The Metabolism of Polyphosphoinositides in Hen Brain and Sciatic Nerve

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1. The distribution of individual phospholipids was determined in hen brain and compared with that in sciatic nerve obtained in a previous investigation. Sciatic nerve is more enriched in the myelinic phospholipids ethanolamine plasmalogen, phosphatidylserine and sphingomyelin, but it contains relatively less triphosphoinositide, and much less diphosphoinositide, than the brain. 2. The course of incorporation of intraperitoneally injected ^{32}P into the acid-soluble phosphorus, phosphoinositides and total phospholipids of hen brain and sciatic nerve was followed. Although the maximum specific radioactivity in sciatic nerve of acid-soluble phosphorus is 4.5 times, and that of triphosphoinositide six times, that in the brain, the relative rate of triphosphoinositide phosphorus synthesis per gram of brain is three times that in sciatic nerve. 3. Administration of the demyelinating agent tri-*o*-cresyl phosphate to hens has no significant effect on the amounts or the rate of ^{32}P incorporation into the total phospholipids of the sciatic nerve. However, the rate of incorporation of ^{32}P into triphosphoinositide, although not its concentration, is raised from the first day after administration of the drug and remains thus 13 and 23 days later. 4. The incorporation of ^{32}P into polyphosphoinositides of hen brain slices *in vitro* was studied. The recovery of triphosphoinositide from the slices is markedly increased in the presence of EDTA, although the rate of incorporation of ^{32}P is unaffected. The incorporation of ^{32}P is dependent on the presence of Mg^{2+} and Ca^{2+} in the medium, and is decreased when Na^+ is replaced with K^+ or cholinium ions.

Recent studies have demonstrated the existence of enzymes in brain tissue preparations that effect the stepwise phosphorylation of monophosphoinositide to diphosphoinositide (Colodzin & Kennedy, 1965; Kai, White & Hawthorne, 1966) and then to triphosphoinositide (Kai, Salway & Hawthorne, 1968). Sheltawy & Dawson (1969) followed the rate of incorporation of intraperitoneally injected ^{32}P into rat brain polyphosphoinositides and compared it with the rate of incorporation into the acid-soluble-phosphorus fraction, and Eichberg & Dawson (1965) and Kai & Hawthorne (1966) observed the distribution of labelled polyphosphoinositides in various subcellular particles of brain. However, studies on subcellular distribution of polyphosphoinositides can sometimes lead to conflicting conclusions on the localization and metabolism of these phospholipids because of the effect of post-mortem hydrolysis on the relative recovery in each subcellular

particle, and on the specific radioactivity of the recovered triphosphoinositide (Eichberg & Dawson, 1965; Kai & Hawthorne, 1966; Sheltawy & Dawson, 1969).

We therefore sought further insight into the metabolism and function of polyphosphoinositides by comparing their rates of synthesis *in vivo* in hen brain and peripheral nerve as well as studying the effect of a demyelinating drug on their synthesis in peripheral nerve. In this latter respect it seemed possible that a demyelinating drug might change the metabolism of one of the few phospholipids that show metabolic activity in adult myelin (Eichberg & Dawson, 1965). We have also studied the effect of the ionic environment on the metabolism of polyphosphoinositides in hen brain slices. The hen was selected for two reasons: first, because of the general need to compare avian and mammalian metabolism; secondly, because of the susceptibility of the hen to peripheral-nerve demyelination after the administration of certain organic phosphorus compounds.

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EXPERIMENTAL

Animals. Domestic hens (Rhode Island Red $\times$ Light Sussex) weighing 2½–3 kg. were used throughout these experiments. When appropriate they were each injected intraperitoneally with 1 mc of inorganic [32 P]phosphate (The Radiochemical Centre, Amersham, Bucks.) in aq. 0.9% NaCl soln. containing 0.45 mg. of KH_2PO_4 /ml.

Treatment of hens with tri-*o*-cresyl phosphate. A sample of the drug was kindly provided by the Industrial Chemical Division, Geigy (U.K.) Ltd., Manchester. This was originally mainly *ortho*, but since it had been prepared in 1964 some compounds of even greater toxicity might have accumulated in the sample. The drug (0.5 ml./kg. body wt.) was introduced by stomach tube, and was followed by a subcutaneous injection of atropine sulphate soln. (40 mg./ml., 0.1 ml./kg. body wt.) to counteract early signs of cholinesterase inhibition. A control group was injected with atropine sulphate soln. only. From day 7 after the administration of tri-*o*-cresyl phosphate, both groups of animals were forced by stomach tube on a liquid mixture containing plain flour (50 g.), corn oil (100 ml.), powdered milk (Glaxo Oster milk no. II, 300 g.) and tap water (750 ml.). Hens received two 50 ml. portions daily, i.e. 280 kcal./hen/day [Report of the Committee on Animal Nutrition (1954). Washington, D.C.: National Research Council] and were allowed free access to water. Symptoms of leg paralysis began to appear in the experimental group from day 12, and were completely obvious by day 20. Inorganic [32 P]phosphate (1 mc/hen) was injected 18.4 hr. before the animals were killed.

Collection of tissues. Animals were killed by decapitation with a guillotine, the heads were frozen immediately in liquid N_2 and the cerebral hemispheres were obtained by chiselling away the skull. The frozen brains were divided into suitable portions for subsequent analysis, weighed as quickly as possible, and crushed under liquid N_2 in a mortar or a thick-walled centrifuge tube cooled in liquid N_2 . Alternatively, in incubation experiments with brain slices, the cerebral hemispheres were dissected out from the decapitated head, and each was divided into two approximately equal portions, weighed and kept in the incubation medium until sliced.

Sciatic nerves were dissected from hens under urethane anaesthesia (1.6 g. of urethane/kg. body wt., injected intraperitoneally as an aq. 50%, w/v, soln.). The nerves were quickly blotted on wet filter paper to free them from contaminating blood, and then frozen in liquid N_2 . Frozen nerves were divided into suitable portions for subsequent analysis, weighed and then crushed under liquid N_2 in a thick-walled centrifuge tube. Care was taken not to allow thawing of any frozen tissue, since this might have accelerated the post-mortem breakdown of polyphosphoinositides (Hayashi, Yagihara, Nakamura & Yamazoe, 1966).

Incubation of brain slices. Small slices were obtained from the cerebral hemispheres (0.5 g. fresh wt.) by using a mechanical chopper (McIlwain, 1961). The slices (about 0.3 mm. thick) were immediately dispersed in the incubation medium with a wide-mouthed 10 ml. Pasteur pipette. The incubation medium (10 ml.) had the following composition: glucose (11.5 mM); NaCl (90 mM); KCl (3.8 mM); CaCl_2 (0.58 mM); MgSO_4 (0.9 mM). Whenever necessary, additions were made to give the following final concentrations: sodium pyruvate (5 mM); sodium fumarate (5.4 mM);

adenosine (1 mM); creatine (1 mM). EDTA (disodium salt, final concn. 1.5 mM) was added to reduce the amount of Ca^{2+} or Mg^{2+} , and when required either one or both cations were added back in the concentrations listed above. When NaCl was replaced with either KCl or choline hydrochloride the iso-osmoticity of the medium was maintained. The medium was finally buffered to pH 7.5 with tris-HCl (38.5 mM), but after gassing with a mixture of $\text{O}_2 + \text{CO}_2$ (95:5) the pH dropped to 6.9.

The slices were allowed to equilibrate with the incubation medium at 37° under $\text{O}_2 + \text{CO}_2$ (95:5) in a Dubnoff shaker for 10 min. The radioactivity experiment then commenced with the addition of 60 μC of inorganic [32 P]phosphate, and unless otherwise specified the reaction was continued under the same conditions for 30 min. At the end of the incubation period, the mixture was centrifuged briefly, the slices were collected, quickly ground with a small amount of acid-washed solvent-extracted sand, and then suspended in 5 ml. of methanol. An equal volume of chloroform was added and the suspension was stored for subsequent examination of the phospholipids.

Extraction and analytical procedures. Lipids were extracted, purified and analysed as described in the preceding paper (Sheltawy & Dawson, 1969). Total phospholipids, extracted with neutral organic solvents, were analysed by the successive-degradative procedure of Dawson, Hemington & Davenport (1962). In some instances the phosphorus-containing products obtained by alkaline ethanolysis were fractionated by paper ionophoresis at pH 9.6 in 0.1 N-diethylamine-acetic acid buffer. In this system the ethanolysis products of phosphatidic acid and monophosphoinositide separated clearly from each other and from the remaining phospholipid products (glycerophosphate, M_F 0.78; glycerylphosphorylinositol, M_F 0.33). Polyphosphoinositides in the acidified solvent extract were fractionated on formaldehyde-treated paper (Sheltawy & Dawson, 1966). Acid-soluble phosphorus was determined in an extract made with aq. 10% trichloroacetic acid (Sheltawy & Dawson, 1969). Radioactivity was determined with a liquid-counter tube (M 6; 20th Century Electronics Ltd., Croydon, Surrey), and counting was continued long enough to yield a significance level better than 5%. All counts were corrected back to the time of the killing of the hen or that of the incubation of the brain slices.

RESULTS

Phospholipids and acid-soluble phosphorus in hen brain and sciatic nerve. In Table 1 the phospholipid composition of hen brain obtained in the present investigation is compared with that of hen sciatic nerve measured in a previous investigation (Sheltawy & Dawson, 1966) and that obtained for hen brain by other workers. As expected, the hen brain contains less of the 'myelinic' phospholipids, i.e. ethanolamine plasmalogen, sphingomyelin and phosphatidylserine, and more lecithin than sciatic nerve, corresponding to the larger myelin component in the latter. The lecithin content is lower than that obtained by other workers for hen brain by silicic acid thin-layer and column chromatography. It is possible that in these latter investigations a

Table 1. *Distribution of phospholipids extracted with neutral chloroform-methanol from hen brain and sciatic nerve*

All values are percentages of the total lipid phosphorus.

	Distribution of phospholipids			
	Brain			Sciatic nerve (Sheltawy & Dawson, 1966)
	Present work	Kreps <i>et al.</i> (1964)	Siek & Newburgh (1965)	
Phosphatidylcholine (lecithin)	23.8	38.6	49.0	14.6
Sphingomyelin	7.2	10.7	3.0	22.1
Phosphatidylethanolamine	16.3	30.8	28.0	4.1
Ethanolamine plasmalogen	23.5			30.3
Phosphatidylserine	8.8	12.5	6.0	11.4
Phosphatidic acid	1.0	0.9	4.0	1.3
Cardiolipin	1.3			0.2
Phosphatidylinositol	1.3	4.5	2.0	0.9
Alkyl ether phospholipid	2.3	—	—	4.5

Table 2. *Concentration of acid-soluble phosphorus and polyphosphoinositides in the nervous system of the hen*

Results are expressed as $\mu\text{g.}$ of phosphorus/g. wet wt., and are the mean of five analyses for brain and ten for sciatic nerve.

	Concn. ($\mu\text{g./g.}$)	
	Brain	Sciatic nerve
Acid-soluble phosphorus	940	390
Total lipid phosphorus	1888	1725
Diphosphoinositide	17.6	0.63
Triphosphoinositide	42.1	28.4

falsely high lecithin value would be produced by autoxidation of the intact phospholipids, which completely alters their running characteristics on silicic acid (Dodge & Phillips, 1966). In contrast with the high concentration of ethanolamine plasmalogen in hen brain, no choline or serine plasmalogens were detected.

Table 2 compares the relative sizes of the acid-soluble phosphorus pools of hen brain and sciatic nerve, that in brain being roughly 2.5 times that in sciatic nerve but similar to that in rat brain (950 $\mu\text{g.}$ of phosphorus/g. wet wt.). Previous studies by Webster (1954) gave a similar size for the acid-soluble phosphorus pool in hen sciatic nerve, and Bodian & Dziewiatkowski (1950) described a similar concentration in guinea-pig sciatic nerve. The concentration of lipid phosphorus is similar in both tissues, whereas that of triphosphoinositide is lower in the peripheral nerve. There is a sharp difference between the diphosphoinositide content of sciatic-nerve and brain material. In the former, diphosphoinositide exists in trace concentrations and was frequently not detected. In brain, on the other

Fig. 1. Incorporation of $[^{32}\text{P}]$ phosphate into the acid-soluble phosphorus, phosphoinositides and total phospholipids of hen brain. Inorganic $[^{32}\text{P}]$ phosphate (1 mc) was injected intraperitoneally. Each point is the mean of two or three determinations. Δ , Acid-soluble phosphorus; $\bullet$, triphosphoinositide; $\circ$, diphosphoinositide; $\blacksquare$, monophosphoinositide; $\square$, total phospholipid phosphorus.

hand, diphosphoinositide constitutes up to 30% of the polyphosphoinositide fraction. Corresponding values for adult rat and guinea-pig brains are 25% and 20% of the polyphosphoinositide fraction respectively (Sheltawy & Dawson, 1969).

Incorporation of ^{32}P into polyphosphoinositides of hen brain and sciatic nerve in vivo. Figs. 1 and 2 show the time-course of incorporation of intraperitoneally injected inorganic $[^{32}\text{P}]$ phosphate into the polyphosphoinositides of hen brain and sciatic nerve obtained under the conditions described to limit post-mortem hydrolysis. The ^{32}P incorporation into the acid-soluble phosphorus pool of each tissue is also given, and for purposes of comparison the uptakes into the monophosphoinositide and total lipid phosphorus fraction are also given. The incorporation of radioactivity into the diphosphoinositide of sciatic nerve could not be measured

Fig. 2. Incorporation of [^{32}P]phosphate into the acid-soluble phosphorus, phosphoinositides and total phospholipids of hen sciatic nerve. Inorganic [^{32}P]phosphate (1 mc) was injected intraperitoneally. Each point is the mean of two or three determinations. ▲, Acid-soluble phosphorus; ●, triphosphoinositide; ■, monophosphoinositide; □, total phospholipid phosphorus.

because the amounts of this phospholipid were so small (Table 2). Also, because of the minimal amounts of radioactivity recovered in the diposphoinositide of brain, the values for this phospholipid are more variable than those reported for mammalian brain (Sheltawy & Dawson, 1969).

Clear differences were observed in isotope-incorporation-time curves for brain and sciatic nerve. Thus the specific radioactivity of the acid-soluble-phosphorus pool in the sciatic nerve reaches a maximum value which is 4.5 times that in the brain of the same animal. Thereafter, the specific radioactivity of the acid-soluble phosphorus falls, rapidly at first and then more slowly. Again, this happens after a longer time-interval in the sciatic nerve than in the brain (Figs. 1 and 2). The specific radioactivities of polyphosphoinositides in both tissues reach a maximum shortly after that of the acid-soluble phosphorus, this maximum being, like the acid-soluble phosphorus, much greater in sciatic nerve than brain. However, the pattern of incorporation of ^{32}P is different in each tissue. In the sciatic nerve, the specific radioactivity of triphosphoinositide reaches maximum values comparable with the maximum of the acid-soluble phosphorus pool, whereas in the brain this is not so. The constant maximum specific radioactivity of brain polyphosphoinositides might indicate a stable pool of this phospholipid, or simply reflect the complexity of brain tissue. This contrasts sharply with the declining amount of label in the triphospho-

inositide of sciatic nerve, which could be due to a rapidly metabolized fraction, or simply be a function of the relative histological simplicity of peripheral nerve.

The incorporation of ^{32}P into monophosphoinositide in both tissues indicates a high rate of metabolism, in contrast with the total phospholipid fraction (Figs. 1 and 2), and with phosphatidic acid (A. Sheltawy & R. M. C. Dawson, unpublished work). It is noteworthy that, whereas the difference in the labelling of the acid-soluble phosphorus pools is reflected in the phosphoinositide labelling, the rate of incorporation of ^{32}P into the total phospholipids does not differ between the two tissues.

*Effect of tri-*o*-cresyl phosphate on polyphosphoinositide metabolism.* In experiments involving the administration of chemical demyelinating agents it is necessary to ensure that any effects produced are not due to changes in the nutritional state (Majno & Karnovsky, 1961). This can result either from loss of appetite or the incapacity of the animals to reach food. It was overcome in this study by force-feeding both the control and the drug-injected groups with the diet described. As shown in Fig. 3, the experimental hens lose weight initially, but this loss does not continue after the start of force-feeding.

A preliminary experiment indicated that no significant difference occurs in either the amounts or the rate of ^{32}P incorporation into brain polyphosphoinositides between the drug-injected and the control animals. Similarly, in the sciatic nerve, no significant difference was detected in the amounts of triphosphoinositide between the drug-injected (28.0 μg . of phosphorus/g.) and the control (27.7 μg .g.) group. Amounts of both acid-soluble phosphorus and individual phospholipids in the sciatic nerve showed some variation, but no significant difference between the two groups was detected.

Table 3 shows the rate of incorporation of ^{32}P into acid-soluble phosphorus, triphosphoinositide and other phospholipids of sciatic nerve in control and drug-injected animals at different periods after administration. From the first day after the administration of tri-*o*-cresyl phosphate, the specific radioactivity of triphosphoinositide is elevated and remains so until 23 days, the longest period studied. If the specific radioactivities of triphosphoinositide at all times after the administration of tri-*o*-cresyl phosphate are compared with those of the control group the elevation is significant ($P=0.02$, Student's *t* test). No consistent stimulation of the same magnitude occurs in the acid-soluble phosphorus fraction, and there is no significant difference in the specific radioactivity of the other phospholipids between the two groups of animals (Table 3).

Incorporation of ^{32}P into hen brain slices. The

Fig. 3. Variation in the weight of hens throughout the experimental period. Results are expressed as a percentage of the starting weight, and each value is the mean of a group of four hens. ●, Normal group; ○, group treated with tri-o-cresyl phosphate.

Table 3. Effect of injection of tri-o-cresyl phosphate on the specific radioactivities of acid-soluble phosphorus and phospholipids of hen sciatic nerve

Tri-o-cresyl phosphate was administered to the experimental group at day 0. Inorganic $[^{32}\text{P}]$ phosphate (1 mc) was injected intraperitoneally 18.4 hr. before killing.

Period (days)	Sp. radioactivity (counts/min./μg. of phosphorus)					
	Control group			Experimental group		
	1	3	23	1	3	23
Acid-soluble phosphorus	27.8	34.5	31.4	29.0	48.2	35.2
Triphosphoinositide	36.3	39.6	40.6	52.7	53.9	52.9
Phosphatidylcholine (lecithin)	2.2	4.8	3.5	2.7	5.4	3.4
Phosphatidylethanolamine	1.8	2.0	2.1	1.6	2.2	1.5
Phosphatidylserine	0.7	0.8	0.5	0.6	1.0	0.6
Phosphatidylinositol	12.1	21.0	—	13.1	24.5	11.7
Phosphatidic acid	8.0	19.4	—	—	15.2	6.0

influence of the ionic composition of the incubation medium, on both the amounts of polyphosphoinositides and the incorporation of ^{32}P into the polyphosphoinositides in hen brain slices, is described in Table 4 and Fig. 4. Results are expressed as total radioactivity recovered in a phospholipid fraction after a given time-interval. It was considered that this method of expression is representative of the rate of reaction, especially with triphosphoinositide, whose specific radioactivity seems to fluctuate according to the extent of polyphosphoinositide hydrolysis occurring during incubation. Amounts of phospholipids recovered after the same reaction period are also shown to permit calculation of specific radioactivities if desired.

When Na^+ is replaced with K^+ , the incorporation of radioactivity into triphosphoinositides is decreased, and this is not simply due to the high concentration of K^+ (Table 4a). When Na^+ is

replaced with choline instead of K^+ , the incorporation is even further decreased.

The inclusion of EDTA in the incubation medium containing Ca^{2+} and Mg^{2+} leads to a marked increase in the recovery of triphosphoinositide, although the amount of ^{32}P incorporated remains virtually unaffected. Mg^{2+} ions are essential for the labelling of triphosphoinositide (Table 4c). One surprising but consistent finding was that in the presence of EDTA the increase in both the maximum ^{32}P incorporation and the recovery of di- and tri-phosphoinositides is dependent on the presence of Ca^{2+} in the incubation medium. When Ca^{2+} is omitted the incorporation of ^{32}P and the recovery of both polyphosphoinositides decreases to a significant extent.

In further experiments, the course of incorporation of ^{32}P into triphosphoinositide, diphosphoinositide, monophosphoinositide and phosphatidic acid was followed in the presence of both EDTA and

Table 4. Influence of the ionic composition of the incubation medium on the recovery and incorporation of ^{32}P into triphosphoinositide and diphosphoinositide of hen brain

The final concentration of individual components of the medium is given in the Experimental section, except for additional K^+ , which is indicated below in parentheses. —, Values not determined. The incubations were continued for 30 min. TPI, Triphosphoinositide; DPI, diphosphoinositide.

Omission	Addition	Radioactivity recovered (counts/min./g. of brain)		Amount of phospholipid recovered ($\mu\text{g. of phosphorus/g. of brain}$)	
		TPI	DPI	TPI	DPI
(a) None	None	2340	—	1.1	—
None	K^+ (87 mm)*	2480	—	2.1	—
Na^+	K^+	1720	—	3.3	—
Na^+	Choline hydrochloride	715	—	1.4	—
(b) None	None	2440	—	3.0	—
Ca^{2+}	None	1630	—	1.8	—
(c) None	None	3400	1740	5.2	2.0
None	EDTA	3750	1750	18.0	2.2
Ca^{2+}	EDTA	1475	556	15.0	1.7
Mg^{2+}	EDTA	1310	1720	10.6	2.6

* Incubation medium was hyperosmotic.

Fig. 4. Incorporation of ^{32}P phosphate into the phospholipids of hen brain slices. Slices were incubated in the presence of $60 \mu\text{C}$ of inorganic ^{32}P phosphate in the medium defined in the Experimental section. ●, Triphosphoinositide; △, phosphatidic acid; ○, diphosphoinositide; ■, total phospholipids.

Mg^{2+} , as well as fumarate, pyruvate, adenosine and creatine (Fig. 4). The rate of labelling appeared

not to decrease over the first hour, in contrast with the rapid fall-off of polyphosphoinositide labelling observed in homogenates and subcellular fractions (Kai *et al.* 1966, 1968). During the incubation period the concentration of triphosphoinositide in the slices remains fairly constant ($15 \mu\text{g. of phosphorus/g.}$), the decrease from the concentration *in vivo* largely occurring before the slices are incubated in the presence of EDTA.

DISCUSSION

Distribution of phospholipids in hen brain and sciatic nerve. It is difficult to understand why the polyphosphoinositides, which are thought to be largely associated with, or to be, components of myelin in the mammalian nervous system (Dawson, 1966; Sheltawy & Dawson, 1966), occur in lower concentrations in hen sciatic nerve than in the whole brain. This difference is apparent whether the concentrations are expressed on a wet-weight basis or as a percentage of the total lipid phosphorus. From the histology of the respective tissues it is to be expected that sciatic nerve would have a greater myelin content and consequently an enrichment in the so-called myelinic lipids. In fact, this is actually seen with ethanolamine plasmalogen, sphingomyelin and phosphatidylserine. Comparison of the lipid composition of myelin isolated from brain and from peripheral nerve showed that the phospholipid distributions were very similar, although the polyphosphoinositides were not examined (Evans & Finean, 1965). A possible explanation is that the

polyphosphoinositides are not components of the myelin sheath itself but are enriched in an associated membrane (Dawson, 1966), which occurs more abundantly in the central nervous system.

Metabolism of acid-soluble phosphorus and polyphosphoinositides in hen brain and sciatic nerve. The larger specific radioactivity of the acid-soluble phosphorus pool in sciatic nerve compared with brain after ^{32}P injection (Figs. 1 and 2) can probably be at least partially explained by the lower pool size in sciatic nerve. However, the maximum specific radioactivity reached by the brain acid-soluble phosphorus is two-ninths of the equivalent value for sciatic nerve, whereas its pool size is only 2.5 times greater. This suggests that the plasma phosphate can probably exchange more rapidly with the acid-soluble pool in the sciatic nerve either because the permeability of the 'plasma-nerve barrier' is lower than that of the 'plasma-brain barrier' or because there is a more rapid metabolism of phosphorus components in the nerve which is responsible for an increased entrance rate (Dobbing, 1961).

The large difference between brain and sciatic nerve in the rate of incorporation of ^{32}P into triphosphoinositide can possibly be largely ascribed to the difference in the specific radioactivities of the precursor acid-soluble phosphorus pools, from which these are synthesized, and also the difference in the triphosphoinositide pool sizes. Both these factors would cause a greater increase in the specific radioactivity of triphosphoinositide in the sciatic nerve per unit weight of the phospholipid synthesized. However, when the rates of synthesis of triphosphoinositide phosphorus in the two tissues are calculated from the incorporation curves, the rate per gram of brain tissue is about three times that in the sciatic nerve. Part of this difference might be accounted for by the greater amount of connective tissue in the sciatic nerve. In this respect Table 2 shows that the nerve has a smaller content of phospholipid than the whole brain in spite of the myelin present.

A similar difference is not seen in the incorporation of ^{32}P into the total phospholipid fraction of sciatic nerve, where the increase in specific radioactivity is similar to that of the phospholipids in brain. This observation, which agrees with similar ones by Bodian & Dziewiatkowski (1950), might be explained both by the equivalence of the phospholipid pool sizes and by the greater myelinic component in sciatic nerve, the bulk of whose phospholipids are metabolically inert (Davison & Dobbing, 1959).

*Effect of tri-*o*-cresyl phosphate on phospholipid metabolism in hen sciatic nerve.* In agreement with previous observations (Webster, 1954; Porcellati & Mastrantonio, 1965), the concentrations of phospholipids examined in this work were not noticeably

altered as a result of administration of tri-*o*-cresyl phosphate. Joel, Moser, Majno & Karnovsky (1967) found no change in major classes of lipids or in their fatty acid composition in hen sciatic nerve with another organic phosphorus nerve poison, Mipafox. On the other hand, Berry & Cevallos (1966) maintained that the concentrations of phosphatidylcholine (lecithin) and choline plasmalogen are decreased in hen sciatic nerve after administration of tri-*o*-cresyl phosphate. In contrast with the latter authors, we could find only small concentrations of choline plasmalogen in normal hen sciatic nerve (Sheltawy & Dawson, 1966).

The evidence on the effect of tri-*o*-cresyl phosphate on the rate of ^{32}P labelling of sciatic-nerve phospholipids is conflicting. In agreement with the original observation of Webster (1954) on the sciatic-nerve total-phospholipid fraction, we found that tri-*o*-cresyl phosphate has no effect on the specific radioactivity of the bulk of the individual phospholipids that constitute this fraction. Porcellati & Mastrantonio (1965), on the other hand, found that di-isopropyl phosphorofluoridate and tri-*o*-cresyl phosphate decreased the specific radioactivities of phosphatidylcholine (lecithin) and phosphatidylethanolamine to 66% of the normal values in hen sciatic nerve, at which values they remained until the animals were completely paralysed.

Majno & Karnovsky (1961) pointed out some complications inherent in studies on the effect of toxic organic phosphorus compounds on the metabolism of sciatic nerve in hens. In the rat, the appearance of pathological lesions in the sciatic nerve was slow, but progressed in an orderly fashion from distal to proximal regions. Biochemical changes such as depression of respiration and of ^{14}C acetate incorporation *in vitro* were noticed in proximal regions of the nerve, whereas in distal regions, where lesions occur, the incorporation of acetate was actually stimulated. By contrast, in the hen, the appearance of lesions is rapid and in single portions of the nerve both normal and degenerating fibres could be seen. The overall biochemical effect is therefore likely to be confusing (cf. Austin, 1957).

Additional complications are the changing nutritional status of the animals, and early signs of cholinesterase inhibition. Majno & Karnovsky (1961) and later Smith (1963) demonstrated that starvation in the hen may depress respiration and incorporation of ^{14}C acetate and of inorganic ^{32}P phosphate *in vitro* into the lipids of the sciatic nerve. Other parts of the nervous system were less readily affected. It is noteworthy that, in the study of Porcellati & Mastrantonio (1965) mentioned above, hens were force-fed only on day 15 after administration of di-isopropyl phosphorofluoridate

and that, by day 20, they had lost 30% of their original weight. In the present study, the weight of hens injected with tri-*o*-cresyl phosphate on day 18 was 92% of that of control animals, and the calorific intake in both groups was constant. The animals also received a suitable dose of atropine sulphate to counteract early signs of cholinesterase inhibition produced by tri-*o*-cresyl phosphate (cf. Porcellati & Mastrantonio, 1965). Previous studies (Nelson & Barnum, 1960) showing a decrease in the synthesis of lecithin in mouse brain after administration of di-isopropyl phosphorofluoridate did not take the precaution of counteracting early signs of cholinesterase inhibition after administration of tri-*o*-cresyl phosphate, although pathological symptoms began to appear only after 12 days. The early stimulation of triphosphoinositide turnover is consistent with the observation of other workers (Majno & Karnovsky, 1958*a,b*) that inhibition of incorporation of acetate into the lipids of the sciatic nerve in drug-treated animals may precede the appearance of any pathological lesions in the nerve.

On reviewing the available data on the relative changes in acetate and phosphate incorporation into the lipids of degenerating nerve, Majno & Karnovsky (1961) made two observations. First, acetate incorporation is easily depressed and might be considered a sensitive indicator of regressive changes in myelin or nerve lipids. Secondly, phosphate incorporation into lipids may be depressed, but also it may rise with reactive and reparative changes of Schwann cells. Thus in Wallerian degeneration, where Schwann cells increase considerably in number, incorporation of phosphate into lipids is stimulated (Magee & Rossiter, 1954; Miani, 1962). In other pathological conditions of the nerve (diphtheric polyneuropathy) and in starvation (Denny-Brown, 1947), where the Schwann cells are injured, the incorporation of phosphate is depressed. In hens poisoned by organic phosphorus compounds, Cavanagh (1954) reported Schwann-cell proliferation and infiltration of macrophages and considered it to be, not a primary cause of myelin degeneration, but rather a secondary effect of axonal damage. Since the effect observed in the present study on the specific radioactivity of triphosphoinositide was a stimulatory one, it might also be accounted for by Schwann-cell proliferation rather than by a primary biochemical lesion produced by tri-*o*-cresyl phosphate in peripheral-nerve myelin.

Incorporation of ^{32}P into the polyphosphoinositides of hen brain slices. In agreement with observations of other workers on guinea-pig brain slices (Hayashi, Nakamura & Yamazoe, 1964), rat brain slices (Hayashi *et al.* 1966) and cat brain slices (Palmer & Rossiter, 1965), slices of hen brain responded to the

replacement of Na^+ with K^+ in the incubation medium by decreasing the rate of incorporation of added ^{32}P into their triphosphoinositide fraction. Replacement with cholinium ions led to an even greater decrease in incorporation (Table 4*a*).

Kai *et al.* (1968) could find little effect of Na^+ or K^+ on the synthesis of triphosphoinositide by purified diphosphoinositide kinase, although in rat brain homogenates the activity was inhibited. Phosphatidylinositol kinase in rat brain homogenates was slightly inhibited by potassium chloride, but at higher concentrations this inhibition disappeared (Kai *et al.* 1966). In mammalian brain replacement of Na^+ with K^+ causes an increase in respiration and aerobic glycolysis, a decrease in anaerobic respiration (Dickens & Greville, 1935; Gore & McIlwain, 1952) and a decrease in the ability to maintain phosphocreatine. The ionic replacement would lead to a stimulation of Na^+ -plus- K^+ -stimulated adenosine triphosphatase in an attempt by the slice to restore ionic gradients across the neuronal membranes, and therefore to a decrease in the energy available for other phosphorylations (McIlwain, 1952). The effect seems to be a general one, as demonstrated by Findlay, Magee & Rossiter (1954) and Palmer & Rossiter (1965), and affects phosphorylations in the whole phospholipid fraction as well as in RNA and phosphoprotein.

The increased recovery of triphosphoinositide from hen brain slices on the addition of EDTA to the medium can probably be explained on the basis of the hydrolytic enzymes present in the tissue. The concentration of this phospholipid found in slices in the absence of EDTA is far below that present in the intact hen brain, presumably owing to catabolism during the preparation and incubation of the slices. EDTA inhibited a purified preparation of triphosphoinositide monoesterase even in the presence of excess of Mg^{2+} required as cofactor for the enzyme (Dawson & Thompson, 1964; cf. Salway, Kai & Hawthorne, 1967). It also severely inhibited triphosphoinositide phosphodiesterase (Thompson & Dawson, 1964), although this enzyme had no absolute requirement for an added metal activator. The addition of EDTA does not affect the rate of labelling of the polyphosphoinositides provided that Ca^{2+} and Mg^{2+} were still added to the incubation medium. Presumably the necessity for Mg^{2+} ions may be associated with its ability to stimulate both phosphatidylinositol kinase and diphosphoinositide kinase (Kai *et al.* 1966, 1968).

The effect of omission of Ca^{2+} in decreasing the incorporation of ^{32}P into triphosphoinositide is more puzzling, since it is inhibitory to both these enzymes in the presence of Mg^{2+} . It can, however, partially activate diphosphoinositide kinase in the absence of Mg^{2+} (Kai *et al.* 1968).

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