Studies on the Hydrolysis of Lecithin by a *Penicillium notatum* Phospholipase B Preparation

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In a previous publication it was reported that the phospholipase B prepared from the mycelium of the mould *Penicillium notatum*, which normally attacks only lysophosphoglycerides, could also hydrolyse lecithin provided that certain activating lipids were present in the system (Dawson, 1957). Two such activating lipids have been isolated from liver tissue and identified as the phosphoglyceride, monophosphoinositide and also a polyglycerol phospholipid (Dawson, 1958*a*). When either of these lipids is added in low concentration to a system containing lecithin and *P. notatum* phospholipase B there is an immediate hydrolysis of the lecithin, with the liberation of glycerylphosphorylcholine and free fatty acids.

In the research reported in the present paper this apparent change of specificity of the enzyme in the presence of the activating lipids has been investigated in some detail to try to determine the nature of its mechanism. The activating effect is confined to a comparatively small group of lipid substances which have, however, very little structural similarities, except that they contain two or more fatty-acyl ester bonds which are necessary for the activation. The activated lecithin hydrolysis is extremely sensitive to the effects of fluoride, and bivalent ions such as Ca^{2+} and Mg^{2+} are also inhibitory.

Monophosphoinositide, one of the activating lipids, has been shown to be attacked by the *P. notatum* phospholipase B preparation, a reaction which can be completely inhibited by the presence of lecithin. However, the possibility of an acyl transferase type of reaction between lecithin and monophosphoinositide has been ruled out by the use of ^{14}C -fatty acid-labelled lecithin as a substrate and the re-isolation of the activating monophosphoinositide from the incubation medium.

By employing a simple ether-extractability test it has been demonstrated that two of the activating lipids, monophosphoinositide and cardiolipin, cause very pronounced effects on the surface of the lecithin particles in an emulsion, changing it from a lipid-like extractable form to that of a non-lipid. It is suggested that the role of these lipids in promoting lecithin hydrolysis may be to introduce,

on the surface of the lecithin particles, certain additional polar groups which are necessary for the enzymic attack.

METHODS

Enzyme preparation, etc. The preparation of *P. notatum* phospholipase B has been described (Dawson, 1957). Attempts to purify the enzyme further than by a simple ammonium sulphate fractionation have met with little success, probably because of the very low protein concentration found in the mould autolysates. Usually the enzyme was stabilized by the addition of glycerol (2 vol.), but the enzyme in aqueous solution retained its activity for many months at -15° .

Incubation. It was convenient to store many of the lipids used in organic solvent solution (chloroform or chloroform-methanol) at -15° . The incubations were performed in 10 ml. capacity glass-stoppered tubes, into which the requisite amounts of lipid solution were pipetted. The solvent was removed *in vacuo* at about 40° : a splash head with a ground-glass joint which fitted into the neck of the tube was convenient for this purpose. Buffer was then introduced and the lipid emulsified by shaking at 37° . After introduction of the enzyme and incubation at 37° for a requisite period, the reaction was stopped by the addition of 0.2 vol. of 5% serum albumin solution and 0.8 vol. of 10% (w/v) perchloric acid. The serum albumin facilitated the removal of the unchanged lipid substrate and the liberated fatty acids by centrifuging and filtration, so that the water-soluble hydrolysis products could be determined.

Preparation of lipids

Ovolecithin. This was prepared from the acetone-insoluble lipids of egg yolk (Hanahan, Turner & Jayko, 1952). A solution of these in chloroform-methanol (1:1, v/v) was passed through an activated-alumina column. The eluate was evaporated to dryness and the lecithin fractionated on a silicic acid column to remove lysolecithin with chloroform-methanol (17:8, v/v) as solvent (Lea, Rhodes & Stoll, 1955). Failure to remove lysolecithin resulted in a very small liberation of glycerylphosphorylcholine in the presence of the phospholipase B preparation which was not observed with pure lecithin. Liver lecithin used in a few experiments was isolated in a similar manner, but inevitably this product would contain a small percentage of choline-containing plasmalogen.

Lecithin labelled with ^{14}C fatty acids. The preparation of this was based on observations made by Bergström, Borgström & Rottenberg (1952), Blomstrand (1955) and Hanahan & Blomstrand (1956). A rat (183 g.) was given by

stomach tube 100 μ C of [2-¹⁴C]stearic acid (30 mg.) dissolved in 1 ml. of warm olive oil. Free access to food and water was allowed. After 7-2 hr. the animal was killed and the liver and small intestine were removed, the latter being cleaned by syringing physiological saline through it. The organs were then homogenized independently in 8 vol. of chloroform-methanol (1:1, v/v) and worked up separately to isolate the lecithin. Each extract was evaporated to dryness *in vacuo* and the residue extracted with ethanol. The ethanol-soluble lipids were evaporated to dryness and the residue extracted with ether. The ethereal solution was cooled to 0° and ether-insoluble lipids removed by centrifuging. The ether-soluble phospholipids were precipitated by adding excess of acetone and then this acetone precipitation was repeated. Lecithin was then prepared from these ether-soluble, acetone-insoluble phospholipids by chromatography on an activated-alumina adsorption column with chloroform-methanol (1:1, v/v) as developing solvent (Rhodes & Lea, 1957). The liver lecithin prepared (4.2 mg. of P) had an activity of 1.95×10^6 counts/min./ μ mole and the intestinal lecithin (1.1 mg. of P) 3.4×10^6 counts/min./ μ mole in a proportional-flow counter of the Robinson (1950) pattern.

Monophosphoinositide. This was isolated from liver tissue (horse, sheep, rat, guinea pig or rabbit), by methods similar to those described in a previous paper (Dawson, 1958a). The methanol-soluble 'kephalins' were adsorbed on an alumina column from chloroform-methanol (1:1, v/v). The 'kephalins' were then eluted with chloroform-ethanol-water (2:5:2, by vol.); the lipid inositol came off the column considerably after the main bulk of the nitrogen-containing kephalins, so that a considerable enrichment of the monophosphoinositide could be obtained by discarding the initial non-inositol-containing fractions coming from the column. The monophosphoinositide-rich fraction was then chromatographed on a silicic acid column with chloroform-methanol (4:1, v/v) as solvent. The nitrogen-containing kephalins emerged from the column first, and eventually the monophosphoinositide. The appearance of the monophosphoinositide could be hastened by changing the solvent to chloroform-methanol (3:2, v/v) as suggested by the work of Hanahan, Dittmer & Warashina (1957). The resultant product contained very little nitrogen or ninhydrin-reacting material. Inositol analysis indicated that it was better than 90% pure. Flame photometry showed the absence of calcium in the preparation, but appreciable sodium (5 atoms/mol.) and a little potassium were present.

Other materials

Tripalmitin was a commercial sample recrystallized twice from ethanol; analysis showed it to contain no phosphorus. Cardiolipin was obtained through the generosity of Dr M. G. Macfarlane and was assumed to have 2 phosphorus atoms/mol. Monoglycerides and diglycerides were kindly given by Dr T. H. Bevan. Lysolecithin was prepared by a method described previously (Dawson, 1956). Other chemicals used were the best commercial samples available. Aluminium oxide (Hopkin and Williams Ltd., 'M.F.C.' 100-200 mesh), and silicic acid (Mallinckrodt) were used for chromatography. Formic acid-NaOH buffers were prepared by titration with use of a pH meter.

Analytical methods

Methods of estimation used for choline, glycerylphosphorylcholine, fatty acids, acyl fatty-ester bonds, inositol and phosphorus have been listed previously (Dawson, 1958a). In the estimation of choline the precipitation of choline periodide tended to be non-quantitative in the presence of appreciable amounts of glycerol which was introduced into the incubation medium by the use of glycerol-stabilized enzyme. Consequently the choline periodide was precipitated out of a solution containing less than 5% of glycerol and 1 hr. or more at 0° was allowed for precipitation.

RESULTS

Nature of the enzymically catalysed reaction

It has already been shown that the enzymic attack of *P. notatum* extracts on lecithin results in the liberation of water-soluble glycerylphosphorylcholine and free fatty acids (Dawson, 1957, 1958a). Further experiments demonstrated that although the extracts contained a glycerylphosphorylcholine diesterase which was not removed by treatment with ammonium sulphate (50 g./100 ml.), this was not appreciably active at the acid pH at which lecithin breakdown occurred.

Difficulty was encountered initially in relating the accumulation of water-soluble phosphorus or choline in the incubation medium with the disappearance of acyl-fatty ester bonds. Results indicated this was due to the presence of glycerol introduced in the enzyme preparation and also volatile fatty acids in the buffers used. Consequently, experiments were performed with freshly prepared enzyme in aqueous solution and no buffer. The incubation media were saturated with carbon dioxide to reduce the pH sufficiently for the reaction to occur. In these circumstances the percentage liberation of water-soluble phosphorus corresponded closely to the percentage decrease of acyl-fatty ester bonds measured by a hydroxamic acid technique (Stern & Shapiro, 1953).

All the available evidence therefore indicates that when the enzyme hydrolyses lecithin in the presence of activating lipid the net reaction occurring is $\text{lecithin} + 2\text{H}_2\text{O} \rightarrow \text{glycerylphosphorylcholine} + 2 \text{ fatty acids}$. It is shown below that in the presence of lecithin no breakdown of activating lipid can be detected.

Lipids which activate lecithin breakdown by Penicillium notatum phospholipase B

Two lipids were isolated from liver, which consistently activated the lecithin -*P. notatum* extract system when added in low concentrations. These were identified as monophosphoinositide and polyglycerol phospholipid (Dawson, 1958a). In the present investigation a survey has been made of various substances, both lipid and non-lipid,

Table 1. *Lipids and non-lipids tested for activation of the lecithin-Penicillium notatum extract system*

Usually lecithin (2.6 μ moles) was emulsified with 1 ml. of 0.2M-acetate buffer (pH 4), and incubated at 37° for 1 hr. with 0.2 ml. of *P. notatum* extract. All results were confirmed in independent runs. Lipids were mixed with the lecithin and a lecithin-lipid emulsion was prepared.

Strong activation

Liver polyglycerol phospholipid
Cardiolipin
Monophosphoinositide

Moderate activation

Tripalmitin
Tristearin
Folch phospholipid fraction I (4 mg. from human brain)*

Low activation

Palmitic acid (2 μ moles)
Oleic acid (2 μ moles)
Elaidic acid (2 μ moles)
Cardiolipin mixed fatty acids (300 μ g.)

No activation

Lipids

Triolein (3 μ moles)
Triacetin (1 μ mole)
Cholesterol (3 μ moles)
Stearic acid (3 μ moles)
Erucic acid (1 μ mole)
1-Monolaurin (1.2 μ moles)
1-Monostearin (2 μ moles)
1:2-Dilaurin (2 μ moles)
1:2-Dipalmitin (1.2 μ moles)
Hexadecanol (1 μ mole)
Octadecanol (1 μ mole)
Phosphatidylethanolamine (3.7 μ moles from liver)†
Lysolecithin (0.7 μ mole)

Non-lipids

Sodium diphenylphosphate (2 mM)
Sodium ethylenediaminetetra-acetate (2 mM)
CaCl₂ (2 mM)
MgCl₂ (2 mM)
MnCl₂ (2 mM)
FeSO₄ (2 mM)
Hexadecyltrimethylammonium bromide (0.017%)
Sodium secondary-alkyl sulphates (0.04%)
Lissapol (0.1%)
Glycerol (5-10%)
Ether (0.2 or 3 ml.)

* Mainly phosphatidylserine plus diphosphoinositide

† Some samples of impure liver phosphatidylethanolamine and synthetic kephalin have given slight activation.

which it was thought might be activators of lecithin breakdown. Only four of the pure compounds tested showed any appreciable activity in the test system; the most active on a molar basis was cardiolipin (2 P atoms/mol. assumed) closely followed by monophosphoinositide, and much less active were the saturated triglycerides, tripalmitin and tristearin (Table 1). Liver polyglycerol phospholipid was not examined in the present work, because its method of isolation from liver (Dawson, 1958a) did not give sufficient quantities for systematic investigation. However, according to all the available evidence cardiolipin probably possesses a structure very similar to that of liver polyglycerol phospholipid (Pangborn, 1947; McKibbin & Taylor, 1952; Macfarlane & Gray, 1957).

Fig. 1 shows the lecithinase activity obtained when various amounts of activating lipid are present in a *P. notatum* extract-lecithin system. In these experiments the lecithin hydrolysis was measured by using an emulsion of lecithin mixed with the activating lipid. Activity was still observed if emulsions of the two lipids in buffer were prepared separately and then mixed, but

generally it was somewhat lower than when the mixed lipid emulsion was used as substrate. The use of liver lecithin in place of ovolecithin did not appreciably affect the pattern of activity observed.

A number of features of the activity curves can be noted. No detectable activity occurs in the absence of activating lipid. In initial experiments very slight activity was observed, but, after purification of the lecithin from contaminating lysolecithin on silicic acid columns, activity was no longer detectable. It was consistently found that a certain minimum amount of activating lipid needed to be added before any breakdown of lecithin occurred; although this minimum threshold value could be lowered somewhat by prolonging the time of incubation, it was still perfectly definite even when large amounts of the enzyme preparation were added to the system. Once the threshold concentration of activating lipid was exceeded, rapid breakdown of lecithin occurred. With emulsions of lecithin plus cardiolipin or monophosphoinositide at higher concentrations, the reaction appeared to proceed at a distinctly slower rate. The activity promoted by tripalmitin was much less marked than that produced by the

other two lipids, even though the incubation time was longer and the highest concentration of enzyme was used. Very similar results were observed with tristearin.

The activating effect of any two activating lipids was greater than the activity produced by either independently. This is clearly demonstrated in the results given in Table 2. Whereas the use of a mixed activating lipid, e.g. tripalmitin + cardiolipin, results in a very appreciable breakdown of lecithin, the addition of either activating lipid at

the same concentration, but independently to the same system, resulted in no glycerylphosphorylcholine release. This is presumably because the minimum threshold concentration of activating lipid was not exceeded. The use of an activating lipid plus a non-activating lipid such as triolein or cholesterol did not show any similar effect.

In Table 1 is given a list of other lipids and non-lipids which have been tested at pH 4.0 under similar experimental conditions and have been found to cause no appreciable activation of the lecithin-*P. notatum* system. It is noteworthy that saturation of the incubation medium with ether or the addition of excess of ether does not result in activation, as has been observed with snake-venom phospholipase A (Hanahan, 1952) and the lecithinases in plants which split off phosphorylcholine or free choline from the lecithin molecule (Kates, 1953; Hanahan & Vercamer, 1954). A very small release of glycerylphosphorylcholine was observed on the addition of high concentrations of oleic, elaidic, palmitic acids or the mixed fatty acids prepared from cardiolipin, but not with stearic or erucic acids. [Epstein & Shapiro (1957) have recently shown that an intestinal lecithinase is stimulated by the addition of fatty acids, although an absolute requirement for these was not demonstrated.] Ca^{2+} ions and detergents showed no activity and were in fact inhibitory when added to a system already activated by the addition of monophosphoinositide or cardiolipin. Water-soluble phosphodiesterases which can stimulate the choline-splitting lecithinase of cabbage (Davidson & Long, 1957) were without activity.

pH optima of the activated lecithin breakdown by *Penicillium notatum* extracts

The pH was determined at which maximum lecithin breakdown occurred in the presence of a constant level of activating lipid. Fig. 2 shows the results obtained with a mixed substrate of lecithin

Fig. 1. Effect of the amount of activating lipid added to lecithin to promote its hydrolysis by *P. notatum* extracts. All tubes contained ovolecithin (2.6 μmoles) and were incubated at 37° in a final medium volume of 1.2 ml. ●, Monophosphoinositide as activating lipid; A: 0.2 ml. of enzyme, 0.08 M-formate buffer (pH 3.3). Incubation, 25 min. B: 0.1 ml. of enzyme, 0.08 M-formate buffer (pH 3.3). Incubation, 35 min. C: 0.04 ml. of enzyme, 0.08 M-formate buffer (pH 3.3). Incubation, 60 min. ○, Cardiolipin as activating lipid; 0.1 ml. of enzyme, 0.08 M-formate buffer (pH 3.3). Incubation, 40 min. △, Tripalmitin as activating lipid; 0.2 ml. of enzyme, 0.08 M-acetate buffer (pH 4.0). Incubation, 60 min.

Table 2. Breakdown of lecithin by *Penicillium notatum* extract in the presence of mixed activating lipids

Lipids mixed with 2.6 μmoles of lecithin were emulsified in 1 ml. of 0.2 M-formic acid-NaOH buffer (pH 3.3) and 0.2 ml. of glycerol-treated *P. notatum* extract was added. Incubation, 65 min. at 37°.

Activating lipids added	Glycerylphosphorylcholine liberated (μmoles)
None	0
Monophosphoinositide (0.2 μmole)	0
Cardiolipin (0.09 μmole)	0
Tripalmitin (1.2 μmoles)	0
Monophosphoinositide (0.2 μmole) + cardiolipin (0.09 μmole)	1.34
Monophosphoinositide (0.2 μmole) + tripalmitin (1.2 μmoles)	0.81
Cardiolipin (0.09 μmole) + tripalmitin (1.2 μmoles)	1.48
Cholesterol (1.2 μmoles)	0
Triolein (1.2 μmoles)	0
Monophosphoinositide (0.2 μmole) + cholesterol (1.2 μmoles)	0
Monophosphoinositide (0.2 μmole) + triolein (1.2 μmoles)	0.05

Fig. 2. pH optima of the monophosphoinositide-promoted lecithinase activity of *P. notatum* extracts in various buffers. The substrate was emulsified with 1 ml. of buffer and the tubes were then incubated at 37° with 0.2 ml. of *P. notatum* extract. ○—○, Sodium formate-formic acid buffers (0.2M). Substrate (2.5 μmoles of lecithin + 0.48 μmole of monophosphoinositide). Incubation, 90 min. ●, Potassium hydrogen phthalate-HCl buffers (0.05M). Substrate (2.5 μmoles of lecithin + 0.27 μmole of monophosphoinositide). Incubation, 120 min. △, Sodium acetate-acetic acid buffers (0.2M). Substrate (2.5 μmoles of lecithin + 0.48 μmole of monophosphoinositide). Incubation, 90 min. ○---○, Disodium hydrogen phosphate (0.2M) + citric acid (0.1M) mixtures. Substrate (2.5 μmoles of lecithin + 0.27 μmole of monophosphoinositide). Incubation, 120 min.

Fig. 3. pH optima of lecithinase activity of *P. notatum* extracts promoted with various activating lipids. ○—○, Substrate, 2.6 μmoles of lecithin + 1 μmole of tripalmitin. Sodium acetate-acetic acid buffers. Incubation, 150 min. at 37°. ●, Substrate, 2.6 μmoles of lecithin + 0.17 μmole of cardiolipin. Sodium formate-formic acid buffers. Incubation, 40 min. at 37°. ○---○, Substrate, 2.5 μmoles of lecithin + 0.48 μmole of monophosphoinositide. Sodium formate-formic acid buffers. Incubation, 90 min. at 37°.

plus monophosphoinositide, and a number of different types of buffer. The optimum pH seems to lie between 3.1 and 3.4 in all of the buffers used, the activity in the citrate-phosphate buffer (McIlvaine, 1921) being somewhat lower than in the phthalate or volatile fatty acid buffers. Whereas in the formate, phthalate and citrate-phosphate buffers little activity was observed at pH 3.8 or 4, appreciable activity was observed at these pH values with acetate buffer. At pH 4.2 no activity was found with any buffer.

The pH optima of the activations produced by various lipids are compared in Fig. 3 for volatile fatty acid buffers. Whereas the pH-activity curve for a mixed cardiolipin plus lecithin substrate is closely similar to that of a monophosphoinositide plus lecithin substrate, the curve for a tripalmitin plus lecithin substrate is quite different, reaching a pH optimum at 4.2.

Fairbairn (1948) reported a pH optimum for *P. notatum* phospholipase B at 4, while Contardi & Ercoli (1933) found an optimum of pH 3.5 for the corresponding enzyme from *Aspergillus oryzae*, which is possibly a reflexion of the acid conditions under which these moulds flourish. With the present enzyme preparation and formate buffers, the maximum hydrolysis of lysolecithin occurred at pH 3.3. This was identical with the lecithin breakdown activated with cardiolipin or monophosphoinositide, but not that activated with tripalmitin. However, whereas appreciable lysolecithin hydrolysis still took place at pH 4.4, no detectable attack of lecithin plus cardiolipin or monophosphoinositide substrate occurred at this pH.

Effect of metals and inhibitors on the reaction

P. notatum phospholipase B is an enzyme which is comparatively insensitive to metal ions or inhibitors (Fairbairn, 1948). It was therefore somewhat surprising to find that the lecithinase activity of the preparation in the presence of monophosphoinositide was completely abolished by the addition of bivalent ions such as Ca^{2+} , Mg^{2+} , Mn^{2+} or Fe^{2+} at a concentration of 2.1 mM. The lecithinase activity produced by the addition of monophosphoinositide seemed more sensitive to the effects of Ca^{2+} than that promoted by the presence of cardiolipin or tripalmitin in the substrate (Table 3). The addition of Ca^{2+} ions at a concentration of 2.1 mM was completely without effect on the lysolecithinase activity of the extract. Results with Mg^{2+} were very similar to those obtained with Ca^{2+} , the most sensitive being again the monophosphoinositide-promoted lecithinase activity. The addition of ethylenediaminetetra-acetic acid in equivalent concentration to that of the added Ca^{2+} or Mg^{2+} ions produced little reversal of the inhibitory effect

of the bivalent ion on the lecithinase activity except when tripalmitin was used as the activating lipid (Table 3). In fact, with cardiolipin-activated lecithinase activity, the addition of ethylenediaminetetra-acetic acid consistently increased the inhibition produced by calcium, even though the chelating agent added on its own was non-inhibitory.

Because of the inhibitory action of bivalent ions on lecithinase activity it was surprising that fluoride was a potent inhibitor of the reaction. This applied whether the preparation was attacking a substrate of lecithin plus cardiolipin, or lecithin plus monophosphoinositide, or lysolecithin on its own. Effects were still apparent on the cardiolipin-activated lecithinase at a level of 0.41 mM fluoride. Fluoride is known to inhibit other esterases such as the alkaline and acid phosphatases, lipases, and cholinesterases, but all these enzymes, in contrast with *P. notatum* phospholipase *B*, are activated by bivalent-metal ions.

Another unexpected finding was the insensitivity of the lysolecithinase activity of the preparation to the action of cyanide. Fairbairn (1948) reported that cyanide was an inhibitor of *P. notatum* lysolecithinase, and it has not been possible to account for this discrepancy. Both the commercial detergents Comprox A, which is a mixture of sodium secondary-alkyl sulphates, and Cetavlon (hexadecyltrimethylammonium bromide) completely inhibited the lysolecithinase and cardiolipin-activated lecithinase of the *P. notatum* extract at concentrations of 0.08 %.

Experiments to show that the activating lipids structure is essential for lecithin hydrolysis

High concentrations of the mixed fatty acids isolated from cardiolipin were found to be weakly active in promoting lecithin hydrolysis. However, calculation showed that this could not possibly account for the activity of the cardiolipin molecule in the lecithin-*P. notatum* extract system. This was

Table 3. *Effect of inhibitors on the lecithinase activity of Penicillium notatum extract promoted with various lipids*

Inhibitor	Concn. (mm)	% of normal activity			
		Lecithin + monophospho- inositide substrate	Lecithin + cardiolipin substrate	Lecithin + tripalmitin substrate	Lysolecithin substrate
Ca ²⁺	1.67	0	14	—	101
	0.83	0-14	82	67	—
Ca ²⁺ + ethylenediamine- tetra-acetic acid	0.83	5	50	94	—
Mg ²⁺	2.1	0	—	—	—
	0.83	14	60	69	—
Mg ²⁺ + ethylenediamine- tetra-acetic acid	0.83	7	71	91	—
NaF	16.4	0	0	—	0
	4.1	0	4	—	0
	2.1	—	30	—	—
	1.1	—	56	—	—
	0.41	—	89	—	—
KCN	1.67	—	—	—	100
	0.83	—	66-93	—	102
Comprox A	0.08 %	—	0	—	0
Cetavlon	0.08 %	—	0	—	0

Table 4. *Experiment to show that hydrolysis by sodium hydroxide of cardiolipin and monophosphoinositide destroys their ability to activate the Penicillium notatum extract-lecithin system*

Lecithin (2.6 μ mole), monophosphoinositide (0.48 μ mole), cardiolipin (0.44 μ mole), 1 ml. of 0.2M-formate buffer (pH 3.3), 0.2 ml. of *P. notatum* extract; incubation, 30 min. (monophosphoinositide) and 120 min. (cardiolipin) at 37°.

Substrate	Glycerolphosphoryl- choline liberated (μ mole)
Lecithin + cardiolipin	0.50
Lecithin + cardiolipin hydrolysed with NaOH	0.01
Lecithin + monophosphoinositide	0.36
Lecithin + monophosphoinositide hydrolysed with NaOH	0.02

confirmed for cardiolipin and also monophosphoinositide in experiments in which the fatty acyl-ester bond was broken by a brief incubation at 37° with methanolic NaOH (Dawson, 1954). The methanol was removed and after the NaOH had been neutralized the mixed hydrolysis products were added to a lecithin emulsion and the whole was incubated with *P. notatum* enzyme. Lecithin hydrolysis occurred only in equivalent control tubes in which the hydrolysis by sodium hydroxide had been omitted (Table 4), indicating that the integrity of lipid structure is essential for activity.

Similarly, the activity shown by the triglycerides, tripalmitin or tristearin is unlikely to be due to hydrolysis products of these compounds, since stearic acid, 1-monostearin and 1:2-dipalmitin were completely without activity (Table 1).

Action of the Penicillium notatum extract on activating lipids

Experiments in which cardiolipin alone was incubated with *P. notatum* extract did not indicate either an increase of non-lipid phosphorus or a decrease of acyl-ester bonds. Similar experiments with tripalmitin again showed no decrease of acyl-ester bonds or an increase of titratable fatty acids. However, when the *P. notatum* extract was incubated with monophosphoinositide it was apparent that there was both a liberation of free fatty acid and non-lipid phosphorus and also a decrease of acyl-ester bonds. This decomposition is illustrated by the experiments reported in Table 5, in which the release of water-soluble inositol from the lipid has been measured. It can be seen that if ovoidlecithin is added to the monophosphoinositide no reaction occurs (Table 5, Expt. 2). However, if the incubation is prolonged so that most of the ovoidlecithin is broken down through the activation effect of the monophosphoinositide, then some liberation of non-lipid inositol does take place

(Table 5, Expt. 1). It has been shown that the water-soluble phosphorus and inositol released from the monophosphoinositide is partly in the form of glycerylphosphorylinositol (Dawson, 1958b) and details of the reaction are being published elsewhere.

Experiments to determine whether there is an acyl transfer between lecithin and monophosphoinositide

The finding that monophosphoinositide was broken down by *P. notatum* extract and that this hydrolysis was completely inhibited by the presence of ovoidlecithin, which in these circumstances broke down itself to produce glycerylphosphorylcholine, suggested the possibility of an acyl transfer of fatty acids from lecithin to monophosphoinositide. *P. notatum* is known to contain an enzyme which can catalyse intra-acyl transfers of fatty acids (Uziel & Hanahan, 1957). Consequently experiments were performed in which lecithin labelled with ¹⁴C-fatty acid, and inactive monophosphoinositide, were incubated with *P. notatum* extract and then the monophosphoinositide was re-isolated from the incubation medium to ascertain whether it possessed any radioactivity. It can be seen from the results in Table 6 that no evidence could be obtained for the transfer of labelled-fatty acid from the lecithin to monophosphoinositide, although it was obvious that a considerable release of free fatty acid from the lecithin had occurred. In these experiments intestinal lecithin was used as well as liver lecithin, because the results of Hanahan & Blomstrand (1956) indicate that after the feeding of saturated [¹⁴C]fatty acid to rats their liver lecithin is predominantly labelled in the β -position, whereas the intestinal lecithin has considerable label in the α -position. The recovery of monophosphoinositide from the incubation medium was low (26–51 %) but its identity was confirmed by inositol assay.

Table 5. *Liberation of acid-soluble inositol on incubating monophosphoinositide with Penicillium notatum extract*

Expt. 1: 1 ml. of acetate buffer (pH 4), 0.2 ml. of enzyme, incubated with substrate 5 hr. at 37°. Expt. 2: 1 ml. of formate buffer (pH 3.3), 0.2 ml. of enzyme, incubated with substrate 20 min. at 37°.

	Acid-soluble inositol liberated (μ g.)
Expt. 1	
1. Monophosphoinositide ($\equiv 45.5 \mu$ g. of inositol) alone	<0.2
2. <i>P. notatum</i> extract alone	<0.2
3. Monophosphoinositide ($\equiv 45.5 \mu$ g. of inositol) + <i>P. notatum</i> extract	37.0
4. As 3, plus ovoidlecithin (1.9 μ moles)	19.3
Expt. 2	
1. Monophosphoinositide ($\equiv 116 \mu$ g. of inositol) alone	<0.1
2. Monophosphoinositide ($\equiv 116 \mu$ g. of inositol) + <i>P. notatum</i> extract	31.0
3. As 2, plus ovoidlecithin (2.6 μ moles)	<0.1
4. As 2, plus lysolecithin (8.4 μ moles)	<0.1

Does the activating lipid cause a precipitation of enzyme on the surface of the lecithin particles?

Kates & Gorham (1957) have recently produced evidence that the ether activation of a phospholipase occurring in plant plastids may be due to its producing a coalescence of lecithin and chloroplast phases. In the present work it was constantly observed that, when enzyme solution was pipetted into control tubes containing only activating lipid (monophosphoinositide or cardiolipin), a marked increase in turbidity occurred. Consequently it appeared possible that the function of the activating lipid in promoting lecithin hydrolysis was in some way to attach or precipitate the enzyme on the surface of the lecithin particle so that the reaction could take place. Experiments were performed therefore in which, after the addition of

enzyme to the incubation medium, the lecithin was partially separated by high-speed centrifuging. The phospholipase *B* content of the supernatant and lipid precipitate were then compared by using lysolecithin as test substrate. The results showed that while there was an even distribution of enzyme between the supernatant and 'precipitate' fractions, 78–87% of the phospholipid was present in the 'precipitate' fraction (Table 7). This suggests that no appreciable precipitation of the phospholipase *B* can have occurred on the surface of the lecithin particle in the presence of activating lipid. It is possible, however, that an enzyme attached to a lecithin plus activating lipid particle may not be available for lysolecithin hydrolysis. Control experiments indicated that lecithin itself did not appreciably affect the assay of phospholipase *B* with lysolecithin as test substrate.

Table 6. *Incubation of labelled lecithin with monophosphoinositide and Penicillium notatum extract followed by re-isolation of the lipids*

Expt. 1: ¹⁴C-Labelled liver lecithin (416 µg. of P) + monophosphoinositide (60 µg. of P) was incubated at 37° for 5 min. in 0.02M-formate buffer at pH 3.3, ±0.5 ml. of aqueous *P. notatum* extract. Total vol., 1 ml. Expt. 2: ¹⁴C-Labelled intestinal lecithin (428 µg. of P) + monophosphoinositide (62 µg. of P) was incubated at 37° for 55 min. in water saturated with CO₂, ±0.5 ml. of aqueous *P. notatum* extract. Total vol., 1 ml. The lipids were re-isolated from a CHCl₃-methanol extract of the incubation medium. They were first applied to an activated alumina column in CHCl₃-methanol (1:1, v/v); the monophosphoinositide and fatty acids remained on the column and were eluted with CHCl₃-ethanol-water (2:5:2, by vol.). They were then applied on a silicic acid column in CHCl₃-methanol (9:1, v/v). The fatty acids were not absorbed; the monophosphoinositide remained on the column and was subsequently eluted with CHCl₃-methanol (3:2, v/v). The radioactivity of the fractions was determined with a mica-window Geiger-Müller tube. Figures in parentheses represent recoveries of phosphorus (µg.).

	Counting rates of re-isolated lipids (counts/min.)		
	Lecithin	Free fatty acid fraction	Monophosphoinositide
Expt. 1. Liver lecithin (containing [¹⁴ C]-fatty acid)			
Incubated without enzyme	13 400 (363)	27	55 (28)
Incubated with enzyme	10 300 (299)	1280	45 (17)
Expt. 2. Intestinal lecithin (containing [¹⁴ C]-fatty acid)			
Incubated without enzyme	9 404 (182)*	18 (0)	21 (31)
Incubated with enzyme	9 350 (187)	5352 (0)	24 (26)

* Some of fraction lost.

Table 7. *Distribution of phospholipase B between lecithin + 'activating lipid' particles and their suspending medium.*

Phospholipids were emulsified in 1 ml. of water and 0.1 ml. of aqueous *P. notatum* extract was added. The particles were centrifuged at 13 000 *g* for 15 min. at 0° and 0.5 ml. of supernatant was withdrawn, leaving 0.6 ml. of a 'precipitate' fraction. Glycerol (2 vol.) was added to both fractions, which were then assayed for enzymic activity and phospholipid concentration.

Particles	Percentage distribution of enzyme concentration		Percentage distribution of phospholipid concentration	
	Supernatant	'Precipitate'	Supernatant	'Precipitate'
Lecithin (2.6 µmoles)	49	51	13	87
Lecithin (2.6 µmoles) + monophosphoinositide (0.8 µmole)	54	46	15	85
Lecithin (2.6 µmoles) + cardiolipin (0.39 µmole)	53	47	22	78

Table 8. Extractability of lecithin into ether from various emulsions

The mixed lipid was emulsified with buffer and shaken with 3 ml. of ether for 3 min.

Lipid emulsified	Aqueous phase	Extractability of lecithin into 3 ml. of ether (%)
Lecithin (2.6 μmoles)	1 ml. of 0.02M-acetate buffer (pH 3.6 or pH 4) or 0.02M-formate buffer (pH 3.3)	85-88
Lecithin (2.6 μmoles) + cardiolipin (0.44 μmole)	1 ml. of 0.02M-acetate buffer (pH 3.6)	0
Lecithin (2.6 μmoles) + cardiolipin (0.44 μmole)	1 ml. of 0.02M-acetate buffer (pH 3.6) + mM Ca ²⁺	67
Lecithin (2.6 μmoles) + monophosphoinositide (0.16 μmole)	1 ml. of 0.02M-formate buffer (pH 3.3)	0
Lecithin (2.6 μmoles) + monophosphoinositide (0.16 μmole)	1 ml. of 0.02M-formate buffer (pH 3.3) + 0.83 mM Ca ²⁺	58
Lecithin (2.6 μmoles) + tripalmitin (1.5 μmoles)	1 ml. of 0.02M-acetate buffer (pH 4)	67-83

Fig. 4. Emulsions of lecithin plus cardiolipin examined with regard to susceptibility to enzymic attack by *P. notatum* extract and extractability into ether. ○—○, Enzymic liberation of glycerylphosphorylcholine; ●, lecithin extracted into ether in the absence of enzyme; ○—○, lecithin extracted into ether in the presence of enzyme; □, lecithin extracted into ether in the presence of boiled enzyme supernatant. Lecithin (2.6 μmoles) mixed with various amounts of cardiolipin was emulsified with 1 ml. of 0.02M-acetate buffer (pH 3.6). *P. notatum* extract (0.2 ml.) or its boiled supernatant was added, and the tube either incubated for 55 min. at 37° or, alternatively, extracted with 3 ml. of ether.

Effect of 'activating' lipid on the physical form of the lecithin emulsion

It was often noticed that a lecithin emulsion containing cardiolipin or monophosphoinositide was less turbid and milky than a corresponding emulsion prepared from pure lecithin. On micro-

Fig. 5. Emulsions of lecithin plus monophosphoinositide examined with regard to susceptibility to enzymic attack by *P. notatum* extract and extractability into ether. ○—○, Enzymic liberation of glycerylphosphorylcholine; ○—○, lecithin extracted into ether in the presence of enzyme; ●, lecithin extracted into ether in the absence of enzyme. Lecithin (2.6 μmoles), mixed with various amounts of monophosphoinositide, was emulsified with 1 ml. of 0.02M-acetate buffer (pH 3.6). *P. notatum* (0.3 ml.) extract was added, and the tube either incubated for 60 min. at 37° or, alternatively, extracted with 3 ml. of ether.

scopic examination it was observed that, whereas the lecithin emulsion consisted of liquid-like globules of a lipid type, that with activating lipid had smaller particles, often with a far more angular and solid appearance. Such changes might be caused by the activating lipid producing an alteration in the surface properties of the lecithin particles. Consequently, experiments were performed to determine whether the activating lipid

caused any difference in the extractability of the lecithin from its emulsion into an organic solvent phase such as ether. Both cardiolipin and monophosphoinositide could completely alter the surface properties of the particles in an ovolecithin emulsion, so that the lecithin was no longer extractable with organic solvents such as ether (Table 8); with tripalmitin, on the other hand, the changes were small or nil. The changes in the surface of the lecithin particles brought about by the presence of cardiolipin or monophosphoinositide could be substantially reversed by the addition of low concentrations of calcium (Table 8), even though the amount of the activating phospholipid present in the lecithin was appreciably in excess of that necessary to cause the alteration in the particle's surface. With simple emulsions of lecithin plus cardiolipin in buffer, about 1% (on a molar basis) of cardiolipin in the lecithin completely prevented any of the lecithin's being extracted into ether (Fig. 4).

Experiments were then performed in which the activity of *P. notatum* extracts incubated with emulsions containing lecithin plus various amounts of monophosphoinositide or cardiolipin was compared with the extractability of these emulsions into ether. The extractability was measured both in the absence and presence of the enzyme, and also on the addition of the enzyme preparation after it had been boiled for 10 min. and the denatured protein removed by centrifuging. Figs. 4 and 5 show the results of such experiments; when sufficient monophosphoinositide or cardiolipin is present for enzymic activity to begin, the lecithin particle is in a form in which it is essentially non-extractable by ether. Experiments were also performed in which the emulsions were treated with the unboiled enzyme preparation at 0° and immediately extracted with ether. This resulted in the extractability of the lecithin in the emulsions being substantially increased (Figs. 4 and 5). However, when the boiled enzyme supernatant was used instead of the enzyme itself, very similar results were obtained (e.g. Fig. 4). This indicates that this effect of the enzyme preparation is due to some other factor in the preparation other than the heat-coagulable protein that it contains. There is, therefore, no evidence from these results that any appreciable amount of the enzyme becomes attached to the lecithin surface; this agrees with the results obtained above when attempts were made to show whether any enzyme was attached to the lecithin surface by direct assay with a lysolecithin substrate.

DISCUSSION

There have been a few isolated indications that phospholipases can attack mixed substrates of

phospholipids more rapidly than the pure phospholipids themselves. Schmidt, Bessman & Thannhauser (1957) for example, found that a phospholipase in rat-intestine mitochondria attacked a mixed substrate of lecithin plus 'kephalin' at a rate 11% faster than the sum of its attacks on each of the individual phospholipid fractions. The present work, however, demonstrates that a lecithinase present in extracts of *P. notatum* mycelium has an absolute requirement for certain other lipids. Five lipids have been shown to be especially active in promoting this latent phospholipase activity, namely cardiolipin, liver polyglycerol-phospholipid, monophosphoinositide and the saturated triglycerides tripalmitin and tristearin.

It is known that *P. notatum* extracts contain a powerful phospholipase *B* which can attack monoacyl-phospholipids such as lysolecithin, but not diacyl-phospholipids like lecithin. However, the present results suggest that this enzyme is responsible for the lecithinase activity of *P. notatum* extracts promoted by the addition of activating lipids. Thus the pH optimum of lecithinase activity in the presence of cardiolipin or monophosphoinositide is exactly the same as the optimum for the phospholipase *B* of the preparation when this is attacking lysolecithin. Moreover, both the activated lecithin attack and the phospholipase *B* activity are powerfully inhibited by low concentrations of fluoride or detergents. If the same enzyme is responsible for both the attack on lysolecithin and on lecithin, this must mean that the effect of the activating lipid in the system is to cause an alteration in the specificity of the phospholipase *B*. The final proof of this will rest on the purification of the phospholipase *B* enzyme. Some success in this direction has been obtained with ammonium sulphate fractionation, but further purification attempts were unsuccessful, possibly because of the very low concentrations of protein present in the solutions. The phospholipase *B* purified with ammonium sulphate was just as effective in attacking lecithin as was the original *P. notatum* extract.

How is it that the activating lipids can promote lecithinase activity in a lecithin-*P. notatum* extract system? The present work has clearly shown that maintenance of the lipid structure of the activating lipids is essential for the development of the enzymic attack. After the fatty acyl-ester bonds had been decomposed by mild hydrolysis with alkali no activity could be demonstrated. Two possibilities therefore suggested themselves: either the lipid was playing some intermediary or coenzyme role in the lecithin hydrolysis or, alternatively, the activating lipid was causing some physical change in the system which allowed the active centre of the enzyme and the

lecithin to come into a correct relationship with one another for satisfactory hydrolysis. No evidence could be obtained for the first alternative. It is to be expected that if the mechanism of the activation were of a coenzyme type, either there would be a transfer of the 'liberated' fatty acids from the lecithin to the structure of the activating lipids, or there would be a breakdown of activating lipid in the presence of the enzyme. No transfer of the ^{14}C -labelled fatty acids from lecithin to monophosphoinositide could be demonstrated in the presence of the enzyme, nor could any attack of the enzyme on the activating lipids, cardiolipin or tripalmitin, be detected. Monophosphoinositide is undoubtedly decomposed by the enzyme preparation, but it is likely that this has no connexion with the activation phenomena; indeed subsequent work, which will be reported elsewhere, has indicated that the phospholipase *B* of pancreas attacks monophosphoinositide in a similar way but possesses no equivalent 'activated' lecithinase activity.

On the other hand, there are a number of experimental indications that the activating lipids cardiolipin and monophosphoinositide can cause profound changes to the surface of the lecithin particle in an aqueous emulsion. This results in a large decrease in the extractability of the lecithin into organic lipid solvents such as ether, light petroleum or chloroform, and a distinct physical change in the appearance of the lecithin particles when they are viewed microscopically. As these changes can be brought about by very small molar percentages of activating lipid, e.g. less than 1% of cardiolipin, it seems likely that these lipids are orientated on the surface of the lecithin particle with their fatty acid residues on the inside and their more polar groups on the outside.

It is suggested that before the enzyme can attack the lecithin particles it is necessary for the particle to become substantially covered with such polar groups, resulting in a complete change in the characteristics of its surface. Such a postulate would explain the peculiar feature of the lecithinase activity whereby a certain concentration of activating lipid in the emulsion is necessary before hydrolysis occurs. The non-extractability of the lecithin particles into ether suggests that at such a point its surface has been substantially covered by the polar groups of the activating lipid. The inhibitory effects of calcium on the enzymic activity can be explained by its action of masking these polar groups on the surface of the lecithin. That such a masking does occur has been demonstrated by the effects of Ca^{2+} ions in reversing the extractability changes brought about by the presence of monophosphoinositide or cardiolipin in the lecithin. Calcium has no inhibitory effect on the lysolecithinase activity of the enzyme preparation

measured with the water-soluble lysolecithin as substrate.

Cationic and anionic detergents have an effect similar to that of monophosphoinositide or cardiolipin in that they prevent the extraction of lecithin from aqueous emulsions into ether. However, such detergents are potent inhibitors of the enzymic activity of the preparation towards lysolecithin, and this probably explains why they do not promote lecithinase activity by the preparation.

One difficulty in accepting the theory that surface changes on the lecithin particle are responsible for the promotion of lecithinase activity is that tripalmitin, which promotes enzymic activity, brings about only a slight reduction of the extractability of the lecithin into ether. It must be remembered, however, that the promotion of enzyme activity is of a considerably lower order than that produced by cardiolipin or monophosphoinositide and occurs at a different optimum pH. Further, it is not to be expected that there would necessarily be a direct correlation of such phenomena as the extraction of lecithin particles into organic solvent and the subtle surface changes necessary for promoting enzymic hydrolysis of the lecithin. That tripalmitin may act by a similar mechanism is suggested by the necessity of a large threshold concentration before activity commences, the fact that the actions of tripalmitin plus either cardiolipin or monophosphoinositide are additive in promoting hydrolysis, and also by the observation that the lecithinase activity promoted by tripalmitin is inhibited by calcium.

SUMMARY

1. Preparations of the phospholipase *B* of *Penicillium notatum* can hydrolyse lecithin to glycerylphosphorylcholine and free fatty acids, if certain activating lipids are added to the system.

2. Cardiolipin, liver polyglycerolphospholipid and monophosphoinositide were the most effective activating lipids; tripalmitin and tristearin were less effective, and certain fatty acids showed very low activity. All other lipids and non-lipid substances tried were ineffective.

3. A certain threshold concentration of activating lipid was necessary before lecithin hydrolysis began. However, lecithin attack occurred if two activating lipids were added at just below their individual threshold concentrations.

4. Optimum lecithinase activity occurred at pH 3.1–3.4 when either cardiolipin or monophosphoinositide was used as the activating lipid, and at pH 4.2 when tripalmitin was used to promote lecithin hydrolysis.

5. The promoted lecithinase activity was strongly inhibited by bivalent-metal ions such as

Ca^{2+} or Mg^{2+} (1.67 mM). These did not inhibit lysolecithin hydrolysis by the enzyme preparation.

6. Fluoride completely inhibited promoted lecithinase activity and lysolecithin hydrolysis at 4.1 mM and inhibition was still apparent at 0.41 mM.

7. Splitting of the acyl fatty-ester bonds in the activating lipids cardiolipin or monophosphoinositide by mild alkaline hydrolysis resulted in a complete loss of the ability to promote lecithin hydrolysis.

8. Monophosphoinositide (but not cardiolipin or tripalmitin) was hydrolysed by the enzyme preparation and this attack was inhibited by lecithin.

9. No transfer of fatty acids from lecithin labelled with ^{14}C -fatty acid to monophosphoinositide could be demonstrated in the presence of the enzyme preparation.

10. The activating lipids caused no detectable precipitation of phospholipase *B* on the surface of the lecithin particles, as measured by the lysolecithinase activity of the particles and supernatant.

11. Small quantities of cardiolipin and monophosphoinositide completely changed the surface character of lecithin particles in aqueous emulsion, so that the lecithin became no longer extractable into organic solvents such as ether.

12. The promotion of lecithinase activity by cardiolipin or monophosphoinositide occurred at a concentration at which the surface of the lecithin particle had changed so that it had become substantially non-extractable into ether. Both the surface change and the enzymic activity could be prevented by the addition of Ca^{2+} ions.

13. It is suggested that cardiolipin and monophosphoinositide promote lecithin hydrolysis by *P. notatum* phospholipase *B* preparations by introducing on the surface of the lecithin particles, certain polar groups which are necessary for the enzymic attack.

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Metabolism of Some ω -Halogenoalkylbenzenes and Related Alcohols in the Rabbit

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A study of the metabolism in the rabbit of the nineteen members of the chloromononitrobenzene series (summarized by Bray, James & Thorpe, 1957b) and of the other monohalogenonitrobenzenes (Bray, James & Thorpe, 1958) showed that in aromatic compounds of this type the halogen is often sufficiently labile to give rise to metabolites in which the halogen is replaced by another group

such as acetylcysteyl or hydroxyl. Acetylcysteyl-dehalogenation, in fact, occurred in sixteen out of twenty-eight compounds examined. In five of the compounds the nitro group was replaced instead of the halogen and only seven of the compounds failed to yield mercapturic acids. Although formation of mercapturic acids by acetylcysteyldehalogenation of nuclear-substituted aromatic com-