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Surface tension
fetus, rabbit
phospholipids
lecithin
sphingomyelin
lung
phosphatidyl-
ethanolamine
phosphatidyl
dimethylethanolamine
phosphatidyl
methylethanolamine
phosphatidyl inositol
serine
cytosine diphosphate

The Biochemical Development of Surface Activity in Mammalian Lung

II. The Biosynthesis of Phospholipids in the Lung of the Developing Rabbit Fetus and Newborn

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Extract

In the lung of the rabbit fetus there was a rise in concentration of total lipids before term, the phospholipids constituting the major fraction. The concentrations of phosphatidylethanolamine and lecithin rose concurrently until day 28 when phosphatidylethanolamine concentration dropped, but lecithin continued to rise to term. From day 28 to term in the nonbreathing fetus there was an increase of 300 % in acetone-precipitated surface-active lecithin found almost entirely in the residual parenchyma after wash with little increase in this fraction in alveolar wash. After breathing for 1 hour there were increases in total alveolar lecithin 3–5 fold over nonbreathing fetal lung while increases in acetone-precipitated alveolar wash lecithin from nonbreathing to breathing lung were 20–30 fold. Enzymatic reactions were studied *in vitro* according to the pathways for the *de novo* synthesis of lecithin and phosphatidylethanolamine as follows:

1. CDP-(1,2-^{14}C) choline + D-α, β-diglyceride → lecithin
2. Phosphatidylethanolamine + 3($^{14}CH_3$)-S-adenosyl-L-methionine → lecithin
3. CDP-(1,2-^{14}C) ethanolamine + D-α, β-diglyceride → phosphatidylethanolamine
4. Phosphatidylethanolamine + (^{14}C)-L-serine → phosphatidyl serine $-CO_2\rightarrow$ phosphatidylethanolamine

CDP-choline incorporation declined steadily during gestation, although at term still showed rapid incorporation. The methylation reaction (2) showed peak incorporation on day 28 of gestation, at beginning viability. CDP-ethanolamine incorporation was the most active *in vitro* pathway studied peaking on days 25–26. Serine incorporation showed little activity, following a pattern of incorporation similar to that of CDP-ethanolamine. Activity of all pathways was found in microsomes. Methylation was also found in mitochondrial fraction of the term fetus and adult and in the cell-free soluble fraction from adult alveolar lavage. Reaction rates were similar from CDP-choline incorporation in both fetal and adult lung homogenate, but fetal lung incorporated methyl groups faster than adult lung. Intermediate compounds of methylation reaction were not found in alveolar wash of fetal lung, but were isolated from adult alveolar wash. Methylation in lung was pH sensitive, peak incorporation was seen at pH 7.8. Addition of ethanol or boiling one minute did not stop methylation. After breathing, those rabbit fetuses delivered by cesarean section after 28 full days of gestation synthesized 100 % of surface-active alveolar lecithin by one hour of breathing, 90 % of incorporation was with ^{3}H-choline, 10 % with ($^{14}CH_3$)-methionine. Much less incorporation into alveolar wash lecithin was seen with the breathing term fetus, but much more surface-active alveolar wash lecithin was isolated than from the fetus of 28 full days of gestation.

Speculation

The most important pathway in rabbit fetal development and in the newborn rabbit for the *de novo* synthesis of surface-active alveolar lecithin is the incorporation of CDP-choline. Although there is good correspondence between the concentrations of lecithin and phosphatidylethanolamine in fetal lung and the enzymatic activities studies *in vitro*, once breathing begins there is little correspondence between *in vivo* and *in vitro* biosynthesis of lecithin by fetuses of the same gestational age. In the rabbit fetus and newborn the methylation reaction appears to be of little significance in the biosynthesis of surface-active alveolar wash lecithin.

Introduction

The surface-active complex presumed to line pulmonary alveoli is rich in phospholipids, with lecithin by far its most abundant surface-active phospholipid. Numerous studies [9, 11, 18, 19, 24, 27, 31, 32, 34, 35] have shown that mammalian lung tissue synthesizes fatty acids and rapidly incorporates radioactive acetate and palmitate into lecithin and other phospholipids, but very little [2, 16] is reported on the *de novo* biosynthesis of lecithin in fetal or adult lung.

Surface activity of saline extract of minced normal mammalian lung first becomes demonstrable late in gestation in the fetus [1, 8, 15, 28, 29] and is characteristic thereafter of normal lung throughout the life of the organism. Previous studies [15] in developing rabbit fetuses described the presence of acetone-precipitated surface-active lecithin in lung parenchyma much earlier in gestation than its appearance in alveolar wash. Once breathing began there was a rapid increase in the proportion of surface-active lecithin to about 50 % of the total lecithin in alveolar wash. In a preliminary report of the *de novo* biosynthesis of phospholipids in mammalian fetal lung, GLUCK and SRIBNEY [16] found that the known principal pathways were active throughout the last third of gestation in the lung of the developing rabbit fetus.

In this report, the *de novo* biosynthesis of lecithin and phosphatidylethanolamine in the lung of the developing fetal rabbit and in the prematurely delivered and full-term breathing animal are described.

Materials and Methods

Rabbits from a pure strain of albino New Zealand whites whose times of mating were known within 8–12 hours were studied. Fetuses between 19 and 31 days of (term) gestation were delivered by cesarean section after electrocution of the doe as previously described [15]. For the various studies, a total of six to eight litters were studied for each day of gestation. In studies of the fetal state, the fetuses were sacrificed by decapitation prior to breathing [15].

Extraction, separation and quantification of phospholipids were done according to the method described by GLUCK *et al.* [14]. (Plasmalogens are not estimated by this procedure.) Extraction of lipids from lungs was done by high speed homogenization at 30,000 rpm in a Virtis '45' homogenizer in chloroform and methanol; separation into groups of lipids (neutral lipids, non-acidic phospholipids and acidic phospholipids) on microcolumns of N, N-diethylaminoethyl (DEAE) cellulose acetate; resolution with thin layer chromatography; and quantification with transmission densitometry. Isolation of individual compounds was done on preparative thin layers of Silica Gel H (Merck and Co., Darmstadt, Germany).

Alveolar lavage was done on intact fetal lungs with a total of 10 ml of 0.9 % NaCl solution in 5–2 ml washings, the lipids extracted and lecithin isolated all as described previously [15].

Separation of lecithin into acetone-soluble and acetone-precipitated fractions also was done as previously described [15], the surface-active lecithin when present separating out in the acetone-precipitated fraction.

For the studies of enzymatic activity, the lungs were dissected out quickly, right and left lungs from each litter divided randomly into two pools and 30 % homogenates of each pool made in ice cold 0.01 M Tris buffer, pH 7.2, with the Virtis '45' homogenizer at 20,000 rpm. Studies on each pool were done in quadruplicate.

The CDP-(1,2-^{14}C)-choline and CDP(1,2-^{14}C)-ethanolamine were synthesized and purified by the methods described by KENNEDY [21] using (1,2-^{14}C)-choline bromide (specific activity 5 mc/mmole) purchased from Nuclear Research Chemicals, Inc., Orlando, Florida, and (1,2-^{14}C)-ethanolamine (specific activity 1.5 mc/mmole) obtained from Volk Radiochemical Co., Skokie, Illinois. (3-^{14}C)-L-serine (specific activity 5 mc/mmole) was obtained from Nuclear-Chicago Corp., Des Plaines, Illinois. The ($^{14}CH_3$)-S-adenosyl-L-methionine (specific activity 46 mc/mmole) was purchased from Tracerlab, Brookline, Massachusetts, and ad-

justed to pH 6.0 with small additions of bicarbonate treated Dowex-2-chloride (Sigma Chemical Co., St. Louis, Missouri). Each batch was checked for purity prior to use by paper chromatography (95 % ethanol/ I N acetic acid/water, 65:1:34, v/v/v) for a single spot of radioactivity on scan of the strip. Nonradioactive S-adenosyl-L-methionine iodide was purchased from CalBiochem, Los Angeles, California.

Radioactivity measurements were made in a Packard Tri-Carb liquid scintillation spectrometer. Samples were evaporated to dryness and dissolved in 5 ml of scintillator solution containing 5 g of 2, 5-diphenyloxazole (PPO) and 0.5 g of 1,4- bis (2-[5-phenyloxazolyl]) benzene (POPOP) per liter of toluene.

For the simultaneous determination of ^{3}H and ^{14}C activity in experiments where both isotopes were used simultaneously, split channel counting was used with a high voltage setting of 6 (approximately 1200 volts). The window settings, calibrated with quenched standards, were: channel 1, 1–30, channel 2, 126–1000, giving 98 % of ^{3}H activity in channel 1 and 90 % of ^{14}C activity in channel 2. Corrections for quenching and for ^{14}C activity in channel 1 and ^{3}H activity in channel 2 were made by adding internal standards (^{3}H and ^{14}C toluene) to each sample. With these settings, the counting efficiencies were about 4 % for ^{3}H and about 28 % for ^{14}C.

Most incubations of reaction mixtures were stopped at 60 minutes by the addition of 3 ml of absolute ethanol. Special instances will be described with results. Extractions of lipids from reaction mixtures, except for special instances, were done according to the procedure described by KENNEDY [22] of repeated 3 ml extractions with absolute ethanol, addition of 10 ml of chloroform to the extract followed by 3 washings with 2 M KCl. The lipids dissolved in the ethanol-chloroform phase while the nonlipid impurities, including the radioactive intermediates, were removed in the KCl phase. Lipids were chromatographed on DEAE cellulose acetate microcolumns [14] and the desired phospholipids eluted from TLC plates.

Protein was determined by the biuret method [17] directly on the reaction mixture homogenate after extraction of lipids. The denatured protein was dissolved in a standard volume of 10 ml 0.2 N NaOH and 1 ml aliquot tested for biuret reaction.

The lung was fractionated into subcellular particles by mincing and homogenizing it in 3 volumes of 0.25 M sucrose in 0.01 M Tris buffer pH 7.4, with a glass homogenizer (Teflon pestle) of the Potter-Elvehjem type. Cellular debris and nuclei were removed by centrifugation at 2°, 800 g, for 15 minutes in a Sorvall Superspeed RC2B automatic refrigerated centrifuge and the mitochondria sedimented from the supernatant at 19,000 g for 20 minutes, resedimented at 8500 g and washed twice. Microsomes were sedimented from the mitochondrial supernatant at 100,000 g for 60 minutes at 2° in the Spinco Model L Ultracentrifuge and washed once. The supernatant from the microsomal preparation and the microsomal and mitochondrial fractions were studied, the latter two fractions resuspended with a Vortex spinner in isotonic sucrose solution in 0.01 M Tris buffer, pH 7.4, in a volume approximately one-fourth the homogenizing solution. Authenticity of the mitochondrial fraction was established by electron microscopy.

In the experiments in which radioactivity in phosphatidyl methylethanolamine, phosphatidyl dimethylethanolamine and lecithin choline were determined, lung homogenate was incubated with ($^{14}CH_3$)-S-adenosyl-L-methionine for 1 hour, the lipids extracted and chromatographed on DEAE cellulose acetate [14], and the three phospholipids separated together from the nonacidic phospholipid fraction by eluting their entire area from preparative thin layer plates. After addition of 3 mg each of nonradioactive choline, methylethanolamine and dimethylethanolamine, as suggested by BREMER and GREENBERG [5], the phospholipids were hydrolyzed by refluxing in 5 ml of 6N methanolic HCl for 3 hours, extracted [37], the hydrolyzed phospholipid bases placed on a 50×1 cm column of Dowex 50–8x and separated by elution with 1.5 N HCl, as described by PILGERAM *et al.* [30]. To each 5 ml fraction from an automatic fraction collector was added 1 g anthracene and 0.1 ml 3 % Tween 80, according to STEINBERG [33], and then it was assayed for ^{14}C activity in the Packard Tri-Carb liquid scintillation spectrometer.

Verification of the bases, methylethanolamine and dimethylethanolamine, was done both by paper chromatography, as described by BREMER and GREENBERG [5] and by gas-liquid chromatography after HONEGGER and HONEGGER [20] and MORGAN *et al.* [26] using a Perkin Elmer Model 881 gas chromatograph with flame-ionization detector on a 6 ft. $\times$ 0.25 in O.D. coiled column of Carbowax 6000 at column temperature 150°, and helium carrier gas at 30 psig.

For the *in vivo* studies of lecithin synthesis by the lung of the newly delivered premature and full-term rabbit fetuses from the onset of breathing, 3 litters of fetuses for each stage were delivered by cesarean section as previously described and each immediately given an intraperitoneal injection containing 1 μM each of ($^{14}CH_3$) methionine (L-methionine-$^{14}CH_3$, Calbiochem, Los Angeles, California) and ^{3}H-choline (choline-methyl-^{3}H-chloride, New England Nuclear, Boston, Massachusetts) precursors of the *de novo* synthesis of lecithin. Each radioactive compound was diluted in 0.9 % saline and sufficient nonradioactive compound added to provide approximately 400,000 CPM/μM. Equimolar

amounts of each solution were combined and diluted in 0.9 % saline so that 0.05 ml of resultant solution for injection contained 1 μM of each precursor.

The fetuses were sacrificed by decapitation [15] and the lecithin isolated from alveolar lavage and from residual lung after lavage as already described. The lecithin was separated into acetone-precipitated and acetone-soluble fractions. Radioactivities for each incorporation were measured by split channel counting with the liquid scintillation spectrometer as already mentioned. For comparison, livers from the fetuses whose lungs were examined also were homogenized, the lecithins extracted and the radioactivities measured.

Results

Levels of Lipid Groups in Lung with Fetal Development

The changes during fetal development in concentrations of total lipids, neutral lipids, nonacidic phospholipids and acidic phospholipids in rabbit lung are shown in table I and figure 1. Values in the table are given both for wet weight and for dry weight of lung because the changing water content of lung during maturation of the fetus makes difficult the interpretation of concentrations of lipid based only on wet weight.

There was a rise in the concentration of total lipids before term. The phosphorus-containing lipids were the major fraction of total lipids in the developing fetal lung and parallelled the changes in total lipids. The nonacidic phospholipid group, principally lecithin, sphingomyelin and phosphatidylethanolamine, contains the largest proportion of phospholipids. The acidic phospholipids include mainly phosphatidylserine and phosphatidyl inositol.

Concentrations of Phospholipids

The concentrations of individual phospholipids for both wet and dry weights of lung are shown in table II. The most striking changes occurred during late fetal development in concentrations of phosphatidylethanolamine and lecithin, shown in figure 2. Between the 22nd and 28th day of gestation, the concentrations of these two phospholipids rose sharply. From the 28th day the phosphatidylethanolamine began to decrease and reached low levels at term while the lecithin concentration continued to rise. The changes seen on the 28th day of gestation coincided with the appearance of normal surface activity of saline extract of minced normal lung (with lowest tension on compression of the surface of the modified Wilhelmy balance less than 15 dyn/cm [15]. Sphingomyelin rose to a peak concentration on the 28th day and levelled off. Phosphatidyl dimethylethanolamine, not found in measureable concentrations in fetal lung, was found in adult rabbit lung,

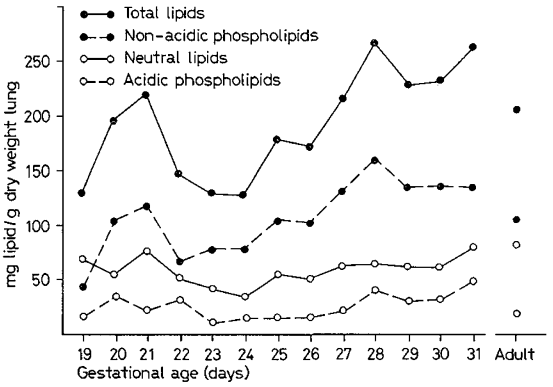

Fig. 1. Changes in concentrations (per unit dry weight) of total lipids and groups of lipids in the lung of the developing rabbit fetus.

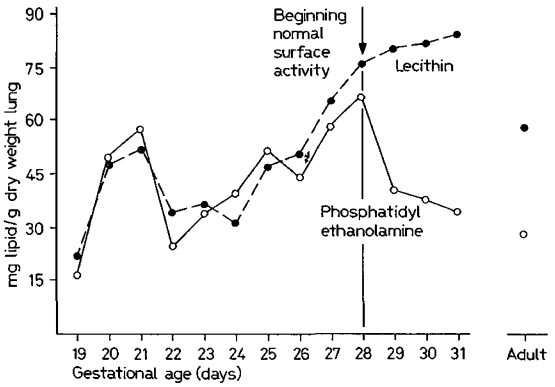

Fig. 2. Concentrations per unit dry weight of phosphatidylethanolamine and lecithin in the lung of the developing rabbit fetus. In the figure, 'beginning normal surface activity' refers to the findings previously described [15] with saline extract of minced lung on the modified Wilhelmy balance. At 28 days of gestation, the lowest tension on compression of the surface was 13.5 dyn/cm (less than 15 dyn/cm considered 'normal' [15] for lung extract).

comprising about 4 % of the nonacidic phospholipid fraction.

Concentrations of Acetone-Soluble and Acetone-Precipitated Lecithin

As previously reported [15], surface activity of lecithin isolated from lung (with lowest tension on compression of the surface of the modified Wilhelmy balance of 0–5 dyn/cm) was found in that fraction precipitated in acetone while the acetone-soluble lecithin

Table I. Concentrations of groups of lipids in lungs of fetal and adult rabbits[1]

Day of gestation of fetus	Total No. of fetuses	mg lipid/g of dry weight lung				mg lipid/g of wet weight lung			
		Total lipids	Neutral lipids[2]	Nonacidic phospho-lipids	Acidic phospho-lipids	Total lipids	Neutral lipids	Nonacidic phospho-lipids	Acidic phospho-lipids
19	34	133.1± 2.8	71.5± 4.2	44.0± 0.8	17.6±2.1	13.2	7.1	4.4	1.7
20	39	196.2± 3.9	56.7± 5.1	103.9± 2.4	35.5±3.4	19.2	5.5	10.2	3.5
21	46	220.0± 3.6	77.9± 2.2	118.8± 3.1	23.3±2.9	21.5	7.6	11.6	2.3
22	33	148.9± 4.2	52.8± 2.8	66.0± 3.4	30.1±1.6	17.2	6.1	7.6	3.5
23	31	131.0± 5.0	42.7± 2.6	77.6± 3.9	10.7±0.5	16.4	5.3	9.8	1.3
24	27	129.1± 2.9	34.9± 1.9	78.5± 3.9	15.7±0.3	15.3	4.1	9.3	1.9
25	28	178.5± 3.7	55.9± 1.6	105.8± 2.8	16.8±1.0	20.9	6.5	12.4	2.0
26	30	173.3± 4.2	53.6± 2.9	103.2± 5.1	16.5±0.9	20.8	6.4	12.4	2.0
27	29	217.2± 6.0	62.5± 5.1	131.0± 4.0	23.7±3.2	25.6	7.4	15.4	2.8
28	36	266.7±13.1	65.0±12.1	160.0± 8.8	41.7±5.8	26.9	6.6	16.1	4.2
29	38	226.8± 4.8	62.4± 5.4	133.9± 6.8	30.5±4.2	26.5	7.5	15.5	3.5
30	22	232.0±11.0	62.0± 5.0	139.5±10.2	33.5±3.8	25.8	7.0	16.1	2.7
31	23	263.5±15.2	80.1± 6.2	138.0±12.1	49.4±5.1	30.8	9.4	15.7	5.7
Adult	6	207.0±21.7	83.2±13.2	104.5± 6.8	19.3±1.3	42.8	17.1	21.7	4.0

[1] Concentrations per g dry weight are shown as means and standard deviations; values per g wet weight are means only.

[2] Includes total of cholesterol, cholesterol esters, free fatty acids, mono-, di- and triglycerides.

Table II. Concentrations of the major phospholipids in lungs of fetal and adult rabbits[1]

Day of gestation of fetus	Total No. of fetuses	mg phospholipid/g *dry weight* lung					mg phospholipid/g *wet weight* lung				
		Nonacidic phospholipids			Acidic phospholipids		Nonacidic phospholipids			Acidic phospho-lipids	
		S	L	PE	PS	PI	S	L	PE	PS	PI
19	34	4.9±0.5	22.5±3.0	16.6±3.8	3.5±1.4	14.1±5.0	0.5	2.3	1.7	0.3	1.4
20	39	6.5±1.6	47.8±7.4	49.6±0.3	11.8±2.9	23.7±3.6	1.4	4.9	3.9	1.2	2.3
21	46	8.5±2.0	52.4±5.8	57.9±9.2	7.8±1.6	15.5±1.2	1.5	5.4	4.8	0.8	1.5
22	33	7.0±0.4	34.4±4.6	24.6±6.1	6.0±0.3	24.1±5.7	1.6	4.2	1.9	0.7	2.8
23	31	6.4±1.3	37.3±5.2	33.9±8.0	5.4±0.8	5.4±1.9	0.9	5.1	3.8	0.7	0.7
24	27	7.7±0.6	31.6±9.1	39.2±7.6	7.9±1.8	7.8±2.1	1.0	3.8	4.5	0.9	0.9
25	28	7.2±2.4	47.2±3.6	51.4±5.2	10.1±2.5	6.8±0.9	1.4	5.4	5.7	1.2	0.8
26	30	8.5±1.5	50.8±1.8	43.9±2.8	11.6±3.8	4.9±0.7	1.8	5.7	4.9	1.4	0.6
27	29	9.2±1.3	65.5±3.2	56.3±2.1	18.9±2.7	4.7±1.2	1.7	7.6	6.1	2.2	0.6
28	36	17.2±3.4	76.3±4.3	66.5±5.0	33.4±4.2	8.3±5.9	2.4	7.4	6.3	3.4	0.8
29	38	13.9±2.1	80.2±0.8	39.8±1.3	21.4±9.4	9.2±7.8	2.7	8.2	4.6	2.4	1.1
30	22	19.8±5.9	82.3±3.5	37.4±0.9	10.1±5.2	13.4±3.8	2.8	8.5	4.8	1.5	1.2
31	23	19.6±3.8	84.2±1.9	34.2±4.7	22.5±1.2	16.0±5.6	2.0	9.2	4.6	3.0	2.2
Adult[2]	6	18.4±2.2	58.0±1.1	28.1±10.2	10.0±2.4	8.9±4.2	4.3	10.9	6.6	2.0	1.9

[1] Concentration per g dry weight are shown as means and standard deviations; values per g wet weight are means only. S = sphingomyelin; L = lecithin; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidyl inositol.

[2] About 4 % (4.9 mg/g dry weight lung) of the nonacidic phospholipids in adult rabbit lung was phosphatidyl dimethylethanolamine, not shown in the table.

fraction was not surface active. Figure 3 shows the concentrations of acetone-soluble and acetone-precipitated lecithin per gram dry weight lung in lung homogenate during development. The rise on day 28 in total lecithin, shown in figure 2, occurred in the acetone-soluble fraction. The increase in concentration of the acetone-precipitated fraction to adult levels occurred on day 29, when surface-active lecithin also was first found in alveolar wash [15]. The highest concentration of acetone-precipitated lecithin in lung was seen at term, day 31, higher than the concentrations in adult rabbit lung.

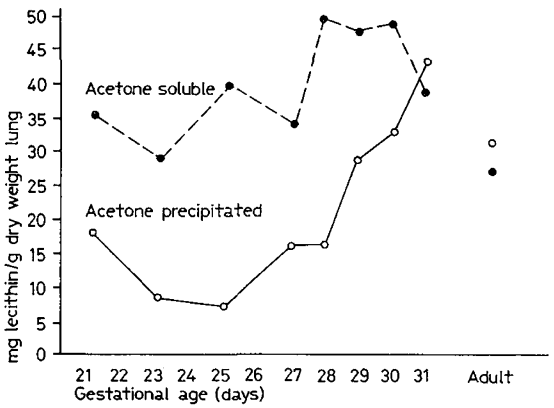

Fig. 3. Concentrations during gestation of acetone-soluble and acetone-precipitated lecithin in whole lung per g dry weight lungs. Of particular significance is the sharp rise between day 28 and term in the acetone-precipitated surface-active fraction of lecithin.

Concentrations of Lecithin in Alveolar Wash and in Lung Parenchyma after Wash

Quantitative measurements late in gestation of lecithin in alveolar wash and in residual lung parenchyma after wash are shown in table III. The marked increase in the nonbreathing fetus in acetone-precipitated lecithin in lung homogenate (fig. 3) was found almost entirely in the residual parenchyma after wash with little increase in this fraction in alveolar wash. After breathing for 1 hour, those stages examined showed increases in total alveolar lecithin of 3–5 fold over nonbreathing fetal lung, while the increases in acetone-precipitated alveolar wash lecithin from nonbreathing to breathing lung were 20–30 fold.

Scheme of Biosynthetic Pathways Studied

Enzymatic reactions were studied *in vitro* according to the pathways for the *de novo* synthesis of lecithin and phosphatidylethanolamine [3, 4, 6, 7, 13, 23, 37, 38] outlined in the scheme below in figure 4. The radioactive precursors are underlined.

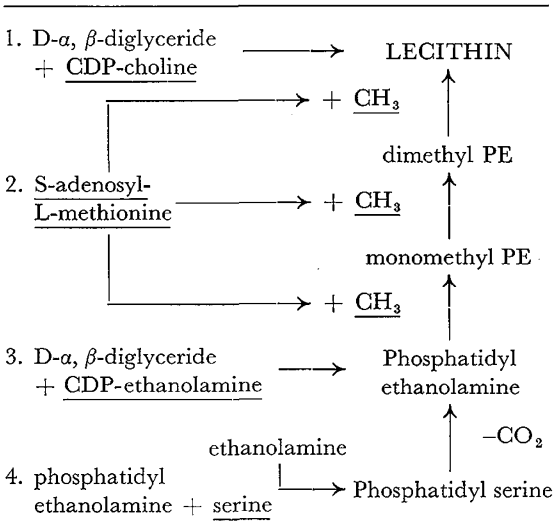

Fig. 4. The known pathways for the *de novo* biosynthesis of lecithin investigated in the present report. The underscored compounds were those labelled with ^{14}C.

Incorporation of CDP-choline into Lecithin

Figure 5 shows the incorporation of CDP-(1,2, ^{14}C)-choline into lung lecithin during development of the rabbit fetus. A high rate of incorporation of this precursor into lecithin was seen in the 21-day fetus, declining steadily to an activity at term approximately 30 % of that seen on day 21. Despite this relative decline, radioactive CDP-choline at term still showed rapid incorporation.

Incorporation of CDP-ethanolamine into Phosphatidyl-Ethanolamine

The incorporation of CDP-(1,2, ^{14}C)-ethanolamine into phosphatidylethanolamine was the most active of the *in vitro* pathways studied. A peak incorporation of CDP-ethanolamine was found on days 25 and 26, approximately twice the peak incorporation of CDP-choline. In contrast to the pattern seen with CDP-choline, CDP ethanolamine incorporation was relatively low in the 21- to 24-day fetus, although active at this time, and then jumped precipitously to peak incorporation on days 25 and 26; dropped abruptly on day 28 to lower levels and demonstrated subsequently, from day 29 through term, low levels of incorporation, as seen in figure 6. Incubated radioactive CDP-ethanolamine also was recovered as radioactive lecithin but no attempt was made to quantify this incorporation both because of the short (1 hour) incubation time and

Table III. Comparison of lecithin concentrations in alveolar wash and in lung parenchyma after wash; concentrations of lecithin in alveolar wash after breathing 1 h

Day of gestation	Number of fetuses	Non-breathed fetal lung			Lung after breathing 1 h	
		Total acetone-precipitated lecithin in residual lung parenchyma after alveolar wash	Total lecithin in alveolar wash	Total acetone-precipitated lecithin in alveolar wash	Total lecithin in alveolar wash	Total acetone-precipitated lecithin in alveolar wash
		mg/g dry weight lung	mg/g dry weight lung	mg/g dry weight lung	mg/g dry weight lung[1]	mg/g dry weight lung
27	29	15	1.5	0.1		
28	36	15	1.5	0.2		
Early 29	33	17	2	0.2	6	2.4
Late 29	29	24	3	0.3	9	5.6
30	22	30	4	0.4		
31	24	40	4	0.35	19	9.8

[1] In the adult lung, the concentration of total lecithin in alveolar wash is 6 mg/g dry weight lung. The extraordinarily high concentrations of alveolar wash lecithin in the term rabbit fetus, breathing for 1 h, decrease and by 6 h of age are about 50 % of the values in the table.

Table IV. Synthesis of phospholipids by subcellular fractions

	Counts/min/mg protein								
	23 day			31 day			Adult		
	Precursors			Precursors			Precursors		
	CDP-chol	CDP-eth	S-ad-L-meth	CDP-chol	CDP-eth	S-ad-L-meth	CDP-chol	CDP-eth	S-ad-L-meth
Mitochondria	0	0	19	14	9	168	78	44	303
Microsomes	960	450	149	384	301	594	464	238	770
Supernatant after microsomal sedimentation	111	29	62	58	22	103	86	19	94
Alveolar wash supernatant	0	0	0	0	0	8	10	4	216

Reaction mixtures and extractions were identical to those described in figures 4, 5 and 7, substituting subcellular fraction for lung homogenate. Specific activities of precursors were CDP-1,2^{14}C-choline, 48,000 cpm/μM; CDP-1,2^{14}C-ethanolamine, 25,000 cpm/μM; ($^{14}CH_3$)-S-adenosyl-L-methionine, 200,000 cpm/μM. The values shown represent the average of 3 determinations. Alveolar wash supernatant was obtained from endobronchial lavage with 0.9 % saline of intact lung. For each fetal lung, a total of 2 ml of saline were used for lavage with 5 gentle to-and-fro washing; 0.5 ml of wash fluid was used per incubation mixture. Adult lung washings were done with a total of 10 ml saline for each lung also washed to-and-fro. Each wash fluid was centrifuged at 1000 g for 20 min at 0–2° and 0.5 ml of cell-free supernatant added to reaction mixtures.

because the methylation of phosphatidylethanolamine was studied independently.

Incorporation of (3-^{14}C)-L-serine into Phosphatidyl-Ethanolamine

The incorporation of (3-^{14}C)-L-serine into radioactive phosphatidylethanolamine is shown in figure 7. This pathway showed relatively little activity compared to the incorporation of CDP-ethanolamine. The pattern of incorporation during gestation of the rabbit fetus was similar to that for CDP-ethanolamine, peak incorporation being seen on day 26. Similar also to incorporation of CDP-ethanolamine, a small amount of radioactivity was found in lecithin on incubating lung homogenate with radioactive L-serine but quantification of incorporation into lecithin was not done.

Incorporation of S-adenosyl-L-methionine into Lecithin

The incorporation of ($^{14}CH_3$) S-adenosyl-L-methionine into lecithin is shown in figure 8. The peak occurred on day 28 of gestation, although only fractionally as active as the peak incorporations of CDP-choline and CDP-ethanolamine. Some important characteristics of this pathway for the conversion of phosphatidylethanolamine to lecithin by three stepwise methylations will be described in later paragraphs.

Combined Timetables of the Various Pathways Studied

Figure 9 is a recapitulation of mean incorporations of radioactive precursors in the pathways studied during gestation, drawn to the scale of the incorporation of CDP-ethanolamine as indicated, to show more easily their temporal relationships. For reference, the concentrations per gram dry weight lung of lecithin and phosphatidyl ethanolamine during gestation also are shown.

Conversion of Precursors to Phospholipids by Fractionated Lung Homogenates

In table IV are shown the results of experiments in which lecithin and phosphatidylethanolamine synthesis were studied in cell fractions. Consistently, most of the activity was found in the microsomal fractions of the different gestational ages examined. The incorporation of radioactive S-adenosyl-L-methionine in the

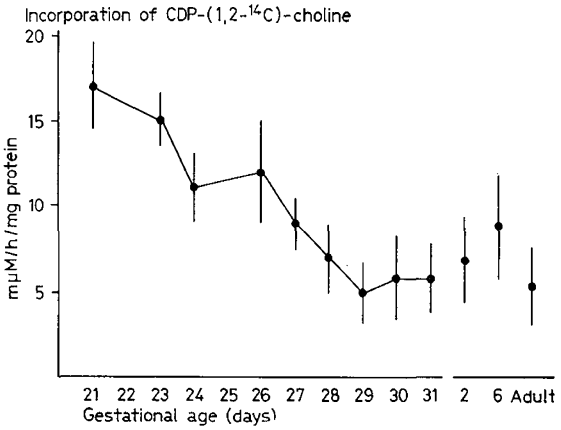

Fig. 5. Incorporation of CDP-(1,2-^{14}C) choline into lecithin. Each tube contained 50 μM of Tris buffer, pH 8.0, 10 μM $MgCl_2$, 5 μM reduced glutathione, 1 μM CDP-(1,2-^{14}C)-choline (specific activity 48,000 cpm) and 0.25 ml 30 % homogenate of lung tissue (in 0.01 M Tris, pH 7.2), in a final volume of 0.55 ml. The tubes were incubated in a metabolic shaker at 37° C for 1 hour after which the reaction was stopped with 3 ml absolute ethanol in each tube. The total lipid fraction was extracted essentially as described by KENNEDY *et al.* [20]. Lecithin and other individual phospholipids were isolated from thin layer plates after initial separation on DEAE cellulose acetate micro-columns as described by GLUCK *et al.* [13]. As explained under Methods, lungs from fetuses of a litter (average 9 fetuses) were pooled and determinations done in quadruplicate. Each point on the figure represents the mean and standard error for 28–46 fetuses from 3–5 litters. A total of 399 fetuses were studied. The numbers 2 and 6 preceding Adult refer to days after birth in this and subsequent figures.

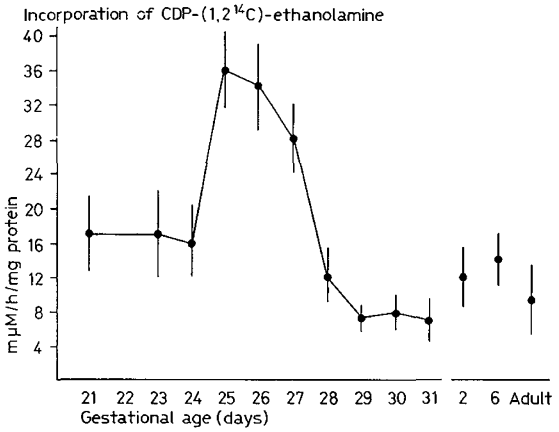

Fig. 6. Incorporation of CDP-(1,2-^{14}C) ethanolamine into phosphatidylethanolamine. Conditions, procedures, reaction mixtures and total numbers of fetuses studied were identical to those for the incorporation of CDP-choline as described in figure 5 except for the substitution of 1 μM CDP-(1,2-^{14}C) ethanolamine (specific activity 25,000 cpm).

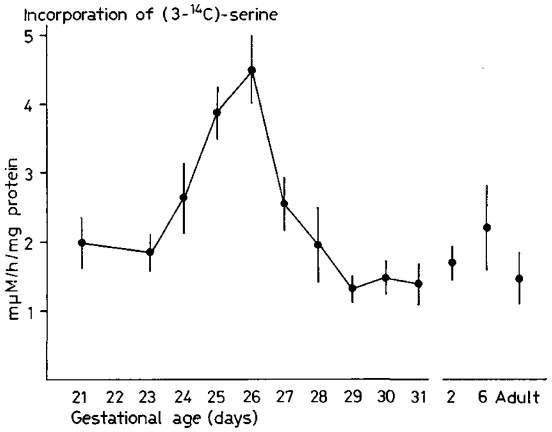

Fig. 7. Incorporation of (3-^{14}C)-L-serine into phosphatidylethanolamine. Each tube contained 25 μM Tris buffer pH 7.4, 1.5 μM $CaCl_2$, 1 μM (3-^{14}C-L-serine (specific activity 78,000 cpm) and 0.25 ml 30 % lung homogenate (in 0.01 M Tris, pH 7.2), in a final volume of 0.65 ml. After incubation at 37° with shaking for 1 h the reaction was stopped with addition of 5.0 ml of 0.3 M trichloracetic acid and the lipids extracted as described in figure 5. All other procedures were identical to those described in figure 5.

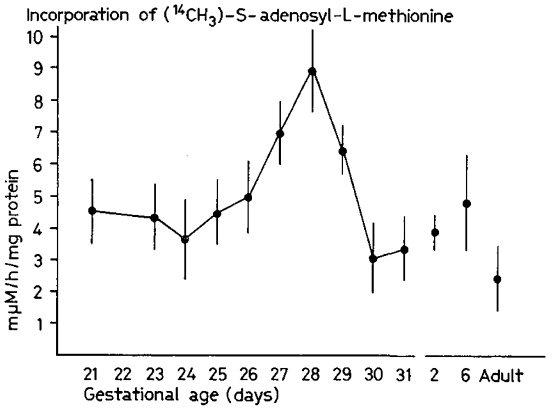

Fig. 8. Incorporation of (^{14}C-CH$_3$)-S-adenosyl-L-methionine into lecithin. Reaction mixtures were identical to those described in figure 5 except for substitution of 1 μM (^{14}C-CH$_3$)-S-adenosyl-L-methionine (specific activity 200,000 cpm) and adjustment of the reaction mixture to pH 7.4 prior to incubation at 37° for 1 h. Three different procedures were compared throughout for stopping the reaction and extracting the lipids: adding 5 ml 5 % TCA and boiling 1 min, followed by the extraction procedure described by KENNEDY *et al.* [20], rapid extraction by the same procedure immediately after adding 3 ml absolute ethanol; and by addition of concentrated HCl and n-butanol as described by BREMER and GREENBERG [31]. The numbers of fetuses and isolation procedures for lecithin are described in figure 5. Duplicate determinations were done with each of the three procedures with close correspondence of values. Thus, each point represents 18–30 determinations.

young fetus was associated with the microsomal fraction, but in the term fetus and adult, some incorporation also was found in the mitochondrial fraction and, in the adult, in the cell-free (soluble) fraction obtained from alveolar lavage of lung.

Comparison of Reaction Rates, Adult vs. Fetus

Comparison of the time curves of incorporation of CDP choline in homogenate of 28-day fetal lung with that of adult lung homogenate in figure 10 shows almost identical incorporations for the two stages.

The rates of incorporation of radioactive methyl groups into lecithin are shown in figure 11, comparing homogenate of adult lung with that from 28-day fetus. As can be seen, the reaction was complete in 15 minutes in 28-day fetal lung but incorporation continued in the adult at one hour.

Radioactivity in Intermediate Phospholipid Bases

Although the intermediate methylated phosphatidyl ethanolamine compounds were not measureably isolated from fetal lung, radioactivity of their bases was recoverable after hydrolysis and elution from a Dowex 50 column as explained under methods and shown in figure 13. Radioactivity of adult lung showed a similar pattern but with much greater incorporation of radioactivity into bases. As described by BREMER and GREENBERG [6], the rate-limiting step in this reaction is the conversion of phosphatidylethanolamine to phosphatidyl methylethanolamine.

Effect of pH on Methylation

As shown by BREMER and GREENBERG [7], S-adenosyl-L-methionine incorporation by rat liver microsomes increased as the pH was increased to a maximum at about pH 10.5. Figure 14 shows the effect of pH on methyl group incorporation by fetal lung and liver. With homogenate of 28-day fetal lung, at pH values of 6.8 and 7.0, virtually no incorporation occurred and peak incorporation was seen at pH 7.8, although the unexplained variability of results beyond pH 8.0 limited the studies as shown. Incorporation of radioactive methyl groups increased with rising pH for 28-day fetal liver homogenate beyond pH 8.0 (to about pH 10).

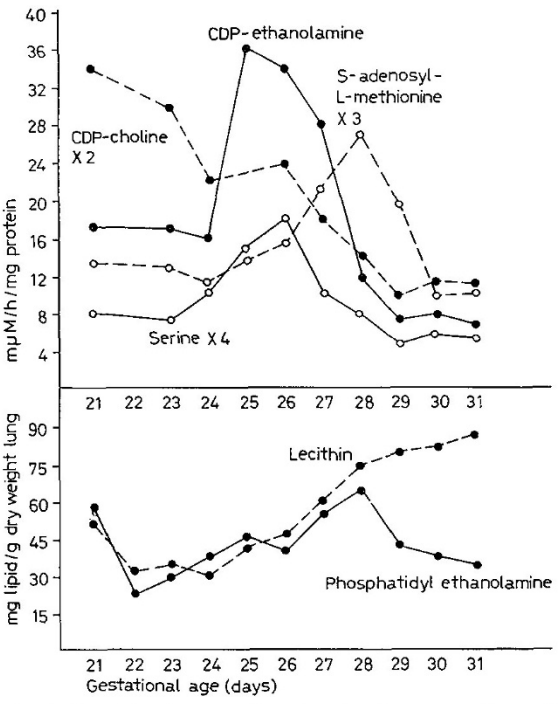

Fig. 9. Combined timetables showing incorporations of precursors into phospholipids during gestation drawn to the same scale as the incorporation of radioactive CDP-ethanolamine. Levels of lecithin and phosphatidylethanolamine during gestation are shown for reference.

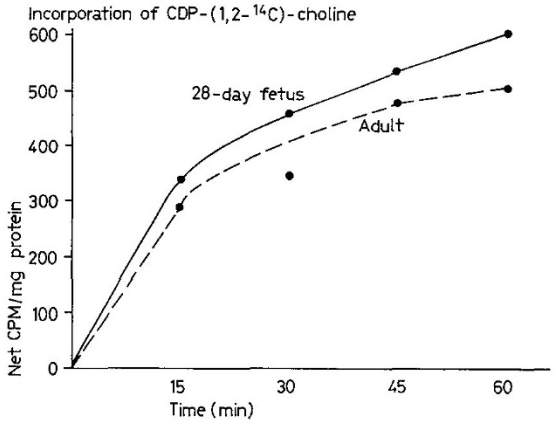

Fig. 10. Comparison of the effect of time on incorporation of CDP-(1,2-^{14}C) choline into lecithin showed no difference between 28-day rabbit fetus and adult rabbit. Incubation conditions were identical to those in figure 5. 1 μM of CDP-(1,2-^{14}C) choline with a specific activity of 48,000 cpm/μM was added to each reaction mixture. Results are averages from 4 litter male fetuses and 3 female adult rabbits, done in triplicate.

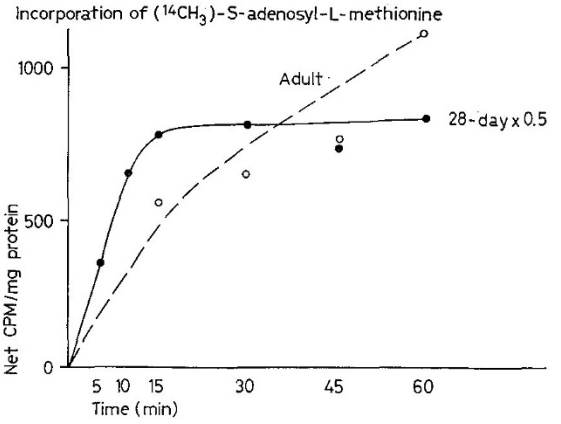

Fig. 11. Comparison between late fetus and adult of the effect of time on incorporation of ($^{14}CH_3$)-S-adenosyl-L-methionine showed that the reaction was complete in 15 min in lung of the 28-day fetus while incorporation by homogenate of adult rabbit lung was much slower. 1 μM of precursor with a specific activity of 200,000 cpm was added to each reaction mixture. All conditions were as described in figure 7. After 1 h incubation, 3 ml absolute alcohol was added, followed by rapid extraction of lipids. Results are averages from 4 litter mate fetuses and 3 female adult rabbits, done in triplicate.

Rabbit. Nonacidic phospholipids from alveolar wash

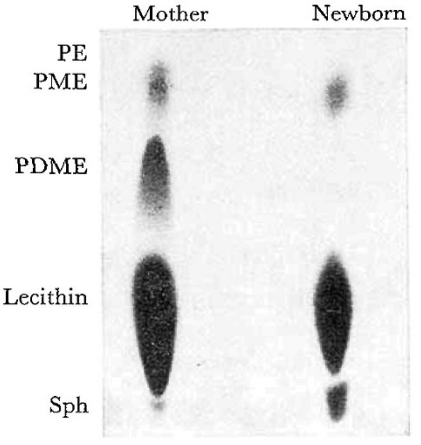

Fig. 12. Portion of thin layer chromatogram (Silica Gel H, $CHCl_3/CH_3OH/H_2O$, 65/25/4, v/v) charred after 50 % H_2SO_4 spray, showing the nonacidic phospholipid fraction recovered from alveolar wash of the lung of a pregnant rabbit and her term fetus. Phosphatidyl methylethanolamine and phosphatidyl dimethylethanolamine were found in the alveolar washings of the adult rabbits but not in those of their fetuses.

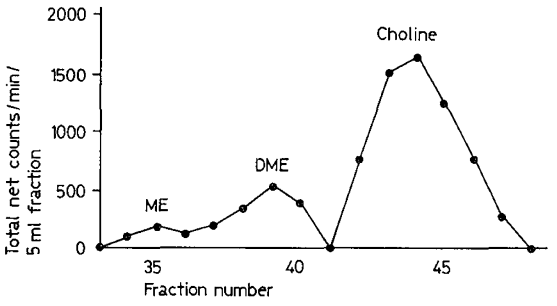

Fig. 13. Radioactivity distributed among the phospholipid bases extracted from a reaction mixture for the incorporation of ($^{14}CH_3$)-S-adenosyl-L-methionine (conditions as described in figure 8), hydrolyzed and separated on a column Dowex-50 as described under Methods. The choline fraction contained 78% of the recovered radioactivity, dimethylethanolamine (DME) 17% and methylethanolamine (ME) 5%. To the reaction mixture was added 1μM of ($^{14}CH_3$)-S-adenosyl-L-methionine with a specific activity of 1,000,000 cpm.

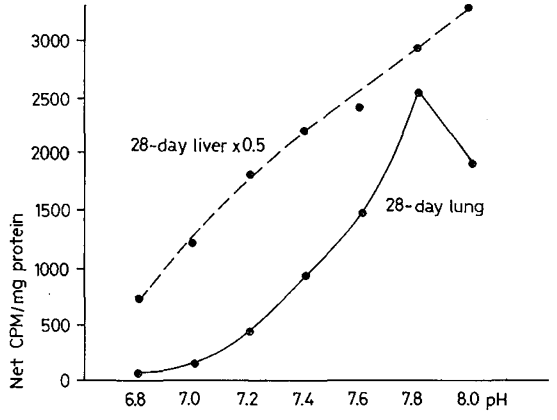

Fig. 14. Effect of pH on the incorporation of ($^{14}CH_3$)-S-adenosyl-L-methionine in 28-day fetal lung and liver. All conditions were identical to those described in figure 7. 1 μM of precursor with a specific activity of 200,000 cpm/μM was added to the reaction mixtures. The pH of reaction mixtures was measured at the end of incubation. Shifts of pH in excess of 0.02 were considered invalid and incubation was repeated with a new mixture.

Comparison of Resistance to Alcohol and Boiling of Liver and Lung Methylating Systems

Important differences between methylating systems in lung and liver included the differences in resistance to alcohol and heat shown in table V. Incubation for one hour of the reaction mixture to which was added 3 ml of absolute ethanol produced good incorporation with lung homogenate in contrast to liver homogenate where no incorporation of radioactive methyl groups occurred. Tissue homogenate boiled for one minute added to the rest of the reaction mixture enhanced incorporation of methyl groups by lung homogenate but there was complete inhibition of incorporation of methyl groups by liver homogenate boiled for 1 minute. Lung homogenate boiled for 15 minutes and added to the reaction mixture showed no incorporation. To assure that the incorporation of methyl groups into phosphatidylethanolamine was not a spontaneously occurring reaction, phosphatidylethanolamine, isolated and purified from lungs of 10-day-old rabbits, was incubated with radioactive S-adenosyl-L-methionine. After one hour, no incorporation of methyl groups had occurred. Neither lung nor liver homogenate showed incorporation of methyl groups when 5% TCA was added to the reaction mixture and boiled one minute.

Lecithin Synthesis in vivo, after the Onset of Breathing

In table VI are shown the average amounts of the fractions of lecithin isolated per lung of the breathing animals studied. As gestation increased, greater amounts of lecithin were recovered although the wet weights of lung were fairly similar.

The incorporation into lecithin of ($^{14}CH_3$)-L-methionine and ^{3}H-choline simultaneously injected into the peritoneum during delivery by cesarean section were compared among full-term and prematurely delivered rabbit fetuses after their onset of breathing and are shown in table VII. The gestational ages of the fetuses included those of full 27 days of gestation (early 28 day), full 28 days of gestation (early 29 day), mature or late 29-day and 31-day (term) fetuses. Radioactivity measurements were made of the acetone-soluble and acetone-precipitated lecithin fractions isolated from alveolar wash and from the residual lung parenchyma after alveolar wash. The average micromols of precursor radioactivity in lecithin from each fraction recovered per lung are shown. These incorporations also are expressed as micromols of radioactive precursor incorporated per hour per micromol of isolated lecithin (using 750 μg/μM) $\times$ 100, which is an indication of the percentage of *de novo* synthesis per hour of each particular lecithin fraction isolated.

This study implied that the lecithin once formed was then not degraded during the period of study—an assumption that is not warranted since there is lecithin turnover. There was also loss of lecithin as a portion of the alveolar layer appeared continuously to be lost and replenished with breathing. This was suggested from suctioning by needle puncture of the tracheas of some breathing animals which yielded radioactive lecithin and a pattern of phospholipids with fatty acid compo-

sition identical to those of alveolar wash. Oropharyngeal aspirations yielded similar findings. Presumably these secretions normally are swallowed by the breathing organism.

In all stages and fractions studied, there was much greater labelling of lecithin with tritiated choline than with ^{14}C-methionine (up to 16 times as much). The total incorporation into lecithin of tritium, adding all lecithin fractions, showed no differences among the fetal stages. Approximately 30 % (0.3 μM) of the radioactivity of the 1 μM of ^{3}H-choline injected was equally recovered after one hour of breathing from the total lung lecithin isolated from each stage, early 29-day gestation, late 29-day gestation and 31-day gestation. Of the total alveolar wash lecithin, that isolated from the early 29-day breathing fetus showed the greatest incorporation of $_{3}$H-choline; the acetone-precipitated fraction was the most heavily labelled.

Although total incorporation of radioactive precursors into lung lecithin was equal for all stages tested, the highest percentages of incorporation, representing *relatively* the most rapid *de novo* biosynthesis were found in the youngest fetuses to survive, those delivered after 28 full days of gestation (early 29th day) who had clinical respiratory distress and recovered. Both ^{3}H-choline and ($^{14}CH_3$)-L-methionine were incorporated rapidly. Of the acetone-precipitated alveolar wash lecithin isolated, 90 % was labelled with ^{3}H-choline. Incorporation of radioactive methyl groups from ($^{14}CH_3$)-methionine also were most active in the early 29th day fetus, with a 10 % incorporation into the acetone-precipitated alveolar wash lecithin. In contrast to the ^{3}H-choline there was little incorporation of ($^{14}CH_3$)-L-methionine into the lecithin in the parenchyma after alveolar wash; although, like the radioactive choline incorporation into the parenchyma, the greatest amount was into the acetone-soluble fraction.

Table V. Comparison of *in vitro* incorporations of ($^{14}CH_3$)-S-adenosyl-L-methionine into lecithin by homogenates of *lung* and *liver* of 10-day-old rabbit under special conditions of incubation

Condition of incubation	Net cpm in lecithin/mg homogenate protein after 1 h incubation	
	Lung	Liver
Normal incubation	980	8800
3 ml absolute ethanol added to reaction mixture	1120	38
Boil homogenate 1 min before adding to reaction mixture	1340	7
Boil homogenate 15 min before adding to reacting mixture	74	6
Reaction mixture (with homogenate) + 3 ml absolute ethanol + boil 1 min	338	13
Reaction mixture without homogenate + ($^{14}CH_3$)-S-ad-L-methionine	0	0
Reaction mixture without homogenate + purified PE + ($^{14}CH_3$)-S-ad-L-methionine	0	0
Reaction mixture (with homogenate) + 5 ml 5 % TCA + boil 1 min	11	0

Reaction mixtures were identical to those described in figure 8. Specific activity of ($^{14}CH_3$)-S-adenosyl-L-methionine added to mixtures was 200,000 cpm/μM. Figures represent averages of 3 duplicate determinations.

Table VI. Lecithin recovered per lung after one hour of breathing from rabbit fetuses delivered by cesarean section

Age of fetus (days of gestation)	No. of litters	No. of fetuses	μgm lecithin recovered per lung[1]			
			Alveolar wash		Lung after wash	
			Acetone-precipitated	Acetone-soluble	Acetone-precipitated	Acetone-soluble
28 day[2]	3	27	20± 9	60± 7	90±14	167±37
Early 29 day	4	33	48± 4	67± 9	122±26	150±51
Late 29 day	3	29	160±12	180±29	380±31	680±69
31 day	3	24	225±14	250± 4	775±83	450±26

[1] Values given are means±standard deviations for determinations which were done on the pooled fetuses in each litter.

[2] All 28-day fetuses died between 18 and 30 min of life. Of these, 8 died at about 20 min; 15 at 18 min; 4 at 30 min.

Total incorporation into alveolar wash lecithin in the term (31 day) fetus after breathing was only about 40 % of that of the early 29-day fetus for both precursors. However the *percent* incorporation by the 31-day fetal lung into the total alveolar wash lecithin isolated was but a small fraction of that of the early 29-day fetus. The highest rates of incorporation seen in the 31-day rabbit were into the acetone-soluble fraction of the parenchyma.

Generally, percent incorporations by the mature 29-day fetus were less than those for the early 29-day organism and more than those for the 31-day fetus.

Those fetuses that were barely 28 days survived less than 30 minutes, the majority expiring by 18 minutes. All the lungs were pooled, regardless of time of death and the averages given were calculated like the others as incorporation per hour. No appreciable incorporation occurred of ($^{14}CH_3$)-L-methionine either into alveolar wash or parenchyma lecithin. Incorporations of ^{3}H-choline for the 20-minute period were almost nonexistent.

Lecithin from the livers of the breathing animals also was isolated and measured for incorporation of radioactive precursors, as shown in table VIII. In all stages

Table VII. In vivo incorporations after 1 h of breathing into lung lecithin of ^{3}H-choline and $^{14}CH_3$-methionine simultaneously injected into rabbit fetuses of different gestational ages at cesarean section

Gestational age and source of lecithin		Average μM lecithin isolated per lung	Average μM ^{3}H-choline incorporated per lung	Average μM ^{14}C-methionine incorporated per lung	Average % incorporation per lung of precursor into lecithin	
					μM ^{3}H-choline incorporated	μM ^{14}C-methionine incorporated
					μM lecithin isolated $\times$ 100	μM lecithin isolated $\times$ 100
28 days of gestation						
Alveolar wash	Acetone ppt.	0.027	0.001		5	
	Acetone sol.	0.080	0.003		4.5	
Residual lung	Acetone ppt.	0.120	0.001		1	
after wash	Acetone sol.	0.223	0.002		1	
	Totals	0.450	0.007	4.5×10^{-4}	1.6	0.1
Early 29 days of gestation						
Alveolar wash	Acetone ppt.	0.060	0.054	0.006	90	10
	Acetone sol.	0.090	0.034	0.004	38	4
Residual lung	Acetone ppt.	0.160	0.041	0.002	25	1.5
after wash	Acetone sol.	0.200	0.160	0.010	80	5
	Totals	0.510	0.289	0.022	57	4.3
Late 29 days of gestation						
Alveolar wash	Acetone ppt.	0.210	0.032	0.005	15	2.5
	Acetone sol.	0.240	0.024	0.004	10	2
Residual lung	Acetone ppt.	0.510	0.096	0.007	19	1.5
after wash	Acetone sol.	0.910	0.145	0.009	16	1
	Totals	1.870	0.297	0.025	16	1.3
31 days of gestation						
Alveolar wash	Acetone ppt.	0.300	0.015	0.001	5	0.5
	Acetone sol.	0.330	0.020	0.004	6	1.5
Residual lung	Acetone ppt.	1.030	0.155	0.010	15	1
after wash	Acetone sol.	0.600	0.132	0.012	22	2
	Totals	2.260	0.322	0.027	14	1.2

3 litters of fetuses (24–33 fetuses) were sudied for each day of gestation.

Table VIII. In vivo incorporation of simultaneously injected ^{3}H-choline and $^{14}CH_3$-methionine into *liver lecithin* by fetuses delivered by cesarean section after 1 h of breathing

	Average μM incorporated/liver of radioactive precursor into total liver lecithin	
	^{3}H-choline	$^{14}CH_3$-methionine
28 day[1]	0.295	0.380
Early 29 day	0.380	0.495
Late 29 day	0.417	0.520
31 day	0.405	0.590

Each incorporation is an average of determinations on 4 livers.

[1] See table VI for description of survival times of 28-day fetuses.

($^{14}CH_3$)-L-methionine was incorporated more rapidly into liver lecithin than was ^{3}H-choline at 1 hour.

Sex Differences in Incorporation of Methyl Groups

BJORNSTAD and BREMER [2] reported that only about half as much radioactivity from methionine is incorporated *in vivo* into liver lecithin in adult male rats as in adult female rats. The same proportions of incorporation into liver lecithin were found in the present study in adult rabbits. Similarly, male adult rabbits showed only about 60 % of the incorporation of ($^{14}CH_3$)-L-methionine into total lecithin of lung as did female adult rabbits. Too few animals were examined to report this finding with statistical significance. In term fetal rabbits comparison of pooled lungs from 6 males with those from 5 females showed no differences in incorporation of methyl groups.

Discussion

Data presented in this report show that the same pathways known in adult animals (principally in liver, but also in other organs) for the *de novo* synthesis of the phospholipids, lecithin and phosphatidylethanolamine, are active in the lung of the developing rabbit fetus. Studied *in vitro*, the rates of incorporation of radioactive precursors by these pathways showed marked variations in their patterns during gestation which generally correlated well with the measured concentrations of lecithin and phosphatidylethanolamine in fetal lung.

The concentration of lipids in lung rose as gestation progressed. The principal increase was found in the largest fraction of lung lipid, the phospholipids. Of the phospholipids, lecithin was present in the greatest concentration, which by term had risen to 50 % of the total phospholipids. The surface-active phospholipids, sphingomyelin, lecithin and phosphatidyl inositol [15] together totaled 70 % of the phospholipids present in fetal lung at term. Phosphatidyl dimethylethanolamine (PDME), identified in adult dog lung and found to be surface active by MORGAN *et al.* [26] was not found in rabbit fetal lung except by radioactivity of its hydrolyzed base. It was never found in alveolar wash of fetal lung. PDME was first recognizably recoverable during the first week of life and thereafter in the rabbit including in alveolar wash, and comprising about 4 % of the nonacidic phospholipids in adult rabbit lung (see table II).

Of the pathways studied *in vitro*, the incorporation of CDP-(1,2-^{14}C)-ethanolamine was the most active during fetal development with peak activity during gestational days 25 and 26. The other pathway for the synthesis of phosphatidylethanolamine, the interchange incorporation and decarboxylation of serine appeared to be only of minor significance in fetal lung for the biosynthesis of phosphatidylethanolamine. The incorporation of CDP-(1,2-^{14}C)-choline overall was the more active *in vitro* fetal pathway for the synthesis of lecithin, although the methylation reaction showed a significant peak of activity around days 28–29, when viability in the rabbit fetus begins.

A good temporal correlation was found between the *in vitro* incorporations of radioactive precursors and the changes in concentrations of lecithin and phosphatidylethanolamine during the period of gestation studied. This was particularly interesting since the concentrations measured represent end points at a given moment of fetal *in vivo* synthesis and might not necessarily have shown correlations with *in vitro* enzymatic measurements. In this regard, the marked drop in concentration of phosphatidyl ethanolamine seen after day 28 of gestation is not wholly explained by the combination of decreased incorporation of precursors plus increased activity of the methylation system. No other studies were done to determine the entire fate of phosphatidylethanolamine, since these would entail *in vivo* incorporation and turnover studies in the intact fetus. However, with this exception, the other changes found can be accounted for by the data obtained on the pathways studied and seem to establish clearly that these are the pathways for the *de novo* biosynthesis of lecithin and phosphatidylethanolamine in fetal rabbit lung.

Studies only of the *de novo* biosynthesis, as described here, give no evaluation of other routes by which lecithin is known to be produced, including exchange reaction between free choline and preformed lecithin [10], and acylation of lysolecithin (from hydrolyzed

lecithin) by acyl CoA [25], neither pathway representing *de novo* biosynthesis. Furthermore, the present study was not concerned with fatty acid incorporation and turnover into preexisting lecithin in the developing fetus, although this will be described in a later report.

Studies of the localization of incorporation activity in subcellular particles of lung showed that both the fetus and the adult incorporated labelled CDP-choline and CDP-ethanolamine into phospholipid principally in the microsomes, as reported by others in liver [36]. Incorporation of ($^{14}CH_3$)-S-adenosyl-L-methionine, reported in liver as a microsomal enzyme reaction [4, 6, 7], showed localization in microsomes in early fetuses studied. However, in the term fetus and in the adult, incorporation was found also in the 'mitochondrial fraction' and additionally in the cell-free supernatant from alveolar wash of the adult rabbit. Unfortunately, the lung is not a mitochondrial-rich tissue and contamination with microsomes could not be ruled out absolutely, although there was electron microscopic confirmation of 'good mitochondrial preparations'. Furthermore, it is possible that there was contamination of both the 'mitochondrial fraction' and the alveolar wash supernatant with lamellar inclusion body [15] content and/or membrane, since the inclusion body appears to sediment in the same centrifugal field as does the mitochondrion. In any event, there is good evidence that the term fetus and adult show incorporation of radioactive methyl groups into phosphatidylethanolamine in the synthesis of lecithin in lung both in microsomes and extramicrosomally.

The effect of time on incorporation of precursors into lecithin was studied with CDP-choline and S-adenosyl-L-methionine, comparing the 28-day fetus with the adult. No difference was noted in reaction rate between the two with CDP-choline. However, a slower rate of incorporation for adult lung tissue was found with S-adenosyl-L-methionine. The methylation reaction was complete by 15 minutes in the fetus while incorporation continued during the hour's study time in the adult tissue. These results might also be due to a difference in availability of endogenous substrate, a possibility which was not examined. Assuming a valid difference in reaction rate, this might account for the lack of measureable phosphatidyl methyl- and dimethylethanolamine in lung (and alveolar wash) of fetal lung except by radioactivity of the hydrolyzed bases. Both of these intermediates readily were recovered from alveolar wash of adult lung.

In a previous report [15] it was shown that surface-active lecithin, if present, can be separated from total lecithin by precipitation with acetone. The acetone-soluble fraction is not surface active. Although this is undoubtedly an artificial separation by metabolic standards, it nevertheless is a valid and most useful functional means of studying that portion of lecithin which actually is surface active. As was reported [15], a surface tension as low as 0 dyn/cm on compression of the surface of the modified Wilhelmy balance is produced by as little of this fraction as 20–25 μg, identical to the 20 μg minimum to form a surface by a synthetic dipalmitoyl lecithin standard studied by FUJIWARA, ADAMS and SCUDDER [12].

It was shown with this method [15] that acetone-precipitated surface-active lecithin was recovered from lung parenchyma of rabbit fetuses considerably before day 29, when it appeared in the alveolar wash, although saline extract of minced lung showed surface activity on the 28th day. Figure 2, the concentrations of *total* lecithin in lung during gestation, shows that the slope of rise of lecithin concentration was sharpest on the 28th day. However, this represented increased concentration of the acetone-soluble fraction, as shown in figure 3, while a sharp increase in concentration of the acetone-precipitated fraction was seen from day 29. This supports the probability that around day 28 a change occurs in intracellular binding of lecithin that permits it to be extracted by stirring minced lung in saline, although it is not recovered by alveolar lavage. Previously [15] also it was shown that in the nonbreathing lung at term only 11 % of the total lecithin in alveolar wash was surface-active acetone precipitated. However, as shown in figure 3, lung homogenate at term contained over 50 % acetone-precipitated lecithin, further evidence for the intracellular storage of surface-active lecithin. The quantitative measurements showed rapidly increasing concentrations of acetone-precipitated (surface-active) lecithin in lung homogenate from day 29 to term; between days 28 and 31, there was a 300 % increase.

The quantitative measurements comparing lecithin in alveolar wash with lecithin in residual lung parenchyma after wash showed this marked increase in concentration to be in the parenchyma after wash, with relatively little increase in alveolar wash lecithin in the nonbreathing fetus. Increases of 3–5 fold in total alveolar lecithin were found in breathing lung after 1 hour, as compared to nonbreathing fetal lung. However, 20–30 fold increases were found in the acetone-precipitated alveolar wash lecithin of the breathing lung compared to the nonbreathing fetal lung. Together, these results are best interpreted as indicative of an intracellular storage of acetone-precipitated surface-active lecithin prior to its appearance in alveoli and its discharge into alveoli following the onset of breathing.

As was described in this report and previously [15], the onset of breathing brought about abrupt changes in the distribution of lecithin and its *de novo* synthesis. In order to understand these changes, it was necessary

to have some assessment of the *in vivo* contribution after breathing of each of the two pathways to the formation of surface-active lecithin in the alveolar wash. The rate of *de novo* synthesis of lecithin in lung was thus measured by the incorporation of ($^{14}CH_3$)-L-methionine and ^{3}H-choline simultaneously injected at birth.

The hazards of this approach, the extrapolations and assumptions in comparing *in vivo* with *in vitro* synthesis are well known. The incorporation of a labelled component into lecithin at a particular rate by a tissue homogenate may be unrelated to the rate of formation of lecithin *in vivo*. The incubation conditions are not comparable, since *in vitro* studies with homogenates are associated with destruction of cell membranes, artificially high concentrations of substrate, artificial intermixing of enzymes by disruption of their normal intracellular locations, uncertainty about essential co-factors and their concentrations, and the presentation to the enzymes under study of substrate in a questionably suitable form.

The intact organism presents another formidable problem, particularly when comparing two precursors, that of the body metabolic pools. This is true of the two labelled precursors in the present study, choline and methionine, which enter different metabolic pools. However, in their studies of the *in vivo* biosynthesis of lecithin in the adult rat, BJORNSTAD and BREMER [2] have shown that these precursors do enter the pathways under consideration (as outlined in fig. 4) and that the lecithins synthesized by the two pathways enter a common metabolic pool. Their studies showed also that rapid equilibration of liver and plasma lecithin takes place, with no significant difference between lecithin synthesized by the methylating pathway and lecithin synthesized from free choline. Injecting the radioactive precursors intraperitoneally, liver lecithin choline was labelled maximally with the methyl group from methionine at 1 hour, while maximal radioactive choline incorporation into liver lecithin occurred at about 2 hours. They further showed that after subcutaneous injection of the precursors there was no difference from intraperitoneal injection with radioactive methionine, while incorporation of radioactive choline was halved in the liver but trebled in extrahepatic tissue.

Accordingly, the intraperitoneal route of injection was chosen to minimize any incorporation advantage into extrahepatic lecithin of choline and after 1 hour, equilibration in body metabolic pools would have been sufficient that assessment of the contribution of each pathway from precursor incorporation to the synthesis of lecithin would have reasonable validity.

It was hoped by delivering progressively more premature rabbit fetuses by cesarean section that a gestational time would be found when after breathing, all or nearly all of the surface active alveolar lecithin would be synthesized *de novo*. It was theorized that this might coincide with that time in the fetus—between the 28th and 29th days of gestation—when surface-active lecithin is just beginning to appear in the alveolus and when the intracellular concentration of acetone-precipitated lecithin begins to increase markedly. This study seemed important in order to get some comparison of this *de novo in vivo* synthesis after breathing, with the *in vitro* synthesis at the same gestational age in fetal lung as well as to identify whether one pathway was clearly predominant in producing the lecithin necessary for alveolar stability with the onset of breathing in the premature animal. It was thus established that those rabbit fetuses delivered by cesarean section after a carefully timed gestation of 28 full days (early 29 days) survived following a period of respiratory distress and synthesized *de novo* 100 % of their surface-active acetone-precipitated alveolar lecithin in one hour.

Incorporation of radioactive choline into lecithin in the early 29-day rabbit fetuses studied accounted for 90 % of the synthesis while only 10 % of the lecithin label was derived from radioactive-L-methionine. These fetuses increased the *proportion* of acetone-precipitated lecithin in their alveolar wash only slowly as compared to the full-term breathing fetus, but the percentage of incorporation of radioactivity in their surface-active alveolar wash lecithin was 20 times that of the breathing term fetus. However, the total amount of surface-active lecithin recovered from alveolar wash was only 20 % that of the term fetus after 1 hour.

The mature, term fetus when delivered by cesarean section breathed easily and in one hour the percentage of acetone-precipitated surface-active lecithin in his alveolar wash increased from about 11 % in the fetal state to about 50 %. In his rate of *de novo* synthesis he showed a relatively slow incorporation in the acetone-precipitated alveolar wash lecithin with both precursors and active incorporation into the lecithin of the residual parenchyma after alveolar wash. As in the early 29-day fetus, about 90% of the incorporation was with radioactive choline. A large amount of surface-active lecithin was recovered from the alveolar wash after 1 hour's breathing.

The average total amount (μM) of radioactivity incorporated per lung was approximately equal among the fetuses studied of early 29 days of gestation, late 29 days of gestation and 31 days of gestation. However, the total micromoles of radioactivity incorporated in the lecithins of alveolar wash was significantly greater in the early 29-day fetus than in the full-term fetus. The greatest total incorporations occurred, in all stages, in the residual lung parenchyma after wash. This fraction would be expected to contain an unknown amount of

blood. No studies were done to define either the metabolic pool of lecithin in the fetus nor to measure liver/plasma equilibration of radioactivity in the fetal rabbit. The data on incorporation into liver lecithin in the present report, considerably higher than that found in the adult rat by BJORNSTAD and BREMER [2], accentuates the need for such studies. Therefore, the data on incorporation by the residual lung parenchyma must be considered an indication of incorporation and must remain partly in doubt until the other studies are done. However, the alveolar lecithin is blood-free and data on incorporation into this fraction are felt to be valid.

As shown in these studies, there was not good correspondence between the *in vitro* enzymatic activity of fetal lung homogenate and the *in vivo* biosynthesis in the breathing fetus of the same gestational age. In addition to problems of *in vitro* versus *in vivo* synthesis discussed above, this suggests that perhaps regulation of fetal enzymatic time tables in some measure may be related to anaerobic metabolism, and with breathing and the onset of aerobic metabolism, different demands and different expressions of enzyme activity are seen, presumably governed in part by aerobic mechanisms. In particular, this seemed true for the methylation reaction.

Special attention in this laboratory was focused on the methylation pathway because the peak of its *in vitro* incorporating activity (28 days) coincided with beginning viability of the rabbit fetus and the onset of normal surface activity of minced lung extract. It seemed even more important to characterize this pathway after findings of MORGAN *et al.* [26] of surface activity of the intermediate, PDME, later confirmed by us [15].

However, as has been discussed, PDME could not be recovered from alveolar wash of the fetal or newborn rabbit but was first found during the first 6 days of age. PDME was identified in homogenates of lung only by the radioactivity of its hydrolyzed base. Furthermore, from *in vivo* studies of synthesis of surface-active alveolar lecithin, the methylating system appeared to have relatively little importance as compared to the incorporation of CDP-choline either in the breathing prematurely delivered or full-term rabbit newborn.

Although the more rapid rate *in vitro* of incorporation of methyl groups by fetal rabbit lung than by adult rabbit lung accounts in part for failure to recover the intermediate compound in the fetus, the principal reason might be that the activity of the methylating enzyme system associated with the alveolar surface is incompletely developed at the time of birth in the rabbit and does not 'mature' until after birth. This is suggested also by the difference in localization and kinetics between the fetal and adult methylating systems. The function of the methylation system in the fetus is possibly in an intracellular synthesis capacity where it might play a role in the storage of intracellular lecithin.

Differences between methylation systems of liver and lung were shown, including resistance of the lung tissue system to addition of alcohol, to brief (1 minute) boiling, and the apparent more circumscribed sensitivity of the lung system to pH. This sensitivity to pH has significant potential clinical implications in the human.

Although the pathways for the *de novo* biosynthesis of lecithin and many basic biochemical, structural and physiological processes seen in the rabbit appear to be shared by the human, the known differences are so profound as to limit suggestions of correspondence between the two species in the biochemical development of the lung. There is extreme telescoping of fetal development in the short gestation of the rabbit which makes it impossible to assess equivalent gestational age in the human. Even more important, however, are the differences in developmental timetables and activities of the biosynthetic pathways between rabbit and human. As will be presented in a subsequent report, in the human there is evidence that the small premature infant for a period after birth synthesizes surface-active alveolar lecithin principally by the methylation pathway while in the rabbit, as presented, the principal pathway of synthesis is by the incorporation of CDP-choline and D-α, β-diglyceride.

Future work requires study of the synthesis and turnover of fatty acids in lecithin during gestation, definition of the structure and the character of the enzyme system responsible for the methylation of phosphatidylethanolamine, and most important, knowledge of the regulatory mechanisms that determine the developmental timetables and interrelationships of the various enzyme systems. Lecithin synthesis in the developing fetal rabbit lung provides an excellent model for the study of biochemical development of the fetus.

Summary

The development of surface activity in the lung of the rabbit fetus was characterized by intracellular storage of surface-active lecithin during the latter third of gestation. During the last tenth of gestation, surface-active lecithin appeared in the alveolar wash during the time of a marked increase in concentration of intracellular surface-active lecithin.

The principal pathways for the *de novo* biosynthesis of lecithin in fetal lung were identified and are (1) the incorporation of CDP-choline + D-α, β-diglyceride, and (2) the triple methylation of phosphatidylethanolamine. The principal pathway for the *de novo* synthesis of phosphatidylethanolamine was found to be the in-

corporation of CDP-ethanolamine + D-α,β-diglyceride, and was the most active of the fetal pathways studied.

During fetal development there was a progressive decline in activity of CDP-choline incorporation. The methylation reaction had peak incorporation coinciding with viability of the rabbit fetus. However, with the onset of breathing, 90 % of the *de novo* synthesis of surface-active alveolar wash lecithin was by incorporation of choline.

The methylation reaction appeared to be of significance in the alveolar layer metabolism of the rabbit after some days of life and thereafter when the surface-active intermediate, phosphatidyl dimethylethanolamine, was recoverable from alveolar wash. Methylation enzymatic activity was present in cell-free alveolar wash of adult rabbit.

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