of the ring are involved is in complete accord with recent ab initio studies of five-membered rings. Cyclopentanone, with a planar trigonal carbon in the ring, is found to have its energy minimum in the T form with interconversion through a planar intermediate,[24] while methylcyclopentane favors the E form with the methyl group occupying the equatorial position.[24]

Our result for the cyclopentyl radical must be compared with the values of 2700–2800 cal/mol reported by Trofimov and co-workers.[7,8] There are not any obvious important differences in experimental procedures and they did not obtain data at sufficiently low temperatures to confirm or deny our observation of a different inversion mechanism. However, the methods of analysis are somewhat different, in that they used a computer program based on a solution of the Bloch equations for the case of two exchanging spins to determine the relative intensities of a broadened and nonbroadened line in the spectrum. We therefore used a similar program to check our results for cyclopentyl and confirmed our original program. Thus we cannot explain the discrepancy except to suggest a systematic error either in programming or in interpretation. The latter might be caused by their large line widths (~25 G).

Finally, we would like to compare our results for radicals with values for inversion barriers in closed-shell compounds. Cyclopentanone has a planar carbon in the ring and is therefore similar to our planar radicals. Both experimental and theoretical results show that the T form is dominant with barriers to planarity of the ring of 2.1 kcal/mol[25] (experimental) and 2.6 kcal/mol[24] (theoretical). Methylenecyclopentane is similarly twisted with a barrier of 1.8 kcal/mol.[25] Therefore, it appears that five-membered rings with a planar trigonal carbon in the ring have barriers for inversion through planar intermediates in the range of 1–2 kcal/mol. On the other hand, substituted cyclopentanes that are nonplanar at the radical site and elsewhere appear to have generally higher barriers that are strongly influenced by the nature of the substituent group. There is no experimental determination of the potential energy surface for methylcyclopentane but the barrier for inversion through a planar intermediate is calculated to be 4.0 kcal/mol.[23]

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Carbon-13 Nuclear Magnetic Resonance Spin–Lattice Relaxation in the *N*-Acylneuraminic Acids. Probes for Internal Dynamics and Conformational Analysis[1a–c]

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Abstract: ^{13}C NMR studies of several *N*-acylneuraminic acid derivatives have been made. Spin–lattice relaxation times (NT_1^{DD} = ca. 0.3 s) indicate that the pyranose ring carbons undergo isotropic rotation and that C-7 and C-8, but not C-9, are isotropic with the ring. A model involving an intramolecular hydrogen bond network is supported by the relaxation data. It is shown that calculated values of T_1^{DD} for nonprotonated carbons agree closely with experiment. The isolation of *N*-acetylneuraminic acid from Oriental birds' nest substance is shown to be a convenient source of this compound.

The acyl derivatives of neuraminic acid, the *N*-acetylneuraminic acid having the widest biological distribution, are α ketosidically linked to the glycoproteins and glycolipids of neuronal and other cell membranes.[2] Representing the most complex group of naturally occurring monosaccharides, the neuraminic acid derivatives contain a variety of functionality unusual in carbohydrate chemistry. Moreover, recent evidence has specifically implicated the acylneuraminic acid moieties of glycolipids and glycoproteins in specific mechanisms of cell function, such as intercellular recognition, hormone reception,

and antigen masking.[2a,c] Therefore, the structures and conformations of the neuraminic acids are of significant interest to organic and biological chemists alike.

NMR represents perhaps the most general technique for the structural analysis of carbohydrates.[3] Early ^1H NMR studies of *N*-acetylneuraminic acid (NeuNAc) succeeded mainly in establishing the pyranose ring conformation and configuration at the anomeric carbon.[4] More recently, though, through the use of superconducting field strengths and computer spin-simulation techniques, Brown and co-workers have completely analyzed the ^1H NMR spectrum of NeuNAc[5] and have derived fundamental conformational information based on the analysis of vicinal coupling constants. ^{13}C NMR spectra of several neuraminic acid derivatives have also been measured. The analysis of these spectra has proved very useful in structural studies of neuraminic acid containing oligo- and polysaccharides.[6] The ability of ^{13}C NMR spin–lattice relaxation times (T_1's) to provide unique dynamical and conformational information for a wide variety of organic and biological molecules is well documented.[7] Therefore, we have investigated, in some detail, the ^{13}C NMR and in particular the T_1 values of the acylneuraminic acids.

The studies presented here are of interest for the following reasons: (1) A versatile technique for the isolation of NeuNAc has been developed and optimized. (2) An analysis of T_1 values as a reflection of molecular dynamics is presented. (3) A conformational model, consistent with the ^1H NMR and with the motional requirements of the T_1 values, is proposed. (4) An analysis of the T_1 values for nonprotonated carbons has proved possible. Furthermore, the application of ^{13}C NMR spin–lattice relaxation techniques to carbohydrate systems has not been extensively investigated.[8] Thus, in order to approach the study of the conformations and dynamics of the complex cell-surface carbohydrates using these powerful techniques, a detailed study of the constituent monosaccharides is clearly required.

Experimental Section

Infrared spectra (IR) were recorded on a Perkin-Elmer Model 735. ^1H NMR were determined on a Varian Associates Model A-60A and referenced to internal DSS. Melting points were measured on a Thomas-Hoover melting point apparatus and are reported uncorrected. Microanalyses were determined by Midwest Microlabs, Indianapolis, Ind. Titrations and pH measurements were done on a Radiometer pH meter Model 25 fitted with a GK2322C combination glass electrode.

Isolation of *N*-Acetylneuraminic Acid (1a). One box of edible birds' nest substance (180 g), which can be purchased at Chinese groceries, was divided into three portions. Each portion was homogenized in 100 mL of water for about 30 s in a Waring blender. The homogenate was added to 6 L of 0.025 M H_2SO_4 and heated with stirring for 2 h at 60–70 °C. Subsequent portions were treated identically. After hydrolysis was completed the mixture was filtered through Whatman No. 2 in a large Büchner funnel. The clear filtrate was then treated by a modification of Svennerholm's method.[9] The filtrate was neutralized to pH 5–6 with saturated $Ba(OH)_2$ solution. After storing for 8–12 h at 4 °C the clear hydrolysate could be decanted from the $BaSO_4$ precipitate. The combined hydrolysates were then passed through coupled columns of Dowex-50-H^+ (3.0 × 30 cm) and Dowex-1-HCO_2^- (3.5 × 50 cm) which were then washed with 4 L of distilled water. The Dowex-1 column was then eluted with 4 L of a 0–2 M linear formic acid gradient and collected in 25-mL fractions. The fractions were treated with Roseman's periodate–resorcinol reagent;[10] tubes with a positive test were pooled and lyophilized. Typical yield was 10–12 g. The white powder was used without purification in subsequent syntheses, but was crystallized from water–methanol–ether (mp 184–186 °C dec) for NMR studies and analysis. Anal. ($C_{11}H_{19}NO_9$) C, H.

Synthesis of Modified Neuraminic Acids. A. Standard literature procedures were used to prepare most of the synthetic neuraminic acids. Methyl β-D-*N*-acetylneuraminate (**1b**) and 2-*O*-methyl-β-D-*N*-acetylneuraminic acid (**1c**) were prepared by the method of Kuhn et al.[11] by treatment with anhydrous methanol in the presence of Dowex-50, **1b** being prepared by stirring in the above mixture for 5 h at room temperature while **1c** required 36–48 h at reflux followed by saponification in aqueous base. The modified Koenigs–Knorr procedures of Meindl and Tuppy were used to prepare 2-*O*-methyl-α-D-*N*-acetylneuraminic acid (**1d**)[12] and 2,3-dehydro-D-*N*-acetylneuraminic acid (**3**).[13] The 2,4,7,8,9-penta-*O*-acetyl-*N*-acetylneuraminic acid (**5**) was prepared by acetylation of **1a** in pyridine–acetic anhydride according to the procedure of Meindl and Tuppy.[12] β-D-*N*-Glycolylneuraminic acid (**2**) was synthesized using Schauer's procedure by the acylation of methyl 2-*O*-methyl-β-D-neuraminate (**6**, see preparation below) with 1,4-dioxolanedione.[14]

B. Methyl 2-*O*-methyl-β-D-*N*-formylneuraminate (**4**) was synthesized by the formylation of **6**, prepared from **1a** in the following manner: 3.0 g of thoroughly dried **1a** was dissolved in 100 mL of CH_3OH and 8.0 mL of acetyl chloride (~5% HCl, see ref 15) and the solution placed in a Wheaton pressure bottle (A. H. Thomas). The bottle was sealed and heated for 3 h in a steam bath. The bottle was cooled and opened and the reddish-brown solution concentrated on a rotary evaporator; methanol was added and reevaporated several times from the product, which was finally concentrated to dryness. The residue was dissolved in water, neutralized to pH 5 with NaOH solution, and filtered through a 0.6 μ Millipore filter. The clear, reddish-brown filtrate was purified by high-pressure liquid chromatography (Waters Associates ALC-100 fitted with Model U6K sample injector, M-6000 pump, and differential refractive index detector) on Porasil C-18 (7 mm × 244 cm) with distilled water as the eluent. The major peak was collected and lyophilized to give 1.4–1.6 g of white powder (40–50% yield based on **1a**) which was the hydrochloride salt of **6** with perhaps some free amine **6**. The reddish-brown product of the methanolysis is not eluted with water from Porasil C-18 but may be conveniently removed after the above purification is completed by flushing with methanol. No apparent degradation of the columns occurred even after several purifications. Characterization: IR (KBr) 1745 cm^{-1}; ^1H NMR (D_2O) δ_H (DSS) 1.4–1.7 (m, 3 H), 3.3 (s, 3 H), 3.8 (s, 3 H), 3.2–4.4 (m, 6 H); ^{13}C NMR δ_C (Me_4Si) 39.9, 52.0, 52.9, 54.3, 63.6, 65.3, 68.3, 69.7, 70.3, 99.8, 170.4.

The synthesis of **4** proceeded as follows: 331 mg of **6**, 2.06 g of dicyclohexylcarbodiimide (Eastman), and 202 mg (0.277 mL) of triethylamine (Aldrich) were dissolved in 30 mL of absolute methanol. After cooling to 0 °C in an ice bath, 460 mg (0.395 mL) of formic acid (Aldrich 97+%) dissolved in 5 mL of methanol was added slowly from an addition funnel to the vigorously stirred solution. After 3 h of stirring the mixture was filtered to remove the dicyclohexylurea and the solvent removed on a rotary evaporator. The residue was triturated with 40 mL of water and filtered, and the filtrate was passed through a column of Amberlite MB-1 (2.8 × 4.0 cm) which was washed with 30 mL of deionized water. After the solution was concentrated to 20 mL on a rotary evaporator the liquid was lyophilized to give a white solid, 277 mg (86% based on **6**) of the formylated derivative **4**. Although essentially pure at this point, the powder could be crystallized from methanol–ether (mp 124–126 °C dec). Anal. ($C_{12}H_{21}O_9N$) C, H. IR (KBr) 1740, 1670 cm^{-1}; ^1H NMR (D_2O) δ_H (DSS) 1.7–2.0 (m, 1 H), 2.2–2.4 (m, 1 H), 3.3 (s, 3 H), 3.4–4.3 (m, 7 H), 3.9 (s, 3 H), 8.1, 8.3 (*E* and *Z* formyl protons, 1 H); ^{13}C NMR, see text.

Sample Preparation for Spin–Lattice Relaxation Experiments. In order to minimize the effects of adventitious paramagnetic ions on relaxation times measured in D_2O solution, special precautions were observed.[16] All glassware and plasticware used in the final stages of preparation, including pipettes, tube, cap, vortex plugs, etc., were soaked for 12–24 h in alkaline EDTA solution, then rinsed thoroughly with deionized water. D_2O (Stohler Isotope 99.8%) was distilled and the distillate extracted three times with 0.05% dithizone solution in CCl_4. Free acids were converted to their sodium salts by titration with NaOH solution to pH 7.8 ± 0.2 followed by lyophilization. D_2O solutions were prepared in a graduated 15-mL conical centrifuge tube fitted with a plastic stopper. Solutions were extracted three times with the dithizone solution by vigorous shaking in the centrifuge tube, centrifuging to separate the layers, and removing the CCl_4 layer with a pipet. The extracted solution was transferred to a 10-mm NMR tube and degassed by purging with nitrogen, approximately 0.5 mg of EDTA was added, and the tube was fitted with a Teflon vortex plug.

^{13}C NMR Spectra. ^{13}C NMR spectra were measured at 25.03 MHz on a JEOL PFT-100 NMR spectrometer equipped with PG-100 pulse programmer and the JEOL sample temperature controller. Samples

Table I. ^{13}C NMR Chemical Shifts of Neuraminic Acid Derivatives

C	1a	1b	1c	1d	2	3	4	5
1	176.8[a]	171.5[b]	175.3[a]	173.5[a]	176.8[a]	169.7[a]	170.4[b]	*f*
2	96.5	95.4	100.6	100.8	96.5	148.1	99.2	99.6
3	39.6	38.8	39.9	40.2	39.6	107.8	39.2	36.7
4	67.4	66.8	67.2	68.3	67.1	67.7	66.2 (66.0)	67.6
5	52.4	52.2	52.1	52.1	52.1	50.1	50.5 (55.1)	49.0
6	70.4	70.2	70.2	72.7	70.1	75.5	69.9	69.6[e]
7	68.6	68.3	68.5	68.3	68.5	68.4	67.9 (67.6)	69.5[e]
8	70.5	70.5	70.2	71.8	70.5	70.0	70.3	70.8[e]
9	63.5	63.3	63.6	62.8	63.4	63.3	63.4	61.6
10	175.5	174.9	174.8	175.2	175.6	174.9	164.8 (167.7)	*f*
11	22.3[g]	22.2[g]	22.3[g]	22.2[g]	61.2[h]	22.4[g]	53.6[c]	*f*
12		53.7[c]	50.6[d]	51.8[d]			51.1[d]	

[a] Carboxylate carbon. [b] Ester carboxyl carbon. [c] Ester methyl carbon. [d] Ketoside methoxy carbon. [e] Tentative assignments. [f] Not assigned owing to complexity of carbonyl and methyl regions of spectrum. [g] Acetyl methyl carbon. [h] Glycolyl hydroxymethylene carbon.

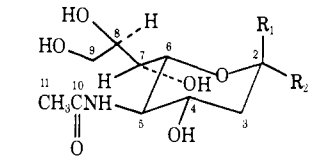
were studied under conditions of complete proton noise decoupling (decoupling power and frequency set to give full decoupling using a 2.5-KHz noise bandwidth) with an internal deuterium field/frequency lock. Chemical shifts were measured relative to the methyl resonance of external acetone and are cross-referenced and reported relative to external Me_4Si. The temperature was controlled at 28.0 ± 1.0 °C.

T_1 values were measured using the fast inversion-recovery method (FIRFT)[17] over a 4000-Hz bandwidth with 8K time domain data points. The $\pi/2$ pulse length was calibrated prior to each set of experiments and varied between 17 and 23 μs. Relaxation times were calculated from sets of spectra representing 10–12 τ values using a least-squares fit to the semilogarithmic plot. Reported values represent the mean of three or more determinations with standard deviations of 10–15%. NOE's ($\eta + 1$) were measured from integrated intensities using the gated decoupling technique of Freeman and Hill;[18] reported values are generally the mean of three or more measurements. T_1^{DD} values were calculated from the experimentally determined T_1 and η values using the equation $T_1^{DD} = 1.988T_1/\eta$. With the exception of the carboxylate carbons the pulse repetition time was $\geq 10T_1$ for all carbons examined; in the case of the carboxylates which have very long T_1 values the repetition times were $\sim 5T_1$ values. The very high ratio of recycle time to decoupler on-time reduces the cross-correlation effect to within experimental error for the measurement (estimated error <4%). These conditions are in accordance with the most recent requirements demonstrated for the gated decoupler sequence.[19]

Results and Discussion

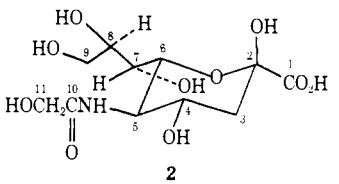
Isolation of *N*-Acetylneuraminic Acid. We have isolated NeuNAc (**1a**) and have synthesized seven acylneuraminic acid derivatives (**1b–d, 2–5**). Synthetic modifications of carbohydrates, especially inherently unstable species such as the neuraminic acids, are often complicated by poor yields owing to side reactions and difficult purifications. The relatively large amounts of modified neuraminic acids that we required for extensive ^{13}C NMR and other studies necessitated abundant quantities of **1a** as a starting material in synthetic schemes. Previously described sources of **1a** were of human (whole blood, milk, meconium) or bovine origin (submaxillary mucin, cow colostrum) and generally somewhat difficult to obtain and prepare.[9,20] Schauer and co-workers have reported the isolation of methoxyneuraminic acid from edible birds' nest substance (BNS) under vigorous methanolysis conditions.[14b] We have found that under substantially milder aqueous hydrolytic conditions **1a** can be isolated from BNS in high yield and without contamination of other acylneuraminic acids. The ease with which it can be handled and the high yields of analytically pure **1a** that it can produce make it likely that BNS will replace the classical sources of NeuNAc.

^{13}C NMR Data. Chemical Shifts and Coupling Constants.

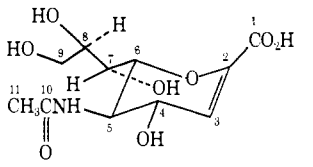


	R_1	R_2
1a	OH	CO_2H
1b	OH	CO_2CH_3
1c	OCH_3	CO_2H
1d	CO_2H	OCH_3

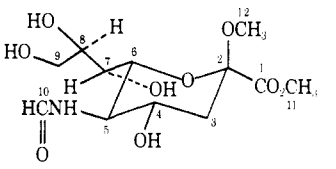
1



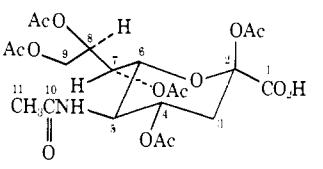
2



3



4



5

The ^{13}C NMR chemical shifts for **1–5** are presented in Table I. These chemical shifts and assignments correspond closely with published results which were based upon empirical chemical shift correlations, off-resonance and specific proton decoupling, as well as chemical modification.[6] The chemical shifts of methyl 2-*O*-methyl-*N*-formylneuraminate (**4**), which we have synthesized by a new and convenient method, are presented here for the first time.[21] The spectrum of **4** shows interesting doublets for four of the resonances, which are recorded in parentheses. These doublets result from isomerization of the formamido group. The *Z* isomer, in which the carbonyl oxygen and C-5 are eclipsed, has the resonances of C-5 and C-10 shifted strongly upfield relative to the *E* isomer.[22] Integration of these resonances indicates that the *Z* isomer predominates in the 85:15 (*Z*:*E*) mixture. Smaller shifts observed at C-4 and C-7 result from conformational interactions discussed below. The chemical shifts for the C-5 resonances, which vary between δ_C (Me_4Si) 49.0 and 52.4, correspond more closely to the *Z* isomer of **4** (δ_C (Me_4Si) 50.5) than to the *E* isomer (δ_C (Me_4Si) 55.1). Since it has been shown that *E*–*Z* isomerization in acetamides can also be resolved in the ^{13}C NMR,[23] it follows that the *N*-acetyl derivatives, which show only one set of resonances, exist exclusively as the *Z* isomers, in agreement with the observed x-ray crystal structure of NeuNAc dihydrate.[24]

Table II. Experimental[a] and Predicted[b] Values for $^3J_{C,H}$ (Hz) and Dihedral Angle (∠) Correlations

	C-1, H-3_{eq}		C-1, H-3_{ax}	
	Exptl $^3J_{C,H}$(∠)	Pred $^3J_{C,H}$(∠)	Exptl $^3J_{C,H}$(∠)	Pred $^3J_{C,H}$(∠)
1c	1.3 (55°)[c]	1.0 (60°)	1.3 (55°)[c]	1.0 (60°)
1d	2.0 (45°)[d]	1.0 (60°)	5.1 (155°)	7.8 (180°)

[a] Calculated from the fully coupled spectra using 8192 time domain points over a 1000-Hz spectral width giving a frequency domain resolution of 0.24 Hz/pt. [b] Based on completely axial or equatorial orientation of C-1 in **1d** and **1c**, respectively. [c] Estimated from line width of unresolved doublet of doublets. [d] Measured from line width of resolved axial coupling doublet.

The ^{1}H NMR studies of Brown et al.[5] established the anti relationships between the axial protons on the pyranose ring. Conformational analysis at the anomeric position (C-2) of the pyranose ring has not, however, been attempted. Lemieux has demonstrated the sensitivity of vicinal ^{13}C-^{1}H coupling constants to the magnitude of the dihedral angle subtended by the planes containing the atoms, and has derived an empirical correlation relating these two quantities.[25] Therefore, we have measured the vicinal coupling between the carboxylate (C-1) and the hydrogens (H-3_{ax} and H-3_{eq}) in the fully coupled spectra of the β (**1c**) and α (**1d**) methyl glycosides. We have applied the Lemieux correlations in order to evaluate the ring conformation at the anomeric position (Table II). The angle of 55° observed in **1c** corresponds closely to the expected value of 60° for an equatorial orientation of C-1. In the α glycoside (**1d**), however, the angles derived from the coupling constants, 155° to H-3_{ax} and 45° to H-3_{eq}, are smaller than the expected angles of 180 and 60°, respectively. The 10° inconsistency in the degree of distortion is well within the accuracy of Lemieux correlation curves. The clear qualitative agreement, though, strongly indicates an angular distortion, probably 15–25°, at this position. Although other mechanisms cannot be ruled out, it seems likely that steric effects between the axial ring hydrogens and the tightly solvated carboxylate anion could make this angular distortion necessary.

^{13}C NMR T_1 Values. We have measured the spin-lattice relaxation times (T_1^{obsd}) and nuclear Overhauser enhancements (η) and present these in Table III. The value of η is a measurement of the fraction of the spin-lattice relaxation due to proton-mediated dipole-dipole interactions. T_1^{obsd} is composed of contributions from this dipole-dipole mechanism and any other mechanism that can compete effectively, according to[7]

$$\frac{1}{T_1^{obsd}} = \frac{1}{T_1^{DD}} + \frac{1}{T_1^{other}} \quad (1)$$

When dipole-dipole interactions with protons contribute exclusively to the relaxation of a carbon the value of $\eta = 1.988$, the theoretical maximum when the extreme-narrowing condition is satisfied. The measurement of η allows the calculation of the dipole-dipole component of T_1^{obsd} when more than one relaxation mechanism contributes. We have calculated T_1^{DD}, using eq 2,[7] and present these values in Table III.

$$T_1^{DD} = \frac{1.988}{\eta} T_1^{obsd} \quad (2)$$

The value of T_1^{DD} for a protonated carbon can be related to the motional characteristics of that carbon with eq 3[7]

$$\frac{1}{T_1^{DD}} = N\gamma_C^2\gamma_H^2\hbar^2\tau_c r_{C-H}^{-6} \quad (3)$$

where N is the number of directly attached protons, γ_C and γ_H are the magnetogyric ratios for carbon and hydrogen, respectively, τ_c is the correlation time defined as the time required for reorientation of the C–H vector through 1 rad, and r_{C-H} is the carbon–hydrogen bond length. Thus when internuclear distances are the same, true for most C–H bonds, then the values of NT_1^{DD} and τ_c are inversely proportional, i.e., longer NT_1^{DD} values indicate shorter correlation times and hence more rapid motion.

Analysis of T_1^{DD} Values. Using these principles, differences in the values of NT_1^{DD} within a given molecule can be used as internal molecular mobility parameters for protonated carbons. Examination of the values for NT_1^{DD} demonstrates that certain generalizations, applicable to all the acylneuraminic acid derivatives (**1a–d, 2–5**), are possible. The long values of NT_1^{DD} observed for methyls are indicative of the rapid internal reorientation generally observed for methyl groups. Equivalence of NT_1^{DD} values for C-3 through C-6 is consistent with a rigid ring undergoing isotropic molecular reorientation.[26] It is also significant to note that C-7 and C-8 have NT_1^{DD} values equivalent to C-3–C-6, indicating that the motion of these carbons is isotropic with the pyranose ring.

Since the value of NT_1^{DD} for C-9 is consistently longer than C-3 through C-8 the presence of internal motion, about the C-8–C-9 single bond, independent of molecular diffusion is indicated. The only apparent exception to this observation is **5**, in which the mass and the steric effects of the acetate minimize the independent motion at C-9. It is important to note that the motion of C-9 in **1d** is not substantially different from that in **1c** despite an intuitively attractive hydrogen bond, between the C-9 hydroxyl proton and the axial carboxylate, available in the α glycoside (**1d**). The longer value for NT_1^{DD} in the hydroxymethylene of the glycolic acid residue of **2** is consistent with the lower energy requirement expected for its sixfold rotational barrier when compared to the threefold barrier at C-9.[27]

The complete analysis of the ^{1}H NMR spectrum of NeuNAc (**1a**) by Brown and co-workers established the conformational relationships between protons of the glycerol side chain based on vicinal coupling constants.[5] They demonstrated that H-6 and H-7 were gauche and that H-7 and H-8 were anti. A conformational model was postulated which involved two intramolecular hydrogen bonds mediated through five-membered ring interactions. In their model the C-7 hydroxyl proton is hydrogen bonded to the pyranose ring oxygen and the C-8 hydroxyl proton is bonded to the oxygen at C-9.

This model, however, is not supported by our measurements since only C-7 would be expected to undergo motion that was isotropic with the pyranose ring; moreover, one would predict equivalent values of NT_1^{DD} for C-8 and C-9. There are two models which, in principle, are consistent with our relaxation measurements. One model in which the C-8 oxygen is hydrogen bonded to the amido N–H and the C-7 hydroxyl proton is bonded to the pyranose ring oxygen conflicts with the ^{1}H NMR, since in all reasonable conformations this model would require an anti relationship between H-6 and H-7. Therefore, we wish to propose the following model (Figure 1) which is consistent with the ^{1}H NMR and the motional constraints required by our measurements of T_1^{DD}. In this model the C-8 hydroxyl proton is intramolecularly hydrogen bonded to the pyranose ring oxygen through a six-membered ring. The oxygen at C-7 can then intramolecularly hydrogen bond with the amido NH. This model meets the requirement that C-7 and C-8 undergo motion isotropic with the ring and predicts independent motion at C-9. It is interesting to note that additional stabilization for this conformation is still possible since the acetamido carbonyl oxygen is favorably placed to interact with the proton on the C-4 hydroxyl in a third intramolecular hydrogen bond mediated through a seven-membered ring.

In retrospect, observed chemical shift dependencies appear to support this model. In addition to the expected sensitivity

Table III. ^{13}C T_1's (s) and η Values of Neuraminic Acid Derivatives Measured in 0.68 M D_2O Solutions

	1a			**1b**			**1c**			**1d**		
C	T_1^{obsd}	η	T_1^{DD}	T_1^{obsd}	η	T_1^{DD}	T_1^{obsd}	η	T_1^{DD}	T_1^{obsd}	η	T_1^{DD}
1	15[a]	1.3	23	14[b]	1.4	20	13[a]	1.4	19	14[a]	1.3	22
2	5.0	1.7	5.9	5.5	1.9	5.8	4.3	1.7	5.1	6.0	2.0	6.0
3	0.13	2.0	0.13	0.17	2.0	0.17	0.12	2.0	0.12	0.15	2.0	0.15
4	0.32	2.0	0.32	0.30	1.9	0.32	0.25	1.9	0.26	0.28[f]	2.0	0.28
5	0.29	2.0	0.29	0.30	2.0	0.30	0.27	2.0	0.27	0.30	2.0	0.30
6	0.29	2.0	0.29	0.32	2.0	0.32	0.25[e]	2.0	0.25	0.32	2.0	0.32
7	0.27	2.0	0.27	0.30	2.0	0.30	0.23	2.0	0.23	0.28[f]	2.0	0.28
8	0.31	2.0	0.31	0.30	2.0	0.30	0.25[e]	2.0	0.25	0.31	2.0	0.31
9	0.22	2.0	0.22	0.20	1.9	0.21	0.18	1.9	0.19	0.20	2.0	0.20
10	6.6	1.7	7.8	7.0	1.4	10	4.8	1.3	7.4	6.3	1.6	7.9
11	1.4	1.6	1.8	1.7	1.7	2.0	1.4	1.6	1.8	1.4	1.7	1.6
12				1.4[c]	1.8	1.6	1.0[d]	1.6	1.3	1.3[d]	1.5	1.7

	2			**3**			**4**[j]			**5**		
C	T_1^{obsd}	η	T_1^{DD}	T_1^{obsd}	η	T_1^{DD}	T_1^{obsd}	η	T_1^{DD}	T_1^{obsd}	η	T_1^{DD}
1	12	1.3	18	19	1.3	29	10	1.6	13		g	
2	5.2	1.6	6.5	6.0	2.0	6.0	5.2	2.0	5.2	2.6	1.7	3.1
3	0.13	1.8	0.14	0.25	2.0	0.25	0.15	1.9	0.16	0.07	2.0	0.07
4	0.28	1.8	0.31	0.27	2.0	0.27	0.30	1.9	0.32	0.12	2.0	0.12
5	0.27	2.0	0.27	0.27	2.0	0.27	0.32	2.0	0.32	0.13	1.9	0.14
6	0.28	1.9	0.29	0.27	1.9	0.28	0.30	2.0	0.30	0.13[h]	1.9	0.14
7	0.25	1.9	0.26	0.24	1.9	0.25	0.29	1.9	0.31	0.12[h]	1.9	0.13
8	0.30	2.0	0.30	0.28	2.0	0.28	0.34	2.0	0.34	0.14[h]	1.9	0.15
9	0.20	2.0	0.20	0.19	2.0	0.19	0.20	2.0	0.20	0.08	1.9	0.08
10	4.8	1.6	6.0	5.4	1.9	5.7	0.37	2.0	0.37		g	
11	0.24[i]	2.0	0.24	1.2[d]	1.9	1.3	1.3[c]	2.0	1.3		g	
12							1.3[d]	2.0	1.3			

[a] Carboxylate carbon. [b] Ester carboxyl carbon. [c] Ester methyl carbon. [d] Methoxy methyl carbon. [e] Overlapping resonances in **1c**. [f] Overlapping resonances in **1d**. [g] Not assigned. [h] Tentative assignments. [i] Glycolyl hydroxymethylene carbon. [j] Measurements reflect values for the major (*Z*) isomer.

of C-4 and C-6 to anomeric configuration (**1c** → **1d**) it is observed that C-8 exhibits a similar anomeric shift but C-7 remains essentially unchanged. This is logical, though, since C-8 interacts much more strongly with the anomeric center through its hydrogen bond to the pyranose ring oxygen. Likewise, the sensitivity of C-4 and C-7 to *E*–*Z* isomerization of the formamido group in **4** reflects the interaction of these positions with the formamide.

The *N*-formyl derivative, **4**, was synthesized specifically to probe the internal motions present at the amido group. Since the carbonyl carbon (C-10) bears a directly attached proton one can compare its T_1^{DD} with the other isotropic carbons (C-3 to C-8). Although the mean C–H distance for formyl and aldehyde type bonds (1.13 Å)[28] is somewhat longer than is observed for the mean C–H bond distances in carbohydrates (1.11 Å),[29] this effect can be included using eq 3. Thus, since the average value of T_1^{DD} for C-4 through C-8 is 0.32 s, one predicts a value of $(1.13/1.11)^6 \times 0.32 = 0.356$ s for the carbonyl carbon. The agreement with the observed value of 0.37 s, while not quantitative, is sufficiently good to indicate that internal motions of the carbonyl carbon have a negligible effect on the T_1^{DD}. Calculations of T_1^{DD} (see next section) for the nonprotonated C-10 in NeuNAc confirm this observation.

The value of T_1^{DD} for the acetamido methyls (C-11), however, is greater than $3 \times T_1^{DD}$ for C-4 through C-8 in the other derivatives. Allerhand has shown that the value of T_1^{DD} for methyls undergoing rapid internal motion about a single C_3 axis reaches a limiting value, T_1^{DD}(limit), equal to three times T_1^{DD} for isotropically reorienting carbons.[8a] The values of T_1^{DD} for the C-11 methyls indicate the presence of an additional axis of motion. Although rotation of the entire acetamido group seems unlikely on steric grounds, it is possible that a librational motion could provide another mode of internal

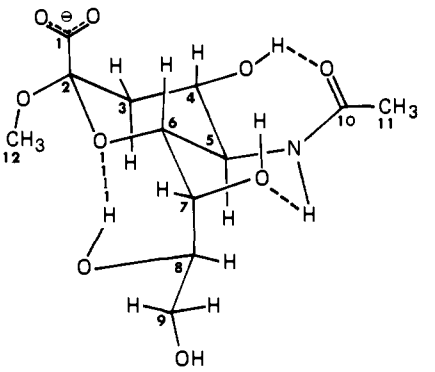


Figure 1. Conformational model of anion of **1d** showing the proposed hydrogen bonds and preferred conformation of the glycerol side chain. Although shown here only for the α anomer, this conformation is independent of anomeric configuration. Construction of CPK models indicates the preferential nature of these hydrogen bonds as well as the lack of unfavorable steric interactions. As measured in D_2O solution the exchangeable protons of the hydroxyls and the amido group would be replaced by deuterons.

reorientation. Librational effects, while not extensively investigated, have been noted in relaxation studies by Levy[30] and by Johnson.[31] Although the effect of this libration on the T_1^{DD} of C-10 in **4** and in **1** (see next section) must be small, its effect on the value of T_1^{DD}(limit) seems substantial. This observation could be related to the dependence of T_1^{DD}(limit) on the angle between the methyl protons and the axes of internal reorientation; motions about a librational axis not coincident with the methyl C_3 axis would affect the value of T_1^{DD}(limit) and predict a longer T_1^{DD} than the limiting value derived for the isolated methyl C_3 reorientation.

Calculation of T_1^{DD} for Nonprotonated Carbons. When carbons bear no directly attached hydrogens the dipole-dipole contribution to the relaxation is derived from other proximate hydrogens, and the value of T_1^{DD} can be calculated from[7]

$$\frac{1}{T_1^{DD}} = \sum_i \hbar^2 \gamma_C^2 \gamma_H^2 \tau_c r_i^{-6} \quad (4)$$

where the contributions of all nearby protons are summed. Using the value of τ_c calculated for protonated carbons with eq 3, with values of r_i measured from molecular models, one can calculate values for T_1^{DD} if the assumption of overall isotropic diffusion holds.

Therefore, we have calculated the values of T_1^{DD} for the three nonprotonated carbons in NeuNAc (**1a**) and present these results in Table IV. From eq 3 we have determined τ_c for **1a** as 1.82×10^{-10} s using an r_{C-H} (1.11 Å) derived as the mean carbon-hydrogen distance in sucrose measured by neutron diffraction.[29] The values for C-1 and C-2 were calculated from eq 4 summing the contributions from all proximate hydrogens (4-5 Å). In these calculations we have also included the small contribution from protons at exchangeable sites (present in a mole fraction of 0.037 from the dissolution of 0.68 M unexchanged **1a** plus the residual protons in the D_2O).

The calculation of T_1^{DD} for C-10 requires a more complex treatment since the freely rotating methyl contributes to the relaxation.[32] Assuming that motion of the acetamido group as a whole does not influence the T_1^{DD} for C-10 in the static conformation shown in Figure 1, the contributions from the isotropic hydrogens were calculated using eq 4. The contribution from the methyl is calculated using

$$\frac{1}{T_1^{DD}} = [(3/2)\cos^2\theta - 1/2]^2 N \gamma_C^2 \gamma_H^2 \hbar^2 \tau_c r^{-6} \quad (5)$$

which includes the angular dependence term.[32b] The angle θ is the angle subtended by the carbonyl carbon-methyl hydrogen vector and the axis of internal reorientation, which in this case was determined as 27° from molecular models.

The results of the calculations of T_1^{DD} for C-2 and C-10 compare favorably with the experimental values and in fact are well within the presumed accuracy of the experimental technique. The close agreement indicates that a single value of τ_c characterizes the motion of these carbons. Furthermore, it confirms the measurement of C-10 in **4**, which indicated that internal motions of the carbonyl carbon do not substantially influence the relaxation. The calculated value for C-1, however, agrees less closely with experiment. Since the calculated value, based on intramolecular interactions, is somewhat longer than the measured T_1^{DD}, it seems likely that intermolecular effects are contributing. Spin-lattice relaxation studies of 0.68 M **1a** in 90% H_2O-10% D_2O showed that the largest T_1 effect occurred at the carboxylate carbon, owing to its greater sensitivity to intermolecular effects. This assumption seems logical for two reasons. The highly solvated carboxylate would generally have more molecules of residual HOD proximate, and since its relaxation is least efficient (longest T_1^{DD}), intermolecular dipole-dipole effects, although small, would compete more effectively with intramolecular mechanisms.

Table IV. Comparison of Calculated and Experimental Values of T_1^{DD} (s) for Nonprotonated Carbons in **1a**

C	T_1^{DD} (calcd)	T_1^{DD} (exptl)
1	28	23
2	5.7	5.9
10	7.9	7.8

Conclusion

Although the specific role played by the acylneuraminic acid residues in the function of membrane glycolipids and glycoproteins is not known, it is clear that their removal drastically alters many of the mechanisms of cell function.[2] In particular, many of the receptor properties of the cell-surface oligosaccharides require NeuNAc residues in terminal positions. The unique and well-defined conformation proposed here could provide a structure for early recognition at the receptor site. Moreover, now that the ^{13}C NMR properties of acylneuraminic acids, chemical shifts, coupling constants, and spin-lattice relaxation times, have been studied, the role of ^{13}C NMR can be expanded to study the very complex structures and interactions of the cell surface carbohydrates.

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Carbon-13 Nuclear Magnetic Resonance of Ganglioside Sugars. Spin–Lattice Relaxation Probes for Structure and Microdynamics of Cell Surface Carbohydrates[1a-c]

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Abstract: The ^{13}C NMR spin–lattice relaxation times (T_1's) have been measured for the constituent sugars of ganglioside head groups. Analysis of the T_1 values for glucose and galactose methyl glycosides, *N*-acetylgalactosamine, fucose, and lactose shows the presence of anisotropic motion in β-linked galactosides and the motional association of the glucose primary hydroxyl group with the pyranose ring. The ^{13}C NMR of *N*-acetylneuraminyllactose (NeuNAc-Lac), the complete carbohydrate head group of ganglioside G_{M3}, was recorded and the T_1 values measured for all protonated carbons. The presence of a mobility gradient, with motion increasing away from the neuraminic acid moiety, is indicated by a comparison of the interresidue T_1's.

The polar head groups of the ganglioside lipids consist of oligosaccharides which extend into the intercellular space from the membrane surface. The carbohydrate portions of gangliosides, which always contain one or more molecules of *N*-acetylneuraminic acid (NeuNAc), have been implicated in mechanisms of hormone reception, metal ion binding, and intercellular recognition and adhesion.[2] The measurement of the ^{13}C NMR spin–lattice relaxation time (T_1) in organic and biological molecules has proved to be a powerful technique for the analysis of molecular dynamics.[3] Therefore, we describe here the first ^{13}C NMR T_1 study of a ganglioside head group, the trisaccharide *N*-acetylneuraminyllactose (NeuNAc-Lac) which represents the entire carbohydrate moiety of ganglioside G_{M3}.

The rationale of this work has been to study systematically the monosaccharides of ganglioside structure and then, building upon this detailed information, to construct experimentally the di- and trisaccharide structure of the G_{M3} polar head group. We have previously demonstrated the potential of ^{13}C NMR T_1 values in the study of the neuraminic acids,[1a] and we report here the results for the remaining carbohydrate components of ganglioside structure as well as the complete head group of G_{M3}, NeuNAc-Lac. These studies demonstrate (1) that substantial differences in the molecular dynamics of the monosaccharides are indicated by their T_1 values. (2) These differences result from internal structural differences in the monosaccharides and therefore are conserved in the oligosaccharides. (3) Analysis of the T_1 values for NeuNAc-Lac indicates the presence of a motional gradient due to segmental motion of the individual residues which increases away from the NeuNAc subunit.

Experimental Section

Materials. The α (**1a**) and β (**1b**) methyl glucopyranosides were obtained from P-L Biochemicals. The α (**2a**) and β (**2b**) methylgalactopyranosides, L-fucose (**3**), *N*-acetyl-D-galactosamine (**4**), and lactose (**5**) were obtained from Sigma Chemical Co. *N*-Acetylneuraminyllactose (**6**) was isolated from bovine colostrum using the ion-exchange procedure of Schneir and Rafelson.[4]

^{13}C NMR. ^{13}C NMR spectra were measured at 25.03 MHz on a JEOL PFT-100 NMR spectrometer equipped with a PG-100 pulse programmer and the JEOL sample temperature controller. Samples were studied under conditions of complete proton noise decoupling with an internal field/frequency lock. Chemical shifts were measured relative to the methyl carbon resonance of external acetone and were cross-referenced to external Me_4Si. Temperature was controlled at 28.0 ± 1.0 °C for all substances except **6**, which was studied at 35.0 ± 1.0 °C.

T_1 values were measured using the fast inversion recovery method (FIRFT)[5] over a 2000- or 4000-Hz bandwidth with 8192 time domain data points. The $\pi/2$ pulse length was calibrated prior to each set of experiments and varied between 18 and 23 μs. Relaxation times were calculated from sets of spectra representing 8–15 τ values using a least-squares fit to the semilogarithmic plot. Reported values represent the mean of three determinations with standard deviations as follows: less than 5% for the monosaccharides (**1–4**), 5–10% for lactose (**5**), and 10–15% for NeuNAc-Lac (**6**). NOE's were determined using the gated decoupling technique[6] with delay times of 8–10 × T_1 in order to minimize cross-correlation effects.[7] Except where noted, η values were 2.0 within experimental error and therefore reported values of $T_1 = T_1^{DD}$.

Sample Preparation. NeuNAc-Lac (**6**) was converted to its sodium salt by titration with sodium hydroxide solution followed by lyophilization. Contamination by paramagnetic ions was minimized using the previously described sample preparation techniques.[8]

Results and Discussion

Interpretation of ^{13}C NMR T_1 Values. When the spin–lattice relaxation behavior of a carbon atom is dominated by the proton-mediated dipole–dipole mechanism ($\eta \simeq 2.0$ for small organic and biological molecules), then $T_1 = T_1^{DD}$. Since T_1 can be related to the motional behavior of a carbon atom with[3a]

$$\frac{1}{T_1} = N\gamma_C^2\gamma_H^2\hbar^2\tau_c r_{CH}^{-6} \qquad (1)$$