Thermotropic Phase Behavior of Mixtures of Long Chain Fatty Acid Species of Cerebroside Sulfate with Different Fatty Acid Chain Length Species of Phospholipid†

J. M. Boggs,* K. M. Koshy, and G. Rangaraj

Department of Biochemistry, Research Institute, Hospital for Sick Children, Toronto, Canada, and Department of Clinical Biochemistry, University of Toronto, Toronto, Canada

Received January 13, 1993

ABSTRACT: The thermotropic phase behavior of asymmetric, long fatty acid chain species of cerebroside sulfate, C24-CBS and C26-CBS, with symmetric species of phosphatidylcholine (PC) containing fatty acid chains of 14–18 carbons in length (diC14-PC, diC16-PC, diC18-PC) and dimyristoylphosphatidylethanolamine (diC14-PE) in 0.1 M KCl was studied by differential scanning calorimetry. Novel cerebroside sulfate (CBS) spin labels containing long chain C24 and C26 fatty acid spin labels with the nitroxide group on the twenty-second carbon were used to study the lipid organization of the gel phases of these mixtures. The phase diagrams of all the mixtures indicated the presence of two immiscible gel phases at low CBS concentrations. All except the C26-CBS/diC14-PC mixture had eutectic phase behavior at low CBS concentrations suggesting that the long fatty acid chain of the CBS species had a destabilizing effect on the gel phase of most of the phospholipids. The C26-CBS/diC14-PC mixture had peritectic phase behavior at low CBS concentrations indicating a stabilizing effect of the CBS C26 acyl chain on diC14-PC. These results are consistent with the relative compatibility of the CBS acyl chain length with the bilayer thickness of the PC; only in the case of the C26-CBS/diC14-PC mixture is the acyl chain of CBS long enough to span the PC bilayer. At intermediate to high CBS concentrations, the CBS and phospholipid (PL) were miscible with the exception of the C24-CBS/diC18-PC combination, which had eutectic phase behavior over a wide concentration range. Thus when the PL acyl chain length was similar to the sphingosine chain length of CBS, CBS bilayers could accommodate symmetric phospholipid molecules better than phospholipid bilayers could accommodate asymmetric molecules of CBS. Use of the spin labels indicated that, at low temperatures and at intermediate to high CBS concentrations, all of the mixtures were in a triple chain mixed interdigitated gel phase which immobilized the spin label. This gel phase slowly transformed over a wide temperature range to a double chain partially interdigitated gel phase in which the spin labels had much more motion. This transformation could be detected as a broad low enthalpy transition by differential scanning calorimetry. In all cases the presence of phospholipid destabilized the mixed interdigitated phase. Stabilization of the partially interdigitated bilayer by intermolecular hydrogen bonding interactions must outweigh the destabilizing forces caused by disruptions in packing and van der Waals interactions between CBS molecules resulting from insertion of molecules of phospholipid into this type of bilayer. Differences in the stability of different lipid combinations in the mixed interdigitated phase are discussed in terms of the relative compatibility of their chain lengths.

Sphingolipids are important constituents of biological membranes, often bearing carbohydrate head groups which are thought to be involved in a variety of cell recognition and receptor functions (Hakomori, 1981). They differ from membrane phospholipids in that they often have long (greater than 20 carbons) saturated fatty acid chains (O'Brien et al., 1964) and are in the gel phase at physiological temperatures. These long fatty acid chain species of sphingolipids have an asymmetric hydrocarbon structure allowing them to form mixed interdigitated bilayers at low temperatures in the gel phase (Levin et al., 1985; Boggs et al., 1988b; Stevenson et al., 1992), like those formed by asymmetric species of phosphatidylcholine (PC)[1] in which one chain is nearly twice the length of the other (Hui et al., 1984; McIntosh et al., 1984). In this kind of bilayer the two shorter sphingosine chains of molecules on opposite sides of the bilayer pack end-to-end while the longer fatty acid chains span the bilayer. However, the sphingolipid species studied to date, C24:0-sphingomyelin (Levin et al., 1985), and the C24:0 and C26:0 species of cerebroside sulfate (CBS) (Boggs et al., 1988b; Stevenson et al., 1992) are not very stable in the mixed interdigitated bilayer. Raman spectroscopic (Levin et al., 1985; Stevenson et al., 1992), spin label (Boggs et al., 1988b), and X-ray diffraction (Stinson & Boggs, 1989) studies indicate that they undergo transformations to partially interdigitated gel phase bilayers as the temperature is raised or, in the case of CBS, with time at low temperatures. This low stability of these sphingolipid species in the mixed interdigitated bilayer may result from insufficient asymmetry; the C24 fatty acid

† This study was supported by the Medical Research Council of Canada. J.M.B. was supported by an MRC Scientist award.

* To whom correspondence should be addressed at the Biochemistry Department, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada M5G 1X8.

[1] Abbreviations: CBS, cerebroside sulfate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; diC14-PC, dimyristoylphosphatidylcholine; diC16-PC, dipalmitoylphosphatidylcholine; diC18-PC, distearoylphosphatidylcholine; diC22-PC, debehenoylphosphatidylcholine; diC14-PE, dimyristoylphosphatidylethanolamine; CBS-22-L-SL, CBS spin label containing 22-doxyllignoceric acid; CBS-22-H-SL, CBS spin label containing 22-doxylhexacosanoic acid; EPR, electron paramagnetic resonance spectroscopy; DSC, differential scanning calorimetry.

0006-2960/93/0432-8908$04.00/0

chain is probably not quite twice the length of the bilayer-penetrating portion of the sphingosine chain, thought to be about 13–14 carbons (Dahlen & Pascher, 1979). It may also result from the ability of various hydroxyl groups and the amide moiety of these lipids to participate in lateral intermolecular hydrogen bonding in the partially interdigitated bilayer. The probability of occurrence of such interactions is likely to be lower in the mixed interdigitated bilayer.

In order to understand how sphingolipids function in membranes it is necessary to determine their phase behavior and structural organization when mixed with symmetric species of glycerophospholipids (PL). If the sphingolipids phase separate into domains or clusters, they could interdigitate with each other. If not, individual molecules might interdigitate into the other side of the PL bilayer. This may depend on the relative lengths of the hydrocarbon chains of the asymmetric sphingolipid species and the symmetric PL species. Since the mixed interdigitated and partially interdigitated bilayers have different thicknesses, the type of structure formed by the sphingolipid might also depend on the dimensions of the bilayer formed by the PL in the mixture.

Significant understanding of how asymmetric PC species behave when mixed with symmetric species of PL and the dependence on the relative dimensions of the two species has been gained by studying their thermotropic phase behavior by differential scanning calorimetry (DSC) (Lin & Huang, 1988; Gardam & Silvius, 1989; Sisk et al., 1990; Bultmann et al., 1991; Mason, 1988; Sisk & Huang, 1992). However, none of these studies included mixtures where the short acyl chain of the asymmetric lipid had a similar length as the acyl chain of the symmetric lipid or where the length of the long chain of the asymmetric lipid was twice the length of the acyl chain of the symmetric lipid. In the present study we report the phase behavior of C24 and C26-CBS when mixed with phosphatidylcholine containing fatty acid chains of 14, 16, and 18 carbons in length in 0.1 M KCl. We have also studied the effect of changing the PL head group from choline to ethanolamine. We compared the behavior of the more symmetric short chain species, C16-CBS, with C24-CBS in mixtures with diC16-PC in 0.1 M KCl earlier (Boggs et al., 1990), and Gardam and Silvius (1989) have reported a complete phase diagram for C24-CBS/diC16-PC in 0.1 M NaCl.

We use spin labels to investigate the structure of the gel phases formed by the mixtures. We showed earlier that a fatty acid spin label with the nitroxide group near the terminal methyl group can detect formation of fully interdigitated bilayers formed by symmetric PL or mixed interdigitated bilayers formed by asymmetric PL (Boggs & Rangaraj, 1985; Boggs & Mason, 1986; Boggs et al., 1989). A spin label of similar acyl chain length as the PL is useful for distinguishing this kind of bilayer from a noninterdigitated bilayer because the nitroxide group near the end of the acyl chain is located near the more ordered apolar–polar interface of an interdigitated bilayer in contrast to the more disordered center of a noninterdigitated bilayer. Thus it is more motionally restricted in the interdigitated bilayer than in the noninterdigitated bilayer.

Use of a stearic acid spin label with the nitroxide group on the sixteenth carbon indicated that asymmetric long chain (C24 and C26) species of cerebroside sulfate in 2 M K^+ and 2M Li^+ also form a mixed interdigitated bilayer at low temperature (Boggs et al., 1988b). However, it was unable to detect formation of this structure in mixtures of CBS with PC (Boggs et al., 1990). The stearic acid spin label is not long enough to span the thickness of the mixed interdigitated bilayer formed by C24 and C26-CBS. If these bilayers are disordered by the presence of PL molecules, the stearic acid spin label may not be motionally restricted and thus is not able to detect a mixed interdigitated bilayer formed by the lipid mixtures. Furthermore, the intermolecular hydrogen bonding and dehydration of the lipid head groups which occur when these CBS species go into the partially interdigitated bilayer (Stinson & Boggs, 1989; Boggs et al., 1988a) results in partial insolubility of the stearic acid spin label (Boggs et al., 1984, 1988b) making it difficult to study the lipid in this phase by this technique. This insolubility also may be partly as a result of its much shorter length compared to the acyl chains of the C24 and C26-CBS species. This spin label is thus not adequate for studying the bilayer organization of CBS in mixtures with symmetric phospholipids of similar chain length as the spin label.

Therefore, in this study we have used novel CBS spin labels containing long chain C24 and C26 fatty acid spin labels with the nitroxide group on the twenty-second carbon, which more closely match the dimensions of the CBS molecules under study. These have better solubility in the different phases of CBS. We show that C26-CBS in 0.1 M K^+ has similar behavior as in 2 M K^+ and Li^+ reported previously (Boggs et al., 1988b; Stevenson et al., 1992), i.e., it forms a mixed interdigitated bilayer at low temperatures, which gradually transforms to a partially interdigitated bilayer as the temperature is increased. This transformation can be correlated with a broad, low enthalpy transition observed by DSC. These spin levels were sensitive to similar behavior in mixtures of CBS with PL at intermediate to high CBS concentrations, allowing us to study the lipid organization in these mixtures. The organization of individual molecules of CBS in phospholipid bilayers, monitored using these CBS spin labels, will be reported separately (Boggs & Koshy, 1993).

MATERIALS AND METHODS

C24:0, C26:0, and C24:0h fatty acid chain species of CBS (C24-CBS, C26-CBS, and C24:h-CBS) were prepared by a semisynthetic procedure as described previously (Koshy & Boggs, 1983; Boggs et al., 1988a). Dimyristoylphosphatidylcholine (DiCl4-PC) and dipalmitoylphosphatidylcholine (diC16-PC) were purchased from Sigma Chemical Co. (St. Louis, MO). Distearoylphosphatidylcholine (diC18-PC), debehenoylphosphatidylcholine (diC22-PC), and dimyristoylphosphatidylethanolamine (diC14-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). All lipids gave single spots by TLC. The CBS spin labels with a C24 (lignoceric acid) or C26 (hexacosanoic acid) chain, each spin labeled at the twenty-second carbon from the carbonyl (CBS-22-L-SL and CBS-22-H-SL, respectively), were synthesized as described (Koshy & Boggs, 1993).

Preparation of Samples for Calorimetry. Solutions of CBS and PC were prepared in chloroform–methanol (2:1, v/v). The actual lipid concentrations of the organic solutions and the ratios of the two lipids in the prepared samples were checked by sulfate and phosphate analyses, respectively (Kean et al., 1968; Bartlett, 1959). Aliquots of the CBS and PC solutions were combined in the desired ratio, and the solvent was removed by evaporation under a stream of nitrogen, followed by evacuation in a lyophilizer at 0.1 Torr for at least 2 h. For some lipid combinations, the dried lipid (2.5 μmol) was then redissolved in 0.5 mL of warm benzene, frozen in a dry ice/acetone bath, and lyophilized as described (Lin & Huang,

1988). Results obtained after this procedure were similar to those obtained when the step of lyophilization from benzene was omitted. To the dry lipid was added 0.2 mL of buffer (10 mM HEPES) at pH 7.4 containing 0.1 M KCl and 2 mM EDTA. The same buffer containing 2 M KCl was used for C24:h-CBS. The lipid was dispersed at a temperature above the lipid phase transition temperature using a vortex mixer by alternately dipping the sample into a 90–95 °C water bath and vortexing for 10 min. The sample was centrifuged in an Eppendorf bench centrifuge, and the wet pellet was loaded into an aluminum DSC pan. The samples were stored in a freezer for at least 24 h before measurement. The effect of storage for longer periods of time (for several months) was examined for some samples; no significant effect was observed. The samples of the pure lipids were treated identically to those of the mixtures.

Calorimetric Measurements. Samples were scanned on a Perkin-Elmer DSC-2 equipped with a Perkin-Elmer data station. Thermograms obtained at heating and cooling rates of 10 °C/min between −3 and 97 °C were used for data analysis and presentation. However, thermograms were also obtained at heating and cooling rates down to 1.25 °C/min. Heating scans after cooling at 1.25 °C/min were similar to those obtained after cooling at rates as high as 40 °C/min. Heating scans at 1.25 °C/min were similar to those at 10°/min. Several heating scans were made on each sample. The first scan was usually only a little different from the reheating scans, as shown for the mixture of C24-CBS with diC16-PC (Boggs et al., 1990), unless otherwise noted. The latter were usually very reproducible. The third heating scan was used for analysis. Some samples were also scanned in a high-sensitivity DSC (Microcal MC-1, Amherst, MA) at a heating rate of 0.5 °C/min; similar results were obtained with regard to the main features of the thermogram. However, the T_m of the broad low enthalpy transition before the main transition was at a 4 °C lower temperature than at the higher heating rate used with the Perkin-Elmer DSC. The temperature of maximum heat capacity, T_m, of the premelt $L_{\beta'}$ to $P_{\beta'}$ transition of PC and of the broad low enthalpy peaks which, on the basis of spin label evidence, were assigned to the transition of the mixed interdigitated to the partially interdigitated gel phase, was determined. Because of the breadth and low amplitude of these peaks, no attempt was made to determine onset and completion temperatures. The onset and completion temperatures, T_o and T_c, were determined only for the heat absorption curve assigned to the gel to liquid crystalline phase transition. Where this overlapped with the peak resulting from the mixed interdigitated to partially interdigitated phase transition, the peaks for each were extrapolated down to the baseline. Since some scans had noisy and/or curving baselines, measurement of onset and completion temperatures at the point of intersection with the baseline was unreliable. Therefore a more accurate measure of the width of the transition, which takes into account the presence of significant shoulders or other peaks in addition to the main transition, was obtained by determining T_o and T_c at a point from the baseline equal to 10% of the total height of the peak. These temperatures were corrected for the width of the transition of the pure lipid components (similarly determined), and theoretical phase diagrams for ideal mixing were calculated by the procedures of Mabrey and Sturtevant (1976). The enthalpies of the pure lipids were determined as described earlier (Koshy & Boggs, 1983). The T_m value obtained on cooling at a rate of 10 °C/min is always 4.5–5 °C below that on heating for any lipid. This difference is designated instrumental hysteresis (Boggs et al., 1989). Any difference greater than this is due to true hysteresis in sample behavior.

Measurement of EPR Spectra. Lipid samples were prepared for measurement of EPR spectra as described for calorimetry except that the spin label was added to the single lipids or lipid mixtures in the organic solvents at a lipid to spin label mole ratio of 100:1. After centrifugation of the lipid dispersions in an Eppendorf bench centrifuge, all but about 50 μL of supernatant was removed, and the lipid pellet was mixed in the remaining 50 μL of supernatant and taken up into a 50-μL glass capillary tube which was sealed at one end and centrifuged at 2000 rpm. The pellet was positioned in the cavity of the EPR spectrometer. Spectra were measured on a Varian E-104B EPR spectrometer equipped with a Varian temperature controller and a DEC LSI-11 based microcomputer system. The maximum hyperfine splitting T_{max} values were measured as described earlier (Boggs et al., 1981) and were used as a relative measure of the degree of motion of the spin label.

From previous studies of C24- and C26-CBS at higher cation concentrations (Boggs et al., 1988a,b), it appears that the lipid freezes initially into the partially interdigitated gel phase on cooling from the liquid crystalline phase and then transforms to the mixed interdigitated phase. Further slow changes in the lipid organization which cause exchange broadening of the spectra, thought to be dehydration of the partially interdigitated phase (Stinson & Boggs, 1989), occur in the temperature range 30–50 °C and inhibit formation of the mixed interdigitated phase at lower temperatures (Boggs et al., 1988b). Therefore, in order to allow complete conversion of C24- and C26-CBS and their mixtures with PL to the mixed interdigitated phase, the samples were cooled rapidly by removing them from the spectrometer at 78 °C and plunging them into ice water for a few minutes before measuring the spectra at low temperatures. However, C24h-CBS in 2 M K^+ (quenched from 92 °C) remains completely in the partially interdigitated phase after this procedure. It can be converted to the mixed interdigitated phase by rapid cooling followed by prolonged (several hours up to 2 days) incubation at temperatures around 20–30 °C as described earlier for the lipid in 2 M Li^+ (Boggs et al., 1988b). This procedure was also tried on the CBS/PL mixtures; however, it did not result in greater motional restriction of the spin label than obtained by cooling rapidly.

Some of the spectra were resolved into components characteristic of different environments (motionally restricted and mobile) by pairwise subtraction of spectra of samples of different lipid composition from each other. The percentage of the probe giving each spectral component was determined in some cases by the method of Brotherus et al. (1980) as described previously (Wang et al., 1989). In most cases, however, the relative amounts of motionally restricted and mobile components were compared in a less quantitative way by measuring the heights, h_r and h_m, of the spectral envelope above the baseline at distances of 12.5 and 8.5 G, respectively, downfield from the point at which the center peak passed through the baseline. The ratio of these two values h_r/h_m was calculated. Although T_{max} values of the restricted and mobile components varied some for the different mixtures, addition of restricted and mobile spectra obtained at different temperatures and with similar variations in T_{max} indicated that this affected the h_r/h_m values by less than 15%. Thus values which differ by more than 15% indicate a difference in amount of the two spectral components.

RESULTS

Six combinations of C24- and C26-CBS with different phospholipids were studied at varying CBS/phospholipid ratios by DSC and by use of spin labels. These included C24-CBS with diC14-PC, diC16-PC, and diC18-PC, and C26-CBS with diC14-PC, diC16-PC, and diC14-PE. Three different types of phase diagrams were obtained: (i) The C24-CBS/diC14-PC, C24-CBS/diC16-PC, C26-CBS/diC16-PC, and C26-CBS/diC14-PE mixtures had eutectic phase behavior over a limited concentration range at low CBS concentrations, (ii) C24-CBS/diC18-PC had eutectic phase behavior over a wide concentration range, and (iii) C26-CBS/diC14-PC had peritectic phase behavior over a limited concentration range at low CBS concentrations.

Eutectic Behavior over a Limited Concentration Range. Calorimetric data for the C24-CBS/diC14-PC and C26-CBS/diC14-PE mixtures will be shown as examples of those combinations having eutectic phase behavior over a limited concentration range. Reheating scans of C24-CBS/diC14-PC mixtures and C26-CBS/diC14-PE mixtures are shown in Figures 1 and 2A, respectively. Aqueous dispersions of C24- and C26-CBS in 0.1 M KCl displayed a large endothermic transition at 59.4 °C (ΔH 13.6 kcal/mol) (Figure 1k) and at 62.2 °C (ΔH 15.0 kcal/mol) (Figure 2Ai), respectively. Each also had a small, broad endothermic transition on heating with a peak maximum at 48–49 °C. This transition was more prominant on reheating than on the first heating scan (Boggs et al., 1990). On cooling, each lipid went through a more complex series of transitions as shown for C24-CBS earlier (Boggs et al., 1990) and for C26-CBS in Figure 2Bi. Thus there was significant hysteresis between heating and cooling scans.

On addition of small amounts of C24-CBS to diC14-PC, two distinct peaks appeared in the thermograms at 19 and 25% (mol %) CBS (Figure 1d,e), the first of which was at a 4 °C lower temperature than pure diC14-PC. Shoulders on the main peak are observed at this lower temperature at 7 and 13% CBS, and a small peak at this temperature may still be present at 41% CBS. Similar behavior occurred when 20–32% CBS was added to diC14-PE except that the two transitions were sharper and more distinct (Figure 2Ab,c). The lower temperature peak at 20% CBS had a T_m 3.5 °C below that of pure diC14-PE, and the second had a T_m similar to that of diC14-PE. For both combinations of lipids, these two transitions also appeared on first heating and on cooling scans (shown for the C26-CBS/diC14-PE combination on cooling in Figure 2Bb,c).

The lowering of the temperature of the main transition at low CBS concentrations indicates eutectic phase behavior. Thus, for the C24-CBS/diC14-PC mixtures there must be a horizontal line of three-phase coexistence at about 21.5 °C, 4 °C below the T_m of diC14-PC, extending from below 10% to at least 40% CBS (Figure 3A). For the C26-CBS/diC14-PE mixture, the eutectic horizontal of three-phase coexistence at about 48 °C probably starts below 10% and does not extend much above 20% CBS (Figure 3B). Thus two solid solutions are present at low CBS concentrations, a phospholipid-enriched gel phase and a CBS-enriched gel phase. The two solid solutions are immiscible in the gel phase but miscible in the liquid crystalline phase. The lowering of T_m can be seen both from the actual peak temperatures and from the onset temperatures shown in Figure 3A,B. However, a single peak with a lower completion temperature was never observed, in contrast to results obtained for mixtures of asymmetric PC's with symmetric PC's by others (Lin & Huang, 1988; Sisk et al., 1990; Bultmann et al., 1991; Gardam & Silvius, 1989). This may be because the actual composition was not studied. Alternatively, the two lipids may not be present in the same bilayer in the added ratio, despite efforts to solubilize and achieve complete mixing (see Materials and Methods). Thus, at low CBS concentrations, two bilayer populations may be present, a smaller population containing mostly phospholipid and a small amount of CBS which destabilizes the phospholipid, lowering its T_m, and the other larger population containing a mixture of phospholipid and the remaining CBS.

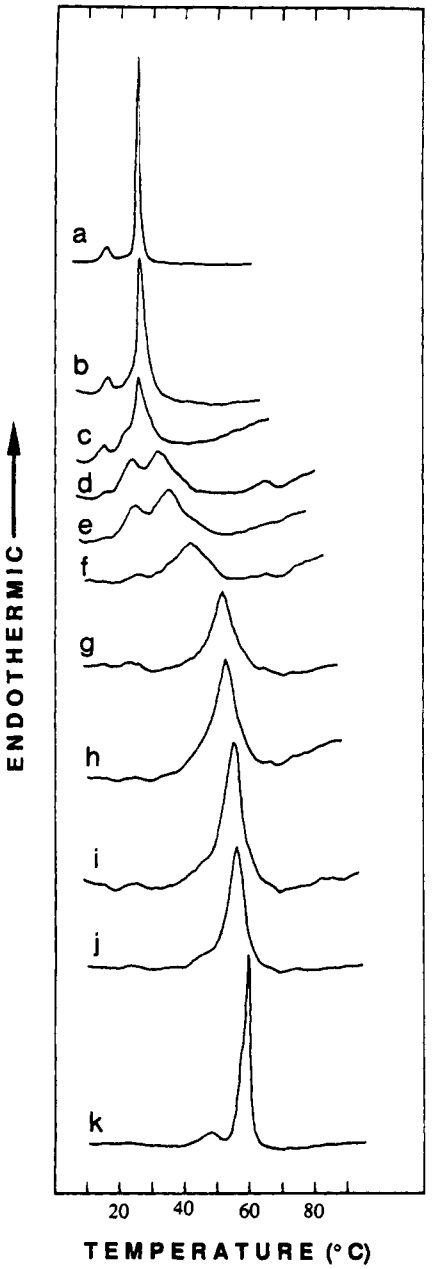

FIGURE 1: DSC thermograms of C24-CBS/diC14-PC mixtures in 0.1 M KCl at a heating rate of 10 °C/min. (a) diC14-PC only; (b) 7% CBS; (c) 13% CBS; (d) 19% CBS; (e) 25% CBS; (f) 41% CBS; (g) 74% CBS; (h) 82% CBS; (i) 88% CBS; (j) 93% CBS; and (k) C24-CBS only. Scans shown are reheating scans. Different amounts of sample were in the pans, so the peak areas cannot be directly compared.

The premelt transition of diC14-PC was retained up to 19% C24-CBS and was lowered in temperature slightly. To account for the behavior of the pretransition of diC14-PC, a second line of three-phase coexistence must be present in the phase diagram of the C24-CBS/diC14-PC mixtures, at about 15 °C, extending from about 19% to at least 40% CBS. Thus

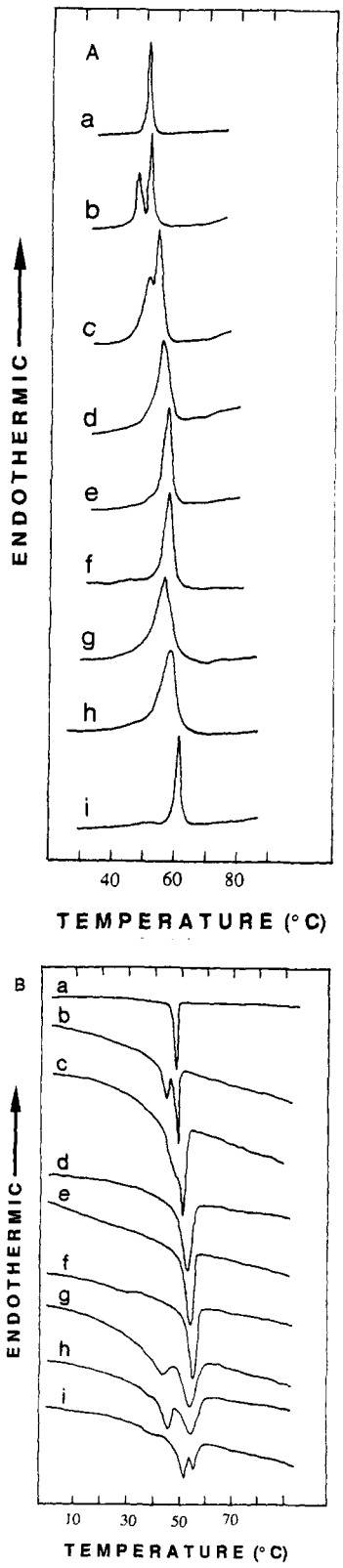

FIGURE 2: DSC thermograms of C26-CBS/diC14-PE mixtures on reheating (A) and on cooling (B): (a) diC14-PE only; (b) 20% CBS; (c) 32% CBS; (d) 48% CBS; (e) 58% CBS; (f) 63% CBS; (g) 71% CBS, (h) 83% CBS; and (i) C26-CBS only. Other details are as given in the caption to Figure 1.

at 15–20 °C the PC-enriched phase is the P_β phase, and below 15 °C it is the L_β phase (Figure 3A).

Peaks with primarily one component were observed at intermediate to high CBS concentrations. For both combinations of lipids, shoulders at lower temperatures than the main peak occurred at 40–48 °C at high CBS concentrations

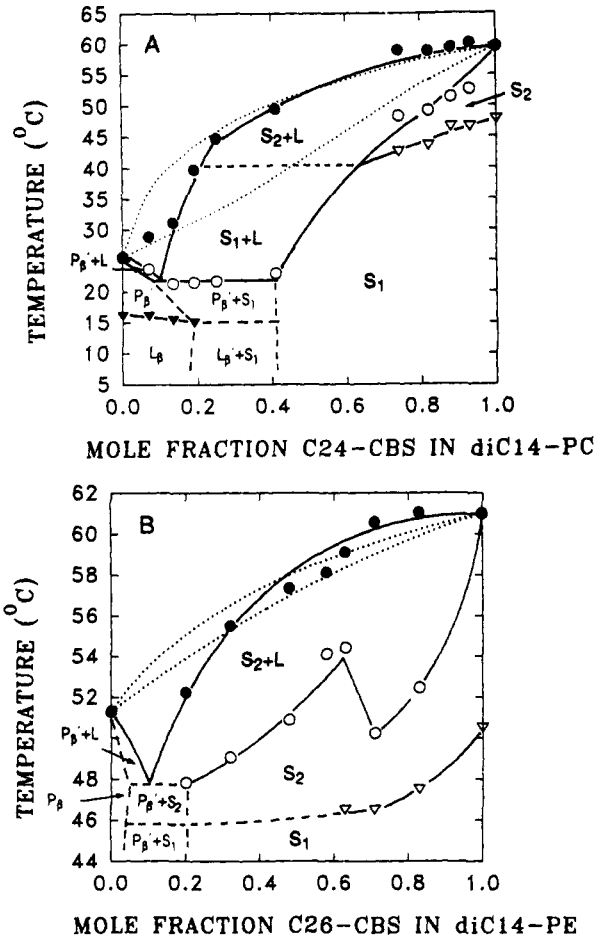

FIGURE 3: Dependence of the onset (○) and completion temperatures (●) for the main gel to liquid crystalline phase transition and T_m values for the premelt transition (▼) and the S_1 to S_2 transition (▽) from reheating scans on the mole fraction of CBS in phospholipid. Solid lines indicate phase boundaries that can be reliably assigned from the calorimetric data, and dashed lines indicate inferred phase boundaries. The eutectic point is estimated. Dotted lines are the theoretical solidus and liquidus curves for ideal mixing. (A) C24-CBS/diC14-PC mixtures. Theoretical curves were calculated using ΔH values of 5.9 and 13.6 kcal/mol for diC14-PC and C24-CBS, respectively. (B) C26-CBS/diC14-PE mixtures. Theoretical curves were calculated using ΔH values of 7.5 and 15.0 kcal/mol for diC14-PE and C26-CBS, respectively.

(Figures 1g–j and 2Af–h). They were more distinct on first heating scans where the main transition was usually a little sharper. They appear to be related to the small 48–49 °C peak of pure C24- and C26-CBS. This conclusion is supported by the similarity in behavior on cooling of the C24-CBS/diC14-PC mixtures containing 88 and 93% CBS and the C26-CBS/diC14-PE mixtures containing 63–83% CBS to pure CBS (shown for the latter combination in Figure 2Bf–h). The presence of the phospholipid greatly decreased the T_m values of the lower temperature transitions on cooling and decreased those of the low enthalpy transitions on heating by a few degrees. The presence of this peak in heating scans indicates that a transition from a gel phase S_1 to another gel phase S_2 occurs. Therefore, a third line of coexistence of the three phases S_1, S_2, and L, the liquid crystalline phase, must be present at about 40 °C at intermediate CBS concentrations for the C24-CBS/diC14-PC mixtures. For the C26-CBS/diC14-PE mixtures, there is a second horizontal line of coexistence of the gal phases P_β', S_1, and S_2 at about 46 °C extending down to low CBS concentrations (Figure 3B). The appearance of the phase diagrams at intermediate and high CBS concentrations indicates mixing of the two lipids in each

of the gel phases, S_1 and S_2. The liquidus line indicates the lipids are miscible in the liquid crystalline phase in both cases.

The C26-CBS/diC14-PE mixture had one interesting difference in behavior from that of the C24-CBS/diC14-PC mixture. The T_m value of diC14-PE containing 71% CBS on reheating and cooling was less than that of the 58 and 63% samples (Figure 2A,Be–g). This resulted from some change in the lipid organization after cycling through the phase transition, rather than an error in sample composition since this was not the case on the first heating scan (not shown). That is, the onset temperatures and T_m values at 71–83% were less on reheating than on the first heating scan, thus causing a discontinuity in the solidus line of the phase diagram in this concentration range (Figure 3B). However, the completion temperature remained high.

Two other lipid combinations, the C24-CBS/diC16-PC and C26-CBS/diC16-PC mixtures, behaved similarly to those discussed above in having eutectic phase behavior over a limited concentration range, from less than 10 to less than 40% CBS, lowering of the premelt transition at low CBS concentrations, occurrence of a transition from the S_1 to S_2 gel phases at intermediate to high CBS concentrations, and in accommodation of significant amounts of PC in the S_1 and S_2 gel phases (data not shown). Low concentrations of C24-CBS lowered the onset temperature of diC16-PC by about 3.3 °C (Boggs et al., 1990). However, C26-CBS lowered it considerably more, by about 7 °C. At 19% C26-CBS in diC16-PC, the thermogram consisted almost entirely of a peak at a lower temperature than diC16-PC. The presence of diC16-PC lowered the temperature of the S_1 to S_2 transition of C24- and C26-CBS by a few degrees. Cooling scans resembled heating scans except for C26-CBS/diC16-PC at 80% CBS where the sample went through the same complex transitions undergone by the pure lipid on cooling (not shown). This was not observed for the C24-CBS/diC16-PC mixture but may have been obscured by the width of the peaks (Boggs et al., 1990).

Eutectic Behavior over a Wide Concentration Range: C24-CBS/diC18-PC Mixtures. Analysis of the mixture of C24-CBS with diC18-PC was complicated by the fact that the T_m values of the pure lipids were only 2 °C apart (Figure 4a,j). Nevertheless, effects of diC18-PC on C24-CBS and of C24-CBS on diC18-PC could be discerned. The premelt was lowered in temperature to 47.5 °C and was present up to 25% CBS. Addition of up to 25% C24-CBS to diC18-PC gave a single sharp peak at a 1–1.5 °C lower temperature than the T_m of pure diC18-PC (Figure 4a–c). At 33–66% CBS there was a distinct shoulder at an even lower temperature (about 4 °C less than the T_m of diC18-PC) (Figure 4d,e,f). This suggested eutectic phase behavior although a single peak at the temperature of the low temperature shoulder in Figure 4d was not observed. However, lowering of the onset temperature occurred over a wide range of compositions (Figure 5A). The low enthalpy peak of pure CBS at about 49.5 °C was lowered to 46.5 °C and was present down to 50% CBS (Figure 5A). On cooling, an additional low temperature, low enthalpy peak was still observed in the presence of PC at 90% CBS only (not shown). Cooling scans at CBS concentrations below 90% CBS resembled heating scans.

These results suggest that there are three horizontal lines of three-phase coexistence, shown in the phase diagram in Figure 5A. The first, for coexistence of $L_{\beta'}$, S_1, and S_2, is at 46.5 °C and extends over the range 10–90% CBS. A second line of coexistence of $P_{\beta'}$, $L_{\beta'}$, and S_2 occurs at about 47.5 °C over a similar conentration range. The eutectic horizontal of coexistence of $P_{\beta'}$, S_2, and L is at about 53.8 °C and extends

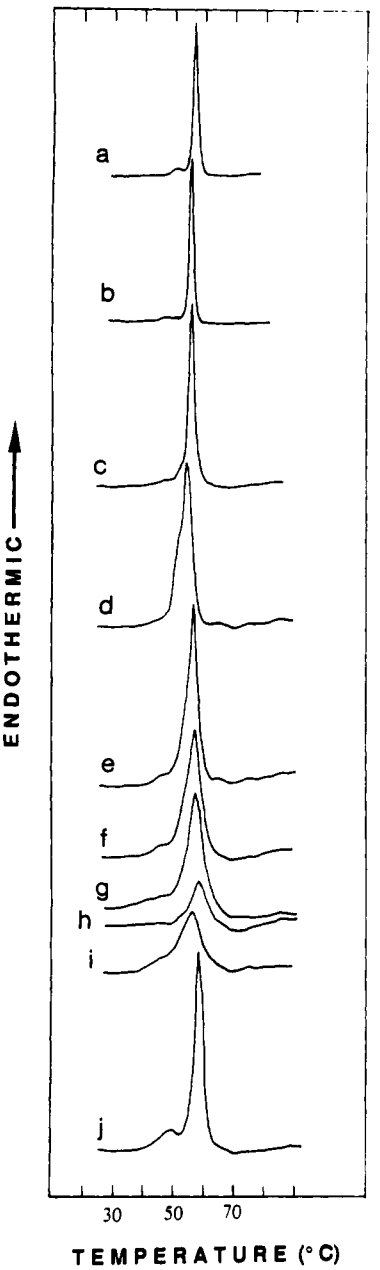

FIGURE 4: DSC thermograms of C24-CBS/diC18-PC mixtures: (a) diC18-PC only; (b) 11% CBS; (c) 25% CBS; (d) 33% CBS; (e) 50% CBS; (f) 66% CBS; (g) 75% CBS; (h) 80% CBS; (i) 90% CBS; and (j) C24-CBS only. Other details are as given in the caption to Figure 1.

from about 33 to 90% CBS with a eutectic composition at about 40% CBS, higher than for the mixture of C24-CBS with the shorter chain PC's. Thus $P_{\beta'}$ contains more CBS and S_2 contains less PC than for the mixtures of C24-CBS with diC14-PC and diC16-PC.

At concentrations above 75% the peaks broadened (Figure 4g–i) so that the completion temperatures were even higher than pure CBS, although the onset temperature remained low (Figure 5A). On the first heating scans, small higher temperature peaks were observed at 75–90% CBS as reported earlier for the mixture with diC16-PC (Boggs et al., 1990; Gardam & Silvius, 1989). They may be due to a less hydrated gel phase of CBS. Although they were not readily apparent on reheating, they probably contributed to the high completion temperatures. Therefore, the completion temperatures were ignored in drawing the liquidus line. A change in sample organization after cycling through the phase transition is

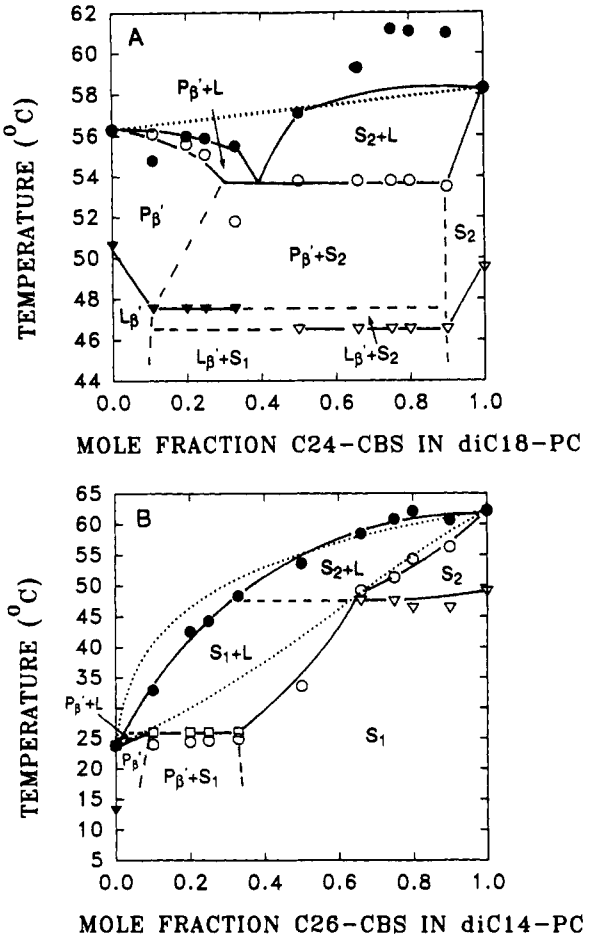

FIGURE 5: (A) Phase diagram for C24-CBS/diC18-PC mixtures. The width of the pure diC18-PC peak in 0.1 M KCl is greater than that of the 10% sample. Thus correction of the onset and completion temperatures for the peak width of the pure lipids resulted in T_c being lower than T_o at 10% CBS. Theoretical curves were calculated using ΔH values of 12.2 and 13.6 kcal/mol for diC18-PC and C24-CBS, respectively. (B) C26-CBS/diC14-PC mixtures. T_m values of the low temperature peaks and shoulders at low CBS concentrations are also indicated (□). The solidus line is based on the onset temperatures at CBS concentrations above 40% and on the T_m values instead of onset temperatures at low CBS concentrations. The peritectic point is based on the T_m values. Theoretical curves were calculated using ΔH values of 5.9 and 15.0 kcal/mol for diC14-PC and C26-CBS, respectively. Other details are as given in the caption to Figure 3.

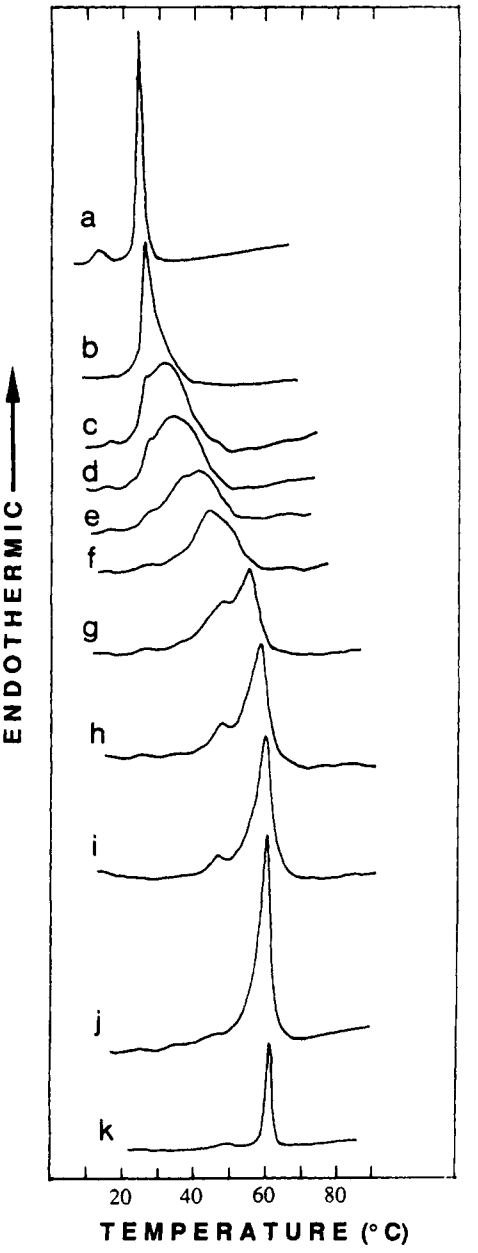

FIGURE 6: DSC thermograms of C26-CBS/diC14-PC mixtures: (a) diC14-PC only; (b) 10% CBS; (c) 20% CBS; (d) 25% CBS; (e) 33% CBS; (f) 50% CBS; (g) 66% CBS; (h) 75% CBS; (i) 80% CBS; (j) 90% CBS; and (k) C26-CBS only. Other details are as given in the caption to Figure 1.

indicated by the fact that at 90% CBS (Figure 4i) the T_m of the main peak was 2.1 °C lower on reheating than on the first heating scan (not shown), similar to the behavior of C26-CBS/diC14-PE samples. Thus its lower T_m on reheating compared to that of the 80% sample in Figure 4h is not due to an error in composition.

Peritectic Behavior: C26-CBS/diC14-PC Mixtures. Like the other lipid combinations, mixtures of C26-CBS with diC14-PC on reheating also had two transitions at 20–33% CBS, although the two peaks were not as well resolved (Figure 6c–e). However, the lower temperature one was at a slightly higher temperature than pure diC14-PC, in contrast to the other lipid combinations. Although the onset temperatures of the mixtures were similar to that of PC up to 33% CBS (Figure 5B), the fact that the T_m's were higher suggests peritectic phase behavior with two solid gel phases coexisting. Thus a horizontal region of three-phase coexistence at about 26 °C over the range of about 10–40% CBS is shown in the phase diagram in Figure 5B. In contrast to C24-CBS, small amounts of C26-CBS stabilized the gel phase of diC14-PC. The premelt was absent even at 10% CBS (Figure 6b). However, since the behavior of the premelt at concentrations below 10% CBS is unknown, the effect of CBS on the Lβ' to Pβ' transition is not indicated in the phase diagram.

A second distinct peak at 45–47 °C was also present from 66–90% CBS (Figure 6g–j). Its size relative to the main peak increased with decrease in amount of CBS. It may have a similar cause as the 49 °C transition of the pure lipid. Alternatively, it could indicate some degree of gel phase separation even at CBS concentrations above the peritectic range with the presence of diC14-PC- and CBS-enriched domains. However, the mixtures with diC14-PC down to 80% and possibly down to 50% went through a similar series of transitions on cooling as pure CBS (not shown). The fact that they showed similar hysteresis in behavior between heating and cooling as pure CBS supports the first explanation for the additional peaks observed on heating. Thus a third horizontal line of coexistence of the CBS-enriched gel phases S_1 and S_2

and the liquid crystalline phase is shown in the phase diagram at about 45 °C in the intermediate concentration range. Cooling scans for samples containing less than 50% CBS resembled heating scans. The phase diagram at intermediate to high concentrations of CBS suggests mixing of the two lipids in the S_1 and S_2 gel phases and in the liquid crystalline phase (Figure 5B).

Organization of the S_1 and S_2 Gel Phases of CBS Determined Using CBS Spin Labels. C24- and C26-CBS have been found to undergo a transition from the mixed interdigitated gel phase to the partially interdigitated gel phase in 2 M K^+ using Raman spectroscopy (Stevenson et al., 1992). However, this transition could not be detected calorimetrically and occurred at lower temperatures than the broad low enthalpy transition at 48–49 °C observed by DSC for the lipids in 0.1 M K^+. Using the fatty acid spin label 16-S-SL, C24h-CBS in both 2M Li^+ and 2 M K^+ also has been shown to undergo a transition from the mixed interdigitated to the partially interdigitated phase (Boggs et al., 1988a,b). In 2 M Li^+ this occurred in a relatively high enthalpy transition at about 50 °C which could be detected by DSC. These results suggested that the 48–49 °C S_1 to S_2 phase transition of C24- and C26-CBS in 0.1 M K^+ might also be a mixed interdigitated to partially interdigitated gel phase transition. This was investigated using long chain CBS spin labels.

The spectrum of CBS-22-L-SL in C26-CBS in 0.1 M K^+ at 7.5 °C is shown in Figure 7A. It resembled that of 16-S-SL in this lipid in 2 M KCl and had a similar T_{max} value of 28.6 G (Boggs et al., 1988b). However, at this temperature the T_{max} values in the noninterdigitated bilayers of diC18-PC and di22-PC were only a little less than those in C26-CBS, although the appearance of the spectra indicates that the spin label had more motion and/or less order in PC than in CBS (shown for diC18-PC in Figure 7B). However, in diC16-PC and diC14-PC the T_{max} values were significantly less (Table I). Furthermore, at a higher temperature, 22.5 °C, there was also a large difference in the spectra of CBS-22-L-SL in C26-CBS and noninterdigitated bilayers such as diC22-PC and diC18-PC (Figure 7C,D). Most of the probe was still significantly motionally restricted in C26-CBS but not in diC22-PC or diC18-PC.

The spectrum of CBS-22-L-SL in C26-CBS can also be compared to that in the partially interdigitated phase of C24h-CBS in 2 M K^+. At this cation concentration, C24h-CBS transforms to the mixed interdigitated phase more slowly than any of the other species we have studied (Boggs et al., 1988b). Therefore, this lipid species can be trapped in a relatively hydrated, partially interdigitated phase in which the spin label is soluble, at low temperatures by fast cooling and can be converted to the mixed interdigitated bilayer by prolonged incubation at room temperature. At 7.5 °C, the T_{max} value of the spectrum of the mixed interdigitated phase was similar to that in C26-CBS (Table I) but was 9–10 G smaller in the partially interdigitated phase (Figure 7E), indicating much greater motion and disorder in the partially interdigitated phase near the end of the C24 chain. A smaller difference of only 4 G was observed earlier for 16-S-SL in the partially interdigitated and mixed interdigitated phases of this lipid (Boggs et al., 1988b). Thus the longer CBS-22-L-SL is better able to distinguish between the partially interdigitated and mixed interdigitated phases of CBS species of similar acyl chain length than 16-S-SL can.

The spectrum of the longer chain CBS-22-H-SL in C26-CBS at 7.5 °C (shown in Figure 8A) indicates that it was a little more ordered than CBS-22-L-SL (Figure 7A). It had a similar spectrum in the mixed interdigitated phase of C24h-CBS in 2 M K^+. However, at this temperature, this spin label was significantly more motionally restricted in the partially interdigitated phase of C24h-CBS than CBS-22-L-SL was. The difference in T_{max} values in the two phases was only about 2 G, making this probe less useful for distinguishing these two phases at this temperature. At a higher temperature, however, the motional restriction was retained in the mixed interdigitated bilayer while the partially interdigitated bilayer became more disordered, thus increasing the difference, as shown for spectra of CBS-22-H-SL at 22.5 °C in Figure 7F,G. The shorter CBS-22-L-SL retained its ability to distinguish between the two phases at the higher temperature (not shown). Thus the similar motional restriction of these probes in C26-CBS to that in the mixed interdigitated phase of C24h-CBS indicates that C26-CBS also formed a mixed interdigitated bilayer in

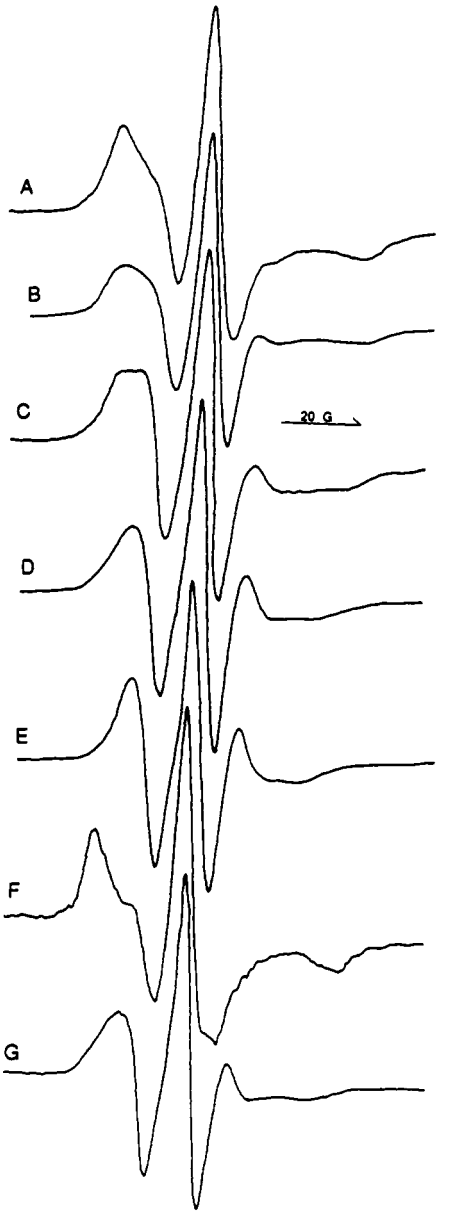

FIGURE 7: EPR spectra of (A–E) CBS-22-L-SL in (A) C26-CBS at 7.5 °C; (B) diC18-PC at 7.5 °C; (C) C26-CBS at 22.5 °C; (D) diC18-PC at 22.5 °C; (E) in the partially interdigitated phase of C24h-CBS at 7.5 °C obtained after rapid cooling from the liquid crystalline phase; (F) CBS-22-H-SL in the mixed interdigitated phase of C24h-CBS at 22.5 °C obtained after incubation at room temperature for 2 h; and (G) CBS-22-H-SL primarily in the partially interdigitated phase of C24h-CBS at 22.5 °C obtained after rapid cooling. C26-CBS is in 0.1 M KCl and C24h-CBS is in 2 M KCl.

Table I: Spectral Parameters and Amount of Motionally Restricted Component, As Determined by Spectral Subtraction, in Spectra of CBS Spin Labels in Lipid Mixtures at 7.5 and 32.5 °C[a]

	7.5 °C			32.5 °C		
		T_{max} (G)[c]			T_{max} (G)[c]	
sample	% motionally restricted spectral component[b]	motionally restricted component	mobile component	% motionally restricted spectral component[b]	motionally restricted component	mobile component
C26-CBS/diC14-PC 9:1	69–70	27.3	20.8			
C26-CBS/diC14-PC 8:2	51–55	25.9	20.8	68	22.6	15.9 (1.8 ns)
C26-CBS/diC14-PC 7:3	36–53	*d*	20.9			
C26-CBS/diC14-PC 1:1	*e*	*e*	20.2	26	22.6	15.9 (1.8 ns)
C26-CBS/diC16-PC 8:2	47	27.0	21.6	35[f]	*d*	19.2
C26-CBS/diC14-PE 8:2	45	*d*	20.8			
C24-CBS/diC18-PC 9:1	76–80	28.6	*d*			
C24-CBS/diC18-PC 8:2	53	30	20.9	54[g]	*d*	19.8
C24-CBS/diC16-PC 8:2	33–44	*d*	20.0	25[f]	*d*	19.2
C24-CBS/diC14-PC 8:2	38–41	*d*	19.9			
C26-CBS		28.7			27.4	
C24h-CBS (mi)[h]		27.7			27.2	
C24h-CBS (pi)[h]			19.3			19.8
diC18-PC		27.6			23.5	
diC16-PC		25.6			23.5	
diC14-PC		24.4			15.5 (1.4 ns)	
diC14-PE		22.0			23.7	

[a] All were in 0.1 M KCl except for C24h-CBS, which was in 2 M KCl. Values at 7.5 °C were obtained using CBS-22-L-SL and cannot be compared to those at 32.5 °C, which were obtained using CBS-22-H-SL. [b] Range of values obtained by subtracting either spectra of other mixtures or spectra of pure lipids in mixed interdigitated or partially interdigitated phase. [c] After spectral resolution except for pure lipids. Value of motional parameter is given in parentheses. [d] Both components could not be determined since the spectrum could be resolved only by subtracting either a motionally restricted or a more mobile spectrum, not both. [e] Not determined, but visual inspection suggests it is low with CBS-22-L-SL. [f] Percent of component resembling spectrum of C26-CBS, not itself 100% motionally restricted at this temperature (see Figure 8B). [g] Percent of component resembling diC18-PC. [h] mi = mixed interdigitated bilayer; pi = partially interdigitated bilayer.

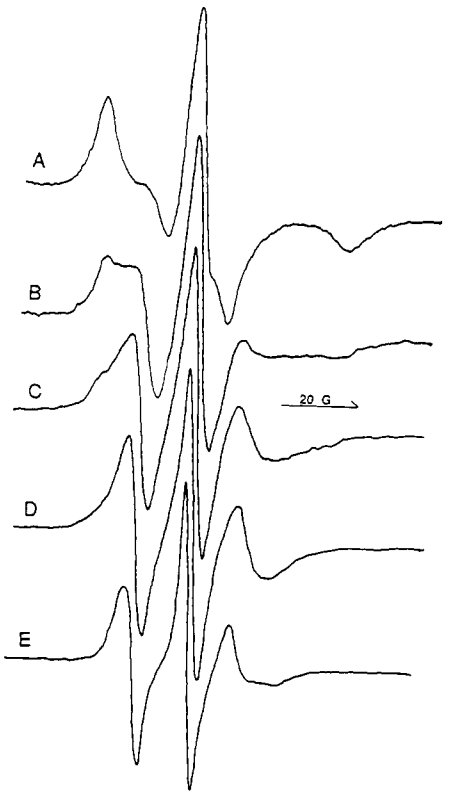

FIGURE 8: EPR spectra of CBS-22-H-SL (A–D) in C26-CBS, 0.1 M KCl, at varying temperatures: (A) 7.5 °C; (B) 32.5 °C; (C) 42.5 °C; (D) 52.5 °C; and (E) in C24h-CBS, 2 M KCl, at 42.5 °C.

0.1M KCl at low temperatures.

Spectra of CBS-22-H-SL in C26-CBS in 0.1 M K^+ with increasing temperature are shown in Figure 8. The spectra acquired a second more mobile component at higher temperatures, and the ratio between the motionally restricted and more mobile components decreased with increasing temperature. At 52.5 °C only a small amount of the motionally restricted component remained (Figure 8D). The more mobile component indicates the presence of a more disordered phase. The spectrum in Figure 8D was similar to although a little more disordered than the partially interdigitated phase of C24h-CBS (shown at 42.5 °C in Figure 8E). This supports the conclusion that C26-CBS went into the partially interdigitated phase with an increase in temperature. The transformation occurred gradually over a wide range of temperatures and was nearly complete by 52.5 °C, near the T_m of the low enthalpy transition observed by DSC. The transition observed by DSC was a broad transition starting at about 30–35 °C. When CBS was scanned at a slow heating rate, 30 °C/h, the T_m of the transition was 4 °C lower than at the higher rate, 10 °C/min, used for the scans shown in the figures. Thus the broad, low enthalpy S_1 to S_2 gel phase transition observed by calorimetry is probably a slow transition from a mixed interdigitated gel phase to a partially interdigitated gel phase, which can take place over a wide temperature range. The main, high enthalpy transition observed by DSC is thus that of the partially interdigitated S_2 gel phase to the liquid crystalline phase. Similar results to those described above for C26-CBS were observed for C24-CBS.

Mixtures of C24- and C26-CBS with Phospholipids. The structure of the phases formed by the mixtures of CBS with phospholipids was then investigated using the long chain CBS spin labels. At 7.5 °C, the shorter chain CBS-22-L-SL was more sensitive to differences in the phase behavior of the different mixtures than CBS-22-H-SL. Spectra of CBS-22-L-SL in several mixtures are shown in Figure 9. The spectra in C26-CBS/diC14-PC 9:1 (Figure 9A) and C24-CBS/diC18-PC 8:2 (Figure 9D) clearly consist of both motionally restricted and more mobile components. Visual inspection of the spectrum of C26-CBS/diC14-PC 8:2 (Figure 9B) and comparison to that of the 1:1 sample (Figure 9C) suggest that the former may also have a more motionally restricted component; the spectrum of the 7:3 sample resembled that of the 8:2 sample

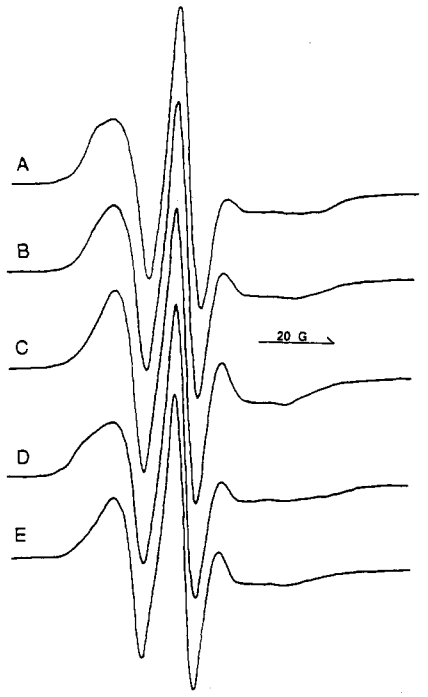

FIGURE 9: EPR spectra of CBS-22-L-SL at 7.5 °C in (A) C26-CBS/diC14-PC 9:1; (B) C26-CBS/diC14-PC 8:2; (C) C26-CBS/diC14-PC 1:1; (D) C24-CBS/diC18-PC 8:2; and (E) C24-CBS/diC16-PC 8:2. All are in 0.1 M KCl.

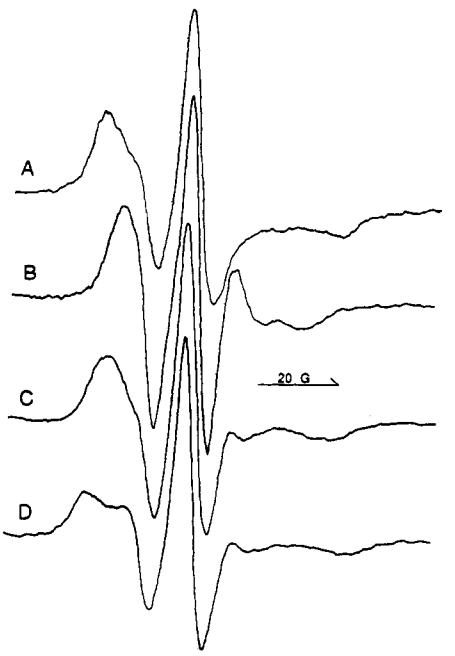

FIGURE 10: Resolved spectral components obtained by subtraction of fractional amounts of EPR spectra of CBS-22-L-SL at 7.5 °C in lipid mixtures from each other. (A) Result obtained after subtracting the spectrum of the C26-CBS/diC14-PC 8:2 sample from that of the C26-CBS/diC14-PC 9:1 sample; (B) result obtained after subtracting the spectrum of the C26-CBS/diC14-PC 9:1 sample from that of the C26-CBS/diC14-PC 8:2 sample; (C) result obtained after subtracting the spectrum of the C26-CBS/diC14-PC 1:1 sample from that of the C26-CBS/diC14-PC 8:2 sample; (D) result obtained after subtracting the spectrum of the C26-CBS/diC14-PC 1:1 sample from that of the C24-CBS/diC18-PC 8:2 sample.

(not shown). However, the spectrum of this spin label in the 1:1 sample did not appear to have much motionally restricted component (Figure 9C), although it indicated more order than in the partially interdigitated phase of C24h-CBS (Figure 7E). The spectrum of the C24-CBS/diC16-PC 8:2 sample (Figure 9E) had less motionally restricted component than that of the C26-CBS/diC14-PC 8:2 sample, and its mobile component resembled the spectrum of the C26-CBS/diC14-PC 1:1 sample.

The presence of a motionally restricted spectral component in the spectra of the C26-CBS/diC14-PC 9:1 and 8:2 samples at 7.5 °C was confirmed by spectral subtraction. The most successful subtraction, resulting in realistic appearing motionally restricted and more mobile spectral components, was achieved by subtracting the spectra of these two samples from each other. The T_{max} value of the motionally restricted component present in the 9:1 sample (Figure 10A) was 27.3 G (Table I), a little less than for pure C26-CBS at this temperature. The T_{max} value of the more mobile component (Figure 10B) was 20.8 G, a little greater than for the 1:1 sample or the partially interdigitated phase of C24h-CBS (Table I). The success of the subtraction indicated that the 8:2 sample must have contained a fairly similar motionally restricted component and the 9:1 sample contained a similar mobile component. The presence of a motionally restricted component in the 8:2 sample was confirmed by subtracting the spectrum of the 1:1 sample from that of the 8:2 sample. The motionally restricted component obtained is shown in Figure 10C. The smaller T_{max} value of 25.9 G and the shape indicated a little more motion and/or less order than the motionally restricted component in the 9:1 sample. Similar results were obtained for the 7:3 sample.

The presence of both a motionally restricted and a more mobile component in the spectra of these samples indicates slow exchange between two different lipid populations, most likely the mixed interdigitated phase and domains of partially interdigitated phase. Domains of noninterdigitated diC14-PC would result in T_{max} values intermediate between those observed. The percentages of each population were estimated as described previously (Brotherus et al., 1980; Wang et al., 1989). The results using this spin label indicated about 70% of the lipid in the C26-CBS-diC14-PC 9:1 sample was motionally restricted while 30% was more mobile at this temperature (Table I). At the 8:2 and 7:3 ratios, the percentage of each population was about 50%. Although the DSC results indicated that the lipids were relatively miscible at this ratio, the presence of clusters of noninterdigitated diC14-PC bilayer cannot be excluded. However, neither the amount of the more mobile component nor its low T_{max} in the 8:2 sample can be accounted for by the presence of the spin label in noninterdigitated PC domains. Although the spin label was also relatively immobilized in PC at this temperature (T_{max} for diC14-PC was 24.4 G), the amount of PC present in the mixtures cannot account for the amount of motionally restricted component in the spectra.

The spectra of the mixtures of C26-CBS with 20% diC16-PC and diC14-PE resembled that of the C26-CBS/diC14-PC 8:2 sample in Figure 9B. The spectrum of the C24-CBS/diC14-PC 8:2 sample resembled that of the C24-CBS/diC16-PC 8:2 sample in Figure 9E. The percent motionally restricted and mobile components in these spectra was estimated by subtraction of the spectra of different mixtures from each other or by subtraction of the spectrum of pure CBS in either the mixed interdigitated or partially interdigitated phase (Table I). The results of these subtractions have less confidence than those shown in Figure 10 because the spectral components differed more from the spectra used for subtraction. Nevertheless, the results of the subtraction plus the appearance of the original spectra indicated that the amount of the mixed interdigitated phase formed at 7.5 °C decreased

Table II: Temperature Dependence of Relative Amounts of Motionally Restricted and Mobile Populations of CBS Spin Labels in Lipid Samples Monitored from Peak Height Ratios, h_r/h_m[a]

sample	h_r/h_m 7.5 °C[b]	22.5 °C[c]	32.5 °C
C26-CBS/diC14-PC 9:1	1.09		
C26-CBS/diC14-PC 8:2	0.82	1.50	0.45
C26-CBS/diC14-PC 7:3	0.75	1.38	0.39
C26-CBS/diC14-PC 1:1	0.55	0.88	0.24
C26-CBS/diC16-PC 8:2	0.91	1.46	0.39
C26-CBS/diC14-PE 8:2	0.76	1.33	0.30
C24-CBS/di18-PC 9:1	1.09		
C24-CBS/di18-PC 8:2	0.86	1.04	0.33
C24-CBS/di16-PC 8:2	0.49	0.83	0.26
C24-CBS/di14-PC 8:2	0.54	0.46	0.19
C26-CBS	2.38		1.16
C24h-CBS (mi)[e]	1.46	4.06	0.61
C24h-CBS (pi)[e]	0.23	0.49	0.41[d]
di18-PC	1.62	1.72	0.75
di16-PC	1.24		0.68
di14-PC	1.02		

[a] All were in 0.1 M KCl except for C24h-CBS, which was in 2 M KCl. Differences in the h_r/h_m values of 15% or more are considered significant. [b] CBS-22-L-SL. [c] CBS-22-H-SL. [d] Goes partly into mixed interdigitated phase at this temperature causing a high value of h_r/h_m. [e] mi = mixed interdigitated; pi = partially interdigitated.

with increase in the amount of PC and was greater for mixtures of C26-CBS with all PL than for C24-CBS with diC14-PC and diC16-PC. This was further confirmed by estimation of the relative amounts of motionally restricted and more fluid populations in the other mixtures from h_r/h_m, the ratios of the heights of the spectral envelope at 12.5 and 8.5 G from the center line, as described under Materials and Methods (Table II, column 1).

The T_{max} value of the motionally restricted component in the spectrum of the C24-CBS/diC18-PC 8:2 sample at 7.5 °C (Figure 10D), as revealed by subtraction of the spectrum of the C26-CBS/diC14-PC 1:1 sample (at 2.5 °C), was 30 G (Table I), even greater than that for the mixed interdigitated phases of pure CBS or the noninterdigitated phase of diC18-PC. The DSC results indicate that phase separation of a CBS-enriched and a diC18-PC-enriched phase occurred. Therefore it is uncertain in this case if the motionally restricted component resulted from a mixed interdigitated CBS phase or a noninterdigitated diC18-PC phase. Although the spin label was not as restricted in either C26-CBS or diC18-PC as in the mixture, subtraction of the spectrum of either gave a realistic appearing mobile component, with subtraction of the spectrum of C26-CBS giving the best result. This allowed an estimate for the amount of motionally restricted component in the spectrum of the C24-CBS/diC18-PC 8:2 sample of 52.5%, too large to be accounted for by the amount of diC18-PC in the sample. Thus it more likely resulted at least partially from a mixed interdigitated CBS phase. Furthermore, a C24-CBS/diC18-PC 9:1 sample contained even more motionally restricted component (Table I), and the degree of immobilization was similar to that of pure CBS (T_{max} = 28.6 G). Thus the mixture of C24-CBS with diC18-PC is in the mixed interdigitated phase more than its mixtures with the shorter chain PC's.

The results of the spectral resolution further indicated that the presence of 20% diC18-PC in the mixed interdigitated bilayer increased T_{max} while the presence of diC14-PC or diC16-PC decreased it (Table I). The resolved spectral components of the partially interdigitated phase in the mixtures of 20–30% phospholipid with C26-CBS and of diC18-PC with C24-CBS indicated that it was more ordered than those of the

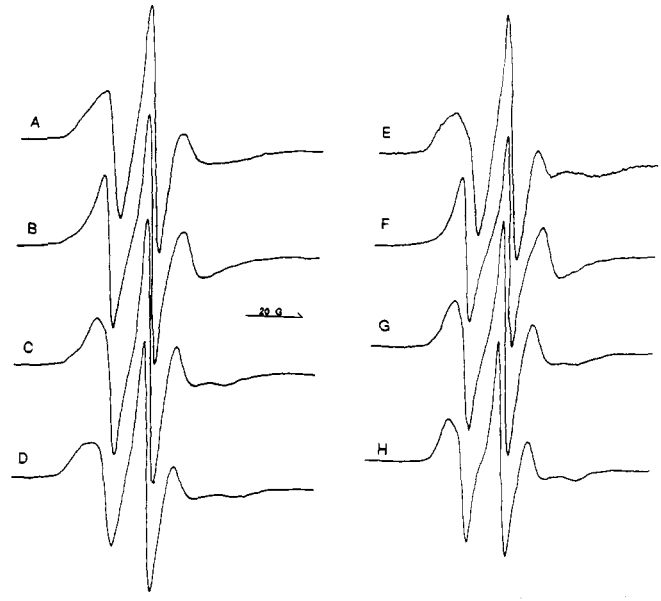

FIGURE 11: EPR spectra of CBS-22-H-SL at 32.5 °C in (A) C26-CBS/diC14-PC 8:2; (B) C26-CBS/diC14-PC 1:1; (C) C24-CBS/diC18-PC 8:2; and (D) diC16-PC. Resolved spectral components obtained by subtraction of fractional amounts of EPR spectra of CBS-22-H-SL at 32.5 °C in lipid mixtures or in pure lipids: (E) motionally restricted component in the spectrum of C26-CBS/diC14-PC 8:2 obtained by subtraction of the spectrum of C26-CBS/diC14-PC 1:1; (F) mobile component in the spectrum of C26-CBS/diC14-PC 1:1 obtained by subtraction of the spectrum in C26-CBS/diC14-PC 8:2; (G) mobile component in the spectrum of C24-CBS/diC16-PC 8:2 obtained by subtraction of the spectrum of C26-CBS; and (H) mobile component in the spectrum of C24-CBS/diC18-PC obtained by subtraction of the spectrum of diC18-PC.

C26-CBS/diC14-PC 1:1 sample, C24h-CBS, or the mixtures of C24-CBS with diC14-PC or diC16-PC (Table I).

Effects of an Increase in Temperature on Mixtures. As the temperature was raised, the amount of motionally restricted component in the spectra of both spin labels in the mixtures decreased. At 22.5 °C no restricted component was readily apparent in the spectra of CBS-22-L-SL in any of the mixtures indicated in Table II except the C26-CBS/diC14-PC 9:1 and C24-CBS/diC18-PC mixtures (not shown). With the longer CBS-22-H-SL, however, a motionally restricted component was still apparent at 22.5–32.5 °C in the spectra of all the mixtures including C26-CBS/diC14-PC 1:1 (Figure 11B). However, it was greatest in the mixtures of C26-CBS with diC14-PC and diC16-PC at CBS concentrations of 70% and above and in the C24-CBS/diC18-PC 8:2 mixture (Figure 11A,C). The h_r/h_m ratios support this conclusion (Table II, columns 2 and 3). Some of the spectra at 32.5 °C were resolved into more restricted and more mobile components by spectral subtraction, confirming that slow exchange between two different lipid populations occurred. These spectral components are shown for the C26-CBS/diC14-PC 8:2 and 1:1 samples in Figure 11E,F. The restricted population had more motion than in the mixed interdigitated phase of C26-CBS at this temperature (Figure 8B) but less motion than would be expected for the partially interdigitated phase or for pure diC14-PC (Table I). The latter is in the liquid crystalline phase at this temperature and gives a sharp three-line spectrum characteristic of more isotropic motion. Therefore the restricted population probably resulted from the mixed interdigitated phase but was disordered by the presence of diC14-PC as it was at 7.5 °C. The more mobile population had less motion than diC14-PC but more than the partially interdigitated phase of C24h-CBS (Table I). Its amount

(Table I) could not be accounted for by the concentration of diC14-PC present in the sample, particularly since the phase diagram indicated no significant degree of phase separation occurred. Therefore, it was probably due to the partially interdigitated phase of CBS, which was disordered by the presence of diC14-PC.

The mixtures of C24- or C26-CBS with diC16-PC could be resolved into their more mobile components by subtraction of the spectrum of pure C26-CBS (Figure 8B) but not by subtraction of the spectrum of pure diC16-PC (Figure 11D), indicating that the more restricted component in these samples is due to the mixed interdigitated phase. The more mobile component in these samples (Figure 11G) is more ordered than that in the mixtures with diC14-PC (Figure 11F) but significantly different from pure diC16-PC, suggesting it is due to the partially interdigitated phase.

The spectrum of C24-CBS/diC18-PC 8:2, on the other hand, could not be resolved by subtraction of the spectrum of C26-CBS, only by that of diC18-PC (which resembled that of diC16-PC in Figure 11D at this temperature). It gave the mobile component shown in Figure 11H. This suggested that the motionally restricted population in this sample at 32.5 °C could have been a domain enriched in diC18-PC supporting the phase diagram in Figure 5A, which indicated that phase separation of PC- and CBS-enriched phases still occurred at this composition. However, the amount of restricted component obtained was too large to be accounted for solely by a PC-enriched domain. Therefore, the presence of some mixed interdigitated phase must also have contributed to the restricted population. The mobile spectral component was probably due to a partially interdigitated population but was more ordered than the others. Indeed, the degree of order of the partially interdigitated phase of the CBS/PC mixtures increased with chain length of the PC (Table I).

Thus a gradual transformation of the mixed interdigitated phase to the partially interdigitated phase with an increase in temperature occurred for the CBS/PL mixtures, as for pure CBS. This was probably the cause of the low enthalpy transitions detected by DSC in these samples at temperatures below the main gel to liquid crystalline phase transition. Although the T_m of these transitions was above 40 °C in all cases, higher than indicated by the gradual increase in motion of the spin label, the peaks observed by DSC were broad and the onset temperatures could not be determined. The spin label results indicate further that the mixed interdigitated phase was destabilized by the presence of PL and transformed to the partially interdigitated phase at a lower temperature for the C24-CBS mixtures than for the C26-CBS mixtures. The mixed interdigitated phase was most stable in the C26-CBS/diC14-PC mixtures (Tables I and II). Thus the boundaries between the S_1 and S_2 phases are probably actually at lower temperatures and vary more with the lipid composition than indicated in the phase diagrams, which are based on the DSC data.

DISCUSSION

Although most mammalian membranes contain relatively low concentrations of acidic glycosphingolipids, CBS is present in myelin at a concentration of 4 wt % of the total lipid (Norton & Cammer, 1984). In addition, myelin contains a high content of galactosylceramide, 23 wt % of the total lipid. In spite of also having a high cholesterol content, a DSC study indicates that some of the lipid in myelin, most likely these glycolipids, is in the gel phase at physiological temperature (Johnston & Chapman, 1988). The receptor activity of these glycolipids could depend on whether they are organized into clusters or dissolved into the PL as single molecules (Grant, 1987). Much of the CBS in myelin has acyl chains of 22–26 carbons, longer than the acyl chains of the surrounding PLs. The participation of the long chain species in transbilayer coupling (Schmidt et al., 1978) would depend on whether they are interdigitated, either as individual molecules across the center of the PL bilayer or in a sphingolipid domain. The results of this study help to understand how long chain species of CBS may behave in myelin and other membranes.

Organization of PL-Enriched Gel Phase. The phase diagrams of all of the mixtures except C26-CBS/diC14-PC were indicative of eutectic phase behavior as found for mixtures of asymmetric and symmetric species of PC where the chain lengths of the two species were significantly different (Lin & Huang, 1988; Gardam & Silvius, 1989; Sisk et al., 1990; Bultmann et al., 1991; Sisk & Huang, 1992). C26-CBS/diC14-PC mixtures, on the other hand, had peritectic phase behavior. Peritectic phase behavior was also found by Mason (1988) for mixtures of asymmetric species of PC with symmetric species of similar chain length as the long chain of the asymmetric species and also by Morrow et al (1992) for mixtures of C24-cerebroside with the monounsaturated species 1-stearoyl-2-oleoyl-PC. Although the eutectic or peritectic concentrations could not be determined with any accuracy for the CBS/PC mixtures, it is clear that this behavior occurred over a more limited concentration range, at low CBS concentrations, than in the studies with PC mentioned above, with the exception of C24-CBS/diC18-PC, where it occurred over a wider range. It indicates the formation of two solid solutions, a PC-enriched gel phase and a CBS-enriched gel phase. These are immiscible in the gel phase but miscible in the liquid crystalline phase.

Thus the PL could accommodate only small amounts of CBS in the gel phase, and it destabilized the PC or PE bilayers, lowering their phase transition temperatures in all cases except C26-CBS/diC14-PC. In that case, low concentrations of CBS stabilized the diC14-PC gel phase, raising its transition temperature. The long C26 chain of CBS is most compatible with the bilayer thickness of diC14-PC. If it interdigitates into the other side of the diC14-PC bilayer, it is long enough to nearly span the bilayer and augment van der Waals interactions between the chains. The greatest decreases in T_m of the phospholipid occurred with C24-CBS in diC14-PC and with C26-CBS in diC16-PC, suggesting that the greatest degree of destabilization occurred in these cases. If the acyl chain of CBS interdigitates into the other side of these bilayers, it might destabilize it because it is not long enough to span the PC bilayer in each of these combinations (Figure 12a). It would disrupt the van der Waals interactions between the saturated PC chains because of the bulky terminal methyl group of the fatty acid and might induce gauche isomerization of the PC in order to minimize the chain length difference. However, C24-CBS had less of a destabilizing effect on diC16-PC and diC18-PC than on diC14-PC. Furthermore, diC18-PC could accommodate more C24-CBS than diC14-PC or diC16-PC. If the acyl chain of C24-CBS interdigitated into the other side of the bilayers of the longer chain PC's it should destabilize them more than diC14-PC. The contrary effect observed suggests that in diC18-PC and diC16-PC, the acyl chain of C24-CBS may insert only to the center of the bilayer (Figure 12a, lower half). This might also destabilize it, either by causing PC molecules surrounding the CBS to protrude out of the bilayer or by causing increased gauche isomerization of the CBS acyl chain in order to minimize exposure of the

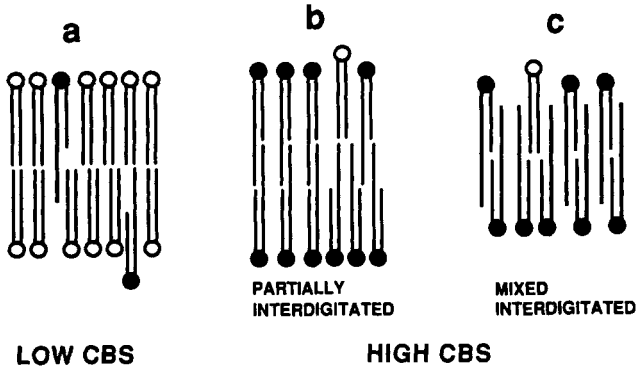

FIGURE 12: Diagrammatic representation of possible modes of packing of symmetric phospholipid molecules with asymmetric CBS molecules. (a) Noninterdigitated gel phase bilayers of symmetric saturated species of PC containing individual molecules of long chain species of CBS, and of (b) partially interdigitated and (c) mixed interdigitated gel phase bilayers of CBS containing individual molecules of symmetric species of PC packed end-to-end with the acyl chain or the sphingosine chain, respectively, of CBS. DiC16-PC and C24- or C26-CBS species are shown with the hydrocaron chains drawn to scale and with the sphingosine chain penetrating 14 carbons into the bilayer, assuming that all chains have a similar degree of trans character as for the pure lipids. However, the fact that the 2-chain of PC penetrates 1.5 carbons less than the 1-chain into the bilayer (Seelig & Seelig, 1990) is not shown. This would reduce the bilayer thickness further. Thus the PC bilayer thickness is actually in between those of diC16-PC and diC18-PC. C24-CBS species are depicted in panel b and in the lower part of panel a. C26-CBS is depicted in panel c and in the top half of panel a. Gauche isomerization and chain tilting may also occur as discussed in the text to minimize chain length differences.

CBS hydrocarbon chains to water. However, less of its hydrocarbon chain would be exposed in the longer chain PC's than in diC14-PC resulting in less destabilization of the former, as observed.

The behavior of CBS-22-L-SL and CBS-22-H-SL in pure PC bilayers was consistent with these conclusions (Boggs & Koshy, 1993). It indicated that the C26 spin label is probably interdigitated across the bilayer center in the gel phase of a diC14-PC bilayer, a diC16-PC bilayer, and possibly a diC18-PC bilayer. The shorter C24 spin label may be interdigitated in diC14-PC but probably not in the longer chain length PC's.

In contrast to its mixtures with diC14-PC, the mixtures of C26-CBS with diC14-PE also had eutectic phase behavior over a limited concentration range despite their expected chain length compatibility. This may be due to disruption of intermolecular hydrogen bonding interactions of phosphatidylethanolamine (PE) by insertion of CBS molecules.

Organization of CBS-Enriched Gel Phase, S_1 and S_2. In S_2 and S_2, which occur above a certain minimal concentration of CBS and over a wide concentration range for all lipid combinations except C24-CBS/di18-PC, CBS and the PL were miscible. Thus CBS bilayers can accommodate the PLs better than the PL bilayers can accommodate CBS. Use of the long chain spin labels shows that C24- and C26-CBS in 0.1 M KCl undergo a transition from the mixed interdigitated to the partially interdigitated gel phase at temperatures well below the gel to liquid crystalline phase temperature. The temperature range over which this transition occurs is similar to that of the broad, low enthalpy transition from 35 to 50 °C observed by DSC. The long chain spin labels were also motionally restricted in mixtures of CBS with PL, indicating that the mixtures also formed the mixed interdigitated bilayer at low temperatures. The presence of the PL disordered the mixed interdigitated phase, with the exception of diC18-PC, which increased the motional restriction of the probe. The greater disorder generally found in the mixtures may account for the inability of the shorter chain spin label 16-S-SL to detect this phase in the mixtures (Boggs et al., 1990).

Like pure CBS, the mixtures also transformed to a partially interdigitated gel phase, S_2, in endothermic transitions which could usually be detected by DSC. Thus both the mixed interdigitated and partially interdigitated gel phases of CBS could accommodate both PC and PE with fatty acid chains of 14–18 carbons in length, with a decrease in the amount of PC incorporated when the PC chain length was increased to 18 carbons. Individual molecules of PC might be incorporated into a partially interdigitated bilayer of CBS by packing end-to-end with the CBS acyl chain as in Figure 12b or into a mixed interdigitated bilayer by packing end-to-end with the short sphingosine chain (Figure 12c). DiC14-PC might be more compatible with CBS in either type of organization than the longer chain length PC's, since the polar head groups of both lipids would be located on the same plane in the former case but not in the latter. The temperature of the transition from the mixed interdigitated to the partially interdigitated gel phase was lower for the mixtures than for pure CBS, and, even at 7.5 °C, the mixtures were partly in the partially interdigitated phase. Thus the presence of PL destabilized the mixed interdigitated bilayer of CBS. Even in cases where the PL was expected to fit into the CBS bilayer most readily, as in the C26-CBS/diC14-PC mixtures, the mixed interdigitated bilayer was destabilized. The mixed interdigitated bilayer may involve a high degree of cooperative, long range organization which is disrupted by the presence of other molecules. Stabilization of the partially interdigitated bilayer by intermolecular hydrogen bonding must outweigh the disruptions in packing and van der Waals interactions (Figure 12b) which must be caused by the presence of PL molecules.

The mixtures of PL with C26-CBS remained in the mixed interdigitated phase to a higher temperature and thus were more stable in the mixed interdigitated phase than those with C24-CBS with the exception of the C24-CBS/diC18-PC mixture. This may be due to the greater asymmetry of C26-CBS compared to C24-CBS. C26-CBS/diC14-PC had the greatest stability in the mixed interdigitated phase of all the combinations studied, as expected because the acyl chain length of di14-PC is most compatible with the mixed interdigitated phase. Although diC14-PE has similar chain length compatibility with CBS, it stabilized the partially interdigitated bilayer more than diC14-PC. This may be due to the occurrence of electrostatic interactions or intermolecular hydrogen bonding between PE and CBS in the partially interdigitated bilayer. These interactions would be less likely to occur in the mixed interdigitated bilayer. However, low concentrations of diC14-PE decreased the T_m value of C26-CBS on reheating relative to the value on the first heating scan. Thus it had some disruptive effect which diC14-PC did not have.

DiC18-PC is not expected to pack as well into either the mixed interdigitated or partially interdigitated bilayers of CBS as shorter chain PC's. In fact, S_1 and S_2 of the C24-CBS/diC18-PC mixture accommodate less PC than in the case of the other mixtures. However, the spin label results indicated that diC18-PC stabilized the partially interdigitated bilayer of C24-CBS less than the shorter chain PC's did. The percent partially interdigitated phase at 32.5 °C was greater for the mixtures of C24-CBS with shorter chain PC's than with diC18-PC. This may be due to inhibition of hydrogen bonding in the partially interdigitated phase by diC18-PC because of protrusion of the PC head groups above the plane of the CBS

head groups. This is supported by the fact that 10% diC18-PC lowers the T_m of the partially interdigitated gel to liquid crystalline phase transition of CBS on reheating.

Coexistence of the PC-Enriched and CBS-Enriched Gel Phases. At low to intermediate CBS concentrations, the noninterdigitated phospholipid gel phase bilayer can coexist with either the mixed interdigitated S_1 phase or the partially interdigitated S_2 phase, depending on the T_m of the phospholipid. Furthermore, both the $P_{\beta'}$ and the tilted $L_{\beta'}$ PC phases can coexist with S_1 and S_2. Since the 2-chain of PC is bent, giving it an effective bilayer penetrating length of 2 carbons less than the 1-chain (Seelig & Seelig, 1980), the bilayer hydrocarbon thickness of the three species of PC is 26, 30, and 34 carbons, respectively. Assuming that the sphingosine chain of CBS penetrates into the bilayer by 13–14 carbons and that the fatty acid chain penetrates its full length, the bilayer hydrocarbon thickness of C24 and C26-CBS should be 38 and 40 carbons, respectively, when partially interdigitated. Domains of this type of bilayer (Figure 12b) should then be most compatible with domains of diC18-PC bilayer and least with diC14-PC. In the mixed interdigitated bilayer structure (Figure 12c), the bilayer hydrocarbon thickness of both C24- and C26-CBS is probably determined by the sphingosine length and thus should be about 28 carbons for both. This is more compatible with the bilayer thickness of diC14-PC and least with diC18-PC. However, the presence of a noninterdigitated bilayer of phospholipid did not stabilize the mixed interdigitated bilayer of CBS, even in the case of diC14-PE, which has a bilayer thickness similar to that of the mixed interdigitated phase of CBS and a high enough T_m to coexist with either S_1 or S_2. Nor does a noninterdigitated bilayer of diC18-PC stabilize the partially interdigitated phase with which it would be more compatible than the mixed interdigitated phase. Furthermore, phase separation occurs in this concentration range even when the dimensions of the PL and CBS gel phases are similar, suggesting that it is due to factors other than bilayer thickness of the two phases. These may include the large difference in structure of the two lipids, the high degree of organization required to form the mixed interdigitated and partially interdigitated bilayers, and the forces which stabilize these phases. These cause them to pack much better with like molecules than with different ones despite a relative similarity in length of one chain.

Using mixtures of an asymmetric lipid which forms a mixed interdigitated bilayer, such as 1-C22,2-C12-PC, with a symmetric lipid, diC17-PC, where the sum of the lengths of the two chains of each species is identical, Huang and coworkers also found that the presence of a noninterdigitated bilayer domain of the symmetric PC did not influence the structural organization of the asymmetric lipid. That is, it did not cause the asymmetric lipid to form a partially interdigitated bilayer, except at low concentrations of the asymmetric lipid, even though the bilayer thickness of the symmetric lipid was similar to that expected for a partially interdigitated bilayer of the asymmetric lipid and considerably greater than that of the mixed interdigitated bilayer (Lin & Huang, 1988; Sisk et al., 1990; Sisk & Huang, 1992; Slater et al., 1992). Thus lipid domains of greatly disparate bilayer thickness can coexist side by side in the same bilayer despite the resulting exposure of hydrocarbon chains at the interfaces. On the other hand, lipids which form completely interdigitated bilayers, either by themselves or in the presence of an amphipathic compound, have been found to influence other lipids of similar chain length to also become interdigitated, providing that they do not participate in intermolecular hydrogen bonding (Lohner et al., 1987; Boggs et al., 1989). In these cases both lipids in the mixture were symmetric chain lipids.

Organization of Liquid Crystalline Phase, L. CBS and the phospholipids generally appeared to be completely miscible in the liquid crystalline phase in all proportions. Greater accommodation of the acyl chain lengths of both lipids to each other and to the bilayer thickness is possible than in the gel phase as a result of alteration in the degree of gauche/trans isomerization (Sankaram & Thompson, 1992). The long chain CBS spin labels had isotropic motion in the liquid crystalline phase of all the PL's, similar to that of short chain CBS spin labels with the nitroxide near the terminal methyl, suggesting that the long acyl chain is not interdigitated into the other side of the bilayer (Boggs & Koshy, 1993). Rather it most likely terminates at the center of the bilayer. Increased gauche isomerization of the CBS chain or trans isomerization of the chains of the surrounding PC molecules might occur, minimizing protrusion of the CBS head group out of the PC bilayer. However, some degree of protrusion is indicated by the fact that the carbohydrate head group of long chain species of glycolipids in liquid crystalline PC bilayers is more exposed at the bilayer surface than that of short chain species (Alving & Richards, 1977; Crook et al., 1986; Stewart & Boggs, 1990, 1993).

Thus the results suggest that, at 4 wt %, CBS is most likely miscible with the other lipids in myelin. If these are in the gel phase, it might interdigitate across the bilayer center, depending on the bilayer thickness of the surrounding lipids. This could allow transbilayer coupling. On the other hand, if the CBS is in liquid crystalline phase domains of myelin lipids, it probably does not interdigitate across the bilayer center, thus increasing its potential receptor activity.

ACKNOWLEDGMENT

We thank Dr. M. Morrow, Memorial University of Newfoundland, for making helpful suggestions.

REFERENCES

Alving, C. R., & Richards, R. L. (1977) *Immunochemistry 14*, 373–381.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Boggs, J. M., & Rangaraj, G. (1985) *Biochim. Biophys. Acta 816*, 221–233.

Boggs, J. M., & Mason, J. T. (1986) *Biochim. Biophys. Acta 863*, 231–242.

Boggs, J. M., & Koshy, K. M. (1993) *Biochim. Biophys. Acta* (submitted).

Boggs, J. M., Stamp, D. & Moscarello, M. A. (1981) *Biochemistry 20*, 6066–6072.

Boggs, J. M., Koshy, K. M., & Rangaraj, G. (1984) *Chem. Phys. Lipids 36*, 65–89.

Boggs, J. M., Koshy, K. M., & Rangaraj, G. (1988a) *Biochim. Biophys. Acta 938*, 361–372.

Boggs, J. M., Koshy, K. M., & Rangaraj, G. (1988b) *Biochim. Biophys. Acta 938*, 373–385.

Boggs, J. M., Rangaraj, G., & Watts, A. (1989a) *Biochim. Biophys. Acta 981*, 243–253.

Boggs, J. M., Wang, H.-Y., Rangaraj, G., & Tümmler, B. (1989b) *Biochim. Biophys. Acta 985*, 199–210.

Boggs, J. M., Mulholland, D., & Koshy, K. M. (1990) *Biochem. Cell Biol. 68*, 70–82.

Bultmann, T., Lin, H., Wang, Z., & Huang, C. (1991) *Biochemistry 30*, 7194–7402.

Brotherus, J. R., Jost, P. C., Griffith, O. H., Keana, J. F. W., & Hokin, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 272–276.

Crook, S. J., Boggs, J. M., Vistnes, A. I., & Koshy, K. M. (1986) *Biochemistry 25*, 7488–7494.
Dahlen, B., & Pascher, I. (1979) *Chem. Phys. Lipids 24*, 119–133.
Gardam, M., & Silvius, J. R. (1989) *Biochim. Biophys. Acta 980*, 319–325.
Grant, C. W. M. (1987) in *Gangliosides and Modulation of Neuronal Functions* (Rahman, H., Ed.) Vol. H7, p 119, NATO ASI Series, Springer-Verlag, Berlin.
Hakomori, S.-I. (1981) *Annu. Rev. Biochem. 50*, 733–764.
Hui, S. W., Mason, J. T., & Huang, C. (1984) *Biochemistry 23*, 5570–5577.
Johnston, D. S., & Chapman, D. (1988) *Biochim. Biophys. Acta 939*, 603–614.
Kean, E. L. (1968) *J. Lipid Res. 9*, 319–327.
Koshy, K. M., & Boggs, J. M. (1983) *Chem. Phys. Lipids 34*, 41–53.
Levin, I. W., Thompson, T. E., Barenholz, Y., & Huang, C. (1985) *Biochemistry 24*, 6282–6286.
Lin, H., & Huang, C. (1988) *Biochim. Biophys. Acta 946*, 178–184.
Lohner, K., Schuster, A., Degovics, G., Muller, K., & Laggner, P. (1987) *Chem. Phys. Lipids 44*, 61–70.
Mabrey, S., & Sturtevant, J. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3862–3866.
Mason, J. T. (1988) *Biochemistry 27*, 4421–4429.
Maulik, P. R., Atkinson, D., & Shipley, G. G. (1986) *Biophys. J. 50*, 1071–1077.
McIntosh, T. J., Simon, S. A., Ellington, J. C., Jr., & Porter, N. A. (1984) *Biochemistry 23*, 4038–4044.
Morrow, M. R., Singh, D., Lu, D., & Grant, C. W. M. (1992) *Biochim. Biophys. Acta 1106*, 85–93.
Norton, W. T., & Cammer, W. (1984) in *Myelin* (Morell, P., Ed.) 2nd ed., pp 147–195, Plenum Press, New York.
O'Brien, J. S., Fillerup, D. L., & Mead, J. F. (1964) *J. Lipid Res. 5*, 109–116.
Sankaram, M. B., & Thompson, T. E. (1992) *Biochemistry 31*, 8258–8268.
Schmidt, C. F., Barenholz, Y., Huang, C., & Thompson, T. E. (1978) *Nature 271*, 775.
Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.
Sisk, R. B., & Huang, C. (1992) *Biophys. J. 61*, 593–603.
Sisk, R. B., Wang, Z., Lin, H., & Huang, C. (1990) *Biophys. J. 58*, 777–783.
Slater, J. L., Huang, C., & Levin, I. W. (1992) *Biochim. Biophys. Acta 1106*, 242–250.
Stevenson, C. C., Rich, N. H., & Boggs, J. M. (1992) *Biochemistry 31*, 1875–1881.
Stewart, R. J., & Boggs, J. M. (1990) *Biochemistry 29*, 3644–3653.
Stewart, R. J., & Boggs, J. M. (1993) *Biochemistry 32*, 5605–5614.
Stinson, R. H., & Boggs, J. M. (1989) *Biochim. Biophys. Acta 986*, 234–240.
Wang, H.-Y., Tümmler, B., & Boggs, J. M. (1989) *Biochim. Biophys. Acta 985*, 182–198.