Metabolism of Cellular Membrane Sulpholipids in the Rat Brain

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1. Metabolism of [^{35}S]sulpholipid was studied in rats of different ages after the injection of [^{35}S]sulphate. 2. The turnover of sulphatide in brain myelin and subcellular fractions was followed for long periods. 3. A small fraction of adult myelin underwent rapid metabolism, the rest being relatively metabolically stable. 4. Fast turnover of brain microsomal sulphatide during development was related to the process of myelination. 5. Turnover of mitochondrial sulphatide was at a lower rate than that calculated for liver mitochondria. 6. The relevance of the results to the metabolism of the whole membrane is discussed.

It has been suggested that the lipids of the central nervous system are almost exclusively associated with membranous structures (Seminario, Hren & Gomez, 1964; Eichberg, Whittaker & Dawson, 1964), for they are located in all subcellular fractions prepared from homogenates with the exception of the soluble fraction, which contains only a very small amount. Probably more than half of the total brain lipid is found in the myelin sheath (Cuzner, Davison & Gregson, 1965*a,b*), the rest being largely distributed among mitochondria, microsomes and synaptic endings (Adams, Davison & Gregson, 1963). Since there is evidence to suggest that lipid forms an integral part of the membrane (Gent, Gregson, Gammack & Raper, 1964) it was decided to study the metabolism of a representative membrane lipid for each of the various subcellular fractions of the brain. For this purpose sulphatide (cerebron sulphuric acid) was chosen, for it appears to be a common constituent of biological membranes (Davison & Gregson, 1962). Moreover, labelled sulphate is readily incorporated into brain sulpholipid, where it possesses the same metabolic characteristics as other membrane lipids (Green & Robinson, 1960; Bakke & Cornatzer, 1961; Cuzner *et al.* 1965*a*).

In the present study [^{35}S]sulphate has therefore been utilized for labelling brain membrane structures. Animals of various ages have been injected with [^{35}S]sulphate and the subsequent fate of labelled sulpholipid has been followed in myelin and subcellular fractions, and a comparison has been made of the turnover of each of the various

membrane structures of the developing brain with that in the adult rat.

METHODS

Albino Wistar rats of either sex were used throughout this work. The animals were given carrier-free [^{35}S]sulphate by intraperitoneal injection, except where stated.

Intraventricular injections were performed on adult animals that had previously had the part of the skull just anterior to the frontal-parietal suture and just lateral to the interfrontal suture thinned with a rose-head dental burr. This operation was performed under pentobarbitone anaesthesia at least 24 hr. and not more than 48 hr. before the injection. For the intraventricular injection animals were anaesthetized and maintained with ether and placed in a head clamp. An Agla micrometer syringe fitted with a 0.5 in. 30 B.W.G. needle clamped in a micromanipulator was centred over the exposed region of the thinned cranium. The needle was inserted to a depth of 3 mm. below the cranium and 10 $\mu\text{l.}$ of carrier-free [^{35}S]sulphate was injected at the rate of 1 $\mu\text{l.}$ every 15 sec.; 1 min. after completion of the injection the needle was slowly withdrawn, the scalp resutured and the animal allowed to revive. The animals normally recovered within 15 min. of completing the injection. All results were calculated in terms of an injected dose of 1 mc of [^{35}S]sulphate.

Preparation of subcellular fractions. Ether-anaesthetized rats were killed by exsanguination. The brains were quickly removed on to preweighed ice-cold containers. After the weighing, the tissues were suspended in 0.32 M-sucrose, containing EDTA (1 mM), by using a Potter-Elvehjem type of homogenizer (Aldridge, Emery & Street, 1960; Webster & Smith, 1964). Subcellular fractions were prepared as described by Cuzner *et al.* (1965*b*).

Preparation of extracts. Fractions were extracted with 19 vol. of chloroform-methanol (2:1, v/v) and each extract was washed exhaustively by repeated partition against water and 'synthetic upper phase' (Folch, Lees & Sloane-Stanley, 1957) until free of water-soluble radioactivity. Mixtures consisting of large volumes of sucrose solution (e.g. clear supernatant fractions) were extracted by the

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method described by Eichberg *et al.* (1964). Where large doses of [^{35}S]sulphate were given by intraperitoneal injection (e.g. adult animals given $11.68\text{ }\mu\text{C}$) rats under ether anaesthesia were perfused with 0.9% NaCl in 0.01% Na_2SO_4 solution for 5 min. before removing and freezing the brain. Brains from these adult animals and from those injected intracerebrally were homogenized in water (10%, w/v) and 1 ml. portions each treated with 1 ml. of cold 20% (w/v) HClO_4 . The acid mixture was centrifuged and the clear supernatant obtained was used for acid-soluble ^{35}S estimations. Portions of the aqueous homogenate were extracted with 19 vol. of chloroform-methanol (2:1, v/v), and water-soluble contaminants were removed by partitioning against water (Folch *et al.* 1957) and repeated washing with 'synthetic upper phase' mixtures containing added Na_2SO_4 (0.01%).

Determination of radioactivity. Dried lipid extracts were dissolved in xylene-phosphor mixture and radioactivity was determined in the Nuclear Enterprise scintillation counter NE5503 as described by Davison & Gregson (1962).

Separation and analysis of sulphatide. Samples of washed chloroform-methanol (2:1, v/v) lipid extract were run on thin-layer plates (Davison & Graham-Wolfaard, 1964) developed with chloroform-methanol-water (17:17:1, by vol.). Sulphatide was recovered by transferring silica zones to a column and eluting with 25 ml. of the developing solvent. Sulphatide was best estimated by sphingosine analysis (Lauter & Trams, 1962). This technique was checked by using ^{35}S -labelled brain lipid extracts: 94% of the total radioactivity was recovered in the area corresponding to sulphatide.

RESULTS

Previous work has indicated that myelin accounts for more than half the lipid of the central nervous system and that labelled precursors incorporated into such lipid undergo only slow turnover (Davison, 1964; Cuzner *et al.* 1965*a,b*). In studying the lipid metabolism of radioactively labelled brain subcellular fractions particular attention has therefore to be paid to separating myelin from each fraction. Thus homogenates of neural tissue in sucrose solution contain myelin fragments of varying size and hence preparations of subcellular fractions by differential centrifugation (e.g. Brody & Bain, 1952) are extensively contaminated (see Laatsch, Kies, Gordon & Alvord, 1962; Gregson, 1965). In the present work myelin has been removed from nuclear and mitochondrial fractions by a single density-gradient centrifugation (Adams *et al.* 1963). The first set of experiments were designed to assess, first, the extent of incorporation of [^{35}S]sulphate into the brain lipids at different ages of rat, correcting the results for brain/body weight ratios, and, secondly, the metabolism of membrane lipid from each of the various cellular fractions of the brain at different stages of development, as judged by uptake of [^{35}S]sulphate *in vivo*.

Incorporation of [^{35}S]sulphate into sulpholipid of rat brain of different ages. After interperitoneal

Fig. 1. Sulphate incorporation into whole-brain lipids. Rats of various ages were given carrier-free [^{35}S]sulphate in 0.9% NaCl solution by intraperitoneal injection. Animals were killed after 2 days and the radioactivity of the total washed brain lipid extract was determined. Results shown have been corrected for the brain/body weight ratio for a standard dose of $10\text{ }\mu\text{C}$ of [^{35}S]sulphate/25 g. body wt. per animal.

injection of [^{35}S]sulphate, the total [^{35}S]sulphate incorporated into whole-brain lipid (Fig. 1) shows a close similarity to that for the incorporation of [$1\text{-}^{14}\text{C}$]galactose into rat-brain neutral glycolipids (Burton, Sodd & Brady, 1958) and for the incorporation of [$1\text{-}^{14}\text{C}$]glucose into cerebroside galactose from the brain of mice of different ages (Moser & Karnovsky, 1959). Thus maximum amounts of [^{35}S]sulphate are taken up into the brain sulpholipids of the 20-day-old rats, whereas only small amounts are incorporated into brain lipids of rats less than 8 days old. The uptake of [^{35}S]sulphate into the lipids of the adult brain, though more than that found for newborn animals, was considerably less than that for 20-day-old rats. These experiments were repeated on separated subcellular fractions to assess the contribution of each to the metabolism of the whole-brain sulpholipid.

Labelling of myelin and brain subcellular-particle lipids in rats of different ages. Brain homogenates in sucrose solution were prepared from rats of different ages 2 days after injection with [^{35}S]sulphate and were separated into myelin, nuclear, synaptic-ending, mitochondrial, microsomal and supernatant fractions. Labelling of whole-brain homogenate lipids varied with age as indicated in Fig. 1, although the extent of incorporation was different in each of the subcellular fractions (Figs. 2 and 3). Thus the amount taken up into the mitochondria diminished with increasing age of injected rats, whereas the amount incorporated into the nuclear, microsomal,

synaptic-ending and supernatant fractions reached a maximum in animals 15–20 days of age. Radioactivity was extensively incorporated into myelin and this accounted for a high proportion of whole-brain ^{35}S -labelled lipid.

These experiments indicated three broad phases of metabolism, which can be correlated with stages in development of the brain. The first, when there is restricted labelling of brain lipid, coincides with

early growth and differentiation before myelination of this part of the central nervous system. The second, represented by rapid sulpholipid synthesis in all fractions (other than mitochondria), parallels the period of rapid myelination (Waelsch, Sperry & Stoyanoff, 1940; Wolman, 1957). The maximal rate of formation of adenosine 3'-phosphate 5'-sulphatophosphate also occurs during this second period (Balasubramanian & Bachhawat, 1961). The final phase is represented by the more steady metabolic state of the adult animal.

Since the amount of [^{35}S]sulphate incorporated after 2 days into each of the brain fractions varies with age, it is necessary to determine whether or not these different uptakes of [^{35}S]sulphate are related to the turnover characteristics of the individual membrane fractions. To elucidate this question metabolism of ^{35}S -labelled lipids of the brain fractions was determined in an extended period after injection of adult, 4-day-old and 12-day-old rats.

Metabolism of sulphatide in brain membrane fractions after the injection of [^{35}S]sulphate into 4-day-old rats. Since myelination does not become histologically detectable in the rat until it is 10 days *post partum* it was hoped that injection of labelled precursors into 4-day-old animals would result in labelling of all brain structures other than myelin. Rats of this age were given $25\mu\text{C}$ of [^{35}S]sulphate

Fig. 2. Sulphate incorporation into the lipids of whole brain and myelin prepared from rats of different ages. Carrier-free [^{35}S]sulphate was injected into groups of animals equivalent to $10\mu\text{C}/25\text{g. body wt.}$ After 2 days rats were killed and brain homogenates separated as described in the text into myelin and subcellular fractions. Results are expressed as lipid radioactivity/mg. of protein corrected for the brain/body weight ratio; myelin (Δ) and whole-brain ($\circ$) lipid radioactivity is shown. Protein was determined by the method of Murphy & Kies (1960).

Fig. 3. Sulphate incorporation into the lipids of brain subcellular fractions. Details were as given in Fig. 2. Results for supernatant (Δ), mitochondria ($\bullet$) and microsomes ($\circ$) are shown.

Fig. 4. Turnover of [^{35}S]sulphatide of neonatal rat-brain membrane fractions. The 4-day-old rats were given $25\mu\text{C}$ of carrier-free [^{35}S]sulphate by intraperitoneal injection. Animals were killed at various times up to 76 days after injection; in the early experiments brains from two to four animals were pooled to allow separation of about 2g. of nervous tissue. Fractionation and determination of radioactivity were as described in the text. Results shown are radioactivity (μC)/whole-brain fraction corrected to 1mc of [^{35}S]sulphate injected. Myelin ($\blacksquare$) was only separated in animals older than 12 days. Radioactivity in mitochondria, including synaptic endings ($\blacktriangle$), microsomes ($\triangle$) and supernatant ($\circ$) are shown. Results for the nuclear fraction are not included.

by intraperitoneal injection and killed at various intervals thereafter. For the first few days after injection two to four rats were killed and the brains pooled for separation into four main fractions: nuclei, mitochondria (including synaptic endings), microsomes and supernatant; myelin, when present, was recovered from the nuclear and mitochondrial pellets. Although there is (Fig. 4) little or no loss of incorporated $[^{35}\text{S}]$ sulphate from whole-brain lipid after injection, the distribution of radioactivity in the individual fractions alters considerably so that the increasing labelling of myelin compensates for turnover in other fractions. Moreover, the rate of turnover of labelled lipid is different for each fraction; for example, most $[^{35}\text{S}]$ sulphate is taken up into the mitochondrial fraction and subsequent loss of radioactivity is at first rapid with an initial half-life of several days, and thereafter metabolism appears to be much slower. Even after 76 days a trace of ^{35}S -labelled lipid is found in the mitochondrial fraction. Maximum incorporation of $[^{35}\text{S}]$ sulphate occurs after 2 days for the supernatant and after 3 days for the microsomal fraction, and again turnover is followed by persistence of radioactivity in each fraction. The nuclear fraction, however, differs from other fractions in that radioactivity, once taken up, remains with little loss. In surviving rats 12 days old or more, myelin was separated from other subcellular fractions. Surprisingly, this fraction contained more than 50% of the whole-brain radioactivity and, moreover, this persisted, with some increase up to the end of the experiment.

Metabolism of sulphatide in brain membrane fractions after the injection of $[^{35}\text{S}]$ sulphate into 12-day-old rats. In this experiment one litter of six young myelinating rats were injected with $10\mu\text{C}$ of carrier-free $[^{35}\text{S}]$ sulphate and animals were killed at intervals up to 31 days after injection. Results for brain fractions were recorded graphically and turnover rates computed. During this short experimental period there was only slow turnover of whole-brain lipids (half-life 30–40 days). Distribution of $[^{35}\text{S}]$ sulpholipid in the brain showed marked variation for each of the subcellular fractions (see Table 1); only one component was detected in the decay curve for loss of radioactivity from the lipids of all fractions but the experiment was of relatively short duration. $[^{35}\text{S}]$ Sulpholipid underwent slow turnover in the nuclear fraction, and no decay (Fig. 5) at all of ^{35}S -labelled myelin was noted (compare with Radin, Martin & Brown, 1957; Davison & Gregson, 1962); the myelin fraction also accounted for the highest proportion of incorporated $[^{35}\text{S}]$ sulphate. The results obtained for the mitochondrial and synaptic-ending fractions were very similar to those obtained in the newborn animals (see Fig. 4 and Table 1). Turnover of microsomal

Fig. 5. Incorporation of $[^{35}\text{S}]$ sulphate into myelin. Animals were injected when 4 days (Δ), 12 days ($\circ$) or more than 1 year ($\bullet$) old. Animals were killed at intervals after injection, the brains were removed and fractionated and lipid radioactivity was determined as described in the text. Results shown are total myelin radioactivity as a percentage of whole-brain radioactivity against time.

and supernatant sulphatide fractions was intermediate between those of the same fractions from neonatal and adult rat brain.

Incorporation of $[^{35}\text{S}]$ sulphate into the sulpholipids of adult brain. Fully mature rats of either sex were given carrier-free $[^{35}\text{S}]$ sulphate by intraperitoneal injection. At various intervals afterwards animals were anaesthetized, perfused for a few minutes to minimize possible contamination by blood $[^{35}\text{S}]$ sulphate and the brains were dissected out. The results showed that equilibrium between brain water-soluble $[^{35}\text{S}]$ sulphate and serum was reached in 4 hr. Graphical analysis indicated an immediate brain extracellular space of approx. 0.0307 ml./g. fresh wt. with a total sulphate space at equilibrium of 0.0618 ml./g. fresh wt., results that are in accord with those of Woodbury (1958).

Relatively small amounts of $[^{35}\text{S}]$ sulphate were, however, incorporated into the brain lipids after intraperitoneal injection; for this reason a reproducible technique for intracerebral injection was developed.

Preliminary experiments with Indian ink indicated that after intracerebral injection there was rapid dissemination of the dose throughout the ventricular system. No damage was noted at the site of the injection, nor were there any discernible abnormalities in treated animals for periods up to a year after injection. It was found that, whereas the rate of incorporation for $[^{35}\text{S}]$ sulphate into sulpholipid after intraperitoneal injection was equivalent to the rate of $0.0015\mu\text{C/hr./mc}$ of injected $[^{35}\text{S}]$ sulphate, that after intracerebral injection was about $0.15\mu\text{C/hr./mc}$ of injected $[^{35}\text{S}]$ sulphate. Intracerebral injections were adopted as a standard procedure for adult animals. Besides this greatly enhanced amount of $[^{35}\text{S}]$ sulphate incorporation

Table 1. *Comparison of turnover rates obtained from biological decay curves for single fractions from brains of rats of various ages*

The decay constant, K , is calculated as the regression coefficient of the lines $\ln(\text{radioactivity } \mu\text{C}) = \ln(a_0 - Kt)$, where t is in days. In the growing animals radioactivity is expressed in terms of the whole brain to allow for the effects of growth. Where the decay of a fraction is biphasic, two constants are calculated, and where necessary the 'fast' phase was corrected for the 'slow' component. The half-life, $t_{1/2}$, in days, was calculated from K . Entry of [^{35}S]sulphate into the slow pool was calculated by extrapolation of the slow-decay curve to $t = 0$, and expressed as a percentage of the total incorporated [^{35}S]sulphate at $t = 0$.

	4-day-old rats		12-day-old rats		Adult rats		
	$-K$	$t_{1/2}$ (days)	$-K$	$t_{1/2}$ (days)	$-K$	$t_{1/2}$ (days)	Entry of [^{35}S]sulphate into slow pool (% of total entry)
Nuclei	0.232	3.0	0.105	6.4	0.263	2.6	—
			—	—	0.038	18.2	—
Mitochondria	—	—	0.703	~1.0	1.99	0.35	15.0
			—	—	0.018	38.6	—
Synaptic endings	—	—	0.035	19.25	0.232	3.0	—
Combined mitochondria and synaptic endings	0.45	1.5	—	—	—	—	—
	0.055	12.5	—	—	—	—	—
Microsomes	0.065	10.5	0.23	3	1.927	0.36	46.0
			—	—	0.077	10.3	—
Supernatant	0.134	5.2	0.254	2.7	2.553	0.27	18.0
			—	—	0.133	5.2	—
Myelin	No detectable decay		—	—	0.266	2.7	3.0
			—	—	0.005	138	—

into brain sulpholipid, the quantity of labelled water-soluble [^{35}S]sulphate fell very rapidly after injection (half-life for acid-soluble sulphate, 1.64 hr.).

Metabolism of [^{35}S]sulpholipid of adult brain membrane fractions. After intracerebral injection [^{35}S]sulphate was incorporated into the lipid of all subcellular fractions in the adult rat brain and the subsequent fate of the [^{35}S]sulpholipid was characteristic for each fraction. Maximal labelling occurred 2–3 hr. after injection in all fractions other than myelin, where peak incorporation was noted 4 hr. after injection (Fig. 5). The myelin fraction accounted for 30–40% and 50–60% of the whole-brain labelled sulpholipid 2 hr. and 4 hr. respectively after injection. The highest specific activity was found in the supernatant fraction (0.296 $\mu\text{C}/\mu\text{mole}$ of sulphatide), whereas the maximal specific activity of the myelin fraction was 0.126 $\mu\text{C}/\mu\text{mole}$ of sulphatide.

The rapid efflux of labelled precursor from the brain of the adult rat has allowed the calculation of turnover rates from data obtained almost immediately after injection as well as from animals from long-term survival experiments. All fractions except synaptic endings showed biphasic decay curves (see also Khan & Wilson, 1965). The supernatant, microsomal and mitochondrial fractions have fast components with half-lives of a few hours (Table 1). By comparison, the fast components for turnover of nuclear and myelin fractions had a

half-life of about 2½ days. Results for the metabolism of the slower components in the decay curves for all fractions were comparable with results obtained for the newborn animals.

DISCUSSION

To study the metabolism of the various brain membrane structures sulphatide has been chosen as a representative lipid. Labelling of non-structural sulphatide appears to be of significance only in very short-term experiments, particularly during a period of active lipid biosynthesis. Acid-soluble [^{35}S]sulphate is rapidly excreted from the body (Dziewiatkowski, 1953; Davison & Gregson, 1962), and after intracerebral or intraperitoneal injection negligible amounts are available in the central nervous system after a day. Synthesis of [^{35}S]sulphatide of high specific activity is therefore restricted to a few hours after injection. In practice, labelling of sulphatide and the subsequent metabolism of [^{35}S]sulpholipid in brain cellular membranes closely resembles that found in similar experiments with ^{14}C - and ^3H -labelled precursors (Khan & Wilson, 1965; M. L. Cuzner, A. N. Davison & N. A. Gregson, unpublished work). Thus it seems that in this type of experiment [^{35}S]sulphatide metabolism of each membrane fraction may accurately reflect the metabolism of the whole basic membrane structure.

Metabolism and growth of the myelin sheath.

Inorganic sulphate is incorporated into the whole-brain sulphatide *in vivo* from the first day *post partum* onwards. The proportion of the dose incorporated varies with age (Fig. 1 and Davison & Gregson, 1962), and appears to be related to the overall rate of deposition and to the total amount of myelin in the brain at any age. Thus the incorporation of [^{35}S]sulphate into lipid is minimal in animals aged up to 10 days, when little myelin is present or being formed, and is maximal at about 20 days, coinciding with maximal overall myelination. In the adult rat the extent of incorporation of [^{35}S]sulphate into brain lipid is greater than would be expected if most of the sulphatide were metabolically stable (Davison & Gregson, 1962). Since labelling of myelin sulphatide accounts for most of the incorporated [^{35}S]sulphate the metabolism of this fraction is considered first.

Morphological evidence (Bunge, Bunge & Ris, 1961) indicates that the myelin sheath is derived from the plasma membrane of oligodendroglia in the central nervous system. This membrane fraction is sedimented in the microsomes of brain homogenate. The rapid turnover of microsomal sulphatide and the delayed appearance of labelled myelin found in the present study in the youngest group and to a smaller extent in the 12-day-old rats can then be explained if the myelin sheath is derived from glial-cell membrane. Sulphatide of this newly formed myelin remains for a prolonged period with little turnover (Table 1 and Davison & Gregson, 1962). This is in contrast with the adult rat, where uptake of [^{35}S]sulphate into myelin sulphatide is at a maximum 4 hr. after injection and there is a subsequent rapid turnover of most of the labelled sulpholipid. It has been found that the proportion of whole-brain labelled sulpholipid in the myelin of the adult animal stays constant up to 70 days after intracerebral injection (Fig. 5). In the initial period after injection turnover in the myelin parallels that in the subcellular fractions so that the proportions labelled stay about the same. Even after 70 days not all the labelled sulphatide is found in the myelin fraction, although myelin accounts for much more of the persisting radioactivity after injection of the younger rats. There is at present no obvious explanation of these results, which underline the differences in metabolism of labelled myelin in young and adult rats. The rapid attainment of maximum labelling, followed by a relatively rapid rate of turnover, suggests that deposition or possibly the major route of synthesis of myelin membrane differs in the adult from that in the young. The period of rapid decay after the rapid incorporation is followed in turn by a period of slow decay. Such a result suggests that myelin sulphatide in the adult animal can be considered to exist in two metabolic compartments: a small but rapidly

exchanging compartment open to a large but slowly exchanging compartment. In a preliminary report of these results this concept was shown diagrammatically (Davison, 1964). From the present results it has been calculated by extrapolation that only 2.9% of the incorporated [^{35}S]sulphate enters the slowly exchanging pool.

On the basis of persistence of isotopic labels in the lipids and proteins in the central nervous system it has been suggested that the myelin sheath is metabolically a relatively inert structure in the adult animal (Davison & Dobbing, 1961). The finding that a part of the [^{35}S]sulphatide incorporated into adult myelin undergoes rapid turnover appears at first to be at variance with this hypothesis. It is thus of great importance to obtain some assessment of the absolute size of this fast pool in the myelin fraction.

From the plot of:

$$\frac{^{35}\text{S incorporation in myelin}}{\int_0^t \text{acid-soluble } ^{35}\text{S} \times dt}$$

against time (t), the initial slope gives an incorporation rate of $0.003075 \mu\text{C/hr.}/\mu\text{C}$ of precursor/g. wet wt. of whole brain equivalent. The values of 2.17 mg. of $\text{SO}_4^{2-}/100 \text{ ml.}$ of rat plasma (Brown & Lewis, 1941) and a brain sulphate space of $0.062 \text{ ml./g. wet wt.}$ give a calculated inorganic sulphate content of rat brain of $1.347 \times 10^{-6} \text{ g./g. wet wt.}$ At zero time after intracerebral injection the [^{35}S]sulphate radioactivity is $102 \mu\text{C/g. wet wt.}$ and the specific activity is $75.82 \text{ c/g. of SO}_4^{2-}$. From this specific activity the incorporation rate is $1.1493 \times 10^{-6} \text{ g. of SO}_4^{2-}/\text{sec./g. wet wt. of whole brain equivalent}$. This incorporation rate can be expressed as $7.4817 \times 10^9 \text{ molecules/sec./g. wet wt. of whole brain equivalent}$. From the decay characteristics of the fast pool of the myelin fraction, the rate of exchange of sulphate in this pool is $0.693 \times N/t, \text{ sec.}^{-1}$. This gives an exchange rate for the fast pool of $1.6797 \times 10^{18} \text{ molecules/sec./mole of sulphatide sulphate in the fast pool}$. Thus the size of this pool is:

$$\begin{aligned} & \frac{\text{observed incorporation rate}}{\text{exchange/mole}} \\ &= \frac{7.4817 \times 10^9}{1.6797 \times 10^{18}} \\ &= 4.4544 \times 10^{-9} \text{ moles of sulphatide sulphate} \end{aligned}$$

Since the total myelin sulphatide in the rat is $2.25 \times 10^{-6} \text{ moles/g. wet wt.}$ (Cuzner *et al.* 1965b) the fast pool as a percentage of the total is only 0.2%.

The small size of the dynamic pool in isolated myelin supports the idea that most of the mature myelin sheath is relatively metabolically stable. The extent of the incorporation and the high

turnover rate into the dynamic pool cannot be explained by the presence of contaminating mitochondria or microsomes, since radioactivity associated with these subcellular fractions is so much less than that of the myelin. Moreover, the calculated size of the dynamic pool of sulphatide precludes it being the complete exterior or interior of the sheaths, since these surfaces would represent some 5–10% of the total myelin membrane. It is possible that the radioactivity of the metabolically active fraction of myelin may be due to sulphatide of a high specific activity or a structure such as the axolemma concentrated in the myelin as prepared. However, a rapidly metabolized polyphosphoinositide component has been described even in a highly purified myelin preparation (Eichberg & Dawson, 1965). Previous work (LeBaron, Hauser & Ruiz, 1962) suggested that the high turnover rate of the phosphoinositides was related to their acting as a 'bridge' between protein and lipid of the membrane structure. Myelin segments, however, are not homogeneous morphologically and it may be that certain regions of the sheath are metabolically active. Thus the paranodal region of fibres in the central nervous system exhibits a region of unspiralling of the myelin lamellae, enclosing oligodendroglial cytoplasm and organelles (Bunge *et al.* 1961; Metuzals, 1964), and may well represent a region of metabolic activity in the central nervous system, as has been suggested for peripheral nerve (Williams & Landon, 1963), and an important site of lipid synthesis. However, although only a small proportion of the myelin is in a dynamic state, this pool still accounts for most of the observed labelling in the adult brain. Thus, although considerable amounts of labelled precursor are incorporated into the central myelin of adult rats (August, Davison & Williams, 1961; Eichberg & Dawson, 1965; Cuzner *et al.* 1965a,b; Ansell & Spanner, 1965), it does not negate the general validity of the hypothesis that most of the myelin sheath is metabolically stable.

Metabolism of microsomal and mitochondrial sulphatide. Sulphatide is a constituent lipid of brain membrane structures other than myelin (Davison & Gregson, 1962). In general, incorporation of [³⁵S]sulphate into these other structures also varies with age (Figs. 3 and 4; see Abdel-Latif & Abood, 1965, for similar experiments with [³²P]-phosphate). Many metabolic and enzyme characteristics of rat brain undergo most rapid change between 10 and 25 days after birth (Flexner, 1955), coinciding with the most rapid structural and functional growth of the brain. Incorporation of [³⁵S]sulphate into the sulphatide of the nuclear, supernatant and synaptic-ending fractions of the brain also changes maximally over this period (Figs. 3 and 4). However, no consistent differences were noted in the metabolism of the brain nuclear

and synaptic-ending fractions prepared from animals of different ages, and since the fractions are heterogeneous no comment will be made about their metabolism.

The observed incorporation pattern of [³⁵S]-sulphate into the brain microsomal sulphatide fraction can be related to the period of myelination. Thus the decreased labelling of microsomal sulpholipid 2 days after injection in young compared with adult animals suggests the utilization of glial membrane only during myelination (see above). The pool of rapidly metabolized sulphatide in both microsomal and brain supernatant fractions may represent active biosynthesis of non-membrane sulpholipid (see Table 1), in conformity with current views on the mechanism of sulphatide synthesis (Goldberg, 1961; McKhann, Levy & Ho, 1965).

There is little evidence from the present experiments of any marked difference in the metabolism of brain mitochondrial sulphatide after the first few days *post partum* (see Fig. 4). Although previous results suggested the possibility of a stable component in brain mitochondria (Davison & Gregson, 1962), no such component was found in the present experiments, in which improved techniques were employed. Khan & Wilson (1965) also failed to demonstrate any persistent labelling in the mitochondrial fraction after the administration of ³H-labelled water. The longest half-life for mitochondrial sulphatide turnover in adult animals was approx. 38.6 days (Table 1). If this more stable sulphatide pool is taken to reflect the behaviour of the whole mitochondrial membrane it appears that turnover in the brain is somewhat slower than the half-life of 10.3 days described for liver mitochondria (Fletcher & Sanadi, 1961). Clearly there are two basically different types of membranous structure, myelin representing the metabolically stable type and mitochondria representing the more labile type (Fleischer & Rouser, 1965).

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