

Ganglioside biosynthesis in mouse embryos: sialyltransferase IV and the asialo pathway

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Abstract The *in vitro* activity of sialyltransferase IV (SAT-IV), which catalyzes the transfer of sialic acid to the terminal galactose of different gangliotetraosylceramides (GA1, GM1a and GD1b), was examined in membrane-enriched preparations from mouse embryos at embryonic day 12 (E-12). Gangliosides GD1a and GT1b were the only reaction products using GM1a and GD1b as substrates, respectively. The K_m values for GM1a and GD1b were 53 μ M and 42 μ M, respectively. Competitive inhibition experiments showed that the same enzyme (SAT-IV) catalyzed sialic acid transfer to the terminal galactose residues of both GM1a and GD1b. Two labeled ganglioside products were obtained, however, using GA1 as a substrate. One product was identified as ganglioside GM1b and the enzymatic reaction for its formation was maximal at pH 6.0, similar to that for GD1a and GT1b formation. The second product, synthesized by a different sialyltransferase, was identified as GD1 α based on results from TLC immunostaining, neuraminidase digestion, and periodate oxidation–borohydride reduction. The pH dependence curve for GD1 α formation had a different shape than that for GM1b formation with a maximum at pH 6.3. GD1 α is apparently synthesized from GM1b by an endosialyltransferase that catalyzes the transfer of a second sialic acid to the internal N-acetylgalactosamine of GM1b. The formation of both GM1b and GD1 α was linear over protein concentration. The ratio of GD1 α /GM1b formation varied from 0.25 to 1.20 depending on the GA1 substrate concentration. We also show that the high SAT-IV activity measured *in vitro* is not correlated with the expression of ganglio-series gangliosides in E-12 mouse embryos measured previously *in vivo*.—Seyfried, T. N., A. M. Novikov, R. A. Irvine, and J. V. Brigande. Ganglioside biosynthesis in mouse embryos: sialyltransferase IV and the asialo pathway. *J. Lipid Res.* 1994. 35: 993–1001.

Supplementary key words GD1 α • GM1b • GD1 • sialic acid

Gangliosides are a family of sialic acid-containing glycosphingolipids that are in high abundance in neural cells and may have a variety of functions in developing and mature tissues (1, 2). The regulation of ganglioside biosynthesis during vertebrate embryonic development, however, is far from clear. In general, the synthesis of the hemato-series gangliosides (GM3 and GD3) predominates during early stages of vertebrate embryogenesis, whereas the synthesis of the more complex ganglio-series gangliosides (GM1, GD1a, GD1b and GT1b) predominates at later stages (3–9). This shift in synthesis from the hemato-

series to the ganglio-series gangliosides is often associated with a reduction in GD3 synthase activity (sialyltransferase II, SAT-II) and an elevation in GM2 synthase activity (GalNAc transferase) (5–7, 10). In other words, the changes in these ganglioside synthetic enzymes measured *in vitro* are correlated with changes in the *in vivo* expression of gangliosides in the “a” (GM3, GM2, GM1, and GD1a) and “b” (GD3, GD2, GD1b, GT1b and GQ1b) biosynthetic pathways (11).

In contrast to these synthetic enzymes and pathways, little is known about the activity of sialyltransferase IV (SAT-IV) or about the expression of gangliosides in the “asialo” biosynthetic pathway during early vertebrate embryogenesis. SAT-IV is proposed to catalyze the synthesis of GM1b, GD1a, and GT1b from GA1, GM1a, and GD1b, respectively (11–16). The “asialo” pathway involves the synthesis of gangliosides GD1 and GM1b through the asialo precursors GA1, GA2, and lactosylceramide (11, 12, 17–22). Previous studies in rat bone marrow cells and in liver Golgi vesicles suggest that the *in vitro* activities of glycosyltransferases in the “asialo” pathway are not correlated with the *in vivo* expression of relevant glycolipid products (20, 21). To explore this phenomenon further, we analyzed SAT-IV activity towards different gangliotetraosyl ceramide substrates in the “asialo”, “a”, and “b” biosynthetic pathways in membrane-enriched preparations from early (E-12) mouse embryos. We find that GD1 α , rather than GD1c, is the synthetic end product when GA1 is used as substrate.

EXPERIMENTAL PROCEDURES

Materials

Cytidine monophosphate, [4-¹⁴C]NeuAc (4.1 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Triton CF-54, 4-chloro-1-naphthol, peroxidase-

Abbreviations: HPTLC, high performance thin-layer chromatography; PBS, phosphate-buffered saline.

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conjugated goat anti-rabbit IgG, neuraminidase (type VI) from *Clostridium perfringens*, sodium borohydride, and sodium metaperiodate were purchased from Sigma Chemical Co., St. Louis, MO. Neuraminidase from *Arthrobacter ureafaciens* was obtained from Calbiochem Corp., La Jolla, CA. Poly(isobutylmethacrylate), high molecular weight, was purchased from Aldrich Chemicals, Milwaukee, WI and gangliotetraosylceramide (GA1) was obtained from Matreya, Inc., Pleasant Gap, PA. Rabbit anti-GA1 antiserum and ganglioside GM1b were gifts from Drs. Robert K. Yu, Toshio Ariga, and Megumi Saito of the Medical College of Virginia, Richmond, VA. The GD1 α standard and anti-GD1 α antibody (KA-17) were gifts from Dr. Yoshio Hirabayashi, Frontier Research Program, RIKEN, Wako, Japan. Standard gangliosides GM1, GD1a, GT1a, GD1b, GT1b, and GQ1b were isolated from adult mouse brain as described previously (23). The gangliosides were separated on Whatman high performance thin-layer chromatography (HPTLC) plates. All other reagents were of analytical grade from commercial sources.

Mice

The mouse strain used in these studies was C3H/DiSn. The animal husbandry conditions were as previously described (24) and the embryos used were collected at E-12. The appearance of a vaginal plug was used to designate day 0 of gestation (E-0). The pregnant mice were killed by cervical dislocation, the uterine horns were removed, the embryos were collected in ice-cold phosphate-buffered saline and then frozen at -70°C . A membrane-enriched P₂ pellet was prepared as described before (25). Briefly, the thawed whole embryos were gently homogenized by hand in 0.32 M sucrose. The homogenate was centrifuged at 1080 g for 10 min. The supernatant was collected and centrifuged at 20,200 g for 45 min to obtain the P₂ pellet. This pellet was resuspended in 0.4–1.0 ml of 0.32 M sucrose and frozen at -70°C if not used immediately. The protein content was determined by the method of Lowry et al. (26).

CMP-NeuAc:glycolipid sialyltransferase assay

The reaction conditions were as follows: the glycolipid substrate (GA1, GM1a, or GD1b), 5–20 nmol; the sialic acid nucleotide donor CMP[4-¹⁴C]NeuAc, 25 nmol; the detergent, Triton CF-54, 50 μg ; cacodylate-HCl buffer, pH 6.0 (10 μmol), containing 1.0 μmol MnCl₂ and 50–100 μg P₂ protein in a total volume of 50 μl . Control tubes contained no glycolipid substrate. The mixture was incubated for 4 h at 37 $^{\circ}\text{C}$. The enzyme reaction was stopped by boiling for 5 min. Carrier gangliosides from mouse brain were then added in 1.0 ml of 0.1 M NaCl. The labeled ganglioside products were desalted using C-18 Bond Elut columns (Analytichem International, Harbor City, CA) as described previously (25). All assays were performed in triplicate.

Estimation and characterization of labeled ganglioside products

The eluates from Bond Elut columns were evaporated, and separated by HPTLC with chloroform-methanol-water 50:45:10 containing 0.02% CaCl₂. The ganglioside bands were visualized with the resorcinol reagent (27) and the radiolabeled ganglioside products were identified through comigration with carrier gangliosides of known structure. Individual ganglioside bands were scraped from the HPTLC plates and the amount of radioactivity in each band was determined using a Pharmacia LKB Liquid Scintillation counter. Alternatively, labeled ganglioside products were quantitated directly on the HPTLC plates, prior to resorcinol spraying, by radiographic scanning of the developed chromatograms using a Bioscan Imaging Scanner (Bioscan Inc., Washington, DC) as previously described (28). Autoradiography of the labeled gangliosides was performed by exposing HPTLC plates to Hyperfilm-³H (Amersham) for 3 days at -70°C .

For structural characterization, the labeled reaction products (combined from about 20 assays) were applied to HPTLC plates and separated as described above. Individual ganglioside bands, located by autoradiography and radiographic scanning, were scraped from the plates and eluted twice for 30 min with 10 ml of chloroform-methanol-water 10:10:3 (v/v) by sonication and vortexing. The eluates were evaporated and repurified using Bond Elut columns as above.

Neuraminidase treatment

The labeled products were evaporated to dryness and treated with neuraminidase as described previously (29). Briefly, dried residues were dissolved in 140 μl 0.1 M sodium acetate buffer (pH 5.0) and the solutions were mixed with 15 μl of *C. perfringens* neuraminidase (1 unit/ml) with no added detergent. The mixtures were incubated for 4 h at 4 $^{\circ}\text{C}$ or for 10 h at 37 $^{\circ}\text{C}$. The reaction products were purified by Bond Elut columns and analyzed by HPTLC, radiographic scanning, and immunostaining.

Immunostaining

Immunostaining for asialo GM1 (GA1) was performed on HPTLC plates according to the method of Saito, Kasai, and Yu (30). After development, the plate was air-dried for 1 h, dipped into a 0.4% poly(isobutylmethacrylate) solution (prepared by diluting 2.5% of the polymer solution in chloroform with n-hexane) for 1 min, and air-dried again for 30 min. The *C. perfringens* or *A. ureafaciens* neuraminidase solutions (75 mU/ml, in 0.1 M sodium acetate buffer, pH 4.8) were applied to each lane on the chromatogram. The lanes were covered with parafilm and incubated at room temperature for 2 h. After development the plate was washed twice with phosphate-buffered saline (PBS, pH 7.4) and air-dried

briefly. The neuraminidase-treated gangliosides were incubated with anti-GA1 antiserum (diluted 1:40 with PBS, containing 1% bovine serum albumin, BSA) for 1 h as described above. After incubation the plate was washed twice with PBS. A peroxidase-conjugated anti-rabbit IgG solution (diluted 1:250 with PBS-1% BSA) was then added to each lane. After 1 h incubation at room temperature, the plate was washed twice with PBS and air-dried. The ganglioside bands were visualized by staining with the following mixture: 10 ml 4-chloro-1-naphthol solution in methanol (3 mg/ml), 25 ml 0.2 M Na₂HPO₄-0.1 M citric acid (pH 5.0), 25 ml water, and 6.6 μl 30% H₂O₂. Immunostaining for GD1α was performed on plastic TLC plates (Nagel Sil G., Germany) according to the procedures of Hirabayashi et al. (31).

Periodate oxidation-borohydride reduction

The presence or absence of sialic acids susceptible to periodate oxidation-borohydride reduction in the purified reaction products of the SAT-IV assay was analyzed as previously described (32, 33). After periodate-borohydride treatment, the labeled reaction products were desalted using Bond-Elut columns (as above) and were then hydrolyzed with mild acid (200 μl of 50 mM HCl for 1 h at 80°C). The released C9 sialic acid and its C7 analogue (N-acetylheptulosaminic acid) were purified by ion-exchange chromatography using AG1-X4 (ACO 200-400 mesh) anion resin (Bio-Rad Laboratories). The purified products were esterified in 50 mM methanolic hydrogen chloride (1 ml) for 1 h at 60°C. The esters were separated on HPTLC plates in 2-butanone-glacial acetic acid-methanol-water 10:4:3:1 (v/v) for 40 min. The esters were visualized by autoradiography and radiographic scanning as described above. As an internal control for the reaction, unlabeled ganglioside GD3 (0.01 μmol) was analyzed along with the labeled SAT-IV reaction products. The unlabeled C9 and C7 GD3 esters were visualized on HPTLC by the resorcinol spray (27).

RESULTS

All of the reactions were conducted in the P₂ fractions prepared from whole mouse embryos as we previously described (25). Whole embryos were used as we found previously that both glycolipid composition and sialyltransferase activity were similar in head and body regions of early mouse embryos (25, 34, 35). Gangliosides GD1a and GT1b were the only radiolabeled products formed in the SAT-IV assay when GM1a and GD1b were used as substrates, respectively. The identity of these products was determined from: *a*) their comigration on HPTLC plates with purified GD1a and GT1b standards; *b*) their conversion to an unlabeled ganglioside that comigrated with purified GM1a (after treatment with *C. perfringens* neur-

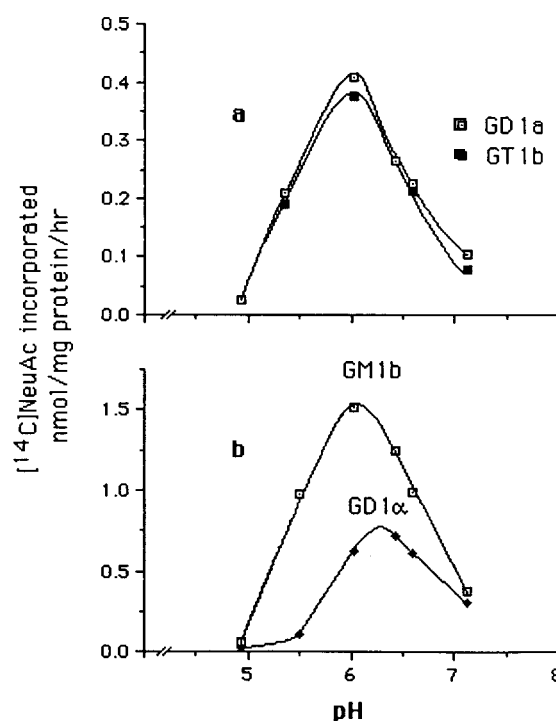


Fig. 1. Effect of pH on the rate of ganglioside formation in E-12 mouse embryos. *a*: Formation of GD1a and GT1b from GM1a and GD1b substrates, respectively. *b*: Formation of GM1b and GD1α from GA1 substrate. The conditions of the assay were as described in Experimental Procedures.

aminidase for 10 h at 37°C); and *c*) their conversion to GA1 (detected by anti-GA1 immunostaining) after treatment on the HPTLC plate with *A. ureafaciens* neuraminidase. The *A. ureafaciens* neuraminidase can remove sialic acid from both internal and external galactose residues as we previously showed (36). The pH dependence curves for GD1a and GT1b formation were narrow and both had a sharp peak at pH 6.0 (Fig. 1).

Competitive inhibition experiments were performed to determine whether the synthesis of GD1a and GT1b was catalyzed by the same or different enzymes (11, 37). Both the GM1a and GD1b substrates were used simultaneously in changeable partial concentrations. The total substrate concentration was maintained at 300 μM and the total reaction velocity (GD1a plus GT1b formation) was determined for each point and designated v_t .

For two independent enzymes, the total reaction velocity, v_t , can be described by the Michaelis-Menten equation:

$$v_t = v_a + v_b = \frac{V_a}{(1 + K_a/[a])} + \frac{V_b}{(1 + K_b/[b])} \quad \text{Eq. 1}$$

where v_a and v_b are partial velocities for substrates *a* and *b*.

If the same enzyme catalyzes the glycosylation of both substrates, v_t can be described as follows:

$$v_t = v_a + v_b = \frac{V_a}{1 + (K_a/[a])[1 + ([b]/K_b)]} + \frac{V_b}{1 + (K_b/[b])[1 + ([a]/K_a)]} \quad \text{Eq. II)}$$

The K_m and V_{max} values were determined in the same membrane-enriched preparation that was used for the competitive inhibition experiments. For GM1a, $K_m = 53 \mu\text{M}$, $V_{max} = 445 \text{ pmol/mg protein/hr}$; for GD1b, $K_m = 42 \mu\text{M}$ and $V_{max} = 385 \text{ pmol/mg protein/hr}$.

The total reaction velocities calculated from equations I and II are shown in Fig. 2. The experimental v_t values matched closely those calculated from equation II. These data suggest that the same enzyme catalyzes the formation of GD1a and GT1b from their exogenous precursors. No ganglioside synthesis was detected in the absence of exogenous substrates.

Two major radiolabeled reaction products were detected when GA1 was used as a substrate in the SAT-IV assay. These products were localized directly on the HPTLC plate by autoradiography, radiographic scanning, and immunostaining using the anti-GA1 antibody (Figs. 3, 4, and 5). Two minor secondary reaction products were also detected on the autoradiogram. One minor product migrated as a double band with standard GM3 and the other migrated slightly behind standard GD3 (Fig. 3). These minor secondary products likely arose from the combined action of endogenous sialidase

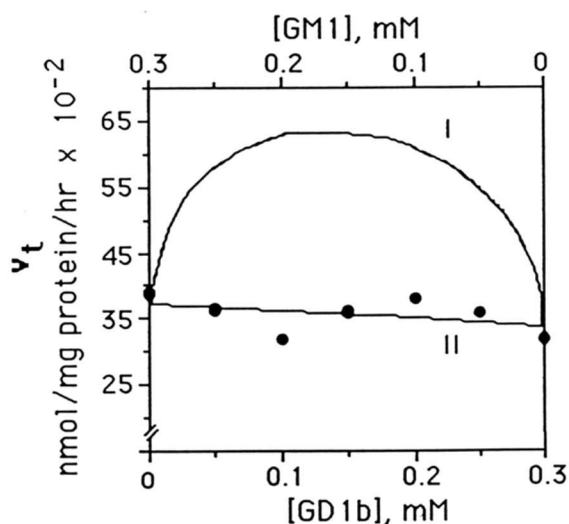


Fig. 2. Competition between GM1a and GD1b in the sialyltransferase assay. Varying partial concentrations of GM1a and GD1b were used, while maintaining the total substrate concentration at $300 \mu\text{M}$. The total reaction velocity for each point (v_t) was determined experimentally (●) and was calculated from equations for either two different enzymes (I, upper curve) or for a single enzyme (II, lower curve).

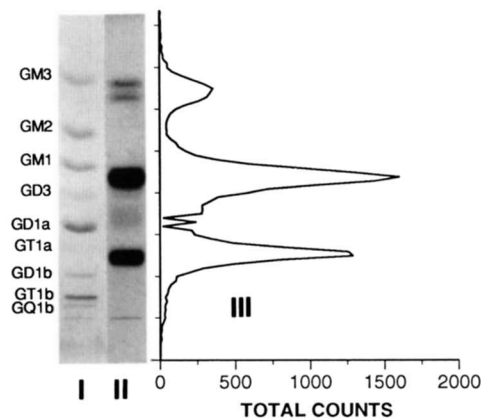


Fig. 3. Reaction products of the SAT IV assay using GA1 as substrate. The total product mixture was separated by HPTLC and visualized by autoradiography and direct radiographic scanning. I, a mixture of ganglioside standards visualized by the orcinol reagent. The GM1 standard is GM1a; II, autoradiogram of the total product mixture; III, radiographic scan (Bioscan) of the total product mixture.

and sialyltransferases (SAT-I and SAT-II) on endogenous substrates in the P2 embryo preparation as we previously described (25).

The major fast-moving reaction product migrated slightly behind GM1a and corresponded to standard GM1b (Fig. 3, Fig. 4aIII, and 4b). The major slow-moving reaction product migrated between GD1a and GD1b (Figs. 3 and 4aI). Both products were purified by preparative TLC as described in Experimental Procedures. Three radioactive bands were detected after the treatment of the

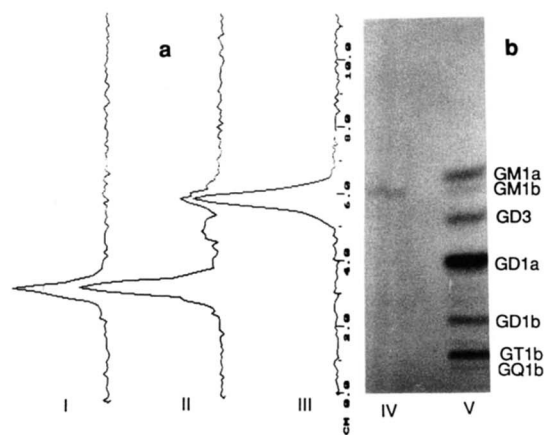


Fig. 4. Biosynthesis of gangliosides from exogenous GA1 in mouse embryos. The individual labeled gangliosides were purified from the total reaction product by preparative TLC and re-chromatographed with standard gangliosides. a: Direct radiographic scanning of the HPTLC plate using Bioscan; b: visualization of the same plate by resorcinol reagent. I, the slow-moving reaction product; II, the same product after treatment with *C. perfringens* neuraminidase for two h at 4°C ; III, the fast-moving reaction product; IV, ganglioside GM1b; and V, a mixture of standard brain gangliosides with added GM1b.

purified slow-moving product with *C. perfringens* neuraminidase under mild conditions (4 h at 4°C) (Fig. 4aII). The first band (major) corresponded to the original unreacted slow-moving product. The second band (minor) migrated between GM1b and GD1a in the same region predicted for GM1 α (31). The third band (minor) corresponded exactly to the purified fast-moving product (GM1b).

Treatment of the combined fast- and slow-moving radiolabeled glycolipids with *C. perfringens* neuraminidase for 10 h at 37°C produced a single nonradioactive product that stained positive with anti-GA1 antibody and migrated on the HPTLC plate with the purified GA1 substrate (Fig. 5aI). Treatment of the entire reaction mixture directly on the HPTLC plate with *C. perfringens* neuraminidase produced three GA1-immunoreactive products (Fig. 5aII). The fastest migrating immunoreactive band corresponded to the purified GA1 substrate and was nonradioactive. The second band was radiolabeled and corresponded to standard GM1b, and the third band was also radiolabeled and migrated between GD1a and GD1b (Fig. 5).

These data indicate that both of the major glycolipid products formed from exogenous GA1 had a ganglio-tetraose backbone and sialic acid residues susceptible to *C. perfringens* neuraminidase. The fast-moving glycolipid contained a sialic acid residue attached to the terminal galactose and was designated GM1b. The slow-moving glycolipid could be formed by the addition of a second sialic acid residue to the newly formed GM1b. It was not clear, however, whether this major slow-moving product

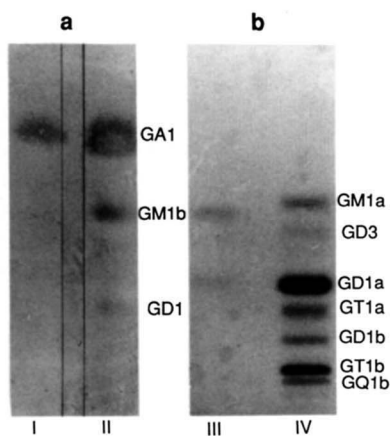


Fig. 5. HPTLC immunostaining of synthesized gangliosides from exogenous GA1 in mouse embryos. Glycosphingolipids were separated by HPTLC and visualized by a: immunostaining with anti-GA1 antibody (as described in Methods) and b: resorcinol reagent. I, the combined slow- and fast-moving reaction products treated with *C. perfringens* neuraminidase prior to spotting on the HPTLC; II, the total reaction product treated with *C. perfringens* neuraminidase directly on the HPTLC plate; III, standard GM1b; and IV, mixture of the brain gangliosides. The faint band migrating with GD1a in III was not resorcinol-positive.

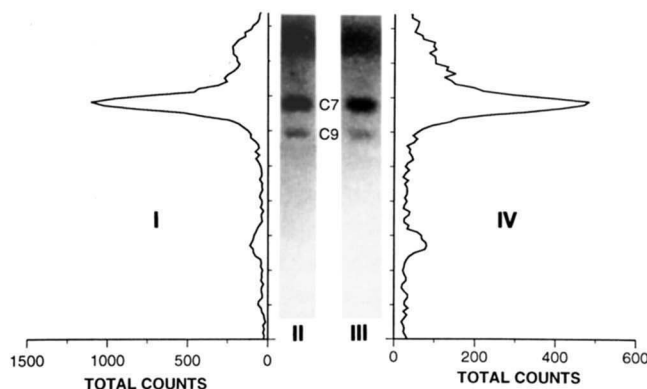


Fig. 6. Periodate-borohydride treatment of GM1b and GD1 α . GM1b and GD1 α were purified from the total product mixture by preparative TLC and subjected to the periodate oxidation-borohydride reduction procedure (see Experimental Procedures). Unlabeled GD3 (0.01 μ mol) was added as an internal control for each reaction. I, radiographic scan of methyl esters derived from treated GM1b; II and III, chromatogram of methyl esters derived from treated GD3 (internal control) visualized by resorcinol reagent: C9 and C7, methyl esters of N-acetylneuraminic acid and N-acetylheptulosaminic acid, respectively; IV, radiographic scan of methyl esters derived from treated GD1 α .

was GD1 α or GD1. GD1 contains a sialosyl-sialosyl linkage attached to the terminal galactose of GA1, whereas GD1 α contains a single sialic acid on the terminal galactose and a second sialic acid attached to the internal N-acetylgalactosamine (GalNAc) (12, 31, 38–40). According to Taki et al. (40), *C. perfringens* neuraminidase can hydrolyze both the terminal and C6-linked NeuAc residues of GD1 α . This could account for the minor amounts of both GM1b and GM1 α that we found using the mild reaction conditions described above.

The periodate oxidation-borohydride reduction procedure of Ando and Yu (32) as modified by Yohe and Yu (33) was used to determine whether the slow-moving reaction product was GD1. This procedure will convert susceptible N-acetylneuraminic acid residues to their 7-carbon (C7) analogue, N-acetylheptulosaminic acid. Internal sialic acids that are part of a sialosyl-sialosyl linkage will be protected and will yield only the C9 N-acetylneuraminic acid. Periodate-borohydride treatment of the purified radiolabeled GM1b reaction product produced only the 7-carbon analogue as expected (Fig. 6, I and II). The radiolabeled C7 analogue migrated exactly with the resorcinol-positive C7 band generated from the internal control GD3 sample. Treatment of the purified radiolabeled slow-moving reaction product also produced only the C7 analogue (Fig. 6, III and IV). This reaction product migrated exactly with the product generated from GM1b conversion indicating that both sialic acids in the slow-moving product are susceptible to periodate oxidation. The absence of a radiolabeled C9 band from the slow-moving reaction product indicates that this product cannot be GD1.

To better define the structure of the slow-moving reaction product we used TLC-immunostaining with the anti-GD1 α monoclonal antibody, KA-17. The results in **Fig. 7** show that the radiolabeled slow-moving reaction product was immunopositive with KA-17. The slight difference in TLC migration between the immunopositive GD1 α band in the GD1 α standard (lane II) and that in the total SAT-IV reaction mixture (lane III) could result from non-lipid impurities in the reaction mixture. The major fast-moving radiolabeled peak (GM1b) was not immunopositive with KA-17. These findings, together with the periodate oxidation-borohydride reduction experiments, indicate that the structure of the slow-moving reaction product is most likely GD1 α rather than GD1.

The formation of GM1b and GD1 α was linear over protein concentrations from 0.3 to 1.5 mg/ml with a constant protein/detergent ratio (**Fig. 8**). The pH dependence curve for GM1b formation was similar to that for GD1a and GT1b formation with a maximal activity around pH 6.0 (**Fig. 1b**). This suggests that the three gangliosides are synthesized by the same SAT-IV enzyme. The pH dependence curve for GD1 α formation, however, had a different shape and a different pH maximum of 6.3. This suggests that GD1 α synthesis occurs through the action of a sialyltransferase different from SAT-IV. The simultaneous formation of GM1b and GD1 α prevented determination of K_m and V_{max} values or competitive inhibition experiments using GM1a and GD1b as the alternative substrates. The rate of GM1b synthesis was greater than that

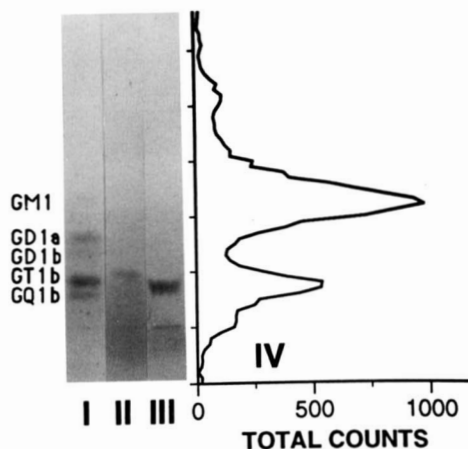


Fig. 7. TLC immunostaining of the reaction products in the SAT-IV assay. I: Ganglioside standard mixture visualized with resorcinol reagent. II: GD1 α standard (10 pmol) visualized by immunostaining with the anti-GD1 α monoclonal antibody, KA-17. III: Anti-GD1 α immunostaining of the *in vitro* reaction products when exogenous GA1 was used as substrate. IV: Radiographic scan of the *in vitro* reaction products in lane III prior to immunostaining. The radiolabeled slow-moving reaction product comigrates with the GD1 α immunopositive band in lane III. The plate (a plastic Nagel Sil G) was developed in one ascending run with chloroform-methanol-water 55:40:10 (by vol) that contained 0.02% CaCl₂.

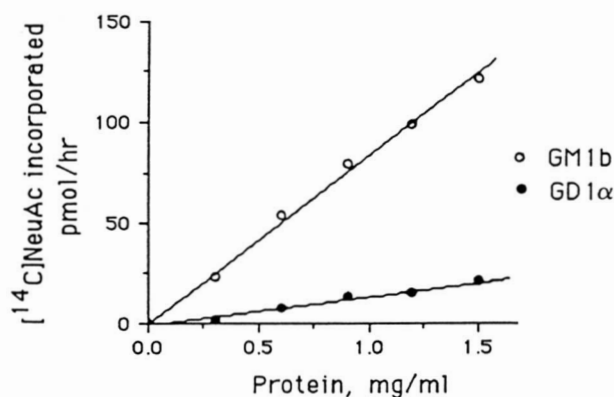


Fig. 8. Effect of protein concentration on the rate of ganglioside formation from gangliotetraosylceramide (GA1) in E-12 mouse embryos.

of GD1a or GT1b synthesis under the same experimental conditions (**Fig. 1**).

The ratio of GD1 α /GM1b formation varied from 0.25 to 1.20 depending on the substrate concentration. GD1 α formation equaled or exceeded GM1b formation at low substrate concentrations (less than 50 μ M) (**Fig. 9**). At higher GA1 concentrations, GM1b was the predominant product of the reaction.

DISCUSSION

Our findings in E-12 mouse embryos suggest that a single enzyme, SAT-IV, catalyzes the transfer of sialic acid to the terminal galactose of GA1, GM1a, and GD1b to form gangliosides GM1b, GD1a, and GT1b, respectively. These results are consistent with previous biochemical findings in rat liver Golgi vesicles (11, 15, 41). The lower rate of GD1a and GT1b synthesis relative to GM1b synthesis may result from steric hindrance of the sialic acid transfer to the terminal galactose of the gangliotetraose backbone in

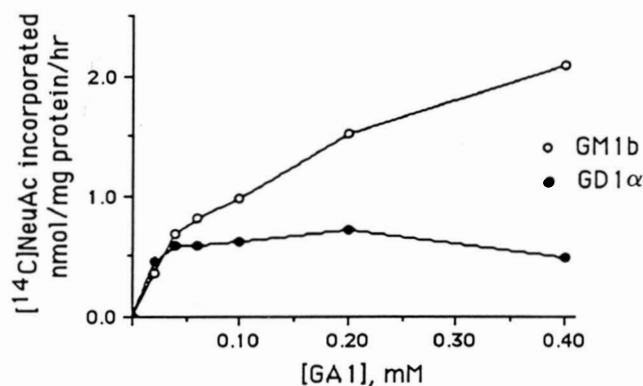


Fig. 9. Effect of substrate concentration on the rate of ganglioside formation from GA1 in E-12 mouse embryos.

the presence of a sialic acid residue bound to the inner galactose as previously suggested (11, 41).

Besides GM1b synthesis, the synthesis of a second ganglioside always occurred in the SAT-IV assay when GA1 was used as a substrate. Pohlentz et al. (11) and Iber and Sandhoff (41) also described the synthesis of a second slow-moving ganglioside product from similar SAT-IV assays in rat liver Golgi vesicles. This ganglioside represented only about 2% of the radioactive ganglioside products using GA1 as substrate and was designated GD1c. SAT-V was proposed to synthesize GD1c from GM1b through the addition of a second sialosyl residue to the terminal sialic acid of GM1b (11, 41, 42). GD1c was thought to represent the end product of the asialo metabolic pathway (11, 41, 42). To avoid confusion with gangliosides synthesized from GT3 in the "c" metabolic pathway, Yu (12) represents GD1, rather than GD1c, as the end product of the "asialo" pathway. In contrast to the findings in rat liver Golgi vesicles, we found significant amounts of the second slow-moving reaction product in the SAT-IV assay from mouse embryo membranes.

Based on evidence from TLC immunostaining, neuraminidase digestion, and periodate oxidation-borohydride reduction, we identified this second ganglioside product as GD1 α rather than GD1. GD1 α was originally characterized in frog brain (39) and more recently in murine T-lymphocytes, murine tumor cells, and as an extremely minor ganglioside in adult bovine brain (31, 38, 40, 43, 44). Because the pH dependence for GD1 α formation was different from the formation of GM1b, GD1a and GT1b, a sialyltransferase different from SAT-IV is likely responsible for GD1 α synthesis. An α 2-6 sialyltransferase, which adds a sialic acid to the 6 position of the internal GalNAc residue, is thought to catalyze the synthesis of GD1 α using GM1b as substrate (31, 45). Although an endosialyltransferase has not been previously characterized for ganglioside biosynthesis, such an enzyme has been characterized for glycoprotein synthesis in fetal calf liver (46). Hidari and coworkers also found a similar enzyme in membrane preparations from rat liver (K. I.-P. J. Hidari, Y. Sanai, and Y. Hirabayashi personal communication). It is not yet clear, however, whether GD1 α synthesis occurs in the mouse embryos *in vivo* or is an artifact formed from the disrupted *in vitro* membrane system. Our results indicate that an endosialyltransferase is active in E-12 mouse embryos and may be important for ganglioside synthesis through the "asialo" pathway.

We previously showed that the predominant gangliosides in E-12 mouse embryos were GD3 (51% of total sialic distribution), GM3 (19%), and GT1b (9.6%) (3). Other gangliosides occurred in much lower amounts, e.g., GM2 (2.6%), GM1 (1.6%), GD1a (3.7%), GD1b (6.3%), and GQ1b (4.5%). These ganglioside distributions were also similar in both neural and non-neural embryo structures suggesting that most undifferentiated embryonic

cells in mice contain GM3 and GD3 as the major ganglioside species (3, 34). Although N-glycolylneuraminic acid (NeuGc) is a major sialic acid in adult mouse non-neural tissues, N-acetylneuraminic acid (NeuAc) is the predominant sialic acid detected in both neural and non-neural embryonic tissues (34). In addition, the high level of GD3 in the mouse embryos was correlated with a high specific activity of sialyltransferase II (605 pmol/mg protein per hr) (25). Other investigators have also reported a positive correlation between GD3 expression and its synthetic rate in vertebrate embryonic tissues (5, 6, 7).

In the present study we found that the high SAT-IV activity in the mouse embryos was not correlated with the expression of relevant ganglioside products, i.e., GM1b and GD1a. As previously shown, GT1b and GD1a are expressed in low amounts (3, 34), and GM1b occurs in only trace amounts. These findings are consistent with results from several previous studies in different mammalian cells and tissues, where high SAT-IV activity measured *in vitro* was not correlated with the expression of relevant gangliosides measured *in vivo* (11, 19, 47).

The discrepancy between SAT-IV activity and product expression may depend on the availability of the substrates for the SAT-IV reaction. In the mouse embryos, for example, the key substrates are either undetectable (GA1) or are expressed in low amounts (GM1a and GD1b). Although LacCer is abundant in E-12 mouse embryos (35), the other glycolipid precursors in the asialo pathway (GA2 and GA1) are undetectable. This could account for the absence of detectable GM1b in the presence of a high SAT-IV activity. Recent studies suggest that the expression of other glycolipids, e.g., sulfoglucuronyl glycolipids and GM4, is also regulated in part by the availability of key upstream metabolic precursors (48, 49). Hence, our findings in mouse embryos support the substrate availability hypothesis of glycolipid regulation. We also realize that multiple regulatory mechanisms are likely for the developmental expression of glycolipids as recently outlined (50).

Little is known about the regulation or function of glycolipids in the "asialo" metabolic pathway. The presence of high SAT-IV activity in E-12 mouse embryos, which do not express detectable GM1b, may offer a model system for examining GM1b function. It would be interesting to determine, for example, whether the addition of appropriate asialo precursors (GA2 or GA1) to embryonic mice might induce a *de novo* expression of GM1b or GD1 α . ■

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