PHYSICAL STUDIES OF CELL SURFACE AND CELL MEMBRANE STRUCTURE

DEUTERIUM NUCLEAR MAGNETIC RESONANCE INVESTIGATION OF DEUTERIUM-LABELLED N-HEXADECANOYLGLACTOSYLCERAMIDES (CEREBROSIDES)

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Summary

1. Deuterium Fourier transform nuclear magnetic resonance spectra of a series of N-palmitoylgalactosylceramides (cerebrosides) specifically labelled with deuterium at one of positions 2', 6', 10' and 16' of the acyl chain, or in the C-6 hydroxymethyl group of the galactose residue, have been obtained using a spin-echo technique at 34.1 MHz with a homebuilt superconducting magnet spectrometer.

2. The effects of temperature and cholesterol on the deuterium spectra have been investigated. The results indicate, when compared at the same reduced temperature, that the hydrocarbon chain organization in the liquid crystalline phase of palmitoylgalactosylceramide is essentially identical to that seen in similar chain length glycerophospholipids. In particular, two sets of quadrupole splittings are seen for a 2'-labelled N-palmitoylgalactosylceramide, indicating non-equivalent deuterons as noted previously for phospholipids.

3. Two sets of quadrupole splittings are observed for the headgroup C-6-labelled N-palmitoylgalactosylceramide. It is proposed that these signals arise from the enantiomeric R and S lipids, and that motion of the hydroxymethyl group is slow (greater than $10^{-5}$ s). These results suggest the presence of a hydrogen bond network in the polar headgroup region.

Abbreviations: PGAC, N-palmitoylgalactosylceramide; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DPPC, dipalmitoylphosphatidylcholine. The nomenclature of lipids is according to the recommendations (1976), IUPAC-IUB Commission on Biochemical Nomenclature, (1976) Lipids 12, 455.
4. The effects of cholesterol on the deuterium spectra of N-palmitoylgalactosylceramide-labelled as C\(^2\)H\(_3\) in the terminal methyl group, at 1:1 mol ratios and in excess water below the crystal to liquid-crystal phase transition temperature (\(T_c\)) of the pure lipid (82 °C), are different to the effects seen with the phosphatidylcholine-cholesterol system. The spectra below \(T_c\) are characterised by two overlapping powder patterns, one with a quadrupole splitting of approx. 6 kHz (fluid liquid-crystalline phase) and one with a quadrupole splitting of about 20–25 kHz (crystal or gel-state lipid). Exchange between these two environments is therefore slow, leading to the possibility of characterising the cerebroside-cholesterol phase diagram using deuterium nuclear magnetic resonance spectroscopy.

Introduction

The structure and organization of cell surface glycolipids, mainly glycosphingolipids, is of particular interest for an understanding of the differences between normal and transformed cell surfaces [1–5]. Early studies showed, for example, that when baby hamster kidney (BHK) fibroblasts were transformed either spontaneously or by a polyoma virus, remarkable decreases in hematoside (GM3 ganglioside, II\(^3\)NeuAc-LacCer) with concomitant increases in GL2 glycolipid (CDH, lactosylceramide, Gal(β1–4)GlcCer, ‘cytolipin H’) occurred, associated with blocked synthesis of the GM3 ganglioside [6]. Similar effects have been seen in a wide variety of other transformed systems, including human malignant tumors and cells [7–11]. It is thought by many workers that such glycolipid changes associated with transformation may be classified as ‘simplification due to blocked synthesis’ due to lack of a ‘glycosyl extension response’ in the transformed cell [3,12–18], and that enhancement of glycolipid synthesis may be dependent on cell-to-cell contact [19,20]. Clearly then, an understanding of the differences in cell membrane structure on transformation due to changes in glycolipid pattern, together with a better understanding of the actual molecular events occurring during ‘cell-cell recognition’, is desirable. In addition, a better understanding of the interactions between glycolipids and other membrane components which lead to the ‘masking’ of many membrane glycolipids in normal cells, but which are absent in some transformed cells leading to their ready agglutinability with lectins, is necessary if it is to be possible to understand the changes in cell structure that occur on malignant transformation. Solution of these problems will clearly require an understanding of the static and dynamic structures of cell membranes and cell surfaces.

In our laboratory we have begun an investigation of the static and dynamic structures of a variety of membranes and membrane surfaces using a combination of nuclear magnetic resonance (NMR) [21–26], neutron diffraction [22] and Raman scattering experiments (Bansil, R., Meadows, M., Rice, D. and Oldfield, E., unpublished results), with particular emphasis on the interactions between membrane lipid and protein constituents and the use of \(^2\)H NMR as a non-perturbing NMR, neutron and Raman spectroscopic probe [22]. To date, both we and others have concentrated on investigation of phospholipid
organization in model systems [27–30], even though most mammalian cell membranes contain substantial glycolipid components. It is particularly surprising that given the central role of glycolipids, especially the more complex glycosphingolipids, in cell growth, recognition and transformation processes [1–5] that so few studies of these systems using non-perturbing spectroscopic techniques have been reported, although recently there have been several X-ray diffraction studies of cerebrosides (N-acylgalactosylceramides) [31,32] together with two studies utilizing the nitroxide spin-probe technique [33,34]. Presumably, difficulties anticipated in the synthesis of say 2H- or 13C-labelled glycolipids have dissuaded most workers from investigating these important systems using NMR techniques, while the general lack of availability of a suitable natural abundance probe such as 31P has prevented others. We find, however, that simple glycolipids (N-acylgalactosylceramides, cerebrosides) are actually far easier to make in high yield in short periods of time than are phospholipids such as the familiar dipalmitoylphosphatidylcholine (DPPC). We are thus currently synthesizing in our laboratory a large range of specifically 2H-labelled mono- and diglycosylceramides (including GalCer, GlcCer, Gal(61→4)GlcCer and Gal(α1→4)GalCer) with a view to investigating the static and dynamic structures of these systems together with their interactions with proteins (lectins) and other lipids, to try to elucidate the role of glycolipids in membrane structure.

In this publication we present preliminary 2H NMR results on a series of specifically 2H-labelled N-hexadecanoylgalactosylceramides (N-palmitoylgalactocerebrosides, PGACs) dispersed in excess water in the presence and absence of the sterol cholesterol, and compare results obtained with this simple glycolipid with previous ones obtained using glycerophospholipids [22].

**Materials and Methods**

*Nuclear magnetic resonance spectroscopy*. Deuterium Fourier transform NMR spectra were obtained at 34.1 MHz using the quadrupole-echo pulse technique [29]. We used a 'home-built' instrument for data acquisition [22], using single-phase detection combined with a spectrum reverse method (Nicolet Instrument Corporation, Madison, WI; software package FT 74) which permitted use of a 100 kHz effective spectral bandwith. The 90° pulse width was approx. 6.5 μs, which corresponds to a radio frequency field strength (γH1) of about 40 kHz. Sample volume was typically 200 μl. Spectral simulations were carried out on a CDC Cyber-175 system, using the laboratory peripherals described previously [25].

*Synthetic aspects*. Galactocerebrosides were isolated from fresh pig brain according to Radin [35], then were hydrolysed to galactosylsphingosine (psychosine), also according to Radin [36,37]. N-Hexadecanoylgalactosylceramide was obtained by reacylating the psychosine with palmitoylchloride in the presence of sodium acetate [36]. Palmitoylchloride was made from palmitic acid (Sigma Chemical Company, St. Louis, MO) by refluxing a benzene solution of the acid with a 10% excess of oxalyl chloride (Aldrich Chemical Company, Milwaukee, WI), in the presence of a trace of pyridine as
catalyst. Solvent was removed on a rotary evaporator and the palmitoylchloride used without further purification. The N-palmitoylcerebroside obtained by acylation of the psychosine was purified chromatographically [36], and was then oxidized to the cerebroside aldehyde using galactose oxidase from Sigma (Sigma Chemical Company, St. Louis, MO). Reduction of the aldehyde with NaB2H4 (Aldrich Chemical Company, Milwaukee, WI) [36] afforded a mixture of R and S, [6-2H]PGAC. The product was purified according to Radin [36]. Hydrocarbon chain-labelled cerebrosides were synthesized by reacylation of psychosine with the appropriate 2H-labelled fatty acid chlorides, which were obtained from the fatty acids as described above. [2,2-2H]palmitic acid was synthesized from palmitic acid by α-exchange according to Oldfield et al. [22] and Aasen et al. [38]. [6,6-2H]- and [10,10-2H]palmitic acids were synthesized by Dr. Tran-Dinh Son of this laboratory using a Kolbe electrolysis procedure [22], and [16-2H3]palmitic acid was obtained from Serdary Research Labs (London, Ontario, Canada).

Cerebroside purity was monitored by thin-layer chromatography, proton NMR spectroscopy at 220 MHz, and field-desorption mass spectrometry. It is estimated that our products contained up to about 5% of a dihydrosphingosine derivative, which however is probably isomorphous with the normal unsaturated derivative [31,32].

Cholesterol was obtained from Nutritional Biochemicals, and was crystallized four times from 95% ethanol.

Results and Discussion

We have synthesized N-palmitoylgalactosylceramides (PGACs) having the following structure

![Structure of N-palmitoylgalactosylceramide](image)

labelled either in the N-acyl chain at one of positions 2′, 6′, 10′ or 16′ or in the galactose headgroup at C-6, and have obtained 2H NMR spectra of these specifically deuterated species in excess water, as a function of temperature and in some instances in the presence of cholesterol.

The theoretical aspects of the 2H NMR spectra are discussed in detail elsewhere (Ref. 22, and references cited therein; Ref. 39, and references cited therein). For the 2H nucleus, with a spin I = 1, the allowed transitions correspond to +1 ↔ 0 and 0 ↔ −1 and give rise to a so-called quadrupole splitting (ΔνQ) of the NMR absorption line, given by

\[ ΔνQ = \frac{3 e^2 q Q}{2 \hbar} \langle P_2(\cos θ) \rangle \]  

(1)
where $e^2qQ/h$ is the deuterium quadrupole coupling constant, which has been found to be about 170 kHz for $^2$H nuclei in aliphatic $^2$H bonds [40,41]; $\theta$ is the angle between the laboratory magnetic field $H_0$ and the principal component of the electric field gradient tensor at the deuterium nucleus; and $\langle P_2(\cos\theta) \rangle$ is the value of $1/2 (3 \cos^2\theta - 1)$ averaged in time over any motions that are rapid compared with 170 kHz.

We present in Fig. 1 $^2$H NMR spectra obtained for PGACs labelled in the terminal methyl group of the N-acyl chain (Fig. 1A), and in the C-6 hydroxymethyl group of the galactose residue (Fig. 1B) in excess water, as a function of temperature. As may be seen from Fig. 1, there are significant changes in the $^2$H NMR spectra of both labelled PGACs between 76 and 90°C.

To date there have been no X-ray crystallographic studies reported on the N-palmitoylgalactocerebroside-water system. Nevertheless, Abrahamsson and coworkers [31] and Reiss-Husson [42] have carried out extensive studies on ox brain cerebroside-water systems. In these natural cerebrosides the fatty acids are predominantly tetracosenoic, 2-hydroxytetracosenoic and 2-hydroxyoctadecanoic acid [31], and crystal to liquid-crystal phase transition has been shown to occur at about 60–70°C [31,42–44], the liquid-crystalline phase forming at high temperature being of the general structural type found in aqueous systems of biological lipids [31]. Our results (Fig. 1 and unpublished data) indicate for the pure lipid species N-palmitoylgalactocerebroside that a crystal to liquid-crystal phase transition occurs at 82 ± 2°C.

We have monitored the crystal to liquid-crystal phase transition by means of $^2$H probes located both in the hydrocarbon chain region (at positions 2', 6', 10' and 16') and in the polar headgroup at C-6. The results shown in Fig. 1A show from room temperature up to about 80°C that high signal-to-noise ratio spectra may be obtained from the terminal methyl-labelled PGAC in the

![Fig. 1. Deuterium nuclear magnetic resonance spectra, obtained by the quadrupole-echo Fourier transform method at 34.1 MHz, of (A) $[16'-^2$H$_3]$palmitoylgalactocerebroside and (B) $[6^-^2$H$_1]$palmitoylgalactocerebroside, at the temperatures indicated. Spectral conditions were typically a 75 ms recycle time, 5 μs 90° pulse widths, $\tau_1 = \tau_2 = 70$ μs, 100 kHz effective spectral width, from 10 000 to 60 000 scans, and a 100 Hz linebroadening from exponential multiplication. Both samples were in excess water.](image)
crystalline phase. The spectra show the characteristic broadened features seen previously with gel-state $^2$H NMR spectra of methyl-labelled glycerophospholipids [24], due to a combination of rapid relaxation and a distribution of quadrupole splittings in the defective gel-like structure [24,25]. In addition there is a central narrower feature in the low temperature spectra of Fig. 1A, for which we have no explanation at this time. The quadrupole splittings in the crystalline phase, obtained from the experimental results of Fig. 1A, vary from approx. 18 kHz at 20°C to approx. 14.5 kHz at 80°C, indicating that there is significant molecular motion in this phase. If there were no fast molecular motion other than methyl group rotation, the measured quadrupole splitting would be approx. 36 kHz [34,41] as is the case for a $^2$H-labelled dimyristoylphosphatidylcholine at liquid nitrogen temperatures [24].

At about 82°C the broad spectra of Fig. 1A collapse to a narrow ($\Delta \nu_Q$ approx. 2.4 kHz) well-resolved spin $I = 1$ zero asymmetry parameter powder pattern, as shown in the top spectrum of Fig. 1A. The 2.4 kHz quadrupole splitting is close to that seen for a variety of glycerophospholipids [22,24,25] when compared at the same reduced temperature of $\theta = 0.023$.

For the C-6 galactose headgroup-labelled cerebroside, no signals are observable with our apparatus at low temperatures (Fig. 1B). This indicates that hydroxymethyl group motion is slow on the timescale of the $^2$H NMR experiment (greater than $10^{-5}$ s), and that the $^2$H NMR spectrum approaches the full powder pattern breadth of 127 kHz. In addition, relaxation may be fast. Under either circumstance our limited 100-kHz spectral widths, limited pulse power and limited spectrometer recovery time (greater than 30 $\mu$s) preclude observation of the rigid $^2$H powder pattern. The hydroxymethyl group in the gel-like phase is rigid, due presumably to the presence of a hydrogen bond network in the region of the polar headgroup. The methyl group in the hydrocarbon chain, however, is rotating rapidly (much less than $10^{-5}$ s) even at some 60°C below the phase transition. Similarly, the methyl groups in a dimyristoylphosphatidylcholine headgroup-labelled phosphatidylcholine are mobile to very low temperatures (Skarjune, R. and Oldfield, E., unpublished results), again due to the lack of any hydrogen bonding interaction. In spectra of the headgroup-labelled species above 82°C there is a large increase in signal intensity and well-resolved overlapping powder pattern spectra (Fig. 1B, 90°C spectrum) are obtained. There are several possible explanations for the appearance of two sets of quadrupole splittings in the high-temperature liquid-crystal phase spectrum of Fig. 1B. First, there may simply be two headgroup conformations, each giving rise to a different quadrupole splitting. A second possibility which we feel is more likely is that during synthesis of the [C-6-$^2$H]PGAC, deuterium is introduced by reduction of a cerebroside aldehyde to form the deuterated hydroxymethyl group, and during this reduction step the chiral $^{1}H^{2}H^{3}OH$ group is introduced, forming the two enantiomeric cerebrosides as shown below:
Although the axis or axes of motional averaging for the $^2$H quadrupolar interaction are unknown, it seems almost certain that the deuteron in structure I will appear motionally inequivalent to that in structure II. We therefore regard the observation of two sets of quadrupole splittings in the 90°C spectrum of Fig. 1B as indicating that the hydroxymethyl group is undergoing very slow motions (much greater than $10^{-5}$ s) due to the presence of strong hydrogen bond interactions within the headgroup region. If the -CH$_2$OH group were undergoing very fast motions (much less than $10^{-5}$ s) then only an exchange-averaged quadrupole splitting would be observed, which is not the case.

Spectral simulation of the 90°C spectrum of Fig. 1B using a Lorentzian contribution to the linewidth such that

$$q(\omega, \Delta \nu_Q) = \frac{\pi}{2} \int_0^{\pi/2} d\theta \sin(\theta/\pi) \left[ \delta^2 + \left( \omega \pm (\Delta \nu_Q/2)(3 \cos^2 \theta - 1) \right)^2 \right]$$

where $\delta$ is the half-width at half-height of the Lorentzian broadening function, $\omega$ is the resonance offset frequency relative to the central frequency $\omega_0$, and $\Delta \nu_Q$ is the quadrupole splitting [25], indicate that the best theoretical fit may be obtained by use of two overlapping powder patterns, consisting of 50% of one component having $\Delta \nu_Q = 9.5$ kHz, $\delta = 225$ Hz and 50% of a second component having $\Delta \nu_Q = 13.5$ kHz, $\delta = 250$ Hz (Fig. 2).
We show in Fig. 2 1H NMR spectra of hydrocarbon chain PGACs, labelled in the N-palmitoyl chain as C2H2 (or C2H3) at positions 2', 6', 10', and 16', as well as in the galactosyl headgroup as -CH2HOH at position 6. The results of Fig. 2, obtained by use of the quadrupole-echo Fourier transform method [29] at 34.1 MHz on PGACs in excess water at 90°C (θ = 0.023) are remarkably similar to those obtained for the glycerophospholipid dimyristoylphosphatidylcholine (DMPC): 29.8 kHz (PGAC) and 31.8 kHz (DMPC) for the 6' label and 25.5 kHz (PGAC) and 24.0 kHz (DMPC) for a 10' label. The results for the terminal methyl-labelled species (PGAC approx. 2.4 kHz, DMPC approx. 3.4 kHz) may reflect a more disordered chain packing in the region of the terminal methyl group of the N-acyl chain in the cerebroside due to the shorter adjacent hydrocarbon chain of the sphingosine backbone.

These results clearly demonstrate that the hydrocarbon chain organization in the glycerophospholipids and glycosphingolipids are very similar. This similarity in membrane structure extends even to the 2'-position, where as shown in Fig. 2B, two sets of quadrupole splittings are seen for the 2'-labelled PGAC. For the glycerophospholipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, and 1,2-dipalmityl-sn-glycero-3-phosphoserine, two sets of quadrupole splittings have been observed in the liquid-crystalline phase [22,45,46]. By analogy with the X-ray crystallographic structure of rac-1,2-dilauroyl-glycero-3-phosphoethanolamine [47] it has been concluded that the sn-2 chain begins parallel to the membrane surface, and is bent perpendicular to it after the C-2' segment. The C-2' deuterons appear motionally inequivalent and give rise to two sets of quadrupole splittings. Our deuterium NMR results suggest that the N-acyl chain in glycosphingolipids may also begin parallel to the membrane surface, and that this unique orientation is a general property of both phospholipid and glycolipid organization in membranes.

The quadrupole splittings of the C-2' segment in PGAC and DMPC are, however, quantitatively rather dissimilar (18 and 25 kHz in PGAC, 12 and 18.2 kHz in DMPC) when compared at the same reduced temperature (θ = 0.023), which indicates that although the main features of glycerophospholipid and glycosphingolipid hydrocarbon chain organization in liquid-crystalline bilayer membranes are very close, there must be small differences in chain organization near the headgroup region. However, because of the P2(cosθ) term in Eqn. 1, these differences may in fact be very small. For example, if we were to assume that the 2'-segment configuration led to a C-2H vector being inclined at an angle of approx. 60° to the axis of motional averaging (as in a gauche bend), then a change in angle (β of Eqn. 3 of Ref. 22) from 59° to 61° would result in a change in ΔνQ from 12.8 to 18.4 kHz, similar to that noted for the DMPC-PGAC results discussed above.

Our spectral simulations for the 2'-labelled PGAC (Fig. 2B) indicate that there are small intensity differences (55% versus 45%) between the two spectral components. In this case, falloff in pulse power may account for these small differences, although only synthesis of an optically active deuterated fatty acid and incorporation into lipid will unambiguously confirm the idea that each quadrupole splitting in the C-2' segment arises from a different deuteron.
**Interactions with cholesterol**

Cerebrosides, together with cholesterol, comprise the largest lipid species in myelin of brain and myelinated nerve fibers [48]. It is somewhat surprising therefore that there have been so few studies of cerebroside-cholesterol interactions [43,49], especially since as seen in this publication and elsewhere [42--44] natural cerebrosides are likely to have solid to fluid phase transitions in the range 60--80°C, considerably above body temperature (37°C).

In Fig. 3 we present $^2$H NMR spectra, obtained by the quadrupole-echo method at 34.1 MHz, of N-palmitoylgalactocerebroside labelled as C$^2$H$_3$ in the terminal methyl group of the N-acyl hydrocarbon chain in the presence of an equimolar quantity of cholesterol, in excess water as a function of temperature. Also presented in Fig. 3 are spectral simulations of the experimental lineshapes, using Lorentzian broadening functions (Eqn. 2). The results of Fig. 3 at low temperatures (20°C) are in qualitative agreement with those of Fig. 1A, however, as the temperature is increased a narrow second component ($\Delta \nu_Q \approx 6.0$ kHz) grows in intensity, in samples containing an equimolar quantity of cholesterol. At 50°C, this narrow component comprises about 12% signal intensity, and above about 65°C it accounts for essentially 100% signal intensity (Fig. 3). These results with the cerebroside-cholesterol mixture are qualitatively different to those seen previously with the phosphatidylcholine-cholesterol system. For the system DMPC-cholesterol (1 : 1) in excess water, and with the

![Fig. 3. Deuterium nuclear magnetic resonance spectra of N-[16'-2H$_3$]palmitoylgalactocerebroside-cholesterol (1 : 1 mol ratio) in excess water at the temperatures indicated, together with spectral simulations of the experimental spectra. The quadrupole splitting of the narrow component in each spectrum is 5.6 kHz except at 20°C ($\Delta \nu_Q = 6.6$ kHz), while that of the broad component was 21 kHz (50°C), 23 kHz (38°C) and 25 kHz (20°C). The relative intensity of the narrow component decreases from 100% at high temperatures to approx. 12% at 50°C, and approx. 5% at 38 and 20°C. The low temperature (20--50°C) spectra used a Lorentzian broadening of 6 kHz for the gel-state component.](image-url)
DMPC labelled as C²H₃ in the 2-chain, we find (Jacobs, R. and Oldfield, E., unpublished results) on cooling the sample from 30 to −20°C ($T_c$, the gel to liquid-crystal phase transition temperature of DMPC is 23°C) that there is simply a broadening of the spectral linewidth, together with a small increase in quadrupole splitting, $\Delta \nu_Q$. However, in the system PGAC-cholesterol (1 : 1) there is clearly a phase separation below about 60°C, into a rigid gel-like phase and a fluid liquid-crystalline phase. These effects are quite reversible.

We have incorporated [3α-²H]cholesterol at 1 : 1 mol ratio into the PGAC system and find that at 84°C, the quadrupole splitting $\Delta \nu_Q$ for the labelled sterol is about 45 kHz (Skarjune, R. and Oldfield, E., unpublished results) which is close to that of the same sterol in a DMPC bilayer ($\Delta \nu_Q \approx 49$ kHz), when compared at the same reduced temperature of $\theta = 0.01$ [22]. This result strongly suggests that the cerebroside-cholesterol system, above the solid to fluid phase transition temperature of the pure glycolipid, possesses the same lamellar structure as does the phosphatidylcholine-cholesterol system [22,50], even though the macroscopic appearance of these systems may be rather unusual in that long cylindrical structures appear [49]. Nevertheless, our results with the labelled cholesterol tend to rule out the possibility of a hexagonal type H₁₁ structure since here a maximum quadrupole splitting $\Delta \nu_Q$ of about 32 kHz would be observed.

The observation of two distinct types of signal in the ²H NMR spectra of Fig. 3, at low temperatures shows that exchange between the fluid liquid-crystalline phase and the rigid crystalline phase is slow. This feature of the cerebroside-cholesterol system may make it possible to deduce the complete lipid-sterol phase diagram, which to date has not been possible for the phosphatidylcholine-cholesterol system [51].

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