Biochemical Development of Surface Activity in Mammalian Lung

III. Structural Changes in Lung Lecithin during Development of the Rabbit Fetus and Newborn

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Extract

This is a report of studies with gas-liquid chromatography of the fatty acids on the α - and β -carbons of surface active and nonsurface active lecithins isolated from alveolar wash and from residual lung after wash in the developing rabbit fetus. The total fatty acids of lecithin show no clear tendency toward greater saturation with development. In alveolar wash, there was greater saturation of fatty acids (65%) compared with total lung (57%). Fatty acids separately determined on α - and β -carbons of lecithins showed significant developmental differences. Differences in surface active acetone-precipitated lecithin and nonsurface active acetone-precipitated and acetone-soluble lecithin were in the β -carbon fatty acids. They were highly saturated (+70%) in surface active lecithin but only about 25% saturated in nonsurface active lecithin. Concentration of acetone-precipitable lecithin in whole lung rises during fetal development to a peak (84%) with breathing. There was a marked increase in acetone-precipitable lecithin in alveolar wash after 1 h of breathing (0.35 mg/g dry weight lung in nonbreathing full term to 9.8 mg/g), a 30-fold increase. During gestation, both α - and β -component palmitic acid ($C_{16:0}$) rose abruptly after day 29 (α -45 to 67% and β -48 to 60%) when alveolar lecithin becomes surface active. The greatest increase in α - and β -palmitic acid followed the onset of breathing, and by day 2 of age, α -palmitic 85%, β -palmitic 62.5%. Thus, dipalmitoyl lecithin is the greatest single identifiable fraction of surface active lecithin isolated from rabbit alveolar wash. Acetone-soluble lecithins, even in the breathing animal, have only about 25% palmitic acid on the β -carbon. Early 29-day fetus, delivered by cesarean section, synthesized *de novo* 100% of his surface active lecithin (CDP-choline + α - β -diglyceride pathway), predominantly α - and β -palmitic acid (69.7 and 61.2%, respectively). Phosphatidyl dimethylethanolamine (PDME), a surface active intermediate in the trimethylation of phosphatidyl ethanolamine (PE) to form lecithin, showed largely an α -palmitic/ β -myristic acid (53/60%) distribution. Since the rate-limiting reaction is the first methyl group in PE, PDME represents the lecithin end product, another pulmonary surface active lecithin with α -palmitic/ β -myristic acids.

Speculation

Use of the β -carbon fatty acids as markers in studying the acetone-precipitated surface active fraction of lecithin isolated from alveolar wash will permit an assessment of the contribution of each of the two major pathways in the biosynthesis of surface active lecithin.

Introduction

The stability of mammalian lung is greatly dependent upon a phospholipid-rich complex, which apparently lines the alveoli and which lowers surface tension on expiration, thus preventing atelectasis. Quantitatively, the largest component of this lining, measured either after tracheal lavage with saline or by saline extraction of minced normal adult mammalian lung, is lecithin.

Previous studies [8, 10, 12] have reported that surface active lecithin when present in a total lecithin fraction can be separated by precipitation in cold acetone. The resultant acetone-soluble fraction is not surface active when measured on a modified Wilhelmy balance although the surface active acetone-precipitable fraction reduces the tension on compression of the surface film to less than 15 dynes/cm, increases the surface tension on expansion of the surface film, and describes a hysteresis during the cycle. In a previous report [7], comparison of fatty acid ester compositions of acetone-precipitated surface active lecithin and non-surface active acetone-soluble lecithin was not made.

In 1961, KLAUS *et al.* [13] noted a similarity in surface activity between synthetic dipalmitoyl lecithin and saline extract of normal beef lung. Subsequently, dipalmitoyl lecithin has been considered by most investigators to be the principal surface active phospholipid in mammalian lung. Since lecithins with specific fatty acid components in general cannot be selectively separated from total lecithin by present methods, the evidence for the presence of dipalmitoyl lecithin comes from the high percentage of palmitic acid found in lecithin isolated from lung [4, 5, 7, 17] and from isolation of dipalmitoyl lecithin in alveolar washings from rabbit lung [2].

The most adequate characterization of the fatty acids esters of lecithin currently requires that those on the β -carbon be examined separately from those on the α -carbon. The finding that the acetone-precipitated fraction contains the surface active lung lecithin, suggested that this fraction in particular be investigated during fetal maturation. This is so because surface activity of lung appears as a phenomenon of late fetal development and increases after breathing. It was thought that these studies might provide basic information about possible specific fatty acids patterns in lecithin structure associated with the development of surface activity in fetal lung.

This is a report of quantitative studies with gas-liquid chromatography (GLC) of the fatty acids on the α - and β -carbons of surface active and nonsurface active lecithin isolated from alveolar wash and from residual lung after wash in the developing rabbit fetus during late gestation. The specificity (or constancy) of fatty

acid composition associated with surface active lecithin synthesized by each of the two pathways are presented.

Materials and Methods

Pooled fetal lungs from a pure strain of albino New Zealand white rabbits were studied on successive days of gestation, from 23 days to term (31 days). Five to eight litters for each day of gestation were examined averaging nine fetuses per litter. All determinations were done in duplicate and some were done in quadruplicate.

Lecithin was isolated, purified, and separated into acetone-soluble and acetone-precipitated fractions as previously described [6, 8, 10]. Separation into these fractions involves adding chloroform in a proportion of about 1:100 (v/w) to purified lecithin to barely moisten (liquify) it and then adding, drop by drop, an excess of cold (0°) acetone until no further precipitation occurs (in proportion of 1 ml acetone/mg lecithin). The mixture is then centrifuged in a refrigerated centrifuge and the supernatant is decanted. Addition of more cold acetone to the precipitate and combining the precipitates is followed by overnight incubation of both tubes at 0–5° and centrifugation at 0°.

Lysolecithin was prepared by the method of LONG and PENNY [15], using 0.5–2 mg purified lecithin emulsified under nitrogen in 2 ml 0.01 M deaerated CaCl_2 solution, pH 6.8, to which was added 1 mg of dried *Naja naja* venom [18]. The solution was overlaid with 10–15 ml redistilled diethyl ether and nitrogen and sealed; the reaction mixture was stirred constantly at room temperature to completeness at 4 h when lysolecithin appeared as a thick colorless gelatinous precipitate. The free fatty acids split from the β -carbon dissolved in the ether phase. The lysolecithin was washed six times with ether and the washings added to the previously removed ether layer.

The optimal time required by phospholipase A (EC. 3.1.1.4) (*Naja naja* venom) to split the β -carbon fatty acid ester bond of lecithin was established by sampling reaction mixtures of varying concentrations of venom and lecithin at 0.5-h intervals. The samples were extracted from the reaction mixtures with methanol and chloroform and applied to thin layers of silica gel H. The reaction was completed (no uncleaved lecithin remaining) after about 3.5 h depending upon the total amounts of lecithin used initially. In the approximate proportions of venom to lecithin described, however, all reactions were complete by 4 h. Results of a sampling at 4-h incubation are shown in figure 1. In the determinations to be described, every reaction mixture was sampled on thin-layer chromatography (TLC) for completeness of cleavage

of lecithin, using approximately 20 μg of lipid. The entire remainder of the reaction mixture was extracted for methanolysis. Recovery of the theoretical maximum of free fatty acids (derived from a test mixture of known composition both by gravimetric and by spectrophotometric measurements) was approximately 90%. Each group of fatty acids was methylated separately by incubation in BF_3 -methanol at 70° for 30 min, similar to the procedure described by METCALFE and SCHMITZ [16].

Analyses of the fatty acid methyl esters were done by dual column gas-liquid chromatography [19] with a flame ionization detector on 6-ft coiled glass columns (id 4 mm) containing 15% diethylene glycol succinate polyester on acid washed and silanized Chromosorb W. The isothermal column temperature was 180°, the in-

jector temperature 290°, and the detector 210°. Samples were dissolved in methylene chloride and approximately 10–30 μg in 0.5–1.0 μl were injected. Carrier gas was helium at 40 pounds per square inch gauge (psig). The flame of the detector was optimized at 35 psig compressed air and 19 psig hydrogen. Recordings were taken at spans of 0–5 and 0–1 mv.

Percentage composition calculations were carried out by multiplication of peak height by width at half-height. Reference standards were obtained from the Hormel Institute and from the National Heart Institute. Quantitative results with National Heart Institute Fatty Acid Standards D and F [11] agreed with the stated composition data with a relative error less than 3% for major components (> 10% of total mixture) and less than 5% for minor mixtures (< 10% of total mixture).

Isolation and verification of PDME from lung and liver were done as previously described [10].

Surface activity measurements were made in standard fashion on a modified Wilhelmy balance, as previously described [8]. On compression of the surface, minimum surface tension below 15 dynes/cm for three consecutive cycles was considered 'normal surface activity'.

Results

Total Fatty Acids in Lecithin

Gross measurements of the total fatty acids of lecithin are shown in table I. No clear tendency toward a greater percentage of saturated fatty acids as gestation progressed was found in whole lung lecithin during representative stages of development; only a slight increase in percentage of palmitic acid was seen. In the lecithin from alveolar wash, there was an appreciable increase in percentage of palmitic acid with fetal development, but no differences were seen in the percentages of saturated fatty acids among the stages compared. The total fatty acids from alveolar wash lecithin, however, showed a higher percentage of saturated fatty acids than were found in whole lung lecithin, confirming the findings of MORGAN *et al.* [17] on adult dog lung.

Comparison of Fatty Acids in Acetone-soluble and Acetone-precipitable Lecithins

Table II shows representative examples of constituent fatty acids on the α - and β -carbons of three groups of lecithins: 1) surface active acetone precipitable; 2) nonsurface active acetone soluble; and 3) nonsurface active acetone precipitable. The latter group are lecithins from liver and kidney which, although insoluble in cold acetone, are not surface active (do not reduce the tension below 15 dynes/cm on compression

Fig. 1. A representative sampling run on a thin layer plate of the lecithin/cobra venom reaction mixture after 4-h incubation is shown on the right. LL: lysolecithin produced. FA: β -carbon fatty acids split from the lecithin (L) isolated from lung. Approximately 20 μg lecithin were applied on silica gel H and chromatographed in chloroform-methanol-water (65:25:4), with charring at 280° after 50% H_2SO_4 spraying.

of the surface in a Wilhelmy balance), while the first two groups are from lung.

Evident is an extraordinarily high percentage of saturated fatty acids on both α - and β -carbons of the surface active lecithins. By contrast, the acetone-soluble lecithins had predominantly unsaturated fatty acids on the β -carbon, although the α -carbon fatty acids resemble those of the surface active lecithin. A very high percentage of saturated fatty acids on the α -carbon and a predominance of unsaturated fatty acids on the β -carbon were found in the acetone-precipitated lecithin from adult rabbit liver. The adult kidney lecithin had a high percentage of unsaturated β -carbon fatty acids but had less than 50% saturated fatty acids on the α -carbon.

In general, the principal differences between the two groups of nonsurface active lecithins include a significantly greater percentage of palmitoleic acid ($C_{16:1}$) in the acetone-soluble lecithins on both α - and β -carbons while the lecithins from liver and kidney had greater concentrations of oleic acid ($C_{18:0}$) and arachidonic acid ($C_{20:4}$).

Quantitative Measurement of Lecithin in the Lung of the Developing Rabbit Fetus, the Newborn, and the Adult

Although much of the data in table III has been presented elsewhere [8, 10, 12], it has not been pre-

sented in unified fashion to show the quantification of the various forms of lecithin. The table presents data showing the rising concentration of acetone-precipitable lecithin in whole lung to a peak with breathing. The most striking finding was the marked increase in acetone-precipitable lecithin in alveolar wash after 1 h of breathing.

Fatty Acids of Alveolar Wash Lecithin: Acetone Precipitated

The fatty acid components on the α - and β -carbons of acetone-precipitated lecithin from alveolar wash during gestation are shown in table IV. Both the α - and β -component palmitic acid ($C_{16:0}$) rose abruptly after day 29, when alveolar lecithin becomes surface active [8]. The greatest increase in α - and β -palmitic acid followed the onset of breathing. These changes are plotted in figure 2.

Other fatty acids also varied during development (table IV), but only the palmitic acid changes correlated with surface activity in alveolar wash lecithin.

Fatty Acids of Alveolar Wash Lecithin: Acetone Soluble

Table V shows the percentage composition during fetal development of α - and β -carbon fatty acids in the nonsurface active acetone-soluble lecithin from alveolar wash. Palmitic acid was the principal α -carbon fatty acid, increasing markedly from day 29. By com-

Table I. Percentage composition of total fatty acids in whole lung and alveolar wash lecithin in rabbits

Fatty acids ¹	Whole lung lecithin, %			Alveolar wash lecithin, %		
	Fetus, 23 day	Fetus, 31 day (term)	Adult rabbit	Fetus, 23 day	Fetus, 31 day (term)	Adult rabbit
$C_{14:0}$, myristic acid	2.2	4.2	1.6	6.9	3.7	2.2
$C_{14:1}$	0.5	1.0	0.6	3.7	1.3	0.1
$C_{14:2}$	0.7	0.4	0.3	—	—	0.7
$C_{16:0}$, palmitic acid	45.3	48.0	49.8	46.7	53.8	60.0
$C_{16:1}$, palmitoleic acid	6.3	12.5	6.7	7.2	11.6	8.3
$C_{16:2}$	—	0.4	—	—	—	—
$C_{18:0}$, stearic acid	3.9	5.1	5.1	11.5	5.8	3.4
$C_{18:1}$, oleic acid	19.3	12.5	15.1	18.6	12.7	13.0
$C_{18:2}$, linoleic acid	9.9	9.5	15.9	5.5	6.8	10.5
$C_{18:3}$, linolenic acid	0.2	0.7	1.5	—	0.6	1.0
$C_{20:0}$, arachidic acid	0.2	0.2	0.1	—	0.3	—
$C_{20:4}$, arachidonic acid	11.3	4.8	3.0	—	3.2	0.5
$C_{22:0}$, behenic acid	—	0.4	0.3	—	—	—
Saturated fatty acids	51.6	57.9	56.9	65.1	63.6	65.6
Unsaturated fatty acids	48.2	41.8	41.3	35.0	36.2	34.1

¹ The convention for identifying fatty acids includes the carbon chain length and, following the colon, the number of unsaturated double bonds.

Table II. Comparison of fatty acid composition of surface active acetone-precipitated lecithins from lung with those of nonsurface active lecithin, including acetone-soluble lecithin from lung and acetone-precipitated lecithins from liver and kidney

Fatty acids	Surface active lecithin, %				Nonsurface active lecithin, %							
	Alveolar wash		Lung after alveolar wash		Alveolar wash		Lung after alveolar wash		Alveolar wash		Lung after alveolar wash	
	acetone precipitated, 2-day-old rabbit	acetone precipitated, 31-day nonbreathing rabbit	precipitated, 31-day nonbreathing rabbit	precipitated, 31-day nonbreathing rabbit	acetone soluble, 2-day-old rabbit	acetone soluble, 2-day-old rabbit	soluble, 2-day-old rabbit	soluble, 2-day-old rabbit	adult rabbit liver	adult rabbit kidney	adult rabbit liver	adult rabbit kidney
	α	β	α	β	α	β	α	β	α	β	α	β
C _{14:0}	9.5	8.7	2.2	6.7	5.7	1.4	3.0	2.1	0.8	12.4	1.7	0.6
C _{16:0}	85.2	62.5	74.4	60.7	77.6	23.8	58.3	22.3	53.2	8.5	23.2	22.6
C _{16:1}	1.5	12.1	3.3	6.4	9.5	20.0	7.9	14.3	1.3	1.4	3.1	0.1
C _{18:0}	1.5	0.4	10.4	2.5	2.9	1.7	7.5	0.9	28.1	2.5	9.3	2.5
C _{18:1}	1.6	6.8	6.5	13.3	0.4	18.9	11.2	20.0	5.7	11.7	16.3	20.2
C _{18:2}	—	8.4	3.0	7.4	3.6	30.5	8.3	37.9	8.4	53.9	21.7	42.3
C _{18:3}	—	—	—	—	—	2.5	—	2.2	—	3.2	—	0.8
C _{20:0}	0.9	0.5	—	—	—	—	1.2	—	—	—	10.9	—
C _{20:4}	—	—	—	2.9	—	1.0	2.4	—	2.6	6.4	13.7	10.9
Total	97.1	72.1	87.0	69.9	86.2	26.2	70.0	25.3	82.1	23.4	45.2	25.7
Saturated, %	3.1	27.3	12.8	30.0	13.5	72.9	29.8	74.4	18.0	76.6	54.8	74.3
Unsaturated, %												

parison, there was an appreciably smaller percentage of palmitic acid in the β -carbon fatty acids of acetone-soluble lecithin. There was a relatively high percentage of β -carbon myristic acid between days 25 and 29. The percentage compositions during late gestation of α - and β -carbon palmitic acid in the acetone-soluble lecithin from alveolar wash of the fetus are plotted in figure 3.

Fatty Acids of Lecithin from Residual Lung Parenchyma after Alveolar Wash: Acetone Precipitated

This fraction representing the surface active parenchymal or 'intracellular' lecithin showed consistently high concentrations of palmitic acid in both α - and β -carbon fatty acids in the fetal state (table VI). After 6 h of breathing by the newborn rabbit, however, there was a 50% drop in concentration of palmitic acid on the α -carbon of the isolated lecithin; this was seen also in the 2-day-old rabbit where it was most marked in the β -carbon palmitic acid. Lecithin from adult rabbit had low β - and high α -carbon palmitic acid. Figure 4 illustrates the percentage composition of the major fatty acid, palmitic acid, on both α - and β -carbons of the surface active acetone-precipitated lecithin from residual lung parenchyma after alveolar wash in the developing rabbit fetus and after birth.

Fatty Acids of Lecithin from Residual Lung Parenchyma after Alveolar Wash: Acetone Soluble

Acetone-soluble lecithin from residual lung parenchyma (table VII) contained from a low of 50 to a high of 72% saturated fatty acids on the α -carbon. Again, the principal fatty acid was palmitic acid. The β -carbon fatty acids differed markedly in percentage composition between nonbreathing and breathing newborn and adult. In particular, postbreathing values showed 75% or more unsaturated fatty acids on the β -carbon, linoleic acid (C_{18:2}) and oleic acid (C_{18:1}) being the

Fig. 2. Changes in percentage concentrations of palmitic acid (C_{16:0}) during fetal development in α - and β -carbons of surface active acetone-precipitated lecithin isolated from alveolar wash. The percentages plotted actually are percentages of palmitic acid in either the α - or β -carbon fatty acids, as labeled and not as percentages of the total fatty acids on the lecithin molecule. The various designations (e.g., fetal states, breathing, lecithin storage in cells, etc.) refer to these experimentally determined states in the fetus and newborn as previously described [8, 10, 12].

Fig. 3. Changes in percentage concentrations of palmitic acid (C_{16:0}) during fetal development, on α - and β -carbons of nonsurface active acetone-soluble lecithin isolated from alveolar wash. See legend of figure 2 for explanation of terms.

Table III. Quantitative measurement of lecithin in the lung of the developing rabbit fetus, the newborn, and the adult¹

Gestational age, days	Whole lung lecithin, mg/g dry wt lung			Alveolar wash lecithin, mg/g dry wt lung			Lecithin from residual lung parenchyma after alveolar wash, mg/g dry wt lung		
	Total	Acetone precipi- tated	Acetone soluble	Total	Acetone precipi- tated	Acetone soluble	Total	Acetone precipi- tated	Acetone soluble
Fetus									
19	22.5±3.0 ²								
21	52.4±5.8	17.5 ³	35 ³	1.0 ³	0.05 ³	0.95 ³	50.0 ³	13 ³	37 ³
22	34.4±4.6								
23	37.3±5.2	8.0	29.0	1.0	0.07	0.93	35.5	9	27
24	31.6±9.1	—							
25	47.2±3.6	7.5	39.0	1.1	0.08	1.02	45.8	16.7	29.1
26	50.8±1.8								
27	65.5±3.2	17.0	34.5	1.2	0.1	1.1	65.2	15.0	50.2
28	76.3±4.3	18.0	50.0	1.3	0.2	1.2	74.9	15.0	59.9
29	80.2±0.8	28.0	48.0	4.1	0.3	3.8	76	24.0	52
30	82.3±3.5	34.0	49.0	4.1	0.4	3.7	78.2	30.0	48.2
31	84.2±1.9	43.0	40.0	4.3	0.35	3.9	79.9	44.0	35.9
Newborn									
1 h breath	84.0±2.1	43.8	41.2	19	9.8	9.0	64.6	34.0	32.0
6 h breath	71.8±	36.0	35	11	5.0	6.0	60.8	31	29
2 day	68	29.6	38.4	8	3.6	4.4	60	26	34
Adult									
	58.0±1.1	28.0	32.0	6	3.4	2.6	52	28	24

¹ Mean measurements from 22–46 fetuses for every value shown.

² Standard deviations are shown for total lung lecithin to indicate the spread of measurements.

³ Only the means are shown for the other values since they represent fractions of the whole lung lecithin.

Fig. 2.

Fig. 3.

Table IV. Percentage composition of fatty acids esterified on α - and β -carbons of acetone-precipitated lecithin from alveolar wash

Fatty acid	Gestational age of nonbreathing fetus, day												Newborn, after 6 h of breathing						2 day		Adult							
	21			23			25			27			28			29			30			31						
	α	β		α	β		α	β		α	β		α	β		α	β		α	β		α	β		α	β		
C14:0	4.8	1.9	9.2	7.2	10.3	6.9	5.6	6.5	8.0	7.0	10.1	9.3	8.5	8.8	11.0	9.7	11.9	17.2	9.4	8.7	5.8	9.4						
C16:0	69.2	77.8	44.2	41.2	33.5	42.7	36.5	38.8	33.7	43.1	45.0	48.2	67.1	60.2	62.0	65.8	79.0	53.2	85.2	62.5	83.8	64.2						
C16:1	3.3	2.8	8.1	9.9	13.1	13.0	14.9	11.6	8.2	11.6	6.5	15.5	6.9	7.4	9.5	8.3	3.6	13.5	1.5	12.0	3.0	4.6						
C18:0	10.5	1.2	10.8	12.3	9.1	8.7	12.7	12.6	8.4	12.2	11.8	3.7	5.8	3.4	4.8	1.6	2.2	0.4	1.5	0.4	2.9	0.4						
C18:1	9.5	12.9	11.5	18.3	16.4	19.3	15.8	20.9	12.8	16.1	11.7	13.5	6.7	12.6	6.2	7.6	1.9	6.8	1.5	6.8	3.5	13.8						
C18:2	1.7	2.2	8.4	7.5	10.3	6.5	11.1	5.2	6.0	6.1	-	7.2	3.2	5.9	5.6	4.4	0.9	7.9	-	8.4	0.7	6.9						
C18:3	-	-	-	1.6	-	1.4	-	2.3	-	-	-	0.8	-	0.4	-	0.2	-	-	-	-	-	-						
C20:0	0.8	-	7.7	-	7.2	-	3.4	-	22.8	1.5	14.8	-	1.9	-	1.1	-	0.4	0.4	0.9	0.5	0.2	0.5						
C20:4	-	1.0	-	3.3	-	1.3	-	1.8	-	2.3	-	1.6	-	1.2	-	0.4	-	-	-	-	-	-						
C22:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6	-	0.2	-	0.2						
Total																												
Saturated, %	85.3	80.9	71.9	60.7	60.1	58.3	58.2	57.9	72.9	63.8	81.7	61.2	83.3	72.4	78.9	77.1	93.5	71.8	97.0	72.3	92.7	74.7						
Unsaturated, %	14.5	18.9	28.0	40.6	39.8	41.5	41.8	41.8	27.0	36.1	18.2	38.6	16.8	27.5	21.3	20.9	6.4	28.2	3.0	27.2	7.2	25.3

Fig. 4. Changes in percentage concentrations of palmitic acid ($C_{16:0}$) during fetal development, on α - and β -carbons of surface active acetone-precipitated lecithin isolated from residual lung parenchyma after alveolar wash. See legend of figure 2 for explanation of terms. Fig. 5. Changes in percentage concentrations of palmitic acid ($C_{16:0}$) during fetal development, on α - and β -carbons of nonsurface active acetone-soluble lecithin isolated from residual lung parenchyma after alveolar wash. See legend of figure 2 for explanation of terms.

Table V. Percentage composition of fatty acids esterified on α - and β -carbons of acetone-soluble lecithin from alveolar wash

Fatty acid	Gestational age of nonbreathing fetus, day										Newborn, after 6 h breathing				2 day				Adult			
	21		23		25		27		28		29		30		31							
	α	β	α	β	α	β	α	β	α	β	α	β	α	β	α	β	α	β	α	β	α	β
C _{14:0}	1.5	2.1	3.8	2.4	2.5	53.4	4.5	59.0	4.5	20.9	1.1	54.7	2.3	2.8	4.1	18.6	6.1	4.5	5.7	1.4	1.8	4.2
C _{16:0}	48.9	31.0	37.3	15.3	36.7	15.1	34.6	14.8	40.7	29.5	65.6	16.9	69.0	35.6	61.1	23.3	69.8	26.1	77.6	23.8	68.8	18.6
C _{16:1}	5.0	0.9	10.2	7.9	9.5	6.2	16.0	7.7	14.0	11.2	7.3	8.0	8.7	18.8	9.3	16.2	11.1	29.6	9.5	20.0	6.9	17.7
C _{18:0}	13.7	2.1	19.4	7.3	13.7	2.2	11.9	4.2	11.4	6.6	11.4	2.0	8.7	2.5	5.1	1.7	4.8	0.6	2.9	1.7	5.3	0.4
C _{18:1}	22.6	24.4	24.1	18.3	17.2	9.3	21.3	8.1	18.9	15.0	9.7	7.7	8.0	18.6	8.1	9.9	6.1	12.5	0.4	18.9	13.0	24.2
C _{18:2}	5.4	15.8	3.0	16.3	20.3	8.0	11.2	6.0	10.3	10.9	4.9	7.7	3.2	18.0	6.8	11.1	2.0	22.3	3.6	30.5	3.5	31.3
C _{18:3}	1.2	6.5	2.0	13.9	-	-	-	-	-	1.7	-	-	-	0.6	-	7.3	-	1.8	-	2.5	0.6	2.7
C _{20:0}	1.6	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	5.3	7.9	-	-	-	-	-
C _{20:4}	-	17.1	-	18.4	-	5.7	-	-	-	3.9	-	2.9	-	2.9	-	3.7	-	2.4	-	1.0	-	0.7
Total																						
Saturated, %	65.7	35.2	60.5	25.0	52.9	70.7	51.0	78.0	56.6	57.0	78.1	73.6	80.0	40.9	75.6	51.5	80.7	31.2	86.2	26.9	75.9	23.2
Unsaturated, %	34.2	64.7	39.3	74.8	47.0	29.2	48.5	15.8	43.2	42.7	21.9	26.3	19.9	58.9	24.2	48.2	19.2	68.6	13.5	72.9	24.0	76.6

Table VI. Percentage composition of fatty acids esterified on α - and β -carbons of acetone-precipitated lecithin from residual lung parenchyma after alveolar wash

Fatty acid	Gestational age of nonbreathing fetus, day										Newborn, after 6 h of breathing				2 day				Adult			
	25		27		28		29		30		31											
	α	β	α	β	α	β	α	β	α	β	α	β	α	β	α	β	α	β	α	β	α	β
C _{14:0}	4.3	19.0	2.8	2.3	0.9	12.3	2.5	4.7	2.1	7.5	2.2	6.7	4.9	10.0	1.9	9.9	2.6	10.0	2.6	10.0	2.6	10.0
C _{16:0}	62.5	45.6	57.3	66.8	69.5	56.8	58.8	59.1	69.0	60.5	74.4	60.7	33.2	57.4	27.8	33.5	44.4	19.5	44.4	19.5	44.4	19.5
C _{16:1}	5.1	4.6	17.1	3.5	1.8	3.9	4.7	4.7	3.2	4.3	3.3	6.4	9.7	6.9	12.4	42.2	9.3	4.5	9.3	4.5	9.3	4.5
C _{18:0}	11.8	1.4	10.2	2.8	13.5	1.3	10.7	1.4	11.2	2.7	10.4	2.5	2.8	1.5	7.6	1.1	11.2	0.9	11.2	0.9	11.2	0.9
C _{18:1}	9.2	17.4	5.7	17.4	8.9	14.7	10.0	17.8	9.4	15.1	6.5	13.3	10.1	13.1	23.0	6.5	16.7	24.8	16.7	24.8	16.7	24.8
C _{18:2}	1.9	7.4	6.8	5.3	3.4	7.4	9.1	8.1	5.0	7.4	3.0	7.4	6.1	8.5	12.7	5.4	8.1	22.9	12.7	5.4	8.1	22.9
C _{18:3}	-	tr	-	-	-	0.1	-	0.3	-	-	-	-	-	-	0.4	-	7.5	2.2	0.4	-	7.5	2.2
C _{20:0}	5.0	tr	-	-	0.9	-	4.0	-	-	-	-	-	17.6	-	14.3	-	-	-	14.3	-	-	-
C _{20:4}	-	4.6	-	1.8	0.9	3.2	-	3.8	-	2.1	-	2.9	15.4	1.9	-	0.9	-	15.1	-	0.9	-	15.1
Total																						
Saturated, %	83.6	66.0	70.3	71.9	84.8	70.4	76.0	65.2	82.3	70.7	87.0	69.9	58.5	68.9	51.6	44.5	58.2	30.4	51.6	44.5	58.2	30.4
Unsaturated, %	16.2	34.0	29.6	28.0	15.3	29.3	23.8	34.7	17.6	28.9	12.8	30.0	41.3	30.8	48.1	55.0	41.6	69.5	48.1	55.0	41.6	69.5

Table VII. Percentage composition of fatty acids esterified on α - and β -carbons of acetone-soluble lecithin from residual lung parenchyma after alveolar wash

Fatty acid	Gestational age of nonbreathing fetus, day										Newborn, 2 day after 6 h of breathing		Adult	
	25		27		28		29		30		31		2 day	
	α	β	α	β	α	β	α	β	α	β	α	β	α	β
C _{14:0}	0.8	23.1	1.0	29.6	1.9	26.7	1.4	10.8	2.1	25.7	2.4	47.1	1.9	3.1
C _{16:0}	51.9	20.9	43.9	12.9	57.5	22.8	57.2	24.3	47.1	22.0	32.3	14.3	55.3	19.7
C _{16:1}	8.0	8.0	5.0	10.7	4.4	9.5	4.9	14.3	6.4	9.1	7.9	10.2	6.5	16.8
C _{18:0}	12.6	1.6	21.3	1.5	13.5	1.1	13.8	1.4	3.7	1.1	6.9	-	9.6	0.3
C _{18:1}	19.9	21.4	20.9	20.6	14.5	24.0	15.4	24.8	16.9	20.8	17.5	10.4	15.4	17.2
C _{18:2}	6.6	15.5	6.9	15.0	7.9	2.0	5.6	23.0	20.0	18.7	19.6	13.2	8.5	30.1
C _{18:3}	-	-	0.8	0.6	-	0.7	-	1.3	0.8	0.5	-	0.5	0.2	2.1
C _{20:0}	-	-	-	-	-	0.6	-	-	-	-	13.3	-	0.5	-
C _{20:4}	-	9.3	-	8.9	-	12.3	1.4	-	2.8	1.9	-	4.2	1.9	10.5
Total	65.3	45.6	66.2	44.0	72.9	51.2	72.4	36.5	52.9	48.8	54.9	61.4	67.3	23.1
Saturated, %	34.5	54.2	33.6	55.8	26.8	48.5	27.3	63.4	46.9	51.0	45.0	38.5	32.5	76.7
Unsaturated, %														

most prevalent. Figure 5 charts the percentage composition changes in α - and β -carbon palmitic acid in the nonsurface active acetone-soluble lecithin from residual lung parenchyma after alveolar wash.

Fatty Acids of Lecithin Synthesized De Novo by the Breathing Fetus

The rabbit fetus delivered by cesarean section after 28 full days of gestation synthesized *de novo* 100% of his acetone-precipitated surface active alveolar wash lecithin after 1 h of breathing [10]. The fatty acids from this lecithin are shown in table VIII and were predominantly α -palmitic/ β -palmitic.

Intermediate Compounds in the Biosynthesis of Lecithin by the Methylation of Phosphatidyl Ethanolamine

Of the two principal known pathways for *de novo* biosynthesis of lecithin in the lung of the rabbit [9, 10], the trimethylation of phosphatidyl ethanolamine (PE) has identifiable intermediate compounds, phosphati-

Table VIII. Percentage composition of fatty acid esters of surface active acetone-precipitated lecithin isolated from alveolar wash of early 29-day gestation rabbit after 1 h of breathing

Fatty acid	α	β
C _{14:0}	12.6	10.5
C _{16:0}	69.7	61.2
C _{16:1}	4.4	4.0
C _{18:0}	6.8	1.9
C _{18:1}	4.2	10.0
C _{18:2}	2.3	8.9
C _{20:0}	-	1.6
C _{20:4}	-	1.8

Fig. 6. Suggested schematic structure of surface active lecithin synthesized by each of the two major pathways for the biosynthesis of lecithin.

(PME), and phosphatidyl dimethylethanolamine (PDME) in lung and liver of adult rabbits

Fatty acid	Total fatty acids in total phospholipid fraction, %		α - and β -carbon fatty acid esters of acetone-precipitated phospholipid, %											
			PE lung			PME lung			PDME lung			PDME liver		
	PE lung	PME lung	PDME lung	α	β	α	β	α	β	α	β	α	β	β
C _{14:0}	8.3	29.6	31.9	4.0	58.7	7.4	56.1	5.9	59.8	1.8	76.6	—	—	—
C _{15:0}	18.0	—	—	8.6	—	—	—	—	—	—	—	—	—	—
C _{16:0}	14.7	25.1	25.5	9.6	8.7	41.9	10.9	52.7	10.6	14.2	12.3	1.7	2.5	2.5
C _{16:1}	3.6	3.7	5.5	1.8	3.2	7.9	4.8	6.5	2.7	1.7	2.4	2.6	2.4	2.4
C _{18:0}	5.2	9.2	11.4	19.1	3.6	9.5	2.9	13.0	1.7	2.6	4.5	8.9	4.5	4.5
C _{18:1}	29.3	17.6	16.3	8.8	7.9	14.4	12.3	11.3	20.0	3.9	1.7	30.9	33.7	—
C _{18:2}	11.8	8.1	5.9	13.6	12.6	11.4	7.1	6.9	0.5	—	—	—	—	—
C _{18:3}	5.3	2.4	18.0	1.9	0.8	—	—	1.4	—	—	—	—	—	—
C _{20:0}	0.3	0.4	1.0	9.6	—	—	2.5	—	—	—	—	—	—	—
C _{20:4}	3.3	3.6	2.6	22.9	4.4	7.5	3.4	2.4	0.8	5.1	—	—	—	—
Total	46.5	64.3	68.8	50.9	71.0	58.8	72.4	71.6	72.1	18.6	91.3	—	—	—
Saturated, %	53.3	36.4	31.3	49.0	28.9	41.2	27.6	28.5	27.9	80.3	8.7	—	—	—
Unsaturated, %	—	—	—	—	—	—	—	—	—	—	—	—	—	—

dyl methylethanolamine (PME) and phosphatidyl dimethylethanolamine (PDME). The PME and PDME are not found in significantly measurable quantities in alveolar wash of rabbit (and sheep) fetuses and newborns, and then not until after several days of life.

Table IX compares the fatty acid components of the acetone-precipitated fractions of adult rabbit PE, PME, and PDME from lung as well as PDME from adult rabbit liver. There was much variation in PE in fatty acid composition from animal to animal; PDME showed little individual variation. Because of the variability, PE isolated from 12 lungs of adult rabbits was pooled and the fatty acids compared with those of PME and PDME.

As seen in table IX, PME and PDME resembled each other in total fatty acid composition while PE was considerably different. In the comparison of the α - and β -carbon fatty acids in the acetone-precipitated fractions, the β -carbon fatty acids of all three compounds were similar. The α -carbon fatty acids of PE differed markedly from those of PE or PDME, while those of the latter two resembled each other.

The α - and β -carbons of acetone-precipitated surface active PDME from adult rabbit alveolar wash showed largely on α -palmitic acid/ β -myristic acid distribution. The β -carbon fatty acids from liver PDME were similar to those of PDME from lung whereas the α -carbon fatty acids were considerably different. Liver PDME was not surface active [8].

Discussion

MORGAN *et al.* [17] first established that there is a significant difference between the phospholipid composition of whole lung homogenates and that of alveolar wash of adult dog lung.

The synthesis of phospholipids in lungs of fetal lambs was studied by CHIDA *et al.* [4] who reported increasing amounts of phospholipids in lung with advancing gestation, similar to findings reported in rabbits [10], with the greatest increases found in the lecithin fraction.

BRUMLEY *et al.* [3] who also studied fetal lambs, found increasing phospholipid concentrations in the lungs after 120 days gestation as well as increasing concentrations of 'disaturated phosphatidyl choline'. These workers, however, did not examine the lecithins in alveolar wash, their studies having been done on homogenates of whole lung.

Surface active lecithin can be separated from the total lecithin fraction by acetone precipitation [10]. The fatty acid ester components of acetone-soluble and acetone-precipitate surface active and nonsurface active lecithins were compared in that report. No sur-

face active acetone-soluble lecithin has as yet been identified in this laboratory, although some acetone-precipitated lecithins are not surface active. Precipitation in cold acetone appears to be a property less easy to define than the property of surface activity. In general, surface activity appears to be associated with a high percentage of saturated β -carbon fatty acids as well as saturated α -carbon fatty acids on the lecithin. Acetone-soluble lecithins (nonsurface active) may have a high percentage of saturated α -carbon fatty acids but the percentage of saturated β -carbon fatty acids is low. The nonsurface active but acetone-precipitated lecithins may also have a low percentage of saturated fatty acids on the β -carbon and highly saturated fatty acids on the α -carbon, which probably accounts for their being nonsurface active. They differ, however, from the acetone-soluble group particularly in a near lack of palmitoleic acid ($C_{16:1}$) on both α - and β -carbons compared with the acetone-soluble lecithins; in addition, they have somewhat greater components of oleic acid ($C_{18:1}$) and arachidonic acid ($C_{20:4}$) on both carbons.

The data in this report show that the appearance late in gestation of normal surface activity in the alveolar wash lecithin from nonbreathed fetal rabbit lung is characterized by marked increases in the percentage of palmitic acid ($C_{16:0}$) esters on both the α - and β -carbons of the surface active lecithin.

From day 25 of gestation till term, the rabbit stores increasing concentrations of lecithin intracellularly in lung [8, 10, 12]. In nonbreathed fetal lungs during this period, surface active acetone-precipitated lecithin isolated from residual lung parenchyma after alveolar wash also showed consistently high percentages of palmitic acid on both α - and β -carbons.

Even at term, only about 11% of alveolar wash lecithin is acetone-precipitated surface active compound [8, 10, 12]. Within an hour after the onset of breathing, this increases to 50% of the lecithin in alveolar wash, essentially the same proportion found throughout the subsequent life of the rabbit. After breathing, however, the fatty acid ester patterns of the lecithins changed markedly from those isolated from nonbreathed fetal lung.

In the full-term rabbit after 6 h of breathing, the percentage of palmitic acid on the α -carbon of the surface active acetone-precipitated alveolar wash lecithin had increased to approximately 85% of all fatty acids esterified on that carbon, but there was no corresponding increase of palmitic acid on the β -carbon. The acetone-precipitated lecithin from residual lung parenchyma after alveolar wash showed a reciprocal change, with a marked drop of about 50% in α -carbon palmitic acid and a slight decrease in that of the β -carbon.

The lack of increase in β -carbon palmitic acid in lecithin from alveolar wash and the lack of a concurrent drop in percentage of β -carbon palmitic acid from residual lung lecithin to match the changes seen in the corresponding α -carbon palmitic acid of the lecithin indicated that significant percentages of fatty acids other than palmitic acid were contained on the β -carbon of the lecithin which passed into the alveolar wash from the cells. The principal fatty acid probably was myristic acid ($C_{14:0}$) which rose in the alveolar wash lecithin fraction to about 17% of the β -carbon fatty acids.

By day 2 a sort of 'steady state' was reached when lecithin compositions became similar to those found in the adult rabbit. Alveolar wash lecithin from 2-day-old rabbits, when compared with alveolar wash lecithin of the 6-h-old newborn, showed a slight increase of β -carbon palmitic acid as well as a sharp drop in the β -carbon palmitic acid of lecithin from residual lung after alveolar wash. In the adult rabbit, β -carbon palmitic acid on the surface active lecithin from residual lung comprised a small percentage of fatty acids, while percentages of both α - and β -carbon palmitic acid were extraordinarily high in the acetone-precipitated surface active alveolar wash lecithin in the 2-day-old and adult rabbits.

These findings, together with those previously reported of the localization [8, 10, 12] and *de novo* biosynthesis of lecithin [10] and the quantitative summary in table III in the present report, show that the rabbit fetus approaching term stores large quantities of surface active dipalmitoyl lecithin as well as other lecithins. This fraction probably includes some palmitoyl-myristoyl lecithins which are released rapidly to the alveolar surface after the onset of breathing. By day 2 of life, the 'steady state', there are relatively few differences between lecithin from alveolar wash and from residual parenchyma after wash in the fatty acids of the 2-day-old rabbit and adult rabbits.

The acetone-soluble lecithin fractions, in alveolar wash and in residual parenchyma after alveolar wash, were not surface active [8]. In both fractions the α -carbon palmitic acid was the most abundant fatty acid found throughout the gestation period. The β -carbon palmitic acid, however, although a major component, was always less than 35% and usually less than 25% of the fatty acids of the β -carbon. Myristic acid ($C_{14:0}$) throughout gestation comprised a significant proportion of β -carbon fatty acids of the acetone-soluble lecithin.

There were high percentages of oleic acid ($C_{18:1}$), both α - and β -carbon components, in the surface active fractions as well as in the nonsurface active ones throughout gestation. The timing and patterns of changes in this fatty acid suggested a possible precursor

role in the production of palmitic acid, perhaps by oxidation of oleic acid; but this remains to be proved.

Other fatty acids also showed transitory increases in concentration suggesting possible special roles at particular times in the lecithin metabolic cycle, including linoleic acid ($C_{18:2}$), palmitoleic acid ($C_{16:1}$), and arachidic acid ($C_{20:0}$). No consistent meaningful pattern, however, was obvious for these acids. The data in this report do not give a clear picture of fatty acid synthesis or incorporation, but indicate only the fatty acid content of lecithin and other phospholipids during gestation and after breathing.

At present, there are no effective technical means to separate the lecithin of a specific composition from total lecithin, nor are there any successful methods known to synthesize specific lecithins containing non-identical α - and β -carbon fatty acid esters. Thus, surface activity of lecithin may be observed with a variety of combinations of α - and β -carbon fatty acids that are 'compatible' and form a compressed layer of low surface tension.

This study demonstrates the specificity of structure of the actual surface active lecithin isolated from alveolar wash. It is not proper to assume, however, even from the extremely high palmitic acid content in surface active lecithin found in the 2-day-old rabbit (85% on the α -carbon and 62% on the β -carbon) that this represents 62% dipalmitoyl lecithin. This is the theoretical maximum, but can range from as low as 47% dipalmitoyl lecithin. From day 30 of gestation throughout the life of the rabbit, a theoretical range between 27 and 64% of the acetone-precipitated surface active lecithin in the alveolar wash is dipalmitoyl lecithin, greater than any other single identifiable fraction.

The major pathway for lecithin synthesis in lung of rabbit was previously shown to be the incorporation of CDP-choline with D- α , β -diglyceride [10]. Direct evidence that synthesis, predominantly of dipalmitoyl lecithin, is associated with this pathway was obtained by identification of the α - and β -carbon fatty acids of the surface active acetone-precipitated lecithin isolated from alveolar wash after 1 h of breathing by the rabbit fetus of 28 full days (early 29 day) gestation. It was shown [10] that such a fetus synthesized *de novo* 100% of its surface active alveolar wash lecithin in 1 h. This lecithin was predominantly α -palmitic/ β -palmitic.

As mentioned earlier, intermediates in the biosynthesis of lecithin are identifiable readily only in the methylation of phosphatidyl ethanolamine (PE). These are phosphatidyl methylethanolamine (PME) and phosphatidyl dimethylethanolamine (PDME). Even in adult alveolar wash, PME is present only in very small amounts compared with PDME. Phosphatidyl dimethylethanolamine cannot be isolated readily from lung of the rabbit fetus [10], when first found in

recoverable amounts in alveolar wash during the first week of life, but PDME is easily isolated from alveolar wash of the adult rabbit.

Since PDME was found to be surface active [8, 17], it was particularly important to characterize its fatty acid components. If any specificity were found of esterified fatty acids associated with PDME, this might provide a marker for assessing the contribution of the methylation pathway in the production and localization of surface active lecithin. This is true since the formation of PME was shown to be the rate limiting step in the methylation of PE [1, 10]. Once the first methyl group is attached to PE, the reaction proceeds easily to completion successively forming PDME and lecithin.

The fatty acid composition of the esters of acetone-precipitated surface active PDME isolated from adult rabbit alveolar wash could not have been predicted from the fatty acid composition of the total lecithin that was isolated. Approximately 50% of the PDME α -carbon esters was palmitic acid; 60% of the β -carbon esters was myristic acid ($C_{14:0}$) and only 10% was palmitic acid. This is evidence suggesting a significant content of surface active palmitoylmyristoyl PDME, or α -16:0/ β -14:0 fatty acid ester configuration. Since the PDME is methylated directly to lecithin, this identifies another specific surface active lecithin of known structure in addition to dipalmitoyl lecithin, palmitoylmyristoyl lecithin. There have been no known previous reports of specificity of lecithin fatty acids associated with the methylation reaction.

These findings suggest that the β -carbon palmitic or myristic acid can act as a marker to indicate the principal pathway of synthesis of the acetone-precipitated surface active lecithin, as shown in figure 6. Use of these markers in assessing the biosynthesis of surface active lecithin under normal and abnormal conditions in the human infant will be described in subsequent reports.

The differences between the fatty acids of PDME and PE (table IX) probably indicate that only a portion of the PE becomes methylated, and that this portion might have a possible specificity of PE structure necessary for methylation.

Summary

In the acetone-precipitated surface active lecithin from alveolar wash of the fetus during late gestation and in the young and adult rabbit, the most abundant identifiable compound is dipalmitoyl lecithin. An intermediate in the methylation pathway, phosphatidyl dimethylethanolamine, another surface active lecithin with an α -16:0/ β -14:0 fatty acid configuration was

identified. From *in vivo* studies it appears that by far the most important pathway for *de novo* synthesis of lecithin by the prematurely born or full-term breathing rabbit is by the incorporation of CDP-choline and D- α , β -diglyceride, which gives predominantly dipalmitoyl lecithin with an α -16:0/ β -16:0 fatty acid pattern.

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