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The Triphosphoinositide Phosphomonoesterase of Brain Tissue

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Two enzymes are responsible for the catabolism of triphosphoinositide by ox-brain tissue (Thompson & Dawson, 1964*a*), and the preparation and properties of triphosphoinositide phosphodiesterase present in brain tissue extracts have been described (Thompson & Dawson, 1964*b*). The present paper reports the preparation and properties of a triphosphoinositide phosphomonoesterase from the same source.

EXPERIMENTAL

Preparation of triphosphoinositide phosphomonoesterase from brain extracts. To 40 ml. of the extract of acetone-dried brain powder (Thompson & Dawson, 1964*a*) was added 20 ml. of 0·132 M-tris–HCl buffer, pH 7·2, and the mixture was cooled to 0°. Solid $(NH_4)_2SO_4$ (10·64 g.) was slowly added with stirring, and the mixture was left for 30 min. at 0°. After centrifuging at 0° for 10 min. at 10000$g_{av.}$ the supernatant was separated and an additional 6 g. of $(NH_4)_2SO_4$ was added. The mixture was stirred until the salt was completely dissolved, kept at 0° for 30 min. and the precipitate recovered by centrifuging at 10000$g_{av.}$. The precipitate was taken up in 3 ml. of 0·132 M-tris–HCl buffer, pH 7·2, and the solution dialysed overnight at 4° against 1 l. of 5 mM-dimethylglutaric acid–NaOH buffer, pH 6·4. A 2½–3-fold concentration of activity and 60% yield were obtained at this point. This 25–40% saturated $(NH_4)_2SO_4$ fraction was then fractionated by density-gradient electrophoresis (Svensson, 1960) as modified by Bangham & Dawson (1962) and Dawson (1963). The light buffer was 2·5 mM-dimethylglutaric acid–NaOH and the heavy component was the same buffer in 45% (v/v) glycerol. The enzyme fraction mixed with the appropriate amount of glycerol and buffer was inserted after about 2–3 cm. of the gradient had been formed. The electrophoresis was carried out for 16–18 hr. at 850 v (5 mA) at the temperature of running tap water. The fractions recovered from the column were assayed for enzymic activity and extinction at 280 mμ. The fractions constituting the main monophosphoesterase peak were combined and stored at −15°.

Conditions of incubation and assay of activity. The incubation medium usually contained: triphosphoinositide (sodium or ammonium salt), 0·43 μmole; 0·132 M-tris–HCl buffer, pH 7·2, 0·3 ml.; 6 mM-$MgCl_2$, 0·15 ml.; 0·2 M-reduced glutathione, 0·1 ml.; enzyme preparation (0·05–0·2 ml.); and water to give a final volume of 1·2 ml. Incubation was carried out for 30 min. at 37°. The subsequent procedure was the same as that described for the phosphodiesterase by Thompson & Dawson (1964*b*), the inorganic phosphate liberated being determined by the Fiske & Subbarow (1925) method, and taken as a measure of enzymic activity. Blanks of substrate alone and enzyme alone released virtually no inorganic phosphate on incubation under the same conditions.

Materials. The preparation or source of the various lipids and other chemicals were as described by Thompson & Dawson (1964*b*). Inositol triphosphate and inositol diphosphate were prepared as described by Dawson & Dittmer (1961).

RESULTS

Preparation of triphosphoinositide phosphomonoesterase. As indicated by Thompson & Dawson (1964*b*), many attempts have been made to obtain a clean separation of triphosphoinositide phosphomonoesterase and triphosphoinositide phosphodiesterase. However, none of these produced a preparation of phosphomonoesterase completely free from phosphodiesterase. The best partial separation was obtained by density-gradient electrophoresis of a 25–40% saturated ammonium sulphate fraction of the crude extract of acetone-dried brain. The ammonium sulphate fractionation resulted in 2½–3-fold purification (60% yield) of the phosphomonoesterase activity of the extract based on protein concentrations measured by extinctions at 260 and 280 mμ. The electrophoresis of this fraction in dimethylglutarate buffer, pH 6·4, resulted in a separation into two protein components that moved towards the anode (Fig. 1). The main triphosphoinositide-phosphodiesterase peak was associated with the faster-moving and larger protein peak. The main triphosphoinositide-monoesterase peak moved more slowly but just in advance of the minor protein peak.

However, Fig. 1 shows that the separation was by no means quantitative and each main enzyme peak was overlapped by a minor peak of the other. Attempts to improve the separation by raising the pH of the electrophoresis buffer were not successful; on decreasing the pH, precipitation of the enzymes occurred.

Nature of the enzymic reaction. The purified enzyme attacked triphosphoinositide in the presence of Mg^{2+} ions and liberated inorganic phosphate and a phospholipid that was identified as monophosphoinositide by alkaline degradation

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(Dawson, Hemington & Davenport, 1962). It is therefore equivalent to the enzyme examined in the crude extracts of acetone-dried brain (Thompson & Dawson, 1964*a*).

Requirement for metal ions. When the crude extract of acetone-dried ox brain was adjusted to pH 5·0 and the precipitate obtained separated by centrifuging, neither the precipitate nor the supernatant contained any appreciable phosphomonoesterase activity. On recombination complete restoration of activity was achieved. It was subsequently found that the supernatant could be largely replaced by Mg^{2+} ions or to a lesser extent by Mn^{2+} ions; on the other hand, Cu^{2+}, Zn^{2+}, Fe^{3+}, Ca^{2+}, Na^{+}, K^{+} and NH_4^{+} ions were not effective. The purified enzyme showed little activity in the absence of added metal ions. Fig. 2 shows the effect of Mg^{2+} ions on the electrophoretically purified enzyme system, the curve exhibiting a sharp increase in activation at about 2 mM. This threshold in the Mg^{2+} ion concentration was roughly proportional to the amount of substrate present. In contrast with findings with the crude enzyme Mn^{2+} ions did not activate. On their own Ca^{2+} ions were completely ineffective, but, by adding a low concentration (1·5 mM) of Mg^{2+} ions, which by itself produced little activation, and then adding Ca^{2+} ions, an activation was produced at a combined concentration of bivalent cations of 2 mM (Fig. 2). Subsequent addition of Ca^{2+} ions inhibited the system.

Replacement of metal ions with activators. The above results suggested that the stimulatory effect of Mg^{2+} ions on the enzymic activity involved two functions, in one of which it could be replaced by Ca^{2+} ions whereas in the other it could not. Fig. 3 shows that the stimulation was also produced by adding palmitoylcholine hydrobromide, stearylamine hydrochloride and cetyltrimethylammonium bromide to the reaction mixture containing suboptimum concentrations (1·5 mM) of Mg^{2+} ions. In the absence of Mg^{2+} ions no enzymic activity was observed at any concentration of cetyltrimethylammonium bromide (Fig. 3), but in the presence of this cationic detergent Mn^{2+} ions were able to replace Mg^{2+} ions. There was a progressive loss of activation by aliphatic amines when the chain length was less than C_9, and hexylamine showed little ability to activate the system (Fig. 4).

When the anionic amphipathic substances stearic acid and sodium dodecyl sulphate were added at various concentrations, they produced no activation, and the latter, when tested in a system which had been stimulated by cetyltrimethylammonium bromide or Mg^{2+} ions, almost abolished the activity.

The effects of other lipids containing no ionizable charged group were variable; cholesterol, triolein and natural diglyceride produced small activations in the presence of suboptimum concentrations of Mg^{2+} ions, but tripalmitin produced no activation. However, activating neutral lipids also had the capacity of removing some of the triphospho-

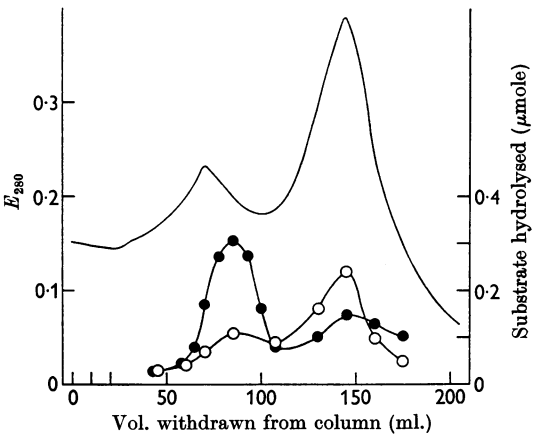

Fig. 1. Gradient-density electrophoresis of 25–40% saturated ammonium sulphate fraction of ox-brain extract. ——, E_{280}; ○, triphosphoinositide-phosphodiesterase activity (Thompson & Dawson, 1964*b*); ●, triphosphoinositide-phosphomonoesterase activity. Electrophoresis was for 17·5 hr. at 850 v (5 mA) in 2·5 mM-dimethylglutaric acid–NaOH buffer, pH 6·4. Samples (0·3 ml.) of the column fractions were assayed for activity. The substrate hydrolysed in the triphosphoinositide-phosphomonoesterase assay was calculated by assuming that only monophosphoinositide is formed and therefore represents a minimum value.

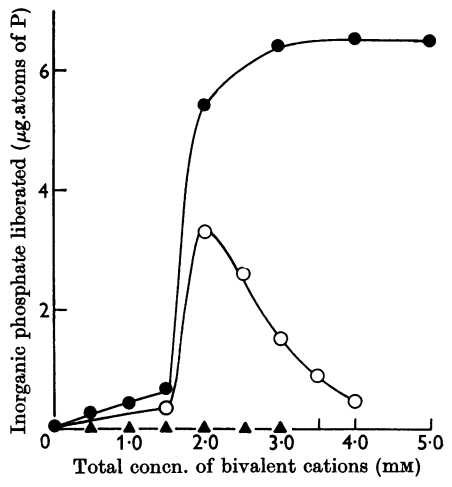

Fig. 2. Effect of Mg^{2+} and Ca^{2+} ions on triphosphoinositide phosphomonoesterase. ●, $MgCl_2$ added; ▲, $CaCl_2$ added; ○, $MgCl_2$ added to give a Mg^{2+} ion concentration of 1·5 mM, then $CaCl_2$ added. The incubation conditions were as described in the Experimental section.

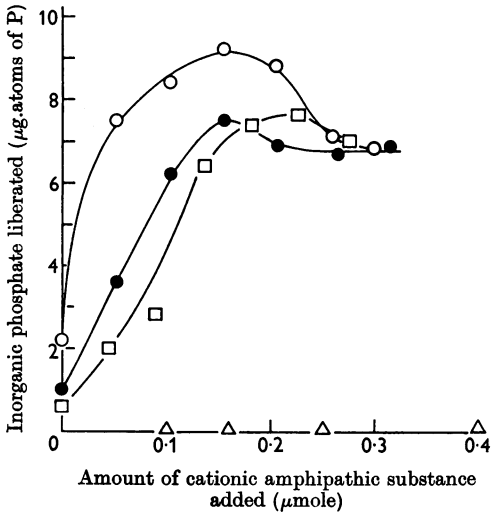

Fig. 3. Effect of amphipathic cations on triphosphoinositide phosphomonoesterase assayed in the presence of suboptimum concentrations of Mg^{2+} ions. ○, Cetyltrimethylammonium bromide added; ●, stearylamine hydrochloride added; □, palmitoylcholine hydrobromide added; △, cetyltrimethylammonium bromide added (no Mg^{2+} ions present). The incubation conditions were as described in the Experimental section except that the concentration of Mg^{2+} ions was decreased to 1·5 mM. Cetyltrimethylammonium bromide and palmitoylcholine hydrobromide were added as aqueous solutions. Stearylamine hydrochloride in ethanol solution was added to the triphosphoinositide solution and evaporated to dryness *in vacuo* before the addition of tris–HCl buffer etc.

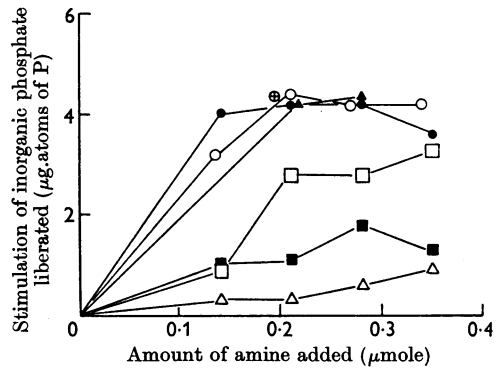

Fig. 4. Effect of added aliphatic amines on triphosphoinositide phosphomonoesterase in the presence of suboptimum concentrations of Mg^{2+} ions. ⊕, Octadecylamine added; ▲, tetradecylamine added; ○, dodecylamine added; ●, nonylamine added; □, octylamine added; ■, heptylamine added; △, hexylamine added. The incubation conditions were as described in the Experimental section except that the concentration of Mg^{2+} ions was decreased to 1·5 mM. Amines dissolved in ethanol were added to substrate solution and evaporated to dryness *in vacuo* before the addition of tris–HCl buffer etc.

inositide substrate from solution, whereas tripalmitin did not. Since the amount of Mg^{2+} ions required for activation was dependent on the amount of substrate present, it would seem that with the neutral lipids the activation was caused by the suboptimum concentration of Mg^{2+} ions becoming optimum with the decreased amount of substrate. A similar activation can in fact be obtained by merely decreasing the substrate concentration.

It was observed that the crude extracts of acetone-dried brain powder contained a substance that also activated the enzymic activity measured in the presence of suboptimum concentration of Mg^{2+} ions. This activator was found in the soluble fraction obtained after precipitating the crude extract at pH 5·0. This pH 5·0 supernatant showed no enzymic activity towards triphosphoinositide when tested in the presence of Mg^{2+} ions and cetyltrimethylammonium bromide. However, it activated the purified enzyme preparation (Fig. 5). That this is not due to traces of Mg^{2+} ions in the preparation is shown by the fact that the activation produced is higher than that obtained with optimum activation by Mg^{2+} ions. In addition, the activating ability was largely retained after prolonged dialysis. The nature of the activating factor has not been deduced, but it would appear that it is not chloroform-soluble and is therefore unlikely to be a lipid. The mechanism of the activation appears to be different from that produced by cetyltrimethylammonium bromide since the addition of sodium dodecyl sulphate to the system does not inhibit.

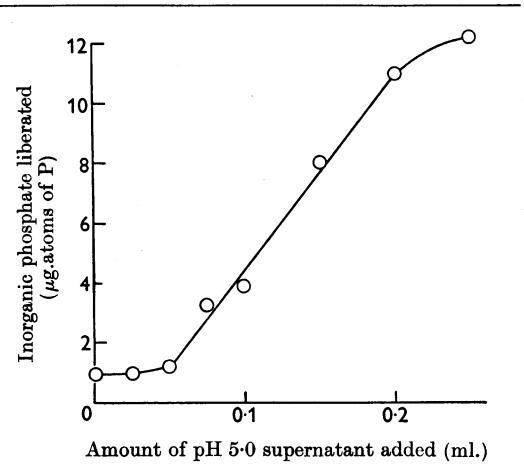

Fig. 5. Effect of adding a pH 5·0 supernatant of crude brain extract on triphosphoinositide phosphomonoesterase in the presence of a suboptimum concentration of Mg^{2+} ions. The incubation conditions were as described in the Experimental section except that the concentration of Mg^{2+} ions was decreased to 1·5 mM.

The only non-lipid macromolecular substances that produced activation in a medium containing suboptimum concentrations of Mg^{2+} ions were the basic proteins histone and protamine (Fig. 6). Here again the activation was inhibited by the addition of sodium dodecyl sulphate.

Effects of sodium chloride and potassium chloride on activators. Initial observations with the crude extract of acetone-dried brain powder showed that the release of inorganic phosphate from triphosphoinositide was markedly stimulated by the addition of sodium chloride or potassium chloride to the reaction mixture (Thompson & Dawson, 1962). This stimulation reached a maximum at a salt concentration of about 0·15 M (Fig. 7) and was not produced by adding sucrose to the same osmolarity. In sharp contrast with these results it was observed with the purified enzyme preparation that sodium chloride at all concentrations up to 0·3 M had no activating effect (Fig. 8*a*). However, in the presence of suboptimum amounts of pH 5·0 supernatant, additions of sodium chloride had a marked

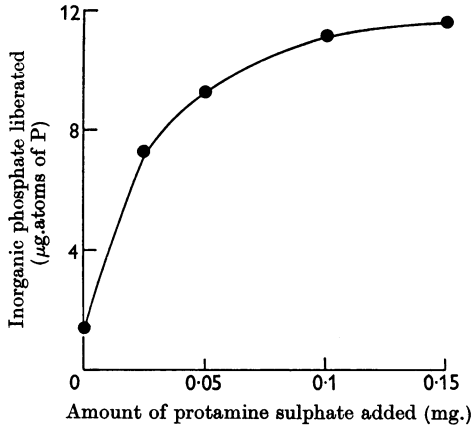

Fig. 6. Effect of protamine sulphate on triphosphoinositide phosphomonoesterase activity in the presence of a suboptimum concentration of Mg^{2+} ions. The incubation conditions were as described in the Experimental section except that the concentration of Mg^{2+} ions was decreased to 1·5 mM.

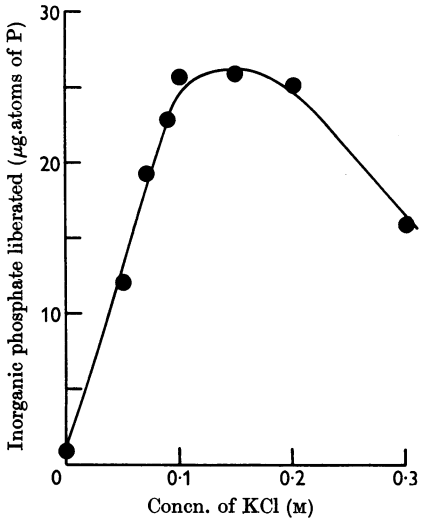

Fig. 7. Effect of KCl on the liberation of inorganic phosphate from triphosphoinositide by extracts of acetone-dried ox brain. The incubation conditions were as described by Thompson & Dawson (1964*a*) but scaled down to a total volume of 2·4 ml.

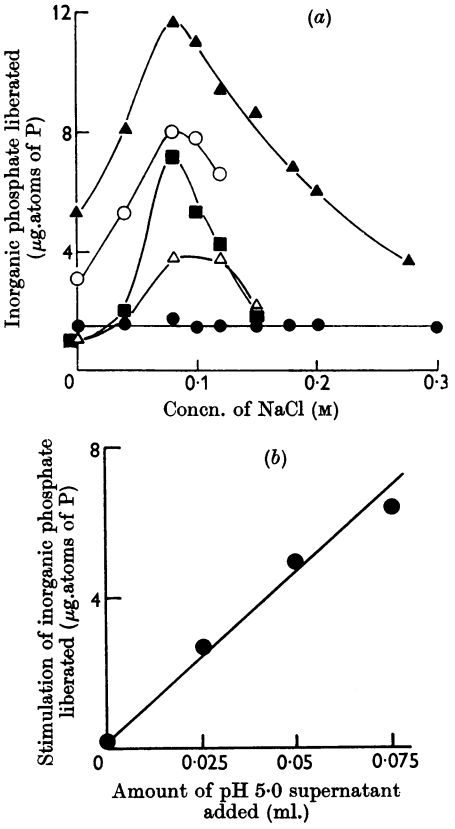

Fig. 8. (*a*) Effect of the addition of NaCl on triphosphoinositide-phosphomonoesterase activity assayed in the presence of suboptimum concentrations of Mg^{2+} ions. The incubation conditions were as described in the Experimental section except that the concentration of Mg^{2+} ions was decreased to 1·5 mM. ●, Basic system; △, basic system + 0·025 ml. of pH 5·0 supernatant; ○, basic system + 0·05 ml. of pH 5·0 supernatant; ▲, basic system + 0·075 ml. of pH 5·0 supernatant; ■, basic system + 0·02 μmole of cetyltrimethylammonium bromide. (*b*) Relationship between the amount (ml.) of pH 5·0 supernatant added to the system and the maximal stimulation produced by NaCl (taken from Fig. 8*a*).

stimulatory effect on the activity of the purified enzyme (Fig. 8*a*). There was a direct proportionality between the amount of pH 5·0 supernatant added and the maximal stimulation produced by the presence of salt (approx. 0·08 M) (Fig. 8*b*). This stimulatory effect of salt was also observed in the presence of a low concentration of cetyltrimethylammonium bromide, which by itself was not sufficient to activate significantly the enzymic activity (Fig. 8*a*).

Specificity. The enzyme preparation (25–40 % saturated ammonium sulphate fraction) was tested for its ability to dephosphorylate the following substrates under the standard conditions described in the Experimental section: monocetyl phosphate, phosphatidic acid, α-glycerophosphate, β-glycerophosphate, phytate, adenosine triphosphate, fructose 1,6-diphosphate, glucose 1-phosphate, glucose 6-phosphate, serine *O*-phosphate, casein, inositol monophosphate, inositol diphosphate and inositol triphosphate. Only inositol triphosphate was attacked, and the liberation of inorganic phosphate was about 30 % of that obtained from triphosphoinositide at the same concentration. Impure preparations of alkaline (calf-intestinal) and acid (human-semen) phosphatases were used to test the susceptibility of triphosphoinositide to these enzymes. Little release of inorganic phosphate occurred compared with that obtained from β-glycerophosphate under the same conditions (Table 1). On the other hand, the crude extract of acetone-dried brain dephosphorylated triphosphoinositide but was without action on β-glycerophosphate (Table 1).

Optimum pH. The activity of the enzyme was assayed in a series of dimethylglutaric acid–sodium hydroxide and collidine–hydrochloric acid buffers (33 mM), and in both maximal liberation of inorganic phosphate was observed at pH 6·8. The activity in tris–hydrochloric acid buffer, pH 7·2, was, however, slightly higher and this was therefore used throughout the investigation.

Effect of inhibitors, etc. Since Mg^{2+} or Mn^{2+} ions are obligatory cofactors for the enzyme, it was not surprising to find that EDTA was an inhibitor. However, the inhibition was not progressive and a residual activity of about 30 % of the original remained as the concentration of EDTA was increased from 0·45 to 4·1 mM (Table 2). This inhibition produced by EDTA was reversed by adding cetyltrimethylammonium bromide to the reaction mixture (Table 2). Sodium fluoride at a concentration of 3·3 mM only slightly inhibited the activity.

Diethyl ether, which markedly inhibited the release of inorganic phosphate from triphosphoinositide by the crude enzyme extract (Thompson & Dawson, 1964*a*), rather surprisingly had only a slight inhibitory action (25 %) when added in amounts equivalent to 33 % of the medium volume (Table 2). Reduced glutathione or cysteine in high concentrations (16·6 mM) produced a variable stimulation of the reaction (Table 3), but usually the inorganic phosphate liberated was approximately doubled. Incubation in the presence of substances that react with thiol groups and in the absence of added reduced glutathione or cysteine gave variable results. Whereas Hg^{2+} ions, *p*-chloromercuribenzoate and phenylmercury acetate strongly inhibited the enzyme at low concentrations, iodoacetate, iodoacetamide and *N*-ethylmaleimide actually caused a slight stimulation.

Table 1. *Action of various enzyme preparations on triphosphoinositide and β-glycerophosphate*

Incubations were carried out in 33 mM-tris–HCl buffer, pH 7·2, for 30 min. at 37°.

Enzyme preparation	Inorganic phosphate liberated (μg.atoms of P): Substrate triphosphoinositide (44 μg.atoms of P)	Substrate β-glycerophosphate (40 μg.atoms of P)
Extract of acetone-dried ox brain	10·7	0
Acid phosphatase (human-semen)	1·5	38·5
Alkaline phosphatase (calf-intestinal; L. Light and Co. Ltd., Colnbrook, Bucks.)	3·8	29·8

Table 2. *Action of inhibitors and other substances on triphosphoinositide phosphomonoesterase*

The incubation conditions were as described in the Experimental section, the 25–40 % saturated $(NH_4)_2SO_4$ fraction being used as enzyme.

	Substance added	Concn. (mM)	Percentage of control activity
Expt. 1	EDTA	0·45	32
		1·25	32
		2·5	29
		4·1	34
Expt. 2	EDTA	4·1	26
	EDTA + 0·31 μmole of cetyltrimethylammonium bromide	4·1	100
Expt. 3	Diethyl ether	8 % (v/v)	75
		25 % (v/v)	76
		33 % (v/v)	76
	NaF	3·3	80

DISCUSSION

The dephosphorylating enzyme was highly specific towards triphosphoinositide. Inositol triphosphate was attacked to a limited extent, but

Table 3. *Action of thiols and of substances that react with thiol groups on triphosphoinositide phosphomonoesterase*

The incubation conditions were as described in the Experimental section, except that reduced glutathione was omitted. The enzyme was a 25–40% saturated $(NH_4)_2SO_4$ fraction.

Substance added	Concn. (mM)	Percentage of control activity
Reduced glutathione	8·3	140
	16·6	178
Cysteine	4·1	163
	8·3	174
	16·6	178
Iodoacetate	1·6	110
	4·1	124
Phenylmercury acetate	0·042	40
	0·17	24
Iodoacetamide	0·41	128
	3·3	137
N-Ethylmaleimide	0·41	120
	1·6	123
p-Chloromercuribenzoate	0·041	36
	0·41	19
	1·6	0
$HgCl_2$	0·041	2
	0·166	4
$CuCl_2$	0·041	58
	0·166	40
	0·41	24

none of the other phosphate esters tested were hydrolysed even though these are known to be substrates for other phosphomonoesterases. It seems reasonable to infer that diphosphoinositide can also act as a substrate since its accumulation as a transient intermediate in the reaction has been demonstrated (Thompson & Dawson, 1964*a*).

The enzyme clearly has an absolute requirement for Mg^{2+} (or Mn^{2+}) ions as a cofactor, since activity was never observed in the complete absence of these ions. However, the stimulation of the activity produced by adding progressive amounts of Mg^{2+} ions was not linear and little activity was observed below a concentration of 2 mM. On adding Mg^{2+} ions to about this concentration a precipitation of the substrate occurred and this was associated with a sudden stimulation of the dephosphorylation. In the presence of concentrations of Mg^{2+} ions below 2 mM, which alone produced little activation, the addition of various other substances produced a marked stimulation of the reaction. For example, Ca^{2+} ions stimulated the reaction when added in amounts equivalent to that of the Mg^{2+} ions required to activate the system, even though they were completely inactive in the complete absence of Mg^{2+} ions. This suggests that the action of Mg^{2+} ions can be divided into two components, one as a completely essential coenzyme and the other as a non-essential factor that can be replaced by other substances. The fact that long-chain bases are efficient activators of the reaction could indicate that the enzyme is unable to dephosphorylate the substrate until the negative charges of the secondary phosphate groups have been decreased or neutralized. A long-chain base such as cetyltrimethylammonium bromide would tend to lie alongside a triphosphoinositide molecule so that the two are orientated with their polar head groups adjacent and their long hydrocarbon chains attracted by van der Waals forces. An anionic amphipathic substance such as sodium dodecyl sulphate did not produce activation, and in fact inhibited the activating effect of cetyltrimethylammonium bromide, presumably by neutralizing the charge effect of this on the substrate.

This hypothesis could also explain why a certain threshold of Mg^{2+} ions is required for activity to begin. The amount of Mg^{2+} ions required to completely neutralize both secondary phosphate groupings on the triphosphoinositide molecule and form the dimagnesium salt would be 0·86 μg.ion in the present system. Stimulation of the system was observed to begin when 0·9 μg.ion was added and this could be correlated with the appearance in the system of a precipitate that was presumably the insoluble dimagnesium salt. If the amount of substrate in the system was decreased the Mg^{2+} ion concentration required to produce activation was proportionally less.

Other activators such as Ca^{2+} ions can only help in this neutralization possibly because the addition of Mg^{2+} ions to the phosphate groups may be an essential prerequisite for the enzymic reaction. Excess of Ca^{2+} ions and other activators may inhibit by displacing these essential Mg^{2+} ions from the substrate.

The activation produced by the basic proteins protamine and histone could also be explained by their action in neutralizing the excess of negative charge on the substrate. Triphosphoinositide is known to occur in Nature in tight combination with protein (Dittmer & Dawson, 1961), and in the present experiments the basic proteins produced a precipitate on being mixed with the substrate. It is presumed that such a combination is through a salt-like linkage of the excess of amino groups on the protein with the phosphate groups on the triphosphoinositide. The activating effect of these basic proteins is again inhibited by sodium dodecyl sulphate, presumably by its competition with the substrate for the basic protein.

The nature of the activator present in the pH 5·0 supernatant of crude brain extracts has not been elucidated. Its activating effect appears to be similar to that of other activators except that no inhibition occurs when sodium dodecyl sulphate is

added to the reaction mixture. Further, unlike the other activators it is completely without action on the triphosphoinositide phosphodiesterase (Thompson & Dawson, 1964*b*).

The addition of sodium chloride appeared to have little activating action unless limited amounts of another activator were present, when a marked stimulation was produced. Univalent cations such as Na^+ or K^+ would tend to neutralize the negative charges on a triphosphoinositide molecule by counter-ion-binding. However, this process would be reversible and, with two negative charges per secondary phosphate group, the chances of these being completely neutralized at any given time would be very limited unless a very high salt concentration were present in the aqueous phase. If part of the charge on the phosphate group is neutralized with another activator so that only one negative charge needs to be taken care of by the Na^+ ions, then the chances of reaction would be much greater. In fact there appears to be an almost linear relationship between the suboptimum amount of activator added and the stimulation produced by sodium chloride (Fig. 8*b*). The reason why sodium chloride cannot activate in the presence of suboptimum concentrations of Mg^{2+} ions may possibly be because a free phosphate group will still be left on the molecule with two negative charges which cannot be effectively neutralized by the Na^+ ions in solution.

No conclusive reason can be given as to why the decrease of the negative charge on the substrate is essential for activity. The enzyme will be negatively charged at physiological pH values and it is reasonable to believe that there will be repulsive forces between the enzyme and the strongly negatively charged region of the substrate on which it is acting which may prevent a favourable orientation of active centre and substrate. The neutralization of the negative charge on the substrate will give it a tendency to form an organized micro-structure with a lipid–water interface but it is impossible to state whether this bears any relation to the activation of enzymic activity.

SUMMARY

1. Triphosphoinositide phosphomonoesterase was isolated from ox-brain extracts by ammonium sulphate fractionation and gradient-density zone electrophoresis. It was slightly contaminated with phosphodiesterase.

2. Triphosphoinositide and, to a lesser extent inositol triphosphate were dephosphorylated.

3. The enzyme had a pH optimum of about 6·8 and required Mg^{2+} (or Mn^{2+}) ions as an essential cofactor.

4. Part of the requirement for Mg^{2+} ions was replaceable by a component of the pH 5·0 supernatant of ox-brain extract and also by substances that would decrease the excess of negative charge on the substrate molecule.

5. Sodium dodecyl sulphate antagonized the effect of all activators except for the ox-brain pH 5·0 supernatant factor.

6. Sodium chloride stimulated the reaction when suboptimum amounts of certain of the substances that replace part of the requirement for Mg^{2+} ions were present.

7. The enzyme required reduced glutathione or cysteine for full activity. *p*-Chloromercuribenzoate, Hg^{2+} ions and phenylmercury acetate were inhibitory in low concentrations. Iodoacetate, iodoacetamide and *N*-ethylmaleimide did not inhibit.

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REFERENCES

Bangham, A. D. & Dawson, R. M. C. (1962). *Biochim. biophys. Acta*, **59**, 103.

Dawson, R. M. C. (1963). *Biochem. J.* **88**, 414.

Dawson, R. M. C. & Dittmer, J. C. (1961). *Biochem. J.* **81**, 540.

Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962). *Biochem. J.* **84**, 497.

Dittmer, J. C. & Dawson, R. M. C. (1961). *Biochem. J.* **81**, 535.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Svensson, H. (1960). In *Analytical Methods of Protein Chemistry*, vol. 1, p. 193. Ed. by Alexander, P. & Block, R. J. Oxford: Pergamon Press Ltd.

Thompson, W. & Dawson, R. M. C. (1962). *Acta neurol. scand.* (suppl. 1), 34.

Thompson, W. & Dawson, R. M. C. (1964*a*). *Biochem. J.* **91**, 233.

Thompson, W. & Dawson, R. M. C. (1964*b*). *Biochem. J.* **91**, 237.