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Hydration and stability of sulfatide-containing phosphatidylethanolamine small unilamellar vesicles

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Abstract

The effect of sulfatide on membrane hydration of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) small unilamellar vesicles (SUVs) was investigated using steady-state and time-resolved fluorescence spectroscopy. The degree of hydration in the headgroup region of the bilayer lipids was assessed with the fluorescence lifetime of *N*-(5-dimethylaminonaphthalene-1-sulfonyl)dipalmitoylphosphatidylethanolamine along with the ratio of its fluorescence intensities measured in samples prepared either in D_2O- or in H_2O-based buffers. Similarly, hydration of acyl chains near the headgroup region and that close to the bilayer center were studied using 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene and 1-palmitoyl-2-[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine as probes. Increasing sulfatide concentration up to 30 mol% resulted in an increase in surface hydration and a decrease in interchain hydration. These were correlated with an increase in bilayer stability of the DOPE/sulfatide SUVs. Moreover, variation of pH was found to affect the hydration and stability of the bilayer vesicles. No further change in headgroup hydration and interchain hydration near the bilayer center was observed at sulfatide concentrations $\geq$30 mol%. At such high sulfatide concentrations, bilayer hydration and stability were no longer pH-sensitive. The effects of sulfatide on hydration and stability of DOPE bilayer vesicles are discussed by taking into account the electrostatic and geometrical properties of the sulfated galactosyl headgroups.

Keywords: Fluorescence lifetime; Membrane hydration; Bilayer stability

1. Introduction

Sulfatide (cerebroside sulfate) has been found in a number of mammalian tissues. In particular, it comprises approximately 6% of the total lipids in brain and is localized mainly to myelin sheath [1,2]. The major mammalian sulfatide consists of monogalactosylceramide in which the C(3) of the galactosyl headgroup is sulfated. It has been suggested that sulfatide is involved in a variety of membrane phenomena such as cell adhesion, platelet aggregation and regulation of enzyme activities [3–5]. On the other hand, sulfatide has often been chosen as the minor lipid component of various liposomes for drug delivery [6–8]. Incorporation of sulfatide into phospholipid such as DOPE vesicles greatly enhances the bilayer

Abbreviations: DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; SUVs, small unilamellar vesicles; PE, phosphatidylethanolamine; PC, phosphatidylcholine; dansyl-PE, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-dipalmitoylphosphatidylethanolamine; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; DPH-PC, 1-palmitoyl-2-[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine; $H_{\|}$, inverted hexagonal phase

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stability and targeting capability of the liposomes [7,8]. It is believed that the hydration level of the negatively charged sulfate headgroup plays an important role in the stabilization of the DOPE/sulfatide vesicles. However, no direct experimental proof exists to support this assertion.

It is well established that there is a 'bound' hydration shell surrounding the headgroups of membrane lipids. In addition, water molecules can also reach deeper into the lipid bilayer and the degree of interchain hydration depends on the packing defects of the acyl chains [9–11]. Since membrane physical parameters such as fluidity and order are strongly correlated only with the interchain hydration, variation of these parameters may not cause any specific change in headgroup hydration [10,11]. The opposite to the above conclusion may also be true. It has been shown that dehydration of the sulfatide headgroup, as induced by protonation of the sulfate group at a reduced pH, does not correlate to the bilayer fluidity of DOPE/sulfatide bilayer vesicles [8].

Water penetration into lipid bilayers generates a dielectric constant gradient from the membrane surface to the bilayer center. Various methods, including neutron diffraction, electron spin resonance, and capacitance techniques, have been used to probe this dielectric constant gradient [12–14]. More recently, it has been shown that information on water penetration into lipid bilayers can be obtained with time-resolved fluorescence spectroscopy [10,11,15,16]. In this work, we have employed the method of fluorescence spectroscopy to study the degree of hydration in DOPE/sulfatide bilayer vesicles at three different levels of depths, namely, the lipid-water interface, the upper acyl chain region which is just below the headgroups and the membrane bilayer center. For this purpose, three fluorescence probes, dansyl-PE, TMA-DPH and DPH-PC, were used and their fluorescence lifetimes were determined as this parameter is known to be very sensitive to the solvent environment of the probes [17–20]. Generally, the greater the degree of hydration, the faster is the excited-state fluorescence decay, or the shorter the fluorescence lifetime. Use of deuterium isotope assay for studying water presence in the vicinity of a fluorophore provides an additional measurement for membrane hydration [21]. In this respect, fluorophores such as dansyl-PE and TMA-DPH have been shown to possess a greater quantum yield in D_2O compared to H_2O due to the reduced rate of nonradiative decay [16,21,22].

The objective of this work was to investigate the correlation between membrane hydration and bilayer stability of DOPE/sulfatide vesicles. The stability of the bilayer vesicles was estimated in terms of the leakage of an encapsulated fluorescent marker, calcein. Our results show that, in a concentration-dependent manner, sulfatide increases the amount of water bound to the lipid-water interface and decreases the interchain hydration. A reduction in pH, which destabilizes the DOPE/sulfatide bilayer vesicles, results in dehydration in the headgroup region but concomitantly more water appears in the acyl chain region. These results provide evidence for the roles played by membrane hydration in the stabilization and pH sensitivity of DOPE/sulfatide bilayer vesicles.

2. Materials and methods

Deuterium oxide, DOPE, calcein, Sephadex G-50, galactocerebroside and sulfatide from bovine brain were purchased from Sigma (St. Louis, MO). The percentage of nonhydroxy and 2-hydroxy fatty acyl chains of the sulfatides was approximately 69.5% and 30.5%, respectively, as determined by thin-layer chromatography using a solvent system of chloroform-methanol-acetone-acetic acid-water (8:2:4:2:1, v/v). DPH-PC, dansyl-PE and TMA-DPH were obtained from Molecular Probes (Eugene, OR). The above chemicals and other reagents of analytical grade were used without further purification. Deionized water was used for all experiments.

2.1. Preparation of bilayer vesicles

DOPE SUVs containing 10–40 mol% sulfatide were prepared by sonication under nitrogen as described by Thulborn and Sawyer [23]. Briefly, various fluorescence probes and appropriate amounts of DOPE and sulfatide were transferred from their respective stock solutions into glass tubes and dried by evaporation under a nitrogen stream. The fluorophore:lipid ratio was 1:200 for DPH-PC, 1:100 for TMA-DPH and 1:75 for dansyl-PE, respectively.

The samples were stored under vacuum for 24 h at 4°C and the thin lipid films formed on the wall of the glass tubes were hydrated with a Tris buffer (10 mM Tris, 150 mM NaCl, pH 6.0 or 7.4). The samples were then sonicated under nitrogen for 10 min (30 s on and 30 s off for each cycle) with a Heat Systems Sonicator XL at maximum power output. Temperature was controlled with an ice-water bath and no lipid degradation was found as examined by thin-layer chromatography. For deuterium isotope exchange experiments, the Tris buffer was also prepared with D_2O in addition to H_2O. The total lipid concentration was 0.2 mM.

Calcein-loaded lipid vesicles were prepared essentially as above, except that the Tris buffer was replaced by an aqueous solution of 175 mM calcein and a higher starting lipid concentration (1 mM) was used. A trace amount of DPH-PC was included to calibrate the concentration of the membrane lipids. The unencapsulated fluorescence dye was eliminated by gel filtration on Sephadex G-50 column using the Tris buffer as eluent. The final lipid concentration was adjusted to 0.2 mM.

2.2. Steady-state fluorescence measurements

Steady-state fluorescence was measured with a Perkin-Elmer LS-50B spectrofluorometer equipped with a thermostatically controlled cell holder. Excitation and emission wavelengths were 335 and 510 nm for dansyl-PE and 360 and 430 nm for TMA-DPH and DPH-PC, respectively. The slit width was 5 nm for both excitation and emission monochromators. The stability of DOPE/sulfatide bilayer vesicles was assayed by the extent of calcein leakage, which was calculated with the following equation:

$$\%\text{release} = (F - F_0)/(F_t - F_0) \times 100,$$

where F is the calcein fluorescence intensity after incubation in the Tris buffer for 8 h, F_0 is the initial fluorescence intensity and F_t is the total fluorescence intensity measured after lysis of the liposomes in the presence of 1% Triton X-100. The release of quenched calcein fluorescence was measured at 516 nm, with the excitation at 495 nm.

2.3. Fluorescence lifetime measurements

Fluorescence lifetimes were determined using a K2 multifrequency phase and modulation fluorometer (ISS, Champaign, IL) equipped with an ISS ADC interface for data collection [24]. The excitation wavelengths were set at 335 nm for dansyl-PE and 360 nm for the DPH-containing fluorophores, respectively. The intensity of the incident light was modulated sinusoidally with a Pockels cell. Short

Table 1
Fluorescence decay analyses of dansyl-PE, TMA-DPH and DPH-PC in sulfatide (30 mol%)-containing DOPE SUVs at pH 7.4 and 25°C

Probe		τ_1	w_1	f_1	τ_2	w_2	f_2	χ^2_{red}	F
dansyl-PE	1 exp	10.57 ± 0.14		1.00				673.50	1
	2 exp	12.96 ± 0.08		0.96 ± 0.01	0.93 ± 0.15		0.04 ± 0.01	1.38	3
	1 Lor	11.46 ± 0.18	8.96 ± 0.33	1.00				154.7	2
	2 Lor	12.93 ± 0.08	0.05 ± 0.00	0.96 ± 0.01	0.74 ± 0.27	0.05 ± 0.01	0.04 ± 0.01	1.55	5
TMA-DPH	1 exp	4.16 ± 0.03		1.00				57.00	1
	2 exp	4.70 ± 0.09		0.93 ± 0.01	1.25 ± 0.30		0.07 ± 0.01	2.29	3
	1 Lor	4.29 ± 0.04	1.05 ± 0.04	1.00				5.25	2
	2 Lor	4.68 ± 0.05	0.49 ± 0.23	0.93 ± 0.01	0.86 ± 0.35	0.05 ± 0.01	0.07 ± 0.01	2.29	5
DHP-PC	1 exp	6.94 ± 0.03		1.00				6.60	1
	2 exp	7.20 ± 0.09		0.96 ± 0.02	2.59 ± 0.75		0.04 ± 0.05	1.57	3
	1 Lor	7.10 ± 0.06	0.56 ± 0.09	1.00				1.59	2
	2 Lor	7.05 ± 0.06	0.44 ± 0.04	0.99 ± 0.01	0.13 ± 0.09	0.05 ± 0.01	0.01 ± 0.01	1.61	5

τ, lifetime centers ($\times 10^{-9}$ s); w, widths at half height of distribution ($\times 10^{-9}$ s); f, fractional intensities; χ^2_{red}, reduced chi-square; F, number of floating parameters. S.E.M. values were from three individual measurements. The average errors in the phase and modulation were 0.2° and 0.004, respectively.

wavelength cut-off filters (KV389 for dansyl-PE and KV418 for TMA-DPH and DPH-PC, Schott Glass Tech.) were used in the emission side to remove contributions from scattered light. In order to eliminate the effects of polarization on lifetime measurements, a Glan Thompson prism polarizer oriented at 55° (magic angle) to the vertical laboratory axis was placed in the fluorescence lightpath [25]. Phase and modulation were collected at 12 frequencies ranging from 2 to 130 MHz. All measurements were obtained by using ρ-bis[2-(5-phenyloxazolyl)]benzene in ethanol as a reference standard, which has a lifetime of 1.35 ns [26]. Temperature was controlled with a circulating water bath and measured with a thermocouple immersed in the sample cuvette. The experimental phase and modulation data were analyzed using a least-squares program from ISS (The ISS187 Decay Analysis Software) as described previously [27]. The estimated uncertainties of phase and modulation measurements were taken as 0.2° and 0.004, respectively.

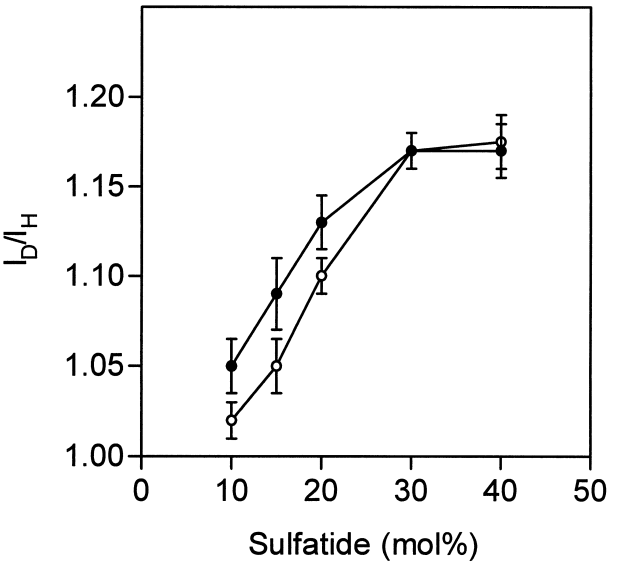

Fig. 2. Effect of sulfatide concentration on the deuterium isotope effect on dansyl-PE in DOPE/sulfatide bilayer vesicles. The dansyl D_2O/H_2O fluorescence intensity ratio (I_D/I_H) was determined at 25°C and pH 7.4 (●) or 6.0 (○). Each point represents the mean ± S.D. of three independent measurements under identical conditions.

3. Results

Phase shifts and demodulations obtained at various modulation frequencies ranging from 2 to 130 MHz were fitted either to single and double exponential decays or to unimodal and bimodal Lorentzian distributions. Typical results are shown in Table 1 and the goodness-of-fit is determined by the value of the reduced χ^2_{red}. Under conditions where χ^2_{red} is indistinguishable, a model with the minimum number of floating parameters is chosen, as it is statistically the most probable model. Therefore, for dansyl-PE in DOPE/sulfatide vesicles, the double exponential model with three floating parameters is apparently superior to the bimodal Lorentzian distribution that has five floating parameters, given essentially the same χ^2_{red} values (Table 1). Similarly, the double exponential model and the unimodal Lorentzian distribution are the most appropriate models for the fluorescence intensity decays of TMA-DPH and DPH-PC, respectively. For dansyl-PE and TMA-DPH, the minor lifetime components (less than 7% of the total intensity) showed no systematic variation for varying sulfatide concentrations. Their physical origin in terms of membrane properties is also unclear [28,29]. Therefore, for comparison purposes, only the main lifetime component has been analyzed.

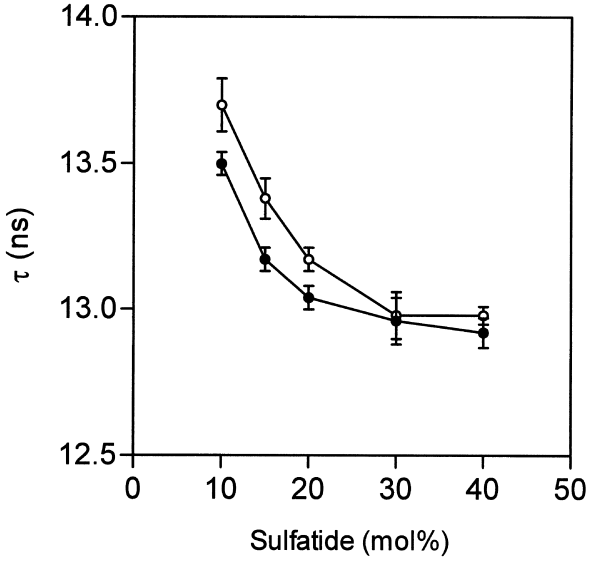

Fig. 1. Effect of sulfatide concentration on the fluorescence lifetime (τ) of dansyl-PE. The experiments were carried out at 25°C and pH 7.4 (●) or 6.0 (○). Each point represents the mean ± S.D. of three independent experiments under identical conditions.

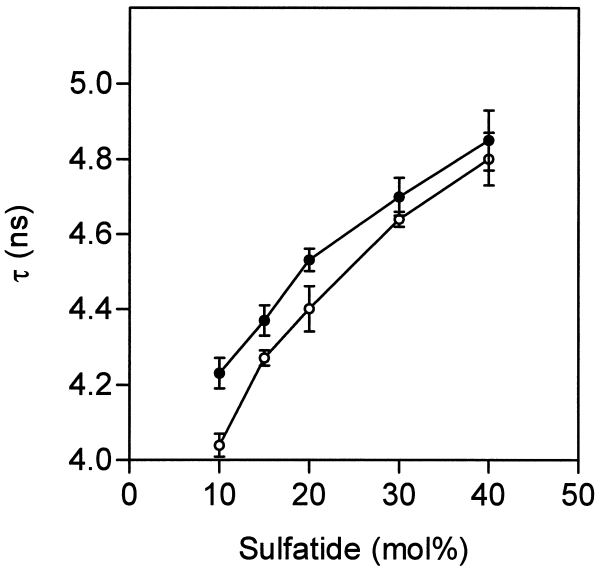

Fig. 3. Effect of sulfatide concentration on the fluorescence lifetime (τ) of TMA-DPH. The experiments were carried out at 25°C and pH 7.4 (●) or 6.0 (○). Each point represents the mean ± S.D. of three independent experiments under identical conditions.

3.1. Effect of sulfatide on dansyl-PE fluorescence emission

Fig. 1 shows the effects of sulfatide on the fluorescence lifetime of dansyl-PE. Increasing the concentration of sulfatide in DOPE bilayers resulted in a modest decrease of the main lifetime component of dansyl-PE from 13.50 ns at 10 mol% sulfatide to 12.96 ns at 30 mol% sulfatide (pH 7.4). This suggests that sulfatide is able to attract more water molecules to the vicinity of the fluorophore at the membrane surface. The additional hydration is believed to be provided mainly by the negatively charged sulfate groups of sulfatide molecules [8]. Fig. 1 also shows that the degree of headgroup hydration was pH-dependent in the sulfatide concentration range of 10–30 mol%, as a simple reduction in pH from 7.4 to 6.0 resulted in a decrease of approximately 0.20 ns in dansyl-PE fluorescence lifetime in an otherwise identical bilayer system. However, the fluorescence lifetime of dansyl-PE became insensitive to pH and remained constant after sulfatide concentration reached 30 mol%, implying that the surface hydration might have arrived at a maximum level under the given conditions.

In order to investigate further the effect of sulfatide on the hydration of bilayer surface, deuterium isotope exchange experiments were carried out at varying pH levels and sulfatide concentrations. A higher yield of fluorescence emission was always observed when H_2O was replaced by D_2O, suggesting that the fluorophore itself be in contact with water (Fig. 2). Since the degree of hydration is proportional to the ratio of fluorescence intensity in D_2O to that in H_2O (I_D/I_H), it can be seen that increasing sulfatide concentration up to 30 mol% resulted in an increase in surface hydration, in agreement with the lifetime experiments. Furthermore, the level of surface hydration was found to be dependent on pH of the medium, with the bilayer being less hydrated at the acidic pH. The deuterium exchange experiments also confirmed that, at sulfatide concentrations higher than 30 mol%, hydration at the bilayer surface reached a maximum level and then became pH insensitive.

3.2. Effect of sulfatide on TMA-DPH fluorescence emission

TMA-DPH, whose DPH fluorescent moiety is directly linked to a cationic head group, can be con-

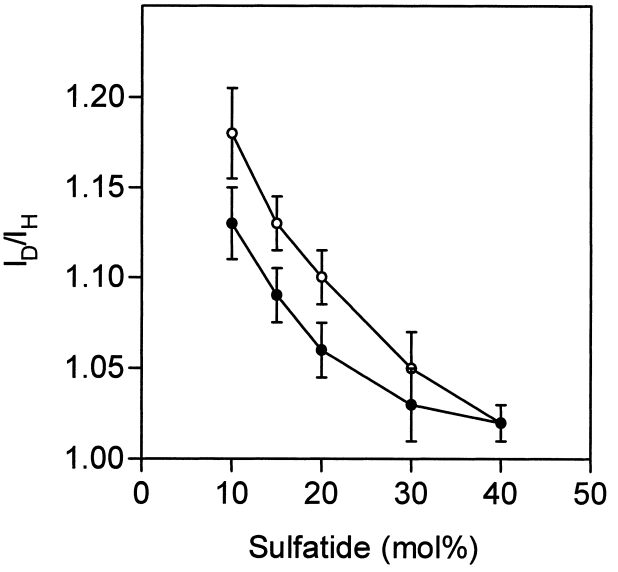

Fig. 4. Effect of sulfatide concentration on the deuterium isotope effect on TMA-DPH in DOPE/sulfatide bilayer vesicles. The DPH D_2O/H_2O fluorescence intensity ratio (I_D/I_H) was determined at 25°C and pH 7.4 (●) or 6.0 (○). Each point represents the mean ± S.D. of three independent measurements under identical conditions.

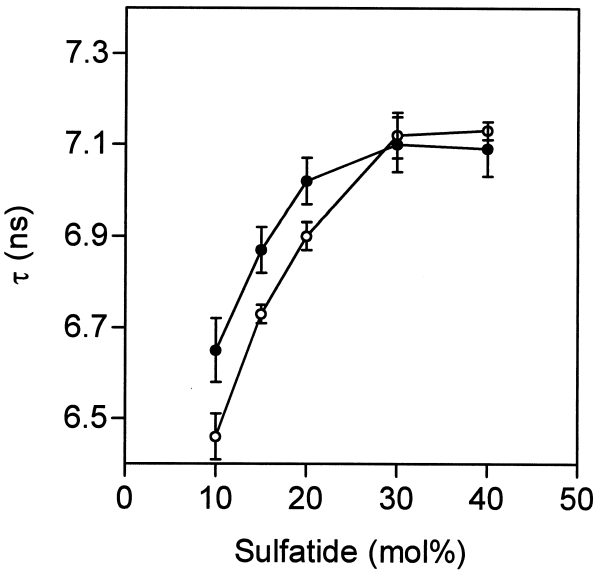

Fig. 5. Effect of sulfatide concentration on the fluorescence lifetime (τ) of DPH-PC. The experiments were carried out at 25°C and pH 7.4 (●) or 6.0 (○). Each point represents the mean ± S.D. of three independent experiments under identical conditions.

sidered as probing the upper acyl chain region (C1 to C10) of the leaflet on which it is bound. The effect of sulfatide concentration on the penetration of water into the DOPE bilayer was first evaluated by studying the TMA-DPH fluorescence lifetime. Fig. 3 shows that increasing sulfatide concentration from 10 to 40 mol% resulted in a steady increase in DPH lifetime, suggesting that the degree of hydration in the upper acyl chain region was reduced by increasing sulfatide concentrations. It can also be seen that below 30 mol% sulfatide, the DPH lifetime was marginally pH sensitive. Since TMA-DPH was known to be sensitive to the presence of deuterium [16], the method of deuterium isotope exchange was again used to confirm the results obtained by lifetime studies. Fig. 4 shows that the TMA-DPH D_2O/H_2O fluorescence intensity ratio decreased upon increasing sulfatide concentration, indicating that dehydration did occur in the upper acyl chain region in the presence of sulfatide.

3.3. *Effect of sulfatide on DPH-PC fluorescence lifetime*

As pointed out earlier, the decay of DPH-PC fluorescence intensity is best described by the unimodal Lorentzian distributions. We have also attempted to fit the data to the uniform or Gaussian distributions; however, there was no better fit in terms of the χ^2_{red} values. Fig. 5 shows that, at pH 7.4, increasing the amount of sulfatide in DOPE bilayer vesicles from 10 to 40 mol% led to an increase in the DPH-PC lifetime distributional center from 6.60 ns to 7.10 ns. This reflects the ability of sulfatide in reduction of water penetration in the bilayer center. A similar effect of sulfatide was observed at pH 6.0 and the hydration level of acyl chains was found to be pH-dependent. This pH dependence of DPH-PC lifetime was abolished at sulfatide $\geq$ 30 mol%.

3.4. *Effect of sulfatide and galactocerebroside on bilayer stability*

The stability of liposomes at pH 6.0 and 7.4 was estimated in terms of the leakage of an entrapped fluorescent marker – calcein, whose fluorescence intensity is independent of pH in the range of 6.0–9.0 [30]. Sulfatide concentration in DOPE bilayer vesicles was varied from 10 to 40 mol%. The percentage of calcein released after 8 h incubation at either pH 7.4 or 6.0 is shown in Fig. 6. It can be seen that the amount of calcein released was substantially re-

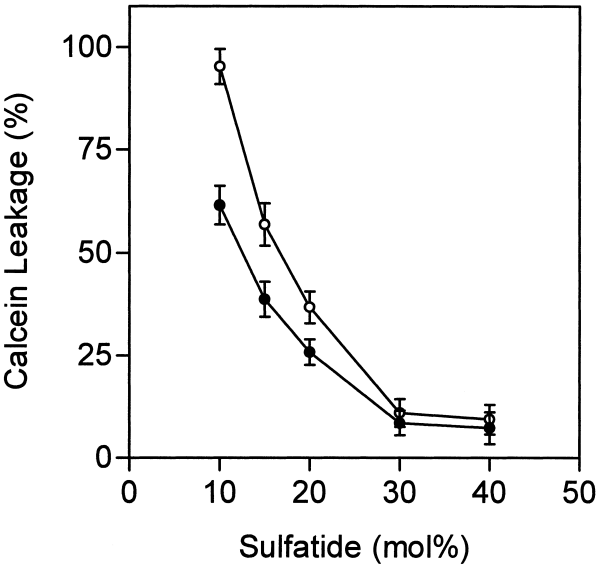

Fig. 6. Effect of sulfatide concentration on the stability of DOPE/sulfatide bilayer vesicles as followed by the release of calcein at 25°C. (●) pH 7.4; (○) pH 6.0. Each illustrated point represents the mean ± S.D. of three independent experiments under identical conditions.

duced upon increasing the content of sulfatide from 10 to 30 mol%, where the effect of sulfatide started to level off. It appeared that a reduction in pH would result in more calcein being released. However, this effect of sulfatide was abolished when its concentration was higher than 30 mol%. On the other hand, a control experiment in the presence of 30 mol% bovine brain galactocerebroside showed that over 95% of calcein were released after 8 h incubation at 25°C, irrespective of the pH values of the incubation media. These results suggest that the negatively charged sulfate group is essential for the stability and pH sensitivity of the sulfatide-containing DOPE SUVs.

4. Discussion

In this study, dansyl-PE, TMA-DPH and DPH-PC were used to obtain information, as a function of sulfatide concentration and medium pH, on surface hydration and water penetration at different depths in bilayers of sulfatide-containing DOPE SUVs. The effects of sulfatide and pH on the structural stability of DOPE vesicles were also examined. It has been found that, at <30 mol% sulfatide, increasing the concentration of the glycosphingolipid resulted in an increase in surface hydration and a concomitant decrease in interchain hydration. These effects were leveled off at ≥ 30 mol% sulfatide. Interestingly, the amount of sulfatide (i.e. 30 mol%) required to reach maximum surface hydration and minimum interchain hydration is consistent with that needed for optimal bilayer stability, suggesting that the stability of the DOPE/sulfatide bilayers is closely correlated with the hydration levels of the membrane surface and acyl chains. This hypothesis has been further tested by studies on the effects of pH on hydration and stability of the bilayer vesicles. Our results showed that the stability of sulfatide-containing liposomes was pH-sensitive at <30 mol% sulfatide, where the surface and interchain hydration could be modulated by variation of pH, confirming that membrane hydration does affect the stability of DOPE/sulfatide bilayer vesicles.

It is proposed that sulfatide stabilizes the bilayer structure of DOPE SUVs by promoting higher water content in the headgroup region while reducing water penetration into the hydrophobic acyl chain region. This statement has been drawn from studies on the fluorescence properties of the three fluorophores employed. For example, a decrease in fluorescence lifetime of dansyl-PE was interpreted as an increase in polarity or hydration in the vicinity of the fluorophore (Fig. 1). Since sulfatide is negatively charged at the headgroup, its presence in the lipid bilayer may affect the D_2O/H_2O fluorescence intensity ratio of dansyl-PE [11]. However, this is unlikely the case here as the results obtained by deuterium isotope exchange experiments are comparable to those obtained by lifetime studies (Fig. 2). It should be pointed out that the decrease in dansyl fluorescence lifetime could not be explained by a conformational change of the PE headgroup. Deuterium nuclear magnetic resonance (^{2}H-NMR) of the deuterated choline group shows that the P^--N^+ dipole tilts with respect to the plane of the PC bilayer surface in the presence of membrane surface charge [31,32]. In this respect, the behavior of phosphoethanolamine headgroup is similar to that of phosphocholine [33]. Since the sulfate group is negatively charged, the N^+ end of ethanolamine headgroup would be forced to reorient toward the membrane interior in the presence of sulfatide. This should bring the dansyl moiety, which is coupled to the tip of the PE headgroup, into a more hydrophobic environment. If such a situation were to occur, the dansyl fluorescence lifetime would increase and its D_2O/H_2O intensity ratio would decrease upon increasing sulfatide concentration in the DOPE vesicles. However, these are just opposite to what have been found in this study. Therefore, the decrease in dansyl fluorescence lifetime and increase in its D_2O/H_2O intensity ratio can only be ascribed to an increase in hydration of the bilayer surface region, where the dansyl group is located.

The increase in surface hydration could be attributed to an increase in sulfatide hydration and/or an increase in DOPE hydration. Although the hydration number of the headgroup of sulfatide is not yet available, its galactose moiety should resemble that of corresponding galactocerebrosides in terms of hydration capacity [34]. Since the hydration number of galactose residues is rather small and not much different from that of PE molecules [34,35], the negatively charged sulfate groups are thus considered to be essential for increase in the surface hydration and

stabilization of the DOPE vesicles. These would seem to be the case, as the galactocerebroside-containing liposomes are extremely unstable compared to the sulfatide-containing liposomes. Moreover, in a similar system, Faure et al. [36] have found that cholesterol sulfate binds ~12 more water molecules at the lipid-water interface than cholesterol does. As far as the hydration of PE headgroup is concerned, there is likely extensive hydrogen bonding between an amine of one PE and the phosphate of another adjacent PE headgroup. The consequence of this hydrogen bonding network among the PE molecules is a very limited capacity to form hydrogen bond between PE and water molecules. Presence of sulfatide might be able to disrupt this hydrogen bonding network and increase the chance for PE to form hydrogen bond with water molecules. This hypothesis is surely supported by the fact that PC added to PE tremendously increases the surface hydration of the mixed phospholipid bilayer vesicles [37].

We have also found in this study that incorporation of sulfatide up to 30 mol% had opposing effects on hydration in the headgroup region with respect to hydration in the hydrophobic core of the bilayers, that is, it increases the surface hydration but decreases the interchain hydration. Considering the long acyl chains of sulfatide, it is not surprising that the behavior of the glycosphingolipid is qualitatively similar to that of long-chain *n*-alkanols [11]. It was assumed that water penetration was reduced because the long chains of methylene groups filled up the void volumes in the acyl chain region [38]. If this is the case, the acyl chain region of the bilayer vesicles should become more rigid. Indeed, introduction of sulfatide into DOPE vesicles apparently reduced the motional freedom of the CH_2 segments as the fluidity of the bilayer acyl chains was decreased (data not shown). An increase in sulfatide mole concentration in the DOPE vesicles from 30 to 40 mol% failed to induce further change in the hydration levels of the bilayer surface and hydrophobic center, suggesting these effects of sulfatide had reached the maximum level (see below for more discussion). However, the increase in hydrophobicity continued at ≥ 30 mol% sulfatide in the acyl chain region near the lipid-water interface, as probed by TMA-DPH (Fig. 3), implying that the dielectric constant gradient set up by water penetration was pushed up towards the lipid-water interface at higher sulfatide concentrations.

A noticeable drop in the amount of bound water molecules in the surface of DOPE vesicles containing less than 30 mol% sulfatide occurred when pH was reduced from 7.4 to 6.0. This was revealed by a longer dansyl-PE fluorescence lifetime and a lower dansyl-PE D_2O/H_2O fluorescence intensity ratio at the acidic pH. A reduction in pH to 6.0 might result in partial protonation of the headgroup of sulfatide, followed by partial dehydration in the bilayer surface due to the loss of charge. It has been shown that dehydration of PE membranes favors the lamellar-hexagonal ($H_{\|}$) phase transition [39,40]. This tendency of phase transition should cause disorder of the acyl chains and thus more water can penetrate into the bilayer acyl chain region. In other words, dehydration in the PE-containing bilayer surface may result in an increase in interchain hydration. This is exactly what has been observed with TMA-DPH and DPH-PC fluorophores (Figs. 3–5). It is in this respect that PE bilayers are different from PC bilayers. PC molecules have no tendency to form nonbilayer hexagonal phase and the surface and interchain hydration of PC bilayers appears entirely uncoupled [11]. On the other hand, it has been suggested that in mixed systems of PE and other lipids, bilayer vesicles become destabilized as their PE component reverts to the hexagonal phase [41]. Our results on bilayer stability at <30 mol% sulfatide, where a reduction in pH from 7.4 to 6.0 gave rise to an increase in calcein release (Fig. 6), confirm that the stability of PE containing vesicles is largely maintained by hydration in the bilayer surface.

The effects of sulfatide on membrane hydration, stability and pH sensitivity of DOPE/sulfatide bilayer vesicles were leveled off at 30 mol% sulfatide. This might be explained in terms of the steric interference of the bulky sulfatide headgroups, which occupy progressively more space at higher sulfatide concentrations. Since the surface areas occupied by a DOPE molecule and a sulfatide molecule are 38 $Å^2$ [42] and 75 $Å^2$ [43,44], respectively, in a DOPE bilayer vesicles containing 30 mol% sulfatide, about 45% of the surface area would be covered by the glycosphingolipid. Under this condition, there might be direct molecular contacts between the hydrated sulfatide headgroups. Due to the geometrical restriction, fur-

ther increase in sulfatide concentration beyond 30 mol% might be difficult to bring more water to the immediate vicinity of the dansyl-PE probes. It should be pointed out that the effect of pH on the stability of DOPE/sulfatide (30 mol%) vesicles was studied before in the presence of 50% human plasma [8]. It has been found that the amount of calcein released from the bilayer vesicles at pH 6.0 doubled that at the physiological pH after 8 h at 37°C. The apparent discrepancy between the present study and the one reported before at 30 mol% sulfatide might be explained by specific interactions between sulfatide molecules and the plasma components, particularly the very low-density lipoprotein particles [45]. The plasma-dependent pH sensitivity of bilayer stability has been ascribed to the possibility of forming sulfatide-enriched lipid domains in the bilayer vesicles [45].

In conclusion, we have completed a systematic study on membrane hydration for bilayer vesicles composed of varying amounts of DOPE and sulfatide. It has been found that the hydration profile of the membrane bilayer is correlated to its stability and pH sensitivity. All these physical parameters are affected by the concentration of the glycosphingolipid present in the lipid bilayer. Particularly, a major change in the structure-function relationship has taken place in the DOPE/sulfatide bilayer vesicles containing about 30 mol% sulfatide. This information should be useful in future for designs of PE-based liposomes for drug delivery, gene transfer and other practical purposes.

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