Isolation of Ceramide Phosphorylethanolamine from the Blowfly *Calliphora erythrocephala*

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The phospholipid ceramide aminoethylphosphonate has been characterized as a constituent of marine organisms (Hori, Itasaka & Inoue, 1966; Simon & Rouser, 1967). Recently we have isolated from rumen protozoa an amino sphingolipid fraction that was shown to be a mixture of ceramide aminoethylphosphonate and an analogue of sphingomyelin, ceramide phosphorylethanolamine (Dawson & Kemp, 1967). Hori, Itasaka & Arakawa (1967) have shown these two sphingolipids to be present either singly or together in several marine invertebrates, but not in two species of fish they examined. The very similar structures of these compounds make it difficult to separate them by techniques such as chromatography and consequently we have examined other possible natural sources for obtaining pure preparations of ceramide phosphorylethanolamine. The characteristic paucity of choline-containing phospholipids in members of the Family Cyclorrhapha and the description by Crone & Bridges (1963) of an alkali-stable lipid in *Musca domestica* that yielded a 'phosphorylethanolamine-like' substance on acid hydrolysis led us to examine the phospholipids of the blowfly *Calliphora erythrocephala*. From this source a new phospholipid has been prepared that we have characterized as ceramide phosphorylethanolamine.

One thousand blowflies (kindly provided by Dr G. D. Greville and Dr E. A. Munn) were immobilized by freezing and homogenized by blending in 1 l. of cold chloroform-methanol (2:1, v/v). The suspension after standing for 15 min. with stirring was filtered through a loosely packed pad of glass wool and the filtrate shaken with 0.2 vol. of 0.145 M-NaCl. The lower chloroform-rich layer was evaporated to dryness in a rotary evaporator and the residue extracted with 20 ml. of chloroform-methanol (9:1, v/v). After filtration through sintered glass, the soluble lipids (37 mg. of P) were applied to a column (18 cm. long $\times$ 4.5 cm. diam.) of silica gel (Mallinckrodt), poured in chloroform-methanol (9:1, v/v) and eluted successively with (a) 400 ml. of chloroform-methanol (4:1, v/v), (b) 200 ml. of chloroform-methanol (7:3, v/v) and (c) 400 ml. of chloroform-methanol (3:2, v/v). The phosphorus-containing peaks eluted by each solvent

were collected, and a portion of the phospholipids was examined by thin-layer chromatography (silica gel H; chloroform-acetone-methanol-acetic acid-water, 10:4:2:2:1, by vol.) after mild alkaline ethanolysis (Dawson, Davenport & Hemington, 1962). The small phospholipid peak eluted by solvent (c) contained appreciable amounts of a ninhydrin-positive alkali-stable phospholipid. This fraction (1.5 mg. of P) was subjected to mild alkaline ethanolysis followed by acid treatment to remove labile phosphoglycerides. The lipid was dissolved in carbon tetrachloride (2.4 ml.), and after the addition of ethanol (21 ml.) and 0.28 N-NaOH (2.7 ml.) the mixture was incubated for 1 hr. at 37°. The incubation mixture was treated with ethyl formate (0.4 ml.) to lower the pH, and chloroform (45 ml.) and 0.1 N-HCl (12 ml.) were added. After shaking at room temperature for 5 min. and allowing the mixture to stand, the lower phase was separated and washed with an equal volume of chloroform-methanol-0.145 M-NaCl (3:45:47, by vol.). The lower phase was evaporated to dryness, and the residual lipids were dissolved in 10 ml. of chloroform-methanol (9:1, v/v) and applied to a column (10 cm. long $\times$ 2 cm. diam.) of silica gel. The column was eluted successively with 50 ml. of chloroform-methanol (9:1, v/v), 100 ml. of chloroform-methanol (4:1, v/v) and 150 ml. of chloroform-methanol (3:2, v/v). Monitoring the effluent showed that a phospholipid was sharply eluted by the last solvent. The column fractions containing this phospholipid were evaporated to dryness and the lipid was redissolved in chloroform, a small precipitate being removed by centrifuging. The lipid in the supernatant (0.7 mg. of P) was examined by thin-layer chromatography on silica gel H plates. In two solvent systems, namely chloroform-methanol-acetic acid-water (65:50:1:4, by vol.) and chloroform-methanol-5 N-NH₃ (65:30:4:7, by vol.), a single spot of ninhydrin-positive phospholipid (R_F 0.25 and 0.18 respectively) was revealed with no evidence of contamination by other phospholipids or non-phosphorus-containing lipids.

The phospholipid obtained on evaporation was a waxy white solid, similar in appearance to sphingomyelin. It was insoluble in acetone, diethyl ether

and light petroleum, slightly soluble in ethanol and readily soluble in chloroform-methanol (1:1, v/v) or pure chloroform. A portion was decomposed by heating for 4 hr. at 100° with methanolic 2N-HCl in a sealed tube. The methanolsate was evaporated to dryness and partitioned between water and diethyl ether. The water-soluble material showed, on paper chromatography in two solvent systems, a single phosphate ester spot that reacted with ninhydrin and was indistinguishable from an authentic phosphorylethanolamine marker (R_F 0.46 in water-saturated phenol-acetic acid-ethanol, 50:5:6, by vol.; R_F 0.23 in butan-1-ol-acetic acid-water, 12:3:5, by vol.). To determine whether the phosphate ester contained aminoethylphosphonate, which mimics phosphorylethanolamine on paper chromatography, it was subjected to strong hydrolysis with aq. HCl (Dawson & Kemp, 1967). After hydrolysis for 42 hr. at 120° in 5N-HCl, paper ionophoresis at pH 3.6 showed that the phosphate ester had been completely broken down into inorganic phosphate and a ninhydrin-positive base migrating to the same position as ethanolamine. This indicated that the phosphate ester produced by acid methanolysis of the isolated phospholipid was pure phosphorylethanolamine and that none of the acid-resistant aminoethylphosphonate was present.

The phospholipid was hydrolysed for 18 hr. at 70° in the aqueous-methanolic HCl reagent devised by Gauer & Sweeley (1965) for the maximal liberation of free long-chain bases from ceramide-containing lipids. The hydrolysate was evaporated to dryness and partitioned between equal volumes of N-HCl and chloroform (McKibbin & Taylor, 1949). The chloroform-soluble material was chromatographed on a silica gel H plate (chloroform-methanol-water, 50:21:3, by vol.) and showed a ninhydrin-positive base that ran to the same position as a marker of sphingosine (R_F 0.43), obtained by an equivalent hydrolysis of authentic sphingomyelin. The infrared spectrum of the new lipid was almost identical with one published for the analogous ceramide aminoethylphosphonate (Hori *et al.* 1966), showing characteristic amide peaks at 545 and 1645 cm^{-1} . However, there is a much more prominent peak at 1030 cm^{-1} , which is to be expected since it is characteristic of P-O-C bonds (Bellamy, 1958), of which ceramide phosphorylethanolamine contains 2/mol. compared with the 1/mol. of ceramide aminoethylphosphonate. Finally, analysis of the phospholipid for nitrogen and phosphorus content gave values that are consistent with ceramide phosphorylethanolamine [Found: N, 4.2; P, 4.2. Calc. for *N*-acyl (C_{18}) sphingosylphosphorylethanolamine: N, 4.1; P, 4.5%]. The fatty acid methyl esters obtained by treating the phospholipid with methanolic 2N-HCl

Table 1. *Fatty acids of ceramide phosphorylethanolamine from Calliphora erythrocephala*

Composition (%) = (area of peak) $\times$ 100/total area of peaks.

Fatty acid	Composition (%)
14:0	Trace
15:0	Trace
16:0	0.6
17:0	Trace
18:0	6.0
19:0	0.9
20:0	51.0
21:0	Trace
22:0	35.0
24:0	6.5

were examined by gas-liquid chromatography (Table 1) with both polar and non-polar columns. The main components of the mixture of saturated fatty acids were stearic acid, arachidic acid, behenic acid and lignoceric acid.

In *Calliphora erythrocephala* it is probable that in the absence of readily available choline the tissue synthesizes ceramide phosphorylethanolamine to replace sphingomyelin in membrane function. Both phospholipids would exist as zwitterions at a physiological pH, and it is known that in the isolated mitochondria of the same species the membrane role of phosphatidylcholine is replaced almost entirely by phosphatidylethanolamine (Dawson, 1966). It is not known whether a similar argument could explain the presence of the ceramide phosphorylethanolamine in rumen protozoa. Here some choline-containing phospholipids are found, but it is conceivable that the protozoal diet, consisting mainly of ruminal bacteria deficient in choline-containing phospholipids, would limit the phosphorylcholine available for sphingomyelin synthesis, so that some phosphorylethanolamine is transferred enzymically to a ceramide acceptor.

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