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The Hydrolysis of Triphosphoinositide by Extracts of Ox Brain

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(Received 7 October 1963)

Sloane-Stanley (1953) reported that brain lipid fractions rich in inositol were rapidly attacked by brain homogenates with the liberation of free and combined phosphate as well as free and combined inositol. Continuing these studies Rodnight (1956) obtained evidence which suggested that the initial product of the breakdown was inositol monophosphate and that its enzymic release was activated by Ca^{2+} ions. However, the interpretation of these results is complicated in view of the recent findings that brain contains at least three

inositol-containing phospholipids, namely mono-, di- and tri-phosphoinositide (Dittmer & Dawson, 1961; Dawson & Dittmer, 1961; Brockerhoff & Ballou, 1961a).

A number of isotopic investigations *in vivo* with labelled phosphate have suggested that brain phosphoinositides are metabolically very active compared with most of the other phospholipids (see, e.g., Ansell & Spanner, 1960). The availability of a method for preparing triphosphoinositide in a relatively pure form (Dittmer & Dawson, 1961) has led us to investigate the enzymic reactions involved in its catabolism and to charac-

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terize the products formed. Preliminary accounts of this work have already been published (Thompson & Dawson, 1962*a*, *b*).

EXPERIMENTAL

Preparation of extracts from ox brain. Ox brain obtained fresh from the slaughterhouse was transported to the laboratory on ice and blood clots were removed by dissection. A portion of the cerebral hemispheres (50 g.) was homogenized in a Waring Blendor with 300 ml. of acetone at -10° . The suspension was filtered through a cooled Buchner funnel under suction and the residue washed with 400 ml. of acetone at -10° followed by 200 ml. of diethyl ether at the same temperature. The solid was rapidly transferred to a desiccator and dried over liquid paraffin and KOH at 4° for 2–3 hr. under suction.

The acetone-dried powder was ground in a chilled mortar and 250 ml. of ice-cold water gradually added to form a smooth suspension. The mixture was kept at 4° for 45 min. with occasional stirring and centrifuged at $10000g_{av}$ for 20 min. The slightly opaque supernatant was dialysed overnight against 6–7 l. of distilled water at 4° . It could be stored frozen at -15° for several months without loss of enzymic activity.

Preparation of triphosphoinositide. Triphosphoinositide was isolated by the method of Dittmer & Dawson (1961). To conserve solvent the initial extraction was carried out with 4 vol. of chloroform-methanol (1:1, v/v) and, after centrifuging, the two phases were decanted away from the triphosphoinositide-containing residue. The triphosphoinositide was eventually precipitated from methanol as the sodium or ammonium salt, and the product used without further purification. The precipitated ammonium salt tended to be colloidal and centrifuged badly; however, by treating the colloidal solution with 0.1 vol. of a saturated solution of NaCl in methanol adequate precipitation was obtained. The final product (yield 12–25 mg./atoms of P/kg. of brain) was dissolved in chloroform and stored at -15° . Analyses (Dawson & Dittmer, 1961) showed that the phosphorus-containing lipids consisted (on a molar basis) of about 85–90% triphosphoinositide with a small amount of diphosphoinositide.

Conditions of incubation and measurement of water-soluble phosphate released. Portions (containing 200 μ g.-atoms of P) of the solution of triphosphoinositide salt were evaporated to dryness *in vacuo* in 30 ml. stoppered tubes. The lipid was dissolved in 4 ml. of 0.05M-tris-HCl buffer, pH 7.2, and incubated at 37° for 25–90 min. with 1 ml. of brain extract in a final volume of 6 ml. After incubation, the tube was cooled in ice, and 1 ml. of 5% (w/v) bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) and 5 ml. of cold 10% (v/v) perchloric acid or 10% (w/v) trichloroacetic acid were added, and the residual triphosphoinositide was co-precipitated with the protein. The mixture was centrifuged and the supernatant decanted through a filter (Whatman no. 531). Appropriate incubation controls were carried through simultaneously. Inorganic phosphate and total phosphate in the solution of acid-soluble phosphate were measured by the method of Fiske & Subbarow (1925).

Isolation of the products of enzymic hydrolysis. (a) Water-soluble products. The trichloroacetic acid extract from the enzymic digestion of triphosphoinositide was washed four

times with an equal volume of ether. The aqueous phase was neutralized with NH_3 , evaporated to 1–2 ml. *in vacuo* and applied to a paper sheet as a band. The phosphate esters were separated by ionophoresis at pH 3.6 and were located and eluted as described by Dawson & Dittmer (1961).

(b) Lipid products. To identical incubation mixtures 10 vol. of chloroform-ethanol (1:1, v/v) was added and filtered. The filtrates were evaporated to dryness in a rotary evaporator, and the residue was treated with 0.5 ml. of light petroleum (b.p. $60\text{--}80^{\circ}$) and then with 10 ml. of acetone. The mixture was kept at 0° for 30 min. and the acetone-soluble neutral lipids were separated by centrifuging. The acetone-insoluble matter was extracted with 10 ml. of chloroform-methanol (2:1, v/v), the suspension centrifuged and the supernatant filtered. The solution was shaken with 2 ml. of 0.1N-HCl and centrifuged, and the lower phase was shaken with 4 ml. of chloroform-methanol-water (3:48:47, by vol.) containing 0.2% of NaCl. After centrifuging, the lower phase, containing the acetone-insoluble phospholipids, was retained for analysis.

The solution of acetone-soluble neutral lipids was evaporated to dryness *in vacuo*, and the residue shaken with 5 ml. of water and 5 ml. of light petroleum (b.p. $60\text{--}80^{\circ}$). After centrifuging, the upper phase containing the acetone-soluble lipids was saved for analysis.

Determinations. Fatty acyl ester, inositol, fatty acid and glycerol were estimated as described by Dittmer & Dawson (1961).

RESULTS

Initial incubations of triphosphoinositide with the extract of acetone-dried brain powder showed that the lipid was rapidly broken down with the liberation of acid-soluble phosphate which was both inorganic and combined (Table 1). The addition of diethyl ether (0.33 vol.) was consistently found to increase the liberation of water-soluble phosphate, but inorganic phosphate was decreased while the liberation of combined phosphate was greatly increased (Table 1).

Ionophoretic separation on paper at pH 3.6 of the water-soluble combined phosphorus compounds liberated in an ether-activated system revealed that the main component was an inositol triphosphate (phosphate:inositol ratio, 2.99, 2.51, 3.04) with smaller amounts of inositol diphosphate (phosphate:inositol ratio, 1.96, 1.71, 1.93). No glycerol was detected in either compound. From identical enzymic hydrolyses an acetone-soluble lipid was isolated which contained no free fatty acid or phosphorus but contained acyl ester and glycerol in the molar ratio of 1.82 and 2.11 in two experiments. This was therefore a diglyceride.

The acetone-insoluble lipid isolated contained phosphate, inositol, glycerol and acyl ester bonds, and appeared to consist of a mixture of phosphoinositides. Studies were therefore made of this lipid fraction during enzymic hydrolysis in the absence of ether. Under these conditions only small amounts of diglyceride were found in the acetone-

soluble lipid fraction. The molar proportions of phosphate:inositol:glycerol:acyl ester in the acetone-insoluble phospholipid changed during the reaction from 3:1.19:0.96:2.33 to 1:0.97:0.85:1.97 at the end of the incubation.

This provided evidence that triphosphoinositide was being converted into monophosphoinositide with the concomitant release of inorganic phosphate. This was confirmed by subjecting the acetone-insoluble phospholipids to alkaline hydrolysis and separating the hydrolysis products by chromatography and ionophoresis (Dawson, Hemington & Davenport, 1962). The positions taken up by the hydrolysis products on the paper indicated a conversion of triphosphoinositide into monophosphoinositide. However, in addition, evidence was obtained for the accumulation and subsequent dephosphorylation of diphosphoinositide as an intermediate in this process (Table 2). Chromatography of the acetone-insoluble lipids on formaldehyde-treated paper (Hörhammer, Wagner & Richter, 1959) showed the accumulation of the

intermediate and the steady accumulation of monophosphoinositide, thus confirming the results shown in Table 2. In Table 3 a comparison is made of the lipid and acid-soluble phosphate products of hydrolysis of triphosphoinositide after incubation in the presence and absence of ether. The addition of ether results in a big increase in the diglyceride and water-soluble phosphate ester formed in the reaction and a decrease in the acetone-insoluble phospholipid and inorganic phosphate liberated. In both incubations the relationship between the amount of diglyceride and water-soluble combined phosphate formed is approximately that to be expected if the triphosphoinositide was being split into a diglyceride and inositol triphosphate (1:3). Similarly, the ratio of inorganic phosphate to acetone-insoluble phospholipid phosphate in both incubations is near to that expected for the dephosphorylation of triphosphoinositide to monophosphoinositide (2:1). This is in spite of the comparatively complicated isolation procedures used for the lipids before analysis, and the likely

Table 1. *Effect of ether on the enzymic breakdown of triphosphoinositide by extracts of ox brain*

Incubation was for 35 min. as described in the text. The amount of ether added was 0.33 vol.

Conditions of incubation		Water-soluble phosphate liberated ($\mu\text{g. atoms of P}$)		
		Total phosphate	Inorganic phosphate	Combined phosphate
Expt. 1 (2.36 μmoles of substrate)	Control	2.90	1.92	0.98
	Ether added	4.88	0.82	4.06
Expt. 2 (1.86 μmoles of substrate)	Control	3.06	2.50	0.56
	Ether added	3.98	1.16	2.82

Table 2. *Formation of phosphoinositides on incubation of triphosphoinositide with brain extracts*

The incubation conditions were as described in the Experimental section. The acetone-insoluble phospholipid was analysed by the method of Dawson, Hemington & Davenport (1962).

Incubation time (min.)	Inorganic phosphate released ($\mu\text{g. atoms of P}$)	Percentage composition of acetone-insoluble phospholipid P		
		Triphosphoinositide	Diphosphoinositide	Monophosphoinositide
0	0	78	15	7
25	1.19	46	29	25
50	3.03	1	34	65
90	3.26	0	3	97

Table 3. *Products of the hydrolysis of triphosphoinositide by brain extracts*

The incubation conditions were as described in the Experimental section. The amounts of acetone-soluble diglyceride were calculated from the glycerol content of the fraction.

Conditions of incubation	Acid-soluble phosphate ($\mu\text{g. atoms of P}$)			Acetone-soluble diglyceride (μmoles)	Acetone-insoluble lipid phosphate ($\mu\text{g. atoms of P}$)
	Total phosphate	Inorganic phosphate	Combined phosphate		
Control	3.07	2.50	0.57	0.13	1.44
Ether added	4.00	1.17	2.83	1.12	0.57

intermediate formation, in the dephosphorylation, of diphosphoinositide, which might undergo some hydrolysis to diglyceride and inositol diphosphate.

DISCUSSION

The observations reported above indicate that two mechanisms exist for the hydrolysis of triphosphoinositide by brain extracts. The first is a cleavage of the lipid into inositol triphosphate and diglyceride (1a) and the second a two-stage dephosphorylation into diphosphoinositide (2a) and finally monophosphoinositide (2b). The finding of small and variable amounts of inositol diphosphate in the incubation mixture also suggests that some of the intermediate diphosphoinositide in mechanism (2) is converted into diglyceride and inositol diphosphate (1b).

The implication is, therefore, that the two methods of degradation (1 and 2) are brought about by independent enzymes, i.e. a phosphodiesterase and a phosphomonoesterase. The separation of the two systems is reported by Dawson & Thompson (1964) and Thompson & Dawson (1964).

The present evidence of an active dephosphorylation of triphosphoinositide in brain tissue can be considered with the circumstantial evidence of a number of workers (Brockerhoff & Ballou, 1961b, 1962; Ellis & Hawthorne, 1962; Wagner, Lissau, Hölzl & Hörhammer, 1961) that the lipid is synthesized in brain tissue by the stepwise phosphorylation of monophosphoinositide. This, coupled with the ^{32}P -incorporation data mentioned above, would suggest that the secondary phosphate groupings of triphosphoinositide undergo rapid metabolic exchange *in vivo*, possibly in a phosphorylation-dephosphorylation cycle. The physiological significance of this rapid metabolism is unknown but it has been suggested that it could be of importance in the transport of cations (Thompson & Dawson, 1962b; Ellis & Hawthorne, 1962).

SUMMARY

1. Sodium triphosphoinositide was rapidly hydrolysed when incubated with dialysed extracts of acetone-dried ox brain.

2. The hydrolysis products were identified as inorganic phosphate, inositol diphosphate, inositol triphosphate, diglyceride and monophosphoinositide.

3. The addition of diethyl ether to the incubation medium greatly increased the amount of diglyceride and inositol phosphoesters formed and decreased inorganic phosphate and monophosphoinositide.

4. Diphosphoinositide accumulated as a transient intermediate during the hydrolysis.

5. The results were interpreted to mean that triphosphoinositide was broken down by the following main pathways:

W. T. held a Postdoctoral Fellowship from the Multiple Sclerosis Society of Canada while this work was in progress. Mr N. Clarke is thanked for valuable assistance and Mr R. W. White for carrying out the inositol assays.

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