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Interdigitated lipid bilayers of long acyl species of cerebroside sulfate. An X-ray diffraction study

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X-ray diffraction analysis was performed on two species of cerebroside sulfate containing lignoceric acid (C24-CBS) or hexacosanoic acid (C26-CBS) hydrated in the presence of Li^+ or K^+ ions. The diffraction patterns indicated that both lipids form bilayers over a wide range of temperatures. A diffraction pattern could not be obtained on this lipid in the low-temperature metastable gel phase formed immediately after cycling through the phase transition as the long range order was too low. However, after storage at 4°C overnight, a more ordered phase was formed, which had a repeat distance of 7.34 nm. Storage at 4°C for a more prolonged period of time resulted in the formation of a very stable 'collapsed' phase in which there was essentially no water between the head groups of adjacent bilayers. The repeat distance, 6.84 nm, was only a little greater than that of the anhydrous lipid, 6.69 nm. The wide-angle diffraction patterns indicated that in these phases, the hydrocarbon chains had an orthorhombic packing at 10°C. On raising the temperature to 50°C, still below the temperature of the transition to the liquid-crystalline phase, the hydrocarbon chains became packed in a hexagonal array. The small-angle reflections were phased and electron density distributions were generated. In the low-temperature phases formed after storage at 4°C, the hydrocarbon chains of unequal length were partially interdigitated, with or without water between the layers. A molecular model of this phase is presented. A liquid-crystalline bilayer structure was observed at 70 to 80°C, which may also be partially interdigitated.

Introduction

Asymmetric synthetic species of phosphatidylcholine (PC), in which one hydrocarbon chain is somewhat longer than the other, are thought to form partially interdigitated bilayers in which a shorter chain from one side is arranged end to end with a longer chain from the other side. When one chain is approximately twice the length of the other and the water content is 10% or greater, a mixed interdigitated bilayer will form in which shorter chains from opposite sides are arranged end to end and the longer chains essentially span the bilayer [1–4]. This latter arrangement provides for each head group a surface area equivalent to the cross-section of three acyl chains. Bilayers of these forms are not restricted to PC and have been observed or inferred in sphingomyelin and various glycosphingolipids [5–8].

Naturally occurring sphingolipids are frequently highly asymmetric with saturated fatty acid chains which are in a gel phase at physiological temperatures. While the sphingosine base is usually 18 carbons long and penetrates into the bilayer to a depth of 13 or 14 carbons [9], the fatty acid chain may be 14 to 26 carbons long and is saturated or mono-unsaturated. Abnormal metabolism of the very long chain fatty acids, which occurs in the hereditary demyelinating disease adrenoleukodystrophy, leads to a 2–5-fold increase in the amounts of C25 and C26 chains in the sphingolipids of myelin and other cell membranes [10,11]. This accumulation of long chain sphingolipids may lead to the formation of regions of interdigitated bilayer in natural membranes, regions which might have important physiological or pathological roles.

Cerebroside sulfate (galactosylceramide I^3-sulfate) is the major charged glycolipid in myelin [12,13]. In the brain the fatty acid chain of cerebroside sulfate (CBS) is

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

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most frequently 24 carbons long although it can be anywhere from 14 to 26 carbons [14]. Semi-synthetic species of CBS with hydroxy and non-hydroxy fatty acids from 16 to 26 carbons in length have been studied in Li^+ and K^+ using differential scanning calorimetry (DSC) and electron spin resonance spectroscopy (ESR). Various stable and metastable phases were observed [8,15,16].

The behavior of a fatty acid spin label in the low-temperature metastable phase of the C24 and C26 species formed after rapid cooling from the liquid-crystalline phase suggested that it was a mixed triple-chain interdigitated bilayer, while higher temperature metastable phases formed on cooling seemed to be partially interdigitated bilayers [8]. Slow cooling or heating allowed the lipid to form a more stable and more ordered phase with a higher enthalpy phase transition to the liquid-crystalline phase. The structure of this phase could not be deduced from use of spin labels since they were insoluble in it. The similarity in behavior of these more stable phases of CBS to that of the crystalline subgel phase of phosphatidylcholine (PC) and the crystalline phase of C24 cerebroside suggested that it was a less hydrated, possibly partially interdigitated bilayer, in which greater intermolecular hydrogen bonding could occur.

In Li^+, which shields the charge of the sulfate less effectively than K^+, and therefore inhibits intermolecular hydrogen bonding, there was less tendency for CBS to go into its more stable phases. Furthermore, even if the C24 species was allowed to go into this phase, a transformation to a less ordered, possibly more hydrated phase occurred at about 50°C. The mobility of the spin label suggested that this phase was partially interdigitated. This behavior did not occur in K^+.

We report here results on the organization of these long chain species of CBS using X-ray diffraction. Both Li^+ and K^+ were used in the hope of observing differences in the organization of the metastable gel state when the charge is shielded to varying degrees. However, it proved impossible to obtain diffraction data on C24 or C26-CBS in the low-temperature metastable gel state which results after cooling rapidly from the liquid-crystalline phase, since the long range order was too low. The order increased after storage at 4°C overnight and the diffraction pattern indicated that both lipids formed more stable phases which are partially interdigitated bilayers at both 10°C and 50°C. Storage at 4°C for a more prolonged period caused them to go into very stable phases in which the partially interdigitated bilayer was almost completely dehydrated. At 80°C a liquid-crystal bilayer results but the exact molecular arrangement cannot be specified. A partially interdigitated bilayer is highly probable since the bilayer thickness seems to be too great for a mixed interdigitated bilayer.

Materials and Methods

Synthetic species of CBS, C24:0 and C26:0-CBS, were prepared as previously described [16,17]. The sphingosine base is 94% dihydroxy 18:1. The majority of the samples were prepared for diffraction experiments as follows. A few mg of CBS was added to 2 M KCl or LiCl (0.7 mg CBS to 40 μl of solution) in a test tube. This was heated in boiling water and then vortexed 15 times. Small quantities were then placed in 2 mm O.D. glass X-ray capillary tubes. These were spun down to the bottom of the tube and most of the excess buffer removed to leave 2 to 3 mg of CBS in each tube. Tubes were sealed, weighed and stored at 4°C until used, at least overnight, some for up to 1 month. A small number of samples were prepared by placing 2–3 mg of CBS directly into the capillary tubes and adding 5 to 6 mg of the salt solution before sealing. These were heated in water at about 98°C and centrifuged from one end of the tube to the other several times before storage at 4°C. Sample weights were checked after each experiment to ensure no loss of water.

Specimen were mounted in a toroidal X-ray camera [18] with three sheets of Kodak DEF-5 X-ray film. Temperatures were controlled to within one degree Celsius. Exposure times ranged from 18 to 48 h. The space surrounding sample, camera and film was evacuated to reduce absorption and scattering by air. Copper K alpha radiation was obtained from a microfocus (Jarrell Ash) generator. Films were developed using standard techniques and reflection intensities obtained from densitometer traces with a Joyce-Loebel model MKIIIC microdensitometer. The background curve was subtracted and integrated intensities, $I(h)$, where h is the diffraction order, were determined with a planimeter. Since these experiments were with unoriented bilayer arrays the magnitude of the structure factor, $|F(h)|$, was set equal to $[h^2\ I(h)]^{1/2}$. The electron density distributions are the Fourier transforms of the structure factors which have both magnitude and phase. Since the bilayers are centrosymmetric the phases must be either + or −. Phase determination proceeded as follows. Models of the density distribution for the different types of interdigitation were constructed which were consistent with the measured d-spacing, the known dimensions of the molecules and the approximate densities of their various components.

Dimensions were obtained by direct measurement of space filling models. Hydrocarbon chain lengths were confirmed by the appropriate formula [19]. For the 26C molecule the chains are approx. 2.0 and 3.4 nm long, assuming that the sphingosine chain penetrates 14 carbons into the bilayer. The longer chain is approx. 0.25 nm shorter in the 24C molecule. The galactopyranosyl-3-sulphate and 3 carbon 'backbone' at the hydrophilic end add 0.7 nm to the length of the mole-

cule and effectively cover the cross-sectional area of the two attached chains. When 26C molecules are in a partial interdigitation model the total bilayer width is about 6.8 nm. This decreases to 5.4 nm in a model with mixed interdigitation. Dipalmitoylphosphatidylcholine and other lipids have densities very close to that of water (see, for example, Refs. 20 and 21). CBS would not be expected to be very different. In our experiments the liquid crystal samples would slowly rise in any excess liquid and the gel samples were seen to be of essentially the same density as water. In our models densities were assigned to bring the overall average to that of water. The dimensions and densities used are consistent with those of (Ref. 6) for sphingomyelins. A layer of water could then be included in the models to make the total of bilayer thickness plus water equal to the observed *d*-spacing. The Fourier transforms of the models could then be used to predict intensities and phases of the reflections. If the predicted intensities from one model were reasonably close to the observed values then the associated predicted phases provided a useful starting point to proceed with the determination of correct phases. Continuous transforms, $F(X)$, of the density distribution, $\rho(x)$ were calculated using the sampling theorem [22,23,24]. A value for the zero-order amplitude is required for this method. This cannot be measured experimentally but can be approximated from the density model previously described.

Since the bilayer separation is varied by using water layers of various thicknesses and since the continuous transform of only the bilayer is desired then the zero order structure factor must be calculated for a 'minus-water' model. The zero-order structure factor then becomes the cumulative difference from water density across the bilayer and will be approximately zero in these experiments. At larger values of reciprocal space where phase uncertainties are greatest the transform is almost independent of the zero-order contribution and hence is most unlikely to be influenced by the approximation of the zeroeth order. At points $X = h/d$ ($h = 0, 1, 2 \ldots n$) the function $F(h)$ $(\sin \pi dX)/\pi \mathrm{d}X$ is laid down. The continuous transform, $F(X)$, is the sum of these functions. Data from an identical bilayer with a different water layer thickness (and thus a different *d*-spacing) can be obtained then a similar continuous transform can be generated and will superimpose over the first transform, but only if the phase assignments are correct. If necessary, such transforms can be calculated for different phase combinations until this condition of superimposition is satisfied. With correct intensities and phases a density distribution can then be obtained and, hopefully, interpreted in terms of molecular arrangement within the bilayer. In this report only relative density distributions are determined.

Wide angle reflections to reveal the packing of the chains in the bilayer were obtained with a position sensitive detector which gave reflections from temperature controlled samples. For these results a line source monochromated with a Franks' camera [25] was use. The detector was placed normal to the wide angle diffracted X-rays to minimize distortion due to paralax.

Results

Freshly prepared samples which had been heated above the transiton temperature gave small and wide angle diffraction patterns that indicated considerable disorder with poorly defined diffraction peaks. This disorder was much smaller after overnight storage and showed no change beyond that. Consequently all reported results are for samples stored at 4°C overnight or longer.

At 10°C the wide-angle pattern from CBS consisted of two sharp symmetrical peaks at spacings of 0.37 and 0.41 nm thus indicating a gel phase with tails perpendicular to the bilayer normal and packed in an orthorhombic lattice. Wide- angle patterns at any given temperature were identical for CBS with either 24 or 26 carbon chains in the presence of either potassium or lithium. At 50°C a gel phase was still observed but a single sharp symmetrical peak in the range from 0.410 to 0.415 nm revealed a rearrangement to a hexagonal packing with the chains still perpendicular. Finally, in the high temperature region (80°C) a single broad diffuse peak centered at approximately 0.43 nm showed that a liquid-crystalline state was present.

The repeat spacing *d* for C26-CBS at 10°C for a sample which had been stored at 4°C only overnight, was 7.34 nm. However, samples stored at 4°C for a more prolonged period of time had a *d* spacing of 6.84 nm, only slightly greater than that of the anhydrous lipid, 6.69 nm, indicating that most of the water had been squeezed out of the region between the head groups. This proved to be a very stable configuration which could only be transformed to the liquid-crystal state by heating the samples in boiling water. Once transformed they would then stay in the more disordered phase at 70 or 80°C. No significant difference was observed at any temperature below the transition temperature for either chain length when potassium was substituted for lithium. In all gel phase distributions with a significant layer of water the bilayer thickness was 0.2 nm less for samples with 24C chains than for those with 26C chains. In those cases where the water layer was approximately zero the total *d* space was 0.2 nm less for the bilayers with the shorter chains.

Model density distributions were created for both species of CBS over a range of possible water layer thicknesses for comparison of predicted intensities with those observed.

Sample density distributions for the C26-CBS in a gel phase in two different types of bilayer organization,

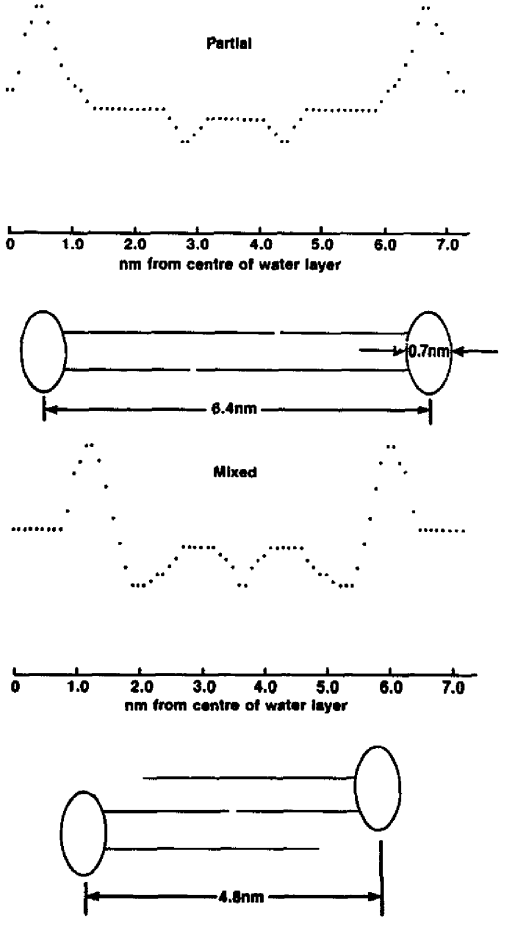

Fig. 1. Densities as used to calculate the predicted intensities in Table I for the larger repeat spacing. Upper half shows the partially interdigitated (Model A) structure. Lower half shows the mixed interdigitated structure (Model B).

the partially (model 'A') and mixed (model 'B') interdigitated bilayers, are shown in Fig. 1. In all cases the overall density was within 3% of that of water. The observed intensities for the two different low temperature phases of C26-CBS with d spacings of 6.84 nm and 7.34 nm at 10 °C are presented in Table I together with the predicted intensities and phases obtained by taking the Fourier transforms of density distributions such as those in Fig. 1. The approximate intensities predicted from model A are closer to the observed intensities than those for model B thus providing support for a partially interdigitated model and suggesting trial sets of phases for the two spacings. Model B is rejected mainly because it predicts very strong 3rd-order reflections which are not observed. All phases are measured relative to the centre of the bilayer.

TABLE I

Comparison of measured intensities with predicted intensities and phases for two d spacings of models A and B.

These are for C26-CBS samples in low temperature ...able phases at 10 °C.

h	d = 6.84 nm					d = 7.34 nm				
	I_{obs}	I_A	I_B	Φ_A	Φ_B	I_{obs}	I_A	I_B	Φ_A	Φ_B
1	100	100	100	–	–	100	100	100	–	–
2	3.2	5.7	0.17	+	+	0.1	2.1	1.0	+	–
3	0.49	0.73	9.4	–	+	0.45	0.022	13.0	+	+
4	0.0	0.14	4.5	+	–	0.57	0.33	1.1	–	–
5	0.12	0.18	0.53	+	–	0.41	1.0	3.0	+	–

In Fig. 2 the continuous transforms generated from these two sets of corrected observed intensities are seen to superimpose when the phases suggested by model A are used. Moreover, all other combinations of phases failed to give reasonable agreement between transforms. The same figure also includes the continuous transform for the same material in the dry state. For this sample the d space was 6.69 nm and the phases were the same as for the sample with spacing 6.84 nm. The phases suggested by the model and supported by the continuous transforms were then used with the observed intensities to create the density distributions shown in Fig. 3 for the three samples of Fig. 2. The overlap of shorter and longer tails of the partially interdigated phase leads

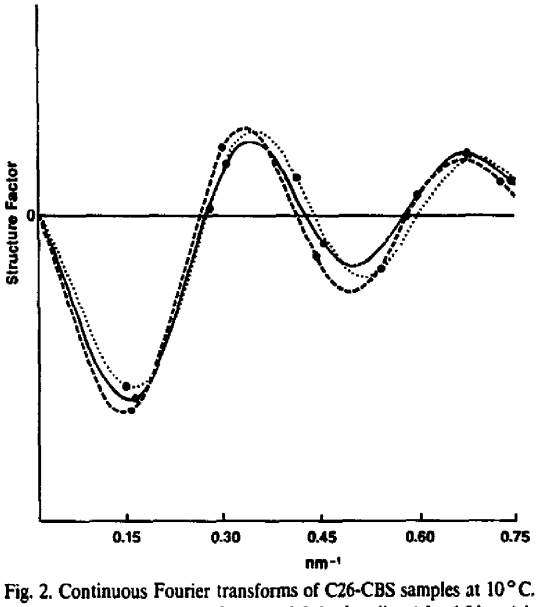

Fig. 2. Continuous Fourier transforms of C26-CBS samples at 10 °C. Solid line (d = 6.69 nm) is dry material, broken line (d = 6.84 nm) is for a sample in the most stable phase after storage at 4 °C for a long time. The dotted line (d = 7.34 nm) is for a sample in a less stable phase after storage at 4 °C overnight. Their observed intensities are in Table I.

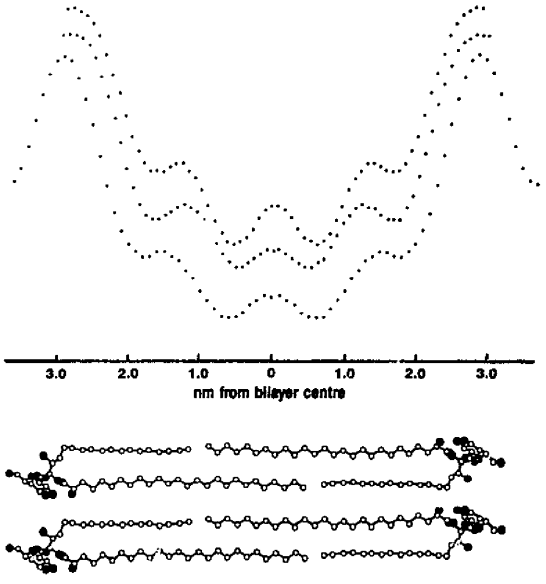

Fig. 3. Relative density distributions of C26-CBS at 10 °C. Upper trace, dry CBS ($d = 6.69$ nm). Middle trace, sample stored several days at 4 °C ($d = 6.84$ nm). Lower trace, sample stored overnight at 4 °C ($d = 7.34$ nm). Proposed partially interdigitated structure shown below.

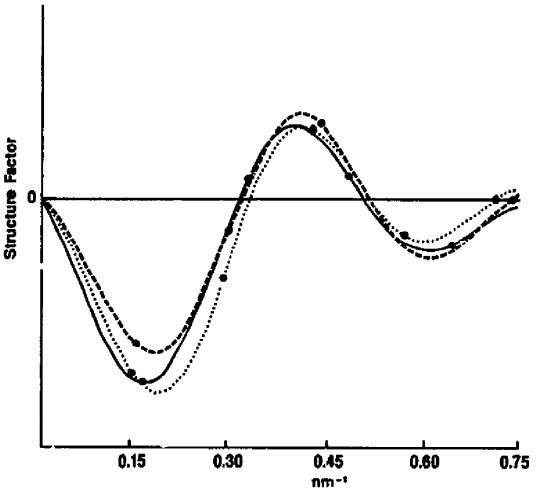

Fig. 4. Continuous Fourier transforms of the liquid-crystal samples listed in Table II. Solid trace ($d = 6.20$ nm) for C24-CBS with potassium. Broken trace ($d = 6.78$ nm) for C24-CBS with lithium. Dotted trace ($d = 7.00$ nm) C26-CBS with lithium.

to a somewhat increased density at the centre of the bilayer with a dip on either side corresponding to the locations of the end methyl groups. The bilayer thickness of 5.8 nm (measured between the highest density points of the lowest trace in Fig. 3) is consistent with molecular dimension of space filling scale models. Details of the molecular arrangement within the high density region are unknown and those shown at the bottom of Fig. 3 are not necessarily correct. Except for the water layer thickness all density distributions were essentially identical for C24- and C26-CBS except for the slight differences imposed by the difference in chain length. The density distributions were also similar at both 10 and 50 °C even though the wide angle patterns showed slightly different tail packing at these two temperatures.

In the liquid-crystalline phase the d spacing for the C24 species was 6.2 nm in the presence of K^+ and 6.78 nm in the presence of Li^+. The greater swelling in the presence of Li^+ may be due to less efficient shielding of the charged sulfate by Li^+ compared to K^+. There is obviously much greater disorder in the hydrocarbon region of the bilayer than in the low-temperature phases, and model density distributions are less predictable and therefore less useful. However, one might logically expect to have high density peaks separated by a narrower low density region than in the case of gel state samples. Table II lists observed intensities for three typical high temperature samples. In each case only two of all the different phase combinations gave density distributions that were believable. Measured intensities from three different liquid-crystal samples were tested with these

TABLE II

Measured intensities used in Figs. 4 and 5
Phases confirmed by Fig. 4.

h	d (nm)	6.20		6.78		7.00	
	T (°C)	80		85		80	
	Ion	K^+		Li^+		Li^+	
	Number of carbons	24		24		26	
		I_{obs}	ϕ	I_{obs}	ϕ	I_{obs}	ϕ
1		100	π	100	π	100	π
2		0.19	0	1.71	π	0.29	π
3		0.16	0	3.98	0	1.14	0
4		0.21	π	1.28	π	0.33	π

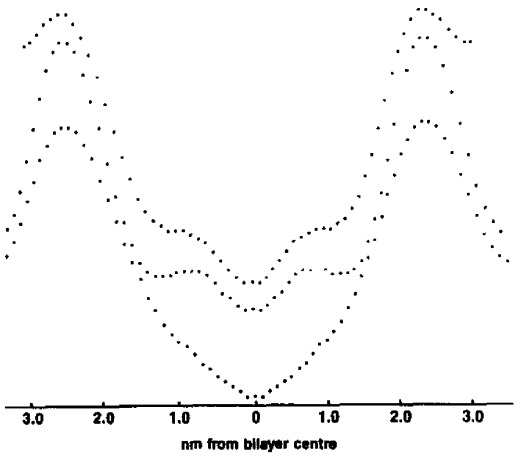

Fig. 5. Relative density distributions of samples in Table II and Fig. 4. All in liquid-crystalline state. Upper ($d = 6.20$ nm) is C24-CBS with potassium. Middle ($d = 6.78$ nm) is C24-CBS with lithium. Lower ($d = 7.00$ nm) is C26-CBS with lithium.

two sets of phase combinations to see which would lead to continuous transforms that would superimpose. The transforms that did superimpose are shown in Fig. 4. The phases confirmed by this figure are those listed in Table II. The density distributions calculated with these phases are shown in Fig. 5 where the width across the bilayer (peak to peak) is seen to be approx. 4.8 nm. The spacing immediately rules out the possibility that there has been a change from partial to mixed interdigitation as the hydrocarbon chains would have to be fully extended in this latter configuration and this is not consistent with the chain disorder indicated by the wide-angle pattern. The presence of a central minimum is unexpected. It suggests that the terminal methyl groups of all the chains must be located in this region. However, it is difficult to conceive of a model in which this might occur with such an asymmetric lipid. It would have to be very disordered. Since only four orders are observed in this high temperature region the resolution must be less than in the gel state. The apparent central minimum in the liquid-crystal state may represent a low resolution averaging of the two minima observed in the gel state. If density distributions are created for the gel phase using only the first four orders of the five observed then they also have a central minimum.

Discussion

The X-ray diffraction results indicate that these long-chain forms of CBS form a lamellar phase above and below the phase transition temperature in agreement with results on natural bovine brain CBS [26]. The results further indicate that a stable phase formed after storage at 4°C overnight is organized as a partially interdigitated bilayer below the transition temperature similar to that of C24:0-cerebroside [7] and possibly also above the transition temperature, like that of long chain forms of sphingomyelin [6]. In addition, the X-ray results show that the most stable phases formed after prolonged storage are almost completely dehydrated. The water between the bilayers seems to be progressively lost with time after cycling through the phase transition. Although the sample studied after storage at 4°C overnight may have lost some water, under these conditions, it is more hydrated than cerebroside even in the presence of high concentrations of monovalent cations. The bilayer repeat was similar in the presence of K^+ or Li^+ below the phase transition temperature but swelling in the liquid-crystalline phase was greater in the presence of Li^+ than K^+. This indicates less shielding of the bilayer surface charge by Li^+ than K^+, consistent with earlier results indicating that Li^+ has a lower binding affinity for CBS than K^+ [27].

Unfortunately, the sample could not be studied in its metastable low-temperature gel phase formed after rapid cooling from the liquid-crystalline phase. Therefore, we could not confirm the formation of a mixed interdigitated bilayer under these conditions, as inferred from results using a fatty acid spin label [8]. Motional restriction of a fatty acid spin labeled near the terminal methyl, 16-doxyl-stearate, occurred in both long chain species of CBS at low temperatures provided that the sample was cooled rapidly from the liquid-crystalline phase. This motional restriction was similar to that which occurs in the mixed interdigitated bilayers of asymmetric species of PC [28] and fully interdigitated bilayers of other lipids [29].

The mixed interdigitated bilayer structure is not very stable for CBS. This is expected since intermolecular bonding between the various hydroxyl groups and the amide moiety cannot occur in this type of bilayer. Indeed, it was clear from the spin label studies that some of the less hydrated phase was present, even after rapid cooling from above the phase transition temperature, as there was an underlying exchange broadened component in the spectra at low temperatures, which increased with time on storage. The X-ray diffraction results indicate that this exchange broadened spectrum arises from the more stable partially interdigitated bilayer as it becomes more dehydrated.

This lipid may also not be sufficiently asymmetric in chain length to be very stable in the mixed interdigitated bilayer. However, C24:0-sphingomyelin of similar degree of asymmetry is also thought to form a mixed interdigitated bilayer at low temperature [5]. It transforms to a partially interdigitated bilayer at higher temperatures, however, indicating that the stability of the mixed interdigitated bilayer is marginal for sphingmyelin. The 10:18 species of PC also has marginal stability in the mixed interdigitated bilayer. The

X-ray pattern shows two coexisting lamellar phases corresponding to the mixed interdigitated bilayer and noninterdigitated bilayer [30], in contrast to the more asymmetric 18:10PC, 18:12PC, or 8:18PC. Interestingly, 18:12PE, whose head group can participate in intermolecular hydrogen bonding is also not very stable in the mixed interdigitated bilayer in contrast to 18:12PC. It forms this kind of bilayer at low temperature but transforms to a non-interdigitated gel phase bilayer at higher temperatures [31].

X-ray diffraction detects a transition at about 50°C from the ordered, less hydrated phase in which the hydrocarbon chains are packed in an orthorhombic lattice, to a less ordered phase in which the chain packing is hexagonal. A transition is also detected at this temperature by calorimetry and use of spin labels for C24-CBS in Li^+. The spin label is soluble in this latter partially interdigitated phase and gives a spectrum similar to a normal gel phase bilayer [8].

In the liquid-crystalline phase, the bilayer thickness is suggestive of a partially interdigitated bilayer. Although this is not supported by the electron density pattern (i.e. there is no region of increased density at the centre of the bilayer), this may be because of the low resolution. The occurrence of a partially interdigitated organization is also supported by the wide angle reflection at 0.43 nm which suggests that this lipid is more closely packed than most lipids in the liquid crystalline phase, where values of 0.45–0.46 nm are usually observed. A similar bilayer thickness was observed for C24-sphingomyelin [6] in the liquid-crystalline phase and the increase in bilayer thickness with increase in fatty acid chain length was greater for this lipid than found for symmetric forms of PC, suggesting that the liquid-crystalline phase of sphingomyelin was more ordered than that of PC.

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