C. (1979) *FEBS Lett. 105*, 58–62.
Stechschulte, D. J., & Austen, K. F. (1974) *J. Immunol. 112*, 970–978.
Sugiyama, K., Sasaki, J., & Yamasaki, H. (1975) *Jpn. J. Pharmacol. 25*, 485–487.
Sullivan, T. J., Greene, W. C., & Parker, C. W. (1975) *J. Immunol. 115*, 278–282.
Vogt, W. (1978) *Adv. Prostaglandin Thromboxane Res. 3*, 89–95.
Volwerk, J. J., Pieterson, W. A., & de Haas, G. H. (1974) *Biochemistry 13*, 1446–1454.
Warner, T. G., & Benson, A. A. (1977) *J. Lipid Res. 18*, 548–552.
Wells, M. A., & Hanahan, D. J. (1969) *Methods Enzymol. 14*, 178–184.
Yang, S. F. (1969) *Methods Enzymol. 14*, 208–211.

Assignment of the Carbon-13 Nuclear Magnetic Resonance Spectra of Gangliosides G_{M4}, G_{M3}, G_{M2}, G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}†

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ABSTRACT: Complete ^{13}C nuclear magnetic resonance assignments are presented for gangliosides in the series G_{M4}, G_{M3}, G_{M2}, G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}. The gangliosides studied are related by the sequential addition of single saccharide residues. The structural relationships among these molecules were confirmed and subsequently utilized to provide the basis for a detailed investigation of ^{13}C NMR oligomer–monomer shielding differences accompanying increasing oligosaccharide complexity. This gradual increase in complexity was reflected in the ^{13}C NMR spectra and proved to be of significant value in the assignment task, resulting in the reassignment of four G_{M1} resonances from our previous work [Sillerud, L. O., Prestegard, J. H., Yu, R. K., Schafer, D. E., & Konigsberg, W. H. (1978) *Biochemistry 17*, 2619–2628]. The carboxyl-containing sialic acids in gangliosides have glycosidic linkage resonance shifts only ~30% as large as those found for neutral hexopyranosides; thus, care must be used in interpreting the ^{13}C spectra of charged oligosaccharides. Secondary structural effects are also found to produce shifts in the resonances of the sialic acid adjacent to the GalNAc residue of G_{M2} and the more complex gangliosides, leading to inequivalence of the sialic acids in G_{D1a}, G_{D1b}, and G_{T1b}.

The gangliosides, glycosphingolipids containing sialic acid, have long been recognized as major membrane components of the cells of nervous tissue (Ledeen & Yu, 1973). More recently, gangliosides have been implicated as participants in important membrane functions of other cell types, where their concentration is far lower than in nervous tissue (Fishman & Brady, 1976). The most widely studied example is the interaction of the monosialoganglioside G_{M1} (II^3NeuAcGgOse$_4$Cer*)[1] (Figure 1) with the protein toxin produced by *Vibrio cholerae* (Cuatrecasas, 1973; Holmgren et al., 1975; Sattler et al., 1977). It has been suggested that specific ganglioside receptors exist in target cells for all of the glycoprotein hormones (Mullin et al., 1977), as well as for many bacterial toxins, interferon (Fishman & Brady, 1976), and lectins (Maget-Dana et al., 1979). Interesting correlations have been reported between ganglioside concentration or complexity and cell differentiation (Dreyfus et al., 1979), cell senescence (Rahmann & Hilbig, 1979), cell transformation (Fishman & Brady, 1976), tumorigenic progression (Morre et al., 1979), and the phylogenetic development of the central nervous system (Hilbig & Rahmann, 1979). Ganglioside G_{D1a} of the thyrocyte specifically binds TSH (Mullin et al., 1976), and it has recently been shown that the binding of FSH to G_{M1} in model membranes specifically alters membrane conductance (Deleers et al., 1979).

In a previous paper, we reported the first complete assignments for the ^{13}C NMR spectrum of ganglioside G_{M1} and demonstrated that ^{13}C NMR is useful for studying the subtle changes in ganglioside structure induced by environmental perturbations such as binding to ligands (Sillerud et al., 1978). We have now extended these results to include G_{M4}, G_{M3}, G_{M2}, G_{D1a}, G_{D1b}, and G_{T1b}. These studies, together with our earlier paper and that of Harris & Thornton (1978) on G_{M1}, G_{M3}, and G_{D1a}, permit a detailed comparison of the complete ^{13}C NMR spectra of seven of the major known gangliosides. Such a comparison confirms most of the earlier assignments, and

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[1] Abbreviations: G_{M1}, II^3NeuAcGgOse$_4$Cer* = Galβ1→3GalNAcβ1→4Gal[3←2αNeuAc]β1→4Glcβ1→1Cer; G_{M2}, II^3NeuAcGgOse$_3$Cer* = GalNAcβ1→4Gal[3←2αNeuAc]β1→4Glcβ1→1Cer; G_{M3}, II^3NeuAcLacCer* = NeuAcα2→3Galβ1→4Glcβ1→1Cer; G_{M4}, I^3NeuAcGalCer* = NeuAcα2→3Galβ1→1Cer; G_{D1a}, IV^3NeuAc,II^3NeuAcGgOse$_4$Cer* = NeuAcα2→3Galβ1→3GalNAcβ1→4Gal[3←2αNeuAc]β1→4Glcβ1→1Cer; G_{D1b}, II^3(NeuAc)$_2$GgOse$_4$Cer* = Galβ1→3GalNAcβ1→4Gal[3←2αNeuAc8←2αNeuAc]β1→4Glcβ1→1Cer; G_{T1b}, IV^3NeuAc, II^3(NeuAc)$_2$GgOse$_4$Cer* = NeuAcα2→3Galβ1→3GalNAcβ1→4Gal[3←2αNeuAc8←2αNeuAc]β1→4Glcβ1→1Cer [forms marked with an asterisk follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (1977)]; Me_4Si, tetramethylsilane.

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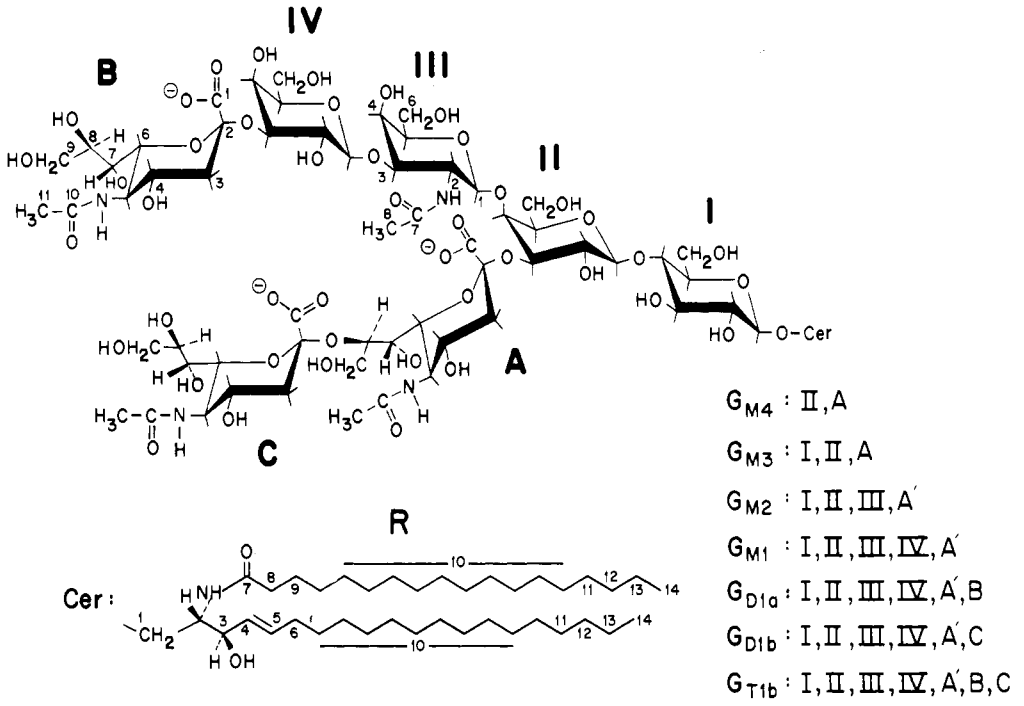

FIGURE 1: Covalent structures of the major gangliosides examined in this study. Shown complete is the oligosaccharide of the most complex member of the series, G_{T1b}. The other gangliosides can be considered "incomplete" precursors of G_{T1b}, as indicated above. All of the gangliosides contain the ceramide portion, residue R. The carbon atoms of the hexopyranoside residues (I, II, III, and IV) are numbered as in residue III. The carbons of the sialic acids (A, B, and C) are numbered as in residue B.

also leads to some reassignments. More interestingly, it reveals several resonances that are sensitive to the presence of ganglioside secondary structure.

Materials and Methods

Reagents. Aldrich (Milwaukee, WI) supplied Me_4Si and deuterium chloride. Deuterium oxide (100%) purified to remove paramagnetic impurities came from Stohler Isotopes (Rutherford, NJ), while carbon-13 depleted $[^{12}C]CD_3OD$ came from Merck (St. Louis, MO). All water was distilled, filtered, and deionized before use. Other reagents used were of the best analytical grade available. Except as noted, all compounds were used as received without further purification.

Purification of Gangliosides. Ganglioside G_{M1} was prepared from bovine brain by neuraminidase treatment of the total ganglioside fraction and isolated according to methods previously reported (Sillerud et al., 1978). Gangliosides G_{D1a}, G_{D1b}, and G_{T1b} were prepared from a mixture of bovine brain gangliosides (Supelco, Supelco Park, PA) by the sequential application of column chromatography on DEAE-Sephadex (A-25, acetate form) and Iatrobeads (6RS 8060, Iatron Laboratories, Inc., Tokyo, Japan). Details of the procedure have been described previously (Momoi et al., 1976; Ando & Yu, 1977; Ledeen & Yu, 1978). Ganglioside G_{M2} was prepared from the brain of a Tay Sachs patient (2.8 years old, female) by a similar procedure (Itoh et al., 1981). Ganglioside G_{M3} prepared from bovine adrenal medulla by the method of Ando & Yu (1977) was a gift from Dr. T. Miyatake of the Jichi Medical School, Japan.

Ganglioside G_{M4} was prepared from chicken brains. Briefly, a total brain ganglioside mixture (about 700 mg) was prepared from 310 g of the white matter of 6–16-week-old chicken brains by the procedure of Ledeen et al. (1973). The gangliosides were separated into four major peaks by a DEAE-Sephadex column (Ando & Yu, 1977). Fractions enriched in G_{M4} were collected, and the material was subjected to further purification by chromatography on an Iatrobeads column (60 g, 1.4 cm i.d. × 80 cm). Gradient elution was carried out between 400 mL of chloroform/methanol/water (75:23:2) and 425 mL of chloroform/methanol/water (50:47:3). Fractions containing G_{M4} were collected, combined, and dried to give about 30 mg of white powder. All the gangliosides, prepared in the above manner, had sodium as the counterion. They were at least 99% pure, as judged by thin-layer chromatography in at least two different solvent systems (Ando et al., 1978).

Carbon-13 Nuclear Magnetic Resonance. Spectra were obtained with a Bruker HX 270 superconducting spectrometer at 67.89 MHz, using quadrature detection in the pulsed Fourier-transform mode. Free-induction decays following a 90° (15-μs) pulse were accumulated in 16384 data points, with an acquisition time of 0.581 s. The sweep width of 14085 Hz (207.46 ppm) resulted in a digital resolution of 0.86 Hz/point (0.013 ppm/point). Complete proton decoupling was accomplished by continuous irradiation of the sample with 1 W of radio frequency noise, with a band width of about 10 ppm centered 5 ppm downfield from the external Me_4Si proton resonance. The field was locked on the solvent deuterium resonance. Pulse delays of up to 2 s were introduced to minimize saturation of quaternary carbons. All chemical shifts were measured by the spectrometer system software by setting to the signal of the ω-1 carbon of the alkyl chain (R-13), to $\delta = 23.45$ with respect to external Me_4Si, as in G_{M1} (Sillerud et al., 1978). The accuracy of measurement was ±0.02 ppm. Similar considerations and parameters were applied to the acquisition of the spectrum of G_{M3} at 90.55 MHz on a Bruker WH360.

Water-soluble gangliosides were initially run as solutions of 100 mg/mL in 200 mM deuterated sodium phosphate buffer, pH 7.1; this buffer was prepared in H_2O, lyophilized, and reconstituted with 100% D_2O. The resulting micellar solutions gave spectra with broad lines. A more satisfactory solvent was found to be a mixture consisting of equal volumes of deuterated phosphate buffer and carbon-13-depleted $[^{12}C]CD_3OD$. The spectral lines in this solvent were about a factor of three narrower than in deuterated phosphate buffer

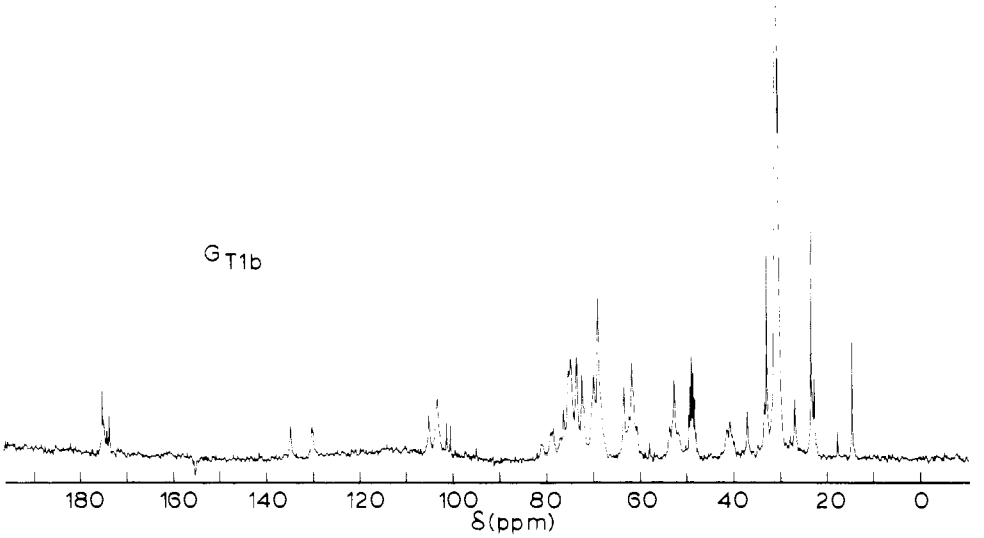

FIGURE 2: Proton-decoupled ^{13}C NMR spectrum of ganglioside G_{T1b} [pH 7.1, 20 °C, 100 mg/mL in methanol/D_2O (1:1 v/v)]. The resonance at 48 ppm is from the residual ^{13}C in the methanol solvent.

Table I: ^{13}C Chemical Shifts and Oligomer–Monomer Shielding Differences for Residue I, β-D-Glucopyranoside, in Gangliosides[a]

carbon no.	G_{M3}	G_{M2}	G_{M1}	G_{D1a}	G_{D1b}	G_{T1b}
I1	103.64	(103.58)	(103.40)	(103.52)	(103.42)	(103.50)
	6.93	(6.87)	(6.69)	(6.81)	(6.71)	(6.79)
I2	(73.95)	74.15	74.09	(73.84)	(73.53)	(73.72)
	(−1.02)	−0.82	−0.88	(−1.13)	(−1.44)	(−1.25)
I3	75.35	(75.31)	(75.23)	(75.21)	(75.05)	(74.96)
	−1.34	(−1.38)	(−1.46)	(−1.48)	(−1.64)	(−1.73)
I4	79.85	79.60	79.80	79.88	79.43	79.44
	9.39	9.14	9.34	9.42	8.97	8.98
I5	75.81	(75.31)	(75.74)	(75.54)	(75.73)	(75.54)
	−0.73	(−1.17)	(−0.80)	(−1.00)	(−0.81)	(−1.00)
I6	61.28	61.14	61.00	61.02	61.02	60.80
	−0.40	−0.54	−0.68	−0.66	−0.66	−0.88

[a] Shielding differences with respect to β-D-glucopyranose. In this and all the following tables, positive values indicate downfield shifts in gangliosides, and all values are in parts per million. Peaks containing two or more incompletely resolved resonances are indicated by parentheses throughout the tables.

alone, an effect attributable to the lower aggregation number of gangliosides in solvents of lower dielectric constant. All other compounds were run at this concentration in deuterated phosphate buffer, pH 7.1. We found a small chemical shift difference of −0.06 ± 0.20 ppm between the resonances of G_{M1} in ^2H_2O (Sillerud et al., 1978) and [^{12}C]CD_3OD/D_2O. For assignment purposes, the component sugars were examined under conditions comparable to those for the gangliosides, and their ^{13}C resonances were assigned as described by Sillerud et al. (1978).

Results

The ^{13}C NMR spectra of all the gangliosides investigated display eight distinct chemical shift regions, assigned (Rosenthal & Fendler, 1976; Sillerud et al., 1978) to the following classes of carbons: carbonyl (δ ~174–176), olefinic (δ ~130–135), anomeric (δ ~100–106), glycosidic ether (δ ~75–78), secondary alcohol and remaining ether (δ ~62–76), primary alcohol (δ ~60–62), amide linkage (δ ~51–54), and methylene and methyl (δ ~14–42). These regions, found for example in the spectrum of G_{T1b} (Figure 2), are completely separate except at δ ~75–76 ppm, where some overlap occurs. The chemical shifts of all the observed peaks are listed in Tables I–VI, together with their proposed carbon assignments. Except as noted, the assignments for G_{M1} correspond to those in our previous paper (Sillerud et al., 1978).

Spectra were examined sequentially in order of increasing ganglioside complexity, from G_{M4} to G_{T1b} (G_{D1a} preceding G_{D1b}). Changes observed in the spectrum upon the addition of a new residue were due either to new signals from the added carbons or to shifts of previously observed signals caused by the presence of the new residue. Signals that remained essentially unchanged when a new residue was added were taken to represent the same carbons in both structures, unless there was evidence to the contrary. All the sugars in gangliosides

Table II: ^{13}C Chemical Shifts and Oligomer–Monomer Shielding Differences for Residues II and IV, β-D-Galactopyranoside, in Gangliosides[a]

carbon no.	G_{M4}	G_{M3}	G_{M2}	G_{M1}	G_{D1a}	G_{D1b}	G_{T1b}	carbon no.	G_{M1}	G_{D1a}	G_{D1b}	G_{T1b}
II1	103.83	103.93	(103.58)	(103.40)	(103.52)	(103.42)	(103.50)	IV1	105.62	105.36	105.50	105.32
	6.64	6.74	(6.39)	(6.21)	(6.33)	(6.23)	(6.31)		8.43	8.17	8.31	8.13
II2	70.02	70.36	(70.78)	70.73	70.76	70.72	(70.35)	IV2	71.69	71.36	71.63	(70.35)
	−2.65	−2.31	(−1.89)	−1.94	−1.91	−1.95	(−2.32)		−0.98	−1.31	−1.04	(−2.34)
II3	76.59	76.62	(75.31)	(75.23)	(75.21)	(75.05)	(74.96)	IV3	(73.59)	76.53	(73.53)	76.45
	3.05	3.08	(1.77)	(1.69)	(1.67)	(1.51)	(1.42)		(0.05)	2.99	(−0.01)	2.91
II4	68.32	68.58	78.33	78.14	78.33	78.88	78.75	IV4	(69.20)	69.20	(69.22)	(69.05)
	−1.19	−0.93	8.82	8.63	8.82	9.37	9.24		(−0.31)	−0.31	(−0.29)	(−0.46)
II5	75.62	76.08	74.89	75.00	(75.21)	(75.05)	74.96	IV5	(75.74)	(75.54)	(75.73)	(75.54)
	−0.24	−0.22	−0.97	−0.86	(−0.65)	(−0.81)	−0.90		(−0.12)	(−0.32)	(−0.13)	(−0.32)
II6	61.77	61.98	62.05	61.83	(61.92)	(61.83)	61.79	IV6	(61.43)	(61.92)	(61.83)	61.79
	0.00	0.21	0.28	0.06	(0.15)	(0.06)	0.02		(−0.34)	(0.15)	(0.06)	0.02

[a] Shielding difference with respect to β-D-galactopyranose.

Table III: ^{13}C Chemical Shifts and Oligomer–Monomer Shielding Differences for Residue III, 2-Acetamido-2-deoxy-β-D-galactopyranoside, in Gangliosides[a]

carbon no.	G_{M2}	G_{M1}	G_{D1a}	G_{D1b}	G_{T1b}
III1	(103.58)	(103.40)	(103.52)	(103.42)	(103.50)
	(7.75)	(7.57)	(7.69)	(7.59)	(7.67)
III2	53.28	52.77	(52.87)	53.30	53.28
	−0.85	−1.36	(−1.26)	−0.83	−0.85
III3	(70.78)	81.52	81.75	81.16	81.05
	(−0.75)	9.99	10.22	9.63	9.52
III4	68.88	68.75	68.54	68.90	68.45
	0.59	0.46	0.25	0.61	0.16
III5	75.57	(75.74)	(75.54)	(75.73)	(75.54)
	0.05	(0.22)	(0.02)	(0.21)	(0.03)
III6	61.43	(61.43)	61.51	61.50	61.48
	0.02	(0.02)	0.10	0.09	0.07
III7	175.52	175.55	175.65	175.64	175.60
	0.68	0.71	0.81	0.80	0.76
III8	(23.45)	(23.45)	(23.45)	(23.45)	(23.45)
	(0.73)	(0.73)	(0.73)	(0.73)	(0.73)

[a] Shielding differences with respect to 2-acetamido-2-deoxy-β-D-galactopyranose.

participate in glycosidic linkages. They will, therefore, be named and referred to as pyranosides.

The chemical shifts of saccharide carbons change in comprehensible patterns when the monomers are linked together to form larger structures. For the presentation of the results, we will make extensive use of the shielding difference, $\Delta = \delta_o - \delta_m$, between equivalent carbon resonances in the ganglioside oligomers, δ_o, and the free monosaccharides, δ_m. The anomeric carbon (C_β) resonance in glycosidically linked hexopyranoses moves ($\Delta_\beta \sim +6$ ppm) downfield, while those carbons (C_γ) adjacent to the site of substitution resonate ($\Delta_\gamma \sim -1$ to -2 ppm) upfield from their position in free saccharides. The glycoside secondary ether carbons ($C_{\beta'}$) across the glycosidic linkage from the anomeric carbon shift ($\Delta_{\beta'} \sim 9$ ppm) downfield. We have used these principles as guides to predict the spectra of the oligosaccharide portion of the gangliosides. We found several instances in which predicted resonances were not found in the experimental spectra. The term "anomalous" is used to designate those resonances whose oligomer–monomer shielding differences fall outside of the expected range. The shifts of the anomalous resonances are interpretable in terms of unique primary and secondary structural features of gangliosides.

The nomenclature relating to complex oligosaccharides as found in gangliosides needs to be clarified for the purpose of ^{13}C NMR so that unambiguous references may be simply made to individual carbon nuclei in the molecules. For this purpose, we propose the following system. The residues in the main neutral saccharide chain are denoted by Roman numerals beginning with the residue closest to the ceramide, residue R; thus, the saccharide residues in what is known as the gangliotetraose are numbered I, II, III, and IV, corresponding to β-D-Glc, β-D-Gal, β-D-GalNAc, and β-D-Gal, respectively (Figure 1). The sole exception is ganglioside G_{M4} in which the ceramide is linked to residue II since residue I is not present. The positions of biosynthetic attachment of the sialic acids begin at carbon 3 of residue II. We label the sialic acid at this site residue A, and subsequent sialic acids B, C, etc. The order of presentation of the data will follow the growth in the complexity of the oligosaccharides from G_{M4} to G_{T1b}. We will defer discussion of the ceramide portion ^{13}C NMR spectra to the end of the results since there is relatively less significant structural variation in the hydrophobic tails.

Ganglioside G_{M4}. The oligosaccharide of G_{M4}, the simplest ganglioside, consists of NeuAc(α2→3)βDGal. The ^{13}C NMR spectrum reflects this simplicity, particularly in the crowded secondary alcohol region (Figure 3). A total of 12 carbons from G_{M4} resonate between 60 and 80 ppm. Our use of a methanol/water solvent mixture provided resolution of at least nine peaks. The assignments for the β-D-Gal, residue II, are shown in Table II, and those for sialic acid (the trivial name for 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosonic acid) residue A, are in Table IV. The tables also show the oligomer–monomer shielding differences, Δ. Positive values of Δ indicate that the resonance for the carbon in a

Table IV: ^{13}C Chemical Shifts and Oligomer–Monomer Shielding Differences for Residues A, B, and C, α-Sialic Acid (5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosidic Acid), in Gangliosides[a]

carbon no.	G_{M4}	G_{M3}	G_{M2}	G_{M1}	G_{D1a}	G_{D1b}	G_{T1b}	carbon no.	G_{D1a}	G_{T1b}	carbon no.	G_{D1b}	G_{T1b}
A1	174.81 [b]	174.60	174.73	174.53	174.83	(174.19)	(174.11)	B1	174.90	174.61	C1	(174.19)	(174.11)
A2	100.59	100.91	102.64	102.51	102.57	101.99	(101.52)	B2	100.71	100.66	C2	101.43	(101.52)
	2.53	2.85	4.58	4.45	4.51	3.93	(3.46)		2.65	2.60		3.37	(3.46)
A3	40.80	40.84	37.76	37.76	38.00	39.41	40.00	B3	40.72	40.69	C3	41.42	41.55
	−0.82	−0.78	−3.79	−3.86	−3.62	−2.21	−1.62		−0.90	−0.93		−0.20	−0.07
A4	68.93	69.20	(69.19)	(69.20)	(69.20)	(69.22)	(69.05)	B4	(69.20)	(69.05)	C4	(69.22)	(69.05)
	−0.05	0.22	(0.21)	(0.22)	(0.22)	(0.24)	(0.07)		(0.22)	(0.07)		(0.24)	(0.07)
A5	52.85	52.97	52.82	51.96	51.89	52.22	52.20	B5	(52.87)	(52.90)	C5	52.81	(52.90)
	0.17	0.29	0.14	0.27	0.34	0.01	0.03		(0.19)	(0.22)		0.13	(0.22)
A6	73.80	(73.95)	73.85	(73.59)	(73.84)	(73.53)	(73.72)	B6	(73.84)	(73.72)	C6	(73.53)	(73.72)
	0.47	(0.62)	0.52	(0.26)	(0.51)	(0.20)	(0.39)		(0.51)	(−0.39)		(0.20)	(0.39)
A7	69.09	69.39	(69.19)	(69.20)	(69.20)	(69.22)	(69.05)	B7	(69.20)	(69.05)	C7	(69.22)	(69.05)
	0.11	0.41	(0.21)	(0.22)	(0.22)	(0.24)	(0.07)		(0.22)	(0.07)		(0.24)	(0.07)
A8	72.46	72.68	72.48	72.86	73.00	77.74	77.14	B8	72.68	(72.53)	C8	72.57	(72.53)
	0.13	0.35	0.15	0.53	0.67	5.41	4.81		0.35	(0.20)		0.24	(0.20)
A9	63.57	63.81	63.90	63.98	(63.60)	62.36	62.50	B9	(63.60)	(63.54)	C9	63.63	(63.54)
	−0.18	0.06	0.15	0.23	(−0.15)	−1.39	−1.25		(−0.15)	(−0.21)		−0.12	(−0.21)
A10	(175.45)	(175.76)	175.73	175.69	175.65	175.84	(175.70)	B10	175.75	(175.70)	C10	175.64	(175.70)
	(−0.21)	(0.10)	−0.07	−0.03	−0.01	−0.18	(−0.04)		−0.09	(0.04)		−0.02	(0.04)
A11	22.67	22.94	22.79	22.78	22.89	22.86	22.79	B11	(23.45)	(23.45)	C11	(23.45)	(23.45)
	−0.44	−0.17	−0.32	−0.33	−0.22	−0.25	−0.32		(0.34)	(0.34)		(0.34)	(0.34)

[a] Shielding differences with respect to α-sialic acid at pH 7.0. [b] No assignment for the carboxyl carbon of monomeric α-sialic acid has been reported in the literature. We were also unable to observe it.

Table V: ^{13}C Chemical Shifts and Shielding Differences for the Ceramide Portion, Residue R, of Gangliosides[a]

carbon no.	G_{M4}	G_{M3}	G_{M2}	G_{M1}	G_{D1a}	G_{D1b}	G_{T1b}
R1	69.90	70.26	70.03	70.15	70.07	70.26	(70.35)
	7.10	7.46	7.23	7.35	7.27	7.46	(7.55)
R2	53.74	54.21	54.02	54.10	54.10	53.95	53.93
	−1.72	−1.25	−1.44	−1.36	−1.36	−1.51	−1.53
R3	72.22	72.28	72.18	72.15	72.27	72.21	72.17
R4	134.83	135.02	134.84	134.89	134.98	135.22	135.05
R5	130.53	130.62	130.65	130.62	130.50	130.37	130.54
R6	33.31	33.32	33.43	33.37	33.98	33.35	33.30
R7	(175.45)	(175.76)	175.20	175.16	175.20	175.44	175.37
R8	37.01	37.17	37.05	37.02	37.07	37.04	37.02
R9	26.97	26.96	27.02	26.96	26.94	26.94	26.92
R10	30.83	30.87	30.90	30.88	30.81	30.89	30.79
R11	30.37	30.33	30.37	30.36	30.38	30.38	30.31
R12	32.70	32.70	32.82	32.82	32.81	32.83	32.80
R13[b]	23.45	23.45	(23.45)	(23.45)	(23.45)	(23.45)	(23.45)
R14	14.57	14.66	14.56	14.55	14.63	14.55	14.58

[a] Shielding differences between gangliosides and ceramide are negligible for carbons R3 to R14. [b] Set at $\delta = 23.45$ to coincide with our previous work on G_{M1} (Sillerud et al., 1978).

Table VI: ^{13}C Chemical Shifts for, and Amounts of, Cis Unsaturation in the Fatty Acyl Chains of the Gangliosides

ganglioside	chemical shift[a] R16, 17	chemical shift[a] R15, 18	% cis
G_{M4}	130.24	27.89	1.8[b]
G_{M3}	130.30	27.95	32 ± 4
G_{M2}	130.23	27.99	20 ± 4
G_{M1}	130.17	28.00	19 ± 5
G_{D1a}	130.15	27.61	25 ± 4
G_{D1b}			[c]
G_{T1b}	130.21	27.94	18 ± 5

[a] Chemical shift in $-(CH_2)_m-\underset{15}{CH_2}-\underset{16}{\overset{H}{C}}=\underset{17}{\overset{H}{C}}-\underset{18}{CH_2}-(CH_2)_n-$.
[b] Average number of cis-unsaturated sites per G_{M4} molecule.
[c] Less than 5%.

ganglioside is found at lower field than that for the monomeric sugar.

The anomeric carbon, II1, of the β-D-Gal residue of G_{M4} is assigned to the peak at 103.83 ppm (Figure 4), giving rise to a linkage shift of $\Delta_\beta = 6.64$ ppm (Table II). Carbon II2, which is γ to two linkage sites (II1 and II3), is assigned to the peak at 70.02 ppm. This gives a linkage shift of −2.65 ppm, indicating that the two γ effects are additive at this position.

Carbon II3 is the site of glycosidic linkage of the sialic acid through carbon A2. We would expect to find the signal for II3 at 82.5 ppm, about 9 ppm downfield from its position in β-D-Gal (73.54 ppm). We have assigned II3 to the peak nearest to this in the spectrum of G_{M4} (Figure 3) at 76.59 ppm. The resulting β shift of only 3.05 ppm is believed to arise from the superposition of two separate perturbations effecting the electron density at the nucleus of carbon II3. We expect the first (+9 ppm) to be the well-established linkage Δ_β, and the second (−6 ppm) to be due to the electric field generated from juxtaposition of the carboxyl carbon A1 on the adjacent Neu-Ac. The chemical shift of carbon II4 (68.32 ppm) is associated with a linkage shift of $\Delta_\gamma = -1.19$ ppm due to a γ effect of the expected magnitude. At a field position of 75.62 ppm, carbon II5 resonates only 0.24 ppm upfield in G_{M4} from the equivalent carbon in β-D-Gal, an effect of the size anticipated for carbons δ to linkages. The chemical shift of II6 is the same as that found in β-D-Gal.

We now turn our attention to the sialic acid moiety in G_{M4} (residue A, Figure 1). A priori, one expects that only carbons A1, A2, and A3 should show values of $\Delta = \pm 1.0$ ppm or

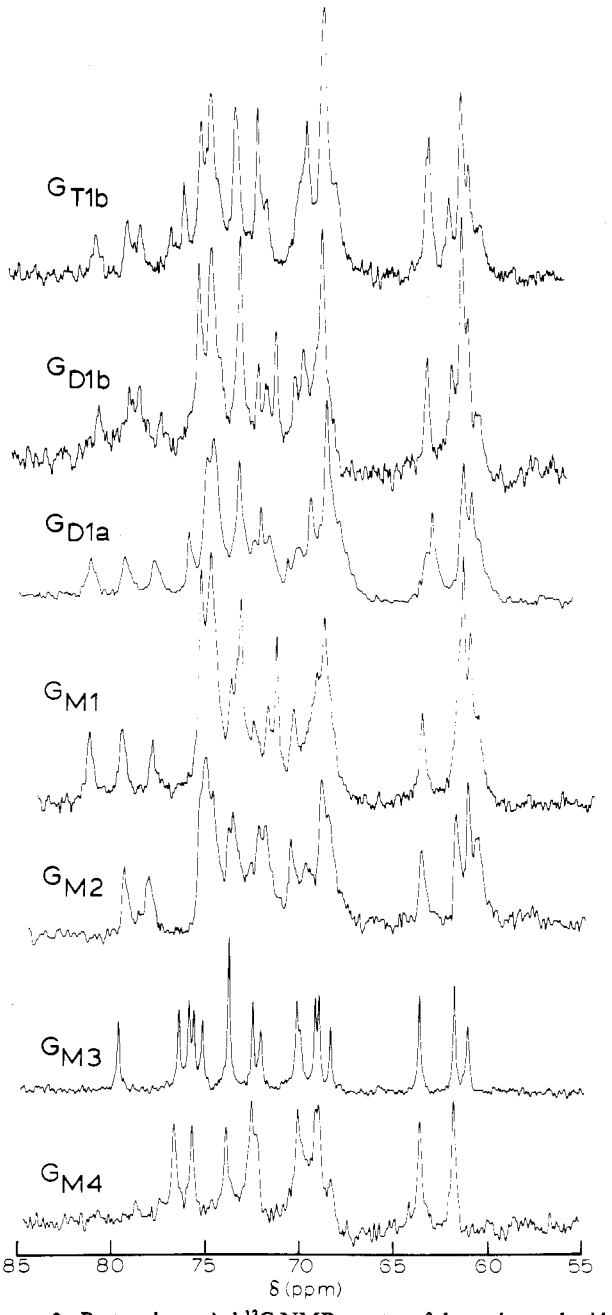

FIGURE 3: Proton-decoupled ^{13}C NMR spectra of the main saccharide region of the gangliosides utilized in this study (pH 7.1, 20 °C).

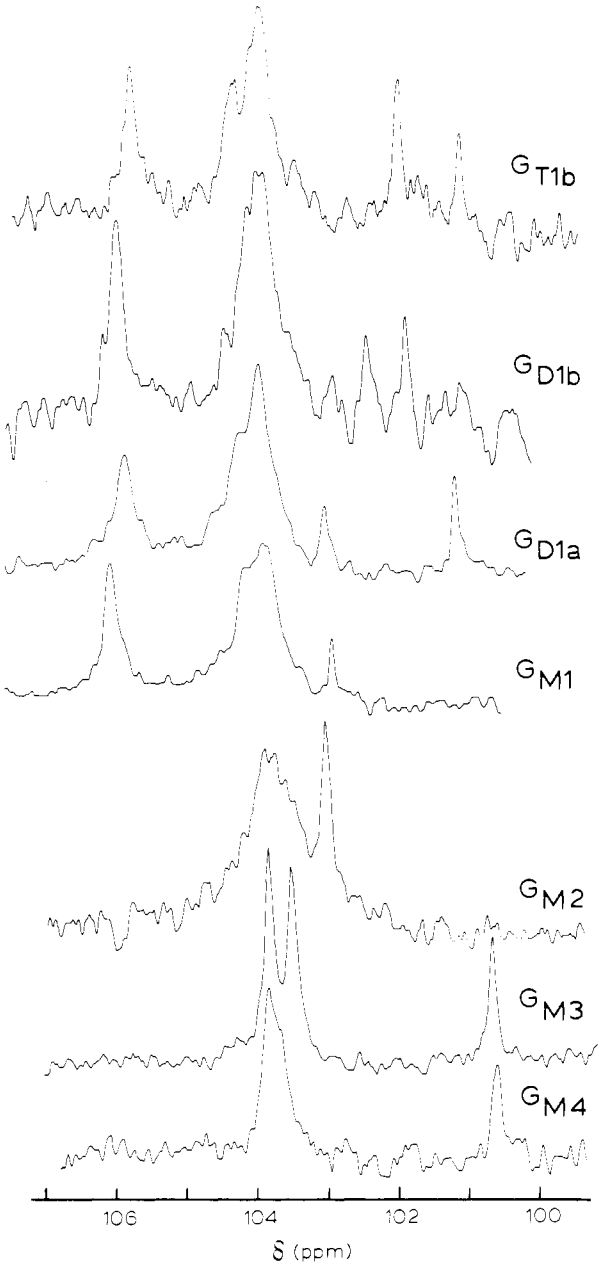

FIGURE 4: Anomeric carbon region of the proton-decoupled natural-abundance ^{13}C NMR spectra of the gangliosides included in the present study (pH 7.1, 20 °C).

greater. Carbon A1 is the sialic acid carboxyl carbon whose field position of 174.81 ppm is very close to that of 174.6 ppm found for α-MeNeuAc (Jennings & Bhattacharjee, 1977). The anomeric carbon, A2, of the NeuAc residue in G_{M4} resonates only 2.53 ppm downfield from its position in the monomer (Table IV). We believe this small Δ value results from the unusual electronic structure of carbon A2 which has three electron-withdrawing groups bound to it. The methylene carbon, A3, also shows a rather small linkage shift of only −0.82 ppm in G_{M4}. The remaining carbons, A4–A11, should resonate at field positions close to those for NeuAc since they are all remote from the site of substitution. We confirmed this by examining the average shielding difference for these carbons in G_{M4} (0.00 ± 0.28 ppm, Table IV).

Ganglioside G_{M3}. The oligosaccharide portion of G_{M3} is the trisaccharide neuraminyllactose [NeuAc(α2→3)DGal(α1→4)DGlc] for which we have already proposed assignments (Sillerud et al., 1978). Only two carbons (I1 and I2) of neuraminyllactose would be expected to resonate at significantly different fields from those in G_{M3}. This compound, then, serves as an excellent model for a large part of the ^{13}C spectrum of G_{M3}. A reflection of their similarity is the small difference of −0.05 ± 0.45 ppm (n = 16) between the chemical shifts of neuraminyllactose (Sillerud et al., 1978) and G_{M3} (Tables I–IV).

In the present series of gangliosides that we have studied, G_{M3} is the simplest member to possess both residues I and II. The β-D-Glc residue I is interposed between the β-D-Gal residue II and the ceramide, R, portion in G_{M3}, and in all the more complex gangliosides (Figure 1). Linkage shifts for the resonances from carbons I1 and I2 in G_{M3} are 6.93 and −1.02 ppm, respectively, values which fall well within the expected ranges for β and γ effects due to substitution at I1. Linkage shifts for carbons I3–I5 follow the expected γ,β′,γ pattern as the result of the attachment of residue II (Table I). The resonance from carbon I6, however, shifts further upfield (−0.40 ppm) than its δ position would predict. One probable reason may be hydrogen bonding between the hydroxyl on I6 and the anomeric oxygen on residue II. The chemical shift and shielding difference patterns for residues II (Table II) and A (Table IV) in G_{M3} follow those discussed above for G_{M4}.

Ganglioside G_{M2}. The oligosaccharide portion of ganglioside G_{M2} results from the β1→4 glycosidic linkage of the 2-acetamido-2-deoxy-β-D-galactopyranoside (GalNAc) residue III to G_{M3}. On the basis of β- and γ-shielding differences due to this linkage, we expected to find changes in chemical shifts of only II3–II5 with respect to G_{M3}. The resonance from carbon II3 in G_{M2} shows a linkage shift of 1.77 ppm, a value 1.28 ± 0.10 ppm smaller than that found for G_{M4} and G_{M3}. It appears that the β and γ effects at this nucleus are additive; the linkage shift is the sum of the β effect of 3.05 ± 0.10 ppm and the γ effect of −1.28 ppm. The field position of the resonance from II4 changes by 10.07 ppm from its chemical shift of 68.45 ± 0.18 ppm in G_{M4} and G_{M3}. The β and γ effects are additive for this site also; the final shielding difference of 8.82 ppm with respect to β-D-Gal is the sum of Δ_β = 9.88 ppm and Δ_γ = −1.06 ± 0.18 ppm. A comparison of the chemical shift of carbon II5 between G_{M4} and G_{M3} on one hand and G_{M2} and the more complex gangliosides (Table II) on the other shows that this carbon resonance experiences a shift, Δ_β = −0.79 ± 0.15 ppm, of the size expected due to its location γ to the linkage at II4.

The GalNAc, residue III, is involved in a β1→4 glycosidic linkage to residue II4 in G_{M2} (Figure 1). The resonance from carbon III1 appears at 103.58 ppm, along with those from carbons I1 and II1 (Figure 4), resulting in a β-linkage shift of 7.65 ± 0.07 ppm in G_{M2} and the higher gangliosides. Replacement of the equatorial hydroxyl of β-D-Gal with an acetamido group shifts the resonance from III2 to a field position characteristic of carbons bonded to nitrogen, 54.13 ppm, in β-D-GalNAc. The resonance from III2 in G_{M2} experiences a γ-linkage shift of −0.85 ppm. Carbons III3–III6 are at positions which are δ or greater with respect to the site of substitution, so that they would not be expected to display resonances with Δ values greater than about ±0.5 ppm. An examination of the spectrum of G_{M2} (Figure 3) indicates that there is no resolved resonance at 71.53 ppm, the chemical shift for III3 in free β-D-GalNAc. We tentatively assign III3 to the peak at 70.78 ppm, with Δ = −0.75 ppm. The resonance from III4 is assigned in a similar fashion to the peak at 68.88 ppm, giving rise to a value of Δ = 0.59 ppm. These shielding differences are somewhat larger than anticipated, but the peaks to which III3 and III4 are assigned are the resolved peaks closest to the chemical sifts for free β-D-GalNAc. We are

preparing the oligosaccharide from G_{M2}, whose ^{13}C NMR spectrum would provide the enhanced resolution necessary to resolve the questions raised here about the assignments of residue III. The remainder of the resonances (III5–III8) from this residue in G_{M2} can be assigned with no difficulty (Table III).

We turn our attention now to the NeuAc residue, A, in G_{M2}. The carboxyl carbon, A1, is assigned on the basis of the observed patterns of carbonyl resonances among the gangliosides. There are only small shielding differences for A1 and the *N*-acetyl carbonyl, A10, on formation of G_{M2}. On the other hand, marked alterations are seen for the field positions of the anomeric, A2, and methylene, A3, carbon resonances of sialic acid in G_{M2} as compared with those observed for G_{M4} and G_{M3}. The Δ_β value for A2 in G_{M3} and G_{M4} of approximately 3 ppm increases to 5 ppm in G_{M2}, at the same time that the Δ_γ value for A3 changes from −0.8 to −3.7 ppm. These correlated changes must arise from long-range effects from some group on residue III since this residue is lacking in G_{M4} and G_{M3}. The trend for A2 and A3 in G_{M2} is also followed in the more complex gangliosides. The remaining sialic acid carbons resonate within 0.2 ppm of their field positions of α-sialic acid (Table IV).

Ganglioside G_{M1}. The assignments for the ^{13}C nuclear magnetic resonances of ganglioside G_{M1} in ^2H_2O have been reported previously (Sillerud et al., 1978). For the most part, spectra recorded in a 1:1 (v/v) mixture of deuterated phosphate buffer (pH 7.4) and [^{12}C]methanol gave chemical shifts whose mean deviation was −0.06 ± 0.20 ppm (n = 45) relative to those previously measured in ^2H_2O (pH 7.5). Exceptions to this trend occur for the ceramide carbons R4 and R5, which experience changes in chemical shifts of −0.33 and 0.58 ppm, respectively, on going from a solvent of higher to one of lower dielectric constant. Similar behavior for R4 and R5 has been reported by Harris & Thornton (1978), who found that these carbon resonances coalesce when gangliosides are transferred from aqueous to hydrocarbon solvents.

A comparison of the assignments of the oligosaccharide portion of G_{M1} reported in Table I with those from our previous work (Sillerud et al., 1978) shows that we have reassigned resonances from 4 of the 37 carbons as a result of an examination of the series of ganglioside spectra reported here. Those carbons reassigned include I1, II2, III3, and IV1. We initially proposed that the single carbon signal at 105.48 ppm should be assigned to I1, the anomeric carbon of the sole β-D-Glc residue in G_{M1}. The signal at 105.62 ppm can now be assigned to the resonance from carbon IV1 of the terminal β-D-Gal residue (Figure 4), while I1 resonates at 103.40 ppm. The gangliosides from G_{M1} to G_{T1b}, containing residue IV, all show a resonance at 105.45 ± 0.14 ppm arising from carbon IV1. The unique chemical shift of carbon IV1 in G_{M1} and the more complex gangliosides is expressed as a deshielding of the IV1 resonance by 1.97 ± 0.21 ppm with respect to the field position of 103.48 ± 0.07 ppm of the related β-D-Gal anomeric carbons II1 and III1. Again, we believe that a long-range effect from residue III may alter the local field experienced by IV1 in much the same way as mentioned above for A2 and A3. The other two resonances reassigned are contributed by carbons II2 and II3 on the β-D-Gal residue at the branch in the oligosaccharide chain. We previously thought that II2 appeared at the same position in G_{M1} as in lactose (71.56 ppm), but the lack of a resonance near this field in G_{M4} makes it clear that reassignment is necessary. Following the results for G_{M4} presented above, we assign the resonance from II2 in G_{M1} to the signal at 70.73 ppm. Carbon II3 participates in the glycosidic linkage of the sialic acid, residue A in G_{M1}. Our previous assignment of II3 to the peak at 81.33 ppm must be changed, again because there is no peak in this region of the G_{M4} spectrum. Even our previous assignment gave rise to a significantly lower Δ value (7.8 ppm) than that expected on the basis of shifts of model compounds (9.5 ± 0.4 ppm). The spectrum of G_{M4} clearly indicates that II3 must resonate upfield of the spectral region characteristic of "normal" glycosidically linked ring carbons. The peak in G_{M4} which is closest to that expected for II3 occurs at 76.59 ppm, giving rise to a value of $\Delta_{\beta'}$ = 3.05 ppm, that is, some 6 ppm lower than that found for neutral linked saccharides. We now believe that the unusual electronic environment of the sialic acid anomeric carbon may be the cause of this markedly reduced value of $\Delta_{\beta'}$ for II3 in all the gangliosides examined so far. The additional presence of a γ effect due to residue III at the site of II3 gives the final chemical shift for II3 of 75.23 ppm ($\Delta_{\beta'}$ = 1.69 ppm) as the superposition of the two shielding contributions. Our reassignment of II3 is supported by an examination of the spectral region, from 78 to 82 ppm, characteristic of nonanomeric ring carbons ($C_{\beta'}$) which take part in glycosidic linkages. One notes (Figure 3) that there is one less peak in this region than expected on the basis of the number of nonanomeric linked carbons in each ganglioside. For example, we expect one peak from II3 in G_{M4} and find none, two peaks from I4 and I3 in G_{M3} and find one, and so forth; the pattern continues through G_{T1b}. Each peak integrates to give a value characteristic of only a single carbon, so that the missing peak is not simply hidden under one of the others as we previously suspected. The solution to this puzzle was found in the spectra of the di- and trisialogangliosides G_{D1a}, G_{D1b}, and G_{T1b}. According to the assignment principles outlined above (see also the results from G_{M4}), we expected to find, for example, two peaks in G_{D1a}, at 75.6 and 76.6 ppm, corresponding to resonances from II3 and IV3, respectively. Such peaks can be clearly seen (Figure 3) shifted upfield from those contributed by the other nonanomeric linked carbons.

Ganglioside G_{D1a}. The core neutral oligosaccharide structure of the gangliosides is complete with the addition of the terminal β-D-Gal residue IV to G_{M2} to form G_{M1}. More complicated ganglioside oligosaccharides are formed from the basic G_{M1} configuration by the addition of one or more sialic acids. We have isolated quantities large enough for ^{13}C NMR of the disialogangliosides G_{D1a} and G_{D1b} and the trisialoganglioside G_{T1b}. The presence of several sialic acids in gangliosides produces ^{13}C NMR spectra which differ from a simple superposition of the components, even when linkage shifts are taken into account. We will present the results for G_{D1a} first.

Most of the ^{13}C nuclear magnetic resonance signals from ganglioside G_{D1a} occur at field positions similar to those found for G_{M1}. Prominent exceptions include carbons IV2, IV3, and IV4, which are shifted in G_{D1a} from the linkage of the second sialic acid residue B at IV3. Furthermore, one expects a doubling of the intensity of the sialic acid resonances, as well as linkage shifts of carbons B2 and B3. We have therefore assigned the G_{D1a} carbon resonances, except those from IV2, IV3, IV4, B2, and B3, by a comparison with the results for G_{M1}. Small differences between the chemical shift patterns in these two gangliosides may yet provide information on effects other than covalent linkage, but in general, the agreement is very good. The results for ganglioside G_{D1a} can be found in Tables I–V.

Five of the carbons in G_{D1a} experience shifts as a result of the glycosidic linkage of residue B, the second sialic acid, to the terminal β-D-Gal residue IV. The resonance from carbon IV2 shifts from 71.69 ppm in G_{M1} to 71.36 ppm in G_{D1a}, with a resulting increase in the magnitude of the oligomer–monomer shielding difference to $\Delta_\gamma = -1.31$ ppm. Comparison with the smaller value of $\Delta_\gamma = -0.98$ ppm in G_{M1} indicates that the two effects from linkages at IV1 and IV3 reinforce one another at IV2. The resonance from carbon IV3 is found at 76.53 ppm, a value which, as discussed above, gives rise to a very low value of $\Delta_\beta = 2.99$ ppm instead of the expected $\Delta \sim 9$ ppm. It should be noted that the resonance from carbon II3 in G_{D1a} is visible at 75.21 ppm. The difference between the field positions of II3 and IV3 in G_{D1a} is 1.32 ppm because II3 experiences both a +3-ppm β' effect due to the linkage of residue A and a γ effect of −1 ppm resulting from the linkage of residue III at II4, while IV3 is shifted from only the β' effect of residue B. Carbon IV4 resonates at 69.20 ppm with a value of $\Delta_\gamma = -0.31$ ppm characteristic of its position γ to the linkage at IV3.

The carbon resonances from the second sialic acid residue B in G_{D1a} overlap those from residue A, with the exception of carbons B2 and B3, which are distinct. We confirmed previous reports (Sillerud et al., 1979; Harris & Thornton, 1978) of the nonequivalence of the resonances from carbons 2 and 3 in residues A and B in G_{D1a}. These carbons resonate at 102.57 and 38.00 ppm in residue A and at 100.71 and 40.72 ppm in residue B, respectively. The differences between the two sialic acid carbon resonances result from differences in the local fields. Residue A is in very close proximity to the *N*-acetyl group from residue III, while residue B has no such neighbor.

Ganglioside G_{D1b}. The other disialoganglioside that we examined was G_{D1b} in which the second sialic acid, residue C, is attached by means of an $\alpha(2\rightarrow8)$ link to the glyceryl side chain of the sialic acid in position A (Figure 1), rather than to residue IV as in G_{D1a}. With this covalent structure in mind, it was expected that the ^{13}C chemical shifts of residues I–IV in G_{D1a} would not differ significantly from those found in the case of ganglioside G_{M1}. The results (Table I–III) confirm the anticipated chemical shift similarities in detail. There is also close agreement among the oligomer–monomer ^{13}C shielding differences for residues I–IV in gangliosides G_{M1}, G_{D1a}, and G_{D1b}. An exception to complete agreement is found only for the resonances from carbons IV2–IV4 in G_{D1a} which are shifted due to the linkage of residue B. Our finding of carbon resonances in G_{D1b} at field positions characteristic of residue IV in G_{M1} is an indication that our method of ganglioside purification effectively resolved G_{D1b} from G_{D1a}.

The sialic acid carbons A2 and A3 in G_{D1b} show ^{13}C shielding differences that are similar to, but not identical with, those found for G_{M2}, G_{M1}, and G_{D1a}. Whereas resonances from A2 and A3 in these latter gangliosides have values of $\Delta_\beta(A2) = 4.51 \pm 0.07$ ppm and $\Delta_\gamma(A3) = -3.76 \pm 0.12$ ppm, respectively (Table IV), we found $\Delta_\beta(A2) = 3.93$ ppm and $\Delta_\gamma(A3) = -2.21$ ppm in G_{D1b}. The different values of Δ for residue A in ganglioside G_{D1b} may again reflect a different orientation of this sialic acid, with respect to a group on residue III, from that found in G_{M2}, G_{M1}, and G_{D1a}. There remains, however, a significant perturbation of the local magnetic field at A2 and A3 due to residue III. The anomeric and methylene carbons of residue A in G_{D1b} display shielding differences that are intermediate between those of G_{M3} and G_{M4} on the one hand and G_{M2}, G_{M1}, and G_{D1a} on the other.

The linkage of the second sialic acid, residue C, to carbon A8 in G_{D1b} should result in resonance shifts for carbons A7, A8, and A9. Our observations (Table IV) indicate that the resonance from A8 undergoes a downfield shift of $\Delta = 5.41$ ppm, a value consistent with a β'-linkage effect, while the peak for A9 shifts upfield by $\Delta = -1.39$ ppm in accord with its position γ to the site of substitution. The signal from A7, however, is assigned to the highest field, resolved peak in the methine carbon spectral region, at $\delta = 69.22$, resulting in a shielding difference of $\Delta_\gamma = 0.24$ ppm, which is the same as that found for A8 in, for example, G_{D1a}. We believe either that the true field position of A7 may yet be found nearer to 67.5 ppm as expected from the γ-linkage effect at this site when the ^{13}C spectrum of the oligosaccharide from G_{D1b} is obtained or that perhaps the carboxyl carbon, C1, from the adjacent residue C may alter the local field at A7, giving rise to the "abnormal" shielding difference for A7.

The second sialic acid, residue C, in G_{D1b} is at a terminal position much like that of residue B in G_{D1a} and is free from any potential interaction with residue III. However, the chemical shifts and shielding differences for the resonances from carbons C2 and C3 are different from those found for the other terminal sialic acid in, for example, G_{D1a} (B2 and B3) and also different from G_{M1}. It is possible that there is a through-space interaction between a group on residue A and carbons C2 and C3, giving rise to this difference (see Discussion and Figure 6). On the other hand, γ effects are dependent on conformation so they may be the reason for the difference. The remaining sialic acid carbons in residue C resonate at field positions not significantly different from those of α-sialic acid.

Ganglioside G_{T1b}. This trisialoganglioside contains the most complex oligosaccharide whose ^{13}C nuclear magnetic resonance spectrum has been obtained to date. The covalent linkage pattern of the neutral saccharide residues I–IV, first completed in G_{M1}, is retained in G_{T1b}. Furthermore, attachment of the three sialic acids, residues A, B, and C, occurs by means of glycosidic linkages that have previously been assigned in G_{D1a}, G_{D1b}, and, for residue A, the lower gangliosides as well. Establishment of a data base from the less complex gangliosides has greatly simplified what would otherwise have been a formidable assignment task. Carbon-13 resonances from the main neutral saccharide chain (residues I–IV) were assigned in analogy with those from G_{D1a}. Chemical shifts and oligomer–monomer shielding differences for these residues in G_{T1b} correlate with the results from G_{D1a} (Tables I–III). The shifts and shielding differences for the sialic acids, A, B, and C, generally also correlate with those of G_{D1a}. The exceptions are again resonances from carbons C2 and C3, whose shifts and shielding differences follow those found for G_{D1b} instead.

Ceramide Moiety. Assignments for the ceramide moiety are given in Tables V and VI. Linkage of R1 produces a downfield shift of 7.35 ± 0.16 ppm, and an upfield shift of R2 of -1.46 ± 0.14 ppm, typical of these carbons β and γ to the site of attachment of the oligosaccharide. The remaining ceramide carbons are assigned without difficulty. We used the penultimate alkyl chain carbon resonance as an internal standard at 23.45 ppm to align the ganglioside spectra with respect to one another.

The structure of the fatty acid, which is amide linked to the sphingosine nitrogen, varies somewhat among the gangliosides examined. The first source of variation is the presence of cis unsaturated olefinic carbons. Nuclei from these carbons resonate at 130.31 ± 0.05 ppm, a value 0.38 ppm smaller than the chemical shift of the trans-unsaturated carbon R5. We

determined the amount of cis-unsaturated fatty acyl chain in the gangliosides by measuring the intensity of the resolved peak at 130.31 ppm and comparing it with that found for carbons R4 and R5 (Koerner et al., 1979). The results (Table VI) are presented as the percentage of ganglioside molecules carrying a single double bond, except for G_{M4}, which has a much higher degree of fatty acyl cis unsaturation (1.8 double bonds per molecule). A separate estimate of cis unsaturation could be made from a measurement of the intensities of the peak at 27.90 ± 0.15 ppm arising from the carbons adjacent to cis-unsaturated sites. The relatively good agreement between these two measurements (Table VI) is reflected in a standard deviation of 21% resulting from their pairwise comparison.

A second source of structural variation among the fatty acyl chains is that of hydroxylation of R8, the carbon α to the carbonyl. Such modification would shift the resonances from carbons R8 and R9 to ~72.6 and ~36 ppm, respectively (Dabrowski et al., 1980). Only ganglioside G_{M4} shows peaks characteristic of an α-hydroxy fatty acid. We found a well-resolved resonance at 35.31 ppm from R9 adjacent to a hydroxyl-bearing R8. From its intensity, we estimated that G_{M4} contains about 43% α-hydroxy fatty acyl chains, in agreement with Cochran et al. (1981). The resonance from hydroxylated R8 is found at 72.46 ppm in G_{M4}, but, since it is unresolved from signals due to A6 and A8, no attempt was made to use it to determine the amount of α hydroxylation.

Discussion

The gangliosides form a class of molecules which are well suited, from the point of view of ^{13}C NMR spectroscopy, to an investigation of the relationship between the covalent structures of oligosaccharides and their nuclear magnetic shielding properties. Gangliosides exist as stable, discreet molecules that can be isolated intact in large quantities without the need for chemical or enzymatic degradation. Their structures differ by single sugar residues. We have exploited the fixed, known relationships among seven of the most common gangliosides as an aid in unravelling the ^{13}C NMR spectra of their oligosaccharides. The members of the series G_{M3}, ..., G_{T1b} are formed, the next from the last, by the biosynthetic addition of a single saccharide residue at a time to an existing oligosaccharide, giving rise to two kinds of ^{13}C resonances not initially present, those from the added residue itself and those from the parent that are now shifted due to electron density changes resulting from the covalent linkage. For example, adding a β-D-GalNAc residue (III) to G_{M3} to form G_{M2} results in a ^{13}C NMR spectrum that now contains not only eight new signals arising from the new residue but also three signals from G_{M3} that are shifted. The primary emphasis of this paper is to account for the field positions of each set of new signals in terms which have general applicability so that the understanding gained can be extended to oligo- and polysaccharides that cannot be examined in the stepwise fashion used here for the gangliosides. Our success in carrying out this program is based on the observation that resonance position shifts due to linkage are confined to those carbons β and γ to the site of substitution. The magnitudes of these effects were established in the early 1970's by Dorman & Roberts (1971) and Colson et al. (1974) for the simple neutral hexose oligomers. A pattern emerges for the chemical shielding of glycosidic linkage participants in the neutral branch, residues I–IV, of gangliosides. Downfield shifts of the anomeric carbons (C_β) in a glycosidic linkage are accompanied by shifts in the same direction of the resonances of the carbon nucleus across the anomeric oxygen ($C_{\beta'}$).

Glycosidic Linkage Shifts. As a first-order assignment aid, the downfield shifts of the resonances of β,β' carbon nuclei involved in glycosidic linkages in oligosaccharides are of significant value. The shifts, Δ, not only are large (~6–10 ppm) and easily measured to good precision (e.g., ±0.04 ppm) but also their magnitude differs for anomeric, C_β, as opposed to nonanomeric, $C_{\beta'}$, ether-linked carbons. For example, on the formation of lactose, the resonance from the anomeric carbon, C_1, of β-D-Gal shifts 6 ppm downfield, while the resonance from the nonanomeric ether carbon, C_4, of the D-Glc residue shifts 9 ppm due to glycosidic linkage. We computed the linkage shifts for all of the involved carbon resonances in the series of gangliosides (Tables I–V).

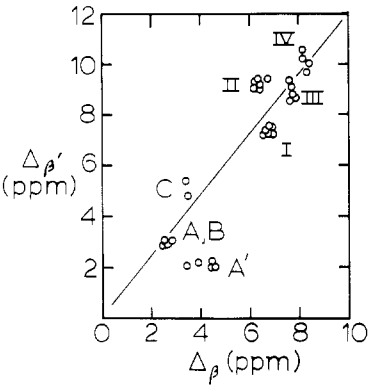

FIGURE 5: Relationship between the oligomer–monomer shielding differences, Δ, for the pairs of ^{13}C resonances of the β and β' carbons participating in glycosidic linkages in the oligosaccharides of the gangliosides included in the present study. The data for sialic acids and neutral saccharides are indicated by symbols corresponding to the structures shown in Figure 1 where A′ refers to the inner sialic acid in G_{M2}, ..., G_{T1b}. The data are taken from Tables I–V.

A search for general properties of oligosaccharide ^{13}C NMR spectra led us to investigate possible correlations among carbon chemical shifts and among oligomer–monomer shielding differences. A plot of the oligomer–monomer shielding difference, Δ_β, of the anomeric carbons against those found for their linkage mates, $\Delta_{\beta'}$, shows several interesting features (Figure 5), the most prominent of which is that when Δ_β is small $\Delta_{\beta'}$ tends also to be small. This suggests that the electronegative anomeric oxygen deshields both nuclei.

The points tend to distribute themselves into distinct, non-overlapping clusters distinguished by the configuration of the saccharide contributing the anomeric carbon, and by the position of attachment on the adjacent ring. We have included the linkage to the ceramide moiety on this plot also, even though the β' carbon is not in a ring structure. We should point out that a similar correlation holds for the chemical shifts (δ_β and $\delta_{\beta'}$) of the resonances of pairs of linked carbons. We chose to present shielding differences rather than chemical shifts to remove biasing from differences in chemical shifts between hydroxymethyl and ring hydroxyl carbon resonances. The largest shielding differences are seen to arise from neutral intersaccharide linkages. Positions of resonance pairs in (Δ_β, $\Delta_{\beta'}$)-space are characteristic of the type of linkage involved and may be used to support our assignments. Furthermore, the positions may be generally useful as aids in the assignment of the ^{13}C NMR spectra of unknown oligosaccharides, once tentative resonance pairing has been proposed.

The evident clustering of the data is described by the means and standard deviations of the points. Linkage of the type II1 → I4, Galβ(1→4)Glc, gives rise to a shielding difference pair of (6.41 ± 0.20 ppm, 9.20 ± 0.21 ppm), while a GalNAcβ-(1→4)Gal link, like III1 → II4, occurs at (7.65 ± 0.07 ppm, 8.98 ± 0.31 ppm). Linkage of the terminal β-D-Gal to residue III, Galβ(1→3)GalNAc, is responsible for the most pro-

nounced deshielding of any pair of β,β' resonances found in the gangliosides, with shielding differences falling at (8.26 ± 0.14 ppm, 9.84 ± 0.32 ppm). These shielding difference pairs may be compared with the smaller values found for the linkage of I1 → R1, in which only one member of the pair, residue I, is in a ring configuration. Here shielding differences of (6.79 ± 0.12 ppm, 7.35 ± 0.16 ppm) indicate that (Δ_β, $\Delta_{\beta'}$) is dominated, in this case, by the nature of carbon R1 as a hydroxymethyl rather than as a ring hydroxyl.

For the most part, shielding differences for the sialic acids are much smaller than those found for the neutral saccharides; however, differentiation among the data clusters proceeds along much the same lines as outlined above for the neutral saccharides. A reversal of the ordering of the linkage types according to the magnitude of Δ occurs when the clusters are examined with attention directed at the ring, or nonring, nature of the β' carbon. Sialic acid (β, β') linkage shifts fall into two separate clusters depending on the nature of the β'-carbon covalent bond participation. At exterior positions, exemplified by residue C, the sialic acid anomeric carbon C2 is linked to an exocyclic hydroxyl (A8) on the glyceryl side chain. It is not particularly surprising, therefore, to find a value of $\Delta_{\beta'}$ for A8 in NeuAc(α2→8)NeuAc (5.56 ± 0.42 ppm) close to that for R1 ($\Delta_{\beta'}$ = 7.35 ± 0.08 ppm). However, we did not expect the small shift (Δ_β = 3.87 ± 0.06 ppm) of the resonance from the anomeric carbon, C2, of sialic acid. In the neutral hexopyranoside residues of gangliosides, resonances from anomeric carbons shift 7.3 ± 0.8 ppm, while those of sialic acid shift only 3.9 ± 0.8 ppm. It is possible that the cause of this −3.4-ppm difference is an inherent property of the NeuAc anomeric carbon which is bound to a negatively charged carboxyl and two electronegative oxygens. Transmission of the carboxyl effect through space or through the glycosidic bond may also account for the lower $\Delta_{\beta'}$ value of 5.6 ± 0.4 ppm found for A8 as compared to $\Delta_{\beta'}$(R1) = 7.4 ± 0.1 ppm.

Even more pronounced is the small size of (Δ_β, $\Delta_{\beta'}$) for inter-ring linkages such as NeuAc(α2→3)Gal found in A2 → II3 and B2 → IV3. Resonances from these carbon pairs show linkage shifts of (3.76 ± 0.83 ppm, 2.03 ± 0.72 ppm) and (2.63 ± 0.04 ppm, 2.95 ± 0.06 ppm), respectively. Noteworthy here, and discussed below, is the relatively large dispersion in Δ_β (σ_β = ±0.83 ppm) for the anomeric carbon A2 of the sialic acid at an interior position in the oligosaccharide chain of the gangliosides. In fact, the data fall into three distinct clusters: (a) that for B2 → IV3 (see above), (b) A2 → II3 (2.69 ± 0.23 ppm, 3.07 ± 0.02 ppm) in the lower gangliosides G_{M4} and G_{M3}, and (c) A2 → II3 (4.19 ± 0.48 ppm, 1.61 ± 0.14 ppm) in the higher gangliosides containing the GalNAc residue III, namely, G_{M2}, G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}. We now shift our attention to γ effects.

Resonances from carbon nuclei which are γ to a linkage site undergo much smaller shifts, Δ_γ, of opposite sign from Δ_β. Among the neutral hexopyranosides in gangliosides, the average of Δ_γ = −1.00 ± 0.13 ppm compares favorably with −1.08 ± 0.22 ppm from the ^{13}C spectrum of lactose. Little variation was noted for Δ_γ with respect to changes in the aglycon. We did observe that Δ_γ for resonances from carbons simultaneously γ to two glycosidic linkages, such as II2, III2, and IV2, was twice as large at Δ_γ = −2.07 ± 0.21 ppm, as from a single adjacent γ carbon. It appears that the resulting Δ is the sum of two γ contributions.

In the sialic acids, Δ_γ refers to the oligomer–monomer shielding difference for the resonances from the methylene carbon. From their position γ to linkages, we expected only small ($\Delta_\gamma \lesssim -1$ ppm) negative shifts for the resonances from

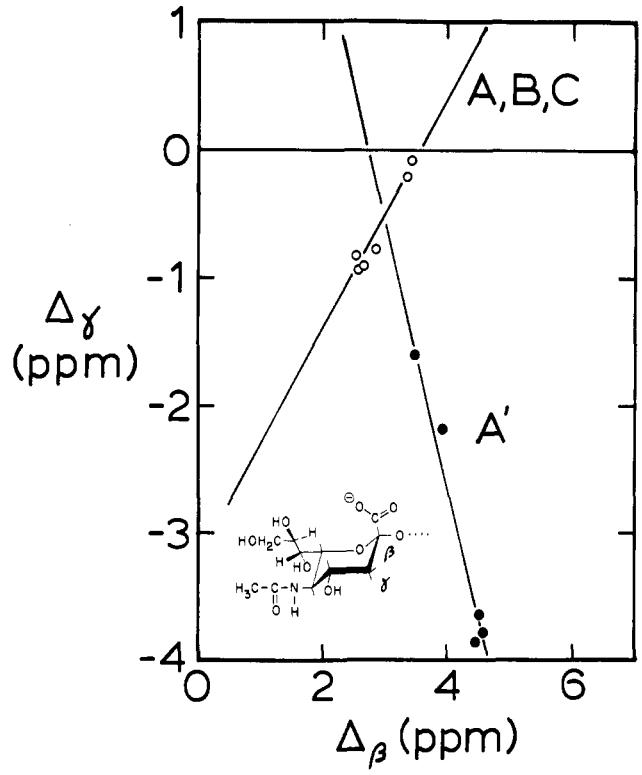

FIGURE 6: Relationships between the oligomer–monomer shielding differences, Δ, for the α-sialic acid anomeric, C_β, and methylene, C_γ, carbon resonances in the gangliosides G_{M4}–G_{T1b}. The open circles refer to data (Table IV) from sialic acids at exterior positions as indicated in Figure 1, while the filled circles refer to data (Table IV) from interior sialic acids, A′, adjacent to a GalNAc, residue III.

carbons A3, B3, and C3. Significant deviations from this simple scheme were found (Table IV). In the lower gangliosides, G_{M4} and G_{M3}, resonances from A3 show Δ_γ = −0.80 ppm. Shielding differences of $\Delta_\gamma(B_3)$ = −0.92 ppm and Δ_γ(C3) = −0.14 ppm were also found for the other sialic acids in exterior positions in the di- and trisialogangliosides. However, the resonance from carbon 3 in residue A in G_{M2} through G_{T1b} was found to shift −1 to −3 ppm, values quite atypical of γ effects in general. Furthermore, a closer examination of the data for carbons 2 and 3, of all the sialic acids in the gangliosides included in the present study, showed that there are correlations (Figure 6) among the shielding differences (Δ_β, Δ_γ). For sialic acids at position A in G_{M4} and G_{M3}, as well as at positions B and C for all the gangliosides examined to date, the linkage shifts fall along a line described by Δ_γ = $0.91\Delta_\beta - 3.26$. A very different behavior is shown by (Δ_β, Δ_γ) for A2 and A3 in G_{M2} through G_{T1b}, where the data fall along the line $\Delta_\gamma = -2.11\Delta_\beta + 5.80$. Prominent among the possible causes for this dichotomy is the change in magnetic environment in the neighborhood of the interior sialic acid, residue A, that accompanies addition of the GalNAc, residue III, to G_{M3} to form G_{M2}. We believe that the anisotropic electromagnetic susceptibility of the *N*-acetyl carbonyl on residue III perturbs the electron density at A2 and A3, giving rise to the observed unique (Δ_β, Δ_γ) for the nearby residue A, an idea proposed earlier by Harris & Thornton (1978). No such through-space interactions are possible for sialic acids at positions B or C or for residue A in the lower gangliosides. We have developed a quantitative theory (L. O. Sillerud, unpublished work) which predicts the shielding differences (Δ_β, Δ_γ) of residue A as a function of the distance from, and orientation of, the carbonyl on residue III. Changes in (Δ_β, Δ_γ), for example, on binding the oligosaccharide from G_{M1} to cholera toxin (Sillerud et al., 1981) can be related, therefore,

to conformational changes in the oligosaccharide. It is of substantial interest to compare these results with those for other protein–ganglioside interactions.

A remaining source of through-space interactions leading to Δ values larger than expected, simply on the basis of covalent structure, is hydrogen bonding. While it is important to exercise great care in attributing small chemical shift differences to hydrogen bonding, the large number of potential donor–acceptor pairs in oligosaccharides makes it virtually certain that at least some of the potential H bonds are formed. We will ignore intermolecular H bonds, since solvent water is likely to effectively compete for them, and concentrate our attention on possible intramolecular H-bonding sites, where covalent constraints are expected to render solvent competition improbable. One such site is between the hydroxyl on carbon I6 and the pyranose ring oxygen in residue II. The shielding difference for carbon I6 of -0.61 ± 0.12 ppm is rather large with respect to its position δ to a linkage site. A similar shift is found in lactose (-0.53 ± 0.02 ppm) (L. O. Sillerud, unpublished experiments). We have also found evidence suggesting an H bond between the hydroxyl on carbon 8 of the sialic acid glyceryl side chain and the carboxyl carbon, from a study of pH effects on the ^{13}C NMR spectrum of α-sialic acid (L. O. Sillerud, unpublished experiments). It is anticipated that other H bonds will be identified in gangliosides as further study of their ^{13}C NMR proceeds.

We have shown that the carboxyl adjacent to the anomeric carbon of sialic acids has a large influence on the resonances of ketosidic linkages. Thus, caution must be exercised when ^{13}C data for neutral saccharides are utilized in the interpretation of aspects of ^{13}C spectra of sialic acid containing oligosaccharides. Our assignments here for the gangliosides have addressed this point and may, therefore, be of significant value both for further work on glycolipids themselves and for the study of more complex and varied oligosaccharides, particularly those from glycoproteins, whose examination by means of ^{13}C NMR is just beginning (Dill & Allerhand, 1979).

We have concentrated our discussion on the analysis of the ^{13}C NMR spectra of the oligosaccharide portions of the gangliosides because that is where the most significant structural variation occurs. Not only does the detailed saccharide sequence determine the ganglioside classification but also it confers *specificity*, on the interactions of gangliosides with ligands, such as proteins, in the role gangliosides play as membrane receptors. Even though the ceramide portions of gangliosides do not share this diversity, they will play an important role in the *function* of these amphiphilic molecules as receptors. In the case of the interaction of cholera toxin with G_{M1}, we have recently shown that the ceramide portion of this ganglioside not only anchors G_{M1} to the membrane but also enables G_{M1} to induce a conformational change in the toxin B subunit that the oligosaccharide from G_{M1} is unable to do (L. O. Sillerud et al., unpublished experiments). Thus, even though there is less structural variation in the ceramide portion of these molecules, there is no less interest in the hydrophobic chains, and hence their ^{13}C NMR spectral assignments.

Acknowledgments

We thank Drs. S. Ando, H. C. Yohe, T. Miyatake, and T. Itoh for preparing some of the glycolipid samples. Our gratitude is extended to Professor Robert G. Shulman for the use of the WH360 spectrometer to obtain the G_{M3} spectrum. It is a pleasure to thank Professors James H. Prestegard and William H. Konigsberg for their encouragement and criticism of this work. We also thank Dr. Ian Armitage and Dr. Theodore Koerner for providing us with a preprint of their work on the ^{13}C NMR of the terminal glycooctapeptide of glycophorin and valuable discussions which enabled us to confirm our sialic acid assignments.

References

Ando, S., & Yu, R. K. (1977) *J. Biol. Chem. 252*, 6247–6250.

Ando, S., Chang, N. C., & Yu, R. K. (1978) *Anal. Biochem. 89*, 437–450.

Cochran, F. B., Yu, R. K., Ando, S., & Ledeen, R. W. (1981) *J. Neurochem. 36*, 696–702.

Colson, P., Jennings, H. J., & Smith, I. C. P. (1974) *J. Am. Chem. Soc. 96*, 8081–8087.

Cuatrecasas, P. (1973) *Biochemistry 12*, 3547–3557.

Dabrowski, J., Egge, H., & Hanfland, P. (1980) *Chem. Phys. Lipids 26*, 187–196.

Deleers, M., Chatelain, P., Poss, A., & Ruysschaert, J. M. (1979) *Biochem. Biophys. Res. Commun. 89*, 1102–1106.

Dill, K., & Allerhand, A. (1979) *J. Biol. Chem. 254*, 4524–4531.

Dorman, D. E., & Roberts, J. D. (1971) *J. Am. Chem. Soc. 93*, 4463–4472.

Dreyfus, H., Louis, J. C., Harth, S., Pettmann, B., Urban, P. F., & Mandel, P. (1979) in *Glycoconjugates: Proceedings of the Fifth International Symposium* (Schauer, R., et al., Eds.) pp 704–705, Georg Thieme Verlag, Stuttgart, Federal Republic of Germany.

Fishman, P. H., & Brady, R. O. (1976) *Science (Washington, D.C.) 14*, 906–915.

Harris, P. L., & Thornton, E. R. (1978) *J. Am. Chem. Soc. 100*, 6738–6748.

Hilbig, R., & Rahmann, H. (1979) in *Glycoconjugates: Proceedings of the Fifth International Symposium* (Schauer, R., et al., Eds.) pp 708–709, Georg Thieme Verlag, Stuttgart, Federal Republic of Germany.

Holmgren, J., Lönnroth, I., Mansson, J.-E., & Svennerholm, L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2520–2524.

Itoh, T., Li, S.-C., Li, Y.-T., & Yu, R. K. (1981) *J. Biol. Chem. 256*, 165–169.

IUPAC–IUB Commission on Biochemical Nomenclature (1977) *Lipids 12*, 455–468.

Jennings, H. J., & Bhattacharjee, A. K. (1977) *Carbohydr. Res. 55*, 105–112.

Koerner, T. A. W. J., Cary, L. W., Li, S.-C., & Li, Y.-T. (1979) *J. Biol. Chem. 254*, 2326–2328.

Ledeen, R. W., & Yu, R. K. (1973) in *Lysosomes and Storage Diseases* (Hers, H. G., & Van Hoof, F., Eds.) pp 105–145, Academic Press, New York.

Ledeen, R. W., & Yu, R. K. (1978) *Res. Methods Neurochem. 4*, 371–410.

Ledeen, R. W., Yu, R. K., & Eng, L. F. (1973) *J. Neurochem. 21*, 829–839.

Maget-Dana, R., Veh, R. W., Sander, M., & Monsigny, M. (1979) in *Glycoconjugates: Proceedings of the Fifth International Symposium* (Schauer, R., et al., Eds.) pp 443–444, Georg Thieme Verlag, Stuttgart, Federal Republic of Germany.

Momoi, T., Ando, S., & Nagai, Y. (1976) *Biochim. Biophys. Acta 441*, 488–497.

Morre, D. J., Kloppel, T. M., & Walter, V. (1979) in *Glycoconjugates: Proceedings of the Fifth International Symposium* (Schauer, R., et al., Eds.) pp 617–618, Georg Thieme Verlag, Stuttgart, Federal Republic of Germany.

Mullin, B. R., Fishman, P. H., Lee, G., Aloj, S. M., Ledley, F. D., Winand, R. J., Kohn, L. D., & Brady, R. O. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 842–846.

Mullin, B. R., Pacuszka, T., Lee, G., Kohn, L. D., Brady, R. O., & Fishman, P. H. (1977) *Science (Washington, D.C.) 199*, 77–79.
Rahmann, H., & Hilbig, R. (1979) in *Glycoconjugates: Proceedings of the Fifth International Symposium* (Schauer, R., et al., Eds.) pp 672–673, Georg Thieme Verlag, Stuttgart, Federal Republic of Germany.
Rosenthal, S. N., & Fendler, J. H. (1976) *Adv. Phys. Org. Chem. 13*, 279–424.
Sattler, J., Schwarzmann, G., Staerk, J., Ziegler, W., & Wiegandt, H. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 159–163.
Sillerud, L. O., Prestegard, J. H., Yu, R. K., Schafer, D. E., & Konigsberg, W. H. (1978) *Biochemistry 17*, 2619–2628.
Sillerud, L. O., Schafer, D. E., Yu, R. K., Prestegard, J. H., & Konigsberg, W. H. (1979) *Biophys. J. 25*, 133a.
Sillerud, L. O., Prestegard, J. H., Konigsberg, W. H., Schafer, D. E., & Yu, R. K. (1981) *J. Biol. Chem. 256*, 1094–1098.

Influence of Phospholipid Structure on Sterol Efflux Induced by Albumin–Phospholipid Complexes†

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ABSTRACT: Sterol release from strain L fibroblasts was measured in serum-free medium supplemented with delipidated human serum albumin and various phospholipids. The sterol molecule appears to preferentially interact with the *sn*-2 acyl chain of the phospholipid. The carbonyl oxygen of the phospholipid acyl ester linkage is not required for sterol–phospholipid interactions, while the phosphate and choline groups are required. In the presence of the human serum albumin–phospholipid complex, phospholipids containing *trans*-acyl groups are significantly more effective at removing cellular sterol than the corresponding *cis*-acyl group.

Many studies of sterol–phospholipid interactions involve protein-free model membrane systems. These systems have produced conflicting evidence regarding the nature of apparent hydrogen bonding between the sterol hydroxyl group and either the carbonyl oxygen atom of the phospholipid acyl ester linkage (Huang, 1976; Brockerhoff, 1974) or the phosphate base group (Darke et al., 1972; Verma & Wallach, 1973). In addition, preferential interactions between the sterol molecule and the acyl chains of the phospholipid have been suggested (Huang, 1977; Bloch, 1979; Seelig & Seelig, 1980; Bush et al., 1980) but not confirmed in biological systems.

In the studies reported here, we have investigated sterol–phospholipid interactions in a lipoprotein-like complex of albumin–phospholipid. Previous work from this laboratory (Chau & Geyer, 1978; Bartholow & Geyer, 1981) has established that homogeneous human serum albumin can bind dipalmitoylphosphatidylcholine and that the resulting complex can cause a synergistic release of sterol from human skin and mouse L fibroblasts in tissue culture. Human serum albumin also binds dilinoleoylphosphatidylcholine, but this combination caused significantly less sterol efflux than the saturated phosphatidylcholine–albumin combination. One explanation for the difference in effects between the saturated and unsaturated complexes is that the double bonds of the polyunsaturated phosphatidylcholine introduced sufficient bending (Spritz & Mishkel, 1969) to interrupt hydrophobic interactions between the phospholipid acyl methylene carbon atoms and the sterol molecule. We have attempted to characterize this sterol efflux in terms of sterol–phospholipid interactions and show that (1) the reduction in sterol efflux observed with the polyunsaturated phosphatidylcholine–albumin complex is related to chain length, (2) hydrogen bonding between the carbonyl oxygen atom of the acyl ester linkage and the sterol hydroxyl is not required for sterol efflux, (3) choline and phosphate groups each contribute to the interaction with the sterol molecule, and (4) there appears to be a preferential interaction between the sterol molecule and the *sn*-2 acyl chain.

Experimental Procedures

Compounds. Crystalline human serum albumin was obtained from Miles Laboratories, horse serum from Grand Island Biological Co., and [1-^{14}C]acetate (56 mCi/mmol) from New England Nuclear Corp. The phospholipids were provided by Calbiochem-Behring Corp., with the exception of dielaideoylphosphatidylcholine, which was obtained from Sigma Chemical Co. A standard lipid mixture (mixture B) was obtained from Supelco while silica gel G plates were obtained from Analabs, Inc.

Preparation of Compounds. Both the human serum albumin and the horse serum were delipidated by the method of Rothblatt et al. (1976), as previously reported (Bartholow & Geyer, 1981). The aqueous proteins were then sterilized by filtration through a 0.22-μm Millipore filter before addition to the cells.

The phospholipids were dissolved in heptane and evaporated to dryness under N_2. NaCl (0.9%) was added to give a concentration of 1 mg/mL. The solution was then sonicated at 60% of full power under N_2 for two 1-min bursts with a Biosonik sonicator having a microtip of 8 mm. The resulting dispersions, prepared in 20-mL sterile glass scintillation vials, were slightly opaque. The sonicated lipids were used immediately after preparation at a final concentration in the incubation medium of 50 μg/mL. Thin-layer chromatography of 50 μg of each phospholipid gave a single spot with iodine visualization.

Cell System. Stock cultures of mouse fibroblasts (L strain) were grown in suspension at 37 °C in 50 mL of modified

† From the Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts 02115. *Received June 2, 1981.* This work was supported by the Harvard Nutrition Fund of the Department of Nutrition.

0006-2960/82/0421-1271$01.25/0