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Polyphosphoinositides in Myelin

By J. EICHBERG* and R. M. C. DAWSON
Biochemistry Department, Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge

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1. On fractionation of guinea-pig forebrain homogenates by differential and gradient-density centrifugation most of the polyphosphoinositides were recovered in the myelin-rich particles. 2. The phospholipids of pure preparations of myelin contained di- and tri-phosphoinositide in proportions 2–3 times greater than in the whole-brain phospholipids. 3. Di- and tri-phosphoinositide appeared in young rat brain during the period of myelination. 4. After the administration of [^{32}P]phosphate to guinea pigs the labelling of the polyphosphoinositides in isolated pure myelin was as great as in the whole brain, whereas little synthesis of the other myelin phospholipids had occurred. 5. When brain subcellular fractions were incubated with [γ-^{32}P]ATP, some triphosphoinositide labelling occurred in the myelin-rich fraction whereas the active labelling of diphosphoinositide was localized mainly in the mitochondrial fraction. 6. The Na^+, K^+ and Mg^{2+} plus Ca^{2+} concentrations in purified myelin have been determined. The Mg^{2+} plus Ca^{2+} content present showed close acid–base equivalence to the polyphosphoinositides. 7. It is concluded that di- and tri-phosphoinositide are rapidly-metabolizing components of the myelin sheath or intimately associated structures.

Since the demonstration of the existence of polyphosphoinositide in cerebral tissues (Folch, 1949) a good deal of indirect evidence has suggested that this is bound to protein (neurokeratin) in the myelin sheath of nerve fibres. This conclusion was based on the differential analysis of white and grey brain matter and the appearance during brain myelination of a trypsin-resistant protein residue that was believed to contain diphosphoinositide (Folch *et al.* 1959). However, as was pointed out by the latter workers, such compilations are wholly speculative. Moreover, recent evidence on the topographical distribution of bound phosphoinositides in the nervous system of various species has led to the suggestion that these compounds are not part of the bulk myelin sheath but that they are located in some other structure of the fibre tracts (Amaducci, Pazzagli & Pessina, 1962; LeBaron, McDonald & Sridhara Ramarao, 1963). The demonstration that the bound polyphosphoinositide fraction consists of two components, di- and tri-phosphoinositide (Dittmer & Dawson, 1961; Dawson & Dittmer, 1961; Brockerhoff & Ballou, 1961), has added a further complication to these considerations.

The development of adequate methods for extracting and estimating polyphosphoinositides (Dawson & Eichberg, 1965) allowed us to extend a previous investigation on the subcellular distribution of lipids in brain (Eichberg, Whittaker & Dawson, 1964) to these phospholipids. It has been shown that the polyphosphoinositides are predominantly associated with the myelin-rich fractions. Moreover, very pure myelin has been isolated by a method based on a procedure developed by Autilio, Norton & Terry (1964), and its phospholipids have been shown to be relatively enriched in polyphosphoinositide.

It has been recognized for some time that the brain polyphosphoinositides are metabolically very active compared with the bulk of the other phospholipids in the tissue (Strickland, 1952; Dawson, 1954; Ansell & Spanner, 1959 LeBaron, Kistler & Hauser, 1960). We have therefore included in these studies an investigation of the relative turnovers *in vivo* of the individual polyphosphoinositides of whole brain and the pure myelin fraction. Data are also included on the metal-ion content of pure myelin. We have also compared the di- and triphosphoinositide content of 2-day-old rat brains (non-myelinated) with the 5-week-old tissue (myelinated) by using methods that prevent the rapid initial post-mortem disappearance of these phospholipids (Dawson & Eichberg, 1965).

* Present address: McLean Hospital Research Laboratory, Belmont, Mass., U.S.A.

METHODS

Subcellular fractions of guinea-pig whole forebrain. These were prepared according to the method of Eichberg *et al.* (1964).

Purified myelin was made by a method based on that worked out by Autilio *et al.* (1964) for ox brain. A homogenate of guinea-pig forebrain [15g.; 10% (w/v) in 0·32M-sucrose] was spun for 11min. at 1000*g* and the pellet resuspended in 0·32M-sucrose (1g. of brain/2ml.). This suspension (8–10ml.) was layered on to 40ml. of 0·8M-sucrose and centrifuged for 30min. at 40000*g* (Spinco model L2 centrifuge, SW 25 head). The myelin band at the junction of the two layers was recovered with a pipette, the cloudy supernatant above the myelin and the bottom pellet being discarded. If 0·65M-sucrose was used at this stage (Autilio *et al.* 1964) some myelin was centrifuged down with the bottom pellet. The myelin band was diluted with 1·5vol. of water and layered on to 0·8M-sucrose and the centrifuging repeated. The myelin band was again recovered, diluted with 2·2vol. of water and centrifuged for 10min. at 1000*g*. The cloudy supernatant was discarded: the crude myelin pellet was diluted to 50ml. with water and the osmotically shocked myelin was centrifuged down for 15min. at 40000*g*. The pellet was resuspended in a minimum volume (6–8ml.) of 0·32M-sucrose and layered on to two tubes each containing three layers: 2ml. of 0·8M-sucrose; 20ml. of continuous sucrose gradient 0·8–0·32M-sucrose; 3ml. of 0·32M-sucrose. After centrifuging (1hr. at 53000*g*) the tube contained two light bands at the top of the continuous gradient and a single thick white myelin band towards the bottom of the continuous gradient. There was no separation into light and heavy myelin as observed by Autilio *et al.* (1964) for ox brain. The myelin was recovered by using a tube cutter and washed by suspending it in 150ml. of water and centrifuging (15min. at 53000*g*). The myelin pellet obtained was rewashed in the same way and resuspended in 6–8ml. of water for analysis etc.

For electron-microscopic examination the myelin was negatively stained by mixing the suspension with an equal volume of 2% (w/v) potassium phosphotungstate and, after spraying and drying on a grid, examined under a Siemens Elmiskop I high-resolution instrument.

Rat brain. Brains were 'dissected' from the frozen heads of young rats (36–48hr. old) that had been totally immersed in liquid N_2 either with or without prior decapitation. As only about 0·2g. of the frozen brain was obtained from a rat, two animals were used for each analysis. Adult (5-week-old) rat brains were obtained as described by Dawson & Eichberg (1965).

Inositol. This was estimated microbiologically as described by Dawson & Eichberg (1965).

Di- and tri-phosphoinositides. These were extracted from whole brain and its homogenates and estimated after acid hydrolysis (Dawson & Eichberg, 1965). When the same method was applied to subcellular fractions, particularly fractions rich in myelin, the preliminary chloroform–methanol extraction removed some polyphosphoinositides. Consequently, in one experiment, the procedure was modified so as to overcome this difficulty. After initial treatment of the fractions with methanol and $MgCl_2$, the residue was immediately extracted with the acidified chloroform–methanol solvent. The methanolic and acidified chloroform–methanol extracts were combined and amounts of chloroform, methanol and conc. HCl were added to produce a solution with the composition chloroform–methanol (2:1, v/v) containing 0·25% (v/v) of conc. HCl and having a final volume 20 times that of the original subcellular fraction. This extract was then washed and subjected to acid hydrolysis. Purified myelin fractions were almost completely soluble in chloroform–methanol (1:1, v/v) and the residue obtained on centrifuging contained virtually no polyphosphoinositide. In such fractions the phospholipids were hydrolysed without prior solvent extraction. An equal volume of conc. HCl was added to a portion of the resuspended myelin preparation and hydrolysis was carried out for 10min. at 100°. The subsequent separation of the hydrolysis products and calculation of the di- and triphosphoinositide concentrations was as described by Dawson & Eichberg (1965). It was possible to run 300μg. of myelin phospholipid P on the paper and still obtain adequate separation of the inositol di- and tri-phosphate from the large amounts of glycerophosphate present in these preparations.

Na^+, K^+ *and* Ca^{2+} *plus* Mg^{2+}. These were determined in purified myelin after this had been prepared with precautions to prevent contamination with metal ions. Thus the sucrose solution used was deionized by passing it through an Amberlite MB-1 analytical resin. All glass vessels and other apparatus used in the fractionation were well rinsed in double-distilled water before use. The final myelin preparation was stored in a polythene bottle. A sample (3–4ml.) was evaporated to dryness in a platinum crucible under an infrared lamp and ashed for 6–7hr. at 500°. To 1ml. of the suspension of ashed myelin was added 0·2ml. of conc. HCl. The mixture was heated at 100° for 15min. and the solution evaporated to dryness *in vacuo* with several additions of small volumes of deionized water. The residue was taken up in 1·0ml. of 0·01N-HCl and portions were used for Mg^{2+} plus Ca^{2+} analysis; $MgSO_4,7H_2O$ was used as a standard. The Ca^{2+} plus Mg^{2+} was estimated by EDTA titration with Eriochrome Black T as indicator (Ames & Nesbett, 1958), and the precautions of Keynes & Lewis (1956). The Na^+ and K^+ were determined in the EEL flame photometer (Evans Electroselenium Ltd., Halstead, Essex).

Specific radioactivities. Samples were digested with perchloric acid (Dawson, 1960) and ^{32}P was assayed in the digest by using a liquid-counter tube (M6) and standard Geiger–Müller counter set-up. The ^{31}P plus ^{32}P was assayed in a portion of the digest by the method of Fiske & Subbarow (1925).

Radioactive materials. Inorganic [^{32}P]phosphate and [γ-^{32}P]ATP were obtained from The Radiochemical Centre, Amersham, Bucks. The labelled phosphate was diluted in 0·9% NaCl containing 0·44mg. of KH_2PO_4/ml. before injection. The [γ-^{32}P]ATP contained about 5% of its radioactivity as inorganic [^{32}P]phosphate at the time of use.

RESULTS

In preliminary experiments the concentrations of polyphosphoinositide in subcellular fractions prepared from four guinea-pig brains by differential and density-gradient centrifuging were measured (Table 1). The polyphosphoinositide in the subfractions (preparations 1–4) was associated with the large easily sedimented particles rather than with

Table 1. *Polyphosphoinositide in subcellular fractions of guinea-pig forebrain*

Results are expressed as μg. of polyphosphoinositide inositol/g. fresh wt. of brain. Results for preparations 1–4 are based on microbiological estimations of inositol in acidified chloroform–methanol extracts. Preparation 5 was analysed by acid hydrolysis and P determinations as described in the Methods section. Values underlined represent primary fractions in the subcellular fractionation (Eichberg *et al.* 1964). N.D., Not determined.

Preparation ...	1	2	3	4	5		
Subcellular fraction					Diphosphoinositide	Triphosphoinositide	Total
Homogenate				121	26·0	52·7	78·7
Crude nuclear	44·7	42·8	51·0	46·2	15·7	38·3	54·0
Large myelin			33·2	31·0	9·5	22·8	32·3
Nuclei			15·3	15·0	1·4	9·1	10·5
Cell debris			1·3	0·8	—	—	—
Crude mitochondrial	10·7	16·2	9·4	3·9	N.D.	N.D.	N.D.
Small myelin			7·2	5·8	3·5	8·1	11·6
Synaptosomes			3·2	4·1	1·4	—	1·4
Mitochondrial			0·8	1·0	—	—	—
Microsomes	0·9	1·3	2·7	1·7	2·6	—	2·6
Supernatant	2·0	N.D.			—	—	—

the microsomes and supernatant. Further subfractionation of the larger particulate matter showed that the myelin-rich fractions contained most of the polyphosphoinositide. Although the nuclei and synaptosomes contained polyphosphoinositide it has to be remembered that these subcellular fractions are contaminated with myelin (Eichberg *et al.* 1964), which might account for at least part of their content. The relatively pure mitochondrial fraction contained only trace amounts of polyphosphoinositide.

The recovery of polyphosphoinositide in the subcellular fractions was lower than that in the whole-brain homogenate (preparation 4 in Table 1). This is probably due to a loss of polyphosphoinositide during the initial chloroform–methanol extraction of myelin-rich subfractions. When the extraction procedure was modified (preparation 5, Table 1), much of this loss was avoided. Nevertheless, the distribution pattern of polyphosphoinositide in the subcellular fractions closely resembled that obtained in the other experiments. When attempts were made to demonstrate enzymic hydrolysis of polyphosphoinositides by standing homogenates for 4hr. at various temperatures, little or no loss could be observed at 0° or 4°, the temperature at which the subcellular fractionation was performed. Standing for this period at room temperature resulted in a 30% decrease in the polyphosphoinositide content. This loss was not prevented by chelators of alkaline-earth metal ions (EDTA, cyclohexane-1,2-diaminetetra-acetate), which are known to inhibit the hydrolytic enzymes that attack triphosphoinositide (Thompson & Dawson, 1964; Dawson & Thompson, 1964). Nor was it stopped by *p*-chloromercuribenzoate or

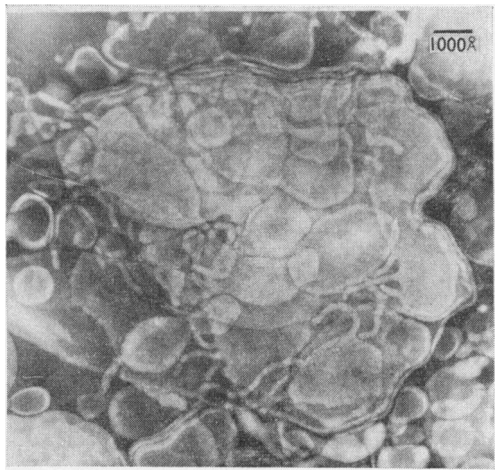

Fig. 1. Negatively-stained electron micrograph of pure myelin fraction.

mercuric chloride, which have been used by Hübscher, Smith & Gurr (1964) to control metabolic activity during the isolation of subcellular particles from intestinal mucosa.

The preparations of myelin examined in the aforementioned studies were still contaminated with non-myelinic elements, and consequently determinations were made on purer preparations obtained by osmotic-shocking and density-gradient centrifugation according to the method of Autilio *et al.* (1964). When examined under the electron microscope such preparations appeared to be entirely membranous (Fig. 1). Table 2 shows the

Table 2. *Polyphosphoinositides in purified myelin*

	Expt. 1			Expt. 2			Expt. 3		
	Diphosphoinositide (μg. of P/g.)	Triphosphoinositide (μg. of P/g.)	Polyphosphoinositide (% of lipid P)	Diphosphoinositide (μg. of P/g.)	Triphosphoinositide (μg. of P/g.)	Polyphosphoinositide (% of lipid P)	Diphosphoinositide (μg. of P/g.)	Triphosphoinositide (μg. of P/g.)	Polyphosphoinositide (% of lipid P)
Guinea-pig forebrain Homogenate	7·2	40·2	2·6	56·8		2·7	4·2	39·2	2·2
Purified myelin from low-speed sediment (Autilio *et al.* 1964)	2·0	7·4	4·7	3·4	11·3	6·7	4·0	11·3	5·8

diphosphoinositide and triphosphoinositide content of three such myelin preparations. The polyphosphoinositides had remained during the fractionation as an integral part of the myelin fraction and, moreover, although the myelin yield was low, they had been enriched 2–3 times compared with their concentration in the phospholipids of the original homogenate (Table 2).

To obtain further evidence that di- and triphosphoinositide were indeed components of myelin the brains of young rats were analysed before the commencement of myelination. These were frozen either *in situ* or immediately after decapitation to prevent the very rapid post-mortem disappearance of polyphosphoinositides that had been observed in the adult rat brain (Dawson & Eichberg, 1965). In rat brains 36–48hr. *post partum* no triphosphoinositide could be detected, but traces of diphosphoinositide were observed (Table 3). This contrasts with the presence of these phospholipids in the brains of 5-week-old rats (Table 3), in which myelination would be essentially complete.

Since it has been suggested that polyphosphoinositides are present in nervous tissue as complex magnesium or calcium salts (Kerr, Kfoury & Djibelian, 1963), the myelin was analysed for $Mg^{2+}+Ca^{2+}$ as well as Na^+ and K^+. These results are presented in Table 4.

Experiments were performed to test whether the myelinic polyphosphoinositides were metabolically active or whether a rapidly renewed portion of these phospholipids in the whole-brain pool was located in a non-myelinic component. The specific radioactivities of the phospholipids of whole brain and purified myelin fractions 3hr. after the administration of inorganic [^{32}P]phosphate are shown in Table 5. In whole brain the polyphosphoinositide P clearly exchanges at a far faster rate than the bulk of the other phospholipid P, although appreciable turnover of the phosphatidic acid and phosphatidylinositol had occurred. The specific radioactivities of the polyphosphoinositide in the myelin fractions were at least as great as that present in the whole brain. In the highly purified myelin fraction (Expt. 2 in Table 5) little labelling of any of the phospholipids, apart from the polyphosphoinositides, was observed. Even the phosphatidic acid and phosphatidylinositol present in the myelin must have turned over at a rate that was very appreciably below that of these phospholipids in other brain fractions.

To test the capacity of brain subcellular fractions to synthesize the higher phosphoinositides the primary fractions obtained by the differential centrifuging of brain homogenates were incubated for 30min. with [γ-^{32}P]ATP. The phospholipids were then extracted and purified and examined for their radioactive content. Table 6 shows the specific

Table 3. *Polyphosphoinositides in the brains of 2-day-old and 33-day-old rats*

Tissue	Treatment	Triphosphoinositide (μg. of P/g. of brain)	Diphosphoinositide (μg. of P/g. of brain)
Brains from young (36–48hr. post-natal) rats	Frozen *in situ* in liquid N_2	0	2·0
		0	0·6
	Decapitated and head frozen in liquid N_2	0	0·5
		0	0·5
Brains from 33-day-old rats	Frozen *in situ* in liquid N_2 (under thiopentone anaesthesia)	31·8–33·5 (2 rats)	11·0–13·4 (2 rats)
	Decapitated and head frozen in liquid N_2	32·6–39·2 (5 rats)	10·5–14·5 (5 rats)

Table 4. *Metal cations and polyphosphoinositides in purified myelin*

	Preparation 1	Preparation 2
Na^+ (μg.atom/g. of brain)	0·120	0·120
K^+ (μg.atom/g. of brain)	0·056	0·057
$Ca^{2+}+Mg^{2+}$ (μg.atom/g. of brain)	0·366	0·388
Triphosphoinositide (μmole/g. of brain)	0·121	0·124
Diphosphoinositide (μmole/g. of brain)	0·055	0·066
Polyphosphoinositide P (acid equiv./g. of brain)	0·770	0·808
$Ca^{2+}+Mg^{2+}$ (base equiv./g. of brain)	0·732	0·776

radioactivities of all the phospholipids of these preparations and the amount of newly synthesized phosphorus-containing compounds present.

Only two phospholipids had been labelled to a significant extent. Phosphatidic acid was labelled in all the particulate preparations, although the microsomes were especially active. There was also an active formation of labelled diphosphoinositide in the whole homogenate and this seemed to be predominantly synthesized in the mitochondria. The only labelled triphosphoinositide formed was found in the crude nuclear fraction, which also contains most of the myelin. However, no triphosphoinositide P could be found in any other fraction, including the whole homogenate, and it is clear that extensive hydrolysis must have occurred during the incubation.

DISCUSSION

The results on the subcellular distribution of polyphosphoinositide in brain need to be interpreted with caution since the polyphosphoinositides found represent those surviving the initial post-mortem hydrolysis (Dawson & Eichberg, 1965). However, it is clear that, under the present conditions of fractionation, a major portion of the remaining polyphosphoinositides and especially triphosphoinositide is found in fractions rich in myelin. Further, a very pure myelin fraction isolated from guinea-pig brain by a complex centrifugation procedure and osmotic shock contains a concentration of polyphosphoinositides in its phospholipids that is some 2–3 times higher than that in the whole-brain phospholipids. It is impossible to say categorically that the myelin prepared is absolutely pure since no methods are available to test this point. Neverthless, under the electron microscope it gives the appearance of being wholly composed of typical myelinic membranes. It is still conceivable that the preparation is contaminated with portions of adjacent structures such as the axolemma. This membrane might become rolled up inside a myelinic particle when the nerve fibre is subjected to the complex shearing forces of homogenization and not be removed during the subsequent purification.

The demonstration of the appearance of triphosphoinositide and diphosphoinositide in rat brain during the early days of post-natal life (36–48hr.–35 days old) is additional evidence that these are myelinic components. Myelin formation in the rat nervous system occurs first in the spinal cord at 2 days, in the cerebellum at about 8 days and then in the cerebrum at 11 days, and is fairly complete by 33 days (Donaldson, 1924). However, such evidence is only circumstantial since it is obvious that some growth of the brain in general will occur during this period as well as myelination.

The reproducible results obtained for the cation content of two preparations of highly purified myelin would tend to rule out the possibility of stray contamination of this fraction with metal ions during the purification. The concentration of Na^+ in myelin is about twice that of K^+, which is the reverse of the situation in whole brain or homogenates (Ames & Nesbett, 1958; Cummins & McIlwain, 1961). This could indicate that the univalent cations in myelin are in equilibrium more with the ions of extracellular fluid rather than those present in the axoplasm.

The amount of Ca^{2+} plus Mg^{2+} present in pure myelin shows a close acid–base equivalence to the polyphosphoinositides present, thus providing good

Table 5. *Incorporation of* ^{32}P *into the phospholipids of whole brain and myelin fractions of guinea pig*

Guinea pigs were injected intraperitoneally with inorganic [^{32}P]phosphate (Expt. 1, 5mc; Expt. 2, 2mc) 3hr. before being killed. Values given are counts/min./μg. of P.

	Expt. 1		Expt. 2	
Phospholipid	Whole forebrain	Crude myelin	Whole forebrain	Pure myelin
Total phospholipid	1·9	1·7		
Polyphosphoinositide				
Inositol triphosphate*	31	32	5·3	6·7
Inositol diphosphate*	35	44	7·9	7·2
Phosphatidylcholine	—	0·4	0·2	0·1
Phosphatidylethanolamine	—	0·4		
Phosphatidylserine	—	0·1	0·1	0·2
Phosphatidylinositol	—	12	6·3	0·1
Phosphatidic acid	—	6	8·8	1·2
Bis(phosphatidyl)glycerol	—	None present	0·9	None present

* Acid hydrolysis of polyphosphoinositides.

Table 6. *Labelling of phospholipids on incubation of subcellular particulate fractions of guinea-pig brain with* [γ-^{32}P]*ATP*

Fractions (equivalent to 0·4g. of brain for whole homogenate and 1·7g. for others) were suspended in 4ml. of a medium containing: KCl (67mM); tris–HCl buffer, pH 7·5 (34mM); $MgCl_2$ (4·2mM); ATP (disodium salt) (4·8mM). [γ-^{32}P]ATP (160μc, 1·23μmoles) was added and the mixture incubated for 30min. at 37°.

	Whole homogenate		Crude nuclear fraction (1000*g*; 11min.)		Crude mitochondrial fraction (17500*g*; 60min.)		Microsomal fraction (100000*g*; 60min.)	
Phospholipid	(counts/min./μg. of P)	(mμg. of P formed)	(counts/min./μg. of P)	(mμg. of P formed)	(counts/min./μg. of P)	(mμg. of P formed)	(counts/min./μg. of P)	(mμg. of P formed)
Phosphatidylcholine	0·1	0	0·1	3	0·1	0	0·1	0
Phosphatidylethanolamine	0·2	3	0·1	3	0·1	0	0·1	0
Phosphatidylserine	0·1	2	1·3	34	0·1	0	3·1	2
Phosphatidylinositol	5·6	9	0·1	0	6·4	8	5·0	1
Phosphatidic acid	40·0	41	23·0	27	120	36	744	53
Cardiolipin	2·8	4	2·2	3	2·0	2	0·1	0
Phosphatidylglycerol	0·1	0	0·1	0	0·8	1		
Alkali-stable phospholipids	0·2	3	0·1	2	0·3	3	4·5	2
Triphosphoinositide	No P present	0	17	16	No P present	0	No P present	0
Diphosphoinositide	2620	216	31	7	1970	84	270	10

evidence that these phospholipids exist in tissues as complexes with alkaline-earth metal ions. Although polyphosphoinositides had previously been isolated from solvent extracts of whole brain tissues as magnesium and calcium salts (Folch, 1949; Kerr *et al.* 1963), the likelihood that these had been formed as artifacts from the free phospholipids and Ca^{2+} plus Mg^{2+} in the extracts was very real.

Our experiments show that the rapid phosphorus exchanges *in vivo* of the polyphosphoinositides of whole brain also occur with the di- and tri-phosphoinositide associated with the myelin fraction. In contrast, the other phosphoglycerides of the purified myelin are labelled to an insignificant degree. This is true even for the phosphatidylinositol and phosphatidic acid, which in the whole brain are labelled as rapidly as the polyphosphoinositides. These results give direct evidence that the bulk of the myelin phospholipids are metabolically inert during adult life, which agrees with the results and theories of Davison (1964) and his colleagues. They noted that when labelled phosphate was injected into baby rats a small proportion of the radioactivity that had been incorporated into the brain phospholipids remained there persistently during growth, especially in the white matter (Davison & Dobbing, 1960*a*,*b*). Further, when a myelin concentrate was isolated from the brain of a young rat, 2 days after injecting [^{32}P]phosphate, its phospholipids had a specific radioactivity slightly more than those of the

other fractions whereas, with an adult rat, the corresponding specific radioactivity was only half that of the other phospholipids (August, Davison & Maurice-Williams, 1961). In these experiments the method of extraction used was likely to have left a major part, if not all, of the polyphosphoinositides in the residue. The turnover of the phospholipids in the myelin fraction observed by these authors is probably mainly due to the presence of non-myelinic components. In our own experiments some of the phospholipids (apart from polyphosphoinositide) in the crude myelin fraction turned over appreciably compared with the same phospholipids in the more highly purified myelin fraction.

The present preliminary results on the incorporation of ^{32}P from terminally labelled ATP into the phospholipids of brain subcellular particles show a rather specific distribution. The major formation of phosphatidic acid took place in the microsomes and this correlates with the results of Hokin & Hokin (1959), who showed that diglycerokinase in brain was mainly located in the microsomal fraction. However, by far the greatest labelling of phospholipid occurred in the diphosphoinositide, and this was localized in the crude mitochondrial fraction. Hawthorne (1964) and colleagues showed that when liver, kidney or brain mitochondria were incubated with inorganic [^{32}P]phosphate and succinate appreciable labelling of the diphosphoinositide occurred. Colodzin & Kennedy (1964) have found an enzyme in brain microsomes that forms diphosphoinositide by catalysing the direct phosphorylation of phosphatidylinositol by ATP.

This different localization of diphosphoinositide synthesis in brain by various workers remains to be explained, but it must be pointed out that the present system is a comparatively complex one and other factors such as the amounts of adenosine triphosphatase and diphosphoinositidases could have a major influence on the amount of diphosphoinositide found labelled in a given fraction.

The only labelled triphosphoinositide recovered was from the crude nuclear fraction, which would also contain most of the myelin in the preparation. However, no triphosphoinositide, labelled or unlabelled, could be recovered from the other subcellular fractions, including the whole homogenate. It is possible therefore that these fractions possess synthetic ability but that this is outpaced by rapid catabolism of the newly synthesized triphosphoinositide. Brockerhoff & Ballou (1962) were unable to demonstrate any labelling of diphosphoinositide or triphosphoinositide in homogenates of rabbit brain incubated with substrate and inorganic [^{32}P]phosphate because of complete breakdown of these substances.

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