A disialoganglioside of the globo-series from chicken skeletal muscle

Somsankar Dasgupta, Jaw-Long Chien, Edward L. Hogan,[1] and Herman van Halbeek*

Department of Neurology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425, and Complex Carbohydrate Research Center and Department of Biochemistry,* The University of Georgia, 220 Riverbend Road, Athens, GA 30602

Abstract We have isolated a disialoganglioside of the globo-series from chicken pectoral muscle. The compound was obtained by extraction followed by ion-exchange and silicic acid column chromatography and judged to be pure by thin-layer chromatography in three solvent systems. The structure of the ganglioside was determined by carbohydrate and ceramide composition analysis, sequential exoglycosidase digestion, methylation analysis, and 500-MHz ^{1}H-NMR spectroscopy to be:

NeuAc α2→6
NeuAc α2→3
Galβ(1→3)GalNAcβ(1→3)Galα(1→4)Galβ(1→4)Glcβ(1→1)Cer.

Analysis of the ceramide moiety indicated d18:1 sphingosine as the long-chain base, and C16:0, C18:0, C18:1, and C20:0 as the prevalent fatty acids. This glycolipid is only the second ganglioside of the globo-series, and the first disialo member of the series, found in chicken muscle.—**Dasgupta, S., J-L. Chien, E. L. Hogan, and H. van Halbeek.** A disialoganglioside of the globo-series from chicken skeletal muscle. *J. Lipid Res.* 1991. **32:** 499–506.

Supplementary key words gangliosides • ^{1}H-NMR • gas chromatography–mass spectrometry

After the discovery of gangliosides in brain by Klenk in 1942 (1), they have been found in virtually all extraneural tissues and in cultured cells (2). Improvements in the methods of purification and characterization have enabled the isolation of numerous gangliosides with diverse structures. In addition to the ganglio-series which comprises the majority of gangliosides in the central nervous system, there are two lacto-series gangliosides which predominate in many extraneural tissues including red blood cells and skeletal muscle. Globo-series gangliosides containing gal-globoside as the core oligosaccharide were found first by Chien and Hogan (3, 4) in chicken skeletal muscle and have subsequently been isolated from human erythrocytes (5) and teratocarcinoma cells (6). Here we report a disialoganglioside of the globo-series in chicken skeletal muscle which resembles compound "DG-4" isolated from human erythrocytes (5).

MATERIALS

Pectoral muscle was obtained from an adult Leghorn chicken purchased at a local supermarket or dissected from an inbred strain of chickens. Extraneous tissues were separated by gross dissection and the muscle was ground and stored at −60°C (4). DEAE-Sephadex A-50 was obtained from Sigma, St. Louis, MO. Sphingosine, dihydrosphingosine, and fatty acid methyl esters were purchased from Supelco, Bellefonte, PA as were gas chromatography packings of 10% DEGS-PS, 3% SP-2340, 3% OV-275, 3% OV-17, and 3% SE-30 (all on Supelcoport support). Sephadex LH-20 was purchased from Pharmacia, Piscataway, NJ and precoated silica gel plates were from Merck, Darmstadt, FRG. Biosil-A was obtained from Bio-Rad, Richmond, CA and neutral glycolipid and ganglioside standards were prepared in our laboratory. β-Galactosidase and β-hexosaminidase were purified from jack bean meal (7, 8) and α-galactosidase from fig powder (9).

Abbreviations: TLC, thin-layer chromatography; THF, tetrahydrofuran; DMSO-d_6, dimethyl-d_6 sulfoxide; TMS, tetramethylsilane; LacCer, Gal(β1→4)Glc(β1→1)Cer; Gb_3Cer, Gal(α1→4)Galβ(1→4)Glcβ(1→1)Cer; nLc_4Cer, Gal(β1→4)GlcNAc(β1→3)Gal(β1→4)Glcβ(1→1)Cer; Gb_4Cer or globoside, GalNAc(β1→3)Gb_3Cer; penta or nLc_5Cer, Gal(α1→3)nLc_4Cer; hexa or nLc_6Cer, Gal(β1→4)GlcNAc(β1→3)nLc_4Cer; Gb_5Cer, IV^3-Gal-globoside; V^3-NeuAcGb_5Cer, Mono-α-(2→3)-sialo-Gal-globoside; $V^{3,6}$-$NeuAc_2$-Gb_5Cer, disialo-Gal-globoside; GLC–MS, gas–liquid chromatography–mass spectrometry; HPLC, high performance liquid chromatography; NeuAc, N-acetyl-neuraminic acid (or sialic acid); PMAAs, partially methylated alditol acetates. All monosaccharides are of the D-configuration. GM1, GD3, GD1a, and GD1b were designated following Svennerholm (1963. *J. Neurochem.* **10:** 613–623).

[1]To whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Isolation of ganglioside

Pectoral muscles from adult Leghorn chickens were separated from extraneous tissues by gross dissection. One kilogram of ground muscle was homogenized in a Waring blender with ten volumes of tetrahydrofuran (THF)–0.01 M KCl 4:1 (v/v). After stirring for 3 h, the mixture was filtered. The extraction was repeated twice with 5 volumes of THF–0.01 M KCl 8:1 (v/v). The filtrates were combined, evaporated to a syrup, and saponified with 200 ml of 0.6 N NaOH in methanol at 37°C for 5 h. After neutralization with 0.1 N HCl, the solvent was evaporated. The residue was suspended in 200 ml of chloroform–acetone 1:1 (v/v) and applied to a silicic acid column (2.5 × 40 cm). Cholesterol, cholesteryl esters, and unesterified fatty acids were eluted with the same solvent and the glycosphingolipids were eluted with 3 volumes of THF–H_2O 7:1 (v/v). After concentrating to dryness, the lipids were dialyzed against distilled water with three changes of water and the retentate was dried with a Savant concentrator. The residue was dissolved in chloroform–methanol 2:1 and applied to a DEAE-Sephadex A-50 column (acetate form, 2.5 × 30 cm). Neutral glycosphingolipids were eluted with methanol and gangliosides were eluted with methanol containing 0.01 M sodium acetate (fraction I) and 0.2 M sodium acetate (fraction II) respectively. Fraction I contained monosialogangliosides. Fraction II contained di- and trisialogangliosides. After the removal of salt by dialysis and drying, the fraction II was further resolved by silicic acid chromatography. The glycolipids were applied in chloroform–methanol 2:1 and eluted from the Biosil-A column (2.5 × 30 cm) with 200 ml of chloroform–methanol–water–ammonium hydroxide 120:70:10:3 followed by 200 ml of chloroform–methanol–water–ammonium hydroxide 120:75:12:4. Fractions (6 ml) were collected and a 50-μl aliquot was taken from alternate tubes to identify the ganglioside by thin-layer chromatography (TLC). The fractions containing the disialoganglioside not previously detected in chicken muscle were pooled and purity was examined by TLC with three solvent systems as described (4)

Carbohydrate composition analysis

Sialic acids were quantitated according to the method of Svennerholm (10) as modified by Miettinen and Takki-Luukainen (11) and analyzed by GLC according to Yu and Ledeen (12). Neutral and amino sugars were determined by gas chromatography, as alditol acetates on a SP-2340 column.

Enzymatic sequencing of the carbohydrate

Glycosyl residues were sequentially cleaved by specific acid exoglycosidases. Sialic acid was released by the neuraminidase from *C. perfringens* (Worthington, Malvern, PA). The incubation mixture contained 40 μg ganglioside, 40 munit enzyme (1 unit hydrolyses, 1 nmol of NeuAc from bovine submaxillary mucin per min at pH 5.0 at 37°C) in 200 μl of 0.05 M sodium acetate buffer, pH 5.0. After incubating for 6 h at 37°C, the reaction was stopped by the addition of 1 ml of chloroform–methanol 2:1. The desialylated glycolipid was recovered in the lower layer and was subsequently treated with β-galactosidase, β-hexosaminidase, and α-galactosidase according to previously published procedures (13).

Permethylation studies

The disialoganglioside (500 μg) was permethylated according to the procedure of Yang and Hakomori (14) and purified on a column of Sephadex LH-20. The methylated glycolipid was treated with 0.6 N H_2SO_4 in 80% aqueous CH_3COOH. After passing through Dowex 1 × 8 cm (formate form), it was reduced and acetylated according to the method of Björndal, Lindberg, and Svensson (15). The partially methylated alditol acetate derivatives were analyzed on a Finnigan Model 3300 gas chromatography–mass spectrometer. The partially methylated neutral alditol acetates were separated on a 3% OV 275 column at 180°C and the amino sugar derivatives were separated on a 3% OV 17 column with temperature programmed at 2°C/min from 180° to 200°C (16–18).

Fatty acid and long-chained base analysis

Fatty acid methyl esters were extracted with hexane after methanolysis of the disialoganglioside (1.5 M anhydrous methanolic HCl, 80°C for 24 h) and analyzed by gas chromatography on a 10% DEGS-PS column at 190°C. The nature of the long chain base was determined as its trimethylsilyl derivative by GC on a 3% SE-30 column (19) after hydrolysis in aqueous methanolic HCl (20).

500-MHz ^{1}H-NMR spectroscopy

The disialoganglioside was subjected to ^{1}H-NMR spectroscopic analysis at 500 MHz. The compound (500 μg) was first converted to the Na^+ form by passing through ion exchange resin AG-50W-X8 (Bio-Rad) in the Na^+ form, in methanol–water. The sample was then deuterium-exchanged by repeated addition and evaporation of methanol-d_4/D_2O, at room temperature. Immediately prior to ^{1}H-NMR spectroscopic analysis, the sample was dissolved in 0.4 ml DMSO-d_6 (Aldrich, 99.96% D) containing 2% D_2O and 1% tetramethylsilane

(TMS) as chemical shift reference, and transferred into a 5-mm NMR tube (Wilmad; 535-PP).

500-MHz one-dimensional ^{1}H-NMR spectroscopy was performed on a Bruker AM-500 spectrometer interfaced with an Aspect-3000 computer. The spectral width was 6025 Hz (12 ppm); 3224 transients were acquired in 16K datapoints, in quadrature detection mode. The probe temperature was kept at either 27°C or 35°C. Further experimental details have been described (21–25). Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation followed by zerofilling to 32K datapoints and Fourier transformation. Chemical shifts (δ) are expressed in ppm downfield from internal TMS with an accuracy of 0.002 ppm.

RESULTS

Thin-layer chromatography of the purified ganglioside

The disialoganglioside was examined for purity by TLC with three solvent systems (see legend to **Fig. 1** for details). As shown in Fig. 1, its R_f was slightly greater than that of GD1b from human brain in solvent systems I and II. However, with solvent III (THF: 0.1% KCl), it migrated significantly slower than GD1b. In each case, only one homogeneous band was detected.

Carbohydrate and lipid composition

The ganglioside contained N-acetylneuraminic acid, galactose, galactosamine, and glucose in a molar ratio of 2.04:3.05:0.95:1, as determined by GC. The acyl groups were mainly palmitic, stearic, and oleic acids, and sphingosine was the major long chain base. (See **Table 1**).

Carbohydrate sequence by serial exoglycosidase digestion

The disialoganglioside was not susceptible to α- and β-galactosidase or β-hexosaminidase. Neuraminidase in the absence of detergent cleaved the sialic acid residues to produce a neutral glycosphingolipid with an R_f close to that of a pentaglycosylceramide (nLc$_5$Cer) from bovine erythrocytes and it co-migrated with standard Gb$_5$Cer prepared by α-neuraminidase digestion of NeuAc Gb$_5$Cer

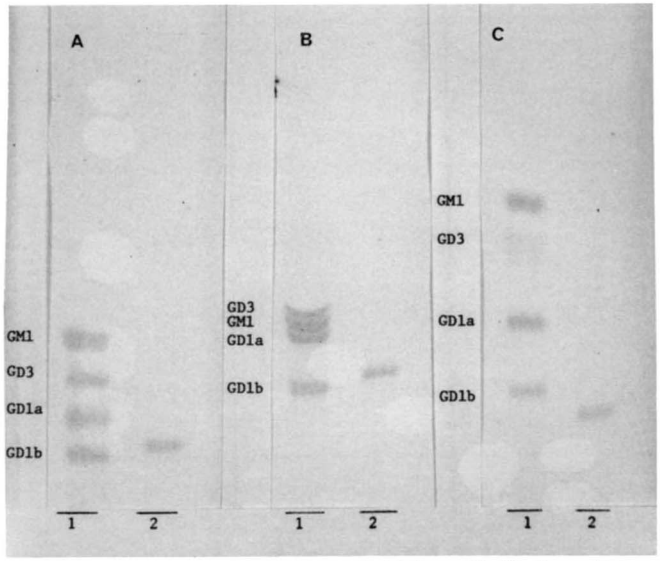


Fig. 1. Thin-layer chromatography of disialoganglioside from chicken muscle. Lane 1: human brain ganglioside standards; lane 2: purified disialoganglioside from breast muscle. Plates are developed in A: chloroform–methanol–0.25% $CaCl_2$ 60:40:9 (v/v/v); B: chloroform–methanol–2.5 N ammonia 60:40:9 (v/v/v); and C: tetrahydrofuran–0.1% KCl 75:15 (v/v).

(Chien, J-L., and E. L. Hogan, unpublished results). In controlled hydrolysis for a short time, an intermediate ganglioside having an R_f identical to that of the monosialopentaglycosylceramide was detected and this was considered an intermediate in conversion to the asialo compound (results not shown). The neutral glycolipid was subsequently hydrolyzed by β-galactosidase to become a tetraglycosylceramide with R_f identical to that of globoside. Further treatment of the glycolipid with β-hexosaminidase, α-galactosidase, and β-galactosidase yielded tri-, di-, and monohexaosylceramide respectively (**Fig. 2**).

Linkages between glycosyl residues

The partially methylated hexitol acetate derivatives were separated by GC and identified based on GC retention times and mass spectra. Four peaks were obtained for the netural PMAAs analyzed on an OV-275 column. They were identified as 2,4,6-tri-O-methylgalactitol-

TABLE 1. Fatty acid and long chain base composition of the disialo-pentaglycosylceramide from chicken skeletal muscle

	C16:0	C18:0	C18:1	C18:2	C20:0	C21:0	C24:0	C26:0	C27:0
					%				
Fatty acid	36.9	20.3	18.6	2.4	10.1	2.7	2.2	2.3	4.5
Long chain base		20.8	79.2						

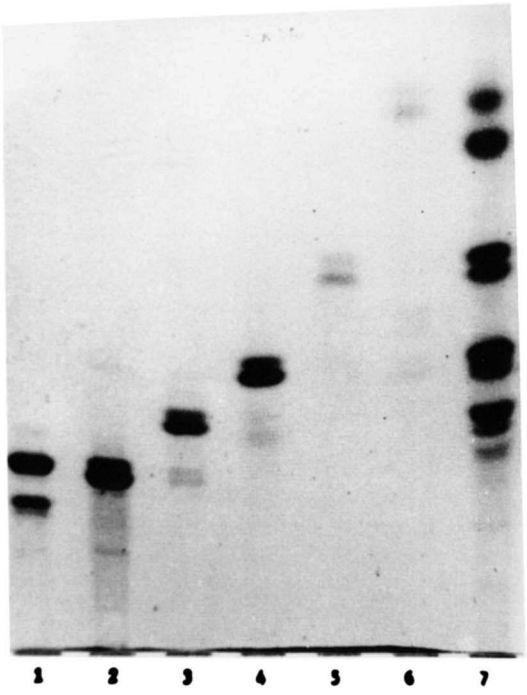


Fig. 2. Sequential exoglycosidase digestion of pentaglycosylceramide from chicken muscle. Lane 1: standard neutral glycolipid from bovine erythrocytes (nLc_5Cer and nLc_6Cer); lane 2: neutral glycolipid obtained from disialoganglioside after neuraminidase hydrolysis; lane 3: lane 2 + β-galactosidase; lane 4: lane 3 + β-hexosaminidase; lane 5: lane 4 + α-galactosidase; lane 6: lane 5 + β-galactosidase; lane 7: standard neutral glycolipid from bovine erythrocytes (GlcCer, LacCer, Gb_3Cer, nLc_4Cer).

1,3,5-triacetate, 2,3,6-tri-O-methylgalactitiol-1,4,5-triacetate, 2,3,6-tri-O-methylglucitol-1,4,5-triacetate and 2,4-di-O-methylgalactitol-1,3,5,6-tetracetate respectively. The amino sugar was identified on OV-17 column as 4,6-di-O-methyl-2-deoxy-2-N-methylacetamido-galactitol-1,3,5-triacetate.

Structural characterization of disialoganglioside by ^{1}H-NMR spectroscopy

The disialoganglioside was subjected to ^{1}H-NMR spectroscopy at 500 MHz. The ^{1}H-NMR spectrum (**Fig. 3a**) revealed a ganglioside of the globo-series. This conclusion was based upon the appearance at δ4.8, of the H-1 signal characteristic for α-linked Gal in globosides (6, 26). The chemical shifts and relevant coupling constants of the structural-reporter groups of the oligosaccharide moiety are listed in **Table 2**. Fig. 3b shows the resolution-enhanced structural-reporter-group regions of the 500-MHz ^{1}H-NMR spectrum of the ganglioside. The purity of the compound as judged from the ^{1}H-NMR spectrum, was >95%.

Comparison of the ^{1}H-NMR data of the disialoganglioside from chicken muscle with those of other gangliosides of the globo-series (6) (Table 2) revealed that our ganglioside has the pentaglycosylceramide Gb_5Cer core sequence:

$$\underset{V}{Gal\beta(1\rightarrow3)}\underset{IV}{GalNAc\beta(1\rightarrow3)}\underset{III}{Gal\alpha(1\rightarrow4)}\underset{II}{Gal\beta(1\rightarrow4)}\underset{I}{Glc\beta(1\rightarrow1)}\underset{R}{Cer}$$

The spectral features characteristic for the presence of the pentasaccharide moiety have been documented (6). In brief, the occurrence of the H-1 signals of Glc-I at $\delta \approx 4.16$, Gal-II at δ4.26, α-Gal-III at δ4.81, GalNAc-IV at $\delta \approx 4.55$, and Gal-V at $\delta \approx 4.22$ (Fig. 3 and Table 2) are typical of the galacto-globoside unit.

The ceramide moiety becomes manifest in the NMR spectrum from its R-5 and R-4 olefinic protons at δ5.516/5.541 and 5.329/5.348 (at 27° and 36°C), respectively, R-1a at δ3.962/3.981 (at 27° and 35°C), while R-11 in the *cis*-fatty acid is found at δ5.306/5.322 (at 27° and 35°C).

The galacto-globoside core element in the chicken muscle ganglioside was found to be extended by two NeuAc residues at the nonreducing end; both are attached to Gal-V, one in α(2→3)-linkage, the other in α(2→6)-linkage, as suggested by the 2,4-di-O-methylgalactose derivative found in the methylation analysis (see above). The $NeuAc^3$ residue is characterized by the H-3eq signal at δ2.77 and the NAc methyl signal at $\delta \approx 1.88$ (compare Table 2, compound V^3-NeuAc-Gb_5Cer). The $NeuAc^6$ residue shows its H-3eq signal at δ2.638, and another NAc methyl signal appears around δ1.88.

The combination of chemical shift values for the disialo-galacto moiety in the chicken muscle ganglioside is novel. Regarding the disialylation of Gal-V, one would expect (6, 25) the Gal-V H-1 doublet to shift downfield by ~0.004 ppm due to the attachment of V^3-NeuAc, and at the same time upfield by -0.03 ppm because of the presence of V^6-NeuAc. The effects are apparently independent of each other and additive, resulting in the position of the Gal-V H-1 signal at δ4.21. The GalNAc H-1 signal undergoes an upfield effect from attachment of both NeuAc residues, to δ4.575.

On the basis of the results of composition analysis, methylation, enzymic sequencing, and ^{1}H-NMR spectroscopy, the chicken muscle disialoganglioside has been assigned the structure:

$$\begin{array}{l} N^6 \\ NeuAc\alpha(2\rightarrow6) \\ \qquad\qquad\diagdown \\ \qquad\qquad\quad Gal\beta(1\rightarrow3)GalNAc\beta(1\rightarrow3)Gal\alpha(1\rightarrow4)Gal\beta(1\rightarrow4)Glc\beta(1\rightarrow1)Cer. \\ \qquad\qquad\diagup \\ NeuAc\alpha(2\rightarrow3) \\ N^3 \end{array}$$

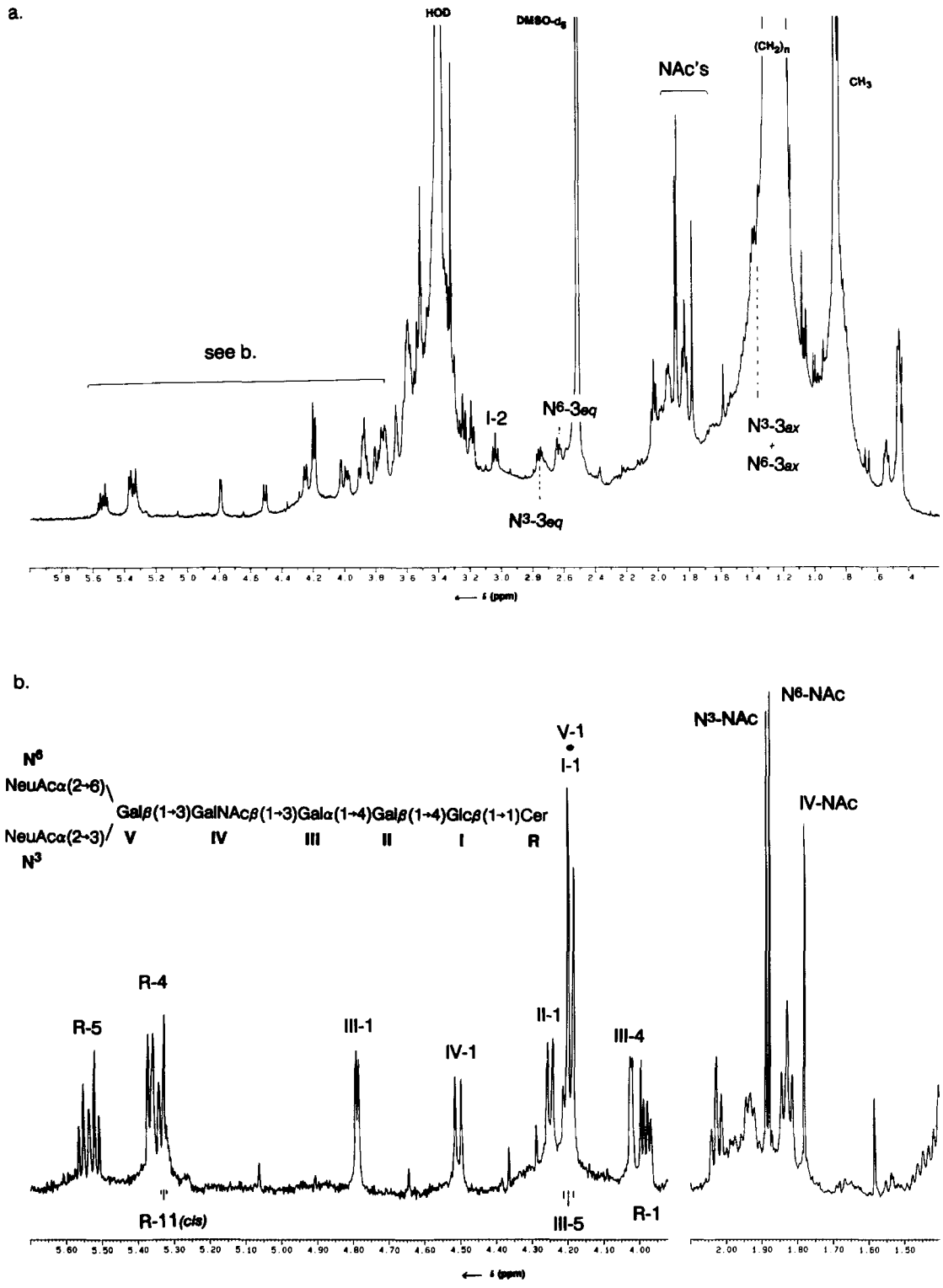


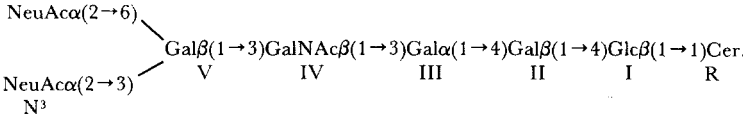
Fig. 3. Overall 500-MHz ^{1}H-NMR spectrum (DMSO-d_6/D_2O 98/2, v/v; pD 6; 27°C); B: structural-reporter-group regions of the spectrum of the disialoganglioside obtained from chicken muscle. The roman numbers in the spectra refer to the corresponding monosaccharide residues in the structure; the arabic numbers refer to the protons within the monosaccharide rings. The relative-intensity scale of the NAc CH_3 region differs from that in the other part in the spectrum by factor 2.

TABLE 2. ^{1}H chemical shifts of structural-reporter groups of constituent monosaccharides and vicinal coupling constants of anomeric protons for the disialoganglioside obtained from chicken muscle, and for structurally related gangliosides of the globo-series[a]

Residue[b]	Reporter Group[c]	Chemical Shift (ppm) and Coupling Constant (Hz) in[c]				
		Gb_4Cer[a,d]	Gb_5Cer[a]	V^3-NeuAc-Gb_5Cer[a]	$V^{3,6}$-$NeuAc_2$-Gb_5Cer	
					27°C	35°C
Glc-I	H-1					
	($J_{1,2}$)	4.16 (7.7)	4.17 (7.3)	4.19 (8.3)	4.172[e]	4.195 (7.9)
Glc-I	H-2	n.d.	n.d	n.d.	3.017	3.045
Gal-II	H-1					
	($J_{1,2}$)	4.26 (7.7)	4.26 (7.3)	4.26 (7.8)	4.229	4.255 (7.3)
Gal-II	H-4	n.d.	n.d.	n.d.	3.793	3.815
Gal-III	H-1					
	($J_{1,2}$)	4.81 (3.6)	4.80 (3.9)	4.81 (2.9)	4.768	4.792 (3.7)
Gal-III	H-4	n.d.	n.d.	n.d.	4.002	4.017
Gal-III	H-5	n.d.	n.d.	4.13	4.178	4.185
GalNAc-IV	H-1					
	($J_{1,2}$)	4.52 (8.1)	4.61 (8.3)	4.57 (8.3)	4.486	4.512 (8.5)
GalNAc-IV	NAc	n.d.	n.d.	n.d.	1.770	1.789
Gal-V	H-1					
	($J_{1,2}$)		4.20	4.24 (7.8)	4.172[e]	4.210 (7.9)
NeuAc-N^3	H-3ax			n.d.	1.35	1.378
NeuAc-N^3	H-3eq			2.77	2.736	2.756
NeuAc-N^3	NAc			n.d.	1.868	1.889
NeuAc-N^6	H-3ax				1.32	1.35
NeuAc-N^6	H-3eq				2.614	2.638
NeuAc-N^6	NAc				1.859	1.881

[a]Data taken from Kannagi et al., 1983 (6); n.d., not determined.

[b]The numbering system used for denoting glycosyl residues in the gangliosides of the globo-series is as follows:



[c]Data were acquired at 500 MHz for solutions of the compounds in DMSO-d_6/D_2O, 98/2 (v/v) at 27°C and 35°C (for the disialoganglioside from chicken muscle), and at 35°C for the reference compounds. Values for coupling constants are in parentheses.

[d]Data are in agreement with those published by Dabrowski, Hanfland, and Egge, 1980, and Dabrowski, 1990 (26, 27).

[e]The anomeric doublets of Glc-I and Gal-V coincide at 27°C (see Fig. 3); however, they are separted at 35°C.

DISCUSSION

The gangliosides of chicken skeletal muscle comprise four different classes, namely, the lacto-series, the ganglio-series, the neolacto-series, and the globo-series (4). The only representative of the globo-series reported to date (4, 28) is V^3-NeuAc-Gb_5Cer. For disialogangliosides, only G_{D3} and G_{Dla} have been previously described. In this study, we have isolated and characterized a disialoganglioside of the globo-series, containing its two sialic acid residues attached to C3 and C6 of the terminal galactosyl residue in Gb_5Cer. Although novel for chicken muscle, a ganglioside with the same carbohydrate structure has been found in human erythrocytes [compound "DG-4" in (5)]. However, compound "DG-4" was not characterized by ^{1}H-NMR spectroscopy, nor have any other oligosaccharides with disialylated galactosyl residues been previously analyzed by this technique.

Cosubstitution of the Gal-V residue of Gb_5Cer by two NeuAc residues in $\alpha(2\rightarrow6)$- and $\alpha(2\rightarrow3)$-linkage manifests itself in the ^{1}H-NMR spectrum by the occurrence of the NeuAc reporter-group signals at unique positions, namely, H-3eq at δ2.638 and 2.756, and the NAc CH_3 signals at δ1.881 and 1.889 ppm. The effects of disialylation on the chemical shifts of neighboring residues are restricted to Gal-V H-1 ($\Delta\delta \sim 0.01$ ppm), GalNAc-IV H-1 ($\Delta\delta \sim 0.1$ ppm) and GalNAc-IV NAc ($\Delta\delta$0.013 ppm).

The disialo globogangliosides $V^{3,6}$ $(NeuAc)_2Gb_5Cer$ from chicken skeletal muscle and human erythrocytes differ considerably in fatty acid and sphingosine base

composition. Eighty-six % of the fatty acids in the chicken muscle ganglioside are 16:0, 18:0, 18:1, and 20:0, while 95% of the fatty acids of the erythrocyte compound are 22:0, 24:0, and 24:1. The long-chain base in the chicken muscle ganglioside is 79% sphingenine (C18:1) and 21% sphinganine (C18:0), while it is entirely sphingenine in the erythrocyte ganglioside "DG-4" (5). These differences may reflect the different microenvironments in the plasmalemmas in which the gangliosides are located.

Controlled hydrolysis of the chicken muscle disialo compound with α-neuraminidase yielded a monosialo derivative that co-migrates with the monosialo ganglioside (V^3 NeuAcGb_5Cer) of chicken muscle (5). This monosialo ganglioside with a terminal α2→3 NeuAc bound to the terminal galactose of GalGb_4Cer was the first globo-series ganglioside characterized. It was found in chicken skeletal muscle (4, 28) and later in human teratocarcinoma cells (6). Both the mono- and disialo derivatives of Gb_5Cer are found in chicken muscle but the neutral glycolipid Gb_5Cer has not yet been identified in this tissue. In human teratocarcinoma cells, Gb_5Cer and its fucosyl derivative (Fuc α1→2 Gb_5Cer, V^2FucIV^3galGbOse$_4$Cer) have been characterized and they may be markers for this neoplasm (6). This 3,6 gal-substituted disialo structure has not previously been recognized in the globo-series or other sialoglycolipids of skeletal muscle (29-33).

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