Study by Infrared Spectroscopy of the Interdigitation of C26:0 Cerebroside Sulfate into Phosphatidylcholine Bilayers†

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ABSTRACT: The insertion mode of the long fatty acid chain of the asymmetric glycosphingolipid C26:0-cerebroside sulfate (C26-CBS) in symmetric matrices of phosphatidylcholines of different acyl chain length has been investigated by transmission and attenuated total reflectance (ATR) infrared spectroscopy. The concentration of C26-CBS in myelin is increased in the demyelinating disease adrenoleukodystrophy. The conformational order and the orientation of the chains of the asymmetric glycosphingolipid have been evaluated for C26-CBS incorporated at 8 mol % in perdeuterated dimyristoylphosphatidylcholine (DMPC-d_{54}) and perdeuterated dipalmitoylphosphatidylcholine (DPPC-d_{62}). The results, for the gel phase, are consistent with interdigitation of the C26-CBS long acyl chain across the bilayer center of an *all-trans*-DMPC bilayer in which DMPC is less tilted than in the absence of CBS. In contrast, in DPPC the results suggest that although the CBS long chain interdigitates across the center of the bilayer, it does not change the tilt angle of the DPPC molecules in the gel phase. Furthermore, in DPPC, C26-CBS is less well oriented than the host DPPC molecules and it increases the *gauche* content of the DPPC acyl chains. The observation of the amide spectral region indicates that exposure of the sphingosine amide moiety to buffer is greater in the longer chain length DPPC bilayer than in the shorter chain length DMPC bilayer. The thermotropic behavior of the lipid mixtures of C26-CBS at 8 mol % in DMPC or DPPC shows that the glycosphingolipid stabilizes the gel phase of the short chain length bilayer while it destabilizes the long chain length one. Our results further demonstrate that, at this concentration, C26-CBS is completely miscible in DMPC and DPPC in the gel and the liquid crystalline phases. The difference in behavior of C26-CBS in DMPC and DPPC is a consequence of the greater mismatch between the C26 chain length and the bilayer thickness of DPPC relative to DMPC. They may help to understand the deleterious effects of glycosphingolipids with very long chain fatty acids in adrenoleukodystrophy.

Cerebroside sulfate (CBS)[1] is a glycosphingolipid, which belongs to the family of complex asymmetric chain length lipids present in cell membranes of a number of tissues. CBS itself is found in relatively large amounts in myelin. Its function is not well understood. However, it is likely that abnormalities in the acyl chain length can disrupt the membrane integrity. Adrenoleukodystrophy is an inherited demyelinating disease which is characterized by the presence of an increase in the amount of very long fatty acid species (C25 and C26) in sphingolipids in myelin and other membranes (Kishimoto et al., 1985).

Hydrated glycosphingolipids generally undergo complex thermotropic behavior which depends on the thermal history of the sample (Bunow, 1979; Ruocco et al., 1981; Barenholz et al., 1983; Boggs et al., 1984; Curatolo & Jungalwala, 1985). X-ray diffraction (Ruocco et al., 1981) and Fourier transform infrared (FTIR) (Jackson et al., 1988) studies of galactosylceramide (cerebroside) have shown that in water it exhibits polymorphism characterized by the conversion of the lipid from a thermodynamically metastable anhydrous form to a more stable, more hydrated form caused by a rearrangement of the hydrogen-bond network formed by intermolecular interactions between the hydroxyl groups of the galactose, the sphingosine hydroxyl group, and the amide moiety (Pascher & Sundell, 1977). The polar head of CBS is composed of galactose sulfated at the 3-hydroxyl, conferring an anionic character to the molecule. DSC and spin-label studies have shown that it also undergoes complex polymorphism (Boggs et al., 1984, 1988a,b). However, unlike cerebroside, the metastable phase of CBS is the more hydrated phase. Upon heating, or on storage at low temperatures, it converts to a more ordered and stable state, which is less hydrated, since X-ray diffraction indicates a decrease in the thickness of the water layer (Stinson & Boggs, 1989). FTIR studies indicate that CBS is also involved in an intermolecular hydrogen-bond network (Tupper et al., 1992).

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[1] Abbreviations: ATR, attenuated total reflectance; C26-CBS, C26:0 cerebroside sulfate; CBS, cerebroside sulfate; DMPC, dimyristoylphosphatidylcholine; DMPC-d_{54}, perdeuterated dimyristoylphosphatidylcholine; DPPC-d_{62}, perdeuterated dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; ESR, electron spin resonance; FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; R, dichroic ratio; T_c, temperature of the gel-to-liquid crystalline phase transition.

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The complexity of the phase behavior of glycosphingolipid systems cannot be attributed only to the reorganization of the hydrogen-bond network, since asymmetric saturated species of sphingomyelin and phosphatidylcholine (PC) exhibit similar dependence on the sample history (Barenholz et al., 1976; Boggs & Mason, 1986; Lewis et al., 1994a). Acyl chain asymmetry in glycosphingolipids as well as phospholipids induces a particular acyl chain packing. Studies by X-ray diffraction and spectroscopic techniques have shown that these lipids give rise to two forms of interdigitated structures (Hui et al., 1984; McIntosh et al., 1984; Levin et al., 1985; Mattai et al., 1987; Reed & Shipley, 1987; Boggs et al., 1988b; Stinson & Boggs, 1989; Stevenson et al., 1992). The triple chain mixed interdigitated structure occurs when two short chains meet end-to-end in the center of the bilayer, while the long chains span across the bilayer. In the double chain partially interdigitated form, the short and long chains meet in the center of the bilayer with, respectively, the long and short chains of an opposing lipid molecule. Asymmetric lipids form mixed interdigitated structures within the condition that the normalized chain length difference, as described by Huang and Mason (1986), is between 0.4 and 0.6. In the liquid-crystalline phase, they form normal fluid bilayers with two chains per headgroup (McIntosh et al., 1984).

In natural membranes, glycosphingolipids are generally present at low concentrations, below 10 mol %, and they probably do not cluster in domains of sufficient size to form the same type of interdigitated bilayer as pure glycosphingolipid systems. Their conformation and organization when mixed into PC bilayers have been investigated by ESR spectroscopy of spin-labeled glycolipids and NMR spectroscopy. Studies of spin-labeled glycolipids in host PC bilayers of symmetric PC molecules show that the conformation of the long acyl chain depends on the difference between the thickness of the membrane and the length of the long chain of the glycosphingolipid. A short acyl chain host PC matrix causes restriction of the motion of the methylene groups near the methyl end of the long chain, at least in the gel phase, suggesting that the long acyl chain of the glycolipid interdigitates across the PC bilayer center, while the short chain meets end-to-end with an acyl chain of the symmetric PC molecule (Grant et al., 1987; Mehlhorn et al., 1988; Florio et al., 1990; Boggs & Koshy, 1994). The conformation adopted by the long chain in the liquid crystalline phase is still not clear. According to one model based on use of glycolipids containing a C24 acyl chain spin-labeled at the 16th carbon, the long acyl chain may interdigitate across the center of the symmetric PC bilayer (Grant et al., 1987; Mehlhorn et al., 1988; Lu et al., 1993; Florio et al., 1990). However, analysis of NMR data from cerebroside containing a perdeuterated C24 chain mixed with PC indicated that the results are inconclusive concerning interdigitation of the C24 chain across the bilayer center (Morrow et al., 1993). Alternatively, the long acyl chain may bend at the center of the hydrophobic core by 90° or by 180°, allowing it to bend up along the other acyl chains in the same monolayer. Other possibilities supported by the use of a CBS spin-label containing a C26 chain, which is spin-labeled near the terminal methyl group, are that the end of the long chain may terminate in the center of the bilayer and either kink up to match the shorter PC chain length or remain relatively extended, thus forcing the glycolipid head group further above the bilayer surface (Boggs & Koshy, 1994).

Studies of the exposure of the glycolipid carbohydrate in liposomes to galactose oxidase or antibodies have shown that the accessibility of the headgroup to the enzyme or antibody is greater when the host matrix has a smaller thickness or when the chain length of the glycolipid increases (Alving & Richards, 1977; Crook et al., 1986; Stewart & Boggs, 1993). The latter suggests that the glycolipid adopts a configuration where the carbohydrate group protrudes at the interfacial surface, while the long acyl chain may terminate in the center of the bilayer rather than interdigitate across the bilayer center.

In the present study, we have used FTIR spectroscopy to investigate further the organization of the C26 acyl chain of CBS incorporated at 8 mol % in a 28- and 32-carbon length PC bilayer, namely, DMPC and DPPC, respectively. This is the first study to examine the packing of an asymmetric lipid in a symmetric PC bilayer using FTIR spectroscopy. Fourier transform infrared spectroscopy is a powerful tool for the investigation of the conformational order and orientation of the glycosphingolipid acyl chains, as well as for the study of the hydrogen-bond network. It allows study of both the gel and liquid crystalline phases. Previous studies of the packing of an asymmetric lipid in a symmetric PC bilayer by NMR spectroscopy have examined only the liquid crystalline phase. In order to be able to follow the CBS and PC components independently, PC molecules with perdeuterated acyl chains have been used in this study. The results also resolve the question of the occurrence of lateral phase separation of low concentrations of CBS in PC.

MATERIALS AND METHODS

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), perdeuterated dimyristoylphosphatidylcholine (DMPC-d_{54}), and perdeuterated dipalmitoylphosphatidylcholine (DPPC-d_{62}) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. C26:0 cerebroside sulfate (C26-CBS) was synthesized as described previously (Boggs et al., 1988a). Lipid mixtures were prepared by dissolving appropriate amounts of the lipids in chloroform/methanol (87/3 v/v) and evaporation of the solvent under a nitrogen stream. Traces of solvent were eliminated under vacuum overnight. The concentration of CBS was 8 mol % in the perdeuterated phosphatidylcholine (PC) matrix. Dry lipid mixtures were hydrated with appropriate volumes of D_2O containing KCl (0.1 M) to yield lipid dispersions containing 20 wt % in total lipids and around 1.6 wt % in CBS. The dispersions were then heated and cooled three times at a temperature about 10 °C above and below the temperature of the sample gel-to-liquid crystalline phase transition. Samples containing CBS were also heated at 90 °C for 10 min in order to ensure complete hydration of the cerebroside sulfate. A drop of approximately 20 μL of lipid dispersion was placed between two CaF_2 windows separated by a 12-μm Mylar spacer in a homemade transmission cell. Spectra were recorded on a Nicolet Magna 550 Fourier transform spectrophotometer equipped with a mercury−cadmium−telluride detector and a germanium-coated KBr beamsplitter. A total of 250 scans were co-added and apodized with a triangular function at a resolution of 2 cm^{-1}.

The CH_2 stretching mode spectral region was corrected by subtracting a straight baseline between 2780 and 3000 cm^{-1}. The frequency of the band due to the symmetric

stretching vibration of the CBS acyl chains was calculated from the center of gravity at 90% of the height of the band, giving a precision of ± 0.2 cm^{-1} (Cameron et al., 1982). The bandwidth of that band was calculated at 65% of its height after a 4-point interpolation of the spectral region, in order to improve the accuracy of the measurement.

Oriented films of multilayers of the lipid mixtures dispersed in water were obtained by spreading the samples with a Teflon bar on one side of germanium attenuated total reflectance plates (50 × 20 × 2 mm, 45° parallelograms) until dry films were obtained by water evaporation. For hydrated samples, the plate was placed vertically in a homemade thermostated cell. The side of the germanium crystal where samples were applied was sealed with Teflon, allowing hydration of the sample with ~600 μL of aqueous phase. These films were then rehydrated with D_2O and reheated in order to ensure a complete rehydration of the films. Order parameters, $f(\theta)$, associated with the lipid acyl chains were determined by using eq 1, which assumes an uniaxial distribution of orientation with respect to the normal of the ATR crystal (Hübner & Mantsch, 1991):

$$f(\theta) = \frac{R - 2}{R + 1.45} \frac{2}{3\cos^2 \alpha - 1} \qquad (1)$$

where R is the dichroic ratio calculated from peak heights of the bands due to the CH_2 and CD_2 symmetric stretching vibrations at 2850 and 2090 cm^{-1}, respectively, measured with the incident radiation polarized parallel ($A_{\parallel}$) and perpendicular ($A_{\perp}$) with respect to the normal of the ATR plate ($R = A_{\parallel}/A_{\perp}$) (Fringeli & Günthard, 1981). The angle α between the transition moment of the symmetric CH_2 and CD_2 stretching vibrations and the chain axis was set to 90°. Only the band due to the symmetric CH_2 stretching mode at 2850 cm^{-1} has been used for the order parameter calculation, in order to minimize errors on peak heights introduced by the contributions of the neighboring bands, like the symmetric CH_3 stretching band at 2880 cm^{-1} and the broad band at 2900 cm^{-1} due to the Fermi resonance between the symmetric CH_2 stretching vibration and the CH_2 bending vibration. ATR spectra were obtained by co-adding 1000 scans with double zero-filling at a resolution of 2 cm^{-1} (Lepla & Horlick, 1990). These conditions were necessary since the bands due to the glycolipid CH_2 acyl chains are weak, the glycolipid being present at only 1.6 wt % in the dispersion.

RESULTS

Thermotropic Behavior of C26-CBS. Figure 1 shows the acyl chain region of the infrared spectrum of pure C26-CBS in dispersion at 1.6% by weight in D_2O, in the gel and liquid crystalline phases. The two main bands observed at 2920 and 2850 cm^{-1} are attributed to the antisymmetric and symmetric CH_2 stretching vibrations, respectively. The two weaker bands at 2955 and 2875 cm^{-1} are due to the asymmetric and symmetric stretching vibrations of the terminal methyl group of the lipid acyl chains, respectively. In this spectral region, two broad features are also present around 2940 and 2900 cm^{-1}, which are due to the Fermi resonance between the symmetric CH_2 stretching vibration and the first overtone of the symmetric CH_3 bending mode and the first overtone of the CH_2 bending mode, respectively. The transition to the liquid crystalline phase is characterized by a shift toward higher frequencies and by the broadening

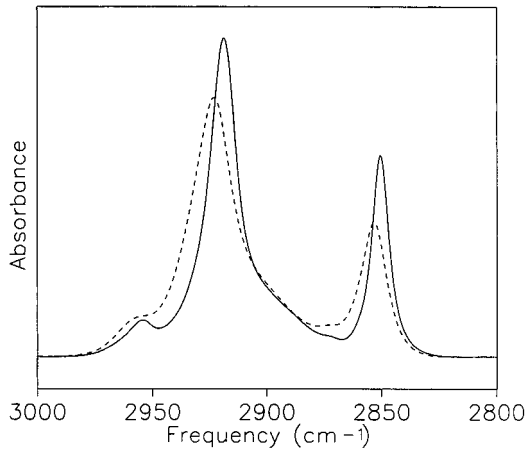

FIGURE 1: Infrared spectra in the acyl chain C−H stretching mode region of pure C26-CBS dispersions in the gel phase at 40 °C (—) and liquid crystalline phase at 75 °C (- - -).

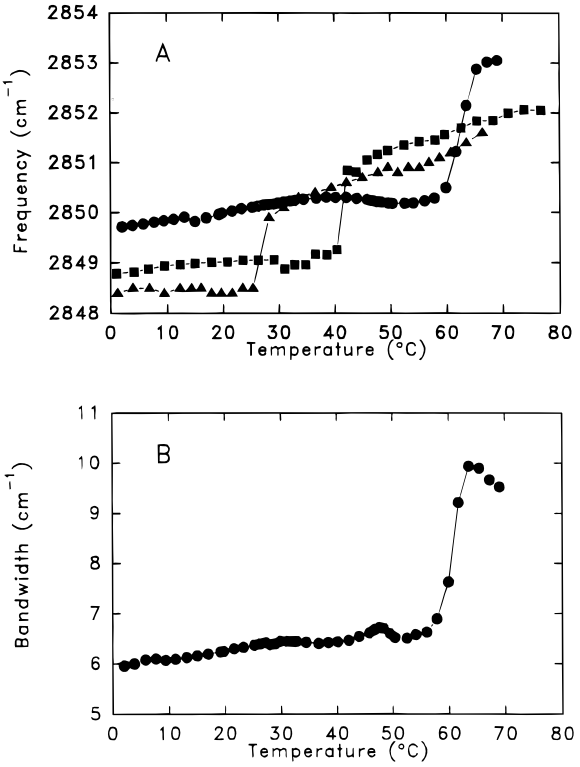

FIGURE 2: Effect of temperature on (A) the frequency and (B) the bandwidth of the band due to the acyl chain CH_2 symmetric stretching vibration of pure DMPC (■), pure DPPC (▲), and pure C26-CBS (●).

of the 2920- and 2850-cm^{-1} bands. The increase of frequency and the broadening of these bands are generally attributed to the diminution of the conformational order of the lipid acyl chains and to the increase of their dynamics. It is thus possible to follow the transition from the ordered gel phase to the disordered liquid crystalline phase from the frequency and the bandwidth of these bands.

Figure 2A shows the temperature dependence of the frequency of the CH_2 symmetric stretching band in the spectra of DMPC, DPPC, and C26-CBS. The gel-to-liquid crystalline phase transition temperature is observed at 24, 42, and 63 °C for pure DMPC, DPPC, and C26-CBS, respectively. These values are in good agreement with differential scanning calorimetry results on DMPC, DPPC (Ladbrooke & Chapman, 1969), and C26-CBS in 0.1 M KCl (Boggs et al., 1993). Figure 2B shows the temperature

dependence of the bandwidth of the CH_2 symmetric stretching band in the spectrum of pure C26-CBS. A pretransition is observed around 48 °C, which corresponds to the broad feature observed around 35−45 °C in the plot of temperature dependence of the CH_2 symmetric stretching band of C26-CBS (Figure 2A). This transition, which has also been detected by Raman spectroscopy for C26-CBS in 2 M KCl (Stevenson et al., 1992) and by DSC in 0.1 M KCl (Boggs et al., 1993), is ascribed to a reorganization of the glycolipid acyl chains from a mixed interdigitated gel state to a partially interdigitated gel state. The former has been detected using spin-labels (Boggs et al., 1988b) and the latter has been detected by X-ray diffraction (Stinson & Boggs, 1989). The increased frequency and bandwidth which occur at about 40−45 °C indicate greater disorder of the lipid chains in the partially interdigitated phase. The subsequent drop in frequency and bandwidth at around 50 °C, before the main transition, indicates greater ordering, which may be due to partial dehydration of the metastable partially interdigitated phase resulting in closer packing of the lipid in a more stable partially interdigitated phase (Boggs et al., 1988a; Stinson & Boggs, 1989). After the gel-to-liquid crystalline phase transition, a decrease of bandwidth is observed between 60 and 70 °C while the frequency of the CH_2 symmetric stretching band does not change. This drop in bandwidth has been found to be reproducible and could be due to restricted motion of the lipid molecules due to further dehydration as the partially melted sample refreezes into an even more stable and less hydrated phase as observed by DSC (Boggs et al., 1984, 1988a). Since this reorganization involves mostly headgroup interactions, it affects the dynamics of the lipid acyl chains but not their conformation.

In both the gel and liquid crystalline phases, the frequency of the CH_2 symmetric stretching band in the spectrum of C26-CBS is significantly greater than the corresponding band in the spectra of DMPC and DPPC, indicating that CBS is more disordered than symmetric chain length phospholipids. In the liquid crystalline phase, the highly asymmetric structure of CBS may not allow this lipid to form a well-ordered bilayer, whether or not it is interdigitated. Figure 2 also shows that the frequency of the CH_2 symmetric stretching band is higher for DPPC than for DMPC. It has already been mentioned by McElhaney and co-workers (Mantsch & McElhaney, 1990; Casal & McElhaney, 1990) that the frequency of this band increases with increasing chain length in diacylphosphatidylcholine, as do the calorimetrically measured enthalpies. It is likely that the probability of *gauche* isomerization increases with chain length, since it has also been shown that the concentration of kink defects in the lipid acyl chains is higher and increases with chain length (Casal & McElhaney, 1990). This observation would partly explain why the frequency of the CH_2 symmetric stretching band is higher in C26-CBS spectra than in those of DMPC or DPPC.

Isotopic Dilution Effect. In the present study, we have used FTIR spectroscopy to study C26-CBS at 8 mol % in DMPC and DPPC in order to determine the conformation and dynamics of the long acyl chain in a symmetric PC environment and the effect of the two lipids on each other. For these experiments, we have used DMPC and DPPC with deuterated acyl chains in order to distinguish the infrared bands due to the CBS acyl and sphingosine chains from the acyl chains of the PC component in the mixture. However, it has been recently reported that for phosphatidylcholine

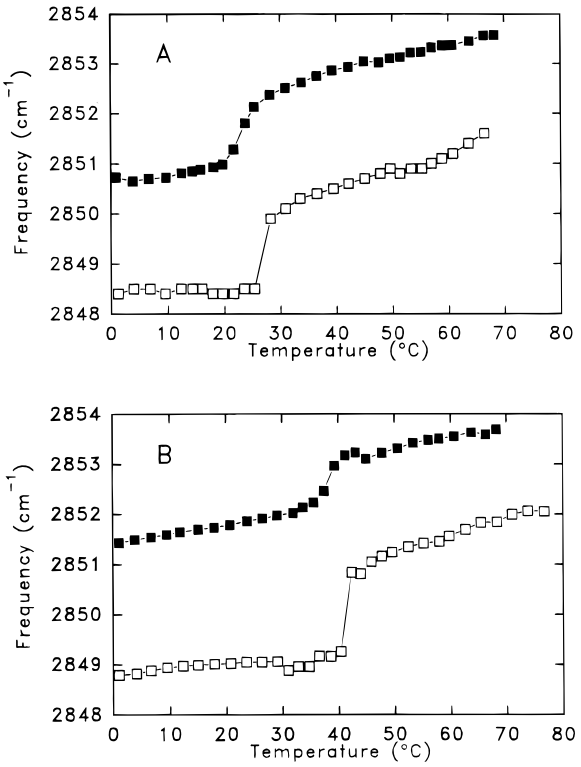

FIGURE 3: Temperature dependence of the frequency of the band due to the acyl chain CH_2 symmetric stretching vibration of (A) pure DMPC (□) and DMPC at 8 mol % in DMPC-d_{54} (■), and (B) of pure DPPC (□) and DPPC at 8 mol % in DPPC-d_{62} (■).

mixtures containing protiated and deuterated acyl chains, isotopic dilution increases the frequency of the bands due to the CH_2 stretching vibrations, compared to the pure protiated lipid (Kodati et al., 1994). Kodati et al. (1994) have shown that this increase in frequency cannot be attributed to conformational disorder as is generally assumed. Furthermore, they observed that the isotopic dilution effect increases linearly when the concentration of the protiated lipid in the deuterated host matrix is decreased down to 20 mol %. In the present study, since the protiated lipid (CBS) is at 8 mol % in the deuterated PC matrix, the magnitude of the isotopic dilution effect must be estimated by using model systems, like DMPC at 8 mol % in DMPC-d_{54} or DPPC at 8 mol % in DPPC-d_{62}, in order to separate isotopic dilution and conformational effects.

Figure 3 shows the temperature dependence of the CH_2 symmetric stretching band in the spectrum of DMPC at 8 mol % in DMPC-d_{54} and DPPC at 8 mol % in DPPC-d_{62}. As seen in Figure 3A, the gel-to-liquid crystalline phase transition temperature (T_c) of DMPC shifts from 24 °C for the pure lipid to 20 °C for the DMPC/DMPC-d_{54} mixture, which is also the transition temperature of pure DMPC-d_{54}. The same shift is observed in Figure 3B between pure DPPC (42 °C) and DPPC/DPPC-d_{62} (38 °C). The shift toward lower temperature of T_c has been observed previously and may be due to the fact that the deuterated acyl chains have a bigger van der Waals volume than nondeuterated acyl chains (Peterson et al., 1975). The incorporation of DMPC or DPPC in the respective perdeuterated matrix, in both the gel and liquid crystalline phases, increases by 2.0−2.5 cm^{-1} the frequency of the CH_2 symmetric stretching band compared to that of pure DMPC and DPPC. Since all lipids in the mixture have the same acyl chain length, the observed shift of the frequency cannot be attributed to conformational

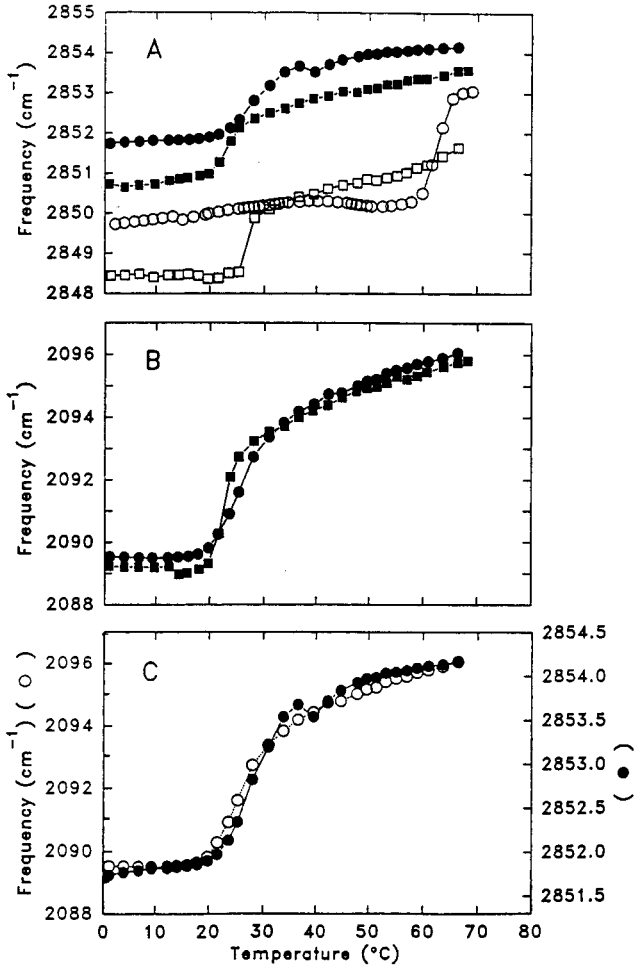

FIGURE 4: (A) Temperature dependence of the frequency of the band due to the acyl chain CH_2 symmetric stretching vibration of pure DMPC (□), pure C26-CBS (○), DMPC at 8 mol % in DMPC-d_{54} (■), and C26-CBS at 8 mol % in DMPC-d_{54} (●). (B) Temperature dependence of the frequency of the band due to the acyl chain CD_2 symmetric stretching vibration of DMPC-d_{54} for the DMPC/DMPC-d_{54} (■) and C26-CBS/DMPC-d_{54} (●) systems. (C) Temperature dependence of the frequency of the band due to the acyl chain CH_2 and CD_2 symmetric stretching vibration of C26-CBS (●) and DMPC-d_{54} (○), respectively, for the C26-CBS/DMPC-d_{54} mixture.

perturbation but rather must be ascribed to isotopic dilution (Kodati et al., 1994). Kodati and co-workers have shown that acyl chains of a protiated lipid isolated in a matrix of perdeuterated acyl chains induce a perturbation of the vibrational interchain coupling. They also showed that this perturbation is not very sensitive to the acyl chain length or to the physical state of the lipids.

C26-CBS in DMPC-d_{54} or DPPC-d_{62}. Figure 4A shows the effect of temperature on the frequency of the CH_2 symmetric stretching band due to the CBS acyl chains for pure CBS dispersions and for CBS/DMPC-d_{54} mixtures, and that due to the DMPC acyl chains for pure DMPC and for DMPC/DMPC-d_{54} mixtures. For the CBS/DMPC-d_{54} system, the gel-to-liquid crystalline phase transition is cooperative and the temperature profile does not show any transition around the T_c of CBS. In the gel phase, the incorporation of CBS in the deuterated PC matrix induces an increase of the frequency of the symmetric CH_2 stretching vibration by about 2 cm^{-1}. The magnitude of this effect is a little less than that observed for the model system DMPC/DMPC-d_{54}, due to isotopic dilution. In the liquid crystalline phase, the increase of frequency of the CH_2 symmetric stretching band of CBS in DMPC-d_{54} at T_c + 10 °C is 1 cm^{-1} as compared to CBS alone, while it is 2.0 cm^{-1} for DMPC in DMPC-d_{54} compared to DMPC alone (Figure 3A), at the same concentration and reduced temperature.

Two explanations may be proposed for this observation. First, the isotopic dilution effect of the CBS chains in the perdeuterated PC matrix could be lowered as a consequence of lateral phase separation. However, phase separation would be expected to be greater in the gel phase and have a greater effect on decreasing the dilution effect than in the liquid crystalline phase. In order to verify whether lateral phase separation occurs in the lipid mixtures, the temperature profiles of the protiated CBS and deuterated DMPC-d_{54} components of the mixture were superimposed (Figure 4C). As seen in Figure 4C, the T_c obtained is the same for each component of the CBS/DMPC-d_{54} mixture. Therefore, these results show that no phase separation occurs in these lipid mixtures.

An alternative explanation is that the smaller difference in frequency between CBS in DMPC-d_{54} and pure CBS, compared to that between DMPC in DMPC-d_{54} and pure DMPC, indicates that, for both the gel and liquid crystalline phases, the CBS acyl chains incorporated in DMPC-d_{54} have a higher conformational order than in pure CBS bilayers (Figure 4A). This would decrease the frequency, partially counteracting the increase caused by the dilution effect. This interpretation is based on the assumption that the isotopic dilution effect is independent of the nature of the low-concentration protiated species. This hypothesis is strongly suggested by the fact that, at 8 mol %, the frequency shift due to the isotopic dilution effect ranges between 2.0 and 2.5 cm^{-1} for both DMPC and DPPC in their respective perdeuterated matrices for the gel and the liquid crystalline phases. Moreover, Kodati et al. (1994) have shown that for decane, hexadecane, and DPPC in the gel and the liquid crystalline phases, the frequency shifts of the CH_2 stretching mode are equally sensitive to the concentration of the respective deuterated species.

Figure 4B shows curves describing the temperature dependence of the frequency of the symmetric CD_2 stretching band around 2090 cm^{-1} for the perdeuterated acyl chains of DMPC-d_{54} in DMPC/DMPC-d_{54} or in CBS/DMPC-d_{54} dispersions. In both the gel-and-liquid crystalline phases, the frequencies for both systems are almost identical. Thus, 8 mol % CBS has little effect on the conformational order of DMPC. As shown in this figure, the gel to liquid crystalline phase transition temperature of DMPC in CBS/DMPC-d_{54} is higher than that of DMPC in DMPC/DMPC-d_{54}. This can also be seen from the CH_2 frequency in Figure 4A. Thus, C26-CBS increases the T_c of DMPC, in agreement with DSC results observed by Boggs et al. (1993).

Figure 5A shows the dependence on temperature of the frequency of the CH_2 symmetric stretching vibration due to the acyl chains of CBS mixed with DPPC-d_{62} and DPPC mixed with DPPC-d_{62}. Comparison of the frequency of the CH_2 symmetric stretching band shows little difference between CBS/DPPC-d_{62} and DPPC/DPPC-d_{62} systems in the gel phase, while in the liquid crystalline phase the frequency of the band due to the CBS acyl chains of the CBS/DPPC-d_{62} mixture is higher by about 0.5 cm^{-1} compared to DPPC/DPPC-d_{62}. As in DMPC, the difference in frequency between C26-CBS in DPPC-d_{62} and CBS alone is less than that between DPPC in DPPC-d_{62} and DPPC alone, especially in the liquid crystalline phase. This suggests that increased

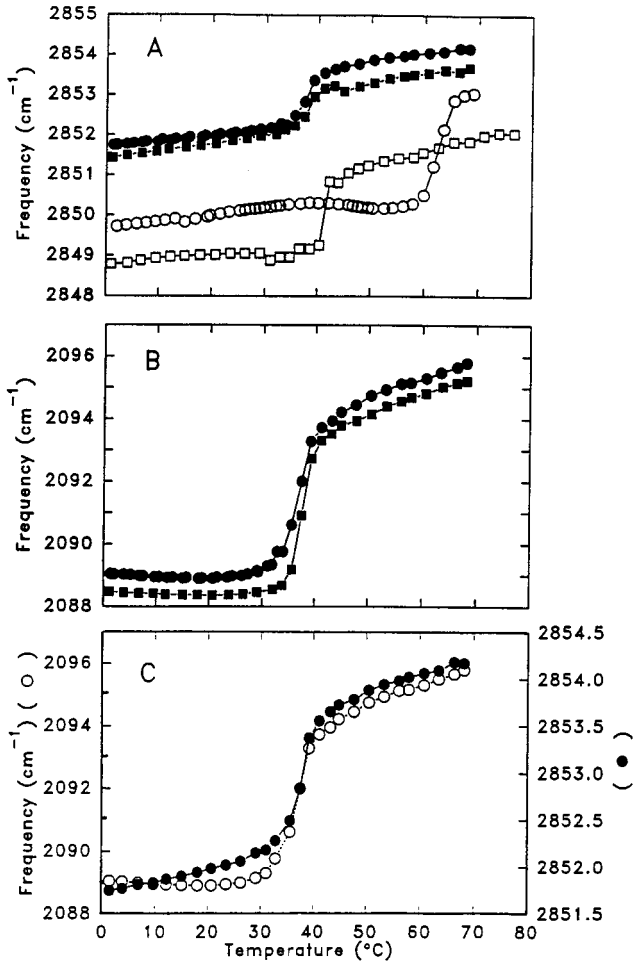

FIGURE 5: (A) Temperature dependence of the frequency of the band due to the acyl chain CH_2 symmetric stretching vibration of pure DPPC (□), pure C26-CBS (○), DPPC at 8 mol % in DPPC-d_{62} (■), and C26-CBS at 8 mol % in DPPC-d_{62} (●). (B) temperature dependence of the frequency of the band due to the acyl chain CD_2 symmetric stretching vibration of DPPC-d_{62} for the DPPC/DPPC-d_{62} (■) and C26-CBS/DPPC-d_{62} (●) systems. (C) Temperature dependence of the frequency of the band due to the acyl chain CH_2 and CD_2 symmetric stretching vibration of C26-CBS (●) and DPPC-d_{62} (○), respectively, for the C26-CBS/DPPC-d_{62} mixture.

ordering of CBS in DPPC occurs and partially counteracts the dilution effect on the frequency. This is supported by the smaller difference in frequency of the CH_2 symmetric stretching band between CBS in DPPC-d_{62} and DPPC in DPPC-d_{62} compared to the difference between CBS alone and DPPC alone. As observed for the CBS/DMPC-d_{54} mixture, Figure 5C shows that no lateral phase separation occurs in the CBS/DPPC-d_{62} mixture, since the T_c obtained on the temperature profiles is the same for each component of the CBS/DPPC-d_{62} mixture.

It is also observed in Figure 5A that the presence of a small quantity of CBS in a matrix of DPPC-d_{62} induces a shift of the transition temperature of the mixture toward lower temperatures of the mixture compared to DPPC/DPPC-d_{62}, in contrast to the behavior of C26-CBS/DMPC-d_{54}. This is in agreement with DSC results, which show that the introduction of C26-CBS at concentrations up to approximately 30 mol % in DPPC decreases its T_c, in contrast to the behavior with DMPC (Boggs et al., 1993). This decrease in T_c can also be seen clearly in Figure 5B, which shows the temperature dependence of the frequency of the band due to the symmetric CD_2 stretching vibration of the perdeuterated PC acyl chain stretching vibration for CBS/DPPC-d_{62} and DPPC/DPPC-d_{62}. These curves indicate that, in both the gel and liquid crystalline phases, the conformational order of the PC chains is slightly higher for the model system DPPC/DPPC-d_{62} compared to the system where the asymmetric CBS is present. It seems that the PC deuterated acyl chains are more perturbed by the presence of the asymmetric glycolipid than by its homologous protiated phospholipid.

Table 1: Order Parameter, $f(\theta)$, and the Average Angle, $\langle\theta\rangle$, Calculated for the CH_2 or CD_2 Symmetric Stretching Vibrations in CBS/DMPC-d_{54} and CBS/DPPC-d_{62} Mixtures, and in Pure DMPC-d_{54} and Pure DPPC-d_{62} Dispersions

	CH_2 chains		CD_2 chains	
	$f(\theta)^a$	$\langle\theta\rangle$ (±1°)	$f(\theta)^a$	$\langle\theta\rangle$ (±1°)
CBS/DMPC-d_{54}	0.70 ± 0.02	26	0.73 ± 0.02	25
DMPC-d_{54}			0.68 ± 0.03	27
CBS/DPPC-d_{62}	0.47 ± 0.03	36	0.58 ± 0.02	32
DPPC-d_{62}			0.65 ± 0.03	29

[a] These values were obtained from an average of at least 6 ATR measurements on either dry or hydrated films at T_c − 20 °C.

ATR Measurements in the Gel Phase. Since the isotopic dilution effect and the increase in disorder that occurs with chain length precludes unambiguous conclusions about the significance of an increase in conformational order of the CBS acyl chains in a deuterated PC lipid bilayer matrix, we have investigated oriented films of CBS mixtures in DMPC-d_{54} or DPPC-d_{62}. By using polarized ATR measurements, it should be possible to determine whether the glycolipid acyl chains are parallel to the chains of the host matrix. Table 1 lists the order parameter, $f(\theta)$, calculated from the symmetric CH_2 and CD_2 stretching vibration of the acyl chains of the protiated, and deuterated components in CBS/DMPC-d_{54} and CBS/DPPC-d_{62} mixtures and in pure deuterated PC dispersions, in the gel phase. The results in this table show that, for the CBS/DMPC-d_{54} system, the order parameter associated with the CH_2 chains of CBS is quite similar to that of the perdeuterated acyl chains of DMPC-d_{54}, while for CBS/DPPC-d_{62} it is lower than that associated with the DPPC-d_{62} matrix. On the other hand, order parameters of the acyl chains of the DMPC matrix are higher than those of DPPC. In addition, the order parameter of the CH_2 chains of CBS is significantly higher in DMPC-d_{54} than in DPPC-d_{62}. Comparison with the order parameter of pure DMPC-d_{54} and DPPC-d_{62} shows that CBS increases the order parameter of DMPC-d_{54} but decreases that of DPPC-d_{62} somewhat.

Acyl chain tilt angles can be calculated from the order parameters if the lipid acyl chains are in the *all-trans* conformation. In the case of a chain orientation distribution of finite width, order parameters obtained by ATR measurements should be interpreted by means of both conformational distribution and tilt angle (Lafrance et al., 1995). By assuming an infinitely narrow orientation distribution of the phospholipid acyl chains, the difference observed between the order parameter values of DMPC-d_{54} and DPPC-d_{62} acyl chains in the presence or in the absence of C26-CBS can be attributed primarily to a difference of the acyl chain tilt angle. Table 1 lists the average angle θ for CBS/DMPC-d_{54} and CBS/DPPC-d_{62} mixtures and for pure DMPC-d_{54} and DPPC-d_{62} dispersions, calculated from

$$f(\theta) = \frac{3\cos^2\theta - 1}{2} \qquad (2)$$

For pure DMPC-d_{54} and DPPC-d_{62} bilayers, the angle θ is $27° \pm 1°$ and $29° \pm 1°$, respectively, between the acyl chains and the normal to the bilayer, in agreement with previous results on pure protiated DMPC and DPPC phases (Fringeli, 1977; Ter-Minassian-Saraga et al., 1988). For the CBS/DMPC-d_{54} mixture, the orientation of the lipid acyl chains is similar for both components, while in the case of CBS/DPPC-d_{62}, the tilt angle of the glycolipid acyl chains with regard to the normal to the bilayer is higher than that of the host matrix. On the other hand, the lower value of the order parameter calculated from the CH_2 groups compared to that of CD_2 chains, for a given system, could be due to the presence of more *gauche* conformers in the protiated acyl chains than in the host matrix with deuterated acyl chains.

The order parameter associated with the CD_2 groups of the acyl chains of DMPC-d_{54} in the presence of CBS is higher than that of pure DMPC-d_{54}. This suggests that the long asymmetric glycolipid induces either a reorientation of the perdeuterated acyl chains of the DMPC-d_{54} matrix or a diminution of the number of *gauche* conformers. The temperature dependence of the frequency of the band due to the CD_2 symmetric stretching vibration shows that, in the gel phase, the frequency for CBS/DMPC-d_{54} is only a little higher than for DMPC/DMPC-d_{54}, suggesting the occurrence of little or no change in the number of *gauche* conformers in perdeuterated acyl chains of DMPC-d_{54} in the presence of CBS (Figure 4B). Therefore, the increase from 0.68 to 0.73 of the order parameter should not be interpreted in terms of a diminution of the number of *gauche* conformers but rather in terms of a reorientation of the acyl chains of DMPC toward the normal to the germanium crystal. This result indicates that the C26-CBS fits well in a 28 chain length matrix provided that the DMPC chains are less tilted and that both chains of CBS are mostly in the *trans* conformation. This interpretation is confirmed by the low difference of the order parameters $f(CH_2)$ (0.70) and $f(CD_2)$ (0.73) for the CBS/DMPC-d_{54} mixture (Table 1).

In contrast, the fit of C26-CBS in DPPC-d_{62} in a 32 chain length PC matrix of DPPC-d_{62} does not seem to be as good. For example, the order parameter of the CBS CH_2 groups is 0.47 compared to 0.58 for the CD_2 groups of the DPPC-d_{62} host matrix. These results show that the CBS acyl chains are either more tilted than those of the DPPC-d_{62} host matrix or more disordered. The lower value of $f(CD_2)$ of DPPC-d_{62} in the mixture with CBS, compared to DPPC-d_{62}, is probably due to an increase in *gauche* content of DPPC rather than a change in tilt angle, as indicated by the increase in frequency of the CD_2 chains of DPPC-d_{62} in the presence of CBS (Figure 5B). The insertion of CBS into a tilted DPPC bilayer combined with the increase in *gauche* content of DPPC will help to minimize the difference between the length of the C26 chain of CBS and the thickness of the 32-carbon-thick DPPC bilayer.

Interfacial Region. Figure 6 shows the amide I′ region infrared spectra of pure CBS bilayers and CBS incorporated in host matrices of DMPC-d_{54} and DPPC-d_{62}, in both the gel and liquid crystalline phases. This vibration is due to the C26-CBS amide group connecting the long fatty acid chain of the glycolipid. For pure CBS in the gel phase, the amide I′ band presents three spectral components with the major one centered at 1618 cm^{-1}. This low value for the frequency of the amide I′ band is due to amide groups hydrogen-bonded with either galactose hydroxyl groups (Tupper et al., 1992) or the sphingosine hydroxyl group of another CBS molecule. There is also a weaker amide I′ band at approximately 1650 cm^{-1}, which can be attributed to non-hydrogen-bonded amide groups. Finally, a component at 1632 cm^{-1}, which becomes more intense in the liquid crystalline phase, could be attributed to amide groups hydrogen-bonded to water molecules. In the liquid crystalline phase, the amide I′ band shifts to 1625 cm^{-1} and becomes much broader, indicating that the intermolecular hydrogen bonding is weakened and is most likely replaced by hydrogen bonds between the CBS amide groups and water molecules. The broadness of the band indicates that there is a wide distribution of hydrogen-bond strength for the liquid crystalline phase.

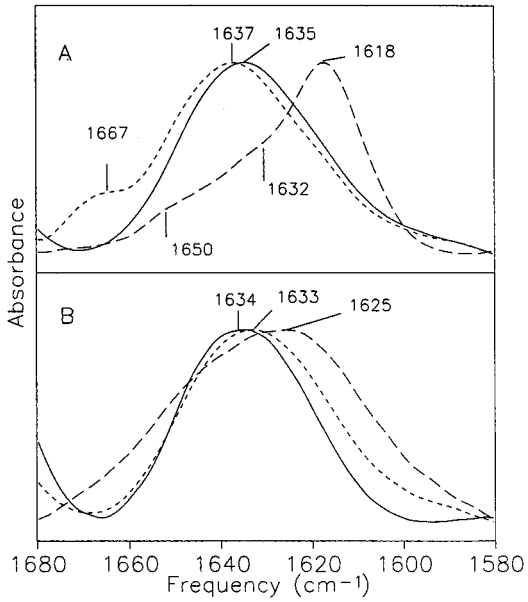

FIGURE 6: Infrared band due to the amide I′ vibration due to the amide groups of C26-CBS in the spectra of pure C26-CBS (— — —), C26-CBS at 8 mol % in DMPC-d_{54} (- - -), and DPPC-d_{62} (—) mixtures, in the (A) gel and (B) liquid crystalline phase, at the same reduced temperature, $T_c \pm 20$ °C (except for pure CBS in the liquid crystalline phase, whose spectrum was recorded at 75 °C).

For CBS in both DMPC-d_{54} and DPPC-d_{62} in the gel phase, the maximum of the amide I′ band appears around 1635 cm^{-1}, indicating that intermolecular hydrogen bonding between CBS molecules has been eliminated, since the CBS molecules are diluted to 8 mol % in the PC matrices. This result also indicates that there is no lateral phase separation in the lipid mixtures, since no intermolecular hydrogen bonding is observed.

As seen in Figure 6A, for the gel phase, when C26-CBS is mixed into DMPC-d_{54}, a small component appears at 1667 cm^{-1}, while for CBS in DPPC-d_{62}, this band is not present. Since this band does not appear in the liquid crystalline phase (Figure 6B) where all the amide groups of the glycolipid should be fully hydrated, it suggests the presence of a population of weakly hydrogen-bonded amide groups or amide groups which are not hydrogen-bonded to water molecules or to lipid groups, in the gel phase of the CBS/DMPC-d_{54} mixture. The difference in frequency between the band at 1667 cm^{-1} observed for a system of amide groups diluted in a PC matrix with the band at approximately 1650 cm^{-1} for pure CBS bilayers, which is also attributed to non-hydrogen-bonded molecules, can be explained by the difference in ionic strength of the interfacial moiety between PCs and glycolipids.

In the case of CBS in DPPC-d_{62}, all the amide groups appear to be exposed to buffer in both the gel and the liquid crystalline phases, since the frequency of the amide I′ band is the same for both phases. The results suggest that in the case of CBS in DMPC-d_{54}, in the gel phase, a small population of the amide groups is more embedded in the interfacial moiety while most of the amide groups are in an environment of lower dielectric constant than in the liquid crystalline phase, since the frequency of the maximum of the amide I′ band decreases considerably from 1637 cm^{-1} for the gel phase to 1633 cm^{-1} for the liquid crystalline phase.

DISCUSSION

The results presented in Figures 4 and 5 show that the difference in the frequency of the symmetric C−H stretching vibration of the acyl chain for pure CBS and CBS mixed with DMPC-d_{54} or DPPC-d_{62} caused by the isotopic dilution effect (Kodati et al., 1994) is partly abolished in the liquid crystalline phase. While this result might be interpreted as due to lateral phase separation, there is reason to believe that this is not the case. Since it has previously been observed that mixtures of lipids of different acyl chain lengths can be immiscible or form microdomains, we want first to highlight that point in the case of C26-CBS incorporated at 8 mol % in PCs. For example, cases of lateral phase separation have been reported in the literature due to mismatch of the acyl chain length for PC mixtures (Huang & Mason, 1986) and differences in head groups for glycolipids in PC matrices (Singh et al., 1992; Barenholz et al., 1983; Gardam & Silvius, 1989). On the other hand, at low concentrations (<20 mol %) in PC, galactosylceramide has been found to be miscible in both the gel and liquid crystalline phases (Lu et al., 1993; Morrow et al., 1992). Our results also indicate that at 8 mol % C26-CBS was miscible in both the gel and liquid crystalline phases of DMPC and DPPC. Frequency values of the CBS CH_2 acyl chain stretching superimpose in the gel phase, before and after heating, indicating that no glycolipid-rich domain occurs during heating or after allowing the dispersion to equilibrate. This is supported by the investigation of the amide I′ spectral region, where no low-frequency component characteristic of self-associated glycosphingolipid amide groups is present for CBS/DMPC-d_{54} and CBS/DPPC-d_{62} in either the gel or liquid crystalline phases. Furthermore, the phase transition temperature of C26-CBS in mixtures with DMPC-d_{54} or DPPC-d_{62} is similar to that of the deuterated DMPC or DPPC in the mixture. Thus, lateral phase separation cannot account for the low frequency value of the CBS CH_2 stretching band observed in the liquid crystalline phase. Compared to galactosylceramide, for which freeze−fracture electron microscopy suggested the formation of gel-phase domains in liquid crystalline-phase PC (Singh et al., 1992), it is less likely that microdomains of CBS could occur at very low concentrations in PC bilayers, since the negatively charged sulfate groups of the galactose in CBS weaken the intermolecular hydrogen-bonding interactions of the galactose. As a result, we expect less tendency for CBS to cluster compared to galactosylceramide.

The difference in frequency of the CBS acyl chain CH_2 band observed in the liquid crystalline phase between pure CBS and CBS in PC is most likely the result of two opposing effects, rather than phase separation: an increase in frequency caused by the dilution effect and a decrease in frequency due to the restriction of the motion of the CBS acyl chain in PC. It therefore appears that CBS is more ordered in PC in the liquid crystalline phase than in the liquid crystalline phase of pure CBS.

This study was carried out primarily in order to determine how an asymmetric glycosphingolipid containing a long acyl chain of 26 carbons can pack into a symmetric PC bilayer of varying thickness. Several models for packing of C26-CBS in a PC bilayer are possible. (i) The CBS long acyl chain may interdigitate across the center of and span the PC bilayer. This would be expected to dynamically and sterically restrict its conformation, which would decrease the frequency of the band due to the CH_2 stretching vibration relative to the model PC system. (ii) Alternatively, the end of the very long acyl chain may terminate in the center of the PC bilayer. In that case, spectroscopic parameters related to the conformational order or orientation of the CBS acyl chains would be expected to indicate more kinks or *gauche* conformers. (iii) We can also imagine that the long acyl chain might bend by 90° in the center of the bilayer. This should decrease the order parameter, which describes the orientation of the acyl chain, relative to that of the PC acyl chains. (iv) Finally, the very long acyl chain of the sphingolipid might bend by 180° in order to minimize chain length differences. The spectroscopic parameter related to the orientation in that case would probably be similar to that of a straight chain spanning the PC bilayer. Effects on the PC chain conformation may also occur.

Using deuterium NMR, Sankaram and Thompson (1992) have shown that, in binary mixtures of lipids of different acyl chain lengths in the liquid crystalline phase, the chain length mismatch is compensated by an increase in *gauche* conformers for the long chain and a decrease for the short one. However, this applies to symmetric lipids whose chains all meet in the center of the bilayer. For an asymmetric chain length molecule whose acyl chain interdigitates across the center of a bilayer thicker than its length, the symmetric lipid chains would be expected to compensate by an increase in *gauche* conformers and the *gauche* content of the long chain of the asymmetric molecule would decrease. Although the frequency of the CH_2 symmetric stretching band suggested that conformational ordering of the CBS CH_2 chain did occur in DMPC and DPPC in the gel and especially in the liquid crystalline phase, it is difficult to ascertain the significance of these changes because of the opposing effects of isotopic dilution and an increase in chain length on frequency. However, the greater disorder of the DPPC-d_{62} CD_2 chains when mixed with CBS compared to mixtures with DPPC in both the gel and liquid crystalline phases is consistent with the model that the CBS acyl chain interdigitates across the bilayer center rather than terminates at the bilayer center. On the other hand, the DPPC chains could become less ordered even if the CBS chain terminates at the bilayer center, simply because of the packing irregularities which an asymmetric molecule such as CBS would cause regardless of which packing model it adopts.

Observation of the wagging progression spectral region would have provided more quantitative information about the effective chain length of the very long CBS acyl chain since the wagging progression is not affected by isotopic dilution (Kodati et al., 1994). This would have allowed us to distinguish between the case where the long acyl chain of the glycolipid spans the PC bilayer giving rise to a spectral wagging progression and the case where the long chain of

CBS bends by either 90° or 180° in the center of the bilayer. The latter would reduce the progression by a factor of 2 or abolish it. Unfortunately, the broad and intense bands due to S═O, as well as PO_2^- stretching vibrations, contribute in that spectral region, precluding any observation of the CH_2 wagging bands.

The DMPC-d_{54} host matrix has the ideal thickness to allow the 26-carbon long acyl chain of CBS to interdigitate across the center of a PC bilayer and span the bilayer. Interdigitation of the long chain of C26-CBS in DMPC-d_{54} gel-phase bilayers is consistent with our ATR results, since $f(CH_2)$ of C26-CBS chains is very similar to $f(CD_2)$ in CBS/DMPC-d_{54}.

The CBS long acyl chain does not fit as well in the 32-carbon thick DPPC gel-phase bilayer as in the 28-carbon DMPC bilayer. The ATR results have revealed that the order parameter of the CBS acyl chains is lower than that of the acyl chains of the DPPC-d_{62} host matrix, thus showing that the CBS acyl chains are either more tilted than those of the DPPC-d_{62} host matrix or more disordered. Results of the amide I′ spectral region in the gel phase suggest that the amide group of CBS is more accessible to buffer when CBS is incorporated in DPPC than in DMPC. These results could be consistent with the model where the long acyl chain of CBS terminates at the center, or around the center, of the DPPC bilayer, thus causing increased exposure of the amide group to the aqueous environment. Alternatively, CBS may be interdigitated in the gel phase of DPPC-d_{62} but both molecules have different tilt angles or conformational order, in order to minimize the chain length mismatch.

This difference between the fit of C26-CBS in DMPC and DPPC host matrices is supported by the thermotropic behavior of the CBS/PC mixtures, since C26-CBS shows peritectic behavior in the presence of DMPC-d_{54} and eutectic behavior with DPPC-d_{62}, as observed in Figures 4 and 5, and in DSC studies (Boggs et al., 1993). C26-CBS stabilizes the gel phase of DMPC bilayers, while it destabilizes that of DPPC bilayers. Consistent with this is the greater disordering of DPPC-d_{62} CD_2 chains caused by CBS in the gel phase compared to DMPC-d_{54} (Figures 4B and 5B).

Less information is available from our study concerning packing of C26-CBS in the liquid crystalline phase. Although the decrease in frequency of the CH_2 symmetric stretching band suggests that ordering of the C26-CBS chains occurs in the liquid crystalline phase of DMPC and DPPC, the magnitude of this effect is difficult to estimate because of the opposing effects of isotopic dilution and the greater length of the CBS acyl chain compared to the C14 and C16 chains of PC. Pure CBS, with its highly asymmetric hydrocarbon structure, probably forms a rather disordered bilayer in the liquid crystalline phase, as shown for asymmetric species of PC (Lewis et al., 1994b). Thus, some ordering of the CBS chains in PC relative to pure CBS would be expected regardless of the type of packing of CBS in PC because of restriction of its motion by the PC chains of the host matrix. The disordering of the DPPC CD_2 chains could be consistent with several models. The frequency of the amide I′ band indicated that all the amide groups of CBS were exposed to water in both DMPC and DPPC in the liquid crystalline phase. However, this also could be consistent with any of the models suggested. It has not yet proved possible to adequately hydrate the ATR films containing acidic lipids on a germanium (or silicon) crystal for study of the liquid crystalline phase. The phase transition of acidic lipids in ATR films is abolished after the first heating scan, suggesting that dehydration of the lipid occurs after going into the liquid crystalline phase (Nabet and Pézolet, unpublished results). Results of Reinl and Bayerl (1993) on PC−PA and PC−PS mixtures in ATR films suggest a similar problem may have occurred in their study. If this problem can be solved, this may help to resolve the question of how CBS packs into a PC bilayer in the liquid crystalline phase.

In summary, the infrared results have shown that 8 mol % C26-CBS is completely miscible in DMPC or DPPC in the gel and liquid crystalline phases. The thermotropism of the lipid mixtures of C26-CBS at 8 mol % in DMPC or DPPC has confirmed that the glycolipid stabilizes the gel phase of the thinner bilayer while it destabilizes that of the thicker one. The FTIR results indicate further that, in the gel phase, the C26-CBS long acyl chain may interdigitate across the bilayer center and span the 28 carbon thick bilayer of tilted DMPC and decrease the tilt of the DMPC molecules. However, it may preferentially end at the center of the 32-carbon thick bilayer of DPPC, causing greater exposure of its amide groups to water, or interdigitate into a bilayer of tilted DPPC molecules. The latter is supported by ATR results. Maintenance of the tilt of DPPC molecules in the presence of CBS would minimize the chain length mismatch. C26-CBS also causes an increase in the *gauche* content of DPPC, which would also help minimize chain length mismatch if the long chain of CBS is interdigitated across the bilayer center. Although this study provides less information about the liquid crystalline phase, the results from the gel phase show that lipid acyl chain conformation and ability of an asymmetric lipid to interdigitate across the bilayer center and cause changes in tilt and conformation of the host matrix lipid depends on the difference between the chain lengths of the glycosphingolipid and the thickness of the lipid bilayer surrounding it. This could help to understand the deleterious effect of sphingolipids with very long acyl chain fatty acids in myelin in adrenoleukodystrophy.

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