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The Physiological Role of Cerebron Sulphuric Acid (Sulphatide) in the Brain

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Sulphur is found in mammalian-brain lipid as the sulphate ester of galactocerebrosides (sulphatides) with cerebronic acid, lignoceric acid or possibly neuronic acid as the fatty acid constituent of the cerebroside (Jatzkewitz, 1960). The sulpholipid has been isolated both as the crystalline form sulphatide A and in association with phospholipid as sulphatide B (Lees, Folch, Sloane-Stanley & Carr, 1959). It has been claimed that brain sulphatides continue to accumulate throughout the

life-span of the rat (Koch & Koch, 1913; Green & Robinson, 1960; Bakke & Cornatzer, 1961; Goldberg, 1961). The continual accretion of sulphatide in the human brain has also been reported (MacArthur & Doisy, 1919; Bakke & Cornatzer, 1961). In conformity with these findings, radioactive sulphate is found to be incorporated in relatively small amounts *in vitro* into the lipids of adult-rat-brain slices (Heald & Robinson, 1961), and after injection of [³⁵S]sulphate into rats or rabbits some labelled sulpholipid can be isolated from the brain (Dziewiatkowski, 1953; Holmgard, 1955; Radin, Martin & Brown, 1957; Green & Robinson, 1960; Bakke & Cornatzer, 1961). It was concluded that this radioactive sulphatide was metabolically inert, for no radioactivity was lost from the brain up to 16 days after injection (Radin *et al.* 1957). These results imply that there is a small but continual accumulation of sulpholipid in the brain and that once this lipid is laid down it is retained possibly for the life-span of the animal (Radin *et al.* 1957; Heald & Robinson, 1961).

Previous work (Davison, Morgan, Wajda & Wright, 1959; Davison & Dobbing, 1960) has suggested that the relative metabolic stability of brain lipids is related to their structural role in the

brain, predominantly in the myelin sheath. Since it was proposed by Koch & Koch (1913, 1917) that sulpholipid may be a constituent of the myelin sheath it was decided to reinvestigate the metabolism of sulphatide in the nervous system of young and old rats and to determine the distribution and accretion of the lipid in the brain throughout life. A preliminary report of some of this work has been published (Davison, 1961*b*).

METHODS

Extraction of sulphatide. Samples of brain were ground up in a glass tissue grinder with 19 vol. of chloroform-methanol (2:1, v/v)/g. wet wt. of tissue. The filtered extract was washed with 0.2 vol. of water, and then twice with equal volumes of synthetic upper phase containing 0.1 M-NaCl (Folch, Lees & Sloane-Stanley, 1957). In some cases proteolipid A and B were decomposed by repeated evaporation of the washed moist chloroform-methanol (2:1, v/v) extract to dryness or by treating the extract with synthetic upper phase containing citrate (Webster & Folch, 1961). Tissue dry weights were determined by drying at 110° to constant weight.

Identification of sulphatide. A 15-day-old rat was injected with 8 μ C of [35 S]sulphate. After 1 week the rat was killed and a washed chloroform-methanol (2:1, v/v) extract of the brain was prepared. A portion (2 ml.) of the extract was hydrolysed at 100° with saturated barium hydroxide solution. After hydrolysis for 3 hr. 92.5% of the radioactivity extractable with chloroform-methanol (2:1, v/v) was lost and most of this radioactivity could be accounted for as barium [35 S]sulphate. In a second experiment 1 ml. of washed chloroform-methanol (2:1, v/v) extract containing the equivalent of 0.093 g. wet wt. of brain was fractionated on an alumina column (1 cm. $\times$ 20 cm.) (Davison & Wajda, 1962). The column was eluted successively as described for small samples of nervous tissue. About 90% of the 35 S was recovered from the fraction containing cerebroside; no radioactivity was found in the cholesterol and choline phospholipid fractions but some radioactivity was associated with the kephalin fraction, which is known to contain some sulphatide (cf. Long & Staples, 1961). Similar fractions of radioactive lipid obtained after incubation of [35 S]sulphate with brain and cord slices indicated that most of the radioactivity was associated with the cerebroside fraction, although here too up to 10% was eluted with the kephalins.

Estimation of lipid sulphate. The following technique was used for the release of lipid sulphate: 2 ml. of washed chloroform-methanol (2:1, v/v) extract containing lipid from at least 0.1 g. wet wt. of brain is pipetted into a test tube fitted by a ground-glass joint to an air condenser (1 cm. $\times$ 25 cm.). Then 0.2 ml. of conc. HCl is added with sufficient methanol to make one phase. A small piece of porous pot is added to the tube. The tube is warmed with shaking for several minutes in a boiling-water bath and then left to reflux for 90 min. at 100°. It is essential to keep the temperature at 100° throughout this period. The inside of the condenser is then washed with 2 ml. of chloroform-methanol (2:1, v/v), the tube is shaken and sufficient water added to allow two phases to separate. The lower chloroform-rich phase is pipetted off and the upper

phase washed successively with 2 ml. of chloroform-methanol (2:1, v/v) and then 2 ml. of chloroform-methanol (17:3, v/v). The washed upper phase is evaporated to dryness in a graduated tube under a continuous stream of nitrogen while being heated in a boiling-water bath. Then 4 ml. of 5% (w/v) trichloroacetic acid solution is added to the tube, which is stoppered, shaken and clarified by centrifuging for 10 min. at approx. 300g.

Sulphate is then estimated turbidimetrically essentially according to 'method B' of Dodgson (1961). A sample (1.5 ml.) of the clear supernatant is next added to 0.5 ml. of 0.5% (w/v) gelatin solution (blank) and to 0.5 ml. of 0.5% (w/v) gelatin in 0.5% (w/v) BaCl₂. Blanks with 1.5 ml. of 5% (w/v) trichloroacetic acid solution and suitable standards are prepared at the same time. The extinction is determined in a Unicam SP. 600 spectrophotometer, fitted with a mask, at 390 m μ .

Hydrolysis was shown to be complete under these conditions by two methods. (i) The liberation of sulphate or loss of radioactive sulphatide from a whole-brain extract was followed for hydrolysis times of 5 to 360 min. (Fig. 1). (ii) In another experiment six samples of a protein-free chloroform-methanol (2:1, v/v) extract from brain (2 ml. containing the equivalent of 0.1185 g. wet wt. of human corpus callosum) was hydrolysed for 90 min. as described above. Five samples were also dried, digested with 0.1 ml. of conc. HNO₃ plus 0.5 ml. of (60%, w/v) HClO₄, and about 10 mg. of pure copper strip was added to the clear digest. The mixtures were evaporated to dryness, 1 ml. of 1 N-KOH was added to each tube and, after centrifuging, the sulphate content of a sample of the supernatant was determined turbidimetrically. There was no significant difference between the results obtained by hydrolysis and by oxidation.

Determination of radioactivity. Lipid extracts were evaporated to dryness and the lipid was dissolved in 10 ml. of xylene containing 2,5-diphenyloxazole (0.3%, w/v) as scintillator solute. The method of determining radioactivity was as described by Davison (1961*a*). A water-cooled Nuclear Enterprise scintillation counter NE 5503, operated at a counting efficiency of 71%, was employed.

Animals. White Wistar rats of either sex were used throughout this work. Rats were given 35 S as carrier-free [35 S]sulphate or [35 S]methionine in 0.9% sodium chloride solution by intraperitoneal injection.

RESULTS

Although relatively simple methods are available for the determination of pure sulphatide (Long & Staples, 1961), its analysis in whole-brain-lipid extracts is more difficult (Green & Robinson, 1960; Bakke & Cornatzer, 1961), since interaction with phospholipids results in the incomplete release of sulphate (Lees *et al.* 1959). Thus the sulphate residue of sulphatide A, but not of sulphatide B, is readily hydrolysed by acid. Incomplete hydrolysis of sulphatide in whole-brain lipid extracts was obtained when established methods for analysis of purified sulphatide were employed (Table 1). Other analytical techniques considered were thought to be unsuitable for routine analysis (e.g. Austin, 1959).

Table 1. *Comparison of methods of hydrolysis of sulphatide*

A protein-free washed chloroform-methanol (2:1, v/v) extract of ox brain was prepared as described in the text. In the first experiment 2 ml. samples of ox-brain extract containing the equivalent of 0.1 g. wet wt. were (a) hydrolysed at 100° with 0.2 ml. of conc. HCl plus methanol to give one phase for 90 min., (b) evaporated to dryness and hydrolysed with 0.15 ml. of water plus 0.15 ml. of conc. HCl at 100° for 90 min. (Long & Staples, 1961), (c) evaporated to dryness and hydrolysed with 0.3 ml. of acetic acid at 100° for 90 min. (Lees *et al.* 1959). After hydrolysis the aqueous solution was washed twice with 2 ml. of chloroform-methanol (2:1, v/v) and once with 2 ml. of chloroform-methanol (17:3, v/v). Sulphate was estimated turbidimetrically as described in the text.

Method of hydrolysis	Concn. of sulphate ($\mu\text{g. of SO}_4^{2-}$ ion/0.8 ml. of extract)	Mean concn. of sulphate ($\mu\text{g. of SO}_4^{2-}$ ion/4 ml. of extract)	Hydrolysis (%)
(a) HCl in chloroform-methanol (2:1, v/v)	65.5 } 67 } 65.5 }	333.5	100
(b) 50% (v/v) conc. HCl on residue	12.25 } 8.75 } 7.5 }	47.5	14.2
(c) Acetic acid on residue	57.5 } 51.5 } 56.0 }	275	82

Fig. 1. Hydrolysis of sulphatide. Washed chloroform-methanol (2:1, v/v) extracts of human white matter were hydrolysed with hydrochloric acid for different times and the liberated sulphate was determined as described in the text. Results are shown as percentages of maximum sulphate liberated (x). A rat-brain extract containing [^{35}S]-sulphatide was also hydrolysed under the same conditions. Results for the latter experiment are expressed as percentage losses of radioactivity (●) from the total lipid extract. Also shown is the release of inorganic sulphate from purified sulphatide A (Δ), prepared according to the method of Lees *et al.* (1959).

The effect of various hydrolysis conditions was next investigated. Hydrolysis of whole-brain lipid extracts by acetic acid at 100° for 90 min. resulted in incomplete hydrolysis, confirming the results of Lees *et al.* (1959). Similar results were obtained when the dried whole-brain lipid residue was hydrolysed with approximately 6N-hydrochloric acid (Table 1). However, when lipid extracts in chloroform-methanol (2:1, v/v) were hydrolysed with hydrochloric acid for 90 min. at

100° complete hydrolysis was attained (Fig. 1). The hydrolysis temperature is critical. Thus at 90° only 70% of the total lipid sulphate was released and at 60° only 58% after 90 min. Evidence for complete cleavage of sulphatide is indicated by the hydrolysis curve (Fig. 1) in which liberated sulphate was followed for hydrolysis periods of up to 360 min. and by the complete loss of [^{35}S]sulphate from labelled sulphatide in whole-brain extracts after hydrolysis for 90 min. (Fig. 1). These findings were also confirmed by the demonstration that total lipid sulphur in a protein-free whole-brain lipid extract could be accounted for by liberated sulphate under the same hydrolysis conditions.

Finally, lipid extracts of human white matter were hydrolysed with hydrochloric acid and parallel samples in sealed tubes with acetic acid and 6N-hydrochloric acid for 48 hr. at 100°. Liberated sulphate was estimated both turbidimetrically and by the much less sensitive chloroanilate method. Reasonable agreement was achieved between the different methods (Table 2).

In preliminary experiments it was found difficult to remove all traces of hydrochloric acid from the crude lipid hydrolysates and, since this interfered with all other methods of sulphate estimation examined except the barium chloride turbidimetric technique of Dodgson (1961), this latter method was adopted with minor modifications. The procedure used throughout this paper (described above) was critically examined and in trial experiments, where small amounts of sulphate were added to brain lipid extracts, $104 \pm 3.2\%$ of added sulphate could be recovered. Further, analysis of a purified preparation of sulphatide A (Lees *et al.* 1959) gave results which were in close agreement with the theoretical value. Neither the presence of proteo-

Table 2. Comparison of methods of estimation of lipid sulphate obtained by hydrolysis of whole-brain lipid extracts by two methods

A chloroform-methanol (2:1, v/v) extract of human white matter containing the equivalent of 0.1 g. wet wt./2 ml. was prepared as described in the text. Hydrolysis with HCl and turbidimetric estimation of sulphate were also as described in the text. A sample (5 ml.) of the extract was evaporated to dryness and hydrolysed with 1 ml. of acetic acid plus 1 ml. of 6N-HCl in a sealed tube for 48 hr. at 100° as described by Green & Robinson (1960). Samples obtained by both types of hydrolysis were analysed by the turbidimetric method (Dodgson, 1961) and by the chloroanilate procedure described by Green & Robinson (1960). Results are given as means $\pm$ S.D., with the number of hydrolyses undertaken in parentheses. For the turbidimetric method and HCl hydrolysis as described in this paper the S.D. was $\pm 3.6\%$ and the S.E.M. $\pm 1.3\%$ of the mean respectively.

Method of hydrolysis	...	...	...	Concn. of sulphate ($\mu\text{g. of SO}_4^{2-}$ ion/g. fresh wt.)	
				HCl in chloroform-methanol	Sealed tube: 1 ml. of acetic acid + 1 ml. of 6N-HCl
Method of analysis					
Chloroanilate (Green & Robinson, 1960)				1055 $\pm$ 70 (2)	847.4 $\pm$ 9.4 (2)
BaCl ₂ -gelatin method (Dodgson, 1961)				827.3 $\pm$ 29.5 (9)	803 $\pm$ 10 (4)

Fig. 2. Lipid sulphate content of rat brain throughout life. Lipid sulphate/g. wet wt. of brain (●) was estimated as described in the text. The results of Koch & Koch (1913) (x) and of Bakke & Cornatzer (1961) (O), expressed as lipid sulphate, are also shown. The mean wet weight of 1-year-old-rat brain was 1.95 ± 0.05 g. and 2-3-year-old-rat brain 2.05 ± 0.16 g.

lipid protein nor prolonged fixation in neutralized formalin-0.9% sodium chloride solution (1:9, v/v) affected the sulphate content of brain as determined by the recommended method.

Effect of age on the sulphatide content of rat brain.

White Wistar rats of different ages up to 3 years old were killed by stunning and exsanguination. Washed chloroform-methanol (2:1, v/v) extracts of each brain were analysed. Fig. 2 shows the lipid sulphate/g. wet wt. of brain plotted against age in days. From these results it appears that the concentration of brain sulphatide remains unchanged in rats more than 100 days old. To facilitate comparison, the analytical figures of Bakke & Cornatzer (1961) and Koch & Koch (1913), expressed as lipid sulphate/g. wet wt. of brain, are also included.

Effect of age on the sulphatide content of human-cerebral white matter and grey matter. Human brains from patients of different ages dying from non-neurological conditions were obtained at

autopsy, and washed chloroform-methanol (2:1, v/v) extracts prepared from grey matter and white matter of the cerebral frontal region as rapidly as possible. Samples were also fixed in neutralized formalin-0.9% sodium chloride solution (1:9, v/v) and stored for periods of up to 6 months. No differences were observed in the sulphatide content of fresh and fixed material under these conditions. For this reason results for the analysis of several preserved brains are also included in Table 3. Dry weights for all samples were determined. For each age group over 18 years old, the values are the means of five subjects.

Regional distribution of sulphatide in human brain.

To provide more information as to the physiological role of sulphatide, samples of tissue from different areas of human brain have been analysed for lipid sulphate. Sulphatide appears to be particularly concentrated in areas of pure white matter whereas less is found in cortical grey matter (Table 4). Since these results suggested that sulphatide may be associated with myelin attempts were made to confirm the possibility by another method. Evidence is accumulating to suggest that the proteolipid soluble in chloroform-methanol (2:1, v/v) is part of the myelin-sheath complex (Greaney, 1961; Koenig, 1959; Amaducci, 1962). Proteolipid was therefore isolated by the method of Kimura & Taketomi (1960) and analysed for sulphatide. Although galactolipid was present little cerebron sulphuric acid was detected. However, proteolipid may only represent a part of the myelin complex and possibly in the extraction process sulphatide is detached from myelin protein.

Distribution of sulphatide in myelin and sub-cellular fractions of rat brain. Adult-rat brain was suspended in 0.32M-sucrose containing phosphate buffer (3 mM) and EDTA (1 mM), the final pH was brought to 7.0-7.3, and the suspension separated

Table 3. *Sulphatide content of human-cerebral white matter and grey matter at different ages*

Both fresh and fixed cerebral cortex were analysed for lipid sulphate by the method described in the text. Dry weights were determined on all samples. Results on each sample represent the average of three estimations. Values for lipid sulphate for ages over 18 years have been grouped together and the mean $\pm$ S.E.M. for five brains is recorded. The concentration of sulphatide is obtained by multiplying that of lipid sulphate by 9.45.

Age or range (years)	No. of patients	Concn. of lipid sulphate in cerebral grey matter		Concn. of lipid sulphate in cerebral white matter	
		(μ g. of SO_4^{2-} ion/g. wet wt.)	(μ g. of SO_4^{2-} ion/g. dry wt.)	(μ g. of SO_4^{2-} ion/g. wet wt.)	(μ g. of SO_4^{2-} ion/g. dry wt.)
2 months	1	92	1060	190	1920
3	1	87	486	783	2460
4	1	108	475	873	3300
19-26	5	94 $\pm$ 15	583 $\pm$ 87	758 $\pm$ 65	2426 $\pm$ 177
31-39	5	174 $\pm$ 21	1101 $\pm$ 127	710 $\pm$ 70	2323 $\pm$ 242
41-58	5	188 $\pm$ 20	1152 $\pm$ 67	766 $\pm$ 52	2483 $\pm$ 273
68-76	5	165 $\pm$ 22	1107 $\pm$ 210	753 $\pm$ 45	2501 $\pm$ 113

Table 4. *Distribution of sulphatide in the adult-human brain*

The sulphatide contents of different areas of human brain were determined as described in the text. The concentration of sulphatide is obtained by multiplying that of lipid sulphate by 9.45.

		Concn. of sulphatide (mg./g. fresh wt. of brain)		
Age of patient (years)	...	74	35	19
Region				
Optic nerve and chiasma		8.85	—	—
Pons		8.75	7.94	6.45
Corpus callosum		13.0	6.99	7.45
Caudate nucleus		0.95	—	1.77
Thalamus (medial)		3.78	3.48	4.31
Cerebellar grey matter		0.95	2.68	1.28
Cerebellar white matter		—	5.7	7.16
Cerebral grey matter		1.01	2.1	1.15
Cerebral white matter		7.5	7.3	8.45

into nuclear, heavy-mitochondrial, light-mitochondrial, microsomal and supernatant fractions by differential centrifuging. The nuclear and heavy-mitochondrial fractions were further separated into myelin and particulate fractions by centrifuging at 0° for 60 min. at 15 000g over 0.8M-sucrose (Davison, Gregson & Williams, 1962). Fractionation was controlled by biochemical analyses and electron microscopy. Each fraction was then analysed for lipid sulphate. About 38.6 % of brain sulphatide was found in the combined myelin fractions and 20.3 % in the light-mitochondrial fraction (Table 5). This latter fraction also contains small myelin fragments. Thus it may be concluded that at least 50 % of the brain sulphatide is located in the myelin sheath although the lipid is also found in mitochondria and microsomes (see below).

Metabolic studies with [^{35}S]sulphate

Incorporation of [^{35}S]sulphate into brain lipid as a function of age. White Wistar rats of different ages

were given intraperitoneal injections of [^{35}S]sulphate in 0.9 % sodium chloride equivalent to 0.32 $\mu\text{C/g.}$ body wt. The animals were killed 2 days after injection, and brain, spinal cord and sciatic nerve were removed and extracted with chloroform-methanol (2:1, v/v) as described above. The extracts were washed repeatedly until all water-soluble radioactive material was removed. Maximum incorporation of radioactive sulphate into the brain occurs in the 15-20-day-old rats (Fig. 3) and similar results have been obtained for the spinal cord and sciatic nerve. In the adult rats, given a dose of [^{35}S]sulphate proportional to body weight (336 g.), much less radioactivity was incorporated into the nervous tissue.

There are at least two possible interpretations of these results. First, it may be that radioactive sulphate was most rapidly incorporated into the developing rat brain as a result of rapid turnover of sulphatide, and that failure to label sulphatide in the adult brain could be ascribed to restricted entry of sulphate into this organ (Heald & Robinson, 1961; Heald, 1960) and not to a generally slow metabolism of adult-brain sulphatide. An alternative hypothesis is that sulphatide is rapidly synthesized only in the developing brain and retained in myelin and other structures which, once formed, do not undergo further dynamic metabolism. In the adult brain the structural elements containing mainly stable sulphatide are relatively inert and therefore do not readily incorporate [^{35}S]sulphate. These two possibilities have been further investigated.

Metabolism of [^{35}S]sulphatide in vitro. In the first experiment in this series a brain suspension from a rat previously injected with [^{35}S]sulphate was incubated at 37° with brain homogenate from adult, 12-day-old and 2-day-old rats. After incubation for 90 min. there was no evidence of any breakdown of sulphatide in any of the preparations (Table 6).

Table 5. *Distribution of lipid sulphate in myelin and subcellular fractions of adult-rat brain*

Adult-rat brains were suspended in 0.32M-sucrose containing 1 mM-EDTA and separated by differential centrifuging at 0–4° (Davison *et al.* 1962). Nuclear and heavy-mitochondrial fractions were refractionated to separate myelin. The lipid in each fraction was extracted in chloroform-methanol (2:1, v/v), washed with water and washed four times with the synthetic upper phase described above. Results for lipid sulphate are the mean of two separate fractionations.

Sedimentation conditions	Fraction	Concn. of whole-brain lipid sulphate in fraction			
		($\mu\text{g. of SO}_4^{2-}$ ion/g. fresh wt.)	(%)	(%) (pooled fractions)	After Green & Robinson (1960) (%)
1000g $\times$ 10 min.	Whole homogenate	295	100	100	100
	{ Heavy myelin*	39	13.2	22.7	33
	{ Debris and nuclei	28	9.5		
5000g $\times$ 10 min.	{ Light myelin*	75	25.4	59.6	50
	{ Heavy mitochondria	41	13.9		
13 500g $\times$ 15 min.	Light mitochondria	60	20.3	14.9	10
20 000g $\times$ 25 min.	Microsomes	44	14.9		
	Supernatant	25	8.4	8.4	7

* Fractions resuspended in 0.32M-sucrose, layered over 0.8M-sucrose and centrifuged at 15 000g for 60 min.: the myelin remains at the interface.

Fig. 3. Incorporation of [^{35}S]sulphate into the brain of rats of different ages. Rats were given doses of [^{35}S]sulphate by intraperitoneal injection and killed after 2 days. Brain lipid was extracted and the radioactivity determined as described in the text. The results for the total radioactivity in washed brain-lipid extract were corrected to the equivalent of a dose of 0.32 μC of $^{35}\text{SO}_4^{2-}$ ion/g. body wt.

In the second series of experiments [^{35}S]sulphate was incubated in a suitable medium with viable brain slices or spinal cord from adult and neonatal rats (Table 7). The results indicate that much less radioactive sulphate was incorporated into the lipid of the adult nervous tissue compared with that from the young. These experiments therefore do not indicate any measurable breakdown of sulphatide in either young or adult brain; moreover, the slow synthesis of [^{35}S]sulphatide in the adult compared with the neonatal rat is similar to the results obtained *in vivo*. Thus the relatively small uptake of [^{35}S]sulphate into the normal lipids of the

adult rat appears to be primarily due to slow metabolism and not to a 'blood-brain barrier' effect (cf. Dobbing, 1961).

Incorporation and persistence of [^{35}S]sulphate into rat neural lipids. After intraperitoneal injection of radioactive sulphate into 15-day-old rats about 0.5% of the dose is incorporated into the brain. Almost all the [^{35}S]sulphate is taken up into the brain lipid and no radioactivity could be detected in brain protein or proteolipid protein. Radioactivity was not detected in the blood after 3 days and free [^{35}S]sulphate rapidly disappears from the brain. After incorporation of radioactive sulphate in the brain there is a slow decline of labelled cerebral lipid with a mean half-life of about 210 days (Table 8).

Turnover of lipid sulphate in the spinal cord appears to be slower compared with the brain; radioactivity appears to persist even longer in the sciatic-nerve lipid. However, these experiments were limited to a period of 210 days and it is possible that some of the sulphatide laid down during development is turning over even more slowly than is suggested by the present results (Davison & Dobbing, 1960).

Only 0.08% of the injected dose of radioactive sulphate is incorporated into the brain of the adult rat (Table 9). There is a slow loss of labelled lipid from brain (mean half-life approx. 210 days) and sciatic nerve, and an even slower loss from the spinal cord.

Persistence of radioactivity in myelin and subcellular fractions of rat brain. Two rats previously injected with 50 μC of [^{35}S]methionine when 16 days old and one rat previously injected with 8 μC of [^{35}S]sulphate when 15 days old were killed after

Table 6. *Incubation of labelled sulphatide with brain suspensions from rats of different ages*

A 13-day-old rat was injected with 0.1 ml. of a solution containing 77 μC of carrier-free [^{35}S]sulphate in 0.9% sodium chloride solution. After 6 days the rat was killed and the brain suspended in 0.32M-sucrose solution, containing phosphate buffer (3 mM) and EDTA (1 mM), equivalent to 100 mg. fresh wet wt./ml. The brain suspension was adjusted to pH 7.4. A portion (1 ml.) of this radioactive suspension was incubated in Warburg flasks at 37° for 90 min. with 0.5 ml. or 1 ml. of brain suspension (100 mg. fresh wt./ml. of 0.32M-sucrose) from rats of different ages. The radioactivity in washed chloroform-methanol (2:1, v/v) extracts, prepared from each flask after incubation, was determined by scintillation counting.

Flask contents	Radioactivity of lipid (counts/min.)
(1) 1 ml. of ^{35}S -labelled suspension	12 826
(2) (1) + suspension of 50 mg. of adult brain	14 660
(3) (1) + suspension of 100 mg. of adult brain	14 996
(4) (1) + suspension of 50 mg. of 12-day-old brain	14 131
(5) (1) + suspension of 100 mg. of 12-day-old brain	14 710
(6) (1) + suspension of 50 mg. of 2-day-old brain	13 056
(7) (1) + suspension of 100 mg. of 2-day-old brain	14 652

Table 7. *Incorporation of [^{35}S]sulphate into rat-brain and spinal-cord lipid in vitro*

The incubation medium used and cortical slices of rat brain were prepared as described by Heald & Robinson (1961). Intact spinal cord was also incubated in the same medium. Each Warburg flask contained 2.5 ml. of medium and the equivalent of 40 μC of carrier-free [^{35}S]sulphate; incubation was for 90 min. at 37° in oxygen as the gas phase. The lipid was extracted from each tissue with chloroform-methanol (2:1, v/v) and washed until free of water-soluble radioactive material before estimation of the lipid radioactivity by scintillation counting.

Age of rat	Tissue	Wet wt. of tissue (mg.)	Oxygen uptake ($\mu\text{moles/g. wet wt.}$)	Radioactivity of lipid sulphate (counts/min./100 mg. fresh wt.)
Adult (over 1 year)	Cortex	73	92	1 850
Adult	Cortex	95	94	1 990
Adult	Cord	110	28.2	58 800
19 days	Cortex	94	71.3	12 060
19 days	Cord	84	47.7	235 000

Table 8. *Incorporation and subsequent persistence of [^{35}S]sulphate into the neural lipids of the growing rat*

A litter of 15-day-old rats (average wt. 25 g.) were each given 0.2 ml. of 0.9% sodium chloride solution containing 8 μC of [^{35}S]sulphate by intraperitoneal injection. Animals were killed at intervals up to 223 days after injection, the brain, cord and sciatic nerve were removed, and the lipid was extracted with chloroform-methanol (2:1, v/v) as described in the text. Water-soluble radioactive material was removed by washing and the radioactivity determined by scintillation counting. Plasma radioactivity was 16 800, 326 and 0 $\mu\text{C}/0.1$ ml. at 4 hr., 7 days and 26 days respectively after injection, and at least 75% of this radioactivity was associated with non-protein material. Counts are corrected for decay. Two samples of each extract were counted, and no difference was obtained in counts on extracts from which proteolipid protein had been removed.

Time after injection (days)	Wet wt. of brain (g.)	Total radioactivity in brain lipid (μC)	Wet wt. of cord (mg.)	Total radioactivity in cord lipid (μC)	Wet wt. of sciatic nerve (mg.)	Total radioactivity in nerve lipid (μC)
4 hr.	1.11	9 750	118	5 200	4	1 210
7	1.30	45 500	129	22 400	16	3 400
26	1.62	41 800	302	22 000	52	4 500
50	1.94	39 800	440	13 500	89	2 910
83	1.86	34 200	610	19 200	126	3 700
104	2.24	27 500	530	13 300	140	2 650
129	1.71	32 500	550	20 400	130	3 350
156	1.96	28 500	584	16 000	121	3 230
223	1.99	23 900	585	13 000	90	3 280

334 and 558 days. The brains were removed, suspended in 0.32M-sucrose, and fractionated by differential centrifuging and further sedimentation over 0.8M-sucrose (Davison *et al.* 1962). The distribution of radioactivity in lipid and protein, 334 days after injection with [^{35}S]methionine, in each

fraction is shown in Table 10. Most of the persisting radioactivity was found in the myelin fractions with both lipid and proteolipid protein [i.e. protein soluble in chloroform-methanol (2:1, v/v)] equally labelled (cf. Davison, 1961*a*). It was, however, unexpected to find radioactivity in the

Table 9. *Incorporation and subsequent persistence of [^{35}S]sulphate into the neural lipids of the mature rat*

In this experiment 5-6-month-old female rats (average wt. 259 g.) were given 0.2 ml. of 0.9% sodium chloride solution containing 8 μC of [^{35}S]sulphate by intraperitoneal injection. Further details were as given in Table 8.

Time after injection (days)	Wet wt. of brain (g.)	Total radioactivity (μC)	Wet wt. of cord (mg.)	Total radioactivity in cord lipid (μC)	Wet wt. of sciatic nerve (mg.)	Total radioactivity in nerve lipid (μC)
4 hr.	1.71	915	494	1210	68	493
1	1.97	750	558	325	82	229
3	2.00	474	582	302	120	164
21	1.90	441	500	221	82	110
54	1.81	360	600	296	120	127
99	1.85	345	530	282	51	37
126	1.98	324	650	276	130	60

Table 10. *Subcellular distribution of persistent radioactivity in rat brain*

Distribution of radioactivity in the lipids and protein, soluble in chloroform-methanol (2:1, v/v), of myelin, and in the subcellular fractions from a rat brain 334 days after intraperitoneal injection with 50 μC of [^{35}S]methionine when the rat was 16 days old. After 334 days the animal was killed and the brain suspended in 0.32M-sucrose. The suspension was separated into myelin and subcellular fractions (Davison *et al.* 1962). Lipid and proteolipid protein soluble in chloroform-methanol (2:1, v/v) was extracted by methods similar to those used by August, Davison & Maurice-Williams (1961). Radioactivity was determined by scintillation counting. Material insoluble in chloroform-methanol (2:1, v/v) contained less than 2% of the total radioactivity.

Fraction	Recovery of radioactivity in chloroform-methanol (%)		
	Total chloroform-methanol extract	Lipid	Proteolipid protein
Myelin separated from nuclei	26.3	13.9	12.1
Myelin separated from heavy mitochondria	42.2	22.0	20.3
Nuclei	1.95	1.95	0
Heavy mitochondria	10.6	10.6	0
Light mitochondria	15.85	15.85	0
Microsomes	0	0	0
Supernatant	5.25	4.1	1.15
Total recovery	103.7	68.4	33.55
Whole brain	100	66.4	33.6

Table 11. *Persistence of radioactivity in myelin and mitochondrial fractions of rat brain*

Rats previously injected when 15-16 days old with 50 μC of [^{35}S]methionine or 8 μC of [^{35}S]sulphate were killed and the radioactivity was determined in the lipid fraction of the pooled myelin and the heavy- plus light-mitochondrial fractions. Details of extraction methods and counting procedure were essentially as described by August *et al.* (1961).

Time after injection (days)	Labelled precursor	Radioactivity recovered in myelin and mitochondrial-lipid fractions (%)	Ratio:	Ratio:
			myelin radioactivity mitochondrial radioactivity	myelin lipid sulphate mitochondrial lipid sulphatide
334	[^{35}S]Methionine	94	1.36	1.28
558	[^{35}S]Methionine	98	2.6	3.3
558	[^{35}S]Sulphate	83	5.0	3.3

lipids of the mitochondrial fractions. This radioactivity could not be accounted for by contaminating myelin fragments in the heavy-mitochondrial fraction, although small myelin fragments were present in the light-mitochondrial fraction and thus some of the persisting ^{35}S in the fraction could be attributed to this labelled myelin. For this reason in the two later fractionations the light-mitochondrial fraction was also layered over 0.8 M-sucrose and centrifuged for 1 hr. at 15 000g at 2°: the myelin fractions were combined and the mitochondrial fractions were also pooled. The percentage of total radioactivity in the lipids of both fractions is shown in Table 11 together with the lipid sulphate content of each.

These results indicate that, though most of the persisting radioactivity, lipid and proteolipid protein is found in the myelin fraction, some ^{35}S also remains associated with mitochondria in the brain. This labelled sulphatide cannot be due to contaminating myelin since only traces of labelled proteolipid protein are found in the mitochondrial fractions from rats killed after 558 days, and, moreover, few myelin fragments can be detected in these latter mitochondrial preparations when examined by electron microscopy.

DISCUSSION

There is good evidence to suggest that all of the lipid sulphur in the mammalian central nervous system is present as the sulphate ester of cerebroside. Thus Green & Robinson (1960) showed that only one spot, with the same R_f as that of authentic cerebrin sulphate, could be detected on paper chromatography of brain lipid extracts. A minor lipid sulphate peak detected by column chromatography (Bakke & Cornatzer, 1961; and this paper) is associated with phospholipid (Jatzkewitz, 1958, 1960). However, there is no evidence at present to suggest that this minor component differs from the sulphatide A except in that it is associated with a small quantity of phospholipid.

To investigate the distribution of sulphatide in the brain at different ages it was first necessary to obtain a reliable method for the routine analysis of this lipid. In the technique finally adopted whole-brain lipid extracts are hydrolysed at 100° after the addition of hydrochloric acid to the washed chloroform-methanol (2:1, v/v) extract. The liberated inorganic sulphate is estimated turbidimetrically with barium chloride (Dodgson, 1961). This technique has been tested against other methods of analysis and found to be satisfactory.

As a result of previous chemical analyses of rat and human brain, during and after development, it has been suggested that there is a continual accretion of sulphatide in the brain throughout

life. This conclusion was apparently supported by metabolic studies with [^{35}S]sulphate (Radin *et al.* 1957). The first analyses of lipid sulphur changes in the rat during development were reported by Koch & Koch (1913). These workers estimated total lipid sulphur and followed its continual accumulation in the brain of rats up to 210 days old, not, as is widely quoted, for the animals' life-span. Later Bakke & Cornatzer (1961) also found a progressive deposition of sulphatide in rats of up to 180 days old. It is apparent that there is an accumulation of sulphatide in the rat brain only until about 100–120 days after birth (see Fig. 2). Similar observations have been recorded for cerebroside in rats (Kishimoto & Radin, 1959), total brain cerebroside slowly accumulating up to the age of 170 days. Although there is an accretion of sulphatide in the rat brain until the age of 4 months our results do not indicate any subsequent increase of the lipid/g. fresh wt. of whole brain. There may be a slight increase in the total amount of sulphatide, however, since the brain weight rises slowly throughout the life-span of the rat (Donaldson, 1911, 1924). Human-brain sulphatide has also been reported to increase in amount throughout life in a linear fashion (MacArthur & Doisy, 1919; Bakke & Cornatzer, 1961; Goldberg, 1961). Although it was concluded by MacArthur & Doisy (1919) that there was no time during life when sulphatide is not being synthesized in the human brain, their own results show no difference in absolute weight of whole-brain lipid sulphate in the three ages they studied (21, 35 and 67 years old). This misconception has remained in the literature unchallenged (Goldberg, 1961), and indeed Bakke & Cornatzer (1961) analysed samples of mixed human brain from subjects of 28, 39 and 60 years of age and also claimed to show that human-brain sulphatide sulphur increased with age in a linear pattern. Nevertheless this conclusion was proposed with reservation since only one sample of mixed tissue was analysed for each age (Bakke & Cornatzer, 1961). The results in the present paper clearly show that the reservation was justified and that there is no significant difference in the human-brain sulphatide content of either cerebral grey matter or cerebral white matter after the age of 30 years.

Since the maximum rate of deposition of sulphatide occurs during the period of myelination it was suggested (Koch & Koch, 1913) that sulphatide is a constituent of the myelin sheath. This possibility has been examined by: (a) study of the distribution of sulphatide in the brain; (b) study of the distribution of lipid sulphate in myelin and subcellular particles from rat brain; (c) isolation and analysis of the myelin proteolipid for sulphatide; (d) investigation of the metabolism of sulphatide.

Table 4 shows that the purest areas of human white matter, such as the corpus callosum and the pons, contain 6–7 times the concentration of sulphatide found in cerebral grey matter. These areas of white matter consist mainly of myelinated fibres and thus the results tend to support the hypothesis that sulphatide is a myelin lipid. However, the experimental observations in the rat do not support this conclusion completely. The pooled myelin fraction obtained from homogenates of whole brain by the present method accounts for some 40 % of the total sulphatide. The light-mitochondrial fraction also contains small myelin fragments which might possibly account for some 10 % of the sulphatide found in this fraction. Thus, in the rat, sulphatide does not appear to be a unique constituent of the myelin sheath.

Metabolic studies. With [^{35}S]sulphate the maximum uptake of the radioactive sulphate into lipid occurred in animals 15–20 days old. Balasubramanian & Bachhawat (1961) found that in the rat brain the formation of 3'-phosphoadenosine 5'-sulphatophosphate was at a maximum at birth and at about 12 days after birth. Myelination commences at about 10 days and is histologically most active at about 20 days after birth (Waelsch, Sperry & Stoyanoff, 1941; Wolman, 1957). This metabolic evidence therefore suggested that [^{35}S]sulphate was being rapidly incorporated into the developing myelin sheaths. Also, the [^{35}S]sulphate, once incorporated, underwent slow turnover somewhat similar to that found for certain phospholipids and other typical myelin lipids (Davison *et al.* 1959). This observation agrees with that of Green & Robinson (1960) who observed very slow turnover after the injection of rats (150 g. body weight) with [^{35}S]sulphate. Thus the small amounts of [^{35}S]sulphate taken up into adult-brain slices and intact cord probably represent slow turnover of sulphatide in the mature-rat brain and not continual accumulation of this lipid. No evidence for any sulphatide degradation could be found on incubation of labelled sulpholipid with suspensions of brain from young and old rats. Moreover, on incubation *in vitro* less [^{35}S]sulphate is taken up by viable adult-brain slices and spinal cord than by similar preparations from young rats.

The present analytical and metabolic results suggest therefore that sulphatide is a characteristic membrane lipid. Cerebroside sulphate is found in rat-brain, liver and kidney mitochondria and microsomes (Green & Robinson, 1960) as well as myelin. The sulphatide found in the microsomal fraction may represent a compartment which undergoes a more rapid turnover in the adult animal, for no detectable radioactivity persists in the fraction. The slow turnover of whole-brain sulphatide may then be taken to reflect the relative

metabolic stability of some part of myelin and also subcellular-particle membrane. Support for this hypothesis comes from a preliminary experiment in which labelled sulphatide was isolated from mitochondrial and myelin fractions of rat brain 334 and 558 days after the injection of [^{35}S]methionine or [^{35}S]sulphate into the neonatal animal.

SUMMARY

1. A relatively simple method for the determination of lipid sulphate in whole brain is described.
2. With this method the sulphatide content of different areas of human brain were estimated.
3. The accretion of brain sulphatide throughout the life-span of human subjects and in rats was investigated. No evidence was obtained for the continual accumulation of the lipid in the brain after development.
4. Maximum incorporation of labelled sulphate *in vivo* occurs in the rat brain at the time of myelination, and after incorporation there is a very slow turnover of this lipid, typical of the general metabolic stability of other constituents of the myelin sheath.
5. Less radioactive sulphate is taken up into the brain of the intact adult rat and this also undergoes slow turnover of sulpholipid. Studies *in vitro* confirm the view that this is the result of slow metabolism of sulphatide and not due primarily to a 'blood-brain barrier' effect.
6. It is suggested that sulphatide is a typical membrane lipid for it is found in mitochondria as well as myelin. Some evidence suggests that the sulpholipid of rat myelin and brain mitochondria is relatively metabolically stable.

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Glutamate-Dehydrogenase Inactivation by Reduced Nicotinamide-Adenine Dinucleotide Phosphate

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There has been considerable interest in the study of glutamate dehydrogenase [L-glutamate-NAD(P) oxidoreductase, EC 1.4.1.3], an enzyme that is readily disaggregated and aggregated (Olson & Anfinsen, 1952; Frieden, 1959*a*). Recent studies have indicated that crystalline glutamate dehydrogenase catalyses both leucine (Struck & Sizer, 1960) and alanine (Tomkins, Yielding & Curran, 1961) deamination, although at a slower rate, and that increased aggregation stimulates and inhibits the glutamate- and alanine-dehydrogenase activities respectively. It has been reported also that diethylstilboestrol and some steroids influence the activity, probably owing to aggregational effects (Yielding & Tomkins, 1960).

Contemporaneously with the demonstration by Inagaki (1959) that glutamate dehydrogenase was less stable to heat in the presence of reduced nicotinamide-adenine dinucleotide, it was shown that enzyme instability induced by substrates and co-factors is a fairly general phenomenon (Grisolia & Joyce, 1959). Such a finding might have important consequences in biology, particularly since it occurs at physiological concentrations and conditions (Tucker & Grisolia, 1962).

Glutamate dehydrogenase was selected for further study because many of its properties are known and because it plays a crucial role in

nitrogen metabolism. Glutamate dehydrogenase is remarkably unstable to reduced nicotinamide-adenine dinucleotide phosphate (Grisolia, Grady, Fernandez & Tucker, 1961; Frieden, 1961); these experiments were carried out at low salt concentrations. However, as the salt concentration increases, the enzyme becomes more stable to NADPH. It appears then that additional knowledge of the stability of this important enzyme is needed, particularly as it is affected by ionic strength.

This paper describes the effects of various salts, of adenosine 5'-diphosphate, of adenosine 5'-triphosphate, of pH and of temperature on enzyme stability to NADPH. It also shows that there is unmasking of SH groups during inactivation together with a change in rotatory power. Although induced inactivation of crystalline glutamate dehydrogenase by NADPH does not yield preparations with grossly changed aggregational properties, aggregation may be related to stability since reagents such as diethylstilboestrol are very effective in potentiating this inactivation. However, activity measurements may be misleading if induced changes in stability by reagents are not taken into consideration. Some of the possible biological implications of these findings are discussed in this paper.