Biosynthesis of phosphatidyl choline during prenatal development of the rat lung

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ABSTRACT The biosynthesis of phosphatidyl choline in rat lung during prenatal development is of interest in connection with the mechanisms of functional development of the lung, particularly in relation to the production of lung surfactant and the control of phospholipid synthesis. The process was studied by determination of the ability of fetal and adult lung slices to incorporate choline-methyl-^{14}C and methionine-methyl-^{14}C into phosphatidyl choline.

The incorporation of radioactivity from methionine-methyl-^{14}C was low in both adult and fetal lung. There was no apparent change in the incorporation during development. The incorporation of choline-methyl-^{14}C into phosphatidyl choline after 60 min of incubation was low for fetal lung at 19 days gestation and increased to adult levels by 22 days gestation.

The incorporation of choline-methyl-^{14}C into phosphoryl choline was high at 19 days gestation and decreased as development progressed. However, the specific activities of the phosphoryl choline were similar in fetal and adult lung, which suggests that the increase in incorporation of choline-methyl-^{14}C into phosphatidyl choline with development results from an increased activity of reactions in the biosynthetic pathway between phosporyl choline and phosphatidyl choline.

KEY WORDS phosphatidyl choline · phosphoryl choline · biosynthesis · rat · lung · fetal development · choline-methyl-^{14}C · methionine-methyl-^{14}C

STUDIES OF THE BIOSYNTHESIS of phospholipids during differentiation and functional development can be useful both for the understanding of the process of differentiation and development and for the information that can be obtained about the control of phospholipid metabolism and its integration with cellular function. The morphology of cellular membranes is known to change during differentiation and development (1). Since phospholipids are a characteristic component of membranes, alterations in phospholipid synthesis may also occur. A detailed study of these alterations should contribute to the understanding of the control of phospholipid synthesis.

Previous studies have shown that phospholipid metabolism does change during functional development of the lung (2). The amount of phospholipid in the lungs of fetal rats increases from 50% of the adult levels at 19–20 days gestation to nearly adult levels by 22 days gestation. The incorporation of ^{32}P into phospholipid by lung slices increases fivefold between 20 and 22 days gestation. Most of the increase in concentration of phospholipid and in the incorporation of ^{32}P resulted from such increases in the choline glycerophosphatides, whose increased biosynthesis may be related to an increased production of surfactant (3), a lipoprotein which contains phosphatidyl choline and apparently functions by lowering the surface tensions within the alveoli (4, 5). The appearance of surfactant activity during development of the rat (3) coincides with the increased biosynthesis of choline glycerophosphatides (2).

Two pathways exist for the biosynthesis of phosphatidyl choline: the formation from choline via cytidine diphosphocholine (6) and the formation by the stepwise methylation of phosphatidyl ethanolamine (7, 8). The cytidine diphosphocholine pathway probably accounts for most of the biosynthesis of phosphatidyl choline in lung slices, since the methylation of phosphatidyl ethanolamine by rat lung has been reported to be low (9, 10). However, Morgan, Finley, and Fialkow (11) have suggested that dipalmitoyl lecithin, thought to be the principal pulmonary surface-active agent, is formed in dog lung by the methylation of phosphatidyl ethanolamine. I have investigated the incorporation of choline-methyl-^{14}C and methionine-methyl-^{14}C into phosphatidyl choline by slices of fetal and adult lung. The results reported in this paper confirm the low activity of the methylation pathway in rat lung, and indicate that the develop-

mental changes in the biosynthesis of phosphatidyl choline involve the cytidine diphosphocholine pathway.

MATERIALS AND METHODS

Pregnant rats of specified delivery dates (±12 hr) were purchased from Charles River Laboratories, Wilmington, Mass. and from Holtzman Company, Madison, Wis. Animals from the two sources gave similar results. Choline-methyl-^{14}C (5.2 mc/mmole) and methionine-methyl-^{14}C (14.75 mc/mmole) were obtained from the New England Nuclear Corp., Boston, Mass. They each gave a single spot when examined by paper chromatography in ethanol–concd NH_4OH (95:5 v/v) and in phenol–water (70:30 w/v). Inulin-carboxyl-^{14}C (3.04 mc/g) and sucrose-^{14}C (337 mc/mmole) were also obtained from the New England Nuclear Corp.

Lungs from the fetal rats and from their mothers were removed and sliced as previously described (2). Slices (200–250 mg) were incubated in a Dubnoff shaker in 2 ml of Krebs bicarbonate medium, pH 7.4 (12), containing 2 mg/ml glucose and 1 μc/ml of choline-methyl-^{14}C (1.9×10^{-4} M) or methionine-methyl-^{14}C (1×10^{-3} M) in an atmosphere of 95% O_2–5% CO_2.

The slices were removed from the incubation medium, rinsed once with 2.0 ml of cold isotonic saline, and extracted by a modification of the procedure of Folch, Lees, and Sloane Stanley (13). The tissue was homogenized in 2.0 ml of methanol and 4.0 ml of chloroform was added. After 1 hr at room temperature, water (1.3 ml) was added and the mixture was vigorously shaken for 1 min. The mixture was separated into three layers by centrifugation: a top aqueous layer, a tissue disc at the interphase, and a bottom chloroform-containing layer. The top and bottom layers were removed and the tissue was extracted again in a similar way. The chloroform extracts were combined and washed with 5.0 ml of "theoretical" upper phase (chloroform–methanol–water 3:48:47). The chloroform was removed by evaporation with a stream of nitrogen and the lipid residue was dissolved in 5.0 ml of chloroform. The aqueous phases and the upper phase washings were combined for the analysis of water-soluble products. The amount of radioactivity in the solutions was determined in a liquid scintillation spectrometer using a standard toluene scintillation fluid for lipid and Bray's solution for the water (14). The counting efficiency was determined by two methods, namely channels ratio and external standard ratio.

Thin-layer chromatography of the lipids was performed by means of the modified systems of Skipski, Peterson, and Barclay (15) as previously reported (2).

The water-soluble metabolic products of choline-methyl-^{14}C were separated by two-dimensional paper chromatography on Whatman No. 3 MM previously washed with 0.5 N acetic acid and water. The papers were developed in the first direction with ethanol-concd NH_4OH–water 61:29:10 and in the second direction with phenol saturated with 0.12% NH_4OH. Choline had an R_f of 0.93 in the first direction and 1.0 in the second direction. Phosphoryl choline had an R_f of 0.29 in the first direction and 0.92 in the second direction. These systems separated choline and its oxidation products from phosphoryl choline and also separated phosphoryl choline from other phosphorus-containing compounds. The radioactive compounds were located by radioautography using Ansco high-speed X-ray film. The areas on the paper corresponding to the exposed areas on the film were cut out and put into counting vials for the determination of radioactivity. After the radioactivity had been determined, the scintillation fluid was removed and the paper was rinsed once with toluene. The dried paper was digested with 70% perchloric acid and total phosphorus was estimated by the method of Bartlett (16) as modified by Galliard, Mitchell, and Hawthorne (17). The scintillation fluid did not remove any radioactivity from the paper.

The inulin and sucrose spaces were determined by incubation of the slices in a Krebs bicarbonate medium that contained 1 μc/ml of either inulin-carboxyl-^{14}C or sucrose-^{14}C. After the desired incubation, the slices were separated from the medium by centrifugation. The tissue was dissolved in 2.0 ml of "NCS",[1] and the total radioactivity was determined in Bray's scintillation fluid. The radioactivity in the incubation medium was also determined with Bray's scintillation fluid. We determined the total water content of the tissue by drying the tissue for 12–16 hr at 110°C.

Tissue potassium was extracted from the slices with 0.75 N nitric acid. Potassium was measured in a Technicon AutoAnalyzer by the method described in the AutoAnalyzer handbook.

RESULTS AND DISCUSSION

Several experiments were performed to assess the integrity of the tissue slice preparations since in the subsequent experiments differences between the in vitro stability of adult and fetal tissues could influence the results. The extracellular space was determined for fetal and adult lung slices after 30- and 120-min incubations with inulin-carboxyl-^{14}C. The determinations at the two times gave the same results (Table 1). There were differences between the adult lung and fetal lung, particularly in the inulin space, but the inulin space did not change from 19 days gestation to 22 days gestation. The tissue potassium

[1] A toluene solution of a quaternary ammonium hydroxide sold by Nuclear-Chicago Corporation, Des Plaines, Ill.

TABLE 1 THE INULIN AND SUCROSE SPACE OF ADULT AND FETAL LUNG SLICES

Incubation Time	Adult	Fetal (Days)		
		19	21	22
min		*% total water content*		
Inulin space				
30	53 ± 5*	34	42	36
		35	44	36
120	51 ± 3	37	32	37
		39	37	34
Sucrose space				
30	55			49
	53			50
120	52			49
	53			43

* SEM for three separate experiments.

concentrations of adult and fetal lung slices before and after incubation are shown in Table 2. In adult slices there is no appreciable change in potassium after a 30 min incubation and a slight decrease after a 120 min incubation. The potassium concentration in fetal slices is increased after 30 min of incubation and remains at this level for 120 min. The slice preparations from both adult and fetal lung appear to be stable for at least 120 min as judged by the above criteria.

We incubated slices of lung from adult and fetal rats for 60 min with choline-methyl-^{14}C to determine the developmental change in the biosynthesis of phosphatidyl choline by the cytidine diphosphocholine pathway. The ability of lung slices to incorporate radioactivity into total lipid was decidedly lower for 19 day old fetal rats than for the mothers (Table 3). The amount of incorporation increased at 20 days gestation and reached adult levels just prior to birth (22 days gestation). Experiments on nonpregnant female rats gave results similar to those reported for the mothers.

The lipid from the above experiments was separated by thin-layer chromatography. Each spot on the thin-layer chromatogram was scraped into a counting vial and the amount of radioactivity in it was determined. 96–98% of the total radioactivity was found in the area of the chromatogram corresponding to the phosphatidyl choline. The total lipid also was hydrolyzed by the method of Dawson (18) as modified by Tarlov and Kennedy (19). This treatment transformed 98% of the total lipid radioactivity to water-soluble radioactivity. Therefore, very little, if any, radioactivity was located in either the fatty acid portion of the lipid or choline plasmalogens. Two-dimensional paper chromatography of the water-soluble hydrolysis products indicated that all of the radioactivity was present as glycerophosphoryl choline. In all subsequent experiments that used choline-methyl-^{14}C, the radioactivity of the total phospholipids was taken as a measure of the incorporation into phosphatidyl choline.

TABLE 2 POTASSIUM CONCENTRATION OF LUNG SLICES BEFORE AND AFTER INCUBATION

Incubation Time	Adult	22 Day Fetal
min	*meq/kg tissue*	
0	35.9	29.8
	34.6	31.2
30	35.0	38.6
	33.5	36.7
120	29.8	37.5
	31.8	37.9

TABLE 3 INCORPORATION OF CHOLINE-METHYL-^{14}C AND METHIONINE-METHYL-^{14}C INTO LIPID BY FETAL AND ADULT LUNG SLICES

Age	Choline Incorporation	Methionine Incorporation
	mμmoles/g wet tissue	
Adult	185 ± 12 (16)*	4.92 ± 0.39 (9)
Fetal		
19†	29.6 (2)	6.30
20	64.6 ± 3.9 (6)	4.09
21	77.6 (2)	5.20 (2)
22	189 ± 14 (7)	4.36

* Average ± SEM with the total number of experiments in parentheses. Each experiment involved duplicate samples incubated for 60 min. "Adult" experiments used a single animal while "fetal" experiments used the combined lungs from two litters. The amount incorporated was calculated by dividing the radioactivity incorporated per g of tissue by the specific activity of the precursor added to the medium.

† Days gestation.

The formation of phosphatidyl choline by the methylation of phosphatidyl ethanolamine, assessed by measurement of the ability of lung slices to incorporate radioactivity from methionine-methyl-^{14}C into total lipid, was quite low in adult lung and in fetal lung at all developmental stages studied (Table 3). Preliminary experiments had indicated that the concentration of methionine used in the incubation medium (1 mM) gave maximal incorporation of radioactivity into lipid from both adult and fetal lung slices. Only 30–40% of the total radioactivity was located in the phosphatidyl choline portion after thin-layer chromatography. The neutral lipids contained 20–30% of the total radioactivity. The lipid also was hydrolyzed with 3 N HCl in a sealed vial for 6–8 hr at 100°C. The fatty acid fraction from this complete hydrolysis contained 30–32% of the total radioactivity in the lipid. Therefore, the values of the incorporation of radioactivity into total lipid reported in Table 3 are maximal estimations of the methylation pathway since a substantial fraction of the total radioactivity is not found in the phosphatidyl choline fraction and some of the radioactivity in the phosphatidyl choline is undoubtedly associated with the fatty acid portion of the molecule.

The developmental pattern in the biosynthesis of phosphatidyl choline from choline-methyl-^{14}C as precursor

(Table 3) coincides exactly with the pattern previously observed with ^{32}P as the precursor (2). The biosynthesis by methylation of phosphatidyl ethanolamine, judged by the incorporation of radioactivity from the methionine-methyl-^{14}C, is very low in adult lung and shows no apparent developmental fluctuation. Thus the changes in the rate of incorporation of radioactivity into phosphatidyl choline by the developing lung result from alterations in the formation of phosphatidyl choline by the cytidine diphosphocholine pathway (6). Choline might be incorporated into phosphatidyl choline by an exchange reaction, which has been reported to occur in vitro (20); however, ^{32}P would not be incorporated by this reaction. Phosphatidyl N, N-dimethyl ethanolamine has been identified in dog lung (11) and there are indications that it also exists in rat lung (2, 21). Morgan et al. suggest (11) that it is formed during the formation of phosphatidyl choline by the methylation of phosphatidyl ethanolamine, but the present results indicate that this pathway is probably of little quantitative significance in rat lung. In addition, the specific activities of phosphatidyl choline and phosphatidyl N, N-dimethyl ethanolamine after the incorporation of palmitate-1-^{14}C into rat lung lipids are not consistent with the assumption that the phosphatidyl *N*,*N*-dimethyl ethanolamine is a precursor of phosphatidyl choline (21).

In order to obtain information on where, in the pathway of choline to phosphatidyl choline, developmental changes occur, we determined the incorporation of choline-methyl-^{14}C into phosphoryl choline and phosphatidyl choline by fetal and adult lung slices after various periods of incubation. In each experiment we used the combined lungs from three pregnant rats and from the corresponding fetuses. Duplicate incubations were analyzed at each time. The rate of incorporation of choline-methyl-^{14}C into lipid by fetal lung slices is low at 19 days gestation, increases as gestation proceeds, and reaches adult levels by 22 days gestation (Fig. 1), a result consistent with the data in Table 3. The amount of radioactivity incorporated into total water-soluble compounds increased rapidly with time of incubation and in the adult reached a constant level by 10 min (Fig. 1). The amount incorporated into fetal lung at 19 and 20 days gestation also increased rapidly in the incubation (compared with lipid incorporation) but did not reach a constant amount even after 180 min of incubation. Three to four times as much radioactivity was incorporated into water-soluble products in 19 day fetal lungs as in lungs from the adult. As gestation progressed to term, fetal lung slices incorporated less radioactivity into water-soluble materials and approached the degree of incorporation observed in adult lung slices.

The fractionation of the water-soluble radioactivity by two-dimensional paper chromatography revealed two

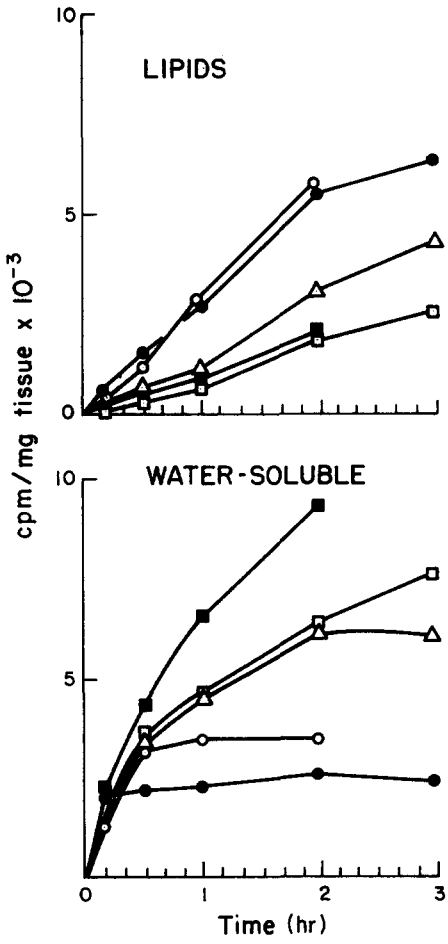

FIG. 1. Incorporation of choline-methyl-^{14}C into phospholipid and water-soluble precursors. The values are the average of eight experiments for the adult and of two, two, and three for 22, 21, and 20 day fetal lungs, respectively. The 19 day value is from a single experiment. The symbols are defined as: ●, adult; ○, 22 day fetal; △, 21 day fetal; □, 20 day fetal; and ■, 19 day fetal.

radioactive areas. One spot had a mobility similar to that of choline; the other, to that of phosphoryl choline. The amount of radioactivity in the phosphoryl choline, calculated from the percentage of the total radioactivity recovered from the chromatogram, was several times higher in the fetal lung than in the adult (Table 4). The amount of radioactivity incorporated into phosphoryl choline was highest for the lung at 19 days gestation and decreased as gestation progressed to term. The specific activities of phosphoryl choline in the fetal lung at all prenatal ages studied were similar to those in the adult lung (Table 5). Thus, the changes in the incorporation of choline-methyl-^{14}C into phosphatidyl choline resulted from differences in the activity of reactions beyond phosphoryl choline. Since the increased radioactivity found in phosphoryl choline in the fetal lung was not due to an increased specific activity, a larger pool of phosphoryl choline must be present in fetal lung. The size of the pool of phosphoryl choline can

TABLE 4 INCORPORATION OF CHOLINE-METHYL-^{14}C INTO PHOSPHORYL CHOLINE

Age	Incubation Period (Min)				
	10	30	60	120	180
			cpm/mg tissue		
Adult	283	310 ± 29 (4)*	350 ± 10 (4)	540 ± 40 (4)	598
	325				733
Fetal					
19†	704	2050	2364	5350	
	896	2298	5210	7750	
20		1129	1050	2620	4080
21		945	2185	2028	2575
22	310	337	545	1510	1850
	484	835	1059	1985	

* Average ± SEM with the total number of experiments in parentheses. Where fewer than three experiments were done, the value for each experiment is given. Each fetal value is the average of duplicate incubations from the combined lungs from three litters.

† Days gestation.

TABLE 5 SPECIFIC ACTIVITY OF PHOSPHORYL CHOLINE

Age	Incubation Period (Min)				
	10	30	60	120	180
			μc/μmole		
Adult	1.14	2.40 ± 0.47 (5)*	2.31 ± 0.18 (5)	3.26 ± 0.40 (5)	2.98 ± 0.26 (4)
Fetal					
19†	0.45	1.74	2.40	4.00	
	0.83	1.51	2.47	3.57	
20		1.27	1.74	2.68	2.30
21		1.47	2.49	2.78	2.16
22		2.19	1.59	2.71	2.55

* Average ± SEM with the total number of experiments in parentheses. Each fetal value is the average of duplicate incubations from the combined lungs from three litters.

† Days gestation.

be calculated from the total radioactivity in phosphoryl choline and its specific activity. The concentration of phosphoryl choline in 19 day fetal lung was about ten times larger than in adult lung and decreased as development progressed to term. There was no consistent change in the size of the pool with increased length of incubation.

In the liver, the rate-determining step in the biosynthesis of phosphatidyl choline is believed by Fiscus and Schneider (22) to be the formation of CDP-choline from phosphoryl chloine and CTP. CDP-choline in turn apparently equilibrates rapidly with phosphatidyl choline (10). Studies of the incorporation of phosphoryl choline-^{32}P and CDP-choline-^{14}C into phosphatidyl choline by brain homogenates at various stages of development have led to the suggestion that low concentrations of CTP: cholinephosphate cytidylyltransferase (EC 2.7.7.15) in neonatal brain may be the rate-limiting segment in the pathway (23). It is not possible from the present results to determine which of the two reactions, the formation of CDP-choline from phosphoryl choline and CTP or the combination of CDP-choline and diglyceride to form phosphatidyl choline, is governing the rate of formation of phosphatidyl choline in the lung, but the rate of phosphatidyl choline biosynthesis does seem to be controlled by one of these reactions.

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