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Studies on Cerebral Sphingomyelin

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Sphingomyelin was first isolated from brain by Thudichum (1884) but little was known of its metabolism until recently. Brady & Koval (1958) and Brady, Formica & Koval (1958) have now carried out extensive investigations on sphingosine biosynthesis, and more recently Sribney & Kennedy (1958) have shown that the biosynthesis of sphingomyelin in liver proceeds by the reaction of a ceramide (*N*-acylsphingosine) with cytidine diphosphate choline. It is quite likely that such a reaction proceeds in nervous tissue (Rossiter, Thompson & Strickland, 1960).

In tissues other than brain the function of sphingomyelin is unknown. In nervous tissue the lipid probably does not make its appearance until the onset of myelination when it rapidly increases in concentration (Folch-Pi, 1955). Earlier investigations (Branté, 1949; Johnson, McNabb & Rossiter, 1949) have shown that the sphingomyelin is preferentially located in the white matter (myelinated fibres), and Finean and his colleagues have suggested that the lipid could fit well into the structure of the repeating units of myelin (Finean & Robertson, 1958). In this sense therefore, white-matter sphingomyelin would appear to have a structural role in the nervous system, although this may not be so for sphingomyelin occurring outside the myelin sheath, e.g. in cerebral cortex. From experiments with [3-^{14}C]serine, Davison, Morgan Wajda & Payling-Wright (1959) have concluded that the sphingomyelin in myelin has a very slow rate of replacement and may even be permanent

when laid down in early life. Indeed, earlier experiments by Dawson (1954*a*) strongly suggested that *in vivo* the uptake of ^{32}P into guinea-pig-brain sphingomyelin was very slight when compared with that of other phospholipids.

The findings of Rennkamp (1949) and Dawson (1958) showed that sphingomyelin can be hydrolysed to yield a mixture of phosphorylcholine and sphingosylphosphorylcholine which can subsequently be isolated. This provides a convenient method for measuring the uptake of isotopic phosphorus into sphingomyelin in small samples of tissue and this paper deals in part with the measurement of the uptake of ^{32}P into rat-brain sphingomyelin *in vivo*. The use of the method to estimate true brain sphingomyelin has also been investigated (cf. Dawson, 1960). A preliminary account of these studies has been given by Ansell & Spanner (1959).

EXPERIMENTAL

Preparation of lipid extracts. These were prepared in two ways. In the majority of experiments with ^{32}P the fresh tissue was homogenized in a mechanical blender with 12% trichloroacetic acid, washed and suspended in acetone, and the residue extracted with $CHCl_3$–methanol (1:1, v/v) as described by Ansell & Dohmen (1956). In other experiments tissue was homogenized in $CHCl_3$–methanol (2:1, v/v), the homogenate filtered and the filtrate dried three times *in vacuo* (Davison & Wajda, 1959). The residue was taken up in $CHCl_3$–methanol (2:1, v/v) and filtered. It was then washed with 0·2 vol. of 0·73% NaCl followed by chloroform–methanol–0·29% NaCl (3:48:47, by vol.) as described by Folch, Lees & Sloane-Stanley (1957), and the lipid solution diluted to standard volume. Lipid solutions prepared in this way were virtually colourless.

Saponification of lipids. Saponification was carried out essentially as described by Schmidt, Benotti, Hershman & Thannhauser (1946). A suitable sample of the lipid solution (e.g. an amount equivalent to 400 mg. of brain tissue) was dried *in vacuo* at $< 40°$ and emulsified with 5 ml. of N-NaOH. After 2–16 hr. at 37° the emulsion was cooled and neutralized with 5N-HCl, and 5·9 ml. of 10% trichloroacetic acid added. After 2 hr. at 4° the suspension was filtered and the residue on the paper washed with 2% trichloroacetic acid until the filtrate was free from chloride. The residue on the paper (lipid stable to dilute alkali and acid) was then dissolved in warm $CHCl_3$–methanol (1:1, v/v). Alternatively the acidified suspension of lipid after saponification was shaken with an equal volume of warm $CHCl_3$–methanol (1:1, v/v), and the $CHCl_3$ layer which was obtained on centrifuging washed by shaking with methanol–water until free from chloride.

Hydrolysis of mild acid- and alkali-stable fraction. The solution of this fraction was then dried under reduced pressure at $< 40°$ and dissolved in 3 ml. of methanolic 2N-HCl. It was then heated at 100–110° in a sealed tube for 1·5 hr. (Dawson, 1958). By adding 3 ml. of $CHCl_3$ and 3 ml. of water and centrifuging, $CHCl_3$-soluble and water-soluble phosphorus fractions were obtained.

Chromatographic separation of water-soluble hydrolysis products. The aqueous phase after hydrolysis of acid- and alkali-stable lipid in 2N-HCl was dried under reduced pressure and stored overnight *in vacuo* over NaOH to remove the last traces of HCl. Single-dimensional chromatography in phenol–water–aq. 18N-NH_3 soln. (80 g.: 20 ml.: 0·3 ml.) was carried out on Whatman no. 1 paper (previously freed from cations by washing with 2N-acetic acid and glass-distilled water). On some occasions sphingosylphosphorylcholine was separated from phosphorylcholine in butan-1-ol–acetic acid–water (Dawson, 1958). Phosphorus compounds were generally located by spraying dried papers with the reagent of Hanes & Isherwood (1949), drying at room temperature and exposing to the unfiltered light from a Hanovia 100w mercury-vapour lamp. The method of Wade & Morgan (1953) was also used when it was desired to elute phosphate esters unchanged from the paper. Amino compounds were detected by spraying with 0·5% ninhydrin in water-saturated butanol, drying at room temperature and then heating the papers at 100° for 2–5 min. The method of Levine & Chargaff (1951) was used to detect choline compounds after destruction of ninhydrin-reacting material (Lea, Rhodes & Stoll, 1955). Radioautographs of chromatograms were prepared with Kodak 'Blue Brand' X-ray film.

Preparation of sphingomyelin. For a large part of the work a sample of sphingomyelin prepared from brain by the method of Klenk & Rennkamp (1941), and kindly donated by Dr J. N. Hawthorne, was used. This contained a small amount of water ($< 5\%$) and had a P content of 3·6% and an N content of 3·1% (N:P ratio, 1·9:1·0). In further experiments sphingomyelin in small amounts was prepared as follows (cf. Davison & Wajda, 1959). Ether-insoluble lipids (500 mg.) from ox brain, kindly donated by Dr P. Kemp, were dissolved in $CHCl_3$–methanol (98:2, v/v), run through a column containing 50 g. of Al_2O_3 (British Drug Houses Ltd. chromatographic, as purchased) and washed through with a further 50 ml. of solvent. All the P was retained. The column was then eluted with 500–600 ml. of $CHCl_3$–methanol (1:1, v/v). According to Davison & Wajda (1959) after C. Long (unpublished work) this elutes choline-containing lipids but no cerebroside or 'kephalin' components. Chromatography of this fraction on silica-impregnated paper in di-*n*-butyl ether–acetic acid–$CHCl_3$–water (40:35:6:5, by vol.) (Marinetti & Stotz, 1956) showed the presence of at least two choline-containing lipids. Accordingly the eluate from the column was dried under reduced pressure and saponified for 8 hr. at 37° in methanolic N-NaOH. The solution was made just acid with HCl and the lipid extracted into $CHCl_3$ by shaking with an equal volume of $CHCl_3$–methanol (1:1, v/v). Then, freed from chloride and water-soluble alkali-labile phosphate by repeated washing with methanol–water, the $CHCl_3$ layer was dried and the residue dissolved in about 10 ml. of light petroleum (b.p. 60–80°)–methanol (9:1, v/v). Phospholipid was precipitated from this solution with acetone, washed with this solvent and dried *in vacuo*. The lipid was then dissolved in the minimum of hot ethyl acetate containing about 5% (v/v) of methanol. On cooling, a white solid crystallized; this was then recrystallized and dried *in vacuo*. From 460 mg. of starting material containing 9·4 mg. of P an amount of lipid equivalent to 2·8 mg. of P was obtained. It was slightly hygroscopic: H_2O content, 6·18% after drying at 100° for 3 hr. *in vacuo* (Found for dried sample: C, 68·67; H, 11·51; N, 3·63;

P, 4·05%; N:P ratio, 1·99:1. Calc. for *N*-stearylsphingomyelin $C_{41}H_{85}O_7N_2P$: C, 65·71; H, 11·44; N, 3·74; P, 4·15%. Calc. for *N*-nervonylsphingomyelin $C_{47}H_{95}O_7N_2P$: C, 67·88; H, 11·53; N, 3·37; P, 3·74%). Elementary analyses were carried out by Dr F. Pascher, Bonn, Buschstrasse 54.

No carbohydrate contaminant was detected by the Molisch reaction. The lipid migrated as a single substance in di*iso*butyl ketone–acetic acid–water (40:30:7, by vol.) (Marinetti & Stotz, 1956) on silica-impregnated paper and gave no reaction with ninhydrin. On hydrolysis with methanolic HCl and subsequent separation of water-soluble P compounds by paper chromatography, 85% of the P present could be recovered as a mixture of phosphorylcholine and sphingosylphosphorylcholine. In all respects this lipid behaved essentially the same as the sample prepared by the method of Klenk & Rennkamp (1941). Determination of the infrared spectra of the two samples by Dr K. J. Morgan showed that the two preparations were indistinguishable. The method described here would appear to provide a convenient method of preparing small amounts of sphingomyelin if the starting material used by Rapport & Lerner (1958) is unavailable. A sphingomyelin has recently been totally synthesized by Shapiro, Flowers & Spector-Shefer (1959).

Phosphorus and nitrogen determinations. P was determined either by the method of Fiske & Subbarow (1925) or, when the amounts were between 1 and 5 μg., by the method of Berenblum & Chain (1938). Spots on chromatograms and appropriate blanks were wet-ashed with $HClO_4$ before P determination. N determinations were carried out by the Conway method as modified by Weil-Malherbe & Green (1955); this method was applied to both lipid samples and aqueous eluates of phosphate esters from chromatograms, sprayed with the $FeCl_3$–sulphosalicylic reagent of Wade & Morgan (1953).

Radioactive assay. This was carried out on aqueous solutions of ^{32}P in an M6 liquid counting tube (20th Century Electronics) with conventional ancillary equipment.

RESULTS

Acid hydrolysis of sphingomyelin

In agreement with the observations of Dawson (1958), the hydrolysis of sphingomyelin with methanolic 2N-hydrochloric acid for 1·5 hr. at 100° in a sealed tube yields two water-soluble phosphate esters which migrate to the same position (R_F 0·95) in phenol–ammonia but which can be separated in butan-1-ol–acetic acid–water. With anhydrous methanol the yield of sphingosylphosphorylcholine was small when compared with the yield of phosphorylcholine, but the amounts could be made equal by the presence of 20% (v/v) water in the methanolic acid (Fig. 1). In this experiment the recovery of phosphorus as a mixture of esters was only about 75% of the total phosphorus in the sphingomyelin, though no phosphorus was detected in the chloroform layer after hydrolysis of the sphingomyelin and extracting with this solvent. The relative proportions of the esters were not affected by increasing the time of hydrolysis at 100–110°, nor was the total yield of water-soluble phosphorus increased.

Acid hydrolysis of acid- and alkali-stable fraction of brain lipids

Although the acid hydrolysis of pure sphingomyelin yielded the greater part of its phosphorus as a mixture of sphingosylphosphorylcholine and phosphorylcholine, this was not true for the alkali-stable fraction of rat-brain lipids. This difference was indeed anticipated because the fraction is known to contain phospholipids other than sphingomyelin (Branté, 1949; Dawson, 1954*b*). When the water-soluble fraction after acid hydrolysis was subjected to single-dimensional chromatography in phenol–ammonia a further phosphorus compound (possibly two) was found with an R_F of about 0·2 (X in Table 1). Further, a considerable amount of phosphorus was found in the chloroform layer after the extraction procedure described in Methods. When methanolic hydrochloric acid containing 20% of water was used there seemed little doubt, to judge from recovery experiments, that a proportion of this chloroform-soluble phosphorus was unchanged sphingomyelin. However, when anhydrous methanolic hydrochloric acid was used, recovery of added sphingomyelin as water-soluble ester phosphorus was good (Table 1), and it was considered that the chloroform-soluble phosphorus must represent some strongly bound lipid phosphorus.

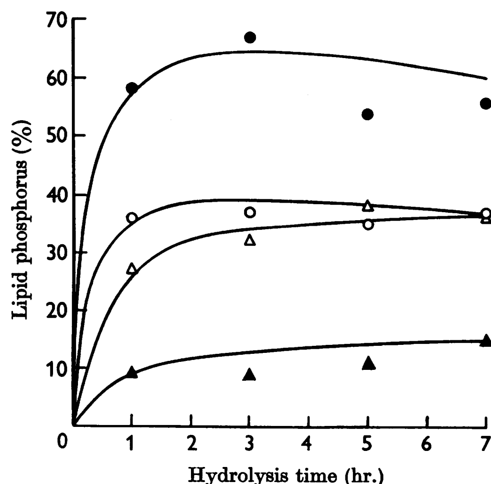

Fig. 1. Hydrolysis of sphingomyelin by hydrochloric acid at 100–110°. (*a*) 2N-HCl in anhydrous methanol: ●, phosphorylcholine; ▲, sphingosylphosphorylcholine. (*b*) 2N-HCl in methanol–water (80:20, v/v): ○, phosphorylcholine; △, sphingosylphosphorylcholine.

Table 1. *Recovery of sphingomyelin phosphorus added to brain-lipid extracts prepared from young rats*

The fractions were separated as described in the text. Values are given as μg. of P/g. of brain

		X	$CHCl_3$ layer	Sphingosylphosphorylcholine–phosphorylcholine	Recovery (%)
Brain lipid ≡ half brain	(*a*)	21·6	26·2	29·6	—
	(*b*)	18·0	23·2	29·2	—
	(*c*)	17·4	24·4	28·8	—
Brain lipid ≡ half brain +29·9 μg. of sphingomyelin P	(*a′*)	17·2	26·2	56·0	88
	(*b′*)	20·0	—	57·4	94
	(*c′*)	16·8	24·8	56·0	91

Table 2. *Effect of chloroform extraction on the amount of sphingosylphosphorylcholine–phosphorylcholine found in the acid hydrolysate of the alkali-stable phospholipid fraction (guinea-pig brain)*

The methanolic-HCl hydrolysate was divided into two parts (*a*) and (*b*). An aqueous suspension of (*a*) was subjected to chromatography in its entirety and (*b*) was extracted with a mixture of chloroform and water to yield two phases; each was separately subjected to chromatography. Values are given as μg. of P/g. of brain.

	X	Sphingosylphosphorylcholine–phosphorylcholine
(*a*) Total hydrolysate	41	174
(*b*) Chloroform phase	None	61
Water phase	41	100
(*a*) Total hydrolysate	49	187
(*b*) Chloroform phase	None	37
Water phase	49	112

However, in the experiments of Dawson (1960 and personal communication) in which the total acid hydrolysate of the acid- and alkali-stable fraction was subjected to chromatography without prior extraction with chloroform, all the phosphorus could be accounted for as 'sphingosylphosphorylcholine–phosphorylcholine' and other water-soluble esters. It was observed from our own experiments that the phosphorus in the chloroform-soluble fraction, when added to the sphingosylphosphorylcholine–phosphorylcholine phosphorus, gave values for guinea-pig-brain sphingomyelin that agreed with those of Dawson (1954*b*) based on total choline in the acid- and alkali-stable fraction (Table 2). Further, when the chloroform-soluble phosphorus was subjected to chromatography either in phenol–ammonia or in butan-1-ol–acetic acid, there was one phosphorus spot which ran to the position of sphingosylphosphorylcholine and reacted with ninhydrin. Subjection of the total hydrolysate to chromatography resulted in a much greater amount of 'sphingosylphosphorylcholine–phosphorylcholine' and no other substances (Table 2) although the chromatogram was rather distorted. Hence the chloroform-soluble phosphorus was behaving in many respects in these experiments like the water-soluble sphingosylphosphorylcholine.

When a sample of the chloroform-soluble fraction from guinea-pig brain was dried and heated in a sealed tube with aqueous 2N-hydrochloric acid at 123° for 46 hr., the phosphorus was rendered water-soluble as inorganic phosphorus. The water-soluble products of this drastic hydrolysis were then subjected to chromatography on potassium chloride-impregnated papers in the butan-1-ol–phenol–formic acid–water system of Bremer & Greenberg (1959). Two ninhydrin-reacting substances were obtained, one of which had an R_F identical with ethanolamine hydrochloride. Choline, which in this system is well separated from ethanolamine, mono- and di-methylethanolamine, could not be detected by the Dragendorff reaction, nor could choline be detected in the hydrolysate by the conventional reineckate method.

From these observations it was concluded that the chloroform-soluble phosphorus obtained on methanolic hydrochloric acid hydrolysis of the alkali-stable brain-phospholipid fraction was not a form of sphingosylphosphorylcholine. It seems unlikely that a phospholipid containing acyl groups would survive saponification and methanolic hydrochloric acid, but attempts to prove this were foiled by the presence of large amounts of methyl esters of fatty acids as contaminants. It is possible that the lipid may be comparable with that recently described by Svennerholm & Thorin (1960).

In a personal communication, Dr R. M. C. Dawson has indicated that he is now of the opinion that his sphingosylphosphorylcholine–phosphorylcholine fraction (Dawson, 1960) may well contain another component.

Estimation of true sphingomyelin in brain

Most estimations of tissue sphingomyelin have been carried out by determining the alkali-stable phosphorus by the method of Schmidt *et al.* (1946) and this has been so for many estimations on nervous tissue (e.g. Johnson *et al.* 1949). True sphingomyelin or mild-alkali-stable choline-containing phospholipid has been determined by prolonged hot alkaline hydrolysis of the alkali-stable

fraction to liberate choline (Branté, 1949; Dawson, 1954b), which can be subsequently estimated.

There are very few published values for the true sphingomyelin content of brain tissue. Branté (1949) gave some values for young rats based on the choline content of the acid- and alkali-stable phospholipid fraction, and Dawson (1954b) has given values for the true sphingomyelin content of adult guinea-pig brain (170 μg. of P/g. of tissue), whereas calculation from the results of Edgar & Smits (1959) shows that adult rabbit telencephalon contains 172 μg. of P/g. as sphingomyelin. Dawson (1960) has estimated the sphingomyelin content of sheep brain to be 207 μg. of P/g. of tissue. The application of the present modification of the Dawson (1958) method has been applied to the determination of sphingomyelin in the brains of rats of different ages after cold extraction with chloroform–methanol, and the results are shown in Table 3, which also lists total lipid phosphorus, acid- and alkali-stable phosphorus and the contributions from the unknown phospholipids. In Table 4 are given values for ox grey and white matter for comparison with the values of Branté (1949).

Nature of the unknown water-soluble phosphate components of the acid- and alkali-stable fraction

The nature of the water-soluble phosphorus-containing acid-hydrolysis products of the alkali-stable fraction (X in the tables) is unknown. The amount obtained was variable and sometimes two distinct spots in addition to phosphorylcholine–sphingosylphosphorylcholine could be seen in the single-dimensional phenol–ammonia chromatograms; more usually there was only one. Two-dimensional chromatography in ethanol–13N-ammonia solution (60:40, v/v) and phenol–ammonia did not resolve the predominant spot further, but in butan-1-ol–acetic acid–water (12:3:5, by vol.) (upper phase) two smaller spots were often seen in addition to the larger one. These additional phosphorus-containing areas did not react with ninhydrin, though the combined areas from a single-dimensional chromatogram contained a large amount of total nitrogen relative to the phosphorus present. Nitrogen:phosphorus ratios varied considerably, however, from 2·3 to 8·5, and it seemed possible that the nitrogen component ran coincidentally with the phosphorus-

Table 3. *Amount of total lipid phosphorus, acid- and alkali-stable lipid phosphorus and sphingomyelin phosphorus in the whole brains of rats of increasing age*

Values are given as μg. of P/g. of fresh brain tissue.

Age	Total lipid	Total acid- and alkali-stable lipid	Sphingomyelin (water-soluble sphingosylphosphorylcholine–phosphorylcholine)	Chloroform-soluble P	X
8 days	760–800 (2)	50–52 (2)	8–9 (2)	15 (2)	9–13 (2)
2 weeks	922–1005 (3)	59–92 (3)	13–16 (3)	19 (3)	6–32 (3)
3–4 weeks	1443 ± 19* (13)	143 ± 1·7* (13)	45 ± 3* (13)	25–27 (3)	28 (3)
5–6 weeks	1460–1511 (2)	143–194 (2)	54–56 (2)	42–44 (2)	24–44 (2)
8–9 weeks	1485–1500 (2)	158–165 (2)	56 (2)	46–47 (2)	27–28 (2)
9–11 weeks	1661 ± 183* (6)	181 ± 18* (6)	56·0 ± 9·3* (6)	44 ± 7* (6)	35 ± 12* (6)
52 weeks	1655–1800 (2)	192–200 (2)	67–72 (2)	40–41 (2)	30–50 (2)

* S.D.

Table 4. *Total alkali-stable phospholipid and sphingomyelin of grey and white matter of the brain*

Values are given as μg. of P/g. of tissue.

	Alkali-stable phospholipid	Sphingomyelin	Chloroform-soluble fraction	X
Present paper				
Ox grey (cortex)	160	23*	60	38
Ox white (corpus callosum)	665	358*	99	107
Calculation from Branté (1949)				
Ox grey	—	164†	—	—
Ox white	—	480 ± 120†	—	—
Man grey	156–300	108 ± 68†	—	—
Man white	—	432 ± 152†	—	—

* Calculated from water-soluble sphingosylphosphorylcholine–phosphorylcholine.
† Calculated from choline estimation.

Table 5. *Effect of trichloroacetic acid precipitation on the recovery of rat-brain phospholipid fractions*

Values are given as μg. of P/g. of tissue.

Age (weeks)	Treatment*	Total lipid	Acid- and alkali-stable lipid	X	Sphingomyelin (water-soluble phosphoryl-choline–sphingo-sylphosphoryl-choline)	Chloroform-soluble P
9–11	Trichloroacetic acid	1330	118	8	43	37
	Direct extraction	1461	170	30	41	34
9–11	Trichloroacetic acid	1405	128	8	43	43
	Direct extraction	1448	184	27	54	45
>11	Trichloroacetic acid	1520	163	21	54	45
	Direct extraction	1720	206	58	64	54
>11	Trichloroacetic acid	1570	153	20	56	51
	Direct extraction	1680	196	38	68	47

* See text.

containing component in phenol–ammonia. There was a considerable decrease in the amount of this unknown phosphorus fraction when the tissue was treated first with trichloroacetic acid, indicating that the component(s) is partially acid-labile. This may be linked with the fact that treatment of brain tissue with trichloroacetic acid decreased the amount of total lipid phosphorus and acid- and alkali-stable lipid phosphorus obtained on subsequent extraction with solvents, in comparison with that obtained by direct solvent extraction of the tissue (see Experimental section) (Table 5). This effect of trichloroacetic acid confirms observations made by Dawson (1954*b*) and Robins, Lowry, Eydt & McCaman (1956). The total decrease in acid- and alkali-stable lipid phosphorus was by no means accounted for by the decrease in X phosphorus, although the amount of sphingomyelin recovered was much less affected by the treatment with trichloroacetic acid. Whichever method was used the sphingomyelin phosphorus, the chloroform-soluble phosphorus and X phosphorus never accounted for the whole of the acid- and alkali-stable phosphorus.

Attempts to separate the phospholipid components of the acid- and alkali-stable fraction by first separating them from fatty acids with silicic acid (Borgström, 1952) and then subjecting a methanol eluate of the phospholipid material to chromatography on silicic acid-treated papers were unsuccessful.

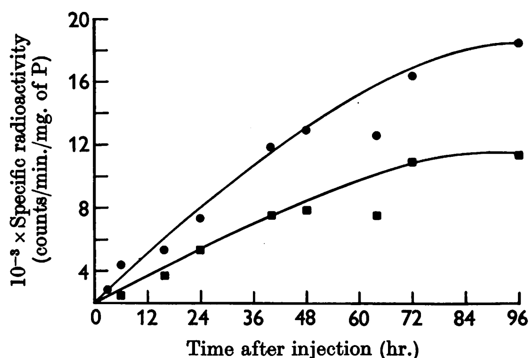

Fig. 2. Uptake of ^{32}P into the cerebral phosphatidylcholine (●) and sphingomyelin (■) of young rats.

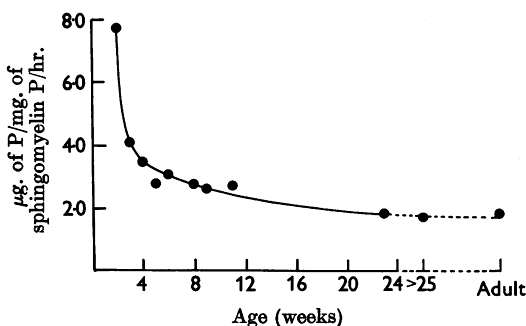

Fig. 3. Exchange of phosphorus in the cerebral sphingomyelin of rats of increasing age.

Uptake of [^{32}P]*orthophosphate into brain sphingomyelin*

When [^{32}P]orthophosphate was injected intraperitoneally into 3- to 4-week-old rats there was a steady uptake into both phosphatidylcholine and sphingomyelin of the brain. Estimation of the specific radioactivity of phosphatidylcholine was by the method of Dawson (1954*c*), and, as discussed elsewhere (Ansell & Spanner, 1960), the incorporation into this lipid in brain followed a different course from that in liver. Fig. 2 shows that young rat brain (now past the stage of maximum rate of myelination which reaches its peak at 14–21 days after birth) incorporated phosphate at a rate approximately half that of phosphatidyl-

choline. This rate must represent a mean for all the brain sphingomyelin wherever it is situated. As discussed below, however, the difficulties of interpretation are ubiquitous.

An attempt is made in Fig. 3 to demonstrate the variation in the rate of uptake with increasing age of the rat. Rats were injected with ^{32}P and killed after 24 hr., over which period the uptake of isotope into sphingomyelin is largely linear (Fig. 2), and the ratio of the specific radioactivity of the sphingomyelin phosphorus to that of the acid-soluble phosphorus was used as an arbitrary measure of phosphate exchange (Dawson & Richter, 1950; Ansell & Dohmen, 1957). This value was calculated as μg. of P incorporated/μg. of sphingomyelin P/hr. and it is apparent that there was a considerably greater rate of incorporation per μg. of P in the young rat than in the old, although, as the amount of sphingomyelin in the brain of the young rat is much smaller (Table 3), it might be inferred that the rates of synthesis of sphingomyelin per gram of brain are not dissimilar.

Although it is difficult to obtain pure 'white matter' from rat brain (Majno & Karnovsky, 1957), an attempt was made to compare the uptake of ^{32}P into the sphingomyelin of grey and white matter in the adult rat brain. Adult rats (about 1 year old) were injected with [^{32}P]orthophosphate (4 μc/g. body weight) and the brains removed after 24 hr. The corpus callosum and optic chiasma of each were pooled to give a total yield of about 10 mg. of white matter for each rat. About 200 mg. of cortical grey matter was also obtained from each brain and the specific radioactivity of the sphingomyelin in each sample determined. There was a definite sphingosylphosphorylcholine–phosphorylcholine spot from grey matter confirming that sphingomyelin occurs outside myelin (cf. Robins, Eydt & Smith, 1956; Edgar & Smits, 1959), and this was significantly radioactive, whereas the spot from white matter was negligibly labelled (Table 6). It would appear from these results that cortical sphingomyelin has a turnover in adult life not shared by its counterpart in the white matter.

Table 6. *Uptake of* [^{32}P]*orthophosphate into the grey- and white-matter sphingomyelin of the adult rat*

The animals received 4 μc of [^{32}P]orthophosphate/g. of body weight and were killed 24 hr. after the injection.

	Specific radioactivity (counts/min./mg. of P) of sphingomyelin	
Rat no.	Cortical grey tissue	Corpus callosum
1	19 500	< 3770
2	18 400	< 1940

DISCUSSION

The present experiments indicate that sphingomyelin represents only a portion of the phospholipid obtained after mild acid and alkaline treatment of brain phospholipids, confirming the earlier observations of Brantė (1949) and Dawson (1954*b*) among others. In the whole brain of adult rats, sphingomyelin phosphorus only accounts for about one-third of the phosphorus stable to mild acid and alkali treatment, whereas for white matter from ox corpus callosum it accounts for 70%. The remaining phosphorus in our experiments gave rise to unidentified water-soluble hydrolysis products and a chloroform-soluble fraction, which did not, however, account for all the phosphorus in the alkali-stable fraction. According to Dawson (1960) sphingomyelin accounts for about 60% of the phospholipid in whole sheep brain which is stable to mild acid and alkali.

Precipitation of brain tissue with trichloroacetic acid before extraction with lipid solvents only slightly affected the recovery of sphingomyelin, but it had an adverse effect on the unknown phospholipid (X) the amount of which was considerably decreased (Table 5). Dawson (1954*b*) also observed this effect of acid on the unknown alkali-stable phospholipid(s). Edgar & Smits (1959) noted that after freeze-drying of the tissue lower values were obtained for this fraction than after direct extraction of the wet tissue. The very recent experiments of Dawson (1960) indicate that direct extraction is the method of choice for the total analysis of tissue phospholipids. The fact that treatment of the tissue with cold trichloroacetic acid reduced the amount of the predominant unknown water-soluble phosphate ester obtained suggests that it may derive from a phospholipid containing an acid-sensitive linkage, e.g. an unsaturated ether as found in plasmalogens.

It is apparent, however, that until the nature of this mild acid- and alkali-stable lipid(s) is known the factors which affect its recovery from tissue samples during an analytical procedure are likely to remain obscure. Carter, Smith & Jones (1958) isolated an acid- and alkali-stable phospholipid from egg yolk which was shown to consist largely of a phosphorylethanolamine derivative of batyl alcohol and they suggested that such a lipid may be present in brain tissue. Very recently Svennerholm & Thorin (1960) have obtained more definite evidence of an acid- and alkali-stable 'kephalin B' from brain which yielded ethanolamine on strong acid hydrolysis and was comparable analytically with the product of Carter *et al.* (1958). In the present work no apparent water-soluble ninhydrin-positive material apart from sphingosylphosphorylcholine was obtained on methanolic hydro-

chloric acid hydrolysis of the acid- and alkali-stable phospholipids of brain, and more than one water-soluble phosphate ester was often obtained in addition to those derived from sphingomyelin. However, it seems possible from our experiments that the chloroform-soluble phosphorus remaining after methanolic hydrochloric acid hydrolysis, which was shown to react with ninhydrin and to yield a component provisionally identified as ethanolamine, might be a lipid similar to that investigated by Svennerholm & Thorin (1960).

Sphingomyelin in the brain of the rat reached its maximal concentration between 9 and 11 weeks of life and was paralleled by the total acid- and alkali-stable lipids (Table 3). The total amount per brain increased beyond this period because the rat brain increases in size for a period of a year or more (Donaldson, 1924). In the mouse the concentration of acid- and alkali-stable lipid is maximal at about 7 weeks after birth (Folch-Pi, 1955) and in the telencephalon of the rabbit, 17–18 weeks after birth; in this species the concentration subsequently falls (Edgar, 1957). The amount of unknown chloroform-soluble phospholipid appeared to reach a maximal concentration before the sphingomyelin, but the scatter of the values in Table 3 precludes a definite conclusion about this.

Experiments indicated that grey-matter sphingomyelin in the adult animal was capable of incorporating labelled phosphate, whereas that of the white matter was relatively inert as far as the phosphate moiety was concerned. That the adult-brain sphingomyelin as a whole is metabolically active is shown in Fig. 3, but it seems highly likely that this phosphate uptake is confined to sphingomyelin occurring outside the myelin sheath. The relatively inert nature of the sphingomyelin of the myelin sheath has been demonstrated by Davison *et al.* (1959), who showed that when [3-^{14}C]serine was injected into the developing rabbit it was incorporated into and retained by myelin sphingomyelin without significant loss for over 6 months. Cerebrosides and cholesterol, which are also characteristic myelin lipids, were shown to be likewise metabolically inert, in that loss of radioactive carbon was negligible once it had been incorporated during the initial accretion. It should be pointed out, however, that the whole acid- and alkali-stable phospholipid fraction was considered as sphingomyelin by Davison *et al.* (1959). The unknown components of this fraction have been shown in recent experiments (unpublished) to be quite significantly radioactive after the injection of labelled phosphate. Davison & Dobbing (1959) have in addition carried out experiments in which ^{32}P was injected into 16-day-old rats and the retention of the isotope by the brain phospholipid investigated. After 70 days a considerable proportion of the incorporated radioactivity remained in comparison with the negligible amount of radioactivity in the liver and kidney. These observations confirmed the early experiments of Changus, Chaikoff & Ruben (1938). In a subsequent investigation Davison & Dobbing (1960*a*) showed that there was a steady loss of radioactivity from the brain lipids until about 100 days after the injection, after which there was no measurable decline. From this they concluded that a proportion of the phosphate moieties of the brain phospholipid was metabolically inert, thus amplifying some conclusions drawn by Thompson & Ballou (1954, 1956). These authors showed that a considerable proportion of the phospholipids of the tissues of rats exposed to tritium when developing had long half-lives (220 days). They did not specifically study brain phospholipids and, as Davison & Dobbing (1960*a*) point out, the 'long-lived' components of Thompson & Ballou could be a mixture of inert and 'short-lived' components. These findings represent in some respects a revival of the concept of Folin (1905).

Discussing their experiments on the persistence of radioactivity in the myelin sheath as opposed to grey matter, Davison & Dobbing (1960*b*) conclude that phospholipids in this tissue are metabolically inert in adult life. It would certainly appear from our own experiments that sphingomyelin of the white matter of the adult rat brain is not particularly active as far as its phosphate metabolism is concerned, but it may not be completely inert. There are certainly great difficulties in interpreting the results obtained after the injection of labelled phosphate as far as brain tissue is concerned (Ansell & Spanner, 1960; Ansell, 1960) because of the chemical and morphological heterogeneity of the tissue. Even the relatively well-established inert brain component, cholesterol (Davison, Dobbing, Morgan & Payling-Wright, 1958) appears to be subject to turnover in adult life (McMillan, Douglas & Mortensen, 1957).

SUMMARY

1. Acid hydrolysis of cerebral phospholipids which are stable to mild acid and alkali hydrolysis has confirmed that sphingomyelin represents only a proportion of this fraction whereas the nature of the remainder is unknown.

2. Sphingomyelin has been determined in the brain tissue of rats and other species and the results compared with those obtained with other methods.

3. The concentration of true sphingomyelin in rat brain reaches its peak 9–11 weeks after birth.

4. The incorporation of labelled phosphate into rat-brain sphingomyelin reached a constant level

after 9 weeks. This metabolism was largely confined to sphingomyelin found in the grey matter.

Note added in proof. The predominant unknown phosphorus compound (X) has now been identified as glycerol-α-phosphate, which suggests that a glycerophospholipid survived the treatment preceding the methanolic hydrochloric acid hydrolysis [cf. Schmidt *et al.* (1959). *Amer. J. Dis. Child.* **97**, 691].

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