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The Diphosphoinositide Kinase of Rat Brain

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1. The supernatant fraction of adult rat brain contains a diphosphoinositide kinase. 2. Formation of triphosphoinositide by the enzyme in the presence of ATP and Mg^{2+} ions was shown with labelled ATP or labelled diphosphoinositide. 3. The kinase was also activated by Ca^{2+}, Mn^{2+} and Co^{2+} ions, but to a smaller extent than by Mg^{2+} ions. 4. In the presence of optimum Mg^{2+} ion concentration the enzyme was inhibited by Ca^{2+} ions. 5. Activity did not depend on thiol groups and the pH optimum was 7·3. 6. The dialysed supernatant fraction had no diglyceride kinase activity and negligible phosphatidylinositol kinase activity. 7. Triphosphoinositide phosphomonoesterase was present but showed little activity under the conditions used to assay the kinase. 8. Diphosphoinositide kinase was purified by ammonium sulphate fractionation, ethanol treatment and chromatography on Sephadex G-200. 9. This purification removed much of the triphosphoinositide phosphomonoesterase.

Labelling experiments *in vivo* and with brain slices have indicated that triphosphoinositide is formed by the stepwise phosphorylation of phosphatidylinositol. The first kinase involved, which produces diphosphoinositide, has been studied by Colodzin & Kennedy (1965), Michell & Hawthorne (1965) and Kai, White & Hawthorne (1966*b*). The second kinase, which forms triphosphoinositide from diphosphoinositide, was first studied by Kai, Salway, Michell & Hawthorne (1966*a*). The two enzymes, which may be called phosphatidylinositol kinase and diphosphoinositide kinase respectively, both require ATP and Mg^{2+} ions, but are otherwise quite different. This paper describes the partial purification and some of the properties of diphosphoinositide kinase. A brief account of the work has been given (Kai & Hawthorne, 1967).

MATERIALS AND METHODS

Reagents. Triton X-100 (*p*-1,1,3,3,-tetramethylbutylphenylpolyethoxyethanol) was obtained from Lennig Chemicals Ltd. (Jarrow, Co. Durham) and Cutscum (iso-octylphenoxypolyethoxyethanol) from Kodak Ltd. (Kirkby, Liverpool). Sigma (London) Chemical Co. Ltd. (London, S.W. 6) supplied the crystalline bovine serum albumin and 6-phosphogluconic acid (sodium salt).

Materials for chromatography. DEAE-cellulose (Whatman DE-52) was obtained from H. Reeve Angel and Co. (London, E.C. 4). Sephadex G-200, CM-Sephadex C-50 and DEAE-Sephadex A-50 were obtained from Pharmacia (G.B.) Ltd. (London, W. 13).

Diphosphoinositide. The diphosphoinositide fraction described by Folch (1949) was purified by the method of Hendrickson & Ballou (1964) on a DEAE-cellulose column. The product was 79% pure (mean value from five preparations) after elution from one such column, the remainder being triphosphoinositide. This material was usually used as substrate for diphosphoinositide kinase. In some experiments (Table 6) completely pure diphosphoinositide was used. This was prepared by rechromatography of the above preparation on DEAE-cellulose.

[^{32}P]*Diphosphoinositide.* The labelled lipid was obtained by incubating rat-brain homogenate (120mg. of protein) with phosphatidylinositol (1mM), terminally labelled [^{32}P]ATP (5·7mM), $MgCl_2$ (30mM), GSH (10mM), Cutscum (2%, w/v) and tris–HCl buffer, pH 7·4 (20mM), in a total volume of 30ml. for 20min. at 37°. The lipid was extracted and washed as described below. After being dried, the lipid was emulsified with 40ml. of 2% (w/v) EDTA (sodium salt) at pH 7·4, to which was added 3·7 μmoles of unlabelled diphosphoinositide as carrier, and dialysed at 4° against 5l. of deionized water overnight, with two changes of water. The resulting sodium salt of diphosphoinositide was then freeze-dried. Pure labelled diphosphoinositide was prepared from this material by chromatography on DEAE-cellulose.

Triphosphoinositide. This was obtained with diphosphoinositide in the preparation described above. It appeared pure by chromatography on formaldehyde-treated paper (Kai *et al.* 1966*b*).

Phosphatidylinositol. This was prepared and purified by the method of Kai *et al.* (1966*b*).

1,2-*Diglyceride.* This was prepared from egg lecithin by the action of phospholipase C and contained 10% of 1,3-diglyceride. It was provided by Dr B. Sedgwick of this Department.

[^{32}P]*ATP.* The preparation and purity of terminally-labelled ATP have been reported by Kai *et al.* (1966*b*).

Fatty acid-free bovine serum albumin. Crystalline bovine serum albumin was treated by the method of Goodman (1957).

Preparation of brain subcellular fractions. Brains from adult rats were homogenized and fractionated by the method

of Nyman & Whittaker (1963). Details were given by Kai *et al.* (1966*b*). For the preparation of the supernatant fraction only, the brains were homogenized in 0·32 M-sucrose containing 20 mM-tris–HCl buffer, pH 7·4, to give a 10% (w/v) homogenate, which was then centrifuged at 105000*g* for 60 min.

Dialysis of soluble enzyme preparations. The preparations were dialysed at 4° against 100 vol. of 20 mM-tris–HCl buffer, pH 7·4. The buffer was changed three times over a period of 20 hr. for the complete removal of sucrose or ammonium sulphate from the enzyme solution.

Preparation of neuronal and glial cell fractions. The method of Rose (1967) was followed.

Assay of diphosphoinositide kinase. Two incubation methods were used. Method A was used for the assay of homogenates and subcellular fractions and has been briefly described by Kai *et al.* (1966*a*). In this method the enzyme preparation was added last to the incubation mixture, which had a total volume of 1 ml. and the following composition: sodium [^{32}P]ATP (5 mM), $MgCl_2$ (30 mM), EDTA (sodium salt, 2 mM), diphosphoinositide (sodium salt, 0·9 mM), tris–HCl buffer, pH 7·4 (20 mM), and the brain fraction (about 2 mg. of protein).

Method B was used for the assay of diphosphoinositide kinase in the supernatant fraction and purer preparations obtained from this fraction. In this method the reaction was started by adding diphosphoinositide. The basal incubation mixture contained [^{32}P]ATP (4 mM), $MgCl_2$ (20 mM), diphosphoinositide (sodium salt, 0·8 mM), GSH (10 mM), tris–HCl buffer, pH 7·4 (50 mM) and enzyme preparation (0·1–0·2 mg. of protein), in a total volume of 1 ml.

After incubation by either of these methods for 5 min. at 37°, lipids were extracted, washed and separated by chromatography on formaldehyde-treated papers, and the radioactivity of triphosphoinositide was determined. All the methods have been described by Kai *et al.* (1966*b*). From the specific radioactivity of the ATP used the enzyme activity was calculated and is expressed as mμmoles of triphosphoinositide formed/min./mg. of protein. All assays were in duplicate or triplicate and individual results differed by less than 10%.

Assay of triphosphoinositide phosphomonoesterase. The method was essentially that of Salway, Kai & Hawthorne (1967*a*) with the addition of $MgCl_2$ (final concn. 7 mM) to the incubation mixture. Tubes were incubated at 37° for 10 min. and the reaction was stopped by the addition of 1 ml. of 10% (w/v) trichloroacetic acid to the 1 ml. of mixture. The precipitate was removed by centrifugation, and P_i was determined in 1 ml. portions of the supernatant by the method of King (1932).

Assay of 6-phosphogluconate dehydrogenase (EC 1.1.1.44). The method of Glock & McLean (1953) was used.

Protein determination. The procedure of Weichselbaum (1946) was followed and, for smaller amounts of protein, that of Lowry, Rosebrough, Farr & Randall (1951).

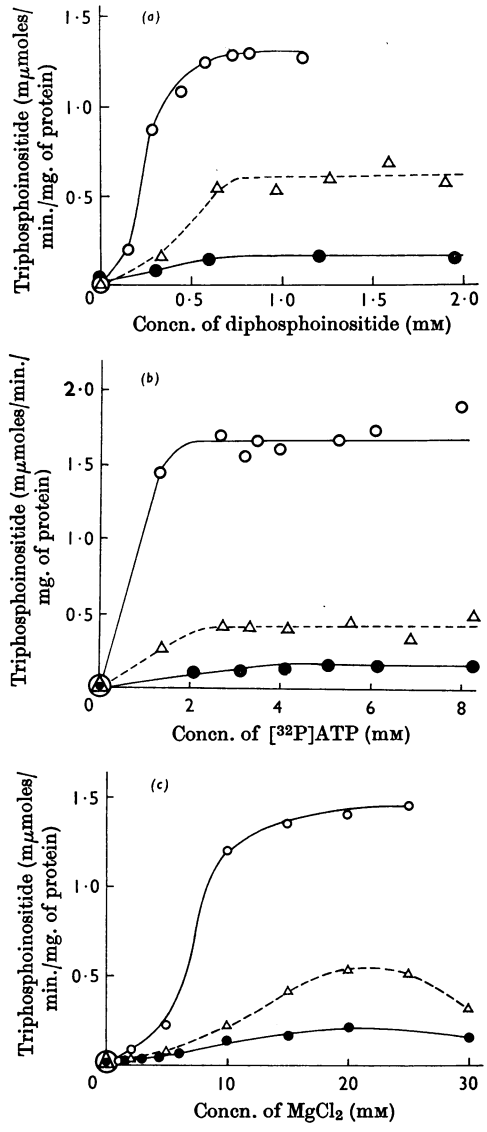

Fig. 1. (*a*) Requirement of diphosphoinositide by kinase in homogenate and supernatant fraction; (*b*) requirement of ATP by kinase; (*c*) requirement of Mg^{2+} ions by kinase. ●, Assays with brain homogenate by method A; ○, assays with supernatant by method B; △, assays by method B in which supernatant was added last rather than diphosphoinositide. For details of the methods see the text.

RESULTS

Properties of diphosphoinositide kinase in brain homogenate

These studies were made to establish conditions for the assay of diphosphoinositide kinase in subcellular fractions from brain. For comparison, results for homogenate and for the supernatant enzyme are plotted together on certain of the Figures.

Diphosphoinositide, ATP and Mg^{2+} *requirements.* Homogenate, unlike the supernatant fraction, showed slight diphosphoinositide kinase activity

in the absence of lipid substrate (Fig. 1*a*), presumably because homogenate contains small quantities of diphosphoinositide. Maximum activity was obtained with 0·6mM-diphosphoinositide and there was no change in activity when this was raised to 2mM. With ATP, maximum activity was reached at 5mM and remained constant up to 8mM (Fig. 1*b*). The kinase reaction required Mg^{2+} ions, the optimum concentration being 20mM in the absence of EDTA (Fig. 1*c*).

Under these optimum concentrations and in the presence of 2mM-EDTA, the activity was proportional to protein concentration in the range 0–4mg. of protein/ml., and the rate of the reaction was constant for 10min.

Effects of Na^+, K^+ *and acetylcholine.* Diphosphoinositide kinase activity in homogenates was decreased by 30–60% on the addition of sodium chloride (150mM), potassium chloride (50mM or 100mM) or the two salts together (120mM sodium chloride + 30mM-potassium chloride). The inhibition was partly reversed by 1mM-ouabain (Kai *et al.* 1966*a*). As shown below, Na^+ and K^+ ions had no effect on the purified kinase. Acetylcholine (0·01 or 0·1mM, each in the presence of 0·1mM-eserine) stimulated the enzyme in homogenates by 30% and 40% respectively. Again there was no effect on the purified kinase.

Effect of detergents. Sodium deoxycholate at concentrations of 1–15mM had no effect on homogenate diphosphoinositide kinase. Triton X-100 inhibited the enzyme by as much as 40% when added in the concentration range 0·5–3·0% (w/v).

Effect of GSH. GSH at concentrations between 3 and 30mM did not affect the kinase. The mean specific activity from seven experiments was 0·20mμmole of triphosphoinositide formed/min./mg. of protein either with or without GSH.

Localization of diphosphoinositide kinase in brain

Subcellular distribution. Most of the diphosphoinositide kinase activity appeared in the supernatant fraction (Table 1), as indicated by the use of 6-phosphogluconate dehydrogenase as a marker enzyme. The fact that the dehydrogenase activity was somewhat higher than the kinase activity in the particulate fractions may be partly explained by the activating effect of freezing and thawing (Table 2). In the experiments of Table 1 the kinase was assayed on fresh fractions, but the dehydrogenase was determined later after storage in the frozen state.

Distribution in cortical cell preparations. Whole rat brains were used for the subcellular fractionations. There was no significant difference in diphosphoinositide kinase activity between grey matter and white matter from the cerebral hemispheres. To obtain further information a cortical cell preparation was separated by density-gradient centrifugation by the procedure of Rose (1967). In this method the original cell suspension was prepared by a very gentle procedure, but when it was centrifuged at 15000*g* for 10min. the supernatant contained about a quarter of the total protein and had higher diphosphoinositide kinase activity than the pellet or any of the fractions from the gradient. These fractions are: (*A*) a surface layer containing myelin, partly disrupted axonal elements, clumps of undisrupted tissue and some

Table 1. *Subcellular distribution of diphosphoinositide kinase and 6-phosphogluconate dehydrogenase in rat brain*

Method A (see the text) was used for assay of the kinase. Relative specific activities were obtained by dividing the specific activity of each fraction by that of the original homogenate.

No. of fractionations	Diphosphoinositide kinase 3		6-Phosphogluconate dehydrogenase 2	
	Rel. sp. activity	Total activity (%)	Rel. sp. activity	Total activity (%)
Homogenate	1·00	100·0	1·00	100·0
Nuclear fraction	0·35	5·5	1·11	12·0
Myelin fraction	0·29	2·4	0·65	4·8
Nerve-ending fraction	0·22	3·6	0·72	11·9
Mitochondrial fraction	0·17	2·8	0·62	11·0
Microsomal fraction	0·45	3·6	0·86	7·4
Supernatant fraction	2·69	64·7	2·35	61·6
Recovery	—	82·6	—	108·7
Sp. activity of homogenate (mμmoles/min./mg. of protein)	0·24		4·0	

Table 2. *Distribution of diphosphoinositide kinase and 6-phosphogluconate dehydrogenase between supernatant and particle fractions from rat brain*

Supernatant and particle fractions were separated by centrifuging the homogenate at 105000*g* for 60min. The values presented are means of two experiments. Method A (see the text) was used for assay of the kinase. Relative specific activities were obtained by dividing the specific activity of each fraction by that of the original homogenate.

	Diphosphoinositide kinase		6-Phosphogluconate dehydrogenase	
	Rel. sp. activity	Total activity (%)	Rel. sp. activity	Total activity (%)
Homogenate	1·00	100	1·00	100
Supernatant	2·46	55	2·47	55
Particles	0·61	43	0·64	45
Particles (frozen and thawed three times)	0·75	—	0·89	—
Recovery	—	98	—	100
Sp. activity of homogenate (mμmoles/min./mg. of protein)	0·28		4·7	

soluble protein; (*B*) a glial-cell-enriched fraction; (*C*) a nerve-cell-body fraction; (*D*) a pellet. Diphosphoinositide kinase activities in these fractions were somewhat variable, but fraction (*A*) always contained the bulk of the recovered activity and had a higher specific activity than the other fractions. Only 0·4% of the total activity was associated with the nerve-cell-body fraction and 7% with the glial fraction.

Properties of diphosphoinositide kinase in supernatant fractions. The supernatant kinase was always dialysed before use. With assay method A, the mean specific activity of four preparations was 0·67mμmole of triphosphoinositide formed/min./mg. of protein before dialysis and 0·61 afterwards. This assay method gives lower activities than assay method B (see below).

Diphosphoinositide, ATP and Mg^{2+} *requirements.* The dialysed supernatant enzyme showed an absolute requirement for diphosphoinositide, ATP and Mg^{2+} ions (Fig. 1). The order in which substrate and enzyme were added to the incubation mixture had a considerable effect on the reaction rate. When enzyme was added last to a preincubated reaction mixture (method A), the activity was less than half that obtained by adding diphosphoinositide to a mixture already containing enzyme (method B). The high activities were also obtained by adding magnesium chloride last. These differences in reaction rate appeared to be due to differences in the degree of dispersion of the diphosphoinositide substrate. In the absence of protein excess of magnesium chloride precipitated the lipid, and in the more active systems the substrate always appeared to be emulsified better.

An approximate K_m value of 0·25mM-diphosphoinositide was obtained for the supernatant enzyme from the data of Fig. 1(*a*). In the basal assay system (method B) 0·1–0·2mg. of protein was used. The rate was proportional to protein concentration in the range 0–0·6mg. of protein/ml. of incubation mixture.

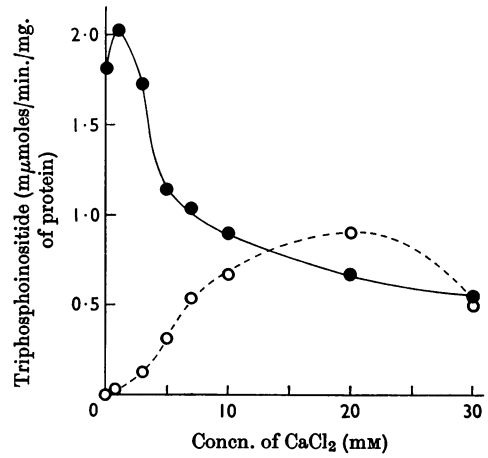

Fig. 2. Effect of Ca^{2+} ions on the kinase. Assays were by method B with 20mM-$MgCl_2$ (●) or without $MgCl_2$ (○). For details see the text.

Effect of other bivalent cations. Fig. 2 shows that Ca^{2+} ions activated the kinase much less effectively than did Mg^{2+} ions. In the presence of optimum Mg^{2+} ion concentration the enzyme was inhibited by Ca^{2+} ions. When the kinase was incubated with 20mM-magnesium chloride before the addition of 5mM-calcium chloride, the degree of inhibition was the same as that caused by preincubation with a magnesium chloride–calcium chloride mixture.

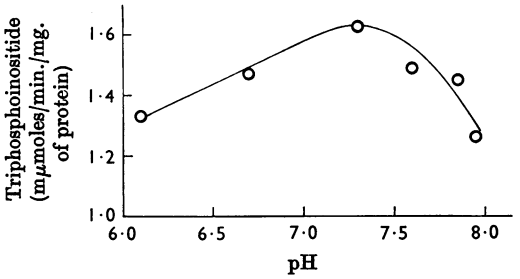

Fig. 3. Effect of pH on the kinase. Assays were made by method B with tris–HCl buffers. For details see the text.

Table 3. *Effect of bivalent cations on diphosphoinositide kinase*

Dialysed supernatant fraction was used as the source of enzyme and $MgCl_2$ was omitted from the incubation mixture. Assay method B (see the text) was employed, but with a 20min. period of incubation. The result with Mg^{2+} ions represents the mean of three experiments.

Bivalent cation added	Concn. of bivalent cation (mM)	Triphosphoinositide formed (mμmoles/mg. of protein in 20min.) 5	20
Mn^{2+}		2·1	1·1
Fe^{2+}		0	0
Co^{2+}		2·1	0
Ni^{2+}		0·4	0
Cu^{2+}		0·3	0
Zn^{2+}		0·4	0
Cd^{2+}		0·2	0
Hg^{2+}		0·4	0
Mg^{2+}		—	20·0

Table 3 shows the effect of other bivalent cations. Of the metal ions tested, only Mn^{2+} and Co^{2+} ions activated the kinase appreciably. At a concentration of 5mM the activity with either of these metal ions was similar to that with 5mM-Mg^{2+} ions. The activity at a concentration of 20mM was much greater with Mg^{2+} ions. There was very little activity with Mn^{2+} or Co^{2+} ions at concentrations of 0·1mM, 0·5mM, 1mM and 2mM.

Effects of Na^+, K^+ *and acetylcholine.* Na^+ ions (25–400mM-sodium chloride) had very little effect on the activity of diphosphoinositide kinase in the dialysed supernatant fraction. A slight inhibition was seen at the highest concentration. The same applied to mixtures of Na^+ and K^+ ions (50mM-sodium chloride + 5–100mM-potassium chloride). These results are in contrast with those for the homogenate, where definite inhibition was seen. Acetylcholine with eserine in the concentrations recorded above for homogenate had no effect on the kinase of the supernatant fraction, though it had stimulated activity in the homogenate.

Table 4. *Effect of thiol reagents on kinase activity*

The kinase was assayed by method B (see the text) but without the addition of glutathione. The incubation was for 20min.

Addition	Concn. (mM)	Activity (%)
None (control)		100
Cysteine	1	51
	10	110
GSH	1	92
	10	99
Iodoacetic acid (sodium salt)	0·1	94
	1	99
	5	95
Iodoacetamide	0·1	93
	1	95
	5	83
N-Ethylmaleimide	0·1	89
	1	74
p-Chloromercuribenzoate	0·1	2
	1	0
GSSG	0·1	83
	1	103

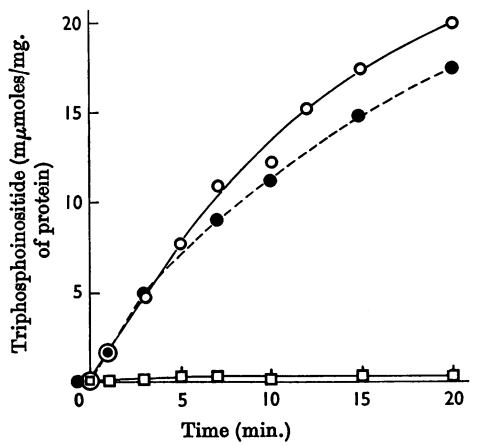

Fig. 4. Time-course of the kinase reaction. Method B was used for the assay at 37° (○), at 0° (□) and with the addition of 0·1mg. of fatty acid-free bovine serum albumin to each tube at 37° (●). For details see the text.

Optimum pH *and effects of thiol reagents.* The kinase was most active at pH 7·3 but there was little variation between pH 7·0 and 7·5 (Fig. 3). When the work began a pH of 7·4 was adopted and this was not changed subsequently.

Table 4 shows the effects of various thiol reagents on diphosphoinositide kinase activity. Neither cysteine nor GSH activated the enzyme, and the only thiol inhibitor that had a marked effect was *p*-chloromercuribenzoate. The unexpected inhibition with 1mM-cysteine was also found for phosphatidylinositol kinase (Kai *et al.* 1966*b*). In another experiment at pH 8·5, similar results to those in Table 4 were obtained with 5mM-iodoacetamide and with 5mM-iodoacetate.

Reaction rate. Fig. 4 shows that the rate was constant for about 5min. and that it then fell somewhat. Addition of fatty acid-free albumin (0·1mg.) did not lengthen the linear part of the curve. This also applied to albumin additions of 0·05mg. and 2·0mg. Kinase activity was negligible at 0°.

The supernatant fraction could be kept frozen for at least 10 weeks without loss of activity. During this period with occasional thawings there was no precipitation of protein.

The specific activity of the supernatant kinase was essentially the same in male and female adult rat brain. The values (mμmoles of triphosphoinositide formed/min./mg. of protein) were: male, 1·58 ± 0·12 (mean ± S.D. of ten preparations); female, 1·54 (mean of four preparations).

Kinase reaction

Effect of P_i. Some labelling of triphosphoinositide might be possible by reversal of the phosphomonoesterase reaction (Dawson & Thompson, 1964) in the presence of [^{32}P]P_i liberated from the labelled ATP. Experiments in which labelled ATP was replaced by [^{32}P]P_i showed that no labelled triphosphoinositide was formed in this way. Formation of labelled triphosphoinositide from [^{32}P]ATP was decreased as expected by the addition of unlabelled ATP. When P_i (10–200mM final concn.) was added to this system there was inhibition, probably owing to the formation of insoluble magnesium phosphate and the resulting removal of Mg^{2+} ions from the kinase.

Formation of triphosphoinositide from [^{32}P]-*diphosphoinositide.* The nature of the kinase reaction was confirmed by a large-scale incubation of ethanol-treated supernatant fraction (see below) with labelled diphosphoinositide and unlabelled ATP. The reaction product was subsequently identified as triphosphoinositide by chromatography on DEAE-cellulose. The incubation mixture had a total volume of 6ml. and contained 3·26mμmoles of [^{32}P]diphosphoinositide ($1·78 \times 10^5$counts/sec.) with ethanol-treated supernatant (1·44mg. of protein). The other components were as follows: ATP (4·2mM), magnesium chloride (20mM), GSH (10mM), EDTA (2mM) and tris–hydrochloric acid buffer, pH 7·4 (50mM). The material extracted from the enzyme preparation by ethanol was emulsified in tris buffer and added back to the incubation mixture, since it partly activated the kinase. The reaction was started by the addition of the labelled diphosphoinositide and the mixture was incubated for 30min.

The amount of triphosphoinositide synthesized

Table 5. *Other kinases in the dialysed supernatant fraction*

Each of the kinases below was assayed in the same dialysed supernatant fraction of rat brain. Except for the substrate, the assay conditions for diphosphoinositide kinase (method B; see the text) were used. Phosphatidylinositol kinase was also assayed under the optimum conditions described in the Materials and Methods section for the preparation of labelled diphosphoinositide. The results appear in parentheses. Diglyceride kinase was estimated by counting the phosphatidic acid area on the usual formaldehyde-treated papers after chromatography as described for the triphosphoinositide assay. Incubation was in all cases at 37° for 5min. The diphosphoinositide used was a preparation containing no detectable triphosphoinositide (see the Materials and Methods section).

	Activity (mμmoles of product/min./mg. of protein)	
Phosphatidylinositol kinase		
1mM-Phosphatidylinositol	0·03	(0)
2mM-Phosphatidylinositol	0·02	(0·01)
Diglyceride kinase		
1mM-Diglyceride	0	
2mM-Diglyceride	0	
Diphosphoinositide kinase		
0·8mM-Diphosphoinositide	1·17	
0·8mM-Diphosphoinositide + 0·1mM-triphosphoinositide	0·89	
0·8mM-Diphosphoinositide + 0·25mM-triphosphoinositide	0·76	
0·8mM-Diphosphoinositide + 0·5mM-triphosphoinositide	0·63	
0·8mM-Diphosphoinositide + 0·8mM-triphosphoinositide	0·56	

Table 6. *Effect of EDTA on diphosphoinositide kinase*

The basal incubation mixture containing 20 mM-$MgCl_2$ was used (assay method B; see the text). The values are means of duplicate determinations.

Concn. of EDTA (mM)	Activity (mμmoles of triphosphoinositide/min./mg. of protein)	
	Dialysed supernatant	Ethanol-treated fraction
0	1·8	4·5
1	1·9	5·0
2	2·3	4·9
3	2·2	5·0
4	1·8	5·2
5	—	5·0

was 157 mμmoles/mg. of protein in the 30 min. period. The value for a similar incubation on a smaller scale with unlabelled diphosphoinositide and labelled ATP was 130 mμmoles/mg.

Product inhibition. When diphosphoinositide kinase was assayed by method B in the dialysed brain supernatant fraction, addition of triphosphoinositide inhibited the enzyme (Table 5).

Contaminating enzymes in the supernatant kinase preparation

The activity of three related enzymes, phosphatidylinositol kinase, diglyceride kinase and triphosphoinositide phosphomonoesterase, was tested in the supernatant fraction.

Phosphatidylinositol kinase and diglyceride kinase. Very little phosphatidylinositol kinase activity was

Table 7. *Triphosphoinositide phosphomonoesterase activity in the kinase system*

As substrate a mixture of diphosphoinositide (0·88 mM) and [^{32}P]triphosphoinositide (0·28 mM) was used. The latter was prepared from labelled diphosphoinositide by using ethanol-treated supernatant as described in the Results section (kinase reaction). The basal incubation system with unlabelled ATP was used (method B; see the text). Tubes were incubated for 5 min. at 37°. Values are means from two determinations. The protein contents of the tubes were as follows: dialysed supernatant, 0·28 mg.; ethanol-treated fraction, 0·22 mg.

	[^{32}P]Triphosphoinositide recovered after incubation (%)	Triphosphoinositide hydrolysed (mμmoles/min./mg. of protein)
Dialysed supernatant	84·1	45
Dialysed supernatant + 2 mM-EDTA	85·8	41
Ethanol-treated fraction	89·7	40
Ethanol-treated fraction + 2 mM-EDTA	87·8	45
Enzyme omitted	100	0

Table 8. *Effect of ethanol and acetone on diphosphoinositide kinase*

The enzyme fraction precipitated when the ammonium sulphate saturation was increased from 20% to 40% was collected by centrifuging at 105 000*g* for 20 min. The supernatant was removed and the pellet cooled to −15° and gently homogenized with acetone or ethanol (1 ml. of solvent/mg. of protein) at the same temperature. The centrifugation was then repeated and the pellet suspended in 20 mM-tris–HCl buffer, pH 7·4 (0·7 ml./mg. of protein). The suspension was then dialysed against 5 l. of the same buffer for 15 hr. with three changes of buffer. The dialysed protein was centrifuged as above and the clear supernatant, which contained about 30% of the original protein, was assayed for diphosphoinositide kinase by method B (see the text). The extracted lipid was added back to some of the incubation mixtures in amounts corresponding to those originally present. For this, the solvent was removed *in vacuo* and the lipid emulsified in the tris–HCl buffer.

Expt. no.	Sp. activity (mμmoles of triphosphoinositide/min./mg. of protein)		% of total activity	
	1	2	1	2
Untreated enzyme	2·2	1·6	100	100
Ethanol-treated enzyme	3·3	3·8	45	61
Ethanol-treated enzyme with extracted lipid	4·2	5·2	57	81
Acetone-treated enzyme	2·7	—	49	—
Acetone-treated enzyme with extracted lipid	3·0	—	56	—

detected. Formation of phosphatidic acid by the diglyceride kinase reaction was not seen at all. For this assay labelled ATP was used with added diglyceride. Results are summarized in Table 5.

Triphosphoinositide phosphomonoesterase. It is known that the supernatant fraction of brain is rich in this phosphomonoesterase (Salway, Kai & Hawthorne, 1967*b*). EDTA was added to the kinase incubation mixtures in an attempt to inhibit the esterase and thus improve the yield of triphosphoinositide. This addition stimulated the kinase (Table 6), but the effect was not due to inhibition of the esterase. As Table 7 shows, only about 15% of the triphosphoinositide was hydrolysed in 5min. under the conditions of the assay.

The phosphomonoesterase was also present in purer preparations of diphosphoinositide kinase (Table 9).

Purification of diphosphoinositide kinase

Ammonium sulphate fractionation. About half of the kinase was precipitated when the ammonium sulphate concentration was increased from 20% of saturation to 40% of saturation. The total recovery of activity was about 70%. Further purification was attempted by various methods. Dropwise addition of acetone at 0° to the enzyme fraction precipitated by 40%-saturated ammonium sulphate caused considerable inactivation. The bulk of the activity was precipitated at acetone concentrations between 33 and 60% (v/v). A similar ammonium sulphate fraction of the enzyme lost 80% of its activity after being heated for 1min. at 50°. At pH5 most of the enzyme was insoluble, but the precipitate had the same specific activity as the original enzyme fraction. Repeated freezing and thawing of the enzyme solution caused considerable coagulation. The resulting protein precipitate had similar kinase activity to the original solution, but could not be redissolved in 20mM-tris–hydrochloric acid buffer, pH7·4.

Treatment with ethanol or acetone. The behaviour on freezing and thawing suggested the presence of lipoproteins, so the enzyme fraction precipitated by 20–40%-saturated ammonium sulphate was treated with lipid solvents at low temperature. Details of the method are given with Table 8. The treated enzyme had a higher specific activity and remained soluble on freezing and thawing, which was a great advantage in further purification work. Activity increased still further when the extracted lipids were emulsified in 20mM-tris–hydrochloric acid buffer, pH7·4, and added back to the enzyme system. The lipids extracted by ethanol were examined by thin-layer chromatography (Prottey & Hawthorne, 1966) and chromatography on formaldehyde-treated paper. Cholesterol, phosphatidylserine and possibly phosphatidylcholine were present, with traces of phsphatidylinositol.

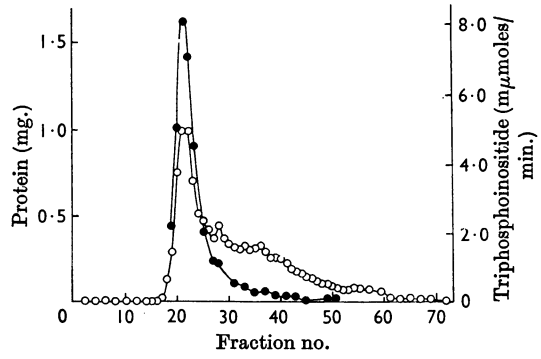

Fig. 5. Gel filtration of ethanol-treated kinase on a Sephadex G-200 column. The column (3·4cm. × 36cm.) was first equilibrated with 20mM-sodium phosphate buffer, pH6·0. The kinase sample (15·5mg. of protein) was applied to the column in 15ml. of 20mM-tris–HCl buffer, pH7·4, and eluted with phosphate buffer at a rate of 2·0ml./cm.^{2} of column/hr. Fractions had a volume of 5ml. and assay method B was used; for details see the text. ○, Protein content of the fractions; ●, kinase activity.

The ethanol-treated fraction was more stable to heat than the original enzyme. The detergent Cutscum (final concn. 0·1–3·0%, w/v) inactivated it by 80–90%. Sodium fluoride caused some inhibition, the values being as follows: 5mM-fluoride, 20% inhibition; 10mM-fluoride, 50% inhibition.

Chromatography on Sephadex G-200. When the ethanol-treated fraction was applied to a column of Sephadex G-200 and eluted with phosphate buffer, most of the enzyme came through at the void volume of the column. Details are given in the legend to Fig. 5, which shows a typical elution pattern. The peak fractions from the column had a higher specific activity than the original ethanol-treated fraction (Table 9). Several other experiments of this sort gave the same elution pattern, which was not changed by omitting the ethanol treatment.

Chromatography on CM-Sephadex C-50. When the peak fractions from the Sephadex G-200 column were chromatographed on CM-Sephadex C-50 no further purification was achieved. When such fractions had been prepared from ethanol-treated enzyme, all kinase activity was eluted at the void volume of the CM-Sephadex C-50 column together with all the protein applied. Elution was with 20mM-sodium phosphate buffer, pH6·0, containing 0·1M-sodium chloride. On the other hand, when the ethanol treatment was omitted much inactive protein was eluted by phosphate buffer without sodium chloride at the void volume, but the kinase was only removed by gradient elution with buffer

Table 9. *Purification of diphosphoinositide kinase*

The values are means ± S.D. for the supernatant. The numbers of experiments are given in parentheses.

Enzyme preparation	Sp. activity (mμmoles of substrate converted/min./mg. of protein)			
	Diphosphoinositide kinase		Triphosphoinositide phosphomonoesterase	
Dialysed supernatant	1·52 ± 0·18	(16)	100	(2)
20–40%-satd. $(NH_4)_2SO_4$ fraction	2·4	(3)	80	(2)
Ethanol-treated fraction	4·0	(3)	42	(2)
Sephadex G-200 eluate	6·6	(1)	20	(1)
CM-Sephadex C-50 eluate	6·5	(1)	—	

containing 0·4 M-sodium chloride. The gradient was obtained by having 80ml. of phosphate buffer in the mixing chamber and 80ml. of the same buffer containing M-sodium chloride in the reservoir. The column measured 1·4cm. diam. × 19cm. long initially, and 9·5mg. of protein was applied. The active fractions were combined and dialysed against 20mM-tris–hydrochloric acid buffer, pH 7·4, but most of the kinase activity was lost after storage in the frozen state for 8 weeks.

Material eluted from Sephadex G-200 columns was also applied to DEAE-cellulose (Whatman DE-52) or DEAE-Sephadex (A-50) columns. The kinase could not be recovered from such columns by elution with 20mM-tris–hydrochloric acid buffer, pH 7·4, containing M- or 2M-sodium chloride.

Table 9 summarizes the purification studies. It also shows that as the kinase activity increased there was a decrease in triphosphoinositide phosphomonoesterase activity.

DISCUSSION

Diphosphoinositide kinase reaction. This work establishes the existence in the supernatant fraction of rat brain of a kinase catalysing the formation of triphosphoinositide by the following reaction:

$$\text{Diphosphoinositide} + \text{ATP} \rightarrow \text{triphosphoinositide} + \text{ADP}. \quad (1)$$

Labelled triphosphoinositide was formed both from [^{32}P]ATP and unlabelled diphosphoinositide and from unlabelled ATP and [^{32}P]diphosphoinositide. Like most kinases, the enzyme was activated by Mg^{2+} ions.

Brain supernatant also contains an active enzyme hydrolysing triphosphoinositide according to reaction (2) (Salway *et al.* 1967*b*):

$$\text{Triphosphoinositide} + H_2O \rightarrow \text{diphosphoinositide} + P_i \quad (2)$$

Reversal of this reaction in the presence of [^{32}P]P_i released from the labelled ATP could also give rise to small quantities of labelled triphosphoinositide. However, no triphosphoinositide was formed when [^{32}P]P_i replaced [^{32}P]ATP, and the purer preparations of diphosphoinositide kinase contained less of the phosphomonoesterase (Table 9). Thus the reversal of reaction (2) appears insignificant.

Purification of diphosphoinositide kinase. Unlike most enzymes acting on lipid substrates, diphosphoinositide kinase is a soluble enzyme. This may be related to the fact that diphosphoinositide is a highly polar lipid. Treatment of the enzyme with ethanol at low temperature produced an unexpected twofold purification. This was not due to removal of lipid, since when the extracted lipid was added back the kinase was activated (Table 8). The ethanol treatment probably caused precipitation of less active protein.

Gel filtration with Sephadex G-200 gave some purification and the behaviour of the enzyme on ion-exchange columns indicated that it is an acidic protein.

Properties of the enzyme. The purified diphosphoinositide kinase was not affected by Na^+ or K^+ ions. The depression of kinase activity in homogenates by these ions may be an indirect effect resulting from changes in the utilization of ATP by other reactions. The kinase activity would appear less if triphosphoinositide phosphomonoesterase was activated by the univalent ions. Activation is possible under certain conditions (Dawson & Thompson, 1964), but the supernatant phosphomonoesterase can be inhibited by Na^+ ions under other conditions (Salway *et al.* 1967*b*).

Hayashi, Yagihara, Nakamura & Yamazoe (1966) showed that the incorporation of [^{32}P]P_i into the lipids of guinea-pig brain slices increased when the slices, which had been incubated in Na^+-free choline–Ringer solution, were transferred to normal Ringer solution for a further incubation. Increases were greatest for diphosphoinositide and triphosphoinositide. The present work and our previous study of phosphatidylinositol kinase (Kai *et al.* 1966*b*) indicate that the Na^+ effect described by

Hayashi *et al.* (1966) is not due to stimulation of the kinases involved in polyphosphoinositide synthesis.

Acetylcholine stimulated the production of triphosphoinositide from labelled ATP in brain homogenates, but was without effect on the soluble preparation of diphosphoinositide kinase. The integrity of certain subcellular membranes may be required for the action of acetylcholine. Durell & Sodd (1966) have shown, for instance, that the synaptosomal fraction is the site of the increased labelling of phosphatidic acid caused by acetylcholine in the presence of $[^{32}P]P_i$.

Activity depended on the order in which diphosphoinositide, magnesium chloride and the kinase were added to the incubation mixture. The enzyme–substrate complex appeared to form less readily with the insoluble magnesium salt of diphosphoinositide, in spite of the fact that the kinase required Mg^{2+} ions. If these ions are involved in the formation of the enzyme–substrate complex, as seems likely, they can be readily displaced by Ca^{2+} ions. Inhibition by Ca^{2+} ions was the same when Ca^{2+} and Mg^{2+} ions were added together as when the kinase was incubated with Mg^{2+} ions before the addition of Ca^{2+} ions.

Localization of the kinase in brain. Diphosphoinositide kinase is a soluble enzyme not obviously associated with any particular subcellular structures. There is no evidence to suggest that the enzyme is associated specifically with the cell body of the neurone or with glial cells. Extracellular fluid occupies about 35% of the total volume of cerebral cortex, whereas neurone-cell bodies occupy less than 5% (Hydén, 1960). It is not yet possible to decide whether the kinase is present only in the cytoplasm or axoplasm.

The subcellular distribution of triphosphoinositide phosphomonoesterase is very similar to that of the kinase (Salway *et al.* 1967*b*).

Comparison with phosphatidylinositol kinase. Diphosphoinositide is formed by the action of phosphatidylinositol kinase in the presence of ATP and Mg^{2+} ions (Colodzin & Kennedy, 1965; Kai *et al.* 1966*b*). Apart from its requirement for ATP and Mg^{2+} ions, diphosphoinositide kinase differs considerably from phosphatidylinositol kinase. The former is a soluble enzyme whereas the latter is particulate (Table 5). Diphosphoinositide kinase is inhibited by Triton X-100 and unaffected by sodium deoxycholate, yet both these detergents stimulate phosphatidylinositol kinase. The activity of phosphatidylinositol kinase depends on thiol groups; that of the present enzyme does not. Finally, though both enzymes are inhibited by Ca^{2+} ions in the presence of optimum Mg^{2+} ion concentration, only diphosphoinositide kinase can be partly activated by Ca^{2+} ions when no Mg^{2+} ions are present.

Rates of formation and breakdown of triphosphoinositide in brain. It is not easy to measure the true rate of triphosphoinositide synthesis in a brain homogenate containing active enzymes that hydrolyse this lipid. The same applies to diphosphoinositide, but the values in Table 10, based on initial rates under optimum conditions, are adequate for comparison with other enzymes in brain. Table 10 shows that triphosphoinositide breakdown is more than 100 times as rapid as its synthesis in adult rat brain, even when the alternative hydrolysis via the phosphodiesterase is ignored. The values are of the same order as those for acetylcholine synthesis and destruction.

Comparable values for the synthesis and breakdown of phosphatidic acid in brain are not yet available, but the work of Hokin & Hokin (1959) suggests that the metabolism of this lipid is quite different. From values based on only the crude microsomal fraction in the presence of detergent, phosphatidate phosphatase was about 12 times as active as diglyceride kinase. This kinase provides only one of the two routes to phosphatidic acid, and

Table 10. *Synthesis and breakdown of brain constituents*

Enzyme	Reaction rate* (mμmoles converted/g. of fresh brain/min.)	Reference
Phosphatidylinositol kinase	6	Kai *et al.* (1966*b*)
Diphosphoinositide kinase	26	Present work: homogenate (Table 1)
Triphosphoinositide phosphomonoesterase	4000†	Salway *et al.* (1967*b*)
Choline acetyltransferase (EC 2.3.1.6)	50‡	McIlwain (1959)
Acetylcholinesterase (EC 3.1.1.7)	6000‡	McIlwain (1959)

* Where necessary rates for fresh rat brain were calculated by using a value of 110 mg. of protein/g. of fresh brain (Ansell, 1961).

† Release of P_i was measured; the rate is based on the release of one phosphate group from each molecule of triphosphoinositide, i.e. conversion into diphosphoinositide.

‡ These values refer to dog brain, but are similar to the values for the rat.

the other route (acylation of glycerophosphate) is likely to be about as active.

Comparison of these rates suggests that phosphatidic acid is involved in the intermediary metabolism of brain lipids but that triphosphoinositide has a different function. It resembles a transmitter substance in that the capacity for its destruction is very great. Triphosphoinositide does not appear to be associated with nerve endings like acetylcholine, but a good deal of evidence (e.g. that of Sheltawy & Dawson, 1966) indicates that it is localized in the myelin fraction. It is possible that its rapid turnover is associated with nerve conduction.

Hodgkin & Keynes (1957) have shown that Ca^{2+} ions enter the squid axon during stimulation and have suggested that the discharge of these ions from the membrane into the axoplasm renders the membrane more permeable to Na^{+} ions. Triphosphoinositide has a greater affinity than EDTA for Ca^{2+} ions (Dawson, 1965) and all the Ca^{2+} and Mg^{2+} ions of purified myelin appear to be bound to polyphosphoinositide (Eichberg & Dawson, 1965) The balance between triphosphoinositide synthesis and breakdown might therefore control the bivalent ion content and permeability of the axonal membrane system.

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