The Phosphatidylinositol Kinase of Rat Brain

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1. The presence of a phosphatidylinositol kinase in homogenates of adult rat brain was shown by using labelled ATP or labelled phosphatidylinositol. 2. The kinase was activated by Mg^{2+} or Mn^{2+} and inhibited by Ca^{2+} , Cu^{2+} , K^+ , Na^+ and F^- . 3. The detergents sodium deoxycholate, Cutscum and Triton X-100 markedly stimulated the reaction; sodium taurocholate, Tween-20 and cetyltrimethylammonium bromide were less effective. 4. The activity of the enzyme was dependent on SH groups. 5. The subcellular distribution of the kinase in brain resembled that of Na^+ -plus- K^+ -stimulated adenosine triphosphatase and 5'-nucleotidase.

Experiments with brain slices have suggested that phosphatidylinositol can be phosphorylated to diphosphoinositide and triphosphoinositide (Brockerhoff & Ballou, 1962), but it has been more difficult to demonstrate these phosphorylations in cell-free preparations. One reason for this is the presence of an active enzyme system dephosphorylating both diphosphoinositide and triphosphoinositide (Dawson & Thompson, 1964). However, the biosynthesis of diphosphoinositide has been observed in subcellular fractions of rat liver by Michell & Hawthorne (1965) and in a microsomal fraction from rat brain by Colodzin & Kennedy (1965).

Further studies on a rat-brain system are now presented. The work has been reported briefly (Kai & Hawthorne, 1966b; Kai, Salway, Michell & Hawthorne, 1966).

MATERIALS AND METHODS

Reagents. Tween-20 was obtained from Honeywell and Stein Ltd., London, W. 1, Triton X-100 (*p*-1,1,3,3-tetramethylbutylphenylpolyethoxyethanol) from Lennig Chemicals Ltd., Jarrow, Co. Durham, and Cutscum (iso-octylphenoxypolyethoxyethanol) from Kodak Ltd., Kirkby, Liverpool. Mr I. Trayer, of the Biochemistry Department of this University, kindly supplied 2-mercaptoethanol, *N*-ethylmaleimide (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) and 3-mercaptopropionic acid (Kodak Ltd.). Crystalline bovine serum albumin and 6-phosphogluconic acid (sodium salt) were the products of the Sigma Chemical Co. Ltd., London, S.W. 6.

Labelled ATP. Terminally labelled [^{32}P]ATP was prepared by the method of Glynn & Chappell (1964). Carrier disodium ATP was added to the 0.25 *N*-HCl eluate from the Dowex 1 column and the mixture then neutralized with NaOH. The ATP had a specific activity of 10^4 counts/sec./ μ mole measured in a liquid Geiger-Müller tube (6% efficiency). The purity of the ATP was checked by descending

chromatography on Whatman no. 1 paper in a solvent system similar to that of Wyatt (1955), except that the Na_2HPO_4 solution was replaced by aq. 5% (w/v) $NaHCO_3$. All the radioactivity was localized in the ATP spot as seen in filtered u.v. light. On storage at 4° for up to 2 weeks there was no significant breakdown of the ATP.

Phosphatidylinositol. This lipid was prepared from ox brain, by making use of the combined supernatants obtained during reprecipitation of the Folch 'diphosphoinositide fraction' (Folch, 1949a,b). The phospholipids from these supernatants were fractionated on an alumina column (Long & Owens, 1962), and the fractions containing phosphatidylinositol were combined and chromatographed on a silicic acid column (Hanahan, 1960) with chloroform-methanol (11:2, v/v), followed by chloroform-methanol (5:2, v/v) for elution. The purity of the phosphatidylinositol eluted by the second solvent was checked by thin-layer chromatography on silica gel H (E. Merck A.-G., Darmstadt, W. Germany) in chloroform-methanol-water (95:35:6, by vol.) or by chromatography on formaldehyde-impregnated papers as described by Kai & Hawthorne (1966a). By both methods, samples containing about 20 μ g. of P gave only one spot.

Phosphatidylinositol was also prepared from Inosithin (American Lecithin Co., New York, N.Y., U.S.A.), a phospholipid fraction from soya beans, by chromatography on silicic acid only, with the above solvent systems. The purified product also gave only one spot on thin-layer chromatography. Formaldehyde-paper chromatography, however, showed traces of other lipids.

For use in the incubation systems, phosphatidylinositol solutions in chloroform were dried down *in vacuo* at 37° and the residual lipid was shaken with sufficient water to give a 10 mM emulsion.

[^{32}P]Phosphatidylinositol. This was prepared by growing the yeast *Schizosaccharomyces pombe* in a medium containing [^{32}P]orthophosphate. The medium was essentially that of Norris & Darbre (1956) with added myo-inositol (100 mg./l.) and less KH_2PO_4 (100 mg./l.). It contained 1 mc of ^{32}P /l. Two 5 l. cultures were grown with continuous aeration at 27°. After 4 days the yeast was

harvested by centrifuging at 15000 rev./min. in the continuous-flow head of the High Speed 18 centrifuge (Measuring and Scientific Equipment Co., London, S.W.1) at a flow rate of 200 ml./min. The cells were stirred with 800 ml. of ethanol and left at room temperature overnight. The mixture was then evaporated to dryness *in vacuo* and the residue was extracted with 600 ml. of chloroform-methanol (2:1, v/v) at room temperature overnight. The insoluble material was removed by filtration through glass-fibre filter paper and re-extracted twice for 4 hr. with 600 ml. of the same solvent mixture. The extracts were combined and washed with 0.2 vol. of 0.05 M-CaCl₂. The lower phase was taken, the solvent removed *in vacuo* and the lipid fractionated on a 10 g. alumina column by the procedure of Long & Owens (1962). Fractions rich in phosphatidylinositol were combined, the solvent was removed *in vacuo* and the lipid was dissolved in 100 ml. of chloroform-methanol (2:1, v/v). This solution was washed with 20 ml. of M-CaCl₂ and the separated lower layer dried down *in vacuo*. The lipid was dissolved in 2 ml. of chloroform and applied to a 4 g. silicic acid column. The silicic acid (100 mesh; Mallinckrodt Chemical Works, New York, N.Y., U.S.A.) was activated at 105° overnight and sieved to remove particles finer than 300 mesh. The calcium salt of phosphatidylinositol was eluted from the column with chloroform-methanol (7:1, v/v) and the peak fractions were combined. The lipid gave only one spot in the chromatographic systems mentioned above and radioautographs (1 µg. of P) of the formaldehyde-impregnated papers also showed only one spot. Radioautographs with 40 µg. of P showed traces of faster-moving materials. No ninhydrin-positive material was seen on the thin-layer plates.

The yield of [³²P]phosphatidylinositol was approx. 400 µg. of lipid P with a specific activity of 35 counts/sec./µg. of P in a liquid Geiger-Müller tube with 6% efficiency. Before use it was dissolved in chloroform-methanol (2:1, v/v) and the solution washed with 0.2 vol. of N-HCl at 4° to remove Ca²⁺. The labelled lipid was emulsified as described above.

Diphosphoinositide. As a reference material for chromatography the diphosphoinositide fraction described by Folch (1949a) was prepared.

Purified diphosphoinositide was prepared on a DEAE-cellulose column by the method of Hendrickson & Ballou (1964), by using a Folch fraction that had been suspended in 2% (w/v) EDTA (sodium salt) at pH 7 and dialysed against deionized water overnight, with several changes of the water.

Preparations of brain homogenates and subcellular fractions. Brains were taken from 250 g. male albino rats within a few minutes of death and homogenized in 0.32 M-sucrose. The dispersions were made in a Teflon-glass homogenizer with a radial clearance of 0.10–0.15 mm. and a pestle rotating at 600 rev./min. Twelve complete strokes of the pestle were made over a period of 1.5 min. at 4°. Further 0.32 M-sucrose was then added to give a 10% (w/v) homogenate. Subcellular fractions were prepared from such rat-brain homogenates by the method of Nyman & Whittaker (1963).

Assay of the kinase reaction. The basic assay system for the synthesis of diphosphoinositide from phosphatidylinositol contained, in a total volume of 1 ml., 0.2 ml. of 10% brain homogenate, sodium [³²P]ATP (6 mM), MgCl₂ (30 mM), tris-HCl buffer, pH 7.4 (20 mM), and phosphatidylinositol (1 mM). These represent optimum concentrations determined as described in the Results section. Tubes

were incubated at 37° for 3 or 5 min. and the reaction was stopped by the addition of organic solvents as described below.

Methods that gave good recoveries of synthesized diphosphoinositide were based on the extraction and washing procedures of Garbus, DeLuca, Loomans & Strong (1963). The two methods used are referred to as methods A and B.

Method A. In this case the reaction was stopped by the addition of 3.75 ml. of chloroform-methanol (1:2, v/v). The remainder of the procedure differed from that of Garbus *et al.* (1963) only in the omission of the two filtration steps. Folch diphosphoinositide fraction was added as a carrier to the final washed lipid extract before paper chromatography.

Method B. After the addition of 3 ml. of methanol followed by 3 ml. of chloroform to the 1 ml. incubation system, the mixture was shaken well and left for 30 min. at room temperature. Then 0.5 ml. of water containing Folch diphosphoinositide fraction (50 µg. of P) and 1.2 ml. of 2 M-KCl-0.5 M-potassium phosphate buffer, pH 7.4, were added. The resulting two-phase mixture was shaken well and the two layers were separated in a bench centrifuge. The clear lower layer was taken for paper chromatography.

Method A gave about 30% higher recoveries of labelled diphosphoinositide than method B.

Diphosphoinositide in the washed chloroform extract was separated by chromatography on formaldehyde-impregnated papers and its radioactivity determined as described by Kai & Hawthorne (1966a). Equilibration of the solvent with ether was omitted. Staining with Nile Blue caused no loss of radioactivity from the paper.

Kinase activity was measured as ³²P incorporated into diphosphoinositide. The number of µmoles of diphosphoinositide synthesized was calculated from this, since the specific activity of the ATP was known.

In some experiments the extracted radioactive lipids were hydrolysed under mild alkaline conditions and the hydrolysis products fractionated on a Dowex 1 (formate form) column as described by Galliard, Michell & Hawthorne (1965). The radioactive compounds co-chromatographed with hydrolysis products of di- and tri-phosphoinositides, confirming the results of paper chromatography.

Assay of enzymes. Several enzymes were assayed as 'markers' to indicate the distribution of various cell components in the different brain subcellular fractions. Succinate dehydrogenase (EC 1.3.99.1) defines the distribution of mitochondria, and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) that of soluble material. Both 5'-nucleotidase (EC 3.1.3.5) and Na⁺-plus-K⁺-stimulated adenosine triphosphatase (EC 3.6.1.3) may be associated with the plasma membrane (for references see Michell & Hawthorne, 1965; Hosie, 1965).

The method for 5'-nucleotidase was based on that used by Michell & Hawthorne (1965). The incubation system contained tris-HCl buffer, pH 7.4 (25 mM), KCl (50 mM), MgCl₂ (15 mM), sodium potassium tartrate (5 mM), AMP (2.5 mM) and brain tissue (0.3–0.7 mg. of protein) in a total volume of 2 ml. After incubation at 37° for 15 min., 1 ml. of 25% (w/v) trichloroacetic acid was added and the tube shaken well. After centrifugation inorganic phosphate was determined on 2 ml. samples of the supernatant by the method of Fiske & Subbarow (1925). Volumes of reagents were adjusted to give a final volume of 5 ml. and, instead of HClO₄, 1 ml. of 5 N-H₂SO₄ was used, to avoid precipitation of

KClO₄. This 5'-nucleotidase method gave a linear response up to 2 mg. of protein and the reaction rate was constant for 25 min. The concentrations of MgCl₂ and AMP were shown to be optima, each representing the centre of a plateau in the concentration curve. Tartrate had no effect on the hydrolysis of AMP by brain preparations over the concentration range 1–20 mM. No release of phosphate from 2-glycerophosphate (5–70 mM) was observed with this assay system.

Na⁺-plus-K⁺-stimulated adenosine triphosphatase was estimated by using incubation systems of the following composition (final volume 1 ml.): (a) tris-HCl buffer, pH 7.4 (30 mM), NaCl (120 mM), KCl (30 mM), MgCl₂ (3 mM), tris-ATP (6 mM), and brain fraction containing 0.3–0.5 mg. of protein; (b) the same system without NaCl and KCl. After incubation at 37° for 2 min., reactions were stopped by the addition of 1 ml. of 10% (w/v) trichloroacetic acid. After shaking and centrifuging, inorganic phosphate was determined in the supernatants (Fiske & Subbarow, 1925) and the Na⁺-plus-K⁺-stimulated adenosine triphosphatase determined as the difference between assays (a) and (b). The reaction rate was constant for 3 min. with brain homogenate containing 0.38 mg. of protein (cf. Swanson, Bradford and Mollwain, 1964). Activity was proportional to protein concentration over the range 0–0.7 mg./ml. and independent of ATP concentration over the range 3–12 mM. The metal ions of system (a) did not affect colour development in the phosphate assay.

Succinate dehydrogenase was determined by the method of Pennington (1961) in the presence of 2 mM-EDTA (Porteous & Clark, 1965).

Assay of 6-phosphogluconate dehydrogenase was by the procedure of Glock & McLean (1953).

Other determinations. Protein, RNA and DNA were all determined as described by Hübscher, West & Brindley (1965).

RESULTS

All the results refer to the synthesis of diphosphoinositide from phosphatidylinositol in systems containing brain homogenate or brain subcellular fractions.

Phosphatidylinositol requirement. The effect of varying the concentration of phosphatidylinositol on diphosphoinositide formation is shown in Fig. 1(a). Without added phosphatidylinositol there was a considerable synthesis of diphosphoinositide. Concentrations of phosphatidylinositol above 1 mM caused only a slight increase in activity, and so this concentration was chosen for the subsequent studies. Addition of purified diphosphoinositide to the basic system containing 1 mM-phosphatidylinositol caused a marked stimulation of labelled diphosphoinositide production. The greatest effect was obtained with 0.7–1.0 mM-diphosphoinositide, which doubled the yield of labelled product.

ATP requirement. This is shown in Fig. 1(b). Optimum activity in a 5 min. incubation is obtained with concentrations of ATP between 5 and 10 mM.

Effects of metal ions, fluoride and EDTA. The phosphorylation was stimulated by Mg²⁺ (Fig. 2a).

Fig. 1. (a) Effect of phosphatidylinositol concentration on diphosphoinositide synthesis. The basic assay system was used, incubations being for 5 min. Lipids were extracted by method A. (b) Effect of ATP concentration on diphosphoinositide synthesis. The basic assay system was used, with incubations for 5 min. and lipid extraction by method B.

Ca²⁺ was inhibitory, both in the presence and absence of Mg²⁺ (Figs. 2b and 2c). A stimulation was observed with 10 mM-manganese chloride, Mg²⁺ being absent (Fig. 2c). Potassium chloride was somewhat inhibitory, except at 25 mM in the presence of the optimum concentration of Mg²⁺. These experiments were done with the basic system incubated for 5 min.

Addition of cupric chloride (0.25 mM) to the basic system gave complete inhibition. Sodium fluoride also inhibited phosphorylation, concentrations of 1 mM and 10 mM giving 70 and 100% inhibition respectively.

Even in the presence of 30 mM-magnesium chloride used in the standard incubation mixture, the addition of EDTA (2 mM) produced a marked stimulation.

Thiol reagents. The results summarized in Table 1 suggest that the activity of the enzyme synthesizing diphosphoinositide is dependent on thiol groups.

Effects of detergents. Table 2 shows the effects on diphosphoinositide formation of various ionic and

non-ionic detergents and also the stimulation by serum albumin. The detergents did not affect the extraction procedure. The effects of the concentration of deoxycholate and of Cutscum on kinase

activity are shown in Figs. 3(a) and 3(b). The cetyltrimethylammonium bromide curve resembled that for deoxycholate in showing a sharp optimum concentration. The effect of Triton X-100 closely resembled that of Cutscum. Deoxycholate partly solubilized the phosphorylating enzyme.

Reaction rate. Under the basic conditions the reaction rate was constant only for about 1 min., and after 2 min. there was no further net synthesis of diphosphoinositide (Fig. 4). At 30° the rate was constant for about 5 min. Addition of Cutscum (1.5%, w/v) at 37° kept the initial rate constant for about 5 min., and enabled synthesis to continue for 10 min. Deoxycholate addition (3 mM) also extended the period of synthesis. These detergent effects are shown in Fig. 5. Addition of EDTA (2 mM) without any detergents allowed synthesis to continue for about 20 min., but when deoxycholate (3 mM) was added as well the rate fell off sharply after 2 min.

Effect on reaction rate of a second addition of ATP. It seemed possible that one reason for the slowing down of the reaction after a few minutes might be lack of ATP. Brain homogenates contain a number of adenosine triphosphatases.

To investigate this, mixtures containing deoxycholate (3 mM) were incubated for 5 min. with different amounts of [32 P]ATP. Then additional ATP was added and the incubation was continued for a further 5 min. The results are given in Table 3. They suggest that lack of ATP is a major factor in

Fig. 2. Effects of Mg²⁺, Ca²⁺, Mn²⁺ and K⁺ concentrations on diphosphoinositide synthesis. The basic assay system was used, with incubations for 5 min. and lipid extraction by method B. (a) Mg²⁺: ●, complete assay system; ○, system without phosphatidylinositol. (b) Ca²⁺ (○) and K⁺ (Δ). (c) Ca²⁺ (Δ) and Mn²⁺ (○): in these experiments MgCl₂ was omitted from the assay system.

Table 1. Effects of thiol reagents on diphosphoinositide synthesis

The basic assay system was used, but with 5 mM-[32 P]-ATP. Cutscum (final concentration 1.5%, w/v) was present. Further additions were as indicated. Each value is the average of two determinations.

Additions	Diphosphoinositide synthesized (mμmoles/mg. of protein/5 min.)
None	1.93
Cysteine (1 mM)	1.61
Cysteine (10 mM)	2.50
Reduced glutathione (1 mM)	2.31
Reduced glutathione (10 mM)	2.74
2-Mercaptoethanol (1 mM)	2.34
2-Mercaptoethanol (10 mM)	2.54
3-Mercaptopropionic acid (1 mM)	2.03
3-Mercaptopropionic acid (10 mM)	2.77
Iodoacetamide (0.1 mM)	1.84
Iodoacetamide (1 mM)	0.88
N-Ethylmaleimide (0.1 mM)	0.64
N-Ethylmaleimide (1 mM)	0.04
p-Chloromercuribenzoate (0.1 mM)	0.05
p-Chloromercuribenzoate (1 mM)	0.04
Oxidized glutathione (0.1 mM)	1.63
Oxidized glutathione (1 mM)	1.48

the slowing down of the reaction with brain homogenate.

Effect of protein concentration. In one experiment the basic incubation system was used and in another EDTA (2mM) was added to the system. With EDTA the reaction rate was proportional to protein

Table 2. *Effects of detergents and albumin on diphosphoinositide synthesis*

The basic assay system was used. Detergents were added to the tubes immediately before the incubation was begun by adding the brain homogenate. Incubation was for 3 min. with Cutscum and Triton X-100, 5 min. in all other cases. The detergent concentrations used were those giving maximum stimulation or, for inhibitory compounds, those giving complete inhibition.

Detergents	Activity (% of control diphosphoinositide synthesis)
Cationic	
Cetyltrimethylammonium bromide (3mM)	300
Hexadecylpyridinium chloride (5mM)	0
Anionic	
Sodium deoxycholate (3mM)	2000-3000
Sodium dodecyl sulphate (5mM)	0
Sodium taurocholate (4mM)	600
Non-ionic	
Tween-20 (0.02%)	500
Cutscum (2.0%)	1200*
Triton X-100 (2.0%)	1100*
Bovine serum albumin (0.3%)	300

* Extraction method A; the remaining assays were done with method B.

Fig. 3. Effects of deoxycholate and Cutscum concentrations on diphosphoinositide synthesis. The basic assay system was used. (a) Deoxycholate (O): the incubation lasted 5 min. and method B was used for lipid extraction. (b) Cutscum (O): the incubation lasted 3 min. and method A was used for lipid extraction. In each Figure the broken line shows the simultaneous production of triphosphoinositide (Δ).

Fig. 4. Rate of diphosphoinositide synthesis at 30° (O) and 37° (●). The basic assay system was used, with extraction method B.

Fig. 5. Rate of diphosphoinositide synthesis with 3mM-deoxycholate (●) or 1.5% (w/v) Cutscum (○). The basic assay at 37° was used. Lipids were extracted by method B in the deoxycholate experiment and by method A in the Cutscum experiment.

Table 3. Effect of a second ATP addition on the rate of diphosphoinositide synthesis

The basic assay system was used with added sodium deoxycholate (3mM) and ATP as indicated (extraction method B). Unlabelled ATP was added as the sodium salt. Values in parentheses show the expected synthesis calculated from the first 5 min. In Expts. 5-7 the final volume was 1.5ml. because of the second ATP addition. Each result is the mean of two determinations.

Expt. no.	[³² P]ATP and time of incubation	Diphosphoinositide synthesized (nμmoles/mg. of protein)
1	6mM, 5min.	0.51
2	10mM, 5min.	0.59
3	6mM, 10min.	0.41 (1.02)
4	10mM, 10min.	0.89 (1.18)
5	6mM, 5min., followed by 6mM, 5min.	1.24 (1.02)
6	6mM, 5min., followed by 10mM, 5min.	1.20 (1.10)
7	6mM, 5min., followed by 10mM (of which 4mM was unlabelled ATP), 5min.	0.76 (0.86)

concentration over the range 0-4mg. of protein. Without EDTA the proportionality extended only over the range 0-2.5mg. of protein and the rate was less.

Labelled phosphatidylinositol as substrate. To provide more conclusive evidence on the nature of the kinase reaction being studied, [³²P]phosphatidylinositol was used as a substrate, with unlabelled ATP.

The following incubation system was used: tris-ATP (10mM); tris-hydrochloric acid buffer, pH 7.4 (20mM); [³²P]phosphatidylinositol (1.3mM); magnesium chloride (30mM); reduced glutathione (10mM); brain homogenate (2.4mg. of protein). The total volume was 1 ml. and tubes were incubated at 37°. In one experiment Cutscum (final concentration 1.5%, w/v) was added and the incubation time was 30min. In the second experiment sodium deoxycholate (3mM) was added and the reaction was allowed to proceed for only 10min. Extraction method A was used and the recoveries of radioactivity from the chromatograms were 45% (Cutscum) and 61% (deoxycholate) respectively. Diphosphoinositide accounted for about 10% of the ³²P on the chromatograms.

At the end of the incubations 25.6 mμmoles of diphosphoinositide/mg. of protein had been produced in the presence of Cutscum (4.8% of the added ³²P). The corresponding value for the deoxycholate experiment was 17.7 mμmoles (3.3% of the added ³²P).

Stability of the enzyme system. If the brain homogenate in 0.32M-sucrose was stored at -20° for up to 6 days, there was no loss of activity.

Subcellular distribution of the kinase system. To obtain a linear rate over a 5 min. incubation period, the activity of phosphatidylinositol kinase in the

subcellular fractions was assayed by incubation at 30°. The basic incubation system was used. No detergent was added to the system. The results are summarized in Tables 4 and 5. Fig. 6 presents the enzymic activities of the various fractions as histograms for easier comparison. If the particulate fractions only are considered, the distribution of phosphatidylinositol kinase resembles that of Na⁺-plus-K⁺-stimulated adenosine triphosphatase most closely. However, there is also considerable kinase

activity in the supernatant fraction, which does not show adenosine-triphosphatase activity. The kinase distribution also somewhat resembles that of 5'-nucleotidase but it is different from that of the other 'marker' enzymes studied.

DISCUSSION

Preparation of phosphatidylinositol. Though silicic acid columns have been widely used for the

Table 4. *Composition of subcellular fractions from rat brain*

Results are means $\pm$ S.D. of percentages of component or activity in the fractions, taking homogenate as 100%, except for 6-phosphogluconate dehydrogenase where the results are means of only two fractionations.

No. of fractionations	...	...	Protein	DNA	RNA	Succinate dehydrogenase	6-Phosphogluconate dehydrogenase
			5	3	3	5	2
Homogenate			100	100	100	100	100
Nuclear fraction			15.9 $\pm$ 1.8	94.7 $\pm$ 10.4	6.4 $\pm$ 3.1	7.6 $\pm$ 2.7	12.0
Myelin fraction			9.5 $\pm$ 1.5	1.2 $\pm$ 1.0	7.1 $\pm$ 0.9	2.7 $\pm$ 1.5	4.8
Nerve-ending fraction			15.9 $\pm$ 0.8	2.4 $\pm$ 0.4	19.5 $\pm$ 2.9	6.6 $\pm$ 0.7	11.9
Mitochondrial fraction			17.7 $\pm$ 2.0	4.0 $\pm$ 0.4	18.6 $\pm$ 1.7	33.5 $\pm$ 5.4	11.0
Microsomal fraction			8.9 $\pm$ 0.9	1.3 $\pm$ 1.2	31.7 $\pm$ 7.5	0.4 $\pm$ 0.2	7.4
Supernatant fraction			24.5 $\pm$ 1.6	0	19.4 $\pm$ 1.4	0.7 $\pm$ 0.7	61.6
Recovery			92.4	103.6	102.7	51.5	108.7
Absolute values:			(mg.)	(μ g. of DNA P)	(μ g. of RNA P)	(μ moles of substrate converted/min.)	
Homogenate, per g. of fresh brain			118.5 $\pm$ 9.5	113.4 $\pm$ 13.2	249.5 $\pm$ 13.6	3.4 $\pm$ 0.6	0.40
Homogenate, per mg. of protein			—	1.22 $\pm$ 0.16	2.18 $\pm$ 0.17	0.038 $\pm$ 0.004	0.004

Table 5. *Subcellular distribution of phosphatidylinositol kinase, 5'-nucleotidase and Na⁺-plus-K⁺-stimulated adenosine triphosphatase*

Results represent (1) relative specific activity (R.S.A.) defined as activity/mg. of protein of fraction divided by activity/mg. of protein of homogenate and (2) percentage of total activity in each fraction, taking homogenate as 100%. Means $\pm$ S.D. of three or four fractionation results are given.

No. of fractionations	...	...	Phosphatidylinositol kinase		5'-Nucleotidase		Na ⁺ -plus-K ⁺ -stimulated adenosine triphosphatase	
			4		3		4	
			R.S.A.	%	R.S.A.	%	R.S.A.	%
Nuclear fraction			1.27 $\pm$ 0.54	20.0 $\pm$ 9.7	0.62 $\pm$ 0.35	12.6 $\pm$ 4.6	1.29 $\pm$ 0.24	21.1 $\pm$ 6.1
Myelin fraction			0.77 $\pm$ 0.23	7.1 $\pm$ 2.5	1.61 $\pm$ 0.11	13.2 $\pm$ 1.1	1.02 $\pm$ 0.44	9.3 $\pm$ 4.8
Nerve-ending fraction			1.36 $\pm$ 0.37	21.8 $\pm$ 5.5	1.38 $\pm$ 0.32	21.7 $\pm$ 5.3	1.87 $\pm$ 0.62	29.6 $\pm$ 9.0
Mitochondrial fraction			0.57 $\pm$ 0.08	10.8 $\pm$ 2.0	0.47 $\pm$ 0.09	7.9 $\pm$ 1.9	0.75 $\pm$ 0.34	12.1 $\pm$ 5.0
Microsomal fraction			1.92 $\pm$ 0.29	17.7 $\pm$ 3.0	1.55 $\pm$ 0.22	12.5 $\pm$ 2.3	1.82 $\pm$ 0.22	15.1 $\pm$ 3.7
Supernatant fraction			0.64 $\pm$ 0.13	15.9 $\pm$ 2.8	0.84 $\pm$ 0.17	19.6 $\pm$ 1.4	0.04 $\pm$ 0.04	0.9 $\pm$ 0.9
Recovery			—	93.3	—	87.5	—	88.1
Absolute values (substrate converted):								
m μ moles/mg. of protein/min.			Approx. 0.054		32.7 $\pm$ 2.7		195 $\pm$ 75	
m μ moles/g. of fresh brain/min.			Approx. 6.3		3600 $\pm$ 600		23000 $\pm$ 7000

Fig. 6. Subcellular distribution of phosphatidylinositol kinase and marker components in rat brain: (a) 5'-nucleotidase; (b) phosphatidylinositol kinase; (c) Na⁺-plus-K⁺-stimulated adenine triphosphatase; (d) 6-phosphogluconate dehydrogenase; (e) succinate dehydrogenase. Relative specific activities (see Table 5) are plotted on the ordinates and percentages of homogenate protein in each fraction on the abscissae. Abbreviations for the subcellular fractions are as follows: N, nuclear; My, myelin; NE, nerve-ending; Mt, mitochondrial; Mc, microsomal; S, supernatant.

purification of phosphatidylinositol, the differences in mobility of the various salt forms of this lipid are not usually recognized. The calcium salt prepared in the experiment with yeast and [³²P]orthophosphate, for instance, was eluted from silicic acid with chloroform-methanol (7:1, v/v). Higher concentrations of methanol have generally been used to elute phosphatidylinositol (Hanahan, 1960). Carter and Weber (1966) have clearly shown that elution of the sodium salt requires higher concentrations of methanol than that of the calcium salt, and have used the difference in behaviour to prepare pure phosphatidylinositol from crude plant lipids.

Extraction of polyphosphoinositides. Diposphoinositide and triphosphoinositide form stable complexes with protein, particularly in the presence of Ca²⁺ (Dawson, 1965). Extraction of these lipids from tissues is therefore difficult and has usually been accomplished by the use of chloroform-methanol mixtures containing hydrochloric acid (Folch, 1952).

To avoid the use of hydrochloric acid, an extraction procedure based on that of Garbus *et al.* (1963) was used in the present study. This involves

addition of methanol-chloroform (2:1, v/v) to the incubation mixture to give a one-phase system, followed by treatment with 2M-potassium chloride in 0.5M-potassium phosphate buffer. Better recoveries of labelled diposphoinositide were obtained by this method than by a method involving trichloroacetic acid precipitation followed by extraction of the precipitate with chloroform-methanol-hydrochloric acid. The effectiveness of the Garbus method is probably due to the high concentration of potassium chloride and potassium phosphate added to the one-phase system. These salts would be expected to act like hydrochloric acid in disrupting ionic bonds between diposphoinositide and positively charged groups in a protein. However, it does not follow that the procedure of Garbus *et al.* (1963) will be more effective than the chloroform-methanol-hydrochloric acid method in the extraction of tissues.

Phosphatidylinositol-kinase reaction. The present work confirms that of Colodzin & Kennedy (1965) on the phosphatidylinositol kinase of rat brain, with the important difference that these workers could detect no synthesis of triphosphoinositide. As

shown in Fig. 3, labelled triphosphoinositide was formed in brain homogenates incubated with [^{32}P]ATP. The difference may be partly due to the fact that Colodzin & Kennedy (1965) used a microsomal preparation. The enzyme catalysing the formation of triphosphoinositide from diphosphoinositide (diphosphoinositide kinase) occurs largely in the supernatant fraction (Kai *et al.* 1966).

The production of labelled diphosphoinositide from both labelled ATP and labelled phosphatidylinositol indicates that the following reaction takes place:

The possibility of other phosphate donors has not been excluded, though the work of Colodzin and Kennedy (1965) makes this unlikely.

The marked increase in diphosphoinositide labelling caused by added diphosphoinositide was unexpected. The added lipid may compete with the labelled diphosphoinositide for sites on hydrolytic enzymes (Dawson & Thompson, 1964).

Using brain microsomes and a crude plant lipid (Inosithin) of unknown phosphatidylinositol content, Colodzin & Kennedy (1965) obtained maximum activity at 5–20 mM-magnesium chloride. In the present study 30 mM-magnesium chloride was required (Fig. 2a). Colodzin & Kennedy (1965) used a lower protein concentration (0.3 mg./ml. of incubation mixture as compared with approx. 2 mg./ml. in the present study), which may affect the Mg^{2+} requirement. The ATP concentrations did not differ.

The activation by low concentrations of EDTA (1–4 mM) in the presence of 30 mM- Mg^{2+} may be due to the removal of Ca^{2+} , since EDTA has a greater affinity for Ca^{2+} than for Mg^{2+} . The activation could also be connected with the fact that the kinase requires thiol groups for activity. Colodzin and Kennedy (1965) also showed that the phosphatidylinositol kinase was a thiol enzyme. Traces of heavy-metal ions, such as Cu^{2+} , which combine with thiol groups, could be removed from the enzyme by EDTA. It has been shown that Cu^{2+} inhibits the

kinase. Another possibility is that EDTA increases the yield of diphosphoinositide by inhibiting the monoesterase and diesterase that attack this lipid (cf. Dawson & Thompson, 1964; Thompson and Dawson, 1964).

Several detergents activated the kinase (Table 2), the most effective being sodium deoxycholate, Cutscum and Triton X-100. There was no obvious relationship between the activity of the detergents and their ionic charges. The enzyme was partly solubilized by deoxycholate. This should be helpful in purification studies. The present system is far from ideal, since the net formation of diphosphoinositide is likely to be decreased considerably by the action of hydrolytic enzymes (Dawson & Thompson, 1964; Thompson & Dawson, 1964). The true rate of the reaction therefore cannot be determined.

Distribution of phosphatidylinositol kinase. Enzymes catalysing the phosphorylation of phosphatidylinositol occur in a variety of mammalian tissues. Galliard *et al.* (1965) showed that the kinase was present in rat liver, where its subcellular distribution suggested that it was a component of the plasma membrane (Michell & Hawthorne, 1965). The enzyme is also present in human erythrocyte membranes (Hokin & Hokin, 1964) and in the brain, kidney, lung, heart and spleen of the rat (Colodzin & Kennedy, 1965).

Table 6 compares the reaction rates in various tissues. As mentioned above, the true rates will probably be much higher, since the results represent net diphosphoinositide production in the presence of hydrolytic enzymes. The results for erythrocyte 'ghosts' were calculated from the data of Hokin & Hokin (1964), assuming that 50% of the 'ghost' dry weight was protein.

The subcellular distribution of brain phosphatidylinositol kinase (Fig. 6) resembles that of Na^+ -plus- K^+ -stimulated adenosine triphosphatase and 5'-nucleotidase, suggesting that the enzyme may be associated with the plasma membrane (cf. Michell & Hawthorne, 1965). Further work is needed on this subject, however, since whole brain provides a complex mixture of cell types, the membranes of which may well differ. In addition, the

Table 6. *Phosphatidylinositol kinase in various tissues*

Source	Reaction rate (μmoles of diphosphoinositide synthesized/mg. of protein/min.)	Reference
Human erythrocyte 'ghosts'	0.06	Hokin & Hokin (1964)
Rat liver	0.5–2.0	Michell & Hawthorne (1965)
Brain microsomal fraction from 20-day-old rat	3.3*	Colodzin & Kennedy (1965)
Adult rat brain	0.05 (1.5†)	Present study

* Rate in the presence of 0.4% Cutscum.

† Initial rate in the presence of 3 mM-deoxycholate (Fig. 5).

assays could be affected to variable degrees by differences in the rates at which diphosphoinositide is hydrolysed or phosphorylated to triphosphoinositide.

It has been suggested that diphosphoinositide and triphosphoinositide may be important in controlling Ca^{2+} concentrations in nervous tissue (Hawthorne and Kemp, 1964). As pointed out by Colodzin and Kennedy (1965), inhibition of phosphatidylinositol kinase by Ca^{2+} may be relevant here. In addition, the hydrolysis of diphosphoinositide to phosphatidylinositol may be activated by Ca^{2+} in the presence of low Mg^{2+} concentrations, like that of triphosphoinositide (Dawson & Thompson, 1964).

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