Preliminary Notes

PN 1330

Isolation and characterization of a diether analog of phosphatidyl glycerophosphate from *Halobacterium cutirubrum* *

In a previous communication[1], it was reported that the lipids of an extreme halophile, *Halobacterium cutirubrum*, contained ether-linked alkyl groups instead of ester-linked fatty acids. Furthermore, it was found that at least 73% of the lipid phosphorus was associated with a single phosphatide component which on acid hydrolysis gave a long-chain diether of glycerol and glycerol diphosphate. Since this phosphatide had an R_F value on silicic acid-impregnated paper in diisobutyl ketone–acetic acid–water (40:25:5, v/v)[2] similar to that of cardiolipin[3], it was considered, tentatively, to be a diether analog of diphosphatidyl glycerol[1].

We have now isolated this phosphatide from the total lipids of *H. cutirubrum* and wish to present evidence establishing its structure as a diether analog of phosphatidyl glycerophosphate.

Isolation of unknown phosphatide as its sodium salt. Total lipids (including carotenoid pigment) were extracted from cells of *H. cutirubrum* as described previously[1]. A chloroform solution of the total lipids (444 mg, containing 20.5 mg P) was concentrated in a stream of N_2 to 3–4 ml, diluted with 10 vol. of methanol, and the mixture was kept at 0° overnight. Some insoluble material was removed by centrifugation and washed three times with 1–2 ml of cold methanol. A 20% aqueous solution of $BaCl_2$ (0.5–0.6 ml) was added dropwise to the combined methanol supernatants until no further precipitation occurred. The mixture was cooled on ice and the barium salt was centrifuged and washed several times with small portions of cold methanol and acetone. It was reprecipitated from 3 ml of chloroform by addition of 30 ml of acetone at 0°, recovered by centrifugation, washed twice with 10 ml of cold acetone, and dried *in vacuo* (yield, 312 mg; %P, 5.62; recovery of P, 86%).

The barium salt was converted to the sodium salt via the free acid, as follows: a solution of 105 mg of barium salt in 15 ml of chloroform was diluted with 15 ml of methanol and acidified with 0.35 ml of 1 N HCl. The mixture was shaken for 15 min, 13.2 ml of water were added, and the chloroform phase was separated by centrifugation and concentrated to dryness *in vacuo* (yield of free acid, 88.4 mg; %P, 6.78). A solution of the free acid in 10 ml of methanol was neutralized to phenolphthalein with methanolic NaOH (alkali consumed, 1.0 equiv/atom P), cleared by centrifugation of a slight precipitate, concentrated to a small volume (approx. 0.5 ml), and diluted with 10 vol. of cold acetone. The precipitate was recovered by centrifugation, washed with cold acetone, and precipitated again from methanol solution with acetone. The sodium salt thus obtained was a white, slightly hygroscopic powder weighing 77.7 mg (86% yield from the barium salt, on basis of P); it was soluble

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in chloroform, methanol, and water, and insoluble in acetone. When chromatographed on silicic acid-impregnated paper with diisobutyl ketone–acetic acid–water as solvent[2], it gave only one spot with R_F 0.68–0.70 identical with that of the major phosphatide in the total lipids.

Analyses: Found: C, 59.50; H, 10.24; P, 6.66; Na, 4.87 %; Na/P atomic ratio, 0.98. $C_{46}H_{94}O_{11}P_2Na_2$ (mol. wt. 930.7) requires C, 59.35; H, 10.18; P, 6.66; Na, 4.95 %; Na/P atomic ratio, 1.00.

The analytical data are in good agreement with those calculated for the disodium salt of a diether analog of phosphatidyl glycerophosphate having C_{20} chains. The previous assignment of C_{17} to C_{18} chains was based on C and H analyses of the glycerol diether[1] and on the relative retention of the latter on a column of SE-52 at 220° (ref. 4), but the data presented here show that the chains are actually C_{20}-branched (see below).

Degradative studies. The sodium salt (25.3 mg; 54.3 μmoles P) was heated under reflux in 4.5 ml of 2.5 % methanolic HCl for 4 h, and the petroleum ether-soluble and methanol–water-soluble products were separated[5]. The water-soluble fraction contained 95.3% (51.7 μmoles) of the total phosphorus in the form of an organic phosphate (less than 0.5% inorganic P was present) which had a phosphorus to glycerol* molar ratio of 2.0:1.2 and most likely was glycerol diphosphate in form of a cyclic phosphate trimethyl ester. After hydrolysis of the phosphate ester in 0.2 N NaOH for 2–3 h at 100°, 68% of the phosphorus was obtained as a barium salt insoluble in boiling water and corresponding in composition and chromatographic properties to barium glycerodiphosphate. [Found: P, 10.95%; P:glycerol molar ratio, 2.00:0.97. $C_3H_6O_9P_2 \cdot 2H_2O$ (mol. wt. 558.1) requires P, 11.10%; P:glycerol molar ratio 2.00:1.00; R_F 0.17 in phenol–water; R_P** 0.41 in butanol–acetic acid–water (5:3:1, v/v); synthetic 1,3-glycerol diphosphate[7] had R_F 0.17 and R_P** 0.42, respectively.]

The ether-soluble fraction was found to be a long-chain diether of glycerol[1] having C_{20}-chains. [Yield, 17.1 mg; 26.3 μmoles; molar ratio diether:sodium salt, 0.97:1.00. Found: C, 78.50; H, 13.63%; mol. wt., 660. $C_{43}H_{88}O_3$ (mol. wt. 652.6) requires C, 79.13; H, 13.59%.] It gave only one peak when subjected to gas–liquid chromatography on silicone SE-52 at 220° (ref. 4), with retention relative to synthetic α,β-di-*n*-octadecyl glycerol ether of 0.64 (corresponding to chains with carbon number[8], 17.2). Its infrared spectrum was identical to that of α,β-di-*n*-octadecyl glycerol[4] except that it contained a doublet at 1385–1375 cm^{-1}, indicative of a *gem*-dimethyl group.

On treatment of the diether with BCl_3 (ref. 9), a long-chain chloride and glycerol were recovered in the molar ratio of 2.00:0.91. Cleavage of the diether with HI (ref. 10) gave an alkyl iodide which showed only one peak on gas–liquid chromatography (butandiol–succinate polymer, 197°) with retention relative to *n*-octadecyl iodide, 0.82, and carbon number[8], 17.4. The nuclear magnetic resonance spectrum of the iodide showed that there were five methyl groups to eleven methylene groups, and that the iodide was on a primary carbon.

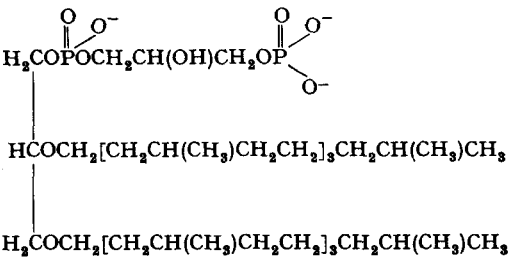
Treatment of the iodide with silver acetate, followed by saponification[10], yielded the corresponding alcohol which was found to have an infrared spectrum and gas–

* Glycerol was determined by the method of LAMBERT AND NEISH[6] after hydrolysis of the sample with 2 N HCl in a sealed tube at 125° for 5 h.

** R_P indicates position of spot relative to inorganic phosphate (R_P, 1.00).

liquid chromatographic properties (carbon number, 17.7, on Apiezon L at 197°) identical with those of dihydrophytol, prepared by hydrogenation of authentic phytol. Mild oxidation of the alcohol with CrO_3 in glacial acetic acid gave the corresponding carboxylic acid, the methyl ester of which had an infrared spectrum and chromatographic properties (carbon number, 17.4, on Apiezon L at 197°) identical with those of the methyl ester prepared in the same way from dihydrophytol.

On the basis of these results, the structure of the major phosphatide in *H. cutirubrum* is most likely a diether analog of phosphatidyl glycerophosphate in which the alkyl chains are dihydrophytyl groups:



Phosphatidyl glycerophosphate has previously been detected as an intermediate in the biosynthesis of phosphatidyl glycerol by chicken liver or rat-liver mitochondria[11]. The high concentration of a diether analog of this phosphatide in *H. cutirubrum* is unusual and may perhaps be connected with the extreme halophilism of this organism.

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[1] S. N. SEHGAL, M. KATES AND N. E. GIBBONS, *Can. J. Biochem. Physiol.*, 40 (1962) 69.
[2] G. V. MARINETTI, J. ERBLAND AND J. KOCHEN, *Federation Proc.*, 16 (1957) 837.
[3] M. G. MACFARLANE, *Biochem. J.*, 78 (1961) 44.
[4] M. KATES, T. H. CHAN AND N. Z. STANACEV, *Biochemistry*, 2 (1963) 394.
[5] M. KATES, *J. Lipid Res.*, in the press.
[6] M. LAMBERT AND A. C. NEISH, *Can. J. Res.*, 28b (1950) 83.
[7] M. KATES AND T. H. CHAN, unpublished results.
[8] F. P. WOODFORD AND C. M. VON GENT, *J. Lipid Res.*, 1 (1960) 188.
[9] W. GERRARD AND M. F. LAPPERT, *J. Chem. Soc.*, (1952) 1486.
[10] K. E. GUYER, W. A. HOFFMAN, L. A. HORROCKS AND D. G. CORNWELL, *J. Lipid Res.*, 4 (1963) 385.
[11] J. Y. KIYASU, R. A. PIERINGER, H. PAULUS AND E. P. KENNEDY, *J. Biol. Chem.*, 238 (1963) 2293.

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