The Polyphosphoinositides and other Lipids of Peripheral Nerves

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(Received 7 December 1965)

1. A detailed lipid analysis of the peripheral nerves of the crab (claw, leg), lobster (claw, leg), cow (splenic), hen, rabbit, sheep and monkey (sciatic) is presented. 2. The so-called 'myelinic lipids', cholesterol, sphingomyelin, ethanolamine plasmalogen and phosphatidylserine, occurred in the highest proportion in the lipids of vertebrate myelinated nerves, whereas the percentage of lecithin was greatest in the lipids of non-myelinated nerve fibres of both vertebrates and invertebrates. 3. Triphosphoinositide was found in all nerves examined and its concentration in the extracted lipids supports the concept that it is predominantly localized in the myelin sheath. 4. In crustacean nerve 12–14% of the phospholipids was in the form of alkyl ether phospholipids, which in the lobster were approximately half choline-containing and half ethanolamine-containing.

A good deal of indirect evidence has suggested that the brain polyphosphoinositides are selectively localized in myelin. Thus they are found in higher concentration in the white matter of brain compared with the grey matter (LeBaron, McDonald & Sridhara Ramarao, 1963), and during the period of myelination there is an appearance in the brain of a trypsin-resistant protein residue believed to contain diphosphoinositide (Folch *et al.* 1959) and also triphosphoinositide (Eichberg & Dawson, 1965). However, a firm conclusion as to their localization cannot be based on such circumstantial evidence, and indeed recent investigations on the anatomical distribution of the polyphosphoinositides in the nervous system have led to the suggestion that they are concentrated in some structure of the fibre tract other than the myelin sheath (Amaducci, Pazzagli & Pessina, 1962; LeBaron *et al.* 1963).

Recent studies on the distribution of polyphosphoinositides in subcellular particles of guinea-pig brain have indicated that these are selectively located in the 'myelin-rich' fractions (Eichberg & Dawson, 1965). Moreover, when pure myelin was prepared by a procedure involving density-gradient centrifugation and osmotic shocking, it contained a two- to three-fold enrichment of polyphosphoinositide in its phospholipids compared with whole-brain homogenates (Eichberg & Dawson, 1965). Unfortunately, the interpretation of such studies is complicated by two factors. First, there is a rapid post-mortem breakdown of triphosphoinositide that occurs within a few minutes of death (Dawson & Eichberg, 1965), followed by a slower

loss during the subsequent fractionation, so that only slightly more than 50% of the original inositide is recovered in the isolated fractions. Secondly, it is difficult to be absolutely certain that there is a complete absence of contaminating membranous structures, e.g. axolemma, from the myelin fraction using present techniques of electron microscopy.

In the present investigation we have attempted to obtain additional evidence as to the anatomical location of polyphosphoinositides in the nervous system by studying their relative concentrations in the myelinated and non-myelinated peripheral nerves of various species. The pattern of distribution obtained is consistent with the assumption that they are components of the myelin sheath of nerve fibres.

In the same study, a detailed analysis of the individual phospholipid classes of peripheral nerve has been made. Previous investigations, summarized by Berry, Cevallos & Wade (1965), have given only limited information and, as emphasized by Rossiter (1962), there is at present a great need for the more complete information given by modern methods of lipid analysis.

EXPERIMENTAL

Animals

The edible crab (*Cancer pagurus*) and the common lobster (*Homarus gammarus*) were purchased locally. On arrival at the laboratory, lobsters were lively and in good condition. However, crabs were invariably near exhaustion and therefore in one case their nerves were analysed soon after the animals were caught near Cromer (Norfolk). The

domestic fowls, rabbit, sheep, cows and monkeys were obtained from the Institute's farm or animal house and all were females.

Dissection and preparation of peripheral nerves

Lobsters were cooled under ice for a maximum of 2 hr. to induce sedation, but crabs were used directly. Their claws and legs were tickled at the distal joint of the meropodite with a pair of forceps. This induced the animals to shed their limbs. The connective tissue between the segments was partially cut and the entire length of the nerve revealed by successively pulling away the segments with the limb held in a straight line. The nerves from four crabs or lobsters were combined to provide sufficient material for analysis. Even so there was not enough available for cerebroside determinations, especially as this occurs in very low concentration in the crustacean nervous system (McMurray, McColl & Rossiter, 1964).

Before dissection of the sciatic nerve, hens were anaesthetized by intraperitoneal injection of 50% (w/v) urethane solution (1.6 g./kg. body wt.), and monkeys by intraperitoneal injection of 6% (w/v) sodium pentobarbital (30 mg./kg. body wt.). Sciatic nerves were dissected out from rabbit and sheep within 5 min. of the animal's death, and the splenic nerve was dissected out within 10–15 min. of the death of the cows. Immediately after separation all nerves were frozen in liquid N₂.

Extraction of lipids

Frozen nerves were weighed and then pounded in a cooled tissue crusher similar to that described by Stone (1938). The resulting disk was ground to a powder in a mortar cooled in liquid N₂. The ground tissue was extracted with 20 vol. of chloroform-methanol (1:1, v/v) and then twice with 20 vol. of chloroform-methanol (2:1, v/v). Chloroform was added to the combined extracts so that the final composition was chloroform-methanol (2:1, v/v) and this was then washed with 0.9% NaCl or 0.04% CaCl₂ (Folch, Lees & Sloane-Stanley, 1957). The residue from the chloroform-methanol extraction of the nerves was extracted three times at 37° with chloroform-methanol (2:1, v/v) containing 0.25% (v/v) of conc. HCl. The combined extracts were washed with N-HCl (Dawson & Eichberg, 1965).

Distribution of individual phospholipids

In chloroform-methanol extracts. This was examined by the successive-hydrolyses procedure developed by Dawson, Hemington & Davenport (1962). In the experiments where CaCl₂ solution was used to wash the lipid extracts, it was noticed that recoveries were slightly lower than usual, and that the fraction that suffered most from this was phosphatidylserine. This could be due to the low solubility of the calcium salt in the hydrolysis mixture (cf. Brockerhoff, 1963). Thus washing with CaCl₂ decreased the concentration of phosphatidylserine P obtained from crab-claw nerve from 7.4 to 2.8% of the total phospholipid P, and with the hen sciatic nerve the concentration was decreased from 12.2 to 7.4%.

All lipid extracts were qualitatively examined by thin-layer chromatography on silica gel H with the solvent system chloroform-methanol-water-acetic acid (65:25:

4:1, by vol.) to reveal any minor lipid component that might have escaped detection. In fact no components were detected other than those already recognized by the hydrolytic procedure.

For the estimation of the relative proportions of choline- and ethanolamine-containing alkyl ether phospholipids in lobster nerve, the phospholipids were initially fractionated by thin-layer chromatography (Parker & Peterson, 1965). The two bands containing the choline and ethanolamine phosphoglycerides were located with marker lanes, and the phospholipid was eluted from the silicic acid after this had been scraped off and inserted in a column. The solvents used were chloroform, chloroform-methanol (2:1, v/v), chloroform-methanol-water-acetic acid (25:15:2:4, by vol.), chloroform-methanol (1:1, v/v) and methanol. The eluted phospholipids contained the diacylated phosphoglycerides, and also their plasmalogen and alkyl ether analogues. The distribution of these components in the sample was determined by the successive-hydrolyses procedure (see above.)

In acidified chloroform-methanol extracts. The polyphosphoinositides were estimated by the acid-hydrolysis procedure of Dawson & Eichberg (1965). This was satisfactory for most lipid extracts but with lipids from crab and cow splenic nerves it was necessary to use the more sensitive procedure of chromatography on formaldehyde-treated paper (Hörhammer, Wagner & Richter, 1959). Before this chromatography, the polyphosphoinositides were converted into their calcium salts. The crude chloroform-methanol-HCl extract was washed with N-HCl in the theoretical upper phase (Dawson & Eichberg, 1965). As much as possible of the lower chloroform-rich phase was separated, thus avoiding the interfacial proteins that would combine with polyphosphoinositides in the presence of Ca²⁺ (Dawson, 1965). This chloroform-rich layer was then washed three times with CaCl₂ in theoretical upper phase (Folch *et al.* 1957). The lower layer was evaporated to dryness and the calcium salt of the polyphosphoinositides dissolved in chloroform-methanol-water (75:25:2, by vol.). This solvent allows quantitative recovery of the calcium salts without necessitating the lengthy procedure adopted by Dawson & Eichberg (1965). Descending chromatography was carried out at 4° with the upper phase of the solvent system butan-1-ol-acetic acid-water (4:1:5, by vol.). The phospholipids were detected by dipping the chromatograms in Nile blue solution (Hack, 1953) and their P content was estimated by the procedure of Dawson *et al.* (1962).

Analytical procedures

Lipid phosphorus. This was estimated by the procedures of Bartlett (1959) and Fiske & Subbarow (1925) for the ranges 0.3–20 and 20–60 µg. of P respectively.

Glycolipids. Of the glycolipids, only cerebroside plus sulphatides were determined, by the procedure of Long & Staples (1961); the lipid galactose values were multiplied by 4.6. Gangliosides have been reported absent from ox sciatic and optic nerves (Lowden & Wolfe, 1964).

Free cholesterol. The presence of polyunsaturated fatty acids leads to erroneous values when the colorimetric method of Zlatkis, Zak & Boyle (1953) is used for estimating cholesterol (Rhodes, 1959). Consequently, the chloroform-methanol extracts were first fractionated on silica-gel G

Table 1. *Lipids of the peripheral nerves of various species*

N.D., Not detected; —, not determined.

Nerve ... No. of animals	Crab (<i>Cancer pagurus</i>)		Lobster (<i>Homarus gammarus</i>)		Cow Spleen	Rabbit Sciatic	Sheep (Welsh Mountain)		Monkey (<i>Rhesus</i>) Sciatic	Domestic fowl (hen) Sciatic
	Claw	Leg	Claw*	Leg*			Sciatic*	Sciatic*		
Cholesterol (free)† (μmoles/g. wet wt.)	7.2	7.1	6.4	7.0	9.1	49.0	51	51	80	52
Cerebrosides + sulphatides (μmoles/g. wet wt.)	—	—	—	—	0.23	15.5	17.8	15.5	20.4	18.2
Total lipid P (μg.atoms/g. wet wt.)	12.1	11.4	13.2	16.6	13.6	63.0	54	54	54	53.5
Lecithin (μg. of P/g. wet wt.)	113	108	125	133	137	255	168	168	218	242.0
Sphingomyelin (μg. of P/g. wet wt.)	49	39	75	66	60	385	427	427	506	367
Phosphatidylethanolamine (μg. of P/g. wet wt.)	31	25	25	26	21	105	63	63	66	68
Phosphatidylserine (μg. of P/g. wet wt.)	22	19	36	29	45	230	248	248	270	188
Ethanolamine plasmalogen (μg. of P/g. wet wt.)	55	51	33	76	50	485	370	370	450	502
Serine plasmalogen (μg. of P/g. wet wt.)	8	3	N.D.	N.D.	N.D.	N.D.	43	43	25	N.D.
Choline plasmalogen (μg. of P/g. wet wt.)	N.D.	4	7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	21
Phosphatidic acid (μg. of P/g. wet wt.)	16	11	8	17	2	26	37	37	3	21
Polyglycerophospholipid (cardiolipin) (μg. of P/g. wet wt.)	0.5	1	1	2	2	N.D.	N.D.	N.D.	N.D.	4
Phosphatidylglycerol (μg. of P/g. wet wt.)	N.D.	N.D.	3	8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Monophosphoinositide (μg. of P/g. wet wt.)	7	10	6	3	15	22	50	50	9	14
Diphosphoinositide (μg. of P/g. wet wt.)	N.D.	N.D.	0.5	0.1	N.D.	2	9	9	6	1.3
Triphosphoinositide (μg. of P/g. wet wt.)	1.0†	1.0†	4	3	0.2	50	43	43	53	24
Alkyl ether phospholipids (μg. of P/g. wet wt.)	55	54	50	61	6	51	32	32	60	73

* The neutral chloroform-methanol extracts were washed with 0.04 M-CaCl₂ solution (Folch *et al.* 1957).

† Only traces of cholesterol ester were detected by thin-layer chromatography.

‡ Detected in one animal only (see the Results section).

plates with the solvent system light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (80:20:1, by vol.). Pure samples of cholesterol were run on outside marker lanes and these were sprayed with phosphomolybdic acid (Barbier, Jäger, Tobias & Wyss, 1959). The corresponding unsprayed areas for the unknowns and a series of standards (20–60 $\mu\text{g.}$) were scraped off and extracted successively with 4 ml. each of chloroform, chloroform–methanol (4:1, v/v) and chloroform again. The combined extracts were evaporated to dryness and free cholesterol was determined by the procedure of Zlatkis *et al.* (1953). The extinctions were measured within 15–30 min. of development since it was noticed that a spectral shift of the $\lambda_{\text{max.}}$ from 560 to 520 $m\mu$ occurred on standing for 2 hr. at room temperature. With this procedure, a straight-line calibration relationship was obtained for the range 7–60 $\mu\text{g.}$ of cholesterol.

RESULTS

The detailed lipid analyses of the peripheral nerves examined in the present investigation are given in Table 1. As is to be expected, the myelinated sciatic nerves contained a far greater concentration of all the major lipid classes (e.g. cholesterol, total phospholipid) than the non-myelinated nerves. However, within each group (myelinated or non-myelinated) the concentrations of total phospholipid and cholesterol were surprisingly similar irrespective of the species.

The molar ratio of total phospholipid to cholesterol is higher in non-myelinated nerves than in the myelinated group. Also, when the actual percentage of individual phospholipids comprising the total phospholipid fraction is considered, there are again clear differences between non-myelinated and myelinated fibres (Fig. 1). Thus lecithin constitutes a lower proportion of the total phospholipids in myelinated nerve, whereas the reverse is true for ethanolamine plasmalogen, phosphatidylserine, sphingomyelin and triphosphoinositide.

Although triphosphoinositide was always detected in lobster nerve, this phospholipid was only found on one occasion in crab nerve and this was when the nerves were obtained for analysis soon after the animals had been caught. Only traces were detected in the splenic nerve of the cow, but as this tissue was not obtained until 10–15 min. after death there is a possibility that the phospholipid may have disappeared *post mortem*. Dawson & Eichberg (1965) demonstrated that a breakdown of triphosphoinositide occurred in the rat brain soon after death. To test whether this occurs also in the peripheral nervous system, the following experiment was conducted. A hen was anaesthetized and the sciatic nerves of both legs were exposed. The nerve from one leg was separated and immediately frozen in liquid nitrogen (control). The hen was then killed and after 13 min. the sciatic nerve was separated from the other leg and

Fig. 1. Distribution of lipids in myelinated and non-myelinated nerve fibres. Non-myelinated nerves ($\square$): A, crab claw; B, crab leg; C, lobster claw; D, lobster leg; E, cow splenic. Myelinated nerves ($\blacksquare$): F, hen sciatic; G, rabbit sciatic; H, sheep sciatic; I, monkey sciatic.

Table 2. *Choline and ethanolamine bases in the alkyl ether phospholipid fraction of lobster nerves*

Nerve ...	Claw	Leg
Total recovered alkyl ethers ($\mu\text{g. of P/g. wet wt.}$)	34	43
(% of total lipid P)	11.0	10.1
Ethanolamine alkyl ethers (% of total alkyl ethers recovered)	54	57
(% of ethanolamine phospho- glycerides)	33	31
Choline alkyl ethers (% of total alkyl ethers recovered)	46	43
(% of choline phosphoglycerides)	23	28

similarly frozen. The concentrations of polyphosphoinositides in the two nerves were determined. In two experiments, the concentrations fell from a mean of 25 $\mu\text{g. of P/g. wet wt.}$ in the controls to 17 $\mu\text{g. of P/g. wet wt.}$ in the other nerves. In brain, the rapidity and extent of this breakdown varies according to the species as shown by comparisons between the rat and guinea-pig brains over short periods *post mortem* (Dawson & Eichberg, 1965) and between ox and human brains after much longer periods *post mortem* (Kerr, Kfoury & Haddad, 1964).

The high concentration of alkyl ether phospholipids in the crustacean nerves was an unexpected finding. It amounted to 12–14% of the total phospholipid fraction whereas in the mammalian and hen nerves it constituted only 1.4–4% of the equivalent fraction. In the lobster the nature of the nitrogenous base of these alkyl ether phospholipids was examined. The alkyl ethers recovered (approx. 80%) were distributed about evenly between the choline-containing and ethanolamine-containing phosphoglyceride fractions (Table 2).

DISCUSSION

The nine different peripheral nerves examined can be broadly classified into non-myelinated and myelinated fibres; the crustacean claw and leg nerves and the ox splenic nerve (Falk, 1908) belong to the first group and mammalian and avian sciatic nerves to the second. The clear differences between these two groups both in the total lipid present and in its percentage composition can largely be ascribed to the preponderance of the lipid-rich myelin sheaths round the axons in the myelinated nerves. Thus it is to be expected that any lipids that are especially enriched or depleted in myelin would cause a reflected difference in the relative compositions of the myelinated and non-myelinated nerves. Lipids characteristic of myelin have until recently been deduced on the basis of

analytical differences between grey and white matter or on the basis of the appearance of specific lipids during myelination. This has led to the concept of the so-called 'myelinic lipids', namely cholesterol, cerebroside, phosphatidylserine, ethanolamine plasmalogen and sphingomyelin (Johnson, McNabb & Rossiter, 1948a, 1949; Branté, 1949). Recently this concept has been largely confirmed and extended by the isolation of more-or-less pure myelin fractions from brain and peripheral nerve and the determination of their lipid distribution (Eichberg, Whittaker & Dawson, 1964; Cuzner, Davison & Gregson, 1965; Norton & Autilio, 1965; Evans & Finean, 1965). In addition to the high proportion of the above-mentioned lipids, myelin also contains some lecithin and phosphatidylethanolamine, although at a lower percentage concentration than the other subcellular fractions. The present results are in agreement with this concept, showing an increased proportion of ethanolamine plasmalogen, phosphatidylserine and sphingomyelin and a lower lecithin proportion in the lipids of myelinated nerves.

The relative concentrations of triphosphoinositide in the peripheral nerves studied suggest that it is enriched in the myelinic sheath. This adds to and confirms the evidence on its localization obtained by studies on brain subcellular fractions and its appearance during myelination (Eichberg & Dawson, 1965). Its presence in the non-myelinated fibres of crustacea could mean that small amounts are present in other structures of the nerve fibres. On the other hand, the morphological distinction between myelinated and non-myelinated fibres is only a relative one, and invertebrate axons are believed to be invested with a few bimolecular layers of lipids that may constitute a rudimentary myelin sheath (Geren & Schmitt, 1954). However, it is not known whether this lipid layer is similar to true myelin in composition or whether it originates from the rudimentary Schwann cells that surround the nerve. There is now little doubt that the true myelin sheath is formed by the outer membranes of a Schwann cell rolling round the axon (Robertson, 1959).

It is difficult to compare the present findings with previous investigations on the lipid composition of peripheral nerve. Previous studies, made with methods of lipid analysis that have now been superseded, tended to describe the phospholipids in groups whose compositions were often ill-defined. To some extent this also applies to the present practice of thin-layer chromatography, which does not distinguish between the diacylated phosphoglycerides and their plasmalogen and alkyl ether analogues, and in unidimensional runs gives poor resolution of the more acidic phospholipids. Our results, as far as comparison is possible, show good

Table 3. Comparison of values obtained for the phospholipid composition of sciatic nerve

Species	Phospholipid composition (% of total lipid P)					
	Present paper		Berry <i>et al.</i> (1965)		Johnson <i>et al.</i> (1948b)	
	Chicken	Rabbit	Chicken	Cat	Rabbit	Cat
Lecithin + lysolecithin	16.0	13.1	33.5	22.0	14.0	13.4
Phosphatidylethanolamine	4.5	5.4	4.1	10.2	39.0	35.6
Ethanolamine plasmalogen	33.0	28.0	28.5	21.0		
Phosphatidylserine	12.2	11.8	12.0	21.0		
Sphingomyelin	24.0	19.8	22.0	22.0	47.0	51.0

agreement with the values obtained by Berry *et al.* (1965) for chicken sciatic nerve (Table 3). However, their values for lecithin are very much higher than ours. They also found a lecithin content in cat sciatic nerve that is again higher than that obtained by Johnson, McNabb & Rossiter (1948b). The lecithin content of rabbit sciatic nerve obtained by this latter group is very similar to our own value (Table 3). The high sphingomyelin content that they obtained would reflect, however, contamination with other phospholipid fractions, e.g. plasmalogens, alkyl ether phospholipids.

In spite of the large differences between myelinated and non-myelinated nerve fibres in their content of lecithin and sphingomyelin, the proportion of the total choline-containing phospholipids was surprisingly constant in the nerves of all species examined ($40 \pm 4.5\%$ s.d. of total lipid P). An analogous case is seen with the erythrocyte membranes of mammals, where the high lecithin content in monogastric animals is largely replaced by sphingomyelin and choline plasmalogen in the ruminant, the total percentage of choline-containing phospholipids remaining constant (Dawson, Hemington & Lindsay, 1960; De Gier & van Deenen, 1961). It is probable that this change in composition produces variations in the packing of the bimolecular layers of lipid. Sphingomyelin can condense into unimolecular films where the surface area occupied by each hydrophobic chain is considerably less than that of lecithin (Dervichian, 1964). There are also considerable differences between the surface potential of lecithin and that of sphingomyelin (Dervichian, 1964) and choline plasmalogen at an air-water interface (Shah & Schulman, 1965). Such variations in the proportions of lecithin to sphingomyelin would not, however, affect the charge balance of the phospholipids, a factor that makes the choline-containing phospholipids ideally suited for forming stable bimolecular laminae (Bangham, 1963).

Alkyl ether phospholipids have been recognized as constituents of the mammalian central nervous system, where they comprise about 2% of the total

lipid P (Svennerholm & Thorin, 1960; Ansell & Spanner, 1961, 1963; Dawson *et al.* 1962). This content is of the same order as that found in the various sciatic nerves as well as the splenic nerve examined in the present study. The finding of a high alkyl ether phospholipid content (12–14% of total lipid P) of crustacean nerve was unexpected. This is not the richest natural source so far described; bovine erythrocyte phospholipids also contain about 10–15% of ethanolamine alkyl ether phospholipids (Hanahan & Watts, 1961), and the phospholipids of the terrestrial slugs *Arion ater* and *Ariolimax columbianus* include 25% of alkyl ethers, about 50% of which is choline-containing and only 13% ethanolamine-containing (Thompson & Hanahan, 1963).

Finally, the separation of nervous-tissue lipids into fractions depending on their polar groups and on broad features of their apolar region, e.g. ester or vinyl ether linkage, represents only an incomplete picture. There is evidence accumulating at present that points to the importance of the fatty acid species of a given phospholipid class in determining its metabolic activity (Collins, 1963; Das, Myers & Crane, 1964).

The authors gratefully thank the Multiple Sclerosis Society of Great Britain for a grant which has provided apparatus and a fellowship for one of us (A. S.). We also appreciate the able technical assistance of Mr Neville Clarke.

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