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# Phosphoinositides

## 3. ENZYMIC HYDROLYSIS OF INOSITOL-CONTAINING PHOSPHOLIPIDS*

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Sloane-Stanley (1953) and Rodnight (1956) described the hydrolysis of ox-brain 'diphosphoinositide' preparations (Folch, 1949) by extracts of brain and other tissues. Dawson (1954) and Hokin & Hokin (1956) showed that the phosphoinositide fraction of several tissues had a high turnover rate, as measured by the incorporation of $^{32}P$. The exact structure of the phosphoinositides involved is not known in every case, but it seems that the phosphate of phosphatidylinositol has a rapid turnover in several tissues (see Hawthorne, 1960*a*).

In view of these observations a study has been made of the hydrolysis of phosphatidylinositol and 'diphosphoinositide' by extracts of animal tissues. With the latter compound, Rodnight (1956) observed enzymic release of both organic and inorganic phosphate. This suggested that more than one enzyme was involved in the degradation and stimulated the present attempt to purify the enzyme catalysing the primary hydrolytic step in the breakdown of phosphoinositides.

Dawson (1959) has shown that phospholipase B preparations from *Penicillium notatum* and ox pancreas attack phosphatidylinositol. A preliminary report on this work has been published elsewhere (Kemp, Hübscher & Hawthorne, 1959).

## MATERIALS AND METHODS

*Analytical methods.* The methods used for the determination of phosphate, nitrogen and glycerol have been described by Hawthorne & Hübscher (1959). Carboxylic ester was determined by the hydroxamate method of Stern & Shapiro (1953) and protein by the Gornal, Bardawill & David (1949) modification of the biuret method. Where solutions were very dilute, the ratio of absorption at 260 and 280 mμ was used to measure the protein content.

*Paper chromatography.* Methods used for the chromatography of glycerol, inositol, choline, ethanolamine and serine have been described before (Hübscher & Hawthorne, 1957). Inositol 1-phosphate and inositol 2-phosphate were separated by chromatography in propan-1-ol–aq. $NH_3$ soln. (sp.gr. 0·88)–water (6:3:1, by vol.) (Pizer & Ballou, 1959). Other solvents for the separation of inositol phosphate and glycerophosphate are described by Hawthorne & Hübscher (1959).

*Ion-exchange columns.* Details of the method for the separation of hydrolysis products of water-soluble phospholipid by ion-exchange chromatography have been published (Hawthorne & Hübscher, 1959).

*Chromatography of glycerides.* Total glycerides in extracts from enzymic digests were prepared by chromatography on alumina according to Trappe (1940). Mono- and diglyceride fractions were obtained from silicic acid columns as described by Barron & Hanahan (1958).

*Ultracentrifugal analyses.* Purified enzyme preparations were studied in the Spinco analytical ultracentrifuge.

*Calcium phosphate gel.* This was prepared as described by Keilin & Hartree (1938).

### *Preparation of phospholipid substrates*

*Phosphatidylinositol.* This was prepared from ox or sheep liver by extraction according to Hawthorne (1960*b*), followed by chromatography on silicic acid by the method of Hanahan, Dittmer & Warashina (1957). The product was further purified on a second silicic acid column (Hanahan & Olley, 1958).

*Diphosphoinositide fraction.* This was prepared from ox brain by the method of Folch (1949). This fraction appears to contain more than one phosphoinositide.

*Cardiolipin.* This was obtained from ox heart by the method of Gray & Macfarlane (1958) and the sample was kindly supplied by Dr M. G. Macfarlane.

*Phosphatidic acid.* This was isolated from ox liver by the method of Hübscher & Clark (1960).

*Phosphatidylcholine.* This was prepared from the acetone-insoluble, alcohol-soluble fraction of ox-brain or ox-liver lipids and purified by chromatography on alumina according to Hanahan, Turner & Jayko (1951).

*Phosphatidylethanolamine.* This was isolated from egg yolk by the method of Rhodes & Lea (1957).

*Phosphatidylserine.* This was obtained from ox brain by the method of Folch (1948).

### *Preparation of phospholipid emulsions*

The phospholipids were dissolved in chloroform and stored in the refrigerator until required. They were obtained in aqueous emulsion, essentially free from inorganic phosphate and other water-soluble contaminants, by the following method. A suitable volume of the chloroform solution was evaporated to dryness under reduced pressure and the residue dissolved in ether. The required volume of water was then added and the ether was evaporated under reduced pressure with constant shaking. When all the ether had been removed, the emulsion was transferred to a

* Part 2: Hawthorne, Kemp & Ellis (1960*a*).

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dialysis sac with an equal volume of 1% ethylenediaminetetra-acetic acid (EDTA) as its disodium salt. It was then dialysed for 72 hr. against distilled water, with frequent changes of water. It was difficult to remove all the inorganic phosphate from the diphosphoinositide fraction by this method. The purified emulsion was stored for use at 4°.

### *Assay of enzymic activity*

The quantity of enzyme used was adjusted to give 20–40% hydrolysis of 12 μmoles of phosphatidylinositol added as an aqueous emulsion. Additions were made in the order: water, sodium acetate buffer (0·5 ml.; 0·4 M; pH 5·7), calcium chloride (40 μmoles), substrate and enzyme. The final volume was 4·0 ml. The tubes were kept for 20 min. at 0°, during which time they were occasionally shaken. Incubation was carried out for 1 hr. at 37°. For the blank experiment, substrate was omitted from the assay system. When the substrate was incubated in the absence of enzyme, neither water-soluble organic phosphate nor inorganic phosphate was liberated.

The reaction was stopped by placing the tubes in an ice bath, adding 0·8 ml. of 50% (w/v) trichloroacetic acid solution and mixing well. After 5 min., 2 ml. of an aqueous suspension of Norit A charcoal (12·5%, w/v) was added. This was required for the production of a water-clear filtrate. Cloudy filtrates could not be used for the determination of inorganic phosphate. The mixture was kept for a further 10 min. at 0°, filtered through a fluted Whatman no. 5 paper, and the filtrate analysed for inorganic and total phosphate.

Enzymic activity was expressed as follows: 1 unit of enzyme is the quantity required for the liberation of 1 μmole of water-soluble organic phosphate/hr. under the above-mentioned assay conditions, with phosphatidylinositol as substrate.

All preparations were carried out in a cold room at 4°. Samples to be assayed at once were kept in ice, otherwise they were quickly frozen and stored at −20°.

### *Preparation of subcellular fractions*

Fresh rat liver was homogenized in ice-cold 0·25 M-sucrose (7 ml./g. of liver) for 45 sec. in a high-speed Waring mixer. The homogenate was then centrifuged for 10 min. at 2° and 730 *g*. The sediment was discarded and the supernatant centrifuged again at 35 000 *g* for 20 min. The material sedimented by this procedure was taken as the mitochondrial fraction. The supernatant was spun at 105 000 *g* for 45 min. to give the microsomal fraction. The resulting supernatant was retained.

All the precipitates were washed by resuspending in the original volume of 0·25 M-sucrose and centrifuging again at the same speed. They were then suspended in a small volume of 0·25 M-sucrose.

### *Acetone-dried powders*

Acetone-dried powders of rat liver and intestinal mucosa and dog liver were prepared by the method of Kaplan & Lipmann (1948).

## EXPERIMENTAL AND RESULTS

Evidence that the purified extract contained more than one enzyme acting on phosphatidylinositol was obtained only in the later stages of this work. In this section therefore the word enzyme is used in the singular to avoid confusion.

In preliminary experiments, homogenates of rat heart or liver were used. These were prepared by homogenizing the organs for 45 sec. in aqueous 0·9% sodium chloride solution (10 ml./g. of fresh tissue) in a high-speed Waring mixer. Acetate buffer was replaced in the assay system by the same molar concentration of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) hydrochloride buffer, pH 7·3. Calcium chloride solution was added to give a final concentration of 1 mM. With 1·0 ml. of heart or liver homogenate and with phosphatidylinositol as substrate, 0·5–1·0 μmole of total water-soluble phosphate was released/hr. Of this, between 10 and 20% was inorganic phosphate. Phosphatidylserine was hydrolysed at only about one-tenth of the rate of phosphatidylinositol.

It was considered likely that the inorganic phosphate was produced by the action of a phosphatase on a possible product of the phospholipase action, inositol monophosphate. To test this, the same assay system and source of enzyme was used, except that 7·0 μmoles of inositol 2-phosphate replaced the lipid substrate. A slow release of inorganic phosphate was observed (0·4–0·6 μmole/hr.). The first aim in purification of the phosphoinositide-splitting enzyme was the removal of this phosphatase.

*Examination of subcellular fractions.* Rat liver was chosen as a more convenient source of enzyme than rat heart owing to its larger size. Subcellular fractions were prepared as indicated above. Each fraction was dialysed against 0·9% sodium chloride solution for 24 hr. at 4° to remove sucrose and soluble phosphoric acid esters. Portions of these preparations equivalent in each case to 0·5 g. of fresh liver, and tris buffer, pH 7·3, were used in the assay. An increase in water-soluble organic phosphate was found with the supernatant fraction (1·3 μmoles/hr.) and the mitochondria (0·16 μmole/hr.). An increase of 0·1–0·2 μmole of inorganic phosphate/hr. was found in all fractions except the mitochondria, where the increase was 0·3 μmole/hr. In some experiments no release of organic phosphate was found with the mitochondrial fraction.

It was concluded that 60–70% of the activity was present in the supernatant fraction.

*Distribution of enzymic activity.* In an assay system with phosphatidylinositol as substrate and acetate buffer, pH 5·4, extracts of various tissues from different animals were tested for activity. Aqueous extracts of acetone-dried powders of dog liver, rat liver and rat intestinal mucosa all released water-soluble organic phosphate, but the specific activity of these extracts was lower than that observed with a fresh extract of rat liver. Activity was also detected in homogenates of sheep heart and rabbit liver as

well as in extracts of fresh brain, kidney and spleen of the rat. In all of these extracts, the specific activity and the total units per gram of wet tissue were lower than the corresponding values for extracts of fresh rat liver.

*Purification of the enzyme.* In the development of the method described below, several different techniques were tried, some of which proved unsuccessful. However, fractionation with ammonium sulphate gave good results. Furthermore, the enzyme could be absorbed by calcium phosphate gel and eluted again, the process giving a substantial purification. The following purification procedure was finally adopted.

*Extraction of the enzyme.* Four to eight livers were removed from male albino rats immediately after death and placed on ice. They were homogenized for 45 sec. in 10 mM-tris buffer, pH 7·3 (8 ml./g. of wet tissue), in the same high-speed mixer. The homogenate in this hypo-osmotic buffer was kept for 2 hr. at 4° to ensure extraction of the enzyme. The particulate matter was then removed by centrifuging at 31 000 *g* for 1 hr. To the supernatant, solid ammonium sulphate was added to give a final concentration of 25%. After 30 min. to 1 hr., the precipitate was separated by centrifuging at 22 000 *g* for 15 min.

*First ammonium sulphate precipitation.* The clear supernatant was made 40% saturated with respect to ammonium sulphate and kept for about 1 hr. at 0°. The precipitate was collected by centrifuging at 2700 *g* for 10 min. and dissolved in 5 mM-tris buffer, pH 7·3, to give a solution containing 5 mg. of protein/ml. If necessary it could be stored overnight at −16° before the next stage was reached. With a glass-electrode pH meter, the solution was adjusted to pH 6·8 by adding a few drops of 0·1 N-acetic acid.

*Calcium phosphate-gel treatment.* Concentrated calcium phosphate gel (23 mg. of tricalcium phosphate/ml. of water) was added (1 mg. of dry gel/mg. of protein) and the mixture stirred gently for 10 min. in an ice bath. It was then centrifuged for 5 min. at 750 *g*. The supernatant was decanted and the gel resuspended in 1·8 mM-sodium acetate–0·18 M-ammonium sulphate solution, pH 7·0. One-half volume of sodium acetate–ammonium sulphate solution was used for elution per volume of solution from which the enzyme was absorbed. The gel was stirred with the solution as before for 10 min. After centrifuging at 750 *g* for 5 min. and removal of the supernatant, the gel was eluted in the same way with 0·5 M-sodium acetate–ammonium sulphate solution, pH 7·0. The gel was again separated from the supernatant by low-speed centrifuging.

*Second ammonium sulphate precipitation.* To the eluate obtained with 0·5 M-sodium acetate–ammonium sulphate solution, 16 g. of solid ammonium sulphate was added/100 ml. After 30 min. the precipitate was collected by centrifuging at 2700 *g* for 10 min. and dissolved in 10 mM-tris buffer, pH 7·3. The solution was stored at −16°.

A flow sheet of the purification procedure is given in Table 1. It will be seen that 49-fold purification of the enzyme was achieved with a recovery of 29%. The original supernatant contained all the activity detected in the homogenate. When stored at −16° the final enzyme preparation was stable for about 10 days.

*Properties of the purified enzyme preparation.* The purified preparation of the enzyme was readily soluble in the assay system buffer to give a completely clear, almost colourless solution. An examination of the preparation in an analytical ultracentrifuge, in 60 mM-phosphate buffer, pH 6·9, containing 150 mM-sodium chloride, revealed four components with molecular weights of (*a*) 60 000 (39·2%), (*b*) 200 000 (39·2%), (*c*) 400 000 (6·2%) and (*d*) 500 000 (15·4%). No attempt was made to correlate the enzymic activity with any one of the components. Assuming that each of the four components was a pure protein, it can be concluded from these data that the preparation was at least 6% and at most 39% pure.

The substrate was added to the assay system as an aqueous dispersion rather than a true solution and thus the enzyme–substrate complex was probably one in which a lipid micelle was involved rather than a single molecule of phosphatidylinositol. The effect of increasing the concentration of phosphatidylinositol is demonstrated in Fig. 1.

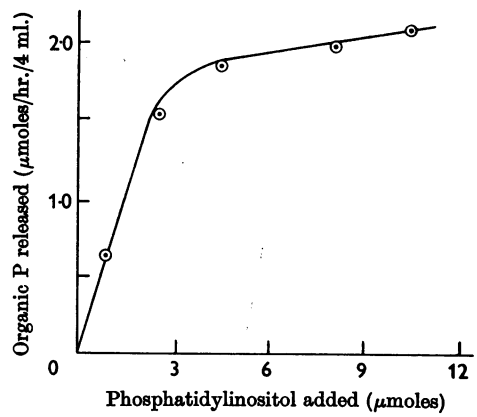


Fig. 1. Effect of substrate concentration on rate of enzymic hydrolysis. Assay system was as described in the text. Enzyme: second ammonium sulphate precipitate, 1 mg. of protein/4 ml.

Table 1. *Flow sheet for enzyme purification*

| Step | Total vol. (ml.) | Protein (mg.) | Units/mg. of protein | Total units | Purification | Recovery (%) |
|---|---|---|---|---|---|---|
| Supernatant | 225 | 2860 | 0·148 | 422 | 3·3 | 100 |
| First ammonium sulphate ppt. | 125 | 625 | 0·490 | 307 | 10·9 | 73 |
| After calcium phosphate-gel treatment* | 73 | 97 | 0·830 | 81 | 18·5 | 19 |
| Second ammonium sulphate ppt. | 73 | 55 | 2·180 | 120 | 48·5 | 29 |

* The activity of this preparation was difficult to determine accurately, possibly because of the presence of excess of $Ca^{2+}$ ions.

There was relatively little increase in reaction rate at substrate concentrations above 3 mM.

When the data of Fig. 1 were treated according to Lineweaver & Burk (1934) a linear relationship was obtained which indicated a $K_m$ value of $3{\cdot}3 \times 10^{-4}$ at pH 5·6. A determination of the Michaelis–Menten constant with a less pure enzyme preparation (first ammonium sulphate precipitate) gave $K_m$ $4{\cdot}4 \times 10^{-4}$, the optimum substrate concentration being $3{\cdot}7 \times 10^{-3}$ M. Further evidence that the assay of the enzyme was carried out under zero-order conditions was obtained from measurements of the rate of release of water-soluble organic phosphate under the conditions of assay. Thus in the presence of 8 mg. of protein from the first ammonium sulphate precipitate organic phosphate was released without initial lag and at a constant rate of 0·9 (±0·1) μmole/10 min. for a period of 50 min.

At low concentrations of protein, the release of water-soluble organic phosphate was proportional to the amount of protein added. Under the assay conditions the rate of release of water-soluble organic phosphate from 8 μmoles of phosphatidylinositol was 1·85 (±0·25) μmoles/hr./mg. of protein of the second ammonium sulphate precipitate when the protein concentration was varied from 0·5 mg. to 2·0 mg./4 ml. of incubation mixture.

The change in activity with the pH of the reaction mixture is shown in Fig. 2. The optimum pH value was 5·7 ± 0·2. A second smaller optimum was present at pH 6·9 ± 0·2. The smaller optimum was consistently observed in varying stages of purification of the enzyme, though the relative activity per milligram of protein of the two optima varied from preparation to preparation under otherwise identical assay conditions. This might suggest the presence of two enzymes acting on the same substrate.

The requirement for metal ions depended largely on the pretreatment of the substrate and enzyme. Thus when an aqueous undialysed emulsion of the substrate and an undialysed enzyme were used, no increase in the rate of reaction was brought about by the addition of $Ca^{2+}$ ions, but an inhibition was obtained at higher concentrations of calcium chloride. These effects are shown with a less purified enzyme in Fig. 3 (*a*). Although the phospholipid used had been prepared by chromatography on two consecutive columns of silicic acid, substantial amounts of $Ca^{2+}$ ions must have been carried through the purification procedure. The requirement for $Ca^{2+}$ ions could readily be shown by using as substrate a preparation which had previously been dialysed for long periods of time against a dilute buffer containing EDTA. The enzyme preparation also contained a small but significant amount of $Ca^{2+}$ ions. Since the enzyme was not stable during dialysis, a complete recovery of activity by the addition of $Ca^{2+}$ ions was difficult to demonstrate. A typical activation of the enzymic reaction by $Ca^{2+}$ ions with a dialysed substrate, but undialysed enzyme (the second ammonium sulphate precipitate), is indicated in Fig. 3 (*b*). Under these conditions, optimum concentrations of calcium chloride were between 5 and 16 mM. Since the concentration of phosphatidylinositol was 3 mM, the molar ratio of $Ca^{2+}$ ions to substrate at maximal activity lies therefore between 1:0·6 and 1:0·2. Of a number of other bivalent ions tested, only barium and magnesium

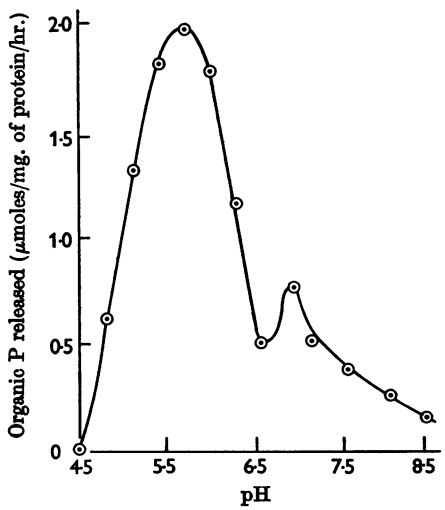


Fig. 2. Effect of pH on rate of enzymic reaction. Assay system was as described in the text, with phosphatidylinositol as substrate and the second ammonium sulphate precipitate.

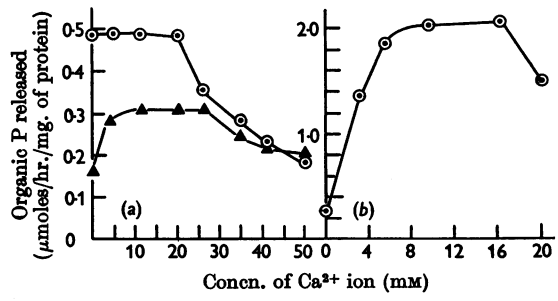


Fig. 3. Activation of enzyme by calcium. (*a*) First ammonium sulphate precipitate; usual assay system. ⊙, Undialysed enzyme; ▲, EDTA-dialysed enzyme. Lipid substrate not dialysed. (*b*) Undialysed second ammonium sulphate precipitate. Assay system was as described in the text but with only 8 μmoles of phosphatidylinositol (dialysed after addition of excess of EDTA).

showed a similar effect, yet in a lesser degree (see Table 2). Some other compounds were tested in the presence of optimum amounts of $Ca^{2+}$ ions to obtain more information about possible active sites of the enzyme. There was a complete inhibition by low concentrations of *p*-mercuribenzoic acid. Neither glutathione nor cysteine when added in the absence of this inhibitor brought about an increase in the rate of reaction. Fluoride ions at 10 mM concentration gave a small, but significant, stimulation in the presence of 10 mM-$Ca^{2+}$ ions. Owing to the insolubility of calcium fluoride addition of fluoride might be expected to decrease the effective $Ca^{2+}$ ion concentration to 5 mM. This decrease would not itself cause the observed increase in rate [see Fig. 3 (*b*)].

The substrate specificity of the enzyme with a less pure preparation was reported previously (Kemp *et al.* 1959). These experiments were repeated with the second ammonium sulphate precipitate prepared as described in the present work. Very similar results were obtained. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, cardiolipin and phosphatidic acid were not attacked by the enzyme. On a molar basis, 'diphosphoinositide' gave twice as much water-soluble organic phosphate as did phosphatidylinositol.

The primary enzymic cleavage of phosphatidylinositol with release of water-soluble phosphate esters could lead to the formation of either glycerylphosphorylinositol or inositol monophosphate. The simultaneous determination of water-soluble organic phosphate liberated and of acyl values in the reaction mixture should therefore give some indication of the hydrolytic pathway.

The results of a study of the release of organic phosphate and fatty acid are given in Fig. 4. After incubation for 60 min. 6·2 $\mu$moles of phosphoric acid ester, but only 3·2 $\mu$moles of fatty acid, were liberated. Since the formation of 1 mole of glycerylphosphorylinositol is accompanied by the liberation of 2 moles of fatty acid, the release of 3·2 $\mu$moles of fatty acid would correspond to the formation of 1·6 $\mu$moles of glycerylphosphorylinositol. If the preparation contained a lipase hydrolysing the diglyceride formed upon release of inositol monophosphate from phosphatidylinositol, the above calculation would not hold. Although small amounts of monoglyceride were found among the reaction products, the separation of water-soluble phosphates described below suggests that the decrease in acyl values is mainly due to the formation of glycerylphosphorylinositol.

*Identification of the reaction products.* A hydrolysis was carried out with phosphatidylinositol as substrate and about 50 times as much enzyme as was usually taken for an assay. The other components of the assay system were increased in amount proportionately. The reaction was stopped after 60 min. by addition of trichloroacetic acid to give a final concentration of 5%. The precipitate was collected by centrifuging and extracted with ether. The aqueous supernatant was extracted with ether to remove trichloroacetic acid. A stream of air was passed through the aqueous solution to remove dissolved ether. After passing the solution through Zeo-Karb 225 ($H^+$ form), the volume was adjusted to 1 l., sodium tetraborate being added to give a final concentration of 5 mM. This solution of

Table 2. *Effect of bivalent metal ions at* pH 5·5

| Concn. (mM) ... | Relative rate 2 | 6 | 10 |
|---|---|---|---|
| Metal added | | | |
| None | 100* | 100* | 100* |
| $Ca^{2+}$ | 325 | 500 | 500 |
| $Mg^{2+}$ | — | 150 | 150 |
| $Ba^{2+}$ | 50 | 100 | 185 |
| $Ni^{2+}$ | 50 | 40 | 30 |
| $Zn^{2+}$ | 25 | — | 15 |
| $Co^{2+}$ | 70 | 55 | 60 |
| $Fe^{2+}$ | 55 | 18 | 18 |
| $Pb^{2+}$ | 26 | 37 | 20 |
| $Cu^{2+}$ | 25 | — | — |

* Arbitrarily set at 100.

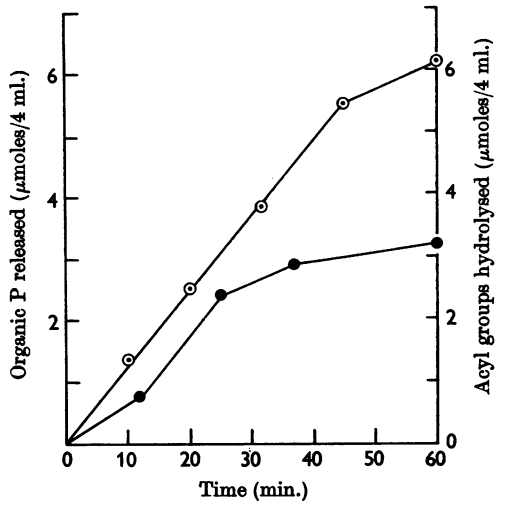


Fig. 4. Time study of water-soluble phosphate and fatty acid release. ⊙, Release of phosphate; ●, hydrolysis of acyl groups. Assay system was as described in the text with omission of charcoal. The trichloroacetic acid precipitate was spun down, washed once with water and extracted with excess of chloroform–methanol (1:1, v/v). The extract was dried and taken up in ethanol–ether (3:1, v/v) for acyl ester determinations. For the calculation of decrease in ester value, the zero-time result was taken as 100%.

phosphate esters was applied to a Nalcite SAR resin column (see Materials and Methods section) and, after the column was washed with 250 ml. of 5 mM-borate, elution was carried out as outlined in Fig. 5. Two main peaks were obtained (1 and 3), which corresponded in their position to glyceryl-phosphorylinositol (peak 1, 41 % of total phosphate) and inositol monophosphate (peak 3, 47·5 %). The first peak contained glycerol, phosphate and inositol in molar ratios 1·0:1·0:0·85. The identity of inositol monophosphate in peak 3 was confirmed by paper chromatography in propan-1-ol–ammonia (sp.gr. 0·88)–water (6:3:1, by vol.). By this method it was shown that the ester isolated was myoinositol 1-phosphate contaminated with about 5 % of the 2-isomer.

The ether extract of the precipitate was washed well with water to remove trichloroacetic acid and twice with 5 % sodium bicarbonate solution to extract free fatty acids. It was then washed with water once more, dried over anhydrous sodium sulphate and chromatographed on silicic acid by the method of Barron & Hanahan (1958). The mono- and di-glyceride fractions weighed 31 and 27 mg. respectively. Assuming a molecular weight of 285 for the fatty acids, analysis for glycerol and acyl groups gave the expected value for acyl groups, but the values obtained for glycerol content were somewhat low, possibly due to incomplete hydrolysis. The diglyceride fraction had a molar ratio of fatty acid to glycerol of 2·5 whereas the corresponding value for the monoglyceride fraction was 1·3. The sodium bicarbonate washings were acidified and the fatty acids were extracted with ether. The ether was removed *in vacuo* and the residue was dried over phosphorus pentoxide. It was then left over solid sodium hydroxide overnight in a vacuum desiccator to remove any volatile acids. The weight of the residue was 50 mg. No phosphate was present in this fraction.

## DISCUSSION

The method used in the present investigation for assaying the enzymic release of water-soluble phosphoric acid esters from phosphatidylinositol has an obvious disadvantage. It determines the total esters released rather than a particular ester. Consequently, if various fractions are assayed during the course of an isolation procedure, little information is obtained about the relative concentration of the two or more enzymes which may be hydrolysing phosphatidylinositol. For further studies on the isolation of enzymes hydrolysing phosphatidylinositol, more specific assay systems will need to be devised.

Our purified preparation had, in fact, two independent hydrolytic actions. This was borne out by

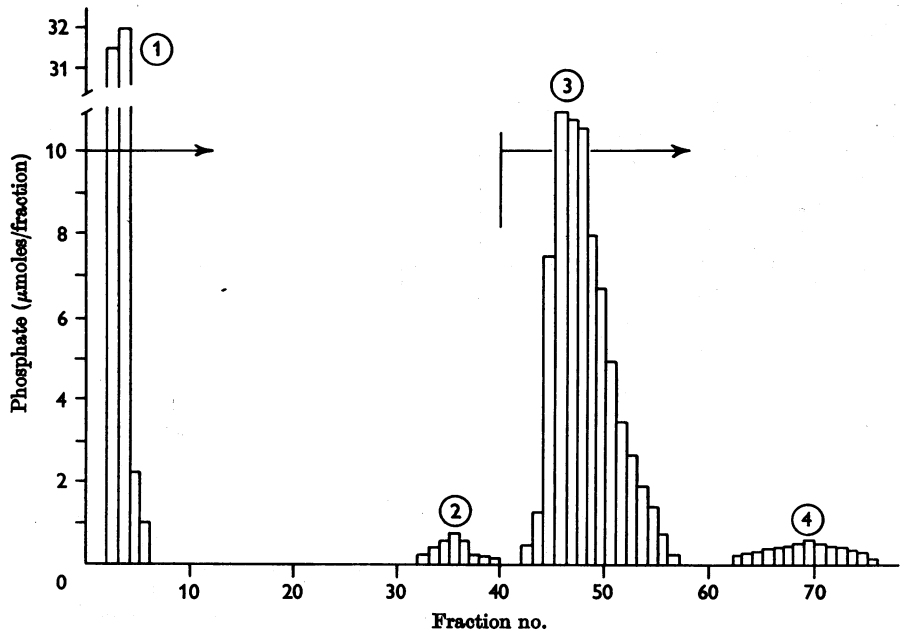


Fig. 5. Chromatography of hydrolysis products from enzymic reaction. Nalcite SAR resin. Eluting mixtures: fractions 1–39, sodium tetraborate (5 mM)–ammonium formate (60 mM); fractions 40–80, sodium tetraborate (5 mM)–ammonium formate (150 mM). Probable identities of peaks are given in the text.

various determinations: the simultaneous release of roughly 6 moles of phosphoric acid ester and 3 moles of fatty acid excluded the formation of inositol monophosphate via glycerylphosphorylinositol. The isolation of glycerylphosphorylinositol, inositol monophosphate, free fatty acid and lower glycerides from the reaction mixture would support this view. Since monoglyceride was found, a lipase may also be present which hydrolyses the diglyceride initially released by the phospholipase. The presence of two pH optima is also of interest in this connexion. The ratio of the activities at the two pH optima varied from preparation to preparation, with identical conditions for assaying. This might be explained by the presence of two enzymes hydrolysing phosphatidylinositol.

The two pathways for the hydrolysis of phosphatidylinositol are given in Fig. 6. It is generally assumed (e.g. Dawson, 1957) that the breakdown of glycerophosphatides in animal tissues occurs as outlined in pathway (A). In fact, the breakdown of a phospholipid in animal tissues by pathway (B) seems to have been described only by Dawson (1959), who showed that a lecithinase B preparation from ox pancreas released inositol monophosphate from phosphatidylinositol. This preparation hydrolysed lysolecithin more rapidly than phosphatidylinositol, whereas the enzymes described in the present work appear specific for phosphoinositides. The pancreas and liver enzymes have similar pH optima and requirement for $Ca^{2+}$ ions. The liberation of phosphorylcholine from lecithin has been reported (Macfarlane & Knight, 1941; Long & McGuire, 1953; Chu, 1949; Hanahan & Vercamer, 1954). However, the sources of the enzymes concerned were extracts of *Bacillus cereus*, *Bacillus mycordes* and *Clostridium perfringens*.

The primary products of the hydrolysis of phosphatidylinositol by pathway (B) are a diglyceride and inositol monophosphate. The diglyceride could either be further degraded to glycerol and free fatty acids by the action of a lipase or it might be used for biosynthesis of phospholipids. D-$\alpha\beta$-Diglyceride is known to be a precursor of phosphatidylcholine and phosphatidylethanolamine (Kennedy, 1956). Free inositol monophosphate has been found in liver (Hübscher & Hawthorne, 1957). The enzyme described here probably accounts for its presence, especially since the biosynthetic pathway to phosphatidylinositol does not seem to include inositol monophosphate as an intermediate (Agranoff, Bradley & Brady, 1958; Paulus & Kennedy, 1960).

In the present investigation it was shown that inositol 2-phosphate is hydrolysed by crude extracts of liver tissue. Liver phosphatidylinositol has the myoinositol 1-phosphate structure (Pizer & Ballou, 1959; Hawthorne, Kemp & Ellis, 1960*b*). The liberation of inorganic phosphate from phosphatidylinositol by crude extracts or subcellular particles of rat liver suggests that the 1-isomer is also hydrolysed. It is not known whether the enzyme catalysing this reaction is one of the unspecific phosphomonoesterases or a specific phosphatase. Pileggi (1959) described a phytase from animal tissues which hydrolysed phytic acid completely, giving free inositol.

In pathway (A) the action of phospholipases A and B gives glycerylphosphorylinositol. The enzyme preparation did not attack lecithin or any of the other glycerophosphatides, though the less pure preparation (Kemp *et al.* 1959) hydrolysed lysolecithin at about 10% of the rate for phosphatidylinositol. It seems, then, that our enzymes are different from the phospholipases in a particulate preparation of the intestinal mucosa of the rat

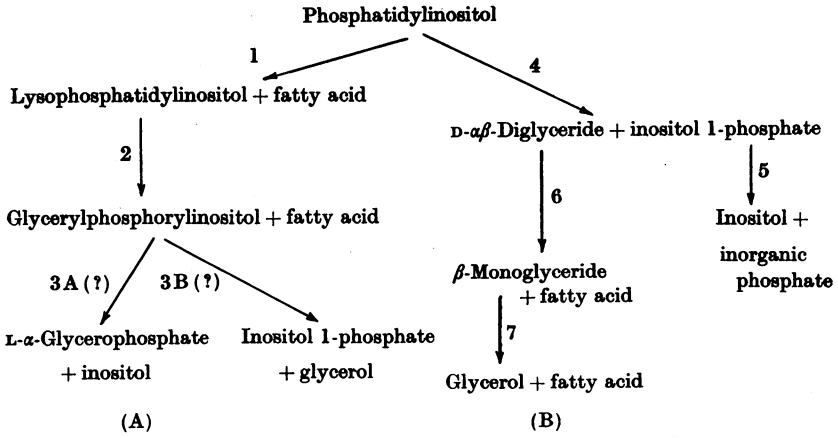


Fig. 6. Two possible pathways for the breakdown of phosphatidylinositol in liver.

(Epstein & Shapiro, 1959). These authors describe lecithinases A and B, which did not require $Ca^{2+}$ ions for their activity. A phospholipase prepared from *Penicillium notatum* also attacked phosphatidylinositol, giving glycerylphosphorylinositol (Dawson, 1959). This enzyme was not activated by calcium, had optimum pH 3·3 and hydrolysed phosphatidylinositol more slowly than lysolecithin. The latter compound inhibited the breakdown of phosphatidylinositol, whereas the liver enzyme was unaffected (Kemp *et al.* 1959). In the presence of phosphatidylinositol or certain other anionic amphipathic molecules, the *P. notatum* enzyme would also attack lecithin (Bangham & Dawson, 1960). Glycerylphosphorylinositol might be further broken down by a diesterase to give either glycerophosphate or inositol monophosphate or both. The diesterases from liver (Dawson, 1956) or nervous tissue (Webster, Marples & Thompson, 1957) gave glycerophosphate and choline as products of the hydrolysis of glycerylphosphorylcholine.

The naming of enzymes attacking phosphoinositides is not easy. The enzyme removing diglyceride from phosphatidylcholine is referred to as lecithinase C by many workers, after the original suggestion of Macfarlane & Knight (1941). The same enzyme is also called lecithinase D (Hanahan, 1957). Since the enzymes described in the present study appear to be specific for phosphoinositides, the name phosphoinositidase seems appropriate for both. The enzyme removing diglyceride would then be a phosphoinositidase D.

## SUMMARY

1. A soluble enzyme which hydrolyses phosphoinositides has been purified 50-fold from rat liver.

2. It hydrolyses phosphatidylinositol and the diphosphoinositide fraction from brain, but does not attack phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid or cardiolipin.

3. For optimum activity $Ca^{2+}$ ions are required. Two pH optima and other properties of the preparation are described.

4. Two independent hydrolytic actions were found to be present in the preparation leading to the formation from phosphatidylinositol of inositol monophosphate (phosphoinositidase C) and of glycerylphosphorylinositol (phosphoinositidases A and B).

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