Biochem. J. (1965) **96**, 634

Diphosphoinositide and Triphosphoinositide in Animal Tissues

EXTRACTION, ESTIMATION AND CHANGES *POST MORTEM*

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(*Received* 1 *January* 1965)

1. A method is presented for the determination of the di- and tri-phosphoinositide in animal tissues. 2. The polyphosphoinositides are quantitatively extracted into chloroform–methanol–hydrochloric acid solvent after a preliminary chloroform–methanol (1:1, v/v) extraction to remove the bulk of the other phospholipids. On washing this extract with N-hydrochloric acid the polyphosphoinositides pass completely into the lower chloroform-rich phase. Their concentrations in the lower phase are determined by chromatography on formaldehyde-treated paper or chromatography and ionophoresis of the acid hydrolysis products. 3. When guinea-pig brain is extracted by the method of Folch (1942), considerable hydrolysis of the triphosphoinositide and accumulation of diphosphoinositide occurs during the initial acetone extraction. 4. The tri- and di-phosphoinositide contents of rat and guinea-pig brain decline substantially within a few minutes after death. 5. The concentrations of tri- and di-phosphoinositide in rat brain are not changed by insulin-hypoglycaemia or electrical stimulation. 6. Examination of frozen rat tissues showed that the brain contained the highest concentration of polyphosphoinositides. Much smaller amounts are present in kidney, and only trace quantities in liver and lung. None could be detected in spleen, heart and skeletal muscle.

The recent recognition that the polyphosphoinositide fraction bound to solvent-extracted brain tissue consisted of two components, namely di- and tri-phosphoinositide, has created a need for a suitable method for quantitatively extracting these phospholipids from tissues and estimating their concentration. Recent reports of a tetraphosphoinositide (Santiago-Calvo, Mulé & Hokin, 1963) have now been withdrawn. Previous estimates of the concentrations of the polyphosphoinositides have usually been based on recovery experiments (Dawson & Dittmer, 1961; Brockerhoff & Ballou, 1962) or total inositol determination without differentiation of the individual components (LeBaron, McDonald & Sridhara Ramarao, 1963). Wagner, Hölzl, Lissau & Hörhammer (1963) introduced a valuable method for the chromatography of phospholipids on formaldehyde-treated paper and used this to obtain an estimate of the polyphosphoinositides in total ether extracts of rat tissues. The concentrations recorded are considerably lower than those found in the present studies.

The methods developed have been applied to sucrose homogenates of brain tissue to test their adequacy for studying the distribution of polyphosphoinositides in subcellular particles of brain tissue. A preliminary account of some aspects of the present work has appeared (Eichberg & Dawson, 1964).

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METHODS

Source of tissues. Ox brain was collected at the slaughterhouse and brought to the laboratory surrounded by ice (45–90 min. *post mortem*). The forebrains of adult guinea pigs were removed after stunning and decapitation and either used directly (4–6 min. *post mortem*) or kept on ice until required.

In one experiment an adult guinea pig was anaesthetized deeply with thiopentone, the skull exposed and the brain frozen *in situ* with liquid N_2. The skull was removed and the frozen brain dissected by judicious chiselling.

Young albino rats (100–120 g.) were decapitated and the head and body immediately frozen in liquid N_2 or they were frozen whole under thiopentone anaesthesia (60 mg./kg.). Tissues (while still frozen) were 'dissected' with a blunt scalpel and transferred to a small cooled tissue crusher in which they were pounded to give a frozen disk (Stone, 1938). With practice the organs could be well separated from adjacent tissue; however, the heart undoubtedly contained more frozen blood than would be present after dissection at room temperature. Electrical stimulation of the brain

was carried out by the technique of Dawson & Richter (1950).

Preparation of homogenates. Brain tissue was mixed with 4vol. of 0·32M-sucrose and homogenized at 4° in an Aldridge, Emery & Street (1960) homogenizer.

Extraction of polyphosphoinositides. Whole or crushed frozen tissues were blended with 10vol. of chloroform–methanol (1:1, v/v) for 1min. in a micro top-drive blender. The homogenate was transferred with washing to a centrifuge tube and, after spinning, the supernatant containing most of the tissue phospholipid was discarded. The residue was washed three times with 10vol. of chloroform–methanol (2:1, v/v) and then extracted three times at 37° for 20min. with four times its packed volume (or at least 10ml.) of chloroform–methanol (2:1, v/v) containing 0·25% (v/v) of conc. HCl (A.R). The successive acid extracts containing the polyphosphoinositides were filtered through a small wad of glass wool in a funnel and combined. The extracts were washed by shaking with 0·2vol. of N-HCl, the upper phase was discarded and the lower phase and interface were washed once with 0·5vol. of chloroform–methanol–N-HCl (3:48:47, by vol.).

Homogenates in 0·32M-sucrose were treated with 4vol. of methanol, heated at 55° for 5min. and then sufficient 0·05M-$MgCl_2$ (0·1–0·5ml.) was added to flocculate the particulate matter and facilitate centrifuging. The precipitate obtained by centrifuging was successively extracted as above with ten times its packed volume of chloroform–methanol (1:1, v/v) and then three times with the same volume of chloroform–methanol (2:1, v/v). The extraction of polyphosphoinositides from the residue was then carried out as above.

Estimation of polyphosphoinositides. (*a*) Acid hydrolysis. The acid-washed lower phase, together with any interfacial material, was evaporated to dryness *in vacuo* at 50° in a stoppered centrifuge tube. Then 2ml. of 5N-HCl was added and the tube heated in a water bath at 100° for 10min. After cooling the hydrolysate was washed with 4ml. of chloroform and, after centrifuging, the upper aqueous layer was removed, leaving any interfacial material. The lower chloroform phase and interface were then washed with 1ml. of 5N-HCl to ensure quantitative recovery of acid-soluble inositol esters. No residual inositol could be detected in the washed interface and lower chloroform phase when these were assayed microbiologically after prolonged acid hydrolysis. The combined aqueous phases were evaporated to dryness *in vacuo*, with the addition of ethanol to control frothing when necessary. The residue was dissolved in a small volume of water (0·5–1ml.) neutralized to pH7 with aq. NH_3, and any precipitate removed by centrifuging. The supernatant was analysed for P, and a portion containing 40–80μg. of P was used for chromatography. It was found that the addition of a small quantity of EDTA (final concn. 1mM) to the supernatant before chromatography virtually eliminated the tendency for some inositol triphosphate to remain at the origin. The paper was developed in the first dimension with a solvent composed of phenol saturated with N-HCl (100vol.), acetic acid (10vol.) and ethanol (12vol.) (16hr., descending). After drying and washing with ether the paper was subjected to ionophoresis in the second dimension in volatile buffer, pH4·2 [water–acetic acid–pyridine (186:10:5, by vol.)] for 55min. at 50v/cm. (Dawson, Hemington & Davenport, 1962).

At the end of this time the paper was dried and the phosphorus-containing spots were located and identified (Dawson & Dittmer, 1961) (Fig. 1) and assayed for P (Dawson *et al.* 1962). The concentrations of triphosphoinositide P and diphosphoinositide P were calculated from the following (Dawson & Dittmer, 1961): triphosphoinositide P = 1·28 × inositol triphosphate P; diphosphoinositide P = inositol diphosphate P − (0·19 × inositol triphosphate P).

(*b*) Alkaline hydrolysis. Alkaline hydrolysis was carried out only on the HCl-washed lower phase since polyphosphoinositides in the interfacial material did not liberate any water-soluble P. Alkaline hydrolysis of the phospholipids with 0·025N-NaOH in 80% (v/v) ethanol and separation of the hydrolysis products were carried out as described previously (Dawson & Dittmer, 1961; Dawson *et al.* 1962).

(*c*) Chromatography on formaldehyde-treated paper. The paper was prepared by a method similar to that described by Hörhammer, Wagner & Richter (1959). Ten sheets of Whatman no. 1 paper (46cm. × 14cm.) were rolled and inserted into a 64oz. wide-necked glass reagent jar. The roll was soaked for 1hr. in a mixture containing 200ml. of formalin (36% formaldehyde solution; A.R.), 10ml. of acetic acid and 0·4g. of NH_4CNS. Surplus solution was drained away and the jar stoppered with a paper strip between the glass lid and the neck. The jar was autoclaved for 3hr. at 123° (18lb./in.^{2}). The papers, still in the jar, were washed with running tap water overnight and then with three changes of distilled water. They were dried in an oven at 70°.

Chromatographic separation of the polyphosphoinositides was successful only if they were first converted into the salt form. The lower chloroform-rich phase obtained by washing the extract with N-HCl was washed three

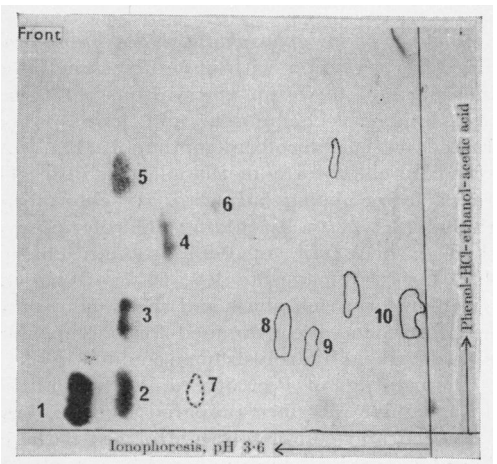

Fig. 1. Separation on paper of acid-hydrolysis products of chloroform–methanol–HCl extracts of brain. Spots outlined with a continuous line were positive on preliminary ninhydrin spraying. Visible spots were detected by an acid molybdate spray followed by irradiation in u.v. light. 1, Inositol triphosphate; 2, inositol diphosphate; 3, inorganic phosphate; 4, glycerophosphate; 5, 6, EDTA (false P colour); 7, inositol monophosphate; 8, aspartic acid; 9, glutamic acid; 10, serine.

times with 0·4vol. of 0·05M-$CaCl_2$–methanol–chloroform (47:48:3, by vol.). This chloroform solution of the calcium salts was relatively stable compared with the acidic polyphosphoinositides and could be stored at 4°. A suitable sample of the solution (5ml., about 20μg. of P) was evaporated to dryness *in vacuo* at 50°, and a known portion of the polyphosphoinositides transferred to the paper for chromatography in either of two ways. (*a*) The residue was treated with 1ml. of acetone, which was removed by boiling. This process was repeated with 1ml. of ethanol. The residue was dissolved in 0·5ml. of chloroform saturated with water, and 0·4ml. was applied to the paper. (*b*) The residue was dissolved in 0·5ml. of chloroform–methanol (2:1, v/v) containing 0·05% (v/v) of conc. HCl. A portion (0·4ml.) was applied to the paper in a cold stream of air and the spot on the paper was treated with strong NH_3 vapour for 15min. It was then left for at least 20min. in air for the excess of NH_3 to disperse.

The chromatogram was developed by ascending chromatography in butanol–acetic acid–water–diethyl ether (20:5:25:6, by vol.; upper phase) for 18hr. The triphosphoinositide (R_F 0·22) and diphosphoinositide (R_F 0·29) spots were located by spraying to detect phosphorus as described above. The spots were cut out along with suitable blanks and analysed as before.

Estimation of inositol. Inositol was assayed by the procedure of Campling & Nixon (1954) with *Kloeckera brevis* as the test organism. Combined inositol was hydrolysed for 18hr. at 105° in 5N-HCl, the acid removed *in vacuo* and the residue made up to a known volume with water. The solution was adjusted to approx. pH5 by the addition of 0·1vol. of 0·2M-sodium acetate–acetic acid buffer, pH5·2, before assay.

RESULTS

Extraction of polyphosphoinositides from brain tissue. (*a*) Extraction with chloroform–methanol. Guinea-pig and ox brain and various rat tissues (heart, lung, spleen, muscle and kidney) were extracted with chloroform–methanol (1:1, v/v) followed by chloroform–methanol (2:1, v/v). The extracts, after being adjusted to chloroform–methanol (2:1, v/v) by adding chloroform, were washed with 0·2vol. of 0·9% sodium chloride (Folch, Lees & Sloane-Stanley, 1957). When the lower chloroform-rich phase and the small proteinaceous interface were examined for polyphosphoinositides by acid hydrolysis none could be detected (less than 1·5μg. of P/g. of brain). In addition, when a similar interface prepared from ox brain was washed and completely hydrolysed with 5N-hydrochloric acid, no inositol could be detected by microbiological assay. Abundant phosphatidylinositol was present in such extracts (Table 1).

When the initial extraction of a guinea-pig brain was performed with chloroform–methanol (2:1, v/v) rather than with chloroform–methanol (1:1, v/v), traces of polyphosphoinositides were detected on acid hydrolysis of the material at the interface formed by washing with 0·9% sodium chloride. No polyphosphoinositide could be detected in the lower chloroform phase.

Table 1. *Extraction of lipid inositol from ox brain by acidified chloroform–methanol solvent*

Ox brain (5g.) was extracted successively with 50ml. of chloroform–methanol (1:1, v/v), then twice with 50ml. of chloroform–methanol (2:1, v/v). The residue was extracted four times for 20min. at 37° with 50ml. of chloroform–methanol (2:1, v/v) containing 0·25% (v/v) of conc. HCl. The extracts were washed (Folch *et al.* 1957) and the lower phases and interfaces assayed for inositol.

	Combined inositol extracted (μg. of inositol)	
	Brain 1	Brain 2
Pooled $CHCl_3$–CH_3·OH extracts	1910	1750
First $CHCl_3$–CH_3·OH–HCl extract	900	800
Second $CHCl_3$–CH_3·OH–HCl extract	160	108
Third $CHCl_3$–CH_3·OH–HCl extract	35	21
Fourth $CHCl_3$–CH_3·OH–HCl extract	0	0
Residue digested with N-NaOH (20hr., 37°)	22	14

(*b*) Extraction with chloroform–methanol containing various additives. The extraction of lipid inositol with acidified chloroform–methanol was a comparatively slow process and three successive extractions for 20min. at 37° were necessary to complete the extraction (Table 1). Digestion of the residue with 5N-sodium hydroxide for 20hr. at 37° showed that less than 1% of the total combined inositol remained in the residue after solvent extraction (Table 1) and this may not have been lipid inositol.

Confirmation of the quantitative extraction of triphosphoinositide from guinea-pig brain by successive extractions with chloroform–methanol–hydrochloric acid at 37° was obtained by examining the solvent-extracted residue by acid hydrolysis for 10min. at 100°; no inositol triphosphate could be detected in a portion representing 0·43g. of brain, whereas the equivalent acidified chloroform–methanol extract contained 15·5μg. of triphosphoinositide P.

Attempts were made to duplicate the extraction of triphosphoinositide with chloroform–methanol acidified with organic acids, e.g. formic acid, citric acid, oxalic acid, rather than hydrochloric acid. Little triphosphoinositide was extracted from brain (Table 2) and the same was true if the acid was replaced with a solvent-soluble base (diethylamine) or salt (lithium chloride). The use of cyclohexane-1,2-diaminetetra-acetate, a potent chelator of the alkaline-earth metal ions, resulted in 26% of the triphosphoinositide being extracted (Table 2). It has been suggested that such metal ions are

Table 2. *Comparison of acidified chloroform–methanol and other solvents as extractants of brain triphosphoinositide*

Ox brain (10g.) was extracted with (*a*) 250ml. of chloroform–methanol (1:1, v/v), (*b*) twice with 100ml. of chloroform–methanol (2:1, v/v) and (*c*) twice with 50ml. of the experimental solvent (given in the Table) at 37° for 15min. The final extracts (*c*) were shaken with 0·2vol. of N-HCl and the lower phase containing the triphosphoinositide was separated from the upper phase and interfacial material.

Expt. no.	Solvent composition	Visual appearance of interface	Total P (μg. of P)	Triphosphoinositide (μg. of P)	Triphosphoinositide extracted (%)
1	100ml. of $CHCl_3$–CH_3·OH (2:1, v/v) +0·25ml. of conc. HCl	++++	663	537	100
	100ml. of $CHCl_3$–CH_3·OH (2:1, v/v) +0·25ml. of water+0·25ml. of diethylamine	Trace	79	16	3
	100ml. of $CHCl_3$–CH_3·OH (2:1, v/v) +0·25ml. of 27·5mM-LiCl	+	41	8	2
	100ml. of $CHCl_3$–CH_3·OH (2:1, v/v) +0·25ml. of water+0·25g. of citric acid	+	17	—	<3
	100ml. of $CHCl_3$–CH_3·OH (2:1, v/v) +0·25ml. of water+0·25g. of oxalic acid	+	51	—	<7
	100ml. of $CHCl_3$–CH_3·OH (2:1, v/v) +0·25ml. of conc. HCl+0·6ml. of pyridine	++++	70	—	10
2	100ml. of $CHCl_3$–CH_3·OH (2:1, v/v) +0·25ml. of conc. HCl	++++	850	593	100
	100ml. of $CHCl_3$–CH_3·OH (2:1, v/v) +0·25ml. of conc. HCl+0·375ml. of pyridine	++++	720	511	86
	100ml. of $CHCl_3$–CH_3·OH (2:1, v/v) +0·5ml. of formic acid	+	64	11	2
	100ml. of $CHCl_3$–CH_3·OH (2:1, v/v) +2·0ml. of 0·14M-sodium cyclohexane-1,2-diaminetetra-acetate	Trace	240	156	26

components of the triphosphoinositide–protein complex (Kerr, Kfoury & Djibelian, 1963). When the hydrochloric acid in the chloroform–methanol mixture was neutralized with 1·5equiv. of pyridine good extraction of triphosphoinositide was still obtained (Table 2). However, further addition of pyridine (to 2·4equiv.) resulted in much less of this phospholipid being extracted.

(*c*) Extraction by Folch's (1942) method. Although little or no polyphosphoinositide is extracted from brain tissue with chloroform–methanol, prior treatment of the tissue with acetone and ethanol as in the method of Folch (1942) results in these phospholipids being solubilized (Dittmer & Dawson, 1961). However, a striking change occurs in both the amount and ratio of triphosphoinositide and diphosphoinositide extracted (Table 3); whereas the guinea-pig brains extracted with acidified chloroform–methanol (Expts. 1 and 3) contain 37·1 and 43·4μg. of triphosphoinositide P/g., those extracted by the Folch procedure followed by acidified chloroform–methanol (Expt. 2) yielded only 16·4μg./g. Diphosphoinositide, on the other hand, showed an increase (from 3 to 22μg. of P/g.) when the Folch procedure was used for the extraction. On the assumption that some enzymic dephosphorylation of triphosphoinositide was occurring during the acetone extraction, in one experiment (Expt. 4) this step was prolonged to 3hr. Virtually all the polyphosphoinositide remaining was diphosphoinositide.

Similar results were obtained with ox brain (Table 3). The triphosphoinositide concentration decreased from 57·2 to 16·5μg. of P/g. if the acetone extraction was prolonged for 3hr. and there was a fourfold increase in the diphosphoinositide concentration.

Behaviour of polyphosphoinositides on washing chloroform–methanol–hydrochloric acid extracts with aqueous solutions. On washing the acidified chloroform–methanol extracts of brain tissue with 0·2vol. of 0·145M-sodium chloride or N-hydrochloric

Table 3. *Extraction of polyphosphoinositides from guinea-pig and ox brain by the present method and the procedure of Folch* (1942)

The pooled guinea-pig brains or ox brain were extracted either with neutral and acidified chloroform–methanol as in the standard procedure (see the Methods section), or with acetone, ethanol and light petroleum according to the method of Folch (1942) followed by chloroform–methanol–HCl. In Expt. 2 the acetone extraction was carried out over 20 min., whereas in Expts. 4 and 6 the extraction was extended to 3 hr. The triphosphoinositide and diphosphoinositide in the extracts were determined after acid hydrolysis (see the Methods section).

Expt. no.	Species and no. of brains	Extracting solvent	Triphosphoinositide (μg. of P/g. wet wt.)	Diphosphoinositide (μg. of P/g. wet wt.)
1	Guinea pig (3)	$CHCl_3$–CH_3·OH (2:1, v/v) containing 0·25% (v/v) of conc. HCl	37·1	3·5
2	Guinea pig (3)	(i) Acetone (20 min.) + ethanol*	0	0
		(ii) Light petroleum	16·4	17·0
		(iii) $CHCl_3$–CH_3·OH (2:1, v/v) containing 0·25% (v/v) of conc. HCl	0	4·5
3	Guinea pig (2)	$CHCl_3$–CH_3·OH (2:1, v/v) containing 0·25% (v/v) of conc. HCl	43·4	2·9
4	Guinea pig (2)	(i) Acetone (180 min.) + ethanol*	0	0
		(ii) Light petroleum	0	10·1
		(iii) $CHCl_3$–CH_3·OH (2:1, v/v) containing 0·25% (v/v) of conc. HCl	0·8	4·2
5	Ox cerebral cortex	$CHCl_3$–CH_3·OH (2:1, v/v) containing 0·25% (v/v) of conc. HCl	57·2	2·8
6	Ox cerebral cortex	(i) Acetone (180 min.) + ethanol*	0	0
		(ii) Light petroleum	13·6	8·6
		(iii) $CHCl_3$–CH_3·OH (2:1, v/v) containing 0·25% (v/v) of conc. HCl	2·9	3·0

* Precipitate that develops on standing added to light-petroleum extract.

Table 4. *Distribution of polyphosphoinositides on washing chloroform–methanol–hydrochloric acid extracts of brain with aqueous solutions*

Pooled guinea-pig brains (11·7 g.) were homogenized in 4 vol. of 0·32 M-sucrose. The homogenate was extracted with chloroform–methanol followed by chloroform–methanol–HCl as described in the Methods section. Portions of the acidified chloroform–methanol extract were shaken with 0·2 vol. of the aqueous solution. Polyphosphoinositides in the lower chloroform phase were examined by alkaline hydrolysis and in the interface by acid hydrolysis.

Aqueous solution used for washing	Fraction examined	Total P (μg. of P/g. of brain)	Triphosphoinositide (μg. of P/g. of brain)	Diphosphoinositide (μg. of P/g. of brain)
N-HCl	Lower $CHCl_3$-rich phase	69·4	31·4	10·6
	Interface	17·0	0	0
0·145 M-NaCl	Lower $CHCl_3$-rich phase	53·0	18·3	9·1
	Interface	28·6	8·3	0·5

acid, the polyphosphoinositides were distributed between the lower chloroform-rich phase and the interfacial proteinaceous material. The polyphosphoinositides in the lower phases were examined by alkaline hydrolysis. Those remaining in the interface were found to liberate no water-soluble P on alkaline hydrolysis and consequently were examined by acid hydrolysis. When hydrochloric acid was used as the washing medium the triphosphoinositide appeared to pass quantitatively into the lower chloroform-rich phase, whereas with the sodium chloride solution considerable triphosphoinositide remained in the interface (Table 4). In both cases the interfaces contained a substantial proportion of phosphorus (equivalent to 10·6 μg. of P/g. of brain) that was not rendered water-soluble on acid hydrolysis. However, when the interface derived from N-hydrochloric acid washing of the acid extract was assayed microbiologically for total inositol only traces could be detected (equiva-

lent to 0·25 μg. of P/g. of polyphosphoinositide P), and even this might have arisen by contamination of the interface with the lower chloroform-rich phase. Consequently it can be assumed that this stable P in the interface is not due to polyphosphoinositide.

Stability of polyphosphoinositides to acid extraction and washing. Tri- and di-phosphoinositide are comparatively labile to acid: e.g. 90% of the polyphosphoinositides in a guinea-pig brain extract was decomposed in 2min. in 5N-hydrochloric acid at 100°, giving predominantly inositol triphosphate and inositol diphosphate respectively.

The acid aqueous upper phase resulting from the washing with hydrochloric acid was reduced in volume by evaporation *in vacuo* and examined by chromatography (phenol–hydrochloric acid–acetic acid–ethanol) and ionophoresis (pH 3·6). Extracts of whole brain and of brain homogenates in 0·32M-sucrose gave a well-defined P spot in the precise position of inositol diphosphate. However, when a sample of this was isolated by preparative paper chromatography it was found to chromatograph with fructose 1,6-diphosphate and not inositol diphosphate: in 2-methylbutan-2-ol–water–toluene-*p*-sulphonic acid (30:15:1, v/v/w; descending, 42hr.) unknown and fructose 1,6-diphosphate had R_F 0·35, inositol diphosphate R_F 0·16. Fructose 1,6-diphosphate moved to the same position as inositol diphosphate on chromatography in the phenol–hydrochloric acid–acetic acid–ethanol solvent and on ionophoresis at pH 3·6.

Inorganic phosphate was the main component of the other phosphorus-containing compounds in the upper aqueous phase. Co-chromatography with markers showed that some phosphorylethanolamine and glycerophosphoric acid were also present. In occasional extracts a trace of inositol triphosphate was seen. However, if this had arisen by decomposition of triphosphoinositide it would only represent a few per cent of the phospholipid present in brain. There were a number of ninhydrin-positive spots: aspartic acid and glutamic acid appeared to be major components.

Nature of phospholipids in acidified chloroform–methanol extract from brain. When guinea-pig brain was extracted exhaustively with chloroform–methanol, the residue still contained 2–3% of the total phospholipid P and this was extracted with the chloroform–methanol–hydrochloric acid solvent. The predominant phospholipids in this latter extract were the polyphosphoinositides, but traces (10%) of other acidic phospholipids were also present, namely phosphatidylserine, cardiolipin, phosphatidylinositol and phosphatidylethanolamine. Equivalent extracts of ox brain also contained polyphosphoinositides as the major components with other acidic phospholipids, principally phosphatidylserine, as minor constituents.

Table 5. *Content of polyphosphoinositides in guinea-pig and ox brain*

The methods of extraction and estimation were as given in the Methods section. Polyphosphoinositides were determined after acid hydrolysis.

		Triphosphoinositide		Diphosphoinositide	
Species	Treatment of brain	(μg. of P/g. of brain)	(% of polyphosphoinositide P)	(μg. of P/g. of brain)	(% of polyphosphoinositide P)
Guinea pig	Fixed in $CHCl_3$–CH_3·OH 4min. *post mortem*	34·0	86·7	5·3	13·3
Guinea pig	Fixed in $CHCl_3$–CH_3·OH approx. 10min. *post mortem*	36·1	91·2	3·5	8·8
Guinea pig	Fixed in $CHCl_3$–CH_3·OH 20min. *post mortem*	35·1	90·8	3·6	9·2
Guinea pig	Fixed in $CHCl_3$–CH_3·OH 120min. *post mortem*	36·1	90·3	3·9	9·7
Guinea pig	Kept on ice; fixed in $CHCl_3$–CH_3·OH 20min. *post mortem*	29·1	90·4	3·1	9·6
Guinea pig	Kept on ice; fixed in $CHCl_3$–CH_3·OH 120min. *post mortem*	36·0	89·1	4·4	10·9
Guinea pig	Brain (under thiopentone anaesthesia) fixed *in situ* with liquid N_2	54·4	83·3	11·0	16·7
Guinea pig	Brains homogenized in 0·32M-sucrose before extraction with $CHCl_3$–CH_3·OH	41·9±4·1*	85·0	7·4±0·6*	15·0
Ox	Carried to laboratory on ice 45min. *post mortem*	57·2	95·3	2·8	4·7

* Mean values for six brains.

Extracts of the homogenates of guinea-pig brain in 0·32M-sucrose contained much more (5–7%) of the total phospholipid in the fraction extracted with chloroform–methanol–hydrochloric acid. On analysis by alkaline hydrolysis (Dawson *et al.* 1962) the mixture was found to contain the following components (μg. of P/g. of brain): phosphatidylethanolamine, 3·0; phosphatidylserine, 31·4; phosphatidylinositol, 3·2; cardiolipin, 1·2; polyphosphoinositides, 46·5; glycerophosphate (probably from decomposition on evaporating slightly acid lower phase), 4·6; alkali-stable phospholipid, 1·4. It seemed possible that the initial treatment of such homogenates with magnesium chloride (see the Methods section) might increase the binding of the acidic phospholipids to the residue, making them less extractable with chloroform–methanol. If EDTA (5mM) or cyclohexane-1,2-diaminetetra-acetate (2·5mM) was added to the initial sucrose homogenate the amount of acidic phospholipids other than polyphosphoinositides extracted by chloroform–methanol–hydrochloric acid was greatly diminished.

Concentration of polyphosphoinositides in brain tissue. Table 5 shows the triphosphoinositide and diphosphoinositide contents of guinea-pig and ox brain that had been homogenized in chloroform–methanol at various times after the death of the animal. The molar concentration of triphosphoinositide in guinea-pig and ox brain was at least six times that of diphosphoinositide. The concentration of each polyphosphoinositide in guinea-pig brain did not vary to any extent between 4 and 120min. *post mortem*. The mean concentrations in six guinea-pig brains that had been homogenized initially in 0·32M-sucrose were slightly higher, and those in a single brain fixed *in situ* with liquid nitrogen appreciably higher (Table 5). This latter result suggested that an initial rapid disappearance of polyphosphoinositide from brain might occur immediately after death.

This possibility was explored further in a series of experiments in which the heads of rats were frozen in liquid nitrogen at various times after decapitation. Dissection and analysis of the brains showed that a very rapid and pronounced decrease of both the triphosphoinositide and diphosphoinositide concentrations had occurred within minutes after decapitation and that the triphosphoinositide/diphosphoinositide concentration ratio decreased (Table 6).

That the stimulation of the brain attendant on decapitation did not cause a change in the concentrations in the brain was shown by freezing the brain *in situ* with liquid nitrogen while the rat was under thiopentone anaesthesia. Such a procedure has led to the much higher recoveries of those cerebral constituents, e.g. phosphocreatine, that are sensitive to stimulation (Kerr, 1935; Dawson &

Table 6. *Polyphosphoinositide content of rat brain at various times* post mortem *and during different physiological states*

The methods of extraction and estimation are given in the Methods section. Polyphosphoinositides were determined after acid hydrolysis.

Treatment of animals and brains	Triphosphoinositide (μg. of P/g. of brain)	Triphosphoinositide (% of polyphosphoinositide P)	Diphosphoinositide (μg. of P/g. of brain)	Diphosphoinositide (% of polyphosphoinositide P)
Rats decapitated; heads frozen immediately in liquid N_2	38·9		11·4	
	32·6		10·5	
	37·2 (37·0)	75·5	14·5 (12·1)	24·5
	39·2		12·0	
Rat decapitated; head frozen in liquid N_2 after 2min.	33·7	76·9	10·1	23·1
Rat decapitated; head frozen in liquid N_2 after 3min.	18·7	70·6	7·8	29·4
Rats decapitated; heads frozen in liquid N_2 after 5min.	14·0	68·6	6·4	31·4
	4·2	44·7	5·2	55·3
Rats decapitated; heads frozen in liquid N_2 after 35min.	4·7	52·2	4·3	47·8
Brain (under thiopentone anaesthesia) frozen *in situ*	33·3	75·5	11·0	24·5
	31·8	70·4	13·4	29·6
Electrical stimulation of brain (2sec., 50v, 50 cyc./sec.) after (1)10sec.; (2) 20sec.; rat decapitated and head fixed in liquid N_2	36·4	76·5	11·2	23·5
	30·6	72·9	12·5	27·1
Insulin administered by intraperitoneal injection: animals in convulsions when decapitated; then heads frozen in liquid N_2	33·7	71·2	15·2	28·8
	34·5	71·1	14·0	28·9

Richter, 1950; Minard & Davis, 1962). The concentrations of triphosphoinositide and diphosphoinositide in the brain were, however, no higher than those of brains fixed in liquid nitrogen immediately after decapitation (Table 6).

The concentrations of the polyphosphoinositides in rat brain were also measured during two physiological states in which the amount of metabolic energy available for their synthesis in the living brain would be limited. Electrical stimulation of the brain with resulting convulsions produced no appreciable decrease in the concentrations, and the same was true when animals were killed during hypoglycaemic convulsions induced with insulin (Table 6).

Concentration of polyphosphoinositides in various tissues of the rat. The concentrations of triphosphoinositide and diphosphoinositide were measured in organs removed from animals that had been frozen immediately after death in liquid nitrogen to minimize post-mortem hydrolysis (Table 7). Apart from brain the only tissue examined that contained appreciable polyphosphoinositide was the kidney. Both liver and lung contained a little diphosphoinositide. No acid-hydrolysis products of polyphosphoinositide could be detected on the chromatograms prepared with acid chloroform–methanol extracts of spleen, heart or skeletal muscle. A small origin spot was visible and it is conceivable that this might contain such hydrolysis products bound in an insoluble form (see the Methods section). However, if this were so the concentration of polyphosphoinositide in the tissue would still be very low compared with that in brain and kidney.

Table 7. *Polyphosphoinositide content of various rat tissues*

Rats (100–120g.) were decapitated and the carcasses frozen whole in liquid N_2. Organs were dissected in the frozen state. N.D., None detected.

Tissue	Triphosphoinositide (μg. of P/g.)	Diphosphoinositide (μg. of P/g.)
Brain	37·0±2·2	12·1±1·2
Liver	0·4	1·1
	1·2	1·2
Kidney	6·3	5·4
	3·5	3·1
	6·8	4·3
Lung	N.D.	2·9
	N.D.	1·6
Spleen	N.D.	N.D.
	N.D.	N.D.
Heart muscle	N.D.	N.D.
	N.D.	N.D.
Skeletal muscle	N.D.	N.D.
	N.D.	N.D.

DISCUSSION

The present results confirm and extend previous observations on the extraction of polyphosphoinositides from tissues by organic solvents (Dittmer & Dawson, 1961). Thus in our extraction technique chloroform–methanol did not extract triphosphoinositide or diphosphoinositide from tissues unless these were initially treated with acetone or ethanol as in the procedure of Folch (1942). LeBaron, Hauser & Ruiz (1962) reported that the proteolipids obtained from chloroform–methanol (2:1, v/v) extracts of ox-brain white matter contained a small percentage of the total polyphosphoinositide present. Our results show that, with whole brain, traces are present when the initial extraction is made with chloroform–methanol (2:1, v/v) rather than with chloroform–methanol (1:1, v/v). Possibly the more polar solvent mixture results in a greater denaturation of tissue protein or, alternatively, white matter behaves differently from whole brain. We have also found that polyphosphoinositides may be extracted with neutral chloroform–methanol from certain subcellular particles from tissues as distinct from the whole tissue (Eichberg & Dawson, 1965).

The chloroform–methanol–hydrochloric acid extraction procedure introduced by Folch (1952) can quantitatively remove polyphosphoinositides from the chloroform–methanol-extracted tissue, provided that the extraction is sufficiently prolonged. Presumably the acid displaces the polyphosphoinositide from some salt-linkage in an insoluble complex. Organic acids such as formic acid do not fulfil the same function, possibly because they are weaker acids than the polyphosphoinositides. The ability of the powerful Ca^{2+} and Mg^{2+} chelator cyclohexane-1,2-diaminetetra-acetate to liberate some triphosphoinositide may be a reflexion of the postulated role of Ca^{2+} and Mg^{2+} in the polyphosphoinositide macromolecular complex of brain (Kerr *et al.* 1963).

Clearly, small proportions of other acidic phospholipids apart from the polyphosphoinositides can remain attached to the residue when brain tissue is extracted with chloroform–methanol, especially when added Mg^{2+} is present in the system. Whether these insoluble phospholipids are parts of specific macromolecular complexes or are produced as artifacts as a result of the phospholipids reacting electrostatically with basic protein during homogenization remains to be ascertained. LeBaron (1963) has interpreted certain data on the extractibility of the triphosphoinositide and protein into acidified chloroform–methanol solvents of various compositions as favouring the existence of a definite complex in the tissue.

The observation that neither of the polyphospho-

inositides is extracted into neutral chloroform–methanol whereas both can be quantitatively extracted into acidified chloroform–methanol provides a suitable starting point for their estimation. The other phospholipids are largely eliminated by the initial extraction, allowing a much more accurate estimate of the polyphosphoinositides, which even in brain amount to only about 2% of the total lipid P. It is necessary to rid the extracts of contaminating inorganic phosphate since excess of this interferes with the subsequent chromatographic estimation. Washing with N-hydrochloric acid provides a convenient way of doing this and it has the added advantage that, unlike other washing media, hydrochloric acid leaves virtually all the polyphosphoinositides in the lower chloroform-rich phase. No appreciable decomposition of the phosphoinositides occurs either during the prolonged acid extraction or during the subsequent acid washing.

Both the separation of the polyphosphoinositides on formaldehyde-treated paper or of the acid-hydrolysis products on ordinary paper are satisfactory procedures for their estimation. For the direct chromatography on formaldehyde-treated paper it is necessary to obtain the phospholipids freely soluble in moist chloroform and thus separated from their macromolecular complex. Difficulty was experienced in obtaining quantitative transfer of the polyphosphoinositides to the paper after concentration; the methods given ensure that a reasonably quantitative transfer to the paper is obtained. It was also necessary to convert the polyphosphoinositides into their calcium salts to achieve stability during storage and also to obtain satisfactory chromatography.

Acid hydrolysis was the only method that could be used to analyse polyphosphoinositides combined in macromolecular complexes, but it had to be assumed that they behaved on hydrolysis in a way similar to the free phospholipids. In general, the acid-hydrolysis method gave slightly higher recoveries than chromatography on formaldehyde-treated paper, even when the phospholipids were free. Breakdown occurred during storage of the acid lower chloroform phase but this did not affect the recovery. The separation was more complicated but no tedious preparation of the paper was necessary.

In general, alkaline hydrolysis was useful only when it was required to assess the concentrations of other phospholipids in the extract. Only the free polyphosphoinositides were decomposed quantitatively and absence of bivalent metal cations was necessary. The breakdown was complex and the relative proportions of di- and tri-phosphoinositide were difficult to compute.

It is clear that a satisfactory estimate of the polyphosphoinositide content of brain can be obtained only if the tissue is frozen *in situ* and subsequently fixed in chloroform–methanol. In rat brain and probably in guinea-pig brain there is a rapid and extensive disappearance of the polyphosphoinositides within minutes after death, after which the concentration remains constant for some time. The disappearance of both triphosphoinositide and diphosphoinositide with the concentration ratio changing in favour of the latter is consistent with the rapid enzymic hydrolysis of triphosphoinositide to monophosphoinositide with diphosphoinositide as an intermediary (Thompson & Dawson, 1964; Dawson & Thompson, 1964).

A similar type of decomposition was observed during the extraction of polyphosphoinositides by the procedure of Folch (1942). If the preliminary acetone extraction was sufficiently prolonged, diphosphoinositide became the predominant polyphosphoinositide. This could possibly explain the initial isolation of diphosphoinositide from such extracts by Folch (1949*a*,*b*) and the comparatively large amounts reported in Folch I and purified diphosphoinositide fractions (Dawson & Dittmer, 1961). It could also account for the loss of phosphatidopeptide P observed to occur when ox brain was pretreated with acetone (LeBaron & Lees, 1962).

The concentration of triphosphoinositide now found in rat brain is some four times that reported by Wagner *et al.* (1963), whereas our diphosphoinositide values are somewhat lower. Probably this can be attributed to the greater care taken in the present work to minimize the rapid hydrolysis that occurs immediately *post mortem* and possibly also by the more quantitative extraction of polyphosphoinositide achieved.

Our values for the concentration of triphosphoinositide in ox brain presumably represent the amount remaining after the immediate post-mortem change, so that the true concentration *in vivo* may well be higher. The total amounts of polyphosphoinositide found in both ox and guinea-pig brain agree reasonably well with the measurement of combined inositol in the phosphatidopeptide fraction recorded by LeBaron *et al.* (1963). Such a phosphatidopeptide fraction probably contains the bulk of the polyphosphoinositides (Dittmer & Dawson, 1961; LeBaron, 1963).

The present values for the concentrations of both triphosphoinositide and diphosphoinositide in rat kidney are about double those reported by Wagner *et al.* (1963). It is again likely that post-mortem changes are the cause of this discrepancy, since the tissues that we examined were quickly frozen *in situ* before dissection. Small quantities of both polyphosphoinositide were found in liver and a trace of diphosphoinositide in lung. Kfoury &

Kerr (1964) have detected diphosphoinositide in pig liver, and Galliard & Hawthorne (1963) have identified diphosphoinositide as a minor component of rabbit-liver mitochondria. No polyphosphoinositide could be detected in the spleen, heart or skeletal muscle of the rat. Because of the limitations of the method it is still possible that trace amounts could be present in these tissues (see Wagner *et al.* 1963). However, before accepting trace amounts as a true constituent of a tissue it would first be necessary to exclude any contribution by neural elements.

Our results do not give any direct indication of the physiological function of triphosphoinositide. In spite of the high metabolic activity of this compound compared with the other phospholipids, its concentration in brain is well maintained even during such physiological emergencies as insulin-hypoglycaemia or convulsions resulting from electrical stimulation. This indicates that it does not act as a readily available source of phosphate bond energy during conditions that interfere with the supply of oxidizable substrate or create an abnormal demand for ATP. The results of Hayashi, Kanoh, Shimizu, Kai & Yamazoe (1962) suggest that ^{32}P incorporation into the triphosphoinositide of rabbit brain is modified by electrical convulsions.

It may be of significance that the two tissues that are the richest in triphosphoinositide, i.e. brain and kidney, are those that are concerned with the active transport of univalent cations. Triphosphoinositide has also been reported as a constituent of the electric organ of *Electrophorus*, another tissue intimately concerned with the physiological sodium pump (Glynn, Slayman, Eichberg & Dawson, 1965). However, in the electric organ of *Torpedo*, participation of triphosphoinositide as a direct cation carrier that is dephosphorylated and subsequently re-formed by phosphorylation has been excluded (Glynn *et al.* 1965).

This investigation was supported (in part) by a U.S. Public Health Service Fellowship (no. 15083) to J. E. We are most grateful to Miss Norma Hemington and Mr N. G. E. Clarke for valuable technical assistance, and to Mr R. W. White for inositol assays.

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