Incorporation of acetate into fatty acids and lecithin by lung slices from fetal and newborn lambs

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ABSTRACT Incorporation of acetate-1-^{14}C into phospholipids and fatty acids by lung slices from fetal and newborn lambs and from ewes was studied in vitro. The distribution of radioactivity in the fatty acids of neutral lipids, phospholipids, and lecithin was determined. Acetate-1-^{14}C was incorporated into myristic, palmitic, and C_{18} fatty acids. Of the lecithin fatty acids, myristic and palmitic were the major radioactive fatty acids.

The results indicate that the lung of fetal lambs is able to synthesize lecithin containing saturated fatty acids, a major constituent of pulmonary surfactant. A marked increase in the rate of acetate incorporation into lecithin was observed during maturation, and these rates were higher than those obtained in the ewes. A possible relationship between developmental changes in lecithin biosynthesis and pulmonary surfactant is discussed.

KEY WORDS incorporation · acetate-1-^{14}C · lecithin · saturated · fatty acids · lung · sheep · fetus · maturation · newborn · pulmonary surfactant

THE INTERNAL SURFACE of the lung is believed to be lined with a surfactant that stabilizes the alveoli during respiration (1). This substance is thought to be a lipoprotein whose activity resides in the phospholipid (2) fraction, particularly in a lecithin (3–6) in the fatty acids of which palmitic acid predominates. The amount of lecithin and the proportion of palmitic acid in it both increase in fetal lung extracts with gestational age, and these increases are closely related to the development of surface activity of the lung (7).

The lung is known to be an active site of lipid synthesis; acetate is incorporated into fatty acids (8–10) and phospholipids (11). The mitochondrial fraction is reported to be the most active subcellular fraction for the synthesis of long-chain fatty acids from acetate (12). The adult lung is also capable of incorporating glucose, palmitic acid, and linoleic acid into phospholipids (10, 13) and specifically into lecithin (14, 15). However no systematic information is available as to which long-chain fatty acids are synthesized in the fetal lung. It is also not known whether lecithin containing saturated fatty acids, especially palmitic acid, is synthesized in the fetal lung itself or transported from other organs.

One purpose of these studies was to determine if the fetal lung can synthesize long-chain fatty acids and lecithin from a simple precursor such as acetate, and to determine possible maturational differences in lecithin synthesis.

MATERIALS AND METHODS

Chloroform, methanol, diethyl ether, pentane, and isobutyl ether were redistilled prior to use. The water used was redistilled and deionized by means of a Deeminizer (Model CL-5, Crystal Research Laboratories, Inc., Hartford, Conn.).

Lung and liver specimens were obtained from ewes and fetal and newborn lambs by surgical biopsy as reported previously (7). Fetal lambs were divided into two groups according to gestational age; those of less than 120 days' gestation were classified as immature fetal lambs, and those of more than 125 days' gestation as mature fetal

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; FFA, free fatty acids; DG, diglycerides; TG, triglycerides; TGFA, triglyceride fatty acids; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine. Fatty acids are designated by number of carbon atoms: number of double bonds.

lambs. The specimens were immediately put into ice-cold Krebs-Ringer bicarbonate buffer (KRB buffer) (16) (pH 7.4) and were rinsed to remove blood.

Lung slices were made from the right lower lobes, the bronchus and trachea being avoided, with the aid of a Stadie-Riggs tissue slicer (Arthur H. Thomas Co., Philadelphia, Pa.) in the cold room at 5°C. The slices were lightly pressed on No. 1 Whatman filter paper and weighed. About 500 mg of slices containing about 40 mg of protein was added to the prepared incubation flask, a 75 ml Erlenmeyer flask equipped with a center well. The time from removal of the specimen to incubation was 10–15 min.

The incubations were carried out at 38°C for 3 hr. The main compartment of each flask contained 50 μmoles of D-glucose, 10 μmoles of sodium glutamate, 10 μmoles (20 μc) of sodium acetate-1-^{14}C (from New England Nuclear Corp., Boston, Mass.), and KRB buffer to bring the final volume to 5 ml. The buffer was gassed with 5% carbon dioxide and 95% oxygen for 20 min before use. The flasks were kept closed during the incubation period and agitated in a water-bath shaker at 60 cycles/min. At the end of incubation, we placed the flasks in a box with solid CO_2 to stop the reaction.

Analysis of Lipids

Tissue and medium were quantitatively transferred to a cup containing five times their volume of chloroform–methanol 2:1 and homogenized. The extract was filtered through Whatman No. 1 filter paper. The residue was homogenized and again extracted with five times its volume of chloroform–methanol 2:1. This was repeated five times and the combined extracts were centrifuged at 2,500 rpm for 10 min at 5°C. The chloroform layer was washed with saline solution three times according to Folch, Lees, and Sloane Stanley (17), which removed 99% of the water-soluble, radioactive materials. The washed chloroform fraction was dried with anhydrous sodium sulfate (18) and evaporated to dryness at 40°C in a rotary flash evaporator. The remaining water-soluble radioactive material was completely removed on a silicic acid column.

Phospholipids were separated from total lipids by silicic acid column chromatography according to Borgström (19). Neutral lipids, cholesterol, and free fatty acids were eluted by chloroform, and phospholipids by methanol. Phospholipids were separated by TLC on basic Silica Gel H plates (20) in a closed glass developing tank with chloroform–methanol–acetic acid–water 60:30:8:4.[1] The R_f values of phosphatidyl serine (PS) and phosphatidyl inositol (PI) were so close that a clear-cut separation of these fractions was not always obtained; the radioactivity for these fractions was therefore expressed as the sum PI + PS. Neutral lipids and free fatty acids were also separated by TLC (21).

Detection of Spots

For the detection of fractions containing fatty acids that were to be analyzed by GLC, plates were sprayed with 2′,7′-dichlorofluorescein (Warner-Chilcott Laboratories, Instruments Division, Richmond, Calif.) and the outlines of the spots were traced under a UV lamp. Usually spots were detected by iodine vapor, 60% sulfuric acid spray, dichlorofluorescein, or 3′,3″,5′,5″-tetrabromophenol sulfonephthalein (Eastman Organic Chemicals, New York). Standard lecithin, sphingomyelin, and cerebroside were obtained through the courtesy of Dr. J. F. Mead, Laboratory of Nuclear Medicine and Radiation Biology, University of California at Los Angeles. Phosphatidyl ethanolamine and phosphatidyl serine were purchased from Applied Science Laboratories Inc., State College, Pa. Phosphat dyl inositol was obtained from Dr. M. Fauré, Pasteur Institute, Paris. Phosphatidic acid was obtained from the General Biochemicals, Chagrin Falls, Ohio; palmitic acid, cholesteryl palmitate, cholesterol, and tripalmitin were commercial samples. They were purified on silicic acid columns according to Dhopeshwarkar and Mead (18). 1,2-Diolein was obtained from Dr. F. H. Mattson, Procter & Gamble Co., Cincinnati, Ohio. 1,3-Dipalmitin was obtained from Dr. C. B. Barrett, Unilever Research Laboratory, Port Sunlight, Great Britain. Monopalmitin was obtained from Sigma Chemical Co., St. Louis, Mo.

TLC of phosphatidic acid took place on Silica Gel H in chloroform–methanol–30% methylamine 65:25:8 (22). TLC of phospholipids or neutral lipids, the radioactivity of which was to be analyzed by means of radiochromatogram scanner, was carried out on a plate 5 cm × 20 cm suitable for the scanner. The amount of lipids applied on the TLC plate was 1.5–2.0 mg. Protein was determined according to Lowry, Rosebrough, Farr, and Randall with bovine serum albumin as standard (23).

Analysis of Fatty Acids

The lipid band (lecithin, phosphatidyl ethanolamine, etc.) was scraped off the TLC plate with a stainless steel spatula, extracted with deaerated chloroform–methanol 1:1 as reported elsewhere (7), and washed with redistilled, deaerated water to remove dichlorofluorescein. The washed chloroform layer was dried over sodium sulfate (18). After centrifugation at 2,000 rpm for 3 min at 5°C, the chloroform was evaporated under nitrogen.

[1] TLC of phospholipids or neutral lipids whose fatty acids were to be analyzed by GLC was performed in nitrogen supplied to the tank via a special glass top (Desaga/Brinkmann, Westbury, N.Y.).

The fatty acids were methylated by BF_3–methanol in a boiling water bath for 10 min (24, 25).

2 ml of redistilled, deionized, deaerated water and 5 ml of pentane were added and mixed thoroughly. The water layer was discarded and the pentane fraction washed twice with water (dichlorofluorescein was thereby completely removed). The pentane fraction was dried over sodium sulfate and evaporated under nitrogen. The methyl esters thus obtained were purified by elution from a Florisil column with 10% diethyl ether in pentane and applied to a Loenco Radiogaschromatograph (Loenco Inc., Altadena, Calif.).

Determination of Radioactivity

The radioactivity of the total phospholipids was determined in a Packard liquid scintillation counter with an efficiency of 60.1%. The radioactivity of each phospholipid fraction was determined as follows. (*a*) Each band on thin-layer plates was scraped off and extracted with chloroform–methanol 1 : 1 as described previously (7). The radioactivity of extracts was determined with the liquid scintillation counter. The recovery of radioactive lipids carried out through extraction was 88–90%. (*b*) The radioactivity of each phospholipid fraction was directly counted by means of a Packard Model 7201 radiochromatogram scanner (26, 27) with an efficiency of 16.4%.[2] Both (*a*) and (*b*) gave satisfactory results, but the results from (*b*) were more reproducible, when determined under the same conditions (i.e., the same collimator sensitivity, gas flow, speed, and thickness of the thin-layer plate). After scanning, the area corresponding to each fraction was cut out and weighed. The peak area was calculated and the radioactivity was computed from the formula: counting rate = (area × conversion factor)/residence time, where conversion factor = (linear range)/(chart width × chart speed), and residence time = slit width/strip speed.

The fatty acids of the lecithin or chloroform–methanol fraction were converted to methyl esters with BF_3–methanol and the radioactivity of the individual fatty acid methyl esters was determined by means of a Loenco Radiogaschromatograph (Model 70, Hi-Flex) with a 5 ft., 0.25 inch I.D. coiled column of 10% diethylene glycol succinate on 60–80 mesh Chromosorb W-HMDS at 175°C.[3] Radioactive fatty acids were identified with radioactive standard palmitic, stearic, oleic, and linoleic acids.[4] The peak area was measured by planimetry and the percentage of radioactivity in each fatty acid was estimated.

[2] In calibration of the radiochromatogram scanner, 8.8 × 10^4 dpm of palmitic acid-1-^{14}C was applied to a thin-layer plate coated with 0.5 mm thick Silica Gel H and chromatographed. The plate was dried and analyzed for radioactivity on the Packard chromatogram scanner model 7201. Efficiency (cpm/dpm) was 16.4%.

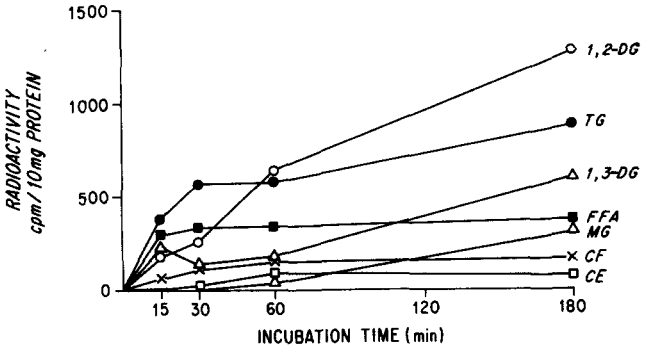

FIG. 1. Incorporation of acetate-1-^{14}C into neutral lipids and FFA of lung slices as a function of incubation time. Lung slices containing 40 mg of protein from mature fetal lambs were incubated with 20 μc of acetate-1-^{14}C in KRB buffer. Lipids were analyzed by means of silicic acid column chromatography and TLC. Radioactivity was determined by liquid scintillation counter and radiochromatogram scanner. Data show the average of two simultaneous experiments.

TG, 1,2-diglyceride (1,2-DG), 1,3-diglyceride (1,3-DG), and FFA were the main fractions into which radioactivity was incorporated. MG, monoglycerides; CF, free cholesterol; CE, cholesteryl esters.

RESULTS

Incorporation of Acetate into Neutral Lipids and Free Fatty Acids

Fig. 1 shows the amount of acetate-1-^{14}C label incorporated into neutral lipids and free fatty acids in the lung slices of a mature fetal lamb as a function of incubation time. Triglycerides (TG), 1,2-diglycerides (1,2-DG), 1,3-diglycerides (1,3-DG), and free fatty acids (FFA) were the main fractions into which radioactivity was incorporated. The activity of FFA reached a steady level at 30 min. TG showed the highest radioactivity for the first 30 min, but 1,2-DG exceeded TG after 60 min.

It is of interest that 1,2-DG, which is an important precursor of both lecithin and TG was very active throughout the entire incubation period, showing the highest radioactivity after 60 min. 1,3-DG was less active.

Incorporation of Acetate into Phospholipids

Fig. 2 shows the amount of acetate-1-^{14}C label incorporated into the phospholipids of the lung slices of the same mature fetal lamb, as a function of incubation time. For the first 30 min, the radioactivity in the solvent front and in 1,2-DG was higher than in lecithin. Although the radioactivity of the solvent front may have been incorporated into phosphatidic acid, we were unable to iden-

[3] The radiogaschromatograph was standardized twice with 2 μl of octadecenoic acid-1-^{14}C (18,800 dpm) at a detector sensitivity of 30 and a thermal conductivity cell sensitivity of 8. The value obtained was 3,850 dpm/cm².

[4] Kindly performed by Dr. J. F. Mead, Laboratory of Nuclear Medicine and Radiation Biology, University of California at Los Angeles.

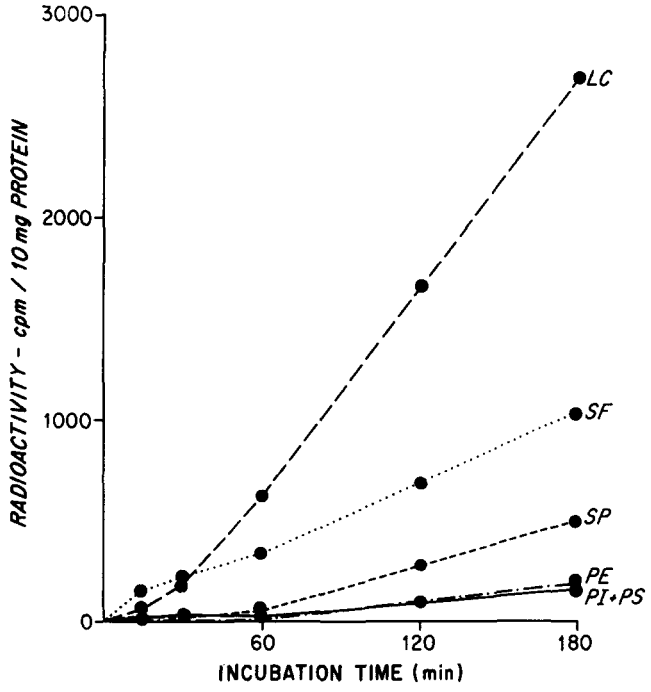

FIG. 2. Incorporation of acetate-1-^{14}C into phospholipids of lung slices of mature fetal lamb as a function of incubation time. See Fig. 1 for experimental details. Data from two experiments. LC, lecithin; SP, sphingomyelin; PI, phosphatidyl inositol; PS, phosphatidyl serine; PE, phosphatidyl ethanolamine; SF, solvent front.

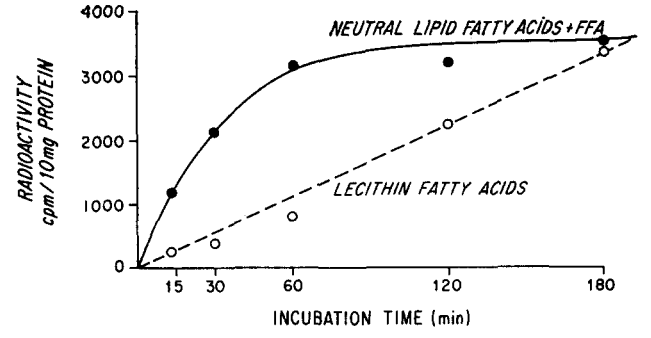

FIG. 3. Incorporation of acetate-1-^{14}C into fatty acids of lung slices from a mature fetal lamb, as a function of incubation time. Data are average of duplicate runs on the lung from one animal.

tify this compound on TLC with three different solvent systems: (*a*) chloroform–methanol–water 65:25:2; (*b*) chloroform–methanol–acetic acid–water 60:25:8:4; and (*c*) chloroform–methanol–30% methylamine, 65:25:8. Lecithin, phosphatidyl serine, phosphatidyl inositol, phosphatidyl ethanolamine, cerebroside, and phosphatidic acid were used as standards. After 30 min, the radioactivity of lecithin rose rapidly, exceeding that both in 1,2-DG and the solvent front which also increased. PE, PI, PS and sphingomyelin showed slight increases in radioactivity. It is noteworthy that phosphatidyl ethanolamine, which is thought to be converted to lecithin through stepwise methylation, showed low radioactivity through the 3 hr incubation.

Incorporation of Acetate into Total Fatty Acids

The incorporation of acetate-1-^{14}C into total fatty acids of the chloroform fraction (neutral lipids, cholesterol, and FFA) and into the lecithin fraction by lung slices from a mature fetal lamb is shown in Fig. 3. The distribution of radioactivity in the fatty acids is given in Table 1. The major fatty acid of the chloroform fraction synthesized in the lung was palmitic acid, which contained 70% or more of the radioactivity. By comparison, in the corresponding fraction from liver slices, only 53.2% of the radioactivity was in palmitic acid and relatively more was present in C_{14} and C_{18} fatty acids. The palmitic acid of the lecithin fraction contained 87–88% of the radioactivity and only trace amounts were present in C_{18} fatty acids. In the methanol fraction, 80% of the radioactivity was in palmitic acid, probably because lecithin fatty acids comprise the major portion of the phospholipid fatty acids.

Influence of Maturation on Incorporation of Acetate into Phospholipids

The incorporation of acetate-1-^{14}C into the phospholipids of the lung slices as a function of maturational age is shown in Table 2. In the immature fetuses, the amounts contained (per 10 mg of tissue protein) in all the phospholipid fractions were 1/3 to 1/2 those present in the mature fetuses, but were higher than those in the ewes. The lung slices from the newborn lambs showed the highest incorporation rates of the four groups studied.

TABLE 1 RELATIVE DISTRIBUTION OF RADIOACTIVITY IN FATTY ACIDS FROM VARIOUS FRACTIONS

	14:0	16:0	16:1	18:0 + 18:1 + 18:2
	% of total radioactivity			
Chloroform fraction				
Pooled immature* fetal lung 146a, 158a†	7.3	70.6	tr.	21.3
Pooled mature fetal lung 164a, 167a	6.4	71.2	tr.	22.0
Pooled mature fetal liver 138a, 161a, 163a	17.3	53.2	tr.	29.3
Methanol fraction				
Pooled mature fetal lung 164a, 167a	12.9	79.7	tr.	7.2
Lecithin fraction				
Pooled mature fetal lung 164a, 167a	11.6	88.2	tr.	tr.
Pooled newborn lung 160, 161	12.5	87.0	tr.	tr.

* Fetal lambs of less than 120 days' gestation are defined as immature and those of more than 125 days' gestation as mature.
† Indicates experiment number.

TABLE 2 INCORPORATION OF ACETATE-1-^{14}C INTO LUNG PHOSPHOLIPIDS AFTER 3 HR OF INCUBATION

Animal No.	Age	Phospholipids					
		Total	SP	LC	PI + PS	PE	SF
		cpm/10 mg of protein					
146a	105*	1,119	46	945	30	49	49
158a	110*	2,061	112	1,529	101	144	175
151a	120*	786	27	528	30	36	165
164a	125*	3,284	276	1,797	184	327	700
167a	135*	4,788	80	3,115	171	209	1,213
138a	1 day	6,091	209	4,437	703	307	435
161a	3 days	10,168	354	6,431	1,402	676	1,305
160a	5 days	34,619	1,514	24,935	7,170	2,134	766
163a	10 days	21,533	733	14,827	4,013	643	1,317
146	adult	872	42	332	38	33	427
155	adult	643	16	251	20	11	345
164	adult	609	80	325	38	37	129

Abbreviations: SP, sphingomyelin; LC, lecithin; PI, phosphatidyl inositol; PS, phosphatidyl serine; PE, phosphatidyl ethanolamine; SF, solvent front.

* Estimated fetal age based on body weight.

DISCUSSION

The results indicate that the lung of fetal and newborn lambs can incorporate acetate into long-chain fatty acids and that 16:0 and 14:0 are the major fatty acids incorporated into phospholipids, especially lecithin. These results do not necessarily represent synthesis of fatty acids in the lung de novo, for acetate incorporation could represent either the synthesis of new fatty acids or the elongation of preexisting ones. However, from the distribution of label shown in Table 1, it appears likely that the majority of acetate incorporated into palmitic acid was through total synthesis via malonyl-coenzyme A and that relatively small amounts of acetate were added to preexisting acyl groups with the elongation of 16:0 to C_{18} fatty acids.

The nonmitochondrial system is known to be a major pathway for the synthesis of long-chain fatty acids by the cell in the liver, mammary gland, adipose tissue, etc. (9, 28, 29). In the lung, however, the mitochondria-rich fraction (in rat) has recently been reported to be the most active subcellular fraction for this synthesis (12, 30). Reiss (31) showed that the mitochondrial fraction from rabbit lung utilized octanoate for synthesis of polar lipids rather than for oxidation, whereas the majority of octanoate was converted into acetoacetate in the mitochondrial fraction from rabbit liver. He further suggested that the synthetic pathway uses up most of the fatty acids available and very little remains for oxidative reactions. It is also possible that the chain-elongation system (the reversal of β-oxidation) of the lung mitochondria may not be as active as in the liver mitochondria. If this is true for the lung tissue from fetal or newborn lambs, the lower percentage of radioactivity of C_{18} fatty acids may be attributed to low activity of the elongation system. In view of the experiments of Reiss (31) and of Tombropoulos (30), lung mitochondria seem to be quite different from liver mitochondria, at least from the standpoint of metabolism of fatty acids and phospholipids. This might explain, to some extent, the difference of distribution of label in fatty acids between lung and liver. Further investigation is underway to determine whether or not lung tissue mitochondria can totally synthesize palmitic acid, mainly through malonyl-coenzyme A.

The fatty acid composition of lecithin from the lung tissue (6) or saline extract of the lung tissue (7, 32) revealed that 16:0 was a major constituent with relatively smaller amounts of other fatty acids. The relatively high percentage of C_{18} fatty acids, especially 18:1 might be due to the influence of FFA or triglyceride fatty acids (TGFA) in the circulating blood, synthesized in other organs (liver, adipose tissue, etc.) or transported through the placenta, because lung tissue has been shown to incorporate FFA or TGFA into phospholipids (33). The lower percentage of 14:0 might be the result of more favorable conversion of 16:0 or C_{18} fatty acids from 14:0 in the intact lung than in the lung slices.

In the time-course experiments, acetate was incorporated into lecithin and 1,2-diglyceride, but relatively small amounts of it were incorporated into phosphatidyl ethanolamine. Although phosphatidyl ethanolamine may not be a major precursor of lecithin (34, 35), except in the liver, it is believed to be converted to lecithin by transmethylation in that organ (36, 37). Our results suggest that the amount of lecithin converted from phosphatidyl ethanolamine in the lung may be much smaller than that from other pathways, such as through the reaction of D-α,β-diglyceride with CDP-choline (38, 39) or acylation of lysolecithin (40–42).

The striking increase observed in the incorporation of acetate into phospholipids, especially lecithin, during maturation is of interest from the standpoint of pul-

monary physiology of the newborn. The fetal lung is known to be filled with fluid (43). After birth, the lung is expanded and the fluid is removed and replaced with air. In the process of aeration, lung surfactant is thought to play an important role. As was vividly described by Felts (10, 33), the lung is an active metabolic unit, engaged in the synthesis of surface active material. The present results suggest that lung tissue of fetal and newborn lambs, like that of rat (8, 10, 33) and rabbit (11), is capable of producing saturated lecithin, an important component of lung surfactant. The surface activity of the saline extract of fetal lung tissue is very low in the immature fetus, but increases with maturation during gestation (7, 44, 45). Concomitantly, the lecithin content and the percentage of palmitic acid in the lecithin fatty acids of the lung saline extract increase during gestation (7). Developmental changes in the activities of the enzymes concerned in the lecithin synthesis may be an important factor. Another factor may be glycolysis in the lung tissue. The metabolism of glucose in phospholipid biosynthesis appears to be essential for providing acetate L-α-glycerophosphate, and a source of NADPH. The latter is obtained, at least in part, from the hexose monophosphate oxidative pathway of glucose degradation, which is active in the lung (10, 33).

Recently, Weinhold and Villee (46) showed an increase during maturation of the ^{32}P-incorporation into phospholipids by rat-liver and rat-lung slices. Gluck and Scribney (47), on the other hand, found an increase in the incorporation of CDP ethanolamine into lecithin in the lung homogenate of fetal rabbits during maturation. These results show a similar tendency to ours, as far as developmental changes in lecithin biosynthesis are concerned.

Morphological changes, such as the changes of alveolar cells or appearance of osmiophilic inclusion bodies, are known to occur in the developing lung of man (48) and sheep (44, 49). The time of onset of cytological changes is closely related to the appearance of surfactant in the lung of fetal lambs (44, 49).

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References

1. Pattle, R. E. 1965. *Physiol. Rev.* **45:** 48.
2. Klaus, M. H., J. A. Clements, and R. J. Havel. 1961. *Proc. Natl. Acad. Sci. U.S.* **47:** 1858.
3. Clements, J. A. 1962. *Physiologist.* **5:** 11.
4. Pattle, R. E., and L. C. Thomas. 1961. *Nature.* **189:** 844.
5. Fujiwara, T., and F. H. Adams. 1964. *Tohoku J. Exptl. Med.* **84:** 46.
6. Fujiwara, T., H. Hirono, and Ts. Arakawa. 1965. *Tohoku J. Exptl. Med.* **85:** 33.
7. Chida, N., F. H. Adams, M. Nozaki, and A. Norman. 1966. *Proc. Soc. Exptl. Biol. Med.* **122:** 60.
8. Lands, W. E. M. 1958. *J. Biol. Chem.* **231:** 883.
9. Popjak, G., and M. L. Beeckmans. 1950. *Biochem. J.* **47:** 233.
10. Felts, J. M. 1964. *Health Physics.* **10:** 973.
11. Nasr, K., and H. O. Heinemann. 1965. *Am. J. Physiol.* **208:** 118.
12. Tombropoulos, E. G. 1964. *Science.* **146:** 1180.
13. Felts, J. M. 1962. *Physiologist.* **5:** 139.
14. Harlan, W. R., Jr., S. I. Said, C. L. Spiers, C. M. Banerjee, and M. E. Avery. 1964. *Clin. Res.* **12:** 291.
15. Heinemann, H. O. 1964. *N.Y. Acad. Med. Bull.* **40:** 74.
16. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. Manometric Techniques. Burgess Publishing Co., Minneapolis, 132.
17. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.
18. Dhopeshwarkar, G. A., and J. F. Mead. 1961. *J. Am. Oil Chemists' Soc.* **38:** 297.
19. Borgström, B. 1952. *Acta Physiol. Scand.* **25:** 101.
20. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. *Biochem. J.* **90:** 374.
21. Skipski, V. P., A. F. Smolowe, R. C. Sullivan, and M. Barclay. 1965. *Biochim. Biophys. Acta.* **106:** 386.
22. Kuhn, N. J., and F. Lynen. 1965. *Biochem. J.* **94:** 240.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* **193:** 265.
24. Metcalfe, L. D., and A. A. Schmitz. 1961. *Anal. Chem.* **33:** 363.
25. Morrison, W. R., and L. M. Smith. 1964. *J. Lipid Res.* **5:** 600.
26. Roberts, S., J. E. Creange, and D. D. Fowler. 1964. *Nature.* **203:** 759.
27. Roberts, S., J. E. Creange, and P. L. Young. 1965. *Nature.* **207:** 188.
28. Brady, R. O., and S. Gurin. 1952. *J. Biol. Chem.* **199:** 421.
29. Martin, D. B., M. G. Horning, and P. R. Vagelos. 1961. *J. Biol. Chem.* **236:** 663.
30. Tombropoulos, E. G. Presented at 2nd International Symposium on Drugs Affecting Lipid Metabolism, Milan, Italy. September 13, 1965.
31. Reiss, O. K. 1965. *Med. Thorac.* **22:** 100.
32. Brown, E. S. 1964. *Am. J. Physiol.* **207:** 402.
33. Felts, J. M. 1965. *Med. Thorac.* **22:** 89.
34. Ansell, G. B., and H. Dohmem. 1957. *J. Neurochem.* **2:** 1.
35. Ansell, G. B., and J. N. Hawthorne. 1964. Phospholipids. Elsevier Publishing Co., New York. 119.
36. Bremer, J., P. H. Figard, and D. M. Greenberg, 1960. *Biochim. Biophys. Acta.* **43:** 477.
37. Gibson, K. D., J. D. Wilson, and S. Udenfriend. 1961. *J. Biol. Chem.* **236:** 673.
38. Kennedy, E. P. 1961. *Federation Proc.* **20:** 934.
39. Kennedy, E. P., and S. B. Weiss. 1956. *J. Biol. Chem.* **222:** 193.
40. Lands, W. E. M. 1960. *J. Biol. Chem.* **235:** 2233.
41. Lands, W. E. M., and I. Merkl. 1963. *J. Biol. Chem.* **238:** 898.

42. Stein, Y., O. Stein, and B. Shapiro. 1963. *Biochim. Biophys. Acta.* **70:** 33.
43. Adams, F. H. 1966. *J. Pediat.* **68:** 794.
44. Orzalesi, M. M., E. K. Motoyama, H. M. Jacobson, Y. Kikkawa, E. O. R. Reynolds, and C. D. Cook. 1965. *Pediatrics.* **35:** 373.
45. Adams, F. H., and T. Fujiwara. 1963. *J. Pediat.* **63:** 537.
46. Weinhold, P. A., and C. A. Villee. 1965. *Biochim. Biophys. Acta.* **106:** 540.
47. Gluck, L., and M. Scribney. Presented at 17th Autumn Meeting of the American Physiological Society, Los Angeles. August 23–27, 1965.
48. Campiche, M. A., A. Gautier, E. I. Hernandez, and A. Reymond. 1963. *Pediatrics.* **32:** 976.
49. Kikkawa, Y., E. K. Motoyama, and C. D. Cook. 1965. *Am. J. Pathol.* **47:** 877.