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Interdigitated lipid bilayers of long acyl chain species of cerebroside sulfate. A fatty acid spin label study

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The metastable phase behavior of semi-synthetic species of cerebroside sulfate (CBS), with hydroxy and non-hydroxy fatty acids from 16 to 26 carbons in length, was compared in Li^+ and K^+ using differential scanning calorimetry. The structure of the metastable and various stable phases formed in the presence of these two cations was investigated using a fatty acid spin label, 16-doxylstearate. A number of stable phases with successively higher phase transition temperatures and enthalpies occur in the presence of K^+ (see the preceding paper). Li^+ prevents formation of the most stable phases with the highest transition temperatures and enthalpies for all species of CBS. However, it does not prevent a transition from the metastable phase to the first stable phase of the longer chain C24 and C26 species. Furthermore, it allows C24:0h-CBS to undergo a similar transition, in contrast to a high K^+ concentration, which prevents it. The spin label has anisotropic motion in the metastable gel phase formed by all species of CBS on cooling from the liquid crystalline phase. The spectra resemble those in gel phase phospholipids. The spin label is partially insoluble in the most stable phases formed by all the lipids, including the unsaturated C24:1 species, preventing further elucidation of their structure using this technique. However, the spin label is soluble in the first stable phase formed on cooling by the longer chain C24:0 and C26:0-CBS in Li^+ and K^+ and by C24:0h-CBS in Li^+, and is motionally restricted in this phase. The motional restriction is similar to that observed in the mixed interdigitated bilayers of asymmetric species of phosphatidylcholine and fully interdigitated bilayers formed by symmetric phospholipids. It strongly suggests that the highly asymmetric long chain species of CBS form a mixed interdigitated bilayer in their first stable gel phases while the metastable phase of these and the shorter chain lipids may be partially interdigitated. The metastable phase of C24:1-CBS is more disordered suggesting that it may not be interdigitated at all. Thus the results suggest that (i) the hydroxy fatty acid inhibits but does not prevent formation of a mixed interdigitated bilayer by long chain species of CBS, (ii) an increase in non-hydroxy fatty acid chain length from 24 to 26 carbons promotes it, and (iii) a *cis* double bond probably prevents any form of interdigitation. These results may be relevant to the physiological and pathological roles of these structural modifications of CBS.

Introduction

Highly asymmetric synthetic species of phosphatidylcholine (PC), in which one chain is nearly twice the length of the other, have been shown by X-ray diffraction to form mixed interdigitated bi-

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Abbreviations: CBS, cerebroside sulfate; PC, phosphatidylcholine; DSC, differential scanning calorimetry; ESR, electron spin resonance.

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layers in the gel phase [1–4]. In this kind of bilayer, the short chains from lipid molecules on each side of the bilayer are packed end to end while the longer chain from lipid on both sides spans the bilayer width. Naturally occurring sphingolipids are often highly asymmetric with saturated fatty acid chains, and are in the gel phase at physiological temperatures. X-ray crystallography indicates that the sphingosine base of 18 carbons penetrates into the bilayer to a depth of only 13 or 14 carbons [6]. The fatty acid chain can vary from 14 to 26 carbons in length, and is either saturated or mono-unsaturated [7]. An increase in the content of long chain α-hydroxy and non-hydroxy fatty acids, especially C25 and C26, in membrane sphingolipids occurs in the demyelinating disease adrenoleukodystrophy, caused by an inherited defect in long chain fatty acid metabolism [8–10]. If long chain sphingolipids can form a region of interdigitated bilayer in natural membranes, it might play a physiological and/or pathological role. Therefore, it is important to determine the structural organization of different sphingolipids.

Synthetic species of sphingomyelin [11,12], cerebroside [13–19], and cerebroside sulfate [20–23] undergo metastable phase behavior and transform into stable phases which have high enthalpy phase transitions. The structural organization of these diffrent phases is beginning to be studied by X-ray diffraction and spectroscopic techniques. X-ray scattering results on a series of synthetic species of sphingomyelin suggest that in the liquid crystalline phase, those in which the fatty acid chain is 20 carbons or greater form a partially interdigitated bilayer in which the long acyl chain of lipid on one side of the bilayer packs end to end with the short sphingosine chain of lipid on the other side [24]. Raman spectroscopy results on C24:0-sphingomyelin in its stable gel phase are consistent with formation of a mixed interdigitated bilayer in this phase [25]. At higher temperatures the results suggested that the lipid goes into a less ordered gel phase which is probably partially interdigitated. X-ray diffraction of C24:0 cerebroside suggested that this lipid forms a partially interdigitated bilayer in both the stable gel (at 22° C) and liquid crystalline phases [19].

Fatty acid spin labels are proving to be a useful technique for detection of mixed or fully interdigitated gel phase bilayers and are able to detect the mixed interdigitated bilayer formed by asymmetric species of PC [28–31]. A spin label located on a fatty acid near its terminal methyl, as in 16-doxylstearate, normally has much more motion and/or less order in a non-interdigitated gel phase bilayer than when the spin label is located close to the carboxyl, as in 5-doxylstearate. However, in the mixed or fully interdigitated bilayer, the spin label group of both 16-doxylstearate and 5-doxylstearate is expected to be located in a similar region of the bilayer, relatively close to the apolar-polar interface. Indeed, we have found that the motion of 16-doxylstearate is restricted to a degree similar to that experienced by 5-doxylstearate in these interdigitated bilayers [28,29]. Similar results are found using the fatty acid spin label, its methyl ester, and a phosphatidylglycerol spin label containing 16-doxylstearate. As with any spectroscopic technique, the observation of motional restriction of 16-doxylstearate cannot yet be considered to be 100% diagnostic of the interdigitated phase. However, it strongly suggests it, particularly when observed in a pure lipid system. Motional restriction of this spin label is very unusual, and in fact, to our knowledge, when it occurs only in the gel phase and not in the liquid crystalline phase, it has been observed only in bilayers which are known to be interdigitated [28,29] or are suspected to be [30]. Therefore, this behavior is strong evidence for formation of a mixed or fully interdigitated bilayer. However, the effect of partial interdigitation on the motion of the spin label is not known and may not be much different from a non-interdigitated bilayer.

In an earlier study of the behavior of this spin label in the metastable and stable phases of synthetic species of cerebroside sulfate we found that the spin label has anisotropic motion in the metastable phase which resembles that of non-interdigitated gel phases of symmetric species of phosphatidylcholine [20,21]. However, in the presence of Na^+, the spin label is frozen out of the stable gel phase preventing further investigation of its structure using spin labels. We have found that there is much less tendency for this to occur in the presence of Li^+ and K^+. Thus in the present study we have used 16-doxylstearate to investigate the structure of the phases formed in the presence of these cations by synthetic species of cerebroside sulfate of varying fatty acid chain length, with and without an α-hydroxy group. The results suggest that long chain C24 : 0 and C26 : 0-CBS can form a mixed interdigitated bilayer while shorter chain C16 : 0 and C18 : 0-CBS may form a partially interdigitated bilayer.

Materials and Methods

Synthetic species of cerebroside sulfate (CBS), C16 : 0, C16 : 0h, C18 : 0, C18 : 0h, C24 : 0, C24 : 0h, C24 : 1, and C26 : 0-CBS, were prepared as described in the preceding paper [23]. The sphingosine base is 94% dihydroxy 18 : 1. The lipid was dispersed at a concentration of 0.7–1.0 mg/0.05 ml Hepes buffer (10 mM) at pH 7.4, containing 1 mM EDTA and the desired concentration of KCl, LiCl, or NaCl. The sample was centrifuged in an Eppendorf bench centrifuge and the wet pellet was loaded into an aluminum DSC pan. Calorimetric measurements were made as described in the preceding paper [23]. The lipid was prepared similarly for measurement of the electron spin resonance (ESR) spectrum except that the lipid and spin label, 16-doxylstearic acid (16-S-SL) (purchased from Syva, Palo Alto, CA), were first dissolved together in chloroform/methanol (2 : 1, v/v and a 200 : 1 mole ratio of lipid to spin label. The solvent was evaporated under nitrogen, the lipid dispersed in buffer, and taken up in 50-μl capillary tubes which were then sealed and centrifuged at 2000 rpm. Spectra were measured on a Varian E-104B ESR spectrometer equipped with a Varian temperature control accessory and a DEC LSI-11 based microcomputer system. The maximum hyperfine splitting T_{max}, and order parameter, S, were measured as described earlier [30]. The T_{max} value can be used as a relative measure of the degree of motion or order of the spin label. Changes in the lipid organization which caused exchange broadening of the spectra occurred to a much lower extent at temperatures below 25 °C. In order to avoid these changes, the ESR samples were cooled rapidly by either removing them from the spectrometer to room temperature or by lowering the setting on the temperature controller to 9 °C. Either way, a temperature of 25 °C was achieved in about 1.5 min, similar to the fastest cooling rate obtainable using DSC (20–40 C°/min). However, the cooling rate for the ESR samples was non-linear. The microwave power used was 10 mW.

Results

Comparison of calorimetric behavior in Li^+ and K^+

The phase behavior of these lipids in Li^+ is a

TABLE I

EFFECT OF STRUCTURAL MODIFICATIONS TO CEREBROSIDE SULFATE ON THERMODYNAMIC PARAMETERS IN Li^+

From heating and cooling scans at 10 C°/min. T_m on cooling has been corrected for instrumental hysteresis by addition of 4.5 C° to values observed.

Lipid	Heating		Cooling	
	T_m (°C)	ΔH (kcal/mol)	T_m (°C)	ΔH (kcal/mol)
C16 : 0	50.6	7.2	49.5	7.4
C18 : 0	51.4	6.9	51.5	6.1
C24 : 0	45, 55.6 [a]	(1 1) [b]	43, 57.1	4.9, 6.5 (11.6) [b]
C26 : 0	59.9	15.7	54.9, 56.7, 60.5	(16.4) [b]
C24 : 1	37.2	4.7	36.2	4.4
C16 : 0h	57.4	6.4	57.3	6.6
C18 : 0h	60.2	6.5	58.8	6.7
C24 : 0h	49.5, 62.3	3.7, 6.2 (9.9) [b]	40.2, 61.5	2.6, 7.2 (9.8) [b]

[a] Major peak is underlined.

[b] Enthalpy value in parentheses is the total enthalpy of all peaks.

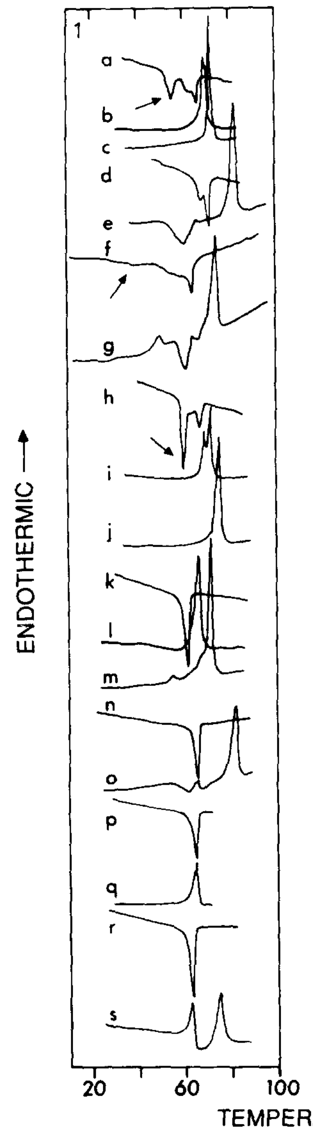

Fig. 1. (Left panel) DSC heating and cooling thermograms of synthetic species of CBS in 2 M K^+ except for (g and h) which are in 0.5 M K^+. Cooling scans have been shifted to the right (higher temperatures) by 4.5 C° to correct for instrumental hysteresis at the heating and cooling rate used, 10 C°/min. (a–c) C24:0-CBS; (d–g) C24:0h-CBS; (h–j) C26:0-CBS; (k–m) C18:0-CBS; (n, o) C18:0h-CBS; (p, q) C16:0-CBS; (r, s) C16:0h-CBS. (a, d, f, h, k, n, p, r) are cooling scans. (b, e, g, i, l, o, q) are the heating scans normally obtained after rapid cooling from the liquid crystalline phase. (s) is the first heating scan obtained after preparation of the sample. (c, j, m) are the heating scans after conversion to the most stable states as described in the preceding paper [23]. A heating scan of C16:0-CBS in its most stable state is not shown. Arrows indicate peak 3′ of the long chain species on cooling.

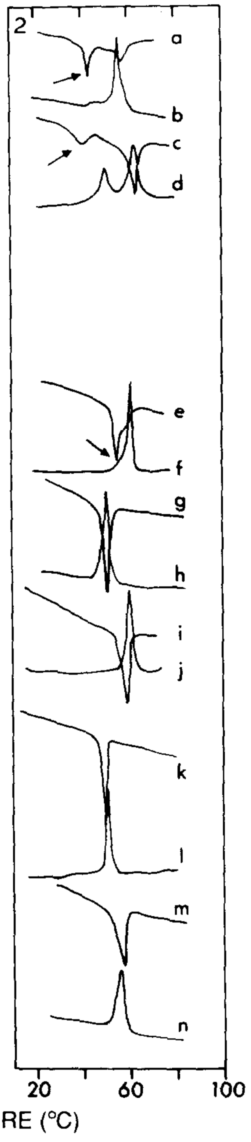

Fig. 2. (Right panel) Summary of DSC heating and cooling thermograms of synthetic species of CBS in 2 M Li^+. Cooling scans have been shifted as in Fig. 1. (a, b) C24:0-CBS; (c, d) C24:0h-CBS; (e, f) C26:0-CBS; (g, h) C18:0-CBS; (i, j) C18:0h-CBS; (k, l) C16:0-CBS; (m, n) C16:0h-CBS. (a, c, e, g, i, k, m) are cooling scans. (b, d, f, h, j, l, n) are heating scans. Arrows indicate peak 3′ of the long chain forms on cooling. The two upper temperature peaks 1′ and 2′ on cooling are not as well resolved as in 2 M K^+.

little different from that in K^+ reported in the preceding and earlier papers [21,23]. Since the spin label is most soluble in the lipids in the presence of Li^+, it is necessary to present the calorimetric behavior in Li^+ and compare the spin label results in the presence of the two cations. The calorimetric behavior of a number of different species of CBS in the presence of 2 M K^+ and Li^+ are summarized and compared on heating and cooling at 10 C°/min in Figs. 1 and 2. The cooling curves have been shifted to higher temperatures by 4.5 C° to correct for the instrumental hysteresis at this heating and cooling rate. Thus the phase transition temperatures on heating and cooling can be directly compared from the figures. A significant difference between the behavior in Li^+ and K^+ is that in Li^+ there is much less hysteresis between heating and cooling scans. With the exception of C26:0-CBS, the calorimetric behavior seen on heating in Li^+ is almost completely reversible on cooling even at this fast heating and cooling rate.

With the exception of C24:0h, the calorimetric behavior on cooling is similar in the presence of either cation. The first two upper temperature transitions seen on cooling for the longer chain lipids in K^+ (Fig. 1 a, d, h) are broad and unresolved for these lipids in Li^+, but clearly still occur (Fig. 2 a, c, e). They become better resolved at slower cooling rates. The enthalpies of the two upper temperature transitions for the longer chain species in Li^+ (Table I) are similar or a little less than those of the liquid crystalline to metastable phase transitions found in K^+ (see the preceding paper). The observation that C24:0h-CBS in Li^+ goes through a third lower temperature phase transition on cooling (peak 3′ indicated by an arrow in Fig. 2 c) like C24:0 and C26:0-CBS (Fig. 1 a, h, Fig. 2 a, e), in contrast to its behavior in K^+ (Fig. 1d), is particularly noteworthy. Thus in Li^+ it behaves similarly to the C24:0 and C26:0 species but in K^+ it behaves similarly to the shorter chain species. Its behavior in Na^+ is similar to that in Li^+ (not shown).

The behavior on heating is much more dependent on the type of cation. The enthalpies in Li^+ on heating (Table I) are less than those observed in K^+ especially for the shorter chain lengths (see the preceding paper). No exothermic transitions are seen on heating in Li^+ and the lipids cannot be converted into the most stable phases found for K^+, which undergo higher temperature, higher enthalpy phase transitions. Another particularly noteworthy difference from K^+ is the observation of two endothermic transitions on heating for C24:0h in Li^+ (Fig. 2 d). This also occurs in Na^+ (not shown). A small second endothermic transition is also observed at a lower temperature than the main transition for C24:0-CBS in Li^+ (Fig. 2 b). These two peaks differ from the multiple peaks seen on heating for these lipids in K^+, in that those in Li^+ are seen even after fast cooling and the ratio of the areas of the two peaks is independent of heating or cooling rate. They seem to be the reverse of the transitions which occur on cooling. Only one endothermic transition is observed for C26:0-CBS in Li^+ on heating (Fig. 2 f). Thus the phase transitions for this lipid are not reversible on heating and cooling.

In 0.5 M K^+ the behavior of C24:0h-CBS is intermediate between that in Li^+ and that in 2 M K^+. As shown in Fig. 1 g, on heating the sample goes through an endothermic transition at a similar temperature as in Li^+ (compare Figs. 1 g and 2 d). This is then followed by an exothermic transition at about 60 °C and a high temperature endothermic transition as in 2 M K^+ (compare Fig. 1 g and e). On cooling there is one sharp exothermic transition followed by two broad ones at lower temperatures (Fig. 1 f). The third one is very broad and indistinct (indicated by an arrow) but probably corresponds to transition 3′ for the other long chain lipids on cooling. No evidence of even a broad third transition is seen for this lipid in 2 M K^+.

TABLE II

EFFECT OF TYPE AND CONCENTRATION OF CATION ON TEMPERATURE OF PHASE TRANSITIONS ON HEATING FOR C24:0h-CBS

	T_1 [a]	T_2 [a]
2 M Na^+	50.7	67.2
0.5 M Na^+	49.4	61.8
2 M K^+	–	81.5
0.5 M K^+	49.5	73.6
2 M Li^+	49.4	61.7

[a] Temperatures of peaks 1 and 2 from heating scans at 10 C°/min.

The phase transition temperatures of CBS are in general very dependent on the type and concentration of cation used [21]. However, as shown in Table II, the temperature of the first endothermic transition on heating for C24:0h-CBS, when observed, is nearly independent of the type and concentration of cation, although that of the second is dependent in the usual way. This suggests that the temperature of the first transition is not affected by the electrostatic repulsion of the head groups while that of the other transition is.

Behavior of 16-doxylstearate in metastable and stable phases

The ESR spectrum of 16-doxylstearate was measured at different temperatures in these various species of CBS in order to investigate the structure of the different phases formed and the nature of the phase transitions observed. Spectra were measured in the gel phases of these lipids by cooling rapidly from the liquid crystalline phase down to room temperature or lower. This allows the spin label to remain soluble in the lipid in the presence of these cations, in contrast to its behavior in Na^+ reported earlier [20,21] where the spin label is frozen out of the lipid. At temperatures in the range of the lowest temperature transition seen on cooling or the exothermic transitions seen for some lipids on heating, some freezing out tended to occur in K^+ and Li^+ also. This tendency is greater in K^+ than Li^+ but much less than that in Na^+ reported earlier. Thus avoidance of slow cooling or slow heating, or of measurement of the spectra at temperatures in the range of these transitions, prevents freezing out of the probe. It presumably also prevents the structural changes which cause insolubility of the probe. These are probably the same structural changes which produce the

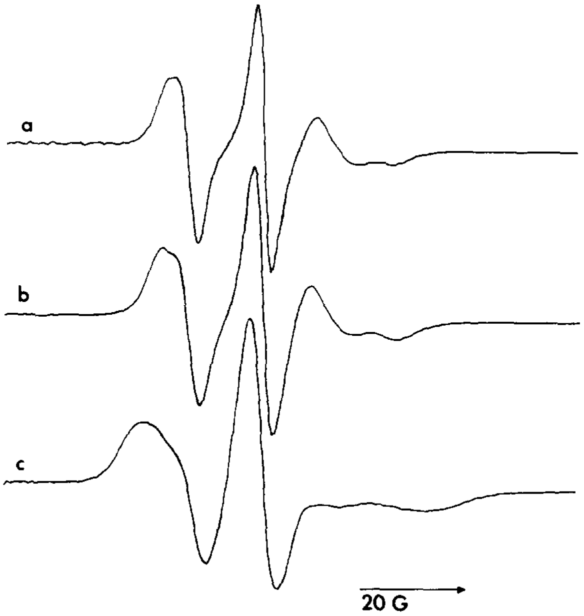

Fig. 3. ESR spectra of 16-doxylstearate in (a) C24 : 0-CBS in 2 M Li^+ at 50 ° C on cooling from the liquid crystalline phase; (b) C18 : 0-CBS in 2 M K^+ at 52 ° C on cooling from the liquid crystalline phase; and (c) C18 : 0-CBS in 2 M K^+ at 9 ° C after rapid cooling from the liquid crystalline phase.

more stable phases B_2 and B_3 for C24 : 0 and C26 : 0-CBS (see the preceding paper), phases B_1 for C18 : 0, C16 : 0, and C24 : 1-CBS, and phase B for all the hydroxy fatty acid species of CBS. Attempts to convert the samples to these phases by the same procedures used for calorimetry cause freezing out of the spin label.

A comparison of the spectra in the gel phases of C24 : 0h and C24 : 0-CBS with those of the shorter chain lipids is particularly instructive since C24 : 0-CBS goes through the third transition on cooling, unlike the shorter chain species, while C24 : 0h does so in Li^+ but not in K^+. We showed earlier that at temperatures in between the first two transitions and the third lower temperature transition for cooling scans of C24 : 0-CBS, such as those shown in Fig. 1 a and Fig. 2 a, the spin label has anisotropic motion, similar to gel phase phospholipids [21]. A typical spectrum in C24 : 0-CBS in Li^+ at 50 ° C is shown in Fig. 3a ($S = 0.344$). A similar spectrum is obtained for C24 : 0h-CBS in Li^+ at this temperature. For C26 : 0-CBS these transitions are too close together to measure the spectrum at an intermediate temperature. A similar spectrum, albeit with somewhat greater order parameter, is obtained in this temperature range for the shorter chain length species as shown for C18 : 0-CBS in K^+ at 52 ° C in Fig. 3 b ($S = 0.411$). Rapid cooling of these shorter chain lipids to lower temperatures gives a spectrum such as that in Fig. 3 c for C18 : 0-CBS in K^+ at 9 ° C. The spin label still has anisotropic motion although the motion is reduced and/or the order parameter is greater than that at higher temperatures. The T_{max} value of the spectrum is 25.0 G for C18 : 0-CBS at 9 ° C. This is similar to the non-interdigitated gel phase of symmetric phospholipids [29].

The spectrum in C24 : 0h-CBS in K^+ at 9 ° C is similar with a T_{max} value of 24.3 G as shown in Fig. 4 a. However, that in C24 : 0-CBS in K^+ (Fig. 4b) is characteristic of more restricted motion with a significantly larger T_{max} value of 28.3 G (Table III). In contrast, in Li^+ where both lipids go through the third transition on cooling, the spectra in both C24 : 0 and C24 : 0h-CBS are similar to that in Fig. 4b with T_{max} values of 28.6 and 28.5 G, respectively (Table III). A similar spectrum was also obtained in C26 : 0-CBS in both cations. The shorter chain length C18 and C16 species all have spectra similar to Figs. 3 c and 4 a with T_{max}

TABLE III

SPECTRAL PARAMETERS OF 16-DOXYLSTEARATE IN DIFFERENT FORMS OF CEREBROSIDE SULFATE MEASURED AT 9 ° C AND 30 ° C

	T_{max} (G)			
	2 M K^+		2 M Li^+	
	9 ° C	30 ° C	9 ° C	30 ° C
C26 : 0	28.6	≈ 26 [a]	28.7	≈ 26 [a]
C24 : 0	28.3	≈ 26 [a]	28.6	≈ 26 [a]
C18 : 0	25.0	21.1	26.6	22.2
C16 : 0	n.d.	20.4	25.2	21.3
C24 : 0h	24.3	21.1	28.5	≈ 26 [a]
C18 : 0h	26.2	22.4	26.2	21.7
C16 : 0h	n.d. [b]	20.8	n.d. [b]	20.8
C24 : 1	21.3	19.9		

[a] Two components were observed at this temperature, one-more immobilized ($T_{max} \cong 26$ G), the other more mobile than in the other lipids. Thus, T_{max} cannot be measured accurately.

[b] n.d., not determined.

values of 25–26.6 G (Table III). In the unsaturated C24 : 1 species the T_{max} value is even less, 21.3 G indicating a lower degree of order.

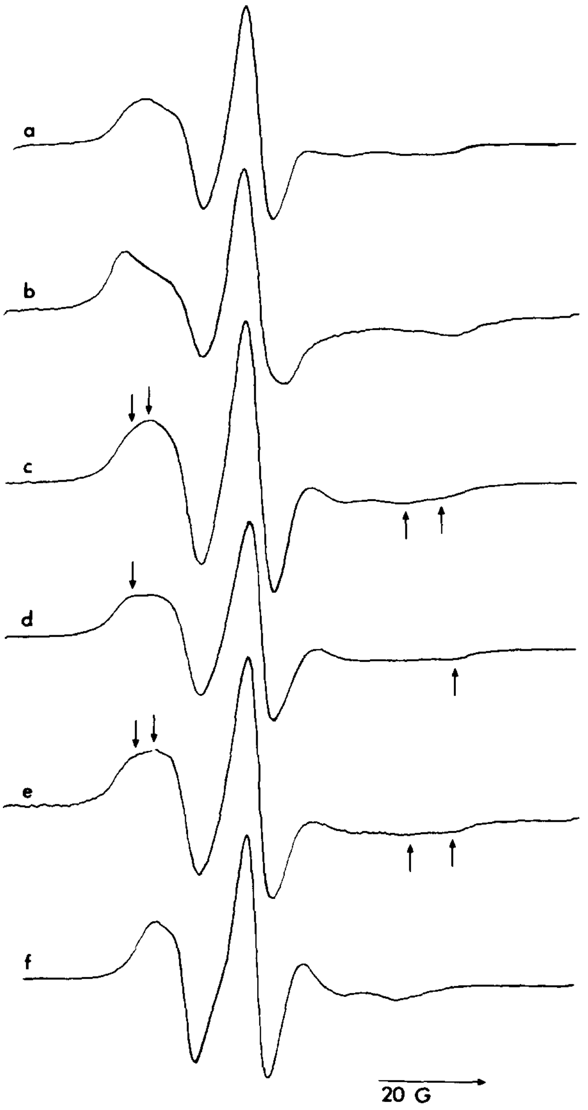

Fig. 4. ESR spectra of 16-doxylstearate in (a) C24 : 0h-CBS in 2 M K^+ at 9°C; (b) C24 : 0-CBS in 2 M K^+ at 9°C; (c) C24 : 0h-CBS in 2 M Li^+ at 19°C at zero time after cooling from the liquid crystalline phase; (d) C24 : 0h-CBS in 2 M Li^+ at 19°C after 50 min at this temperature; (e) C24 : 0-CBS in 2 M K^+ at 29°C on heating from a low temperature, (f) C24 : 0h-CBS in 2 M K^+ at 29°C on heating from a low temperature. (c) and (d) are plotted out at the same instrument sensitivity so that the spectral intensities can be directly compared. Arrows indicate the presence of two components, one characteristic of more restricted motion than the other.

These results indicate that during the third transition on cooling, when the long chain species go into the more stable B_1 phase, the motion of the spin label becomes more restricted than in the metastable phase adopted by the shorter chain species. Furthermore, on cooling from the liquid crystalline phase down to a low temperature, C24 : 0h-CBS in K^+ remains in the same kind of phase as the metastable A phase of the shorter chain species, but in Li^+ it goes into the same kind as the stable B_1 phase of the longer chain species, as suggested by the calorimetric behavior.

This third transition of C24 : 0h-CBS in Li^+ is less cooperative and slower than for the C24 : 0 and C26 : 0 species, as is evident from the broadness of the peak and the lower temperature at which it occurs (Fig. 2 c). If the sample is cooled rapidly to a low temperature it takes time for the spin label to become motionally restricted. When C24 : 0h-CBS in Li^+ is cooled rapidly to 19°C, the spectrum initially has two components, the major one with anisotropic motion, and another with less motion, as indicated by the arrows in Fig. 4 c. After incubation at this temperature for 50 min, the proportion of the component with less motion is considerably increased (Fig. 4d).

On heating of the longer chain samples, the spectra still have detectable components characteristic of restricted motion up to 35–45°C. However, they also acquire increasing amounts of a component characteristic of greater motion. Such a two component spectrum is shown for C24 : 0-CBS in K^+ at 29°C in Fig. 4 e and is compared to the single component spectrum with anisotropic motion of the metastable phase of C24 : 0h-CBS in K^+ in Fig. 4 f. Since exchange broadening and spectral distortion also tend to occur at these temperatures, it is difficult to investigate the changes which occur in the temperature range of 35–50°C and the nature of this more mobile component. This exchange broadening occurs for all species of CBS in K^+, including C24 : 1. When it occurs for any species of CBS in K^+, it is maintained up until the highest temperature phase transition, where the lipid goes into the liquid crystalline phase.

Exchange broadening also tends to occur to some extent for the longer chain lipids in Li^+. The spectrum of the spin label in C24 : 0h-CBS in Li^+

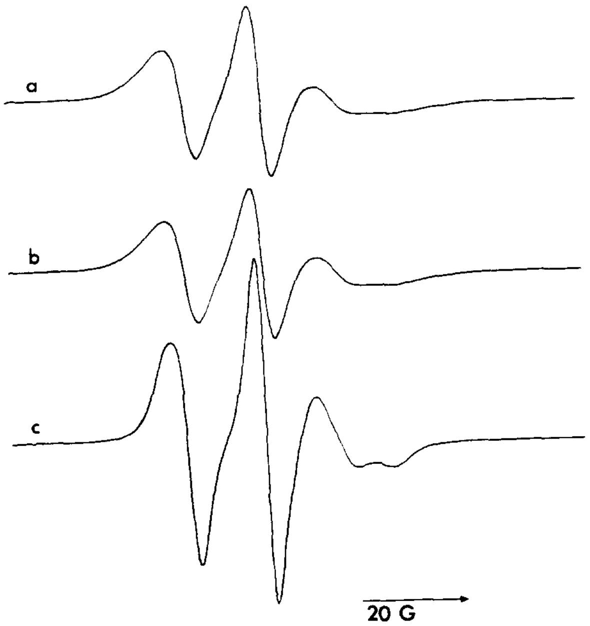

Fig. 5. ESR spectra of 16-doxylstearate in C24 : 0h-CBS in 2 M Li^+ at (a) 39 ° C at zero time after heating from a low temperature; (b) at 39 ° C after 1.5 h at this temperature; (c) at 49 ° C on heating from 39 ° C after 1.5 h incubation at that temperature. All are plotted at the same instrument sensitivity so that their spectral intensities can be directly compared.

on heating up to 39 ° C is shown in Fig. 5 a and after 1.5 h at this temperature in Fig. 5 b. The broad, rather featureless character of the spectrum is caused by some exchange broadening and/or other distortion of the spin label motion as a result of some freezing out of the probe. This conclusion is supported by the reduction in height which occurs after incubation at this temperature (Fig. 5 b). However, in contrast to K^+, the exchange broadening and spectral distortion disappear for C24 : 0 and C24 : 0h-CBS in Li^+ when they are heated up to temperatures around 50 ° C, i.e. above the first endothermic transition of these lipids seen in Fig. 2 b and d. The spectrum then becomes sharper and is again characteristic of anisotropic motion, as shown for C24 : 0h-CBS in Li^+ at 49 ° C in Fig. 5 c. Similar results are found for C24 : 0-CBS in Li^+. The spectra resemble those seen on cooling for these lipids (Fig. 3 a). These results suggest that during the first lower temperature transition on heating in Fig. 2 b and d, C24 : 0 and C24 : 0h-CBS in Li^+ go from a stable B_1 phase in which the spin label is motionally restricted, back into one in which it has anisotropic motion, i.e. similar to the metastable phase A. Thus the phase behavior seen on cooling is reversible. No such transition is seen for the longer chain lipids in K^+ or for C26 : 0-CBS in Li^+, either by calorimetry or from the fatty acid spin label spectra. Thus the transition from A to B_1 on cooling may not be reversible on heating in these cases.

Dependence of behavior on cation concentration

The rate of the third transition (peak 3′) of the long chain species on cooling depends on cation concentration. However, it increases at lower cation concentrations in contrast to some other structural changes of these lipids whose rate decreases at lower cation concentrations, such as the exothermic process on heating for the hydroxy fatty acid species [23]. C18 : 0-CBS in Na^+ undergoes a second exothermic transition on cooling during which the spin label is frozen out [21]. The rate of this transition also decreases with decrease in Na^+ concentration. The increased rate of transition 3′ of the longer chain species at lower cation concentrations is suggested by results shown in Fig. 6 and Table IV, which show that the temperature difference between transitions 1′ and 3′ (see Fig. 6) on cooling decreases with decrease in cation concentration for C26 : 0 and C24 : 0-CBS in

TABLE IV

DEPENDENCE OF TEMPERATURE DIFFERENCE BETWEEN PEAKS 1′ and 3′ ON COOLING OF LONG CHAIN CEREBROSIDE SULFATE ON TYPE AND CONCENTRATION OF CATION

	Cation	ΔT [a] (C°)
C24 : 0-CBS	2 M Na^+	13.8
	0.5 M Na^+	11.2
	0.01 M Na^+	10
	2 M K^+	10.8
	0.5 M K^+	8.6
	2 M Li^+	14
C26 : 0-CBS	2 M Na^+	6.7
	0.1 M Na^+	4.4
	2 M K^+	6.8
	0.1 M K^+	3.8
	2 M Li^+	4.5

[a] $\Delta T = T_1' - T_3'$ from cooling scans at 10 C°/min.

K^+ and Na^+. This is a result of an increase in the temperature of transition 3′ and not a decrease in the temperature of transition 1′, as can be seen by comparing the temperatures of these transitions on cooling to that on heating (Fig. 6). In some cases, as for C26 : 0-CBS in 0.1 M Na^+ (Fig. 6 h) and 2 M Li^+ (Fig. 2 e), transition 3′ overlaps transitions 1′ and 2′. The temperature difference for C26 : 0-CBS is less than that for C24 : 0-CBS under all conditions (Table IV). These results suggest that increased electrostatic repulsion and increased fatty acid chain length both promote transition 3′ and stabilize phase B_1 of the long chain species in which the spin label is motionally restricted.

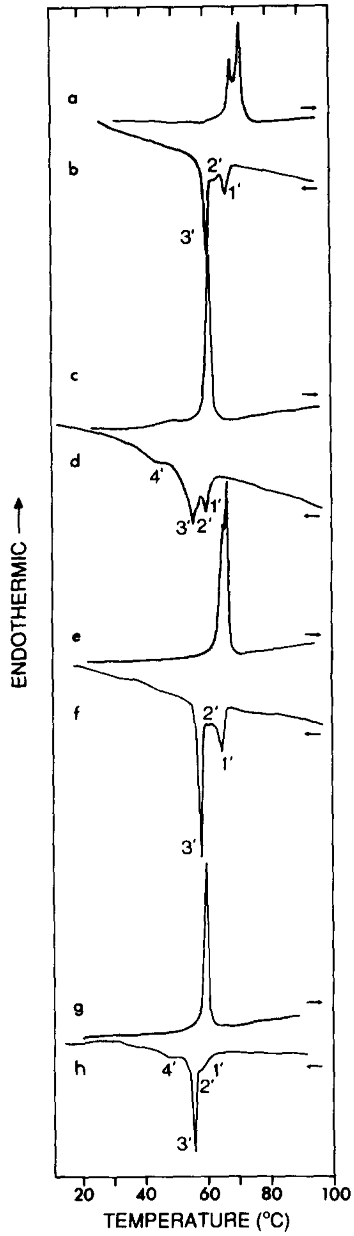

Fig. 6. DSC heating and cooling thermograms of C26 : 0-CBS in (a, b) 2 M K^+; (c, d) 0.1 M K^+; (e, f) 2 M Na^+; (g, h) 0.1 M Na^+. (b, d, f, h) are cooling scans; (a, c, e, g) are heating scans. Cooling scans are shifted to the right by 4.5 C° as in Figs. 1 and 2. Peaks 1′, 2′, 3′, and 4′ on cooling are indicated. Arrows indicate the direction of the temperature change. Peak 2′ sometimes overlaps with 3′ and in (h) both peaks 1′ and 2′ overlap with 3′.

These results also indicate that the process occurring during transition 3′ of the long chain species on cooling is different from those which occur during the exothermic transition on heating for the hydroxy fatty acid species, and during the second exothermic transition on cooling for C18 : 0-CBS in Na^+ [21] (and in K^+ at slow cooling rates). These later two processes, in which the fatty acid spin label is frozen out, are favored by shielding of the charge and reduced electrostatic repulsion. However, the spin label is also frozen out of the long chain lipids if they are cooled slowly or incubated in the range of transition 3′. Results shown in Fig. 6 d, h for C26 : 0-CBS in 0.1 M cation suggest that the process which causes freezing out of the spin label from the long chain lipids on slow cooling is different from that which occurs during transition 3′ causing motional restriction of the spin label. A fourth low enthalpy (about 2 kcal/mol) exothermic transition is observed on cooling in these cases (peak 4′). A similar transition occurs for C24 : 0-CBS in low K^+ [21]. This may be the transition leading to one of the more ordered phases, B_2 and B_3, in which the spin label is insoluble for the long chain species. It resembles that which occurs on cooling for C18 : 0-CBS in Na^+ [21] and may be similar to that which occurs on heating for the hydroxy fatty acid species in K^+. However, the latter two are higher enthalpy transitions, about 5 kcal/mol for C18 : 0 and C18 : 0h-CBS, and 10 kcal/mol for C24 : 0h-CBS. At high cation concentrations, transition 4′ may occur either simultaneously with transition 3′, or more slowly and uncooperatively, so that it is not observed calorimetrically. The enthalpy is not large enough to determine if it might occur simultaneously with transition 3′ in 2

M K^+ from enthalpy measurements. However, the fact that on rapid cooling, the spin label is motionally restricted but not frozen out, suggests that transition 3′ takes place but not 4′. Thus transition 4′ may be slower than 3′.

Discussion

The motional restriction of 16-S-SL which occurs in the stable gel phase B_1 of long chain C26 : 0 and C24 : 0-CBS in both K^+ and Li^+ and in C24 : 0h-CBS in Li^+, but not in the shorter chain C18 and C16 species, is similar to that which occurs in the mixed interdigitated bilayer of highly asymmetric species of PC (18 : 10PC, 18 : 12PC, and 10 : 18PC) and in fully interdigitated bilayers of symmetric species of PC and phosphatidylglycerol [28,29]. This strongly suggests that the long chain, highly asymmetric species of CBS also form a mixed interdigitated bilayer, at least at temperatures below 35–45 ° C. If the sphingosine chain penetrates to a depth of only 13 or 14 carbons into the bilayer as suggested by previous studies [6], a C24 acyl chain might be just long enough to allow the lipid to form a mixed interdigitated bilayer. The C26 fatty acid would probably stabilize such a bilayer better.

The smaller value of T_{max} in the metastable gel phase A of the shorter chain lipids in K^+ and Li^+ and of C24 : 0h-CBS in K^+ suggests that these lipids may be partially interdigitated. Although the T_{max} values in the metastable phase are similar to that of a non-interdigitated gel phase formed by a symmetric lipid [29], a non-interdigitated bilayer formed by these asymmetric lipids would be expected to be much more disordered and have a lower T_{max} value than is obtained. The somewhat larger value of T_{max} in the asymmetric C18-CBS compared to the more symmetric C16-CBS is consistent with a greater degree of partial interdigitation in the former. The much smaller value of T_{max} for C24 : 1-CBS indicates that it forms a more disordered gel phase bilayer and therefore may not be interdigitated at all. We reported earlier [29] that the spectrum of 16-S-SL in 18 : 14PC at 4 ° C has two components, one with a T_{max} value of 26.6 G and the other 21.9 G, suggesting that it has two populations, one of which may be partially interdigitated, and another which may not be interdigitated but rather is disordered. X-ray diffraction shows that this lipid does not form a mixed interdigitated bilayer in contrast to the more asymmetric 18 : 10PC and 18 : 12PC [1]. Although it has not yet been established that 18 : 14PC is partially interdigitated, X-ray diffraction results are consistent with this type of organization [26]. Therefore, a value of 25–26.6 G at low temperature may be characteristic of partial interdigitation while a lower value of 21–22 G might be expected for an asymmetric lipid which is not at all interdigitated. However, this must be confirmed by further studies of partially interdigitated lipids, when such are identified.

The changes in lipid organization on slow cooling or slow heating which occur during transformation to the most stable phases in which the probe is insoluble, i.e. the B_1 phase of the shorter chain lipids and the C24 : 1 species, the B_2 and B_3 phases of the non-hydroxy fatty acid species, and the B phase of the hydroxy fatty acid species, may involve varying degrees of dehydration and/or more ordered forms of molecular packing. Such structural changes lead to the subgel phases of symmetric and asymmetric species of PC [32,33] in which 16-doxylstearate is insoluble [29], and to a crystalline form of C24 : 0 cerebroside [19]. It is noteworthy that under some conditions the asymmetric 10 : 18PC also undergoes an exothermic transition on heating from a mixed interdigitated phase to a more stable phase with a higher transition temperature and enthalpy, in which the spin label is insoluble [29]. The fact that, like the saturated species of CBS, C24 : 1-CBS also undergoes a transformation on heating to a phase with a higher enthalpy phase transition, in which the probe is insoluble, suggests that the changes which lead to insolubility of the probe in CBS involve mainly increased interactions between the head groups rather than more ordered packing of the chains, thus supporting the involvement of dehydration. This is also supported by the fact that this transformation occurs more readily at higher cation concentrations, which would decrease electrostatic repulsion.

However, the reason for the dependence of this behavior on the type of cation is not entirely understood. The probe is frozen out of the non-hydroxy species of CBS most readily in Na^+ [21]

suggesting that in Na$^+$ the lipids go directly into their most stable phases on cooling, even though the phase transition temperatures (which are higher for the more stable phases) are all less in Na$^+$ than in K$^+$. Thus the rate of formation of these stable phases, in which the probe is insoluble, by the non-hydroxy fatty acid species decreases with the type of cation in the order Na$^+ >$ K$^+ >$ Li$^+$. However, the stable B phase of the hydroxy fatty acid lipids does not form in Na$^+$, only in K$^+$. The size of these cations and their binding affinity to CBS increases in the order Li$^+ <$ Na$^+ <$ K$^+$, while their degree of hydration decreases in the order Li$^+ >$ Na$^+ >$ K$^+$ [34]. The inhibition of formation of the most stable phases by the more hydrated Li$^+$ is consistent with the idea that these phases are dehydrated. However, the differences in dependence of the rate of formation of these phases on Na$^+$ and K$^+$ for the hydroxy and non-hydroxy fatty acid species is puzzling.

Based on the motion of the spin label in the different phases of CBS we suggest the following model, depicted in Fig. 7, for the changes in structure which occur during the phase transitions. The long chain lipids freeze from the liquid crystalline phase C into a partially interdigitated metastable gel phase A in the two stage transition detected calorimetrically as peaks 1′ and 2′. The first stage of this transition may be reformation of *trans* chains while the second stage may be partial interdigitation of the *trans* chains. During the third transition detected calorimetrically (peak 3′) for C26 : 0 and C24 : 0-CBS in K$^+$ or Li$^+$ and C24 : 0h-CBS in Li$^+$ but not K$^+$, the partially interdigitated bilayer transforms into the mixed interdigitated bilayer B$_1$, with additional release of heat because of the greater van der Waals interactions between the long chains when they span the bilayer. This transition is reversible on heating for the C24 lipids in the presence of Li$^+$ but apparently not for C26 : 0-CBS. Thus the first endothermic transition seen on heating for the C24 lipids in Li$^+$ is the transition of the mixed interdigitated bilayer back to the partially interdigitated bilayer while the second transition is that of the partially interdigitated phase to the liquid crystalline phase. The single transition observed on heating for C26 : 0-CBS in Li$^+$ is that of the mixed interdigitated phase B$_1$ to the liquid crystalline phase. This accounts for the greater enthalpy of the latter compared to the C24 species in Li$^+$, and for the lower enthalpy of the main transition of the C24 species in Li$^+$ compared to K$^+$. If these samples in K$^+$ are held in the range of the transition temperature of A to B$_1$ or B$_1$ to the liquid crystalline phase for some time they transform to the more stable, possibly less hydrated and/or better ordered B$_2$ and B$_3$ phases.

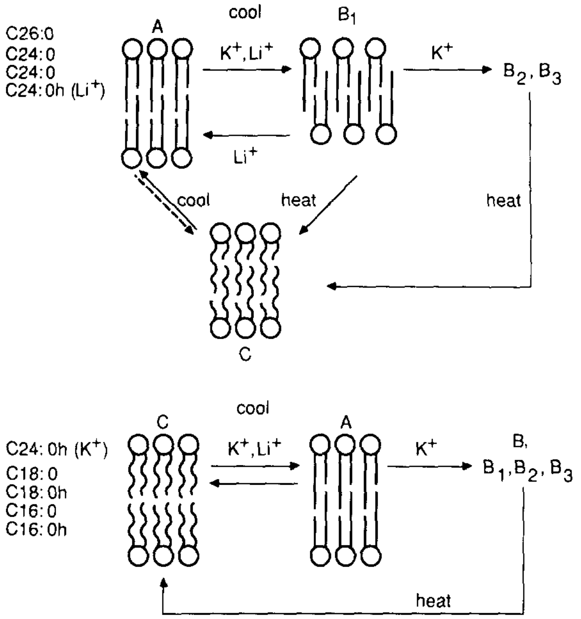

Fig. 7. Diagrammatic representation of the phase behavior suggested to occur for the different species of CBS in K$^+$ and Li$^+$. State C is the liquid crystalline phase. State A is the first metastable phase. The various B states are more stable states. States B$_2$ and B$_3$ for the long chain species, state B for the hydroxy fatty acid species, and states B$_1$, B$_2$, and B$_3$ for the shorter chain species may be less hydrated or more ordered forms of the partially interdigitated and mixed interdigitated phases shown.

All of the shorter chain hydroxy and non-hydroxy fatty acid species in K$^+$ and Li$^+$ and C24 : 0h-CBS in K$^+$ freeze into the partially interdigitated metastable phase A on fast cooling and remain in this phase down to low temperatures. This occurs in a two stage transition for C24 : 0h-CBS but in a single stage transition for the shorter chain species. This transition is reversible for all of these species in Li$^+$. In K$^+$, on reheating or during slow cooling, the partially interdigitated phase transforms into more stable, better ordered and/or less hydrated B phases. The fact that the shorter chain hydroxy fatty acid species behave similarly to the long chain C24 : 0h-CBS in K^+ suggests that these more stable phases are partially interdigitated for C24 : 0h-CBS as well as the shorter chain hydroxy and non-hydroxy fatty acid species. This is supported by the results on C24 : 0h-CBS in 0.5 M K^+ on heating, where an endothermic transition at the temperature of the transition from the mixed interdigitated to the partially interdigitated bilayer is followed by an exothermic transition. This suggests that it is the partially interdigitated bilayer which goes through the exothermic transition to the B phase at higher K^+ concentrations also.

According to this model, the high enthalpy exothermic transition seen on heating for C24 : 0h-CBS and the other hydroxy fatty acid species is not the same process as that which gives peak 3′ on cooling for C24 : 0 and C26 : 0-CBS, i.e. it is not a transition from a partially interdigitated phase to a mixed interdigitated phase, even though the enthalpies are similar [21,23]. The dissociation of two exothermic peaks in the temperature range of peak 3′ on cooling for the long chain species at low cation concentrations supports the conclusion that there are two processes going on in this temperature range. One (transition 3′) is formation of a mixed interdigitated bilayer and the other (transition 4′) is the process which leads to a bilayer structure in which the spin label is insoluble. This latter process can occur for the nonhydroxy fatty acid species on cooling or heating but only on heating for the hydroxy fatty acid species. The former process, the transition to the mixed interdigitated bilayer, occurs only for the long chain non-hydroxy fatty acid species and not for the hydroxy fatty acid species in K^+. The enthalpy change involved in transition 4′ is less for the mixed interdigitated bilayer than similar transitions of the partially interdigitated bilayer. This may be because the former is already more ordered or at a lower energy level than the latter.

The increased rate of transition 3′ at lower cation concentrations is consistent with formation of a mixed interdigitated bilayer since the greater separation of the head groups in this kind of bilayer should be favored by increased electrostatic repulsion. Conversely, decreased electrostatic repulsion and/or increased intermolecular hydrogen bonding should favor formation of a partially interdigitated rather than a mixed interdigitated bilayer. Thus C24 : 0h-CBS forms a partially interdigitated bilayer in K^+ which shields the negative charge better than Li^+. In Li^+, where there is more electrostatic repulsion, it forms a mixed interdigitated bilayer at low temperatures but then goes into a partially interdigitated bilayer at higher temperatures, allowing hydrogen bonding interactions to reoccur. This may explain why the neutral and strongly hydrogen bonded C24 : 0 cerebroside apparently forms only a partially interdigitated and not a mixed interdigitated bilayer in its stable phase [19], while the less interactive (as inferred from its lower transition temperature [27]) C24 : 0-sphingomyelin forms a mixed interdigitated bilayer in the gel phase [25].

The important points in this model which are supported most strongly by the present data are the occurrence of a mixed interdigitated bilayer for the long chain species at low temperatures and the transformation back to a gel phase bilayer which is not of the mixed interdigitated type on heating for C24 : 0-CBS and C24 : 0h-CBS in Li^+. The model of partial interdigitation for the metastable phase is less well supported but is reasonable based on results on other spingolipids and structural considerations. As indicated above, other important points for which there is little direct evidence, because of the problems with probe solubility, are whether the mixed interdigitated structure is maintained up to the transition to the liquid crystalline phase for the long chain lipids in K^+ and for C26 : 0-CBS in Li^+, whether C24 : 0h-CBS in K^+ transforms to a mixed interdigitated bilayer during the exothermic transition on heating, and the structure of the most stable phases, B for the hydroxy fatty acid species in K^+, and B_2 and B_3 for the non-hydroxy fatty acid species. However, we consider the model as presented to be most consistent with all the data. X-ray diffraction is of course necessary to confirm this model and is in progress. Preliminary results on the long chain species are consistent with the important features of the model (Stinson, R. and Boggs, J.M., unpublished data).

The results presented in this and the preceding paper [23] show that fatty acid hydroxylation, chain elongation, and unsaturation are very important structural modifications for this lipid. Hydroxylation of the fatty acid inhibits the organizational changes which give rise to the most stable and ordered phase, in which the spin label is insoluble, for all chain length species. It also affects formation of the mixed interdigitated phase for the C24 form. It prevents formation of this phase at high K^+ concentrations, where shielding of the negatively charged sulfate occurs, but only inhibits it in Li^+ and Na^+, where increased electrostatic repulsion occurs. Furthermore, it promotes deinterdigitation on heating. Participation of the fatty acid hydroxyl groups in intermolecular hydrogen bonding with the free hydroxyl group on the sphingosine base and with the amide linkage of adjacent molecules may stabilize the partially interdigitated bilayer for C24:0h-CBS. These effects of the hydroxy fatty acid are very cation dependent. Thus the phase behavior of the hydroxy fatty acid species is even more sensitive to the type and concentration of cation present than the non-hydroxy fatty acid species. An increase in chain length to 26 carbons stabilizes the mixed interdigitated phase, while unsaturation of the C24 chain prevents formation of this phase and probably also of the partially interdigitated bilayer. These results may be related to the physiological and pathological effects of chain hydroxylation, unsaturation, and elongation in certain conditions.

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References

1 Hui, S.W., Mason, J.T. and Huang, C. (1984) Biochemistry 23, 5570–5577.
2 McIntosh, T.J., Simon, S.A., Ellington, Jr., J.C. and Porter, N.A. (1984) Biochemistry 23, 4038–4044.
3 Huang, C.-H. and Mason, J.T. (1986) Biochim. Biophys. Acta 864, 423–470.
4 Mattai, J., Witzke, N.M., Bittman, R. and Shipley, G.G. (1987) Biochemistry 26, 623–633.
5 Karlsson, K.-A. (1982) in Biological Membranes, Vol. 4 (Chapman, D., ed.), pp. 1–74, Academic Press, New York.
6 Dahlen, B. and Pascher, I. (1979) Chem. Phys. Lipids 24, 119–133.
7 O'Brien, J.S., Fillerup, D.L. and Mead, J.F. (1964) J. Lipid Res. 5, 109–116.
8 Miyatake, T., Ariga, T., Atsumi, T. and Komiya, Y. (1978) Adv. Exp. Med. Biol. 100, 585–599.
9 Tsuji, S., Suzuki, M., Ariga, T., Sekine, M., Kuriyama, M. and Miyatake, T. (1981) J. Neurochem. 36, 1046–1049.
10 Kishimoto, Y., Moser, H.W. and Suzuki, K. (1985) in Handbook of Neurochemistry, Vol. 10, ed. (Lajtha, A., ed.), pp. 125–151, Plenum Press
11 Barenholz, Y., Suurkuusk, D., Mountcastle, D., Thompson, T.E. and Biltonen, R.L. (1976) Biochemistry 15, 2441–2447.
12 Estep, T.N., Calhoun, W.I., Barenholz, Y., Biltonen, R.L., Shipley, G.G. and Thompson, T.E. (1980) Biochemistry 19, 20–24.
13 Ruocco, M.J., Atkinson, D., Small, D.M., Skarjune, R.P., Oldfield, E. and Shipley, G.G. (1981) Biochemistry 20, 5957–5966.
14 Freire, F., Bach, D., Correa-Freire, M., Miller, I. and Barenholz, Y. (1980) Biochemistry 19, 3662–3665.
15 Barenholz, Y., Freire, E., Thompson, T.E., Correa-Freire, M.C., Bach, D. and Miller, I.R. (1983) Biochemistry 22, 3497–3501.
16 Bunow, M.R. (1979) Biochim. Biophys. Acta 574, 542–546.
17 Curatolo, W. (1982) Biochemistry 21, 1761–1764.
18 Curatolo, W. and Jungalwala, F. (1985) Biochemistry 24, 6608–6613.
19 Reed, R.A. and Shipley, G.G. (1987) Biochim. Biophys. Acta 896, 153–164.
20 Koshy, K.M. and Boggs, J.M. (1983) Chem. Phys. Lipids 34, 41–53.
21 Boggs, J.M., Koshy, K.M. and Rangaraj, G. (1984) Chem. Phys. Lipids 36, 65–89.
22 Boggs, J.M., Rangaraj, G., Moscarello, M.A. and Koshy, K.M. (1986) Biochim. Biophys. Acta 816, 208–220.
23 Boggs, J.M., Koshy, K.M. and Rangaraj, G. (1988) Biochim. Biophys. Acta 938, 361–372.
24 Maulik, P.R., Atkinson, D. and Shipley, G.G. (1986) Biophys. J. 50, 1071–1077.
25 Levin, I.W., Thompson, T.E., Barenholz, Y. and Huang, C. (1985) Biochemistry 24, 6282–6286.
26 Mattai, J., Sripada, P.K. and Shipley, G.G. (1987) Biochemistry 26, 3287–3297.
27 Boggs, J.M. (1987) Biochim. Biophys. Acta 906, 353–404.
28 Boggs, J.M. and Rangaraj, G. (1985) Biochim. Biophys. Acta 816, 221–233.
29 Boggs, J.M. and Mason, J.T. (1986) Biochim. Biophys. Acta 863, 231–242.
30 Boggs, J.M., Stamp, D. and Moscarello, M.A. (1981) Biochemistry 20, 6066–6072.
31 Kubesch, P., Boggs, J.M., Luciano, L., Maas, G. and Tummler, B. (1987) Biochemistry 26, 2139–2149.
32 Serralach, E.N., De Haas, G.H. and Shipley, G.G. (1984) Biochemistry 23, 713–720.
33 Stumpel, J., Eibl, H. and Nicksch, A. (1983) Biochim. Biophys. Acta 727, 246–254.
34 Abramson, M.B., Katzman, R., Curci, R. and Wilson, C.E. (1967) Biochemistry 6, 295–304.