Ganglioside hydration study by ^{2}H-NMR: dependence on temperature and water/lipid ratio

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Abstract Dynamic properties of ^2H_2O in samples of ganglioside aggregates hydrated at water/lipid ratios ranging from 25:1 to 8000:1 mole/mole were studied by using deuterium nuclear magnetic resonance (^{2}H-NMR). We present a physical model for the interpretation of the measured spin-spin relaxation times (T_2). For all the concentrations studied the model provides evidence for the existence of at least two kinds of water environments: one in which the rotational correlation time is in the range of 10^{-9} to 10^{-8} s, and a second in which it lies between 10^{-11} to 10^{-10} s. A detailed study on the temperature dependence was performed for two of the concentrations, one corresponding to the hexagonal phase (100:1 mole/mole) and the other involving a micellar phase (200:1 mole/mole). In the 100:1 ^2H_2O/ganglioside molar ratio sample, most of the water is tightly bound to long cylindrical structures. For the 200:1 sample, there are on average approximately 30 water molecules tightly bound to the polar head group of each ganglioside molecule. The relative number and dynamics of molecules in this environment are essentially insensitive to temperature variations in the range 220–300K. The rest of water molecules are also influenced by the aggregate, having a different mobility from that observed in the free liquid state.—**Arnulphi, C., P. R. Levstein, M. E. Ramia, C. A. Martín, and G. D. Fidelio.** Ganglioside hydration study by ^{2}H-NMR: dependence on temperature and water/lipid ratio. *J. Lipid Res.* 1997. **38:** 1412–1420.

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Water molecules in the vicinity of macromolecules occupy special sites with preferential orientations characterized by anisotropic and restricted motions; these features differentiate the bound from the unbound molecules (1).

Gangliosides belong to a family of complex glycosphingolipids (2–4) containing one or more sialic acid in their bulky polar head group. They are strong amphipathic molecules having hydrophilic and hydrophobic regions of similar length as displayed in **Fig 1.** This strong amphipathic characteristic provided by the carbohydrate residues of the polar head group influences their interfacial, topological, and thermotropic properties (3, 5, 6).

Gangliosides present an interesting lyotropic behavior. At water/lipid molar ratios higher than 140:1, they arrange themselves in globular or toroidal micelles (7). The average diameter of the smallest spherical micelle is 6.1 nm (8). However, at lower molar ratios, an hexagonal (H_I) mesophase structure is observed. In this phase the ganglioside molecules form hexagonally packed rod-like structures in which the nonpolar lipid chains radiate from the center of the rods, with the sugar groups on the cylinder surface in contact with water. X-ray diffraction studies (7) give the total radius of a ganglioside cylinder as 3.1 nm at 16°C and 2.8 nm at 55°C.

Gangliosides are particularly concentrated in membranes of neural tissue (9). These lipids have been involved in the modulation of processes taking place at the cell surface such as adhesion, membrane-mediated transfer of information, cell ligand-associated-events, and intracellular response mechanisms (4, 9, 10).

Recently, it has been reported that for low amounts of gangliosides, the glycolipid acts as phospholipase A_2 and C inhibitor in mixed phospholipid–glycosphingolipid interfaces (11–14). This observation was seen to be independent of the topological state of the phospho-

Abbreviations: ^{2}H-NMR, nuclear magnetic resonance of deuterium; TBG, total bovine brain gangliosides; FFT, fast Fourier transform; FID, free induction decay; H_I, hexagonal I type; H_{II}, hexagonal II type; G_{M1}, Gal(β1–3)GalNac(β1–4)Gal(3–2α)NeuAc(β1–4)Glc(β1–1′)N-acylsphingosine; G_{D1a}, NeuAc(α2–3)Gal(β1–3)GalNac(β1–4)Gal(3–2α)NeuAc(β1–4)Glc(β1–1′)N-acylsphingosine; G_{D1b}, Gal(β1–3)GalNac(β1–4)Gal(β3–2α)NeuAc(8–2α)NeuAc(β1–4)Glc(β1–1′)N-acylsphingosine; G_{Tlb}, NeuAc(α2–3)Gal(β1–3)GalNac(β1–4)Gal(3–2α)NeuAc(8–2α)NeuAc(β1–4)Glc(β1–1′)N-acylsphingosine.

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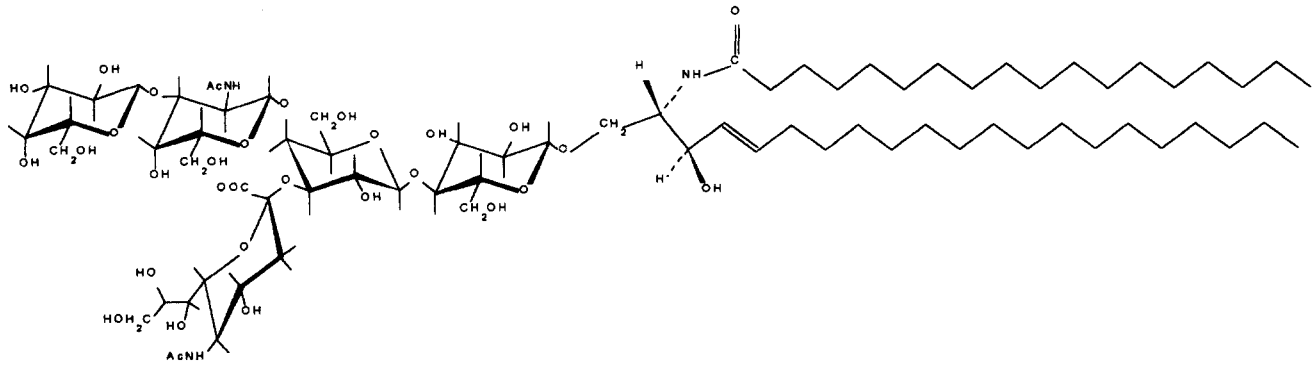

Fig. 1. G_{M1} ganglioside. Notice the size of the polar head group relative to the hydrocarbon chains.

lipid substrate aggregate (15). It has been associated with the anti-inflammatory effect induced by gangliosides in vivo and in vitro tests (16). This particular property of restraining the activity of lipolytic enzymes induced by these complex glycosphingolipids may be correlated with both a higher interfacial micropolarity (17, 18) and a higher dielectric constant gradient from the polar head group to the inner hydrophobic core found for ganglioside-containing aggregates (19).

The aim of the present work was to study the dynamic state of the hydration water in ganglioside aggregates utilizing nuclear magnetic resonance (NMR). To avoid complex NMR spectra resulting from protons (^1H) belonging to the hydrocarbon chains and carbohydrate moieties, we have performed the experiments in deuterated water samples. As ^2H nuclei possess a quadrupole moment which makes them very sensitive to local electric field gradients, it is possible to estimate the anisotropy of the motion of the water molecules in the hydration shell of the aggregates. The observed NMR signal comes from the deuterons in the water molecules and those deuterons appearing in the amide, hydroxylic, and carboxylic groups as a result of exchange with the water molecules.

Previous work performed on proteins (20) and on phospholipids (21–23) showed that the number and dynamic state of water molecules affected by the biological surface are not constant but rather depend on the molar water/lipid ratio. Consequently, we investigated samples covering a wide range of concentrations.

MATERIALS AND METHODS

Samples were prepared for water/lipid molar ratios ranging from 25:1 to 8000:1 for studies at room temperature. Two ratios from different lyotropic phases were selected, namely 100:1 and 200:1, for detailed investigation of their temperature dependence.

Total bovine brain gangliosides (TBG) formed from 21% of G_{M1}, 42% of G_{D1a}, 18% of G_{D1b}, 19% of G_{T1b}, were purified to better than 99% using the procedure described by Fidelio, Ariga, and Maggio (24). Gangliosides were hydrated with ^2H_2O (99.9%, Sigma, Chemical Co., St. Louis, MO) at the indicated mole ratio.

Purified TBG samples of known weight were placed in an Eppendorf tube and centrifuged for 30 sec at 13,000 *g* in order to accumulate the powder at the bottom of the tube. Then an appropriate amount of ^2H_2O was added and the sample was centrifuged again for 2 min at 13,000 *g*. All the samples were heat treated at 50°C for 30 min to achieve complete homogenization. They were then left at room temperature for 24 h before performing the NMR measurements.

NMR data were recorded with a Bruker MSL-300 spectrometer with a 7.2 T magnet. The resonance frequencies are 46.073 and 300.13 MHz for deuterium and hydrogen nuclei, respectively. The samples were gradually cooled from room temperature to 220 K.

For the sample with a water/ganglioside molar ratio of 200:1, the NMR spectra were obtained from fast Fourier transforms (FFT) of the free induction decay (FID) signals after excitation by a resonant $\pi/2$ radio frequency pulse. For these experiments the pulse width was 8 μs, the acquisition dead time 22 μs, the sampling time 6 μs, and the acquisition time 25 ms; 100 scans were taken with a recycling time of 4 s. The spectra were fitted by means of Lorentzian profiles for which the linewidth $\Delta\nu$ is related to T_2 by $\Delta\nu = 1/\pi T_2$ (for our samples, $T_2^* \sim T_2$ (25)).

For the sample with 100:1 ^2H_2O/TBG molar ratio, the longitudinal spin-lattice relaxation time T_1 was measured using the inversion recovery sequence ($180°-\tau_d-90°$) and the spin–spin relaxation time T_2 by means of

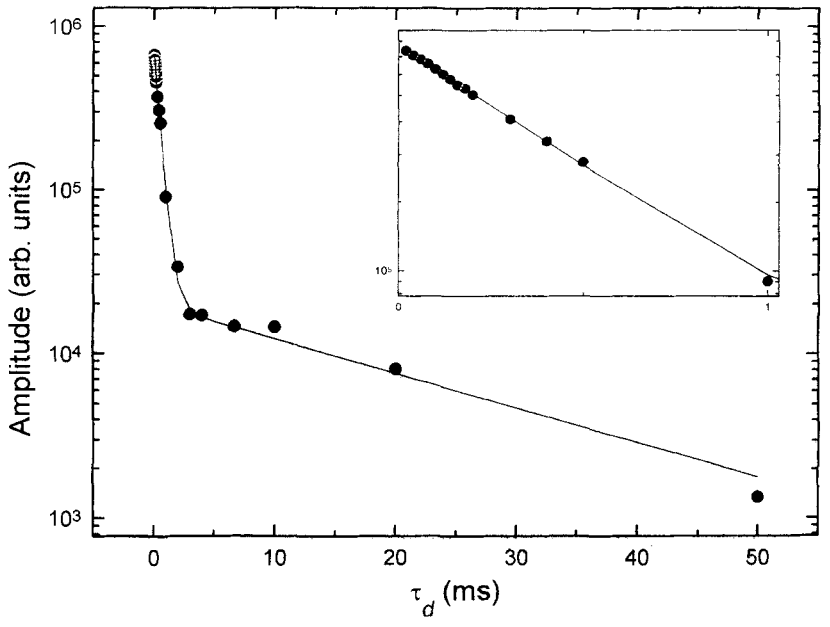

Fig. 2. Spin-echo amplitude as a function of the delay time between 90° and 180° pulses in the 100:1 molar water/lipid sample at 280 K. The solid line was obtained by least-squares fitting of the experimental data (●) to equation 3. The parameters obtained from this fit are T_{2l} = 20.70 ms, $P_l = 1.99 \times 10^4$, T_{2b} = 487 μs, $P_b = 6.98 \times 10^5$. The insert shows in detail the short delay time behavior.

a spin echo (90°–τ_d–180°) sequence, both as a function of temperature.

THEORY

The ^{2}H-NMR spectra are interpreted on the assumption that the dominant relaxation mechanism is the quadrupolar interaction (26). In the thermodynamic regime where a spin temperature is well defined, the inverse of T_1 and T_2 can be related to the molecular reorientational correlation time τ_c by:

$$\frac{1}{T_1} = \left(\frac{3\pi^2}{100}\right)\frac{2I+3}{I^2(2I-1)}\chi^2\left(1+\frac{\eta^2}{3}\right)\left[\frac{2\tau_c}{(1+\omega_0^2\tau_c^2)}+\frac{8\tau_c}{(1+4\omega_0^2\tau_c^2)}\right] \quad Eq.\ 1)$$

$$\frac{1}{T_2} = \left(\frac{3\pi^2}{100}\right)\frac{2I+3}{I^2(2I-1)}\chi^2\left(1+\frac{\eta^2}{3}\right)\left[3\tau_c+\frac{5\tau_c}{(1+\omega_0^2\tau_c^2)}+\frac{2\tau_c}{(1+4\omega_0^2\tau_c^2)}\right] \quad Eq.\ 2)$$

where $\chi = (e^2q_{zz}Q)/h$ is the quadrupole coupling constant; q_{zz} is the principal component of electric field gradient tensor; η is the asymmetry parameter, and Q is the nuclear electric quadrupole moment. For this work χ = 240 kHz.

RESULTS AND DISCUSSION

For each spin-echo experiment we obtained a biexponential decay of the magnetization amplitude as illustrated in **Fig. 2** for the 100:1 ^2H_2O/TBG molar ratio sample. The decay of the echo amplitude $M(\tau_d)$ after a (90°–τ_d–180°) pulse sequence can be represented by the expression:

$$M(\tau_d) = P_l \exp(-\tau_d/T_{2l}) + P_b \exp(-\tau_d/T_{2b}) \quad Eq.\ 3)$$

This result suggests the existence of two well-differentiated environments with relaxation times T_{2l} and T_{2b}. The weighting factors P_l and P_b are related to the number of molecules in each defined environment. Nonlinear least-squares fittings of the experimental decay to equation 3 allows us to obtain the parameters T_{2l}, T_{2b}, P_l, and P_b. On the other hand, the Fourier transform of the FID can be deconvoluted in two Lorentzian lines (27, 28) as shown in **Fig. 3** for the 200:1 sample. This shows that the effects of field inhomogeneity can be neglected; that is $T_2^* \sim T_2$.

These findings indicate that there are at least two environments for the water molecules and that the systems are in the slow exchange regime. Therefore:

$$\frac{1}{\tau_l}+\frac{1}{\tau_b} \ll \frac{1}{T_{2b}}-\frac{1}{T_{2l}} \quad Eq.\ 4)$$

where τ_b, τ_b are the residence times of molecules in each of the two environments and T_{2b}, T_{2l} are the spin-spin relaxation times corresponding to the environments defined as tightly bound *b* and loosely bound *l*, respec-

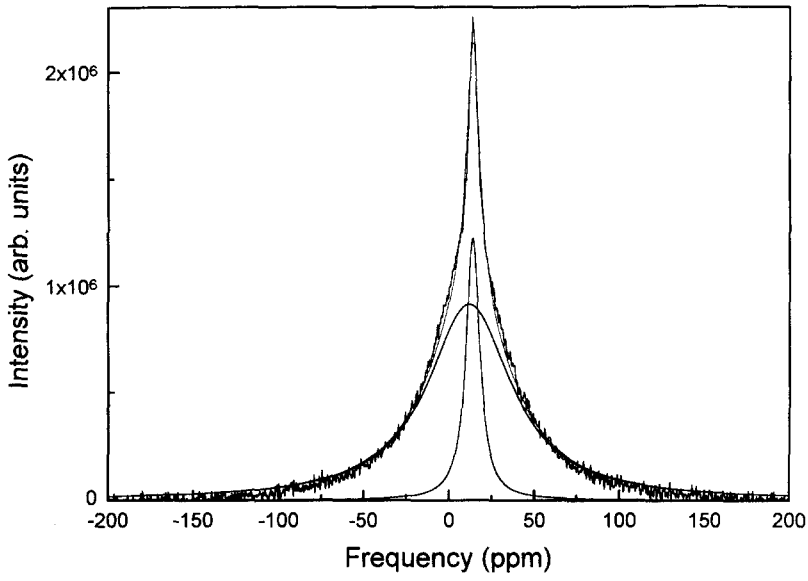

Fig. 3. Frequency spectrum obtained by Fourier transformation of an FID from a single 90° pulse (100 acquisitions), for the 200:1 molar water/lipid sample at 259 K. A good fit is reached by adding two Lorentzian profiles for which one has $\Delta\nu$ = 394 Hz and represents 18% of the total area and the other has $\Delta\nu$ = 841 Hz and corresponds to 82% of the total area. The former is assigned to tightly bound nuclei; the latter to loosely bound nuclei.

tively. The rotational correlation times τ_c of these two populations fall between those values found for solid water (10^{-6} s) and liquid water (10^{-12} s) (29). For example, in the 100:1 sample at 280 K the rotational correlation times τ_c, calculated from T_{2b}, T_{2l} by solving equation 2, are 2.1×10^{-9} s and 4×10^{-11} s, respectively. By contrast, for systems in the rapid exchange regime, as it is usually assumed for biological systems, the whole system would relax with a single characteristic time. We did not reach this regime even in the 8000:1 water/lipid molar ratio sample.

Figure 4 summarizes the spin-spin relaxation time data as a function of water/lipid molar ratio obtained by fitting of the experimental data to equation 3. As can be seen, T_{2b} (solid triangles) and T_{2l} (open circles), vary with the amount of water in the system indicating that the dynamic state of each population depends on the extent of hydration. It is interesting to note that there is a clear discontinuity in the T_{2l} behavior at the 140:1 water/lipid ratio where the hexagonal I to micellar solution phase transition occurs as established by Curatolo, Small, and Shipley (7) using calorimetric and X-ray diffraction techniques. The spin-spin relaxation times T_{2b} of tightly bound water molecules for H_I are systematically lower than those in the micellar phase. This can be interpreted as an indication of anisotropic motion of the water molecules at the surface of the long cylinders which does not permit a complete averaging

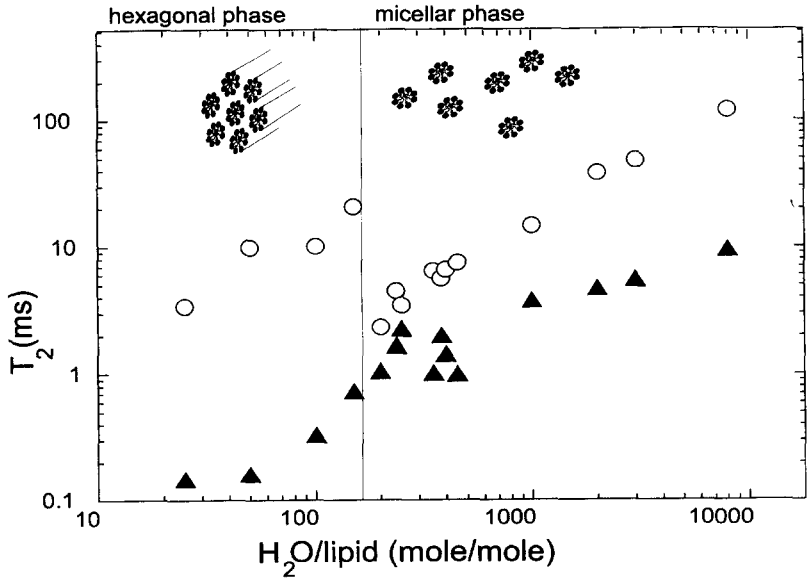

Fig. 4. Room temperature spin-spin relaxation times (T_2) of each microenvironment as a function of water/lipid molar ratio. The vertical line indicates the ratio at which the hexagonal I to micellar solution phase transition occurs, according to Curatolo et al. (7). Short T_2 values are associated with longer correlation times and are assigned to ^2H_2O tightly bound to ganglioside molecules. Solid triangle: tightly bound nuclei. Open circle: loosely bound nuclei.

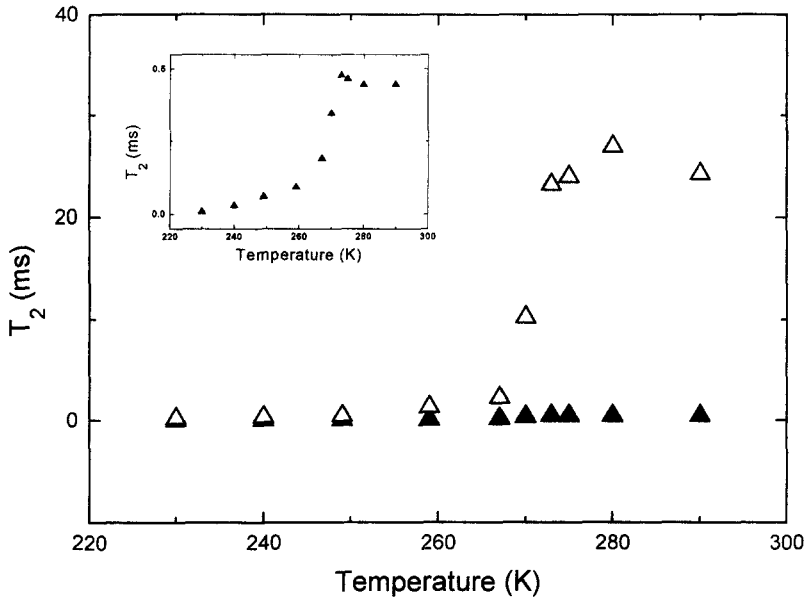

Fig. 5. Spin-spin relaxation times of each microenvironment in the 100:1 molar water/lipid sample as a function of temperature. T_{2b} is assigned to ^2H_2O tightly bound to the aggregate (solid triangle) and T_{2l} (open triangle) is assigned to more mobile nuclei. The insert shows T_{2b} using an amplified scale.

of the electric field gradient. This can be compared with the relatively isotropic motion of the water molecules in the globular or small cylindrical micelles. The relative population corresponding to the short T_2 in the H_I phase is much larger than in the micellar phase leading to the conclusion that most of the water is strongly affected by the aggregate. In this phase it is possible to see a small proportion of molecules having a high mobility; these are not distinguished in the micellar phase. For all the concentrations analyzed in the H_I phase, we found at most 10 deuterium nuclei per lipid molecule. There are three possible locations for these deuterium nuclei. One involves water molecules in contact with the hydrophobic part of the amphiphiles, known as the "wet" region (19, 30) which should have higher mobility than water in the hydrophilic region where a complex network of hydrogen bonds is formed. For these molecules a strong temperature dependence would be expected near the gel-liquid crystalline phase transition as the mobility of the hydrocarbon chains is strongly affected in this regime. The phase transition temperatures (T_m) range from 270 to 300 K for the gangliosides present in the TBG mixture (31). However, a temperature dependence study we have performed in a pure ganglioside G_{MI} (with T_m = 292.3 K) does not support this hypothesis. Another possibility would involve water traveling, and acting as a lubricant, between the cylinders of gangliosides in the hexagonal phase. For this case we see no special reason to have a much longer T_2 than that for the tightly bound water molecules. Thus, the most likely explanation involves the deuterium nuclei located in the exchangeable sites of the highly mobile exocyclic hydroxy methyl groups of galactose, N-acetyl-D-galactosamine and sialic acids. It has been observed by ^{13}C-NMR that these groups exhibit rapid reorientations (32) that average the interactions and account for longer T_2 values. In the micellar solution phase, the chemical exchange between deuterium nuclei in these groups and those in the more abundant hydration shell produces a broadening of the individual signals, leading to an apparent T_2 that is shorter than that observed in the H_I phase. At higher water/lipid ratios, a fast exchange regime is reached with the loosely bound water molecules. Under this condition, the signal with T_{2l} involving more than 170 deuterium nuclei, is probably the exchange-narrowed collapsed line coming from the hydroxy methyl groups and the loosely bound water molecules.

Figure 5 shows T_2 versus temperature for each environment in the 100:1 ^2H_2O/lipid molar ratio sample. The shorter T_2 value associated with the first hydration shell does not change significantly over the range of temperatures studied. On the contrary, the mobile population labeled as "*l*", which represents approximately 3% of the deuterium nuclei, undergoes a marked change in its T_2 value. This T_{2l} value decreases dramatically as temperature falls from room temperature to $T_f \sim 270$ K, in which both environments have essentially the same value. As we mentioned before, the hydroxy methyl groups seem to be the responsible for the longer T_2 signal in the hexagonal phase. This hypothesis is supported by the fact that there are on average five of carbohydrate groups per ganglioside molecule in a TBG sample (each containing only one exchangeable site). This represents 2.5% of the ^2H added in the 100:1 ^2H_2O/TBG molar ratio sample. **Figure 6** shows the tem-

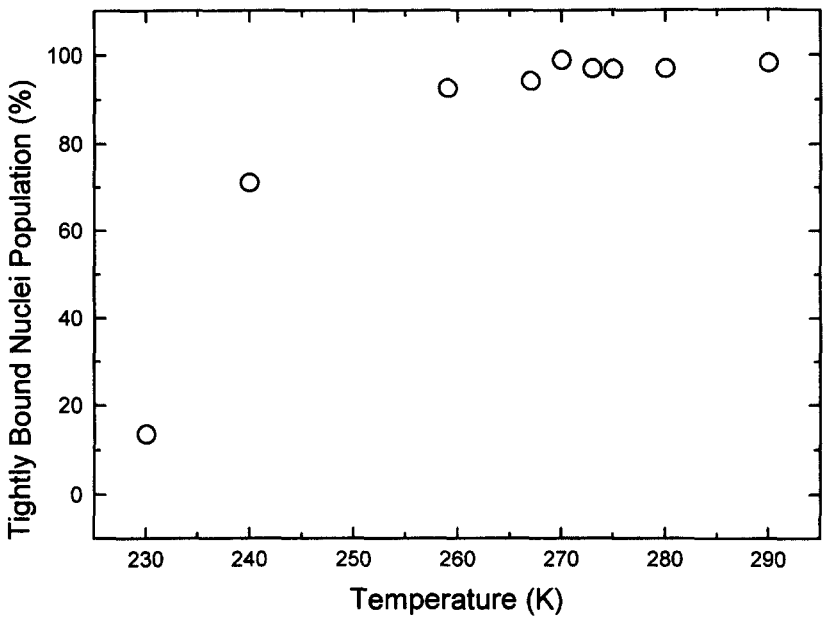

Fig. 6. Temperature dependence of the magnetization amplitude of tightly bound deuterium nuclei in the 100:1 molar ratio water/lipid sample.

perature dependence of the tightly bound population. This population diminishes as the temperature decreases below 270 K and the system becomes more rigid. This apparent loss of signal intensity may be explained by an abrupt decrease of T_2. Nuclei relaxing with T_2 in the order of the dead time of the spectrometer do not contribute to the signal.

In the 200:1 ^2H_2O/TBG molar ratio sample, lineshape analysis also makes it possible to distinguish two populations of molecules (**Fig. 7**); one is much more sensitive to the temperature variation than the other. This population is associated with the more mobile water molecules at high temperatures but becomes more rigid as temperature falls below the melting point of water T_f. In order to determine the proportion of nuclei related to each microenvironment, the spectra were fitted to two Lorentzian lineshapes. The linewidths $\Delta\nu$ of these curves provide the correlation times for molecules in each environment. We find that the tightly bound molecules have a correlation time τ_c equal to 2×10^{-9} s at 279 K whereas the loosely bound molecules have a correlation time of 6×10^{-10} s at the same temperature.

The areas under the curves allow us to determine the

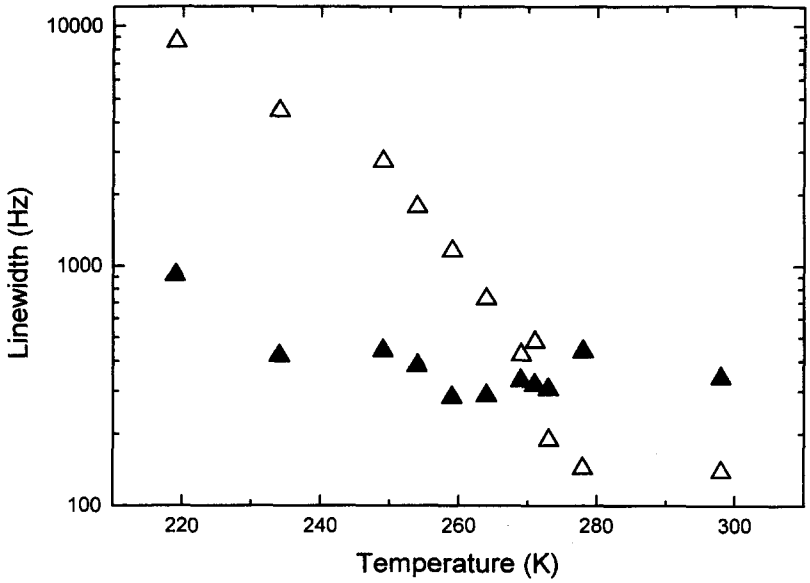

Fig. 7. ^{2}H-NMR linewidth as a function of temperature in the 200:1 molar water/lipid sample. Solid triangles, tightly bound nuclei; open triangles, loosely bound nuclei.

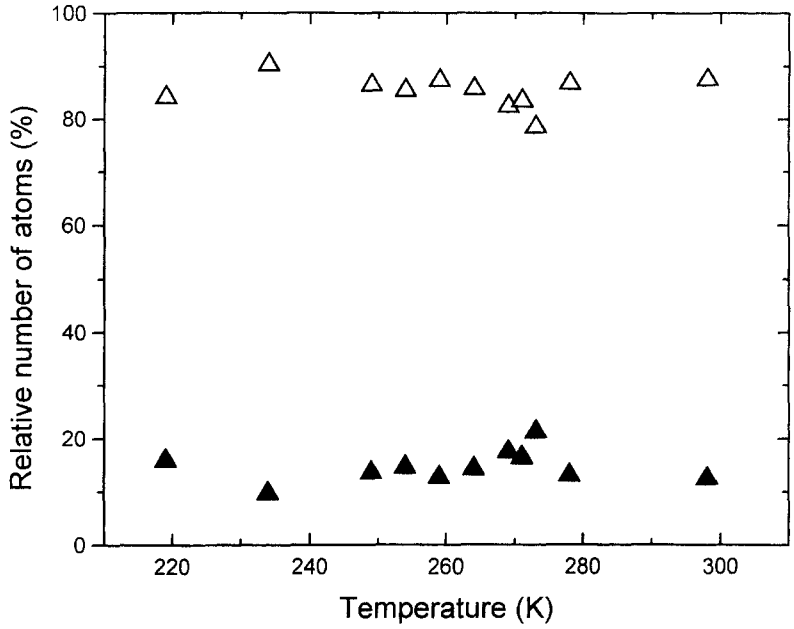

Fig. 8. Relative number of atoms in each microenvironment, as calculated from the areas of the Lorentzians, as a function of temperature for the 200:1 molar water/lipid sample. Solid triangles, tightly bound nuclei; open triangles, loosely bound nuclei.

relative number of nuclei in each microenvironment (**Fig. 8**). In the 200:1 molar ratio sample, an average of 16% of ^2H nuclei belongs to the less temperature-sensitive water population giving an estimate of 32 molecules of water strongly bound to each ganglioside molecule. These results are in complete agreement with the results of calorimetric experiments (33) where the number of unfreezable molecules of water per lipid molecule was found to be 22–30 in G_{MI}, and 33–40 in a $G_{DIa} + G_{DIb}$ mixture. It should be noted that there are about 25 exchangeable $^1H/^2H$ sites at the oligosaccharide polar head group which, under the experimental conditions used, cannot be distinguished from water. Consequently, we can say that there are at least 20 molecules of water tightly bound to each ganglioside molecule. This is as expected, well above the 11 water molecules found to be tightly bound to phosphatidylcholine (21).

CONCLUSIONS

Previous evidence of a large amount of structured water associated with ganglioside polar head groups (33) has been confirmed in this work. In addition, a more detailed dynamic study of the complex hydration shell of gangliosides was performed. The results are in full agreement with a model consisting of two hydration populations surrounding the aqueous ganglioside aggregates. As the NMR data are obtained from the resonance signal of ^2H nuclei, we can conclude that the bound shell is formed by tightly associated water molecules at the oligosaccharide polar head group on the gangliosides. Also, there is an overlapping contribution to this NMR signal arising from ^2H which has replaced labile ^1H in the OH, NHCO, and COOH exchangeable positions at the carbohydrate groups of the gangliosides. The other well-differentiated hydration environment, visualized in the micellar solution phase, is constituted of water molecules which, although much more mobile than the first group, do not have the dynamic characteristics of bulk free water. Water molecules from both populations undergo exchange between the defined shells by translational diffusion at a rate slower than the fast exchange regime usually assumed in biological systems.

The enhancement of phospholipase A_2 and phospholipase C activities against phospholipid bilayers has been correlated with the formation of hexagonal phase H_{II} induced by diacylglycerol (34). Accordingly, it has been observed that inhibition of these activities is produced by gangliosides, which in an independent study were found to inhibit the temperature or composition induced H_{II} phase formation (35, 36). Thus, a simple mechanism involving the inhibition by gangliosides of the H_{II} phase formation by opposing geometrical constrains can be invoked in order to interpret the changes in phospholipases A_2 and C activities. On the other hand, the fact that the inhibition of these activities is independent of the topological state of the substrate (15), occurring in monolayers, micelles, and vesicular aggregates, leads us to think of a less curvature-dependent mechanism. In this sense, the fact that the phos-

pholipase A_2 binding occurs with dehydration of the microinterphase (37) suggests that the high capacity of ganglioside to structure water plays a major role in the inhibitory process. Probably, the complex network of hydrogen bondings generated by the water tightly bound to the oligosaccharide groups alters the availability of the substrate.

Further experiments using lipids with packing parameters leading to structures similar to those formed by gangliosides, but with different hydration properties, will help to clarify the importance of each mechanism.

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