Incorporation of Injected [^{32}P]Phosphate into the Phosphoinositides of Subcellular Fractions from Young Rat Brain

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1. Six subcellular fractions were prepared from the brain of 3-week-old rats after the injection of [^{32}P]orthophosphate into the subarachnoid space. The fractions were characterized by chemical and enzymic determinations and by electron microscopy. 2. The highest concentration of phosphoinositides was found in the microsomal fraction. 3. After an exchange period of 4 hr. *in vivo*, the specific radioactivity of phosphatidylinositol was high in the nuclear, mitochondrial and supernatant fractions, and that of diphosphoinositide was high in the nuclear, microsomal and supernatant fractions. Triphosphoinositide specific activity was highest in the myelin fraction. 4. Specific activities (counts/100 sec./ μg . of P) were in the following order for all fractions except myelin: diphosphoinositide > phosphatidylinositol > triphosphoinositide. For myelin the order was: diphosphoinositide > triphosphoinositide > phosphatidylinositol. 5. Phosphatidylinositol extracted from a tissue fraction by acidified chloroform-methanol had roughly twice the specific activity of that extracted by neutral solvents. The same applied to diphosphoinositide.

The three inositol lipids of brain, phosphatidylinositol, diphosphoinositide and triphosphoinositide, have closely related structures. Isotope-incorporation experiments in a number of Laboratories led to the suggestion that biosynthesis of triphosphoinositide involves two successive phosphorylations, that of phosphatidylinositol to diphosphoinositide and that of diphosphoinositide to triphosphoinositide (Wagner, Lissau, Hölzl & Hörhammer, 1962; Ellis & Hawthorne, 1962; Brockerhoff & Ballou, 1962*a,b*). In addition, the rapid incorporation of [^{32}P]phosphate *in vivo* into the 4- and 5-phosphate groups of triphosphoinositide (I) prompted speculations about the physiological significance of this lipid, possibly in connexion with cation transport (Thompson & Dawson, 1962; Ellis & Hawthorne, 1962).

Colodzin & Kennedy (1964) have shown that a microsomal fraction from brain phosphorylates phosphatidylinositol to diphosphoinositide. A similar phosphorylation has been described with rat-liver mitochondria (Galliard & Hawthorne, 1963). Apart from these two brief reports, there is little information on the labelling of phosphoinositides in different subcellular fractions.

In the present work the labelling from inorganic [^{32}P]phosphate *in vivo* of the three phosphoinositides in subcellular fractions of rat brain is compared. Young rats were chosen, in which myelination was still in progress. The synthesis of inositol lipids is likely to be more active at this stage than in the adult (Agranoff, 1960).

EXPERIMENTAL

Subarachnoid injections. Three-week-old rats weighing about 40 g. were injected by the method of Ansell & Chojnacki (1963). The animals were anaesthetized by the intraperitoneal injection of Avertin (2.5%, w/v, in aq. 0.9% NaCl solution; Winthrop Laboratories, New York, N.Y., U.S.A.) at a dose of 0.05 ml./10 g. body wt. After the forehead was shaved, a 20-gauge needle fitted to an Agla micrometer syringe (Burroughs, Wellcome and Co., Beckenham, Kent) was inserted obliquely into the subarachnoid space between the frontal lobes. Occasionally, when the skulls were opened later, traces of pricking were seen on the brain surface, but the bulk of the needle tip must have been in the subarachnoid space. A volume of 30 μl . of carrier-free [^{32}P]orthophosphate solution (50–160 μC) was

injected in this way. Then 4 hr. later the rats were killed and their brains removed, this equilibration period being sufficient to give significant labelling of di- and tri-phosphoinositide.

Subcellular fractionation. For each experiment three brains (including cerebellum and medulla oblongata) were used. The combined weight was approx. 4.2 g. The fractionation was done at 4°. The brains were homogenized in 0.32M-sucrose-0.05 mM-HgCl₂ adjusted to pH 7.4 by the addition of a small quantity of solid tris, the final volume being 50 ml. The HgCl₂ was added in the hope of inhibiting triphosphoinositide hydrolases (Thompson & Dawson, 1964; Dawson & Thompson, 1964). However, at concentrations greater than 0.05 mM, HgCl₂ affected the fractionation process, causing partial precipitation of protein. It was successfully used in the fractionation of intestinal mucosa at a concentration of 2 mM (Hübscher, Smith & Gurr, 1964). In the present work only the initial homogenization was done in the presence of HgCl₂.

A 10 ml. portion of the homogenate was set aside and the remainder treated by the method of Whittaker (1959) and Nyman & Whittaker (1963) for the preparation of nuclear, myelin, nerve-ending ('synaptosomal'), mitochondrial, microsomal and supernatant fractions. The myelin, synaptosomal and mitochondrial fractions obtained by density-gradient centrifugation were collected as the interfacial band including the corresponding supernatant layer and were not further spun down as pellets. Nuclear and microsomal fractions were obtained as pellets and resuspended by homogenization in 0.1M-sucrose. Samples of each fraction were taken for protein determination by the biuret method of Weichselbaum (1946) and the lipids were extracted from the remainder.

To obtain some indication of the purity of the subcellular fractions several chemical and enzymic assays were made with brains from rats that had not received [³²P]phosphate. To overcome any inhibition by Hg²⁺ ions, L-cysteine (final concn. 2 mM) was added to the enzyme assay system. Succinate dehydrogenase was measured as succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride oxidoreductase (EC 1.3.99.1) by the method of Pennington (1961). Glucose 6-phosphatase (*D*-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) was assayed by the method

of Swanson (1955), but did not prove to be active enough to act as a marker enzyme in the brain subcellular fractions (cf. Aldridge & Johnson, 1959). Cholesterol was estimated by the method of McIlwain & Rodnight (1962), and DNA by that of Burton (1956). Dr J. B. Finean of this Department also examined the fractions by electron microscopy.

Extraction of lipids. The sucrose suspension of each subcellular fraction was first mixed with trichloroacetic acid solution (final concn. 5%, w/v). The mixture was centrifuged at 2500g for 15 min. and as much as possible of the supernatant was then drained off. The precipitate was triturated with 20 ml. of acetone and centrifuged as before. The residue was next extracted with 20 ml. of ethanol, the acetone and ethanol extracts being combined. After these preliminary extractions the residue was extracted three times at 37° for 20 min. with 20 ml. of acidified chloroform-methanol [400 ml. of chloroform-methanol (2:1, v/v) mixed with 1 ml. of conc. HCl (cf. Dittmer & Dawson, 1961)]. These extracts were combined and the residual solid was discarded. The acetone-ethanol and chloroform-methanol-HCl extracts were separately treated as follows. Each was evaporated to dryness *in vacuo* and the residue dissolved in 10 ml. of chloroform-methanol (2:1, v/v). This solution was shaken with 2 ml. of 10 mM-CaCl₂ and centrifuged (Folch, Lees & Sloane-Stanley, 1957). The aqueous layer was removed and replaced by a similar volume of fresh upper layer. After shaking, the mixture was centrifuged and the washed chloroform layer evaporated to dryness *in vacuo*. The residual lipid was dissolved in approx. 0.2 ml. of chloroform-methanol (2:1, v/v) for application to paper chromatograms.

Paper chromatography. Formaldehyde-treated papers (Hörhammer, Wagner & Richter, 1959; Wagner, Hölzl, Lissau & Hörhammer, 1963) were prepared as follows by a slight modification of the method of Letters (1964). Fifty sheets of Whatman 3MM paper (23 cm. × 50 cm.) were wrapped in a protective sheet of paper and soaked overnight in 2.7 l. of a mixture of aq. 40% (w/v) formaldehyde and acetic acid (2:1, v/v) in a 5 l. beaker covered with a glass plate. The covered beaker and its contents were then transferred to an oven at 110° for 5 hr. Excess of formaldehyde vapour was removed by attaching a water pump to the vent at the top of the oven. After 5 hr. the oven was

Table 1. *Components of brain subcellular fractions*

Experimental details are given in the text. The values for protein are means ± S.E.M. of seven determinations, three to four brains being combined for each determination.

	Protein (mg./g. of fresh brain)	DNA (μg. of DNA P/g. of fresh brain)	Succinate dehydrogenase (activity as % of homogenate)	Cholesterol (mg./g. of fresh brain)	Lipid inositol	
					(μmoles/g. of fresh brain)	(μmoles/100 mg. of protein of fraction)
No. of rats	22	6	3	6	9	
Homogenate	161 ± 20	127	100	12.0	1.38	0.85
Nuclear fraction	19 ± 3	133	9.5	1.4	0.14	0.72
Myelin fraction	23 ± 2	1.5	3.2	2.9	0.20	0.97
Nerve-ending fraction	36 ± 2	2.6	17.0	3.6	0.36	1.05
Mitochondrial fraction	27 ± 2	4.3	65.7	1.5	0.27	1.10
Microsomal fraction	17 ± 1	1.1	0	2.1	0.21	1.30
Supernatant	29 ± 1	2.6	0	0.4	0.05	0.22
Recovery (%) ...	94	114	95	99	94	

turned off and allowed to cool overnight. The papers were then washed for 8 hr. in running tap water followed by five washes in deionized water. They were then allowed to stand overnight in deionized water and were finally hung up to dry at room temperature. As much as 100 μ g. of lipid P could be applied to these papers in a band 2.5 cm. wide.

The papers were chromatographed at room temperature by the descending technique with the upper phase of butan-1-ol-acetic acid-water (4:1:5, by vol.) equilibrated for 2 days with 0.125 vol. of diethyl ether (Letters, 1964); this produced two layers, of which the upper was used. Chromatography took 12-16 hr., after which the papers were dried and radioautographs prepared by superimposing them on X-ray film in cassettes for 3-5 days. The film was then developed and fixed in the usual way. For non-radioactive spots or confirmation of the presence of lipids, the chromatograms were soaked for 30 min. in a solution (0.002%, w/v) of Nile Blue in 0.1N-H₂SO₄ and washed for 1 min. in running tap water. Spots containing 1 μ g. of phospholipid P could be detected by this method. The identity of the spots corresponding to phosphatidylinositol, diphosphoinositide and triphosphoinositide was confirmed by determination of P/inositol ratios. Values of 0.78, 1.8 and 3.2 respectively were obtained. For these determinations the areas of the chromatograms containing the phospholipids were cut out. Paper blanks of the same size were cut from parts of the chromatogram between the inositol spots.

Phosphate estimation. Control areas and lipid-containing areas from the chromatograms were cut into small pieces and digested with 1.4 ml. of 72% (w/v) HClO₄, together with 0.02 ml. of 1% (w/v) ammonium molybdate as catalyst. The colourless digest was then treated by the method of Bartlett (1959) for the determination of phosphate. The accurate measurement of 0.5 μ g. of P was possible by this method.

Measurement of radioactivity. The samples from the phosphate determination were counted in a 10 ml. liquid Geiger-Müller tube with 6% efficiency. Since there were considerable variations in specific activity between experiments, results were always related to the specific activity of homogenate phosphatidylinositol (see Table 3).

Inositol estimation. Samples were dissolved in 2-3 ml. of 6N-HCl and hydrolysed in sealed tubes at 110° for 24 hr. Inositol was estimated in the hydrolysate by the microbiological method of Norris & Darbre (1956).

RESULTS

Electron microscopy of the subcellular fractions indicated some contamination of the nuclear fraction with myelin, but the remaining fractions were relatively homogeneous. Table 1 gives further information on the composition of the fractions. Mitochondrial and nerve-ending fractions contained the bulk of the succinate dehydrogenase, and almost all the DNA was in the nuclear fraction. All the membrane fractions contained lipid-bound inositol, but the quantity per mg. of protein was lowest in the nuclear fraction and highest in the microsomal fraction.

Table 2 gives the distribution of phosphatidyl-

Table 2. *Distribution of phosphoinositides among brain subcellular fractions*

	Phosphatidylinositol			Diphosphoinositide			Triphosphoinositide		
	(μ moles/100 mg. of protein)	(% recovery)	(enrichment)	(μ moles/100 mg. of protein)	(% recovery)	(enrichment)	(μ moles/100 mg. of protein)	(% recovery)	(enrichment)
			richment			richment			richment
Homogenate	1.11 $\pm$ 0.12 (5)	100	1.00	109 $\pm$ 16 (5)	100	1.00	27 $\pm$ 4 (5)	100	1.00
Nuclear fraction	0.85 $\pm$ 0.06 (5)	8	0.76	35 $\pm$ 6 (5)	3	0.32	approx. 10* (5)	6	0.38
Myelin fraction	1.20 $\pm$ 0.16 (5)	13	1.08	69 $\pm$ 17 (5)	8	0.63	25 $\pm$ 13 (5)	12	0.92
Nerve-ending fraction	1.50 $\pm$ 0.20 (5)	28	1.35	125 $\pm$ 25 (5)	23	1.14	24 $\pm$ 6 (5)	17	0.88
Mitochondrial fraction	1.34 $\pm$ 0.01 (5)	21	1.21	133 $\pm$ 16 (5)	21	1.22	22 $\pm$ 3 (5)	13	0.80
Microsomal fraction	1.75 $\pm$ 0.17 (5)	16	1.58	137 $\pm$ 24 (5)	13	1.25	43 $\pm$ 10 (5)	15	1.53
Supernatant	0.21 $\pm$ 0.01 (5)	4	0.19	12 $\pm$ 2 (4)	2	0.11	Trace (5)	—	—
Recovery of P (%)	92			71			63		
Recovery of radioactivity (%)	95			85			69		

* Values ranged from 0 to 30.

Table 3. *Relative specific activities of the phosphoinositides in brain subcellular fractions*

Experimental details are given in the text. The values are given relative to the specific activity of homogenate phosphatidylinositol and are expressed as means $\pm$ s.e.m., with the numbers of experiments in parentheses, three brains being used for each experiment. The actual specific activities of phosphatidylinositol in the homogenate varied from 190 to 1720 counts/100 sec./ μ g. of P. The dose of [32 P]phosphate injected into each rat varied between 50 and 160 μ c.

	Phosphatidylinositol	Diphosphoinositide	Triphosphoinositide
Homogenate	100 (5)	135 $\pm$ 35 (5)	93 $\pm$ 22 (5)
Nuclear fraction	134 $\pm$ 6 (5)	228 $\pm$ 41 (5)	approx. 26 (2)
Myelin fraction	59 $\pm$ 6 (5)	150 $\pm$ 27 (5)	141 $\pm$ 46 (4)
Nerve-ending fraction	79 $\pm$ 7 (5)	139 $\pm$ 32 (5)	65 $\pm$ 8 (5)
Mitochondrial fraction	131 $\pm$ 9 (5)	141 $\pm$ 39 (5)	61 $\pm$ 11 (5)
Microsomal fraction	106 $\pm$ 9 (5)	177 $\pm$ 45 (5)	80 $\pm$ 26 (5)
Supernatant	144 $\pm$ 9 (5)	approx. 261 (3)	—

inositol, diphosphoinositide and triphosphoinositide among the various subcellular fractions. Results are also calculated to show the relative enrichment in a particular fraction when compared with the brain homogenate. For all three phosphoinositides the microsomal fraction showed the greatest enrichment. The nerve-ending and mitochondrial fractions also showed significant enrichment with respect to phosphatidylinositol. Microsomal, mitochondrial and nerve-ending fractions contained relatively more diphosphoinositide than the homogenate, whereas only the microsomal fraction showed enrichment of triphosphoinositide. As shown in Table 2, recoveries of both phosphate and radioactivity were poor with diphosphoinositide and triphosphoinositide.

The relatively large standard errors in Table 2 are probably due to biological variation. Myelination is still taking place in 3-week-old rats, and inositol lipid concentrations may well depend on the extent of myelination and thus vary from animal to animal. Among the particulate fractions only the mitochondrial fraction shows relatively constant phosphatidylinositol/protein ratios. This is probably because lipid and protein synthesis are closely integrated in the mitochondrion. It may also reflect the absence from mitochondria of enzymes hydrolysing phosphatidylinositol.

The specific activities of the various phosphoinositides (Table 3) are given as percentages of that of the homogenate phosphatidylinositol for comparison. Values correspond to the total inositide extracted by the solvents used (cf. Table 4). There was considerable variation among the subcellular fractions. Specific activities of diphosphoinositide and triphosphoinositide represent averages of all the phosphates in each molecule. They were usually in the sequence: diphosphoinositide > phosphatidylinositol > triphosphoinositide. The specific activity of phosphatidylinositol was high in supernatant, nuclear and mitochondrial fractions. It

Table 4. *Comparison of specific activities of the phosphoinositides extracted by acidified chloroform-methanol and those extracted by acetone followed by ethanol*

Experimental details are given in the text. Values represent the specific activity of the phosphoinositide extracted by acidified chloroform-methanol divided by the specific activity of the phosphoinositide extracted by acetone and ethanol, and are expressed as means $\pm$ s.e.m. of five experiments.

	Specific activity ratio	
	Phosphatidylinositol	Diphosphoinositide
Homogenate	2.0 $\pm$ 0.13	2.3 $\pm$ 0.06
Nuclear fraction	2.3 $\pm$ 0.19	approx. 2.7
Myelin fraction	1.7 $\pm$ 0.11	1.6 $\pm$ 0.09
Nerve-ending fraction	1.7 $\pm$ 0.06	1.3 $\pm$ 0.04
Mitochondrial fraction	1.6 $\pm$ 0.10	0.8 $\pm$ 0.01
Microsomal fraction	2.3 $\pm$ 0.15	3.1 $\pm$ 0.50
Supernatant	2.0 $\pm$ 0.07	—

was relatively low (59% of homogenate) in the myelin fraction.

The specific activity of diphosphoinositide was higher in most of the cell fractions than in the homogenate. Nuclear, microsomal and supernatant fractions appeared to be most active. For triphosphoinositide, only the myelin fraction showed a higher specific activity than the homogenate.

Though most of the phosphoinositides appeared in the acidified chloroform-methanol extract, small amounts were found in the combined acetone and ethanol extracts. In all cell fractions except mitochondria the less firmly bound phosphatidylinositol and diphosphoinositide (acetone-ethanol extract) had consistently lower specific activities, approximately half those of the corresponding lipids in the acidified chloroform-methanol extract.

Table 4 summarizes the results. The differences were most marked in the microsomal and nuclear fractions.

DISCUSSION

From the protein and phosphatidylinositol values given in Tables 1 and 2, the content of phosphatidylinositol P/g. of fresh rat brain was 55.3 μ g. This was for 3-week-old rats. The corresponding value for adult guinea-pig forebrains was calculated from the results of Eichberg, Whittaker & Dawson (1964), who used a different analytical method, as 56.8 μ g./g. Higher values were obtained by Wagner *et al.* (1963) and Margolis & Heller (1965) for adult rat brain (93 μ g./g. and 108 μ g./g. respectively).

The greatest enrichment value for phosphatidylinositol was given by the microsomal fraction. A similar conclusion for adult guinea-pig forebrain was reached by Eichberg *et al.* (1964). Recoveries of lipid inositol (Table 1) by the microbiological assay were about 70% of the inositol values calculated from the phosphoinositide contents in Table 2. The reason for this is not known.

Hörhammer, Wagner & Hölzl (1960) reported the absence of polyphosphoinositide from the grey matter of ox brain, though small quantities are found in the grey matter of adult cat brain (P. Kemp & M. Kai, unpublished work). Eichberg & Dawson (1964) found most of the polyphosphoinositide of adult guinea-pig brain in the myelin-rich fractions. In the present experiments with 3-week-old rats, polyphosphoinositides were found in mitochondrial, microsomal and nerve-ending fractions, as well as in the myelin fraction.

Post-mortem enzymic breakdown of triphosphoinositide and diphosphoinositide is known to occur (Eichberg & Dawson, 1964). The addition of mercuric chloride to the sucrose solution used in our initial homogenization does not seem to have prevented this breakdown. A value of 15.1 μ g. of polyphosphoinositide P/g. of fresh brain can be calculated from Table 2, whereas the value for adult rat brain fixed in liquid air was 49.1 μ g. of P/g. (Eichberg & Dawson, 1964). This loss seems unavoidable in subcellular fractionation studies, and will mean that the values for triphosphoinositide in Table 2 are low whereas those for diphosphoinositide may be high. Phosphatidylinositol values are unlikely to be affected by the relatively small amounts of polyphosphoinositide involved.

Specific activities expressed as counts/100sec./ μ g. of P for homogenate were in the order: diphosphoinositide > phosphatidylinositol > triphosphoinositide. Wagner and his colleagues (Wagner *et al.* 1962; Hölzl & Wagner, 1964) performed similar experiments with adult rats, except that the [32 P]phosphate was injected subcutaneously and the brains

were frozen quickly in liquid air after death. With a 4hr. equilibration they obtained the sequence: triphosphoinositide > diphosphoinositide > phosphatidylinositol. This sequence was also obtained at 6hr. after the subarachnoidal injection of [32 P]-orthophosphate into rabbits (Ellis & Hawthorne, 1962). The difference may be due to the fact that young rats, in which myelination was still in progress, were used in the present experiments. The specific activity of triphosphoinositide may also be affected by the enzymic breakdown referred to above. It is not easy to decide whether this breakdown would lower or raise the specific activity. If the newly synthesized triphosphoinositide is more readily hydrolysed, a lowered specific activity would be expected. It is equally difficult to predict what effect the hydrolysis would have on diphosphoinositide specific activity. An appreciable rise seems unlikely. Brockerhoff & Ballou (1962a,b) and Rossiter, McMurray & Palmer (1964), using rabbit- and rat-brain slices respectively, expressed their results as molar radioactivities and obtained the sequence: triphosphoinositide > diphosphoinositide > phosphatidylinositol. If the results of Brockerhoff & Ballou (1962a,b) are calculated as counts/100sec./ μ g. of P, the sequence obtained, as in the present work, is: diphosphoinositide > phosphatidylinositol > triphosphoinositide.

An unexpected finding in the present study was the high specific activity of phosphatidylinositol and diphosphoinositide in the nuclear and supernatant fractions. The most highly labelled triphosphoinositide, on the other hand, was found in the myelin fraction. Phosphatidylinositol specific activity was lowest in this fraction. Preliminary experiments with 1hr. and 2hr. equilibration periods gave very little labelling of the phosphoinositides.

Phosphatidylinositol extracted by acidified chloroform-methanol has about twice the specific activity of the same lipid in the acetone and ethanol extracts. The same applies to diphosphoinositide. These results suggest that the metabolically active phosphoinositides are bound more firmly to protein.

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