SUMMARY

1. The activities of succinate dehydrogenase, malate dehydrogenase and aconitate hydratase were measured on fractions of tobacco-leaf extracts prepared by differential centrifuging. The dehydrogenases were also measured in fractions produced when the chloroplast and mitochondrial preparations were resolved by sucrose gradient centrifuging into chloroplasts, chloroplast fragments and mitochondria.

2. All the succinate dehydrogenase of the extracts behaved as if it were attached to mitochondria. It sedimented along with the succinate-oxidase system, and the succinate-dehydrogenase activity was sufficient to account for its expected role in this oxidase system.

3. Most of the malate dehydrogenase was recovered in the supernatant fraction. The small proportions (5–19 %) present in the mitochondrial and chloroplast preparations were reduced by washing. More enzyme appeared to be washed out of the chloroplasts and mitochondria as they sedimented in sucrose gradients.

4. Most of the aconitate-hydratase activity of leaf extracts was recovered in the supernatant fraction. The rest occurred in the mitochondrial fraction and could be partially removed by washing.

5. It is suggested that some of the malate dehydrogenase that was recovered in the supernatant fraction originated in chloroplasts and mitochondria, and that some of the aconitate hydratase originated in mitochondria.

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Phosphoinositides

5. THE INOSITOL LIPIDS OF OX BRAIN*

BY R. B. ELLIS,† T. GALLIARD AND J. N. HAWTHORNE

Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham 15

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An enzyme from liver that hydrolyses inositol phospholipids was described by Kemp, Hübscher & Hawthorne (1961*b*). During studies of its action on the brain 'diphosphoinositide' fraction prepared by the method of Folch (1949), phosphate esters were observed which were more difficult to remove

from ion-exchange resins than the expected inositol diphosphates (Kemp, Hawthorne, Sammons & Ellis, 1961*a*). Further investigation showed that they were inositol triphosphates (Ellis, Kemp & Hawthorne, 1960), confirming results from other Laboratories (Grado & Ballou, 1960; Dittmer & Dawson, 1960).

A more complete study of the hydrolysis of the Folch 'diphosphoinositide' fraction is now presented, from which it is concluded that ox brain

* Part 4: Hawthorne (1961).

† Present address: Department of Physiological Chemistry, Medical Center, U.C.L.A., Los Angeles 24, Calif., U.S.A.

contains at least three phosphoinositides. A preliminary report of this work has appeared (Ellis & Hawthorne, 1961).

MATERIALS AND METHODS

'Diphosphoinositide' fraction. This was prepared as described by Folch (1949). Ox brains were obtained from the slaughter-house, packed in ice and extracted within 2 hr. from death. Four brains (about 1 kg. of material) were used for most preparations, though 20 were used in one case. The yields at the various stages were close to the values given by Folch (1949). After dialysis of an aqueous suspension against de-ionized water for 6 days the material contained 1.9 μ moles of organic phosphorus, only traces of inorganic phosphorus, 1.8 μ moles of carboxylic ester and 0.04 μ mole of aldehyde/mg. of lipid. Appreciable amounts of carbohydrate were present. The three preparations of the 'diphosphoinositide' fraction used in this study are referred to in the text as A, B and C (see Tables 1 and 2).

Analytical methods. Phosphorus was determined by the method of King (1932) and glycerol by either that of Burton (1957) or that of Hanahan & Olley (1958), with mannitol standard. Sodium borate and ammonium formate, in the concentrations used for the ion-exchange chromatography, were shown not to interfere with the assay of glycerol.

Two methods were used to determine inositol after hydrolysis with 6N-HCl in a sealed tube at 110° for 18 hr.: the periodate oxidation method of Böhm & Richarz (1954) or the sensitive microbiological assay method of Norris & Darbre (1956), with *Schizosaccharomyces pombe* as test organism. The effect on the microbiological assay of the reagents used as eluting agents in the ion-exchange separations was examined. Solutions containing 1 μ g. of inositol in varying concentrations of the different reagents were used as test solutions in the assay. The following values represent the highest concentrations of the reagents that allowed full growth of the organism, compared with growth on a standard solution of inositol in water: 2 mM-LiCl; 50 mM-ammonium formate; 50 mM-formic acid; 0.5 mM-sodium tetraborate. A solution of 0.2M-NaCl caused no inhibition. Glycerol and inositol were detected qualitatively by descending paper chromatography with propan-2-ol-acetic acid-water (3:1:1, by vol.) and Whatman no. 54 paper. The spots were made visible with the alkaline AgNO₃ reagents of Trevelyan, Procter & Harrison (1950). The hydroxamate method of Stern & Shapiro (1953) was used for the determination of carboxylic ester bonds and the results were related to standards of triolein or stearylcholine iodide. The dimethylacetal of palmitaldehyde was the standard used in the estimation of aldehyde residues by a Schiff reagent method (see McIlwain & Rodnight, 1962).

Deacylation of phospholipids by a mild alkaline hydrolysis. The technique of Dawson (1954) as modified by Hübscher, Hawthorne & Kemp (1960) was used but, to avoid the intractable emulsions formed in the ether-water distribution after alkaline hydrolysis, the mixture was added to a CHCl₃-methanol-water system so that the final proportions (by vol.) of these solvents were 10:5:3. The upper phase was removed and the lower phase washed with fresh 'upper phase'. The combined upper phases were diluted to 1 l. and made 5 mM with respect to borate as previously

described (Hübscher *et al.* 1960). Small amounts of CHCl₃ that separated on dilution did not affect the Dowex columns to which such hydrolysates were applied.

Resin columns. De-ionized water was used in all work with resins. Dowex 1 (10% cross-linked; 200-400 mesh) was suspended in water and the supernatant decanted just before all the resin had settled. This process was repeated about ten times so that the finest particles were rejected and faster-flowing columns could be prepared. The resin was then washed successively with 2N-HCl, water, 3M-ammonium formate and water. For the fractionation of phosphate ester mixtures containing about 100 mg. of phosphorus, a column 35 cm. $\times$ 1.6 cm. internal diameter was filled to a height of 25 cm. with resin. Flow rates with a hydrostatic head of 6 ft. were 1-1.5 ml./min. The effluent was collected in 25 or 50 ml. fractions and a portion from each was analysed for phosphorus. Peak fractions were pooled.

Removal of inorganic salts from combined fractions. To avoid interference with yeast assay for myo-inositol some fractions were diluted with four times their volume of water and passed through a column (14 cm. $\times$ 1.3 cm.) of Dowex 1 (Cl⁻ form; 10% cross-linked; 200-400 mesh). The salts present were washed out with 5 mM-LiCl and the phosphates then eluted with a stronger solution of this salt. The fractions containing phosphorus were evaporated carefully to dryness *in vacuo* with additions of dry acetone when most of the water had been removed. They were then kept in a vacuum desiccator over concentrated H₂SO₄ overnight before extraction of the LiCl with dry ethanol. The ethanol-insoluble residue was washed twice with small portions of ethanol before being dissolved in water.

Further hydrolysis by phosphomonoesterases. Acid phosphatase was a purified specimen from human semen. The assay system containing enzyme (1.06 mg. of protein per test), 0.4M-sodium acetate buffer, pH 5.4 (1 ml.), and ester containing about 300 μ g. of phosphorus in a total volume of 3 ml. was incubated at 37°. One-tenth of this amount of enzyme released all phosphate from sodium glycerophosphate (300 μ g. of phosphorus) in 30 min. Samples (0.4 ml.) were withdrawn at various times and their inorganic phosphate content was determined. For all samples, the liberation of inorganic phosphate had reached a maximum by 8 hr. and did not increase during a further 16 hr. Alkaline phosphatase, prepared from intestine, was a commercial sample. The assay system contained enzyme (0.5 mg.), 0.1 M-ethanolamine-HCl buffer, pH 9.5 (1 ml.), 5 mM-magnesium acetate (1 ml.) and 300 μ g. of phosphorus of the phosphate under study, in a final volume of 5 ml. Inorganic phosphate determinations were made on 0.5 ml. samples. Constant values were reached after 9 hr. and did not increase during a further 15 hr. With both phosphatases, substrate and enzyme blanks showed no release of inorganic phosphate during 24 hr.

RESULTS

Separation of hydrolysis products on Dowex 1. Previous work had shown that a solution of ammonium formate containing sodium tetraborate (up to 150 and 5 mM respectively) would elute the deacylation products of most known phospholipids labile to alkali in the following order: glycerylphosphorylcholine; glycerylphosphorylethanolamine;

glycerylphosphorylinositol; glycerylphosphorylserine; deacylation product of diphosphatidylglycerol and glycerophosphoric acid (Hawthorne & Hübscher, 1959; Hübscher *et al.* 1960). With phospholipids extracted from tissues such as liver, this completed the elution of phosphate esters from the column. With brain, on the other hand,

further elution with formic acid-ammonium formate mixtures produced several more compounds, all of which contain inositol. These compounds are the major hydrolysis products of the 'diphosphoinositide' fraction.

The water-soluble phosphates released from the Folch 'diphosphoinositide' fraction by mild alkaline hydrolysis, under the conditions given above, separated readily on columns of Dowex 1 (formate form). Fig. 1 shows one such separation.

Elution was continued with 0.1M-formic acid containing increasing concentrations of ammonium formate. Four inositol derivatives were thus eluted (Fig. 1). A fifth inositol-containing substance was obtained with 0.3M-lithium chloride and a sixth with 2N-hydrochloric acid. These last two peaks together represented only 4% of the applied phosphorus.

Though there was some variation from one preparation of the 'diphosphoinositide' fraction to another in the pattern of peaks eluted before inorganic phosphate, peaks 6-11 always appeared. Peak 9 was the major component. In some experiments peaks 7 and 8 ran as a single broad peak. Recovery of phosphorus applied to the column was between 91 and 98%, though this is subject to some error because of slight variations in fraction size. During removal of salts on a second column (see Materials and Methods section) peaks 3 and 4 were each eluted as two peaks and so probably do not represent pure compounds.

Analysis of fractions. Table 1 summarizes the analyses of the fractions obtained from a typical hydrolysate of the Folch 'diphosphoinositide' fraction (preparation A). Fig. 1 refers to the same hydrolysis. Peak 2 was not analysed owing to lack of material and peak 5 was shown to be entirely inorganic phosphate.

Glycerol determinations were made both on the intact compounds as eluted and also after strong

Fig. 1. Mild alkaline hydrolysis of undialysed Folch 'diphosphoinositide' fraction (preparation A). The hydrolysate contained 105 mg. of phosphorus and was applied to a Dowex 1 column (formate form; 10% cross-linked; 200-400 mesh; 25 cm. $\times$ 1.6 cm.). The resin retained all the phosphate present in the hydrolysate. Peak 5 (broken line) represents inorganic phosphate. Peak 11 (1.2 mg. of phosphorus), which does not appear in the Figure, was eluted with 2N-HCl (250 ml. collected as one fraction). Solutions A-H used for elution had these compositions:

	Ammonium formate (mM)	Sodium tetraborate (mM)	Formic acid (M)
A	60	5	—
B	120	5	—
C	180	5	—
D	200	—	0.1
E	300	—	0.1
F	400	—	0.1
G	500	—	0.1
H	0.3M-LiCl	—	—

Table 1. *Hydrolysis of Folch 'diphosphoinositide' fraction (preparation A)*

Analysis of peak fractions shown in Fig. 1. Figures represent molar ratios.

Peak no.	...	...	1	3	4	6	7	8	9	10	11
Phosphate:glycerol before hydrolysis			2.2	2.1	2.5	4.1	0	0	6.0	0	0
Phosphate:glycerol after hydrolysis			1.0	1.1	1.1	2.3	0	0	3.1	0	0
Phosphate:inositol (<i>S. pombe</i>)			{ 0.9 1.0	0 0	0 0	2.0 1.7	2.6 2.9	Little growth	3.1 { 2.8 3.0	5.5 { 7.5 3.5	15 —
Phosphate:inositol (periodate)*			1.0	0	0	2.1	2.0		2.9	—	—
Phosphate:inositol (<i>S. pombe</i>)†			1.1	—	—	1.8	4.2		2.9	6.0	—

* Analyses of fractions from another hydrolysis ('diphosphoinositide' fraction, preparation B). In this experiment peaks 7 and 8 were not separated.

† Hydrolysis of 'diphosphoinositide' fraction (preparation C) from 20 brains. Peaks 7 and 8 were not separated.

acid hydrolysis (6*N*-hydrochloric acid, 18 hr., sealed tube at 110°). The analysis depends on the production of formaldehyde by periodate oxidation of the primary alcohol groups of glycerol. As will be seen from Table 1, the glycerol values of peaks 1, 6 and 9 double after hydrolysis. From this it is inferred that the usual 1-3-glycerophosphate structure occurs in the phospholipids from which these compounds are derived. Periodate would not attack 2-glycerophosphate.

The two methods of analysis for inositol agreed well for peaks 1, 6 and 9: molar proportions of glycerol:phosphorus:inositol are 1:1:1, 1:2:1 and 1:3:1 respectively. Consistently poor results were obtained with peaks 7, 8 and 10, which did not contain glycerol. It seems likely that the main component of peaks 7 and 8 is an inositol diphosphate, and peak 10 appears to be largely inositol triphosphate. Phosphate:inositol molar ratios were often too high for these compounds, however, when the microbiological assay was used for inositol determination. There was also considerable variation from one analysis to the next. These compounds always gave a faint yellow colour after acid hydrolysis, and the glycerol-containing esters remained colourless. Unidentified contaminants may therefore have been present. Internal standards of inositol gave the theoretical response, so the high phosphate ratios cannot be explained by growth-inhibition.

Action of acid and alkaline phosphatases (orthophosphoric monoester phosphohydrolases). To obtain further information about the chemical structures of the inositol esters liberated by alkaline hydrolysis, the esters were incubated with phosphatases specific for the monoester linkage. Conditions are given in the Materials and Methods section. Preparation A of the 'diphosphoinositide' fraction was used.

The peak 1 ester, which gave the correct analysis for glycerylphosphorylinositol, was not attacked by the phosphatases, as would be expected. Approximately half of the phosphate was liberated from the peak 6 ester (acid phosphatase, 51%; alkaline phosphatase, 54%), indicating that one of the two phosphate groups is in monoester linkage. This confirms the glycerylphosphorylinositol monophos-

phate structure suggested by the analytical results. The phosphate of peaks 7 and 8 was entirely in the monoester form (acid phosphatase, 95%; alkaline phosphatase, 98%), confirming the inositol diphosphate structure. The compound of peak 9 was only slowly attacked by acid phosphatase. With alkaline phosphatase, however, a plateau in the release of inorganic phosphate was reached at 67%. This would be expected from a glycerylphosphorylinositol diphosphate.

In all the Folch 'diphosphoinositide' fractions studied, glycerylphosphorylinositol, glycerylphosphorylinositol monophosphate and glycerylphosphorylinositol diphosphate were the major inositol-containing hydrolysis products. They separated well from other compounds on the Dowex columns and gave the expected analytical results. Appreciable amounts of other inositol phosphates were also obtained, however (peaks 7, 8, 10 and 11, Fig. 1). These gave less satisfactory results.

Characterization of peaks 7 and 8 (Fig. 1). Peaks 7 and 8 appeared to represent isomeric inositol diphosphates. As can be seen from Table 2, there was little difference between hydrolysis for 10 min. at 10° and the usual hydrolysis, in the amount of phosphorus recovered as inositol diphosphate. It can be concluded that the peak is not due to further hydrolysis of another phosphate ester such as glycerylphosphorylinositol diphosphate. The remaining possibility was that peaks 7 and 8 were due to water-soluble phosphates contaminating the original lipid. Table 2 shows that this was so. The inositol diphosphate peak was not found in a hydrolysate of 'diphosphoinositide' fraction (preparation C) which had been dialysed by a method based on that of Folch (1949). A suspension of the lipid in water was cooled to 0° and mixed with 0.1 vol. of 10*N*-hydrochloric acid. After being stirred vigorously for 5 min. at 0° the suspension was dialysed against running de-ionized water at about 12°. The hydrolysis of this lipid was performed in the usual way. In this experiment there was an unusually low recovery (70%) of the phosphate loaded on the column. The reason for this is unknown.

Table 2. *Composition of various preparations of the Folch 'diphosphoinositide' fraction*

Details of the various preparations are given in the text. Abbreviations: GPI, glycerylphosphorylinositol; GPS, glycerylphosphorylserine; GPIP, glycerylphosphorylinositol monophosphate; IP₂, inositol diphosphate; GPIP₂, glycerylphosphorylinositol diphosphate.

'Diphosphoinositide' preparation	Peak P (% of organic P of hydrolysate)					0.4 <i>N</i> -HCl eluate
	GPI	GPS	GPIP	IP ₂	GPIP ₂	
A (Fig. 1)	16	—	8	17	36	4
C (Undialysed, from 20 brains)	3	—	16	7	55	5
C (Acid-dialysed)	—	—	11	0	41	12
C (10° hydrolysis)	—	—	32	6	58	4
Supernatant in preparation C	12	53	0	0	0	0

Peaks 10 and 11 (Fig. 1). In some separations the elution with 0.3M-lithium chloride was omitted. The 0.4N-hydrochloric acid eluate then represented the combined peaks 10 and 11. As can be seen from Table 2, dialysis did not remove the phosphate eluted by 0.4N-hydrochloric acid. However, inositol:phosphate ratios in undialysed preparations were too high for the inositol triphosphate expected in this fraction, suggesting the presence of other phosphate esters.

To study these contaminants, samples from the 0.4N-hydrochloric acid eluate were hydrolysed in sealed tubes at 100° for 16 hr. with 0.5N-sulphuric acid. After neutralizing with saturated barium hydroxide solution and centrifuging to remove barium sulphate, samples were taken for paper chromatography in the following solvents: (a) propan-2-ol-acetic acid-water (3:1:1, by vol.); (b) propan-2-ol-aq. hydrochloric acid (17 ml. of propan-2-ol and 41 ml. of conc. hydrochloric acid made to 250 ml. with water). For the separation of the intact phosphates solvent (c) propan-2-ol-aq. ammonia (sp.gr. 0.88)-water (7:1:2, by vol.) and solvent (a) were used. After descending chromatography the papers were viewed in ultraviolet light (Chromatolite, Hanovia Ltd., Slough, Bucks.). For the detection of phosphates, the spray described by Hanes & Isherwood (1949) was used and for sugars the aniline hydrogen phthalate reagent described by Partridge (1949). The silver nitrate dip of Trevelyan *et al.* (1950) and a ninhydrin spray were also used.

Chromatography of the hydrolysed peak in solvent (a) showed the presence of inositol and ribose in approximately equimolar quantities, together with a smaller amount of glycerol. Two minor ninhydrin-reacting spots were not identified. Examination in u.v. light of chromatograms developed with solvent (b) showed guanine to be the major absorbing spot. Chromatography in solvent (c) of the unhydrolysed 0.4N-hydrochloric acid fraction, after removal of hydrochloric acid *in vacuo*, showed three phosphate spots, two of which were u.v.-absorbing.

Both intact and hydrolysed fractions gave a u.v.-absorption spectrum suggesting a nucleotide derivative. No such spectrum was obtained with the corresponding peak from a hydrolysate of acid-dialysed 'diphosphoinositide' (preparation C). Determination of inositol and phosphate after hydrolysis in this case confirmed the removal of contaminants. Microbiological assay gave a phosphate:inositol molar ratio of 3.18:1.

Since this inositol triphosphate did not appear to be a contaminant of the original lipid, the possibility that it arose from breakdown of glycerylphosphorylinositol diphosphate on the resin column was investigated. A sample of this substance from peak 9 and representing 34 mg. of

phosphorus was rechromatographed on a Dowex 1 column similar to that used in the acid-dialysed 'diphosphoinositide' fraction experiment. Elution with 0.1M-formic acid-0.3M-ammonium formate removed no phosphate from the column, but complete recovery of the glycerylphosphorylinositol diphosphate was obtained with 0.1M-formic acid-0.5M-ammonium formate. Further elution with 0.4N-hydrochloric acid gave only traces of phosphate (less than 0.7% of the applied phosphorus). It can be concluded therefore that the inositol triphosphate is not a breakdown product of glycerylphosphorylinositol diphosphate produced during resin chromatography.

Composition of supernatant lipid obtained during purification. The final stages of the Folch (1949) preparation of the 'diphosphoinositide' fraction involve reprecipitation ten times from chloroform by the addition of methanol. The combined supernatants from this procedure were concentrated and hydrolysed under mild alkaline conditions. Analysis of the hydrolysate by chromatography on Dowex 1 showed that no higher inositides were present. The results are given in Table 2 (supernatant in preparation C) and show that phosphatidylserine and phosphatidylinositol were major components. Some phosphatidylethanolamine was also present. Less than 5% of the inorganic phosphate associated with crude 'diphosphoinositide' fraction remained in the supernatant.

DISCUSSION

It can be concluded from these results that the 'diphosphoinositide' fraction prepared from ox brain by the method of Folch contains three inositol lipids, with structures (I), (II) and (III) respectively. Similar conclusions have been reached by Dittmer & Dawson (1961) and Brockerhoff & Ballou (1961). The positions of the phosphate groups on the *myo*inositol ring were established by the work of Tomlinson & Ballou (1961). Though the name diphosphoinositide has been used to mean the

- (I) Phosphatidylinositol ($R_1 = H$, $R_2 = H$)
- (II) Diphosphoinositide ($R_1 = PO_3^{2-}$, $R_2 = H$)
- (III) Triphosphoinositide ($R_1 = PO_3^{2-}$, $R_2 = PO_3^{2-}$)

fraction of brain phospholipids prepared by Folch, it should now be applied only to a compound of structure (II), since the major component of the Folch fraction is a triphosphoinositide (Table 2).

Our preparations contain less diphosphoinositide relative to triphosphoinositide than those of Dawson & Dittmer (1961). This may be explained by post-mortem enzymic changes, since brain tissue contains enzymes capable of removing the monoesterified phosphate from compounds (II) and (III) (Thompson & Dawson, 1962).

The present work provides no information about the positions of the fatty acids in compounds (II) and (III). Previous work (Kemp *et al.* 1961*a*) showed that considerable quantities of diglyceride were released from Folch 'diphosphoinositide' fractions on acid hydrolysis and that the overall fatty acid:glycerol ratio was 2:1. The presence of the L-3-glycerophosphate structure also suggests that the usual 'phosphatidyl' residue is present, as in phosphatidylinositol (I). Dawson & Dittmer (1961) came to the same conclusion. Our earlier work (Kemp *et al.* 1961*a*) showed that 'diphosphoinositide' preparations from ox brain contained large proportions of stearic acid (49% of total fatty acids), thus resembling liver phosphatidylinositol. This should reflect the fatty acid composition of the brain inositides *in situ* because there was no loss of higher inositides during the fractionation (see Table 2: supernatant in preparation C).

The inositol diphosphate found in hydrolysates of undialysed Folch 'diphosphoinositide' fractions appears to be a contaminant of the original lipid, rather than a hydrolysis product. Brockerhoff & Ballou (1962) also found an unexpectedly large quantity of inositol diphosphate in hydrolysates of rabbit-brain lipid, but could not explain its origin. When they incubated brain slices with [³²P]orthophosphate, these compounds gained higher specific activities than would be expected if they originated from triphosphoinositide. These authors had only dialysed the brain lipid overnight. Several days are needed for complete removal of contaminants. If traces of nucleotides, for example, were present as contaminants of the original lipid, a relatively high specific activity might be expected in the inositol diphosphate fraction.

The demonstration of inositol diphosphate as a contaminant of the diphosphoinositide fraction provides the first direct evidence that inositol diphosphate occurs free in brain tissue. It is probably produced by the enzymic hydrolysis of triphosphoinositide. If, as the evidence suggests, there are two isomeric diphosphates (peaks 7 and 8, Fig. 1), two hydrolytic enzymes may be involved, catalysing reactions (1) and (2).

Thompson & Dawson (1962) have described an enzyme hydrolysing triphosphoinositide according to reaction (1). The second hydrolysis could be due to an unspecific phosphatase that would finally produce free inositol. A diphosphate could also arise from diphosphoinositide by a reaction analogous to (1).

The last fraction of the hydrolysate eluted from Dowex 1 appears to contain inositol triphosphate together with nucleotide(s) and other contaminants. These contaminants can be removed by dialysis of the acidified lipid before hydrolysis. The last peak then represents pure inositol triphosphate, the origin of which is obscure. Milder hydrolysis produced as much triphosphate as the usual method (Table 2), indicating that it did not arise from glycerylphosphorylinositol diphosphate during the alkaline hydrolysis. Two possibilities remain: that the triphosphate comes from a type of inositol lipid not yet described, or that it is produced by hydrolysis of glycerylphosphorylinositol diphosphate on the Dowex column. Rechromatography of glycerylphosphorylinositol diphosphate gave no evidence of such hydrolysis. It seems likely therefore that ox brain contains small quantities of a phosphoinositide of unknown structure that releases inositol triphosphate on mild alkaline hydrolysis. The work of Klenk & Hendricks (1961) on human brain may relate to a similar compound.

When the Folch fraction I of ox-brain kephalin is purified by successive precipitations (Folch, 1949) the soluble material consists largely of phosphatidylinositol and phosphatidylserine. This confirms the results of Dittmer & Dawson (1961). It is significant that the large quantity of inorganic phosphate contaminating crude 'diphosphoinositide' fractions remains with the higher inositides of the precipitate and not with the phosphatidylinositol.

The presence of three closely related phosphoinositides in brain immediately suggests a biosynthetic scheme (3) involving the successive phosphorylation of phosphatidylinositol. This has been confirmed by studies of incorporation of ³²P *in vivo* into these lipids

(Ellis & Hawthorne, 1962). The specific activities were in the following order: (I) < (II) < (III), and the monoesterified phosphate groups in compounds (II) and (III) on carbon atoms 4 and 5 of the inositol were much more highly labelled than the phosphate group on C-1 of the inositol. Similar results have been obtained by Wagner, Lissau, Hölzl & Hörhammer (1962) and by Brockerhoff & Ballou (1962).

Triphosphoinositide is an unusual phospholipid in that it has two monoesterified phosphate groups. The fact that these groups have a much higher turnover than the rest of the molecule points to an important function of this lipid in nervous tissue. It is likely to be a more efficient carrier of cations than phosphatidic acid, for instance, since it has five replaceable hydrogen atoms instead of two.

SUMMARY

1. The 'diphosphoinositide' fraction was prepared from ox brain by the method of Folch (1949) and hydrolysed under mild alkaline conditions. The hydrolysis products were separated by ion-exchange chromatography and analysed.

2. The major hydrolysis product was glycerylphosphorylinositol diphosphate. Glycerylphosphorylinositol monophosphate and glycerylphosphorylinositol were also present.

3. It is concluded that ox brain contains three phosphoinositides: phosphatidylinositol, diphosphoinositide [1-phosphatidyl(inositol 4-phosphate)] and triphosphoinositide [1-phosphatidyl(inositol 4,5-diphosphate)].

4. Inositol di- and tri-phosphate were also found in hydrolysates of the Folch 'diphosphoinositide' fraction. The free diphosphates occur in ox brain. The triphosphate may come from a new type of phosphoinositide.

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