*Biochimica et Biophysica Acta*, 986 (1989) 241–249
Elsevier



BBAMEM 74612

# The ordering and dynamics of the terminal methyl group on the lignoceric acid chain in cerebroside sulfate

K.R. Jeffrey [1], J.M. Boggs [2], K.M. Koshy [2] and A.P. Tulloch [3]

[1] *Biophysics Interdepartmental Group, Department of Physics, University of Guelph, Guelph, Ontario,* [2] *Department of Biochemistry, Hospital for Sick Children, Toronto, Ontario, and* [3] *National Research Council, Plant Biotechnology Institute, Saskatoon, Saskatchewan (Canada)*

(Received 24 May 1989)

Key words: Cerebroside sulfate; Lignoceric acid; Phase transition; NMR, $^2$H-

**Cerebroside sulfate containing lignoceric acid deuterated at the terminal methyl group was prepared by a semi-synthetic method in order to study the ordering and motion of the fatty acyl chain by deuterium NMR spectroscopy. The lipid was hydrated with either 2 M KCl or 2 M LiCl. The NMR results are consistent with the formation of an interdigitated bilayer in the gel phase as suggested by spin label and X-ray diffraction studies, although they do not delineate whether the interdigitation is of the partial or mixed triple-chain type. In the low-temperature stable phase formed by this lipid on slow cooling from the liquid-crystalline phase, which has been shown by X-ray diffraction to be partially interdigitated, the dominant molecular motion is reorientation of the lipid about its long axis. The motion had a correlation time of the order of the deuterium quadrupole coupling constant ($10^{-6}$ s). The rate of axial diffusion is greater than that found for symmetric phospho- or glycosphingo-lipids in their crystalline subgel phases, but less than that of a lipid which forms a non-interdigitated gel phase bilayer such as DPPE, or a lipid which forms a fully interdigitated gel phase bilayer like DHPC. The rate of motion appears to be greater in a low temperature, more hydrated metastable phase formed by this lipid after cooling rapidly from the liquid crystalline phase. This phase is thought to be a mixed interdigitated bilayer based on spin label studies. The disorder observed at the C-terminal methyl group in the $L_\alpha$ phase, which is probably partially interdigitated according to X-ray diffraction studies, is greater than that of a non-interdigitated bilayer formed by a symmetric lipid. In the CBS/$Li^+$ system at high temperature, there is evidence for a liquid-crystalline to liquid-crystalline phase transition, in which the average orientation of the terminal methyl group changes.**

## Introduction

Generally acidic glycosphingolipids such as the sulfatides and gangliosides are found in relatively small amounts [1] in the extracellular monolayer of the plasma membrane. In myelin [2], however, cerebroside sulfate (galactosylceramide $I^3$-sulfate) is the major charged glycolipid representing 3.8 wt% of the total lipid, almost as high as the phosphatidylserine content. CBS consists of a number of lipid species because both the fatty acyl chain and the sphingosine base can vary. The fatty acyl chain of CBS found in the brain varies in length from 14 to 26 carbons but lengths greater than 22 predominate [3]. The fatty acid can be monounsaturated and/or α-hydroxylated. The predominant non-hydroxy fatty acid is 24 carbons long with one unsaturated carbon bond (24 : 1) while the major hydroxy lipid chain is 24 : 0. The sphingosine base of glycosphingolipids varies from 14 to 22 carbons in length and is predominantly unsaturated with up to three free hydroxy groups. In bovine brain CBS, the starting material used to prepare the lipid for the measurements reported in this paper, the sphingosine base is 94% dihydroxy 18 : 1, 4% dihydroxy 18 : 0, 2% dihydroxy 18 : 2, and 1% dihydroxy 16 : 0 [4].

Only recently has there been much effort [5–9] to study the physical properties of cerebroside sulfate in

Abbreviations: CBS, cerebroside sulfate (galactosylceramide $I^3$-sulfate); NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine; NLGS, *N*-lignocerylgalactosylsphingosine (cerebroside); NPGS, *N*-palmitoylgalactosylsphingosine (cerebroside); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; PC, phosphatidylcholine; EFG, electric field gradient.

Correspondence: K.R. Jeffrey, Biophysics Interdepartmental Group, Department of Physics, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

an attempt to further our understanding of their role in membranes and the relationship between their structure and function. The fatty acyl chains in CBS are generally much longer than the sphingosine moiety and the packing of such asymmetric molecules in the ordered gel and disordered liquid-crystal phase is not clear. The phase behavior of CBS containing lignoceric acid (24:0) has been investigated using DSC and a fatty acid spin probe, 16-doxylstearic acid [5,6] and is very dependent on the heating and cooling rates and sample history [5–8]. In 2M $K^+$, which helps shield the charge repulsion of the sulfate, DSC indicates that on heating there is a phase transition in the region of 68–69°C with an enthalpy of about 15.5 kcal/mol, provided the sample has been cooled and reheated relatively rapidly (10 C°/min) [7]. ESR indicates that this is the main gel to liquid-crystalline phase transition. On cooling ESR suggests that the conformation of the acyl chains in the gel phase depends on the cooling rate. On very rapid cooling to 10°C, the behavior of 16 doxylstearic acid suggests that the CBS/$K^+$ solution may form a mixed interdigitated bilayer [10,11] where the short chains from lipid on each side of the bilayer meet in the center and the long C24:0 chain passes all the way through the bilayer [8]. Such a structure is formed by asymmetric forms of PC such as 18:10 PC [12,13] and by C24:0 sphingomyelin [14].

However, if C24:0-cerebroside sulfate is cooled and reheated more slowly (less than 1.25 C°/min), it goes into a more ordered phase in which the spin label is insoluble. The temperature and enthalpy of the transition of this phase to the liquid-crystalline phase are increased to 71.1°C and 18.8 kcal/mol [7]. X-ray diffraction studies [15] indicated this low temperature phase is partially interdigitated i.e., the sphingosine chain of the lipid on one side of the bilayer is packed end to end with the fatty acid chain on the other side of the bilayer. Its behavior is similar to that of C24:0 cerebroside, NLGS, where again X-ray diffraction [16] has shown that the fatty acid and sphingosine chains are partially interdigitated. In addition X-ray diffraction [15] shows that dehydration of CBS lipid bilayers may also occur upon storage at low temperature.

DSC indicates the possibility that on cooling C24:0-CBS transforms from the liquid-crystalline phase into the gel phase via a series of transitions. Similar behavior observed in NLGS [16] bilayers suggests that the formation of the low-temperature gel state progresses stepwise through at least one intermediate state.

To date there has been little investigation of the ordering and dynamics of the sphingolipids or of the asymmetric lipids of any type in their various phases using NMR spectroscopy. Wide-line deuterium NMR has proven to be a powerful method for the study of ordering and motion in lipids [17–20] at the molecular or sub-molecular level. In the present context, deuterium is the preferred nucleus for several reasons: (1) it can be easily introduced at specific sites in the molecule (2) it has spin $I = 1$, and the resulting quadrupolar interaction reflects the time averaged site symmetry, (3) measurements of the spin-lattice relaxation time can determine with the aid of a suitable model the time scales of the molecular motions and (4) the spectral linewidths ($\approx$ 170–200 kHz) are in a convenient range for modern Fourier Transform spectrometers. In summary, deuterons acts as an excellent probe of the local environment at specific molecular sites.

In this study cerebroside sulfate containing lignoceric acid deuterated at the terminal methyl group was synthesized in order to study the ordering and motion of the fatty acyl chain by deuterium NMR. The characteristic deuterium NMR spectra are used to identify the long range ordering associated with each phase of the CBS system. The terminal methyl on the lignoceric acid moiety is located in the very interesting region extending beyond the adjacent sphingosine base where the effects of interdigitation may be felt. The lipid was studied in the presence of either 2 M KCl or 2 M LiCl. $Li^+$ has a lower affinity for the sulfate group of CBS and shields the charge less effectively than $K^+$. Thus the transition temperature and enthalpy are lower in 2 M $Li^+$. Because of the greater charge repulsion between the CBS head groups in $Li^+$ there is a greater tendency for the lipids to stay in any metastable phase formed on rapid cooling of the sample.

## Materials and Methods

### *Synthesis of deuterium labelled CBS*

Cerebroside sulphate containing [24-$^2H_3$]lignoceric acid was prepared by deacylation of the bovine brain CBS and reacylation as described [5,7]. The [24-$^2H_3$]lignoceric acid was prepared by elongation of the [12-$^2H_3$]dodecanoic acid, prepared as described [21], by the reaction with morpholinocyclohexane, followed by alkaline cleavage of the $\alpha$-diketone and reduction of the introduced carbonyl with cyanoborodeuteride [17]. The fatty acid was purified by crystallization from ethanol at 10°C.

### *NMR measurements*

The deuterium NMR spectra were obtained using a home build spectrometer and either a superconducting magnet operating at 6.3 T (280 MHz for protons) or at 8.5 T (360 MHz for protons). The NMR signal was collected using a digital oscilloscope (Nicolet model 2090) which was interfaced to an IBM/pc computer. Software written specifically for the spectrometer not only allowed the collection of the free induction decay signal and subsequent Fourier transformation to obtain the spectrum but also control of the sample temperature and pulsing of the spectrometer so that data collection

over the period of several days could take place under computer control. The quadrupolar echo [22] pulse sequence $(\pi/2)_x$-$\tau$-$(\pi/2)_y$-$\tau$-acquire was used to obtain the spectra. Typically the $\pi/2$ pulse width was 3 $\mu$s and the separation between the pulses, $\tau$-, was 45 $\mu$s. Because the deuterium NMR spectra from the methyl group do not cover a wide spectral range (less than 50 kHz), the use of radio frequency pulses as short as 3 $\mu$s leads to little loss in signal intensity in the wings of the spectra [23].

Spin-lattice relaxation time ($T_1$) measurements were made by initially inverting the broadline spectrum using a $\pi$ pulse then waiting a variable length of time before measuring the amplitude of the nuclear signal. Measurements of the signal amplitude were made by integrating a portion of the quadrupolar echo for typically 15 different delay times between the $\pi$ pulse and the subsequent quadrupolar echo. This method gives a $T_1$ averaged across the quadrupolar powder pattern.

The NMR samples were made by placing 20 mg of deuterated lipid with 200 $\mu$l of buffered ionic solution, either 2 M KCl or LiCl containing 10 mM Hepes buffer adjusted to pH 7.4, in a 8 mm glass tube which was sealed. The sample was thoroughly mixed by heating to about 80 °C, vortexing and centrifuging lightly. The cycle was repeated about 10 times. Independent measurements were carried out on two sample for each ionic solution.

The sample was held within a thick walled copper container to eliminate any temperature gradients. The sample temperature could be read to within 0.1 C° and was electronically regulated to approximately 0.2 C° over the period of time needed to obtain a spectrum or relaxation time measurement. During a typical temperature run the sample was heated to the liquid crystal phase and measurements carried out by lowering the temperature a few degrees at a time down to 0 °C or below before returning stepwise to the liquid-crystal phase. The sample would stay at each temperature for about 2 h.

In one series of experiments an attempt was made to trap the intermediate metastable phase observed by ESR [6]. The sample was removed from the NMR probe and heated to about 90 °C then plunged into an ice bath. The sample after remaining at 0 °C for several minutes was quickly transferred back into the probe which was held at 10 °C and a spectrum taken.

## Results

Typical $^2$H-NMR spectra for the terminal methyl group on the lignoceric acid chain of CBS in 2 M KCl are shown in Fig. 1. In the temperature region above $\approx$ 70 °C a narrow axially symmetric powder pattern is observed. This spectrum is characteristic of the liquid-crystalline phase where the chain have melted and there is motion which is axially symmetric about the bilayer normal [19]. Below $\approx$ 45 °C there is a much broader powder pattern where the characteristic peaks and shoulders are not very well defined as shown in Fig. 1. These spectra are characteristic of the gel and crystalline phases in hydrated lipid systems [18]. The lack of

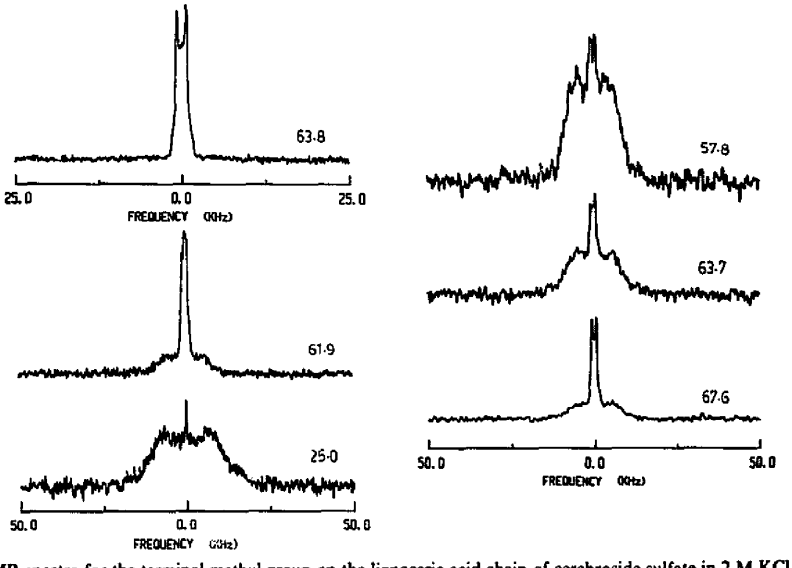


Fig. 1. Deuterium NMR spectra for the terminal methyl group on the lignoceric acid chain of cerebroside sulfate in 2 M KCl. The temperatures at which the spectra were recorded are indicated for each spectrum. The spectra on the left were recorded on a cooling run while these on the right on heating.

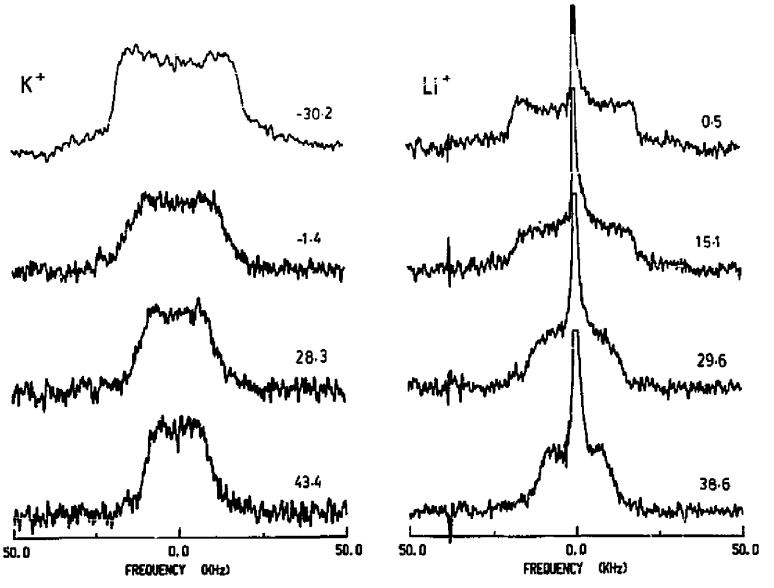


Fig. 2. Deuterium NMR spectra for the methyl group in the gel phase. The spectra on the left are for cerebroside sulfate in 2 M KCl solution and on the right for 2 M LiCl. A small residual $D_2O$ peak has been removed for the KCl spectra. The central peak in the LiCl spectra is larger than that expected from the $D_2O$ in the ionic solution and may indicate the formation of small lipid vesicles.

sharp peaks are a result of the presence of molecular motions which have time scales of the order of $\omega_Q$ [24]. The effective quadrupolar splitting is about 17 kHz at 45°C as shown in Fig. 2 and decreasing the temperature brings about a further increase in the width of the powder pattern. At −30°C there are sharp edges with a splitting of about 38 kHz.

Upon cooling, the spectra in the region between 64 and 58°C the high- and low-temperature spectra appear superimposed as shown in Fig. 1. The first indication of the gel phase in the NMR spectra appears at 64°C and by 60°C, 70% of the original liquid-crystalline phase has transformed to the gel phase. However, some of the liquid crystal lingers on to much lower temperatures. This shows that the lipid transforms from the liquid crystalline phase to the gel phase during the set of two higher temperature transitions seen by DSC and in agreement with spin label results [6,8]. At the slow cooling rate used in NMR measurements, the third low-temperature transition seen by DSC may overlap with the other two, since no further pronounced changes are observed in the NMR spectra on cooling below 25°C. Alternatively, the changes in molecular dynamics which occur at the lower transition observed on the DSC scan have little influence on the lipid chains at the center of the bilayer.

The hysteresis associated with the transformation is explicitly set out by comparing the spectra recorded on cooling with those obtained upon heating as shown in Fig. 1. On heating, the first indication of the liquid-crystalline phase appears at about 55°C but there is gel phase still evident at 69°C. Again the transition takes place unevenly over this temperature range. The last 70% of the original gel phase transforms to the liquid-crystal phase in the temperature range from 67 to 69°C. These observations are in essential agreement with the DSC results where a single peak is seen in the DSC scan on heating in the region from 69 to 72°C depending on the sample history.

An approach to the analysis of deuterium NMR spectra which does not depend on any assumption about the possible molecular motions taking place is to use the method of moments [18]. The $n$th moment of the spectrum with lineshape $f(\omega)$ is defined by

$$M_n = \int_0^\infty d\omega \omega^n f(\omega) / \int_0^\infty d\omega f(\omega) \quad (1)$$

This particular definition of the moments means that even though the deuterium spectra are symmetric, the odd moments are not zero. The first moments of the experimental deuterium spectra are shown in Fig. 3 as a function of temperature. The sudden increase or decrease in the first moment is associated with the change from those spectra characteristic of the liquid crystal to those of the gel phase. The data graphically demonstrate the hysteresis in the transformation between the gel phase and liquid-crystalline phases on heating and cooling. The phase transition is about 5 C° lower on cooling. Within the gel phase there is a steady increase

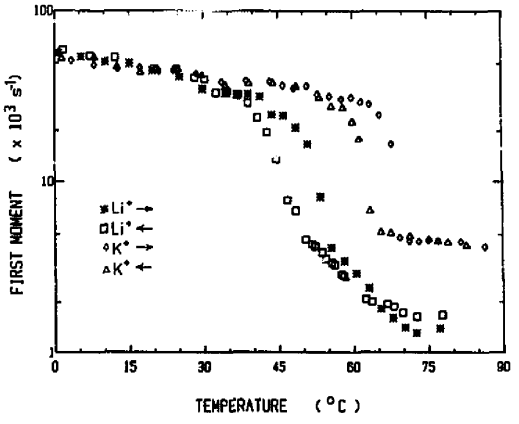


Fig. 3. The first moment of the deuterium NMR spectra in 2 M KCl and 2 M LiCl. The data obtained on cooling the $Li^+$ sample (□) and those obtained on heating (•) are shown. Data for $K^+$ solutions on cooling (△) are indicated and on heating (◇) are presented. The transition from the gel to liquid crystal in the region shows considerable hysteresis for CBS in either ionic solution.

in width of the powder pattern with decreasing temperature, but no distinct transitions are apparent.

The phase behavior in the presence of 2 M LiCl is similar but not identical to that found with KCl. Spectra characteristic of the $L_\alpha$ and gel phases are observed in the CBS/$Li^+$ system as in the CBS/$K^+$ system. However, in the presence of $Li^+$ ions, there is an isotropic peak superimposed on the deuterium powder pattern at all temperatures. Part of this signal is a result of $D_2O$ in the LiCl solution but there is an additional component which must arise from molecules which isotropically tumble in a time frame short compared to $1/\omega_Q$. One possibility is that the $Li^+$ ions induced the formation of a distribution of small lipid vesicles. Breakdown of large multilamellar vesicles of asymmetric forms of PC and sphingomyelin into small vesicles has been observed to occur at the phase transition by electron microscopy [12,25].

The transition from the $L_\alpha$ to the gel phase in the presence of $Li^+$ ions has a similar hysteresis to that found for KCl solutions. On cooling the first indication of the gel phase occurs at 43°C while on heating the gel phase does not completely disappear until 51°C. The first moments shown in Fig. 3 emphasize the hysteresis at the gel phase boundary. The rapid change in the first moment at the main chain melting transition is very evident and matches the behavior for the samples in KCl solution but at a lower temperature. Within the gel phase below 40°C there is a progressive increase in the width of the deuterium powder pattern with decreasing temperature as shown in Fig. 2. The plot of the first moments shown in Fig. 3 seems to indicate that the gel phase behavior of the deuterium spectra are very similar for CBS in the two solutions. However, close inspection of the deuterium spectra shows that there is more intensity in the wings of the spectra for the $Li^+$ samples. The second moments for the $Li^+$ samples are larger than for the $K^+$ samples. The sharp edges at a separation of about 40 kHz characteristic of slowing down of the axial rotation of the lipid molecule appear at a higher temperature in the $Li^+$ samples.

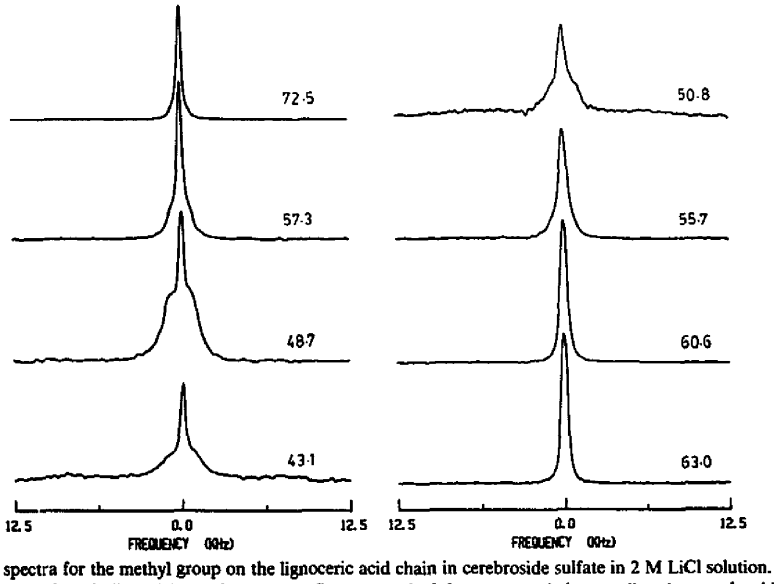


Fig. 4. Deuterium NMR spectra for the methyl group on the lignoceric acid chain in cerebroside sulfate in 2 M LiCl solution. The temperatures at which the spectra were recorded are indicated for each spectrum. Spectra on the left were recorded on cooling the sample while those on the right on heating.

Spectra in the liquid-crystalline phase for the CBS/$Li^+$ system are very different that those observed for the CBS/$K^+$ system. Above $\approx 60°C$ there is no observable quadrupolar splitting as shown in Fig. 4. The NMR spectrum consists of a single narrow isotropic line even though X-ray diffraction studies indicated that the CBS/$Li^+$ system remains multilamellar at this temperature [15]. In the region about 50 °C there is a superposition of a narrow line and a broader one. In Fig. 4 the spectrum taken at 48.7 °C shows two components one having half-width equal to 0.3 kHz and the other 2.5 kHz. The broader component again does not have any distinguishable quadrupolar splitting but the width of the line is similar to that found for CBS in $K^+$ solutions. The broader component is more prominent on cooling than on heating as seen from Fig. 4. Because the width of this component is relatively narrow in comparison with typical gel phase spectra, it is reasonable to assume that the sample is still in a liquid-crystalline phase. The first moment at high temperature for the LiCl samples as shown in Fig. 3 is significantly smaller than for the KCl samples and emphasizes the fact that the liquid crystal behavior of CBS in the two ionic solutions is very different.

In an attempt to observe the metastable state seen using ESR spin probe the temperature of the samples was reduced as rapidly as possible from 90 to 0 °C and the spectra recorded at 10 °C. Fig. 5 compares spectra

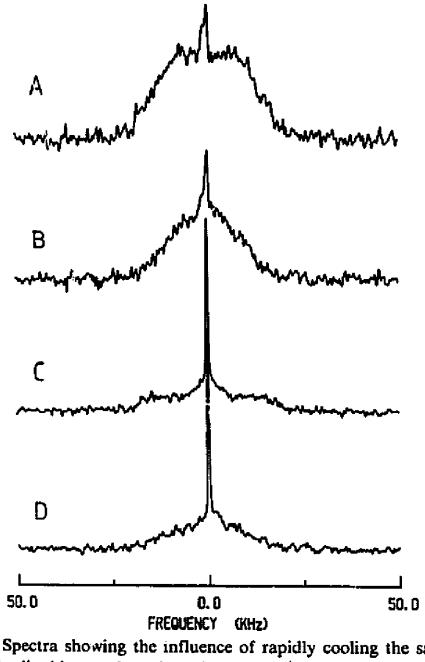


Fig. 5. Spectra showing the influence of rapidly cooling the samples from the liquid crystal to the gel phase. $K^+$ sample: slow (A) and rapid (B). $Li^+$ sample: slow (C) and rapid (D).

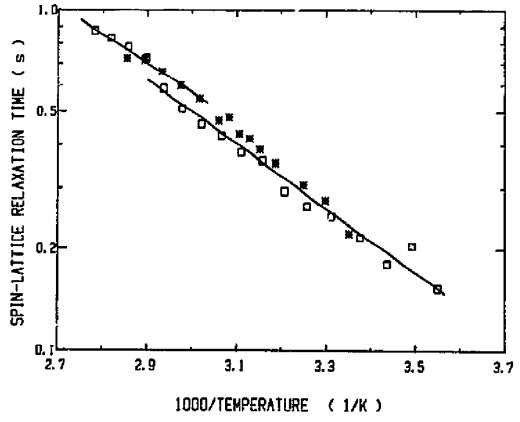


Fig. 6. The spin-lattice relaxation time as a function of the reciprocal temperature in 2 M KCl. The data obtained on cooling (•) and on heating (□) the sample are presented. The lines are least-squares fits to the data above and below the transition temperature.

for both $K^+$ and $Li^+$ samples when they have been cooled rapidly (seconds) and very slowly (days). The spectra taken immediately after rapid cooling have a distinctly different shape and the decay of the NMR signal amplitude with spacing between the pulses in the quadrupolar echo sequence is more rapid for those samples that have been cooled quickly.

Fig. 6 shows the deuterium spin-lattice relaxation times as a function of reciprocal temperature for cerebroside sulfate in the 2 M KCl solution. The data follows an Arrhenius temperature dependence with a discontinuity at the phase transition from the liquid crystal to gel phase. The hysteresis observed at the phase transition in the spectra is also apparent from the relaxation time. The fact that $T_1$ increases with increasing temperature means that the correlation time of the molecular motion dominating the relaxation behavior is short compared to the Larmor precession frequency ($2\pi \times 41$ MHz) [18]. The activation enthalpy for the molecular motion can be obtained from the slope of the least-squares lines shown on the diagram. The values in the two phases are the same within experimental error and equal to $4.0 \pm 0.1$ kcal/mol.

## Discussion

Deuterium NMR has made a very significant contribution to our understanding of the molecular dynamics in lipid/water mixtures [26,27]. Detailed examinations of the deuterium lineshapes and relaxation time measurements relevant to this study of CBS have been carried out on the following lipid systems; *N*-palmitoylgalactosylceramide [28], DMPC [27,29,30,31] and DPPE [32]. As well, there have been studies of the influence of cholesterol [33], lipid mixtures [34,35] and integral membrane proteins [36].

Simulations [26,27,29] of the deuterium spectra have used models which take into account the following types of motion of the lipid chains: (i) rotational diffusion both about and (ii) perpendicular to the long axis of the lipid molecule and (iii) *trans-gauche* isomerization as well as (iv) methyl group reorientation about the terminal C-C bond if appropriate.

*The gel phase*

The deuterium NMR spectra within the gel phase for the cerebroside sulfate in either ionic solution do not exhibit the sharp peaks and shoulders usually associated with quadrupolar powder patterns when the molecular motions are either very fast or very slow with respect to the deuterium quadrupole coupling constant. There must, therefore, be molecular motions which have correlation times of the order of $\omega_Q$ ($10^6$ s) present in the gel phase. Below $-30\,^\circ$C for the KCl solution and $0\,^\circ$C for the LiCl solution there are prominent edges in the spectra, the precursors of peaks, separation by about 40 kHz. This value is close to 127/3 kHz expected when there is only methyl group reorientation about the terminal C-C bond in the lignoceric acid chain. At the lowest temperatures investigated in these experiments the only motion not frozen out, therefore, is the reorientation of the terminal methyl group about the last C-C bond in the chain. This occurs at a higher temperature in $Li^+$ than in the $K^+$ solutions.

Methyl group reorientation dominates the spin-lattice relaxation time over the entire temperature range investigated. The spin-lattice relaxation times allow a determination of the correlation time for the motion. The relaxation is due to that part of the quadrupolar interaction which is made time dependent by the molecular motion. It is possible to express the relaxation rate $1/T_1$ in terms of the change in the second moment $\Delta M_2$ of the lineshape resulting from the molecular motion.

$$1/T_1 = \Delta M_2\{(\tau_c/(1+\omega^2\tau_c^2)+4\tau_c/(1+4\omega^2\tau_c^2))\} \qquad (2)$$

For methyl group reorientation, $\Delta M_2 = (8/9)M_2(\mathrm{RL})$ where $M_2(\mathrm{RL})$ is the second moment at very low temperature where all motion has ceased. From the measured value of $T_1$ the correlation time at room temperature is about $10^{-11}$ s. From spectral simulation in DMPC [31] the correlation time for the methyl group reorientation at room temperature was about $2 \cdot 10^{-11}$ s and the activation energy was 2.4 kcal/mol. However, from the slope of the $T_1$ [31] versus the reciprocal of the temperature plot the activation energy is calculated to be 4 kcal/mol as found in this study of CBS.

Returning to the first moment of the spectra, it can be seen that there is a decrease by about a factor 2 within the gel phase over the temperature region investigated ($-30$ to $45\,^\circ$C). The factor of two suggests that reorientation about the long axis of the lipid molecule becomes of the order of the quadrupolar interaction in this temperature region; that is, the reorientation rate is about $10^6$ s$^{-1}$. Molecular motion within the gel phase has been studied in a number of lipid systems using deuterium NMR. The simulations indicate that for NPGS [28], rotation of the molecule about the long axis is very slow on the NMR time scale and the lineshape is primarily determined by *trans-gauche* isomerization. In this situation spectra having an effective asymmetry parameter $\eta = 1$ are observed in the gel phase just below the transition to the liquid-crystal phase. On the other hand in DPPE [32], the rotation about the long axis has a rate of about $10^6$ s$^{-1}$, a frequency of the order of the quadrupole interaction, while the *trans-gauche* isomerization rate is about an order of magnitude less. The simulation of the DMPC spectra [27,29] shows that the correlation times for *trans-gauche* isomerization and long axis rotation are of the same order of magnitude at the high temperature limit of the gel phase. DHPC [37] which is fully interdigitated in the gel phase, persists in axial diffusion down to at least $-21\,^\circ$C. A comparison of the spectra shown in Fig. 2 with the spectra published in the papers noted above reinforces the conclusion that while there is probably a combination of molecular motions responsible for the observed lineshapes, the rotational frequency for the reorientation about the long axis is of the order of the quadrupolar interaction and dominates the spectral changes as in the case of DPPE. Although axial diffusion of DPPE slows down at low temperature as does CBS, the rotation frequency at the gel to liquid-crystal transition appears to be greater for DPPE than for CBS judging from the spectral linewidth. The slower rotational frequency at the phase transition for CBS may be caused by interdigitation of the asymmetric chains. Interdigitation of either the partial or mixed type might be expected to slow axial rotation relative to either non-interdigitated or fully interdigitated bilayers. Axial rotation of an asymmetric lipid in a partially or mixed interdigitated bilayer would require a simultaneous and concerted rotation of lipid molecules on both sides of the bilayer.

X-ray diffraction shows that there is a transformation from hexagonal to orthorhombic packing of the lipid chains in the gel phase of these CBS ionic solution mixtures. Such a change has been observed using deuterium NMR in rubidium stearate/water mixtures [38]. Because the rotation about the long axis of the molecule only has two fold symmetry in the orthorhombic phase, the deuterium powder pattern is axially asymmetric and shows the characteristic shoulder as well as peak and step. For the rubidium stearate system the asymmetric powder pattern is most evident when the deuterons are substituted into the middle of the acyl chain. The asymmetry in the powder pattern was not

evident in the terminal methyl group spectrum. The gel phase spectra for the terminal methyl group reported in this paper also show no evidence of the hexagonal to orthorhombic transition.

To have a more detailed analysis of the lipid dynamics in CBS/ionic solution mixtures, it will be necessary to have spectra and relaxation time data for deuterons substituted at other positions on the acyl chain. Such experiments are now in progress.

*The liquid-crystal phase*

For CBS in $K^+$ ionic solution there is a well defined axially symmetric quadrupolar powder pattern typical of the liquid-crystal phase for most lipids. The observed splitting, 1.7 kHz, is significantly smaller than that seen for the terminal methyl in DPPC (3 kHz), and in DMPC (5 kHz). The resulting very small $S_{C\text{-}D}$ order parameter reflects the motional freedom of the terminal methyl group. In the $L_\alpha$ phase there is rotation of the lipid molecule about the long axis of the molecule, *trans-gauche* isomerization and reorientation of the terminal methyl group. All of these motions are rapid on the NMR time scale. The methyl group rotation projects the quadrupolar tensor along the terminal C-C bond while the rotation about the long axis projects the resultant tensor along the bilayer normal. The observed quadrupolar splitting depends on the factor $\langle(3\cos^2\alpha - 1)/2\rangle_{avg}$ where $\alpha$ is the angle between the terminal C-C bond and the bilayer normal. The *trans-gauche* isomerization causes this C-C bond to be oriented predominately at angles of 35.3, 90 and 144.7° with respect to the bilayer normal. If the probabilities of the C-C bond being at these orientations are $P_1$, $P_2$ and $P_3$ then the observed quadrupolar splitting is given by

$$\Delta\nu_Q = (e^2qQ/8h)[P_1 + P_3 - P_2] = (e^2qQ/8h)[1 - 2P_2] \qquad (3)$$

because $P_1 + P_2 + P_3$-1. The observed quadrupolar splitting of 1.7 kHz can be interpreted as meaning that $P_2 = 0.46$. The small splitting is a result of the terminal C-C being oriented at 90° with respect to the bilayer normal nearly 50% of the time.

As shown in Fig. 4, the spectra in the presence of $Li^+$ ions and in the region just above the gel phase consist of two components. One of the component spectra may have a quadrupolar splitting similar to that seen with the $K^+$ ions but the peaks are not resolved and a comparison of the width can only be made. At higher temperatures where there is a single component, if there is any quadrupole splitting it is extremely small and can not be resolved with the present equipment. One possible explanation of these results in that $Li^+$ ions at high temperature in the liquid-crystalline phase induce the formation of a long range lipid structure with a small radius of curvature. The diffusion of the lipid molecules over these highly curved surfaces averages the quadrupolar splitting to a very small value. The other possible interpretation arises from the application of Eqn. 12. If the terminal C-C bond can have the three possible orientations listed above and is oriented perpendicular to the bilayer normal 50% of the time then the predicted quadrupolar splitting is zero.

The observation of two components in the spectra in the region from 43 to 57 °C indicates the possibility that there are in fact two liquid-crystalline phases in the presence of $Li^+$ ions. DSC studies showed that CBS in 2 M LiCl has a greater number of distinguishable transitions on cooling than in 2 M KCl. It has three overlapping broad peaks in the range 45–57 °C, a distinct sharp one at 41 °C, and possibly a broad one at 37 °C. The high temperature ones may include a liquid-crystalline II to liquid-crystalline I phase transition. The sharp peak at 41 °C corresponds to the main chain melting transition. On the first heating scan there is primarily, a sharp transition at 54 °C and a shoulder at 60 °C. On reheating scans there is in addition, a broad transition at 45 °C. The shoulder at 60 °C may represent the reverse liquid-crystalline I to liquid-crystalline II phase transition.

*The gel to liquid-crystal phase transition region*

The NMR results show the coexistence of the gel and liquid-crystal phases over an appreciable temperature range. In the case of DPPC it is now recognized that the coexistence is a result of sample impurities and that the more refined the sample the narrower the transition region [39]. The samples used in these experiments do not consist of a single-lipid type because of the variation in the sphingosine base in the starting bovine CBS. It is not surprising therefore to observe a significant transition region. The sphingosine base is dominated by dihydroxy 18 : 1 chains and this is reflected in the fact that 70% of the lipid is transformed over a 3 C° range but the deuterium NMR is sufficiently sensitive to show a mixture of phases over a much wider temperature range as in Fig. 1.

Usually the NMR spectra were collected over a long period of time and jumps in temperature of 2–3 C° were the largest taken. The samples stayed at one temperature for 1–4 h before proceeding to the next. This probably allowed transformation of the sample to its most stable phase. While hysteresis was present in both ionic solutions there was no indication of the formation of metastable phases with the usual slow changes in sample temperature. The DSC and ESR measurements which showed the metastability involved much more rapid changes in sample temperature. Quickly cooling the NMR samples from the liquid-crystalline to 10 °C in the gel phase did result in changes in the deuterium NMR spectra as shown in Fig. 5. An interpretation of these changes is, however, not immediately evident. The narrowing of the spectra suggests greater disorder in the

lipid chain on rapid cooling of the sample. The spin label ESR study [8] suggested that the lipid formed a mixed interdigitated bilayer following similar treatment.

Confirmation of the mixed interdigitation by X-ray diffraction was not possible because the bilayers formed on rapid cooling were too disordered to give well defined diffraction peaks [15]. However, the motion of the terminal methyl is expected to have a greater range of freedom in a mixed interdigitated bilayer than in a partially interdigitated one. Thus the observed narrowing of the NMR spectra following rapid cooling is consistent with this model.

Spectra with a greater signal to noise ratio and detailed simulations of the deuterium NMR spectra are necessary for a better understanding of the molecular behavior in the gel phase.

## Conclusions

This is the first $^2$H-NMR study of a highly asymmetric glycosphingolipid. The phase boundaries are consistent with previous DSC and ESR results and the NMR data clearly show which transition is the one associated with melting of the fatty acid chains. The NMR results are consistent with the formation of an interdigitated bilayer in both the gel and liquid-crystalline phases although they do not delineate whether the interdigitation is of the partial or mixed type. Axial diffusion in the gel phase is greater than that of a symmetric phospholipid in its crystalline subgel phase but less than that of a non-interdigitated bilayer such as DPPE or a fully interdigitated bilayer such as DHPC. The disorder measured at C-terminal methyl group in the $L_\alpha$ phase is greater than that of a non-interdigitated bilayer formed by a symmetric lipid. There is evidence for a liquid-crystalline to liquid-crystalline phase transition in the CBS/$Li^+$ system at high temperature. The two phases are differentiated by the average orientation of the terminal methyl group.

## References


1 Karlsson, K.-A. (1982) in Biological Membranes (Chapman, D., ed.), Vol. 4, pp. 1–74, Academic Press, New York.
2 Norton, W.T. and Cammer, W. (1984) in Myelin (Morell, P., ed.), 2nd Edn., pp. 147–195, Plenum Press, New York.
3 O'Brien, J.S., Fillerup, D.L. and Mead, J.F. (1964) J. Lipid Res. 5, 109–116.
4 Karlsson, K.-A. (1970) Chem. Phys. Lipids 36, 6–43.
5 Koshy, K.M. and Boggs, J.M. (1983) Chem. Phys. Lipids 34, 41–53.
6 Boggs, J.M., Koshy, K.M. and Rangeraj, G. (1984) Chem. Phys. Lipids 36, 65–89.
7 Boggs, J.M., Koshy, K.M. and Rangaraj, G. (1988) Biochim. Biophys. Acta 938, 361–372.
8 Boggs, J.M., Koshy, K.M. and Rangaraj, G. (1988) Biochim. Biophys. Acta 938, 373–385.
9 Ruocco, M.J. and Shipley, G.G. (1986) Biochim. Biophys. Acta 859, 246–256.
10 Hwang, C. and Mason, J.T. (1986) Biochim. Biophys. Acta 864, 325–359.
11 Slater, J.L. and Huang, C.-H. (1988) 325–359.
12 Hui, S.W., Mason, J.T. and Huang, C. (1984) Biochemistry 23, 5570–5577.
13 McIntosh, T.J., Simon, S.A., Ellington, J.C., Jr. and Porter, N.A. (1984) Biochemistry 23, 4038–4044.
14 Levin, I.W., Thompson, T.E., Barenholtz, Y. and Huang, C. (1985) Biochemistry 24, 6282–6286.
15 Stinson, R.H. and Boggs, J.M. (1989) Biochim. Biophys. Acta 986, 234–240.
16 Reed, R.A. and Shipley, G.G. (1987) Biochim. Biophys. Acta 896, 153–164.
17 Smith, I.C.P. (1985) in Structure and Properties of Cell Membranes (Benga, G., ed.) pp. 237–260, CRC Press Inc., Boca Raton.
18 Davis, J.H. (1983) Biochim. Biophys. Acta 737, 117–171.
19 Seelig, J. (1977) Q. Rev. Biophys. 10, 353–418.
20 Griffin, R.G. (1981) Methods Enzymol. 72, 108–174.
21 Tulloch, A.P. (1983) Prog. Lipid Res. 22, 235–256.
22 Davis, J.H., Jeffrey, K.R., Bloom, M., Valic, M.I. and Higgs, T.P. (1976) Chem. Phys. Lett. 42, 390–394.
23 Bloom, M., Davis, J.H. and Valic, M.I. (1980) Can. J. Phys. 58, 1510–1517.
24 Spiess, H.W. and Sillescu, H. (1981) J. Magn. Reson. 42, 381–389.
25 Hui, S.W., Stewart, T.P. and Yeagle, P.L. (1980) Biochim. Biophys. Acta 601, 271–281.
26 Wittebort, R.J., Olejniczak, E.T. and Griffin, R.G. (1987) J. Chem. Phys. 86, 5411–5420.
27 Meier, P., Ohmes, E., Kothe, G., Blume, A., Weldner, J. and Eibl, H.-J. (1983) J. Phys. Chem. 87, 4904–4912.
28 Huang, T.H., Skarjune, R.P., Wittebrot, R.J., Griffin, R.G. and Oldfield, E. (1980) J. Am. Chem. Soc. 102, 7379–7381.
29 Meier, P., Ohmes, E. and Kothe, G. (1986) J. Chem. Phys. 85, 3598–3614.
30 Westerman, P.W., Vaz, M.J., Strenk, L.M. and Doane, J.W. (1982) Proc. Natl. Acad.. Sci. USA 79, 2890–2894.
31 Mayer, C., Muller, K., Weisz, K. and Kothe, G. (1988) Liq. Cryst. (UK) 3, 797–806.
32 Blume, A., Rice, D.M., Wittebrot, R.J. and Griffin, R.G. (1982) Biochemistry 24, 6220–6230.
33 Blume, A. and Griffin, R.G. (1982) Biochemistry 24, 6230–6242.
34 Blume, A., Wittebrot, R.J., Das Gupta, S.K. and Griffin, R.G. (1982) Biochemistry 24, 6243–6253.
35 Lewis, B.A., Das Gupta, S.K. and Griffin, R.G. (1984) Biochemistry 23, 1988–1993.
36 Meier, P., Sachse, J.-H., Brophy, P.J., Marsh, D. and Kothe, G. (1987) Proc. Natl. Acad. Sci. USA 84, 3704–3708.
37 Ruocco, M.J., Makriyannis, A., Siminovitch, D.J. and Griffin, R.G. (1985) Biochemistry 23, 4844–4851.
38 Jeffrey, K.R. and Wong, T.C. (1984) Mol. Phys. 50, 289–306.
39 Albon, N. and Sturtevant, J.M. (1978) Proc. Natl. Acad. Sci. USA 75, 2258–2260.