Pediat. Res. 14: 254–258 (1980)

liposome
lung
phosphatidylcholine
phospholipase A
respiratory distress syndrome
surfactant

The Action of Lung Lysosomal Phospholipases on Dipalmitoyl Phosphatidylcholine and its Significance for the Synthesis of Pulmonary Surfactant

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Summary

Subcellular fractions were prepared from rabbit lung and characterized by marker enzyme assays. Phospholipases A of lysosomal type (active at pH 4.0 in the absence of Ca^{2+}) show a peak of specific activity in fraction P3 (13000 × *g*; 20 min; pellet). About 70% of the phospholipase A activity of P3 can be obtained in a soluble extract. The rates of hydrolysis of dipalmitoyl (saturated) and unsaturated phosphatidylcholine molecules, presented to enzyme preparations in liposomes of varying composition, are shown in Tables 3 to 5. Although the rate for unsaturated molecules is unaffected by liposome composition, saturated molecules are hydrolyzed significantly faster in 5 mol % dipalmitoyl phosphatidylcholine liposomes than in 95 mol %. With enzyme preparation I (60% A_1; 40% A_2) the increase was 6-fold ($P < 0.01$). Preparations I and II hydrolyzed both kinds of molecules at similar rates in 50 mol % dipalmitoyl phosphatidylcholine liposomes, but preparation III, with a higher proportion of A_2 (80%), gave a notably lower rate of hydrolysis of saturated molecules. Phosphatidylglycerol (10 mol %) also decreased the rate of hydrolysis of saturated molecules. These results are discussed in relation to fluidity of the liposomes and to the functions of the lysosomal-type phospholipases A of the lamellar inclusion bodies of the lung.

Speculation

In lamellar bodies, phospholipase A_2 will preferentially hydrolyze unsaturated phosphatidylcholine, yielding a lysolipid which can be reacylated to produce dipalmitoyl phosphatidylcholine; phospholipase A_1 will require further inhibition, possibly provided by the phosphatidylglycerol of surfactant. Failure of A_2 action or failure of the inhibition of A_1 would be expected to lead to a deficiency of surfactant and thus to the respiratory distress syndrome in the newborn.

Pulmonary surfactant, the agent that maintains the stability of the alveoli of the lung against collapse, is a mixture of lipids with a small amount of protein. The principal lipid, comprising at least 40% of the total, is 1,2-dipalmitoyl phosphatidylcholine (15, 22). Most mammalian phosphatidylcholines have at least one unsaturated fatty acid residue (at position 2), but this major component of surfactant has two saturated acids. This unusual composition is necessary for its surfactant function (40). The respiratory distress syndrome of the newborn (hyaline membrane disease) results from a deficiency of pulmonary surfactant (4, 13, 20).

The source of the extracellular surfactant is to be found in the lamellar inclusion bodies of the cuboidal (type II) pneumonocytes of the alveolar epithelium (4, 9, 13, 20, 21). Cytochemical studies on lung sections and enzyme assays performed on isolated lamellar body preparations have shown the presence in these inclusions of numerous lysosomal hydrolases (16). The origin and purpose of this lysosomal involvement is unsure, but it seems likely that lysosomes take up newly synthesized surfactant by an autophagic mechanism and process it to the characteristic lamellar bodies before it is released from the cell by exocytosis (16, 34, 36).

The lysosomal hydrolases present in lamellar bodies include two enzymes capable of catabolizing the phosphatidylcholine of surfactant, phospholipases A_1 (EC 3.1.1.32) and A_2 (EC 3.1.1.4) (17). Phospholipase A_1 hydrolyzes the fatty acid residue at position 1 of phosphatidylcholine and other phospholipids; phospholipase A_2 removes the acid at the 2-position. The lysosomal forms of these enzymes have maximum activity near pH 4, are inhibited by Ca^{2+}, and require the phospholipid for hydrolysis to be in the form of a bilayer, such as the lamellae of the lung inclusion bodies or the liposomes (lipid vesicles) used as the assay substrate.

Aside from their catabolic role, these enzymes could function in the normal process of surfactant biosynthesis. The lysosomal phospholipase A_2 of the lamellar bodies in particular may be concerned with the remodeling of unsaturated phosphatidylcholine into the dipalmitoyl species by removing the unsaturated fatty acid from the 2-position so that it may be replaced with a palmitoyl residue (37).

Under normal conditions, the dipalmitoyl phosphatidylcholine is not degraded by these enzymes, but under abnormal conditions, the situation could arise where surfactant is synthesized at a normal rate, but is broken down intracellularly by phospholipases before it can be released to the alveolar space. This paper describes the preparation of soluble lysosomal phospholipases from rabbit lung, gives an analysis of their action on dipalmitoyl and unsaturated phosphatidylcholines presented to the enzymes as liposomes of mixed composition, and discusses the relevance of these findings to the roles of phospholipases A in the normal and pathologic metabolism of pulmonary surfactant.

MATERIALS AND METHODS

PREPARATION OF A LYSOSOMAL FRACTION FROM RABBIT LUNG

Lungs from New Zealand White rabbits were homogenized in cold 0.25 M sucrose in 50 mM Tris-HCl (pH 7) (10 ml/g) for 2 × 30 sec with a Silverson homogenizer (Silverson Machines Ltd., London, United Kingdom), and the homogenate was filtered through four layers of muslin. A scheme of differential centrifugation was then applied as follows (see Table 1): 600 × *g*, 10 min to yield pellet P1 consisting of cell debris and nuclei; 5000 × *g*, 20 min giving P2, mitochondrial fraction; 13000 × *g*, 20 min giving P3, lysosomal fraction; 40,000 × *g*, 30 min giving P4, microsomal fraction, and the final supernatant, S. All pellets were resuspended in 1 mM disodium EDTA in 1 mM $NaHCO_3$. All fractions and a sample of the homogenate were subjected to five cycles of freezing and thawing and then dialyzed overnight against 1 mM EDTA: 1

mM $NaHCO_3$. These fractions were analyzed for protein content and characterized by assays of marker enzymes.

ANALYSIS OF LUNG FRACTIONS

Protein was estimated by the procedure of Lowry *et al.* (25) as modified by Campbell and Sargent (7) with bovine serum albumin as standard (Armour Pharmaceutical Co., Chicago, IL). Lysosomal acid phosphatase (EC 3.1.3.2) was determined as that activity sensitive to inhibition by tartrate (see Ref. 3), with 4-nitrophenyl phosphate as substrate (Sigma Technical Bulletin 104; Sigma London Chemical Co. Ltd., Poole, Dorset, United Kin}. Cathepsin D (EC 3.4.23.5) was assayed according to Barrett (2) with bovine hemoglobin (gift of Dr. A. J. Barrett) as substrate. Total arylsulphatase (EC 3.1.6.1) was determined by the method of Worwood *et al.* (42) which yields a combined value for arylsulphatases A and B. The substrate was 2-hydroxy-5-nitrophenyl sulphate (Sigma London Chemical Co. Ltd.). Succinate dehydrogenase (EC 1.3.99.1) was assayed according to Pennington (29) as modified by Porteous and Clark (31). NADPH cytochrome reductase (EC 1.6.2.4) was assayed with cytochrome c (Sigma London Chemical Co. Ltd.) as acceptor (30). Lactate dehydrogenase (EC 1.1.1.27) was determined according to Sigma Technical Bulletin 340-UV.

PREPARATION OF EXTRACT CONTAINING LYSOSOMAL PHOSPHOLIPASES

After freezing and thawing and dialysis of fraction P3 from rabbit lung, soluble phospholipases A were recovered by centrifugation at 90000 × *g* for 2 hr. The soluble extract of P3 was dialyzed against 20 mM Tris-HCl (pH 7.5), applied to a column (1.5 × 32 cm) of Ultrogel AcA54 (LKB Instruments Ltd., Croydon, United Kingdom), and eluted with this buffer. Fractions of 1 ml were collected.

PHOSPHOLIPASE ASSAYS

The assay of lysosomal phospholipases A_1 (EC 3.1.1.32) and A_2 (EC 3.1.1.4) in rabbit lung fractions was performed as previously described (17, 43), except that an improved chromatographic system was used: hexane:chloroform:methanol:acetic acid:water, 100:75:25:2:1, v/v. Sensitivity to inhibition by Ca^{2+} ions was investigated in 0.1 M sodium acetate buffer (pH 4.0) containing either 5 mM disodium EDTA or 20 mM calcium acetate. Incubations were also carried out in 0.1 M Tris-HCl (pH 9.0 at 37°C) containing 5 mM $CaCl_2$ to determine nonlysosomal phospholipases A_1 and A_2.

For assays on column eluates, the substrate was 1-palmitoyl-2-[1-^{14}C]oleoylglycerophosphocholine (55 Ci/mole) (Applied Science Laboratories, State College, PA) diluted to 0.50 Ci/mole with Sigma type III-E phosphatidylcholine. Assay conditions were similar to those described for lysosomal enzymes above, except that 0.1 ml of column fraction was incubated in a final volume of 0.2 ml.

The investigations of the activity of the enzymes against dipalmitoyl and unsaturated phosphatidylcholines were carried out with 1,2-di-[1-^{14}C]palmitoyl-glycerophosphocholine (88 Ci/mole) and [*U*-^{14}C]phosphatidylcholine (from *Chlorella pyranoidosa*; 1765 Ci/mole) (both from NEN Chemicals GmbH, Dreieichenhain, West Germany), the latter diluted to 88 Ci/mole with Sigma type III-E phosphatidylcholine (from egg). These preparations were then diluted to 1.0 Ci/mole with either Sigma type III-E phosphatidylcholine or Sigma synthetic 1,2-dipalmitoyl phosphatidylcholine or a mixture of the two to produce substrates with different proportions of saturated and unsaturated phospholipid, namely 5, 40, 50, 60 and 95 mol % dipalmitoyl phosphatidylcholine. The effect of phosphatidylglycerol on the hydrolysis of saturated and unsaturated phosphatidylcholines was investigated on mixtures of 9 parts of 50 mol % dipalmitoyl phosphatidylcholine with 1 part (mole/mole) of phosphatidylglycerol (prepared from egg phosphatidylcholine by Lipid Products, Redhill, Surrey, United Kingdom). Liposomes of these substrates were prepared at a phospholipid concentration of 0.1 mM in 80 mM sodium acetate (pH 4.0) containing 10 mM disodium EDTA, as follows. The lipids were dried from chloroform solution in a stream of N_2. The buffer (N_2 flushed) was added, and the tube was warmed at 45°C for 5 min. The contents of the tube were mixed by vortexing and then ultrasonicated for 1 min at 1.5 A in an MSE Ultrasonic Power Unit. After rewarming to 45°C, the substrate was dispensed in 0.1-ml aliquots to tubes at 37°C. The reaction was started by the addition of 0.1 ml of enzyme preparation.

Incubation proceeded for 1 hr at 37°C with continuous shaking, and then the reaction was stopped by the addition of 0.7 ml of chloroform: methanol (1:2, v/v), and the lipids were extracted by the method of Bligh and Dyer (5). Fatty acids and phospholipids were separated by TLC in hexane:diethyl ether:acetic acid (69:29: 2, v/v) (12) on silica gel G (Eastman Chromagram 13191 from Kodak Ltd., Liverpool, United Kingdom). The spots were excised, and the radioactivity was measured in toluene scintillant (3.4 g/ liter 2,5-diphenyloxazole and 0.05 g/liter 1,4-bis-(4-methyl-5-phenyloxazolyl-2)-benzene, both from Sigma London Chemical Co. Ltd). The percentage of breakdown of labeled phospholipid was then determined, due allowance being made for differences in specific activity between substrate and product (17). If dipalmitoyl and unsaturated phosphatidylcholines were not distinguished by the enzymes, the percentage of breakdown of each species at fixed phospholipid and enzyme concentrations would always be the same, irrespective of liposome composition. Differences between mean values of percentage breakdown were examined for significance by Student's *t* test.

Table 1. *Protein content of fractions from rabbit lung*

Fraction	Protein content (% of homogenate)
P1 600 × *g*, 10 min, pellet	18
P2 5000 × *g*, 20 min, pellet	3.9
P3 13000 × *g*, 20 min, pellet	1.9
P4 40000 × *g*, 30 min, pellet	2.0
S Final supernatant	65
Total recovery	91

RESULTS

PREPARATION OF A LYSOSOMAL FRACTION FROM RABBIT LUNG

Table 1 shows a typical protein distribution in fractions prepared from rabbit lung homogenate by differential centrifugation, and Figure 1 shows the profiles of marker enzymes. The profiles of succinate dehydrogenase and NADPH cytochrome reductase indicate that mitochondria and microsomes are concentrated in fractions P2 and P4, respectively. The lysosomal markers acid phosphatase and cathepsin D have highest specific activity in P3, but significant activity in P2 and P4. Arylsulphatase shows maximum specific activity in P2. Lysosomes thus appear to concentrate in P3 and to a lesser extent in P2 which also contains mitochondria. Phospholipases A of lysosomal type (active at pH 4.0 in the absence of Ca^{2+}) show a peak of activity in P3, although much A_1 activity is found in the final supernatant. Lysosomal phospholipases A, in contrast to nonlysosomal types, are sensitive to inhibition by Ca^{2+} (11, 39), and Table 2 shows the differences in calcium sensitivity between the phospholipases from P3 and S which suggest that the activity at pH 4.0 in the supernatant is not principally of lysosomal origin. The distributions of phospholipases of the nonlysosomal type (active at pH 9.0 in the presence of Ca^{2+}) differ from those of the lysosomal enzymes and show peaks of activity in the microsomal fraction, P4.

PREPARATION OF SOLUBLE LYSOSOMAL PHOSPHOLIPASES

About 70% of the phospholipase A activity of fraction P3 is obtained in the soluble extract after centrifugation. A typical

elution profile of this material after application to Ultrogel AcA54 is shown in Figure 2. Fractions 28 to 31 provided Enzyme Preparation I, containing phospholipases A_1 and A_2 in the ratio 60:40. Other extracts gave slightly different profiles, and from two of these were obtained preparation II (A_1:A_2 = 40:60) and preparation III (A_1:A_2 = 20:80).

THE ACTION OF LYSOSOMAL PHOSPHOLIPASES A ON DIPALMITOYL AND UNSATURATED PHOSPHATIDYLCHOLINES

The rates of hydrolysis of dipalmitoyl (saturated) and unsaturated phosphatidylcholine molecules presented to enzyme preparation I in liposomes of varying composition are shown in Table 3. Whereas the rate for unsaturated molecules is unaffected by liposome composition, saturated species are hydrolyzed 6-fold faster in 5 mol % dipalmitoyl phosphatidylcholine liposomes than in 95 mol % ($P < 0.01$). The trends in the center of the composition range were confirmed by enzyme preparation II (Table 4). Preparation III, which contained a higher proportion of phospholipase A_2 (80%), gave somewhat different results (Table 5), most notably the low rate of hydrolysis of saturated molecules in 50 mol % dipalmitoyl phosphatidylcholine liposomes. The effect of 10 mol % phosphatidylglycerol on hydrolysis rates in 50 mol % dipalmi-

Table 2. *Inhibition of lung phospholipases A by Ca^{2+}*

Fraction	Activity in the presence of Ca^{2+} (% of EDTA control)	
	Phospholipase A_1	Phospholipase A_2
P3	0	36
S	87	81

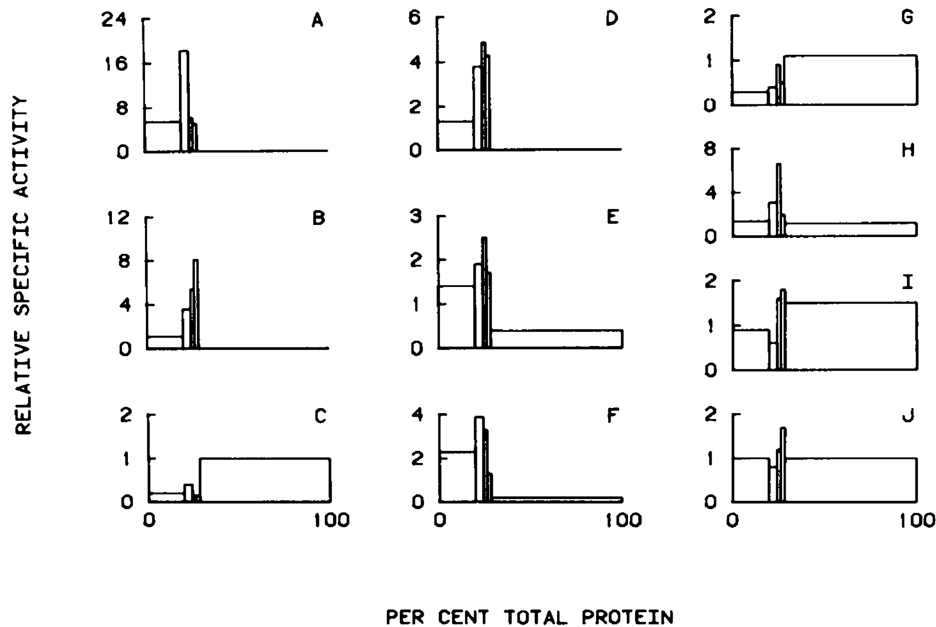

Fig. 1. Enzyme profiles of rabbit lung fractions. Fractions were prepared and assayed as described in "Materials and Methods." Enzyme activities are expressed as relative specific activities (specific activity of fraction/specific activity of homogenate). *A*, succinate dehydrogenase; *B*, NADPH cytochrome reductase; *C*, lactate dehydrogenase; *D*, acid phosphatase; *E*, cathepsin *D*; *F*, arylsulphatase; *G*, phospholipase A_1 (pH 4); *H*, phospholipase A_2 (pH 4); *I*, phospholipase A_1 (pH 9); *J*, phospholipase A_2 (pH 9). Sequence of fractions: P1, P2, P3, P4, S.

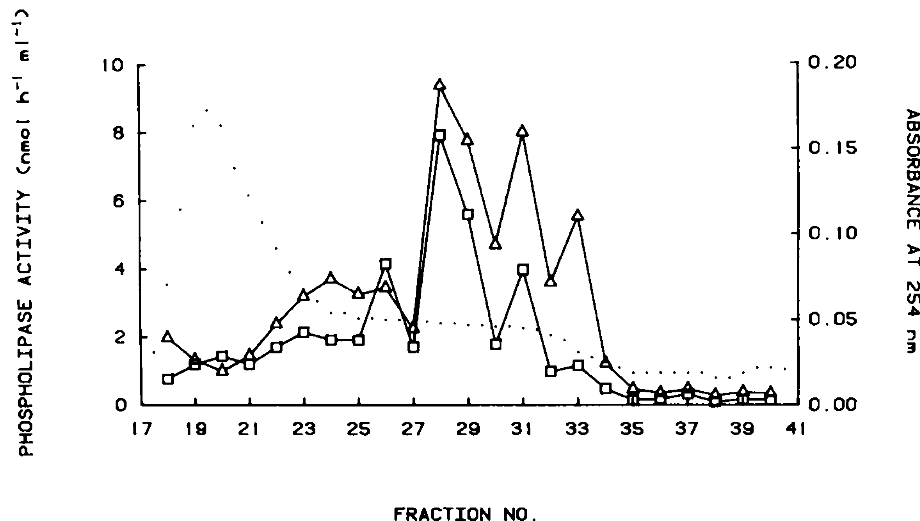

Fig. 2. Gel chromatography of soluble lysosomal phospholipases A from rabbit lung. The soluble extract of fraction P3 was chromatographed on a 1.5- × 32-cm column of Ultrogel AcA54 as described in "Materials and Methods." Fractions (1 ml) were analyzed for phospholipases A_1 (△) and A_2 (□) and monitored for protein content at 254 nm with a 3-mm light path (· · · ·). The void volume elutes at fraction 18.

Table 3. *Rates of hydrolysis of saturated and unsaturated phosphatidylcholines presented to lung lysosomal phospholipases A (preparation I)*[1] *in liposomes of varying composition*

Liposome composition (mol % DPPC)[2]	Rates of hydrolysis[3] (%/hr)		
	Saturated	Unsaturated	P
5	16.44 ± 2.30[4]	5.37 ± 0.44	<0.01
50	7.92 ± 1.78	6.87 ± 2.79	NS[5]
95	2.64 ± 1.42	6.07 ± 0.93	<0.01

[1] A_1:A_2 = 60:40.
[2] Dipalmitoyl phosphatidylcholine.
[3] $n = 4$.
[4] Mean ± S.E.
[5] Not significant ($P > 0.05$).

Table 4. *Rates of hydrolysis of saturated and unsaturated phosphatidylcholines presented to lung lysosomal phospholipases A (preparation II)*[1] *in liposomes of varying composition*

Liposome composition (mol % DPPC)[2]	Rates of hydrolysis[3] (%/hr)		
	Saturated	Unsaturated	P
40	4.22 ± 1.08[4]	4.14 ± 1.51	NS[5]
50	3.75 ± 0.73	4.16 ± 0.93	NS
60	3.08 ± 0.29	4.22 ± 0.98	NS

[1] A_1:A_2 = 40:60.
[2] Dipalmitoyl phosphatidylcholine.
[3] $n = 4$.
[4] Mean ± S.E.
[5] Not significant ($P > 0.05$).

Table 5. *Rates of hydrolysis of saturated and unsaturated phosphatidylcholines presented to lung lysosomal phospholipases A (preparation III)*[1] *in liposomes of varying composition*

Liposome composition (mol % DPPC)[2]	Rates of hydrolysis[3] (%/hr)		
	Saturated	Unsaturated	P
5	8.90 ± 0.88[4]	6.81 ± 0.97	NS[5]
50	2.68 ± 0.30	12.89 ± 0.61	<0.001
95	2.18 ± 0.68	9.71 ± 0.68	<0.001

[1] A_1:A_2 = 20:80.
[2] Dipalmitoyl phosphatidylcholine.
[3] $n = 4$.
[4] Mean ± S.E.
[5] Not significant ($P > 0.05$).

Table 6. *Effect of phosphatidylglycerol on the hydrolysis of phosphatidylcholines*[1] *by lung lysosomal phospholipases A*[2]

Liposome composition (mol % phosphatidylglycerol)	Rates of hydrolysis[3] (%/hr)	
	Saturated	Unsaturated
0	7.92 ± 1.78[4]	6.87 ± 2.79
10	3.16 ± 0.58	6.58 ± 0.37
P	<0.05	NS[5]

[1] Fifty mol % dipalmitoyl phosphatidylcholine liposomes.
[2] Preparation I, A_1:A_2 = 60:40.
[3] $n = 4$.
[4] Mean ± S.E.
[5] Not significant ($P > 0.05$).

toyl phosphatidylcholine liposomes, with preparation I, is shown in Table 6.

DISCUSSION

The different rates of hydrolysis of saturated and unsaturated phosphatidylcholines could be due to the chemical differences between the molecules or to a marked physical difference known to exist between the substrate liposomes. Dipalmitoyl phosphatidylcholine is solid in liposomes at 37°C, the temperature of its transition to the fluid state being 41.4°C (26). The transition temperature for egg phosphatidylcholine, on the other hand, is near −10°C (8), so this lipid is fluid in liposomes. When the dipalmitoyl species is diluted with egg phosphatidylcholine, its transition broadens, and the temperature of the transition is lowered until at compositions of less than about 70 mol % dipalmitoyl phosphatidylcholine the lipid is fluid at 37°C (23). Those mixtures that are below the transition temperature are probably mosaics composed of areas of solid dipalmitoyl and fluid unsaturated phosphatidylcholine. Above that temperature, there will be limited miscibility of the fluid phases (24, 38).

The results with liposomes containing 5 mol % dipalmitoyl phosphatidylcholine show that chemically the saturated species is susceptible to attack by phospholipases A (Table 3), although perhaps phospholipase A_2 attacks less readily than A_1 (Table 5). However, the physical condition of the liposomes does affect the rate of hydrolysis of the saturated molecules (Tables 3 to 5). As the proportion of saturated molecules is increased, the solidity of the liposomes increases, and the rate of hydrolysis of dipalmitoyl phosphatidylcholine falls. Similar changes in enzyme activity caused by alterations in the fluidity of lipids have been described, but only for nonlysosomal phospholipases (18, 19, 28, 41).

Preparations of pulmonary surfactant contain 40 to 60% disaturated phosphatidylcholine (15, 22). It is difficult to obtain intracellular surfactant free of contaminating lipids, but it must certainly contain more than 60% disaturated phosphatidylcholine. If this is the composition of the lamellae, then those phosphatidylcholine molecules remaining unsaturated will be attacked preferentially by the lamellar body phospholipase A_2 (Table 5), whereas the phospholipase A_1 will attack the dipalmitoyl molecules as well (Tables 3 and 4). The presence of phosphatidylglycerol, which constitutes up to 10% of surfactant, could modify this picture. Phosphatidylglycerols are freely miscible with phosphatidylcholines of similar fatty acid composition (10), so they do not affect fluidity, but unlike phosphatidylcholines they are negatively charged and thus alter the charge of the lamellae (or substrate liposomes). Table 6 shows the inhibitory effect of the addition of 10 mol % phosphatidylglycerol on the hydrolysis by enzyme preparation I of saturated molecules in liposomes of 50 mol % dipalmitoyl phosphatidylcholine.

The *de novo* pathways of phosphatidylcholine synthesis in the lung produce principally unsaturated molecules (32, 33, 35) which must be converted to the dipalmitoyl species by a remodeling process (6, 37). This starts with phospholipase A_2 action on the unsaturated lipid yielding 1-palmitoyl-2-lysophosphatidylcholine which can be turned into the required product by the introduction of a palmitoyl residue at the vacant 2-position of molecule. The lung has two mechanisms for achieving this, either transfer from palmitoyl-CoA (reacylation) or from a second molecule of the palmitoyl lysophosphatidylcholine (transacylation). The preferential attack of lamellar body phospholipase A_2 on unsaturated phosphatidylcholine yields as the major product 1-palmitoyl-2-lysophosphatidylcholine, the substrate for the remodeling enzymes. The principal site of remodeling processes is probably the endoplasmic reticulum, where many enzymes of the *de novo* pathway and the reacylating enzyme (lysophosphatidylcholine: acyl-CoA acyltransferase, EC 2.3.1.23) are found. The transacylating enzyme (lysophosphatidylcholine:lysophosphatidylcholine acyltransferase, EC 2.3.1.-) is, however, free in the cytosol, and it has been suggested (1) that it might be able to act on the products of lamellar body phospholipase A_2 action *in situ*, although the mechanism by which this might occur is unclear. In any case, it seems probable that the lysolipid will be returned to the synthetic pathways for remodeling. Failure to remodel effectively results in a form of respiratory distress in which normal amounts of surfactant phosphatidylcholine are produced, but with a decreased proportion of saturated molecules (27).

Clearly, the presence of phospholipases in lamellar bodies rep-

resents a threat to the integrity of the surfactant; phospholipase activity might be a contributory factor in the etiology of the respiratory distress syndrome of the newborn. The deficiency of phosphatidylglycerol in the lung lipids of infants with respiratory distress (14) would exacerbate any such catabolic action of the phospholipases.

CONCLUSION

These studies of lysosomal phospholipases A from rabbit lung have shown significant changes in the rate of catabolism of dipalmitoyl phosphatidylcholine in response to changes in the proportions of saturated and unsaturated phosphatidylcholine molecules in the substrate liposomes. The data suggest that the phospholipase A_2 of lamellar bodies will preferentially hydrolyze unsaturated molecules and might thus assist in surfactant synthesis.

Phospholipases A_1 and A_2 in lamellar bodies also represent a threat to the integrity of the surfactant and thus a possible contributory factor to the respiratory distress syndrome of the newborn. Phosphatidylglycerol, a constituent of surfactant, is inhibitory to these enzymes.

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43. The specific radioactivities given in "Materials and Methods" in Ref. 17 should be as follows: for 13 Ci/mole, read 0.25 Ci/mole, and for 25 Ci/mole, read 0.50 Ci/mole.
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46. This research was supported by the Sir Halley Stewart Trust and by the Medical Research Council, who defrayed part of the expenses.
47. Received for publication Dec 13, 1978.
48. Accepted for publication May 15, 1979.

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0031-3998/80/1403-0254$02.00/0