The Triphosphoinositide Phosphodiesterase of Brain Tissue

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The results described in the preceding paper (Thompson & Dawson, 1964) suggested that two enzymes are responsible for the degradation of triphosphoinositide by extracts of ox brain. The present paper describes the isolation and properties of a brain triphosphoinositide-phosphodiesterase preparation that was free from phosphomonoesterase activity.

EXPERIMENTAL

Preparation of triphosphoinositide phosphodiesterase from brain extracts. The dialysed aqueous extract of acetone-dried brain powder (Thompson & Dawson, 1964) was held in a water bath at 50° for 30 min. After cooling in an ice bath 0·25 vol. of 0·132 M-tris–HCl buffer, pH 7·2, was added. Saturated $(NH_4)_2SO_4$ solution (neutralized to pH 7) at 4° was slowly added with stirring to give 30% saturation. After standing for 30 min. in ice, the precipitate was removed by centrifuging at 0° at 10000$g_{av.}$ for 20 min. The supernatant was brought to 50% saturation with the $(NH_4)_2SO_4$ solution, and after standing for 30 min. the precipitate was collected by centrifuging as above. The 30–50% saturated $(NH_4)_2SO_4$ precipitate was dissolved in 0·02 M-tris–HCl buffer, pH 7·2 (volume equal to that of the original extract), and dialysed overnight against 1 l. of 5 mM-tris–HCl buffer, pH 7·2, at 4°. The non-diffusible material was centrifuged in a Spinco centrifuge at 105000$g_{av.}$ for 60 min. The water-clear colourless supernatant was collected, diluted with an equal volume of water and stored at 4°.

Conditions of incubation and assay of activity. The sodium or ammonium salt of triphosphoinositide (40 μg.atoms of P) was dissolved in 0·3 ml. of 0·132 M-tris–HCl buffer, pH 7·2. An activator [e.g. 0·3 ml. of 0·075% cetyltrimethylammonium bromide (0·62 μmole)] was added to obtain maximal activity. Incubation with 0·2 ml. of enzyme preparation was carried out for 30 min. at 37° in a final volume of 1·2 ml. The reaction was stopped by cooling the incubation tube in ice and immediately adding 0·2 ml. of 5% (w/v) bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) followed by 1 ml. of ice-cold 10% (v/v) perchloric acid. The precipitate was centrifuged and the supernatant filtered. A 1·5 ml. sample of the filtrate was assayed for combined phosphate by the method of Fiske & Subbarow (1925). As soon as it had been established that no inorganic phosphate was liberated by the enzyme preparation only total phosphate in the filtrate was measured. Appropriate incubation controls containing enzyme alone and substrate alone were carried through the same procedure. The substrate controls usually contained some combined phosphate (about 3–5 μg.atoms of P) but the enzyme controls contained none.

Electrophoretic mobilities were measured as described by Bangham & Dawson (1959).

Materials. The preparation and storage of triphosphoinositide were as described by Thompson & Dawson (1964). A 'diphosphoinositide' preparation was isolated by the procedures of Folch (1942, 1949*a*, *b*). The calcium (magnesium) salt of the isolated lipid was converted into the acid form by dissolving the dialysed and freeze-dried product in wet chloroform and adding 0·5 vol. of methanol and 0·2 vol. of N-HCl. After being shaken, the mixture was centrifuged and the lower phase evaporated to dryness *in vacuo* at room temperature. The residue was extracted with 5 ml. of methanol. The methanolic solution was cooled to 0° and treated with methanolic 0·1 N-NaOH until precipitation was complete. The precipitate was centrifuged, dissolved in chloroform and stored at −15°. Analysis (Dawson, Hemington & Davenport, 1962) showed it to contain about 27% (on a molar basis) of diphosphoinositide, the remainder being practically all triphosphoinositide.

Lecithin, phosphatidylethanolamine and diglyceride were prepared from egg yolk (Dawson, 1963). Sphingomyelin was isolated by the procedure of Davenport & Dawson (1962). Phosphatidylinositol was prepared from ox liver by the method of Dawson (1958): it was free from nitrogen-containing phospholipids. Phosphatidic acid was prepared as described by Dawson, Hemington & Lindsay (1960). Palmitoylcholine hydrobromide and a preparation of brain gangliosides were kindly given by Dr V. P. Whittaker. Histone isolated from calf thymus was a gift from Dr J. A. Lucy.

RESULTS

Comments on the preparation of triphosphoinositide phosphodiesterase. Extensive attempts were made to separate the two enzymes in brain extracts that attack triphosphoinositide (Thompson & Dawson, 1964) by protein-fractionation techniques. The methods tried included precipitation of the protein at various pH values and with acetone and ammonium sulphate, adsorption and elution from calcium phosphate gel, and absorption into columns of CM-Sephadex C-50, SE-Sephadex C-50, DEAE-Sephadex A-50, Sephadex G-100 and Sephadex G-200. At best only partial separation of the enzymes could be achieved without worthwhile purification. Considerable losses of enzymic activity also occurred.

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The method finally adopted to give a preparation free from phosphomonoesterase was heat treatment of the crude extract of acetone-dried brain powder at 50°. This resulted in a much greater denaturation of the phosphomonoesterase, so that by choosing an appropriate time this enzyme could be completely destroyed with an approximately 40% loss of phosphodiesterase activity. The differential heat lability of the two enzymes occurred at temperatures down to 20°, but at this temperature a much longer time was required to remove the phosphomonoesterase. The denaturation of both enzymes at 47° was more rapid at pH 5·8 than 6·2.

An ammonium sulphate (30–50% saturation) fractionation of the heat-treated enzyme resulted in a small increase in specific activity, and the final clear solution obtained after high-speed centrifuging gave a negligible enzyme blank. No significant loss of activity was observed on storage at 4° unless the preparation became contaminated with micro-organisms.

Nature of enzymic reaction. The enzyme preparation released combined water-soluble phosphate but no inorganic phosphate when incubated with triphosphoinositide. It was established that the enzyme showed the same phosphodiesterase activity as the crude brain extract (Thompson & Dawson, 1964). Only inositol triphosphate could be detected on examination of the water-soluble phosphate esters by paper ionophoresis at pH 3·6. Presumably little inositol diphosphate was formed because no diphosphoinositide accumulated in the absence of triphosphoinositide-phosphomonoesterase activity.

Activators of the enzymic reaction. When triphosphoinositide was incubated with the enzyme preparation there was always an initial small but rapid liberation of acid-soluble combined phosphate which then stopped almost completely (Fig. 1). When this combined phosphate was examined by paper ionophoresis it all migrated to the position of inositol triphosphate. In the presence of cetyltrimethylammonium bromide, the release of combined phosphate was greatly increased (Fig. 1), although once again the reaction did not go to completion. This stimulation was also observed when the cetyltrimethylammonium bromide was added after the initial basic hydrolysis had occurred. The stimulation brought about by cetyltrimethylammonium bromide did not occur until an initial threshold concentration had been reached, and with increasing concentrations the activity passed through a maximum (Fig. 2). If the concentration of substrate was doubled then the concentration of activator required to produce maximum activity was also approximately doubled (Fig. 2).

A similar stimulation of the reaction was produced by other cationic amphipathic substances, namely cetylpyridinium bromide, palmitoylcholine and stearylamine. When a series of long-chain amines were tested, no effect was observed until the chain length was C_{18} (Fig. 3). Tetramethylammonium bromide, butyrylcholine, tyramine and stearamide were unable to activate the reaction.

The reaction was also stimulated by the addition of Mg^{2+} or Ca^{2+} ions, similar patterns of activation occurring on increasing the concentration of the ions, with a lag phase, then a rapid increase to maximal stimulation followed by an equally rapid

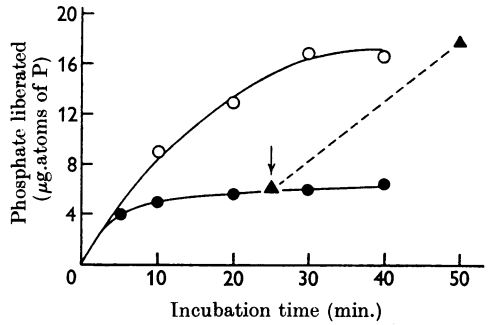


Fig. 1. Hydrolysis of triphosphoinositide by a phosphodiesterase preparation from ox brain. The incubation conditions were as described in the Experimental section. ●, Basic system; ○, 0·62 μmole of cetyltrimethylammonium bromide added; ▲, 0·62 μmole of cetyltrimethylammonium bromide added to the basic system after incubation for 25 min.

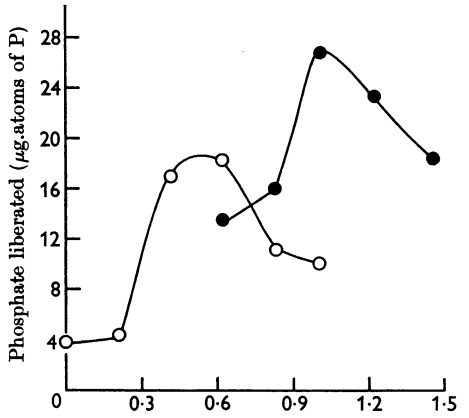


Fig. 2. Effect of cetyltrimethylammonium bromide concentration on triphosphoinositide-phosphodiesterase activity at two substrate concentrations: ○, 0·43 μmole of triphosphoinositide/1·2 ml.; ●, 0·86 μmole of triphosphoinositide/1·2 ml. The incubation conditions were as described in the Experimental section. The cetyltrimethylammonium bromide was added in aqueous solution.

decline (Fig. 4). Neither bivalent cation at its optimum concentration stimulated the reaction to the same extent as the optimum amount of a suitable cationic amphipathic substance.

The activating effects of Mg^{2+} ions and cationic amphipathic substances were not additive; in fact, Mg^{2+} ions inhibited the reaction when added in the presence of an optimum activating concentration of cetyltrimethylammonium bromide. It was found that UO_2^{2+} ions, which readily bind with negative phosphate groups on a lipid surface (Kruyt, 1949; Bangham & Dawson, 1959), did not stimulate the reaction. However, UO_2^{2+} ions in low concentrations (0·33 mM) completely inhibited the enzyme reaction in a cetyltrimethylammonium bromide-activated system.

The basic proteins protamine and histone also increased the amount of combined phosphate liberated from triphosphoinositide, the stimulation reaching a maximum when about 0·5 mg. had been added to the basic system.

In the absence of an added activator the relationship between the amount of enzyme added to the system and the water-soluble phosphate liberated from triphosphoinositide was not linear (Fig. 5). However, in the presence of optimum activating amounts of cetyltrimethylammonium bromide or phosphatidylethanolamine plus sodium chloride (see below) the relation was approximately linear up to 0·2 ml. of enzyme added (Fig. 5).

Activation with sodium chloride. When sodium chloride was added to the reaction mixture a very small stimulation of the activity was observed. However, in the presence of lecithin or phosphatidylethanolamine there was an appreciable activation, even though these phospholipids, by themselves, had little effect on the reaction (Fig. 6). Sphingomyelin, phosphatidylinositol and phosphatidic acid, and the non-phosphorus-containing lipids cetyl alcohol, stearic acid, dilaurin, cholesterol, tripalmitin, natural diglyceride and

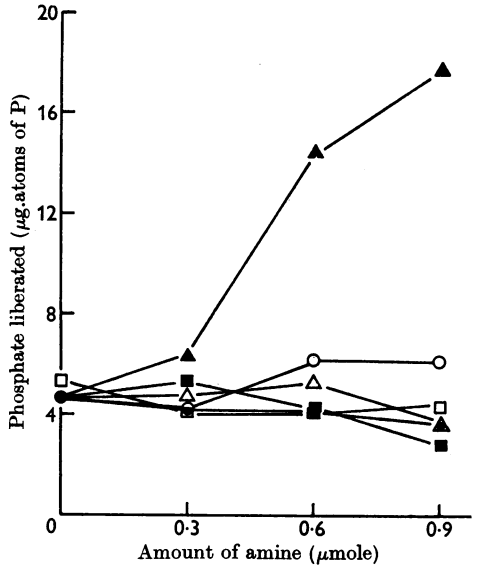


Fig. 3. Effect of long-chain aliphatic amines on triphosphoinositide phosphodiesterase. The incubation conditions were as described in the Experimental section. ●, *n*-Hexylamine added; ○, *n*-nonylamine added; △, *n*-dodecylamine added; ■, *n*-tetradecylamine added; □, *n*-hexadecylamine added; ▲, *n*-octadecylamine added. The amines dissolved in methanol were mixed with the substrate solution (in chloroform) and evaporated to dryness before buffer etc. were added.

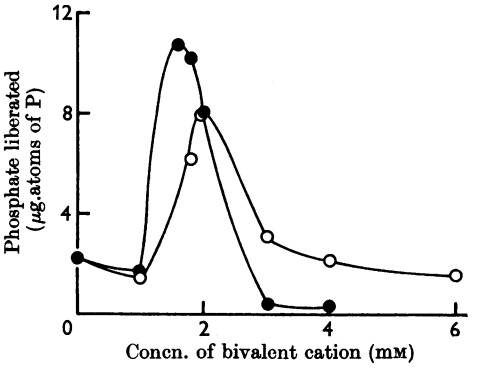


Fig. 4. Effect of bivalent cations on triphosphoinositide phosphodiesterase. The incubation conditions were as described in the Experimental section. ●, $CaCl_2$ added; ○, $MgCl_2$ added.

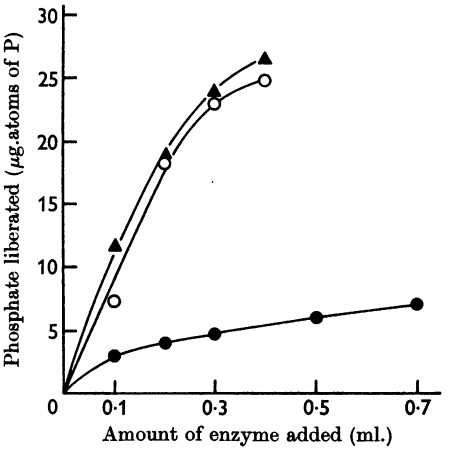


Fig. 5. Effect of enzyme concentration on triphosphoinositide phosphodiesterase in the absence and presence of added activators. The incubation conditions were as described in the Experimental section. ●, No additions; ○, 0·1 μmole of phosphatidylethanolamine + NaCl (0·125 M) added; ▲, 0·62 μmole of cetyltrimethylammonium bromide added.

stearamide, did not activate either in the presence or absence of sodium chloride. When magnesium chloride was added to a system containing phosphatidylethanolamine or lecithin at concentrations up to 10 mM no stimulation comparable with that produced by sodium chloride was observed. The phospholipids almost completely inhibited the activation produced by 1 mM-magnesium chloride.

Inhibitory effect of anionic amphipathic molecules. When a long-chain alkyl sulphate (hexadecyl or dodecyl) was added to the enzyme system the activity was inhibited, both in the basic system and in the presence of cetyltrimethylammonium bromide as activator (Fig. 7). The activation produced by cetyltrimethylammonium bromide was eliminated when approximately an equimolar amount of long-chain alkyl sulphate or dicetyl phosphate was added; stearic acid was slightly less effective (Fig. 7). A preparation of ox-brain ganglioside was also a potent inhibitor of the enzyme activated with cetyltrimethylammonium bromide, 150 μg. almost abolishing the activation.

Specificity. The enzyme did not attack a preparation containing mainly triphosphoinositide with some diphosphoinositide (27 % on a molar basis) when this was in the form of an insoluble calcium (magnesium) salt, but did so after conversion into the water-soluble sodium salt. The phosphate esters produced consisted of inositol triphosphate and inositol diphosphate in the molar ratio of 5:1. Since sodium triphosphoinositide gives only inositol triphosphate under these circumstances, it seems reasonable to conclude that diphosphoinositide was attacked by the enzyme preparation.

When lecithin, sphingomyelin, phosphatidylethanolamine or phosphatidylinositol was incubated with the enzyme preparation under conditions that produced a large breakdown (46 %) of triphosphoinositide, no liberation of acid-soluble phosphate could be detected, either in the absence or presence of the optimum concentration of cetyltrimethylammonium bromide. Phosphatidylinositol was not significantly hydrolysed by the enzyme preparation under conditions that enabled the phosphatidylinositol phosphodiesterase of liver or pancreas to attack this substrate (Dawson, 1959; Kemp,

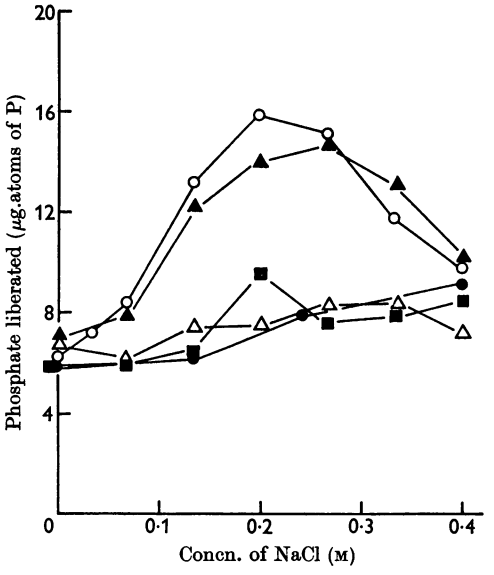


Fig. 6. Effect of NaCl on triphosphoinositide phosphodiesterase in the presence and absence of other phospholipids. The incubation conditions were as described in the Experimental section. ●, No lipid apart from substrate added; ○, 0·1 μmole of phosphatidylethanolamine added; ▲, 0·1 μmole of lecithin added; ■, 0·1 μmole of phosphatidylinositol added; △, 0·1 μmole of sphingomyelin added. The lipids, dissolved in chloroform, were mixed with substrate solution and evaporated to dryness before the addition of buffer etc.

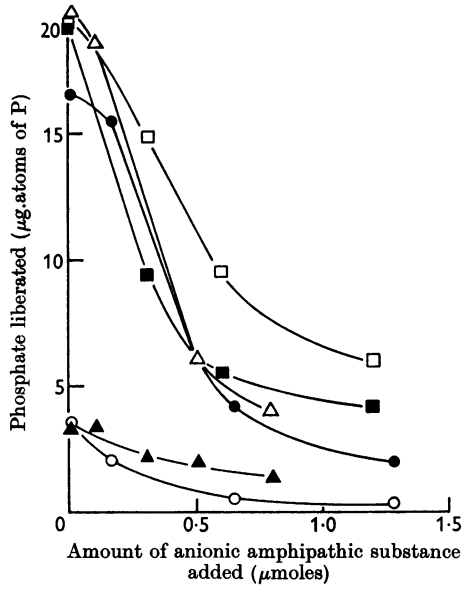


Fig. 7. Inhibitory effect of anionic amphipathic substances on triphosphoinositide phosphodiesterase. The incubation conditions were as described in the Experimental section. Basic system (no activator): ○, sodium hexadecyl sulphate; ▲, sodium dodecyl sulphate. Basic system + 0·62 μmole of cetyltrimethylammonium bromide: ●, sodium hexadecyl sulphate; △, sodium dodecyl sulphate; □, stearic acid; ■, dicetyl phosphate. Sodium dodecyl sulphate was added in aqueous solution, and sodium hexadecyl sulphate was added to the substrate in ethanol solution, and then the mixture was evaporated to dryness *in vacuo*. Stearic acid and dicetyl phosphate were added to the substrate in chloroform solution.

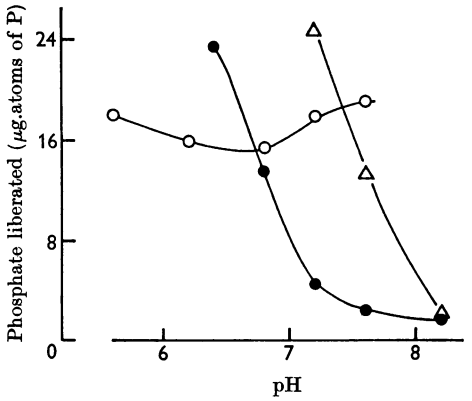


Fig. 8. Effect of buffer composition and pH on triphosphoinositide-phosphodiesterase activity. The incubation conditions were as described in the Experimental section. ○, ββ′-Dimethylglutaric acid–NaOH buffer (33 mM); ●, collidine–HCl buffer (33 mM); △, tris–HCl buffer (33 mM).

Hübscher & Hawthorne, 1961). When diphenyl phosphate was incubated with the enzyme no evidence was obtained for the liberation of phenol as measured by the method of King (1951). The enzyme did not liberate choline from glycerylphosphorylcholine (Dawson, 1956).

Effect of pH. The activity was highly dependent on the type of buffer used and consequently no pH optimum could be determined (Fig. 8). Tris–hydrochloric acid and collidine–hydrochloric acid buffers were examined at a constant base concentration and dimethylglutaric acid–sodium hydroxide buffer at a constant acid concentration of 33 mM. However, in other experiments, similar pH–activity curves were obtained with dimethylglutaric acid–sodium hydroxide buffers in which either the base or the acid concentration was kept constant at 33 mM.

Effect of inhibitors, etc. No absolute requirement for metal ions was found for the enzyme preparation. Although it was possible to stimulate the activity with Mg^{2+} and Ca^{2+} ions (see above), this action could be entirely replaced by the addition of a cationic amphipathic substance. It was surprising therefore to find that EDTA was a potent inhibitor of the reaction: 0·5 mM-EDTA virtually abolished the activity (Table 1).

If the EDTA was chelating a metal ion that is essential for enzymic activity this ion must be tightly combined with the enzyme. Thus dialysis of the enzyme for 72 hr. against 5 mM-tris–hydrochloric acid buffer, pH 7·2, at 4° resulted in no loss in activity. Further, EDTA still inhibited the diesterase activity of the enzyme purified by electrophoresis (Dawson & Thompson, 1964), which was likely to remove any remaining metal ions from the preparation.

Table 1. *Action of inhibitors and other substances on triphosphoinositide phosphodiesterase*

The incubation conditions were as described in the Experimental section, with 0·62 μmole of cetyltrimethylammonium bromide added as activator.

Substance added	Concn. (mM)	Percentage of control activity
EDTA	0·5	4
	1	4
1,10-Phenanthroline	0·5	110
	1	119
	4	110
8-Hydroxyquinoline	0·5	81
	1	76
	4	84
NaF	1	105
	3	78
Reduced glutathione	1·7	76
	6·8	65
Cysteine	1·25	71
	5	42
Iodoacetate	0·1	98
	0·5	101
Iodoacetamide	1	101
	5	92
Phenylmercuric acetate	0·01	23
	0·05	15
	0·1	15
N-Ethylmaleimide	1	82
	5	66
p-Chloromercuribenzoate	0·1	38
	0·5	22
$HgCl_2$	0·05	12
	0·1	10
$CuCl_2$	0·05	70
	0·1	47
Diethyl ether	25% (v/v)	78

When the EDTA was added to the enzyme and the mixture dialysed for 65 hr. with frequent changes of water, the preparation did not readily attack triphosphoinositide even when activating amounts of cetyltrimethylammonium bromide were present. However, activity was restored by the addition of Mg^{2+}, Ca^{2+} or Zn^{2+} ions at a concentration of 0·1 mM, which suggested that inhibitory amounts of EDTA were still left in the preparation after the dialysis.

In contrast, 1,10-phenanthroline at a concentration of 4 mM had no inhibitory effect, and 8-hydroxyquinoline inhibited only very slightly (Table 1). Sodium fluoride inhibited slightly at a concentration of 3 mM.

The addition of either reduced glutathione or cysteine at a concentration of 5 mM did not activate

the enzyme; in fact an inhibition was usually noted (Table 1). Iodoacetate (0·5 mM) and iodoacetamide (1 mM) were not inhibitory, but in contrast phenylmercuric acetate, Hg^{2+} ions and *p*-chloromercuribenzoate inhibited at low concentrations.

Diethyl ether (final concn. 25%, v/v) added to the system produced only slight inhibition of the cetyltrimethylammonium bromide-activated enzyme and none of the non-activated enzyme.

DISCUSSION

The enzyme preparation was free from triphosphoinositide-phosphomonoesterase activity, and of the phospholipid and water-soluble phosphodiester substrates tested only triphosphoinositide and diphosphoinositide were attacked. It is therefore clearly different from the phosphatidylcholine cholinephosphohydrolase (phospholipase C) which splits a diglyceride from lecithin, and in certain circumstances, from phosphatidylethanolamine. In addition there is no evidence that it will attack phosphatidylinositol to any appreciable extent, which suggests it is different from the enzyme that hydrolyses this latter substrate into inositol monophosphate and diglyceride (Dawson, 1959; Kemp *et al.* 1961). However, in view of the exacting physicochemical requirements for the form of the substrate in phospholipase reactions (Bangham & Dawson, 1959, 1962; Dawson, 1963), it is advisable to exercise some reservation in describing the specificity of such enzymes towards various substrates.

Unlike triphosphoinositide phosphomonoesterase the enzyme does not have an absolute requirement for an exogenous metal ion. However, the strongly inhibitory effect of EDTA at low concentrations suggests that a metal ion may be involved in the reaction. If this is so it must be tightly bound to the enzyme, since it is not removed by the purification and activity is not lost by prolonged dialysis or after electrophoretic separation of the enzyme.

In the absence of an added activator the enzyme appears to attack only about 10–15% of the substrate before hydrolysis almost ceases. This is not due to denaturation of the enzyme on a highly negatively charged lipid (see Fraser, Kaplan & Schulman, 1955), since once the activity has ceased it can be restored by the addition of an activator. The reaction involved appears to be the same as that in the activated system since electrophoretic separation of the water-soluble phosphate produced indicates that this is all inositol triphosphate. Since inositol triphosphate was not inhibitory at concentrations in excess of those formed in the reaction, the cessation of the reaction was not due to this product. Diglyceride prepared from ovolecithin was not inhibitory when added in amounts equivalent to those produced by the hydrolysis, but was so when added in excess. It is difficult, however, to come to a firm conclusion on this evidence, since the insoluble diglyceride released in the reaction may bear a physicochemical orientation to substrate and enzyme that is different from that taken up when it is intimately mixed with the substrate by evaporation from the same solution.

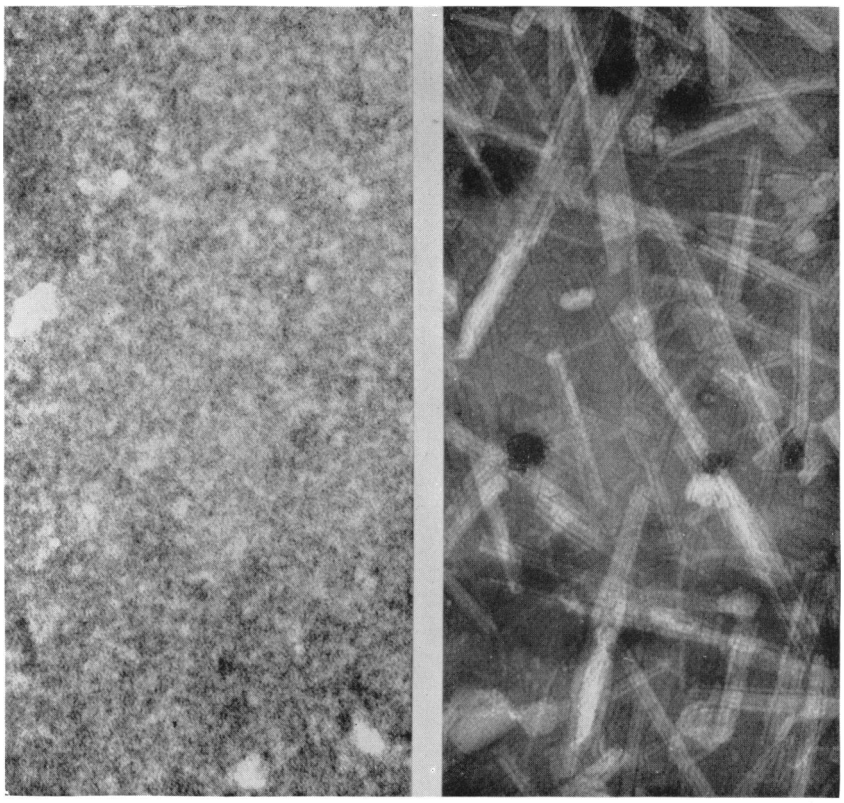
When solutions of triphosphoinositide (ammonium salt) were examined by electron microscopy (negative staining with 1% potassium phosphotungstate) in the absence of buffer, no discernible micro-structure was visible, as it is with many so-called soluble lipids such as lysolecithin. Presumably the repulsive effect of the strongly charged phosphate groups is too great to allow the hydrophobic hydrocarbon chains to come together to form an organized micro-structure.

The substances that activate the reaction are all of a type which would tend to combine preferentially with the substrate and to decrease or neutralize the excess of negative charge of its phosphate groups. Thus the amphipathic cations would probably line up alongside the triphosphoinositide molecules with the polar head groups adjacent and the hydrocarbon chains attracted to one another by van der Waals forces. Eventually an organized structure would be produced and precipitation would result. This is not seen at the point when cetyltrimethylammonium bromide is added at optimum activating concentrations, but on examination by electron microscopy a well-defined organized structure is seen (Plate 1). Further addition of cetyltrimethylammonium bromide produces a visible precipitate. The fact that the cetyltrimethylammonium ion is preferentially combining with substrate is shown by the observation that doubling the substrate concentration makes it necessary to double the cetyltrimethylammonium bromide added to produce optimum activation.

The Mg^{2+} and Ca^{2+} ions would presumably carry out the same function by forming salts with the secondary phosphate groups. It is significant that on adding Mg^{2+} ions the commencement of activation is just coincident with the precipitation of the substrate, presumably as the dimagnesium salt.

Protamine and histone, being very basic proteins, would also act on the triphosphoinositide in a similar way, and they cause some precipitation of the substrate in the incubation tube.

It is difficult to see why the combination of sodium chloride and lecithin or phosphatidylethanolamine should act in a similar manner. Possibly the positively charged groups on each of these phospholipid molecules can line up with a negatively charged phosphate group on the sub-



(*a*) (*b*)

Plate 1. Electron micrographs after negative staining with potassium phosphotungstate of (*a*) ammonium triphosphoinositide; (*b*) ammonium triphosphoinositide (40 μg.atoms of P) + cetyltrimethylammonium bromide (1·04 μmoles). Magnification $\times$ 130 000.

strate, and the Na^+ ions in high concentration then decrease the negative charge (on the substrate) still further by counter-ion-binding.

The anionic amphipathic substances sodium hexadecyl sulphate and ganglioside, which act as potent inhibitors of the reaction, presumably compete with the substrate for the activators available and thus prevent the necessary decrease of the negative charge on its phosphate groups. How the decrease of the negative charge on the substrate influences the enzymic reaction cannot be answered from the present observations. Presumably the substrate in its natural state exists with its phosphate groups tightly bound to a protein, possibly through two Mg^{2+} ion–inorganic phosphate bridges (Dittmer & Dawson, 1961; S. E. Kerr, personal communication). Electrophoretic separation of the enzyme (Dawson & Thompson, 1964) indicates that this would possess a net negative charge at pH 7·2, so that the enzyme and the free lipid substrate would tend to repel one another. The decrease in the negative charge on the substrate could well decrease the energy required for the active centre of the enzyme to come into a favourable stereochemical orientation to the substrate. However, the effect is a subtle one and does not appear to be exactly equivalent to the precise requirements for a certain substrate ζ-potential shown by other phospholipases (Bangham & Dawson, 1959, 1962). Thus, in additional experiments, electrophoretic-mobility measurements on the particles formed from triphosphoinositide and optimum activating amounts of stearylamine or Mg^{2+} ions showed that these were still strongly negatively charged ($-18{\cdot}0$ and $-8{\cdot}9\,\mu$/sec./v/cm. respectively). Moreover, the electrophoretic mobilities of stearylamine-plus-triphosphoinositide particles were almost identical with those of palmitylamine-plus-triphosphoinositide particles, even though the latter amine was consistently shown not to act as an activator. Presumably this extreme sensitivity to chain length may reflect a sensitive requirement for the precise orientation of substrate and activator molecules alongside one another.

SUMMARY

1. Triphosphoinositide phosphodiesterase free from phosphomonoesterase activity was isolated from ox brain.

2. The enzyme removed diglyceride only from triphosphoinositide and diphosphoinositide.

3. No absolute requirement for metal ions was found, but the dialysed enzyme was inhibited by 0·5 mM-EDTA.

4. Hydrolysis of triphosphoinositide was limited unless a cationic amphipathic substance was present as activator.

5. Histone, protamine, Ca^{2+} ions and Mg^{2+} ions also activated at certain concentrations.

6. Sodium chloride in the presence of lecithin or phosphatidylethanolamine produced a marked activation; the phospholipids alone did not.

7. Anionic amphipathic substances were potent inhibitors of the enzyme.

8. It is suggested that the activators produce their effect by decreasing the excess of negative charge on the phosphate groups of the substrate molecule which allows the enzyme to come into a favourable stereochemical orientation.

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