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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]

**Michael F. Czarniecki[1d] and Edward R. Thornton***

*Contribution from the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104.* *Received May 5, 1977*

**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 $\beta$-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 $\alpha$ (**1a**) and $\beta$ (**1b**) methyl glucopyranosides were obtained from P-L Biochemicals. The $\alpha$ (**2a**) and $\beta$ (**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 $\mu$s. Relaxation times were calculated from sets of spectra representing 8–15 $\tau$ 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, $\eta$ 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]

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

Table I. $^{13}C$ $T_1$ Values (s) for Monosaccharides[a] as 1.0 M $D_2O$ Solutions, 28 °C

| C | 1a | 1b | 2a | 2b | 3a | 3b | 4a | 4b |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.74 | 0.82 | 0.86 | 0.87 | 1.13 | 1.16 | 0.54 | 0.55 |
| 2 | 0.74 | 0.81 | 0.80 | 0.86 | 1.09 | 1.10 | 0.52 | 0.52 |
| 3 | 0.76 | 0.75 | 0.82 | 0.84 | 1.17 | 1.08 | 0.55 | 0.54 |
| 4 | 0.74 | 0.74 | 0.83 | 0.75 | 1.15 | 0.97 | 0.53 | 0.52 |
| 5 | 0.76 | 0.73 | 0.80 | 0.86 | 1.06 | 1.13 | 0.55 | 0.54 |
| 6 | 0.40 | 0.36 | 0.53 | 0.53 | 0.99 | 0.99 | 0.39 | 0.45 |
| 7 | 2.3 | 2.5 | 2.5 | 2.5 | | | 12[b] | 12[b] |
| 8 | | | | | | | 1.9 | 1.9 |

[a] Except where noted $\eta \simeq 2.0$; therefore, $T_1 = T_1^{DD}$. [b] $\eta = 1.5$, $T_1^{DD} = 16$ s.

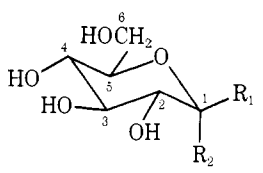
$T_1$ values are a sensitive probe for molecular motions. When the carbon-hydrogen bond distances are equal, the equation can be simplified for protonated carbons to the relationship[3b]

$$\frac{1}{NT_1} \propto \tau_c \qquad (2)$$

where $\tau_c$ is the time required for the reorientation of a C-H vector through one radian relative to the external magnetic field and $N$ is the number of directly attached protons.

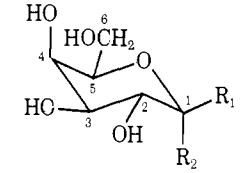
Values of $NT_1$ can then indicate changes in overall molecular motion that may be due to molecular weight changes, solvation, or intermolecular effects. On the other hand, when values of $NT_1$ within a molecule are compared they can demonstrate the presence of anisotropy of molecular reorientation, independent rotation, or segmental motion. Therefore, $NT_1$ can be used as an internal molecular mobility parameter.

**Monosaccharides.** We have measured the spin-lattice relaxation times of the hexose carbohydrate constituents of gangliosides. Presented in Table I are the $T_1$ values for the $\alpha$ and $\beta$ methyl-D-glucosides (Glc **1a,b**), the $\alpha$ and $\beta$ methyl-



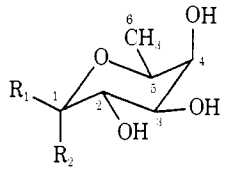
Glc

| | $R_1$ | $R_2$ |
|---|---|---|
| **1a** | H | $OCH_3$ |
| **1b** | $OCH_3$ | H |



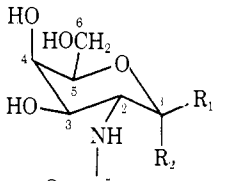
Gal

| | $R_1$ | $R_2$ |
|---|---|---|
| **2a** | H | $OCH_3$ |
| **2b** | $OCH_3$ | H |



Fuc

| | $R_1$ | $R_2$ |
|---|---|---|
| **3a** | H | OH |
| **3b** | OH | H |



GalNAc

| | $R_1$ | $R_2$ |
|---|---|---|
| **4a** | H | OH |
| **4b** | OH | H |

D-galactosides (Gal **2a,b**), *N*-acetyl-D-galactosamine (GalNAc **4a,b**), as well as the interesting 6-deoxy sugar L-fucose (Fuc **3a,b**) which occupies terminal positions in many glycoproteins. The chemical shifts and assignments are in agreement with previously published studies.[9] Two reports have recently presented $T_1$ values for glucose derivatives and our results are in qualitative agreement.[10,11]

Analysis of the $NT_1$ values for **1-4** provides insight into their internal and overall molecular dynamics. Methyl groups exhibit the long $NT_1$ values characteristic of their expected rapid internal reorientations. It is interesting to note that C-6 in L-fucose (**3a,b**) has a substantially shorter value of $NT_1$. This may be a reflection of two effects; since it experiences more eclipsing interactions for motion about its single bond, its rotational energy barrier is higher. Secondly, it has only one axis for internal reorientation while the other methyls have two degrees of internal motional freedom.

The effect of anisotropic motion on $T_1$ values is related to the angle between the axis of preferred molecular reorientation and the C-H vectors in the molecule. When a C-H bond lies on or near an anisotropic axis, molecular reorientations about this axis effect little or no reorientation of the C-H bond relative to the magnetic field. Its effective correlation time is therefore slower since only motions with components perpendicular to the preferred axis will orient the C-H vector. The effectively slower motion results in a shorter value of $T_1$ for carbons whose C-H bonds lie along an anisotropic axis than for those with other orientations.

The $T_1$ value for C-4 of the $\beta$-methyl galactoside (**2b**) is anomalously shorter than that for the other ring carbons, and although the difference is small it was highly reproducible and was well outside experimental error. It is even more interesting that the $\alpha$ anomer (**2a**) does not exhibit this effect, and therefore it cannot be ascribed to an unexpected bond-length change due to a different C-4 stereochemistry. Consequently, we would like to suggest that the introduction of a $\beta$ anomeric substituent affects the inertial axes sufficiently to cause molecular diffusion to be preferred along an axis that runs approximately on the long axis of the molecule, which would lie near the C-H bond of C-4. Although the effect is small, Deslauriers et al. have calculated effects on $T_1$ values for various ellipsoids and have shown that measurable effects can occur even for small deviations from isotropicity.[12] One would predict that increasing the size of the $\beta$ substituent would increase the relative difference between ring carbons and C-4, and in fact this is observed in lactose (next section). In principle, the $\beta$ glucoside (**1b**) would also undergo a similar anisotropic diffusion. The C-H vector for C-4, however, is not favorably oriented to the long axis of the molecule to make detection unequivocal. The effect observed for the galactosides is also seen in fucose.

We have sought to probe the dynamics of the hydroxymethylene groups in the monosaccharides (**1, 2, 4**) in order to understand the ways in which structural differences affect internal molecular motions. Our measurements demonstrate, somewhat surprisingly, that the $NT_1$ values for galactose derivatives (**2, 4**) indicate a substantially greater degree of motional freedom for C-6 than is observed in glucose derivatives (**1**). This suggests that rotation about the C-5, C-6 bond in glucose is restricted by some intramolecular interaction not present in galactose, an idea which is supported by the near equality of the glucose C-6 $NT_1$ values to those of the ring carbons, indicating that C-6 is essentially isotropic with the ring. An explanation involving differences in principal rotation axes between glucose and galactose seems less likely because

**Table II.** $^{13}C$ $T_1$ Values (s) for Lactose (**5**)[a] as 0.50 M $D_2O$ Solution, 28 °C[b]

| C | αGlc | βGlc | Gal |
|---|---|---|---|
| 1 | 0.34 | 0.41 | 0.37 |
| 2 | 0.43 | 0.39 | 0.39 |
| 3 | 0.40 | 0.42 | 0.40 |
| 4 | 0.35 | 0.39 | 0.30 |
| 5 | 0.41 | 0.35 | 0.38 |
| 6 | 0.18[c] | 0.18[c] | 0.27 |

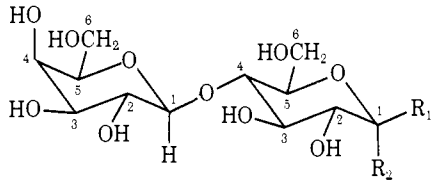
[a] For all resonances, $\eta \simeq 2.0$, therefore, $T_1 = T_1^{DD}$. [b] Chemical shifts and assignments corresponded to published data; see ref 17. [c] Unresolved resonances.

of the isotropicity of glucose C-6 and also because one sees the same effect for both anomers.

Analysis of the rotameric population of C-6 in **1a,b** by $^1H$ NMR[13] and optical rotation studies[14] indicates that the hydroxyl would be favorably placed to hydrogen bond intramolecularly to the pyranose ring oxygen. Although formally a five-membered ring, somewhat unfavorable for hydrogen bonds due to deviations from linearity and distance requirements, we would like to suggest that a more geometrically favorable seven-membered ring[15] hydrogen bonding system could be formed through an intercalated molecule of $H_2O$ (see Figure 1). In either case, however, the stabilizing influence of this intramolecular hydrogen bond could hinder internal reorientation of C-6.

On the other hand, $NT_1$ values for C-6 in galactose derivatives indicate the presence of greater internal motion, yet the galactose derivatives should, in principle, be able to interact identically with the ring oxygen. The differences observed must, however, be related to the different C-4 stereochemistry in glucose and galactose, since in all other respects the monosaccharides are the same. It has been suggested that the axial hydroxyl and the pyranose ring oxygen can form an intramolecular hydrogen bond.[14,16] The presence of this interaction, which could be mediated through a five-membered ring or more likely a seven-membered $H_2O$ intercalated ring, would reduce the effectiveness of the C-6 interaction since it would compete more effectively because of its rigid stereochemical relationship with respect to the pyranose ring oxygen. The resulting effect on the galactose C-6 would reduce the intramolecular hindrance to motional freedom relative to glucose.

**Lactose.** The sequence of hexoses in all gangliosides is initiated by the disaccharide lactose which is glycosidically linked from the glucose residue to the lipid portion of the molecule. Therefore, we have studied the $T_1$ values of lactose (**5**) to probe



**5**

| | Gal | Glc |
|---|---|---|
| | $R_1$ | $R_2$ |
| α | H | OH |
| β | OH | H |

its motional behavior and in particular to compare its dynamics with its constituent monosaccharides.

Our results, presented in Table II, demonstrate that many of the relaxation and motional characteristics observed in the monosaccharides are conserved in lactose. Overall molecular reorientation, reflected in the absolute values of the $T_1$, is

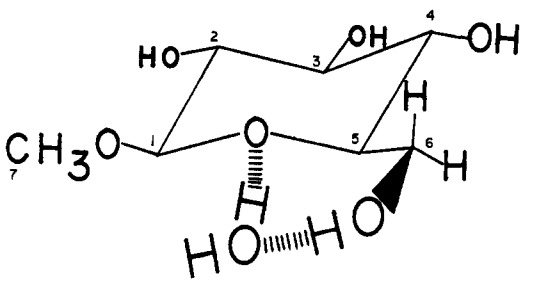
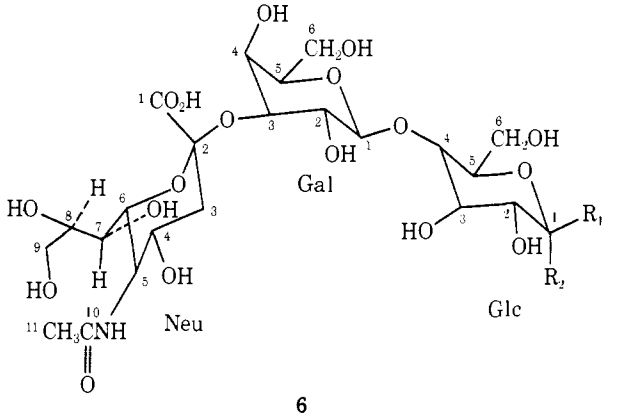


**Figure 1.** Suggested intramolecular hydrogen bonding of methyl β-D-glucoside (**1b**) mediated through an intercalated $H_2O$ molecule. Space-filling models (CPK) demonstrate van der Waals contact and near linearity of the proposed hydrogen bonds.

slower, a direct effect of the higher molecular weight of **5**. The preferred axis for molecular reorientation appears to lie along the long axis of the molecule and is therefore reflected in the shorter $T_1$ at C-4 of the galactose residue. The apparent difference between the relaxation times observed in α and β C-1 resonances may also be a reflection of their differing C–H orientations relative to the anisotropic axis. It is interesting to note that Allerhand's $T_1$ study of sucrose[18] did not reveal anisotropic motion. It now seems probable that the lack of a suitably oriented C–H vector prevented the observation of anisotropy in that system.

The value of $NT_1$ for the glucose residue C-6 is substantially shorter than $NT_1$ for C-6 in the galactose residue. This is consistent with the measurements in the monosaccharides which indicated a greater motional freedom in galactose hydroxymethylene positions relative to glucose. Moreover, the conservation of this effect in lactose strengthens our argument that the $T_1$ differences resulted from the internal structural differences of the sugars.

***N*-Acetylneuraminyllactose.** We have studied the $T_1$ values for the carbon atoms in NeuNAc-Lac (**6**) in order to probe the



**6**

| | $R_1$ | $R_2$ |
|---|---|---|
| α | H | OH |
| β | OH | H |

dynamics of ganglioside polar head groups. NeuNAc-Lac is the entire carbohydrate portion of ganglioside $G_{M3}$, and therefore provides a clear opportunity to study the motions of the complex cell surface carbohydrates. Some of the chemical shift values for NeuNAc-Lac have been previously reported by Eschenfelder et al.,[19] and our values (Table III) are identical with theirs when a small correction (−0.7 ±0.1 ppm) is made for different referencing systems.

In our construction of a motional picture for dynamics of the cell surface carbohydrates we have examined the $T_1$ values of the individual hexoses, neuraminic acids, and the disaccharide lactose. Approximately one-half of the resonances of NeuNAc-Lac can be rigorously assigned on the basis of

**Table III.** $^{13}C$ NMR Chemical Shifts ($\delta_C$ ($Me_4Si$)), Partial Assignments[a] (C), and $T_1$ Values (s)[b] for 0.4 M NeuNAc-Lac (**6**) in $D_2O$, 35 °C

| $\delta_C$ ($Me_4Si$) | C | $T_1$ | $\delta_C$ ($Me_4Si$) | C | $T_1$ | $\delta_C$ ($Me_4Si$) | C | $T_1$ |
|---|---|---|---|---|---|---|---|---|
| 175.2 | Neu-10 | *c* | 74.9 | I | 0.20 | 67.7 | III | 0.14 |
| 174.0 | Neu-1 | *c* | 74.5 | I | 0.24 | 63.0 | Neu-9 | 0.13 |
| 102.8 | Gal-1 | 0.16 | 74.0 | I | 0.23 | 61.2 | Gal-6 | 0.14 |
| 100.0 | Neu-2 | *c* | 73.0 | II | 0.18 | 60.4 | Glc-6[f] | 0.09 |
| 95.9 | Glc-1$\beta$ | 0.24 | 71.9 | III | 0.16 | 52.0 | Neu-5 | 0.13 |
| 92.0 | Glc-1$\alpha$ | 0.11 | 71.4 | I | 0.27 | 39.8 | Neu-3 | 0.05 |
| 78.5 | II | 0.17 | 70.2 | I | 0.23 | 22.4 | Neu-11 | 0.75[e] |
| 75.6 | I | 0.20 | 69.5[d] | I + II | 0.19 | | | |
| 75.3 | II | 0.18 | 68.4[d] | III | 0.12 | | | |

[a] For explanation of Roman numerals, see text. [b] Except where noted, $\eta \simeq 2.0$; therefore, $T_1 = T_1^{DD}$. [c] Nonprotonated carbon, not measured. [d] Resonance integrates for two carbons. [e] $\eta = 1.7$, $T_1^{DD} = 0.88$ s. [f] Unresolved $\alpha$ and $\beta$ anomers.

chemical shift. Analysis of the $T_1$ values for these well-resolved resonances provides a unique probe of the dynamics of the trisaccharide. The more complex region of the spectrum (65–80 ppm) corresponds to the area of hydroxymethine carbons. Although resonance overlaps decreased the possible accuracy of $T_1$ values in this region, it was observed that the resonances could be segregated into three classes which corresponded to those $T_1$'s that could be unambiguously assigned (Glc = I, Gal = II, Neu = III). While the assignments are not rigorous this classification does demonstrate the different $T_1$ values characteristic of the monosaccharide residues.

Examination of the $NT_1$ values for the hydroxymethylene groups (Glc-6, Gal-6, Neu-9) demonstrates consistency with the previous studies on internal motions. The longer $NT_1$ values observed in the neuraminic acid and galactose residues compared to the glucose are consistent with the previously demonstrated internal motions of the monosaccharides. The presence of anisotropic motion roughly along the molecular length is suggested by the striking difference between the $T_1$ values in the $\alpha$ (C–H equatorial) and $\beta$ (C–H axial) anomers of the glucose residue, the close correspondence between the equatorial C–H and the preferred axis resulting in a longer effective correlation time and therefore shorter $T_1$ in the $\alpha$ anomer.

Of particular interest is a comparison of the $T_1$ values for the ring carbons between residues. The increase in $NT_1$ observed proceeding from the neuraminic acid (Neu-3, Neu-5) to the galactose (Gal-1) to the glucose (Glc-1$\beta$, C–H off anisotropic axis) indicates segmental motion of the residues. This confirms Allerhand and Doddrell's original demonstration of segmental motion in oligosaccharides, from their $^{13}C$ NMR PRFT (partially relaxed Fourier transform) study of stachyose.[20] Unlike the segmental motion in stachyose, however, which radiated from the central residues, the neuraminic acid molecule in NeuNAc-Lac is the least mobile and appears to anchor one end of the molecule in solution. This is probably the result of the highly solvated negatively charged carboxylate that NeuNAc bears at physiological pH. In $G_{M3}$, though, the glucose is also immobilized owing to its glycosidic bond to the lipid portion of the ganglioside. The carbohydrate structure, stabilized by solvation at one end and the membrane surface at the other, would be conformationally immobile. In view of the receptor properties ascribed to gangliosides,[2a] a well-defined and stable carbohydrate structure might be essential.

Recent studies have indicated that high-resolution $^{13}C$ NMR spectra can be obtained for a variety of purified gangliosides.[21] Therefore, further $^{13}C$ NMR studies of these important biomolecules are in progress.

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