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Effect of Calcium on the Dynamic Behavior of Sialylglycerolipids and Phospholipids in Mixed Model Membranes. A ^2H and ^{31}P NMR Study†

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ABSTRACT: DTSL, a sialic acid bearing glyceroglycolipid, has been deuteriated at the C3 position of the sialic acid headgroup and at the C3 position of the glycerol backbone. The glycolipid was studied as a neat dispersion and in multilamellar dispersions of DMPC (at a concentration of 5–10 mol % relative to phospholipid), using ^2H and ^{31}P NMR. The quadrupolar splittings, $\Delta\nu_Q$, of the headgroup deuterons were found to differ in the neat and mixed dispersion, suggesting different headgroup orientations in the two systems. In DTSL–DMPC liposomes, two quadrupolar splittings were observed, indicating that the axial and equatorial deuterons make different angles with respect to the axis of motional averaging. The splittings originating from the equatorial and axial deuterons were found to increase and decrease with increasing temperature, respectively, indicating a temperature-dependent change in average headgroup orientation. Longitudinal relaxation times, T_{1Z}, were found to be short (3–6 ms). The field dependence of T_{1Z} suggests that more than one motion governs relaxation. At 30.7 MHz a T_{1Z} minimum was observed at approximately 40 °C. At 46.1 MHz the T_{1Z} values were longer and increased with temperature, demonstrating that the *dominant* rigid-body motions of the headgroup at this field are in the rapid motional regime ($>10^8$ s^{-1}). DTSL labeled at the glycerol C3 position was studied in DMPC multilamellar dispersions. Whereas two quadrupolar splittings have been observed for other glycolipids labeled at this position, only a single $\Delta\nu_Q$ was observed. This shows that the orientation of the C2–C3 segment of DTSL relative to the bilayer normal differs from that of other glycolipids. T_{1Z} values were short (3–7 ms) and increased with temperature, demonstrating that motion is in the rapid motional regime. Quadrupolar splittings and T_{1Z} values were also obtained for the headgroup-labeled DTSL in the presence of 5 and 50 mM Ca^{2+}. As the Ca^{2+} concentration was increased, the ratio of outer to inner quadrupolar splittings increased, suggesting a small change in headgroup orientation. From the longitudinal relaxation times the rate of the dominant headgroup motion(s) appeared to decrease. The DTSL–DMPC liposomes were also studied by ^{31}P NMR and by two-dimensional solid-state ^{31}P NMR, the latter technique giving information on the orientational exchange of phospholipid molecules. DTSL appeared to alter the headgroup orientation of DMPC and also to increase the rate of orientational exchange. The latter most likely reflects an increase in the rate of lateral diffusion of the phospholipid. Ca^{2+} was found to reverse both of these effects partially.

Glycolipids are an important class of lipid found in animals, plants, and microorganisms (Gigg, 1980). Glycolipids can be divided into two distinct classes: glycosphingolipids, the carbohydrate-bearing lipids of animal cells, and glycoglycerolipids, which occur in plants (Quinn & Williams, 1978), bacteria (Rogers et al., 1980), and mycoplasma (Wieslander et al., 1978). Glycosphingolipids have been implicated as specific recognition sites for a variety of important cellular processes, including immune recognition (Hakomori, 1984b), cell–cell interaction (Critchly et al., 1979), and binding of viruses and proteins (Fishman & Brady, 1976). Both classes of lipids have the potential to modulate membrane physical properties (Curatolo, 1987a). This is particularly clear for such organisms as *Acholeplasma laidlawii*, where the glycoglycerolipids are the major constituent lipid of the cellular membrane (Rottem, 1980; Razin, 1980). The biological roles of glycolipids depend not only on the structure of the surface carbohydrate but also on its accessibility to external factors. Thus, the spatial organization of the membrane surface, which will be determined by such factors as the orientation, ordering, and dynamics of the carbohydrate residues, is expected to be of critical importance in understanding cell-surface phenomena.

^2H NMR[1] is a powerful technique for studying the anisotropic molecular environment of biological and model mem-

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[1] Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DTSL, 1,2-di-*O*-tetradecyl-3-*O*-α-D-sialyl-*sn*-glycerol; [$^2H_2$-NeuAc]DTSL, 1,2-di-*O*-tetradecyl-3-*O*-α-D-[3,3-$^2H_2$]sialyl-*sn*-glycerol; [$^2H_1$-3G]DTSL, 1,2-di-*O*-tetradecyl-3-*O*-α-D-sialyl-*sn*-[3-$^2H_1$]glycerol; β-DTGL, 1,2-di-*O*-tetradecyl-3-*O*-β-D-glucopyranosyl-*sn*-glycerol; α-DTML, 1,2-di-*O*-tetradecyl-3-*O*-α-D-mannopyranosyl-*sn*-glycerol; NMR, nuclear magnetic resonance.

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branes and has been applied extensively to the study of glycolipid headgroups in cerebrosides (Skarjune & Oldfield, 1979; 1982), in glycoglycerolipids (Jarrell et al., 1986, 1987a,b; Renou et al., 1989; Carrier et al., 1989), and in model glycolipid systems (Ram & Prestegard, 1988). Detailed information on the orientation, conformation, and motion of monosaccharide headgroups (Jarrell et al., 1986, 1987a,b), disaccharide headgroups (Renou et al., 1989; Carrier et al., 1989), and the gycerol backbone (Jarrell et al., 1987a,b; Auger & Jarrell, 1990; Auger et al., 1989, 1990a,b) has been obtained. These studies demonstrate the power of ^{2}H NMR in providing details relating to cell-surface topology.

The current study extends the data base on uncharged mono- and diglycosylglycerolipids to a sialic acid containing glycolipid, 1,2-di-*O*-tetradecyl-3-*O*-α-D-sialyl-*sn*-glycerol (DTSL). Sialic acid, an important constituent of glycoproteins and complex glycosphingolipids such as GM_1, has been associated with a number of biological and immunological phenomena (Corfield & Schauer, 1982). Sialyloligosaccharides have been identified as receptors for the human influenza virus (Weis et al., 1988) and as oncogenic markers (Hakomori, 1984; Feizi, 1985). Furthermore, sialic acid is negatively charged at physiological pH, making DTSL the first charged glycolipid to be studied by ^{2}H NMR. We have elected to study DTSL, labeled in the sialyl residue at the C3 position and in the glycerol backbone at the C3 position, at low concentration in phospholipid bilayers composed of DMPC. This allows us to study the behavior of a charged monosaccharide headgroup in a bilayer environment and to examine the effect of the adsorption of Ca^{2+} to the membrane surface, a process which can alter membrane properties (McLaughlin, 1977; Altenbach & Seelig, 1984; MacDonald & Seelig, 1987a) and is of importance physiologically (McLaughlin, 1977). Studies on the model lipid DTSL should also provide a basis for understanding the behavior of sialic acid in more complex lipids. In addition, we utilize a combination of one- and two-dimensional ^{31}P NMR (Fenske & Jarrell, 1991) to probe the effect of the charged DTSL on phospholipid headgroup orientation and orientational exchange. In the latter case, information can be obtained regarding the effect of DTSL and Ca^{2+} on liposome size and/or on the rate of phospholipid lateral diffusion. Thus, a combination of ^{2}H and ^{31}P NMR data provides details on the rates and modes of reorientation of functional groups within individual molecules, on the diffusive motions of phospholipids, and on changes in membrane morphology.

Materials and Methods

L-α-Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids, Birmingham, AL. 1,2-Di-*O*-tetradecyl-3-*O*-α-D-[3,3-$^2H_2$]sialyl-*sn*-glycerol and 1,2-di-*O*-tetradecyl-3-*O*-α-D-sialyl-*sn*-[3-$^2H_1$]glycerol were synthesized as previously described (Roy et al., 1990).

DTSL (5–10 mg) was dispersed in approximately 7 mL of 10 mM Tris-HCl/0.15 M NaCl, pH 7.6, prepared in deionized distilled water, at 30–40 °C and vortexed until homogeneous. The lipid was pelleted by ultracentrifugation at 45 000 rpm at 4 °C for 1–2 h. This process was repeated once to twice more, the supernatant was removed, and the DTSL pellet was dried under vacuum. The DTSL and the desired quantity of DMPC were then codissolved in MeOH, the solvent was removed under a stream of Ar at 40–50 °C, and residual traces of MeOH were removed under high vacuum overnight. The lipid was hydrated with at least a 3-fold excess of 10 mM Tris-HCl/0.15 M NaCl/0.02% Na_4EDTA, pH 7.6 or 10 mM Tris-HCl/0.15 M NaCl, pH 7.6 at 30–35 °C, followed by five cycles of vortex–freeze–thaw until a homogeneous dispersion was obtained. The sample was then lyophilized and rehydrated with an equivalent volume of deuterium-depleted water, with vortex–freeze–thawing as above. The deuterium-depleted water exchange was repeated once.

^{2}H NMR spectra were acquired at 30.7 MHz on a "home-built" spectrometer operated by a Nicolet 1280 computer or at 46.1 MHz on a Bruker MSL-300. Spectra were recorded using the quadrupolar echo pulse sequence (Davis et al., 1976) with full phase cycling (Perly et al., 1985) and quadrature detection. The $\pi/2$ pulse length was 2.5 μs (30.7 MHz) or 3.9 μs (46.1 MHz) (5-mm solenoid coil), the pulse spacing was 60 μs, and the recycle time was 50 ms. Longitudinal relaxation times, T_{1Z}, were obtained using the standard inversion–recovery pulse sequence coupled with the quadrupolar echo pulse sequence (Perly et al., 1985). The frequency of the spectrometer was carefully set at the center of the quadrupolar powder pattern. The samples were equilibrated at a given temperature for 20–30 min prior to data acquisition; temperatures are accurate to ±0.5 °C. In general, spectra were not folded about the Larmor frequency, except for some T_{1Z} measurements, where they were folded to increase S/N. No spectral distortions were introduced by the folding procedure. Quadrupolar splittings were measured from the 90° oriented sample ("dePaked") spectra which were calculated from the powder spectra as described by Bloom et al. (1981).

^{31}P NMR spectra were acquired at 121.5 MHz on a Bruker MSL-300 spectrometer. One-dimensional spectra were recorded using a Hahn echo pulse sequence (Rance & Byrd, 1983) with WALTZ decoupling (gated on during acquisition). The ^{31}P $\pi/2$ pulse length was 4.0–8.0 μs (10-mm solenoid coil), the pulse spacing was 60 μs, and the recycle time was 5.0 s. The longer $\pi/2$ pulses were due to the presence of 0.15 M NaCl in the DTSL–DMPC samples, which prevented perfect tuning of the probe. Because of this, the ^{31}P and ^{1}H pulse lengths were measured individually for each sample. Two-dimensional spectra were recorded as described by Fenske and Jarrell (1991), using the basic NOESY pulse sequence with TPPI (used on Bruker spectrometers) to give quadrature detection in both dimensions (Bodenhausen et al., 1984).

$$[(\text{preparation})\text{–}90°\text{–}t_1(\text{evolution})\text{–}90°\text{–}t_{mix}\text{–}90°\text{–}t_2(\text{detection})\text{–}(\text{delay})]$$

WALTZ ^{1}H decoupling was gated on during the evolution and detection periods. Preparation of the system was achieved by including 16 dummy scans at the beginning of each serial file, t_{mix} varied from 50 μs to 100 ms, and the recycle delay was either 2 or 3 s. The data sets were 256 points in the F_2 dimension, and 64 points were zero-filled to 256 points in the F_1 dimension. Between 128 and 256 transients were recorded for each serial file in a given 2D experiment. For most experiments, the spectral width in both dimensions was 50 kHz. Other parameters were as described for the one-dimensional experiments.

Results and Discussion

Multilamellar Dispersions of [$^2H_2$-NeuAc]DTSL. Prior to examination of DTSL–DMPC mixtures, preliminary studies were performed on neat DTSL dispersions. ^{2}H NMR spectra of [$^2H_2$-NeuAc]DTSL, hydrated with a 10-fold excess of 10 mM Tris-HCl/0.02% Na_2EDTA, pH 7.6, prepared in deuterium-depleted water, are shown in Figure 1 at 30, 40, and 50 °C. It should be stressed that the polymorphic form favored under these conditions is not known. At 50 °C, the spectrum is indicative of axially symmetric motion. Only one quadrupolar splitting, $\Delta\nu_Q$, of 18 kHz is observed, suggesting that both

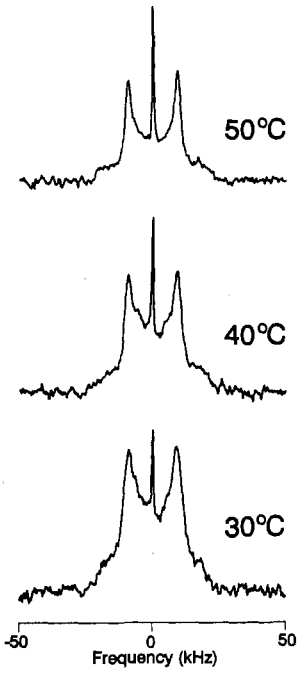

FIGURE 1: ^2H NMR spectra (30.7 MHz) of [$^2H_2$-NeuAc]DTSL dispersions as a function of temperature. Number of acquisitions = 150 000 (bottom) or 100 000 (center and top).

deuterons make essentially the same angle with respect to the axis of motional averaging. A slight change in the lineshape occurs at the lower temperatures, which could indicate either the onset of asymmetric motion or the presence of two Pake doublets with slightly different splittings. The latter could occur from a temperature-dependent change in headgroup orientation, which is observed in the mixed lipid systems (discussed below), and thus seems more likely. Partially relaxed spectra of [$^2H_2$-NeuAc]DTSL at 30 °C (not shown) revealed a null point at a delay time <5 ms, indicating a longitudinal relaxation time $T_{1Z} < 7$ ms.

[$^2H_2$-NeuAc]DTSL, 10 mol % in multilamellar dispersions of DMPC, was hydrated with unbuffered deuterium-depleted water. Figure 2 shows ^2H NMR spectra at 30 and 40 °C (left column) and the corresponding 90° oriented sample spectra ("dePaked") (right column). Two quadrupolar splittings are observed, of approximately equal intensity, indicating that the two deuterons at C3 of the sialyl headgroup are inequivalent (make different angles with respect to the axis of motional averaging). Thus, the headgroup orientation in the mixed lipid systems is different from that in pure DTSL, where only one splitting (18 kHz) is observed (Figure 1). This is in agreement with studies involving neutral glycolipids. Jarrell et al. (1986) calculated a different headgroup orientation for β-DTGL in neat dispersions and dispersed at 10 mol % in DMPC. Skarjune and Oldfield (1982) reported a disordering of a glucocerebroside headgroup when it was dispersed in phospholipid. These studies indicate that the physical properties of any one surface constituent will be sensitive to the presence of other constituents, i.e., to the specific membrane composition. In the case of DTSL, one may speculate that differences in the surface charge density in the two systems may contribute to the change in headgroup orientation, because this parameter is known to affect the orientation of phosphatidylcholine headgroups (Seelig et al., 1987b). However, this does not explain the composition-dependent change in headgroup orientation of the neutral glycolipids, and therefore other considerations, such as geometrical packing constraints or changes in hydrogen bonding, may be involved as well.

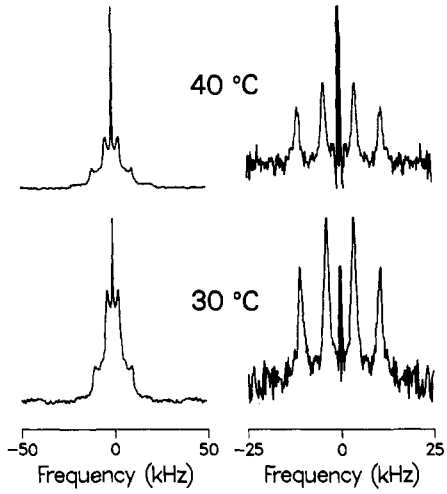

FIGURE 2: ^2H NMR spectra (30.7 MHz) of [$^2H_2$-NeuAc]DTSL at a concentration of 10 mol % in DMPC multilamellar dispersions as a function of temperature. The powder spectra are shown in the left column, and the corresponding dePaked spectra, calculated from the powder spectra, are shown in the right column. Note the increase in the isotropic component at 40 °C. The number of acquisitions was 100 000.

The isotropic component observed in the spectra shown in Figures 1 and 2 increased in intensity with increasing temperature. Although a portion of this component may originate from residual water, the majority is due to degraded lipid. By 70 °C, the sample in Figure 1 gave a completely isotropic spectrum (not shown), which remained isotropic upon cooling, even after extended periods at −20 °C. Thin-layer chromatography of this sample revealed lipid degradation with release of the headgroup (hence the isotropic signal). For this reason, quadrupolar splittings and T_{1Z} values obtained for the [$^2H_2$-NeuAc]DTSL–DMPC sample (Figure 2) are not given as lipid degradation products may affect these parameters. A slight increase in the quadrupolar splittings with time was observed during an inversion recovery experiment at 40 °C (not shown). The most likely source of the lipid degradation observed in these two samples is acid hydrolysis of the sialyl headgroup by residual ammonium ion (the lipid was prepared as the ammonium salt). Sialic acid compounds are sensitive to acidic conditions. An efficient way of removing the residual ammonium was via ultracentrifugal buffer exchanges as described under Materials and Methods. Samples prepared from lipid pretreated in this manner (the [^2H-NeuAc]DTSL and the [$^2H_1$-3G]DTSL described in the following sections) were found to be stable under the present conditions (temperatures in the 30–50 °C range).

Multilamellar Dispersions of [^2H-NeuAc]DTSL and DMPC. The following ^2H NMR studies were performed on a DTSL sample which was a mixture of [$^2H_2$-NeuAc]DTSL and [$^2H_1$-NeuAc]DTSL, henceforth named [^2H-NeuAc]-DTSL. During the deuteriation of sialic acid, the axial deuterons at the C3 position are exchanged first, followed by the equatorial deuterons (Roy et al., 1990). To facilitate ^2H resonance assignments, a differential labeling of the axial and equatorial positions was required. Thus, the above mixture was prepared by not allowing the second exchange to go to completion. Figure 3 shows several ^2H NMR spectra of [^2H-NeuAc]DTSL, 5 mol % in multilamellar dispersions of DMPC. Several differences are apparent from the spectra in Figure 2. The intensity of the outermost splitting is greatly reduced, allowing assignment of the 7-kHz splitting to the axial deuteron and the 25-kHz splitting to the equatorial deuteron. The splittings observed over 30–50 °C are listed in Table I.

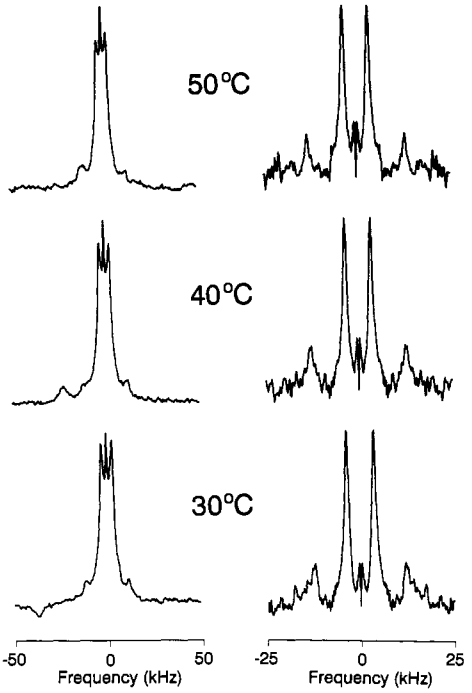

FIGURE 3: ^{2}H NMR spectra (30.7 MHz) of [^{2}H-NeuAc]DTSL at a concentration of 5 mol % in DMPC multilamellar dispersions as a function of temperature. The powder spectra are shown in the left column, and the corresponding dePaked spectra, calculated from the powder spectra, are shown in the right column. The number of acquisitions was 1×10^6.

Table I: Quadrupolar Splittings, $\Delta\nu_Q$ (in kilohertz), of [^{2}H-NeuAc]DTSL in DMPC Multilamellar Dispersions as a Function of Temperature and Ca^{2+} Concentration

	[Ca^{2+}] (mM)		
T (°C)	0	5	50
30	24.2, 7.4	24.6, 7.0	24.6, 6.6
40	25.4, 7.0	25.8, 6.6	25.4, 6.2
50	26.2, 6.6	26.6, 6.6	27.0, 5.8

As the temperature is raised from 30 to 50 °C, the outer quadrupolar splitting increases from 24.2 to 26.2 kHz and the inner splitting decreases from 7.4 to 6.6 kHz. If the only effect of increasing temperature were a decrease in molecular order, the splittings would be both reduced and scaled by a constant factor and the ratio of outer to inner quadrupolar splittings, $\Delta\nu_Q$(o/i), would remain constant. The change in this ratio (with the two splittings varying in opposite directions) suggests a temperature-dependent average headgroup orientation. Unfortunately, with only two observables (quadrupolar splittings), the headgroup orientation cannot be determined directly. Thus, we cannot compare the orientation of the charged DTSL headgroup with those previously determined for monoglycosylglycerolipids (Jarrell et al., 1986, 1987a,b) and diglycosylglycerolipids (Renou et al., 1989; Carrier et al., 1989).

While the residual quadrupolar interactions can provide information on the orientation and ordering of a molecular group, the rates of molecular motion can be probed through measurement of relaxation times. The longitudinal relaxation times, T_{1Z}, for the C3 deuterons of the DTSL headgroup were measured at 30, 40, and 50 °C, at both 30.7 and 46.1 MHz. The T_{1Z} values at 30.7 MHz (listed in Table II) were extremely short, clustering close to 3 ms over the range 30–50 °C, suggesting the presence of a T_{1Z} minimum at approximately 40 °C. If a single motion was governing relaxation, this would indicate that the correlation time was approaching

Table II: Longitudinal Relaxation Times, T_{1Z} (in milliseconds), of [^{2}H-NeuAc]DTSL in Multilamellar Dispersions of DMPC as a Function of Temperature and Ca^{2+} Concentration

	[Ca^{2+}] (mM)		
T (°C)	0	5	50
	30.7 MHz		
30	3.0 ± 0.1	3.3 ± 0.2	3.6 ± 0.1
40	3.7 ± 0.1	2.9 ± 0.1	nd[a]
50	3.1 ± 0.1	3.5 ± 0.1	nd[a]
	46.1 MHz		
30	4.0 ± 0.2	5.4 ± 0.3	4.8 ± 0.2
40	4.9 ± 0.3	4.3 ± 0.1	5.0 ± 0.2
50	6.0 ± 0.1	5.2 ± 0.1	4.1 ± 0.1

[a] nd = not determined.

the Larmor frequency. However, at 30.7 MHz, the shortest relaxation time is expected to be on the order of 2 ms (Davis, 1983). That the T_{1Z} values are significantly longer at the "minimum" demonstrates the presence of more than one motion (with different correlation times). This is also suggested by the data at 46.1 MHz. For a single correlation time, an increase in ω_0 would shift the motions into the slow motional regime, where the T_{1Z}'s decrease with increasing temperature. However, at 46.1 MHz, the T_{1Z} values are longer (4–6 ms) and increase with temperature. This anomaly can be explained if several motions contribute to relaxation, with a different blend of motions sampled at the higher field. The temperature dependence of T_{1Z} implies that the *dominant* motion at 46.1 MHz is in the rapid motional regime. Assuming that such is the case will allow us to draw some inferences regarding the effects of Ca^{2+} on lipid headgroup mobility (see below). The presence of complex headgroup motions in DTSL agrees with ^{2}H NMR relaxation data obtained for a related glucolipid, β-DTGL, where several motions were necessary to explain the headgroup relaxation in the liquid crystalline state (Winsborrow et al., 1991).

The relaxation times for DTSL are shorter than those measured for the headgroups of any other glycoglycerolipids except gentiobiose (Carrier et al., 1989). At 30.7 MHz, the headgroup motions of the other monoglycosylglycerolipids (Jarrell et al., 1986, 1987a) and a lactosyl glycerolipid (Renou et al., 1989) were in the rapid motional regime, whereas the motions of the gentiobiose headgroup were in the slow motional regime (Carrier et al., 1989). In the present case, some of the DTSL headgroup motions at 30.7 MHz appear to be very close to the region of the T_{1Z} minimum. Thus, the mobility of the DTSL headgroup is intermediate between that of gentiobiose and those of the other glycolipids.

The adsorption of Ca^{2+} to membrane surfaces, an important physiological phenomenon (McLaughlin, 1977), has been studied by Seelig and co-workers (Altenbach & Seelig, 1984; Borle & Seelig, 1985; Seelig et al., 1987; Macdonald & Seelig, 1987a,b) and others (Roux & Bloom, 1990), using a phosphocholine headgroup selectively deuteriated at the α and β positions as a sensitive probe of the surface charge of the membrane. The phospholipid headgroup orientation changes in response to a change in the surface potential which can be altered by the adsorption of ions or by the presence of charged amphiphiles. In light of the potentially important role of the surface potential in modulating membrane surface phenomena, it was of interest to examine the effect of ion binding on the orientation and dynamics of the charged sialyl headgroup. The temperature dependence of $\Delta\nu_Q$ and T_{1Z} for [^{2}H-NeuAc]-DTSL, 10 mol % in multilamellar dispersions of DMPC, was studied in the presence of Ca^{2+} concentrations in the physiological range, 5–50 mM. ^{2}H NMR spectra were acquired at

Ca^{2+} concentrations of 5 and 50 mM (not shown), corresponding to DTSL:Ca^{2+} molar ratios of approximately 9:1 and 1:1, respectively. The spectra are very similar to those in Figure 3, as are the $\Delta\nu_Q$ values obtained from the dePaked spectra (Table I). The same temperature dependence of the two splittings is observed in both cases. However, Ca^{2+} does appear to have a small but discernible effect on the ^2H spectrum. At all temperatures, the ratio of outer to inner $\Delta\nu_Q$ ($\Delta\nu_Q$(o/i)) shows an increase with increasing Ca^{2+}. For example, at 30 °C, $\Delta\nu_Q$(o/i) values of 3.27, 3.51, and 3.73 were obtained for Ca^{2+} concentrations of 0, 5, and 50 mM, respectively. This indicates a Ca^{2+}-dependent headgroup orientation. On the basis of the small changes in the actual $\Delta\nu_Q$ values (less than 1 kHz), a large change in headgroup orientation is unlikely. Thus, Ca^{2+} concentrations up to 50 mM induce only small changes in DTSL headgroup orientation. We have not studied higher Ca^{2+} concentrations, but the changes in $\Delta\nu_Q$ observed here are of the same order of magnitude (ca. 1 kHz) as observed for phospholipids by Altenbach and Seelig (1984) for Ca^{2+} concentrations up to 50 mM. It would be of interest to see if charged glycolipid headgroups also change orientation in a quantitative manner with membrane surface charge.

Two other observations can be made on the basis of these results. At these Ca^{2+} concentrations, there is no observable separation of crystalline DTSL–Ca^{2+} domains, in which case we could expect a broadening of "solid" DTSL and a loss of signal intensity due to liquid crystalline phase. However, we would not be able to detect small quantitites of "solid" DTSL, and it is possible that higher Ca^{2+} concentrations would cause such separation. Essentially identical results were obtained for mixtures of POPC with the anionic lipids phosphatidylglycerol (Macdonald & Seelig, 1987a) and cardiolipin (Macdonald & Seelig, 1987b). Furthermore, only a single quadrupolar splitting is detected in the presence of Ca^{2+}; thus, "free" and "bound" Ca^{2+} are in fast exchange, and the residence time of a Ca^{2+} ion at an individual binding site must be short ($\ll 10^{-6}$ s) on the NMR time scale.

Increasing concentrations of Ca^{2+} clearly affect headgroup mobility as they reverse the temperature dependence of T_{1Z} at 46.1 MHz (Table II). In the absence of Ca^{2+}, the T_{1Z} values increase with temperature (rapid motional regime). In the presence of 5 mM Ca^{2+}, a T_{1Z} minimum is observed at 40 °C. As the Ca^{2+} concentration is increased to 50 mM, the T_{1Z} values appear to decrease with increasing temperature (the values at 30 and 40 °C are the same within experimental error) (slow motional regime). Although the T_{1Z} data are limited by the number of measurements, they are consistent with a reduction in the rate of headgroup reorientation due to increased Ca^{2+} concentration, which suggests an electrostatic interaction between Ca^{2+} and the sialyl headgroup.

Multilamellar Dispersions of [$^2H_1$-3G]DTSL and DMPC. The ordering and dynamics of the glycerol backbone of DTSL were probed via deuterium labels at the glycerol C3 position. [$^2H_1$-3G]DTSL (where each lipid molecule is singly deuteriated, and each position is 50% labeled) was studied at a concentration of 5 mol % in DMPC multilamellar dispersions. Spectra were acquired at 30–50 °C, at both 30.7 and 46.1 MHz. Only a single, relatively temperature-invariant splitting was observed, with values of 24.6, 25.4, and 25.2 kHz at 30, 40, and 50 °C, respectively. A representative spectrum is shown in Figure 4, along with the dePaked spectrum. At both fields, the T_{1Z} values increase with temperature, indicating that the dominant glycerol backbone motions are in the short correlation time regime (Table III). The quadrupolar

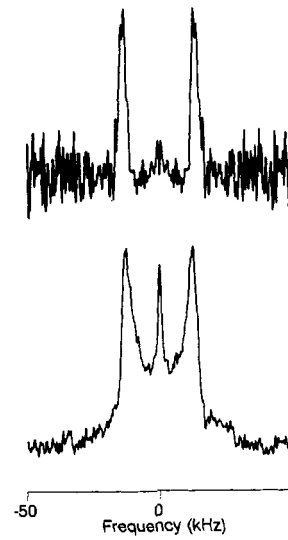

FIGURE 4: ^2H NMR spectrum (46.1 MHz) of [$^2H_1$-3G]DTSL at a concentration of 5 mol % in DMPC multilamellar dispersions at 30 °C (bottom). The corresponding dePaked spectrum is shown above. Number of acquisitions = 884656.

Table III: Temperature and Magnetic Field Dependence of Longitudinal Relaxation Times, T_{1Z} (in milliseconds), for 5 mol % [$^2H_1$-3G]DTSL in Multilamellar Dispersions of DMPC

T (°C)	30.7 MHz	46.1 MHz
30	3.2 ± 0.2	4.6 ± 0.3
40	4.5 ± 0.3	7.0 ± 0.4
50	5.0 ± 0.3	7.1 ± 0.1

splittings obtained for DTSL are significantly smaller than those obtained for other monoglycosylglycerolipids; in the case of the latter lipids, two quadrupolar splittings are observed. This suggests a different orientation of the glycerol C2–C3 segment in DTSL from that of either β-DTGL or α-DTML (Jarrell et al., 1987b). The $\Delta\nu_Q$ values of the β-DTGL are 32.3 and 29.3 kHz for the pure lipid and 29.6 and 27.1 kHz for 20 mol % β-DTGL in DMPC (Jarrell et al., 1987b; Auger et al., 1989). For α-DTML, $\Delta\nu_Q$ = 29.9 and 27.8 kHz for the pure lipid (Jarrell et al., 1987b). The values obtained for DTSL (approximately 25 kHz) may suggest a decrease in the order of the glycerol segment as well as a change in orientation. The T_{1Z} values obtained for [$^2H_1$-3G]DTSL in the liquid crystalline state are very similar to those obtained for the glycerol C3 segment of β-DTGL in the gel state (Auger et al., 1990a) and liquid crystalline state (Winsborrow et al., 1991).

^{31}P NMR Studies of DTSL–DMPC Multilamellar Dispersions. The DTSL–DMPC mixtures were examined by ^{31}P NMR to ascertain the effect of the negatively charged glycolipid on the behavior of the phospholipid. Scherer and Seelig (1989) have shown that negatively charged amphiphiles reduce the residual $\Delta\sigma$ of neutral membrane phospholipids via surface charge induced changes in the headgroup orientation. ^{31}P NMR spectra, obtained at 30 °C, are shown in Figure 5. It is evident that all the phospholipids are in liquid crystalline environments, giving spectra characteristic of axially symmetric motion in bilayer lipids. In Figure 5, the bottom spectrum was obtained from pure DMPC, while the spectrum directly above is DMPC containing 5 mol % [$^2H_1$-3G]DTSL. A significant change in lineshape is observed, and the $\Delta\sigma$ is reduced. This could result from a change in headgroup orientation, increased amplitude of motional averaging, reduction in the mean size of the liposome population, or an increase in the rate of lateral diffusion. However, it is extremely unlikely that the incorporation of DTSL into DMPC liposomes at this low

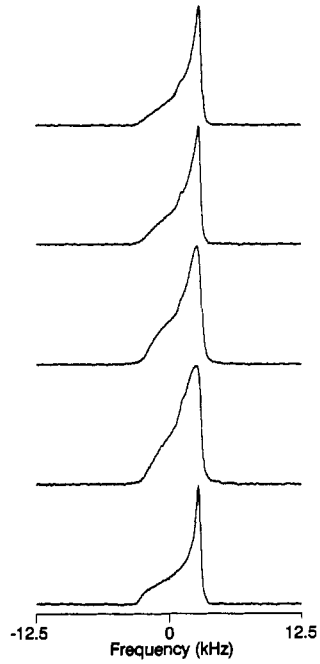

FIGURE 5: ^{31}P NMR spectra (121.5 MHz) of DMPC, DMPC containing 5 mol % [$^2H_1$-3G]DTSL, DMPC containing 5 mol % [^2H-NeuAc]DTSL, DMPC–[^2H-NeuAc]DTSL + 5 mM Ca^{2+}, and DMPC–[^2H-NeuAc]DTSL + 50 mM Ca^{2+} (bottom to top), all acquired at 30 °C.

concentration results in a change in particle diameter sufficient to affect the NMR spectrum. No such evidence is obtained with mixtures of phospholipids with negatively charged phosphatidylserine (Tilcock et al., 1984), phosphatidylglycerol (Borle & Seelig, 1985; Macdonald & Seelig, 1987a), cardiolipin (Macdonald & Seelig, 1987b), and cationic and anionic amphiphiles (Seelig et al., 1987; Scherer & Seelig, 1989). The middle spectrum of Figure 5 is DMPC containing 5 mol % [^2H-NeuAc]DTSL. As expected, the lineshape is very similar to that of the [$^2H_1$-3G]DTSL-containing sample. The top two spectra represent the DMPC–[^2H-NeuAc]DTSL samples containing 5 and 50 mM Ca^{2+}, respectively. The spectra are similar to that of DMPC, which argues against a reduction in liposome size as the source of the lineshape changes observed in the absence of Ca^{2+}. This is supported by the ^2H NMR results. If the effect of Ca^{2+} on the ^{31}P spectra originated from liposomal size changes, similar effects should be observed in the ^2H spectra. However, the quadrupolar splittings observed in the absence and presence of Ca^{2+} are very similar. Two explanations of the ^{31}P spectra present themselves. First, and most likely, the DMPC headgroup orientation may be altered by the negatively charged DTSL, an effect which is neutralized by the addition of positively charged Ca^{2+}. The Ca^{2+} could interact with the DTSL, as suggested by the ^2H T_{1Z} results at 46.1 MHz, or with the DMPC. Ca^{2+} is known to bind to POPC bilayers (Altenbach & Seelig, 1984), and the presence of additional positive charge at the membrane surface should increase the absolute value of $\Delta\sigma$ (Scherer & Seelig, 1989). A second possibility is for the DTSL to induce increased motional averaging, via an increase in the phospholipid lateral diffusion rate, in the mixed lipid system. A significant increase in diffusion rate would be required to significantly alter the 1D spectrum, however, and thus this possibility does not appear as likely.

These questions may be addressed by solid-state 2D ^{31}P NMR. By employing the pulse sequence originally developed by Jeener et al. (1979) to measure chemical exchange in liquids, it is possible to probe the slow orientational exchange

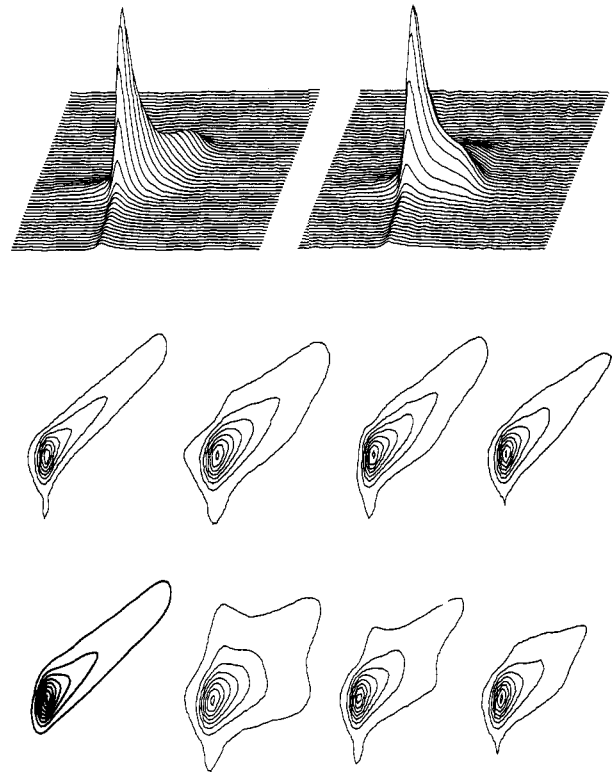

FIGURE 6: 2D ^{31}P NMR spectra of DTSL–DMPC mixtures. Stacked plots of 5 mol % DTSL in DMPC at 30 °C for t_{mix} = 50 μs (top left) and 100 ms (top right). The plot width is ±10 kHz in both dimensions. Contour plots for DMPC, DTSL–DMPC, DTSL–DMPC + 5 mM Ca^{2+}, and DTSL–DMPC + 50 mM Ca^{2+} for t_{mix} = 50 μs (center row, left to right). Bottom row: Same as above, except t_{mix} = 1 ms. The scale of the contour plots is slightly greater than that of the stacked plots.

of phospholipids which results from lateral diffusion over the curved surfaces of membranes (Fenske & Jarrell, 1991). The rate of orientational exchange will depend on at least two factors: the curvature of the membrane surface (and therefore its radius) and the rate of phospholipid lateral diffusion. Without additional information it is not possible to separate these two contributions; nevertheless, a change in orientational exchange resulting from variation in membrane composition indicates an effect on membrane structure not easily observed by many other techniques.

2D ^{31}P spectra were acquired for DMPC, 5 mol % DTSL in DMPC, and 10 mol % DTSL in DMPC in the presence of 5 and 50 mM Ca^{2+}. Representative spectra for DMPC and DPPC are given in Fenske and Jarrell (1991). Representative spectra of DTSL–DMPC liposomes are given in Figure 6. These spectra are difficult to compare directly due to the different lineshapes observed for the different samples (Figure 5), which also complicates the use of spectral simulations as described in Fenske and Jarrell (1991). Nevertheless, several qualitative conclusions can still be made, and it is possible to compare the spectra in a more quantitative manner using an empirical approach to be described below.

Initially, we consider the spectra obtained at short mixing times (50 μs and 1 ms). For DMPC, and for both values of t_{mix}, all spectral intensity is located on the diagonal, indicating that essentially no orientational exchange is observed on these short time scales. For t_{mix} = 50 μs, the addition of 5 mol % DTSL results in a significant increase in the width of the diagonal, indicating an increase in exchange at this frequency. This broadening is reduced by the presence of Ca^{2+}, slightly more effectively at the higher concentration. The same trends are observed at t_{mix} = 1 ms, but the effects are more dramatic.

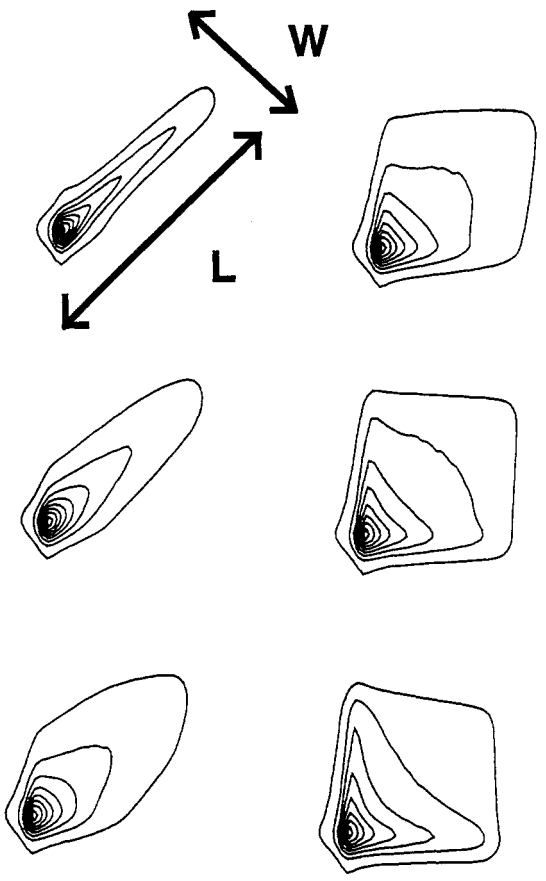

FIGURE 7: Theoretical 2D exchange spectra for $t_{mix}/\tau_d = 0, 0.1, 0.3$ (top to bottom, left column) and 0.8, 1.5, 5.0 (top to bottom, right column).

Significant exchange is observed for 0 mM Ca^{2+}, slightly less for 5 mM Ca^{2+}, and even less for 50 mM Ca^{2+}. However, the exchange at 50 mM Ca^{2+} is still greater than in the absence of DTSL. Thus, the incorporation of DTSL into DMPC liposomes results in a significant increase in the rate of orientational exchange, an effect which is partially reversed by Ca^{2+}.

In order to obtain a more quantitative comparison of the orientational exchange rates observed in DMPC and DMPC–DTSL multilamellar dispersions, an empirical method is necessary to "follow" the exchange process through a series of spectra corresponding to different mixing times. Consider the series of simulations, shown in Figure 7, which were obtained as described in Fenske and Jarrell (1991). The simulations assume spherical liposomes having a single radius of curvature and show the expected change in the 2D spectrum (contour plots) as a function of the ratio of mixing time, t_{mix}, to the correlation time for exchange, τ_d. As t_{mix} increases, the length L of the 2D spectrum decreases (measured parallel to the diagonal) and the width W of the spectrum increases (measured orthogonal to the diagonal). Thus, the exchange should be adequately described by the ratio L/W as a function of t_{mix}; two such plots are shown in Figure 8 for $\tau_d = 1$ and 10 ms. In constructing such plots, the length and width were measured from the lowest contour, and the width was the maximum width for that spectrum. Consideration of Figure 8 reveals that τ_d is given by the point where the curve begins to level off, and thus it should be possible to obtain approximate τ_d values from such a plot.

In order to test this approach, the 2D ^{31}P spectra obtained previously for DMPC and DPPC (Fenske & Jarrell, 1991) were analyzed as shown in Figure 9. The curve of L/W for

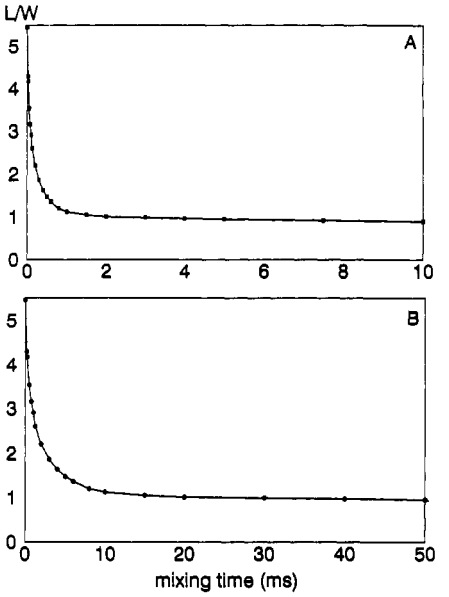

FIGURE 8: Theoretical plots of L/W as a function of t_{mix}, obtained from the simulated spectra in Figure 7, for $\tau_d = 1$ ms (A) and 10 ms (B). Each point represents a different simulation.

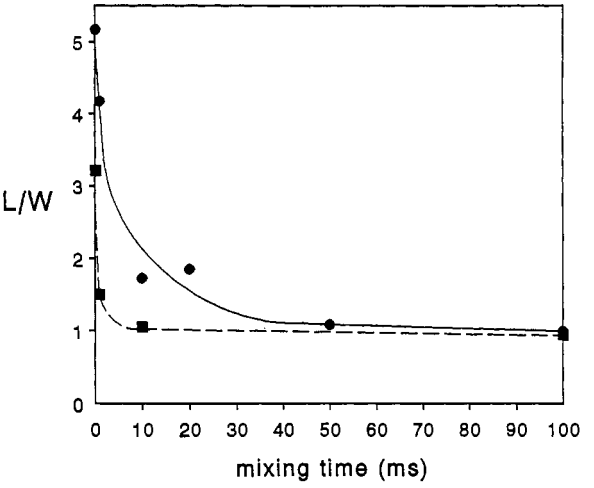

FIGURE 9: Plots of L/W as a function of t_{mix} for DMPC at 30 °C (solid circles and solid line) and DPPC at 45 °C (solid squares and dashed line) (Fenske & Jarrell, 1991).

DMPC is seen to level off at a t_{mix} of approximately 50 ms, and that of DPPC at approximately 10 ms. These values are in excellent agreement with the correlation times obtained via spectral simulations, which were 44 and 8 ms, respectively (Fenske & Jarrell, 1991). The curves obtained for the DMPC–DTSL liposomes are shown in Figure 10. The exchange is seen to level off most rapidly in the absence of Ca^{2+}, giving $\tau_d = 10$ ms (Figure 10A). The samples containing 5 and 50 mM Ca^{2+} level off somewhat more slowly, giving τ_d values of approximately 20 ms (Figure 10B). The rate of orientational exchange is increased significantly by the presence of DTSL, an effect which is partially reversed by Ca^{2+}. The results obtained from the empirical approach agree with the conclusions arrived at from consideration of the exchange observed at short mixing times and in addition yield estimates of the correlation times for exchange.

As mentioned above, there are at least two mechanisms by which the rate of orientational exchange as measured by the ^{31}P NMR approach could be modified. The first is by a change in particle radius; a decrease in radius would increase membrane curvature, allowing more angles with respect to the external magnetic field to be sampled by a phospholipid dif-

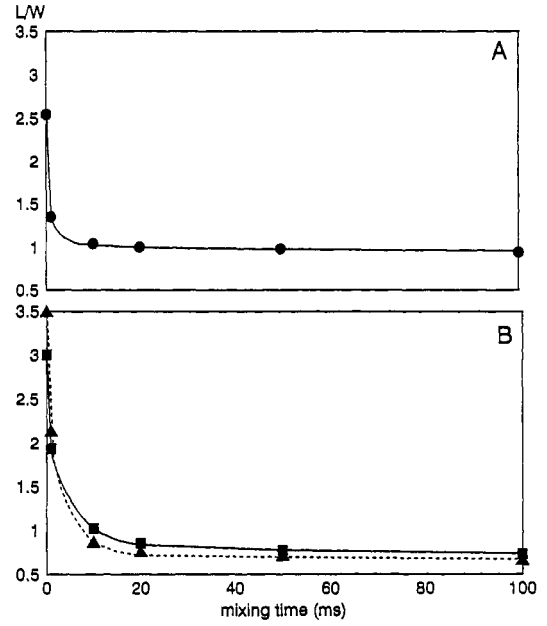

FIGURE 10: Effect of Ca^{2+} on orientational exchange in DTSL–DMPC mixtures. L/W as a function of t_{mix} for $[Ca^{2+}]$ = 0 mM (A), 5 mM (B, solid squares and solid line), and 50 mM (B, solid triangles and dashed line).

fusing through a given distance. However, as discussed above, charged amphiphiles do not seem to affect the size of PC liposomes (Seelig et al., 1987; Scherer & Seelig, 1989), whereas they *are* known to affect the headgroup orientation. In addition, a further mechanism would then be necessary to explain the decrease in exchange observed in the presence of Ca^{2+}. Nevertheless, we cannot rule out absolutely a decrease in liposome radius sufficient to affect the 2D spectrum but not the 1D spectrum.

A second explanation is that charged DTSL alters the packing of PC, leading to an increase in the rate of lateral diffusion. In this regard, it may be significant that the rate of lateral diffusion of diacylglycoglycerolipids is a factor of 2–6 times greater than that of lecithins (Wieslander et al., 1981; Lindblom et al., 1981). While Ca^{2+} does not appear to cause partial phase separation of the DTSL into DTSL-rich regions (which could explain the partial return of diffusion rates to those observed in the absence of DTSL), it must partially shield the net negative charge, possibly reducing any DTSL-induced disruption of DMPC packing.

One conclusion which follows from the 2D studies is that the change in lineshape observed in the 1D spectra *cannot* result from the changes in orientational exchange and therefore must result from changes in phospholipid headgroup orientation. The correlation times estimated for the DTSL-containing samples fall in the range of 10–20 ms. These are intermediate between those obtained for DPPC (τ_d = 8 ms) and DMPC (τ_d = 44 ms) (Fenske & Jarrell, 1991), both of which have essentially identical 1D ^{31}P NMR lineshapes. Thus, the changes in orientational exchange rates are not sufficient to alter the 1D spectra.

Conclusions

In aqueous dispersions, the headgroup orientation of DTSL alone differs from that when mixed with DMPC. This behavior is reminiscent of that seen for other glycolipids (Jarrell et al., 1986; Skarjune & Oldfield, 1982). The rate of headgroup reorientation was reduced relative to that reported for most other glyceroglycolipids, with the exception of one having gentiobiose as the headgroup. While the average orientation of the headgroup was found to be temperature-dependent, that of the glycerol C2–C3 segment was not and differs from that of other glycolipids.

DTSL appears to induce a change in the headgroup orientation of DMPC in DTSL–DMPC dispersions (5–10 mol % DTSL) relative to that of DMPC alone. The presence of DTSL also increases the rate of DMPC orientational exchange, which is most likely due to an increase in the rate of phospholipid lateral diffusion. The latter result suggests that the DTSL is dispersed homogeneously in the DMPC matrix.

The presence of physiological concentrations of Ca^{2+} (in the 5–50 mM range) affects the behavior of both DTSL and DMPC. As the Ca^{2+} concentration is increased, a small change in DTSL headgroup orientation occurs, and the rate of headgroup reorientation is reduced, moving from the short correlation time regime at 0 mM Ca^{2+}, through a T_1 minimum at 5 mM Ca^{2+}, to the long correlation time regime at 50 mM Ca^{2+}. With respect to the DMPC, Ca^{2+} was found to partially reverse the effects caused by the DTSL. The ^{31}P spectra in the presence of Ca^{2+} were similar to that of neat DMPC, and the rates of orientational exchange were reduced, with the correlation time τ_d for exchange increasing from approximately 10 ms in the absence of Ca^{2+} to 20 ms in the presence of Ca^{2+}. These effects of Ca^{2+} most likely proceed via alterations in the membrane surface charge density rather than by partial phase separation of the DTSL.

The application of 1D and 2D ^{31}P NMR in the current study points out that additional information on membrane dynamics and morphology can be obtained, in principle, from 2D chemical exchange spectroscopy. Molecular orientation and ordering can be deduced from the classical ^2H and ^{31}P 1D studies, while global membrane phenomena, such as changes in diffusion rates or membrane morphology, can be probed by 2D methods. Thus, 2D exchange NMR, in conjunction with other techniques, offers a powerful additional approach to questions of membrane structure.

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Registry No. DMPC, 18194-24-6; DTSL, 92131-78-7; Ca, 7440-70-2.

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