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BIOSYNTHESIS *in vitro* OF MONO- AND DI-SIALOSYLGANGLIOSIDES FROM GANGLIOTETRAOSYLCERAMIDE BY CULTURED CELL LINES AND YOUNG RAT BRAIN. STRUCTURE OF THE PRODUCTS, AND ACTIVITY AND SPECIFICITY OF SIALOSYLTRANSFERASE*

PIERRE STOFFYN AND ANNE STOFFYN

Eunice Kennedy Shriver Center for Mental Retardation, Walter E. Fernald State School, Waltham, Massachusetts 02154, and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115 (U.S.A.)

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ABSTRACT†

Incubations *in vitro* of G_{A1}, labeled with ^3H in the terminal D-galactopyranosyl group, with nonradioactive CMP-NeuNAc in the presence of homogenates of $C2_1$ rat brain glial cells, NIE mouse neuroblastoma cells, 3T3 mouse fibroblasts, SV 40-transformed 3T3 cells, chick embryo fibroblasts, Rous sarcoma virus-transformed chick embryo fibroblasts, and 9-day old rat brain resulted in all cases in the formation in high yield of G_{M1b}, in which the neuraminidase-labile NeuNAc group is linked at O-3 of the terminal D-galactosyl residue, as shown by permethylation studies. No trace of the naturally occurring neuraminidase-stable G_{M1a} was detected in any case. In addition, with NIE cells, and normal and RSV-transformed chick embryo fibroblasts, a disialosylganglioside (G_{D1}) differing from G_{D1a} and G_{D1b}, and bearing only one substituent at O-3 of the terminal D-galactopyranosyl residue was formed. It was also biosynthesized from G_{M1b} and CMP-NeuNAc by NIE and chick embryo cells but not by $C2_1$ cells, or rat brain. However, $C2_1$ cells and rat brain were capable of synthesizing G_{D1a} from G_{M1a}. Periodate oxidation degraded both NeuNAc groups in G_{D1} to a 7-carbon fragment, indicating lack of substitution at O-8. G_{M1b} could not be detected as a natural product in rat brain.

*Preliminary communications have been presented, see refs. 1 and 2.

†Abbreviations: G_{A1}, gangliotetraosylceramide, β-D-Gal*p*-(1→3)-β-D-GalNAc*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-Cer; G_{M1a}, II^3-*N*-acetylneuraminosylgangliotetraosylceramide; G_{M1b}, IV^3-*N*-acetylneuraminosylgangliotetraosylceramide; G_{D1a}, IV^3-*N*-acetylneuraminosyl-II^3-*N*-acetylneuraminosylgangliotetraosylceramide; G_{D1b}, II^3-(*N*-acetylneuraminosyl)$_2$-gangliotetraosylceramide; G_{D1}, disialosylganglioside of unknown structure; G_{A2}, gangliotriaosylceramide, β-D-GalNAc*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-Cer; G_{M2}, II^3-*N*-acetylneuraminosylgangliotriaosylceramide; $C2_1$, rat-brain glial cells; NIE, mouse neuroblastoma cells; 3T3, mouse fibroblasts; SV40-3T3, 3T3 cells transformed by Simian virus 40; CEF, chick-embryo fibroblasts; and RSV-CEF, CEF cells transformed by Rous sarcoma virus.

INTRODUCTION

The altered distribution of glycolipids in cancer cells and cells transformed by oncogenic agents suggests that they participate in the control of cell behavior through information carried in their carbohydrate moieties, and immunological and enzyme-substrate mechanisms have been proposed[3-7]. The understanding, in molecular terms, of the role of glycolipids requires the knowledge of the exact chemical structure of the carbohydrate moiety of glycolipids, as well as precise information about the activity and specificity of glycosyltransferases in terms of both substrates and products formed from these substrates. Until now, the chemical structure of the products biosynthesized *in vitro* has not been characterized precisely because these products are obtained usually in very small quantities. On the other hand, cultured cells are difficult to grow in amounts sufficient for the separation, in chemically pure form, of their glycolipid components in quantities adequate for the undertaking of structural studies by classical methods.

However, positional isomerism that depends on animal species and organ of origin has been shown in the carbohydrate chain of glycolipids[8-13] and changes in the proportion of isomers, which might result from alteration of the specificity of glycosyltransferases by oncogenic agents, may evoke altered response in cell-to-cell interactions. It is possible that the changes in glycolipid pattern that have been observed so far in transformed cells may not be limited to quantitative variations but may involve structural modifications as well. In order to explore this possibility, a systematic investigation has been undertaken of the biosynthetic capability of transformed *vs.* nontransformed cells by the stepwise application of a methodology that permits the establishment of the precise chemical structure of glycolipids biosynthesized *in vitro*[14]. Thus, the biosynthesis of mono- and di-sialosylgangliosides, and the activity and specificity of sialosyltransferases present in homogenates of $C2_1$, NIE, 3T3, SV 40-3T3, CEF, and RSV-CEF cells, and infant-rat brain tissue were investigated. These reactions are of particular interest because they lead to the formation of polar molecules that may have particular functions as receptors at the cell surface[15], and because alternate pathways have been proposed for the synthesis of gangliosides[16-19].

EXPERIMENTAL

Materials and methods. — T.l.c. was performed on prescored plates coated with Silica Gel G (Uniplate, Analtech, Inc., Newark, DE 19711) in (*A*) 60:35:8 (v/v) chloroform–methanol–water; (*B*) 7:3 (v/v) 1-propanol–5M ammonia; (*C*) 8:5:1:1 (v/v) ethyl acetate–1-propanol–2-propanol–water; and (*D*) 500:9 (v/v) acetone–5M ammonia. Sephadex G-25 was purchased from Pharmacia, Fine Chemicals AB, Uppsala (Sweden); *Clostridium perfringens* and neuraminidase sialosyllactose from Sigma Chemical Co., St. Louis, MI 63778; G_{M1a} and G_{D1a} from Supelco, Inc., Bellefonte, PA 16823; bovine brain gangliosides from Serdary Research Laboratories,

London, Ontario (Canada); sodium borotritide and CMP-[^{14}C]NeuAc from New England Nuclear, Boston, MA 02118; and galactose oxidase from A. B. Kabi, Stockholm (Sweden). Radioautography was done on Kodak X-Omat R/XR-1 X-ray film, and tritium radiation was detected by the intensification procedure of Randerath[20] except for preparative t.l.c. G_{D1b} was a gift from Dr. Robert McCluer.

Standard *O*-methyl-D-galactose derivatives[8] and CMP-NeuAc[21] were prepared as previously described. G_{M1a} was treated with neuraminidase to free it from any neuraminidase-labile gangliosides, and was then purified by preparative t.l.c. in solvent *A*. G_{A1} was prepared by hydrolysis of bovine brain gangliosides with 0.1M hydrochloric acid for 1 h at 100° and purified by preparative t.l.c.[18].

Preparation of labeled precursors. — G_{A1} and G_{M1a} were labeled with tritium in the terminal D-galactopyranosyl group by oxidation with D-galactose oxidase, followed by reduction with sodium borotritide of high specific activity[22,23] under conditions previously described[14]. The products were purified by repeated t.l.c. in solvents A and B. Proof of the selective labeling of the terminal D-galactopyranosyl group was obtained by radioautography of the t.l.c. plate in solvent *C* of the total hydrolyzates with 2M hydrochloric acid for 3 h at 100°, which revealed only radioactive D-galactose. Per-*O*-methylation, followed by hydrolysis, revealed only 2,3,4,6-tetra-*O*-methyl-D-galactose, as detected by radioautography of the t.l.c. plate after irrigation in solvent *D*.

Hydrolysis with neuraminidase. — The radioactive mono- and di-sialosylgangliosides, products of the incubations, were submitted to the action of neuraminidase from *C. perfringens* at 37° in an incubation mixture containing the enzyme (0.25 units) in 0.2M acetate buffer at pH 5.2 (0.5 mL), which contained 2mM Ca^{2+}. G_{M1a} (100 μg) was added as internal standard, as well as a few drops of toluene. Two more portions of the enzyme (0.25 units) in water (0.1 mL) were added at 24-h intervals, and the incubation was continued for 48 h. The reaction was stopped by addition of 2:1 (v/v) chloroform–methanol (10 mL) and the mixture was passed through a column containing Sephadex G-25 Superfine (3 g) equilibrated in 40:20:3 (v/v) chloroform–methanol–water. Elution with this solvent was continued until all radioactivity was recovered in the effluent (~20 mL). The solvent was evaporated under vacuum, and the residues were chromatographed on Silica gel G in solvents *A* and *B*. G_{A1}, G_{M1a}, and G_{D1a} were used as separate standards. The radioactive products were detected by radioautography. The nonradioactive internal and separate standards were revealed with the anthrone–sulfuric acid reagent.

Methylation. — Radioactive glycolipids were methylated by the method of Hakomori[24] under the specific conditions previously described[14].

Culture of cell lines. — 3T3 mouse fibroblasts and SV 40-transformed 3T3 cells. The untransformed 3T3 Swiss mouse fibroblasts and the SV 40-transformed 3T3 cells were cultured in the laboratory of Dr. Harvey Shein at McLean Hospital. Cells were grown in 0.95-L Brockway bottles in 1066 medium (60 mL) supplemented with 7.5% fetal calf serum at 37°, in an atmosphere of 19:1 air–CO_2. The cells were maintained and subcultured by trypsinization as previously described[25]. Several

times a year, tests were performed for the detection of mycoplasma as previously described[26]. The cells were prepared for biosynthetic incubations when they were confluent by microscopic examination. The growth medium was decanted and the cell layer washed three times with a phosphate-buffered salt solution (pH 7.2). The cells were then scraped from the growth surface into the salt solution, collected by gentle centrifugation, and kept at $-70°$.

Lines of transformed cells of neural origin. $C2_1$ glial cell-lines derived by cloning rat brain glial tumors induced by intravenous injection of *N*-nitrosomethylurea, and the NIE neuroblastoma cell-line derived from the Bar Harbor mouse neuroblastoma C 1300 were also prepared in Dr. Shein's laboratory and kept at $-70°$. These cloned cell lines were cultured in Falcon, plastic tissue-culture flasks in Dulbecco's modification of Eagle's medium, supplemented with 10% fetal calf serum, penicillin G (50 units), and streptomycin sulfate (50 μg/mL) in 9 : 1 air–CO_2. The medium was changed on alternate days. Glial cell clones were collected at confluency and neuroblastoma at stationary phase.

Primary chick-embryo fibroblasts and Rous sarcoma virus-transformed derivatives. — These cultures were prepared in the laboratory of Dr. P. W. Robbins, Biology Department at Massachusetts Institute of Technology. Cultures of chick-embryo cells of the C/O or C/B phenotypes[27] were prepared and maintained as described[28]. One half of the cell suspensions used in preparing the primary "normal" control cultures were infected with Rous sarcoma virus PR RSV-C (Prague strain RSV) by adding the cells (at a concentration of 10^7 cells/mL) suspended in growth medium to an equal amount of virus ($1 \cdot 10^7$ – $5 \cdot 10^7$ focus-forming units/mL). The suspension was mixed on a magnetic stirrer for 1 h at room temperature[29]. The cells in both uninfected and infected suspensions were further diluted with growth medium to $5 \cdot 10^5$/mL and seeded in 0.95-L Brockway bottles at 37° in an atmosphere of 19 : 1 air–CO_2. Under these conditions, 90% of the cells in the RSV-infected cultures exhibited transformation within 48 h. Accordingly, cells from uninfected and RSV-infected cultures were harvested for biochemical incubation when confluent. The culture medium was decanted, the cells rinsed three times with phosphate-buffered saline solution at pH 7.2, and scraped from the growth surface with the same solution, collected by gentle centrifugation, and kept at $-70°$.

Incubations. — Thawed cell-pellets containing ~10 mg of protein were washed with a solution (10 vol.) containing (per L): NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.15 g), and KH_2PO_4 (0.2 g). The washed cells collected by gentle centrifugation were ruptured at 0° in a Potter–Elvejem homogenizer in 0.3M sucrose (1.2 mL) containing 10mM EDTA and 0.1% (v/v) 2-mercaptoethanol[30]. Washing the frozen cells, in several cases, increased the apparent sialosyltransferase activities by 20–30% as compared to unwashed cells. Typical incubation mixtures were prepared by adding the cell homogenate and a 2% aqueous solution of CMP-NeuAc (0.5 mL) to a suspension containing the precursor radioactive glycolipid (0.5 μmol; specific activity 0.2 Ci/mmol), Triton CF-54 (4 mg), and Tween 80 (2 mg) in 0.5M cacodylate buffer, pH 6.5, (0.5 mL). Incubations of nonradioactive glycolipid precursor and

CMP-[^{14}C]NeuAc were performed under identical conditions, except that 1 μmol of glycolipid precursor and 5–10 μCi of CMP-[^{14}C]NeuAc were used. In double-label experiments, the tritium-labeled glycolipid precursor and CMP-[^{14}C]NeuAc were used in the aforementioned amounts. The incubations were carried out for 3 h at 37°. The glycolipid products were purified as previously described[14].

Periodate oxidation of [^{14}C]G_{M1} *and* [^{14}C]G_{D1}. — Each of the radioactive G_{M1} and G_{D1} ($1.5 \cdot 10^6$ c.p.m.) was dissolved with standard G_{D1b} (0.2 mg) in methanol (0.1 mL), and 0.2M sodium acetate buffer (pH 4.4) (0.5 mL) was added, followed at 0° by 0.5M sodium metaperiodate (0.1 mL). The solution was kept for 48 h at 4°, and then 0.25M barium acetate (0.3 mL) was added, and the barium periodate and iodate precipitated in the cold were filtered off on a layer of Celite analytical filter-aid. The pH of the solution was adjusted to 6.5 with 0.1M sodium hydroxide, and sodium borohydride (25 mg) was added. The solution was kept for 18 h at 4°, and the excess of borohydride was decomposed with glacial acetic acid (~100 μL). The solution was then passed through a column containing AG 50 cation-exchange resin (5 mL, H^+, 100–200 mesh) prepared in 1 : 1 (v/v) methanol–water, and eluted with the same solvent mixture (10 mL). The total effluent was evaporated to dryness on a rotary evaporator, and boric acid was eliminated by repeated coevaporation with methanol. The residue, transferred with methanol into a tube (7 mm diam. × 15 cm), was dried under a stream of nitrogen, and then under vacuum, and a 3% solution (1.5 mL) of hydrogen chloride in methanol was added. The tube was sealed and heated for 1 h at 100°. The tube previously cooled in solid carbon dioxide was opened, and an excess of silver carbonate was added, followed by acetic anhydride (0.1 mL). After being kept for 2 h at room temperature, the suspension was filtered through a pad of Celite, and the filtrate, plus the washings with methanol, were passed through a column of AG 50 cation-exchange resin (0.5 mL, H^+, 100–200 mesh) prepared in methanol. The eluate and washings with methanol (2 mL) were evaporated under a stream of nitrogen, and the residue was transferred with a small amount of methanol to the origin of a t.l.c. plate. The chromatogram was developed with 1 : 1 (v/v) ethyl acetate–methanol. The radioactive spots were revealed by radioautography, and the plate was sprayed with Ehrlich reagent to reveal the standards.

Standards of methyl (methyl 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosid)onate (methyl ester methyl glycoside of *N*-acetylneuraminic acid) and of the C-7 derivative methyl (methyl 5-acetamido-3,5-dideoxy-L-*arabino*-heptulopyranosid)onate were prepared by methanolysis and re-*N*-acetylation; or periodate oxidation, methanolysis, and re-*N*-acetylation, respectively, of sialosyllactose.

Search for G_{M1b} *as a natural constituant of rat brain.* — Since G_{M1b} could not be separated clearly from G_{M1a} by any chromatographic system tried, an indirect way was attempted to detect G_{M1b} in rat brain. A total lipid extract of the brains of ten one-month-old rats was prepared, and the gangliosides were extracted into an upper phase according to Folch *et al.*[31]. After evaporation of the solvents, the lipids were separated by preparative t.l.c. in solvent *A*. Narrow strips of the t.l.c. plates were snapped off, and the glycolipids were detected with the anthrone–sulfuric acid reagent.

The remainder of the band corresponding to standards of G_{M1a} and radioactive G_{M1b} was scraped from the plate, and the gangliosides were extracted from the silica with solvent *A* (150 mL). After evaporation of the solvent, aliquots of the residue were treated with neuraminidase as just described. The lipids were extracted from the incubation mixture, purified by passage through a column of Sephadex-G-25, and examined by t.l.c. in solvent *A*. For control, unchanged G_{M1a} recovered from the t.l.c. plates was treated a second time with neuraminidase, and the product was examined by t.l.c.

RESULTS

As shown in Fig. 1 (lanes 1–4), incubations *in vitro* of radioactive G_{A1} with CMP-NeuAc in the presence of homogenates of $C2_1$, NIE, CEF, and RSV-CEF resulted in the formation of two radioactive bands that migrated on t.l.c. close to standards of G_{M1a} and G_{D1a}, and are therefore tentatively named G_{M1} and G_{D1}.

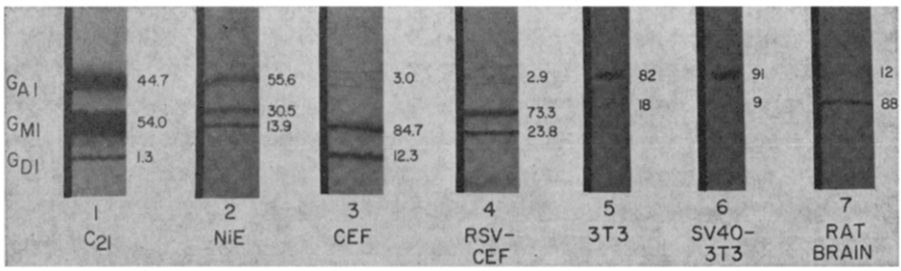

Fig. 1. Radioautograms of sections of preparative t.l.c. plates of glycolipids from biosynthetic incubations of [^{3}H]G_{A1} with nonradioactive CMP-NeuAc in the presence of homogenates of: (1) $C2_1$, (2) NIE, (3) CEF, (4) RSV-CEF, (5) 3T3, and (6) SV 40-3T3 cells, and (7) rat brain tissue. The upper band corresponds to unchanged G_{A1}, the two lower bands are tentatively labeled G_{M1} and G_{D1}. Numbers in the margins indicate the percentage of c.p.m. in each band (not necessarily obtained under optimal conditions).

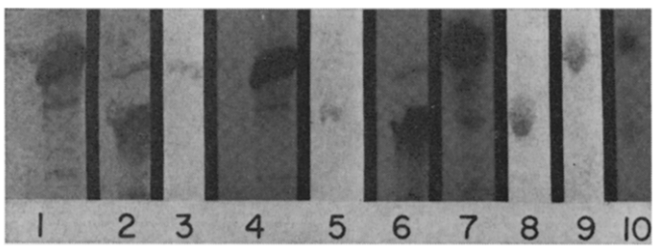

Fig. 2. Radioautograms of G_{M1} and G_{D1} separated and recovered from preparative t.l.c. and compared by cochromatography with standards of G_{M1a} and G_{D1a}: (1) [^{3}H]G_{M1} obtained with CEF cells (right), cochromatographed with a standard of G_{M1a} (left), the spots were applied with partial overlap; (2) [^{3}H]G_{D1} obtained with CEF cells (right), cochromatographed with a standard of G_{D1a} (left); the spots were applied with partial overlap; (3) and (4) [^{3}H]G_{M1} (4) as seen separated from the standard G_{M1a} (3); (5) and (6) [^{3}H]G_{D1} (6) as seen separated from the standard G_{D1a} (5); (7) [^{3}H]G_{M1} obtained with $C2_1$ cells; (8) standard G_{D1a}; (9) standard G_{M1a}; and (10) ^{3}H-labeled lower-band formed with $C2_1$ cells.

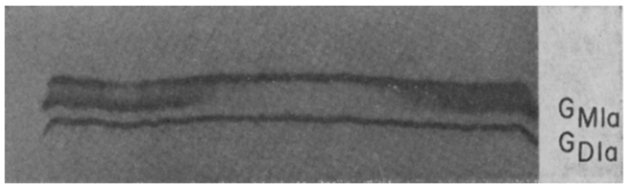

Fig. 3. Radioautogram of t.l.c. plates of products of incubation of [^{3}H]G_{M1a} and CMP-NeuAc with $C2_1$ cells.

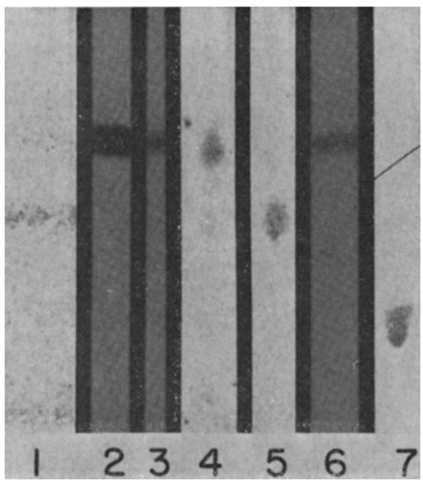

Fig. 4. Radioautograms of t.l.c. plates of products of degradation of G_{M1} and G_{D1} with neuraminidase: (1) Internal standard of G_{M1a}; (2) [^{3}H]G_{A1} from [^{3}H]G_{M1}; (3) [^{3}H]G_{A1} standard; (4) G_{A1} standard; (5) G_{M1a} standard; (6) [^{3}H]G_{A1} from G_{D1}; and (7) G_{D1a} standard.

With 3T3, SV 40-3T3, and rat brain, only G_{M1} was formed (Fig. 1, lanes 5–7). Control incubations carried out with boiled enzyme or without addition of CMP-NeuAc showed only the unchanged precursor G_{A1}.

Cochromatography of radioactive G_{M1} obtained with all cells (Fig. 2; lanes 1, 3, and 4) with a standard of G_{M1a} indicated that the G_{M1} detected by radioautography migrates slightly slower than G_{M1a} detected with the anthrone–sulfuric acid reagent. On the other hand, in repeated cochromatographies, G_{D1} obtained with CEF, RSV-CEF, and NIE migrated well ahead of G_{D1b}, but just behind a standard of G_{D1a} (Fig. 2; lanes 2, 5, and 6). The standards and radioactive compounds seem to exclude each other from the silica gel; in fact, no radioactivity is found to coincide with G_{M1a} and G_{D1a} standards. In contrast, the lower band obtained with $C2_1$ cells seems to be an artifact of chromatography since, when rechromatographed, it moves as G_{M1} (Fig. 2; lanes 9 and 10).

It is of interest to note that t.l.c. of the lipid fraction obtained from incubation mixtures often results in chromatography artifacts with doubling of bands which, in fact, correspond to the same substance. This is strikingly illustrated in Fig. 3 in which G_{M1a} separates into two bands near the edges of the plates.

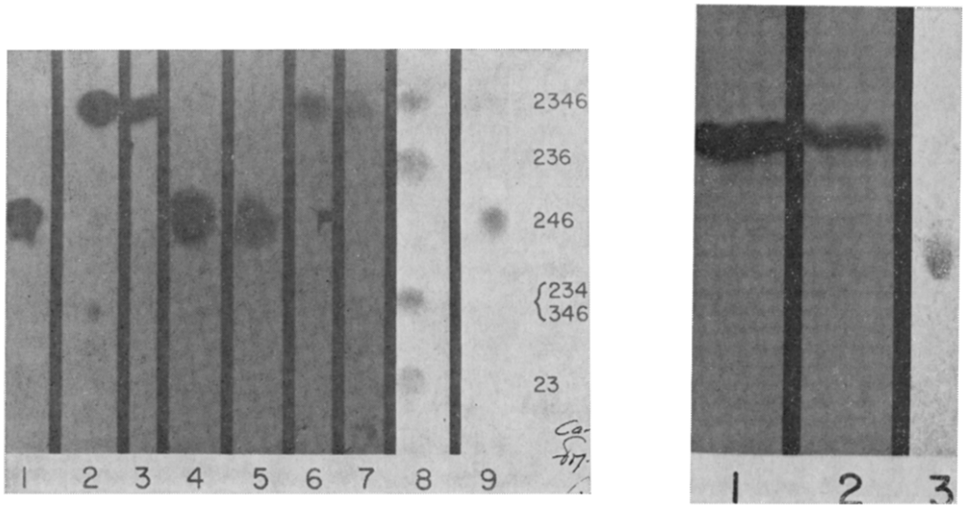

Fig. 5. Radioautograms of t.l.c. plates of ^{3}H-labeled methyl ethers of D-galactose derived from: (1) [^{3}H]G_{M1} ($C2_1$ cells); (2) [^{3}H]$G_{A1\ precursor}$; (3) [^{3}H]G_{A1} from [^{3}H]G_{M1} ($C2_1$ cells) treated with neuraminidase; (4) [^{3}H]G_{M1} (CEF cells); (5) [^{3}H]G_{D1} (CEF cells); (6) [^{3}H]G_{A1} from [^{3}H]G_{M1} (CEF cells) treated with neuraminidase; (7) [^{3}H]G_{A1} from [^{3}H]G_{D1} (CEF cells) treated with neuraminidase; and (8) and (9) standard *O*-methyl-D-galactoses. Figures in the margin refer to the position of the *O*-methyl substituents.

Fig. 6. Radioautograms of t.l.c. of products of incubation of: (1) [^{3}H]G_{M1b} with CMP-NeuAc and $C2_1$ cells; (2) precursor [^{3}H]G_{M1b}; and (3) G_{D1a} standard.

As shown in Fig. 4 (lanes 2 and 6), treatment of radioactive G_{M1} and G_{D1} with neuraminidase liberated in all cases the radioactive precursor G_{A1}. No neuraminidase-stable, radioactive G_{M1a} was detected in any case, although an internal standard of G_{M1a} was found unchanged (Fig. 4, lane 1).

Permethylation–hydrolysis of G_{M1} and G_{D1} obtained in all incubations gave only radioactive 2,4,6-tri-*O*-methyl-D-galactose (Fig. 5; lanes 1, 4, and 5), whereas an identical treatment of the precursor G_{A1}, and also of G_{A1} recovered after neuraminidase treatment of both G_{M1} and G_{D1}, gave 2,3,4,6-tetra-*O*-methyl-D-galactose (Fig. 5; lanes 2, 3, 6, and 7). These data establish the substitution at O-3 of the terminal D-galactopyranosyl residues by *N*-acetylneuraminosyl groups, in G_{D1} as well as in G_{M1}. The observation that no 2,3,4,6-tetra-*O*-methyl-D-galactose was detected in lanes 1 and 4 (Fig. 5) confirms that no G_{M1a} is formed in the biosynthetic incubations.

When radioactive G_{M1b} was reincubated with CMP-NeuAc and a homogenate of $C2_1$ cells, no radioactive product was detected except for the precursor G_{M1b} (Fig. 6, lane 1), but, as shown in Fig. 7 (lane 1), a radioactive product that moves on the chromatograms like G_{D1} was formed with CEF cells (and also with RSV-CEF and NIE cells). In double-label incubations of [^{3}H]G_{A1} and CMP-[^{14}C]NeuAc with CEF cells (Fig. 7, lane 2), both G_{M1} and G_{D1} were formed. The ratio of ^{14}C to ^{3}H in G_{D1} was found to be twice the ratio measured in G_{M1} (0.290 *vs.* 0.140 $\pm 5\%$). A

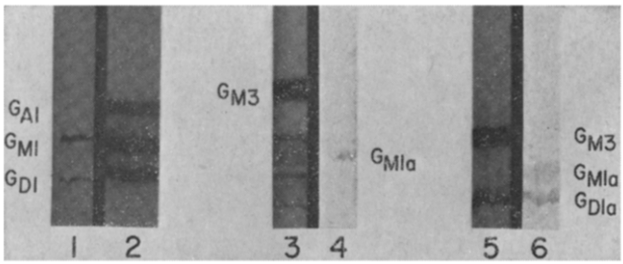

Fig. 7. Radioautogram of t.l.c. plates of products of incubation of: (1) [^{3}H]G_{M1b} with nonradioactive CMP-NeuAc and CEF cell homogenate; (2) [^{3}H]G_{A1} with CMP-[^{14}C]NeuAc and CEF cells; (3) CMP-[^{14}C]NeuAc with CEF cells without addition of exogenous glycolipid acceptor; (4) inner standard of G_{M1a}; (5) CMP-[^{14}C]NeuAc with NIE cells without addition of exogenous glycolipid acceptor; and (6) inner standards of G_{M1a} and G_{D1a}.

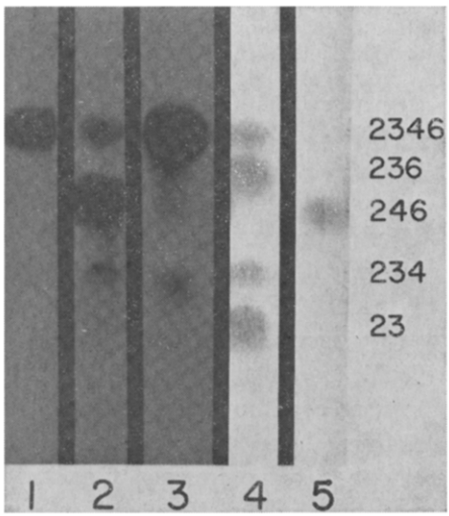

Fig. 8. Radioautogram of t.l.c. plates of ^{3}H-labeled methyl ethers of D-galactose derived from: (1) precursor [^{3}H]G_{M1a}; (2) product of incubation of [^{3}H]G_{M1a} with CMP-NeuAc and $C2_1$ cells; (3) G_{M1a} resulting from treatment of [^{3}H]G_{D1a} with neuraminidase; and (4) and (5) standard *O*-methyl-D-galactoses. Figures in the margin refer to the positions of the methyl substituents.

control incubation carried out under identical conditions, but without addition of exogenous G_{A1}, showed little radioactivity at the positions of G_{M1} and G_{D1} (Fig. 7, lane 3); [^{14}C]G_{M3} was the main product of this reaction, presumably endogenous lactosylceramide being the precursor. Hence, the increased ratio of ^{14}C to ^{3}H in G_{D1} as compared to G_{M1} in the double-label experiment, was not due, to a large part, to products formed from endogenous acceptors, but indicates that G_{D1} is indeed a disialosylganglioside that can be formed from G_{A1}, as well as from G_{M1b}, in the presence of NIE and CEF cells but not of $C2_1$ cells. $C2_1$ cells, however, were capable of catalyzing the biosynthesis of G_{D1a} from G_{M1a} (Fig. 3). The structure of the product G_{D1a} was demonstrated by permethylation–hydrolysis which gave 2,4,6-tri-*O*-methyl-D-galactose (Fig. 8, lane 2), whereas permethylation–hydrolysis of the pre-

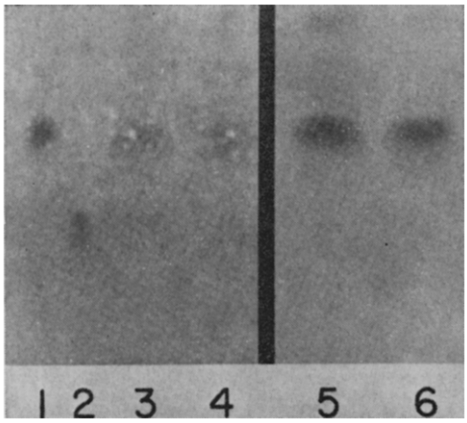

Fig. 9. T.l.c. plates of products of periodate oxidation of G_{M1} and G_{D1}: (1) Methyl (methyl 5-acetamido-3,5-dideoxy-L-*arabino*-heptulopyranosid)onate; (2) Methyl (methyl 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosid)onate; (3) product of oxidation of a mixture of [^{3}H]G_{M1} and G_{D1b} standard; (4) product of oxidation of a mixture of [^{3}H]G_{D1} and G_{D1b} standard; (5) radioautogram of (3); and (6) radioautogram of (4). The spots of (1), (2), (3), and (4) were detected with the Ehrlich reagent.

cursor [^{3}H]G_{M1a} and of [^{3}H]G_{M1a}, recovered after neuraminidase treatment of [^{3}H]G_{D1a}, gave 2,3,4,6-tetra-*O*-methyl-D-galactose (Fig. 8, lanes 1 and 3).

Incubations of homogenates of NIE cells with CMP-[^{14}C]NeuAc, without addition of exogenous glycolipid acceptor, gave products that migrated like G_{M3} and G_{D1a} derived from endogenous acceptors (Fig. 7, lanes 5 and 6), no G_{M1a} being formed. G_{M1} and G_{D1}, biosynthesized under the same conditions but starting from nonradioactive G_{A1} and CMP-[^{14}C]NeuAc, and thus labeled with ^{14}C in the neuraminosyl groups, were submitted to periodate oxidation in the presence of an inner standard of nonradioactive G_{D1b}. After methanolysis and re-*N*-acetylation, the products were compared by t.l.c. with standards of the methyl ester methyl glycoside of *N*-acetylneuraminic acid and its C7 fragment, obtained by periodic acid oxidation of sialosyllactose. Whereas the internal standard of G_{D1b} gave a mixture of both derivatives detectable with the Ehrlich reagent (Fig. 9, lanes 3 and 4), the radioactivity derived from the neuraminosyl groups of G_{D1} was found only in the C_7 fragment (Fig. 9, lane 6). In the same way, the C_7 fragment was obtained from G_{M1} (Fig. 9, lane 5).

Search for G_{M1b} in rat brain tissue. — A first treatment with neuraminidase of monosialosylgangliosides from rat brain, under conditions that lead to complete degradation of G_{M1b} into G_{A1}, liberated a very small proportion of material migrating on t.l.c. like G_{A1}, and being revealed with anthrone–sulfuric acid as a barely detectable blue band above a strong band of unchanged G_{M1a}. A second treatment with neuraminidase of G_{M1a}, recovered after the first treatment, gave again a very faint band corresponding to G_{A1}. This indicates that G_{M1a}, even in the absence of detergents, is not completely resistant to neuraminidase. Therefore, it appears that most of the

very small proportion of material migrating like G_{A1}, observed after a first treatment with neuraminidase of total monosialosylgangliosides of rat brain, resulted from a partial degradation of G_{M1a}.

DISCUSSION

The present experimental data indicate that, in the presence of all cells used as sources of sialosyltransferases, G_{A1} incorporates a neuraminosyl group from CMP-NeuAc at O-3 of the terminal D-galactopyranosyl group to form exclusively the neuraminidase-labile G_{M1b}. These results confirm the previous demonstration of the structure of G_{M1b} biosynthesized by rat brain homogenate[32] and extend to several cell types the observation of the unique formation of this ganglioside from G_{A1} *in vitro*. No trace of the naturally occurring, neuraminidase-stable G_{M1a} was detected as a product of any of the biosynthetic reactions. Hence, in the tissues tested, the biosynthesis of G_{M1a} does not proceed *via* G_{A1}. The present experiments failed to demonstrate the existence of G_{M1b} in rat brain. Treatment with neuraminidase of total monosialosylgangliosides indicates that G_{M1b}, if present in rat brain, would be only a very minor component. The observation that very little or no detectable monosialosylganglioside was formed by incubations of homogenates of various cells and rat brain tissue with CMP-[^{14}C]NeuAc, whereas G_{M1b} was formed in high yield by similar incubation mixtures to which exogenous G_{A1} had been added, indicates that G_{A1} is not available as an endogenous precursor in the cell homogenates. With all cells, G_{M3} was the main product formed from endogenous acceptors and, with some cells, a considerable proportion of G_{D1a} was also formed. This is consistent with the observations reported by Ng and Dain[33].

The second product of the biosynthetic incubations of G_{A1} and CMP-NeuAc with NIE and CEF cells (G_{D1}) appears clearly to be a disialosylganglioside. Indeed, it was produced from [^{3}H]G_{A1} and also from the intermediate [^{3}H]G_{M1b} and non-radioactive CMP-NeuAc. When CMP-[^{14}C]NeuAc was used in these incubations, the products formed were doubly-labeled, thus confirming the linking of [^{14}C]NeuAc to [^{3}H]G_{A1} or [^{3}H]G_{M1b}, respectively. Moreover, the ratio of ^{14}C to ^{3}H in G_{M1b} and G_{D1}, obtained in the double-label experiments from [^{3}H]G_{A1} and CMP-[^{14}C]-NeuAc indicates that G_{D1} had incorporated two [^{14}C]NeuAc groups per [^{3}H]G_{A1}, as compared to one for G_{M1b}. However, this disialosylganglioside is different from the known G_{D1a} and G_{D1b} in its chromatographic mobility. In addition, it is completely degraded by *C. perfringens* neuraminidase with liberation of G_{A1}, under conditions that cleave only one NeuAc residue from G_{D1a} or G_{D1b} with liberation of the neuraminidase-stable G_{M1a}.

Kaufman *et al.*[16] reported that ceramidetetrahexoside (G_{A1}) is an acceptor for NeuAc from CMP-NeuAc with embryonic chicken brain as a source of sialosyl-transferase, and that two products were formed, a mono- and a di-sialosylganglioside. These gangliosides were found to lose, respectively, 70 and 66% of their sialic acid component upon treatment with neuraminidase. The discrepancy between these

figures and our data, which show a complete loss of neuraminosyl groups, apparently is not due to tissue specificity. It results most likely from the experimental conditions of Kaufman *et al.*[16], who starting from nonradioactive G_{A1} and CMP-[^{14}C]NeuAc dealt with mixtures of products resulting from the incorporation of NeuAc into both exogenous and endogenous glycolipid receptors, the latter ones giving a proportion of neuraminidase-stable ganglioside. Indeed, G_{M1} biosynthesized from [^{3}H]G_{A1} in the presence of a homogenate of 12-day-old chicken-embryo brain was found to be totally degraded by neuraminidase with liberation of G_{A1}.

Permethylation–hydrolysis of G_{D1} showed that only one position of the terminal D-galactopyranosyl group was substituted, as 2,4,6-tri-*O*-methyl-D-galactose was obtained. On the other hand, the second NeuAc group being not linked to the inner D-galactose residue, as indicated by its lability to neuraminidase, suggests that the two NeuAc groups may be linked together to form a (2→8)-disialosyl chain bound to O-3 of the terminal D-galactose residue. If that were the case, G_{D1} might still be considered as a possible precursor of a trisialosylganglioside. However, periodate oxidation of G_{D1} labeled at its NeuAc groups, with ^{14}C failed to show the existence of a NeuAc residue resistant to the oxidant, whereas, under the same conditions, G_{D1b} used as an internal standard gave a C_9 and a C_7 derivative of neuraminic acid. The position of attachment of the second NeuAc group remains unknown.

It is of interest that NIE and CEF cells have the enzymic capability to synthesize G_{D1} from G_{M1b}, and from G_{A1}. $C2_1$ and 3T3 cells do not catalyze this reaction. On the other hand, $C2_1$ cells are capable of synthesizing G_{D1a} from G_{M1a}. These observations suggest that the sialosyltransferase involved in the biosynthesis of G_{D1} from G_{M1b} is different from the one synthesizing G_{M1b} from G_{A1}, and G_{D1a} from G_{M1a}. The latter two reactions may be catalyzed by the same sialosyltransferase. This point remains to be established by competitive inhibition experiments.

The data provided in the present work suggest that, in the tissues examined, neither G_{M1b} nor G_{D1} are natural intermediates in the metabolism of complex gangliosides. Recently, however, the natural occurrence of G_{M1b} has been demonstrated in tumor cells[24]. It is conceivable that G_{M1b} is not detectable in brain because of very rapid further metabolism. This possibility, however, appears unlikely, considering that incubations of [^{3}H]G_{M1b} for 1 h at 37° with an homogenate of 10-day-old rat brain at the natural pH of the tissue, and also at pH 6.5 with an homogenate of $C2_1$ cells (Fig. 6) left this ganglioside intact. Our observations support the arguments presented by Kaufman *et al.*[16] in favor of the view that the sialosyl group that is attached to the inner D-galactose residue of G_{M1a} is incorporated by a specific sialosyltransferase, which uses lactosylceramide as a precursor of G_{M3}, which in turn accepts 2-acetamido-2-deoxy-D-galactosyl and D-galactosyl groups. These arguments were based on the observation that lactosylceramide is a poor acceptor of 2-acetamido-2-deoxy-D-galactose from UDP-2-acetamido-2-deoxy-D-galactose, as compared to G_{M3} which accepts a 2-acetamido-2-deoxy-D-galactosyl group in higher yield. This hypothesis has been further substantiated by Cumar *et al.*[35] who observed that G_{A2} and G_{M2} are poor acceptors of sialic acid. Such indirect evidence could, however, be some-

times misleading for two reasons: (a) The quality of a glycolipid as an acceptor in biosynthetic incubations *in vitro* may depend on its molecular species, *i.e.*, its fatty acid composition, as shown by Kemp and Stoolmiller[36] (and confirmed by us[37]) for the biosynthesis of G_{M3} from lactosylceramide. Stearoyllactosylceramide incorporated only 5% of NeuAc *in vitro*, as compared to the lignoceroyl derivative, chick embryo fibroblasts being the enzyme source. Therefore, the use, as acceptors, of glycolipids isolated from sources other than the tissue used for the examination of glycosyltransferase activities may give false information. (b) As exemplified in the present and previous work, high transferase activity may be observed by use of exogenous substrates, but may result in the formation of products that do not exist in the tissues used as a source of glycosyltransferase. It is, therefore, clear that in order to validate a quantitative evaluation of the activity of transferases, for example in transformed *vs.* nontransformed cells, it is indispensible to establish the precise structure of the products obtained, and then to correlate the results of this investigation with analyses of the quantitative distribution and structure of the glycolipids actually present in dynamic equilibrium in the tissue. This requirement, combined with the difficulties of assaying insoluble, membrane-bound transferases, makes the investigation of the function and modulation of the function of these enzymes in different tissues during development and in cancer cells a very complex task. The method used in the present work, with its capability to discriminate products formed from exogenous and endogenous precursors, and to establish the exact structure of these products, constitutes a tool for the determination of the factors that control the activity and the specificity of glycosyltransferases *in vitro* (activators and inhibitors).

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