Thermotropic Behavior of Mixtures of Glycosphingolipids and Phosphatidylcholine: Effect of Monovalent Cations on Sulfatide and Galactosylceramide†

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ABSTRACT: The thermotropic behavior of both sulfatide (3-sulfogalactosylceramide) and galactosylceramide in dielaidoylphosphatidylcholine (DEPC) liposomes was studied, using steady-state fluorescence polarization of parinaric acid isomers. The glycosphingolipid (GSL) concentration of the liposomes was varied from 0 to 100%, and phase diagrams were constructed. The data indicate that sulfatide and DEPC are immiscible in the gel phase at sulfatide mole ratios of less than 0.30. The temperature of onset of the gel–liquid-crystalline phase transition is higher in K^+-containing buffer than in osmotically equal Na^+-containing buffer. Similar measurements, using galactosylceramide, a neutral GSL, indicated that this lipid and DEPC are immiscible in the gel phase at galactosylceramide mole ratios of less than 0.40. In contrast to the results obtained with sulfatide, onset temperatures are identical in Na^+- or K^+-containing buffers. The phase properties of sulfatide/DEPC mixtures are shown to depend on the cation only when the sulfatides contain hydroxy fatty acids. Our observations indicate that physiologically relevant concentrations of monovalent cations affect motion and distribution of sulfatide in biological membranes and further implicate this GSL as an important determinant of function of the Na^+,K^+-ATPase. A preliminary report of these data has appeared elsewhere [Rintoul, D. A., Welti, R., & Song, W. (1988) *Biophys. J. 53*, 126a].

Glycosphingolipids are ubiquitous components of the outer leaflet of eukaryotic plasma membranes. This location and the abundance of different carbohydrate structures found on these cell surface lipids implicate GSLs[1] as important determinants of surface membrane function. In recent years, GSLs have been associated with various membrane-mediated functions, including differentiation, transformation, metastasis, and immune system functions [for review, see Hakomori (1981, 1986) and Curatolo (1987b)]. In addition, the negatively charged GSLs, including sulfatide and the gangliosides, have been shown to have ion-binding potential by a variety of techniques (Curatolo, 1987a). This property and a positive correlation between sulfatide content and Na^+,K^+-ATPase content in eukaryotic membranes [for review, see Hansson et al. (1979)] have led to the hypothesis that sulfatides are involved in potassium binding during the transport of sodium and potassium by the Na^+,K^+-ATPase (EC 3.6.1.3). Koshy and Boggs (1983) found that the transition temperature of *N*-palmitoyl sulfatide, as measured by differential scanning calorimetry, is higher in potassium-containing buffers than in equivalent sodium-containing buffers. Furthermore, it has been shown recently (Boggs et al., 1984) that the phase behavior of several synthetic sulfatides is altered in high-K^+ buffers; both the temperature and the enthalpy of the transition are higher in 2 M K^+ than in either 2 M Na^+ or 2 M Li^+. However, no information exists regarding monovalent cation effects on the thermotropic behavior of this lipid in mixtures with phospholipids. Similarly, the thermotropic behavior of the uncharged GSL, galactosylceramide, has not been studied in aqueous dispersions containing various cations. The present study was designed to fill this gap in the available information regarding GSL–phospholipid interactions. We have used fluorescence spectroscopic techniques and parinaric acid probes of membrane structure in order to further elucidate the interactions of GSLs with physiological concentrations of cations in artificial phosphatidylcholine membranes. We have determined that the transition temperature of mixtures of sulfatide and DEPC is higher in potassium-containing buffer than in osmotically equal sodium-containing buffer. This effect is confined to sulfatides that contain hydroxy fatty acids. In addition, our observation that a negatively charged GSL is immiscible in the gel phase with phosphatidylcholine is contrary to the hypothesis that only neutral GSLs would exhibit this behavior (Thompson & Tillack, 1985). The immiscibility suggests that domains of sulfatide may exist in biomembranes, leading to interesting ramifications regarding ion or ligand (Roberts et al., 1986) binding to this cell surface molecule. These observations will aid us in examining the interaction(s) of sulfatide with the Na^+,K^+-ATPase during ion transport.

Materials and Methods

Reagents and Supplies. Parinaric acid isomers were purchased from Molecular Probes (Junction City, OR) and used without further purification. Sulfatide and galactosylceramide were purchased from Sigma Chemical Co. (St. Louis, MO), characterized by TLC, and used without further purification. DEPC was purchased either from Sigma or from Avanti Polar Lipids, Inc. (Pelham, AL). The DEPC purchased from Sigma was found to be contaminated with approximately 10% of a lysophospholipid. Pure DEPC was prepared by preparative

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[1] Abbreviations: GSL, glycosphingolipid; DEPC, dielaidoylphosphatidylcholine (di-9-*trans*-octadecenoylphosphatidylcholine); DPPC, dipalmitoylphosphatidylcholine (dihexadecanoylphosphatidylcholine); cPnA, *cis*-parinaric acid (9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid); tPnA, *trans*-parinaric acid (9,11,13,15-*all-trans*-octadecatetraenoic acid); HFA, 2-hydroxy fatty acid; HPTLC, high-performance thin-layer chromatography; NFA, normal fatty acid; PC, phosphatidylcholine; PA, phosphatidic acid; TLC, thin-layer chromatography.

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TLC on silica gel G; the solvent system was chloroform/methanol/ammonium hydroxide (65/25/5 v/v/v). DEPC from Avanti was characterized by TLC and used without further purification. Sulfatide fractions containing only hydroxy fatty acid (HFA) or normal fatty acid (NFA) were prepared by preparative TLC. Commercially available mixed sulfatide (8–12 mg) was spotted on a preabsorbent 500-μm silica gel G TLC plate (Analtech, Inc., Newark, DE). Plates were developed in chloroform/methanol/ammonium hydroxide (65/25/5 v/v/v), dried in the fume hood, and sprayed with distilled water to visualize the lipid bands. Bands were marked and scraped from the plate; the silica gel was extracted twice with 11.5 mL of chloroform/methanol/water (5/5/1.5 v/v/v). The extracts were dried under a stream of nitrogen, resuspended in chloroform/methanol (1/1 v/v), and analyzed by analytical TLC using HPTLC plates (Analtech) and the same solvent system as described above for the preparative TLC. Purity was assessed by spraying the HPTLC plates with 50% sulfuric acid in water, charring for several hours at 120 °C, and scanning with a Kontes densitometer. These analyses indicated that the HFA-sulfatide fraction containing 2–5% NFA sulfatide; NFA-sulfatide fractions typically contained no detectable HFA sulfatide (data not shown). The HFA and NFA sulfatide fractions were assayed for sphingosine by using a modification of the method of Naoi et al. (1974). Phospholipase D was prepared from Savoy cabbage, purchased locally, by using the method of Yang (1969). All solvents were HPLC grade and were purchased from Fisher Scientific (St. Louis, MO).

Preparation of Liposomes. Phospholipid, with or without varying amounts of glycosphingolipid (200 nmol total), was dried under nitrogen from chloroform/methanol (2/1 v/v) solutions and further dried in vacuo in a glass desiccator for 15–20 min. The residue was dissolved in 10 μL of absolute ethanol containing 1.0 nmol of parinaric acid. This solution was injected into 2.5 mL of buffer (50 mM HEPES, pH 7.2, and either 100 mM KCl or 100 mM NaCl) while vortexing at a temperature at least 15 °C above the phase-transition temperature of the lipid mixture (Batzri & Korn, 1973). These unilamellar liposomes were then used for measurements of steady-state fluorescence anisotropy as described previously (Welti & Silbert, 1982; Rintoul et al., 1986). Analysis of liposomes prepared by this technique indicated that the final preparations contained 95–100% of the original phospholipid (as assayed by phosphate content) and 98–100% of the original glycosphingolipid (as assayed by sphingosine content).

Fluorescence Anisotropy Measurements. Liposomes, prepared as described above, were placed in a 3.0-mL quartz cuvette in the thermostated, temperature-controlled cuvette chamber of a Spex 1902 Fluorolog spectrofluorometer. This instrument was equipped with quartz polarizers obtained from Spex (Edison, NJ). The limiting fluorescence anisotropy (for fluorescein in alkaline glycerol at 10 °C; Chen & Bowman, 1965) was 0.35 for this instrument. The samples were excited at 320 nm when tPnA was used and at 325 nm when cPnA was used; bandpass for both excitation wavelengths was 5.0 nm. Fluorescence emission was monitored at 420 nm (bandpass 20.0 nm) for both probes. Fluorescence emission parallel and perpendicular to the vertically polarized excitation light was monitored as the sample was cooled at a rate of 0.75 °C/min, controlled by a circulating water bath and linear temperature programmer. Data were collected and analyzed by using a computer curve-fitting and smoothing program previously described (Welti et al., 1981). Steady-state fluorescence anisotropy (r) of the parinaric acid probes, as measured by this technique, is correlated with membrane lipid order (Sklar, 1976; Welti & Silbert, 1982). This ratio can vary from a theoretical minimum of 0 (completely anisotropic motion of the probe) to a theoretical maximum of 0.4 (essentially no probe motion during the lifetime of the excited state). No corrections for scattering depolarization were required for any of the experiments described in this paper, as preparations of liposomes prepared by these techniques are optically clear at low lipid concentrations. Since the ethanol injection method, as used for the preparation of these liposomes, yields small, unilamellar vesicles, the transition widths observed for pure lipids (e.g., pure DEPC) are broader than those reported for large vesicles of the same lipids. Data obtained while the samples were heated were equivalent to those obtained while the samples were cooled for all samples examined, including those containing 100% of either of the glycosphingolipids. These observations imply that the structures formed are at equilibrium and do not exhibit metastable behavior at these scan rates and in these temperature ranges.

Phospholipase D Hydrolysis. Liposomes, prepared as described above but containing 100 nmol of phospholipid and varying amounts of glycosphingolipid, were incubated with 25 mg of crude phospholipid D for 1 h at room temperature. The buffer used in these experiments was identical with the KCl-containing buffer used for fluorescence measurements, with the addition of 0.2 M calcium chloride, which is required by the phospholipase. The reaction mixtures were extracted with 9 volumes of chloroform/methanol (2/1); a two-phase system was formed by the addition of 2 volumes of 0.88% (w/v) KCl containing 0.01 N HCl. After centrifugation at 1000g at room temperature, the lower (chloroform) phase was collected and the solvent was evaporated under nitrogen. The residue was dissolved in a small volume of chloroform/methanol (2/1); aliquots were applied to a TLC plate (250-μm-thick silica gel G) along with appropriate standards and developed in chloroform/methanol/water (65/25/5). The plate was sprayed liberally with 50% H_2SO_4 in water (v/v) and charred at 140 °C for several hours to visualize the lipid-containing regions. These charred spots were identified by comparison to the standards and removed from the plate by scraping. These silica gel fractions containing hydrolyzed phospholipid were assayed for inorganic phosphate content as described by Ames (1966); standard curves were constructed by using DEPC spotted on TLC plates, charred and scraped as described.

Analytical Methods. Fatty acid compositions of the lipids used in this study were determined by gas–liquid chromatography of the fatty acid methyl esters after transesterification of lipid in 1 N methanolic HCl and extraction with pentane as previously described (Rintoul et al., 1979). Phosphate was assayed as described by Ames (1966). Parinaric acid concentrations were determined by absorbance, using known extinction coefficients (Sklar, 1976).

Results

Phase diagrams were constructed as described by Sklar et al. (1979) and Welti (1982). This involved cooling the samples and determination of the onset temperature of the thermotropic phase transition using tPnA; this temperature was assumed to be the highest temperature at which solid-phase lipid was present. Determination of the temperature at which no further change in anisotropy could be detected with cPnA was assumed to be the lowest temperature at which fluid-phase lipid was present. Thus, these data were used to define the solidus and fluidus lines of the phase diagram, respectively. All data shown are averages of duplicate and, in most cases, triplicate de-

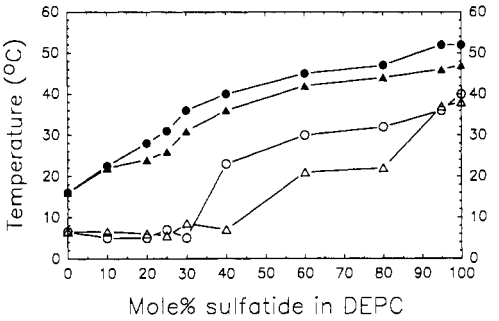

FIGURE 1: Phase diagram of DEPC and sulfatide: effect of monovalent cations. Mixtures of DEPC and sulfatide were prepared and analyzed as described under Materials and Methods. The samples were cooled from 60 to 1 °C at a rate of 0.75 °C/min; fluorescence emission was monitored throughout the cooling cycle. Samples were prepared in HEPES-buffered (50 mM, pH 7.2) solutions containing 100 mM NaCl (triangles) or 100 mM KCl (circles). Steady-state fluorescence anisotropy was calculated and smoothed curves were generated by the computer program previously described (Welti et al., 1981); data shown are averages of duplicate or triplicate analyses. Transition onsets were determined by using *trans*-parinaric acid (filled symbols); transition completions were determined by using *cis*-parinaric acid (open symbols).

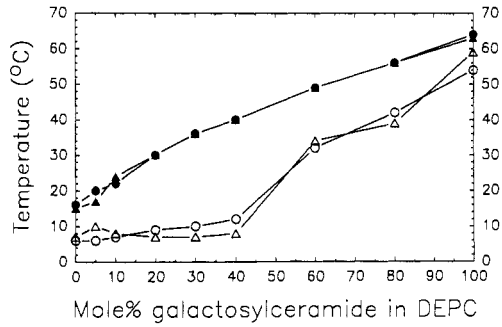

FIGURE 2: Phase diagram of DEPC and galactosylceramide: effect of monovalent cations. Mixtures of DEPC and galactosylceramide were prepared and analyzed as described in the legend to Figure 1, except that all samples were prepared at 80 °C and cooled from 80 to 1 °C at a rate of 0.75 °C/min. Data shown are averages of duplicate or triplicate analyses. Triangles represent data from samples prepared in NaCl buffer; circles represent data from samples prepared in KCl buffer. Open symbols are transition completions determined by using *cis*-parinaric acid; filled symbols are transition onsets determined by using *trans*-parinaric acid.

terminations. The phase diagrams for bovine brain sulfatide and DEPC, in buffer containing either 100 mM KCl or 100 mM NaCl, are shown in Figure 1. The fluidus line for sulfatide concentrations at and above 20 mol % is at a higher temperature in the KCl buffer than in the NaCl buffer. In addition, the solidus line is horizontal below 30 mol % sulfatide in potassium-containing buffer; the horizontal portion extends to 40 mol % in the sodium-containing buffer, indicating immiscibility of sulfatide and PC in the solid phase. These data indicate that transition onset and completion temperatures of mixtures of DEPC and sulfatide are influenced by relatively low concentrations of monovalent cations.

In order to determine if this monovalent cation effect was specific for sulfatide, we constructed a phase diagram for galactosylceramide and DEPC under similar conditions. These data are shown in Figure 2. While these lipids also exhibit solid-phase immiscibility at low GSL mole ratios, transition onset and completion temperatures of these mixtures were similar in potassium- and sodium-containing buffers, in contrast to the data in Figure 1. Since sulfatide is a sulfated galactosylceramide, these data suggest that the negatively charged sulfate on the head group is the basis for the cation specificity shown in Figure 1.

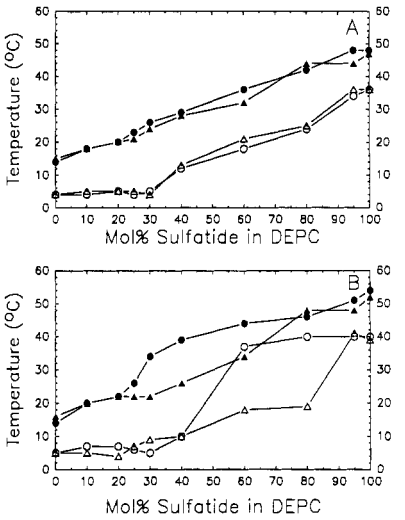

FIGURE 3: Phase diagram of DEPC and sulfatides containing non-hydroxy fatty acids (A) or hydroxy fatty acids (B): effect of monovalent cations. Sulfatide fractions containing either HFA or NFA were prepared by preparative thin-layer chromatography as described under Materials and Methods and assayed for sphingosine content. Liposomes containing DEPC and NFA sulfatides (A) or DEPC and HFA sulfatides (B) were prepared and analyzed by the procedures described in the legend to Figure 1; buffer solutions contained 50 mM HEPES (pH 7.2) and either 100 mM NaCl (triangles) or 100 mM KCl (circles). Data shown are averages of duplicate determinations. Transition completions were determined with *cis*-parinaric acid (open symbols); transition onsets were determined with *trans*-parinaric acid (filled symbols).

Previous workers had noted that glycosphingolipids containing hydroxy fatty acids have significantly different physical properties than similar glycosphingolipids containing normal fatty acids [for review, see Curatolo (1987a)]. Since the sulfatide used in the experiments shown in Figure 1 contained approximately 35% hydroxy fatty acid species, it was of interest to determine if the cation effect seen in liposomes containing DEPC and mixed acyl-chain sulfatides could be detected in both purified HFA and NFA sulfatides. Sulfatides were separated into hydroxy and non-hydroxy species by preparative TLC. The phase diagrams for these purified sulfatides and DEPC are shown in Figure 3. It is apparent that the NFA sulfatides (Figure 3A) exhibited similar phase properties in either potassium- or sodium-containing buffers. However, the HFA species, which are the minor species in the mixed sulfatides used to obtain the data in Figure 1, had significantly different phase behavior in these two buffers (Figure 3B). In particular, transition onset temperatures are significantly higher in potassium-containing buffers at ratios greater than 20 mol %, and transition completion temperatures are greatly increased in potassium-containing buffer at sulfatide concentrations between 40 and 90 mol %. It also should be noted that the transition temperatures of the two types of purified sulfatides were different; HFA sulfatides melted between 40 and 54 °C (in KCl buffer); NFA sulfatides melted between 36 and 48 °C in the same buffer. Furthermore, the fatty acid compositions of the mixed sulfatide and the mixed galactosylceramide were drastically different (Table I). Specifically, most of the fatty acid in the galactosylceramide used in this experiment consisted of relatively short chain species, while long-chain species dominated the fatty acid composition of the sulfatide. Additionally, TLC analysis indicated that this lot of galactosylceramide contained only 22% HFA. In order to eliminate the possibility that the lack of cation effect in DEPC/galactosylceramide dispersions was due to these differences in fatty acid composition, similar experiments, using

Table I: Fatty Acid Composition of Glycosphingolipids[a]

sample	% HFA	16:0	18:0	18:1	18:2	20:0	20:1	22:0	22:1	24:0	24:1	>24 carbons
sulfatide (mixed)	35	1.85	4.79	0.37	0.12	0.58	0.30	3.0	0.04	3.03	60.11	25.8
sulfatide (non-OH)		0.7	2.94		0.1	0.41	0.17	5.15	0.36	1.07	76.4	13.8
sulfatide (OH)		4.03	9.69	0.1	0.6	0.4	0.3	3.1	0.6	16.2	38.8	26.0
GalCer (mixed)	22	4.3	56.0			0.15		6.6				33.0
GalCer (non-OH)		2.02	5.3	1.29	0.67	0.26	0.98	6.5	5.0		53.3	24.6
GalCer (OH)		2.8	2.52	2.03	1.25		0.3	0.84		67.53		22.5

[a] Hydroxy and non-hydroxy sulfatides were purified by preparative TLC from the mixed sulfatides, as described under Materials and Methods. Hydroxy and non-hydroxy galactosylceramides were purchased from Sigma, as was the galactosylceramide mixture; 200 nmol of each lipid was transesterified for 5 h at 70 °C in 1 M methanolic HCl. Fatty acids were extracted with pentane; hydroxy fatty acids were acetylated in 5:1 pyridine–acetic anhydride mixture overnight at room temperature. After evaporation of these solvents, aliquots of the fatty acid methyl esters were analyzed on a 6-ft SP-2330 GLC column at 185 °C. Carrier gas used was helium at 40 psi. Amounts of each methyl ester were determined by using a Spectra-Physics chromatography integrator; identification was based on retention times of known standards and log-linear extrapolations of the retention times of these standards.

HFA- and NFA-galactosylceramides (fatty acid composition shown in Table I) and DEPC, were performed as described for the sulfatide/DEPC mixtures. Phase diagrams constructed for HFA-galactosylceramide and DEPC were nearly identical in K^+- and Na^+-containing buffers. Similarly, phase diagrams for mixtures of NFA-galactosylceramide and DEPC were unaffected by the species of monovalent cation present.

Previous investigators have disagreed on the micellar or lamellar nature of pure sulfatide dispersions or dispersions of sulfatide and PC [for review, see Curatolo (1987a)]. Therefore, we investigated this question in ethanol-injected dispersions, using phospholipase D as a probe of phospholipid exposure to the aqueous phase. It was reasoned that this enzyme, which converts phospholipids such as PC to phosphatidic acid (PA), would not penetrate a closed lamellar vesicle. Half of the PC in lamellar phase structures should be hydrolyzed by this enzyme, whereas all of the PC should be converted to PA if the structures were micellar. Mixtures of DEPC and glycosphingolipid (containing a constant amount of PC) were prepared and incubated with enzymes as described under Materials and Methods. Results are shown in Figure 4. Similar preparations, incubated with 1.0 mL of diethyl ether, showed 100% hydrolysis of the phospholipid, indicating that the enzyme was capable of completely hydrolyzing this amount of phospholipid. The presence of phosphatidylethanol in the reaction mixtures can be attributed to transphosphatidylation reactions utilizing the ethanol remaining from the injection step. It can be seen that approximately 50% of the phospholipid was hydrolyzed in preparations containing up to 90% glycosphingolipid. Although there are differences between the preparations containing galactosylceramide and those containing sulfatide, in general there were no major breaks in the curves which would indicate a transition from lamellar to a micellar phase. These data are consistent with the hypothesis that ethanol-injected dispersions of these glycosphingolipids and DEPC are lamellar at all glycosphingolipid levels up to 90 mol %. In addition, the level of hydrolysis attained (approximately 50%) indicates that, at all glycosphingolipid concentrations and with both glycosphingolipids, these structures are unilamellar vesicles.

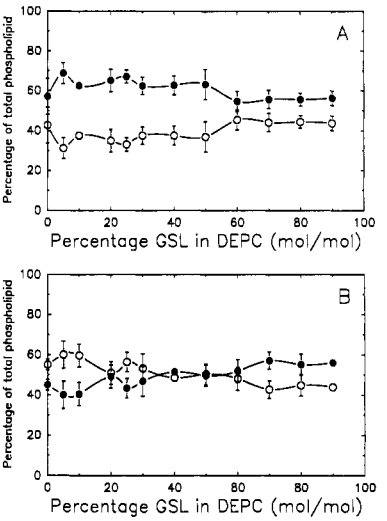

FIGURE 4: Use of phospholipase D to determine orientation of PC in mixtures of DEPC and glycosphingolipids. Liposomes containing DEPC and either galactosylceramide (A) or sulfatide (B) were prepared in 50 mM HEPES and 100 mM KCl by using DEPC (100 nmol) and various amounts of glycosphingolipid. These preparations were digested with 25 mg of crude phospholipase D, extracted, and analyzed as described under Materials and Methods. Data shown are means and standard deviations of four separate experiments. Open symbols represent phosphatidylcholine; filled symbols represent phosphatidic acid and phosphatidylethanol.

Discussion

Gel-Phase Immiscibility of GSL and DEPC. The phase diagrams shown in Figures 1 and 2 indicate that both sulfatide and galactosylceramide exhibit gel-phase immiscibility with DEPC at levels of GSL below 30 mol %. Previous workers [reviewed by Thompson and Tillack (1985) and Curatolo (1987a)] observed similar phase behavior in mixtures of galactosylceramide and phosphatidylcholine (PC). In mixtures of a disaturated PC [diplamitoylphosphatidylcholine (DPPC)] and bovine brain galactosylceramide, Maggio et al. (1985) found gel-phase immiscibility below 40 mol % galactosylceramide. Using a mixed-acid lecithin (1-palmitoyl-, 2-oleoyl-PC) and bovine brain galactosylceramide, Curatolo (1986) observed gel-phase immiscibility below 70 mol % galactosylceramide. The observation that sulfatide and DEPC exhibit gel-phase immiscibility (Figure 1) is somewhat surprising, since the bulk of previous evidence would indicate that charged glycosphingolipids (particularly gangliosides) are miscible in PC bilayers [for review, see Thompson and Tillack (1985)]. Our data suggest that glycosphingolipid head-group size as well as charge may be an important determinant of domain formation in simple PC bilayers. In the case of sulfatide, the ionic composition of the aqueous phase is an additional factor that can affect this clustering (see discussion below). The observation of gel-phase immiscibility in sulfatide/DEPC systems indicates that this glycosphingolipid behaves similarly to neutral glycosphingolipids such as galactosylceramide or lactosylceramide. An implication of this behavior is that sulfatide might exist as gel-phase domains in otherwise fluid biomembranes; changes in the extracellular ion composition might modulate this behavior.

Effects of Monovalent Cations on Sulfatide/DEPC Phase Properties. Previous workers have observed that sulfatide dispersions exhibit thermotropic gel to liquid-crystalline phase

transitions and that monovalent cations exert a significant effect on the behavior of these dispersions (Boggs et al., 1984). Free hydroxyl groups, which are found in both the fatty acid and the sphingosine portion of brain glycosphingolipids (Svennerholm & Stallberg-Stenhagen, 1968), participate in intermolecular hydrogen bonding and result in a more stable membrane bilayer. However, the repulsion between GSLs with negatively charged head groups can interfere with the formation of this hydrogen-bonding network. The effect of monovalent cations on sulfatide order is attributed to shielding of the negatively charged head group that then enables the hydrogen bonding to occur in sulfatide dispersions (Boggs et al., 1984). Specifically, these workers noted that the melting temperature of sulfatide dispersions, in 2 M salt, increased in the order $Li^+ < Na^+ < K^+ < Rb^+$; this order corresponds to a decrease in hydration radii of these cations. Since it had been hypothesized that sulfatide might be involved in ion discrimination by the Na^+,K^+-ATPase (Hansson et al., 1978, 1979), it was of interest to determine if these two monovalent cations could affect sulfatide behavior at physiologically relevant concentrations and in structures that more closely resembled biomembranes. As shown in Figure 1, sulfatide/DEPC dispersions in 100 mM potassium are characterized by a higher melting temperature and a less extended range of gel-phase immiscibility when compared to similar preparations in 100 mM sodium. These effects are specific for sulfatide, since similar dispersions of galactosylceramide and DEPC do not exhibit this behavior (Figure 2).

Structure of GSL/DEPC Dispersions Produced by the Ethanol Injection Method. Previous reports contain contradictory conclusions regarding the lamellar or micellar nature of phospholipid/sulfatide mixtures or pure sulfatides. For example, one calorimetric study of sulfatide/PC mixtures, utilizing sonicated dispersions of *N*-stearoyl sulfatide and DPPC (Cestaro et al., 1981), described the formation of a sulfatide-rich lamellar phase below 20 mol % sulfatide and the appearance of a new micellar phase at higher sulfatide concentrations. Later work by this group, using other lipids (sonicated bovine brain sulfatide and egg PC), indicated that unilamellar vesicles are formed in mixtures below 30 mol %, vesicles and micelles are found between 30 and 70 mol %, and micelles only are found above 70 mol % sulfatide (Cestaro et al., 1984). In the latter study these workers used a variety of measurements in order to conclude that mixtures of egg PC and sulfatide were micellar at sulfatide levels of greater than 30 mol %. These data agree with earlier X-ray diffraction data (Abrahamsson et al., 1972) which indicated that pure sulfatide is micellar at high water contents. Boggs et al. (1984) and Ruocco and Shipley (1986), in contrast, have equally convincingly concluded, in part on the basis of identical techniques such as X-ray diffraction, that pure sulfatide is lamellar. These discrepancies may be due in part to differences in sample preparation techniques or buffer salts. Our data, based on the accessibility of DEPC to phospholipase D (Figure 4), are consistent with the hypothesis that dispersions of DEPC and either sulfatide or galactosylceramide, prepared by ethanol injection, are lamellar at all glycosphingolipid levels up to 90 mol %.

Properties of Sulfatides Containing Hydroxy and Non-Hydroxy Fatty Acids. The data in Figure 3 indicate that cation-induced differences in the melting temperatures of DEPC/sulfatide mixtures are confined to those sulfatides that contain hydroxy fatty acids. It had previously been reported (Koshy & Boggs, 1983; Boggs et al., 1984) that transition temperatures of synthetic HFA and NFA sulfatide are higher in potassium-containing buffers than in sodium-containing buffers. Our data (Figure 1) indicate that the transition temperature of a dispersion of mixed sulfatides was approximately 5 °C higher in 100 mM KCl than in 100 mM NaCl, in agreement with previous measurements. However, in dispersions of pure HFA or NFA sulfatide (Figure 3), the transition temperatures were only slightly affected by the type of cation present. This could be due to the formation of nonlamellar phases in the mixed-sulfatide dispersions or could be linked to interdigitation of acyl chains of unequal length (Boggs et al., 1984). In this regard, it should be noted that the HFA and NFA sulfatide preparations used in our study contained a variety of acyl chains and that the acyl-chain composition of the NFA fraction was significantly enriched for longer chain species (Table I). It is not likely that the sulfatide dispersions exhibited metastable phase behavior (Boggs et al., 1984), since the temperature scans were performed at relatively low rates. Additionally, as mentioned above, data obtained while the samples were heated were equivalent to those obtained while the samples were cooled for all samples examined. Regardless of the complexities of the phase behavior of sulfatide dispersions, the data presented in Figures 1 and 3 clearly indicate that the phase properties of sulfatide/phospholipid dispersions are a function of the monovalent cation present in the aqueous phase. The effect of physiologically relevant concentrations of K^+ on transition temperatures and on the appearance of the phase diagram for these mixtures seems to be dependent on the presence of hydroxy fatty acids. Previous papers have described additional surface properties of other glycosphingolipids that are due to the presence of hydroxy fatty acids in these lipids [for review, see Thompson and Tillack (1985) and Curatolo (1987a)]. The effect of hydroxy fatty acids on antibody binding to sulfatide in PC liposomes was recently determined by Crook et al. (1986). These authors showed that the presence of hydroxy fatty acids in the sulfatide fraction actually inhibited the binding of high-affinity antibody to the liposomes, suggesting that alterations in the acyl-chain region of these lipids must alter surface exposure or head-group conformation. This is in direct contrast to the observations of Kannagi et al. (1983), who found that hydroxy fatty acids were responsible for high reactivity of antibody to the tumor antigen Gg_3Cer in murine lymphoma cells. This inconsistency may be due to differences in the avidity or specificity of the antibodies used or to interference by other glycolipids in the tumor cell membrane. Regardless of the basis of these results, our data indicate the existence of another modulator of hydroxy acyl sulfatide domain formation in membranes, that being the concentration of potassium ion. In this regard, the observations of Maggio et al. (1987) might be relevant. These workers presented data consistent with the presence of two populations of sulfatide in DPPC/sulfatide mixtures. One population exhibited a higher enthalpy and transition temperature in the presence of low levels of calcium; the calorimetric properties of the other population were unchanged by these levels of calcium. This effect of a divalent cation could be explained if calcium selectively shielded the sulfatide containing hydroxy fatty acid in a manner analogous to the shielding by monovalent cations.

Conclusions. Our data indicate that physiological levels of potassium can influence the phase properties of low levels of sulfatide in DEPC bilayers. Additionally, the data are consistent with the presence of lamellar phase structures at all sulfatide concentrations up to 90 mol % in these ethanol-injected dispersions. The effects of potassium are restricted to sulfatide and are not observed in similar lamellar phase

preparations of galactosylceramide and DEPC. Furthermore, the effects of potassium are confined to those sulfatides that contain hydroxy fatty acids. These observations, coupled with previous observations on antibody recognition of glycosphingolipids (Crook et al., 1986; Kannagi et al., 1983), indicate that acyl-chain composition can influence the membrane surface properties of this small, negatively charged glycosphingolipid. Additionally, the apparent selectivity of these glycosphingolipids with regard to monovalent cations is consistent with the hypothesis of Karlsson (1976) that sulfatides are involved in ion discrimination by the Na^+,K^+-ATPase. Further experiments, aimed at elucidation of the contribution of lipid structure to these biological functions, are currently under way.

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Registry No. DEPC, 52088-89-8; ATPase, 9000-83-3; Na, 7440-23-5; K, 7440-09-7; sulfatide, 85496-63-5; galactosylceramide, 85305-88-0.

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