BIOSYNTHESIS OF O-ACYL ETHANEDIOL PHOSPHORYLCHOLINE IN A CELL-FREE SYSTEM FROM RAT LIVER*

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SUMMARY

1-O-Hexadecanoyl [U-^{14}C]ethanediol can serve as substrate in the formation of 1-O-hexadecanoyl ethanediol 2-phosphorylcholine by particulate cell-free preparations from rat liver. Catalytic activity is largely associated with the microsomal fraction. The reaction requires CDPcholine and Mg^{++}. Phosphatidylcholine cannot substitute for CDPcholine, but Mn^{++} is almost as effective as Mg^{++}. Ca^{++} inhibits the reaction. The acyl ethanediol phosphorylcholine produced was identified by repeated cochromotography with authentic diol phospholipid to constant specific radioactivity, and by enzymatic and chemical degradations.

Phospholipids derived from short-chain diols are commonly found in biological systems (2-4). They usually occur at low levels (4). At somewhat higher levels, acyl diol phosphorylcholines possess strongly lytic activity exceeding that of lysolecithins (5-7). The metabolic turnover rates of these diol-derived phospholipids are much higher than those of the corresponding glycerolipids (8). Rapid turnover and concomitant loss of diol ester precursor through lipase hydrolysis (unpublished data) have made *in vivo* metabolic studies difficult.

In the present communication we report on a first successful attempt to synthesize a short-chain diol-derived phospholipid in an *in vitro* system. We show that rat liver microsomes are capable of catalyzing cholinephosphate transfer from CDPcholine to O-acyl ethanediol, reminiscent of the pathway of phosphatidylcholine formation from diacyl glycerol (9, 10).

*"Naturally occurring diol lipids. XIV." For the preceding paper in this series, see reference 1. Abbreviations: PC, lipids containing phosphorylcholine; CDP, cytidine 5'-diphosphate; CMP, cytidine 5'-monophosphate; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Preparation of lipid standards: 1-O-Hexadecanoyl ethanediol was prepared according to standard procedures (11). 1-O-Hexadecanoyl ethanediol phosphorylcholine was synthesized by condensation of the ethanediol monoester with β-bromoethyl dichlorophosphate (12), followed by hydrolysis of the chloride and reaction with trimethylamine (4).

Synthesis of 1-O-hexadecanoyl [U-^{14}C]ethanediol: [U-^{14}C]ethanediol (1.81 mCi, 30 mCi/mmole) supplied as ethanol solution (Dhom Products, North Hollywood, Calif.), 70 mg of hexadecanal prepared by dimethylsulfoxide oxidation (13) of hexadecyl methanesulfonate (14), 40 mg of p-toluenesulfonic acid, and 30 ml of dry benzene were heated to reflux temperature in a 50 ml three-necked flask equipped with water separation head, reflux condenser, inlet tube for dry nitrogen, heating mantle, and stirrer. Approximately 20 ml of the benzene/ethanol/water azeotrope were distilled off over a period of 2-3 hrs. After cooling, 2% aqueous potassium carbonate was added until neutral, and the products were extracted with diethyl ether and dried in vacuo. The mixture of excess aldehyde and labeled 2-pentadecyl-1,3-dioxolane was reduced with 100 mg of lithium aluminum hydride in 50 ml of anhydrous diethyl ether for 2 hrs (15), and the long-chain cyclic acetal was separated from hexadecanol by TLC; developing solvent, hexane-diethyl ether, 90:10 (v/v). The dry ethanediol cyclic acetal was dissolved in 10 ml of freshly distilled methylene chloride-ethyl acetate, 1:1 (v/v), and ozone was bubbled through the solution (20 ml/min) at -16 to -18°C for 40 min (16). Removal of the solvent and purification by TLC (hexane-diethyl ether, 50:50, v/v) produced 1.08 mCi of 1-O-hexadecanoyl [U-^{14}C]ethanediol in an overall yield of 60%. The radiopurity of the labeled diol monoester was better than 99.9%.

Preparation of subcellular fractions: Livers were obtained from five male rats (150 g) of the Sprague-Dawley strain (Dan Rolfsmeyer Co., Madison, Wis.). All procedures of subcellular fractionation were carried out at 0°C. The fresh tissue was rinsed with ice-cold 0.25 M sucrose solution and then minced in a Sorvall Omnimixer at high speed for 10 sec using 2 volumes of 0.25 M sucrose. The mixture was gravity filtered through a fiberglass screen, and the cells were ruptured by rapid decompression after being equilibrated at 1000 psi in a nitrogen bomb for 30 min (17). Nuclei and cell debris were obtained by centrifugation (Sorvall RC 2-B) at 1000 x g for 10 min, the mitochondrial fraction at 20,000 x g for 15 min. The microsomal fraction was prepared at 105,000 x g for 2 hrs (Beckman L2-65B, type-30 fixed angle rotor). Each cellular fraction was washed once with 0.25 M sucrose solution and stored at -30°C for not more than one month. Protein was determined by the Biuret method (18).

Incubations: Subcellular fractions were brought to ice water temperature and resuspended in 0.25 M sucrose solution as needed. The fractions were shaken in a supermixer before each transfer. All incubations were carried out in 30 ml screw cap vials at 37°C using an Eberbach water bath shaker (180 excursions/min, 1 1/8 inch stroke). Protein and cofactor solutions were preincubated for 2 min. Substrate in ethanol was added, the complete mixture was shaken in a supermixer for 5 sec, and then incubated for 10

min. Incubations were terminated by addition of 18 ml of chloroform-methanol, 10:8 (v/v), and shaking in a supermixer.

Lipid fractionation and analysis: O-Hexadecanoyl ethanediol, O-hexadecanoyl ethanediol phosphorylcholine, and ethanediol (7.5 mg of each) were added to the extract, and the mixture was treated with 1 g of Silica Gel H (E. Merck, Darmstadt, Germany) (4). The adsorbent was filtered off on a sintered glass funnel and washed three times with chloroform-methanol-water, 50:40:10 (v/v/v). The crude lipid residue was fractionated on 0.3 mm layers of Silica Gel H; developing solvent (A).** The diol PC fraction (Rf 0.23) was eluted, O-hexadecanoyl ethanediol (7.5 mg) and ethane diol (7.5 mg) were added and the mixture was rechromatographed using developing solvent (B). The diol PC fraction (Rf 0.30) was recovered and rechromatographed up to three more times using solvent systems (C), (D), and (B), until constant specific activity was attained in at least two subsequent fractions.

The purified acyl ethanediol phosphorylcholine from the microsomal preparation was hydrolyzed by phospholipase C from Bacillus cereus as described previously (4) and the hydrolysis product was cochromatographed with hexadecanoyl ethanediol (5 mg) using hexane-diethyl ether, 50:50 (v/v) as developing system in TLC. Methanolysis (4) followed by acid-catalyzed acetalation of the resulting ethanediol with hexadecanal (4) produced labeled long-chain cyclic acetal which was purified by TLC (hexane-diethyl ether, 90:10, v/v) and then cochromatographed with 5 mg of authentic 2-pentadecyl-1,3-dioxolane; developing solvent in TLC was benzene.

Gas chromatography of acyl ethanediol acetates was done on a Victoreen 4000 instrument using an aluminum column, 180 cm x 0.4 cm, packed with ethylene glycol succinate (10% EGSS-X) on Gas-Chrom P, 100-120 mesh (Applied Science Laboratories, State College, Penn.). The column was operated at 220°C, and fractions were collected in glass tubes permitting recoveries of 80%. Radioactivities were determined with a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.) in Aquasol (New England Nuclear, Boston, Mass.) for phospholipids (counting efficiency, 71%) and in toluene solutions of Permablend I (Packard) for neutral lipids (efficiency 91%).

RESULTS AND DISCUSSION

O-Hexadecanoyl [U-^{14}C]ethanediol was incubated with subcellular preparations from rat liver in the presence of CDPcholine and magnesium ions, and incorporation of label into acyl ethanediol phosphorylcholine (diol PC) was followed. As shown in Table

**The following TLC solvent systems were used (proportions by volume): (A), chloroform-methanol-ammonia (15 N), 10:90:8; (B), chloroform-methanol-water, 65:35:8; (C), methanol-water-acetic acid, 90:10:8; (D), chloroform-methanol-ammonia (15 N), 65:35:8. Phospholipid fractions were eluted from the adsorbent with chloroform-methanol-water, 50:40:10.

I, cholinephosphate transferase activity was substantial in the total homogenate and in the mitochondrial fraction, and was most pronounced in the microsomal fraction. Hence, we studied diol PC formation in the microsomal system in greater detail.

Preliminary experiments had shown that short incubation periods (10 min) and low microsomal protein concentrations (0.1

TABLE I. Incorporation of O-hexadecanoyl [U-^{14}C]ethanediol into O-acyl ethanediol phosphorylcholine in cell-free systems from rat liver

Incubation Conditions[a]	Radioactivity[b]
Complete Systems	
Total homogenate	385
Nuclei and debris	201
Mitochondrial fraction	332
Microsomal fraction	654
Supernatant	24
Boiled microsomal fraction	38
Microsomal System - Deletions and Additions	
- CDPcholine	79
- Mg^{++}	71
- Microsomal fraction	0
+ Tween 20 (0.4 mg)	597
- CDPcholine + rat liver glycerol PC (1 mg) + Tween 20 (0.4 mg)	84
+ 1,2-Diacyl glycerol[c] (1 mg) + Tween 20 (0.4 mg)	273
- Mg^{++} + Mn^{++} (5 mM)	588
+ Ca^{++} (0.2 mM)	412
+ Ca^{++} (1.0 mM)	176

[a]The complete incubation system contained: protein, 0.1 mg in 0.6 ml of 0.25 M sucrose; CDPcholine (Sigma Chemical Co., St. Louis, Mo.), 0.5 mg; $MgCl_2$, 20 μmoles, equivalent to a 10 mM concentration; glutathione (Sigma), 10 μmoles; Tris buffer (pH 7.2), 1.4 ml of a 0.1 M solution; O-hexadecanoyl [U-^{14}C]ethanediol, 1.32×10^6 dpm (20 nmoles) in 25 μl of ethanol. The mixture (2 ml) was incubated for 10 min at 37°C.

[b]Radioactivities (dpm/incubation) associated with the acyl ethanediol PC fraction after two purifications by TLC using solvent systems (A) and (B)**. Values given are averages of at least two incubations differing by less than ± 5%.

[c]Prepared from rat liver glycerol PC by phospholipase C hydrolysis.

mg) favored net diol PC synthesis. Even under these conditions, more than 90% of the substrate suffered lipase hydrolysis, thus reducing the amount of diol monoester actually available for diol PC synthesis. The remainder of radioactivity recovered, was largely associated with unmetabolized substrate and with the diol PC fraction.

Incubations were terminated by addition of chloroform-methanol, and lipid extracts were purified with Silica Gel H - avoiding partition with water - in order to preclude loss of water-soluble diol PC (4). Thus, it was necessary to remove residual sucrose and other nonlipid contaminants, as well as labeled substrate and free ethanediol by repeated thin-layer chromatography in the presence of unlabeled ethanediol, hexadecanoyl ethanediol and hexadecanoyl ethanediol phosphorylcholine as carriers. The labeled diol PC fraction was identified by cochromatography with synthetic hexadecanoyl ethanediol phosphorylcholine to constant specific activity using various neutral, acidic and basic developing systems**. It is evident from Table II that the diol PC fraction contained substantial amounts of labeled and unlabeled contaminants after the initial TLC purification, but that constant specific radioactivity was attained after the second fractionation. Hence, all values given in Table I are those determined after two purifications of the acyl ethanediol phosphorylcholine produced in the cell-free rat liver systems.

For further characterization of the diol phospholipid and for localization of the label, the pure acyl ethanediol phosphorylcholine fraction was cleaved by phospholipase C (4), and the resulting hydrolysis product was cochromatographed with hexadecanoyl ethanediol. The labeled monoester fraction was subjected to methanolysis, then to acetalation with hexadecanal (4),

TABLE II. Sequential chromatography of acyl ethanediol phosphorylcholine produced in the microsomal system[a]

Fractionation[b]		Diol PC Fraction[c]		
No.	Solvent	dpm	mg	dpm/mg
1	A	5427	43.41	
2	B	654	4.38	149
3	C	500	3.45	145
4	D	399	2.91	137

[a]The microsomal system was that of Table I, footnote a.

[b]Developing solvents in TLC are given in the Experimental Section**

[c]Radioactivities (dpm/incubation) and weights (mg) given are those obtained after fractionation. Hexadecanoyl ethanediol phosphorylcholine (7.5 mg), hexadecanoyl ethanediol (7.5 mg) and ethanediol (7.5 mg) were added as carriers before the first fractionation, hexadecanoyl ethanediol (7.5 mg) and ethanediol (7.5 mg) also before the second fractionation.

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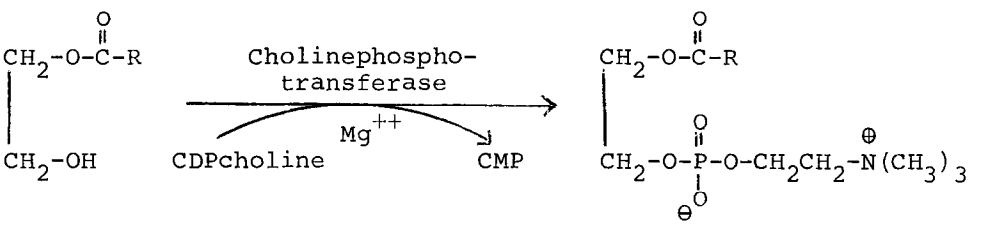
and the long-chain diol acetal was isolated and cochromatographed with 2-pentadecyl-1,3-dioxolane. In the course of this degradation, 71% of the label present in the diol PC fraction was recovered as long-chain ethanediol acetal, demonstrating that ^{14}C had been retained in the diol backbone during diol PC synthesis. Alternatively, when the ethanediol monoester obtained through phospholipase C hydrolysis was acetylated and subjected to gas chromatography, more than 95% of the label recovered was associated with the hexadecanoyl ethanediol acetate fraction, supporting the concept of a direct cholinephosphate transfer to hexadecanoyl ethanediol and the absence of substantial acyl exchange on the phospholipid.

In the microsomal system, incorporation of hexadecanoyl [U-^{14}C]ethanediol into hexadecanoyl ethanediol phosphorylcholine requires CDPcholine as well as magnesium ions (Table I). An attempt to substitute phosphatidylcholine for CDPcholine as

cholinephosphate donor (19,20) was unsuccessful. On the other hand, Mg^{++} could be replaced by Mn^{++} essentially without loss of activity. Furthermore, 1,2-diacyl glycerol addition depressed diol PC synthesis and increasing concentrations of Ca^{++} were inhibitory. This strongly suggests that acyl ethanediol PC synthesis from acyl ethanediol is catalyzed by an enzyme system identical or similar to 1,2-diacyl-glycerol: CDPcholine cholinephosphotransferase (EC 2.7.8.2) (9,10).



This cholinephosphotransferase also appears to catalyze choline phospholipid formation from 1-alk-1'-enyl 2-acyl glycerol (21), 1-alkyl 2-acyl glycerol (22) and 2-acyl 1,2-hexadecanediol (23). 1-Alkyl glycerol (24) or 1-alkyl ethanediol (24,25) cannot be utilized as substrate, but cholinephosphate transfer from CDPcholine to acyl ethanediol occurs readily in the microsomal rat liver system. More detailed work is in progress.

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REFERENCES

1. Baumann, W. J., Madson, T. H., Chang, N., Bandi, P. C., and Schmid, H. H. O. (1975) Biochem. Biophys. Res. Commun. 66, 717-724.
2. Vaver, V. A., Shtshennikov, V. A., and Bergelson, L. D. (1967) Biokhimiya 32, 1027-1031.

3. Bergelson, L. D., Vaver, V. A., Prokazova, N. V., Ushakov, A. N., Rozynov, B. V., Stefanov, K., Ilukhina, L. I., and Simonova, T. N. (1972) Biochim. Biophys. Acta 260, 571-582.
4. Baumann, W. J., Schupp, E., and Lin, J. T. (1975) Biochemistry 14, 841-847.
5. Baer, E. (1953) J. Am. Chem. Soc. 75, 5533-5539.
6. Baer, E., Duke, A. J., and Buchnea, D. (1968) Can. J. Biochem. 46, 69-74.
7. Reman, F. C., Demel, R. A., de Gier, J., van Deenen, L. L. M., Eibl, H., and Westphal, O. (1969) Chem. Phys. Lipids 3, 221-233.
8. Lin, J. T., and Baumann, W. J. (1974) Biochem. Biophys. Res. Commun. 59, 100-107.
9. Kennedy, E. P., and Weiss, S. B. (1956) J. Biol. Chem. 222, 193-214.
10. Weiss, S. B., Smith, S. W., and Kennedy, E. P. (1958) J. Biol. Chem. 231, 53-64.
11. Baumann, W. J., Schmid, H. H. O., Ulshöfer, H. W., and Mangold, H. K. (1967) Biochim. Biophys. Acta 144, 355-365.
12. Hirt, R., and Berchtold, R. (1958) Pharm. Acta Helv. 33, 349-356.
13. Mahadevan, V., Phillips, F., and Lundberg, W. O. (1966) Lipids 1, 183-187.
14. Baumann, W. J., and Mangold, H. K. (1964) J. Org. Chem. 29, 3055-3057.
15. Schupp, E., and Baumann, W. J. (1973) J. Lipid Res. 14, 121-124.
16. Baumann, W. J., and Madson, T. H. (1974) J. Lipid Res. 15, 528-529.
17. Hunter, M. J., and Commerford, S. L. (1961) Biochim. Biophys. Acta 47, 580-586.
18. Layne, E. (1956) Methods in Enzymology 3, 447-454.
19. Diringer, H., and Koch, M. A. (1973) Z. Physiol. Chem. 354, 1661-1665.
20. Ullman, M. D., and Radin, N. S. (1974) J. Biol. Chem. 249, 1506-1512.
21. Kiyasu, J. Y., and Kennedy, E. P. (1960) J. Biol. Chem. 235, 2590-2594.
22. Snyder, F., Blank, M. L., and Malone, B. (1970) J. Biol. Chem. 245, 4016-4018.
23. Chang, N., Bandi, P. C., and Schmid, H. H. O. (1975) Biochem. Biophys. Res. Commun. 62, 204-211.
24. Snyder, F., Malone, B., and Piantadosi, C. (1974) Arch. Biochem. Biophys. 161, 402-407.
25. Schmid, H. H. O., Bandi, P. C., Chang, N., Madson, T. H. and Baumann, W. J. (1975) Biochim. Biophys. Acta, in press.