Raman Spectroscopic Study of Semisynthetic Species of Cerebroside Sulfate: Two Types of Hydrocarbon Chain Interdigitation†

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ABSTRACT: Raman spectroscopy was used to study the phase behavior of several semisynthetic species of the acidic glycosphingolipid cerebroside sulfate (CBS) which occur in myelin. The C–H stretching mode region at 2800–3100 cm^{-1} of C18:0-CBS, C24:0-CBS, and C26:0-CBS, and the α-hydroxy fatty acid species C18:0h-CBS, was studied in the presence of 2 M Li^+ and 2 M K^+. Earlier studies have shown that K^+ shields the negative charge on the sulfate more effectively than Li^+, thus promoting intermolecular hydrogen-bonding interactions between the lipid molecules. Indeed, a novel broad background feature was present in the Raman spectra from 2900 to 3200 cm^{-1}, which was attributed to O–H stretch associated with intermolecular hydrogen bonding between lipid hydroxyl groups. After subtraction of this broad feature, the intensities of the lipid C–H stretching vibrational transitions could be determined. These indicated that in K^+, the degree of order (intrachain conformation and lateral chain–chain interactions) of C18:0-CBS, whose hydrocarbon region is fairly symmetrical in chain length, is similar to that of the symmetric chain length glycerolipid dipalmitoylphosphatidylcholine, while the degree of order is lower in Li^+, as a result of the increased lateral charge repulsion of the head groups in Li^+. Two phase transitions were observed for the highly asymmetric species C24:0-CBS and C26:0-CBS in K^+ but only one transition in Li^+. At the first transition of these lipids in K^+, the order decreases from values characteristic of gel-phase bilayers, to a value intermediate between that of the gel phase and the liquid-crystalline phase, while at the second transition the order decreases to a value characteristic of the liquid-crystalline phase. The first transition is ascribed to a transition from a mixed interdigitated gel state to a partially interdigitated gel state, while the second transition is ascribed to the liquid-crystalline phase. In Li^+, the Raman data indicate that the gel phase of the asymmetric species has the same degree of order as a mixed interdigitated bilayer. If the lipids transform to a partially interdigitated bilayer gel phase in Li^+, they do not do so until just before the transition to the liquid-crystalline phase. This difference in behavior in Li^+ compared to K^+ is attributed to increased charge repulsion and decreased intermolecular hydrogen bonding between the head groups, which stabilizes the mixed interdigitated bilayer relative to the partially interdigitated gel-phase bilayer.

Cerebroside sulfate (CBS)[1] is an important sphingolipid in myelin (3.8 wt % of the total lipid) (Norton & Cammer, 1984). It occurs in a number of other membranes as well (Karlsson, 1982). The fatty acids in myelin CBS can vary from 14 to 26 carbons in length with the predominant chain length being 24 carbons. They are either saturated or monounsaturated (O'Brien et al., 1964). An increase in the content of very long chain fatty acid species (C25 and C26) occurs in membrane sphingolipids in the demyelinating disease adrenoleukodystrophy (Kishimoto et al., 1985).

Since the sphingosine chain of 18 carbons is thought to penetrate into the bilayer to a depth of about 13 or 14 carbons (Dahlen & Pascher, 1979), the longer chain species are highly asymmetric in their hydrocarbon structure, similar to asymmetric species of phosphatidylcholine (PC), which have been shown by X-ray diffraction (Hui et al., 1984; McIntosh et al., 1984) to form "mixed interdigitated" bilayers in which the short chains pack end-to-end and the long chains span the bilayer. Indeed, use of spin labels indicates that, under certain conditions, the C24 and C26 species of cerebroside sulfate also can form both mixed interdigitated bilayers and "partially interdigitated" bilayers (Boggs et al., 1988b). In the latter, the short sphingosine chain of a lipid molecule on one side of the bilayer is packed end-to-end with the long acyl chain of another lipid molecule on the other side of the bilayer. X-ray diffraction can detect the partially interdigitated bilayer formed by this lipid but has not detected the mixed interdigitated structure (Stinson & Boggs, 1989). An X-ray diffraction study of the C24 species of cerebroside by Reed and Shipley (1987) showed that it also forms a partially interdigitated bilayer. However, use of Raman spectroscopy indicates that the C24 species of sphingomyelin undergoes a transformation at temperatures below the main gel to liquid crystalline phase transition from an ordered phase, thought to be a mixed interdigitated bilayer, to another phase, thought to be a partially interdigitated bilayer (Levin et al., 1985). Raman spectroscopy indicates that the latter is less ordered than the mixed interdigitated bilayer but more ordered than the liquid-crystalline

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[1] Abbreviations: CBS, cerebroside sulfate; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; ESR, electron spin resonance; NMR, nuclear magnetic resonance.

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phase bilayer (Levin et al., 1985; Huang et al., 1983).

In the present study, we have used Raman spectroscopy to measure changes in the conformational properties with temperature of C18:0, C24:0, and C26:0 species of CBS. Since about half of the CBS in myelin contains fatty acid chains which are hydroxylated at the α-carbon (O'Brien et al., 1964; Svennerholm & Stallberg-Stenhagen, 1968), providing opportunities for increased intermolecular hydrogen-bonding interactions with the hydroxyl group present on the sphingosine chain, we also attempted to study one hydroxy fatty acid species, C18:0h-CBS. The lipids were studied in the presence of 2 M Li^+ and 2 M K^+. This cation concentration results in a smaller number of sharper phase transitions than observed at lower cation concentrations (Boggs et al., 1984). K^+ shields the charge on the sulfate more effectively than Li^+, thus promoting intermolecular hydrogen-bonding interactions between the lipid molecules and favoring more stable phases with higher transition temperatures and enthalpies (Boggs et al., 1984, 1988a,b).

Materials and Methods

Materials. Semisynthetic species of CBS (galactosylceramide I^3 sulfate) containing stearic acid (C18:0-CBS), lignoceric acid (C24:0-CBS), hexacosanoic acid (C26:0-CBS), and α-hydroxy stearic acid (C18:0h-CBS) were prepared from bovine brain CBS as described earlier (Koshy & Boggs, 1982, 1983; Boggs et al., 1984). They were chromatographically pure and contained the expected amounts of galactose and sulfate, determined by the methods of Kushwaha and Kates (1981) and Kean (1968), respectively. Dipalmitoylphosphatidylcholine (DPPC) and phosphatidylinositol were kind gifts from Dr. K. Keough, Memorial University of Newfoundland.

Sample Preparation. CBS was suspended in 2 M KCl or 2 M LiCl prepared in triply distilled water. The other lipids were suspended in triply distilled water. The water or cation solution (10 μL) was added to the dry lipid (1.5–2 mg) in a capillary tube sealed at one end. The tube was sealed at the other end and the lipid was hydrated and mixed by immersing the lower half in an ultrasonic bath for 3–5 min. The tube was then centrifuged in an International Equipment Co. bench centrifuge (model CL) for 1–2 h to sediment the lipid. Samples were stored at −10 °C after preparation. Annealing of the sample by storage at low temperatures converts it into a more ordered, less hydrated state (Boggs et al., 1988a,b; Stinson & Boggs, 1989). Raman spectroscopic measurements on annealed samples also indicated greater order, consistent with these other studies. However, in the present study, we were interested in the structure of the more hydrated phases. Therefore, in order to ensure complete hydration for measurement of the spectra, the samples were heated to 90 °C in a water bath for 2–5 min and then inserted into the sample holder at 10 °C.

Raman Spectroscopy. Vibrational Raman spectra in the C–H stretching region, 2700–3200 cm^{-1}, were obtained at a scanning rate of 0.5 cm^{-1}/s using a home-built Raman spectrometer (Stevenson, 1988) consisting of an argon ion laser (either Spectra Physics model 165 or Continental Laser Corporation 3000 series, used interchangeably) operated with a prism to select the 514.5-nm line, a temperature-controlled sample holder placed at the focus of the laser beam, wide-aperture collection optics consisting of a cassegrain telescope assembly (Applied Photophysics Ltd., model 6500), a Czerny-Turner double monochromator with 1800 line/mm holographic gratings (Spex Industries model 14018), and a thermoelectrically cooled photomultiplier tube (RCA 31034 with Ga–As photocathode) operated with a pulse shaper/discriminator in the photon-counting mode. The laser beam was filtered with a narrow band-pass filter in order to eliminate interfering plasma emission lines. Laser power at the sample was limited to 60–100 mW to minimize heating. 180° scattering geometry was used.

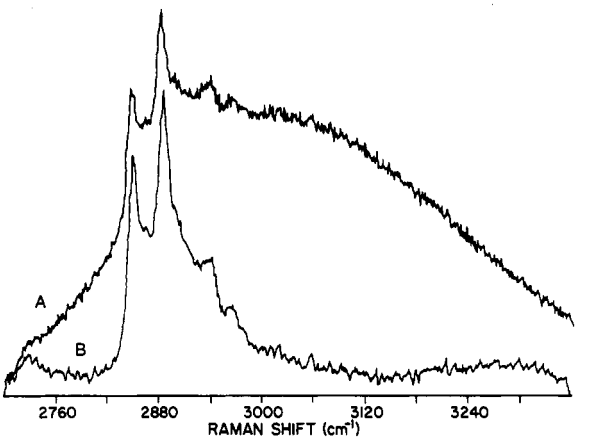

FIGURE 1: Raman spectrum of C18:0-CBS in 2 M Li^+ at 20 °C (A) before and (B) after smoothing the data and subtracting a fitted background profile.

Spectra were recorded in an ascending temperature mode; the equilibration time at each temperature was 6 min before recording the spectrum. The temperature was monitored by a thermistor (Fenwall) inserted into the thermostatically controlled sample holder near the sample position but out of the light path. Repeated runs on the same sample gave reproducible temperature profiles.

The spectra were digitally recorded on a tape drive and downloaded to a VAX 8800 for analysis. The spectra were smoothed by simple running averages using three or five points, and a broad background feature at about 2900–3200 cm^{-1} was subtracted from the spectra (Stevenson, 1988). The peak maxima at approximately 2845, 2885, and 2935 cm^{-1} were located, and the intensities, I, were determined as the peak heights with respect to the baseline, at the associated wavenumbers. The ratios $R_1 = I_{2935}/I_{2885}$ and $R_2 = I_{2845}/I_{2885}$ were calculated.

Results

Figure 1 shows the spectra of C18:0-CBS in 2 M Li^+ before and after smoothing the data and subtracting a fitted background profile, in order to remove the broad background feature at about 2900–3200 cm^{-1} and centered at 3000 cm^{-1}. This broad feature resembled the free water O–H stretch normally observed at about 3400 cm^{-1}. It is most likely due to O–H stretching associated with the CBS O–H groups or CBS head group–water interactions. It was not present in DPPC or phosphatidylinositol (not shown), in pure water or ice, D_2O, or the KCl or LiCl solutions used. It also was not observed in other hydroxylated solvents or compounds such as ethanol or 3 M sucrose in water. A water O–H band was present for the other phospholipids and for sucrose, but it was much less intense than the C–H region and was adjacent to it, not superimposed with it.

The broad feature was even more intense for the hydroxy fatty acid species of CBS, C18:0h-CBS, than for the nonhydroxy fatty acid species (compare Figures 1A and 2A) and was centered at 2900 cm^{-1}, at virtually the same frequency as the C–H stretching modes. Therefore, a reliable spectra of the lipid C–H stretching region could not be obtained by subtraction of the broad feature for the hydroxy fatty acid

FIGURE 2: Changes in the raw Raman spectra for C18:0h-CBS in 2 M Li^+ with temperature: (A) 20.6 °C, (B) 41.7 °C, (C) 64 °C.

species. For all species of CBS, the broad feature changed dramatically upon the lipid gel to liquid crystalline phase transition. It decreased in intensity and broadened further while a shoulder at 3400 cm^{-1} was unchanged as shown in Figure 2 for C18:0h-CBS. The feature at 3400 cm^{-1} is likely the O–H stretching mode associated with the excess water present, emerging from the background at higher temperature (Figure 2C).

In D_2O the broad feature was still present at the same frequency and intensity. Assuming that significant proton–deuteron exchange between the lipid and water hydroxyl groups had not occurred, this result suggests that the feature arises from the O–H groups of the lipids rather than an O–D stretch between oxygens of the lipid and deuterium of the water.

In the subtracted spectrum of C18:0-CBS in Li^+ shown in Figure 1B, containing the C–H stretching mode region between 2800 and 3100 cm^{-1}, three spectral features predominanted. The two most intense were relatively sharp bands at about 2845 and 2885 cm^{-1}, attributed to the symmetric and asymmetric C–H stretching modes, respectively, for the coupled methylene groups of the lipid hydrocarbon region. The third, weak broad band centered at about 2935 cm^{-1} is assigned partly to a Fermi resonance component of the symmetric C–H stretching modes of the hydrocarbon chain terminal methyl groups. Changes in the intensities of these lines were evident in the spectra of C18:0-CBS in 2 M K^+ at different temperatures shown in Figure 3 as the temperature was increased to above the phase transition temperature at about 60 °C. As is standard practice in lipid Raman spectroscopy (Levin, 1984), the chain packing characteristics of CBS were monitored by comparing the intensities of the 2885- and 2935-cm^{-1} features and the intensity of the 2885-cm^{-1} feature to the relatively constant intensity of the 2845-cm^{-1} feature. Although the interpretation of changes in these intensities is complex, by comparing the values with those of well-characterized systems, it has been suggested that the ratio $R_1 = I_{2935}/I_{2885}$ may reflect intrachain conformational disorder in addition to lateral chain–chain interactions, while the ratio $R_2 = I_{2845}/I_{2885}$ may

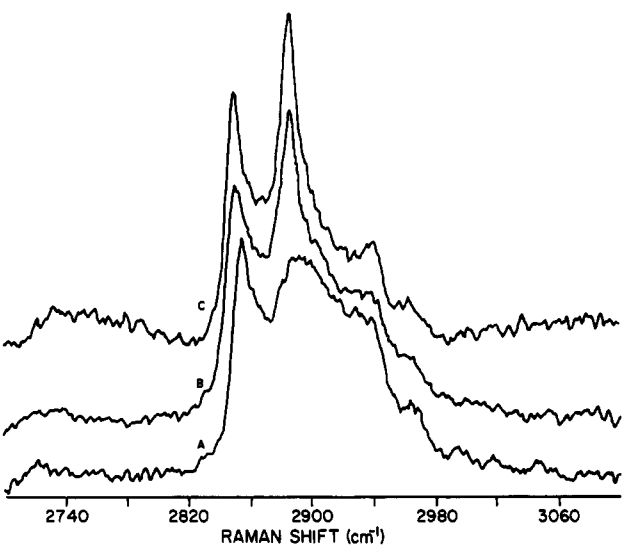

FIGURE 3: Changes in resolved Raman spectra of C18:0-CBS in 2 M K^+ with temperature, illustrating the changes in the C–H stretch vibrational modes as the chains develop gauche conformers: (A) 63.4 °C, (B) 58.6 °C, (C) 20.3 °C.

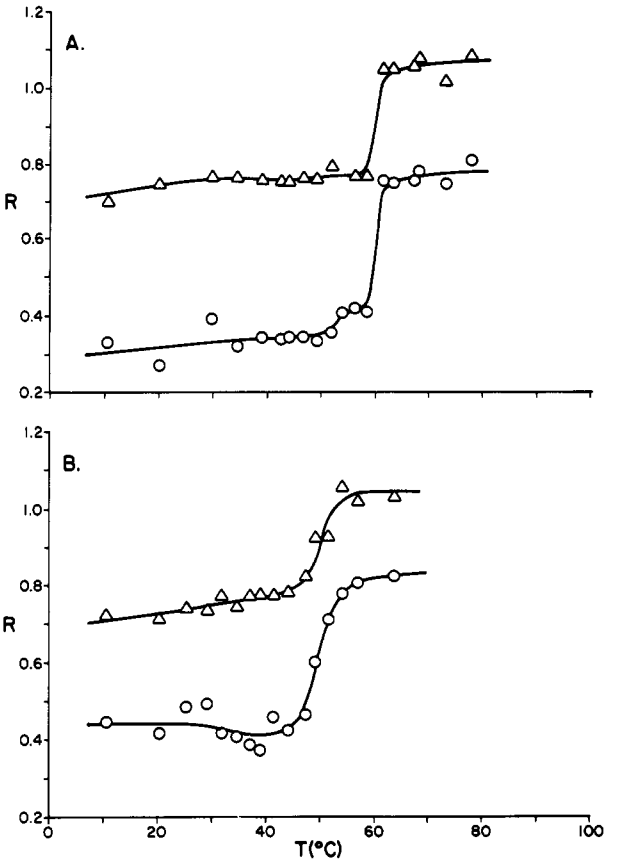

FIGURE 4: Temperature profiles generated from Raman data for C18:0-CBS; (○) R_1, (△) R_2. (A) in 2 M K^+; (B) in 2 M Li^+.

be more a measure of lateral chain–chain interactions with less contribution from intrachain conformation (Levin et al., 1985). Both ratios increase as disorder increases.

Temperature profiles derived from the Raman peak height intensity ratios, R_1 and R_2, are shown for C18:0-CBS in 2 M K^+ and 2 M Li^+ in Figure 4, panels A and B, respectively. The scatter in the data for the CBS samples is due to the difficulty in subtracting the broad feature and illustrates the experimental error involved in performing this subtraction. Considerably less scatter occurred for measurements of DPPC

Table I: Raman Peak Height Ratios for CBS and DPPC in the Gel and Liquid-Crystalline Phases

	gel phase[a]		liquid-crystalline phase		
system	R_1	R_2	temp (°C)[b]	R_1	R_2
DPPC	0.30	0.70	60	0.78	1.06
C18:0-CBS					
2 M K^+	0.31	0.72	80	0.79	1.08
2 M Li^+	0.44	0.72	63	0.82	1.02
C24:0-CBS					
2 M K^+	0.32	0.70	78	0.79	1.00
2 M Li^+	0.31	0.71	72	0.75	1.02
C26:0-CBS					
2 M K^+	0.28	0.69	72	0.72	1.01
2 M Li^+	0.28	0.71	79	0.74	1.03

[a] All measured at 11 °C. [b] The temperature of measurement, about 10–20 °C above the phase transition temperature.

(not shown), for which no background feature was present. The use of two different intensity ratios helped to ascertain whether a fluctuation in the temperature profile was a result of this scatter or a real change in lipid conformation. The profiles for C18:0-CBS displayed clearly discernible order/disorder transitions at similar temperatures for R_1 and R_2, centered at 51 °C for Li^+ and 60 °C for K^+. The R_1 and R_2 values were relatively invariant with temperature above and below the phase transition. In the gel phase, the values for R_1 for C18:0-CBS in K^+ were similar to that of DPPC and lower than in Li^+, while the values for R_2 were similar for all three lipids (Table I), suggesting that the greater shielding of the sulfate by K^+ may have increased intrachain order more than lateral interactions. In the liquid-crystalline phase, C18:0-CBS in Li^+ may also have had more chain conformational disorder than in K^+ or than DPPC, judging from R_1 values, but R_2 values were smaller, indicating that it may have had greater lateral order.

Temperature profiles for C24:0-CBS in K^+ are shown in Figure 5A. In contrast to those for C18:0-CBS, the R_1 profile shows two distinct order/disorder transitions at 41 and 62 °C. This behavior is similar to that reported by Levin et al. (1985) for C24:0-sphingomyelin. In the first transition, R_1 increased from 0.32 to 0.45. In the second, it increased sharply to 0.65 at the transition temperature and then continued to increase more gradually with increase in temperature above the transition temperature. The first transition was also seen in the R_2 profile, where a distinct increase from 0.70 to 0.82 occurred, but a distinct second transition was not observed. At temperatures above the temperature of the second transition observed from R_1, the R_2 values increased gradually with temperature with the same slope as observed for the R_1 values. However, even at a temperature 20 °C above the transition temperature, the R_2 values did not reach those observed for DPPC or C18:0-CBS. Thus, at the first transition of C24:0-CBS, there was an abrupt increase in both intrachain and lateral disorder, while at the second transition the increase in disorder which occurred may have been the result of an increase in intrachain conformational disorder more than a decrease in the lateral interactions, in contrast to the gel to liquid crystalline phase transition of DPPC or C18:0-CBS. At low temperatures below the first transition, the R_1 and R_2 values were similar to those of DPPC. At temperatures between those of the first and second transitions, they were intermediate between values typical of an ordered gel phase and the liquid-crystalline phase (Table I). Thus C24:0-CBS could occur in two gel phases, one more ordered than the other.

In Li^+ only one distinct transition at 53 °C was observed for C24:0-CBS from both the R_1 and R_2 profiles (Figure 5B). However, it was broader than those of the other lipids. The

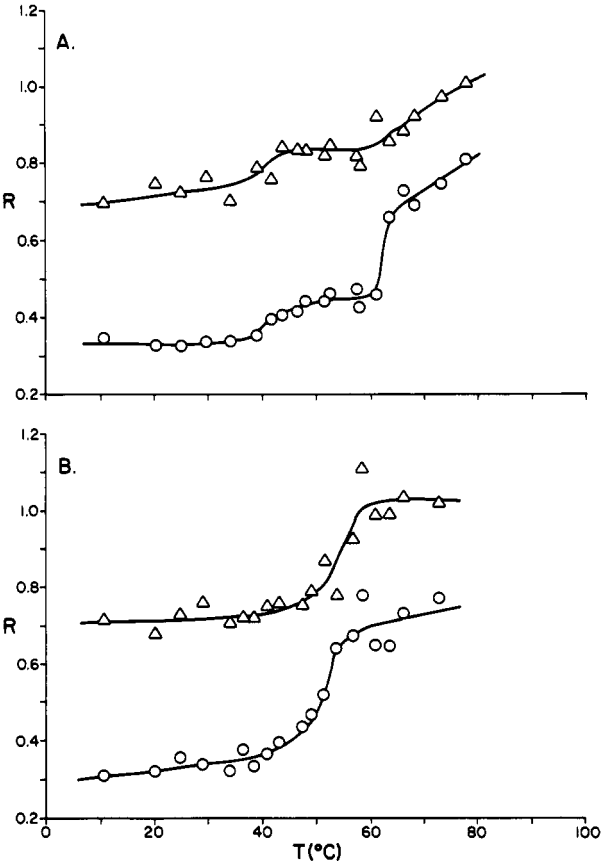

FIGURE 5: Temperature profiles generated from Raman data for C24:0-CBS; (○) R_1, (△) R_2. (A) in 2 M K^+; (B) in 2 M Li^+

R_1 and R_2 values at low temperatures were similar to those observed for DPPC and the more ordered phase of this lipid in K^+ (Table I). The values did not increase to the level of the less ordered gel phase seen for this lipid in K^+ until the temperature was close to the main transition temperature. The values above the temperature of this transition were characteristic of the liquid-crystalline phase, suggesting that this single transition was a gel to liquid crystalline phase transition. However, as in K^+ the liquid-crystalline phase of this lipid in Li^+ was more ordered than DPPC and C18:0-CBS.

C26:0-CBS behaved similarly to C24:0-CBS in K^+ and Li^+ as shown in Figure 6. In K^+ (Figure 6A), the first transition occurred at a lower temperature, between 10 and 30 °C, than in C24:0-CBS. It also seemed broader, although this may have been due to the smaller number of data points at low temperatures and the scatter in the data. However, the low values of R_1 and R_2 at 10 °C indicated that this lipid was also in the more ordered gel phase at this temperature. It transformed to the less ordered gel phase at temperatures well below the main gel to liquid crystalline phase transition. Unlike C24:0-CBS, the main transition could be seen distinctly from increases in both R_1 and R_2 values. In the liquid-crystalline phase, both R_1 and R_2 were lower than for DPPC or C18:0-CBS. In Li^+ (Figure 6B), only one transition was observed. The low values of R_1 and R_2 at temperatures below this transition indicated that it also formed the more ordered gel phase in Li^+ and that it did not transform to the less ordered gel phase until near the temperature of the main transition. The values in the liquid-crystalline phase were similar to or possibly a little greater than those in K^+.

Discussion

The broad background feature underlying the spectra of

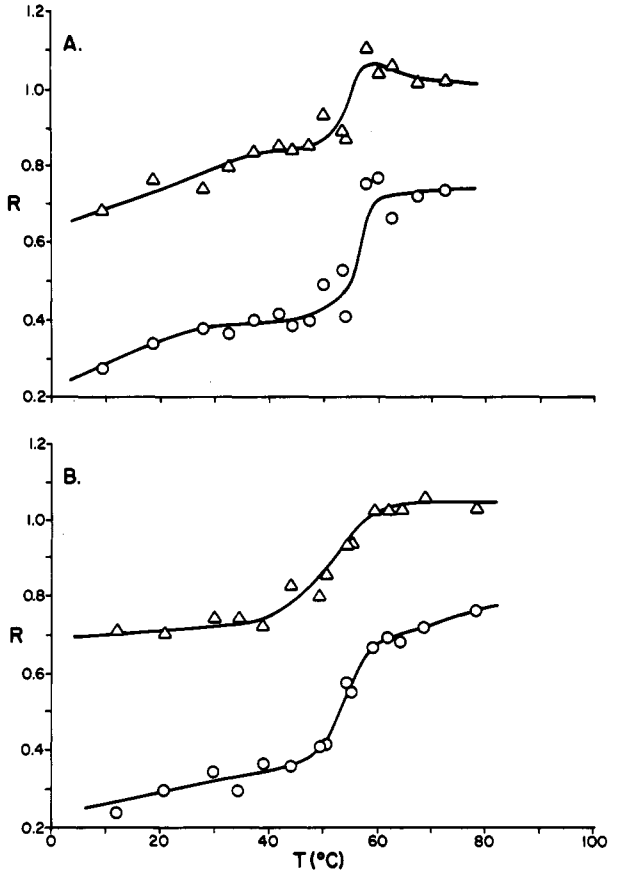

FIGURE 6: Temperature profiles generated from Raman data for C26:0-CBS; (○) R_1, (△) R_2. (A) in 2 M K^+; (B) in 2 M Li^+.

CBS in the 2900–3200-cm^{-1} region was reminiscent of the free water O–H stretch at about 3400 cm^{-1} but differed in shape and was shifted downward in frequency to 3000 cm^{-1} for the non-hydroxy fatty acid species and further to 2900 cm^{-1} for the hydroxy fatty acid species. Although this feature was a nuisance, it is also of interest since it may be due to O–H stretch associated with intermolecular hydrogen bonding of O–H groups of CBS to other molecules of CBS. This is supported by the fact that its intensity was greatest in the gel phase, where intermolecular hydrogen bonding should be strongest; that it was greater for the hydroxy fatty acid species than for the non-hydroxy fatty acid species; and that it remained at the same frequency in the presence of D_2O. The extra hydroxyl group on the fatty acid chain is thought to participate in and strengthen the intermolecular hydrogen-bonding network for CBS as inferred from the fact that it increases the transition temperature of CBS (Boggs et al., 1984, 1988a).

The R_1 and R_2 values of the more ordered and less ordered gel phases of C24:0- and C26:0-CBS in K^+ were similar to those reported for C24:0 sphingomyelin by Levin et al. (1985) and attributed by them to a transformation from a mixed interdigitated bilayer to a partially interdigitated bilayer. Thus the Raman spectra of the C–H stretching region of CBS are consistent with spin label results indicating that, in K^+, C24:0- and C26:0-CBS form a mixed interdigitated bilayer gel phase at low temperatures, which transforms to a partially interdigitated bilayer gel phase at temperatures well below the temperatures of their transitions to the liquid-crystalline phase. The low R_1 and R_2 values at low temperatures in Li^+ indicate that these lipids are also in the mixed interdigitated phase in this cation. However, if the mixed interdigitated gel phase of this lipid in Li^+ also transforms to the partially interdigitated gel phase, it must do so just before the transition to the liquid-crystalline phase, in contrast to its behavior in K^+. Thus the transformation to the partially interdigitated gel phase occurs at a lower temperature in the presence of K^+ than in Li^+ and at a lower temperature for C26:0-CBS in K^+ than for C24:0-CBS.

The Raman results indicate further that the gel phase transforms to a liquid-crystalline phase which may also have some degree of partial interdigitation, since R_1 and R_2 are less than values found for DPPC or C18:0-CBS in the liquid-crystalline phase. X-ray diffraction studies of C24:0-sphingomyelin (Maulik et al., 1986) and C24:0- and C26:0-CBS (Stinson & Boggs, 1989) in the liquid-crystalline phase support this suggestion.

However, Raman spectroscopy cannot prove that a mixed interdigitated bilayer occurs since such a bilayer is not more ordered, with regard to chain conformation or lateral interactions, than a noninterdigitated gel-phase bilayer formed by a symmetric chain lipid such as DPPC. This was shown by Huang et al. (1983) in comparisons of asymmetric chain length PC's with symmetric chain length PC. Raman spectroscopy has shown that a partially interdigitated bilayer is more disordered than either a mixed interdigitated or noninterdigitated bilayer (Levin et al., 1985; Huang et al., 1983), however, lending increased support to the diagnosis of a partially interdigitated bilayer from the Raman results for C24 and C26:0-CBS at intermediate temperatures in the gel phase. This is also supported by X-ray diffraction results on these species (Stinson & Boggs, 1989).

Fatty acid spin labels of length similar to the long chain of the asymmetric lipid, and having the nitroxide group located near the terminal methyl, can detect formation of the mixed interdigitated bilayer, because the spin label group locates in it near the polar/apolar interface, a more ordered region of the bilayer than its normal location near the center of a noninterdigitated bilayer. Such spin labels become motionally restricted in C24:0-CBS and C26:0-CBS at low temperatures (Boggs et al., 1988b), as they do in a number of interdigitated and mixed interdigitated bilayer systems (Boggs & Mason, 1986; Boggs et al., 1989), leading to the suggestion that long-chain species of CBS also form a mixed interdigitated bilayer. This could not be confirmed by X-ray diffraction (Stinson & Boggs, 1989), however, as the phase which restricted the spin label motion only occurred under certain conditions, such as after rapid cooling from the liquid-crystalline phase. Under these conditions, the samples had low long-range order, and a diffraction pattern could not be obtained. The long-range order increased after storage at low temperatures, and the diffraction pattern indicated that the lipid was then in a partially interdigitated phase. It was possible to obtain Raman and ESR spectra at low temperatures immediately after cooling from the liquid-crystalline phase. The high degree of order detected under these conditions with the nonprobe Raman spectroscopic technique supports the spin label results indicating the formation of a mixed interdigitated bilayer and lends confidence to those results.

A transition from a mixed interdigitated bilayer to another gel phase at temperatures below the main transition to the liquid-crystalline phase can also be detected by use of spin labels. However, for C24:0- and C26:0-CBS the spin label becomes insoluble in this phase, making it difficult to determine the temperature of this transition for comparison with the Raman results or to extract further information about its structure with this technique (Boggs et al., 1988b). On the other hand, the hydroxy fatty acid species C24:0h-CBS can

occur in two different gel phases in both of which the spin label is soluble. In one, the spin label is motionally restricted, consistent with formation of a mixed interdigitated bilayer, while the other has more motion consistent with formation of a partially interdigitated bilayer (Boggs et al., 1988b). Deuterium NMR spectroscopy of C24:0-CBS containing a fatty acid chain deuterated at the terminal methyl group provided information about the motional dynamics of the lipid and was consistent with the formation of interdigitated gel phases but could not delineate the structure of the phase (Jeffrey et al., 1989). Thus Raman, ESR, and NMR spectroscopy and X-ray diffraction have provided complementary information to help understand the phase behavior of this lipid.

Endothermic transitions at temperatures below the main transition temperature have been detected for C24:0-CBS by differential scanning calorimetry in Li^+ (Boggs et al., 1988b) and at low K^+ concentrations, but not at high K^+ concentrations (Boggs et al., 1984). No such transitions have been detected for C26:0-CBS in either cation (Boggs et al., 1988b). The transitions observed by calorimetry for C24:0-CBS in Li^+ and low K^+ concentrations may be due to the transformation to a partially interdigitated bilayer seen by Raman spectroscopy. The behavior of spin labels in these samples supports this conclusion. At high K^+ concentrations for C24:0-CBS and for C26:0-CBS, this transformation may not be of sufficiently high enthalpy or cooperativity to detect calorimetrically.

The apparent transformation of CBS in K^+ and of sphingomyelin from a mixed interdigitated to a partially interdigitated gel-phase bilayer indicates that these lipids are unstable in the mixed interdigitated bilayer. This may partly be a result of insufficient chain length asymmetry for the C24:0 and C26:0 species, since this kind of bilayer is most stable when one chain is twice the length of the other (Xu & Huang, 1987). The 10:18 species of PC, which is less asymmetric than 8:18 PC or 18:10 PC because of the inequivalence of the *sn*-1 and *sn*-2 chains (Seelig & Seelig, 1980), also has marginal stability in the mixed interdigitated bilayer and transforms to the partially interdigitated gel-phase bilayer, while 18:10 PC and 8:18 PC occur only in the mixed interdigitated bilayer in the gel phase (Shah et al., 1990).

CBS may also be unstable in the mixed interdigitated bilayer because of its high propensity for intermolecular hydrogen bonding. This can take place only in the partially interdigitated bilayer and is promoted by shielding of the charge on the sulfate (Boggs et al., 1988b). Thus K^+, which has a higher affinity for the sulfate than Li^+, stabilizes the partially interdigitated bilayer better than Li^+ and causes the transition from the mixed to the partially interdigitated bilayer at a lower temperature than occurs in Li^+. Interestingly, 18:12 phosphatidylethanolamine, whose head group can participate in intermolecular hydrogen bonding (Boggs et al., 1987), is also not very stable in the mixed interdigitated bilayer, in contrast to 18:12 PC. It transforms to a noninterdigitated gel-phase bilayer at intermediate temperatures (Mason & Stephenson, 1990). C24:0 cerebroside, which is a stronger hydrogen bonder than CBS, has been observed only as a partially interdigitated bilayer in the gel phase (Reed & Shipley, 1987).

C26:0-CBS, which is more asymmetric than C24:0-CBS, would be expected to be more stable in the mixed interdigitated phase. However, the Raman results indicate that it transforms to the partially interdigitated phase at a lower temperature than C24:0-CBS in K^+. Possibly the increased van der Waals interactions between the longer chains in the partially interdigitated bilayer help to stabilize the hydrogen-bonded phase even more than they stabilize the mixed interdigitated phase. In Li^+, however, C26:0-CBS is as stable as C24:0-CBS in the mixed interdigitated bilayer.

These saturated long-chain species of CBS occur normally in myelin and other membranes and are below their phase transition temperatures at physiological temperature, in contrast to most species of naturally occurring glycerol-based lipids. Abnormal metabolism of the very long chain fatty acids in the hereditary demyelinating disease adrenoleukodystrophy causes a 2–5-fold increase in the amounts of C25 and C26 chains in the sphingolipids of myelin and other cell membranes (Tsuji et al., 1981; Miyatake et al., 1978). This accumulation of long-chain sphingolipids may lead to formation of interdigitated bilayer domains in biological membranes. The long-chain fatty acids of these lipids may also interdigitate into the other side of a noninterdigitated bilayer in a less ordered form of interdigitation (Mehlhorn et al., 1988). These interdigitated regions might have important physiological or pathological roles.

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Role of Carbohydrate Modification in the Production and Secretion of Human Granulocyte Macrophage Colony-Stimulating Factor in Genetically Engineered and Normal Mesenchymal Cells†

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ABSTRACT: Colony-stimulating factors (CSFs) are a group of acidic glycoproteins which stimulate the proliferation and differentiation of hematopoietic progenitor cells in vitro and stimulate hemopoiesis in vivo. Human GM-CSF contains two N-linked carbohydrate side chains of the complex acidic type and several sites of O-linked carbohydrate clustered on serine and threonine residues near the N-terminus of the molecule. Previous studies have failed to detect a significant functional role for the carbohydrate modification characteristic of human GM-CSF. Using permanent cell lines and transient expression systems which produce moderate to high levels of native or carbohydrate-deficient forms of the growth factor, the role of carbohydrate modification in the biosynthesis and secretion of GM-CSF was studied. Unlike a number of other secreted glycoproteins, the transit time and secretory efficiency of several carbohydrate-deficient mutants of GM-CSF are indistinguishable from those of the native growth factor in BHK, 293, COS, and ldlD cells. Furthermore, normal human endothelial cells and fibroblasts, which normally produce the growth factor, can synthesize and secrete GM-CSF that lacks all forms of carbohydrate modification. These studies help to point out the range of roles played by carbohydrate modification in the biosynthesis, assembly, and secretion of glycoprotein hormones.

Granulocyte macrophage colony-stimulating factor (GM-CSF) is an acidic glycoprotein required for the survival, proliferation, and differentiation of hematopoietic progenitor cells in semisolid culture systems and participates in the functional activation of mature blood cells (Metcalf, 1986; Clark & Kamen, 1987). Recently, recombinant human (hu) GM-CSF was shown to stimulate hematopoiesis in vivo and is thus a useful therapeutic agent in the recovery of bone marrow function following cytotoxic therapy (Groopman et al., 1987) and in a number of conditions of bone marrow failure (Vadhan-Raj et al., 1987).

The primary structure of hu GM-CSF contains 144 amino acids, including a 17-residue secretory leader sequence, resulting in a predicted molecular mass of 14 465 daltons for the mature polypeptide. When analyzed by a number of physicochemical means, natural and recombinant hu GM-CSF is quite heterogeneous. Initial studies using inhibitors of N-linked glycosylation, such as tunicamycin or 2-deoxyglucose, suggested that much of the size and charge heterogeneity characteristic of the CSFs is due to variable degrees of sialic acid containing glycosylation (Tsuneoka et al., 1981). This has now been clearly demonstrated using site-directed mutants of human (Donahue et al., 1986; Kaushansky et al., 1987) and murine (Miyajima et al., 1986) GM-CSF.

The functional role of the carbohydrate modification of hu GM-CSF is not clearly understood. Several recent studies have suggested that forms which lack some or all of the carbohydrate modification normally present on GM-CSF are fully active in a number of biological assays (Sparrow et al., 1985; Kaushansky et al., 1987; Moonen et al., 1987). In fact, most reports suggest that hu GM-CSF which lacks N-linked carbohydrate has a significantly enhanced specific activity in vitro when compared to the native recombinant growth factor (Kaushansky et al., 1987; DeLamarter et al., 1987; Moonen et al., 1987). In addition, although the initial tissue distribution

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