomal markers, *N*-acetyl-β-glucosaminidase, aryl sulfatase, and acid phosphatase. These enzymes were also enriched in fraction 3 and acid phosphatase showed a slight enrichment in fraction 4 of lung (1.5-fold over cell-free homogenate). The marker used for endoplasmic reticulum, NADPH cytochrome *c* reductase, was most enriched in fraction 4 (4–5-fold over cell-free homogenate). The three more rapidly sedimenting fractions also contained significant amounts of this enzyme.

The activity of PAPase in the subcellular fractions was not parallel to the apparent distribution of any single subcellular organelle. Its distribution was consistent with association of the enzyme with all of the organelles. Less than 10% of the total recovered PAPase activity was in the soluble fraction 5 of lung. In liver, 12% of the enzyme was soluble, consistent with previous reports (29, 30).

In some preparations, a lamellar body fraction was isolated from a 1,600–16,000 *g* pellet as previously described (26). Less than 0.2% of the total recovered PAPase activity was found in this fraction and therefore it was not included in the reported fractionation scheme.

A quantitative interpretation of the data in Figs. 6 and 7 appears in **Table 2**, which gives an estimated contribution of each subcellular organelle to the PAPase activity in fractions 1–4. This interpretation was derived from the following analysis.

The specific activities of microsomal, mitochondrial, and lysosomal PAPase in fraction 1 were designated A, B, and C, respectively. The sum of these unknowns is equal to the total specific activity of fraction 1. The specific activities of fractions 2–4 were then defined in terms of these three unknowns by using ratios of the appropriate marker enzymes to define the contribution of each subcellular organelle relative to fraction 1. Thus:

$$\text{Specific activity of fraction 1} = A + B + C$$

$$\text{Specific activity of fraction 2} = a_2A + b_2B + c_2C$$

$$\text{Specific activity of fraction 3} = a_3A + b_3B + c_3C$$

$$\text{Specific activity of fraction 4} = a_4A + b_4B + c_4C$$

where

$$a_n = \frac{\text{specific activity of NADPH cytochrome } c \text{ reductase in fraction n}}{\text{specific activity of NADPH cytochrome } c \text{ reductase in fraction 1}}$$

$$b_n = \frac{\text{specific activity of succinate cytochrome } c \text{ reductase in fraction n}}{\text{specific activity of succinate cytochrome } c \text{ reductase in fraction 1}}$$

$$c_n = \frac{\text{specific activity of } N\text{-acetyl-}\beta\text{-glucosaminidase in fraction n}}{\text{specific activity of } N\text{-acetyl-}\beta\text{-glucosaminidase in fraction 1}}$$

These equations were solved for the unknowns A, B, and C, which then allowed calculation of the contribution of microsomes, mitochondria, and lysosomes to PAPase activity measured in each fraction by the microsomal assay. Since the assay was designed to optimize microsomal activity, the absolute amounts of mitochondrial and lysosomal activities may be underestimated by this analysis in both tissues. Comparison of the two tissues, however, shows that the total microsomal PAPase activity found in the four membranous fractions of lung was 2.9 times greater than the total

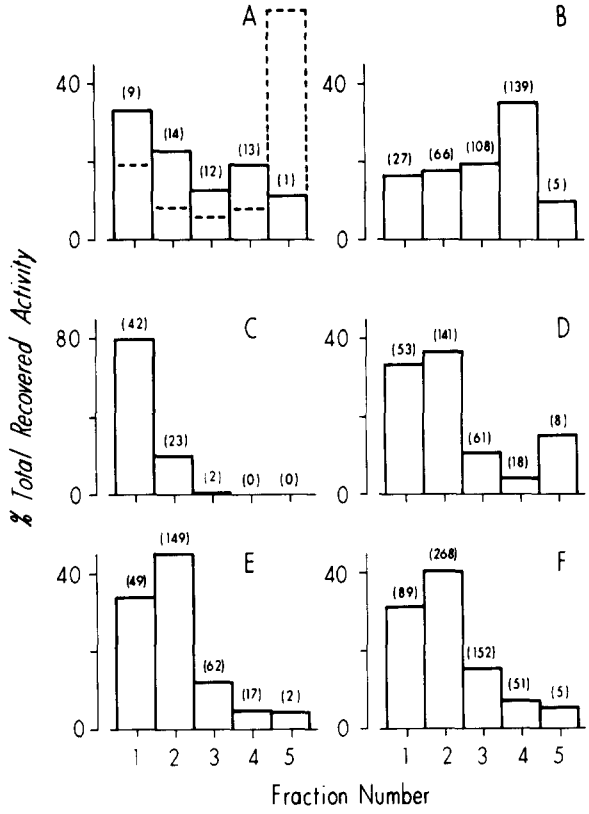

Fig. 7. Distribution of PAPase, protein, and marker enzymes in subcellular fractions of rat liver. Details as in Fig. 6. Specific activity of cell-free homogenate was: *A*, 5; *B*, 31; *C*, 9; *D*, 28; *E*, 23; *F*, 52. Enzyme recoveries from cell-free homogenate were 75% or more and protein recovery was 76%.

microsomal activity found in the corresponding fractions of liver, starting with the same amount of cell-free homogenate protein. The mitochondrial activity, in contrast, showed no difference in the total amount measured by this assay in the two tissues. Measurable lysosomal activity was two-fold higher in lung than in liver.

DISCUSSION

The presence of PAPase of high specific activity in a microsomal fraction of lung is consistent with the high rate of de novo phosphatidylcholine biosynthesis which is presumably required for maintenance of the rapidly degraded phosphatidylcholine component of pulmonary surfactant. Implication of the enzyme in surfactant synthesis requires several lines of evidence. *1*) The specificity of the enzyme towards lipid substrates must be evident. *2*) The cellular and subcellular sources of the enzyme must be established. *3*) The appearance of the enzyme during gestation should coincide with the appearance of surfactant in the fetal lung. This coincidence has been recently reported for the enzyme from rabbit lung (31). In the

TABLE 2. Estimated contribution of endoplasmic reticulum, mitochondria, and lysosomes to PAPase activity in membranous subcellular fractions of rat lung and liver

Fraction	Endoplasmic Reticulum		Mitochondria		Lysosomes		Total	
	Specific[a] Activity	Units[b]	Specific Activity	Units	Specific Activity	Units	Specific Activity	Units
Lung								
1	10.4	47	4.1	19	6.7	30	21	96
2	12.7	62	4.3	21	10.3	51	27	134
3	20.4	51	2.0	5	9.7	24	32	80
4	27.7	125	0.5	2	2.9	13	31	140
Total units		285		47		118		450
Liver								
1	2.1	17	4.4	37	2.6	22	9	76
2	5.5	20	2.4	9	6.5	23	14	52
3	8.6	21	0.2	1	3.0	7	12	29
4	12.1	41	0	0	1.0	3	13	44
Total units		99		47		55		201

[a] nmol PA hydrolyzed per min per mg total protein in fraction.

[b] nmol PA hydrolyzed per min per total fraction derived from 50 mg of cell-free homogenate protein. Liver data were normalized to 85% recovery of cell-free homogenate protein which was the recovery in lung. (Actual liver recovery was 76%.)

present study we present evidence regarding points 1 and 2.

The substrate specificity of the highly active lung enzyme is similar to that of the liver enzyme. Phosphatidic acid and lysophosphatidic acid are hydrolyzed at comparable rates and the concentration effects of these substrates are similar in the two tissues. Comparable rates of hydrolysis of PA and LPA have also been reported for a preparation of PAPase which was purified six-fold from rat liver microsomes (27). Activity of lung microsomes towards glycerophosphate is relatively low in the concentration range that is optimal for PA. Greater than 10-fold higher concentrations of α- or β-glycerophosphate is required to produce comparable rates of phosphate release. Thus the lung enzyme appears highly specific for PA or LPA relative to soluble substrates.

Inhibition of PA hydrolysis by fluoride and lack of inhibition by tartrate are consistent with lung microsomal PAPase being a biosynthetic enzyme rather than a lysosome-derived degradative enzyme.

The subcellular source of the PAPase activity in the microsomal fraction of the lung was established by comparing the distribution of PAPase in several differential centrifugal fractions of rat lung and liver with the distribution of marker enzymes which are believed to be exclusively located in specific subcellular organelles. The distribution of marker enzymes in the fractions from lung or liver shows that the differential centrifugal scheme used here yielded fractions from either tissue that were similar in their content of lysosomes, mitochondria, and endoplasmic reticulum. Most significantly, fraction 4 from both tissues was most enriched in the marker enzyme for endoplasmic reticulum and contained relatively insignificant amounts of the enzymes used as markers for mitochondria or lysosomes. Thus the PAPase in this fraction would seem to be most probably derived from endoplasmic reticulum, and the high specific activity of this fraction from lung compared to liver would seem to be properly interpreted as reflecting an increased activity of a lung "microsomal" enzyme. In addition, the quantitative analysis of the membranous fractions (Table 2) demonstrates that the total amount of PAPase activity in each fraction can be accounted for by a contribution from each subcellular organelle, which is proportional to the amount of the appropriate marker enzyme in each fraction. This analysis allows an estimate of the total microsomal, mitochondrial, and lysosomal PAPase in all four membranous fractions from each tissue, and thus the total amount of enzyme associated with each organelle as measured by the assay used here. The association of the PAPase with all three of the organelles studied has been accepted for a number of years (29, 30). Our analysis is an attempt to quantitatively estimate the distribution of activity among these organelles. This is important in implicating the highly active lung PAPase in surfactant lipid synthesis, which is believed to occur on the endoplasmic reticulum of the alveolar Type II epithelial cell (2). Comparison of estimated total microsomal enzyme in lung and liver (Table 2) confirms what is suggested by comparison of the raw data from fraction 4, i.e., the higher activity per mg of lung extract

compared to liver extract derives primarily from an increase in the microsomal enzyme. Of the 249 additional enzyme units recovered from 50 mg of lung homogenate compared to 50 mg of liver homogenate, 186 units or 75% of the increase results from an increased microsomal activity. Thus the higher PAPase activity in lung is not a generalized increase in PAPase associated with all organelles, but an increase that primarily reflects an increased activity of the endoplasmic reticulum.

Our finding of less than 0.2% of the total recovered lung PAPase in an isolated lamellar body fraction is in apparent disagreement with the results of Meban (32) who reported histochemical evidence for localization of PAPase in the perilamellar membrane of lamellar inclusion bodies of alveolar Type II epithelial cells of hamster lung. The histochemical procedure of that study utilized osmotic damage to the lamellar inclusion bodies of fixed lung tissue to provide a point of entry for the diffusion of phosphatidic acid substrate into the tissue. The substrate emulsion was believed to enter the damaged lamellar vacuoles, but it was not established that the substrate permeated the tissue beyond the limiting membrane of the lamellar bodies. The localized deposit of lead phosphate in the perilamellar membrane suggests, in fact, that this membrane served as a diffusion barrier to the PA substrate which may not have permeated the rest of the cell. Hydrolysis of PA accumulated at the perilamellar membrane may have been by soluble phosphohydrolases or enzymes present in the membrane itself. The quantity of enzyme acting at the perilamellar membrane cannot be compared to the quantity present elsewhere in the cell by this technique.

More recently, biochemical assay of PAPase in isolated lamellar bodies of pig lung (33) and detection of PAPase together with highly saturated phosphatidylcholine in a 105,000 *g* pellet of human amniotic fluid (34) have led to speculation that lamellar bodies might be the site of biosynthesis of pulmonary surfactant (34). The PAPase in amniotic fluid was believed to have been secreted by fetal lungs in association with pulmonary surfactant, although earlier studies reported that an increase in PAPase activity of amniotic fluid during gestation preceded the increase in surfactant phosphatidylcholine by 1–2 weeks (35, 36). The role of PAPase in amniotic fluid remains to be established.

In the lamellar body study (33), 0.3% of the total lung PAPase was found in an isolated lamellar body fraction, essentially in agreement with our present findings. The authors of the pig lung study, however, interpreted their data to indicate that 40% of the PAPase in Type II cells of the alveolar epithelium was associated with lamellar bodies. This calculation required three assumptions. The first was that dipalmitoyl phosphatidylcholine in the lung is an exclusive biochemical marker for lamellar bodies. This is clearly not true, since a major amount of dipalmitoyl phosphatidylcholine in the intact lung exists extracellularly as secreted surfactant (37) and it has not been established that lamellar bodies are the exclusive intracellular site of dipalmitoyl phosphatidylcholine. Thus the first assumption resulted in an overestimation of the total content of lamellar bodies in the lung. Unfortunately, no biochemical marker exclusive for lamellar bodies is presently known.

Second, the authors assumed that all of the PAPase in the isolated lamellar body fraction was integral to the lamellar body structure rather than due to contamination with enzyme from other organelles. We have reported (26) that a lamellar body fraction from rat lung was contaminated by microsomes to an extent of 14%, enough to account for all of the PAPase present in that fraction. In our present study, lamellar bodies were contaminated with microsomes up to about 25%,[2] accounting for a major amount of the lamellar body-associated PAPase. The lamellar bodies of pig lung were shown to be less than 6% contaminated with microsomes and were free of mitochondria. Thus the high specific activity of PAPase in that preparation could not be accounted for by contamination with either microsomes or mitochondria. The pig lung homogenate was prepared with a blade-type homogenizer, a procedure that has been reported to release lysosomal enzymes (38) and to disrupt the structure of lamellar bodies (39). Assessment of lysosomal contamination of lamellar bodies is complicated by the observation of DiAugustine that a number of lysosomal hydrolases copurified with lamellar bodies from rabbit lung (40). In addition, histochemical studies have shown that certain hydrolytic activities were selectively localized in lamellar bodies of fixed lung tissue (41–43). The work of Meban (32), discussed above, suggests that histochemical localization of activities in lamellar bodies must be interpreted with caution, since the peculiar osmotic properties of the lamellar inclusions may render them selectively permeable to histochemical substrates. It is unclear whether lysosomal hydrolases in isolated lamellar bodies are derived from lysosomes or are integral components of the lamellar bodies themselves. Several authors have suggested that lamellar bodies may be a form of lysosome (40–42). Lysosomes of liver contain PAPase activity (29, 30), and our

[2] Mavis, R. D., J. N. Finkelstein, and B. P. Hall, unpublished observations.

present data suggest PAPase is also associated with lung lysosomes. Thus the possible lysosomal character of lamellar body-associated PAPase must be investigated before it can be assigned a function.

The third assumption of Spitzer et al. (33) was that PAPase was evenly distributed throughout the total lung cell population and that Type II alveolar cells, which make up 5–10% of total lung cells, contain only 5–10% of the total lung PAPase activity. Even distribution of PAPase in the many diverse cell types of lung seems unlikely. Type II alveolar cells, which are the most active of the lung cells with respect to lipid synthesis (2), might be expected to contain a major rather than a minor fraction of lung PAPase.

Thus we feel that a more conservative interpretation of the findings of Spitzer et al. (33), Garcia et al. (26), and our present study with respect to PAPase in lamellar bodies is that the relatively small amount of enzyme that is isolated with these unique organelles is unlikely to be functional in the relatively rapid synthesis of pulmonary surfactant phosphatidylcholine.

The microsomal PAPase characterized here is 63% of the total lung enzyme according to the analysis in Table 2 and thus could play a major role in lung lipid synthesis, of which surfactant synthesis is apparently the most active pathway (2). In addition to its high activity, the lung microsomal enzyme is distinguished from the liver enzyme by differences in its pH profile and thermolability. These differences could be due to differences in the membranous environment or differences in the structure of the enzymes. Whether the high activity in lung reflects a different PAPase or a larger amount of the same enzyme found in liver can be resolved only by further comparison of more highly purified preparations of the enzymes.

The cellular distribution of the microsomal lung PAPase, studied here in fractions of whole lung homogenate, remains to be determined. Studies on isolated Type II cells are obviously essential in further implication of this enzyme in surfactant synthesis.

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