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Physical Studies of Cell Surface and Cell Membrane Structure. Deuterium Nuclear Magnetic Resonance Studies of *N*-Palmitoylglucosylceramide (Cerebroside) Head Group Structure†

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ABSTRACT: Deuterium Fourier-transform nuclear magnetic resonance spectra of *N*-palmitoyl[2,3,4,6,6-$^2H_5$]glucosylceramide, *N*-palmitoyl[1-^2H]glucosylceramide, *N*-palmitoyl[5,6,6-$^2H_3$]glucosylceramide, and *N*-palmitoyl[6,6-$^2H_2$]glucosylceramide have been obtained at 55.3 MHz (corresponding to a magnetic field strength of 8.5 T) for lipids as multilamellar dispersions in excess water at 90 °C, above the gel to liquid-crystal phase transition temperature (T_c = 82 °C). Spectra were also obtained for these same lipids dispersed with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, and cholesterol, all in excess water at 90 °C. The results are analyzed in terms of a model in which the lipid undergoes fast axial diffusion, together with a "wobbling" of the polar head group, by mathematical methods similar to those used previously for the choline and ethanolamine head groups in phosphatidylcholines and phosphatidylethanolamines [Skarjune, R., & Oldfield, E. (1979) *Biochemistry 18*, 5903-5909]. However, contrary to the results obtained in the previous study, which indicated many possible conformations for the choline and ethanolamine head groups, results with labeled cerebrosides yield at most a few orientations for the glucose head group in each of the systems studied. Furthermore, where multiple solutions do occur, they fall within a narrow orientational subspace so that all solutions exhibit the same general features. We also show that the order parameter describing the head group wobble is fully determined for each system, and it indicates a rather mobile structure for the cerebroside head group, in a variety of environments. In each system studied, the polar head group projects essentially straight up from the bilayer surface into the aqueous region, thereby permitting maximum hydration of the four glucose hydroxyl groups by bulk water molecules.

Knowledge of the structure and dynamics of cell surface glycosphingolipids may be of considerable importance for understanding functional differences between normal and transformed cell membranes (Hakomori, 1973, 1975; Tooze, 1973; Clarkson & Baserga, 1974; Lee & Smith, 1974). To date, many studies have shown that these lipids undergo dramatic changes in composition upon malignant transformation (Hakomori & Murakami, 1968; Hildebrand et al., 1971, 1972; Seifert & Uhlenbruck, 1965; Kostic & Buchheit, 1970; Karlsson et al., 1974). These changes involve primarily a simplification of the (polar) carbohydrate residues (Tooze, 1973; Hakomori, 1970, 1971; Robbins & Macpherson, 1971; Sakiyama et al., 1972; Critchley & Macpherson, 1973; Kijimoto & Hakomori, 1972; Nigam et al., 1973). This suggests that important biological properties of the glycosphingolipids may be associated with the polar head group region of these molecules. Clearly then, it should be of considerable interest to examine the head groups of various glycosphingolipids by nuclear magnetic resonance (NMR)[1] spectroscopic and other physical methods in order to obtain an understanding of the static and dynamic structures of these species, an approach that might eventually provide valuable insights concerning cellular recognition processes at the molecular level.

In this paper we present the results of deuterium (^2H) NMR experiments with aqueous multilamellar dispersions of *N*-palmitoylglucosylceramide (PGLC) specifically labeled with deuterium in the carbohydrate ring. In contrast to work previously done with ^2H-labeled phosphatidylcholine and phosphatidylethanolamine, which yielded poorly defined results (Seelig et al., 1977; Seelig & Gally, 1976; Skarjune & Oldfield, 1979a), we show in this publication that all solutions for the glucose head group region of PGLC in a variety of environments show the same general orientational features. We use a very simple model of molecular motion in which the lipid molecules undergo fast axial diffusion, with the sugar moiety

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[1] Abbreviations: PGLC, *N*-palmitoylglucosylceramide; [2,3,4,6,6-$^2H_5$]PGLC, *N*-palmitoyl[2,3,4,6,6-$^2H_5$]glucosylceramide; [1-^2H]PGLC, *N*-palmitoyl[1-^2H]glucosylceramide; [5,6,6-$^2H_3$]PGLC, *N*-palmitoyl[5,6,6-$^2H_3$]glucosylceramide; [6,6-$^2H_2$]PGLC, *N*-palmitoyl[6,6-$^2H_2$]glucosylceramide; NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; SGADC, *N*-(2-hydroxystearoyl)galactosyldihydroceramide; PGAC, *N*-palmitoylgalactosylceramide.

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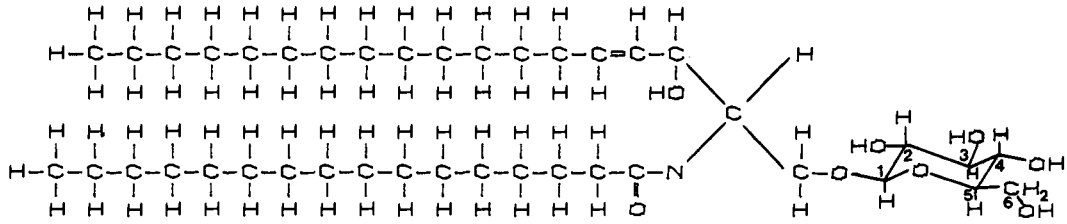

FIGURE 1: Structure of PGLC showing the numbering of carbon atoms in the glucose head group.

undergoing an additional "wobbling" motion. Our results suggest that the ^2H NMR method should permit determination of polar head group organization in more complex glycolipids, both in model systems and in biological membranes themselves.

Experimental Procedures

Synthetic Aspects. Natural cerebroside was isolated from beef brain by the procedure of Radin (1976) and was hydrolyzed to ceramide by the methods of Carter et al. (1961). Ceramide was then converted to sphingosine by using the psychosine procedure of Radin (1974). The sphingosine thus obtained was converted to labeled glucosylpsychosine by the scheme of Pascher (1974) with the following minor modifications: (1) the quantities of reagents were adjusted to be compatible with sphingosine, (2) hydrolysis of the N-protecting group was performed by the procedure of Radin (1974), except that methanol was substituted for butanol, water was eliminated from the hexane partitionings, and the refluxing was carried out for 1 h, and (3) intermediates were purified by column chromatography only. Reacylation of glucosylpsychosine was according to Radin (1972).

[*2,3,4,6,6-$^2H_5$*]-α-D-*Glucose* was prepared according to Koch & Stuart (1977, 1978a,b) by catalytic exchange with deuterized Raney nickel. Decolorizing charcoal was added to the glucoside hydrolysis reaction during the last 30 min, and deionization of the sugar solutions was carried out with Amberlite MB-3 whenever the conductivity significantly exceeded that of distilled water. The sugar was crystallized by adding either ethanol or glacial acetic acid to the thick sugar syrup.

[*1-^2H*]-α-D-*Glucose* was prepared by $NaBD_4$ reduction of β-gluconolactone. The procedure was essentially that of Lowenstein (1963) except for modifications as follows: (1) Dow-Corning antiform A was added to the reaction mixture, (2) the solution was deionized with Amberlite MB-3, and (3) the glucose was crystallized by adding glacial acetic acid to the thick syrup.

[*5,6,6-$^2H_3$*]-α-D-*Glucose* was prepared from β-glucuronolactone by the procedures of Fieser et al. (1956) (1,2-*O*-isopropylidene-α-D-glucofuran-5-ulosurono-6,3-lactone), Onodera & Kashimura (1972) (1,2-*O*-isopropylidene-α-D-*xylo*-hexofuran-5-ulosurono-6,3-lactone), and Mackie & Perlin (1965b) ([5,6,6-$^2H_3$]-α-D-glucose). Column purification was used for the intermediates, and the final product was crystallized as above.

[*6,6-$^2H_2$*]-α-D-*Glucose* was prepared by $NaBD_4$ reduction of 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone according to Mackie & Perlin (1965a).

Acetobromo sugars were prepared according to Barczai-Martos & Korosy (1950). Palmitoyl chloride was used in the reacylation step (Skarjune & Oldfield, 1979b), and the basic structure of the final products is shown in Figure 1. The purity of the final compounds, and their intermediates, was monitored by thin-layer chromatography, ^1H and ^{13}C NMR, and field-desorption mass spectrometry. It is estimated that ~5% of the cerebroside consisted of the dihydrosphingosine analogue (Skarjune & Oldfield, 1979b).

NMR Spectroscopy. ^2H Fourier-transform NMR spectra were taken at 55.3 MHz with a "home-built" Fourier-transform NMR spectrometer that consists of an 8.5-T, 3.5 in. bore high-resolution superconducting solenoid (Oxford Instruments, Osney Mead, Oxford, U.K.), together with a variety of digital and radio-frequency electronics. We used a Nicolet 1180 computer, 293B pulse programmer, and Model NIC-2090 dual-channel 50-ns transient recorder (Nicolet Instrument Corp., Madison, WI) for experiment control and rapid data acquisition, together with a Diablo Model 40 disc system for data storage (Diablo Systems, Inc., Haywood, CA). In order to generate radio-frequency pulses of power sufficient to cover the entire ^2H spectral width (≃3-μs 90° pulse widths), we used an Amplifier Research (Amplifier Research, Souderton, PA) Model 200L amplifier to drive a retuned Henry Radio (Los Angeles, CA) Model 2006 transmitter to an ~1000–1500-W output power level. Deuterium NMR spectra were recorded on this instrument by using an 800-μL sample volume and a quadrupole-echo (Davis et al., 1976) pulse sequence. The 90° pulse width varied between 2.0 and 4.5 μs. The 90° pulse widths and phase quadrature between the two radio-frequency pulses were established by viewing quadrature free-induction decay signals of *S*-[*methyl-$^2H_3$*]methionine. The same settings were used for data acquisition on the ^2H-labeled cerebroside dispersions. In essentially all cases, no phase corrections were necessary after Fourier transformation. Typically, data were collected with sampling rates of 5 μs/point. The instrument zero frequency was established with a 1% D_2O reference. The sample temperature was regulated by means of a heated air flow, the temperatures reported being measured with a calibrated Doric trendicator (San Diego, CA) with a copper–constantan thermocouple. Gas-flow temperatures were measured next to the sample, and separate experiments indicate that this temperature is accurate to ±2 °C over the entire sample volume. Between 4000 and 100 000 scans were accumulated by using typically an 85-ms recycle time. Samples contained between 20 and 200 mg of lipid dispersed in excess deuterium depleted water (Aldrich Chemical Co., Milwaukee, WI) above the gel to liquid-crystal phase transition temperature (T_c = 82 °C). Spectra were recorded at 90 °C. Calculations were performed on a Control Data Corp. Cyber-175 computer, interfaced to a Tektronics 4006-1 graphics terminal and Model 4662 interactive digital plotter (Tektronix, Beaverton, OR) in our laboratory.

Computational Aspects. The mathematical analysis of the cerebroside head group conformation is somewhat similar to that for the choline and ethanolamine head groups in phosphatidylcholine and phosphatidylethanolamine (Seelig et al., 1977; Seelig & Gally, 1976; Skarjune & Oldfield, 1979a) but with the advantage that the cerebroside head group almost certainly contains a *rigid* carbohydrate ring. Because the carbohydrate ring exists as a well-defined structure (Pascher & Sundell, 1977; Abrahamsson et al., 1977), the results of NMR experiments with deuterium labels at the several different sites should therefore strongly reflect the orientation of the sugar residue. The major limitation in the previous work

with the choline and ethanolamine head groups was that there were too few NMR parameters to describe a structure in which there are clearly numerous likely possibilities for rotational isomerism (Skarjune & Oldfield, 1979a).

In order to interpret our ^2H NMR data, we have therefore developed the following simple model for head group motion. First, as with other liquid-crystalline lipids, the cerebroside molecules rotate rapidly about their long molecular axes (Skarjune & Oldfield, 1979a,b). This motion is not perfectly collinear with the bilayer normal, and a symmetric wobble of the long axis, or the polar head group, about the bilayer normal may be introduced to account for such disorder. Analogous calculations with phospholipids used a two-site "enantiomeric" exchange motion to describe the motions of the choline and ethanolamine head groups (Seelig et al., 1977; Seelig & Gally, 1976; Skarjune & Oldfield, 1979a). This model was suggested by examination of X-ray crystallographic structures of related compounds, which showed two enantiomeric molecules in the unit cell (Abrahamsson & Pascher, 1966). In the case of cerebroside X-ray crystallographic studies, two independent molecules in the unit cell are also seen, but the differences reflect packing effects in the nonpolar region of the molecule; i.e., the head group regions are very similar (Pascher & Sundell, 1977). We use only a single head group in our model. Thus, the motional model we have chosen is one in which there is a fixed head group conformation and the presence of fast rotation about the bilayer normal, together with a head group wobble of undetermined amplitude about the rotation axis. Previous work (Skarjune & Oldfield, 1979b) has suggested that the hydroxymethyl group of the carbohydrate is effectively rigid relative to the ring; however, recent results indicate the possibility that a two-site exchange occurs for this group both above and below the phase transition temperature (R. G. Griffin, private communication). As a result, no assumption is made about the hydroxymethyl group motions, and the results from deuterium labels at this position are not used in the calculations. The model considers only head group motions, and the remainder of the molecule may undergo multisite reorientations such as the gauche–trans isomerizations thought to occur in the fatty acid side chain (Huang et al., 1980).

In earlier work with the choline head group, the all-trans conformation was chosen as the reference for which all torsion angles were 0° (Skarjune & Oldfield, 1979a). The choline head group is essentially a straight chain, so there is no ambiguity in choosing an all-trans conformation. However, the rigid nonplanar sugar ring in the cerebroside head group requires a different definition of the reference conformation. Analogous to the treatment of choline, in which the glyceryl C_1C_2 was included in the reference, C_1 and C_2 of sphingosine will be part of the PGLC reference. Proceeding toward the sugar moiety, all torsion angles are made trans (i.e., 0°). The carbohydrate ring is oriented so that C_1 and C_2 of the glucose residue lie in the plane defined by the previous trans torsion angles. Furthermore, the hydroxymethyl group is oriented so the $C_4C_5C_6O_6$ torsion angle is trans. This arbitrary reference conformation is illustrated in Figure 2.

Also included in Figure 2 is the labeling scheme used for the atoms and torsion angles and the coordinate system of the bilayer normal. C_2 of sphingosine (C_{2S}) is the origin of the coordinate system. While free rotations about the bilayer normal make this coordinate system axially symmetric, the x and y axes are differentiated to define various rotations and to aid in visualizing the bilayer plane. Thus, all head groups may be freely rotated about the z axis.

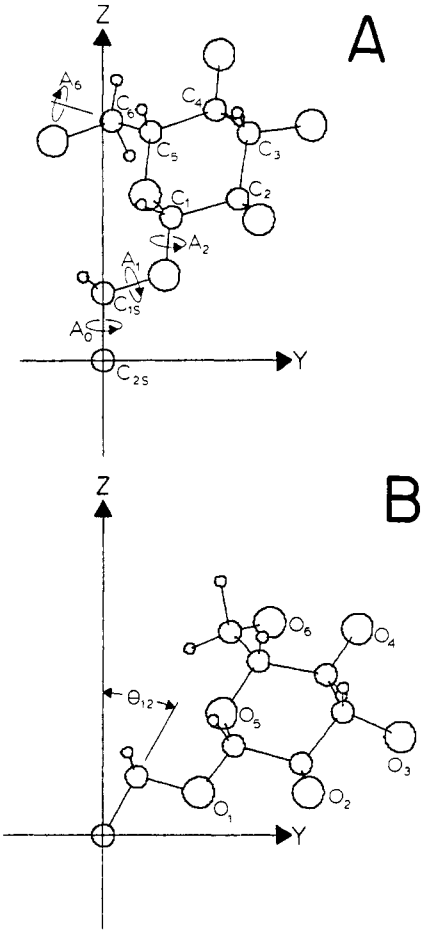

FIGURE 2: Torsion angle reference conformation and coordinate system. (A) Reference conformation showing labeling for torsion angles and carbon atoms. C_{2S}, C_{1S}, O_1, C_1, and C_2 lie in the yz plane. The z axis is the bilayer normal. (B) Rotation about x axis showing the definition of θ_{12} and labeling scheme used for the oxygen atoms.

The z axis passes through C_{1S} in the reference conformation; however, the $C_{2S}C_{1S}$ bond vector should be free to have any arbitrary orientation. To do this, we defined θ_{12} as the angle between the z axis and the $C_{2S}C_{1S}$ bond vector. The head group conformation can be completely defined by specifying θ_{12}, A_0, A_1, A_2, and A_6. Note, however, that no attempt will be made to determine the torsion angles for the hydroxyl protons as these are of course rapidly exchanged with surrounding water protons. Also, A_6 will not be included in the calculations for the reasons outlined above.

It is important to know the coordinates of atoms in the head group after arbitrary rotations of θ_{12} and the A_0–A_6 torsion angles. These locations must be derived from crystal structures, which in general will be defined in a different coordinate system. The derivation is readily obtained in three steps: (1) the reference coordinates of C_{2S}, C_{1S}, O_1, C_1, and C_2 are coplanar and may be calculated from the crystal bond lengths and angles, (2) the reference orientation of the carbohydrate ring may be found by performing rotations which result in O_1, C_1, and C_2 being coincident with the coordinates determined in step 1, and (3) the coordinates are then calculated for arbitrary rotations of θ_{12}, A_0, A_1, A_2, and A_6.

There are two appropriate crystal structures available: *N*-(2-hydroxystearoyl)galactosyldihydroceramide (SGADC; Pascher & Sundell, 1977) and glucosylphytosphingosine hydrochloride (Abrahamsson et al., 1977). The first of these is a cerebroside closely related to PGLC, and the second is a psychosine with a glucose head group. SGADC was used for

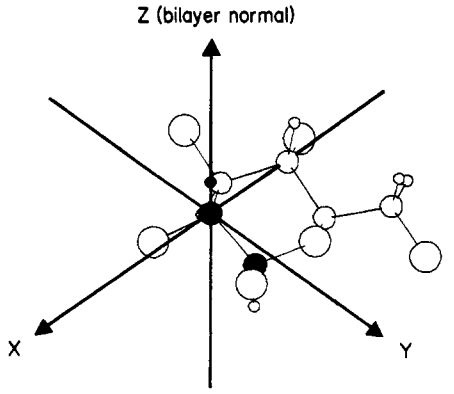

FIGURE 3: (θ, A_r) reference conformation and coordinate system with C_2 at origin, D_2 along the z axis, and C_1 in the yz plane (solid circles). The z axis is the bilayer normal.

determining coordinates for the sphingosine region of the reference conformation while the psychosine was used to calculate the glucose coordinates.

In the previous choline head group calculations, the conformations were tested by rotating about all adjustable torsion angles within a small window about the crystallographic values (Seelig et al., 1977; Seelig & Gally, 1976; Skarjune & Oldfield, 1979a). Despite the fact that a very small fraction of the total conformational space was examined, a large number of conformations consistent with the NMR results were obtained. Anticipating the advantage offered by the several ring deuterons for determining the orientation of the carbohydrate ring, we thought it possible to examine the entire conformational space without finding unreasonably large numbers of conformational solutions. However, with three torsion angles freely variable over 360° and θ_{12} variable over 180°, the total number of conformations for 1° increments is 8.4×10^9. To reduce this number significantly without sacrificing a complete search of ring orientations, we therefore devised an alternative approach. This is accomplished by defining another coordinate system based only upon the orientation of the carbohydrate ring; Figure 3 is a schematic representation. In the new coordinate system, C_2 is at the origin, and the z axis is defined by the C_2H_2 vector. The C_2C_1 vector is taken to lie on the positive y side of the yz plane, and the x axis is chosen to complete a right-handed Cartesian coordinate system. If A_6 is defined as in the previous coordinate system, this new reference orientation is unambiguous. Only three angles, θ, A_r, and A_6, are needed to completely specify the orientation of the carbohydrate ring (hydroxyl torsion angles neglected as before). θ is a positive rotation about the y axis, and A_r is a rotation about the C_2H_2 bond axis. As in the torsion angle coordinate system, the z axis is defined to lie along the bilayer normal, and the x and y axes are presented for mathematical and visual convenience. Arbitrary θ, A_r, and A_6 rotations will generate any possible orientation of the sugar ring, and the resulting orientations of the various CD vectors can be used to calculate the expected quadrupole splittings since

$$\Delta\nu_Q = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)\left(\frac{3}{2}\cos^2\theta - \frac{1}{2}\right)S_{BN} \qquad (1)$$

where e^2qQ/h is the quadrupolar coupling constant, θ is the angle between the CD vector and the bilayer normal, and S_{BN} is the order parameter describing the magnitude of the rotational wobble. The quadrupolar coupling constant appropriate for labeled carbohydrates was measured from a ^2H NMR spectrum of anhydrous, polycrystalline [2,3,4,6,6-$^2H_5$]-α-D-glucose and was estimated to be 164 kHz.

That this approach is more efficient than the torsion angle search is immediately apparent from the requirement of only three angles to describe the ring orientation. Neglecting A_6 as before, the total number of conformations at 1° increments is only 64 800, a much more tractable number.

Our computer program performs the calculations as follows: For each possible positive value of S_{BN} (positive and negative splittings are indistinguishable), the program mathematically rotates the carbohydrate ring about all possible orientations and determines which of these could yield the experimental data. Recalling that the C_2D_2 vector is aligned along the bilayer normal in the reference structure, it is clear that there is a simple relationship between the values of θ, S_{BN}, and the splitting expected for this label. Furthermore, the choice of the C_2D_2 vector as the A_r rotation axis makes the splitting of deuteron 2 (D2) independent of the value of A_r. The calculations are based upon the experimental spectra obtained from N-palmitoyl[2,3,4,6,6-$^2H_5$]glucosylceramide ([2,3,4,6,6-$^2H_5$]PGLC). Solving eq 1 for θ indicates that there are a maximum of four values of θ for a given splitting and S_{BN}. With the experimental spectrum potentially consisting of five different splittings, a maximum of 20 values of θ are possible for each value of S_{BN}. Each of the [2,3,4,6,6-$^2H_5$]PGLC experimental splittings is assigned to D2 for each value of S_{BN}. The possible values of θ are then calculated, and subsequent rotation of A_r yields all possible orientations of the carbohydrate ring. The expected splitting for all nonhydroxyl deuterons is calculated by means of eq 1 for each of these. By using 1° increments, this procedure reduces the number of orientations to be tested by a factor of about 18, yet it does not exclude any orientational space where solutions may be found. Each time a splitting is assigned to D2, the remaining experimental splittings are assembled into all possible combinations of two to test for consistency with the calculated splittings for deuterons 3 and 4 (D3 and D4). In this way, every possible orientation that will yield three of the five experimental splittings for D2, D3, and D4 is considered to be a tentative solution. Additionally, each solution found by this procedure must give calculated values for deuterons 1 and 5 (D1 and D5), which are consistent with the values obtained from experiments with dispersions of N-palmitoyl[1-^2H]-glucosylceramide ([1-^2H]PGLC), N-palmitoyl[5,6,6-$^2H_3$]-glucosylceramide ([5,6,6-$^2H_3$]PGLC), and N-palmitoyl[6,6-$^2H_2$]glucosylceramide ([6,6-$^2H_2$]PGLC). Note that while "exact" values of θ can be calculated, A_r must be incremented. Experience has shown that 1° increments provide the best combination of resolution and reasonable computer execution time. However, it can be shown from eq 1 that a splitting can change as much as 3338 Hz for only a 1° change in CD vector orientation. Moreover, the crystallographic structures used to generate the reference coordinates report calculated fractional coordinates for the protons, so a certain amount of flexibility must be allowed in the CD vector reference orientations due to uncertainty in the X-ray data. The preliminary calculations allow a $\pm 3338 S_{BN}$-Hz spread about each of the experimental splittings in which the calculated splittings must fall to be considered consistent. This assures that valid solutions are not "jumped over" during the incrementing and allows an uncertainty in the crystal structures on the order of a few degrees. Once the general region of the orientational space in which solutions may be found is determined, the spread is increased from a low value in increments of 100 Hz to $\pm X S_{BN}$ Hz where X is the minimum value that produces solutions. When these calculations are performed over the entire orientational space for the carbohydrate ring (θ, $0 \rightarrow 180°$; A_r,

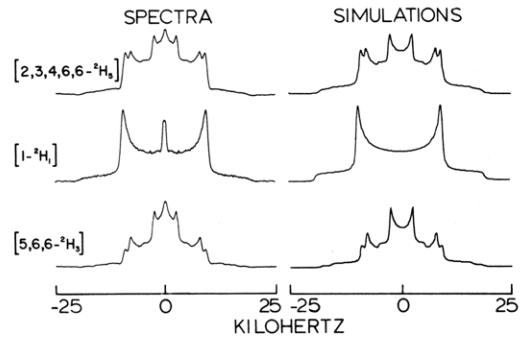

FIGURE 4: Experimental ^2H NMR spectra of labeled PGLC's. Splittings (in hertz) are 5200, 16 000, and 18 500 (three resonances) for [2,3,4,6,6-$^2H_5$]PGLC, 19 200 for [1-^2H]PGLC, and 5200, 16 000, and 18 500 for [5,6,6-$^2H_3$]PGLC.

Table I: Experimental Quadrupolar Splittings (kHz) for Labeled *N*-Palmitoylglucosylceramide[a]

PGLC label	PGLC[b]	PGLC: CHOL[c]	PGLC: DPPC[d]	PGLC: DPPE[e]
[2,3,4,6,6-$^2H_5$]	5.2	5.8	2.4	4.9
	16.0	12.0	11.75	13.5
	18.5	16.0	12.0	14.0
	18.5	16.0	12.25	14.0
	18.5	16.0	12.5	14.0
[1-^2H]	19.2	17.0	12.5	14.0
[5,6,6-$^2H_3$]	5.2	5.8	2.4	4.9
	16.0	12.0	12.0	13.5
	18.5	16.5	13.5	13.5
[6,6-$^2H_2$]	5.2	5.8	2.4	4.9
	18.5	12.0	12.0	13.5

[a] Quadrupole splittings were determined by means of spectral simulations (Figure 4 and additional unpublished data). All spectra were recorded at 90 ± 2 °C by means of the quadrupole-echo method. All samples were dispersed in excess water; the estimated error is ±0.5 kHz. [b] Pure *N*-palmitoylglucosylceramide (PGAC). [c] Equimolar mixture of PGLC with cholesterol (CHOL). We did not determine if excess cholesterol is present outside the bilayer. [d] 17 wt % PGLC in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. [e] 17 wt % PGLC in 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine.

$0 \rightarrow 360°$), sets of angles that are possible solutions to the head group orientation are obtained. Furthermore, the entire positive range of S_{BN} is incremented during the calculations, and its value for each solution is noted.

Results and Discussion

We show in Figure 4 typical experimental ^2H NMR spectra of dispersions of [2,3,4,6,6-$^2H_5$]PGLC, [1-^2H]PGLC, and [5,6,6-$^2H_3$]PGLC, at 90 °C. Splittings are determined by comparison with spectral simulations (Figure 4) and are listed in detail in Table I. The spectra of Figure 4 (and other unpublished results) show the presence of a small but relatively broad central component. Simulations including this resonance indicate that the worst case relative intensity (i.e., assuming no contribution from residual HO^{2}H) is about 5% of the total intensity, for each spectrum. When the cerebroside was very carefully repurified by column chromatography, no difference in the spectra was apparent. These facts suggest that the central intensity may be due to a small ($\lesssim$5%) amount of cerebroside that is not incorporated into the normal bilayer structure but has perhaps been incorporated into some micellar or other disordered structure, which permits averaging of the quadrupole splittings to the small line widths (<1 kHz) observed. The excellent agreement between the rest of the ^2H spectra and their simulations, obtained with $\eta = 0$ values, strongly suggests, however, that most ($\gtrsim$95%) of the ^2H line shape is characterized by a zero asymmetry parameter.

Table II: Calculated Head Group Orientations for Pure PGLC[a]

	θ	A_r	S	spread
I	98	352.0	0.32	1300
II	103	17.0–18.0	0.31	1300
III	82	172.0	0.32	1300
IV	77	197–198	0.31	1300

[a] All angles in degrees. Spread in hertz.

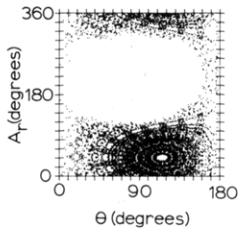

FIGURE 5: Computer-generated plot showing the total (θ, A_r) orientational space mapped when the torsion angles of Figure 2 are incremented over their total 360° ranges and unlikely conformations (as defined in the text) are excluded.

Table II lists the results of the computer search described above. In this search, all possible combinations of assignments were considered, as well as values of the order parameter (S_{BN}) ranging from 0.0 to 1.0, in increments of 0.01. These are analogous to the "quasi-conformations" seen for choline and ethanolamine in a related paper (Skarjune & Oldfield, 1979b); however, in the present case, the advantages offered by the larger number of NMR parameters and the rigidity of the carbohydrate ring have combined to yield only four solutions, each with a spread of no more than 2° in the A_r dimension and discrete values in the θ dimension.

These results actually represent two sets of symmetry-related solutions. Equation 1 is symmetric about $\theta = 90°$, so a CD vector oriented at $\theta°$ with respect to the bilayer normal will give the same splitting as another vector oriented at $180° - \theta°$. This symmetry is generated in the glucose head group when an additional rotation about A_r of 180° is added. Thus, in Table II the members of the two pairs, I–III and II–IV, are complementary with respect to θ and supplementary with respect to A_r. In the reference coordinate system used in the calculations, this relationship will always hold, and the best refinement possible with no further assumptions is a single pair of solutions.

However, it seems reasonable to suggest that all head group orientations may not be possible for the cerebroside molecule. For example, conformations that would result in a penetration of the polar glycolipid head group into the nonpolar bilayer matrix would not be expected. A 180° rotation of A_2 (Figure 2) would be an example of such an unlikely conformation. Specifically, we shall consider any conformation that generates a negative z coordinate, in the coordinate system of Figure 2, for one or more atoms directly bound the carbohydrate ring, to be energetically unlikely and will therefore be deleted. For each set of angles (θ_{12}, A_0, A_1, A_2) in the coordinate systm of Figure 2, it is possible to determine the corresponding value of (θ, A_r) in the coordinate system of Figure 3. Figure 5 shows the results of a computer mapping between the two coordinate systems. In these calculations, θ_{12}, A_0, A_1, and A_2 were incremented in 15° steps over their entire ranges, and (θ, A_r) values were calculated for each conformation. If a particular set of torsion angles led to a likely conformation according to the criterion above, the appropriate (θ, A_r) point was plotted; points were not plotted for unlikely conformations. Figure 5 shows the results for the total search, for PGLC. It is clear that there is a sizable (θ, A_r) subspace that is unpopulated

Table III: Characteristics of the PGLC Conformational Solutions Calculated[a]

system[b]		θ (deg)	A_r (deg)	S_{BN}	D1 (kHz)	D2 (kHz)	D3 (kHz)	D4 (kHz)	D5 (kHz)	spread (kHz)
PGLC	I	98	352	0.32	19.6	18.5	18.2	18.7	18.9	1.3
	II	103	17–18	0.31	19.1	16.0	18.9	18.9	18.2	1.3
PGLC:CHOL[c]		99	356–357	0.28	17.2	16.0	16.4	16.4	16.9	1.6
PGLC:DPPC[d]	I	99	9–10	0.21	12.7	12.0	12.8	12.2	12.9	2.8
	II	98	12–14	0.21	12.5	12.3	12.8	11.9	12.9	2.8
	III	96	16–17	0.21	12.2	12.5	12.7	11.5	12.9	2.8
	IV	100	6–7	0.21	12.8	11.8	12.8	12.4	12.9	2.8
PGLC:DPPE[e]	I	98	40	0.24	14.0	14.0	14.3	13.9	13.3	1.3

[a] All angles in degrees. The quadrupole splittings and spread are in kilohertz. [b] Samples were all dispersed in excess water and spectra recorded at 90 °C. Systems containing two lipid components were mixed by dissolution in chloroform–MeOH, followed by lyophilization and a 24-h period of evacuation. Equilibration with H_2O was carried out at 100 °C. [c] Equimolar PGLC:cholesterol. [d] 17 wt % PGLC in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. [e] 17 wt % PGLC in 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine.

according to the above criterion. More importantly, the results of Figure 5 show that two of the four solutions listed in Table II fall within the unpopulated subspace. By neglecting these, the number of glucose orientations is limited to solutions I and II in Table II, and the calculated parameters are listed in Table III. Good agreement exists between the experimental and calculated splittings that are consistent with the ±1300 S_{BN}-Hz spread allowed.

A "picture" of one of the PGLC head group orientations, showing two views of the glucose ring relative to the bilayer normal, is given in Figure 6A, and it is apparent that the head group is essentially fully extended. This orientation suggests to us that interaction with the water layer is the major determinant of head group orientation. The other solution looks very similar, the primary difference being a clockwise rotation of the ring of 25° in the first view in Figure 6A. What is perhaps surprising is the S_{BN} of 0.32, which is significantly smaller than the 0.66 value determined for pure DPPC bilayers (Gally et al., 1975; Seelig & Gally, 1976). We therefore repeated our calculations with a different glucoside crystal structure (methyl β-D-glucopyranoside hemihydrate; Jeffrey & Takagi, 1977) to check for consistency. Although the CD vector orientations are somewhat different in the two calculated structures, values of S_{BN} and θ are essentially identical.

Lipid Mixtures. Intact biological membranes contain a wide variety of lipids and sterols, and cerebrosides are generally dispersed at rather low concentration, except in the case of the myelin membrane. It is therefore of some interest to examine the effects of changing the lipid composition around the cerebroside upon the head group orientation. We have therefore carried out some preliminary work with [2,3,4,6,6-$^2H_5$]PGLC dispersed with various phospholipids and cholesterol. At a concentration of 17 wt % PGLC dispersed in each of dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and dipalmitoylphosphatidylethanolamine (DPPE) just above the phase transition temperature of the phospholipid component, liquid-crystalline spectra were obtained, despite the fact that the phase transition temperature of pure PGLC is some 20–60 °C higher than the gel to liquid-crystal phase transition temperatures of the lipids investigated. This concentration of cerebroside is slightly higher than the ~13 wt % galactocerebroside found in myelin (McIlwain & Bachelard, 1971), suggesting that cerebrosides in biological tissues will also be in a liquid-crystalline state. Furthermore, the observed patterns (data not shown) of the quadrupole splittings when compared with those seen in pure PGLC dispersions suggest that the head group orientations are different. To test this idea, we have carried out calculations for PGLC dispersed in DPPC, DPPE, and cholesterol, all in excess water at 90 °C. The phospholipid systems contained

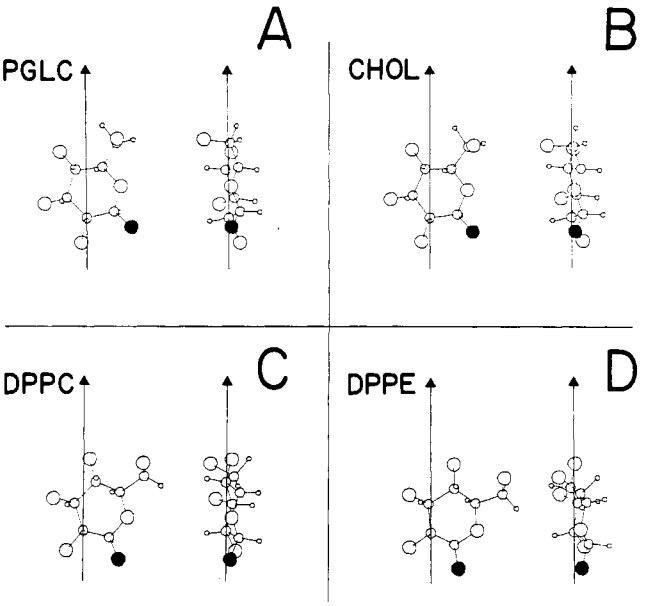

FIGURE 6: Typical PGLC head group orientations at 90 °C for dispersions of (A) PGLC (solution I), (B) 1:1 mole ratio PGLC:CHOL, (C) 17 wt % PGLC in DPPC (solution III), and (D) 17 wt % PGLC in DPPE. The arrows represent the bilayer normal, and the solid circles are the glycosidic oxygens. The structure on the right of each pair is generated by a 90° rotation of the left-hand structure about the bilayer normal. The hydroxymethyl group is shown to aid visualization of the ring, but the A_6 torsion is arbitrary.

17 wt % PGLC while we used a 1:1 mole ratio with cholesterol. The splittings obtained from the experimental spectra are listed in Table I, and the characteristics of the solutions are listed in Table III. Typical head group orientations are shown schematically in Figure 6. Note that all of the orientations are essentially fully extended with respect to θ, the major differences between the various systems being in the degree of extension with respect to A_r. In the case of the DPPC and DPPE mixtures, the A_r extension is essentially complete while the cholesterol mixture is somewhat less extended. In the phospholipid dispersions, there are roughly five phospholipid molecules per cerebroside, so it is possible that the more numerous choline and ethanolamine head groups "crowd" the glucose head groups into a more extended orientation.

Note also that the values of S_{BN} are lower for PGLC dispersed with each of the other lipids, especially the phospholipids (Table II). Considering the qualitative similarity of the orientations of Figure 6, the primary effect of dispersing cerebroside with other liquids is therefore to increase the disorder in the sugar–lipid head group region, due presumably to the increased overall "fluidity" of the lipid bilayer in the region of the sugar head group.

Our calculations for cerebroside show that the carbohydrate head group is essentially fully extended away from the bilayer surface for each of the systems we have examined. The observation of multiple solutions is of limited significance since all orientations have the same general appearance (Figure 6). Thus, these results, based on a search of the entire orientational space of the head group, are far more revealing than similar searches performed for the choline and ethanolamine head groups, which examined a more limited conformational space (Seelig et al., 1977; Seelig & Gally, 1976; Skarjune & Oldfield, 1979a).

Conclusions

The results discussed in this paper represent the first attempt at detailing the structure of a glycolipid in the sugar head group region, in the liquid-crystalline phase. With use of four selectively deuterated glycolipids, and a simple motional model involving only fast axial lipid diffusion together with a wobbling of the sugar residue, the orientation of the sugar residue may be confined to a narrow orientational subspace and the order parameter describing the head group fluctuations determined.

The general features derived from the calculations indicate that the carbohydrate head group is extended away from the bilayer to allow maximum interaction with the bulk water layer. Furthermore, the head group order parameter is quite small, suggesting large amplitude fluctuations of the sugar moiety.

Our results also indicate that ~17 wt % glycolipid may be readily dispersed into liquid-crystalline phosphatidylcholine and phosphatidylethanolamine bilayers and that these lipids have a small but measurable effect on the cerebroside head group structure. Similar experiments and calculations could yield the orientation of cerebroside head groups in a variety of environments, giving information on, for example, protein–lipid interactions, both in model systems and in biological membranes themselves. A similar approach should also be applicable to determining the structures of individual monosaccharide units in more complex glycosphingolipids.

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