The Association of the Sulphogalactosylglycerolipid of Rat Brain with Myelination

By JOANNE PIERINGER,*†‡ G. SUBBA RAO,† PAUL MANDEL† and
RONALD A. PIERINGER†‡

†Centre de Neurochimie du CNRS, 67000 Strasbourg, France, and

‡Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140, U.S.A.

(Received 5 January 1977)

The sulphogalactosylglycerolipid of rat brain is closely associated with the process of myelination, as demonstrated by the following observations. 1. The lipid is barely detectable in rat brain before 10 days of age, accumulates rapidly between age 10 and 25 days, and remains relatively constant in amount (between 0.3 and 0.4 μmol per brain) thereafter into adult life. 2. The activity of adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase is almost absent before 10 days of age, attains a maximum at age 20 days, and slowly decreases thereafter with increasing age. This developmental pattern correlates well with that of other myelin-specific metabolites. 3. Both the concentration of the sulphogalactosylglycerolipid and the activity of sulphotransferase are greatly decreased in the non-myelinating jimpy mouse. 4. The myelin fraction of rat brain contains most of the sulphogalactosylglycerolipid. The lipid occurs in a diacyl and an alkylacyl form. Determinations of the relative amount of each type in brain showed about a 1:1 mixture in both 21-day-old and adult rats. Rats injected with $\text{H}_2^{35}\text{SO}_4$ at 20 days of age lost ^{35}S from the diacyl form at a higher rate than from the alkylacyl compound over a 21-day period. These data suggest that the diacyl form has a higher turnover than the alkylacyl derivative. The percentage of the total sulpholipid content of brain contributed by the sulphogalactosylglycerolipid is 16% in 21-day-old rats and 8.4% in adult rats.

The novel sulphogalactosylglycerolipid of rat brain has been found to exist in both a diacyl (Flynn *et al.*, 1975) and an alkylacyl (Levine *et al.*, 1975) form. The diacyl derivative was partially characterized as a 3'-sulphogalactosyldiacylglycerol and was shown to be biosynthesized from 1,2-diacyl-3- β -D-galactopyranosyl-*sn*-glycerol and adenosine 3'-phosphate 5'-sulphatophosphate in the presence of a 1% Triton X-100 extract of microsomal fraction from brain (Flynn *et al.*, 1975; Subba Rao *et al.*, 1977). The alkyl ether form had chromatographic properties (Levine *et al.*, 1975) similar to those of the 2-acyl-1-alkyl-3- β -D-(3'-sulphogalactosyl)-*sn*-glycerol of testis and spermatozoa (Ishizuka *et al.*, 1973; Kornblatt *et al.*, 1974).

Our previous studies have shown the galactosyldiacylglycerol of rat brain to be an excellent marker metabolite of myelination (Inoue *et al.*, 1971; Deshmukh *et al.*, 1971). Because the galactosyldiacylglycerol is a direct precursor of the sulphogalactosylglycerolipid, we initiated studies on the possible association of the latter with the process of

myelination. Our results of this investigation are reported here.

Materials and Methods

Animals

Studies on the quantity of sulphogalactosylglycerolipid and of the adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase activity in brains of rats of different ages were carried out on an inbred line of rats (similar to the Sprague-Dawley strain) maintained at the Centre de Neurochimie, Strasbourg, France. All litters were of mixed sex and were adjusted to eight pups immediately after birth. Other investigations of rats used females purchased from West Jersey Biological Supply Farm, Wenonah, NJ, U.S.A. Pregnant female mice carrying the sex-linked jimpy mutation, which is characterized by a severe lethal deficiency of myelin in the central nervous system (Sidman *et al.*, 1964), were obtained from the Jackson Laboratories, Bar Harbor, ME, U.S.A. These animals were the source of the jimpy male and sibling control mice used in our studies.

* Present address: Philadelphia College of Osteopathic Medicine, Department of Physiological Chemistry, 48th and Spruce Streets, Philadelphia, PA 19139, U.S.A.

Chromatography systems

The following systems were used: I, silicic acid (either Unisil, Clarkson Chemical Co., Williamsport, PA, U.S.A., or SilicAR, Mallinckrodt, New York, NY, U.S.A.) column (2 cm $\times$ 15 cm) eluted successively with 300 ml of chloroform, 500 ml of acetone and 300 ml of methanol; II, precoated thin layers of silica gel 25 or 60 (E. Merck, Darmstadt, Germany) developed with chloroform/methanol/water (65:25:4, by vol.); and III, Whatman no. 1 paper developed (ascending) with 1 M-ammonium acetate (pH 7.5)/ethanol (3:7, v/v).

Assay of sulphogalactosylglycerolipid

Rat brains and fractions of brains were extracted with 20 vol. of chloroform/methanol (2:1, v/v) overnight at 4°C by the procedure of Folch *et al.* (1957). Chromatographically pure [^{35}S]sulphogalactosylglycerolipid biosynthesized *in vivo* by rat brain was added (usually 10000 d.p.m. of specific radioactivity approx. 1000 d.p.m./nmol) at the beginning of the extraction. After washing with water and theoretical upper phase (chloroform/methanol/water, 3:48:47, by vol.) the lipids in the chloroform phase were dried and placed on a column of silicic acid (system I). The sulphoglycerolipids were eluted with acetone and were further purified on thin layers of silica gel (system II). The lipids were detected with iodine vapour. The spot containing ^{35}S (as detected by a Geiger counter or by counting the radioactivity of a portion of the eluted material in a liquid-scintillation spectrometer) was eluted thoroughly with chloroform/methanol (2:1, v/v), and the quantity of sulphur present in the sulphogalactosylglycerolipid (both diacyl and alkylacyl forms present) was determined by the method of Kean (1968). Two or three concentrations of each sample were assayed in duplicate or triplicate for determination of sulphur content. Pure cerebroside sulphate (Applied Sciences Laboratories, State College, PA, U.S.A.) from ox brain was used as the standard. The percentage recovered was calculated from the amount of ^{35}S in the final purified lipid. The small amount of ^{35}S -labelled lipid added at the beginning was subtracted from the final total quantity of sulpholipid. The presence of the added radioactive sulphogalactosylglycerolipid proved essential, because incomplete elution was noted from the silicic acid column by acetone (as much as 5% loss here) and particularly from the silica gel of the thin-layer plate (as much as 25% loss here). Even well-defined spots of pure ^{35}S -labelled lipid placed directly on the silica gel in most cases could not be completely removed from the silica gel. The diacyl and alkylacyl forms of the sulphogalactosylglycerolipid were readily separated from other lipids in the acetone fraction of system I, but not from each other

by system II (Flynn *et al.*, 1975). Because the Kean (1968) method measures compounds containing lipid-soluble sulphur, care was taken to prevent contamination by traces of dish-washing detergents.

Preparation of ^{35}S -labelled diacyl and alkylacyl derivatives of sulphogalactosylglycerol

In a typical preparation, six rats (19 days old) were injected intracerebrally with 0.25 mCi (10 μl) of carrier-free $\text{Na}_2^{35}\text{SO}_4$ (New England Nuclear Corp., Boston, MA, U.S.A.). The brains were excised 18 h after injection and extracted with 20 vol. of chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957). The lipid in the chloroform extract was purified by silicic acid column chromatography and t.l.c. (systems I and II). Over 2.6×10^6 d.p.m. of [^{35}S]sulphogalactosylglycerolipid and 12.9×10^6 d.p.m. of cerebroside sulphate were recovered. The specific radioactivity of the sulphogalactosylglycerolipid was usually 1000–3500 d.p.m./nmol. The compounds were shown to be pure by treatment of the lipids with mild alkali (deacylating conditions), by the technique described by Ambron & Pieringer (1971). The ^{35}S -labelled compound that chromatographs with standard cerebroside sulphate (R_F 0.23) remained soluble in chloroform, and the ^{35}S -labelled compound at the R_F (0.31) of sulphogalactosylglycerolipid became soluble in water. On rechromatography of the chloroform-soluble ^{35}S -labelled compound in system II, it ran the same distance as cerebroside sulphate and not as sulphogalactosylglycerolipid. Chromatography of the water-soluble ^{35}S -labelled material in system II revealed two ^{35}S -labelled compounds; one remained at the origin with standard 3'-sulphogalactosylglycerol (Flynn *et al.*, 1975) and the other ran the same distance (R_F = 0.22) as 1-alkyl-3-O-(3'-sulphogalactosyl)-sn-glycerol, the deacylated form of the sulpholipid from testis (Ishizuka *et al.*, 1973; Kornblatt *et al.*, 1974). The 1-alkyl-3-O-(3'-sulphogalactosyl)-sn-glycerol ran at the solvent front and the 3'-sulphogalactosylglycerol had R_F 0.69 in system III.

Quantitative analysis of the diacyl and alkylacyl derivative of 3'-sulphogalactosylglycerol

The diacyl and alkylacyl forms of the sulphogalactosylglycerolipid were most readily separated by chromatographing the deacylated derivatives of the lipid in systems II and III (R_F values given above). The two compounds on the chromatograms were detected by exposure to iodine vapour and by measuring the intensity of radioactivity with a Geiger counter or liquid-scintillation spectrometer. The compounds were eluted from the chromatogram with water/methanol (1:1, v/v), and the galactose content of each was determined by the technique of Walborg

& Christensson (1965). Recovery was monitored by measuring the quantity of ³⁵S at the beginning and end of the procedure. Pure galactose (Pfanstiehl Laboratories, Waukegan, IL, U.S.A.) was used as the standard. Standard galactosylglycerol gave the same absorption as an equivalent amount of galactose, indicating that the glycosidic bond of this type of carbohydrate need not be broken before subjecting it to the Walborg & Christensson (1965) technique. All determinations were done in triplicate.

Preparation of fractions of rat brain

Homogenates of brains from ten rats (21 days old) were fractionated by modifications of the procedures of Lapetina *et al.* (1967) and Eichberg *et al.* (1964) described by Inoue *et al.* (1971). All fractions were resuspended in 0.32M-sucrose, homogenized and diluted to a known volume. Each fraction was divided into several portions and stored at -20°C. These samples were assayed for protein content (Lowry *et al.*, 1951) and succinate dehydrogenase, acetylcholinesterase and 2':3'-cyclic AMP phosphohydrolase activities.

The specific activity of 2':3'-cyclic AMP phosphohydrolase (marker for myelin; Prahaska *et al.*, 1973) was highest in the fractions designated 'small myelin' and 'large myelin' (Table 1). The total activity of the enzyme was greatest in small myelin and microsomal fractions. There was significant activity in the nuclei and M₂ (synaptic-vesicle) fraction as well as in the large myelin. Some contamination with myelin was indicated by 2':3'-cyclic AMP phosphohydrolase activity in these fractions.

Succinate dehydrogenase activity (marker enzyme for mitochondria; Ellman *et al.*, 1961) was located primarily in the mitochondrial fraction (M₁-C). Since the activity of this enzyme in the other fractions was relatively low, there was little contamination of mitochondria in these fractions.

The largest amount of acetylcholinesterase activity (marker enzyme for microsomal and soluble fractions; Lee & Lardy, 1965) was found in the microsomal fractions. The specific activity of this enzyme was highest in the microsomal and M₂ fractions (the latter consisted of synaptic vesicles and membranes). The specific activity of acetylcholinesterase in the myelin fractions was about half that in the microsomal, indicating some contamination of these fractions with microsomal material.

Assay for adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase activity

Brains of three to eight littermate rats were prepared as a 10% (w/v) homogenate in 0.32M-sucrose. The homogenate was centrifuged at 10000g for 15 min at 4°C. The supernatant was centrifuged at 100000g

Table 1. Relative enrichment of fractions from rat brain as determined by marker enzymes and the distribution of sulphogalactosylglycerolipid in the fractions. Fractions were prepared from brains of 21-day-old rats and the activities of enzymes and the concentration of sulphogalactosylglycerolipid were determined as described in the Materials and Methods section.

Fraction	Total protein (mg/fraction)	2':3'-Cyclic AMP phosphohydrolase		Succinate dehydrogenase		Acetylcholinesterase		Sulphogalactosylglycerolipid		
		Total activity (μmol/fraction)	Specific activity (μmol/min per mg of protein)	Total activity (μmol/fraction)	Specific activity (μmol/min per mg of protein)	Total activity (μmol/fraction)	Specific activity (μmol/min per mg of protein)	(μmol/mg of protein)	(μmol/total)	(% of total)
Nuclei	38.2	75.6	1.98	1.53	0.040	3.20	0.084	0.032	1.26	11.1
Cell debris	36.4	24.0	0.66	0.51	0.014	1.16	0.032	0.031	1.12	9.9
Large myelin	15.4	64.2	4.17	0.02	0.001	1.79	0.116	0.136	2.10	18.6
Small myelin (M ₁ -A)	42.0	307.0	7.31	0.13	0.003	5.29	0.125	0.087	3.64	32.3
Synaptic membrane (M ₁ -B)	22.8	28.3	1.24	1.14	0.050	1.98	0.087	0.037	0.84	7.4
Mitochondria (M ₁ -C)	25.7	4.9	0.19	7.20	0.280	0.18	0.007	0.003	0.08	0.7
Synaptic vesicles (M ₂)	35.1	66.3	1.89	0.07	0.002	6.11	0.174	0.020	0.70	6.2
Soluble (M ₃)	95.2	0	0	0	0	4.38	0.046	0.009	0.84	7.4
Microsomal fraction	146.3	231.2	1.58	1.46	0.010	32.77	0.224	0.003	0.42	3.7
Supernatant	166.9	0	0	0.50	0.003	9.51	0.057	0.002	0.28	2.5

for 1 h. The pellet from this centrifugation (microsomal fraction) was resuspended in 10 ml of 0.32 M-sucrose and freeze-dried overnight. The protein content of the freeze-dried microsomal preparation was determined and the material was stored in a desiccator at -20°C .

A Triton X-100 (Rohm and Haas Co., Philadelphia, PA, U.S.A.) extract of the freeze-dried microsomal fraction contained the enzyme for the biosynthesis *in vitro* of the sulphogalactosylglycerolipid. A weighed portion of this microsomal preparation was reconstituted with water to the original concentration before freeze-drying. Enough material was used to give a final concentration of about 2 mg of protein/ml. An equal volume of 1% (w/v) Triton X-100 was added, and the mixed suspension was centrifuged at $100\,000g$ for 30 min. Protein concentration was measured in the supernatant, which was used as the source of enzyme for the biosynthetic assay.

The assay for the enzymic synthesis of the sulphogalactosylglycerolipid was carried out as follows: $0.1\text{ }\mu\text{mol}$ of galactosyldiacylglycerol [extracted from spinach (Kates, 1970) and purified by systems I and II] dissolved in chloroform was placed in each tube. Solvent was removed by evaporation and the lipid was suspended in 1% Triton X-100. Then $10\text{ }\mu\text{mol}$ of imidazole buffer, pH 7.0, $1\text{ }\mu\text{mol}$ of EDTA, $1\text{ }\mu\text{mol}$ of NaF and enzyme solution were added to the lipid emulsions. Three different concentrations of enzyme, ranging from 20 to $150\text{ }\mu\text{g}$ of protein, were used in each experiment. Finally $200\,000\text{--}300\,000\text{ d.p.m.}$ of adenosine 3'-phosphate 5'-sulphatophosphate (specific radioactivity $6\times 10^6\text{--}11\times 10^6\text{ d.p.m./}\mu\text{mol}$; New England Nuclear Corp.) was added. The tubes, which contained a total volume of 0.2 ml, were incubated for 10 min at 37°C . Then 2 ml of chloroform/methanol (1:1, v/v) and 0.5 ml of water were added to each tube. The solutions were thoroughly mixed and centrifuged for 10 min at about 2000 rev./min (PR-II rotor, International centrifuge). The chloroform phases were washed three times with chloroform/methanol/water (3:48:47, by vol.). The amount of radioactivity in 0.2 ml of each chloroform phase was counted in a liquid-scintillation spectrometer.

A portion (0.5 ml) of each chloroform phase was deacylated as previously described (Ambron & Pieringer, 1971). After chloroform and water were added to the methanolic solutions of deacylated material, approx. 0.2 g of Dowex 50 (H^+ form) was added to each tube before centrifugation at $900g$ for 10 min. Then 1 ml of each aqueous layer was taken for determination of radioactivity.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard.

Determination of radioactivity

All samples were counted for radioactivity in a Beckman LS-250 liquid-scintillation spectrometer (counting efficiency 92%). The scintillation fluid contained 0.55% diphenyloxazole and 0.0125% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene/Triton X-100 (3:1, v/v).

Results

Quantity of sulphogalactosylglycerolipid in brains of rats of different ages

The amount of sulphogalactosylglycerolipid was determined in brains of rats of 25 different age groups, varying between 0 and 70 days (Fig. 1). The lipid was barely detectable before 10 days of age, increased sharply in amount per brain between 10 and 22 days, and in general thereafter changed little in concentration as the animals became older (to 70 days).

Adenosine 3'-phosphate 5'-sulphatophosphate galactosyldiacylglycerol sulphotransferase activity in brains of rats of different ages

The activity of the enzyme catalysing the synthesis of sulphogalactosylglycerolipid was relatively low before 10 days of age, but increased by more than 4-fold between 10 and 22 days of age (Fig. 2). After reaching a maximum at about 22 days, the activity of the enzyme gradually diminished with increasing age. At 73 days of age the specific activity of the enzyme was about half that from the 22-day-old animals (Fig. 2).

Fig. 1. Amount of sulphogalactosylglycerolipid in brains of rats of various ages

Each determination was carried out on brains pooled from at least four rats from litters that were adjusted at birth to eight pups per litter. The assay for the sulphogalactosylglycerolipid is described in the Materials and Methods section.

Fig. 2. Adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase activity in brains of rats of various ages

The assay for the sulphotransferase is described in the Materials and Methods section and was performed on brains pooled from at least four rats from litters adjusted at birth to eight pups per litter. Averages from triplicate samples of each enzyme preparation were plotted.

Determination of the content and enzymic synthesis of the sulphogalactosyldiacylglycerolipid of normal and jimpy mutant mice

The non-myelinating jimpy mutant mouse had about a 7-fold lower concentration of sulphogalactosyldiacylglycerolipid in its brain than its normal sibling littermate (Table 2). The ages chosen (20–23 days) for assay would be expected to give the largest possible differences in sulphogalactosyldiacylglycerolipid concentration between the mutant and normal mice. At 20 days of age the mutant has approached the terminal stage of life, owing to insufficient myelination (death usually occurs at about 25 days), whereas the normal mouse has nearly completed the period of maximum myelination. The low amount of sulphogalactosyldiacylglycerolipid in the brain of the jimpy mouse may be attributed to a defect in enzymic synthesis of the lipid. A greatly diminished adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase activity exists in the brains of these mice (Table 2); a 3–9-fold lower activity was noted in the jimpy mouse compared with the control in each of the three age groups tested.

Determination of sulphogalactosyldiacylglycerolipid in fractions of rat brain

Myelin-enriched fractions ('small' and 'large') of brains from 21-day-old rats contained more than 50% of the total sulphogalactosyldiacylglycerolipid (Table 1). At 21 days of age more of the sulphogalactosyldiacylglycerolipid was associated with the small myelin than with the large myelin fraction. This finding is in agreement with studies on the myelin marker metabolite galactosyldiacylglycerol (Inoue *et al.*, 1971), which is a direct precursor of sulphogalactosyl-

Table 2. Amount of sulphogalactosyldiacylglycerolipid and activity of adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase in brains of jimpy and normal mice

The brains of jimpy and sibling normals of the same age were extracted with chloroform/methanol (2:1, v/v). The sulphogalactosyldiacylglycerolipid in these extracts was purified and assayed at two different concentrations in duplicate as described in the Materials and Methods section. Each value was obtained from the brain of one animal. The activity of the sulphotransferase responsible for the synthesis of sulphogalactosyldiacylglycerolipid was measured in brains from jimpy and sibling normals of the same age, as described in the Materials and Methods section. Values represent averages of triplicate assays of each brain preparation.

Age (days)	Sulphogalactosyldiacylglycerolipid ($\mu\text{mol/g}$ wet wt.)		Adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase (pmol/h per mg of protein)	
	Jimpy	Normal	Jimpy	Normal
19	—	—	0.51	4.74
20	0.059; 0.03	0.45	0.90; 0.72	2.66
21	0.05	0.37	—	—
23	0.068	0.49	1.14	3.26

glycerolipid. The concentration of galactosyldiacylglycerol was also considerably higher in the small myelin fraction at 21 days of age (Inoue *et al.*, 1971). At a later age the relative percentage of galactosyldiacylglycerol increased in the large myelin fraction.

Relative amounts of the diacyl and alkylacyl derivatives of sulphogalactosyldiacylglycerol and of sulphogalactosyldiacylglycerolipid and cerebroside sulphate in brains of 21-day-old and adult rats

Our initial studies (Flynn *et al.*, 1975) demonstrated the presence of sulphogalactosyldiacylglycerol in the brains of 22-day-old rats. However, Levine *et al.* (1975) found the monoalkyl ether form of the lipid in brains of adult rats. In neither study was the possibility of the presence of the other form of the sulphogalactosyldiacylglycerolipid excluded. The possibility that the molar ratios of the two forms of the lipid might change with age was tested by measuring the amounts of the diacyl and alkylacyl forms of sulphogalactosyldiacylglycerolipid in 2021-day-old rats and in four adult (3–4-month-old) rats. The group of younger rats were injected intracerebrally with approx. $50 \mu\text{Ci}$ of carrier-free $\text{H}_2^{35}\text{SO}_4$ /rat at 20 days of age. The adult rats were not injected with the $\text{H}_2^{35}\text{SO}_4$, but, for ease of detection and calculation of recovery, known small amounts of pure cerebroside

[^{35}S]sulphate (from rat brain) and [^{35}S]sulphogalactosylglycerolipid were added at the start of the extraction procedure. The cerebroside sulphate and sulphogalactosylglycerolipid were extracted from the brains with chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957), purified by systems I and II and then assayed for sulphur content by the technique of Kean (1968).

The molar ratio of sulphogalactosylglycerolipid to cerebroside sulphate in a representative sample of total glycolipids applied to system II was 0.47/2.93 μmol (1:6.23) respectively in the 21-day-old rat brain. The sulphogalactosylglycerolipid contributes 16% to the total sulpholipid content of the brain of the 21-day-old rat. In the adult rat brain the sulphogalactosylglycerolipid/cerebroside sulphate molar ratio was 0.416/4.51 μmol (1:10.85). In the adult brain the sulphogalactosylglycerolipid contribution to total sulpholipid content is therefore 8.4%.

A portion of the sulphogalactosylglycerolipid from 21-day-old and adult rats was deacylated with mild alkali, and the resulting water-soluble sulphogalactosylalkylglycerol and sulphogalactosylglycerol were separated by system II. The two radioactive derivatives of the sulphogalactosylglycerolipid were detected with iodine vapour and then eluted from the silica gel with water and water/methanol (1:1, v/v). When the deacylated compounds from the 21-day-old rats were assayed for galactose content (Walborg & Christenson, 1965), the [^{35}S]sulphogalactosylglycerol spot (57800 c.p.m.) contained 109.4 nmol (specific radioactivity = 528 c.p.m./nmol) and the [^{35}S]sulphogalactosylalkylglycerol spot (59700 c.p.m.) contained 106.3 nmol (specific radioactivity 563 c.p.m./nmol). On the basis of the specific radioactivity of the sulphogalactosylglycerolipid determined by sulphur analysis before deacylation, the 57800 c.p.m. of the fully deacylated derivative should have produced 99 nmol and the 59700 c.p.m. of the deacylated alkyl ether compound should have yielded 102.2 nmol. These results show that in the 21-day-old rat brain there are nearly equal amounts of the two forms of the sulphogalactosylglycerolipid, that each has approximately the same specific radioactivity, and that the compounds both have a 1:1 molar ratio of sulphate to galactose. The ratio of the diacyl to the alkylacyl derivative of the sulphogalactosylglycerolipid in the adult rat brain was determined by similar procedures. The galactose content of the sulphogalactosylglycerol spot was 116 nmol and that of the sulphogalactosylalkylglycerol was 114 nmol, i.e. a ratio of 1:1.

Relative turnover of the diacyl and alkylacyl derivatives of sulphogalactosylglycerolipids in rat brain between 21 and 41 days of age

The finding of approximately equal proportions of the alkylacyl and diacyl forms of sulphogalactosyl-

glycerol in the adult and in 21-day-old rats does not rule out the possibility that the two forms of the lipid may be metabolized at different rates. To test this possibility, 24 female rats (20 days old) were injected intracerebrally with 10 μl (250 μCi) of carrier-free $\text{H}_2^{35}\text{SO}_4$. The amounts of $\text{H}_2^{35}\text{SO}_4$ actually reaching brain tissue was somewhat less than intended, because of slight bleeding at the point of injection in some rats. Groups of four rats were killed at ages 21, 24, 27, 30, 35 and 41 days, i.e. 1, 4, 7, 10, 15 and 21 days after injection.

The [^{35}S]sulphogalactosylglycerolipid (mixture of diacyl and alkylacyl forms) was extracted from the brains of each group and purified on systems I and II. The total radioactivity in the purified lipid was highest in brains of rats 1 day after the injection of $\text{H}_2^{35}\text{SO}_4$ (1.14×10^6 c.p.m.) and varied from 3.1×10^5 c.p.m. to 1.24×10^5 c.p.m. at other time-intervals. The decrease in radioactivity is due to the turnover of the sulphogalactosylglycerolipid and the variations might be due to the different amounts of $\text{H}_2^{35}\text{SO}_4$ reaching the brain. Because of the difficulties in injecting an exact amount of $\text{H}_2^{35}\text{SO}_4$, it was considered appropriate to measure the relative turnover of the diacyl and alkylacyl derivatives of sulphogalactosylglycerol by determining the ratio of radioactivities in the two lipids. Portions of the labelled sulphogalactosylglycerolipid purified from the various groups were therefore deacylated with mild alkali. The water-soluble [^{35}S]sulphogalactosylglycerol and [^{35}S]sulphogalactosylglycerol ether were separated in system III and located by a radiochromatogram scanner (Baird Atomic Accessories, Bedford, MA, U.S.A.). The quantity of radioactivity in each spot was determined in a liquid-scintillation spectrometer (Beckman Instruments) (Table 3). Because much of our other work used 21-day-old rats that had been injected with $\text{H}_2^{35}\text{SO}_4$ at age 20 days, we have listed several values for 1 day after injection. The general trend of the ratios of the radioactivities of the two sulphogalactosylglycerolipids shows an increasing proportion of the alkylacyl to the diacyl lipid with increasing time after injection. At 1 day after injection, the ratio is almost 1:1, with the diacyl compound being slightly greater, but at 21 days after injection the ratio has shifted to a larger proportion of the alkyl ether form. The values show that the diacyl form loses radioactivity faster and therefore has a higher turnover than the alkylacyl form.

Discussion

The results of these studies show that the sulphogalactosylglycerolipid and the sulphotransferase that synthesizes it in brain are closely associated with the process of myelination. The activity of adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacyl-

Table 3. *Relative turnover of the diacyl and alkylacyl forms of sulphogalactosylglycerol in brains of rats*

Female rats (20 days old) were injected intracerebrally with 10 μ l (250 μ Ci) of carrier-free $\text{H}_2^{35}\text{SO}_4$. Groups of four were killed at the ages indicated. The [^{35}S]sulphogalactosylglycerolipid was extracted from the brains and purified as described in the Materials and Methods section. Portions of the sulphogalactosylglycerolipid from different groups of rats were deacylated and the radioactivity in the deacylated diacyl and alkylacyl derivatives was determined as described in the text. The radioactivity in the samples taken for deacylation varied, and the actual counts from a typical experiment are given in the Table. The quantities in parentheses are values obtained from other experiments on 21-day-old rats.

Age (days)	Time after injection (days)	Sulphogalactosylglycerolipid			
		Diacyl		Alkylacyl	
		(c.p.m.)	(%)	(c.p.m.)	(%)
21	1	1000	64.1	559	35.9
		(57800)	(49.2)	(59700)	(50.8)
		(1232)	(53.7)	(1062)	(46.3)
24	4	904	44.7	1115	55.3
27	7	2267	38.2	3660	61.8
30	10	661	36.0	1176	64.0
35	15	817	37.5	1364	62.5
41	21	1391	26.3	3619	73.7

glycerol sulphotransferase follows the same pattern of development observed for enzymes which synthesize two other myelin constituents, cerebroside (Neskovic *et al.*, 1972) and sulphatides (McKhann & Ho, 1967). The deficiency of sulphogalactosylglycerolipid and the galactosyldiacylglycerol sulphotransferase activity in the jimpy mouse is similar to that previously described for cerebroside and sulphatides (Nussbaum *et al.*, 1969), UDP-galactose-ceramide galactosyltransferase (Neskovic *et al.*, 1972) and adenosine 3'-phosphate 5'-sulphatophosphate-cerebroside sulphotransferase (Sarlieve *et al.*, 1972). The galactosyldiacylglycerol of brain, which is a direct precursor of sulphogalactosylglycerolipid, has also been shown to be a good marker metabolite for myelination (Inoue *et al.*, 1971; Deshmukh *et al.*, 1971).

In all of the parameters used in the present study to demonstrate the association of the sulphogalactosylglycerolipid with myelination, parallel results have been obtained previously for the galactosyldiacylglycerol (Inoue *et al.*, 1971; Deshmukh *et al.*, 1971). In addition to establishing that galactosyldiacylglycerol is a substrate in the formation of sulphogalactosylglycerolipid *in vitro*, the temporal data obtained *in vitro* in the present work when compared with our previous studies shows a probable precursor-product relationship between galactosyldiacylglycerol and sulphogalactosyldiacylglycerol. The concentration of galactosyldiacylglycerol in rat brain dramatically increases between 10 and 20 days of age, reaches a maximum of 1280 nmol per brain at 20 days of age and equally dramatically decreases to 680 nmol per brain by 23 days of age (Inoue *et al.*, 1971). The sulphogalactosylglycerolipid attains its highest

concentration (approx. 400 nmol per brain) at 22–25 days of age. Although galactosyldiacylglycerol is attacked by lipase and galactosidase enzymes in brain (Subba Rao *et al.*, 1970), the precipitous fall in the concentration of galactosyldiacylglycerol within 3 days may be attributed largely to its conversion into sulphogalactosyldiacylglycerol rather than to its degradation, because there is no concomitant large change in the hydrolytic-enzyme activity during this time (Subba Rao *et al.*, 1970). The galactosyltransferase that synthesizes the galactosyldiacylglycerol attains a maximum activity at about 17 days of age (Wenger *et al.*, 1968, 1970), whereas the adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase reaches its maximum at 20 days. In both systems the enzyme attains a maximum about 3 days before the highest concentration reached by the product of the reaction. The temporal sequence of the maximum activities and concentrations (17 days galactosyltransferase; 20 days galactosyldiacylglycerol; 20 days sulphotransferase; 23 days sulphogalactosylglycerolipid) indicates that the regulation of the sulphotransferase may be related to the concentration of one of its substrates, the galactosyldiacylglycerol.

The contribution of the sulphogalactosylglycerolipid to the total sulpholipid concentration in brain is highest during the period of active myelination (16% in the 21-day-old rat). Similarly galactosyldiacylglycerol is about 25% the concentration of cerebroside in the 20-day-old rat, but this percentage decreases considerably in the adult (Inoue *et al.*, 1971). These observations suggest that the glycerol glyco- and sulphoglyco-lipids have a more rapid turnover than their sphingolipid counterparts. The

major structural differences between the two types of lipid is the fatty acid ester bond of the glycerolipids compared with the fatty acid amide bond of the sphingolipids. The fatty acid ester is susceptible to degradation through the action of a brain lipase (Subba Rao *et al.*, 1970). This lipase, which is non-specific with respect to lipid substrate, steadily increases in activity with age (Subba Rao *et al.*, 1970). The higher activity in older animals may explain the decrease in glycerol sulphoglyco- and glyco-lipids relative to their sphingolipid counterparts as the animal ages. The ability of the body to degrade the glycerol sulphoglyco- and glyco-lipids to water-soluble compounds by another pathway (lipase) would aid the prevention of excessive build-up of these lipids in various sphingolipidosis diseases.

The careful technical assistance of Ms. E. Cabacungan and Ms. H. Hudin contributed greatly to the completion of these studies. J.-A. P. and R. A. P. are grateful for the help given to them while on sabbatical leave by the members of the lipid and tissue-culture groups at the Centre de Neurochimie. This investigation was supported by U.S. Public Health Service Research Grants NS-10221 and AI-05730, by Research Career Development Award (to R. A. P.) 1K4 NS 12776, and by an Institutional Grant from the American Cancer Society. Part of this work was supported by grants from the French Commissariat à l'Energie Atomique, Département de Biologie, and from the Délégation Générale à la Recherche Scientifique et Technique (ACC 'Membranes Biologiques').

References

- Ambron, R. T. & Pieringer, R. A. (1971) *J. Biol. Chem.* **246**, 4216–4225
- Deshmukh, D. S., Inoue, T. & Pieringer, R. A. (1971) *J. Biol. Chem.* **246**, 5695–5699
- Eichberg, J., Jr., Whittaker, V. P. & Dawson, R. M. C. (1964) *Biochem. J.* **92**, 91–100
- Ellman, G. L., Courtney, K. D., Andres, V., Jr. & Featherstone, M. (1961) *Biochem. Pharmacol.* **7**, 88–95
- Flynn, T. J., Deshmukh, D. S., Subba Rao, G. & Pieringer, R. A. (1975) *Biochem. Biophys. Res. Commun.* **65**, 122–128
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Inoue, T., Deshmukh, D. S. & Pieringer, R. A. (1971) *J. Biol. Chem.* **246**, 5688–5694
- Ishizuka, I., Suzuki, M. & Yamakawa, T. (1973) *J. Biochem. (Tokyo)* **73**, 77–87
- Kates, M. (1970) *Adv. Lipid Res.* **8**, 225–265
- Kean, E. L. (1968) *J. Lipid Res.* **9**, 319–327
- Kornblatt, M. J., Knapp, A., Levine, M., Schachter, H. Murray, R. K. (1974) *Can. J. Biochem.* **52**, 689–697
- Lapetina, E. G., Soto, E. F. & DeRobertis, E. (1967) *Biochim. Biophys. Acta* **135**, 33–43
- Lee, Y.-P. & Lardy, H. A. (1965) *J. Biol. Chem.* **240**, 1427–1436
- Levine, M., Kornblatt, M. J. & Murray, R. K. (1975) *Can. J. Biochem.* **53**, 679–689
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McKhann, G. M. & Ho, W. (1967) *J. Neurochem.* **14**, 717–724
- Neskovic, N., Sarlieve, L. & Mandel, P. (1972) *Brain Res.* **42**, 147–157
- Nussbaum, J., Neskovic, N. & Mandel, P. (1969) *J. Neurochem.* **16**, 927–934
- Prahaska, J. R., Clark, D. A. & Wells, W. W. (1973) *Anal. Biochem.* **56**, 275–282
- Sarlieve, L., Neskovic, N., Rebel, G. & Mandel, P. (1972) *Neurobiology* **2**, 70–82
- Sidman, R. L., Dickie, M. M. & Appel, S. H. (1964) *Science* **144**, 309–311
- Subba Rao, K., Wenger, D. A. & Pieringer, R. A. (1970) *J. Biol. Chem.* **245**, 2520–2524
- Subba Rao, G., Norcia, L. N., Pieringer, J.-A. & Pieringer, R. A. (1977) *Biochem. J.* **166**, 429–435
- Walborg, E. F., Jr. & Christensson, L. (1965) *Anal. Biochem.* **13**, 186–193
- Wenger, D. A., Petitpas, J. W. & Pieringer, R. A. (1968) *Biochemistry* **7**, 3700–3707
- Wenger, D. A., Subba Rao, K. & Pieringer, R. A. (1970) *J. Biol. Chem.* **245**, 2513–2519