METHYLATION OF PHOSPHATIDYL MONOMETHYLETHANOLANINE IN LIVER PREPARATIONS*

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Recent reports have indicated that lecithin ("phosphatidyl choline") can be formed by stepwise methylation of the nitrogenous moieties of intact phospholipids (Bremer et al., 1960; Artom, 1960; Gibson et al., 1961). With liver microsomes, the additions of non-labeled synthetic DME-, or MME-containing phospholipids (but not that of phosphatidyl EA) stimulated the incorporation of C^{14} from Me-methyl-C^{14} into lecithin (Bremer and Greenberg, 1961; Cooksey and Greenberg, 1961). Attempts to demonstrate more directly a methylation of C^{14}-labeled phosphatidyl EA by liver homogenate, or by its fractions, were not successful (Artom, 1962). On the other hand, such preparations actively converted labeled phosphatidyl DME into lecithin (Artom and Lofland, 1960; Artom, 1962). Similar evidence for the methylation of intact phosphatidyl MME is presented in this communication.

Methods. MME-1,2-C^{14} (synthesized from labeled ethylene dioxide and unlabeled methylamine by Dr. J. C. Leak of Atomic Accessories, Inc.) was incubated in a Dubnoff shaking apparatus with various types of rat liver preparations. The lipids and proteins were precipitated with colloidal Fe_2O_3 and $MgSO_4$. The precipitate was washed first with a solution of unlabeled MME, then

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repeatedly with water, and was finally extracted overnight with ethanol and ether at room temperature. The lipid extract was hydrolyzed, and the products were fractionated and treated, as shown in Fig. 1. On the separated fractions the radioactivity was determined with a Q-gas flow counter.

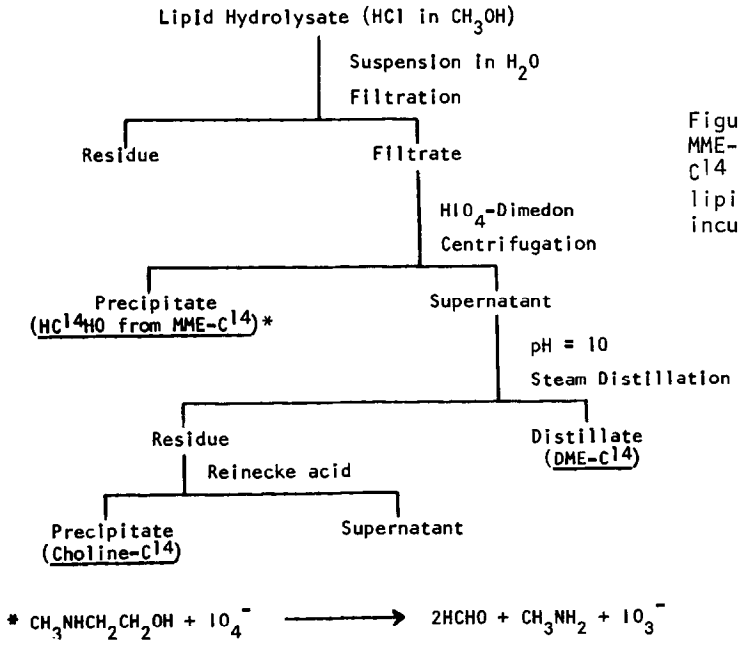

Figure 1. Determination of MME-C^{14}, DME-C^{14}, and choline-C^{14} in the hydrolyzate of lipids from liver preparations incubated with MME-1,2-C^{14}.

In several experiments, thin-layer chromatography of the unhydrolyzed lipids was carried out also. The plates were developed with a solvent (chloroform/methanol/7N NH_4OH:60/35/5) which effectively separates MME-, DME-, and choline-containing phospholipids.* The radioactive spots were scraped into vials, containing a scintillating thioxotropic gel suspension, and were counted in a liquid scintillation counter.

Incorporation of MME-1,2-C^{14} into Liver Lipids. The results of experiments in which MME-1,2-C^{14} was incubated with liver slices or liver homogenate, respectively, are exemplified in Table I. After incubation with liver slices,

*Thus in one experiment, Rf values of 0.37, 0.54, and 0.72 were obtained for samples of synthetic L-α-dipalmitoyl-lecithin, L-α-N-methyl-distearoyl-, and L-α-N,N-dimethyl-distearoyl cephalin, respectively.

the C^{14} was distributed among the MME-, DME-, and choline moieties. When Me was not the limiting factor, most of the radioactivity was present in phosphatidyl choline.

TABLE I. Incorporation of MME-1,2-C^{14} into Rat Liver Lipids.

The system for exp. 4 contained 5 mg reduced glutathione, 1.5 mg each penicillin G and dihydrostreptomycin, liver slices (approx. 400 mg), and 6.1 μmoles (=1.3 μcuries) MME-1,2-C^{14} in 0.5 ml of 0.15 M Tris HCl and 2.5 ml of Krebs-Ringer-phosphate (pH 7.5). The system for exp. 7 contained the same amounts of glutathione, penicillin, and dihydrostreptomycin, 20 μmoles $CaCl_2$, rat liver homogenate (nuclei, red cells and cell fragments removed - frozen preparation: 36 mg protein), and 0.85 μmoles (=1.7 μcuries) MME-1,2-C^{14} in 0.5 ml of 0.15 M Tris HCl, 0.5 ml Krebs-Ringer-phosphate, and 2.4 ml of 0.25 M sucrose (pH 8.4). Non-isotopic additions were: 25 μmoles Me (exp. 4), or 3.5 μmoles AdMe (exp. 7). Incubation at 37° in air for 2 hours.

Exp. No.	Preparation	Non-isotopic additions	MME-1,2-C^{14} incorporated into lipids	% of lipid C^{14} in Phosphatidyl-		
				MME	DME	Choline
			milliμmoles per g. liver			
4	Slices	None	127	75	15	10
4	Slices	Me	197	38	21	41
7	Homog.	None	56	97	1	2
7	Homog.	AdMe	79	10	7	83

Incorporation of MME-C^{14} into the lipids of liver homogenates was often quite low, but was markedly stimulated by the addition of Ca^{++}. With the Ca-enriched homogenate, or its fractions, practically all the radioactivity was present in phosphatidyl MME. When, however, unlabeled AdMe had been added also, a notable portion of the lipid C^{14} was found in the DME-, and, more especially, in the choline moiety. The finding that the addition of Me (slices), or of AdMe (homogenate), markedly increased the total incorporation of C^{14} into the lipids should be noted, although its interpretation remains doubtful.

Identification of Biosynthetic Phosphatidyl MME. Upon elution from a silicic acid-celite column* as well as on thin-layer chromatography with two different solvents, the radioactive lipid obtained by incubating MME-1,2-C^{14} with liver homogenate (Ca-stimulated, but without addition of AdMe) behaved in the same manner as synthetic N,N-dimethyl distearoyl α-cephalin. When digested by phospholipase A (from Crotalus adamanteus), C (from cabbage), or D (from Cl. Welchii), both materials gave products (lysophospholipid, free MME, or phosphoryl MME, respectively) with the same chromatographic and chemical properties.

Methylation of Phosphatidyl MME-. The biosynthetic material was emulsified in Ringer solution containing 1% albumin, distributed into three flasks, A, B, and C, and then incubated with rat liver homogenate. A control sample of the lipid emulsion was incubated without the homogenate, then 0.5 ml of glacial acetic acid and the homogenate were added in succession. The values determined on flasks A, B, and C were corrected on the basis of the small amounts of radioactivity found in the "DME" and "choline" fractions of the control. The corrected results obtained in one such experiment are shown in Table II.

It is apparent that while little or no radioactive lecithin was found in the absence of added AdMe, a readily demonstrable methylation to phosphatidyl DME and phosphatidyl choline occurred in the flasks, where non-isotopic AdMe had been added. The lack of an isotope dilution effect in Flask C to which unlabeled MME had been added, seems to exclude the remote possibility that isotopic MME was first split off, methylated to choline, and then reincorporated into a phospholipid. A substantially identical distribution of the radioactivity was obtained by thin-layer chromatography of the lipid extracts.

*Similar columns have been used for the isolation of phosphatidyl MME from Neurospora crassa (Hall and Nyc, 1961) and from Cl. butyricum (Goldfine, 1962).

TABLE II. Methylation of Phosphatidyl MME-1,2-C^{14} by Rat Liver Homogenate.

The system consisted of 5 mg reduced glutathione, 1.5 mg each penicillin G and dihydrostreptomycin, 20 mg bovine serum albumin, rat liver homogenate (nuclei, red cells and cell fragments removed - fresh preparation: 50 mg protein), and emulsified lipids containing biosynthetic phosphatidyl MME-1,2-C^{14} (78 millimoles and 6.7 millimicuries) in 1.5 ml of 0.25 M sucrose and 2.5 ml of Krebs-Ringer-phosphate (pH 7.6). Non-isotopic additions were 3 μmoles AdMe, and/or 25 μmoles MME. Incubation at 37° in air for 2 hrs.

Exp. and Flask No.	Preparation	Non-isotopic additions	% of lipid C^{14} in Phosphatidyl-		
			MME	DME	Choline
6-A	Homog.	None	99	0	1
B	Homog.	AdMe	86	3	11
C	Homog.	AdMe, MME	78	6	16

Discussion. In the experiments, in which emulsified phosphatidyl MME was incubated with AdMe and liver homogenate (Table II), the percent of C^{14} incorporated into phosphatidyl choline was lower than in experiment no. 7 (Table I) in which free MME was the isotopic substrate. The discrepancy might perhaps be ascribed to imperfect dispersion of the phospholipid with a consequently inadequate contact between enzyme and substrate.*

Methylation of the emulsified phosphatidyl MME was also less extensive than the methylation of phosphatidyl DME observed in previous experiments (Artom and Lofland, 1960) in which as much as 60 percent of the labeled substrate was converted to lecithin. It should be noted that in the present experiments, after incubation with labeled phosphatidyl MME the counts were consistently higher in the lecithin than in the phosphatidyl DME fraction.

*In an attempt to improve the emulsification, in one experiment small amounts of octanol and Na_2SO_4 were added to the system. These additions (which strikingly stimulate the activity of phosphatidyl serine decarboxylase (Kanfer and Kennedy, 1962)) almost completely inhibited the methylation of phosphatidyl MME by our preparations.

In conclusion, it is believed that, while identification of phosphatidyl EA as the direct acceptor in the initial methylation step toward the formation of lecithin may still be regarded as doubtful, our previous and present results demonstrate unequivocally the occurrence in liver preparations of the two subsequent steps, involving the methylation of MME- and DME-containing phospholipids, respectively.

References

Artom, C., Fed. Proc., 19, 233 (1960); 21, 297 (1962)
Artom, C., and Lofland, H.B., Biochim. Biophys. Res. Commun., 3, 244 (1960)
Baer, E., and Pavanaram, S.K., J. Biol. Chem., 236, 1269 and 2410 (1961)
Bremer, J., and Greenberg, D.M., Biochim. Biophys. Acta, 46, 205 (1961)
Bremer, J., Figard, P.H., and Greenberg, D.M., Biochim. Biophys. Acta, 43, 477 (1960)
Cooksey, K.E., and Greenberg, D.M., Biochim. Biophys. Res. Commun., 6, 256 (1961)
Gibson, K.D., Wilson, J.D., and Udenfriend, S., J. Biol. Chem., 236, 673 (1961)
Goldfine, H., Biochim. Biophys. Acta, 59, 504 (1962)
Hall, M.O., and Nyc, J.F., J. Lipid Res., 2, 321 (1961)
Kanfer, J.N., and Kennedy, E.P., J. Biol. Chem., 237, PC270 (1962)
Wolf, B., and Nyc, J.F., J. Biol. Chem., 234, 1068 (1959)