Hydration and Order in Lipid Bilayers†

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ABSTRACT: The relationship between membrane lipid bilayer hydration and acyl chain order was investigated using time-resolved fluorescence spectroscopy. The degree of hydration in the head group region was assessed from fluorescence lifetime data along with fluorescence intensity measurements in D_2O, relative to H_2O buffer, using *N*-(5-dimethylaminonaphthalene-1-sulfonyl)dipalmitoylphosphatidylethanolamine (dansyl-PE). The degree of hydration in the acyl chain region was estimated from its effect on the fluorescence lifetime of 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine (DPH-PC), and acyl chain order was determined from time-resolved anisotropy measurements of the DPH-PC. Comparisons of *sn*-2 unsaturation with *sn*-1,2 diunsaturation in phosphatidylcholine (PC) bilayers with the *same* number of double bonds/PC revealed a marked difference in interchain hydration and acyl chain order but little difference in terms of head group hydration. For diunsaturated dioleoyl-PC (DOPC) bilayers with two double bonds/PC, the DPH-PC fluorescence lifetime data indicated a greater level of interchain hydration than 1-palmitoyl-2-docosahexaenoyl-PC (PDPC) with six double bonds/*sn*-2 chain. By contrast, the head group hydration for DOPC was markedly less than for PDPC. A similar lack of correlation of effects on the two regions of the bilayer was found with cholesterol, it having opposite effects on interchain and head group hydration. When DPH-PC fluorescence lifetime data for bilayers composed of a range of different lipids was plotted as a function of acyl chain order, a strong correlation of interchain hydration with acyl chain order was revealed that was *independent* of lipid composition. By contrast, there was a complete lack of correlation of acyl chain order with head group hydration of lipids of varied composition. These results are suggestive of head group and interchain hydration being "uncoupled" and may explain why effects on bulk membrane physical parameters, such as acyl chain order or the related parameter membrane fluidity, fail as a reliable predictor of functional effects on membrane proteins.

Water plays a key role in cell structure and function and provides the driving force in the formation of the basic lipid bilayer structure of the cell membrane. In its close interaction with both proteins and lipids, it may be considered to form an integral part of the molecule in question, and as such it may potentially dictate key structural properties of the molecule. Any alteration to the degree of hydration, particularly at the protein−lipid interface in cell membranes, could potentially lead to modifications to protein structure that could in turn modify function. This potential in allosteric regulation by such a perturbed water layer around proteins and lipid bilayers was recently emphasized (Rand, 1992; Colombo et al., 1992).

It is well established that there is a "bound" hydration shell surrounding the head group of membrane phospholipids that plays an important role in bilayer stability [reviewed by Rand and Parsegian (1989)]. The waters involved participate in a hydrogen bond network between the phospholipid molecules forming a "hydration layer" (Prats et al., 1987; Raghavan et al., 1992; Slater et al., 1993; Teissie et al., 1990) or "water network" (Rand et al., 1988). Several laboratories have published graphical representations of the arrangement of bilayer phospholipids that include head group hydration calculated from molecular dynamics energy minimization procedures [see, for example, Hauser et al. (1981); Bassolino-Klimas et al. (1993); Damodaran et al. (1992); Vanderkooi (1991); Raghavan et al. (1992); Venable et al. (1993)]. This theoretical approach has provided evidence for the existence of hydration shells around the ammonium hydrogens and nonesterified head group oxygens of DLPE,[1] which overlap with that of the adjacent lipid (Damodaran et al., 1992), and in another study energy minimized multibilayer crystal structures of DMPC were derived that included hydrogen bonded water bridging structures (Vanderkooi, 1991).

There are three regions in the phospholipid head group region that may be involved in interaction with water molecules, the ester and phosphate oxygens of all phospholipids, and the carboxyl, amino, or tetramethylammonium groups for PS, PE, and PC, respectively (Gruen et al., 1983; Boggs, 1987). For PC, the latter would not appear to be

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[1] Abbreviations: dansyl-PE, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)dipalmitoylphosphatidylethanolamine; di-18:2-PC, dilinoleoyl-PC; di-18:3-PC, dilinolenoyl-PC; DOPC, dioleoyl-PC; DLPE, dilauroyl-PE; DMPC, dimyrisotoyl-PC; DPH-PC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-PC; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoyl-PC; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PAPC, 1-palmitoyl-2-arachidonoyl-PC; PC, phosphatidylcholine; PDPC, 1-palmitoyl-2-docosahexaenoyl-PC; PE, phosphatidylethanolamine; PLPC, 1-palmitoyl-2-linoleoyl-PC; POPC, 1-palmitoyl-2-oleoyl-PC; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; PS, phosphatidylserine; TMA-DPH, 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene; χ^2_R, reduced χ^2.

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important since computations of the energy of binding of water to the tetramethylammonium group suggest that it is comparable to water−water interactions (Port & Pullman, 1973). Using Fourier transform infrared spectroscopy of $^{13}C{=}O$-labeled phospholipids, hydrogen bonding to the carbonyl groups involving water has been demonstrated (Blume et al., 1988), confirming earlier ^{13}C-NMR chemical shift measurements of the carbonyl resonances (Smaby et al., 1983; Yeagle & Martin, 1976; Schmidt et al., 1977). Also various direct interlipid hydrogen bonding possibilities exist, for example, between the phosphate or carbonyl oxygen of any phospholipid and an adjacent amine of PE or PS (Boggs, 1987). Since water also hydrogen bonds to these groups, a hydrogen bond-bridge may be formed between phospholipids not otherwise able to directly hydrogen bond, such as PC, forming the basis for the hydration layer.

A large number of studies have addressed the issue of the number of waters bound/phospholipid molecule. For example, using X-ray diffraction, 7 and 10 waters/DLPE were found for gel and liquid crystalline phase lipid, respectively. Half of this water was associated with the interlamellar space between adjacent bilayers, while the rest intercalated into the bilayer itself (McIntosh & Simon, 1986). For DOPC, the values are 9 and 20 H_2O, respectively, shown using differential scanning calorimetry (Ulrich et al., 1994). This type of data, among a number of other important parameters pertaining to hydrogen bonded water interactions between opposing bilayer surfaces, has been reviewed in a comprehensive survey of the major phospholipids (Rand & Parsegian, 1989). More recently, from molecular dynamics computations, estimates of the waters/phospholipid have begun to provide values for the extent of phospholipid hydration close to experimentally determined values [see, for example, Damodaran et al. (1992); Bassolino-Klimas et al. (1993); Vanderkooi (1991); Vanderkooi (1994); Raghavan et al. (1992)].

Although the water molecules of the hydration layer are distinct from bulk water, there appears to be a rapid exchange between the two, as shown using ^2H-NMR (Lazrak et al., 1987; Pope & Cornell, 1979). Early studies distinguished tightly bound and weakly associated water molecules (Ladbrooke & Chapman, 1969; Small, 1967; Finer & Darke, 1974). Indeed the first layer of water makes a major contribution to the dipole potential, as influenced by the carbonyl groups (Gawrisch et al., 1992). However, since both the conformation and dynamics of the PC head group and hydration curves for D_2O, as studied by ^2H-NMR, obey a smooth exponential dependence on varied hydration (Ulrich & Watts, 1994; Volke et al., 1994a,b), the idea of "tightly bound" and "weakly bound" waters appears to be artificial. Nevertheless, the first few waters bind tightly to the phosphate group (Cevc, 1992) and are motionally restricted (Salsbury et al., 1982; Volke et al., 1994a).

Water molecules also exist deeper within the lipid bilayer, fitting between acyl chain packing "defects" constituting an "interchain hydration" and distinguishing it from head group hydration. These water molecules fit into "holes" or defects due to trans-gauche "kinks" in the acyl chains (Trauble, 1971) and probably move between these defects by a "hopping" mechanism (Franks & Lieb, 1981; Lieb & Stein, 1969, 1971). In a recent neutron diffraction study with DOPC and small peptides, it was calculated that there were ~2 waters/peptide from which it was suggested that water, as well as residing in hydrocarbon core packing defects, was directly associated with the peptide, possibly with a tryptophan (Jacobs & White, 1989). It was suggested that such an effect, if enhanced by larger peptides, would be important for the insertion of helices into bilayers and for helix−helix interactions involving hydrogen bonding. Also, it was suggested that a lack of consideration of hydrogen bonding in transbilayer helixes might lead to errors in hydrophobicity plots.

While it is clear that the hydration layer contributes significantly to the strength of lipid−lipid interactions and protein−lipid interactions, there are fundamental issues that remain to be explored. One is the relative contribution of the different types of hydrogen bond interactions (direct and via hydrogen bonded water, between different phospholipid moieties, etc.). Another aspect is the relative contribution to the degree of hydration of major membrane components such as the major phospholipid classes, cholesterol, and the degree of fatty acyl unsaturation. Much less is known about interchain hydration than head group hydration, and whether it forms part of the extended-hydrogen bond network continuum at the surface remains to be determined. It is also not clear to what extent acyl chain order relates to either the degree of head group or interchain hydration, one of the goals of the present study being to gain a better understanding of this relationship.

There are a number of approaches to the study of hydration in membranes, including neutron diffraction (Simon & McIntosh, 1986; Franks & Lieb, 1979; Knott & Schoenborn, 1986; Worcester & Franks, 1976; Wiener et al., 1991; Scherer, 1989) and capacitance (Simon et al., 1982) techniques. Water in the excited state solvent cage of a suitably placed fluorophore, such as DPH or anthroyl stearate fluorophores, shortens the fluorescence decay rate, providing another approach. The fluorescence lifetime of a fluorophore in lipid bilayer depends on its surrounding dielectric environment provided that no excited state reactions such as excimer formation occur. Small alterations in the degree of hydration have been shown to significantly affect the fluorescence lifetime due to fluorophore excited state-water interactions (Straume & Litman, 1987a,b; Fiorini et al., 1987−1989; Ivessa et al., 1988; Trotter & Storch, 1989; Sommer et al., 1992; Mitchell & Litman, 1994), so that fluorescence lifetime measurements can be used to infer the presence of water in the immediate fluorophore vicinity. Fluorophores that contain an ionizable group, such as dansyl-PE, have a greater quantum yield in D_2O compared to H_2O due to a reduced rate of nonradiative decay (Stryer, 1966; Lawaczeck, 1979, 1984; Ho & Stubbs, 1992). The limitation is that the method is only applicable to fluorophores that can donate a proton, which unfortunately rules out fluorophores that locate in the interchain region of the bilayer.

In a previous study from this laboratory on unsaturation and cholesterol in lipid bilayers, the rate of lipid desorption as a measure of the contribution of hydrogen bonding to the effects on lipid−lipid head group interactions was investigated (Slater et al., 1993). It was shown that increased phospholipid unsaturation increased the rate of desorption while disordering the acyl chain region; by contrast, cholesterol had little effect on desorption in spite of its strong lipid ordering effect (Slater et al., 1993). Also, ethanol increased the rate of desorption while only marginally disordering the lipids at the concentration used. Again, in

Table 1: Fluorescence Decay Analyses of DPH-Type Fluorophores and Dansyl-PE in LUV of POPC at 37 °C[a]

		τ_1	w_1	f_1	τ_2	w_2	f_2	χ^2_R
DPH-PC	1 exp	6.51 ± 0.08		1.00				46.1
	1 Lor	6.78 ± 0.04	0.95 ± 0.10	1.00				3.76
	2 exp	7.41 ± 0.02		0.86 ± 0.01	3.47 ± 0.18		0.14 ± 0.01	0.83
	2 Lor	7.40 ± 0.02	0.13 ± 0.07	0.86 ± 0.01	3.44 ± 0.18	0.01 ± 0.01	0.14 ± 0.01	0.92
DPH	1 exp	7.74 ± 0.02		1.00				34.1
	1 Lor	7.87 ± 0.03	1.40 ± 0.19	1.00				1.84
	2 exp	8.33 ± 0.09		0.93 ± 0.01	2.80 ± 0.19		0.07 ± 0.01	1.06
	2 Lor	8.32 ± 0.08	0.11 ± 0.09	0.93 ± 0.01	2.76 ± 0.18	0.01 ± 0.01	0.07 ± 0.01	1.15
TMA-DPH	1 exp	2.97 ± 0.14		1.00				61.6
	1 Lor	3.04 ± 0.08	1.15 ± 0.10	1.00				4.10
	2 exp	3.42 ± 0.12		0.89 ± 0.01	0.86 ± 0.06		0.11 ± 0.01	2.12
	2 Lor	3.43 ± 0.05	0.45 ± 0.11	0.88 ± 0.02	1.02 ± 0.20	0.22 ± 0.18	0.12 ± 0.02	1.31
dansyl-PE	1 exp	13.75 ± 0.10		1.00				500
	1 Lor	13.88 ± 0.02	3.96 ± 0.74	1.00				96.9
	2 exp	14.33 ± 0.11		0.97 ± 0.01	1.38 ± 0.14		0.03	1.52
	2 Lor	14.38 ± 0.13	1.54 ± 0.26	0.96 ± 0.01	1.54 ± 0.17	0.06 ± 0.06	0.04 ± 0.01	0.89

[a] τ, lifetime centers ($\times 10^{-9}$ s); w, distributional width ($\times 10^{-9}$ s); f, fractional intensities; χ^2_R, reduced χ^2. Data were collected for 10−12 frequencies between 2 and 140 MHz. Standard errors were from three individual measurements. The χ^2_R values were taken from a representative data set.

spite of the fact that *both* cholesterol and unsaturation strongly modulate acyl chain order, another study found that increased unsaturation increased a parameter defined as "quantifying the bilayers capacity to utilize an increase in phospholipid acyl chain packing free volume for accommodation of expanded meta II (rhodopsin) conformation", while cholesterol was without effect (Mitchell et al., 1992). These results taken together suggest that acyl chain order and hydration as it affects lipid−lipid interactions are not simply related quantities.

The primary aim of this study was to gain a better understanding of the relationship between hydration and order, in particular with respect to variation in the level of *sn*-2 and *sn*-1,2 diunsaturation, cholesterol, PE, and PS using fluorescence approaches. The results revealed that while unsaturation increased both interchain and head group hydration, cholesterol, PE, and PS had opposite effects on these two regions. A strong correlation between interchain hydration and acyl chain order was revealed that was independent of lipid composition. By contrast, head group hydration assessed either by effects on the fluorescence lifetime or deuterium isotope exchange showed no correlation with acyl chain order for varying lipid composition. One conclusion that arises from this study is that it is quite possible that bilayers differing in lipid composition may have a similar value of membrane order but different levels of head group hydration. This would explain why acyl chain order is such a poor predictor of the influence of different lipids on membrane protein functioning.

MATERIALS AND METHODS

Materials. Phospholipids and DPH-PC were obtained from Avanti Polar Lipids (Birmingham, AL). DPH, TMA-DPH, and dansyl-PE were from Molecular Probes (Eugene, OR). Cholesterol was from Sigma Chemical Co. (St Louis, MO), and D_2O (99.9%) was from Cambridge Isotope Laboratories (Woburn, MA). All other chemicals used were of analytical grade and were obtained from Fisher Scientific (Malvern, PA).

Phospholipid Vesicles. Briefly, aliquots of the required phospholipid in chloroform and fluorophore were placed in a test tube, and the solvents were removed under a stream of nitrogen. Probe/lipid concentration ratio was 1:400 for all probes except dansyl-PE, which was 1:100. Buffer (10 mM Tris-HCl and 150 mM NaCl, pH 7.4) was then added to give a total lipid concentration of 2 mM. After vortexing, the sample was passed through a 0.1 mm filter using the Avestin Liposofast extruder (Avestin, Ottawa, Canada) as described (MacDonald et al., 1991). Samples were diluted to a final lipid concentration of 200 μM. For the deuterium solvent isotope exchange measurements, the buffer was made up in D_2O.

Fluorescence Measurements. Fluorescence data were collected using an SLM 48000 multifrequency phase-modulation fluorimeter. The excitation was provided by a Liconix HeCd laser (Model 4240NB, 325 nm), sinusoidally modulated by a RF frequency from 2 to 150 MHz. For lifetime measurements, the phase shift and demodulated emission was observed through a 430 nm redpass filter and a Glan-Thompson polarizer set at the magic angle. For time-resolved anisotropy measurements, a T-format setup was used. The principle and operation details are as published elsewhere (Gratton & Limkemann, 1983; Lakowicz, 1983; Lakowicz & Maliwal, 1985). An aqueous solution of rabbit liver glycogen was used as a lifetime reference. All the measurements were performed at 37 °C. The experimental error used in data analysis was the averaged error over the range of frequency collected in data acquisition, usually near 0.2° in the phase and 0.002 in the modulation. Each measurement was repeated three times on separate vesicle preparations, and the results are the mean of three determinations.

Data Analysis. Phase and modulation data were analyzed using the GLOBALS UNLIMITED software (Laboratory of Fluorescence Dynamics, University of Illinois, Urbana−Champaign, IL) (Beechem & Gratton, 1988; Beechem, 1990). The data were fit to both exponential and distributional analytical forms and compared on the basis of χ^2_R and residuals (see Table 1). The analysis of the time-resolved anisotropy followed published procedures (Kawato et al., 1977; Dale et al., 1977; Lakowicz & Prendergast, 1978; Stubbs et al., 1981; van der Meer et al., 1984; Hare, 1983) using a hindered rotator model. For DPH-PC in lipid vesicles, as previously characterized (Parente & Lentz, 1985), it was found that the model with two rotational correlation times and a residual anisotropy was most appropriate based

Table 2: Analysis of Fluorescence Anisotropy Decay of DPH-PC in LUV of POPC and DOPC at 37 °C[a]

model	ϕ_1	ϕ_2	ϕ_3	r_∞	χ^2_R
		POPC			
ϕ	0.58				465.8
ϕ, r_∞	1.00			0.14	51.5
2ϕ	0.49	9.52			3.10
2ϕ, r_∞	0.32	4.33		0.06	0.32
3ϕ	0.30	3.86	81.2		0.31
		DOPC			
ϕ	0.47				495.8
ϕ, r_∞	0.74			0.10	156.2
2ϕ	0.30	5.92			1.30
2ϕ, r_∞	0.26	4.60		0.02	0.37
3ϕ	0.01	1.23	7.72		0.35

[a] ϕ, rotational correlation time ($\times 10^{-9}$ s); r_∞, residual anisotropy; χ^2_R, reduced χ^2.

on the residual and χ^2_R (see Table 2). There are three components in this model: two are rapid decaying components, recovered as rotational correlation times ($\phi_{1,2}$), and the third is a constant component-residual anisotropy (r_∞). The decay curve can be expressed as

$$r(t) = (r_0 - r_\infty)[f_1 \exp(-t/\phi_1) + f_2 \exp(-t/\phi_2)] + r_\infty$$

where r_0 is the limiting anisotropy, $\phi_{1,2}$ are the rotational correlation times, and r_∞ is the residual anisotropy that is related to the acyl chain order. This has previously been shown to be appropriate for DPH-PC (Squier et al., 1991). Data fit to this model proved satisfactory based on the random distribution of errors and low χ^2_R. In general, more complex decay models such as triple exponentials did not show improved χ^2_R and residuals. The averaged experimental error for phase and modulation was used for data analysis, usually 0.2° in the phase and 0.008 in the modulation.

An order parameter $S_{DPH\text{-}PC}$ was used to characterize the acyl chain structural order from anisotropy measurements on DPH-PC and was calculated according to

$$S = (r_\infty/r_0)^{1/2}$$

The order parameter S of an acyl chain segment or fluorophore probe is defined by $S = (3\langle\cos^2\theta\rangle - 1)/2$, where $\langle\cos^2\theta\rangle$ is the time-averaged cosine of the angles made with the bilayer normal.

RESULTS

The purpose of this study was to determine the impact of varied unsaturation, cholesterol, PE, and PS levels, such as would be encountered in biological membranes, in terms of hydration at the head group and interchain regions. To accomplish this, the fluorescence lifetime of dansyl-PE and DPH fluorophores was measured in lipid vesicles of defined composition. Fluorescence decay data were subjected to various analytical forms as shown in Table 1. For DPH-PC and DPH, it can be seen that a distributional analysis was no better than a two-exponential. By contrast, for TMA-DPH and dansyl-PE, Lorentzian distributional analyses were superior due to head group location providing fluorophore excited state solvent cage environmental heterogeneity. For examination of the effects of hydration, the principle parameter in question is the excited state lifetime in the case of DPH-PC or the lifetime distribution center for dansyl-PE, and since the lifetime centers for the distributional analyses were not significantly different from the fluorescence lifetime obtained from a exponential analysis, the latter was considered adequate for comparison purposes.

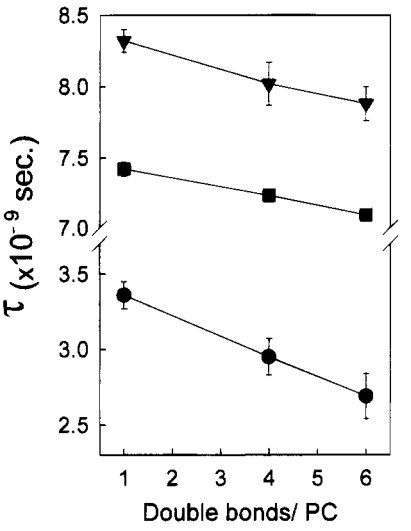

FIGURE 1: Fluorescence lifetimes (major lifetime component of a biexponential fit) of DPH (triangle), DPH-PC (square), and TMA-DPH (circle) in LUV phospholipids with varied unsaturation in *sn*-2 chain. Phospholipids include POPC, PAPC, and PDPC. Data are the mean of three separate measurements ± SD. Other details are as described under Materials and Methods.

Acyl chain order was determined from time-resolved anisotropy measurements of DPH-PC. Rapid local conformational motions, arising mainly from kink diffusion, as measured by ^{2}H-NMR can be distinguished from the "rigid body angular motion", which describes the rocking fluctuations of the entire acyl chain (Peterson & Chan, 1977; Jahnig et al., 1982). The motion of DPH attached to the *sn*-2 position of PC provides an appropriate model for the latter type of motion, reflecting the time-averaged orientation of the entire chain. If the motion is considered to be confined within a cone (Kinosita et al., 1977; Kawato et al., 1977; Engel & Prendergast, 1981), one may obtain an order parameter $S_{DPH\text{-}PC}$ for this type of motion (Heyn, 1979; Jahnig, 1979; van der Meer et al., 1984). ^{2}H-NMR information on local regions along an unsaturated chain [see, for example, Baenziger et al. (1992, 1991)] indicate regions of low and high local order; by contrast, DPH probes reflect a general disordering with increased unsaturation as shown with DPH (Stubbs et al., 1981) and here with DPH-PC (see below). Of the various analyses examined for the decay of DPH-PC anisotropy, two-rotational correlations time plus r_∞ gave the best fit to the data as shown in Table 2. From r_∞ values, $S_{DPH\text{-}PC}$ values were calculated (see Materials and Methods).

Effects of cis-Unsaturation. The effect of increasing *sn*-2-PC unsaturation on the fluorescence lifetime of DPH, TMA-DPH, and DPH-PC is shown in Figure 1, and the effects of varied *sn*-2 and *sn*-1,2 unsaturation on the fluorescence lifetimes of DPH-PC and dansyl-PE are compared in Figure 2, panels a and b. The *sn*-2 unsaturation series included POPC, PLPC, PAPC, and PDPC while the *sn*-1,2 unsaturation series included DOPC, di-18:2-PC, and di-18:3-PC. Overall, there was a reduced lifetime for increased unsaturation. Increased unsaturation introduces packing defects into the lipid bilayers, which can accom-

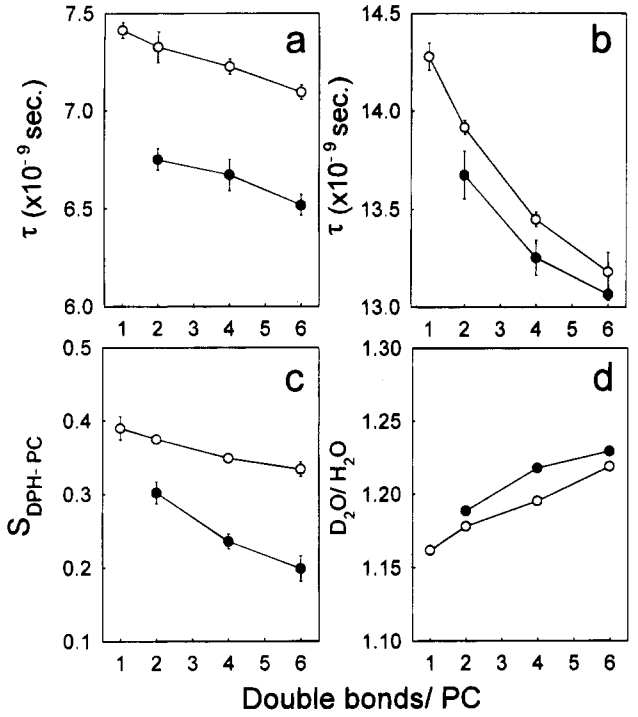

FIGURE 2: Effect of altered lipid unsaturation at *sn*-2 (open circle) and *sn*-1,2 (solid circle) on (a) fluorescence lifetime of DPH-PC; (b) fluorescence lifetime of dansyl-PE; (c) $S_{DPH\text{-}PC}$; (d) fluorescence intensity ratio of dansyl-PE in lipid bilayers made in D_2O:intensity in H_2O (excitation 340 nm, emission 528 nm). The *sn*-2 unsaturated lipids include POPC, PLPC, PAPC, and PDPC; the *sn*-1,2 diunsaturated lipids include DOPC, di-18:2-PC, and di-18:3-PC. Data are the mean of three separate measurements ± SD. Other details are as described under Materials and Methods.

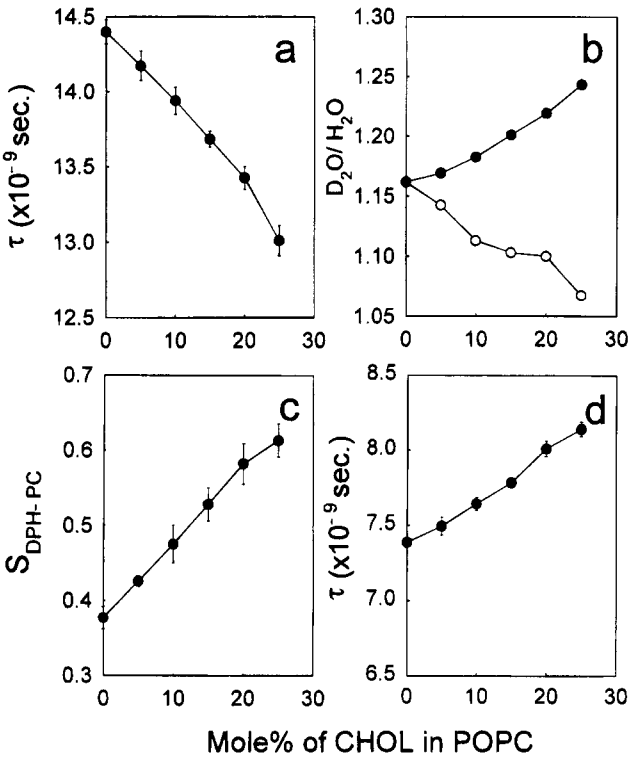

FIGURE 3: Effect of varied cholesterol (0−25 mol %) on POPC bilayers: (a) fluorescence lifetime of dansyl-PE; (b) fluorescence intensity ratio of dansyl-PE (solid circles) in lipid bilayers made in D_2O:intensity in H_2O (excitation 340 nm, emission 528 nm), and D_2O/H_2O ratio of TMA-DPH (open circles, excitation 360 nm, emission 430 nm); (c) $S_{DPH\text{-}PC}$; (d) fluorescence lifetime of DPH-PC. Data are the mean of three separate measurements ± SD. Other details are as described under Materials and Methods.

modate more water. These water molecules when in the excited state of a fluorophore solvent cage will shorten the lifetime; the results indicating that interchain hydration increases deep in the bilayer as well as nearer the surface. Considering the size, location, and tethering aspects, dansyl-PE and DPH-PC appeared to be the best fluorophore combination for comparison of effects on the head group and interchain regions of the bilayer.

The effects of varied *sn*-2 and *sn*-1,2 unsaturation on acyl chain order are shown in Figure 2c. Superficially, a decrease in order for increased unsaturation paralleled a reduction in lifetimes, a result that may be rationalized on the basis of increased packing defects in the bilayer accommodating more water. However, on comparing *sn*-1,2 unsaturation with *sn*-2 unsaturation with the *same* number of double bonds/PC molecule, there was a significant difference in terms of DPH-PC, but only a marginal difference in terms of dansyl-PE fluorescence lifetimes.

The effect of unsaturation at the head group region was also assessed using the solvent isotope effect. By measuring the fluorescence intensity of dansyl-PE (excitation 340 nm, emission 528 nm) in D_2O and comparing it to that in H_2O as a D_2O/H_2O ratio, the effect of increased unsaturation directly on the degree of hydration in the dansyl-fluorophore vicinity could be assessed. The results in Figure 2d show a D_2O/H_2O ratio greater than unity, indicative of the presence of water in the excited state solvent cage, and an *increase* in the D_2O/H_2O ratio with unsaturation showing an increase in hydration. Comparison of the effects of *sn*-1,2 diunsaturation with *sn*-2 unsaturation with the *same* number of double bonds/PC again revealed only marginal differences in terms of the D_2O/H_2O fluorescence intensity ratio and therefore in head group hydration.

Another notable difference was that DOPC bilayers with a total of two double bonds, one acyl or one peracyl chain, had a lower DPH-PC fluorescence lifetime compared to PDPC with six double bonds/*sn*-2 chain. By contrast, for DOPC bilayers the dansyl-PE lifetime was greater than for PDPC. Also, there was a 1×10^{-9} s difference in the dansyl-PE lifetime between POPC and PDPC compared to DPH-PC ($\sim 0.3 \times 10^{-9}$ s difference).

Effects of Cholesterol. Cholesterol was found to decrease the dansyl-PE fluorescence lifetime (Figure 3a) and increase the dansyl-PE D_2O/H_2O fluorescence intensity ratio (Figure 3b), indicating an increase in head group hydration. The generally held view is that the 3-β-hydroxyl group of cholesterol is located near the carbonyl group of the phospholipid glycerol backbone, forming a hydrogen bond (Brockerhoff, 1975; Yeagle et al., 1975; Worcester & Franks, 1976; Franks, 1976). Furthermore, several studies from other laboratories have shown that cholesterol has negligible effects on lipid head group dynamics (Brown & Seelig, 1978; Ghosh, 1988; Milburn & Jeffrey, 1989; Han & Gross, 1991). Indeed, measurement of rotational correlation times of dansyl-PE in POPC LUV also showed that cholesterol has no affect on dansyl moiety rotational motion (unpublished results). Therefore, cholesterol probably acts as a spacer molecule that increases the separation between phospholipid head groups and reduces intermolecular interactions.

While cholesterol and unsaturation behaved in a qualitatively similar manner at the head group region, opposite

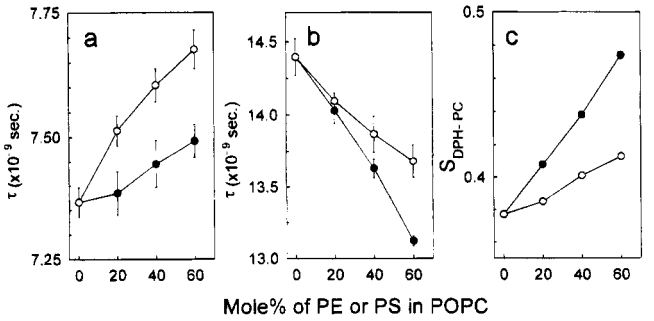

FIGURE 4: Effect of increased POPE (solid circle) and POPS (open circle) in POPC bilayer vesicles on: (a) fluorescence lifetime of DPH-PC; (b) fluorescence lifetime of dansyl-PE; (c) S_{DPH-PC}. Data are the mean of three separate measurements ± SD. Other details are as described under Materials and Methods.

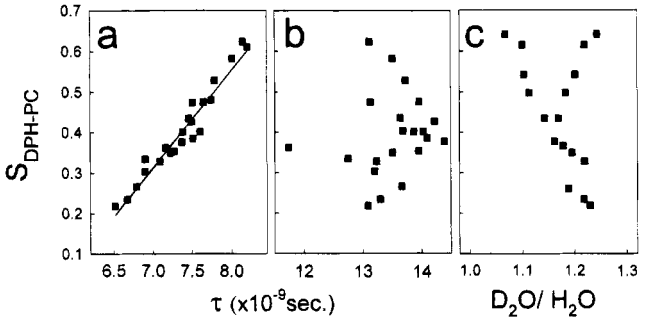

FIGURE 5: Plot of acyl chain order parameter (S_{DPH-PC}) as a function of (a) fluorescence lifetime of DPH-PC in PC bilayers with varied cholesterol, PE, and PS and for *sn*-2 and *sn*-1,2 diunsaturated-PCs (the line has a correlation coefficient of 0.94); (b) fluorescence lifetime of dansyl-PE; (c) D_2O/H_2O fluorescence intensity ratio of dansyl-PE.

effects were found on the acyl chain region. Thus, cholesterol increased acyl chain order (Figure 3c) and the fluorescence lifetime of DPH-PC, reflecting decreased interchain hydration (Figure 3d). The DPH of DPH-PC is anchored too deep in the bilayer to be used in the deuterium isotope effect (D_2O/H_2O) unlike TMA-DPH, which can be used since it is positioned nearer the surface. The TMA-DPH D_2O/H_2O fluorescence intensity ratio decreased with added cholesterol, *opposite* to the effect with dansyl-PE (Figure 3b), confirming the opposite effects on hydration that cholesterol has on the two regions of the bilayer. TMA-DPH is often used as a probe of the head group region, but this result would indicate that it is in fact probing deeper in the bilayer, within the C1−C10 region.

Effects of PS and PE. Both increased PE in POPE/POPC and PS in POPS/POPC bilayers were found to increase the fluorescence lifetime of DPH-PC and to reduce the lifetime of dansyl-PE as shown in Figure 4a,b. Thus, both PE and PS had opposite effects on hydration in the head group and interchain regions as with cholesterol. Also PE and PS had an ordering effect on the lipid bilayers (Figure 4c), although the effect was relatively small.

Correlation of Hydration with Acyl Chain Order. The finding that cholesterol, PS, and PE, but not unsaturation, have opposite effects on head group and interchain hydration suggests that the two regions in question are in some way "uncoupled". In Figure 5a, a plot of acyl chain order (S_{DPH-PC}) for the different lipid systems as a function of interchain hydration (DPH-PC fluorescence lifetime) reveals a strong correlation between the order and interchain hydration that was *independent* of lipid composition. Although the fact that the correlation is derived from the same probe weakens the argument, we have recently found that perturbation by a series of *n*-alkanols does not reveal a interchain hydration/acyl chain correlation even though the data are again from DPH-PC lifetime and order. This suggests that the lifetime and probe anisotropy, from which acyl chain order is derived, are not linked parameters.

For head group hydration, acyl chain order only showed a correlation with the dansyl-PE fluorescence lifetime (Figure 5b) or the D_2O/H_2O fluorescence intensity ratio (Figure 5c) *within* lipid compositional alterations of the *same type* not across different lipid types.

DISCUSSION

In this work, we have investigated the effects of increased levels of *sn*-2 unsaturation and *sn*-1,2 diunsaturation, cholesterol, PS, and PE on hydration and acyl chain order. Increased unsaturation elevated both head group and interchain hydration; by contrast, cholesterol, PE, and PS also increased head group hydration but had the opposite effect of decreasing interchain hydration. Also *sn*-1,2 diunsaturation (in PC) was more potent at disordering and hydrating the acyl chain region than *sn*-2 unsaturation, yet there was little difference between the two in the effects on hydration at the head group region. Overall, the results provide evidence that acyl chain and head group regions are structurally uncoupled.

In this study, it was found that unsaturation decreased the fluorescence lifetime of DPH-PC in a manner that was consistent with the introduction of acyl chain defects, introduced by the inflexible cis-double bonds, able to harbor water molecules within the bilayer. This result would be consistent with the literature. For example, while increasing PC unsaturation reduces the gel-to-liquid crystalline phase transition temperature (T_C), after a certain point it increases again (Litman et al., 1991; Keough et al., 1987; Keough, 1990) indicating that acyl chains with multiple double bonds take on compact and relatively inflexible configurations. Another study showed that 16:0-γ(Δ9,12,15)18:3-PC has a higher T_C and is less ordered in the liquid crystalline phase than the *isomeric* form 16:0-γ(Δ6,9,12)18:3-PC (McCabe et al., 1994). Also experimental evidence for two 18:2(Δ6,9) interchangeable alternate conformations has been found using ^{2}H-NMR (Baenziger et al., 1992). For three or more double bonds/chain, a rapid "jump" motion between these types of conformations should not be able to occur (Baenziger et al., 1992), a result suggested to be consistent with theoretical structures proposed elsewhere based on 1,4-pentadiene (Baenziger et al., 1991, 1992) as proposed for arachidonyl and docosahexaenoyl-PC and most likely of a helical nature [see, for example, Applegate and Glomset (1986); Stubbs and Smith (1984); Stubbs (1989, 1992)].

The effect of increased unsaturation was to increase *both* head group and interchain hydration as probed by dansyl-PE and DPH-PC fluorescence lifetimes, respectively. This contrasted dramatically with the effects of increased cholesterol, PS, and PE levels, which had opposite effects on head group and interchain hydration. In previous studies, increased *sn*-2 unsaturation was shown to decrease both TMA-DPH and DPH fluorescence lifetimes from which an increased hydration *within* the bilayer was inferred (Straume

& Litman, 1987a,b), although in these studies head group and interchain hydration were not distinguished. More recently, using the ESR approach, "hydrophobicity" profiles of frozen liposome systems were obtained, and cis-double bonds were found to increase this quantity (Subczynski et al., 1994). The results of the present study show that increased lipid disorder results from increased unsaturation and that this parallels an increased interchain hydration. In addition, it was unexpectedly found that DOPC bilayers were *more* disordered than bilayers of PAPC or PDPC, yet accommodated *less* water molecules in the head group region. Although this would at least be consistent with early monolayer data showing that DOPC had smaller molecular area at the air−water interface than PAPC and PDPC (Demel et al., 1972). Overall, increasing *sn*-2 and *sn*-1,2 unsaturation decreased acyl chain order ($S_{DPH\text{-}PC}$) in proportion to the double bonds/PC, although differences in order for bilayers of lipids differing by 1−2 double bonds/PC were not resolvable. By contrast, differences in head group hydration as reflected on dansyl-PE lifetime were resolvable, even for lipid pairs differing by as little as 2 double bonds/PC.

Cholesterol profoundly orders the hydrocarbon region of the membrane, although it has a negligible effect on the head group motional rates of phospholipids in the liquid crystalline phase [see, for example, Brown and Seelig (1978); Ghosh (1988); Milburn and Jeffrey (1989); Han and Gross (1991)]. In a recent study, we found that cholesterol had surprisingly little impact on the desorption rates of phospholipids from lipid bilayers (Slater et al., 1993). Thus hydrogen bonding involving the 3-β-hydroxyl group must at best be of comparable strength to that in the hydration layer involving hydrogen bonded water between phospholipid head groups. This is supported by the finding that cholesterol has been noted to have very little effect on membrane dipole potential or interbilayer hydration pressure (McIntosh et al., 1989). The ordering effect on the acyl chain region directly results in a decreased water permeability (Lazrak et al., 1987; Finkelstein, 1976) and decreased depth to which water penetrates into the lipid bilayer [see, for example, Simon et al. (1982)] and also an increased hydrophobicity in the acyl chain region (Subczynski et al., 1994). In the present study, this effect was seen as a marked increase in the fluorescence lifetime of DPH-PC. By contrast, at the head group region, the decreased dansyl-PE lifetime and the increased D_2O/H_2O dansyl fluorescence intensity ratio suggest the opposite of *increased* hydration. Probably this result is best rationalized on the basis of the relatively small head group volume of cholesterol acting as a phospholipid "spacer" as with PE.

The finding of a strong correlation of acyl chain order with interchain hydration and a lack of any correlation between head group hydration and order (Figure 5a−c) has certain implications. Thus, upon modifications to the acyl chain region, resultant alterations to acyl chain order cannot be used as a reliable indicator of likely effects at the head group region, at least in terms of hydration. This applies not only to compositional changes in the membrane lipids but also to the effects of external agents such as alcohols and anesthetics. The results also reveal a strong lipid *type* specificity with respect to changes in the degree of head group hydration. Overall, the results may explain why acyl chain order or the related parameter membrane fluidity, as blanket terms, often fail as reliable predictors of functional effects on membrane proteins, which may also be sensing altered hydration at the head group region of the bilayer.

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