Characterization and Developmental Expression of a Novel Sulfotransferase for the Biosynthesis of Sulfoglucuronyl Glycolipids in the Nervous System*

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Sulfoglucuronyl glycolipids (SGGLs) are temporally and spatially regulated molecules in the developing nervous system. A novel sulfotransferase (ST) from rat brain which catalyzes the terminal step in the biosynthesis in vitro of SGGLs is described. The enzyme catalyzes a transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate to a hydroxyl group on carbon 3 of the terminal glucuronyl residue in IV3β-glucuronyl neolactotetraosylceramide (GlcAnLeOse4Cer) and IVβ-glucuronyl neolactohexaosylceramide (GlcAn-LeOse6Cer) to form 3-sulfated glucuronyl glycolipids. The enzyme is highly specific for glucuronyl glycolipids (GGs) and requires the free-COOH group of the terminal glucuronic acid for reactivity. GGL:ST present in the microsomal membranes requires Mn2+ ions and a nonionic detergent, Triton X-100, for activity. The optimal pH is 7.2 with Tris-HCl buffer and $K_v$ values were 7 μM for 3'-phosphoadenosine 5'-phosphosulfate and 29 μM for GlcAnLeOse4Cer. GGL:ST was shown to be different from previously well studied galactocerebroside: sulfotransferase for the synthesis of myelin membrane-specific lipid sulfatide. This conclusion was based upon several criteria, i.e., including different requirements of incubation conditions for maximal activity, substrate competition experiments, different effects of heat, dithiothreitol, NaCl, and pyridoxal phosphate, as well as different profiles of expression of activity during development of the nervous tissues. The two enzymes were also partially resolved on a pyridoxal phosphate-ligated agarose column. Studies on the developmental expression of the GGL:ST in the rat cerebral cortex and cerebellum showed that it is not a regulatory enzyme controlling the expression of SGGLs in these neural tissues.

Sulfoglucuronyl glycolipids (SGGLs)1 are specifically expressed in the mammalian nervous system (1-4). Two glycolipids of this family have been characterized; one is 3-sulfoglucuronyl neolactotetraosylceramide (SGGL-1) and the other 3-sulfoglucuronyl neolactohexaosylceramide (SGGL-2). Monoclonal antibody HNK-1, which reacts with the terminal 3-sulfoglucuronyl residue of these glycolipids also recognizes a carbohydrate epitope on a subset of human lymphocytes, including natural killer cells (5) and an important group of glycoproteins and proteoglycans in the nervous system involved in cell-cell interactions (6-10). SGGLs localized in the peripheral nervous system have been implicated in the immunopathogenesis of a human disease called paraproteinemia with peripheral neuropathies, due to abnormal circulating IgM antibodies, reacting with the same terminal sugar of these lipids as HNK-1 (1, 2, 4, 11). The HNK-1-reactive carbohydrate is also involved in neuron-astrocyte and astrocyte-astrocyte cellular adhesion in the developing nervous system and acts as a ligand in cell interactions (12). The HNK-1 carbohydrate epitope has been a useful marker for tracing migration of neural crest cells in the developing embryo and is functionally important in the interaction of neural crest cells with extracellular matrix during migration (13, 14).

The complete biosynthesis of SGGLs is not known. Previously we have characterized a novel and specific enzyme glucuronyltransferase from embryonic chicken and rat brain, involved in the synthesis of glucuronyl neolactotetraosylceramide (GGL-1) and glucuronyl neolactohexaosylceramide (GGL-2) from their respective precursors nLeOse4Cer and nLeOse6Cer (15, 16). In this report we have studied the properties of a novel sulfotransferase involved in the terminal step for the synthesis of SGGLs from the GGLs. It is shown here that the GGL:sulfotransferase (GGL:ST) is different from the well studied galactocerebroside:sulfotransferase (GalCer:ST) which is involved in the biosynthesis of sulfatides.

SGGLs are maximally expressed during development of rat cerebral cortex only during the pre- and perinatal period and completely disappear from the postnatal and adult cortex (3, 17). However, in the rodent cerebellum and peripheral nervous system they increase with development after birth and are maximally expressed in the adult (18, 19). The mechanism of regulation of such differential expression and its physiological significance in these neural areas is unclear. Studies on the developmental expression of the enzyme nLeOse4Cer:glucuronyltransferase in the rat cortex and cerebellum indicated that

PAP$^{35}$S, 3'-phosphoadenosine 5'-phosphosulfate; CHAPS, 3-[N,N-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; GM$_3$, IP(3'NeuAcLeCer); GM$_4$, IP(4NeuAcGgOse4Cer); GM$_{14}$, IP(4NeuAcGgOse4Cer); GD$_{14}$, IP(4NeuAcGgOse4Cer); GT$_{14}$, IV(4NeuAc); GgOse4Cer.

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1 The abbreviations used are: SGGLs, sulfoglucuronyl glycolipids; ED, embryonic day; PD, postnatal day; GalCer, galactocerebroside; GGLs, glucuronylglycolipids; GGL-1 or GlcAnLeOse4Cer, IVβ-glucuronyl neolactotetraosylceramide; GGL-2 or GlcAnLeOse6Cer, IVβ-glucuronyl neolactohexaosylceramide; GEA, glucuronic acid; HPTLC, high-performance thin-layer chromatography; nLeOse4Cer, neolactotetraosylceramide; nLeOse6Cer, neolactohexaosylceramide; SGGL-1, 3-sulfoglucuronyl neolactotetraosylceramide; SGGL-2, 3-sulfoglucuronyl neolactohexaosylceramide; ST, sulfotransferase;
the activity of the enzyme was not correlative with the expression of SGGLs (16). Considerable activity of the glucuronyl-
transferase was present in the adult rat cerebral cortex, even
though SGGLs were almost completely absent by postnatal
day 15 (PD 15). It was therefore of importance to determine
if GGL:ST was involved in the regulation of SGGLs. The
developmental expression of GGL:ST in the rat cerebral
cortex and cerebellum was evaluated. The results show that
like the glucuronyltransferase, the sulfotransferase is not a
regulatory enzyme controlling the expression of SGGLs. Part
of this work has been previously reported in abstract form
(20).

MATERIALS AND METHODS

Sprague-Dawley albino rats of various ages including timed preg-
nant dams were purchased from Charles River Laboratory (Wilmington,
MA). Radioactive 3'-phosphoadenosine 5'-phosphosulfate (PAP35S; specific activity, 2.5 Ci/mmole) was purchased from Du
Page New England Nuclear. Nonradioactive PAPS as well as other chem-
icals for lipid assays were purchased from Sigma. All other chemicals for lipid assays were purchased from Sigma.

Enzyme Preparation—Neural tissues were homogenized in 10 vol-
tumes of 0.2 M sucrose in 10 mM Tris-HCl buffer, pH 7.4, with a
Potter-Elvehjem homogenizer at 4 °C. The homogenate was centrifuged at 1,000 X g for 10 min, followed by 12,000 X g for 30
min, to remove nuclear and crude mitochondrial fractions. The mi-
crosomal fraction was yielded by centrifuging the post-mitochondrial supernatant at 100,000 X g for 60 min. Protein concentra-
tions were determined by using the Bicinchoninic acid reagent (Pierce Chemical Co.).

Assay for GlcAlcOse6-Cer:ST and GalCer:ST—The complete in-
cubation mixture contained, in a volume of 100 µl, 100–250 µg of
microsomal proteins or total homogenate proteins from appropriate
neural tissue, 100 µM Tris-HCl buffer, pH 7.2, 100 mM NaCl, 10 mM
MnCl2, 2.5 mM ATP, 10 mM MnCl2, 10 mM; dithiothreitol, 1 mM;
bovine brain GalCer, 5 µg; GGL-1, 4 pg; PAP35S (0.1 µCi, 0.45 nmol); bovine serum albumin, 40 µg; and phosphatidylcholine, 10 µg, and 50 µl of the
eluant fraction, in a total volume of 100 µl. The mixture was incubated at 37 °C for 1 h. The radioactive lipid products were separated from
the radioactive nucleotide precursor by a reversed-phase Cle-BondElut cartridge (Analytichem International, Harbor City, CA) essentially as described previously (22). A portion of the combined radioactive lipids, sulfatide
and SGGL-1, eluted from the cartridge was counted in a scintillation counter, and the rest of the fraction was further separated on a
HPTLC plate developed with chlorormethane, methanol, 0.25% CaCl2
(5:4:1). The plate was autoradiographed with an x-ray film, and the
amount of radioactivity in the two lipids was determined.

RESULTS

Optimal Conditions for Rat Brain Sulfotransferases—A va-
riety of conditions were tested for the optimal requirements of the enzyme GlcAlcOse6-Cer:sulfotransferase (GGL:ST) with PAPS as the donor and GlcAlcOse6-Cer (GGL-1) as the acceptor. The results were also compared with the well
known GalCer:ST (Table 1). Although the requirements of cofactors of both the sulfotransferases were similar, there was a
quantitative difference in the activity of the enzyme with
GGL-1 and GalCer as acceptors. GGL:ST was much more sensitive to omission of Mn2+ and Triton X-100; whereas GalCer:ST was more sensitive to omission of ATP from the

| Condition | GlcAlcOse6-Cer:ST | GalCer:ST |
|-----------|------------------|-----------|
| Complete  | 100%             | 100%      |
| -ATP      | 42               | 11        |
| -Mn2+     | 16               | 64        |
| -Triton X-100 | 0.01       | 4.5       |

*Complete reaction mixture for GGL:ST in 100 µl consisted of 250 µg of microsomal protein from 8-day-old rat cerebral cortex; Tris-
HCl buffer, pH 7.2, 100 mM; Triton X-100, 1 µM; ATP, 2.5 mM;
MnCl2, 10 mM; GGL-1, 4 pg; and PAP35S (approximately 106 dpm,
50 µCi/pmol). The incubation was for 45 min at 37 °C.

 Solubilization and Separation of Sulfotransferases—Microsomal
membranes from 30 rat whole brains (PD 14–16) were prepared as
previously described. The membranes were solubilized with 40 ml of 1%
Triton X-100 in 20 mM Tris-HCl buffer, pH 7.2, 10 mM MnCl2,
25% glycerol, and 1 ml dithiothreitol (solution A), overnight, at 0–
4 °C. The mixture had a protein:Triton X-100 ratio of 5:1. The
supernatant was centrifuged at 50,000 X g for 1 h. The supernatant had
approximately 95% and 80% of the total microsomal GalCer:ST and
GGL:ST activity, respectively.

Aminooxyl agarose (30 ml; Affi-Gel 102, Bio-Rad) suspended in
50 ml of water, was mixed with 0.5 g of pyridoxal 5-phosphate, and
the pH was adjusted to 7.0 by adding NaOH (24). The mixture was
incubated at the dark at room temperature, overnight. The resulting
Schiff base was reduced by addition of 50 mg of solid sodium boro-
hydride at 4 °C. After the reduction, the derivatized agarose was washed with 0.3 M NaCl (24).

A pyridoxal phosphate-ligated agarose column (2 X 8 cm) was pre-
equilibrated with solution A. The microsomal membrane proteins
solubilized in solution A, 40 ml, were loaded onto the column at a
rate of 30 ml/h. The eluant was diluted 10-fold with solution A, but
without Triton X-100 such the Triton X-100 concentration from 1 to 0.1%) and reloaded onto the column. The column was then
washed with 150 ml of solution A containing 0.1% Triton X-100, and
10 ml fractions were collected. The bound proteins were then eluted with 80 ml of a linear gradient of zero to 0.2 M NaCl, and 3 ml
fractions were collected. The activities of the GGL:ST and GalCer:ST
were measured in the collected fractions as follows. Tris-HCl buffer,
pH 7.2, 100 mM; MnCl2, 10 mM; ATP, 10 mM; dithiothreitol, 1 mM;
phosphatase, 0.1 µCi, 0.45 nmol; bovine brain GalCer, 5 µg; GGL-1, 4 pg; Triton X-100, 1 µM; bovine serum albumin, 40 µg; and phosphatidylcholine, 10 µg, and 50 µl of the
eluant fraction, in a total volume of 100 µl. The mixture was incubated at 37 °C for 1 h. The radioactive lipid products were separated from
PAPS and other water soluble components by a C8-BondElute cartridge (22). A portion of the combined radioactive lipids, sulfatide
and SGGL-1, eluted from the cartridge was counted in a scintillation
counter, and the rest of the fraction was further separated on a
HPTLC plate developed with chlorormethane, methanol, 0.25% CaCl2
(5:4:1). The plate was autoradiographed with an x-ray film, and the
amount of radioactivity in the two lipids was determined.

| Condition | GlcAlcOse6-Cer:ST | GalCer:ST |
|-----------|------------------|-----------|
| Complete  | 100%             | 100%      |
| -ATP      | 42               | 11        |
| -Mn2+     | 16               | 64        |
| -Triton X-100 | 0.01       | 4.5       |

*Complete reaction mixture for GGL:ST in 100 µl consisted of 250 µg of microsomal protein from 8-day-old rat cerebral cortex; Tris-
HCl buffer, pH 7.2, 100 mM; Triton X-100, 1.0%; ATP, 2.5 mM;
MnCl2, 10 mM; GGL-1, 4 pg; and PAP35S (approximately 106 dpm,
50 µCi/pmol). The incubation was for 45 min at 37 °C. For GalCer:ST the incubation conditions were the same, except
Triton X-100 was 0.2%, MnCl2 was 15 mM and, instead of GGL, 5 µg
of GalCer was used.

*100% activity for GGL:ST represents 108 pmol/mg/h and that for GalCer:ST 7.2 pmol/mg/h. The assay was done in duplicate and the
results are average of the two determinations.
reaction mixture. The specific activity of GGL:ST was about 15-fold higher than GalCer:ST, when 4-8-old rat brain microsomal membrane proteins were used as the enzyme source. Since GGL:ST almost absolutely required detergent Triton X-100 for the reactivity, a variety of other detergents were tested. Triton X-100 and Triton CF-54 were most effective compared to other detergents, such as CHAPS (23%) and octylglucoside (11%), however, Tween, deoxycholate, and tau-rodoxycholate were ineffective. The effect of varying the concentration of Triton X-100 on the activity of GalCer:ST (Fig. 1). GGL:ST was highly stimulated with 0.1% Triton X-100 and was inhibited with higher concentration of the detergent, whereas GalCer:ST was only moderately affected with the detergent.

GGL:ST required Mn$^{2+}$ for activity. The activity was completely abolished in the presence of 10 mM EDTA. Other metal ions such as Mg$^{2+}$ and Ca$^{2+}$ were partially effective, but Cu$^{2+}$, Hg$^{2+}$, and Zn$^{2+}$ were completely ineffective. The optimal concentration of Mn$^{2+}$ was 10 mM, at higher concentration it was inhibitory (Fig. 2). Mg$^{2+}$, 20-25 mM, was about 80% as effective as Mn$^{2+}$. The activity profile of GalCer:ST on varying the concentration of Mn$^{2+}$ and Mg$^{2+}$ ions was significantly different from that of GGL:ST (Fig. 2). The stimulation of GalCer:ST was not as strong by these metal ions.

GGL:ST showed maximal activity at pH 5.8 with sodium cacodylate buffer (Fig. 3), however, at the same pH sodium acetate was not as effective. With Tris-HCl, the activity was maximal at pH 7.2, and it was slightly higher than that at pH 5.8 with sodium cacodylate. The GalCer:ST activity was not as sensitive to pH changes and type of buffer used as the GGL:ST. With sodium acetate and cacodylate the GalCer:ST activity was maximal at pH 5.8, however, with Tris-HCl, similar levels of GalCer:ST activity was observed between pH 7.2 and 8.0.

The GGL:ST activity was linear up to about 1 h, thereafter the rate of reaction gradually declined. The activity increased linearly with 15-200 µg of microsomal protein used. The K$_m$ of PAPS as the substrate for the activity of GGL:ST was 7.4 µM ($V_{max}$ = 97.4 pmol/mg/h). The K$_m$ of GGL-1 as the substrate for the GGL:ST was 29 µM ($V_{max}$ = 125 pmol/mg/h).

**Product Identification**—The radioactive products formed after the enzymatic reaction of PAP$^{35S}$S with GGLs, were analyzed after HPTLC, followed by autoradiography and immunoreactivity with monoclonal antibody HNK-1 (Fig. 4). The autoradiogram of the products after the reaction showed two bands which comigrated with standard SGGL-1 and SGGL-2 (Fig. 4C). These radioactive bands on the HPTLC plate reacted with monoclonal antibody HNK-1 (Fig. 4B, lane P) indicating that the radioactive products formed were SGGLs. In the orcinol-sprayed HPTLC plate (Fig. 4A, lane P) the products were not visualized, because the amounts formed were not sufficient enough for the orcinol reaction.

To identify the point of attachment of the sulfate group in the in vitro biosynthesized radiolabeled SGGLs, periodate oxidation of the vicinal hydroxyl groups followed by sodium borohydride reduction was carried out. The radiolabeled lipids before (Fig. 5B, lane B) and after the oxidation-reduction procedure (Fig. 5B, lane A) were analyzed by HPTLC followed by autoradiography. The autoradiogram showed that both radioactive SGGLs did not change their mobility after the oxidation-reduction reaction. The HPTLC plate after spraying with orcinol reagent is shown in Fig. 5A. The major nonradioactive lipids G$_{SM1}$ and G$_{DA}$ (originating from the microsomal members) as well as the substrates GGLs (migrating with G$_{SM1}$ and G$_{DA}$) in the reaction mixture (Fig. 5A, lane B) had reacted with the periodate and were no longer present after the reaction (Fig. 5A, lane A), indicating the effectiveness of the reaction. The migration pattern of SGGLs did not change after the periodate reaction, and no other radioactive products were formed (Fig. 5B, lane A) indicating that these compounds do not have reactive vicinal hydroxyl groups in the sugar chain. Therefore, the sulfate group must...
Fig. 4. HPTLC analysis of substrates and reaction products of GGL:sulfotransferase in vitro. The glycolipids were separated on an HPTLC plate with chloroform, methanol, 0.25% CaCl2 (5:4:1) as the developing solvent. The plate was subjected to autoradiography and immuno-overlay as in C and B, respectively. The lipids were visualized on an identical HPTLC plate after spraying the plate with orcinol reagent and heating, as in A. In A: Lane S1, ganglioside standards for reference; from top to bottom: GM3, GM1, GD1b, GD1a, and GT3h, respectively; S2, substrates GlcAnLcOse4Cer (GGL-1, major band) and GlcAnLcOse&er (GGL-2) minor band migrating near GD2.lane; B, reaction product visualized after HPTLC analysis of substrates and reaction products. The same lane P in A and B was exposed to x-ray film.

Fig. 5. Linkage analysis of GGL:sulfotransferase reaction products formed from GlcAnLcOse,Cer and GlcAnLcOse&er.Cer. The radioactive [35S]sulfate-labeled SGGLs as well as the nonradioactive gangliosides from the microsomal membranes were oxidized with 0.5 M sodium metaperiodate for 48 h at 0–4 °C, followed by reduction with sodium borohydride. The isolated lipids were analyzed by HPTLC and radioautography. A, reaction product visualized after orcinol spray; lane GGL, standard GlcAnLcOse,Cer; lane B, lipid products of the sulfotransferase reaction before oxidation; lane A, lipid reaction products of the sulfotransferase after oxidation; S, standard SGGL-1; G, standard gangliosides as in Fig. 4. B, radioautogram of lanes B and A.

be linked to the 3 position of the terminal glucuronyl residue of SGGLs.

Substrate Specificity—The activity of the 4-day-old rat brain microsomal sulfotransferase was determined with different glycolipids as acceptors, under the reaction conditions as described in Table I for GGL-1 (Table II). The maximal activity was found only with GGL-1 (GlcAnLcOse,Cer) as the acceptor. The activity was drastically reduced with 6-methylGlcAnLcOse,Cer and GlcLcOse,Cer, as substrates, indicating that the enzyme was highly specific for the terminal GlcA of the free-COOH group was required for the reaction. Substitution of the –COOH of GlcA with either –COOCH3 or –CH2OH made the substrates ineffective as acceptors of sulfate by the sulfotransferase. In 4-day-old rat brain, little activity of sulfotransferase was seen with galactosylceramide (GalCer), lactosylceramide, asialo-GM1, and monogalactosyl diglyceride, whereas other glycolipid substrates were completely ineffective.

Identity GGL-ST from Other Sulfotransferases—Competition experiments were performed to determine the identity of GGL:ST from other sulfotransferases. Rat brain homogenate or microsomal fraction was incubated with PAP35S, GGL-1, and varying amounts of galactosylceramide. Alternately, incubations were performed by keeping a constant level of galactosylceramide and varying the amounts of GGL-1 (Table III). After incubation, the radioactive products were isolated and separated on HPTLC, and the amount of incorporation was determined in each product formed. The results showed that by varying the amounts of galactosylceramide and keeping the same level of GGL-1, the incorporation into sulfatide or SGGL1 was not affected (Table IIIa). Similarly, keeping the same level of GalCer and varying the levels of GGL-1 also did not affect the incorporation into SGGL-1 and sulfatide, (Table IIIb). These results suggested two separate sulfotransferases for these two substrates. However, when GalCer was incubated with varying amounts of lactosylceramide, the in-
corporation into both sulfatide and sulfated lactosylceramide was affected indicating that the same enzyme may be active for the latter two substrates (Table IIIc).

**Heat Inactivation Profile**—13-day-old rat brain microsomal membranes were incubated at 45 °C for various time periods between 0 and 20 min and then assayed for the sulfotransferase reaction with GGL-1 and GalCer as acceptors. The GalCer:ST activity was more susceptible to heat inactivation than GGL:ST, e.g., after 5 min at 45 °C, about 80% of the original GGL:ST activity remained intact, whereas only 40% of the original GalCer:ST activity remained.

**Effects of Dithiothreitol, NaCl, and Pyridoxal Phosphate on the Sulfotransferases**—The effect of dithiothreitol, at different concentrations, on the GGL:ST and GalCer:ST was studied. Dithiothreitol, 0.5 mM, stimulated GGL:ST about 3-fold but had little effect on GalCer:ST. The effect of NaCl on the activities of these enzymes, in the presence and absence of dithiothreitol, is shown in Fig. 6. In the absence of dithiothreitol, 100 mM NaCl reduced the activity of GGL:ST to 25% of the original, whereas GalCer:ST was not negatively affected up to 150 mM NaCl (Fig. 6A). In the absence of dithiothreitol, GGL:ST was also more negatively affected than GalCer:ST, by increasing concentration of NaCl in the incubation mixture (Fig. 6B). Pyridoxal 5-phosphate has been shown to be a potent inhibitor of GalCer:ST (23). GGL:ST was also found to be inhibited by pyridoxal 5-phosphate, however, it was somewhat less susceptible to the inhibitor than GalCer:ST. For example, in the presence of 0.1 mM of the inhibitor, only 10% of the original GalCer:ST remained, but 37% of the original GGL:ST remained.

**Partial Separation of GGL:ST from GalCer:ST**—In order to resolve the activity of GGL:ST from GalCer:ST, PD 15 rat brain microsomal membranes were solubilized in a solution containing 1% Triton X-100 and chromatographed on a pyridoxalphosphate-ligated agarose column (22) (Fig. 7). Under the column conditions given under “Materials and Methods,” the activity of GalCer:ST did not readily bind to the column and was mostly eluted from the column in the loading fraction (fraction zero) and during column wash (fractions 1–15). Whereas, GGL:ST was relatively more bound to the column and was eluted with a gradient of 0–2 M NaCl, and 3-ml fractions were collected. The activity of the GGL:ST and GalCer:ST was measured as described in the text.

**Activity of GGL:ST during Development of the Rat Cerebral Cortex and Cerebellum**—The activity of the GGL:ST was measured in the homogenates of rat cerebral cortex and cerebellum from embryonic day 17 (ED 17) to postnatal day 60 (PD 60) (Fig. 8). The specific activity of GGL:ST in the cerebral cortex increased from ED 17 and reached a maximum at around PD 5 and then declined (Fig. 8A). However, a significant activity (~30% of the maximal) still remained in the cerebral cortex from PD 30 to PD 60.

In the cerebellum, the specific activity of GGL:ST was high at ED 21, the first point measured. The activity slightly increased to a maximum around PD 5, then declined and reached a plateau level between PD 15 and 60 (Fig. 8A).

The GalCer:ST activity was also measured in the cerebral cortex and cerebellum during development (Fig. 8A). In both of these areas, the activity was very low during neonatal period and increased only after PD 10 and reached maximum around PD 15.

The total activity of GGL:ST in the cerebral cortex increased with age and reached a maximum at PD 15, remained about the same until PD 30, and then slightly declined (Fig. 8B). In the cerebellum, also the total activity of GGL:ST increased postnatally and reached a maximum at PD 15 and then slightly declined (Fig. 8B).

**Expression of Sulfotransferases in Different Neural Areas**—The specific activity of GGL:ST in homogenates of PD 25 rat gray matter was 2.5-fold higher than that in white matter (Table IV). The GalCer:ST, however, was 4-fold higher in the white matter than in the gray matter (Table IV). In PD 13 rat, the specific activity of GGL:ST was highest in cerebral cortex and lowest in the spinal chord, whereas GalCer:ST was affected indicating that the same enzyme may be active for the latter two substrates (Table IIIc).
in the cerebral cortex differentiation stages in the cerebral cortex and cerebellum.

It was concluded that GlcA-transferase was not a regulatory enzyme controlling the differential expression of SGGLs in the cortex and cerebellum. Here we have characterized the activity of GGL:ST involved in the last step for the synthesis of SGGLs.

A variety of sulfotransferases have been reported in the nervous system, however, studies on sulfotransferase related to glycolipid acceptors are limited to GalCer:ST. The latter enzyme has been studied in the nervous system and in kidney where it is more preponderant than in other tissues (22-28). In the nervous system, GalCer:ST is mostly localized in the Golgi apparatus of oligodendrocytes in the central nervous system and in Schwann cells in the peripheral nervous system (27, 28). The major function of this enzyme in the cells is to synthesize sulfatide which is a characteristic and abundant lipid of myelin membrane. The enzyme GalCer:ST is expressed maximally in the central nervous system during the period of active myelination in which the rodents reaches a maximum around PD 15-20 (29, 30).

In this report we have described some of the properties of a novel GGL:ST, which are different from those of GalCer:ST. For example, the GGL:ST had a strict requirement for a divalent metal ion, especially Mn²⁺, but Mg²⁺ and Ca²⁺ were also partially effective (Fig. 2). Metal ion requirement for GalCer:ST was not absolute (Fig. 2), which confirmed the previous similar observations (26). The activity of GGL:ST in vitro was also dependent upon the optimal presence of a nonionic detergent such as Triton X-100 or CF-54 (0.1% for about 125 μg of protein); at higher concentration than 0.1%, Triton X-100 was inhibitory. The activity of GalCer:ST was not as sensitive to the detergent concentration (Fig. 1).

The GGL:ST transferred radioactive ³²SO₄ from PAPS specifically to the hydroxyl group on carbon 3 of the terminal GlcA residue of GGL-1 and GGL-2. The activity was drastically reduced when other structural analogs of GGL-1, such as 6-methylGlcAnLcOse4Cer or GlcnLcOse4Cer, were used as acceptors. This showed requirement of a free -COOH group in the terminal GlcA residue, for the transfer of SO₄. No other side products were formed from GGLs by the GGL:ST. Little sulfotransferase reaction activity was seen with other lipid acceptors, when microsomal enzyme from 4-day-old rat brain was used as the enzyme source (Table II). This suggested that at this age, GGL:ST was the major sulfotransferase in the cerebral cortex and other lipid acceptors were not utilized by the enzyme, under the conditions of the assay. Competition experiments with GalCer also showed that GGL:ST activity in vitro was not affected by the presence of GalCer, similarly GalCer:ST activity was not affected by the presence of GGL-1 (Table III, a and b), indicating two separate sulfotransferases. However, GalCer:ST activity was affected by the presence of Cer-Glc-Gal (ceramide lactoside) in the reaction mixture (Table IIIc), indicating GalCer:ST also catalyzed the transfer of SO₄ to ceramide lactoside. It has been reported, with purified preparations of GalCer:ST from rat brain and kidney, that GalCer and ceramide lactoside were equally good acceptors but little activity was seen with psychosine and monogalactosyldiglyceride (26). Studies on heat inactivation and effects of sulfahydral reagent dithiothreitol, NaCl (Fig. 7), and pyridoxal phosphate on the activities of GGL:ST and GalCer:ST clearly showed that these two enzyme activities are most probably due to two different enzyme proteins. Both of these sulfotransferase activities were partially resolved on

![Fig. 8. A, specific activities of GGL:ST and cerebroside:ST in homogenates of rat cerebral cortex and cerebellum during embryonic (E) and postnatal development. Zero represents day of birth. Values at each age are the average of two determinations with three separate groups of animals. The values varied within ±14% of the average value. B, total activity of GGL:ST in homogenates of rat cerebral cortex and cerebellum during development.](image)

**TABLE IV**

*Specific activity of GlcAnLcOse4Cer:ST and GalCer:ST in homogenates of various neural area of rat*

| Neural tissue | GlcAnLcOse4Cer:ST | GalCer:ST |
|---------------|------------------|----------|
| Postnatal 25 days |                 |          |
| Gray matter    | 60               | 10       |
| White matter   | 24               | 42       |
| Postnatal 15 days |             |          |
| Cerebral cortex | 115              | 24       |
| Cerebellum     | 55               | 12       |
| Pons           | 64               | 106      |
| Spinal cord    | 19               | 241      |

activity was highest in the spinal chord and lowest in the cerebellum.

**DISCUSSION**

The expression of SGGLs in the mammalian cerebral cortex, as determined by biochemical methods, is limited to embryonic development and is no longer detectable shortly after postnatal development (3, 17). In the cerebellum, however, these glycolipids are robustly expressed during neonatal development, followed by a significant decline by PD 7 and a second burst of expression near PD 20 (18). Specific glycosyltransferases and glycosidases are possibly involved in the regulation of expression of SGGLs during the neural cell differentiation stages in the cerebral cortex and cerebellum. In addition, these enzymes are possibly regulated differently in the cerebral cortex versus cerebellum to account for the unique temporal expression of SGGLs in these areas of the nervous system. Previously, we have reported on the expression and regulation of UDP-glucurononateolactotetraosylceramide glucuronyltransferase during development of the nervous system (16).
a phosphopyridoxylagarose column, leading to the conclusion that these lipid sulfotransferases are two different proteins.

The expression profiles of GGL:ST and GalCer:ST in the rat cerebral cortex and cerebellum during development are remarkably different (Fig. 8). As previously reported by others (29, 30), the specific activity of GalCer:ST was negligible neonatally and reached a maximum near PD 15–20 (Fig. 8). The specific activity of GGL:ST in the cerebral cortex increased sharply during embryonic and neonatal development and reached a maximum at PD 5. The specific activity in the cerebellum also increased neonatally but not as sharply as in the cerebral cortex and reached a maximum around PD 5. The total and specific activities of GGL:ST do not correspond to the amount of SGGLs in the rat cerebral cortex and cerebellum during development of these tissues (3, 18).

SGGLs almost completely disappeared from the cerebral cortex by PD 20, however, a significant level of GGL:ST activity remained in the cortex. In the cerebellum, the amount of SGGLs increased during postnatal development, but the specific activity of GGL:ST declined. These results suggest that GGL:ST is not a regulatory enzyme which controls the expression of SGGLs in these neural tissues. We have previously reported similar results with the expression of UDP-glucuronate:neolactotetraosylceramide glucuronyl transferase involved in the synthesis of GGLs (16). In fact, analyses of the availability of the precursors nLcOse,Cer and nLcOse,Cer in these tissues during development correlated much better with the expression of SGGLs, suggesting that enzymes involved in the synthesis of these precursors control the differential expression of SGGLs in these tissues (16).

Available evidence indicates that SGGLs are the products of and primarily localized in the neuronal cells rather than in glial cells at least in the central nervous system (17). Immunoocytochemical localization and analysis of SGGLs in Purkinje cell abnormality murine mutants clearly showed their expression only in Purkinje cells in the cerebellum (18, 31, 32). Comparisons of the activity of GGL:ST and GalCer:ST in the gray matter, which is enriched in neuronal cells, and in white matter, which is enriched in oligodendroglial cells, indicate that GGL:ST is enriched in gray matter, whereas GalCer:ST is enriched in white matter. Van der Pal et al. (30) have reported that the activity GalCer:ST was 5-fold higher in the spinal cord than in cerebral cortex at PD 15. We confirm these results, however, about 5-fold higher GGL:ST activity in the gray matter than in the spinal cord corroborates the conclusion that these two sulfotransferases are different. Previously, it has been reported that the levels of SGGLs in spinal cord are extremely low.

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