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IDENTIFICATION OF THE SIALOSYL–SIALOSYL LINKAGE IN BIOSYNTHESIZED GANGLIOSIDES

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ABSTRACT

A technique was developed to examine the sialosyl–sialosyl linkage in the minute quantities of complex gangliosides biosynthesized *in vitro*. The disialoganglioside, G_{D3}*, and the trisialoganglioside, G_{T1a}, were biosynthesized as secondary products by the reaction of CMP-(4-^{14}C)sialic acid with the glycolipid substrates, lactosyl ceramide and G_{M1} ganglioside, respectively. Following periodate oxidation–borohydride reduction, the intact sialic acid residue, and the susceptible sialic acid residue converted into an *N*-acetylheptulosaminic acid residue, were released by hydrolysis, esterified, separated, and analyzed for radioactivity. Analysis of the biosynthesized G_{D3} and G_{T1a} gangliosides indicated radioactivity in both the susceptible and the nonsusceptible sialic acid residues, demonstrating that a sialosyl-(2→8)-sialosyl linkage had been synthesized. When the primary products (G_{M3} and G_{D1a}, respectively) of the synthesis just described were examined, radioactivity was detected, as expected, only in the susceptible sialic acid residue. Because the *N*-acetylneuraminic acid and *N*-acetylheptulosaminic acid derivatives formed in the reaction were identified by chromatography, the standards used for comparison were verified by g.l.c.–m.s.

INTRODUCTION

Product identification of gangliosides biosynthesized *in vitro* usually has relied on comparison of chromatographic mobilities with those of known gangliosides[2–4]. Owing to the very small quantities of biosynthesized products, standard chemical techniques cannot be used to determine the exact chemical structure that has actually been synthesized. Recently, a technique has been developed to determine the glycosyl linkages in biosynthesized glycolipids[5]. Glycolipids were biosynthesized by use of a labeled glycolipid-precursor and an unlabeled sugar-nucleotide donor. Using specific

*The ganglioside nomenclature used here is that described by Svennerholm[1]. The gangliosides are designated as follows: G_{M3}: II^{3}NeuAc-LacCer; G_{M1}: II^{3}NeuAc-GgOse$_4$Cer; G_{D3}: II^3(NeuAc)$_2$-LacCer; G_{D1a}: IV^{3}NeuAc, II^{3}NeuAc-GgOse$_4$Cer; and G_{T1a}: IV^3(NeuAc)$_2$, II^{3}NeuAc-GgOse$_4$Cer.

glycosidases and permethylation prior to hydrolysis, examination of the degradation products allowed determination of the anomeric configuration and position of attachment of the new residue in the biosynthesized glycolipid. This method was extended to demonstrate the linkage of a sialic acid residue to *O*-3 of the terminal D-galactose residue of gangliotetraosylceramide[6]. A remaining problem is the demonstration of a (2→8)-linkage between sialosyl moieties in the more complex gangliosides produced in biosynthesis *in vitro*.

The sialosyl-(2→8)-sialosyl linkage in complex gangliosides was determined by employing the selective reaction of periodate on vicinal diol groups[7-9]. Linkage of the external sialic acid residue to *O*-8 of the penultimate sialic acid residue prevents the latter residue from being susceptible to periodate oxidation, whereas location of the linkage elsewhere does not. Following periodate oxidation–borohydride reduction, the susceptible sialic acid residues were reduced to a 7-carbon analog, which is *N*-acetylheptulosaminic acid for *N*-acetylneuraminic acid.

Cumar *et al.*[10] have shown that *in vitro, de novo* synthesized gangliosides can serve as substrate for additional incorporation of sugar residues. Recently, we demonstrated[4] the serial synthesis of sialosyl residues resulting in a radioactive label in both sialosyl residues. Based on chromatographic comparison with standards, the biosynthesized ganglioside was presumed to contain a sialosyl-(2→8)-sialosyl linkage. We report here an extension of the periodate oxidation–borohydride reduction technique[7-9] to the very small quantities of biosynthesized gangliosides. Thus, the *in vitro* synthesis of a sialosyl-(2→8)-sialosyl linkage could be verified in gangliosides in which both linked sialosyl-residues both contain a radioactive label.

MATERIALS AND METHODS

Materials — The following compounds were obtained: CMP-(4-^{14}C)NeuAc (specific activity 1.68 Ci/mol) from New England Nuclear (Boston, MA 02118); *N*-acetylneuraminic acid (type IV) and *N*-acetylneuraminosyllactose (type 1 from bovine colostrum) from Sigma (St. Louis, MO 63178); and lactosylceramide from Miles Laboratories Inc. (Elkhart, IN 46515). The monosialoganglioside, G_{M1}, was isolated in this laboratory[8] from a mixture of bovine brain gangliosides purchased from Supelco (Bellefonte, PA 16823). All other chemicals used were of reagent grade, and solvents were redistilled prior to use.

Biosynthesis of gangliosides. — The gangliosides, G_{D3} and G_{T1a} were biosynthesized *in vitro* by use of the appropriate glycolipid acceptors and CMP-(4-^{14}C)-sialic acid as the source of radioactivity. The disialoganglioside, G_{D3}, was obtained as a secondary product of the synthesis of G_{M3} from lactosylceramide by the procedure of Kaufman *et al.*[11]. The trisialoganglioside, G_{T1a}, was obtained from G_{M1} *via* G_{D1a}, by the procedure previously reported[4]. The synthesis of G_{T1a} was also performed with 11-day-old rat-brain membranes as the enzyme source, and the same membrane isolation and incubation system reported for chick brain[4]. In all cases, the time of

enzyme incubation was extended to 4 h to increase the yield of secondary product, G_{D3} or G_{T1a}.

Isolation and analysis of the product gangliosides. — The total ganglioside fraction was isolated from the incubation mixture as previously described[4]. In some cases, G_{T1a} samples from several incubation mixtures were pooled for analysis because of the low levels of label incorporated. The specific, radiolabeled-ganglioside products and additional, carrier ganglioside (equivalent to 15–30 μg of lipid-bound sialic acid) were isolated by preparative t.l.c. on Silica gel 60 plates (E. Merck, Darmstadt, Germany) with 10:9:2 (v/v) chloroform–methanol–water, containing 0.02% calcium chloride dihydrate as the developing solvent. The addition of carrier was necessary for the adequate recovery of gangliosides from the subsequently employed desalting columns[12]. Following location of the gangliosides on the chromatograms by a water spray, the appropriate areas of the silica gel were scraped from the plate. The gangliosides were extracted by repeated suspension and centrifugation of the gel (4 times at 1000*g* for 10 min) in 2-mL aliquots of 1:4 (v/v) chloroform–methanol. The combined aliquots from each sample were dried under nitrogen and then under vacuum. The dried ganglioside samples were subjected to periodate oxidation–borohydride reduction according to the conditions of Ando and Yu[8]. Reaction products were desalted by the Sephadex G-50 column procedure reported previously[13]. The isolated ganglioside products were hydrolyzed with mild acid (200 μL of 50mM hydrochloric acid, 1 h, 80°), and the released sialic acid and its analog were isolated by ion-exchange chromatography under the following conditions: the hydrolyzate (200 μL) was diluted to 1 mL with distilled, de-ionized water, and the diluted solution applied to a 0.5-mL bed-volume column of AG 1-X4 (AcO, 200–400 mesh) anion-exchange resin (Bio-Rad Laboratories, Richmond, CA 94804). The column was rinsed with distilled de-ionized water (10 mL), and then eluted with 0.3M acetic acid (12.5 mL). The eluate was lyophilized to remove both water and acetic acid.

The free sialic acid and its 7-carbon analog were separated directly by ascending paper chromatography in 1:2:1 (v/v) 1-butanol–1-propanol–0.1M hydrochloric acid as the developing solvent[14], and made visible by the resorcinol–trichloroacetic acid spray[15]. Additional experimentation indicated that a better resolution could be achieved by converting the acids to their methyl esters, followed by separation on cellulose t.l.c. or silica gel high-performance t.l.c. (h.p.t.l.c.) plates (Merck). Esterification was performed for 1 h at 60° in 50mM methanolic hydrogen chloride (1 mL). The solvent was evaporated under nitrogen at room temperature, and the residue was then applied directly to the chromatogram in a small quantity of methanol. The esters were separated on 10 × 20-cm cellulose t.l.c. plates by development in 1:2:1 (v/v) 1-butanol–1-propanol–0.1M hydrochloric acid for 4 h. In addition, the esters could also be separated on 10 × 10-cm silica gel h.p.t.l.c. plates developed in 5:2: (v/v) 1-butanol–acetic acid–water for 2 h, or in 10:4:3:1 (v/v) 2-butanone–glacial acetic acid–methanol–water for 40 min. The esters were made visible on cellulose t.l.c. plates by the resorcinol–trichloroacetic acid spray[15] and on h.p.t.l.c. plates

by the resorcinol–hydrochloric acid spray[16]. Resorcinol-positive areas were isolated and analyzed for radioactivity as previously reported[4]. The recovery of radioactivity for the entire method was estimated to be ~70%, based on the isolated gangliosides.

Preparation and analysis of standards. — Standard *N*-acetylneuraminic acid was obtained commercially as indicated in the Materials section. Standard *N*-acetylheptulosaminic acid, the 7-carbon analog of *N*-acetylneuraminic acid, was prepared from commercial *N*-acetylneuraminosyllactose (15 mg) dissolved in 0.1M sodium acetate buffer (2 mL), pH 4.4. After addition of 0.5M sodium periodate (270 μL), the mixture was kept for 48 h at 4°. The reaction was stopped by the addition of 10M ethanediol (120 μL). After 3 h at 4°, 0.2M sodium hydroxide (650 μL) was added, followed by 9% sodium borohydride (240 μL), and the mixture was kept overnight at 4°. The borohydride reduction was stopped by the addition of 2M acetic acid (400 μL), and the sample was desalted on a Sephadex G-25 column (1.5 cm × 85 cm) with 0.1M acetic acid as the eluent. Following lyophilization, the sample was hydrolyzed with 50mM hydrochloric acid (1 mL) for 1 h at 80°. The hydrolyzate was diluted to 10 mL with distilled water and applied to a 5-mL bed-volume column of AG 1-X4 (AcO^-, 200–400 mesh) anion-exchange resin. The column was rinsed with distilled water (100 mL), the *N*-acetylheptulosaminic acid was eluted with 0.3M acetic acid (125 mL), and the eluate was lyophilized.

The identity of standard sialic acid and its 7-carbon analog was verified by g.l.c.–m.s. The samples were treated for 2 h at 100° with 3% hydrogen chloride in absolute methanol, trifluoroacetylated[17], and injected into the gas chromatograph–mass spectrometer (Shimadzu-LCB 9000 and MSPAC-300M computer system, Shimadzu Seisakusho Ltd., Kyoto, Japan) *via* a 3% OV-1 column; the oven temperature was programmed from 140 to 210°, and the spectra were normalized by computer.

RESULTS

Analysis of the standards by mass spectrometry. — The mass spectra of the trifluoroacetylated derivatives of neuraminic and heptulosaminic acid are shown in Fig. 1, and the probable fragments corresponding to *m/z* values indicated are shown in Table I. As expected, the derivative of sialic acid was identified as pentakis(trifluoroacetyl)neuraminic acid methyl ester methyl glycoside. The derivative of the periodate–borohydride–treated sialic acid from *N*-acetylneuraminosyllactose was identified as tris(trifluoroacetyl)heptulosaminic acid methyl ester methyl glycoside.

Separation of N*-acetylneuraminic acid,* N*-acetylheptulosaminic acid, and their esters.* — Initial analysis of the biosynthesized gangliosides was achieved by separation of the free acids by paper chromatography as shown in Fig. 2a. This chromatogram required a long developing time and gave diffuse spots resulting in poor separation. The free acids were therefore converted into the esters, which were separated by cellulose t.l.c. as shown in Fig. 2b. In the final procedure, the esters were separated by h.p.t.l.c. as shown in Fig. 3. The h.p.t.l.c. procedure was faster and resulted in less diffusion than either paper chromatography or cellulose t.l.c., and gave excellent

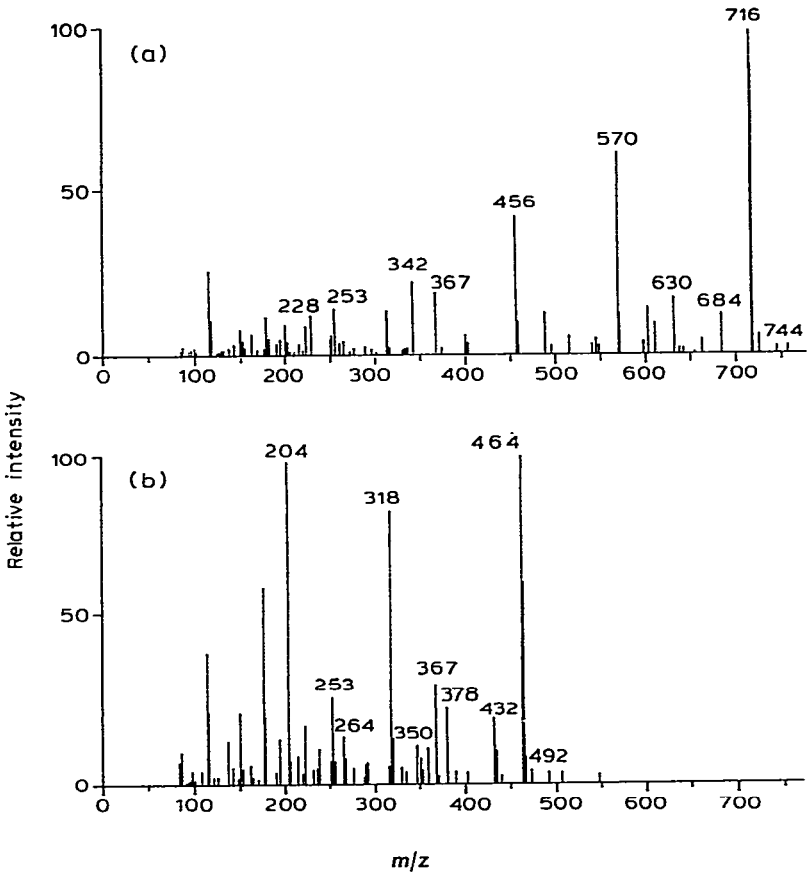

Fig. 1. Mass spectra of the trifluoroacetyl derivatives of (a) neuraminic acid methyl ester methyl glycosides, and (b) heptulosaminic acid methyl ester methyl glycoside.

TABLE I

ION FRAGMENTS OBSERVED IN THE MASS SPECTRA OF THE TRIFLUOROACETYL DERIVATIVES OF NEURAMINIC ACID METHYL ESTER METHYL GLYCOSIDE AND HEPTULOSAMINIC ACID METHYL ESTER METHYL GLYCOSIDE

Neuraminic acid		*Heptulosaminic acid*	
m/z	*Probable fragment*	m/z	*Probable fragment*
744	M−·OCH_3	492	M−·OCH_3
716	M−·CO_2CH_3	464	M−·CO_2CH_3
684	716−CH_3OH	432	464−CH_3OH
630	744−CF_3CO_2H	378	492−CF_3CO_2H
570	684−CF_3CO_2H	367	464−CF_3O·
456	570−CF_3CO_2H	350	464−CF_3CO_2H
367	716−CF_3O· and $CF_3\dot{C}O_2$-NH-CH_2-O_2CCF_3	318	432−CF_3CO_2H
		264	378−CF_3CO_2H
342	456−CF_3CO_2H	253	367−CF_3CO_2H
253	367−CF_3CO_2H	204	318−CF_3CO_2H
228	342−CF_3CO_2H		

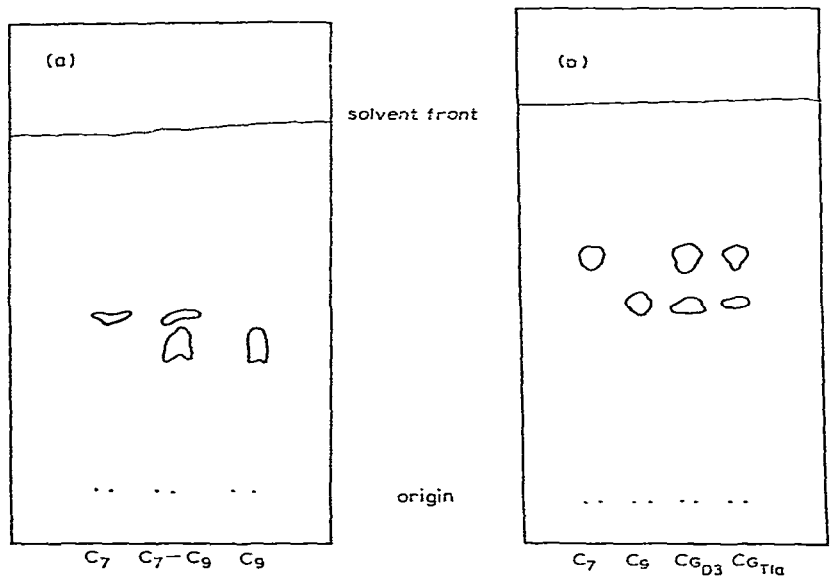

Fig. 2. Diagrams of (a) a paper chromatogram of sialic acid and its 7-carbon analog, and (b) a cellulose-t.l.c. of the methyl esters of sialic acid, its 7-carbon analog, and samples obtained from periodate–borohydride treated gangliosides. In (a), C_7 designates *N*-acetylheptulosaminic acid, C_9 *N*-acetylneuraminic acid, and C_7–C_9 a mixture of both. In (b), C_7 and C_9 designate the methyl esters of *N*-acetylheptulosaminic acid and *N*-acetylneuraminic acid, respectively; CG_{D3} and CG_{T1a} the samples derived from periodate–borohydride treated gangliosides, G_{D3} and G_{T1a}, respectively. Both chromatograms were developed ascending in 1:2:1 (v/v) 1-butanol–1-propanol–0.1M hydrochloric acid, and the spots were made visible by the resorcinol–trichloroacetic acid spray[12].

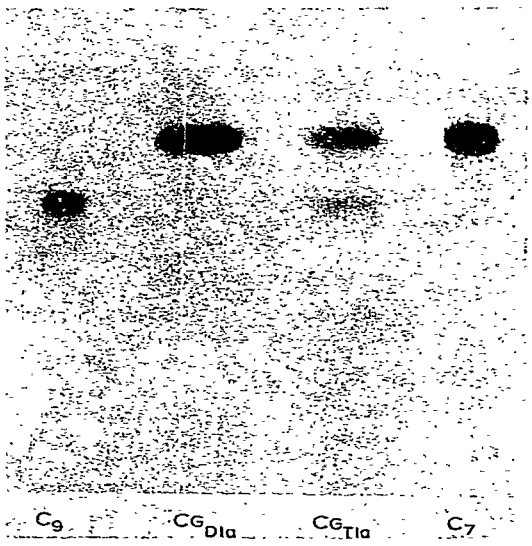

Fig. 3. H.p.t.l.c. plate of a chromatogram of the methyl esters derived from: C_9, *N*-acetylneuraminic acid; CG_{D1a}, sample from periodate–borohydride treated G_{D1a}; CG_{T1a}, sample from periodate–borohydride treated G_{T1a}; and C_7, *N*-acetylheptulosaminic acid. The chromatogram was developed in 10:4:3:1 (v/v) 2-butanone–acetic acid–methanol–water and the spots were made visible by the resorcinol–hydrochloric acid spray[13].

TABLE II

DISTRIBUTION OF RADIOACTIVE LABEL IN PERIODATE SUSCEPTIBLE (C_7) AND NONSUSCEPTIBLE (C_9) SIALIC ACID IN THE *in vitro* BIOSYNTHESIZED GANGLIOSIDES

Ganglioside		*Ratio of radioactivity*[a] (C_7:C_9)	
Chick brain	G_{M3}	95:5 ± 1.0	(4)
	G_{D3}	54:46 ± 1.1	(9)
	G_{D1a}	95:5 ± 1.3	(9)
	G_{T1a}	78:22 ± 8.0	(6)
Rat brain	G_{D1a}	97:3 ± 0.8	(6)
	G_{T1a}	85:15 ± 2.0	(8)

[a]Values are given as percentages of total radioactivity ± the standard error. Numbers in parentheses indicate the number of samples analyzed.

resolution. Separation of free *N*-acetylneuraminic acid and *N*-acetylheptulosaminic acid on h.p.t.l.c. plates employing various solvent-systems was unsuccessful owing to excessive tailing of the free acids. Attempts to generate the esters directly by methanolysis of intact gangliosides were also unsuccessful, and resulted in mixtures of methyl ester, methyl glycosides, and methyl ester methyl glycosides of sialic acid and its 7-carbon analog that were difficult to separate and identify.

Distribution of the radioactivity in N-*acetylneuraminic acid and* N-*acetylheptulosaminic acid residues obtained from periodate–borohydride-treated gangliosides.* — The label distribution was determined as indicated in Table II. The primary ganglioside products, G_{M3} and G_{D1a}, contain sialic acid residues that are susceptible to periodate oxidation. Therefore, nearly all the label was found in the 7-carbon analog after periodate–borohydride treatment of these gangliosides. The results with G_{M3} and G_{D1a} indicate the quantitative nature of the experimental conditions employed. Both secondary products, G_{D3} and G_{T1a}, showed the label in both susceptible and non-susceptible sialic acid residues, which is indicative of the sialosyl-(2→8)-sialosyl linkage. In the compounds from both species examined (rat and chick), the trisialoganglioside G_{T1a} contained less radioactivity in the nonsusceptible sialic acid residue than might be expected. This is probably due to cold, endogenous G_{D1a} acting as a substrate for G_{T1a}, in addition to the G_{D1a} synthesized *in vitro*, *de novo*.

DISCUSSION

A method to determine specifically the sialosyl–sialosyl linkage in gangliosides biosynthesized *in vitro* was developed. It is based on the periodate oxidation–borohydride reduction procedure, in which an *N*-acetylneuraminic acid residue without substitution at OH-8 gives the 7-carbon analog, *N*-acetylheptulosaminic acid. *N*-Acetylneuraminic acid substituted at OH-8 remains intact. Polysialogangliosides

were obtained as secondary products from *in vitro, de novo* synthesized substrates, resulting in sialosylsialosyl residues that are radiolabeled in both residues. Following periodate oxidation–borohydride reduction, the radioactivity was found in both the intact sialic acid and its 7-carbon analog, indicating that a sialosyl-(2→8)-sialosyl residue had been synthesized.

The method described herein is based on two assumptions. The first is that adequate synthesis of the secondary product gangliosides could be achieved. Previous reports on brain ganglioside sialyltransferases indicates that the conditions for *in vitro* biosynthesis are quite similar[2-4,11]. In addition, we have already demonstrated the incorporation of radioactive sialic acid into the trisialoganglioside G_{T1a} by use of the monosialoganglioside G_{M1} as the initial substrate[4]. The second assumption is that the cold, endogenous ganglioside corresponding to the *in vitro, de novo* synthesized substrate (*i.e.*, the primary product) would not interfere competitively in the synthesis of the secondary product ganglioside. Total, competitive interference by the endogenous ganglioside with the *in vitro, de novo* substrate would lead to the erroneous conclusion that the incorrect structure had been synthesized. The effect of unlabeled endogenous substrate is apparent in both syntheses. The effect[18] on G_{D3} synthesis was small, presumably because of the small amounts of endogenous G_{M3}. However, the distribution of the label in the trisialoganglioside G_{T1a} was strongly slanted toward periodate-susceptible sialic acid residues. G_{D1a}, the intermediate in the synthesis of G_{T1a}, is a major brain ganglioside, and G_{T1a} is a minor one[18]. Therefore, the application of this analytical method to G_{T1a} was an excellent test of the second assumption.

The method described herein may be used to firmly establish the pathways of ganglioside biosynthesis by permitting direct examination of the actual product synthesized. Coupled with the use of neuraminidase[4,19], this method can clearly indicate whether or not an α-sialosyl-(2→8)-sialosyl linkage has been synthesized. The method should prove useful in additional studies on the biosynthesis of other gangliosides that contain α-sialosyl-(2→8)-sialosyl linkages and, in particular, for determining the different pathways and products proposed for higher and lower vertebrates[9]. Extension of this technique to biosynthesized sialoglycoproteins would permit the structural determination, in these compounds, of sialosyl linkages that heretofore could not be determined because of the extremely small quantities of labeled sialoglycoproteins that can be isolated.

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