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Water Dynamics in Glycosphingolipid Aggregates Studied by LAURDAN Fluorescence

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ABSTRACT   We have characterized the fluorescence properties of 6-dodecanoyl-2-dimethylamine-naphthalene (LAURDAN) in pure interfaces formed by sphingomyelin and 10 chemically related glycosphingolipids (GSLs).[1] The GSLs contain neutral and anionic carbohydrate residues in their oligosaccharide chain. These systems were studied at temperatures below, at, or above the main phase transition temperature of the pure lipid aggregates. The extent of solvent dipolar relaxation around the excited fluorescence probe in the GSLs series increases with the magnitude of the glycosphingolipid polar headgroup below the transition temperature. This conclusion is based on LAURDAN's excitation generalized polarization (GP_{ex}) and fluorescence lifetime values found in the different interfaces. A linear dependence between the LAURDAN GP_{ex} and the intermolecular spacing among the lipid molecules was found for both neutral and anionic lipids in the GSLs series. This relationship was also followed by phospholipids. We conclude that LAURDAN in these lipid aggregates resides in sites containing different amounts of water. The dimension of these sites increases with the size of the GSLs polar headgroup. The GP function reports on the concentration and dynamics of water molecules in these sites. Upon addition of cholesterol to Gg_4Cer, the fluorescence behavior of LAURDAN was similar to that of pure cerebrosides and sphingomyelin vesicles. This observation was attributed to a change in the interfacial hydration as well as changes in the shape and size of the Gg_4Cer aggregates in the presence of cholesterol. After the addition of cholesterol to gangliosides, the changes in the LAURDAN's spectral parameters decrease progressively as the polar headgroup of these lipids becomes more complex. This finding suggests that the dehydration effect of cholesterol depends strongly on the curvature radius and the extent of hydration of these lipid aggregates. In the gel phase of phrenosine, GalCer, Gg_3Cer, sulfatide, and sphingomyelin, the excitation red band (410 nm) of LAURDAN was reduced with respect to that of LAURDAN in the gel phase of pure phospholipids. This observation indicates a local environment that interacts differently with the ground state of LAURDAN in GSLs when compared with LAURDAN in phospholipids.

INTRODUCTION

Glycosphingolipids (GSLs) are ubiquitous components of animal cell membranes that are particularly abundant in the nervous system (Tettamanti, 1988). GSLs are involved in a large variety of physiologically important phenomena (Tettamanti, 1988; Maggio, 1994). For example, the simple polar headgroup GSLs play a role in stabilizing membranes (Pascher, 1976), perhaps through interlipid hydrogen bonds

Abbreviations used: GSLs, glycosphingolipids; Cer, ceramide: (*N*-acyl-sphingosine); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; GalCer, Galβ1→1′Cer; Gg_3Cer (asialo G_{M2}), Gal-NAcβ1→4Galβ1→4Glcβ1→1′Cer; Gg_4Cer (asialo G_{M1}), Galβ1→3Gal-NAcβ1→4Galβ1→4Glcβ1→1′Cer; G_{M3}, NeuAc2α→3Galβ1 4Glcβ1→1′Cer; G_{M2}, Gal-NAcβ1→4Gal(3←2αNeuAc)β1→4Glcβ1→1′Cer; G_{M1}, Galβ1→3Gal-NAcβ1→4Gal(3←2αNeuAc)β1→4Glcβ1→1′Cer; G_{D1a}, NeuAc2α→3Galβ1→3Gal-NAcβ1→4Gal(3←2αNeuAc)β1→4Glcβ1→1′Cer; G_{T1b}, NeuAc2α→3Galβ1→3GalNAcβ1→4Gal(3←2αNeuAc8←2αNeuAc)β1→4Glcβ1→1′Cer; LAURDAN, 6-dodecanoyl-2-dimethylamine-naphthalene; Phre, phrenosine: Galβ1→1′(N-[α-OH]acyl-sphingosine); Sphin, egg yolk sphingomyelin; Sulf, HSO_3→3Galβ1→1′Cer.

(Pascher, 1976; Boggs, 1987; Curatolo, 1987). Neutral GSLs and gangliosides are implicated in cell recognition and signal transduction processes (Tettamanti, 1988; Maggio, 1994). These lipids occur in relatively large amounts in specialized membranes such as myelin (Norton and Cammer, 1984; Ledeen, 1989; Yu and Saito, 1989; Maggio et al., 1986). In particular, gangliosides are enriched in nerve cells where they represent between 2% and 10% of the total lipids. On the neuronal surface >30% of the sialic acid content is supplied by these lipids (Maggio, 1994; Curatolo, 1987). However, the effects of these lipids on the structure and function of cell membranes remain obscure.

Previous work from our laboratory has elucidated several biophysical properties of a series of chemically related GSLs ranging from simple cerebrosides to complex gangliosides (Maggio, 1994; Maggio et al., 1981, 1986). Gangliosides contain one or more sialic acids attached to a neutral oligosaccharide chain resulting in a series of polar headgroups of different complexity. The hydrophilic portion of these gangliosides reaches a length similar to that of the hydrocarbon portion and, in turn, dramatically influences the surface, thermotropic, and topological properties of ganglioside micelles (Maggio, 1994; Maggio et al., 1981, 1985, 1986, 1988; Fidelio et al., 1986). The number and type of carbohydrates in the polar headgroup of these GSLs also have a direct influence on the extent of the interfacial hydration or micropolarity (Montich et al., 1985 and 1988)

and on the polarity gradient taken from the polar headgroup to the hydrophobic core of the lipid aggregate (Bagatolli et al., 1995). (The term aggregate was used in the text as a general term. In Table 3 the shape of the aggregates is specified for each lipid.)

To study the polarity of the lipid interfaces, we used LAURDAN, which is an amphiphilic fluorescence probe synthesized by Prof. G. Weber to study the dipolar relaxation processes (Weber and Farris, 1979). In solvents of high polarity, LAURDAN shows a considerable shift of its emission spectrum to longer wavelengths due to dipolar relaxation processes (Parasassi et al., 1991). When the local environment of LAURDAN is a phospholipid phase, the emission depends strongly on the packing of the lipid chains. At temperatures below the phase transition (gel state) the emission maximum is near 440 nm. At temperatures above the phase transition (liquid crystalline state) the emission maximum is red-shifted to ~490 nm (Parasassi et al., 1991; Parasassi and Gratton, 1995). The steady-state fluorescence parameter known as the generalized polarization (GP) quantitatively relates these spectral changes by taking into account the relative fluorescence intensities of the blue and red edge regions of the emission and excitation spectra, respectively (Parasassi et al., 1991; Parasassi and Gratton, 1995). For phospholipids, a characteristic GP value was found for the gel and liquid crystalline phases. However, the observation of a GP value intermediate between the gel and liquid crystalline values is not a final proof of the coexistence of separate phospholipid domains. The LAURDAN GP spectra profile in phospholipid vesicles allows determination of phase coexistence (Parasassi et al., 1991). Parasassi et al. have determined that in phospholipid membranes, the solvent dipolar relaxation process is independent of the type of polar headgroup and of pH within the range pH 4–10 (Parasassi et al., 1991). The GP values obtained for phospholipids undergoing phase changes are related to the number and motional freedom of water molecules around the fluorescent group (Parasassi et al., 1991). In a recent study by two-photon fluorescence microscopy, heterogeneous GP distributions in pure DLPC and DOPC vesicles in the liquid crystalline state were reported (Parassasi et al., 1997). This heterogeneity was found in individual vesicles and was attributed to a distribution of different LAURDAN sites, characterized by a different number of adjacent water molecules.

In a previous report we described the fluorescence properties of LAURDAN in pure G_{M1} and Gg_4Cer aggregates and in mixed aggregates of DPPC/Gg_4Cer, and we compared these results with the calorimetric data measured for these interfaces (Bagatolli et al., 1997). For pure G_{M1} and Gg_4Cer dispersions, we demonstrated that LAURDAN exhibits a progressive red shift of its emission below the gel-to-liquid crystalline transition temperature depending on the size of the polar headgroup of these GSLs. This effect was attributed to an increase in the water content of these interfaces compared to phospholipids (Bagatolli et al., 1997). LAURDAN shows a high partition coefficient into lipid aggregates and is virtually insoluble in water. The fluorescent moiety of LAURDAN has been reported to be located at the hydrophilic-hydrophobic interface of the phospholipid bilayer (Parasassi et al., 1991). In GSLs the location of the LAURDAN fluorescent group is in the hydrophilic-hydrophobic interface near the upper part of the ceramide moiety, with the lauric acid tail anchored in the GSLs acyl chains region (Fig. 1). This conclusion is based on acrylamide quenching of the LAURDAN fluorescence inserted in GSL aggregates (Bagatolli and Fidelio, unpublished results) and on thermodynamic considerations and rules out the possibility that the LAURDAN molecule is located in the polar headgroup region of GSLs near bulk water.

Various features of the auto-aggregation behavior of GSLs were theoretically predicted and experimentally stud-
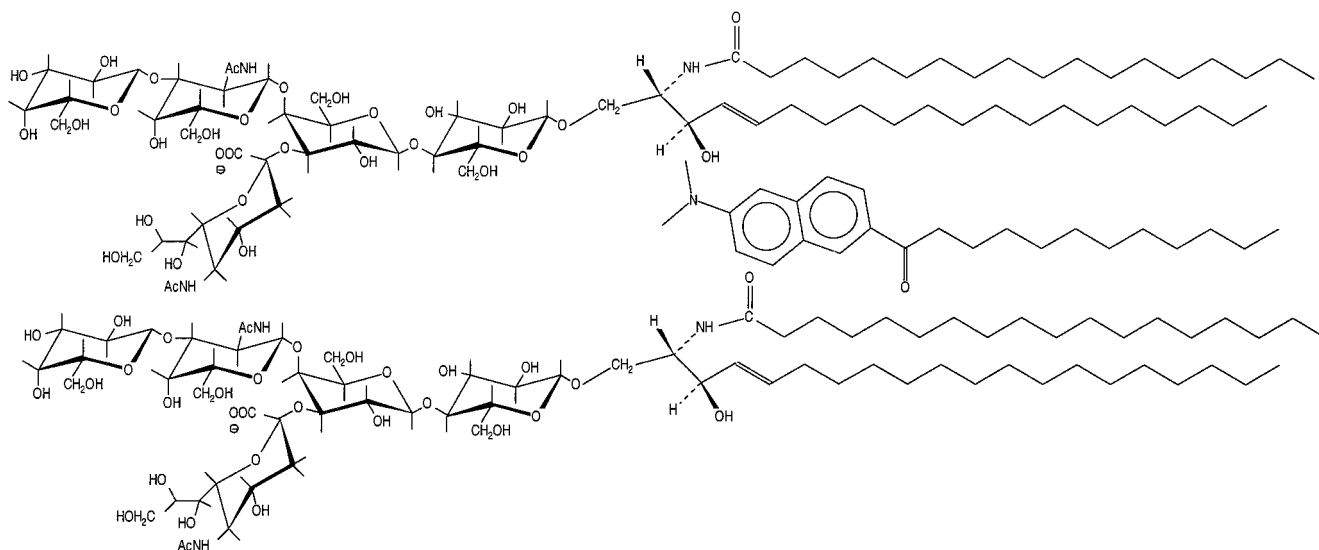

FIGURE 1 Schematic representation of the LAURDAN location in GSLs interfaces. The lipids in the figure correspond to G_{M1} molecules.

ied, i.e., shape, aggregation number, aggregate molecular weight, hydrodynamic radius, etc. (Maggio, 1985, 1994; Cantù et al., 1986). The effects of water environment on the GSLs organization has been studied with monolayers spread on subphases containing solutes that affect water structure (see review article, Maggio, 1994). Also, it was reported that a considerable amount of non-freezable water is associated with the polar headgroup of the more complex GSLs (Bach et al., 1982; Curatolo, 1987; Arnulphi et al., 1997). However, there is a lack of information about the interfacial water content and dynamics in the GSLs series. Since LAURDAN is particularly sensitive to interfacial water content and dynamics in phospholipid vesicles (Parasassi and Gratton, 1995), we studied its behavior in interfaces containing simple and complex GSLs, such as Phre, GalCer, Gg_3Cer, and Gg_4Cer, and gangliosides such as G_{M3}, G_{M2}, G_{M1}, G_{D1a}, and G_{T1b}. For comparison, Sulf and Sphin were also included. These studies were performed below, at, and above the main phase transition temperature of these pure lipid aggregates.

We also measured the effect of cholesterol in Gg_4Cer, G_{M1}, G_{D1a}, and G_{T1b} to ascertain the effects of this molecule on the fluorescence characteristics of LAURDAN. In phospholipid systems, cholesterol decreases the water content in the liquid crystalline phase (Parasassi et al., 1994). The lack of information about the effect of cholesterol in pure Gg_4Cer and ganglioside aggregates motivated us to determine whether similar effects can be detected in these lipid systems.

The spectral behavior of LAURDAN in the different lipid aggregates is quite complex. However, we are able to explain LAURDAN excitation and emission features using a single model based on the effect of surrounding water on LAURDAN's fluorescence properties. This unified interpretation allows determination of the water content at the membrane interface. We believe that this is the first comprehensive measurement of water penetration and dynamical properties of water in these lipid aggregates.

MATERIALS AND METHODS

Materials

LAURDAN was purchased from Molecular Probes Inc. (Eugene, OR), DPPC was supplied by Avanti Polar Lipids (Alabaster, AL), and sphingomyelin was purchased from Sigma (St. Louis, MO). Phre, GalCer, Sulf, Gg_3Cer, Gg_4Cer, G_{M3}, G_{M2}, G_{M1}, G_{D1a}, and G_{T1b} were obtained as previously described (Maggio et al., 1978; Fidelio et al., 1991).

Sample preparation

Stock solutions of LAURDAN were made in absolute ethanol, Gg_3Cer, Gg_4Cer, G_{M3}, G_{M2}, G_{M1}, G_{D1a}, and G_{T1b} in chloroform/methanol/water (60:30:4.5, v/v) and Phre, GalCer, Sulf, Sphin, and DPPC in chloroform/methanol (2:1, v/v). Lipid-probe dispersions were made with a lipid/probe ratio of 400:1 (LAURDAN final concentration was 1 μM). The solvent was evaporated under a stream of N_2 and the lipid was heated to 55°C for 1 h and dried under high vacuum for at least 4 h. The dry lipid mixtures were hydrated in 20 mM TrisClH-50 mM NaCl, pH 7.4 following the same

procedure described by Maggio et al. (1985, 1988). All samples were prepared and stored in the dark and spectra were measured immediately after preparation.

Fluorescence measurements

Steady-state excitation and emission spectra were measured with a SLM 4800C fluorometer (SLM-AMINCO, Rochester, NY) equipped with a xenon-arc lamp using 8-nm bandwidth. The excitation spectra were corrected with a quantum counter (rhodamine B in ethylene glycol 3 g/ml) in the reference cell and the emission spectra were corrected for instrument response with SLM software. We carried out a blank subtraction (lipid without probe) for all samples.

The excitation generalized polarization (GP_{ex}) is given by:

$$GP = \frac{I_B - I_R}{I_B + I_R}$$

where I_B and I_R are the fluorescence intensities at the maximum emission wavelengths in the gel and in the liquid crystalline phase, respectively (excitation GP). In addition, a GP value can also be obtained using the fluorescence intensities at the maximum excitation wavelengths in the gel phase and in the liquid crystalline phase (emission GP) (Parasassi et al., 1990, 1991, 1993). To obtain GP_{ex} spectra of gangliosides and Gg_4Cer at different temperatures, the intensities of the emission at wavelengths of 440 and 490 nm were selected and GP_{ex} values were calculated using excitation wavelengths from 320 to 420 nm. For Phre, GalCer, Sulf, Gg_3Cer, and Sphin we selected the 430- and 490-nm emission wavelengths. To obtain the GP_{em} spectra at different temperatures the 390- and 360-nm wavelengths were chosen, and GP_{em} values were calculated using emission wavelengths from 420 to 520 nm.

The temperature was controlled by a refrigerated thermocirculating bath and the temperature was directly measured in the sample cuvette by a digital thermometer. LAURDAN fluorescence lifetimes were measured using a multifrequency phase and modulation fluorometer (ISS K2, ISS Inc., Champaign, IL) equipped with a xenon arc lamp as light source and the emission at wavelengths >380 nm was observed through a KV399 cutoff filter. A solution of 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene (dimethyl-POPOP) in ethanol was used as a reference (τ = 1.45 ns) (Lakowicz, 1983). Data were collected until the standard deviation from each measurement of phase and modulation were at most 0.2° and 0.004, respectively. Phase and modulation data were analyzed using either discrete exponentials (Jameson et al., 1984) or distribution models (Gratton and Parasassi, 1995). In all cases the data fit best (as judged by the reduced chi-square) to two components: a major component corresponding to a Lorentzian distribution and a minor discrete exponential component.

Intermolecular distance calculations

The intermolecular distances (ID) between the lipid molecules were calculated using the molecular areas of the GSLs and phospholipids previously reported for lipid monolayers (Maggio et al., 1985; Fidelio et al., 1986). This intermolecular distance is that between two neighboring ceramide moieties in GSLs or between two neighboring diacyl-glycerol residues in phospholipids. This parameter was calculated subtracting the diameters associated with the surface area circumscribed by the rotation of the lipid molecule and the rotation of the ceramide backbone, respectively. The dependence of the GP_{ex} with the ID was similar in the range of surface pressures from 5 to 30 mN/m.

RESULTS

LAURDAN fluorescence behavior in pure ganglioside micelles

The emission spectra of LAURDAN in pure ganglioside gel phase micelles show a dramatic red shift (almost 60 nm)

compared with the characteristic LAURDAN emission spectra found for phospholipid vesicles in the gel phase (Fig. 2 and Table 1). An exception to this behavior is found for the ganglioside G_{M3} (Fig. 2 and Table 1), in which LAURDAN shows an emission maximum centered at 432 nm in the gel state and a red shift of 54 nm after the phase transition. The bandwith of LAURDAN emission spectra in G_{M3} gel phase micelles is clearly larger than that found for LAURDAN in DPPC vesicles below the main phase transition temperature (Fig. 2).

The excitation spectra of LAURDAN inserted in these ganglioside micelles in the gel state are characterized by two bands, with the red band as a maximum (Table 1), but the intensity of this band is lower than that found in phospholipid in the gel state (compare the LAURDAN excitation spectra in G_{M3} and DPPC aggregates, Fig. 5) in agreement with our previous report (Bagatolli et al., 1997).

The fluorescence lifetime of LAURDAN in the gel phase state of G_{T1b}, G_{D1a}, G_{M1}, and G_{M2} micelles was similar (Table 1). However, the LAURDAN GP_{ex} value decreases as $G_{M2} > G_{M1} > G_{D1a} > G_{T1b}$ (Fig. 3). At a temperature below the main phase transition, the lifetime value found for LAURDAN in G_{M3} is higher than that of LAURDAN in the others gangliosides and lower than that found for LAURDAN in DPPC in the gel state (Table 1). From Fig. 3 it is evident that the LAURDAN GP_{ex} is able to detect the main phase transition temperature only for the simpler G_{M3} ganglioside, in keeping with the calorimetric data (see Table 1). In addition, the LAURDAN GP spectra (emission and excitation) in all gangliosides studied show a liquid crystalline profile independent of the temperature (not shown) as pre-

viously reported for LAURDAN inserted in G_{M1} (Bagatolli et al., 1997).

LAURDAN fluorescence behavior in pure neutral GSLs, sulfatide, and sphingomyelin vesicles

The LAURDAN emission maxima and the lifetime values for Gg_3Cer and Gg_4Cer vesicles in the gel state are between those found for gangliosides group and the simple polar headgroup GSLs (GalCer, Phre, Sulf, see Table 1). The LAURDAN behavior in Gg_3Cer and Gg_4Cer vesicles is similar to that found for G_{M3} micelles. Therefore, these last three lipids were distinguished as the transition group of all the series of lipids studied, i.e., an intermediate LAURDAN fluorescence behavior is obtained compared with that found for more complex gangliosides and the simple polar headgroup GSLs. The broad gel-to-liquid crystalline transition observed for this group of lipids by DSC (Maggio et al., 1985) is in agreement with the temperature variation of LAURDAN GP_{ex} (see Table 1 and Figs. 3 and 4). The emission red-shifts upon phase transition are 50 nm for Gg_3Cer (similar to G_{M3}, see above) and 32 nm for Gg_4Cer (Table 1). The bandwith of the emission spectra of LAURDAN in Gg_3Cer and Gg_4Cer vesicles at temperatures below the main phase transition temperature is broader than those found for DPPC, similar to that found in G_{M3} micelles (see above). The LAURDAN GP_{ex} values found in the gel phase indicate decreases in the following order: $Gg_3Cer > G_{M3} > Gg_4Cer$. This behavior is also dependent on the size of the polar headgroup of these lipids (Maggio, 1994) in agreement with that found in the complex GSLs.

For LAURDAN inserted in pure Gg_4Cer and G_{M3} aggregates in the gel state, the excitation maximum corresponded with the excitation red band as previously described (Bagatolli et al., 1997). However, for LAURDAN inserted in Gg_3Cer below the main phase transition temperature, the excitation maximum is located in the blue band (Fig. 5).

The LAURDAN excitation spectra in interfaces conformed by pure cerebrosides, Sulf, and Sphin below the main transition temperature also display the blue band as excitation maximum, similar to that observed for LAURDAN inserted in pure Gg_3Cer vesicles in the gel phase (Table 1). The intensity of the red excitation band is considerably lower than the blue band in the gel state and disappears in the liquid crystalline state (Fig. 6). The bandwith of the LAURDAN emission spectra below the main transition temperature in these lipids is similar to that found for LAURDAN in DPPC vesicles in the gel state (not shown). LAURDAN is sensitive to the main phase transition in all these lipids (Fig. 4) showing higher GP_{ex} values for cerebrosides and Sulf in the gel phase as compared with DPPC. The LAURDAN lifetime values in the gel phase of these lipids was between 6 and 6.9 ns, similar to the values found for LAURDAN in phospholipids in the gel phase.
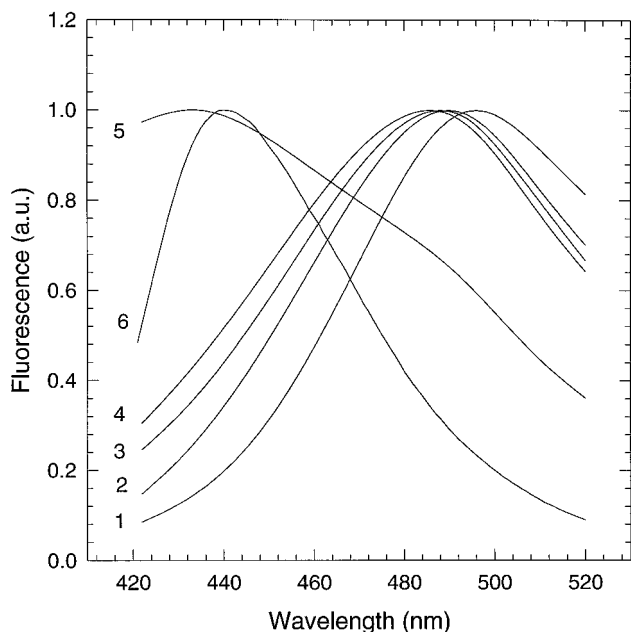

FIGURE 2   Normalized LAURDAN emission spectra in G_{T1b} (1), G_{D1a} (2), G_{M1} (3), G_{M2} (4), G_{M3} (5), and DPPC (6) pure aggregates below the main phase transition temperature.

TABLE 1 LAURDAN fluorescence parameters in GSLs series, Sulf, Sphin, and DPPC

Lipid	Emission Maximum (nm)		Excitation Maximum (nm)		τ^* (ns)		$T_m^{\#}$ (°C)	$T_m^{\S}$ (°C)
	Gel phase	L.C. phase	Gel phase	L.C. phase	Gel phase	L.C. phase		
G_{T1b}	496	498	392	376	3.93	2.33	—	7.3
G_{D1a}	490	492	390	376	3.93	2.56	—	15.2
G_{M1}	488	490	396	370	3.95	2.61	—	19.3
G_{M2}	486	490	396	374	3.41	2.70	—	29.3
G_{M3}	432	486	394	368	5.40	2.36	33	35.3
Gg_4Cer	454	486	394	368	4.65	2.94	53	54.0
Gg_3Cer	434	484	368	364	5.35	2.50	60	60.8
Sulf	425	489	368	366	6.00	2.25	50	50.2
GalCer	426	482	368	360	6.64	2.15	65	66.7
Phre	424	484	366	358	6.70	2.46	63	64.9
Sphin	429	485	368	366	6.82	3.09	38	39.0[¶]
DPPC	440	490	392	360	6.75	2.62	42	41.4

The transition temperatures sensed by LAURDAN are compared with those previously measured by DSC.
*Center of value of the Lorentzian distribution.
[#]Calculated from LAURDAN data using dG/dT.
[§]Taken from Maggio et al., 1985.
[¶]Taken from Calhoun and Shipley, 1979.

The GP_{ex} and GP_{em} spectra of LAURDAN in GalCer, Phre, Sulf, and Sphin show a particular behavior (Fig. 7 shows the GalCer data as a representative of this group of lipids). In the gel phase state, the LAURDAN GP spectra are wavelength-independent, similar to the phospholipids case (Parasassi et al., 1991). However, as shown in Fig. 7, a similar profile of LAURDAN GP spectra is obtained at the transition temperature and in the liquid crystalline state, contrasting with that found for LAURDAN in phospholipids (Parasassi et al., 1991).
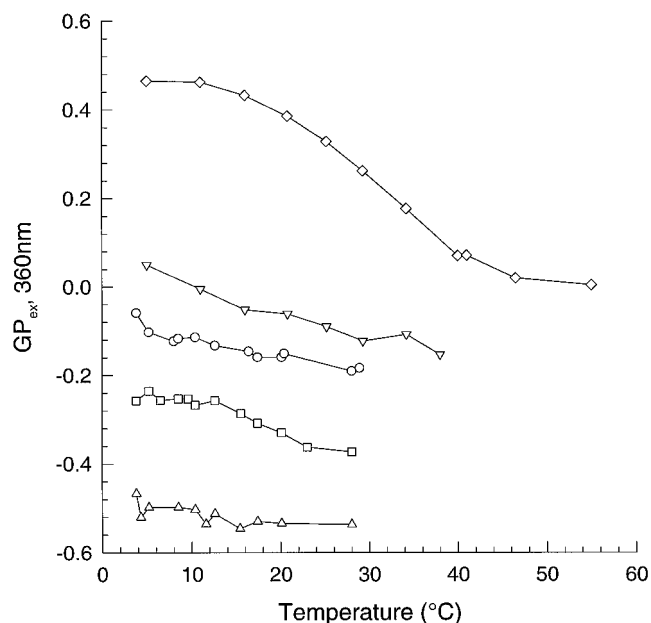
LAURDAN fluorescence behavior in aggregates of Gg₄Cer or complex gangliosides mixed with cholesterol

In Table 2 we show the LAURDAN emission and excitation maxima and the lifetime values in Gg_4Cer, G_{M1}, G_{D1a} and G_{T1b} mixed with 20% of cholesterol (Chol) at temperatures below and above the corresponding main phase transition temperatures of these pure GSLs aggregates. As shown in Fig. 8 (A and B), LAURDAN only detects the main phase
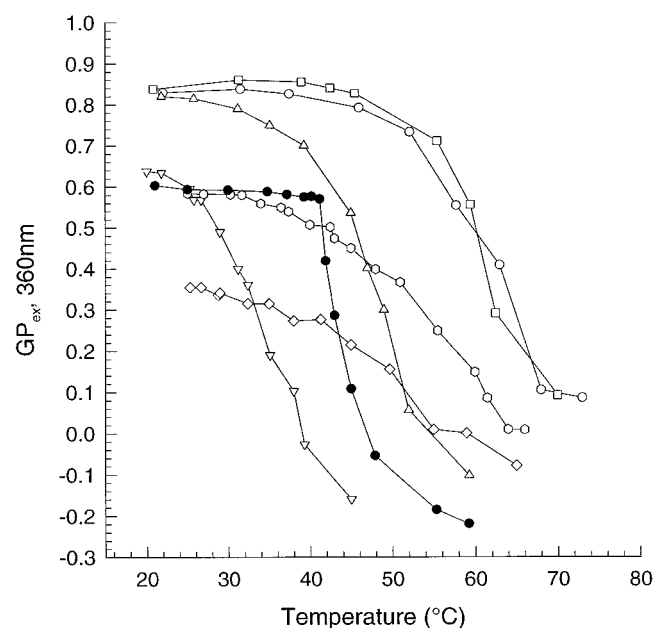
FIGURE 3 GP_{ex} values as a function of temperature for LAURDAN in micelles of: G_{T1b} (△), G_{D1a} (□), G_{M1} (○), G_{M2} (▽) and G_{M3} (◇). The GP_{ex} values were calculated using an excitation wavelength of 360 nm.

FIGURE 4 GP_{ex} values as a function of temperature for LAURDAN in vesicles of: Sulf (△), Phre (□), GalCer (○), Sphin (▽), Gg_4Cer (◇), Gg_3Cer (○) and DPPC (●). The GP_{ex} values were calculated using an excitation wavelength of 360 nm.
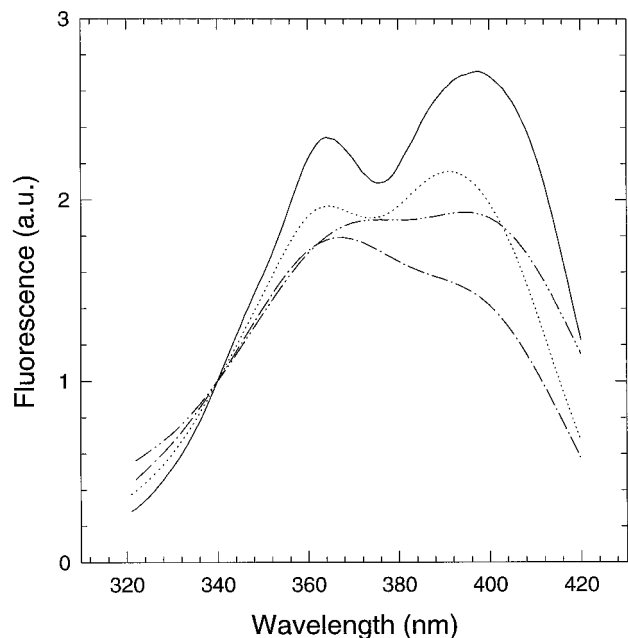
FIGURE 5 Normalized LAURDAN excitation spectra (at 340 nm) in DPPC (—), Gg_4Cer (········), Gg_3Cer (—·—) and G_{M3} (—··—··), pure aggregates below the main phase transition temperature.

FIGURE 7 GP_{ex} (continuous line) and GP_{em} (dotted line) of LAURDAN in pure GalCer vesicles below (*a*), at (*b*), and above (*c*) the main phase transition temperature. Similar spectra profiles were found in pure Gg_3Cer, Sulf, Phre, and Sphin vesicles.

transition temperature of the Gg_4Cer/Chol mixture, exhibiting a smoother curve as compared with Gg_4Cer pure vesicles. The GP_{ex} values for all the samples increases with the addition of 20% Chol at temperatures below and above the lipid phase transition found for pure GSL aggregates. Particularly in G_{M1} and Gg_4Cer aggregates at low temper-
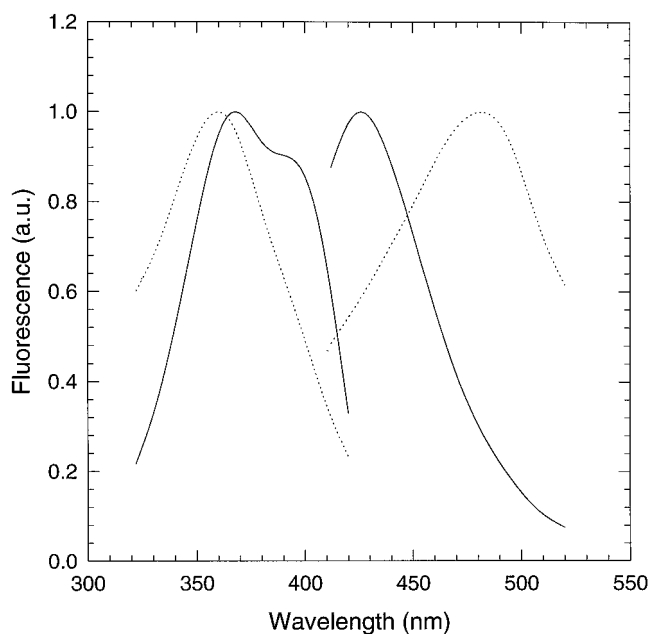

FIGURE 6 Normalized excitation and emission spectra of LAURDAN in pure GalCer vesicles below (*continuous line*) and above (*dotted line*) the main phase transition temperature. The same excitation spectra features were found in pure Gg_3Cer, Sulf, Phre, and Sphin vesicles.
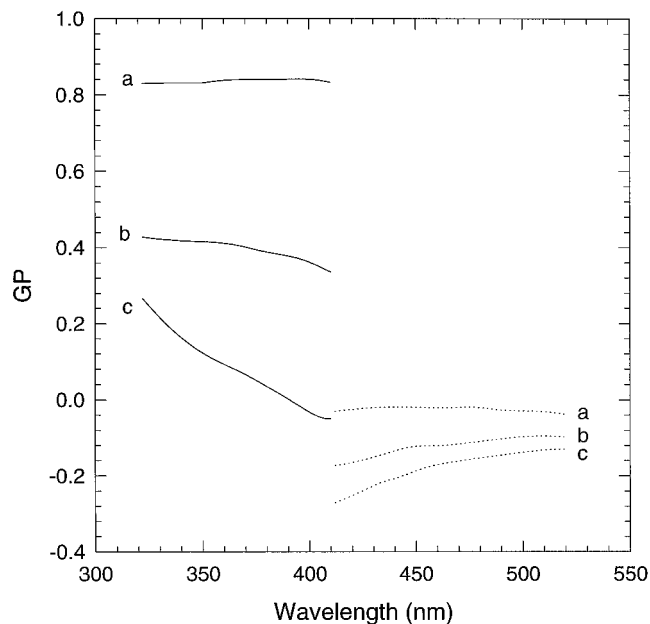
atures, the effect of Chol causes a strong blue shift in LAURDAN emission maximum (Table 2). In addition, the LAURDAN excitation spectrum at low temperatures in the Gg_4Cer/Chol mixed aggregates shows an excitation maximum situated on the blue side (Table 2), similar to that observed for the simple polar headgroup GSLs and Sphin (Table 1). The LAURDAN lifetime values in the presence of 20% Chol are also reported in Table 2.

The LAURDAN emission and excitation GP spectra in all GSLs/Chol mixtures show a liquid crystalline spectra profile similar to that described for pure G_{M1} (Bagatolli et al., 1997). Only for Gg_4Cer/Chol samples at low temperatures do the LAURDAN GP_{ex} spectra show a wavelength-independent profile (Fig. 9) similar to that found for simple polar headgroup GSLs and Sphin vesicles in the gel state.

DISCUSSION

The fluorescence behavior of LAURDAN inserted into interfaces conformed by GSLs of different polar headgroup complexity and also Sphin can be considered in terms of three groups. Table 3 is a descriptive summary of our results. As shown in Table 3, in the first group, the emission spectral shift is responsible for the low LAURDAN GP_{ex} values indicating significant solvent dipolar relaxation processes below the phase transition temperature. The rotational correlation time of tightly bound water molecules associated with the G_{M1} interface in the gel state, as measured by ^{2}H-NMR technique, is 0.7 ns (C. Arnulphi and G. D. Fidelio, unpublished results). This correlation time is similar to the dipolar relaxation time reported by LAUR-

TABLE 2 LAURDAN fluorescence parameters in GSLs/Chol mixed aggregates

Lipid (% of Chol = 20)	Emission Maximum (nm)		Excitation Maximum (nm)		τ^* (ns)	
	Gel phase	L.C. phase	Gel phase	L.C. phase	Gel phase	L.C. phase
G_{T1b}	496	498	392	376	3.93	2.33
G_{T1b}/Chol	496	496	392	372	3.95	2.83
G_{D1a}	490	492	390	376	3.94	2.57
G_{D1a}/Chol	488	490	390	372	4.06	3.00
G_{M1}	488	490	396	370	3.95	2.62
G_{M1}/Chol	434	474	396	368	4.90	3.10
Gg_4Cer	454	486	394	368	4.65	2.94
Gg_4Cer/Chol	424	484	370	368	6.98	2.60

*Center of value of the Lorentzian distribution.

DAN in phospholipid liquid crystalline state (Parasassi and Gratton, 1992). The relaxation of water orientation causes the red shift in the complex ganglioside micelles in the gel phase, i.e., the dynamics of water in the GSLs interface are in the same time range as the LAURDAN fluorescence lifetime. It is noteworthy that the calorimetric transition temperature for these gangliosides can be accurately determined (see Table 1). Since LAURDAN inserted in these ganglioside micelles always shows a red-shifted emission spectrum, the spectroscopic observation of phase transition and phase coexistence cannot be realized.

From the point of view of LAURDAN fluorescence, the second group has a behavior intermediate between the first group, described above, and sphingolipids with simpler polar headgroups such as cerebrosides, Sulf, and Sphin (third group, see Table 3). In both groups LAURDAN detects the main broad transition temperatures of these lipids, in agreement with those obtained from DSC (Maggio et al., 1985; Calhoun and Shipley, 1979; see Table 1).

The lack of sensitivity of LAURDAN's GP spectra to coexistence of different phases at the lipid transition temperatures is common to all the GSLs series studied here (see Fig. 7, *curve b* as an example). For LAURDAN in phospholipids, we can easily distinguish between a homogeneous liquid crystalline phase and a mixed phase of coexisting domains using the LAURDAN GP spectra (Parasassi et al., 1991). The red band of the LAURDAN excitation spectrum is mainly populated by LAURDAN molecules stabilized by interactions with phospholipids in the gel phase, showing a blue-shifted emission spectrum and a high GP_{ex} value. In other words, exciting LAURDAN in its red band photoselects molecules surrounded by phospholipids in the gel phase. If phospholipids in the gel phase are not present, the intensity of the red band dramatically decreases, showing a red-shifted emission spectrum and a low GP_{ex} value (Parasassi and Gratton, 1995). Thus, moving the excitation toward the red in the phospholipid liquid crystalline phase results in a decrease in the calculated LAURDAN GP_{ex} value. In phospholipid bilayers composed of coexisting phases, however, excitation toward the red would result in an increase in the LAURDAN GP_{ex} value. Similar reasoning can be made with the GP_{em} parameter taking into account that the wavelength dependence of GP_{em} and GP_{ex}

values is opposite (Parasassi and Gratton, 1995). LAURDAN's excitation band at 390 nm in GSLs aggregates in the gel state is less intense than it is in phospholipids in the gel state (Fig. 5). The lack of clear excitation spectra separation, i.e., higher differences between the intensity of the red excitation band in the gel and the liquid crystalline phases, prevents the strong photoselection of LAURDAN molecules that is found for the case of phospholipids in the gel state (Parasassi et al., 1991). Photoselection of LAURDAN molecules surrounded by lipids in the gel phase is a necessary condition to obtain the characteristic slope of the LAURDAN GP spectra at the phospholipid phase transition temperature (Parasassi and Gratton, 1995).

The meaning of LAURDAN GP_{ex} in GSLs and phospholipids aggregates

The concentration and dynamics of water molecules near the polar headgroup, together with the inherent topological features of each particular GSLs interface, are responsible for the fluorescence behavior of LAURDAN, in particular, below the gel-to-liquid crystalline transition temperature. The position and surface orientation of hydrogen-bond donor and acceptor groups at the sphingolipid interfaces as well as the relative size of the polar headgroup with respect to the hydrocarbon portion and the membrane curvature appears to be critical for polar head interactions (Maggio, 1994). For example, gangliosides form different kinds of micelles depending on the size of the polar headgroup, whereas Gg_3Cer and Gg_4Cer adopt small vesicle shapes (Maggio et al., 1988). The size of Gg_3Cer and Gg_4Cer vesicles are in between those found for small micelles of gangliosides (diameter < 6–7 nm) and the larger vesicles found for GalCer or Sulf (diameter ~ 500 nm) (Maggio et al., 1988).

We found a complete correspondence between these observations and the LAURDAN results in the GSLs series. The reasons for LAURDAN sensitivity to different phospholipid phases (gel and liquid crystalline) can now be extended to the GSLs series. For example, LAURDAN is able to show the phase transition temperature in GalCer, Phre, Sphin, Sulf, Gg_3Cer, Gg_4Cer, and G_{M3} because the
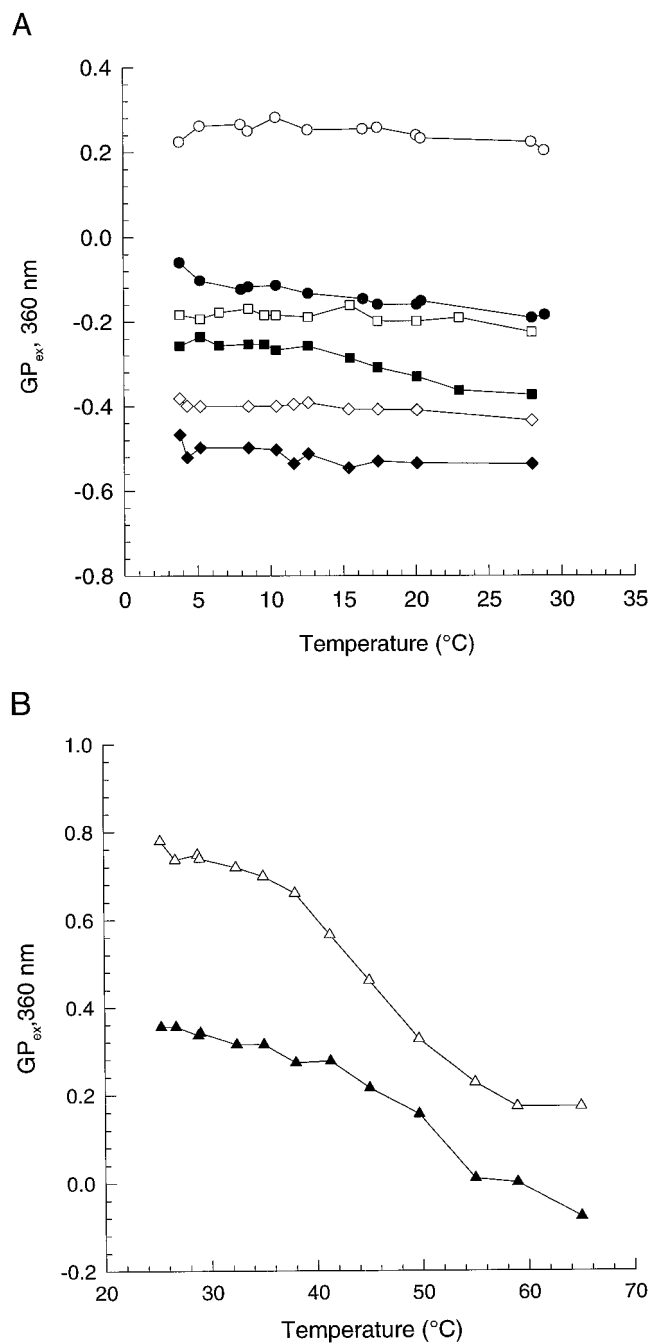
A

B

FIGURE 8 GP_{ex} values as a function of temperature for LAURDAN in GSLs aggregates with (*empty symbols*) and without (*filled symbols*) cholesterol. (*A*) G_{T1b} ($\diamond$), G_{D1a} ($\square$), and G_{M1} ($\bigcirc$) micelles, and (*B*) Gg_4Cer ($\triangle$) vesicles. The GP_{ex} values were calculated using an excitation wavelength of 360 nm. In the mixtures the cholesterol percentage was 20%.

FIGURE 9   LAURDAN GP_{ex} spectra in Gg_4Cer (*continuous line*) and $Gg_4Cer:Chol$ (20%) (*dotted line*) aggregates below (*a*), at (*b*), and above (*c*) the main phase transition temperature of pure Gg_4Cer.

Using the intermolecular separation among GSLs (calculated from planar monolayers data, see Material and Methods) we show in Fig. 10 that there is a linear correlation between LAURDAN GP_{ex} and the intermolecular separation in the GSLs series. We found two straight lines depending on the net charge of the lipid molecules (neutral or anionic). Depending on the size and charge of the lipid polar headgroup, sites of different size are available for water penetration in the aggregates modifying the LAURDAN spectral behavior. The size of the sites depends on the size of the polar headgroup, which in turn influences the topology of each lipid aggregate. As a consequence, the type of polar head determines the concentration and dynamics of water molecules in these sites. For example, in gangliosides the geometrical restrictions in these high curved aggregates favors water penetration, while in the simple polar headgroup GSLs this possibility is strongly reduced. It is important to mention the good correlation between the LAURDAN GP_{ex} measured at different temperatures in DPPC (gel and liquid crystalline state) and in the neutral Gg_4Cer (in the gel and near the transition temperature) with the calculated intermolecular distance (see Fig. 10). The same phenomenon is observed for the charged lipids (G_{M3} in the gel and the liquid crystalline state).

Our results suggest that the linear dependence between the LAURDAN GP_{ex} and the intermolecular spacing among anionic or neutral lipids is a general relationship; i.e., it is dependent on the size of these sites and reflects the water content and dynamics at this location. For instance, the interfacial region of sphingolipids is substantially more polar compared to the glycerol-based lipids. This is due to the presence of both donor and acceptor hydrogen-bonding

phase transition appreciably increases the water content of the lipid aggregate. Our data show that the solvent dipolar relaxation processes below the phase transition increase with the size of the polar headgroup of these lipids. This observation is also valid in the liquid crystalline state (see Figs. 3 and 4). Therefore, the LAURDAN GP_{ex} parameter in this series decreases as the size of the GSLs polar residue increases (Table 3).
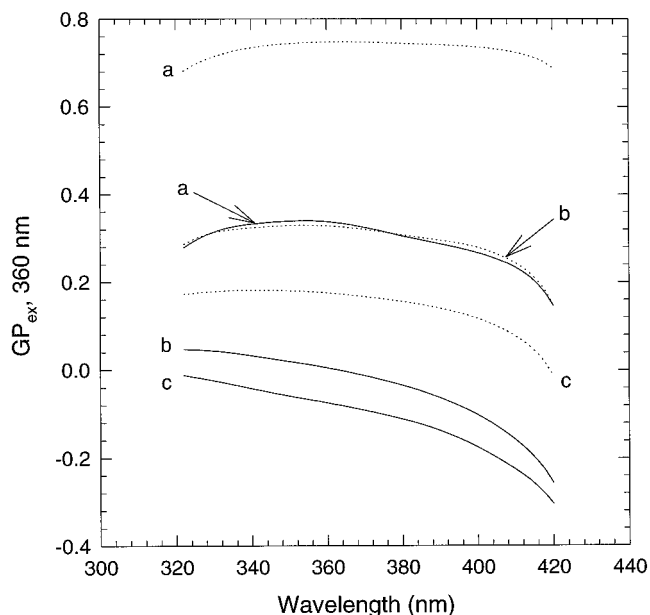
TABLE 3　Descriptive summary of LAURDAN results

Lipids	First Group				Second Group			Third Group			
	G_{T1b}	G_{D1a}	G_{M1}	G_{M2}	Gg_4Cer	Gg_3Cer	G_{M3}	Phre	GalCer	Sulf	Sphin
Number of sugars in the polar headgroup	7*	6*	5*	4*	4	3	3*	1	1	1	PC#
Net Charge	−3	−2	−1	−1	0	0	−1	0	0	−1	0
Shape of the aggregates	Micelles				Small vesicles		Micelles	Vesicles			
LAURDAN emission (gel phase)	Red emission spectra with maximum near 490 nm				Emission spectra with maximum between 430 to 450 nm			Blue emission spectra with maximum near 425 nm			
Location of excitation maximum (gel phase)	Red band (390 nm)				Gg_4Cer and G_{M3}, red band Gg_3Cer, blue band			Blue band (365 nm)			
LAURDAN GP spectra profile compared to phospholipids (Parasassi et al., 1991)	Shows liquid crystalline profile below, at, and above the phase transition temperature				Shows liquid crystalline profile below, at, and above the phase transition temperature			Shows wavelength independence in the gel state. Shows liquid crystalline profile at and above the phase transition temperature			
LAURDAN sensitivity of phase transition	No				Yes			Yes			
Range of GP_{ex} values (gel phase)	−0.5–0.1				0.3–0.6			0.6–0.85			
Range of LAURDAN lifetime values (gel phase)	3.5–4 ns				4–5.5 ns			6–7 ns			

#This sphingolipid has a phosphoril choline as a polar headgroup.
*Including the sialic acid.

moieties in the hydroxyl group of the sphingosine base and the amide-linked fatty acyl chain. These structural differences induce a more condensed, less hydrated, and less fluid surface phase (Maggio, 1994). The comparison between LAURDAN GP_{ex} behavior in simple polar headgroup GSLs and DPPC at low temperatures is in complete agreement with this last observation (see Fig. 10).

We have no evidence that the polar residues (e.g., –OH) of the lipid headgroups in the GSL series contribute to the dipolar relaxation process: 1) the location of the LAURDAN probe is in the top part of the ceramide moiety (see Fig. 1), far away from the lipid polar headgroups; 2) if the polar residues (–OH) of the lipid headgroup contribute to the dipolar relaxation process, we would not have observed the linear relationship between the GP_{ex} and intermolecular distance in the GSLs series (–OH-containing polar headgroup) and DPPC (non–OH containing polar headgroup); 3) in phospholipids the dipolar relaxation process is independent of the chemical nature of the polar headgroup (Parasassi et al., 1991); and 4) sphingomyelin and Gg_3Cer have an –OH group in the top part of the ceramide residue and Gg_3Cer has several –OH residues in the polar headgroup (Gg_3Cer has three sugars in the polar headgroup) but we found similar LAURDAN GP_{ex} values among DPPC, Gg_3Cer, and sphingomyelin. All those observations rule out the possibility that the –OH residues are participating in the dipolar relaxation process.

Taking into account the position of LAURDAN in these interfaces, we only compare the water content at this location in the lipid interfaces. The water content at this location is different from the amount of non-freezable water bound to the polar headgroup previously reported (Bach et al., 1982; Arnulphi et al., 1997).

Comparison between LAURDAN fluorescence excitation spectra in phospholipids and in simple polar headgroup GSLs

As mentioned in the Results section, the LAURDAN excitation spectra in the simple polar headgroup GSL vesicles
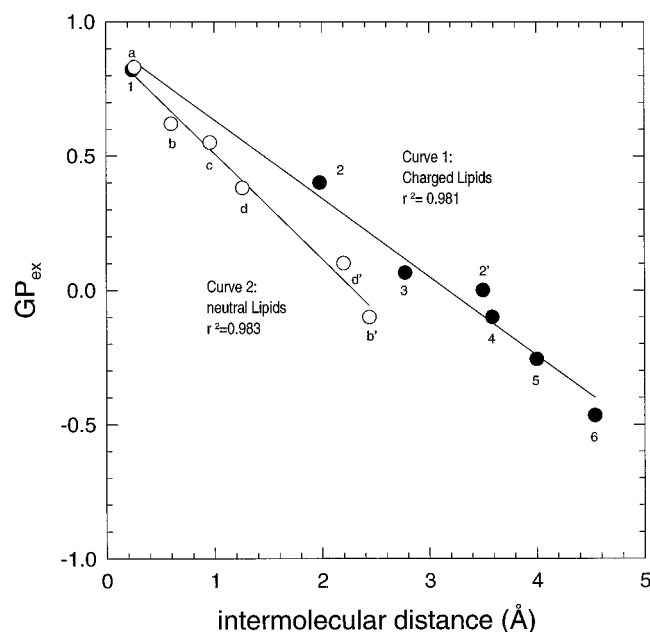
FIGURE 10　Variation of LAURDAN GP_{ex} with the calculated intermolecular distance (see Materials and Methods) of anionic GSLs (*filled symbols*) and neutral GSLs and phospholipids (*empty symbols*). Neutral lipids: GalCer (*a*); DPPC below (*b*), and above (*b'*) the transition temperature; Gg_3Cer (*c*); Gg_4Cer below (*d*) and at (*d'*) the transition temperature. Anionic lipids: Sulf (1); G_{M3} below (2), and above (2') the transition temperature, G_{M2} (3); G_{M1} (4); G_{D1a} (5); G_{T1b} (6).

do not have the same characteristic of the excitation spectra found in phospholipids. In the simple polar headgroup GSLs the LAURDAN excitation maximum remains in the blue side independent of the phase state, while in phospholipids the excitation maximum changes depending on the phase state (see Table 1 and Fig. 6). A remarkable finding is the comparison between the LAURDAN excitation spectra in phospholipids and in Sphin. In particular, this lipid possesses the same polar headgroup as the 1,2-diacyl-sn-glycero-3-phosphocholine, but the hydrocarbon portion is a ceramide moiety, as in GSLs. The LAURDAN excitation spectra distinguish between the interface formed by ceramide-based lipids and glycerol-based lipids in the gel phase when the polar headgroup is the same as it occurs between Sphin and DPPC (compare Fig. 5 with Fig. 6). The major difference between the simple polar headgroup ceramide-based lipids and most phospholipids lies in the extensive hydrogen bonding capacity of these ceramide-based lipids (Maggio, 1994; Boggs, 1987; Barenholz and Thompson, 1980). This hydrogen-bonded network includes the upper part of the ceramide moiety and the polar headgroup of GSLs (Maggio, 1994; Boggs, 1987). This situation has been studied for GSLs with one or two carbohydrates residues and cerebroside sulfatide (Maggio, 1994). Therefore, we conclude that in Sphin (and in the simple polar headgroup GSLs) the interactions between the upper portion of the ceramide moiety and LAURDAN are different from those found with the glycerol backbone moiety in phospholipids. These two environments have different influences on the ground state of LAURDAN.

Effect of cholesterol

In phospholipid vesicles, cholesterol modifies the gel and the liquid crystalline phases differently. The disorder of the gel phase is increased by cholesterol addition, while the liquid crystalline phase is ordered (Vist and Davis, 1990). In Gg$_4$Cer and gangliosides, in particular G$_{M1}$, at low temperatures, the addition of cholesterol is followed by a strong increase in the LAURDAN GP$_{ex}$ value, which is interpreted as a decrease in the dipolar relaxation process, i.e., a decrease in water content. In fact, the effect of cholesterol in the complex GSLs in the gel phase should be compared with the effect of cholesterol in the liquid crystalline phase of phospholipids, because both pure phases allow water to penetrate into the membrane.

LAURDAN shows the same spectral features found in simple polar headgroup GSLs when Chol is mixed with Gg$_4$Cer. We also believe that this strong change in the hydration of the lipid aggregates may be followed by changes in the shape of the aggregates. The dehydration effect is also found to a lesser extent in the G$_{M1}$/Chol mixture. The emission blue shift of LAURDAN in the Gg$_4$Cer/Chol and G$_{M1}$/Chol aggregates at low temperatures is similar to that induced by cholesterol in the phospholipid liquid crystalline state (Parasassi et al., 1994). In the more

complex gangliosides (G$_{D1a}$ and G$_{T1b}$) the effect of Chol on decreasing the water content is less evident but it is still present, probably because water is very abundant in these interfaces. These findings indicate that the effect of cholesterol on reducing the water content in gangliosides and Gg$_4$Cer interfaces decreases with the extent of the polar headgroup and suggests that the dehydration effect of cholesterol depends strongly on the curvature radius together with the extent of hydration of these lipid aggregates.

For phospholipids the molecular area measured in monolayer experiments depends on the physical state of the acyl chains rather than the type of polar headgroup. This is in contrast to the monolayer behavior of the GSLs used here, in which the molecular area depends on both the physical state of the acyl chains and on the size of the polar headgroup. (Maggio, 1994). In addition to the molecular area, the phase transition temperature and the ΔH_{cal} obtained by DSC and the critical packing parameter of these lipids show a correlation with the size of the polar headgroup (Maggio, 1994). Considering all these findings, we conclude that the gel phase of Gg$_4$Cer and complex gangliosides is different from the phospholipid gel phase. In fact we showed, using the LAURDAN fluorescence, that the water dipolar relaxation process in the gel phase of Gg$_4$Cer and G$_{M1}$ is different from that found in phospholipid vesicles in the gel phase. Furthermore, we previously showed that the fluorescence lifetimes of the probe diphenylhexatriene (DPH) measured in the gel phase of G$_{M1}$ and Gg$_4$Cer pure aggregates are similar to that found in DPPC in the liquid crystalline phase (Bagatolli et al., 1997).

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