Formation of Acidic Phospholipids in Rabbit Lung during Perinatal Development

MIKKO HALLMAN^{1,451} AND LOUIS GLUCK

Division of Perinatal Medicine, Department of Pediatrics, School of Medicine, University of California, San Diego, La Jolla, California, USA

Summary

Phospholipids were measured in the lamellar bodies and alveolar lavage of perinatal rabbits. Between 28 and 31 fetal days, phosphatidylinositol (PI) increased, and phosphatidylserine decreased. Small amounts of phosphatidylglycerol (PG) appeared at term. After birth, PG markedly increased and PI concomitantly decreased. PG increased prematurely after removal from the uterus of fetuses 29 days gestation or more. Fatty acid analysis of PG and PI revealed the presence of 62 to 63%, and 58 to 59%, of saturated species, respectively. As measured in total lung homogenate, PI increased between 28 and 31 fetal days, and PG increased between term and the first neonatal day.

Glycerol incorporation into PG increased at birth, according to the measurement of lung slices. *myo*-Inositol incorporation into PI increased in the fetus parallel to accumulation of surfactant.

The development of the following enzyme activities was studied in subcellular fractions derived from lung homogenates: phosphatidate cytidyltransferase, cytidine 5'-diphosphate (CDP)-diglyceride:inositol transferase, glycerophosphate phosphatidyltransferase, and phosphatidylglycerophosphatase. As measured in the microsomal fraction, all the activities increased between 27 and 30 fetal days. Shortly after birth, phosphatidate cytidyltransferase and glycerophosphate phosphatidyltransferase increased further, whereas the two other enzyme activities decreased somewhat. Cytidine 5'-diphosphate diglyceride:inositol transferase increased after the newborn period. There were only small developmental changes in K_m 's. In contrast to microsomes, the mitochondrial activities mainly decreased during development. Phosphatidylglycerophosphatase was the only activity that was present in significant amounts in lamellar bodies.

It is proposed that the developmental changes in the acidic surfactant phospholipids are due to changes in the biosynthesis rates in type II cell microsomes. This may be controlled both by changes in the enzyme activities (phosphatidate cytidyltransferase, cytidine 5'-diphosphate diglyceride:inositol transferase, and glycerophosphate phosphatidyltransferase) and by changes in substrate concentrations (*myo*-inositol and *sn*-glycerol-3-P).

Speculation

Besides surfactant lecithin, phosphatidylglycerol is critical to alveolar stability and its absence may precipitate respiratory distress syndrome in the newborn. Surfactant phosphatidylglycerol and phosphatidylinositol serve as indices of fetal lung maturity or help to specify diagnosis of respiratory distress in the neonate. Because several hormones seem to regulate individual surfactant phospholipids, the measurement of these components (lung profile) could be a way to analyze consequences of hormonal or nutritional balance and eventually correct it if necessary.

biosynthesis and regulation of the major surfactant compound, lecithin [phosphatidylcholine (PC)] and its main component, disaturated PC (47). These studies were greatly stimulated by the introduction of the lecithin/sphingomyelin (L/S) ratio as a prenatal index of fetal lung maturity (14). Thus, the widely accepted concept that a lack of surfactant causes the respiratory distress syndrome (RDS) (2, 14) is based largely upon studies on PC. Knowledge of the other surfactant components is far less complete.

Surfactant PG recently was shown to be an indicator of lung maturity; it is lacking in RDS but present in healthy newborns and in diseases other than RDS (18). The other phospholipids of lung effluent are present in RDS, although in reduced (PC) or increased (sphingomyelin, phosphatidylserine, and phosphatidylethanolamine) quantities (18, 35). PG may also be useful in prenatal amniotic fluid analyses because its presence indicates lung maturity despite high-risk pregnancy or when blood or meconium contamination of the amniotic fluid hampers the maturity tests (22). This epidemiologic evidence, supported by surface activity measurements, has led to the postulate that PG is a surfactant component essential in stabilizing the alveoli, particularly during acute stresses. Absence of PG at birth could precipitate RDS with transitional, or, exceptionally, even with an L/S ratio of two or more.

In adult rodents, PG is located preferentially in the surfactant fractions, namely on the extracellular lining of the airways and in intracellular lamellar bodies of type II alveolar cells (19). According to some authors, lamellar bodies contain high specific activities of PG synthesizing enzymes (4, 26, 41). However, quantitatively, by far most of the biosynthetic activity has been found in microsomal fractions and in mitochondria (19, 41).

Phosphatidate cytidyltransferase (EC 2.7.7.41) (I), glycerophosphate phosphatidyltransferase (EC 2.7.8.5) (II), and phosphatidylglycerophosphatase (EC 3.1.3.27) (III) catalyze the biosynthesis of PG as follows (46).

- (I) Phosphatidic acid + cytidine triphosphate $\rightarrow$ cytidine 5'-diphosphate (CDP)-diglyceride + PP_i
- (II) *Sn*-Glycerol-3-phosphate + CDP-diglyceride $\rightarrow$ phosphatidylglycerolphosphate + cytidine monophosphate
- (III) Phosphatidylglycerolphosphate $\rightarrow$ PG + P_i

In addition to PG, biosynthesis of phosphatidylinositol (PI) requires CDP-diglyceride in a reaction catalyzed by CDP-diglyceride:inositol transferase (EC 2.7.8.11) (IV):

- (IV) *Myo*-Inositol + CDP-diglyceride $\rightarrow$ PI + cytidine monophosphate

In the present communication, we have studied the formation of phospholipids in the lung during perinatal development. The above-mentioned enzyme activities were studied particularly in a fraction derived from crude microsomes. We further document the successive appearance of surfactant components and propose a mechanism for stepwise development of surfactant PI and PG.

Biochemical research on surfactant has been focused on the

MATERIALS AND METHODS

New Zealand albino rabbits were used. The time of conception was known to within 2 hr. The day of conception was considered day zero. Under light ether anesthesia, 20 to 30 ml of air was injected IV into the does. The fetuses were removed quickly from the abdominal cavity, and their necks and abdominal aortae were cut before the onset of breathing. Only when specifically indicated, were the preterm fetuses allowed to breathe room air. The number of animals in each litter was 5 to 15. For isolation of the subcellular organelles, the number of animals was as follows: for 28-day-old or younger fetuses, the whole litter; from 29-day-old fetuses to 8-day-old rabbits, 3 to 8 animals.

Reference phospholipids, except for phosphatidylmethylethanolamine (Sigma Chemical Co., St. Louis, MO) and CDP-diglycerides (Serdary Research Laboratories, London, Ontario, Canada) were products of Supelco. CDP-diglycerides were purified by lipid extraction at pH 8, followed by acidification of the water phase and reextraction at pH 2, according to Eichberg and Hauser (12). The purified CDP-diglyceride thus obtained migrated as a single spot in thin-layer chromatography. Phosphatidylglycerolphosphate was derived from diphosphatidylglycerol using *Bacillus cereus* phospholipase C (EC 3.1.4.3) (Sigma Chemical Co.) (10) and subsequently isolated on two-dimensional thin-layer chromatography. The identity of the lipid was verified by analyzing the chemical composition (39, 40); the molar ratio of phosphorus:glycerol:fatty acid was 1.00:96:1.02, i.e., close to the theoretical, 1:1:1. bis(Glycerol) phosphate was prepared by deacylation of phosphatidylglycerolphosphatase as described by Dawson (9). Phospholipase D (cabbage) (EC 3.1.4.4) and *sn*-glycerol-3-P were purchased from Calbiochem. Phospholipase A₂ (*Naja Naja* venom) (EC 3.1.1.4) was the product of Sigma Chemical Co.

The radioactive isotopes were purchased from New England Nuclear (Boston, MA) except for [1,3-³H]glycerol, which was supplied by Amersham/Searle. Radioactive phosphatidylglycerolphosphate was synthesized in the presence of *sn*-[U-¹⁴C]glycerol-3-P, CDP-diglyceride, and mercuric chloride using the membrane fraction derived from *Escherichia coli*, essentially according to Chang and Kennedy (7). The radioactive lipid was isolated using two-dimensional thin-layer chromatography. To further confirm the identity of the radioactive lipid, it was deacylated according to Dawson (9). The resulting compound was isolated on a thin-layer chromatogram as described previously (19); 94% of the radioactivity was associated with bis(glycerol)phosphate standard.

PHOSPHOLIPID ANALYSIS

The total lipids were extracted according to Bligh and Dyer (5) using chloroform:methanol (2:1, v/v). Phospholipids were separated by two-dimensional thin-layer chromatography on laboratory-made (15) silica gel H (E. Merck) plates as described previously (30), and the individual lipids were quantified on the basis of phosphorus contents (27). Disaturated PC was isolated according to Mason *et al.* (33).

The fatty acid components of the phospholipids were analyzed by gas-liquid chromatography (15). The acidic phospholipids on thin-layer plates were identified by spraying with water or methanol. The individual phospholipids were eluted using a solution containing chloroform:methanol:H₂O:58% NH₄OH (3:6:0.5:0.5 v/v). The eluate was neutralized using 0.1 M Na-citrate (pH 2.0) and the lipids were recovered by extraction according to Bligh and Dyer (5). One-half of the specimen was treated with phospholipase A₂ followed by fatty acid analysis of lysophospholipids (1-position) and free fatty acids (2-position). The other one-half was analyzed without phospholipase treatment. The individual fatty acids were expressed as percentage of total fatty acids. The results were acceptable only when the mean fatty acid composition of lysophospholipids and free fatty acid fractions were similar to the total fatty acid composition (difference in individual fatty acids less than $\pm 4\%$).

ISOLATION OF LUNG FRACTIONS

Before isolation, the animals were exsanguinated through the abdominal aorta. The lungs were chilled in ice cold 0.27 M sucrose:0.01 M Tris-HCl:0.1 mM EDTA (pH 7.4). All subsequent steps took place at 0 to 4°C. After removal of the large bronchi, the tissue was weighed and minced with scissors. The tissue was homogenized with three complete strokes of a motor-driven Teflon pestle homogenizer (clearance 0.15 to 0.23 mm; Arthur Thomas Co.). The final homogenate contained 3 ml of 0.27 M sucrose:Tris:EDTA per 1 g of fetal lung or 4 ml of 0.27 M sucrose:Tris:EDTA per 1 g of postnatal lung. After centrifugation at 900 $\times g$ for 10 min, the resulting supernatant was poured through double-thickness of cheesecloth. The supernatant, called cell-free homogenate, was thereafter spun at 8000 $\times g$ for 10 min to obtain lamellar body-mitochondrial pellet and a new supernatant containing microsomes. The lamellar body-mitochondrial pellet was resuspended in 0.27 M sucrose:Tris:EDTA and layered on the top of the discontinuous density gradient containing 4 ml of 1.3 M sucrose:Tris:EDTA 4 ml of 0.55 M sucrose:Tris:EDTA and 1 ml of 0.45 M sucrose:Tris:EDTA. The gradients were centrifuged at 85,000 g_{av} for 60 min using SW-27 rotor (Beckman Instruments). The resulting pellet was resuspended in 0.27 M sucrose:Tris:EDTA and washed once by sedimentation at 10,000 $\times g$ for 10 min. The sediment was the mitochondrial fraction. Lamellar body fraction was collected as the layer containing 0.45 M sucrose:Tris:EDTA, diluted with excess of 0.27 M sucrose:Tris:EDTA, and sedimented at 10,000 $\times g$ for 10 min.

For isolation of microsomes, the 8000 $\times g$ supernatant was further spun at 10,000 $\times g$ for 10 min. The resulting supernatant was layered over a discontinuous density gradient containing 4 ml of 1.3 M sucrose:Tris:EDTA and of 4 ml of 0.55 M sucrose:Tris:EDTA. After centrifugation at 100,000 g_{av} for 2 hr in SW 27 rotor, the material between 1.3 and 0.55 M sucrose:Tris:EDTA was recovered, diluted to 0.27 M sucrose:Tris:EDTA, and sedimented at 230,000 g_{av} for one hr using a Ti 50 rotor.

Endobronchial lavage was performed through a catheter inserted into the trachea. First, the lungs were gently inflated-deflated with air. This was followed by four lavagings each time with 1.5 to 75 ml of 0.15 M NaCl (20°C), depending on the size of the animals. This procedure removes more than 70% of the phospholipids that can be collected during 10 washings. The combined lavage fluid was centrifuged for 10 min at 200 $\times g$ to remove any cells. When indicated, the surfactant phospholipids of the lavage fluid were further purified using density gradient centrifugation (21).

ASSAYS OF PHOSPHOLIPID BIOSYNTHESIS

Slice Experiments. The lungs were rapidly removed and chilled in ice-cold Krebs-Ringer phosphate buffer. Most of the bronchial tissue was removed. Lung slices weighing from 45 to 65 mg were prepared using a Stadie-Riggs slicer (Arthur Thomas Co., Philadelphia, PA). The slices were incubated in Krebs-Ringer bicarbonate containing 6.9 mM glucose and either 0.15 mM [1,3-³H]glycerol, *myo*[2-³H]inositol (0.15 mM) or 0.15 mM *sn*-glycerol-3-P (specific activity, 46 μ Ci/ μ mole). Incubation took place for 30 min at 37°C with shaking in flat-bottomed flasks, each containing one slice and 0.7 ml of incubation medium, under a slow stream of 95% O₂:5% CO₂. After the incubation, the lipids were extracted, and total phospholipid content and radioactivity associated with PG and PI were measured.

Measurements of Enzymatic Activities. The assays were done in conical flasks at 37°C with shaking, volume, 0.07 to 0.10 ml. Reactions were stopped by the addition of 0.6 ml methanol, 1.2 ml of chloroform, and 0.5 ml of 100 mM Na-citrate (pH 2.0) containing 10 mM of the unlabeled precursor. The lipid phase was removed, and the upper phase was reextracted with 1 ml of theoretical lower phase (chloroform:methanol:water, 86:14:1 v/v). The resulting lower phase was combined with the previous

lower phase and extracted with 0.2 ml of 100 mM Na citrate (pH 2.0). Thereafter, the radioactivity associated with the lower phase was measured. The enzyme activities were optimized with respect to pH, except when specifically indicated.

Phosphatidate cytidyltransferase activity was assayed by measuring the rate of cytidine 5'-triphosphate (CTP) incorporation into CDP:diglyceride. The final reaction mixture contained 100 mM Tris:Maleate (pH 7.0), 0.55 mM phosphatidic acid (prepared by sonication in the presence of Cutscum), 0.2 μ g/ml Cutscum, 3.0 mM [5- ^{3} H]CTP (specific activity, 17.1 μ Ci/ μ mol), 40 mM MgCl_2 , and 0.8 to 1.5 mg/ml protein. The rate of incorporation was linear for 10 min. In the results shown, the incubation time was 5 to 10 min. The reaction was started by addition of the enzyme preparation followed by addition of MgCl_2 . Omission of MgCl_2 inhibited the activity by 60%. More than 90% of the radioactive lipid formed was recovered with CDP:diglyceride after two-dimensional thin-layer chromatography. Therefore, in routine assays the radioactivity was measured in the reextracted lipid phase. Each preparation was also assayed in the absence of phosphatidic acid and detergent.

CDP-Diglyceride:inositol transferase activity was assayed by measuring the rate of incorporation of *myo*-inositol into PI in the presence of CDP-diglyceride. The reaction mixture in routine assays contained 0.3 mM CDP-diglyceride, 150 mM Tris-Cl (both pH 7.5 and pH 8.5), 5.6 mM reduced glutathione, 1.5 mM *myo*-(2- ^{3} H)-inositol (specific activity, 8.8 to 9.6 μ Ci/ μ mol), 5 mM MnCl_2 , and 0.2 to 0.4 mg/ml protein. CDP-Diglyceride was first added in chloroform, which was then evaporated. Freshly prepared MnCl_2 was added last, immediately before incubation, followed by brief sonication in a water bath (model 8845-3; Cole-Palmer Instrument Co., Chicago, IL) at 0°C. The rate of incorporation was linear for 20 min. The incubation time was 10 min. More than 95% of the radioactive lipid formed during the incubation coincided with authentic PI in two-dimensional thin-layer chromatography. Therefore, in routine assays, the radioactivity was measured in the reextracted lipid phase.

Freshly prepared microsomes showed little if any incorporation of inositol into PI in the absence of CDP-diglyceride. Addition of detergents failed to increase the activity. Manganese (4 to 7 mM MnCl_2) was required for maximum activity. Further addition of Mg^{++} did not improve the incorporation rate.

Glycerophosphate phosphatidyltransferase was assayed by measuring the rate of incorporation of *sn*-[U - ^{14} C]glycerol-3-P into phosphatidylglycerolphosphate and PG in the presence of CDP-diglyceride (18). The reaction mixture contained 0.2 mM CDP-diglyceride, 80 mM Tris-Cl (pH 7.4), 6 mM reduced glutathione, 0.45 mM *sn*-glycerol-3-P (15.3 μ Ci/ μ mole), and 0.8 to 1.5 mg/ml protein. The incubation time was 10 min. In the presence of repurified CDP-diglyceride, 90 to 97% of the radioactive lipid was associated with PG and phosphatidylglycerolphosphate. Therefore, in routine assays the radioactivity was measured in total lipid extract. Only when specifically indicated were the phospholipids isolated using two-dimensional thin-layer chromatography and the radioactivity associated with PG, phosphatidylglycerolphosphate, diphosphatidylglycerol, and bis(monoacylglycerol) phosphate measured as described previously (18).

Phosphatidylglycerophosphatase activity was measured by incorporation of phosphatidyl[^{14} C]glycerolphosphate into PG essentially as described by Johnston *et al.* (26). The incubation mixture contained 0.1 mM phosphatidylglycerolphosphate (specific activity, 1.2 μ Ci/ μ mole), 0.18% Triton X-100, 90 mM Tris-maleate (pH 6.8), protein (0.08 to 0.10 mg/ml). After the incubation and extraction of lipids, phospholipids were isolated using two-dimensional thin-layer chromatography, and the radioactivity associated with PG and phosphatidylglycerolphosphate was measured. The recovery of radioactivity in PG and phosphatidylglycerolphosphate spots was 79 to 86%. This somewhat low figure is largely explained by the fact that the loss of phosphatidylglycerolphosphate during the lipid extraction was 9 to 13%. However, the recovery of PG was always close to 100%.

Other Methods. The activities of succinate dehydrogenase (EC

1.3.99.1), and rotenone-insensitive NADPH:cytochrome *c* reductase (EC 1.6.2.4) were measured according to Sottocasa (43). Protein was quantified according to Lowry *et al.* (32). Phospholipid radioactivity was measured according to Webb and Mettrick (48). External standardization was used to correct the quenching. The results are expressed as means $\pm$ S.E. Statistical significance was calculated using the *t* test (Student distribution).

RESULTS

Microsomes, mitochondria, and lamellar bodies were examined for contamination using mitochondrial (succinate dehydrogenase) and microsomal (NADPH:cytochrome *c* reductase) marker enzymes. Table 1 shows the distribution of the marker enzymes in mitochondria and in microsomes. On the basis of succinate dehydrogenase activities, there was less than 5% mitochondrial contamination in microsomal fraction, regardless of the source of the organelles. There was somewhat more microsomal contamination in mitochondrial fractions. However, due to low fetal activity of NADPH:cytochrome *c* reductase, microsomal contamination could not be evaluated in most fetuses. Lamellar bodies were not contaminated by mitochondria (succinate dehydrogenase activity in lamellar bodies less than 5% of that in mitochondria), and there were small amounts of microsomal contamination (NADPH:cytochrome *c* reductase activity in lamellar bodies 5.4 to 9.5% of that in microsomes).

Table 2 illustrates total phospholipid/protein ratios in lamellar bodies and in alveolar lavage from different age groups. An increase in phospholipid content was noted between the 28th and 30th fetal days. Phospholipid content in lamellar bodies exceeded those in alveolar lavage regardless of the age. We further purified alveolar lavage by density gradient centrifugation. The surfactant thus obtained had at least as high a phospholipid protein ratio as lamellar bodies. On the other hand, the phospholipid composition of crude alveolar lavage and its fraction isolated by density gradient were similar (data not shown). These measurements suggest that a remarkable part of proteins recovered during alveolar lavage come from sources other than lamellar bodies.

PHOSPHOLIPID COMPOSITION

The composition of individual phospholipids in the lung during perinatal development are shown in Table 3. The results resemble those from Gluck *et al.* (16). However, in the present study the acidic phospholipids, PG, bis(monoacylglycerol)phosphate, and diphosphatidylglycerol (cardiolipin) have been analyzed in detail. The percentage amounts of PC and PG increased during development. PI increased between days 26 and 27 and 30 and 31 in the fetus. The contents of phosphatidylethanolamine and diphosphatidylglycerol decreased somewhat, whereas changes in sphingomyelin and phosphatidylserine were more variable.

Table 4 presents phospholipid composition in lamellar bodies and alveolar lavage from 28- and 30-day-old fetuses. Phospholipids 2 hr after delivery by cesarean section are presented, too. Sphingomyelin, phosphatidylethanolamine, and phosphatidylserine were significantly higher, but PC and PI were significantly lower in alveolar lavage than in lamellar bodies. In 30-day-old animals 2 hr after delivery by cesarean section, the composition of alveolar lavage had become similar to that in lamellar bodies. However, in 28-day-old animals, even after air-breathing, the phospholipid composition as compared between alveolar lavage and lamellar bodies remained different. In 30-day-old animals, PG increased at birth, whereas in 28-day-old animals it remained absent.

In 28-day-old fetuses, the total phospholipids recovered by alveolar lavage (0.8 ± 0.3 nmoles/mg residual lung protein) were less and remained smaller after 2 hr of air breathing (1.5 ± 0.6 nmoles/mg residual lung protein) than in 30-day-old ones (3.3 ± 0.5 nmoles/mg protein; 7.3 ± 1.2 nmoles/mg protein after 2 hr of air-breathing).

The development of the acidic surfactant phospholipids in lamellar bodies is shown in Figure 1. Diphosphatidylglycerol was

Table 1. Distribution of succinate dehydrogenase and NADPH:cytochrome *c* reductase between mitochondria and microsomes as an index of microsomal and mitochondrial contamination¹

Age	N ²	Succinate dehydrogenase (nmoles × min ⁻¹ × mg ⁻¹ protein)		Mitochondrial contamination in microsomes (%)	NADPH:cytochrome c reductase (nmoles × min ⁻¹ × mg ⁻¹ protein)		Microsomal contamination in mitochondria (%)
		Mitochondria	Microsomes		Mitochondria	Microsomes	
Fetus							
24–27 days	4	119.2	3.8	3.2 (1.3–5.4) ³	n.m. ⁴	n.m.	
28–31 days	3	110.0	2.7	2.5 (0.0–4.2)	n.m.	n.m.	
Newborn							
0–2 days	3	127.5	2.9	2.0 (1.2–3.2)	13.5	121.4	10.9 (5.9–14.2)
4–8 days	3	158.2	3.0	1.7 (1.0–2.3)	13.2	127.0	10.3 (9.4–11.0)
Adult	3	139	3.2	3.8 (2.8–5.1)	6.7	80.1	8.0 (6.1–10.3)

¹ The contamination was calculated using the following equations:

$$\begin{cases} \text{succinate dehydrogenase}_{M_i}/x = \text{succinate dehydrogenase}_{M_s}/(1-y) \\ \text{NADPH:cytochrome } c \text{ reductase}_{M_i}/(1-x) = \text{NADPH:cytochrome } c \text{ reductase}_{M_s}/y \end{cases}$$

where *x* = the portion of the mitochondrial fraction representing pure mitochondria, and *y* = the portion of the microsomal fraction representing pure microsomes.

² *N*, number of assays.

³ Numbers in parentheses, range.

⁴ n.m., not measured.

Table 2. Phospholipid:protein ratio in lamellar bodies, alveolar lavage, and purified surfactant¹ recovered from rabbit lung

Age	Lamellar bodies (nmoles phospholipid/mg protein)	Alveolar lavage (nmoles phospholipid/mg protein)	Purified surfactant (nmoles phospholipid/mg protein)
Fetus			
28 days	4413 ± 306 ² (4) ³	886 ± 83 (4)	n.m. ⁴
29–30 days	7770 ± 377 (5)	1896 ± 82 (6)	10202 (2)
Newborn			
0–2 days	7811 ± 402 (7)	2834 ± 190 (11)	11030 (3)
Adult	8100 ± 395 (5)	3417 ± 239 (7)	n.m.

¹ Purified surfactant was derived from alveolar lavage as previously described (21).

² Mean ± S.E.

³ Numbers in parentheses, number of assays.

⁴ n.m., not measured.

never detected, and bis(monoacylglycerol)phosphate was always less than 1.0% of the total phospholipids (data not shown). The changes in the other acidic phospholipids are shown. Toward term, the concentration of phosphatidylserine decreased, and PI increased. The second change took place after birth; PG increased from the low fetal values, and PI decreased. Throughout development, there were only small changes in the sum of the three phospholipids.

The ratio of PG in homogenate (Table 3) and lamellar bodies (Fig. 1) was studied. In the fetus, the ratio was at least one, however, at birth is decreased sharply below one. This suggests that the neonatal increase in lung PG was due mainly to an increase in surfactant PG.

Table 5 shows the fatty acid components of surfactant PG and PI. Fatty acids associated with 1- and 2-position were measured separately. More than 40% of the total fatty acids associated with the 2-position of PG were saturated. The corresponding percentage for PI was somewhat lower. Palmitate was always the major fatty acid constituting more than 75% of total saturated fatty acids. As shown in Table 5, the phospholipids were isolated from animals of different ages. Our analysis failed to detect any age-dependent changes in fatty acids. However, the data are insufficient to permit

critical evaluation of developmental trends in fatty acid composition.

LUNG SLICE EXPERIMENTS

Phospholipid incorporation in lung slices was studied using the following isotopes: [1,3-³H]glycerol, *myo*[2-³H]inositol and *sn*[U-¹⁴C]glycerol-3-P. The apparent rates of *sn*-glycerol-3-P incorporation into PG ranged between 0.1 and 0.5 pmoles PG × min⁻¹ × nmoles⁻¹ phospholipid-P. It increased significantly at birth. However, glycerol incorporation into PG was markedly higher than that of *sn*-glycerol-3-P. Therefore, the penetration of *sn*-glycerol-3-P into the cell may be slow and seriously limit the incorporation. The apparent rates of glycerol incorporation into PG are shown in Figure 2. The rate slightly decreased (*P* < 0.025) between the 26th and 28th fetal days. At birth, the activity doubled. Thereafter, only small changes in PG incorporation were detected. After phospholipase D treatment, 79 to 86% of the radioactivity associated with PG was released from the lipid, indicating that most of the glycerol was incorporated in the headgroup glycerol.

The apparent rate of *myo*-inositol incorporation into PI almost doubled between the 26th and 30th days. Thereafter, activity

revealed little change. (Fig. 2). All of the radioactivity was released from the lipid during phospholipase D treatment. Neither PG nor PC contained any radioactivity.

THE ACTIVITIES OF PHOSPHOLIPID SYNTHESIZING ENZYMES

Phosphatidate Cytidyltransferase. Addition of phosphatidic acid to the reaction mixture stimulated [^3H]CTP incorporation into CDP-diglyceride. Mere dispersion of phosphatidic acid by sonication stimulated the rate of incorporation less (1.2- to 1.8-fold higher rate than without phosphatidic acid) than when both phosphatidic acid and a nonionic detergent, Cutscum, were dispersed together (4.8- to 9.7-fold higher rate than without phosphatidic acid and Cutscum).

Figure 3 shows phosphatidate cytidyltransferase activities as measured in microsomal and mitochondrial fractions as a function

of age. Microsomal activity increased markedly during the perinatal period, whereas after the first neonatal day, there were only small changes. Mitochondrial phosphatidate cytidyltransferase revealed little change during development.

Table 6 shows the apparent K_m 's for CTP as measured in mitochondrial and microsomal fractions from different age groups. The preparations studied revealed only small differences in the apparent K_m .

CDP-Diglyceride:Inositol Transferase. Figure 4 shows CDP-diglyceride:inositol transferase activity in microsomal fraction as a function of age. The assays were done both in physiological pH (7.4) and in optimal pH (8.5). Inasmuch as the fatty acid structure of the natural precursor is unknown, both egg lecithin derivative of CDP-diglyceride and dicaproyl CDP-diglyceride were used in the assay. The egg lecithin derivative of CDP-diglyceride gave a

Table 3. *Phospholipid composition in the lung during perinatal development*

% phospholipid phosphorus										
Age	N ¹	PC	Sphingo- myelin	Phosphati- dylethanol- amine	Phosphati- dylserine	PI	PG	bis-(Mono- acylglycerol) - phosphate	Diphospha- tidylglycerol	Recovery ²
Fetus										
24-25 days	6	41.9 ± 1.3 ³	7.2 ± 0.4	35.6 ± 1.8	9.4 ± 0.5	3.6 ± 0.1	0.8 ± 0.3	n.m. ⁴	1.5 ± 0.2	90.4 ± 3.9
26-27 days	6	46.3 ± 1.8	8.1 ± 0.6	26.4 ± 0.8	13.6 ± 1.4	3.1 ± 0.3	0.8 ± 0.2	n.m.	1.7 ± 0.2	89.9 ± 4.4
28 days	5	45.9 ± 0.9	9.5 ± 0.3	25.7 ± 1.0	12.5 ± 1.0	4.6 ± 0.1	0.5 ± 0.2	n.m.	1.3 ± 0.3	91.7 ± 3.7
29 days	4	51.1 ± 2.3	8.9 ± 0.5	22.4 ± 1.3	10.6 ± 0.3	4.8 ± 0.2	0.6 ± 0.1	0.4 ± 0.3	1.2 ± 0.1	93.4 ± 1.0
30-31 days	7	51.7 ± 1.9	9.4 ± 0.5	20.5 ± 0.7	10.4 ± 0.9	5.5 ± 0.2	0.9 ± 0.1	0.6 ± 0.4	1.0 ± 0.4	90.0 ± 5.7
Newborn										
1 days	4	53.0 ± 2.6	8.3 ± 0.2	20.9 ± 0.5	10.0 ± 0.4	4.5 ± 0.3	1.7 ± 0.2	0.8 ± 0.0	0.8 ± 0.2	87.3 ± 4.0
2-4 days	6	53.1 ± 2.5	9.2 ± 0.4	19.7 ± 0.9	9.4 ± 1.0	4.0 ± 0.2	2.5 ± 0.2	1.0 ± 0.5	1.1 ± 0.1	94.2 ± 4.4
Adult	5	54.0 ± 1.9	11.2 ± 0.3	17.1 ± 1.1	9.1 ± 0.6	3.6 ± 0.1	3.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.2	90.9 ± 2.9

¹ N, number of individual assays performed in total lung homogenate.

² The sum of individual phospholipid-P in percentage of total chloroform soluble phosphorus.

³ Mean ± S.E.

⁴ n.m., not measured.

Table 4. *Phospholipids in alveolar wash and in lamellar bodies from 28- and 30-day-old fetuses and newborns delivered by cesarean section*

% of phospholipid-P										
Age	N ¹	Disaturated PC (% of total PC)	PC	Sphingo- myelin	Phosphati- dylethanol- amine	Phosphati- dylserine	PI	PG	bis- (Mono- acylgly- cerol) phosphate	Recov- ery (% of CHCl ₃ - soluble phospho- rus)
28 days old										
Lamellar bodies	4	53.6 ± 2.0 ²	70.5 ± 2.2	7.1 ± 0.5	8.4 ± 0.5	4.4 ± 0.5	9.0 ± 0.7	0.2 ± 0.3	0.4 ± 0.4	92 ± 4
Alveolar wash	6	45.2 ± 2.0	49.3 ± 2.3	20.5 ± 1.9	13.3 ± 1.1	9.8 ± 0.9	5.9 ± 0.4	0.3 ± 0.2	0.9 ± 0.4	89 ± 5
Two hr after delivery by cesarean section:										
Lamellar bodies	2	55.0	70.1	6.6	8.9	4.8	9.3	0.2	0.1	94
Alveolar wash	11	48.8 ± 2.7	61.2 ± 1.9	14.5 ± 1.1	10.0 ± 0.9	6.3 ± 0.7	7.0 ± 0.4	0.0 ± 0.1	1.0 ± 0.0	91 ± 6
30 days old										
Lamellar bodies	6	60.0 ± 3.6	77.5 ± 2.9	1.0 ± 0.7	5.8 ± 0.3	1.7 ± 0.3	13.2 ± 1.1	0.6 ± 0.4	0.2 ± 0.1	98 ± 4
Alveolar wash	7	51.2 ± 0.9	63.5 ± 3.4	12.0 ± 0.7	8.0 ± 0.2	6.9 ± 0.8	8.0 ± 0.5	0.4 ± 0.5	1.2 ± 0.6	93 ± 2
Two hr after cesarean section:										
Lamellar bodies	2	57.2	76.8	1.8	5.3	1.9	12.0	2.9	0.3	94
Alveolar wash	6	56.9 ± 2.7	73.0 ± 3.1	3.0 ± 0.2	6.4 ± 0.3	2.6 ± 0.1	11.8 ± 1.0	2.2 ± 1.0	1.0 ± 0.0	92 ± 4

¹ N, Number of assays.

² Mean ± S.E.

Fig. 1. The contents of the acidic phospholipids in lamellar bodies during the perinatal development. Phosphatidylglycerol (Δ), phosphatidylinositol ($\bullet$), phosphatidylserine ($\circ$), and the sum of the three acidic phospholipids ($\times$). B, birth; A, adult.

Table 5. The percentage of fatty acid composition of surfactant PG and PI¹

	PG		PI	
	1-position	2-position	1-position	2-position
Lamellar bodies				
	5 assays		6 assays	
14:0	0.7 $\pm$ 0.6 ²	2.0 $\pm$ 0.2	1.5 $\pm$ 0.2	1.8 $\pm$ 0.2
16:0	73.8 $\pm$ 4.9	36.1 $\pm$ 2.2	62.5 $\pm$ 2.9	27.7 $\pm$ 1.1
16:1	0.6 $\pm$ 0.7	10.7 $\pm$ 0.8	0.9 $\pm$ 0.0	7.4 $\pm$ 1.1
18:0	8.2 $\pm$ 1.5	3.2 $\pm$ 0.7	14.2 $\pm$ 1.2	7.9 $\pm$ 0.6
18:1	12.4 $\pm$ 0.6	40.7 $\pm$ 3.0	6.9 $\pm$ 0.4	30.5 $\pm$ 1.1
18:2	0.8 $\pm$ 0.1	6.1 $\pm$ 0.5	7.0 $\pm$ 0.0	16.6 $\pm$ 1.2
Others	3.5 $\pm$ 1.9	1.2 $\pm$ 0.7	7.0 $\pm$ 1.0	8.1 $\pm$ 0.1
Saturated	82.7 $\pm$ 4.2	41.3 $\pm$ 2.5	78.2 $\pm$ 3.4	37.4 $\pm$ 1.6
Alveolar wash				
	5 assays		5 assays	
14:0	2.9 $\pm$ 0.2	2.9 $\pm$ 0.6	2.0 $\pm$ 0.3	2.0 $\pm$ 0.4
16:0	73.8 $\pm$ 3.1	36.2 $\pm$ 1.1	64.8 $\pm$ 2.9	31.7 $\pm$ 1.9
16:1	2.2 $\pm$ 0.9	8.3 $\pm$ 0.9	1.3 $\pm$ 0.1	8.0 $\pm$ 0.7
18:0	7.0 $\pm$ 0.8	4.0 $\pm$ 0.2	12.4 $\pm$ 2.9	5.3 $\pm$ 0.5
18:1	11.4 $\pm$ 0.5	41.1 $\pm$ 2.7	13.7 $\pm$ 1.0	29.8 $\pm$ 1.6
18:2	1.0 $\pm$ 0.9	5.7 $\pm$ 0.4	0.8 $\pm$ 0.2	17.0 $\pm$ 1.1
Others	1.7 $\pm$ 0.8	1.8 $\pm$ 0.3	5.0 $\pm$ 0.7	6.2 $\pm$ 0.8
Saturated	83.7 $\pm$ 3.6	43.1 $\pm$ 1.5	79.2 $\pm$ 2.9	39.0 $\pm$ 2.3

¹ PG's were obtained from 2-hr to 8-day-old animals, PI's were obtained from 30-day-old fetuses to 8-day-old newborns.

² Mean $\pm$ S.E.

lower activity than dicaproyl CDP-diglyceride. The developmental pattern was fairly similar regardless of the assay conditions used; the activity increased during the late fetal period and transiently decreased during the first neonatal days. CDP-diglyceride: inositol transferase activity in mitochondria was 20% or less than that in microsomes and thus could be due mainly to microsomal contamination (data not shown).

Table 6 shows the apparent K_m 's for *myo*-inositol and CDP-diglyceride as measured in microsomes from different age groups. Only small changes were detected. In three instances, we measured K_m using dialyzed microsomal membranes. This did not have an effect on K_m , suggesting that no endogenous *myo*-inositol was present.

Glycerophosphate Phosphatidyltransferase. Figure 5 shows the development of the rate of *sn*-glycerol-3-P incorporation into phospholipids in lung microsomes, mitochondria, and cell-free homogenates. In cell-free homogenate, glycerophosphate phos-

Fig. 2. The apparent rates of incorporation of 0.15 mM *myo*-[2-³H] inositol (specific activity, 51 mCi/mmole) into phosphatidylinositol ($\bullet$) and 0.15 mM [1,3-³H]glycerol (specific activity, 308 mCi/mmole) into phosphatidylglycerol ($\circ$) of lung slices obtained from perinatal rabbits. Incubation in Krebs-Ringer bicarbonate was done in flat-bottomed tubes under 95% O₂:5% CO₂ for 30 min. For other details, see "Materials and Methods." The results are expressed as the mean $\pm$ S.E. of 4 to 8 measurements run in duplicate. B, birth.

Fig. 3. The activities of phosphatidate cytidyltransferase in mitochondria ($\circ$) and microsomes ($\bullet$) during perinatal development. The results are the means $\pm$ S.E. of four to seven assays run in duplicate. Whenever two to three assays were performed, only means are shown. Phosphatidic acid and Cutscum were added to the reaction mixture. For details, see "Materials and Methods." B, birth; A, adult.

Table 6. Apparent K_m 's for enzyme activities involved in the synthesis of PG and PI¹

Enzyme	Substrate	Age	K_m (mM)	
			Microsomes	Mitochondria
Phosphatidate cytidyltransferase	Cytidine triphosphate	Fe 27 D ²	0.85	n.m. ³
		Fe 31 D	1.13	n.m.
		Adult	1.09	n.m.
Glycerophosphate phosphatidyltransferase	<i>Sn</i> -Glycerol- 3-P	Fe 24-27 D	0.02	0.02
		NB 1 D ⁴	0.03	0.03
		Adult	0.03	0.03
	CDP-digly- ceride (egg lecithin) ⁵	Fe 27 D	0.07	0.10
		Adult	0.07	0.08
CDP-diglyceride Inositol Transferase	<i>Myo</i> -Inositol	Fe 26-29 D	0.09	n.m.
		Adult	0.07	n.m.
	CDP-digly- ceride (egg lecithin) ⁵	Fe 29 D	0.09	n.m.
		Adult	0.10	n.m.

¹ The enzyme activities were assayed in the presence of increasing concentrations of the substrate (from 1 μ M to 6 mM, seven to 12 different concentrations), and K_m was calculated using Lineweaver-Burk plot. Substrate inhibition was not detected.

² Twenty-seven-day-old fetus.

³ n.m., not measured.

⁴ One-day-old newborn.

⁵ Incubation medium containing CDP-diglyceride was prepared by sonication using a micro tip (Cell disruptor; Heat Systems, Ultrasonics, Inc., NY).

phosphatidyltransferase gradually fell between the 24th fetal day and term. After birth, there was a transient increase in the activity. On the other hand, the mitochondrial activity revealed a gradual fall throughout development. In the incubation conditions used, no diphosphatidylglycerol formation was detected.

In contrast to mitochondria, microsomal glycerophosphate phosphatidyltransferase was low in the 26- to 27-day-old fetus, but increased almost 3-fold during the last fetal days and the first neonatal day. Besides that increase, only small changes in the activity were detected. Even in the adult, the microsomal activity was 62 to 43% of the corresponding mitochondrial activity. As shown in Figure 5, dicaproyl CDP-diglyceride gave the highest reaction rate. However, in mitochondria CDP-diglyceride derived from egg lecithin was almost as effective as the dicaproyl derivative.

Table 6 shows K_m 's for *sn*-glycerol-3-P and CDP-diglyceride as measured in different age groups. Again, only small changes among the different age groups were seen.

Table 7 illustrates the distribution of the label between phosphatidylglycerolphosphate and PG. In the present conditions, most of the label was associated with PG. However, the PG: phosphatidylglycerolphosphate ratio of the label increased during the last fetal days and decreased in the newborn. No label was associated with either diphosphatidylglycerol or bis(monoacylglycerol)phosphate. The demonstration of diphosphatidylglycerol synthesis in mitochondria requires a longer incubation time (19). bis(Monoacylglycerol)phosphate formation was only found in lysosomes (data not shown).

Phosphatidylglycerophosphatase. Table 8 shows the activities in microsomal and lamellar body fractions. In microsomes phosphatidylglycerophosphatase increased 3.5-fold between the 24th and 26th and 30th and 31st fetal days. After birth, the activity fell to 70% of its highest fetal value. The corresponding changes in lamellar bodies were smaller. Furthermore, the specific activity of phosphatidylglycerophosphatase associated with lamellar body fraction was 44% or less of the corresponding microsomal activity. Comparison between microsomal glycerophosphate phosphatidyltransferase and phosphatidylglycerophosphatase activities revealed that the latter exceed the former by at least 90-fold.

DISCUSSION

In the present communication, we have further characterized the sequential changes that take place in the acidic phospholipids during perinatal development in the rabbit. In the lamellar body fraction and the alveolar lavage, phosphatidylserine, PI, and PG, in that order, each were the prominent acidic phospholipid at the particular stage of development. A fall in one phospholipid was accompanied by an increase in another phospholipid. The same phenomenon was observed among the acidic phospholipids in human amniotic fluid, suggesting similar development and further confirming the continuity between the fetal lung secretions and the amniotic fluid (18).

Earlier study on lipids of the lung and alveolar lavage failed to disclose similar changes in the acidic phospholipids (42). This may be due to differences in the methods of lipid analysis.

MECHANISM OF ACIDIC SURFACTANT PHOSPHOLIPID FORMATION

The mechanism underlying the successive development and the mutual relationship between surfactant PI and PG is poorly known (20, 28, 36). In the present communication, we have investigated this problem by studying the apparent rates of PG and PI synthesis in lung slices using glycerol, *myo*-inositol, and *sn*-glycerol-3-P as precursors. Comparison of the changes in PG and PI contents (Table 3) and those in the corresponding incorporation rates (Fig. 2) revealed some similarity. The measured changes could be due to changes either in transport and distribution, in precursor pools, or in activities of enzymes. To evaluate this question, we further studied the development patterns of four enzyme activities involved in the formation of PG and PI in subcellular fractions of the lung. During fetal development, microsomal phosphatidate cytidyltransferase (I) and CDP-diglyceride:inositol transferase (IV) increased in activity parallel to increase in lamellar body PI. At term and after birth, microsomal glycerophosphate phosphatidyltransferase (II) and phosphatidate cytidyltransferase (I) increased. At the same time, PG appeared in surfactant.

Mitochondria and lamellar bodies contain enzymatic activities involved in PG synthesis, too (4, 19, 26, 41). Even though mito-

Fig. 4. CDP-diglyceride:inositol transferase activities in microsomes during perinatal development. Each assay was run in four different conditions as shown in the Figure. For further details, see "Materials and Methods" and Figure 3.

Fig. 5. Glycerophosphate phosphatidyltransferase activities in microsomes, mitochondria, and in cell-free homogenate during perinatal development. Dicaproyl CDP-diglyceride (○) or CDP-diglyceride derived from egg lecithin (●), were used as precursors. For further details, see "Materials and Methods" and Figure 3.

chondrial glycerophosphate phosphatidyltransferase exceed that in microsomes by at least 1.6-fold, the mitochondrial activity showed no correlation to the amount of surfactant PG, but fell rather than increased during development. Furthermore, mitochondria isolated from various tissues possessed appreciable quantities (comparable to those in the lung) of phosphatidate cytidyltransferase, glycerophosphate phosphatidyltransferase, and phosphatidylglycerophosphatase (46). These enzymes apparently participate in the synthesis of cardiolipin (diphosphatidylglycerol), a characteristic mitochondrial structural component. Cardiolipin

synthesis expectedly reflects the rate of biosynthesis of mitochondrial membranes. Therefore, the activities that we found in mitochondria may only reflect an autonomous biosynthesis characteristic to these organelles.

Lamellar bodies isolated by the present method possessed appreciable quantities of phosphatidylglycerophosphatase activity, whereas the other three enzyme activities measured were low throughout and probably solely reflected microsomal contamination. However, even phosphatidylglycerophosphatase in lamellar bodies had a lower specific activity than that in the microsomal fraction. In addition, the estimated protein content of lamellar bodies (0.04% of whole-lung protein) is much lower than that of microsomes (21% of whole-lung protein) or mitochondria (18% of whole-lung protein) (19). Therefore, the contribution of lamellar body associated phosphatidylglycerophosphatase to the total activity is small, indeed. Furthermore, phosphatidylglycerophosphatase never seemed to be rate limiting because it always exceeded the activity of glycerophosphate phosphatidyltransferase by at least 90-fold, and phosphatidylglycerophosphate was not detected in measurable quantities in the lung. Thus the role of lamellar body associated activity remains open. According to recent evidence, lamellar body associated phosphatase(s) exhibit a low substrate specificity (4, 11, 26). Its role may indeed prove to be other than biosynthesis of surfactant phospholipids. In addition, the possibility that some of the enzyme activities found in the lamellar body fraction represents lysosomal contamination has not been rigorously disproven.

Kinetic studies using radioactive lipid precursors *in vivo* support in view of the present study that PG and PI synthesis take place in microsomal membranes and that these phospholipids are transported from there to lamellar bodies and eventually released to alveolar spaces (1, 8, 25). According to the proposed mechanism for surfactant PG and PI synthesis, microsomal CDP-diglyceride:inositol transferase and glycerophosphate phosphatidyltransferase activities compete for their common substrate, CDP-diglyceride. Even though the specific activities of these microsomal enzymes change parallel to surfactant composition during the perinatal period, CDP-diglyceride:inositol transferase as compared to glycerophosphate phosphatidyltransferase was inappropriately high to totally account for the dramatic increase in surfactant PG. The finding that microsomal glycerophosphate phosphatidyltransferase had higher affinity for CDP-diglyceride than CDP-diglyceride:inositol transferase (Table 6) can partially explain this discrepancy

Table 7. The activity of glycerophosphate phosphatidyltransferase in microsomal fraction and in mitochondria from developing rabbits¹

Age	N ²	PG/PGP ³	
		Microsomal fraction	Mitochondria
Fetus			
24-26 days	2	1.8	3.4
28-29 days	3	2.4	4.2
30 days	3	3.0	6.3
Newborn			
1-3 days	3	1.2	4.7
Adult female	2	1.6	2.8

¹ Distribution of the radioactive label between PG and phosphatidylglycerol phosphate. The assay of glycerophosphate phosphatidyltransferase was performed as described in "Materials and Methods." After 10 min of incubation with the radioactive precursor, the lipids were isolated by two-dimensional thin-layer chromatography and assayed for the radioactivity. In the present conditions, no radioactivity was associated with either cardiolipin or bis(monoacylglycerol) phosphate.

² N, number of assays.

³ PGP, phosphatidylglycerol phosphate.

Table 8. The activity of phosphatidylglycerophosphatase in lung microsomal fraction and lamellar bodies from developing rabbits

Age	N ¹	Phosphatidylglycerolphosphate formed (nmoles $\times$ min ⁻¹ $\times$ mg ⁻¹ protein)	
		Ms ²	LB
Fetus			
24-26 days	2	12.1	
28-29 days	3	15.7	6.6
30-31 days	3	42.0	13.5
Newborn			
1-3 days	2	29.3	10.9
Adult female	3	21.8	9.5

¹ N, Number of assays.

² Ms, microsomal fraction, LB, lamellar bodies.

since intracellular CDP-diglyceride concentrations are characteristically low (45). However, in the adults, there was no correlation between the acidic surfactant phospholipids and the microsomal biosynthetic activities (Fig. 6).

The fact that the ontogeny of acidic surfactant phospholipids cannot solely be explained on the basis of changing enzyme activities, may be owing to some of the following possibilities. The alternative that factors other than biosynthesis rate, such as changes in intracellular transport or affinity of phospholipid binding protein regulate surfactant composition, has no experimental support (29). Increase in lung PG was preferentially, if not only due to induction of surfactant PG, whereas lung fractions, other than surfactant, contain only small amounts of this phospholipid (19). Surfactant composition may also be dependent on catabolism rates of individual components. Again, the evidence of Jobe and Gluck (24) fails to support this alternative. It is further possible that microsomal CDP-diglyceride:inositol transferase mostly originates from other than alveolar type II cells. Moreover, the distribution of the enzymes between various lung cells may change during development. These possibilities need to be evaluated by comparative studies using subcellular fractions derived from isolated type II alveolar cells. Finally, changes in substrate concentration may control the biosynthetic rates.

As studied in various species, *myo*-inositol concentration in the fetus is high. In the rat serum, it falls sharply at birth (6), whereas in the human, the corresponding change is more gradual (31). In contrast to *myo*-inositol, serum glycerol increases at birth (38). These changes could contribute to the characteristic alterations in the acidic surfactant phospholipids. However, serum levels may not necessarily reflect the levels at the biosynthetic site. Indeed, discrepancies between serum and tissue levels of *myo*-inositol have been found (37). Furthermore, even if serum glycerol is utilized effectively for surfactant biosynthesis (34), factors regulating intracellular *sn*-glycerol-3-P concentration are complex and may not correlate with serum glycerol levels. The possibility that availability of substrates, particularly *myo*-inositol, plays an additional role in the ontogeny of surfactant phospholipids remains an attractive alternative that needs to be studied further.

ROLE OF ACIDIC SURFACTANT PHOSPHOLIPIDS

Disaturated PC is synthesized by a series of enzymatic reactions that characteristically change during development (for review, see Ref. 47). According to Stern *et al.* (46), phosphocholine cytidyltransferase, catalyzing the formation of CDP-choline, may be rate limiting in *de novo* formation of PC in the lung. As measured in the lung supernatant, the activity increased at birth. This was due to enzyme activation rather than synthesis of new enzyme protein (13). Of the activators tested, PG was the most effective, followed by PI and phosphatidylserine. However, the enzyme activity obtained from adults was little affected by addition of phospholipids,

possibly because the acidic phospholipids already were present in optimal concentration. This finding illustrates a mechanism controlling biosynthesis of various surfactant phospholipids.

It is tempting to speculate that during the late fetal period the successive increase in phosphatidylserine, PI and PG activates phosphocholine cytidyltransferase and thereby increases surfactant PC synthesis.

The crucial role of disaturated PC in achieving low surface tension on peripheral airway lining is accepted widely (17). However, it has become increasingly evident that disaturated lecithin alone cannot perform all surfactant functions; in particular, it does not spread effectively on the alveolar surface. We have proposed that physicochemical properties of surfactant are modified by changes in the composition of acidic phospholipids (18, 21). To further test this hypothesis, Ikegami *et al.* (23) used mixtures of dipalmitoyl PC and various acidic phospholipids to restore lung pressure-volume characteristics in surfactant depleted lungs. Combination of PG with disaturated PC was the most effective, followed by PI + disaturated PC, and phosphatidylserine + disaturated PC. In addition, Bangham *et al.* (3) found evidence, that a mixture of PG and dipalmitoyl PC improves spreading of disaturated species on surface and allows dipalmitoyl PC enriched monolayer to be repeatedly compressed to a minimum surface tension of about zero dynes $\times$ cm⁻¹ (3). These studies need to be extended to involve other surfactant components, too. Furthermore, the potential role of the acidic phospholipids in modifying the secretion of surfactant from epithelial cells to alveolar spaces entirely remains to be studied.

The ontogeny of surfactant is a complex process involving development of functions such as biosynthesis, intracellular transport, release from the intracellular storage, and spreading on alveolar surface at birth. Accordingly, there is no single specific etiology of surfactant deficiency in RDS because the cause varies during development. The stepwise appearance of acidic surfactant phospholipids as shown in the present study, further implies the complexity of regulation. Measurement of acidic phospholipids, not just disaturated PC, may better differentiate between the actions of hormones that control lung development. Monitoring of the lung profile (L/S ratio, percentage of disaturated PC, PG,

Fig. 6. Correlation between the ratio of microsomal glycerophosphate phosphatidyltransferase to CDP-diglyceride:inositol transferase (nmoles $\times$ min⁻¹ $\times$ mg⁻¹ protein:nmoles $\times$ min⁻¹ $\times$ mg⁻¹ protein) and the ratio of phosphatidylglycerol to phosphatidylinositol in lamellar bodies (% of lipid-P:% of lipid-P). The enzyme activities were assayed in the presence of dicaproyl CDP-diglyceride (pH 7.4 and pH 7.5, respectively). B, birth; A, adult.

and PI) in the amniotic fluid enables even more exact evaluation of the degree of functional development than the L/S ratio alone (30). Hopefully, a better understanding of both the ontogeny of surfactant and the risks involved in medical interference with lung maturation enables further reduction of the incidence of RDS in those cases where prevention of prematurity fails.

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- Requests for reprints should be addressed to: Mikko Hallman, M.D., Department of Pediatrics, C/019, University of California, San Diego, La Jolla, California 92093 (USA).
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