The Action of Phospholipase C on Ethanolamine Plasmalogen (2-Acyl-1-alkenylglycerylphosphorylethanolamine)

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1. The phospholipase C of *Bacillus cereus* attacks the ethanolamine plasmalogen of brain to yield a plasmalogenic diglyceride (2-acyl-1-alkenylglycerol). 2. This plasmalogenic diglyceride is analogous to the material obtained by the action of the phospholipase C of *Clostridium welchii* on the choline plasmalogen of heart.

The phospholipase C (EC 3.1.4.3) of *Clostridium welchii* catalyses the hydrolysis of phosphatidylcholine in the presence of Ca^{2+} to yield a D-2,3-diglyceride and phosphorylcholine (Macfarlane & Knight, 1941). The same enzyme catalyses the hydrolysis of sphingomyelin (Macfarlane, 1948; Sribney & Kennedy, 1958) and choline plasmalogen (Kiyasu & Kennedy, 1960; Gottfried & Rapport, 1962; Norton, Gottfried & Rapport, 1962); the products are phosphorylcholine and, respectively, a ceramide (*N*-acylsphingosine) and a plasmalogenic diglyceride (2-acyl-1-alkenylglycerol). Ethanolamine phospholipids are only slightly attacked (Bangham & Dawson, 1962), but the degree of hydrolysis is enhanced in the presence of choline phospholipids (de Gier, de Haas & van Deenen, 1961; Bangham & Dawson, 1962).

The analogous phospholipase from *Bacillus cereus* is much more active towards ethanolamine phospholipids (Chu, 1949; Robinson, Harrison & Poole, 1957; Kushner, 1962) and other phospholipids (Houtsmuller & van Deenen, 1963). There have been no previous reports on the action of phospholipase C of *B. cereus* on ethanolamine plasmalogen (2-acyl-1-alkenylglycerylphosphorylethanolamine) itself, though it is likely that the mixture of phospholipids used as a substrate by Chu (1949) and Robinson *et al.* (1957) contained phospholipids of this type.

The studies described in the present paper stemmed from a desire to prepare a plasmalogenic diglyceride for use in investigations on plasmalogen biosynthesis in nervous tissue.

METHODS

Phospholipase C preparations. A culture of *B. cereus* (strain 654) was kindly provided by Dr D. S. Robinson, M.R.C. Cell Metabolism Research Unit, Oxford, and grown by Dr N. Blakeborough of the Biological Engineering Section of the Chemical Engineering Department of this

University. The peptone broth described by Heimpel (1955) was used and growth was allowed to proceed for 12–15 hr. at pH 7.5–8.5. After removal of the organism by filtration the supernatant was dialysed for 16 hr. against running tap water and for 2 hr. against distilled water. On freeze-drying the dialysed solution, a fluffy buff-coloured powder was obtained which was used without further purification. This was dissolved in water just before use. Attempts at fractionation of the supernatant with $(\text{NH}_4)_2\text{SO}_4$ (Heimpel, 1955; Robinson *et al.* 1957) did not yield a product of higher specific activity in terms of protein.

Phospholipase C preparations from *Cl. welchii* were either purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.) or obtained (α -toxin) from Burroughs Wellcome (Beckenham, Kent).

Analytical methods. Phospholipids were analysed by the hydrolytic procedure of Dawson, Hemington & Davenport (1962), as modified for plasmalogen determination by Ansell & Spanner (1963). Vinyl ether groups were measured either by the spectrophotometric method of Gottfried & Rapport (1962) or by the titrimetric method of Rapport & Franzl (1957). The correction factor of Rapport & Norton (1962) was found necessary for the spectrophotometric method but not for the titrimetric procedure. Acyl ester groups were determined by the method of Stern & Shapiro (1953), total aldehydes by the method of Gray & Macfarlane (1958) and phosphorus by the method of Fiske & Subbarow (1925).

Miscellaneous chemicals. Isobutyl vinyl ether and 2-ethylhexyl vinyl ether were purchased from K & K Laboratories Inc. (Jamaica 33, N.Y., U.S.A.) and octadecyl vinyl ether was provided by Antara Chemicals (New York 14, N.Y., U.S.A.). Other reagents were of A.R. quality wherever possible. The small amount of ethanol used to stabilize the CHCl_3 was not removed.

Preparation of choline lipids from heart. The method used for the extraction of phospholipids was that adapted by McMurray (1964) from the method of Gray & Macfarlane (1958).

Fresh minced ox heart (700 g.) was homogenized with 1.5 l. of acetone in a Waring Blendor. After the mixture had been filtered, the solid residue was re-extracted with acetone and the damp powder stored for 16 hr. in a vacuum desiccator over CaCl_2 and paraffin wax. The yield was about 265 g.

A 50g. sample of this acetone-dried powder was dispersed in 400 ml. of CHCl_3 -methanol (2:1, v/v), the extract filtered and the extraction repeated twice more with 200 ml. of the same solvent. After the extract had been evaporated to dryness under reduced pressure, the residue (5g.) was dissolved in 22 ml. of light petroleum (b.p. 60–80°) and any insoluble residue removed by centrifugation. Phospholipids were precipitated by adding 120 ml. of acetone and cooling the mixture to -20° for 7 hr. The acetone-insoluble material was washed with acetone, dried *in vacuo*, dissolved in CHCl_3 , re-dried and finally dissolved in 12 ml. of CHCl_3 -methanol (2:1, v/v).

In a typical preparation a sample of this solution (7.7 ml. $\equiv$ 48.5 mg. of P) was fractionated on a column of alumina by the method of Davison & Wajda (1959). Choline-containing phospholipids were eluted with CHCl_3 -methanol (1:1, v/v); 561 μg . atoms of P were obtained in this fraction together with 680 μmoles of ester groups and 224 μmoles of vinyl ether groups. Phospholipid analyses of the modified hydrolytic procedure of Dawson *et al.* (1962) demonstrated the presence of 45.8% of phosphatidylcholine, 37.8% of choline plasmalogen (2-acyl-1-alkenylglycerylphosphorylcholine), 15.0% of sphingomyelin and 1.4% of a saturated ether phospholipid (2-acyl-1-alkoxyglycerylphosphorylcholine). The phosphatidylcholine and plasmalogen values corresponded closely with those expected from total ester and vinyl ether values.

Ethanolamine plasmalogen. This was prepared from brain white matter by the method of Ansell & Spanner (1963).

RESULTS

Activity of B. cereus phospholipase C towards ethanolamine plasmalogen

A suitable incubation mixture was prepared as follows. Ethanolamine plasmalogen (15 μmoles) was solubilized in 5 ml. of 0.3% sodium chloride solution containing 30 mg. of sodium deoxycholate. To this solution were added 5 ml. of 0.01M-borate buffer, pH 7.4, and 5 ml. of water containing 5 mg. of enzyme, and the mixture was incubated at 37° . Samples (1 ml.) were taken and shaken with 4 ml. of chloroform-methanol (2:1, v/v) and phosphorus estimations carried out on the chloroform phase. There was a steady loss of lipid phosphorus, over a period of 6 hr., of 0.25 μg . atom of lipid P/hr. (derived from determinations at 1, 1.5, 2, 3 and 4 hr.).

The concentration of Na^+ in the medium contributed by all the components was 4.6 mM. This was not sufficient to inhibit the enzyme (Chu, 1949), a finding confirmed by decreasing the Na^+ concentration still further. A phospholipase C solution, prepared from a culture of *B. cereus* by the precipitation of active protein with ammonium sulphate, and kindly supplied by Professor van Deenen, was of approximately the same activity.

Requirement for Ca^{2+} . It is known that the phospholipase C of *Cl. welchii* is strongly activated by Ca^{2+} and to a smaller extent by Mg^{2+} (Macfarlane & Knight, 1941; Macfarlane, 1948; Chu, 1949).

Chu (1949) also found that the enzyme from *B. cereus* was activated by Ca^{2+} whether lecithin or cephalin was the substrate; the optimum concentration lay between 1 and 4 mM. In our system the addition of calcium chloride up to a maximum concentration of 10 mM had no effect on the rate of release of water-soluble phosphorus. However, the addition of EDTA (1 mM) completely abolished enzyme activity. This implies that the Ca^{2+} or other cation remained bound to the enzyme protein during the dialysis procedure and was present in sufficient amount to give optimum concentration on dilution in the reaction mixture. Since the addition of calcium chloride (5 mM) or zinc chloride (5 mM) (Ottolenghi, 1964) failed to reverse the inhibition caused by 1 mM-EDTA, it is likely that the EDTA complexes formed with the endogenous cations remain bound to the enzyme.

The phospholipase C preparations of Robinson *et al.* (1957) and Houtsmuller & van Deenen (1963) were also active in the absence of added Ca^{2+} . The determination of the endogenous Ca^{2+} in our preparation and that of Houtsmuller & van Deenen (1963) showed that the final concentration in the reaction mixture used was about 70 μM . This is very much less than the optimum found by Chu (1949). The possibility therefore exists that the active cation may not be Ca^{2+} (see Ottolenghi, 1964).

Product of the reaction

Removal of phosphorylethanolamine from the ethanolamine plasmalogen should yield 2-acyl-1-alkenylglycerol. After incubation the difference between the lipid phosphorus and the total vinyl ether groups in the chloroform layer should therefore be a measure of the removal of phosphorylethanolamine from ethanolamine plasmalogen. Measurement of the vinyl ether groups by the spectrophotometric method of Gottfried & Rapport (1962), however, suggested that during incubation they were lost at the same rate as lipid phosphorus (Table 1). On the other hand, measurement of free and bound aldehyde by the method of Gray & Macfarlane (1958) indicated that the vinyl groups were intact after incubation of the ethanolamine plasmalogen with the enzyme (Table 1).

The implication of these observations was that a neutral plasmalogen, e.g. 2-acyl-1-alkenylglycerol, does not react with iodine under the conditions of assay in the method of Gottfried & Rapport (1962). A suggestion to this effect was made in a review by Rapport & Norton (1962), who attributed the lack of reactivity to the relative insolubility of neutral plasmalogen in the methanol of the reaction mixture. The samples in our experiments seemed completely soluble; further, isobutyl vinyl ether, 2-ethylhexyl vinyl ether and octadecyl vinyl

Table 1. *Parallel between the loss of lipid phosphorus from ethanolamine plasmalogen and the lack of reactivity of vinyl ether groups in the spectrophotometric method*

Ethanolamine plasmalogen (15 μ moles) was incubated with the phospholipase C of *B. cereus* in borate buffer as described in the text.

Incubation time (hr.)	Percentage of starting material estimated as:		
	Lipid phosphorus	'Vinyl ether groups' (spectrophotometric assay)	Bound aldehyde
1	79	88	99
1.5	71	77	101
2	59	60	101
2.5	52	57	99
4	47	54	101
6	30	42	—

ether, all of which dissolved well in methanol, did not react in the spectrophotometric system. However, it was confirmed that the titrimetric procedure of Rapport & Franzl (1957) works very well with the synthetic vinyl ethers and with the ethanolamine plasmalogen. It also gave values for the vinyl ether content of the product from the action of the phospholipase on ethanolamine plasmalogen that agreed with values for the total bound aldehyde content (see below).

It was therefore decided to carry out a large-scale preparation of the product of the dephosphorylation of ethanolamine plasmalogen and to compare it with the product of the action of the phospholipase C of *Cl. welchii* on choline plasmalogen.

Preparation of plasmalogenic diglyceride (neutral plasmalogen) from mixed choline phospholipids and ethanolamine plasmalogen

From choline lipids. The method was essentially that of Kiyasu & Kennedy (1960). An amount of mixed choline lipids (see the Methods section) ($\approx 99 \mu\text{g. atoms of P}$) was dissolved in 4 ml. of diethyl ether and incubated at room temperature with 4 ml. of 0.15M-tris-hydrochloric acid buffer, pH 7.4, containing magnesium chloride (25 mM) and 7.8 mg. of *Cl. welchii* α -toxin for 16 hr. The mixture was agitated with a magnetic stirrer. Analysis of the ether phase showed that it contained all the acyl ester groups and total aldehyde of the original lipid but no phosphorus. After removal of the ether, the lipid was dissolved in 5 ml. of benzene and an amount equivalent to 90% of the starting material was applied to a short column of silicic

Fig. 1. Chromatography on silicic acid of the dephosphorylated mixed choline phospholipids from heart (—) compared with that of the ethanolamine plasmalogen of brain (---). A, Lipid containing aldehyde and ester groups; B, lipid containing only ester groups. The details are given in the text.

acid previously equilibrated with the same solvent. Gradient elution of the material from the column was performed by running chloroform-benzene (1:1, v/v) into a mixing chamber originally containing 200 ml. of benzene. Analysis of the 10 ml. fractions showed that there were two peaks, one containing only ester groups and the other bound aldehyde and ester groups (Fig. 1). The dephosphorylated product of sphingomyelin remained adsorbed to the silicic acid under the conditions used. When the contents of tubes 6–14 (see Fig. 1) were pooled the molar ratio of ester to bound aldehyde was found to be 1.0:1.04; in three subsequent experiments the ratios were 1.0:0.98, 1.0:0.99 and 1.0:1.02. No reaction occurred in the spectrophotometric assay of Gottfried & Rapport (1962), but no free aldehyde was present.

From ethanolamine plasmalogen. Ethanolamine plasmalogen (100 μ moles) was dissolved in 22.5 ml. of a solution of sodium deoxycholate (2 mg./ml.) and 7.5 ml. of 1.2% (w/v) sodium chloride. To this were added 30 ml. of 0.1M-borate buffer, pH 7.4, and 30 ml. of a 0.1% solution of the *B. cereus* enzyme preparation. After 23 hr., 360 ml. of chloroform-methanol (2:1, v/v) was added, and the mixture was then shaken and centrifuged and the chloroform layer isolated. The solvent was removed *in vacuo* and the lipid residue dissolved in 5 ml. of

benzene. All of this solution was fractionated on a silicic acid column as described above for the preparation from choline lipids. One peak was obtained (Fig. 1), corresponding exactly to that of the aldehyde-containing material from the choline lipids. When the contents of tubes 5-12 were pooled, analysis gave a molar ratio of ester to bound aldehyde 1.0:0.96. A second preparation gave an identical ratio (Table 2).

The recoveries of bound aldehyde as neutral plasmalogen were 60-70% of the aldehyde in the original starting material.

Experiments in which mixtures of brain phosphatidylcholine and ethanolamine plasmalogen were incubated with the phospholipase C of *Cl. welchii* in borate buffer showed that only the choline lipid was hydrolysed. The same result was obtained when the enzyme was dissolved in tris buffer and shaken with a mixture of the two phospholipids dissolved in diethyl ether.

Properties of the plasmalogenic diglyceride

The properties of the neutral plasmalogen shown in Table 2 are those expected of 2-acyl-1-alkenylglycerol, assuming that the positions of the aldehyde and the fatty acid groups were the same as on the parent plasmalogen. The inability to react under the conditions of the iodometric

spectrophotometric assay was anomalous, but the compounds could be assayed by the titrimetric procedure. They also reacted in the specific colour reaction described by Norton (1959). When the compounds were used as substrates for the plasmalogenase described by Ansell & Spanner (1965) the vinyl ether linkage was cleaved in the absence of Mg^{2+} , though Mg^{2+} enhanced it. Plasmalogenic diglyceride from both choline plasmalogen and ethanolamine plasmalogen was hydrolysed with the liberation of free aldehyde, whereas the intact choline plasmalogen is not a substrate for the brain enzyme (G. B. Ansell & S. Spanner, unpublished work).

DISCUSSION

It has been shown in the present paper that the phospholipase C of *B. cereus* attacks an ethanolamine plasmalogen to yield a neutral lipid similar to that produced by the action of the phospholipase C of *Cl. welchii* on choline plasmalogen. As far as we know, the action of phospholipase C on purified ethanolamine plasmalogen has not been described previously. Nevertheless, the substrates used in many earlier investigations must have contained this phospholipid. Kiyasu & Kennedy (1960) used the enzyme from *Cl. welchii* to prepare plasmalogenic diglyceride from the mixed choline lipids of heart. Rapport and his co-workers (Gottfried & Rapport, 1962; Norton *et al.* 1962) prepared the same material from a pure choline plasmalogen and deacylated it with alkali to yield a glycerol 1-alkenyl ether.

The phospholipase C obtained from *B. cereus* by different workers seems to vary enormously in terms of activity/unit dry wt. of the preparation. The preparation described in the present paper released 0.4 μ mole of water-soluble phosphate/mg. dry wt./hr. from ethanolamine plasmalogen. This was similar to the activity shown by a preparation provided by Professor van Deenen and also to that reported by Kushner & Feldman (1958). The preparation used by Robinson *et al.* (1957) hydrolysed only 3 $m\mu$ moles of ethanolamine phospholipid/mg. dry wt./hr., whereas that of Chu (1949) was nearly 40 times as active as our preparation. Presumably, differences in strain and methods of preparation account for the variations in activity.

It was found unnecessary to add Ca^{2+} to the reaction mixture in order to activate the enzyme; the preparations of Robinson *et al.* (1957) and Houtsmuller & van Deenen (1963) were also active without additional Ca^{2+} . On the other hand, low concentrations of EDTA completely inhibited activity, indicating a requirement for and the presence of a bivalent cation. This bivalent cation must have remained bound to the preparation

Table 2. *Properties of neutral lipid prepared by the action of phospholipase C on brain ethanolamine plasmalogen and mixed choline lipids from heart*

Results are expressed in μ moles/ μ mole of lipid; the ester values have been adjusted to the theoretical.

	Lipid from brain ethanolamine plasmalogen	Lipid from mixed choline lipids	
		Diglyceride	Plasmalogenic diglyceride
Acyl ester	1.00	2.00	1.00
Total aldehyde	0.96	0	1.01
Free aldehyde	0	0	0
Vinyl ether groups*	0.96	0	0.96
Glycerol released on saponification		1.02	0
Reaction with Hg^{2+} †	+	—	+
R_f on thin-layer chromatogram‡		0.51	0.56

* Titrimetric method of Rapport & Franzl (1957).

† Colour reaction of Norton (1959).

‡ In light petroleum (b.p. 60-80°)-acetone-acetic acid (45:15:1, by vol.) on silica gel G (E. Merck A.-G., Darmstadt, Germany).

during the dialysis. Determinations of Ca^{2+} indicated the presence of about $9\mu\text{g.}$ of $\text{Ca}^{2+}/\text{mg.}$ dry wt. of the preparation, which on dilution in the reaction mixture would give a very low concentration of the ion (about $70\mu\text{M}$). Sheldon (1960) found that Zn^{2+} was the most potent activator of the phospholipase C of *Cl. welchii*. Ottolenghi (1964) reported that Zn^{2+} is the likely metal ion cofactor for the enzyme from *B. cereus* and that it remains bound to the protein during purification procedures. It is possible, therefore, that some bivalent cation other than Ca^{2+} may be present in sufficient amounts in the preparations used by us and others.

Plasmalogenic diglycerides from both ethanolamine and choline plasmalogens are suitable as substrates for studying the action of enzymes cleaving the vinyl ether linkage. The commercially available unsaturated ethers, isobutyl vinyl ether, 2-ethylhexyl vinyl ether and octadecyl vinyl ether, all yield acetaldehyde on hydrolysis; they are not therefore strictly comparable with unsaturated ethers of long-chain aldehydes.

A plasmalogenic diglyceride from brain ethanolamine plasmalogen would also be suitable for studying the reaction with cytidine diphosphate esters of phospholipid bases (cf. Kiyasu & Kennedy, 1960).

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