Biosynthesis of Liver Microsomal Phosphatidyl Cholines During the Development of Choline Deficiency[1] (34849)

DARREL J. RYTTER AND W. E. CORNATZER

Guy and Bertha Ireland Research Laboratory, Department of Biochemistry, University of North Dakota School of Medicine, Grand Forks, North Dakota 58201

Phosphatidyl choline biosynthesis in liver microsomes is known to occur by two major different pathways: The Kennedy (1) pathway involves cytidine diphosphocholine and α-β-diglyceride to form phosphatidyl choline. The Greenberg (2) pathway involves the methylation of phosphatidyl ethanolamine from adenosylmethionine to form phosphatidyl choline. The specificity of the incorporation of 1,2-^{14}C-choline and 1,2-^{14}C-ethanolamine into these phosphatidyl choline fractions as a means of measuring these two biosynthetic pathways of lecithin synthesis has been determined (3). Phosphatidyl choline of fraction one and two are chiefly incorporated from 1,2-^{14}C-ethanolamine and provides a lecithin rich in polyunsaturated fatty acid (3, 4). The phosphatidyl cholines of fraction 3 and 4 are mainly synthesized by the 1,2-^{14}C-choline pathway (3). The biosynthesis of the phosphatidyl choline fraction have not been determined in microsomes from choline-deficient animals with a fatty liver.

Methods. Choline chloride-1,2-^{14}C-(specific activity 2.5 mCi/mmole) was purchased from Mallinckrodt Nuclear, St. Louis, Missouri. Ethanolamine-1,2-^{14}C-(specific activity 3.7 mCi/mmole) was purchased from the New England Nuclear Corp., Boston, Massachusetts.

Female albino rats of Sprague-Dawley strain weighing 176 $\pm$ 19 g were divided into two groups. Group I served as controls and was fed a standard laboratory chow (Purina Laboratory Chow) obtained from the Ralston Purina Co., St. Louis, Mo. The animals of Group II were fed 5% casein–5% fat diet (5, 6) plus 1% guanidoacetic acid for 7 days. The fat was Crisco, Proctor and Gamble Co., Cincinnati. Wire-bottomed cages were used to house individual rats, and the rats were allowed free access to food and water. The fatty acid composition of the Laboratory Chow and fat used in the diet has been determined (6).

At the end of the dietary regimen, the control and choline-deficient rats were injected intraperitoneally with 3.33 μCi/100 g of body weight of the isotopic compounds. The rats were killed by decapitation at 1, 2, 3, and 4 hours after injection of the isotopic compounds. The livers were removed, rinsed with cold water, blotted, weighed, and homogenized with ice-cold 0.25 *M* sucrose in a Potter-Elvehjem homogeinzer with a Teflon pestle. The microsomal fraction was isolated by differential centrifugation (7). The nuclear and mitochondria was separated from the homogenate by centrifuging for 10 min at 14,500 $\times$ g. The supernatant solution was centrifuged at 78,450 $\times$ g for 45 min to sediment the microsomal pellet. The method of Folch *et al.* (8) was employed to extract and purify the lipids from microsomes. The lipids were stored in a dilute chloroform solution under nitrogen at $-18°$. The total phospholipid phosphorus (9, 10) and radioactivity were determined on an aliquot of the chloroform solution. Phosphatidyl cholines were isolated from the lipid extract by thin-layer chromatography by the method of Parker and Peterson (11) using solvent chloroform:methanol:acetic acid:water, 65:25:4:1.4 (v/v/v/v). Phospholipids were identified by comparison with purified phospholipid standards. Plates

[1] Supported in part by a grant from U. S. Atomic Energy Commission AT(11-1) - 1513.

were sprayed with 0.008% rhodamine 6G solution and viewed under ultraviolet light to identify and outline the band of gel containing the phosphatidyl cholines. The silica gels containing the phosphatidyl cholines were scraped into a flask containing 20 ml of chloroform–methanol (2:1, v/v). The phosphatidyl cholines were eluted from the gel by filtration of the chloroform–methanol solution with the aid of a sintered-glass funnel (medium porosity). The gel was washed twice with chloroform–methanol–water (200:97:3) and once with methanol. Quantitative recovery of phosphatidyl choline was possible with this elution procedure. Recovery values represented 94% of the total phosphorus. The filtrate was washed with 0.2 vol of 0.04% calcium choloride solution. A dilute solution of phosphatidyl choline extract in chloroform was stored under dry nitrogen at —18°. On an aliquot of the chloroform solution the total phosphatidyl choline phosphorus (9, 10) and radioactivity was determined. Fractionation of phosphatidyl choline fractions were carried out by thin-layer chromatography on silica-gel H impregnated with silver nitrate (12). The phosphatidyl choline fractions were identified by spraying with 0.01% methanolic solution of 2,7-dichlorofluorescein and viewing under ultraviolet light (12). The phosphatidyl choline fractions were scraped into tubes containing 15 ml chloroform:methanol, 2:1 (v/v) and 9 ml 0.04% $CaCl_2$ added, shaken, and filtered with sintered-glass funnel (medium porosity) to remove the silver ion and collect the lipid–silica gel. The lipids were extracted from the gel with 15 ml chloroform:methanol:water, 200:97:3 (v/v/v), and 5 ml methanol; and chloroform was added to adjust the ratio of 2:1 (chloroform:methanol). The solution was shaken, centrifuged, and aspirated to remove the methanol–water phase. Fifteen milliliters of methanol and 9 ml of 0.04% $CaCl_2$ was added, and the washing procedure was repeated. Fifteen milliliters of chloroform was added to the lipid extract, cooled in a refrigerator, and the water layer was removed by aspiration. The volume of lipid extract was diluted to 50 ml with chloroform. Recovery values represented 90–95% of the phosphorus applied to the silica gel impregnated with silver nitrate. Samples were taken for lipid phosphorus (9, 10) and radioactivity. All radioactivity measurements were made in a Packard Tri-Carb liquid scintillation counter. The scintillation solvent consisted of 0.4% of 2,5-diphenyloxazol (PPO) and 0.005% of 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP) in toluene. Specific activity is expressed as counts/minute per μg of phosphorus.

Results and Discussion. The incorporation of inorganic phosphate ($^{32}P_i$) into total liver phospholipids in rats fed the stock diet is similar in animals fed the choline-deficient diet for 7 days (13). Liver phospholidid synthesis does not decrease until the histological picture changes to cirrhosis (14, 15). The ratio of liver phospholipid P/protein N is unchanged in rats fed the 5% casein–5% fat diet compared to a stock diet, 25% casein–5% for 7 days (14). The liver of the animals fed the 5% casein–5% fat-supplemented with guanidoacetic acid for 7 days showed a 3- to 4-fold increase in neutral lipids when compared to those rats fed stock diet (6, 16).

The incorporation of 1,2-^{14}C-choline into total phospholipid P, total lecithin P and the phosphatidyl choline-P fractions of liver microsomes at 1, 2, 3, and 4 hr after intraperitoneal injection of the isotope in choline-deficient and control animals fed stock diet is shown in Table I.

The incorporation of 1,2-^{14}C-choline into the total phospholipid-P and total lecithin P of liver microsomes is greater in the choline-deficient than in the control animals. The major incorporation of 1, 2-^{14}C-choline was in lecithin fractions 3 and 4 in both choline-deficient and control animals. It has been shown that 85% of the total lecithin P of liver microsomes are found in these two fractions (17) and that these lecithins are synthesized by the choline pathway (3). It is apparent from the data in Table I that the lecithin fractions 3 and 4 from the choline-deficient animals have a greater incorporation of the isotope as shown in the specific activity values than the controls. This observation would suggest that the CDP-choline-

TABLE I. Effect of Choline Deficiency on the Incorporation of 1,2-^{14}C-choline into Phosphatidyl Choline Fractions from Liver Microsomes of Female Rats.

Expt. conditions	Time after injection (hr)	No. of animals	Specific activity (cpm/μg P)[a]					
			Total phospholipid-P	Total lecithin-P	Phosphatidyl choline-P fractions			
					1	2	3	4
Controls	1	3	7.2 ± 1.5	4.9 ± 0.2	2.1 ± 0.8	2.7 ± 1.2	5.0 ± 0.4	8.0 ± 0.2
	2	3	55.0 ± 22.7	80.6 ± 10.8	51.0 ± 3.4	50.1 ± 8.3	73.3 ± 15.8	139.4 ± 29.8
	3	3	57.7 ± 12.3	75.7 ± 14.4	24.1 ± 12.6	30.3 ± 11.9	66.5 ± 18.0	122.0 ± 16.7
	4	3	62.8 ± 3.0	85.3 ± 2.8	33.3 ± 3.8	40.4 ± 12.2	77.8 ± 3.8	122.4 ± 10.0
Choline-deficient	1	3	545 ± 10	1083 ± 107	548 ± 83	498 ± 104	1037 ± 116	1326 ± 120
	2	3	449 ± 10	837 ± 85	590 ± 69	388 ± 102	750 ± 87	1202 ± 59
	3	3	366 ± 20	767 ± 78	410 ± 48	359 ± 36	664 ± 19	797 ± 75
	4	3	332 ± 75	693 ± 225	403 ± 78	380 ± 31	584 ± 42	718 ± 47

[a] Numbers preceded by ± are standard deviations.

TABLE II. Effect of Choline Deficiency on the Incorporation of 1,2-^{14}C-ethanolamine into Phosphatidyl Choline Fractions from Liver Microsomes of Female Rats.

Expt. conditions	Time after injection (hr)	No. of animals	Specific activity (cpm/μg P)[a]					
			Total phospholipid-P	Total lecithin-P	Phosphatidyl choline-P fractions			
					1	2	3	4
Controls	1	3	305 ± 82	105 ± 29	99.4 ± 27.7	73.6 ± 25.0	59.6 ± 12.2	44.7 ± 24.3
	2	3	275 ± 98	154 ± 40	85.5 ± 13.2	64.7 ± 15.5	112.6 ± 10.3	101.4 ± 22.3
	3	3	377 ± 16	253 ± 11	280 ± 65	188 ± 8	231 ± 20	115 ± 10
	4	3	308 ± 10	241 ± 16	346 ± 46	189 ± 9	194 ± 43	137 ± 39
Choline-deficient	1	3	334 ± 46	25.0 ± 6.6	18.0 ± 6.2	17.9 ± 5.9	11.0 ± 2.7	8.2 ± 3.1
	2	3		23.4 ± 3.4	10.8 ± 1.8	13.4 ± 3.3	19.0 ± 2.3	24.8 ± 4.3
	3	3	415 ± 17	48.6 ± 2.0	57.5 ± 15.7	26.7 ± 3.1	27.1 ± 5.2	16.7 ± 5.4
	4	3	328 ± 69	63.9 ± 10.3	90.9 ± 20.4	41.1 ± 1.0	41.2 ± 10.4	24.7 ± 6.3

[a] Numbers preceded by ± are standard deviations.

α-β-diglyceride pathway is more active in the choline-deficient animals. Wilgram, Holoway, and Kennedy (18) have shown that the concentration of CDP-choline per liver was 0.36 μmoles in choline-deficient and 0.32 μmoles in control liver. The incorporation data and the CDP-choline-concentration analysis (18) would support the idea that the biosynthesis of lecithin by the CDP-choline-α-β-diglycerol is not deficient in the production of a fatty liver.

The incorporation of 1,2-^{14}C-ethanolamine into the total phospholipid-P, total lecithin P, and the phosphatidyl choline-P fractions of liver microsomes at 1, 2, 3, and 4 hr after intraperitoneal injection of the isotope into choline-deficient and control animals fed stock diets is shown in Table II. There is very little difference between the incorporation of 1,2-^{14}C-ethanolamine into the total lipid P of choline-deficient animals as compared to controls. The greatest incorporation of 1, 2-^{14}C-ethanolamine is in the lecithin fractions 1 and 2 in both control and choline-deficient animals. This confirms a previous observation (3) and these lecithin fractions 1 and 2 contains the polyunsaturated fatty acids. The biosynthesis of these fractions 1 and 2 are chiefly from the methylation of phosphatidyl ethanolamine (3, 4). It is apparent from the data of Table II that the specific activities of the lecithin fractions of the liver microsomes of the choline-deficient are much smaller than the control animals. This observation would suggest an impairment in the formation of phosphatidyl choline from phosphatidyl ethanolamine in choline deficiency.

Summary. The incorporation of 1,2-^{14}C-choline and 1,2-^{14}C-ethanolamine into the total phospholipid-P, total lecithin P, and the phosphatidyl choline-P fractions of liver microsomes at 1, 2, 3, and 4 hr after intraperitoneal injection of the isotopic compounds in choline-deficient animals was studied. The incorporation of 1,2-^{14}C-choline into the total phospholipid P and lecithin P of liver microsomes was greater in the choline-deficient than in the control animals. The greatest incorporation occurred in lecithin fractions 3 and 4 in the choline-deficient animals. The greatest incorporation of 1,2-^{14}C-ethanolamine was in the lecithin fractions 1 and 2 in both controls and choline-deficient animals. There was a decrease in the incorporation of 1,2-^{14}C-ethanolamine into the lecithin fractions of microsomes from the choline-deficient animals.

1. Kennedy, E. P., and Weiss, S. B., J. Biol. Chem. **222**, 193 (1956).

2. Bremer, J., and Greenberg, D. M., Biochim. Biophys. Acta **37**, 173 (1960).

3. Rytter, D., Miller, J. E., and Cornatzer, W. E., Biochim. Biophys. Acta **152**, 418 (1968).

4. Balint, J. A., Beeler, D. A., Treble, D. H., and Spitzer, H. L., J. Lipid Res. **8**, 486 (1967).

5. Cornatzer, W. E., Sarosi, G. A., and Newland, J. R., Proc. Soc. Exp. Biol. Med. **107**, 463 (1961).

6. Glende, Eric A., Jr., and Cornatzer, W. E., J. Nutr. **86**, 178 (1965).

7. deDuve, C., and Berthet, J., *in* "International Review of Cytology" (G. H. Bourne and J. F. Danielli, eds.), Vol. 3, p. 225. Academic Press, New York (1954).

8. Folch, J., Lees, M., and Stanley, G. H. S., J. Biol. Chem. **226**, 497 (1957).

9. Miller, J. E., and Cornatzer, W. E., Proc. Soc. Exp. Biol. Med. **118**, 948 (1965).

10. Fiske, C. H., and SubbaRow, Y., J. Biol. Chem. **66**, 375 (1925).

11. Parker, F., and Peterson, N. F., J. Lipid Res. **6**, 455 (1965).

12. Arvidson, G. A. E., J. Lipid Res. **6**, 574 (1965).

13. Artom, C., and Cornatzer, W. E., J. Biol. Chem. **171**, 779 (1947).

14. Cornatzer, W. E., Annals N. Y. Acad. Sci. **57**, 919 (1954).

15. Cornatzer, W. E., and Walser, A. H., Proc. Soc. Exp. Biol. Med. **116**, 893 (1964).

16. Artom, C., *in* "Phosphorus Metabolism" (William D. McElroy and B. Glass, eds.), Vol. 2, p. 203. Johns Hopkins Press, Baltimore, Maryland (1952).

17. Miller, J. E., and Cornatzer, W. E., Lipids **4**, 19 (1969).

18. Wilgram, G. F., Holoway, C. F., and Kennedy, E. P., J. Biol. Chem. **235**, 37 (1960).

Received Feb. 17, 1970. P.S.E.B.M., 1970, Vol. 134.