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Assignment of the ^{13}C Nuclear Magnetic Resonance Spectrum of Aqueous Ganglioside G_{M1} Micelles†

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ABSTRACT: This article describes the natural-abundance Fourier-transform carbon-13 nuclear magnetic resonance spectrum, at 67.88 MHz, of aqueous micelles of bovine brain ganglioside G_{M1} of purity greater than 99%. Assignments are given for every carbon nucleus in the molecule, on the basis of a comprehensive study of the relevant mono-, di-, tri-, and polysaccharides, including several containing sialic acid (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosonic acid), and phospho-, sphingo-, and glycosphingolipids. These assignments represent an extension of the ^{13}C nuclear magnetic resonance data from monosaccharides and lipids to complex oligosaccharides and glycolipids. They also form the basis for interpretation of spectral perturbations induced in G_{M1} by titration with paramagnetic europium(III). The single sialic acid in G_{M1} was found to be α-glycosidically linked in the oligosaccharide from considerations of its unique anomeric chemical shift. The sialic acid carboxyl and glyceryl side chain, along with additional ligands donated by the 2-acetamido-2-deoxy-β-D-galactopyranoside and terminal β-D-galactopyranoside residues in the oligosaccharide portion of G_{M1}, were found to be intimately involved with cation binding. It is proposed that the higher affinity, compared with monomeric sialic acid, of G_{M1} for cations may result from these additional oligosaccharide groups, which may effectively compete for water ligands in the metal cation coordination sphere.

Gangliosides are membrane glycosphingolipids containing a hydrophilic oligosaccharide portion glycosidically linked to a hydrophobic ceramide, which presumably anchors the ganglioside molecule in the membrane. Several classes of gangliosides are differentiated by the number and position of attachment of one or more sialic acid residues, giving rise to mono-, di-, and trisialogangliosides, denoted as G_M, G_D, and G_T, respectively (Svennerholm, 1964). The presence of the sialic acid confers a negative charge on the oligosaccharide portion of the molecule at physiological pH, which engenders a strong interaction with cations of biological significance, like calcium (Abramson et al., 1972).

Although gangliosides have been known to be constituents of plasma membranes for a number of years, their function has only recently begun to be understood. With their hydrophilic oligosaccharide portions projecting out of the membrane, gangliosides are well suited to act as receptors for biological signal molecules (Fishman & Brady, 1976). Recently, gangliosides have been implicated as membrane receptors for a variety of endogenous and exogenous proteins, including hormones (Aloj et al., 1977, and references cited therein) such as thyrotropin, luteinizing hormone, and follicle-stimulating hormone (Ledley et al., 1976), interferon (Kohn et al., 1976), and the bacterial toxins elaborated by *Vibrio cholerae* (Moss et al., 1977, and references cited therein) and *Clostridium tetani* (Helting et al., 1977; Ledley et al., 1977). The specificity of the protein-ganglioside interaction is determined by the carbohydrate pattern of the ganglioside, each protein binding

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preferentially to one class of gangliosides. There is a large body of evidence which indicates that cholera toxin, for example, binds most strongly to the monosialoganglioside G_{M1}[1] (Cuatrecasas, 1973), and that this binding is the first step in the mechanism of action of this toxin (Fishman & Brady, 1976).

Studies beyond this of ganglioside structure and function using physical techniques have not been widespread. Carbon-13 NMR offers several unique advantages in furthering an atomic description of the molecule (Allerhand, 1975), one of which is the nondestructive nature of the method. The large range of ^{13}C chemical shifts encountered makes this nucleus ideal for unraveling the spectra of large complex molecules like ganglioside G_{M1}, which may contain 50 or more different types of carbon atoms. Furthermore, a large body of data has been accumulated on monosaccharides (Bundle et al., 1973; Dorman & Roberts, 1970; Walker et al., 1976), oligosaccharides (Colson & King, 1976; Colson et al., 1974; Dorman & Roberts, 1971), and polysaccharides (Bhattacharjee et al., 1975; Bundle et al., 1974), as well as phospholipids (Shapiro et al., 1975) and sphingolipids; such data form a basis for the interpretation of carbon-13 NMR spectral information in terms of structural features.

Carbon-13 NMR is of great value in determining, in particular, the nature of metal binding sites (Behr & Lehn, 1973; Czarniecki & Thornton, 1977), the linkage pattern of oligosaccharides (Jennings & Smith, 1973), the conformation of the glycosidic bonds (Perlin et al., 1970), and solution-structural features like hydrogen bonding (Czarniecki & Thornton, 1976) and segmental mobility (Gent & Prestegard, 1977). To realize the maximum amount of information about G_{M1}, one must first completely assign the ^{13}C NMR spectrum. Although the assignments are the primary focus of this paper, some structural consequences of these assignments will be discussed. We were able to utilize the spectral perturbations produced by paramagnetic europium(III) to investigate those G_{M1} groups which participate in binding metal cations.

Ganglioside G_{M1} is an amphiphilic molecule which forms aqueous micelles having a mass of (250 ± 50) kdaltons (Yohe & Rosenberg, 1972) and containing roughly 160 molecules. Since the critical micelle concentration is only $(9 \pm 1) \times 10^{-5}$ M, the NMR experiments, which were run around 5×10^{-2} M, were done on G_{M1} samples predominantly in micellar form.

The ganglioside G_{M1} used in this study was prepared from a natural mixture of gangliosides by neuraminidase digestion, which removes sialic acids from polysialogangliosides G_T and G_D, but does not further degrade the monosialogangliosides G_{M1} and G_{M2}. The number of sialic acid residues on gangliosides correlates directly with the amount of 20-carbon sphingosine in the molecule (Yohe et al., 1976), so that our G_{M1} will have a sphingosine chain length formed from an average of the chain lengths of the tri-, di-, and monosialogangliosides. In addition, Yohe & Rosenberg found that a single double bond linked C_{41} and C_{42} in 97% of the molecules, with the rest existing as dihydrosphingosine. The results of Svennerholm (1964) show that the other lipophilic side chain in G_{M1} from bovine brain is predominantly (85%) octadecanoic acid, while the remaining 15% is divided among longer (20-, 22-, and 24-carbon) chains.

[1] Abbreviations used: NMR, nuclear magnetic resonance; G_{M1}, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; G_{M2}, *N*-acetylgalactosaminyl(*N*-acetylneuraminyl)-galactosylglucosylceramide; Me_4Si, tetramethylsilane; CPK, Corey-Pauling-Koltun.

Materials and Methods

Sources of Materials. Aldrich (Milwaukee, Wis.) supplied *N*-acetyl-D-neuraminic acid and *N*-acetyl-D-galactosamine as synthetic products, as well as ^2H_2O of 99.8% deuterium enrichment. D-Glucose, D-galactose, lactose, neuraminyllactose, ceramides, sphingomyelin, cerebrosides, and neuraminidase (*Clostridium perfringens,* type VI) were purchased from Sigma Chemical Co., St. Louis, Mo. Dihydroglucosylceramide and dihydrolactosylceramide were obtained from Miles Laboratories (Kankakee, Ill.). Anhydrous europium(III) chloride was supplied by Alfa Inorganics, Beverly, Mass. All other reagents used were of the highest purity available.

Preparation and Purification of Beef Brain Ganglioside G_{M1}. A sample, kindly provided by Dr. Herbert C. Yohe, of mixed beef brain gangliosides, isolated from gray matter according to Folch et al. (1951) and purified by silicic acid column chromatography (Svennerholm, 1972), was used as the starting material. The sample, about 900 mg, was dissolved in 100 mL of 0.1 M sodium acetate buffer, pH 5.0, containing 0.1% $CaCl_2 \cdot 2H_2O$. One unit of neuraminidase was added. The surface of the solution was layered with a small amount of toluene to retard bacterial growth. The mixture was then incubated at 37 °C for 2 days. During the incubation 3 more units of the enzyme were added at different intervals. At the end of the incubation, 3 mL of 0.5 M ethylenediaminetetraacetic acid (tetrasodium salt) was added, and the solution was dialyzed against cold water for 2 days. The retentate was then lyophilized to give a crude G_{M1} fraction. The sample was dissolved in 200 mL of chloroform:methanol:water (30:60:8, v/v) and applied to a column of DEAE-Sephadex (A-25, acetate form, 120 mL) packed in the same solvent (Ledeen et al., 1973). After elution with 1 column volume each of chloroform:methanol:water (30:60:8) and methanol, G_{M1} was eluted with 2.5 column volumes of 0.07 M sodium acetate in methanol (Ando & Yu, 1977). The solvent in this last fraction was evaporated and the residue dissolved in 50 mL of water and dialyzed. After lyophilization, about 250 mg of G_{M1} was recovered as a white powder. Silica gel thin-layer chromatographic analysis indicated that the purity of G_{M1} was over 99%, with the remaining 1% consisting of G_{M2}. This procedure resulted in the sodium salt of the sialic acid of G_{M1}.

Carbon-13 Nuclear Magnetic Resonance. Since direct comparison of a number of spectra from model compounds was essential to the success of this endeavor, a set of standard instrumental conditions was chosen, as far as practical, in an attempt to minimize the systematic rather than the actual differences among compounds. All compounds were run as solutions in ^2H_2O, except for the water-insoluble ceramide and glucosyl- and lactosylceramides, which were run in pyridine: C_6D_6 (1:1, v/v). These latter compounds showed obvious solvent-induced downfield shifts of about 1–2 ppm in the glycosyl portion of the molecule reminiscent of those seen for sialic acid in hexadeuteriodimethyl sulfoxide (Holmquist & Ostman, 1975). Chemical shifts were measured relative to the penultimate carbon of the acyl chain of lipid-containing species, to the exocyclic carbon of the saccharides, and to internal dioxane, which resonates 67.40 ppm downfield from external Me_4Si. The chemical shifts have been corrected so that they may be directly compared with those observed by other investigators using an external Me_4Si reference. Solution concentrations ranged from 25 to 100 mg/mL, depending on the amount of material available. Significant line broadening, accompanied by higher solution viscosity (Chupriyanova et al., 1976), was noted for ganglioside concentrations above 150 mg/mL. Spectra were obtained at both 20 and 67.88 MHz.

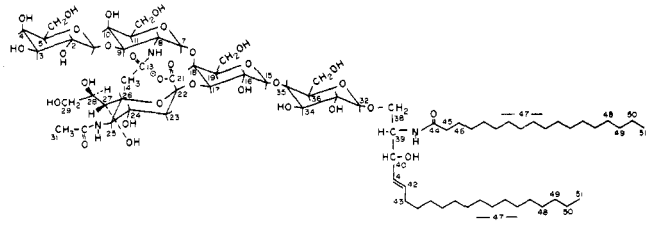

FIGURE 1: The structure of ganglioside G_{M1}. The numbering system treats separately each carbon species which gave rise to a distinct carbon-13 NMR signal.

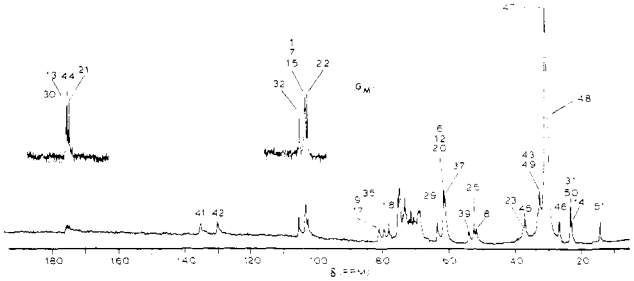

FIGURE 2: The proton noise decoupled carbon-13 NMR spectrum at 67.88 MHz of aqueous bovine brain ganglioside G_{M1} micelles (sodium salt, pH 7.1, 86 mM, 42 °C). The carbon numbers are given in Figure 1 and represent the assignments. The assignments for carbons resonating between 65 and 78 ppm are given in Figure 4. The spectrum below was recorded with a 0.5-s acquisition time, while the insets were recorded with an additional pulse delay of 6.4 s to reveal slowly relaxing quaternary carbons.

The 20-MHz spectra were taken with the aid of a Varian CFT 20 at a temperature of 32 °C, using a sweep width of 4 kHz. Spectra were accumulated using a 90° pulse with an acquisition time of 1.023 s under conditions of proton noise decoupling. The field was stabilized by locking on the solvent deuterium resonance. The 8-mm spinning sample tubes, containing approximately 1 mL of solution, were fitted with vortex plugs. Accumulation times of from 1 to 4 h were necessary to give adequate signal-to-noise ratios. A 1.0-s pulse delay was sometimes added to minimize saturation of slowly relaxing nonprotonated carbons, particularly the carbonyl, carboxyl, and anomeric carbons of sialic acid.

The 67.88-MHz spectra were obtained at the Southern New England High Field NMR facility, utilizing a Bruker HX270 superconducting spectrometer equipped with a Nicolet BNC12 computer, disk mass storage, and quadrature detection. The decoupling power of from 4 to 5 W raised the sample temperature from ambient 19 °C to an estimated 40 °C. A sweep width of 15 kHz was used, with an acquisition time of 0.54 s. Pulse delays of up to 6.4 s were introduced in order to minimize saturation of the slowly relaxing carbons. Excellent signal-to-noise ratios were obtained after the accumulation of from 3000 to 10 000 pulses, for approximately 1.5 mL of solution in 10-mm spinning sample tubes fitted with vortex plugs. Optimal line broadening was used to improve sensitivity. Samples were run in 1 mM ethylenediaminetetraacetic acid (disodium salt) at pH 7.1, to avoid shifts due to metal-ion impurities which may have been present in the ^2H_2O. Digital difference spectra were obtained with the aid of the spectrometer system software. For the paramagnetic metal-ion titrations of G_{M1}, europium(III) chloride (100 mM in ^2H_2O) was added to the NMR samples to give the concentrations indicated. The standard deviation of the G_{M1} chemical shifts from 10 separate determinations at 67.88 MHz was ±0.06 ppm for all peaks except for carbons C_{46} and C_{47}, which had statistical errors of ±0.14 ppm.

Results

The structure of ganglioside G_{M1} is shown in Figure 1, while the Fourier-transform ^{13}C NMR spectrum of aqueous G_{M1} micelles is shown in Figure 2. Most of the oligosaccharide resonances occur in the range from 60 to 105 ppm, while most of the ceramide carbons resonate between 10 and 56 ppm. The G_{M1} spectrum shows about 40 resonances from the approximately 50 carbon types which are expected to yield distinct peaks.

Oligosaccharide Assignments (C_1–C_{37}). The oligosaccharide portion of G_{M1} contains four saccharide species, which, because of their participation in glycosidic linkages, will be named and referred to as glycosides. They include β-D-galactopyranoside (C_1–C_6, C_{15}–C_{20}), β-D-glucopyranoside (C_{32}–C_{37}), 2-deoxy-2-acetamido-β-D-galactopyranoside (C_7–C_{12}), and sialic acid (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosidic acid) (C_{21}–C_{31}). Spectra of these four monosaccharides were compared with those in the literature, to gain some understanding of the range of variation of sets of chemical shifts of the same molecules reported by different laboratories. Constant spectral offsets of up to 1 ppm were found and compensated for, so that direct comparisons could be made among compounds.

When monosaccharides are linked into oligosaccharides, there are characteristic shifts of the carbons β and γ to the site of substitution in linkages. In particular, the carbon (C_β) directly involved in an intersaccharide linkage is shifted 5–10 ppm downfield with respect to its monomeric resonance position, while the adjacent carbons (C_γ) experience a small upfield shift of −0.7 ± 0.6 ppm (Colson & King, 1976). Shifts of carbon δ to linkages are insignificant, so that the carbons in the oligosaccharide portion of G_{M1} which are δ or ε to linkages can be assigned on the basis of a comparison with the monosaccharide spectra. These are carbons C_3–C_6 of β-D-galactopyranoside, C_{11}–C_{14} of 2-acetamido-2-deoxy-β-D-galactopyranoside, C_{24}–C_{31} of sialic acid, and the exocyclic carbons C_6, C_{12}, C_{20}, and C_{37}.

To assign the remaining carbons it was necessary to examine the extensive literature on di-, oligo-, and polysaccharides, as well as to obtain spectra from lactose, neuraminyllactose, and glucosyl- and lactosylceramides. A comparison of the chemical shifts of β-D-galactopyranose, α-sialic acid, sialic acid α-methyl glycoside, β-D-glucopyranose, neuraminyllactose, lactose, dihydroglucosylceramide, and dihydrolactosylceramide with those of the oligosaccharide portion of G_{M1} appears in Table I. The neuraminyllactose used in this study was a mixture of trisaccharides from bovine colostrum, with about 85% of the sialic acid linked (2 → 3) to the galactose ring of lactose and the remaining 15% linked (2 → 6). Significant (~20%) quantities of *N*-acetyl-D-glucosamine were found in the equilibrium mixture of synthetic *N*-acetyl-D-galactosamine. Our assignments in Table I agree with those in the literature, where previous ^{13}C NMR data exist. The data in Table I reflect our measurements of the chemical shifts, except for α-sialic acid (Jacques et al., 1977) and sialic acid α-methyl glycoside (Jennings & Bhattacharjee, 1977).

β-D-Galactopyranoside (C_1–C_6). The terminal β-D-galactopyranoside residue is necessary for the binding to cholera enterotoxin (Cuatrecasas, 1973), so that it presents an interesting and important assignment problem. The anomeric carbon C_1 is involved in a (1 → 3) link to 2-acetamido-2-deoxy-β-D-galactopyranoside, so that C_1 and C_2 should be shifted downfield and upfield respectively, while the rest of the

TABLE I: Carbon-13 Assignments for the Oligosaccharide Portion of Ganglioside G_{M1} and Related Model Compounds at pH 7.[a]

C no.	G_{M1}	I	II	III	IV	V	VI	VII	VIII	IX
1	103.37	97.29	103.31							
2	71.56	72.74	71.51							
3	73.39	73.65	73.21							
4	69.40	69.40	69.11							
5	75.63	75.96	75.61							
6	61.84	61.84	61.81							
7	103.37					96.14				
8	51.86					54.44				
9	81.33					71.84				
10	68.97					68.60				
11	75.63					75.83				
12	61.84					61.72				
13	175.68					175.16				
14	23.10					23.01				
15	103.37	97.29	103.31	103.21	104.65					
16	71.56	72.74	71.51	71.56	71.76					
17	81.33	73.65	73.21	79.16	74.13					
18	78.31	69.60	69.11	69.10	69.60					
19	75.17	75.96	75.61	75.56	75.95					
20	61.84	61.84	61.81	61.82	61.84					
21	174.76			174.31			174.6	174.77		
22	102.65			103.21			101.9	98.42		
23	37.78			40.31			41.3	41.49		
24	69.40			69.43			69.5	69.40		
25	52.56			52.72			53.2	53.07		
26	73.39			73.63			73.8	73.63		
27	68.97			68.96			69.5	69.40		
28	72.95			72.42			72.9	72.42		
29	63.72			63.89			63.9	64.10		
30	175.82			175.00			176.1	176.10		
31	23.10			22.61			23.3	23.25		
32	105.48		96.31	96.39	105.09				96.31	105.20
33	73.96		74.39	74.39	74.13				74.91	74.13
34	75.17		74.91	74.88	74.59				76.21	74.64
35	79.77		79.04	79.16	81.40				70.31	70.50
36	75.17		75.23	75.29	74.59				76.21	74.64
37	61.46		61.21	61.27	61.67				61.31	61.46

[a] Shifts in ppm; Me_4Si = 0.00 ppm. I, β-D-galactopyranose; II, lactose (Dorman & Roberts, 1971); III, neuraminyllactose; IV, lactosylceramide; V, 2-acetamido-2-deoxy-β-D-galactopyranose (Bundle et al., 1973); VI, α-methyl-D-sialic acid (Jennings & Bhattacharjee, 1977); VII, α-D-sialic acid (Jaques et al., 1977); VIII, β-D-glucopyranose (Walker et al., 1976); IX, β-D-glucosylceramide.

carbons, C_3–C_6, should resonate at their unlinked field position. The conventions adopted here are that positive glycosidic linkage shifts (Δ's) are downfield, with increasing chemical shifts. The glycosidic linkage shifts are summarized in Table II.

There are three peaks in the anomeric carbon region of the spectrum, from 100 to 106 ppm. No peaks appear at field positions between 90 and 100 ppm, where free anomeric carbons resonate, confirming that our G_{M1} sample was pure and contained no free monosaccharides or other hydrolysis products. One expects, then, that the three anomeric signals would correspond to the three sugar types; and the intensity ratios found (1:3:1) follow this expectation (Figure 1). The three anomeric carbons in β-D-galactopyranoside rings, C_1, C_7, and C_{15}, are then assigned to the peak at 103.37 ppm. The nitrogen bound to C_8 apparently has no influence on the chemical shift of C_7, so that it resonates at 103.37 ppm along with C_1 and C_{15}; in lactose, where the nonreducing saccharide is β-D-galactopyranose, we found C_1 to occur at 103.31 ppm (Table I), while Colson & King (1976) report a value of 103.3 ppm for the equivalent carbon in glycosidically linked 2-acetamido-2-deoxy-β-D-galactopyranose.

The β-D-galactopyranoside C_2 should be shifted about −1 ppm in G_{M1} with respect to β-D-galactopyranose (Dorman & Roberts, 1971). In lactose this carbon resonates at 71.51 ppm, which is −1.2 ppm from the β-D-galactopyranose signal. The G_{M1} resonance at 71.56 ppm is therefore assigned to C_2. The remaining terminal β-D-galactopyranoside carbons, C_3 to C_6, are assigned on the basis of the field position of the resonance in β-D-galactopyranose, and are reported in Table I. It should be noted that there are two peaks separated by 0.4 ppm in the G_{M1} spectrum near 61 ppm, the field position expected for the exocyclic sugar carbons C_6, C_{12}, C_{20}, and C_{37}. The presence of both galactosyl and glucosyl residues in G_{M1} leads to a natural assignment of the high- and low-field peaks to the respective exocyclic carbons, since in β-D-glucopyranose and β-D-galactopyranose these carbons resonate at 61.31 and 61.84 ppm, respectively, a separation of 0.5 ppm, in agreement with Bundle et al. (1973), while in G_{M1} these peaks appear at 61.46 and 61.84 ppm, respectively.

2-Acetamido-2-deoxy-β-D-galactopyranoside (C_7–C_{14}). The anomeric 2-acetamido-2-deoxy-β-D-galactopyranoside carbon, C_7, is assigned as above to the G_{M1} peak at 103.37 ppm. The deoxy carbon, C_8, appears at 54.44 ppm in 2-acetamido-2-deoxy-β-D-galactopyranose, and there is a signal in the G_{M1} spectrum at 54.14 ppm; before a final choice is made,

TABLE II: Glycosidic Linkage Shifts of Carbons from Ganglioside G_{M1} and Model Compounds at pH 7.

Carbon no.	Ganglioside shift[a]	Model shift	Reference
1	6.1	6.2 ± 0.2	Dorman & Roberts, 1970
2	−1.2	−1.0 ± 0.2	Colson & King, 1976
7	7.2	7.3 ± 0.2	Colson & King, 1976
8	−2.6	−0.5 ± 0.3	Egan et al., 1977
9	9.5	9.5 ± 0.4	Colson & King, 1976
10	0.4	−1.2 ± 0.2	Colson & King, 1976
15	6.1	6.2 ± 0.2	Colson & King, 1976
16	−1.2	−1.2 ± 0.2	Colson & King, 1976
17	7.8	9.5 ± 0.4	Colson & King, 1976
18	8.9	9.5 ± 0.4	Colson & King, 1976
19	−0.8	−1.0 ± 0.2	Colson & King, 1976
21	0.0	0.0 ± 0.3	Egan et al., 1977
22	4.2	4.2 ± 0.3	Jennings & Bhattacharjee, 1977
23	−2.1	−1.0 ± 0.3	Jennings & Bhattacharjee, 1977
32	9.1	9.0 ± 0.1	Table I
33	−0.9	−1.2 ± 0.2	Colson & King, 1976
34	−1.0	−1.2 ± 0.2	Colson & King, 1976
35	9.5	9.5 ± 0.4	Colson & King, 1976
36	−1.0	−1.2 ± 0.2	Colson & King, 1976
38	6.7	6.8 ± 0.2	Colson et al., 1974
39	−1.6	−1.4 ± 0.3	Egan et al., 1977

[a] Data from Table I. The standard deviation of the G_{M1} shifts is ±0.1 ppm.

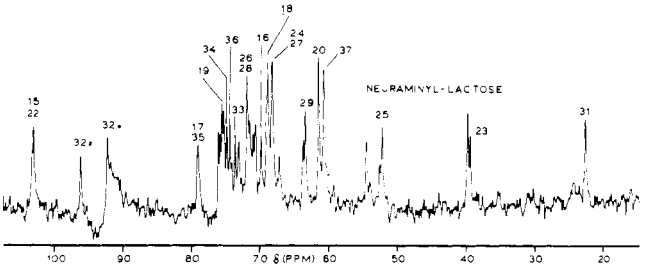

FIGURE 3: The proton noise decoupled carbon-13 NMR spectrum at 67.88 MHz of neuraminyllactose (pH 7.0, 26 mM, 40 °C) isolated from bovine colostrum bearing ~85% (2 → 3) linkages and ~15% (2 → 6) linkages. The numbering system corresponds to the ganglioside numbering in Figure 1. Only the peaks corresponding to (2 → 3) linkages are assigned. A substantially unassigned spectrum has been previously reported by Eschenfelder et al. (1975).

however, there are two shielding effects to take into consideration, since this position is unique in that it is adjacent to two linked carbons, C_7 and C_9.

For acetamido containing disaccharides one expects to see shielding of −1 to −2 ppm, due to the (1 → 4) linkage at C_7. The linkage at C_9 will also contribute an upfield shift, but these shifts are steric in origin, so they should not be strictly additive (Colson & King, 1976). The G_{M1} resonance at 51.86 ppm is therefore assigned to C_8. Carbon C_9 of G_{M1} is involved in a link to C_1 of the terminal β-D-galactopyranoside; it is thus expected to shift downfield by 9–10 ppm and is assigned to the peak in the G_{M1} spectrum at 81.33 ppm. The other two G_{M1} resonances in this region, at 78.32 ppm and 79.77 ppm, would give abnormal linkage shifts if they were assigned to C_9.

Carbon C_{10} is shifted from 68.60 ppm in 2-acetamido-2-deoxy-β-D-galactopyranose to 68.97 ppm in G_{M1}, an abnormal positive 0.4 ppm shift for C_{10} due to the linkage at C_9. Carbons C_{11} and C_{12} appear at the same field positions, 75.63 ppm and 61.84 ppm, respectively, where they are found in 2-acetamido-2-deoxy-β-D-galactopyranose, i.e., 75.83 ppm and 61.72 ppm. The acetyl carbons C_{13} and C_{14} resonate at 175.31 ppm and 23.10 ppm, respectively. The two peaks in the neighborhood of 23 ppm are due to acetyl methyls such as C_{14}, as well as the ceramide alkyl chain penultimate carbons C_{50}. Comparison of spectra taken with pulse delays of 0.0 and 6.4 s reveals that the peak at 23.10 ppm relaxes more slowly than that at 23.45 ppm. The more mobile acetyl methyls, C_{14} and C_{31}, would be expected to relax more slowly than those methylene carbons, such as C_{50}, bound into the long alkyl chains of the ceramide. Consequently, C_{14} is assigned to the peak in G_{M1} at 23.10 ppm.

β-D-Galactopyranoside (C_{15}–C_{20}). In the ganglioside molecule, this galactosyl residue forms the junction of the two hydrophilic arms consisting of, on the one hand, the disaccharide composed of the terminal β-D-galactopyranoside and 2-acetamido-2-deoxy-β-D-galactopyranoside and, on the other, sialic acid. Since three of its six carbons participate in linkages, only the exocyclic carbon C_{20} will not be shifted in the G_{M1} spectrum, as compared with β-D-galactopyranose. The shifts in this galactopyranosyl residue to be anticipated on complex oligosaccharide formation like that found in G_{M1} have not previously been reported. Fortunately, we have been able to predict these shifts on the basis of previous structural studies of oligosaccharides and then to test our predictions to a large extent on the trisaccharide neuraminyllactose (Figure 3), which constitutes one arm of G_{M1}. This trisaccharide provides a good model for assigning the remaining oligosaccharide carbons except C_{18}, C_{19}, C_{32}, and C_{33}.

The galactopyranoside anomeric carbon C_{15} resonates at 103.21 ppm in neuraminyllactose and is therefore assigned to the G_{M1} peak at 103.37 ppm, along with the other galactopyranosidic anomeric carbons. Carbon C_{16} again presents an unusual assignment problem in that, like C_8, it is between two carbons which participate in linkages and might therefore experience shifts from both links; in neuraminyllactose, however, this carbon resonates at 71.56 ppm, which is the same as in lactose and −1.2 ppm from its position in β-D-galactopyranose.

Carbons C_{17} and C_{18} are unique in that both are linked to anomeric carbons, as well as being α to linked carbons. Carbon C_{17} participates in a link to C_{22} of sialic acid, and appears in G_{M1} at 81.33 ppm along with C_9, another galactopyranosidic residue. Carbon C_{17} resonates at 79.16 ppm in neuraminyllactose. The discrepancy of 2.2 ppm may be due to steric effects from the second anomeric site at C_{18}. Carbon C_{18} is linked to C_7 of 2-acetamido-2-deoxy-β-D-galactopyranoside and appears at 78.32 ppm. Carbon C_{19} resonates at 75.17 ppm in G_{M1}. Carbon C_{20} is unshifted at 61.84 ppm.

5-Acetamido-3,5-dideoxy-D-glycero-D-galacto-nonulopyranosidic Acid (C_{21}–C_{31}). The linkage of the anomeric carbon C_{22} of sialic acid to C_{17} of G_{M1} can be expected to shift the carboxyl carbon C_{21} of the sialic acid by −1 to −2 ppm; thus the carboxyl carbon C_{21} resonates at the highest field of the four carbonyl carbons in G_{M1} and may be assigned to the peak at 174.76 ppm. This carbon is the only free carboxyl carbon in the ganglioside G_{M1} molecule; all the rest are amide carbons, so that C_{21} is expected to have a unique chemical shift. This assignment was confirmed by the use of europium(III) chloride as a shift reagent. Since the only ionizable group in the G_{M1} molecule is the sialic acid carboxyl C_{21}, this anionic site must be involved in the interaction with cations. This carbon should then experience the largest shift upon the addition of a paramagnetic cation. The G_{M1} peak at 174.76 ppm

TABLE III: Assignments of the Ceramide Carbons of Ganglioside G_{M1} and Related Model Compounds

Carbon no.	δ^a (ppm)	Glucosyl-ceramide	Lactosyl-ceramide	Ceramide	Cerebro-sides	Sphingo-myelin	Egg lecithin[b]
38	68.97	70.96	70.45	62.25	69.12	65.31	
39	54.14	55.33	55.13	55.71	54.03	54.85	
40	72.12	72.19	72.61	72.60	71.99	71.85	
41	135.22			133.07	134.97	135.08	
42	130.04			131.70	129.47	130.02	130.19
43	32.91			32.51	32.65	32.61	28.41
44	175.31	173.62	173.61	175.89	176.70	175.31	175.17
45	37.16	35.21	35.32	35.82	35.16	37.30	35.33
46	27.03	26.73	26.73	26.09	26.82	26.74	26.68
47	31.18	31.25	31.15	31.13	31.02	31.15	30.95
48	30.81	30.85	30.78	30.76	30.65	30.76	
49	32.91	32.12	32.53	33.02	32.95	33.14	33.13
50	23.45	23.33	23.28	23.23	23.37	23.27	23.82
51	14.49	14.66	14.75	14.68	14.49	14.63	15.05

[a] With respect to Me_4Si at 0.00 ppm. [b] Assignments of Shapiro et al. (1975).

undergoes the largest Eu^{3+}-induced shift, thus confirming its assignment to C_{21}.

The anomeric carbon of sialic acid, C_{22}, is nonprotonated, so that it will have a longer spin–lattice relaxation time than the other anomeric carbons in G_{M1}. The spin–lattice relaxation behavior of aqueous ganglioside G_{M1} micelles will be reported separately (Sillerud et al., in preparation); it was clear, however, from spectra taken with pulse delays of 0 and 6.4 s, that the highest field anomeric peak in the G_{M1} spectrum at 102.65 ppm relaxed much more slowly than the other anomeric signals. Carbon C_{22} may therefore be assigned to this peak. It is well known that axial anomeric carbons resonate at a higher field than equatorial carbons (Colson et al., 1974). An axial carbon linkage for sialic acid has not been observed (Ledeen & Yu, 1976), and model building convinces one that such a linkage for sialic acid in G_{M1} is impossible. The high field position of C_{22} is consistent with its being the only α-linked anomeric carbon in G_{M1}.

Carbon C_{23} of monomeric sialic acid has no directly bound hydroxyl, giving it a unique chemical shift of 41.49 ppm. Of the two G_{M1} peaks in this region, both lie at higher field positions (37.16 and 37.78 ppm), so that this carbon must experience an upfield shift of 1.7 to 2.3 ppm, possibly due to the involvement of C_{22} in linkage formation, or to steric effects at this crowded junction, perhaps from the hydroxyl at C_{16}. The remaining carbons in sialic acid, C_{24} through C_{31}, appear at field positions in the G_{M1} spectrum characteristic of the sodium salt of the α-methyl glycoside (Jennings & Bhattacharjee, 1977), and their assignments are reported in Table I, along with the relevant neuraminyllactose resonances.

β-D-Glucopyranoside (C_{32}–C_{37}). The anomeric carbon C_{32} of the single β-D-glucopyranosidic residue in G_{M1} participates in the linkage which joins the oligosaccharide moiety to the ceramide portion of the molecule. The assignment of C_{32} to the peak at 105.48 ppm is facilitated by an examination of the spectrum of glucosylceramide, in which only this carbon resonance appears in the spectral region characteristic of linked anomeric carbons at 105.20 ppm. The β-D-galactopyranosidic carbon C_{15} appears at 104.60 ppm in lactosylceramide, while C_{32} resonates again at 105.09 ppm, confirming the assignments of all the anomeric carbons in the oligosaccharide.

The linkage of C_{32} results in a −0.9-ppm upfield shift of C_{33} from 74.91 ppm in β-D-glucopyranose to 73.96 ppm in G_{M1}. Carbon C_{34} is similarly shifted upfield from 76.21 to 75.17 ppm as a result of the linkage of C_{35} to C_{15}. Carbon C_{35} is unique in G_{M1} as a linked carbon, by virtue of its nature as a β-D-glucopyranosyl rather than β-D-galactopyranosyl carbon. Carbon C_{35} is equatorial rather than axial like the β-D-galactopyranosyl carbon in the equivalent ring position. Carbon C_{35} appears at 79.77 ppm in G_{M1}, a shift of 9.5 ppm from its position at 70.31 ppm in β-D-glucopyranose. Carbon C_{36} is shifted from 76.21 ppm to 75.17 ppm, due to the involvement of the adjacent C_{35} in a link with C_{15}. The final carbon in the oligosaccharide of G_{M1} is the exocyclic β-D-glucopyranosyl C_{37}, which resonates at the unique field position of 61.46 ppm, characteristic of β-D-glucopyranose and upfield from the β-D-galactopyranosyl exocyclic carbons C_6, C_{12}, and C_{20} at 61.84 ppm. Two resonances appear at 61.67 ppm and 61.84 ppm, respectively, in lactosylceramide, but only one at the lower field position in glucosylceramide.

The Ceramide Moiety of G_{M1} (C_{38}–C_{51}). The ^{13}C NMR assignments of a natural mixture of ceramides can be found in Table III. Once again, the large range of chemical shifts inherent in ^{13}C NMR spectroscopy provides a number of well-resolved resonances even for this complex molecule, which constitutes the hydrophobic portion of ganglioside molecules. Ceramide consists of the long-chain alkyl amino alcohol, sphingosine, amide-linked to a fatty acid. Most of the sphingosine has either 18 or 20 carbons, while the predominant fatty acid is octadecanoic acid. Sphingosine contains one trans double bond as well as two free hydroxyl groups.

It is to the first of the hydroxylated carbons, C_{38}, that we now turn our attention. Like those nonanomeric oligosaccharide carbons which are involved in linkages to other sugars, C_{38} will be subject to a shift of about 7 ppm when the spectrum of G_{M1} is compared with that of the free ceramide. The chemical shift of C_{38} in free ceramide is 62.25 ppm, a shift reminiscent of a hexopyranose exocyclic hydroxymethyl carbon, exemplified by C_6. In a (1 → 6)-linked disaccharide, this carbon shifts 6.8 ppm (Colson et al., 1974), so that C_{38} should resonate near 69 ppm in G_{M1}. The G_{M1} peak at 69.40 ppm is clearly C_4, while the peak at 70.74 ppm is C_{24}. Carbon C_{38} is assigned to the G_{M1} peak at 68.97 ppm. This assignment is strengthened by the appearance of C_{38} at 69.12 ppm in cerebrosides.

The second ceramide backbone carbon, C_{39}, resonates at a field position characteristic of a carbon adjacent to a nitrogen (54.14 ppm) and explains the third of the three peaks in this region in the G_{M1} spectrum, the other two arising from oligosaccharide carbons C_8 and C_{25}. There is a small upfield shift of −1.6 ppm of C_{39}, which is due to the linkage of C_{38} in G_{M1}.

Ceramide carbon C_{40} can be assigned to the G_{M1} peak at 72.12 ppm, on the basis of an examination of the spectra of ceramide (72.60 ppm), glucosylceramide (72.19 ppm), and lactosylceramide (72.61 ppm). The two trans double-bonded carbons C_{41} and C_{42} are separated by 5.2 ppm, as a result of the hydroxyl group on the adjacent C_{40}, which polarizes the double bond. It is clear from spectra of hydroxyalkenes (Stothers, 1972) that the carbon (C_{41}) nearest the hydroxyl will be the farthest downfield (135.22 ppm), and that C_{42} will appear upfield. The carbon, C_{43}, adjacent to the double bond resonates at 32.91 ppm in G_{M1}, while it is found at 32.5 ± 0.1 ppm in Table III for the other four sphingolipids. The fatty acid carbonyl C_{44} resonates at 175.31 ppm, while the adjacent C_{45} is found at 37.16 ppm, close to 37.78 ppm of C_{23}. In some spectra of G_{M1} these two peaks are more clearly resolved than in Figure 1. In Table III, carbon C_{46} is seen in G_{M1} at 27.03 ± 0.14 ppm (see Materials and Methods), and in the other sphingolipids at 26.63 ± 0.27 ppm.

The remaining carbon classes in the ceramide alkyl chains have unique chemical shifts resembling those seen in phospholipids like lecithin and sphingomyelin. The methylene chain carbons C_{47} contribute an intense broad envelope at 31.18 ± 0.14 ppm, and the chain terminal carbons C_{49}, C_{50}, and C_{51} resonate in the sequence 32.91, 23.45, and 14.49 ppm, respectively. Finally, in the spectra of the sphingolipids there appears a peak or shoulder on the upfield side of the main methylene (C_{47}) peak. Although this peak is more sharply resolved in some spectra than in others, it is clearly present in all of them. It is assigned to C_{48}, the third carbon from the terminal methyl of the alkyl chain (Batchelor et al., 1974), with a chemical shift 0.37 ppm upfield from the methylene (C_{47}) peak at 31.18 ppm.

Europium Titration of G_{M1} ^{13}C Spectra. In order to determine the details of the interaction of G_{M1} with metal ions, the influence of paramagnetic europium on the ^{13}C NMR spectrum of G_{M1} was examined. The resonances altered include those shown in Figure 4. The effect of the europium was to shift certain resonances and broaden others. Many resonances assigned to the sialic acid portion of G_{M1} were altered. A comparison between the effect of europium on the sialic acid carbons of G_{M1} and its effect on the spectrum of monomeric sialic acid is given in Table IV. Computer-generated difference spectra revealed that there are additional resonances altered by europium, including C_2, C_3, C_{11}, C_{12}, C_{13}, and C_{14}, which are assigned to oligosaccharide carbons other than those in sialic acid.

TABLE IV: Effects of Europium(III) on the ^{13}C NMR Spectra of Ganglioside G_{M1} and β-D-Sialic Acid.

Carbon no.	Effects[a] on G_{M1} spectrum		Effects[a] on β-D-sialic acid spectrum	
2	0.3	B		
3	0.9	S		
11	1.0	S		
12	0	B		
13	*b*	B, S		
14	1.1	B, S		
21	*b*	B, S	8.4	S
22	1.0	B, S	6.3	S
23	0.3	B	−0.4	B
24	0.6	B, S	0.3	N
25	1.1	B, S	0.6	B
26	0.9	B, S	1.1	B, S
27	0.4	B	4.4	S
28	0.4	B	0.8	S
29	0	N	−0.9	B, S
30	*b*	B	−0.4	N
31	1.1	B, S	0.1	N

[a] Values are europium-induced shifts in ppm. B denotes broadening, S denotes significant shifting, relative to the strongest shift observed for the compound (1.1 ppm for the ganglioside, 8.4 ppm for the sialic acid), and N denotes no change in the spectrum. An aliquot of a 100 mM europium chloride stock solution was added to 86 mM ganglioside or sialic acid to give a final europium concentration of 35 mM at pH 7.0. The ganglioside carbon resonances influenced by europium ion were determined from computer-generated difference spectra. [b] Carbons 13, 21, and 30 were the most sensitive of the ganglioside carbons to europium ion, shifting 1.1 ppm at 5 mM and broadening beyond detection at 35 mM europium chloride.

Discussion

The natural-abundance ^{13}C NMR spectrum of the sodium salt of ganglioside G_{M1} has been obtained from a solution of aqueous micelles. The assignment of the spectrum made extensive use of model saccharides and lipids. Several unique features of the assignments arising from the branched and N-acetylated nature of the oligosaccharide and from the linkage to the free hydroxyl of ceramide have been interpreted in terms of linkage shifts at the β and γ carbon positions adjacent to the linkage. A crucial factor in the assignment of about half of the oligosaccharide carbons was the ability to build up the pattern of linkage shifts from an examination of the spectra of related but simpler oligosaccharides. As a result, assignments are reported for several saccharides and sphingolipids of biological interest that have not previously been communicated in the literature, including neuraminyllactose, ceramide, cerebroside, sphingomyelin, and dihydroglucosyl-, and dihydrolactosylceramides.

These assignments were greatly aided by obtaining ^{13}C spectra at high field (67.88 MHz), where a number of saccharide resonances were sufficiently resolved to remove ambiguity in resonance chemical shifts. High-field ^{13}C NMR was ideally suited to an investigation of this nature. Spectra of the more complex model compounds taken at lower field (20 MHz) lacked adequate resolution necessary for a satisfactory assignment of every carbon. Furthermore, the much greater sensitivity of the high-field instrument enabled spectra to be recorded from small amounts (~25 mg) of rare or expensive compounds in only 1 to 2 h of accumulation. This paved the way for further studies of the relaxation behavior of several of these compounds.

The assignment process was iterated four times, with an eye to minimizing the difference between a ganglioside resonance and its chemical shift in one or several of the model compounds, unless there was an obvious reason, such as a linkage, for the discrepancy. The preponderance of unshifted peaks was considered assigned in this fashion when the difference fell below the difference (0.4 ppm) between the closest resolved peaks in the G_{M1} spectrum. Small steric or conformation shifts of this order can be found in spectra from many series of related compounds. A good example is given by the C_6 carbons of the (1 → 2)-, (1 → 3)-, and (1 → 4)-linked β-D-glucopyranosyl-L-rhamnopyranose disaccharides, which vary from 17.9 ppm in the (1 → 2)-linked to 18.2 ppm in the (1 → 4)-linked species (Colson & King, 1976). The elucidation of these small effects in terms of the conformation of the oligosaccharide of G_{M1} is both interesting and important, but outside the scope of the present discussion.

Those oligosaccharide shifts arising from linkages are, however, a central portion of this investigation and, conse-

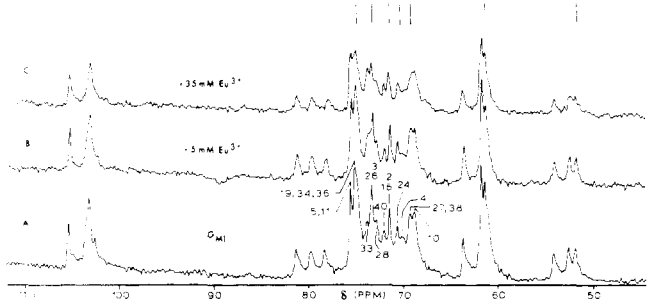

FIGURE 4: The proton noise decoupled ^{13}C NMR spectrum at 67.88 MHz of ganglioside G_{M1} (86 mM, 42 °C) expanded to emphasize the oligosaccharide region. (A) The spectrum in the absence of added paramagnetic ions; the numbers are the assignments corresponding to carbons in Figure 1. (B) As in A, but in the presence of 5 mM $EuCl_3$, pH 7.1. (C) As in A, but with 35 mM $EuCl_3$, pH 7.0. Those peaks altered by Eu^{3+} are indicated at the top of the figure.

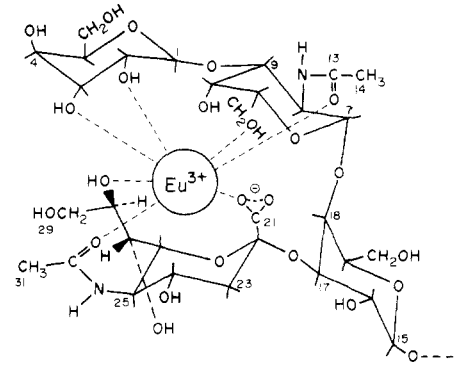

FIGURE 5: Proposed structure of the metal cation-binding site on G_{M1} based on the spectral perturbations produced by paramagnetic europium in the ^{13}C NMR spectrum. The 2-acetamido-2-deoxy-β-D-galactopyranoside ring is flipped over to account for the broadening of C12 and C13 simultaneously. The β-D-galactopyranoside (C1–C6) and 2-acetamido-2-deoxy-β-D-galactopyranoside rings are in a favorable position, along with an oxygen-rich surface, which competes with solvent water for the coordination sphere of the cation. Many more saccharide ligands are available for complexation with the cation in G_{M1} than is the case for monomeric sialic acid. It is felt that this may account for the greater affinity for cations displayed by G_{M1} as compared with monomeric sialic acid.

quently, will be treated further. The linkage shifts are dominated by the changes in electron density at the nucleus of interest resulting from the covalent bonding of the monomeric sugars. The sign and magnitude of the shifts are determined by the carbon type (i.e., anomeric, exocyclic, or cyclic) in the monomer, and by proximity to, and steric effects from, axial or equatorial substituents. Investigations of disaccharides have revealed general patterns for the linkage shifts, which can be compared with the results for the oligosaccharide portion of G_{M1}, in order to strengthen the basis for the ganglioside assignments and to extend the results for disaccharides to complex oligosaccharides. The shifts for the carbons β to linkages are the largest, and are characteristic of the type of linked carbon. Pyranose ring carbons experience larger downfield shifts upon linkage than the anomeric carbons on the juxtaposed saccharide, because of the reduction in electron density from the electron-withdrawing anomeric oxygen. The shifts found for the cyclic carbons C_9, C_{17}, C_{18}, and C_{35} average 8.9 ± 0.8 ppm, a value which is in substantial agreement with that of 9.3 ± 0.7 ppm found for a variety of hexopyranose disaccharides (Colson & King, 1976). A smaller shift of 7.0 ± 1.3 ppm is in evidence for the anomeric carbons C_1, C_7, C_{15}, C_{22}, and C_{32} in G_{M1}. This, too, compares well with the value of 7.5 ± 0.5 ppm reported by Colson & King (1976). The linkage shift for C_1 is 6.1 ppm in G_{M1}, which is the same shift found in lactose (6.0 ppm), even though the latter is (1 → 4)-linked, while the terminal β-D-galactopyranoside in G_{M1} is (1 → 3)-linked.

The γ linkage shifts in G_{M1} average −1.1 ± 0.3 ppm (Table II), except for C_8 and C_{23}, which have about twice that value, and C_{21}, which shows no shift at all. Steric effects may be responsible for the relatively large shifts of C_8 and C_{23} compared with those for the other γ carbons. The remaining shifts in Table II are well supported by the model compounds, with the prominent exceptions of C_{17} and C_{18}. These carbons display substantially smaller shifts than the expected 9.5 ppm, most likely as a result of their presence at the crowded junction of the two arms of the oligosaccharide. No suitable model could be found for C_{32}, but there is no doubt about the large value of its linkage shift, since the spectra of glucosyl- and lactosylceramides show the same linkage shift as seen in G_{M1}.

Metal Cation-Binding Site on G_{M1}. The binding of calcium and magnesium by gangliosides has been well established (Abramson et al., 1972; Behr & Lehn, 1973), but those groups on the molecule which directly interact with these metal ions have not yet been determined. The sialic acid, with its negative charge at physiological pH, must play an important role, since it has been shown to bind cations. With the proper choice of paramagnetic cation, ^{13}C NMR can provide precise information about those ganglioside atoms which occupy the binding site and coordinate with the cation. The affinities of G_{M1} for various cations, such as calcium and magnesium, are around 1.5×10^3 M^{-1} (Behr & Lehn, 1973). The atoms involved in calcium binding by β-sialic acid have been investigated by Czarniecki & Thornton (1976) and Jaques et al. (1977). However, the naturally occurring isomer in G_{M1} is α-sialic acid, so that a different shift pattern should emerge from that found for a solution of sialic acid, which at equilibrium is about 92% β anomer. This strong predominance of the β anomer is accentuated upon the addition of calcium, since the affinity of β-sialic acid is 99 ± 31 M^{-1} while that of the α anomer is less than 2.5 M^{-1} (Behr & Lehn, 1973; Jaques et al., 1977), so that the equilibrium is shifted to an almost total preponderance of the β anomer. Consequently, in an effort to understand the metal-binding properties of gangliosides, a number of laboratories have investigated the binding of cations to β-sialic acid. Cation binding to the natural α anomer is more relevant biologically, but because of the instability of the α anomer in solution, only binding to the α-methyl glycoside has been studied.

The α-methyl glycoside of sialic acid binds calcium poorly (K_A = 2.5 M^{-1}, Jaques et al., 1977) so that gangliosides would be poor calcium ligands if the sialic acid were the only available coordination moiety. In spite of this, the affinity of G_{M1} for calcium is even larger than that found for β-sialic acid. It seemed likely that ^{13}C NMR could provide some understanding of the nature of this discrepancy. The addition of successive amounts of $EuCl_3$ to the G_{M1} solution resulted in spectra with two types of alterations: downfield shifts of *N*-acetyl carbons and broadening of hydroxylated carbon resonances (Table IV and Figure 4). The influence of Eu^{3+} on β-sialic acid resembles its influence on G_{M1} (Table IV). One of the primary differences, however, lies in the opposite effect of Eu^{3+} on C_{23} and C_{24} in G_{M1} and β-sialic acid. The Eu^{3+} ion is much closer to C_{24} than C_{23} in G_{M1} (Figure 5), while just the reverse is true in β-sialic acid. In sialic acid the carboxylate anion, the ring oxygen, and the glyceryl side chain participate in Eu^{3+} binding.

An examination of the changes in the spectrum of G_{M1} on Eu^{3+} binding reveals that in the α anomer of sialic acid the carboxylate carbon C_{21} is once again involved. In addition, the anomeric carbon C_{22} is shifted downfield. Resonances from carbons C_{24}, C_{25}, C_{27}, and C_{28} also broaden, so that the picture of the Eu^{3+} binding site which emerges is one involving the negative carboxylate and the glyceryl side chain, reminiscent of the complex of Eu^{3+} with free sialic acid, but not involving the ring oxygen (Figure 5). On the basis of an examination of CPK models of G_{M1} we believe that, while the resonances from C_{27} and C_{28} are broadened by Eu^{3+} indicating that both carbon nuclei are close to the cation, the conformational restrictions on the glyceryl chain of sialic acid allow the hydroxyls on either C_{27} or C_{28}, but not both, to coordinate with Eu^{3+}. The pattern of shifts seen in β-sialic acid is consistent with intimate involvement of the hydroxyl on C_{27} in cation binding. In G_{M1}, with its α-linked sialic acid, C_{28} is in a more favorable position for coordination with the europium ion.

There are more resonances broadened by Eu^{3+} than reside solely in the sialic acid regions; these include C_2, C_3, C_{11}, and C_{12}. Carbons C_{13} and C_{14} are shifted. The involvement of these additional groups in binding cations is felt to provide a logical explanation for the fact that G_{M1} has a higher affinity for cations than have monomeric sialic acids. Those carbons shifting at 5 mM Eu^{3+} are limited to C_{13} and C_{21}, while at 35 mM Eu^{3+}, C_{30} shifts as well, indicating that C_{30} is less tightly associated with the cation. Further examination of a CPK model of G_{M1} reveals that C_{13} and C_{21} are within Van der Waals contact distance of about 0.28 nm of each other, while C_{30}, at 0.55 nm, is about twice as far from C_{21} as is the C_{13} carbonyl oxygen. The differences in shifts among the oligosaccharide carbonyls are therefore in accord with distances found from CPK models.

Influences on other G_{M1} carbons, including carbons C_2 and C_3 on the terminal β-D-galactopyranoside, and carbons C_{11} and C_{12} on 2-acetamido-2-deoxy-β-D-galactopyranoside, also appear only when the concentration of shift reagent is raised to 35 mM. Examination of CPK models again reveals a rationale for these spectral changes in terms of coordination of oligosaccharide oxygens with Eu^{3+}. The binding site is revealed as an oxygen-rich coordination surface on the ganglioside head group. The most stable and common lanthanide complexes are those with chelating oxygen ligands (Cotton & Wilkinson, 1972) with coordination numbers from 7 to 9. The hydroxyls on β-D-galactopyranoside carbons C_2 and C_3, as well as on C_{28} of the glyceryl side chain of sialic acid and C_{12} in 2-acetamido-2-deoxy-β-D-galactopyranoside, are in a favorable position in G_{M1} to substitute for water ligands in the Eu^{3+} hydration sphere.

The terminal β-D-galactopyranoside extends out from the rest of the molecule, in a favorable position for its oxidation at C_6 by galactose oxidase. Such a configuration removes C_1 and C_4–C_6 from influence by Eu^{3+}. The neuraminidase-resistant C_{22} to C_{17} bond is buried at the junction of sialic acid with the remaining sugars of the oligosaccharide. One should also note the specificity of the paramagnetic effect of Eu^{3+}, which is evident in the lack of alteration of resonances assigned to C_{15}–C_{20} and C_{32}–C_{51}.

In order for both the glyceryl side chain of sialic acid and C_{12} and C_{13} of 2-acetamido-2-deoxy-β-D-galactopyranoside to contribute oxygen ligands, the latter ring must be flipped 180° around the C_1–C_9 and C_7–C_{18} bonds. The hydrogen bonds which could be formed in this conformation of the oligosaccharide include C_6-OH to C_{13}-carbonyl, and C_{12}-OH to C_{19}-OH. An internal hydrogen bond in sialic acid from C_{27} to NH may be successfully competed for by the binding of a cation to C_{27}, with the result of a conformational change in the glyceryl side chain of sialic acid. Such a conformational change on calcium binding has been invoked to explain the change in binding stoichiometry and affinity of G_{M1} as the cation concentration increases (Behr & Lehn, 1973; Abramson et al., 1972). The proposed change in the sialic acid hydrogen-bonding pattern is consistent with such a conformational change. In summary, the cation binding site is visualized as an oxygen-rich surface presented by the oligosaccharide portion of G_{M1}, involving not only the sialic acid but also 2-acetamido-2-deoxy-β-D-galactopyranoside and the terminal β-D-galactopyranoside, which substitutes effectively for the hydration sphere of the cation. This presentation of additional oxygen ligands by G_{M1} may account for the larger affinity shown by G_{M1} for cations than that of β-sialic acid.

Note Added in Proof

While this manuscript was in press we learned from Dr. Phil Pfeffer that the assignments given by Dorman & Roberts (1971) for lactose carbons C_3 and C_5 (G_{M1} carbons C_{34} and C_{36}) must be reversed. This reassignment was based on the observation of a much larger hydrogen/deuterium isotope shift for lactose C_3 than for C_5. We have corrected the Table I entries for lactose and neuraminyllactose to reflect this fact.

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Mechanism of the Stereospecific Irreversible Inhibition of Bacterial Glutamic Acid Decarboxylase by (*R*)-(−)-4-Aminohex-5-ynoic Acid, an Analogue of 4-Aminobutyric Acid†

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ABSTRACT: 4-Aminohex-5-ynoic acid inhibits bacterial glutamic acid decarboxylase in a time-dependent irreversible manner. The inhibition is stereospecific and requires the abstraction of the propargylic hydrogen from 4(*R*)-(−)-4-aminohex-5-ynoic acid. This leads to the generation of a reactive alkylating agent in the active site which can react with a nucleophilic residue. At complete inhibition, there is incorporation of one molecule of inhibitor per pyridoxal binding site. If the decarboxylation of glutamate occurs with retention of configuration, the irreversible inhibition of this enzyme by the 4-(*R*) isomer can be rationalized on the basis of reversibility of the protonation step in the normal catalytic mechanism.

We reported previously that (±)-4-aminohex-5-ynoic acid is a suicide enzyme inactivator of GABA-aminotransferase from *Ps. fluorescens* (Jung and Metcalf, 1975) and that this compound decreases brain GABA-aminotransferase activity when administered to rats or mice by a systemic route (Jung et al., 1977). During the in vivo investigations, it was found that there is also a decrease in brain glutamate decarboxylase activity, and the mechanism of the action of the GABA-aminotransferase inhibitor on glutamate decarboxylase from mammalian brain and of *Escherichia coli* was investigated. The present work discloses that this GABA analogue is a catalytic irreversible inhibitor of bacterial glutamate decarboxylase and that the inhibition requires the abstraction of the hydrogen α to the acetylenic group (propargylic hydrogen) from 4(*R*)-4-aminohex-5-ynoic acid. The proposed mechanism of inactivation is based on the reversibility of the protonation step during the normal decarboxylation of L-glutamic acid to GABA and should apply to the inhibition of other α-amino acid decarboxylases.

Materials and Methods

Chemicals. L-Glutamate and (±)-2-methylglutamate were purchased from Sigma Chemical Co. (St. Louis, Mo.). Pyridoxal phosphate, dithiothreitol, and ruthenium dioxide were obtained from Merck (Darmstadt). [1-^{14}C]-DL-Glutamic acid (sp. act. 50 Ci/mol) was bought from New England Nuclear.

Enzyme. Type II glutamate decarboxylase of *E. coli* was obtained from Sigma (sp. act. 2.5 units/mL). This preparation was used as such or in a purer form readily obtained by heating a solution (10 mg/mL) of the commercial enzyme in 0.2 M pyridine hydrochloride, pH 4.5, at 37 °C for 1 h; centrifugation and dialysis at room temperature against 0.1 M pyridine hy-

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[1] Abbreviations used are: GABA-aminotransferase, 4-aminobutyric acid-2-ketoglutarate aminotransferase (EC 2.6.1.19); glutamate decarboxylase, L-glutamic acid carboxylyase (EC 4.1.1.15); GABA, 4-aminobutyric acid; Py CH, the phosphorylated pyridoxilidene moiety; Me_3Si, trimethylsilyl.

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