Synthesis and turnover of cerebroside sulfate of myelin in adult and developing rat brain

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Abstract The turnover of cerebroside sulfate (sulfatide) was followed in both microsomal and myelin fractions of developing and adult rat brains after an intracerebral injection of $Na_2{}^{35}SO_4$. In the adult rats, the specific radioactivity of sulfatide of the microsomal fraction reached a maximum 12 hr after the injection, and after 3 days it was reduced to less than 30% of the maximum. In contrast, the specific radioactivity of the myelin sulfatide did not reach a peak until 3 days after the injection and remained essentially at the same level for as long as 6 months. In the case of 17-day-old rats, the specific radioactivity of myelin sulfatide reached a maximum level around 12 hr after the injection and then appeared to decline. The decline was most marked 2–6 days after the injection, suggesting an apparently rapid turnover of myelin sulfatide. When a correction was made for deposition of newly formed sulfatide, the results indicated that the turnover of myelin in the developing animals was also relatively slow. In vitro experiments with purified myelin and 3′-phosphoadenosine-5′-[^{35}S]phosphosulfate showed that myelin does not catalyze the galactocerebroside sulfotransferase reaction. This enzyme was found mainly in the microsomal fraction. In vivo studies suggested that a transfer of sulfatide molecules from the endoplasmic reticulum to myelin might occur. In order to obtain direct evidence for such a transfer, rat brain slices after pulse labeling with $Na_2{}^{35}SO_4$ were washed free of the isotope and reincubated with nonlabeled Na_2SO_4. The specific radioactivity of the microsomal sulfatide declined, with a concomitant rise in the specific radioactivity of the myelin sulfatide. These observations are therefore consistent with the postulate that myelin sulfatide is probably synthesized in the endoplasmic reticulum.

Supplementary key words [^{35}S]sodium sulfate · 3′-phosphoadenosine-5′-[^{35}S]phosphosulfate · microsomes · in vitro labeling studies · transfer of lipids between membranes

Metabolic studies of myelin using various radioactive precursors of lipids and proteins have been interpreted in the past to indicate that myelin in the adult animal is rather an inert structure (1). In those studies the precursor was injected into the animals either intravenously or intraperitoneally. However, recent studies in which there was intracerebral or intraventricular administration of the precursors of phospholipids have given results indicating that a substantial part of the phospholipid molecules in the adult myelin is in a fairly rapid equilibrium with other components of brain tissue (2–4). It appears that the route of administration of the isotope and the biological property of the precursor used may account to some extent for the contradictory results. The very long half-lives found for various myelin lipids after intravenous or intraperitoneal injection could be due to incorporation of the small percentage of the total radioactive precursors available to brain for exchange or turnover, due to the blood–brain barrier, and reutilization of the degradation products, which may persist for a long time in the animal body. The intracerebral route of injection of isotope could be more advantageous for turnover studies in brain because after such an injection the tissue is subjected to a sudden pulse of radioactivity, and greater incorporation is obtained in the subcellular fractions, which allows observations over longer periods (4). Cerebroside sulfate (sulfatide) is particularly abundant in myelin (5). The turnover of sulfatide in rat brain myelin and other subcellular fractions has been studied previously by Davison and Gregson (6). In their studies, sodium [^{35}S]sulfate was injected intraperitoneally into the developing animals and intracerebrally into the adult animals. It was suggested that most of the adult rat brain myelin sulfatide was metabolically stable; however, 0.2% of the total pool of myelin sulfatide was rapidly turning over. In view of the opposite results obtained with phospholipids in myelin after intraventricular injection of precursors (2–4), it seemed appropriate to reinvestigate the sulfatide turnover in highly purified myelin as well as in microsomes and other subcellular membranes in rat brain.

It has been shown that the greatest amount of labeling occurs in the sulfatide of myelin compared with other subcellular fractions after an injection of sodium [^{35}S]sulfate (6). It has been suggested that myelin does not contain the

Abbreviations: TLC, thin-layer chromatography; PAPS, 3′-phosphoadenosine-5′-phosphosulfate.

enzyme responsible for the synthesis of sulfatide (7, 8) and that the sulfatide is mainly synthesized in the endoplasmic reticulum (microsomes) and is transported to the myelin membrane via a lipoprotein (9). The experiments described in this paper demonstrate that the galactocerebroside sulfotransferase reaction is not catalyzed by purified myelin. It also describes the in vitro synthesis of sulfatides in myelin of rat brain slices. Turnover studies in vivo and pulse–chase experiments in vitro with slices provide evidence to indicate that microsomal sulfatide could be the precursor of sulfatide in myelin.

MATERIALS AND METHODS

Intracerebral injections

Female adult albino rats weighing 200 ± 5 g were anesthetized with ether, and each was injected intraventricularly with 100 μCi of sodium [^{35}S]sulfate (sp act 738 mCi/mmole) in a 10-μl volume using a Stoelting stereotaxic instrument essentially as previously described (4). The young animals (17 days old) of either sex weighing 30 ± 3 g were injected intracerebrally with 75 μCi of sodium [^{35}S]sulfate per animal. The site of injection in these cases was as close as possible to the left lateral ventricle as judged by the locations of sutures on the skull. The injection needle was introduced vertically into the brain about 2–3 mm below the surface of the skull, using a stereotaxic instrument.

Preparation of subcellular fractions after in vivo injection

The methods for the preparation of purified myelin as well as microsomes and supernatant solution have been previously described (4) and were used with slight modifications. In this procedure the myelin is isolated from the crude nuclear fraction and constitutes approximately 70% of the total myelin (4). The crude myelin fraction obtained after the second 0.8 M sucrose gradient centrifugation was resuspended in 0.32 M sucrose by homogenization in a hand-operated homogenizer and centrifuged at 755 *g* (avg) for 11 min. The supernate was discarded and the pellet was rewashed four times as described above. The washed pellet was then osmotically shocked and applied to a continuous linear gradient of 0.32–0.8 M sucrose as previously described (4). In the case of young animals (17–22 days old), the washing was done as described above; however, the myelin pellet was packed each time by continuing the spinning of the rotor for 2 min more at 3000 *g* (avg) after the first spin for 11 min at 755 *g* (avg). This washing procedure was essential to remove traces of microsomal contamination of myelin. The myelin and microsomal pellets were suspended in 1.5 ml of 0.32 M sucrose. The supernatant solution above the microsomal pellet was carefully removed together with the floating lipids and was lyophilized, and the powder was then redissolved in 1.5 ml of water. The mitochondrial fraction was prepared from the brain homogenate essentially as described by Eichberg, Whittaker, and Dawson (10). The mitochondria (P_2C fraction of Eichberg et al. [10]) were washed by resuspending the pellet in 20 ml of 0.32 M sucrose by homogenization and centrifuging for 10 min at 6000 *g* (avg). The supernate was discarded and the pellet was washed three more times as just described. The washed pellet was suspended in 1.5 ml of 0.32 M sucrose.

Aliquots of the subcellular fractions were saved for the marker enzyme assays as well as protein determinations.

Acid-soluble sulfate

Cold trichloroacetic acid (10 ml, 10% w/v) was added to an aliquot of rat brain homogenate (1 ml) and allowed to stand for 30 min in the cold. The mixture was centrifuged to remove the precipitated material. The supernate was washed four times with equal volumes of water-saturated diethyl ether to remove the trichloroacetic acid. The washed water phase was then adjusted to pH 7.4 by blowing ammonia vapor from a pipet. Radioactivity in an aliquot of the water phase was determined. An equal aliquot was used for the determination of phosphorus by the method of Bartlett (11).

Isolation of sulfatides and determination of specific radioactivity

The subcellular fractions in 0.32 M sucrose were extracted with 20 vol of chloroform–methanol 2:1 (v/v) as described previously (4). The extract was washed three times with 0.2 vol of a mixture containing chloroform–methanol–water–10 mM sodium sulfate 3:45:46:1 (by vol) and three times with the same mixture but without sodium sulfate. The washed extract was made up to 20 ml with chloroform–methanol 2:1 (v/v), and 1 ml was taken for the determination of radioactivity. Suitable aliquots were also removed for the determination of sulfatide in the extract. The method of Kean (12) was used for this assay. All assays were done in triplicate for each sample and an average value was taken. The remainder of the extract was taken to dryness under N_2, redissolved in chloroform, and then passed through a small (5 × 20 mm) column of silicic acid as described by Vance and Sweeley (13). The nonpolar lipid fraction was eluted with chloroform, and the galactolipid fraction was eluted with a mixture of acetone–methanol 9:1. 80–90% of the radioactivity applied to the column was recovered in the acetone–methanol fraction. The rest of the radioactivity was eluted in the methanol fraction. Specific radioactivity of the sulfatide was determined in the glycolipid fraction. The glycolipid fraction was further purified by thin-layer chromatography on silica gel G plates, and the sulfatide spot was eluted from the plate as described by Nagai and Kanfer (7). The specific radioactivity of the sulfatide in the eluted sample was again determined.

Synthesis of sulfatide in vitro by isolated subcellular particles

The subcellular particles were prepared as described in the preceding sections. The incubation conditions were similar to those described by Farrell and McKhann (14) for the rat brain microsomal system. The incubation system contained 100 mM Tris–HCl, pH 7.1 (at 37°C), 2.5 mM ATP, 20 mM $MgCl_2$, 0.23 μCi of [^{35}S]PAPS, 0.08 mg of galactocerebroside, 0.2% Triton X-100, and enzyme source in a final volume of 0.5 ml. The mixture was incubated in a shaker bath for 1 hr at 37°C. After the incubation, 10 ml of chloroform–methanol 2:1 (v/v) was added.

The sulfatides were extracted, the extracts were washed, and the radioactivity in the extract was determined as described above.

Incubation of slices and pulse–chase experiments

Slices were prepared from brains of young rats (16–21 days old) by removing the entire brain and slicing it at 0–4°C with a microtome (15) into slices about 0.5 mm thick. The slices were about 3 × 3 mm. They were kept suspended in cold Krebs modified medium containing 15.7 mM KH_2PO_4, 111.2 mM NaCl, 5.4 mM KCl, 1.3 mM $MgCl_2$, 4.0 mM $NaHCO_3$, and 15.0 mM glucose. The pH of the medium was adjusted to 7.4 with NaOH. Equal amounts of slices (0.5–1 g) suspended in 2 ml of the above medium were incubated with 100 μCi of sodium [^{35}S]sulfate at 37°C with shaking under a gas phase of 95% O_2–5% CO_2.

In the pulse–chase experiments, after the incubation with the radioactive precursor for the desired time interval, the slices were washed in the cold with 25 ml of the Krebs medium containing 2 mM sodium sulfate, and the slices were collected by centrifugation at 3000 *g* (avg) for 10 min. The supernate was discarded, and the pelleted slices were resuspended in 25 ml of the Krebs medium containing 2 mM sodium sulfate and recentrifuged. The washing procedure was repeated three times. These washed slices were then resuspended in 2 ml of fresh Krebs medium containing 2 mM sodium sulfate and reincubated for the desired length of time as before but without the isotope. The effect of the washing procedure on the ability of the slices to synthesize sulfatide was determined as follows. The slices were washed after the initial incubation exactly as in the case of the pulse–chase experiment with 25 ml of Krebs medium but without 2 mM sodium sulfate and were reincubated for the desired time periods with 2 ml of Krebs medium (without 2 mM sodium sulfate) and with the same amount of fresh sodium [^{35}S]sulfate added originally. After the incubation, the latter slices were washed twice with Krebs medium containing 2 mM sodium sulfate. The slices that were incubated as such were washed only once. The washed slices from both the experiments were then resuspended in 25 ml of 0.32 M sucrose, allowed to equilibrate for 15 min, and centrifuged. The slices were washed once more in 0.32 M sucrose. These were homogenized in 20 vol of 0.32 M sucrose in a motor-driven Teflon Duall homogenizer with 0.008-inch clearance. Myelin, microsome, and supernatant fractions were isolated as described in the preceding section. However, in some experiments when brains from younger animals (16–18 days old) having small amounts of myelin were used for making slices, the homogenate was centrifuged at 13,000 *g* (avg) for 30 min in a Sorvall refrigerated centrifuge to obtain a combined nuclear and mitochondrial fraction. The supernate was recentrifuged at 166,500 *g* (avg) for 1 hr in a Beckman model L2-65B ultracentrifuge (Ti-50 rotor) to collect the microsomal pellet and high-speed supernate. The 13,000 *g* pellet, which contained virtually all the rat brain myelin, was resuspended in 5–7 ml of 0.32 M sucrose, layered on 30 ml of 0.8 M sucrose, and spun for 30 min at 80,000 *g* (avg) in an SW-27 rotor of the Beckman ultracentrifuge. The myelin-rich layer at the interface was collected, diluted with 1.1 vol of water, relayered on 0.8 M sucrose, and recentrifuged as above. The crude myelin was then treated as described in the preceding section to obtain purified myelin.

The subcellular fractions obtained from slices were treated as described above for the assay of marker enzymes, extraction of lipids, and determination of specific radioactivity in sulfatide.

Determination of radioactivity

Samples in organic solvents were evaporated to dryness, a toluene-base scintillation solution was added, and the samples were counted in a Tri-Carb scintillation counter (4). The radioactivity in the acid-soluble sulfate samples was determined by using Bray's solution (16). Corrections were made for the amount of radioactivity lost due to radioactive decay of ^{35}S from the time of injection.

Marker enzyme assays

NADPH–cytochrome *c* reductase and cytochrome *c* oxidase were determined as described previously (4). Protein was measured by the biuret method (17) with crystalline bovine serum albumin as standard.

Materials

Sodium [^{35}S]sulfate (sp act about 700 mCi/mmole) and [^{35}S]PAPS (sp act 708 mCi/mmole) were purchased from New England Nuclear, Boston, Mass. Sulfatide and galactocerebroside used as standard were purchased from Supelco, Bellefonte, Pa. The purity of these lipids was checked by TLC. Unisil (activated silicic acid) was from Clarkson Chemical Co., Williamsport, Pa. Uniplates coated with silica gel G were from Analtech, Inc., Newark, Del.

RESULTS

Comments on the purity of subcellular fractions

The myelin fraction was purified extensively by washing the crude myelin fraction with 0.32 M sucrose and isolating the fraction after centrifugation at very low centrifugal force (755 *g* avg). This procedure eliminates possible contamination by synaptosomal or microsomal fractions. The microsomal marker enzyme NADPH–cytochrome *c* reductase assays showed that the myelin thus isolated had less than 2% contamination by the microsomal proteins. This enzyme activity differed with different microsomal preparations and age of the animals. The average values were within 0.15–0.3 absorbance units of reduced cytochrome *c*/mg protein/min measured in a 1-cm light path in a final volume of 1 ml for the brain microsomal preparations. The washed mitochondrial preparations obtained from brain were contaminated up to about 10–15% with the microsomal protein. There was negligible mitochondrial contamination (cytochrome *c* oxidase activity) in the myelin, microsomal, or supernatant fraction. The myelin fraction reported in in vivo experiments was isolated from the nuclear fraction. This myelin fraction is more easily freed of microsomal contamination compared with the myelin isolated from the crude mitochondrial fraction. However, in the slice experiments, when very young animals (16–18 days old) were used, the myelin was isolated from the combined nuclear and crude mitochondrial pellet and then purified.

Incorporation of sodium [^{35}S]sulfate in vivo in adult animals

The incorporation of sodium [^{35}S]sulfate, after intraventricular injection, into different rat brain subcellular fractions is shown in Figs. 1 and 2. The results shown for myelin and microsomes (Fig. 2) are based on the specific activity obtained after isolation of sulfatide by purification through silicic acid column chromatography and thin-layer chromatography as described in Methods. However, the amounts of sulfatide present in the supernatant and mitochondrial fractions were very small; hence, values shown in the figures are based on the sulfatide isolated after silicic acid column chromatography. In any case, the values of the specific radioactivities of sulfatide determined on the chloroform–methanol extract and after silicic acid column chromatography and thin-layer chromatography were not significantly different. The radioactivity present in the chloroform–methanol lipid extract of different subcellular fractions was found to be present only in the sulfatide, as shown by TLC of the lipid extracts in two different systems (13, 18). Radioactivity could not be detected in the area cochromatographing with cholesterol sulfate in the propanol–ammonia–water 160:26.6:13.3 (by vol) system (18). The specific radioactivity in the acid-soluble pool was determined based on the amount of total phosphate in the acid-soluble fraction of the rat brain homogenate and thus does not represent true but a relative specific radioactivity. It is expected that the amount of acid-soluble sulfate should be smaller than the amount of acid-soluble phosphorus, hence the true specific radioactivity should be higher than measured.

The radioactivity in the acid-soluble pool declined rapidly with time. 1 day after the injection, the remaining radioactivity was only 10% of the radioactivity at 3 hr after the injection. The rapid loss of radioactivity in the acid-soluble pool indicated a rapid metabolism and/or elimination of the injected sodium sulfate by brain. The specific radioactivity of the microsomal sulfatide reached maximum around 6 hr after the injection and then rapidly declined. The decrease of the microsomal specific radioactivity in the initial time period was faster than at later time periods, and radioactivity could still be detected in the microsomal pool 197 days after the injection. However, the radioactivity in the acid-soluble pool was practically completely gone by 20 days. The specific radioactivity of the sulfatide in the high-speed supernatant fraction (Fig. 2, ×) reached a maximum around 12 hr after the injection and then declined rapidly, as in the case of microsomal sulfatide. The specific radioactivity in the myelin, however, was low in the initial time periods and reached maximum after 3 days. Thereafter, the specific radioactivity declined slightly and then remained practically unchanged. There appeared to be only a slight loss of specific radioactivity even after 197 days. The rate of incorporation of radioactivity in the mitochondrial sulfatide was very similar to that found in the microsomal fraction. The total amount of incorporation in the mitochondrial fraction was very small. When the entire experiment was repeated with two separate groups of animals, essentially similar results were obtained.

Incorporation of sodium [^{35}S]sulfate in vivo in developing animals during myelination

The initial rate of incorporation of sodium [^{35}S]sulfate into the microsomal, myelin, and high-speed supernatant fractions as well as the radioactivity in the acid-soluble sulfate of young animals is shown in Fig. 3.

It has been shown previously (6, 19) that the maximum amount of sodium [^{35}S]sulfate incorporation into sulfatide of brain takes place during the period of active myelination. The incorporation is maximal when the rats are about 20 days old, whereas only a small amount of radioactivity is incorporated into brain lipids of rats less than 8 days old and adult rats. In the present study, rats were injected intracerebrally when they were 17 days of age. The specific radioactivities of sulfatides of all the subcellular fractions were higher in the young animals compared with adults. The radioactivity in the acid-soluble pool declined

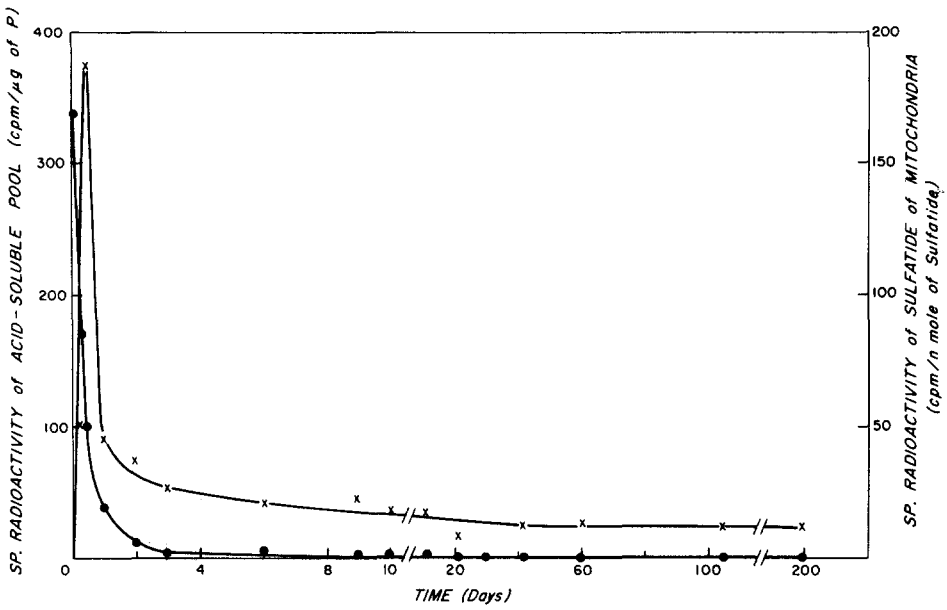

Fig. 1. Specific radioactivities of sulfatides in the mitochondrial fraction (×) and acid-soluble sulfate (●) of brain after intracerebral injection of sodium [^{35}S]sulfate into adult rats.

very rapidly. 3 hr after the intracerebral injection, most of the radioactivity in the acid-soluble pool was cleared from the brain (Fig. 3). The specific radioactivity of the microsomal sulfatide reached a maximum 3 hr after the injection and then declined gradually with time. The specific radioactivity of the supernatant fraction showed some variations, and the peak of incorporation could not be precisely determined. However, the specific radioactivity of the myelin sulfatide appeared to reach a maximum at a later time, around 11–12 hr after the injection. The results of the incorporation of [^{35}S]sulfate into myelin, microsomal, and supernatant fractions at longer time periods are shown in Fig. 4. The specific radioactivity of the microsomal sulfatide declined rapidly, and 3 days after the injection the specific radioactivity in this fraction was only 18% of the maximum. The specific radioactivity of the myelin sulfatide also declined with time after reaching a maximum. The rate of this decline appeared to be maximal 2–6 days after the in-

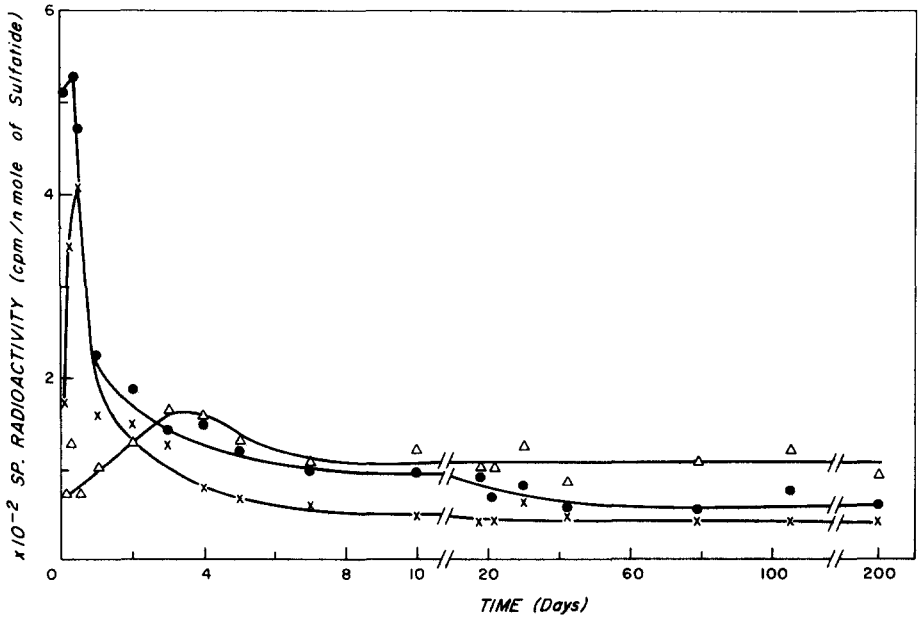

Fig. 2. Specific radioactivities of sulfatides in the microsomal (●), myelin (△), and supernatant (×) fractions of brain after an intracerebral injection of sodium [^{35}S]sulfate into adult rats. Myelin was isolated from the crude nuclear pellet.

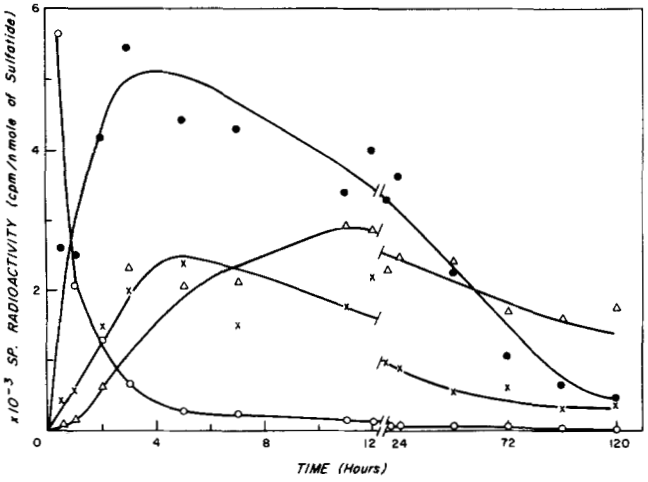

Fig. 3. Specific radioactivities of sulfatides in the microsomal (●), myelin (Δ), supernatant (×), and acid-soluble (○) fractions of brain after intracerebral injection of sodium [^{35}S]sulfate into 17-day-old rats. Specific radioactivity in the acid-soluble fraction is expressed as cpm/μg of phosphorus. Brains from three animals were pooled for each time point, and myelin was isolated from the crude nuclear fraction.

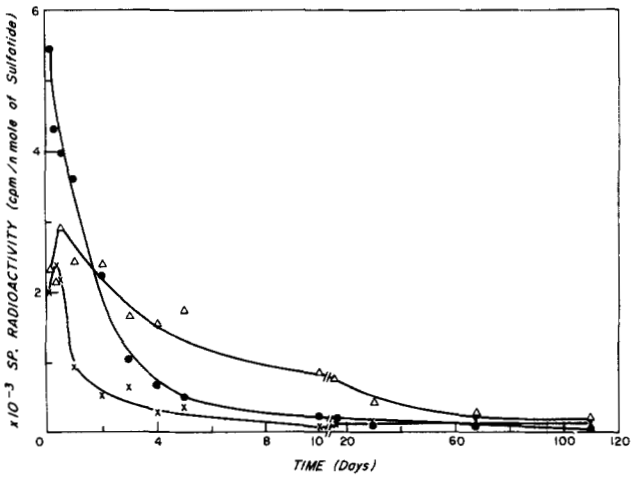

Fig. 4. Specific radioactivities of sulfatides in the microsomal (●), myelin (Δ), and supernatant (×) fractions of brain after intracerebral injection of sodium [^{35}S]sulfate into 17-day-old rats. Myelin was isolated from the crude nuclear pellet.

jection. The injected animals were 19–23 days of age during this period. Hauser (20) reported rapid changes in the concentration of sulfatide in the whole brain during maturation. His results showed a rapid deposition of sulfatide in animals between 17 and 25 days old. The deposition of sulfatide continued until maturity, though not at the same rate. Though these results were based on the determination of sulfatides of the whole brain, it is known (5) that most cerebral sulfatide is present in myelin. In view of these observations, the apparent decline in specific radioactivity of myelin and microsomal sulfatides in the case of developing animals could at least in part be explained as being due to the rapid deposition of newly formed nonradioactive sulfatide. The rate of deposition of sulfatides in brain was estimated by determining the amount of sulfatide in an aliquot of the rat brain homogenates at different time intervals. These values were used to correct the specific radioactivities of myelin and microsomal fractions as due to the deposition of newly synthesized nonradioactive sulfatides. The corrected values are replotted in Fig. 5. It is evident from the figure that though microsomal sulfatide lost its specific radioactivity with time, even after accounting for the deposition of newly synthesized material, the rate of fall of the specific radioactivity of myelin sulfatide appeared to be slow.

Synthesis of sulfatides by subcellular fractions in vitro from 3′-phosphoadenosine-5′-[^{35}S] phosphosulfate

The incubation conditions for the synthesis of sulfatide in vitro from [^{35}S]PAPS have been well worked out by Farrell and McKhann (14), and essentially their system was used. The total incorporation by microsomes and purified heavy myelin isolated from adult rats under different conditions is given in Table 1. It is evident that the maximal incorporation takes place with the microsomal fraction in the presence of the nonionic detergent Triton X-100 and externally added galactocerebroside, while there is negligible synthesis by myelin under the same conditions. The incorporation was linear at least up to 2 hr and up to 2.5 mg of microsomal protein. In a separate experiment, various subcellular fractions were isolated from brains of 21-day-old rats. The mitochondrial and myelin fractions were made as free from microsomal contamination as possible, and they were incubated with [^{35}S]PAPS under the conditions previously described. The amount of contamination in each subcellular fraction by the microsomes was determined by NADPH–cytochrome *c* reductase assay, and the incorporation due to

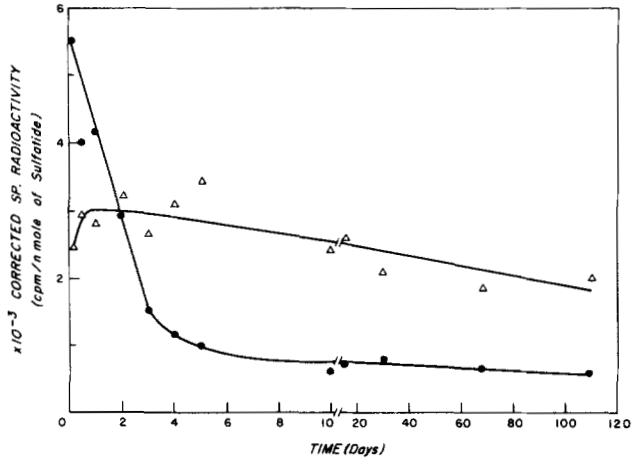

Fig. 5. Specific radioactivities of sulfatides in the microsomal (●) and myelin (Δ) fractions of brain after intracerebral injection of sodium [^{35}S]sulfate into 17-day-old rats, corrected for dilution by newly formed nonradioactive sulfatide.

TABLE 1. Incorporation of [^{35}S]PAPS into sulfatides by microsomes and myelin

Condition	Total Radioactivity
	cpm
Microsomes alone	5,175
Microsomes + Triton X-100	6,440
Microsomes + galactocerebroside	5,055
Microsomes + Triton X-100 + galactocerebroside	12,290
Myelin alone	80
Myelin + Triton X-100	175
Myelin + Triton X-100 + galactocerebroside	250

Microsomes (1.6 mg of protein) and purified myelin (1.9 mg of protein) isolated from adult rat brain were incubated for 1 hr at 37°C in a medium containing 100 mM Tris–HCl buffer, pH 7.1, 2.5 mM ATP, 20 mM $MgCl_2$, and 0.46 μCi of [^{35}S]PAPS in a final volume of 0.5 ml. Triton X-100 when added to the medium was in a final concentration of 0.2% (w/v). The amount of galactocerebroside when added to the medium was 0.08 mg suspended in a final concentration of 0.002% of Triton X-100. The myelin was purified from the nuclear pellet.

such contamination was calculated. The results of the incorporation of [^{35}S]PAPS into sulfatide of different subcellular fractions are given in Table 2. The maximal incorporation occurred with the microsomal fraction. Significant incorporation also occurred with synaptosomal and nuclear fractions. However, these two fractions were not purified and contained appreciable amounts of microsomes. Mitochondria and myelin incorporated a small amount of radioactivity, but this could be accounted for by microsomal contamination.

The chloroform–methanol extract of each of these subcellular particles after incubation was chromatographed by TLC (7). The plates were scanned with a TLC radioscanner (Varian Aerograph) as well as radioautographed. In the case of the nuclear and microsomal fraction, all the radioactivity appeared to be present in the area corresponding to the sulfatide spots. However, in the case of the synaptosomal fraction, 80% of the radioactivity appeared together with the sulfatide spots and the rest of the radioactivity moved with the solvent front. The incorporation in mitochondrial and myelin fractions was too low for chromatographic analysis.

The incorporation of [^{35}S]PAPS into sulfatide of myelin, isolated from the nuclear fraction of 21-day-old rat brains, was studied in vitro by adding increasing amounts of microsomes to a constant amount of purified myelin. The amount of incorporation into sulfatide increased linearly with the amount of microsomes added to myelin. When such a linear relationship was extrapolated to zero microsomal contamination, the amount of synthesis by myelin alone was zero.

Pulse–chase experiments

The myelin isolated from the slices had less than 3% contamination by microsomal proteins as measured by the

TABLE 2. Incorporation of [^{35}S]PAPS into sulfatides of various subcellular fractions

Fraction	Total Radioactivity	Specific Activity	Calculated Total Radioactivity That Could Be Due to Microsomal Contamination
	cpm	*cpm/mg protein*	*cpm*
Nuclei	1,665	1,040	2,872
Mitochondria (purified)	500	215	2,261
Synaptosomes	3,420	4,275	2,888
$Myelin_1$ (purified from crude mitochondrial fraction)	290	483	745
$Myelin_2$ (purified from crude nuclear fraction)	295	214	591
Microsomes	12,370	8,138	
Supernate	0	0	0

The incubation conditions were the same as described in the text. The amounts of protein used for the assay were (mg): nuclei, 1.6; mitochondria, 2.32; synaptosomes, 0.8; $myelin_1$, 0.6 mg; $myelin_2$, 1.38; microsomes, 1.52; and supernate, 0.31. The subcellular fractions were isolated from 21-day-old rats according to Eichberg et al. (10) and purified as described in the text.

NADPH–cytochrome *c* reductase assays. The specific activity of the latter enzyme in the microsomes isolated after incubation of the slices at various time intervals did not appreciably change and thus still could be used as a convenient marker enzyme.

Results of a pulse–chase experiment shown in Fig. 6 are representative of several such experiments performed by varying experimental conditions slightly. The specific radioactivities of the sulfatides of the microsomal, myelin, and supernatant fractions from slices increased almost linearly up to 5 hr, even though the slices after 2 hr of incubation were extensively washed and reincubated in the presence of fresh isotope. This incorporation indicated intactness of the slices to form sulfatide even after long time periods. When the washed slices were reincubated with nonradioactive sodium sulfate, the specific radioactivity of the sulfatide of the microsomal fraction decreased. However, the specific radioactivity of the sulfatide of myelin fractions continued to increase at least up to 3 hr after the removal of the labeled precursor. The specific radioactivity of the sulfatide of the supernatant fraction increased up to 2 hr after the removal of the precursor and then decreased. The incubations were not continued for more than 5 hr because in other experiments it was noticed that the rate of formation of sulfatide in the subcellular fractions of the slices began falling off after 5 hr. There was no significant difference in the incorporation when myelin was isolated either from the crude nuclear pellet or from the crude mitochondrial fraction. The specific radioactivi-

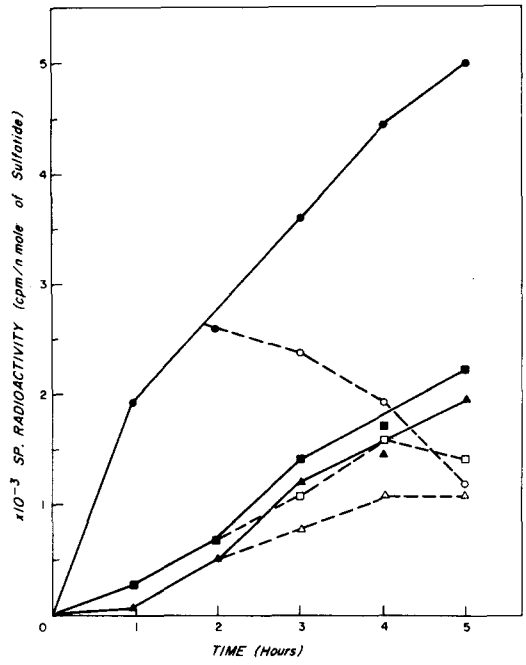

Fig. 6. Specific radioactivities of sulfatides in the microsomal (● and ○), myelin (▲ and △), and supernatant (■ and □) fractions of rat (18 days old) brain slices after pulse labeling with sodium [^{35}S]sulfate. After 2 hr of labeling with the precursor, the slices were washed free of the isotope and reincubated either in the presence of the original amount of radioisotope (closed symbols) or in the presence of nonradioactive sodium sulfate (open symbols). Myelin was isolated from the combined nuclear and mitochondrial pellet.

ties of sulfatides of microsomes, supernate, and particularly myelin isolated from slices of brain of young adult animals were slightly lower than those of the developing animals. However, overall results obtained were very similar.

DISCUSSION

In our studies, after an intraventricular injection of sodium [^{35}S]sulfate into adult rats (Figs. 1 and 2), almost 90% of the radioactivity in the acid-soluble pool disappeared within 1 day after the injection. After 3 days, only a trace of radioactivity in the acid-soluble pool could be detected. The rate of loss of radioactivity in the acid-soluble pool was considerably more rapid than that observed when either [^{32}P]phosphate, [^{14}C]glycerol, or [2-^{14}C]ethanolamine was injected and the radioactivity was determined under similar conditions (4). In the latter three cases, significant radioactivity and, presumably, the precursor were present in the brain even after 7–20 days, and measurable radioactivity could still be found at longer time periods up to 3 months. It appears from these results that metabolism of sodium sulfate and its eventual elimination from rat brain is a fairly rapid process compared with phospholipid precursors described. The incorporation of sodium [^{35}S]sulfate into microsomal, mitochondrial, and supernatant fractions of brain appeared to be complete within 12 hr, and at longer time periods the specific radioactivity began to decline in all these fractions. The incorporation of [^{35}S]sulfate in myelin sulfatide was a slow process; the maximum incorporation took place 3 days after the injection, and even after 6 months the specific radioactivity of the myelin sulfatide was diminished only slightly (about 30% of the maximum). The rate of appearance of labeled sulfatides in these fractions suggested that there could be a precursor–product relationship among sulfatides of microsomes, supernate, and myelin. If it is assumed that the sulfatides are synthesized in the endoplasmic reticulum and are transferred to other subcellular fractions through the cytosol fraction, then at longer time periods the specific radioactivity of the sulfatide in the supernatant fraction should reach a value equal to or slightly higher than that of the microsomal fraction. The specific radioactivity of the sulfatide in the supernatant fraction declined more rapidly than microsomal sulfatide, and at longer time periods the specific activity in the supernatant fraction was always lower than the microsomal sulfatides (Fig. 2). However, the microsomes examined in these experiments were not fractionated and thus may have contained other non–endoplasmic reticulum membranes, such as plasma membranes, and small fragments of myelin (21). At longer time periods, the specific radioactivity of the sulfatide in the latter two subfractions should be higher than that of pure endoplasmic reticulum. The observed higher specific radioactivity of the sulfatide of the total microsomal fraction at longer time periods could be due to these contaminants. The incorporation of [^{35}S]sulfate into sulfatide of myelin continued even when there was negligible acid-soluble precursor available to the brain. This would indicate that the myelin was receiving its complement of sulfatide from other membrane-bound lipids. Once it was incorporated into myelin the sulfatide appeared to have little turnover. These results, in general, agree with the conclusion of Davison and Gregson (6) that most of the adult rat brain myelin is relatively metabolically stable. However, they have reported the presence of a small but rapidly exchanging pool in the adult myelin based on the observation that in adult rats there was a maximum incorporation of sodium [^{35}S]sulfate into sulfatide of myelin at 4 hr after an intraventricular injection. In our experiments, such a rapid uptake of precursors into myelin could not be demonstrated.

The total amount and the rate of incorporation of sodium [^{35}S]sulfate into sulfatides of microsomes and myelin of developing brain were relatively high compared with adult animals (Figs. 3 and 4). Again, the rate of elimination of injected precursor, as represented by the radioactivity in the acid-soluble pool, also appeared to be faster in the developing animals (Fig. 3). The specific radioactivity of the sulfatide in myelin of developing animals continued to increase, even though the water-soluble precursor, as

measured in the acid-soluble pool, was almost negligible. The rate of formation of sulfatide in myelin would indicate that the microsomal sulfatide could be the precursor of myelin sulfatide.

The specific radioactivity of the microsomal as well as myelin sulfatide after an intracerebral injection of sodium [^{35}S]sulfate into developing animals appeared to decline with time after reaching a maximum (Fig. 4). However, as shown in Fig. 5, this rate of decline in myelin could be due to rapid deposition of newly formed sulfatide during this developing period (20) with little breakdown or exchange but effectively lowering the specific radioactivity.

The results obtained indicate that the myelin sulfatide of the adult as well as developing rat brain is very stable, and once deposited in myelin it appears to undergo very slow turnover or exchange with other subcellular fractions. The phospholipids of myelin, however, appeared to turn over, though at different rates (4, 22). This would suggest that myelin metabolically is not a single entity and that different constituents turn over at their own specific rates rather than as a single unit.

Previous studies (8, 14, 19, 23) have indicated that the enzyme galactocerebroside sulfotransferase is mainly localized in the microsomal and synaptosomal membranes as well as to a lesser extent in other subcellular particles, including myelin. Recently, Neskovic, Sarlieve, and Mandel (24) reported the presence of sulfotransferase activity in myelin and "myelinlike" fractions. However, in all the previous studies, myelin was not extensively purified. The results in Tables 1 and 2 indicate that the enzyme is localized only in the microsomal fraction and possibly to a very small extent in the synaptosomal fraction. The incorporation observed in other subcellular fractions, viz., nuclei, mitochondria, and myelin, could be due to the presence of microsomal contamination of the subcellular fractions. It is assumed that the microsomal marker enzyme NADPH–cytochrome *c* reductase is present neither in other subcellular particles nor in myelin. It has been shown previously that mitochondria from brains or liver were unable to synthesize major nitrogen-containing phospholipids in vitro (21, 25). The results in this paper demonstrate that isolated mitochondria and myelin do not contain the enzyme galactocerebroside sulfotransferase.

Herschkowitz et al. (9) found that after an intraperitoneal injection of sodium [^{35}S]sulfate in young rats, the microsomal sulfatide had the highest specific radioactivity. When the "chasing" dose of unlabeled sodium sulfate was injected after 2 hr, there was a sharp drop in the specific radioactivity of the microsomal sulfatide, while the incorporation into myelin sulfatide appeared to increase. Similar results were obtained in pulse–chase experiments in vitro with slices from rat brain (Fig. 6). When the slices were pulse labeled with sodium [^{35}S]sulfate, the specific radioactivity of the sulfatide was in the order of microsomal > supernatant > myelin. After 2 hr of incubation, when the slices were washed free of the precursor and reincubated with nonlabeled sodium sulfate, the incorporation of radioactivity in the microsomal sulfatide appeared to stop and the specific radioactivity began to decline gradually. The specific radioactivities of the sulfatides of the supernatant and myelin fractions, however, continued to increase. The decline in the specific radioactivity of the microsomal sulfatide after the removal of the radioisotope could be due either to dilution of the microsomal pool by the newly synthesized material of lower specific radioactivity or to exchange of sulfatide between the microsomal fraction and other subcellular particles. However, for the time period considered, the contribution by the newly synthesized sulfatide with nonlabeled sulfate would amount to only a small percentage of the total microsomal sulfatide pool, and calculations show that a decline in specific radioactivity of the microsomal sulfatide could not be explained totally as due to this contribution. Again, the breakdown of the microsomal pool by sulfatases would cause a decrease in the total radioactivity but not specific radioactivity, unless it is assumed that there are two pools of the microsomal sulfatide, one small rapidly turning over pool that is preferentially hydrolyzed while the other metabolically more stable pool remains unaffected. So far there has been no evidence available for the presence of such separate pools in the microsomal fraction. The continuous increase in the specific radioactivity of the sulfatide of myelin after the removal of the precursor can be explained as due to exchange of the sulfatide from the more rapidly labeled endoplasmic reticulum, possibly through the supernatant fraction. It is to be expected that the rise in the specific radioactivity of the myelin sulfatide will not be as large as the fall of that of the microsomes because the total sulfatide pool of myelin is 5–10 times larger (5) than the microsomal sulfatide pool. Eichberg et al. (10) have shown that myelin and microsomes isolated from guinea pig brain contained about equal amounts of lipid phosphorus, viz., about 16 μg/mg of nondiffusible solids. Cuzner, Davison, and Gregson (5) also found that total phospholipids were about equal in rat brain myelin (0.63 μmole) and microsomes (0.767 μmole) per milligram of dried lipids. If the data are replotted taking into consideration this difference in pool size, it can be demonstrated that the increase of radioactivity in myelin is roughly accounted for by the decrease of radioactivity in microsomes. It could be argued that the rise in the specific radioactivity of the myelin sulfatide could be due to a precursor pool in the slices that could not be affected by the change in the incubation medium and is independent of the microsomal sulfatide precursor pool. However, so far there has been no evidence for such an independent pool.

The results obtained here thus, in general, support the hypothesis originally put forward by Dawson (26) that complex lipids are synthesized in the endoplasmic reticulum, and intracellular transport and exchange of the newly synthesized material could be carried out through the intermediation of a soluble lipoprotein in the cyto-

plasm. This hypothesis is supported by results from many different laboratories (27–29) that demonstrated the exchange of phospholipids between microsomes and mitochondria as well as synaptosomes in vitro and possibly in the whole cells (30–32). The results of Herschkowitz et al. (9) on the synthesis of sulfatide of myelin in vivo also support such a hypothesis. However, so far it has not been possible to demonstrate a direct exchange of phospholipids or sulfatide (personal observations) between microsomes and myelin (27). Though Pleasure and Prockop (33) claimed to demonstrate a transfer of [^{35}S]sulfatide from the microsomal fraction to myelin of sciatic nerves of chick embryos in vitro, these results should be considered with caution because the amount of radioactivity used to demonstrate the exchange was very low and myelin was not checked for microsomal contamination after the incubation.

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