Effect of *n*-Alkanols on Lipid Bilayer Hydration†

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ABSTRACT: The effect of a homologous series of aliphatic *n*-alkanols on the presence of water within the head group and acyl chain region of lipid bilayers was investigated using time-resolved fluorescence spectroscopy according to a previously published approach [Ho, C., Slater, S. J., & Stubbs, C. D. (1995) *Biochemistry 34*, 6188−6195]. Upon addition of *n*-alkanols to phosphatidylcholine bilayers the fluorescence lifetime of *N*-[5-(dimethylamino)naphthalene-1-sulfonyl]dipalmitoylphosphatidylethanolamine (dansyl-PE) decreased, indicative of an increased water content within the head group region, the effect being a linear function of *n*-alkanol chain length (C1−C8), based on the total *n*-alkanol concentration. The fluorescence lifetimes of 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine (DPH-PC) and *N*-[[4-(6-phenyl-1,3,5-hexatrienyl)phenyl]propyl]trimethylammonium *p*-toluenesulfonate (TMAP-DPH), and the fluorescence intensity ratio of the latter in D_2O compared to that in H_2O, were used to probe the level of water in the acyl chain region. There was a decrease in the lifetime and an increase in the D_2O/H_2O fluorescence intensity ratio upon addition of short-chain *n*-alkanols (C1−C3), suggestive of increased water content. By contrast, long-chain *n*-alkanols (C4−C8) increased the lifetime and decreased the ratio, suggestive of decreased water content. Acyl chain order, determined from DPH-PC fluorescence anisotropy, was decreased by all *n*-alkanols, indicating that the effects were not probe-dependent. The effects of short- and long-chain *n*-alkanols on the fluorescence lifetime of the tryptophans of gramicidin, incorporated into phosphatidylcholine bilayers as a model membrane protein, were similar to those obtained with TMAP-DPH and DPH-PC; ethanol decreased and hexanol increased the lifetime. Thus the effect of *n*-alkanols and general anesthetics on changes in the amount of water that may be accommodated within the acyl chain region of the bilayer is not predictable on the basis of the magnitude of effects on head group region or acyl chain order/fluidity.

The mechanism of ethanol intoxication and general anesthetic action has long been considered to involve some critical perturbation of the cell membrane structure. In addition, *n*-alkanols are often used to investigate the effect of such a perturbation, apart from the obvious interest in the effect of ethanol itself. Also, long-chain *n*-alkanols are often used to model general anesthetic−membrane interactions, and use of a homologous series of *n*-alkanols provides a test whether effects are related to the hydrophobicity of the compound. Historically studies of the effects of *n*-alkanols on membranes have concentrated on acyl chain order or membrane "fluidity" (Goldstein, 1984; Ueda, 1991). However, this idea has been criticized, mainly on the basis of the finding that acyl chain disordering by pharmacologically relevant concentrations of *n*-alkanols and anesthetics tends to be small (Franks & Lieb, 1994; Franks & Lieb, 1982) and that nonlipid (protein) hydrophobic sites could also serve as primary targets (Franks & Lieb, 1984; Rooney et al., 1993; Covarrubias et al., 1995; Slater et al., 1993a, 1995; Wood et al., 1991; Forman et al., 1995). However, measurement of the effects of alcohols and anesthetics solely in terms of bulk or average physical properties such as acyl chain order or "fluidity" is clearly an oversimplification. A number of other types of perturbation of membrane structure have therefore been investigated as likely to be critically involved in alcohol and anesthetic perturbation. Among these are effects on the water content of the lipid bilayer (Stubbs & Rubin, 1993), alteration of which would impact on hydrogen-bond interactions in the head group region and on lipid head group mobility [see for example Ulrich and Watts (1994)].

There is a critical level of hydration required to establish the conditions for lipid molecules to form a lipid bilayer. This hydration participates intimately in the lipid bilayer structure (Newman & Huang, 1975; Small, 1967; Ladbrooke & Chapman, 1969; Finer & Darke, 1974; Jendrasiak & Hasty, 1974; Ueda et al., 1986; Sen & Hui, 1988). It has been known for many years that water diffuses across lipid bilayers (Bittman & Blau, 1972; Demel et al., 1968, 1972), and in fact water is able to exchange quite rapidly between the interior and exterior of vesicles (Bittman & Blau, 1972; Finkelstein & Cass, 1968) and is therefore found even within the hydrophobic region (Simon & McIntosh, 1986). Water molecules are found within the head group region of lipid bilayers (Griffith et al., 1974; Gruen et al., 1983; Ulrich & Watts, 1994; Damodaran & Merz, 1994; Damodaran et al., 1992; Wiener & White, 1992; Rand & Parsegian, 1989) and participate in a hydrogen-bond network between the phospholipid head group ester and phosphate oxygens and the carboxyl, amino, or tetramethylammonium groups of phosphatidylethanolamine, -serine, and -choline, respectively

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(Boggs, 1987). This system of interlipid head group hydrogen-bond bridges involving water has been termed a "hydration layer" (Teissie et al., 1990) and it has quite distinct properties from bulk water. The penetration of water into the bilayer sets up a so-called "dielectric constant gradient" from a dielectric constant value at the membrane surface of ~70 to ~5 at the bilayer center (Zannoni et al., 1983; Fiorini et al., 1987). Experimental evidence for the existence of water molecules within the lipid bilayer is available from neutron diffraction, although being time-averaged the degree of penetration water may be overemphasized (Worcester & Franks, 1976; Franks & Lieb, 1979; Zaccai et al., 1975; Blechner et al., 1990), and from ESR studies (Griffith et al., 1974). Using this approach, recent studies have shown that the introduction of small peptides, consisting of just three amino acids, can cause a shift of water deeper into the bilayer (Jacobs & White, 1989).

Another approach that provides evidence for water penetration into the lipid bilayer is fluorescence spectroscopy, based on effects on lifetime and intensity (Perochon et al., 1992; Fiorini et al., 1987, 1989; Williams & Stubbs, 1988; Ho & Stubbs, 1992; Ho et al., 1992, 1995). While fluorophores can be localized at discrete depths within the lipid bilayer to monitor water content, effects on the fluorophore properties, such as intensity, lifetime, and anisotropy, may be difficult to unambiguously assign to changes in the water content of the bilayer. To address this question, the deuterium isotope effect provides a useful approach for assessing water presence in the fluorophore vicinity. In this method, the fluorescence intensity of the fluorophore in a H_2O-based buffer is compared to that in a D_2O buffer (Stryer, 1966). For some fluorophores the fluorescence intensity is greater in D_2O compared to H_2O. Thus, if the fluorophore is exposed to water the D_2O/H_2O fluorescence intensity ratio is greater than unity, and from changes in the ratio, effects on the water content in the fluorophore vicinity may be inferred (Nakanishi et al., 1980; Ho et al., 1995; Ho & Stubbs, 1992; Kranz et al., 1981; Jain & Vaz, 1987; Maeda-Yorita & Massey, 1993; Konopasek et al., 1995). In principle, providing a fluorophore has an exchangeable proton and can be made to locate in the area of interest, then water accessibility may be determined. Using this method, we have explored the effects of varied lipid composition on the head group region and that just below this region using dansyl-PE[1] and TMA-DPH (Ho & Stubbs, 1992; Ho et al., 1995). Thus with increased cholesterol, for example, the water content below the head group region was reduced, in keeping with the results of other studies (Simon et al., 1982), while the water content in the region probed by dansyl-labeled PE in the head group region increased. If a fluorophore is buried more deeply in the bilayer and/or does not show an appreciable deuterium isotope effect, the presence of water may only be inferred (indirectly) by an effect on the fluorescence lifetime, if other excited-state reactions do not take place.

Studies of the more commonly investigated short-chain *n*-alkanol, ethanol, indicate that it participates in the interlipid hydrogen-bond network in the head group region, thereby displacing water and weakening overall lipid−lipid interactions (Ueda, 1991; Urry & Sandorfy, 1991; Curatola et al., 1991; Slater et al., 1993b; Barry & Gawrisch, 1995). Recently direct NMR evidence for ethanol binding to the lipid−water interface of phospholipid bilayers has been provided (Barry & Gawrisch, 1995). While it was shown that little or no ethanol bound in the hydrocarbon interior of the bilayer, it significantly modified the lipid head group orientation and disordered the entire length of the hydrocarbon chains. In recent studies from this laboratory the phospholipid desorption rate from donor vesicles to acceptor vesicles (or bovine serum albumin) was used as a measure of the overall forces holding a phospholipid in a lipid bilayer. Ethanol was found to enhance the desorption rate. Interestingly, neither the effect of ethanol or varied PC unsaturation on the desorption rate correlated with acyl chain lipid order and the effect was attributed to phospholipid−water hydrogen-bond disruption (Slater et al., 1993b).

The present work was directed to the question of the effects of *n*-alkanols on the extent of water residing within the head group and acyl chain regions compared with those on lipid order. Following the previously developed approach (Ho et al., 1995), the effects of a series of *n*-alkanols were determined using dansyl-PE, DPH-PC, TMAP-DPH, and gramicidin (tryptophan) fluorescence lifetimes and D_2O/H_2O fluorescence intensity ratios. The results were consistent with *n*-alkanols increasing the level of water residing in the head group region. By contrast, while short-chain (C1−C3) *n*-alkanols increased water content of the acyl chain region, long-chain *n*-alkanols (C4−C8) decreased the water content.

MATERIALS AND METHODS

Materials. All phospholipids and DPH-PC and dansyl-PE fluorophores were obtained from Avanti Polar Lipids (Alabaster, AL). TMAP-DPH was from Molecular Probes (Eugene, OR). Lipid concentrations were routinely checked by phosphorus analysis (Bartlett, 1959). *n*-Alkanols and gramicidin D were from Sigma Chemical Co. (St Louis, MO). Deuterium oxide was from Cambridge Isotope Laboratories (Andover, MA). All other chemicals used were of analytical grade except for solvents, which were of HPLC grade and were obtained from Fisher Scientific (Malvern, PA).

Phospholipid Vesicles. Large unilamellar vesicles (LUV, 100 nm diameter) were prepared as described elsewhere (MacDonald et al., 1991). Briefly, aliquots of the required phospholipid in chloroform together with the fluorophore were placed in a test tube and the solvents were removed by a stream of nitrogen. The fluorophore:lipid ratio was 1:400 for DPH-PC and TMAP-DPH and 1:100 for dansyl-PE. 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 brief vortexing, the sample was passed through a 100 nm polycarbonate filter using an Avestin Liposofast extruder (Avestin, Ottawa, Canada). Samples were diluted to final lipid concentrations of 50 and 200 μM for steady-state

[1] Abbreviations: dansyl-PE, *N*-[5-(dimethylamino)naphthalene-1-sulfonyl]dipalmitoylphosphatidylethanolamine; DPH-PC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; LUV, large unilamellar vesicles; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PDPC: 1-palmitoyl-2-docosahexaenoylphosphatidylcholine; S_{DPH-PC}, order parameter for 1-palmitoyl-2-[[2-4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine; χ^2_R, reduced chi-square; TMAP-DPH, N-[[4-(6-phenyl-1,3,5-hexatrienyl)phenyl]propyl]trimethylammonium *p*-toluenesulfonate; τ, fluorescence lifetime.

Table 1: Fluorescence Decay Analyses of DPH-PC and Dansyl-PE in POPC LUV 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						46.1
1 Lor	6.78 ± 0.04	0.95 ± 0.10					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	0.05 ± 0.07	0.86 ± 0.01	3.44 ± 0.18	0.01 ± 0.01	0.14 ± 0.01	0.92
				Dansyl-PE			
1 exp	13.75 ± 0.10						500
1 Lor	13.88 ± 0.02	3.96 ± 0.74					96.9
2 exp	14.33 ± 0.11		0.97 ± 0.01	1.38 ± 0.14		0.03 ± 0.01	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 chi-square. Data was collected for 10−12 frequencies between 5 and 130 MHz; standard errors (std dev) were from three separate measurements. The χ^2_R values were taken from representative single data points.

fluorescence anisotropy and lifetime measurements, respectively. For the deuterium isotope exchange measurements, samples were diluted to the desired concentration with D_2O and H_2O buffers.

Incorporation of Gramicidin. POPC bilayers with gramicidin (channel form) incorporated (1:10 gramicidin:phospholipid molar ratio) were prepared as described previously (LoGrasso et al., 1988; Cox et al., 1992). Briefly, gramicidin in trifluoroethanol was added to POPC dried under a stream of nitrogen. Buffer (10 mM Tris-HCl and 150 mM NaCl, pH 7.4) was added after sample was thoroughly dried. Then the sample was subjected to 10 s of sonication with full power (Fisher Scientific Dismembrator, microtip). After centrifugation at 1100*g* for 5 min to remove unincorporated material, the amount of gramicidin incorporation into the vesicles was determined using a lipid phosphorus assay (Bartlett, 1959), while gramicidin was determined from its absorption at 280 nm using a standard curve (in methanol).

Fluorescence Measurements. Fluorescence data were collected using an SLM 48000 multifrequency phase-modulation fluorometer. The details of phase and modulation method have been described previously (Lakowicz & Maliwal, 1985; Gratton & Limkemann, 1983). The excitation was provided by a Liconix HeCd laser (Model 4240NB, 325 nm), sinusoidally modulated by a RF frequency from 5 to 130 MHz. For lifetime measurements, the phase-shifted and demodulated emission was observed through a 430 nm red-pass filter and a Glan-Thompson polarizer set at the magic angle. 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, usually near 0.2° in the phase and 0.002 in the modulation. Steady-state anisotropies were measured using a T-format configuration with excitation wavelength set at 360 nm from a xenon lamp. The vertical and horizontal emission components were collected through 430 nm red-pass filters and the anisotropy was determined as previously described (Stubbs et al., 1984). Steady-state emissions were measured using magic-angle optics.

Fluorescence lifetime measurements of gramicidin tryptophans were carried out at the Regional Laser and Biotechnology Laboratories (RLBL) at the University of Pennsylvania. A time-correlated single photon counting system was used, based on a cavity-dumped dye laser synchronously pumped by a Nd−YAG laser (Coherent, Santa Clara, CA). The time base for the instrument was calibrated by an optical delay line and an instrument function width of ≈36 ps (full width at half-maximum) was obtained. The output from the dye laser was frequency-doubled to produce 295 nm emission. Emission at 340 nm was collected through a magic-angle polarization configuration and a low-dispersion monochromator. The signal was monitored and collected by a microchannel plate photomultiplier tube and TAC (O'Connor & Phillips, 1984).

Data Analysis. Phase and modulation data were analyzed using the Globals Unlimited software (Laboratory of Fluorescence Dynamics, University of Illinois, Urbana−Champaign, IL) (Beechem, 1990; Beechem & Gratton, 1988). Total fluorescence intensity decay was fit to both exponential and distributional analytical forms:

$$I(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$$

or

$$I(t) = \int_0 a_1(\Omega) \exp[-t/\tau_1(\Omega)] + a_2(\Omega) \exp[-t/\tau_2(\Omega)]\, d\tau$$

Ω is a Lorentzian distributional function, and selection was made by comparison on the basis of χ^2_R and residuals.

The fluorescence decay of dansyl-PE was found to be best described using a bimodal Lorentzian distributional analysis. This seems reasonable considering the wide orientational freedom enjoyed by the fluorophore at the membrane surface (see Table 1 for comparison of different fitting models). For DPH-PC, there was a minor lifetime component (less than 5% of the total intensity), as expected from a DPH-type probe (Parasassi et al., 1991; Lentz & Burgess, 1989), that showed no systematic variation for varying experimental conditions. For DPH-PC, as previously discussed (Ho et al., 1992), with DPH anchored at PC *sn*-2 chain, a discrete (exponential-type) fluorescence decay analysis was found to be appropriate (see Table 1 for comparison of fits to various models). Gramicidin tryptophan excited-state decays were analyzed using RLBL analysis software. The measured fluorescence time profiles were fitted to a four-lifetime decay function, using iterative deconvolution, to give an adequate χ^2_R and residuals, as seen in Table 2.

An order parameter S_{DPH-PC}, used to characterize the acyl chain structural order, was calculated from steady-state anisotropy measurements on DPH-PC, as described elsewhere (Heyn, 1979; Jahnig, 1979). Briefly, order parameters were calculated from $S = (r_\infty/r_0)^{1/2}$, where r_0 is the limiting anisotropy in the absence of rotational motion and r_∞ is the residual anisotropy. A modified Perrin equation, $r_s = [(r_0 - r_\infty)/(1 + \tau/\phi)] + r_\infty$, was used to obtain r_∞. In this

Table 2: Fluorescence Decay of Gramicidin Tryptophans in LUV Made from POPC at 37 °C

model	τ_1	f_1	τ_2	f_2	τ_3	f_2	τ_4	f_4	χ^2_R
1 exp	1.77								40.6
2 exp	3.12	0.19	0.57	0.81					3.24
3 exp	3.81	0.08	1.00	0.37	0.19	0.55			1.19
4 exp	4.24	0.04	1.27	0.18	0.34	0.26	0.04	0.52	1.01

[a] τ, fluorescence lifetime; f, fractional intensity; χ^2_R, reduced chi-square.

equation the first term represents the kinetic contribution, and the second term, the structural one. The value of r_o, 0.393, for DPH-PC was taken from previously published data (Squier et al., 1991). The use of steady-state rather than time-resolved measurements was justified on the basis of invariance of the fluorescence lifetime:rotational correlation time (τ/ϕ) for different ethanol concentrations (data not shown).

RESULTS AND DISCUSSION

In this study the effects of *n*-alkanols on level of water residing in the lipid head group and interchain region of the bilayers and on average lipid acyl chain order was investigated in POPC bilayers. To accomplish this, effects on the fluorescence lifetime of dansyl-PE were used to assess the level of hydration in the lipid head group region while TMAP-DPH and DPH-PC were used to assess water presence deeper within the acyl chain region. This approach follows similar studies in this laboratory (Ho et al., 1995; Ho & Stubbs, 1992) and others (Fiorini et al., 1987; Lakowicz et al., 1989; Straume & Litman, 1987) where the level of hydration in the locality of the fluorophore in question was under investigation; generally, the greater the hydration level, the faster the excited-state decay (shorter fluorescence lifetime). Also, effects on the level of hydration were determined using the deuterium isotope effect with the fluorophore TMAP-DPH.

Effect of n-Alkanols on the Fluorescence Lifetime of DPH-PC. In a previous study from this laboratory the fluorescence decay of DPH attached to the *sn*-2 position of PC was used to assess the level of water in the acyl chain region, in the ~C3−C12 region (Ho et al., 1995). The effect of *n*-alkanols on the fluorescence lifetime of DPH-PC in POPC vesicles is shown in Figure 1a. The short-chain *n*-alkanols (methanol−propanol) decreased the fluorescence lifetime, a result that is consistent with increased water content. However, long-chain *n*-alkanols (butanol−octanol) increased the fluorescence lifetime. The results are also shown in terms of the mole fraction of alcohol in the membrane in Figure 1b. The *slopes* of the plots from Figure 1a were a linear function of *n*-alkanol chain length, as shown in Figure 1c, for the respective decrease (short-chain *n*-alkanols) and increase (long-chain *n*-alkanols) in τ_{DPH-PC}. By contrast, the *potency* of the effects, here defined as the $\Delta\tau_{DPH-PC}$ for the same mole fraction of each *n*-alkanol in the membrane (arbitrarily set to the mole fraction of ethanol that produced a 1% $\Delta\tau_{DPH-PC}$) was not linear, and the long-chain *n*-alkanols in particular showed a reduced potency of the effect on τ_{DPH-PC} (see Figure 1d). Similar effects have been demonstrated previously in the literature. For example the effect of *n*-alkanols on the liquid crystalline−hexagonal ("Hex_{II}") nonbilayer phase transition is also a nonlinear function of *n*-alkanol chain length (Hornby & Cullis, 1981). Similar

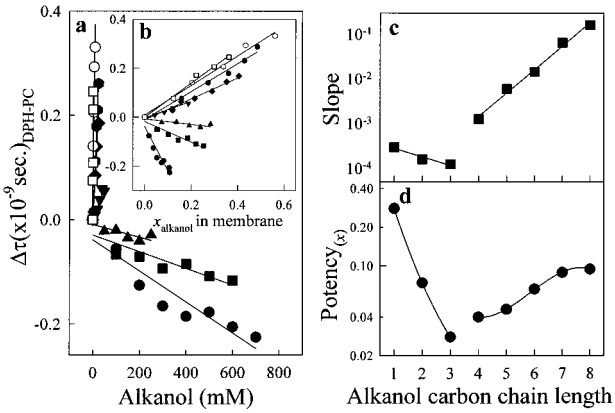

FIGURE 1: Effect of *n*-alkanols on the fluorescence lifetime of DPH-PC. (a) Effects in terms of $\Delta\tau_{DPH-PC}$ in POPC bilayers, prepared as described under Materials and Methods, as a function of total *n*-alkanol concentration: methanol (●); ethanol (■); propanol (▲); butanol (▼); pentanol (◆); hexanol (⬢); heptanol (○); octanol (□). (b) Effect of *n*-alkanols on $\Delta\tau_{DPH-PC}$ as a function of the mole fraction of *n*-alkanol in membrane [x = moles of *n*-alkanol in membrane/(moles of *n*-alkanol in membrane + moles of lipid)]. (c) Effect of *n*-alkanols on the $\Delta\tau_{DPH-PC}$ calculated from the slopes of the effect for each *n*-alkanol from panel a ($\Delta\tau_{DPH-PC}$/[*n*-alkanol]). (d) Effect of *n*-alkanols on $\Delta\tau_{DPH-PC}$ as a function of *n*-alkanol chain length in terms of potency, based on $\Delta\tau_{DPH-PC}$ for each *n*-alkanol at the same mole fraction in membrane (the mole fraction of ethanol in the membrane that produces a 1% $\Delta\tau_{DPH-PC}$). Experimental details are as described under Materials and Methods.

contrasting effects of short- and long-chain *n*-alkanols were found on the MI−MII equilibrium of rhodopsin in retinal rod outer segment discs (Mitchell et al., 1996). The authors showed that, by plotting their data using the mole fraction of alcohol in the bilayer, the effects switched from a sharp discontinuity (between short- and long-chain *n*-alkanols) to a continuous decline.

The increased water content in acyl chain region by short-chain *n*-alkanols is believed to be due to the increased headgroup spacing which in turn allows increased water molecule penetration. The potency of this effect *decreased* upon increasing *n*-alkanol chain length due to the progressive increased presence of methylene groups reducing the void volume in acyl chain region (Mitchell et al., 1996), with the effect leveling off at C7−C8.

The question arises of distinguishing between altered water content and the presence of the alcohol itself. Other studies indicate that both short, and long-chain *n*-alkanols are anchored at the membrane surface (Rowe et al., 1987; Pope et al., 1984; Brasseur et al., 1985) so that the alcohol presence in the hydrocarbon region of the bilayer would be in the form of its hydrocarbon chain. Therefore the decreased fluorophore lifetime induced by short-chain *n*-alkanols would be unlikely to be due to increased presence of the alcohol hydroxyl group in this region. However, to confirm this point water accessibility was assessed by measuring the deuterium isotope effect. Although this was not possible with DPH-PC, by contrast, TMAP-DPH had a measurable deuterium isotope effect. Accordingly, as with DPH-PC the short-chain *n*-alkanol ethanol decreased the TMAP-DPH lifetime and the long-chain *n*-alkanol increased the lifetime (Figure 2). The TMAP-DPH D_2O/H_2O intensity ratio (at 430 nm, for 360 nm excitation) increased with the short-chain *n*-alkanol and decreased with the long-chain *n*-alkanol (Figure 3), compatible with decreased and increased water adjacent to the fluorophore, respectively. With all probe-dependent methods the possibility of probe-generated arti-

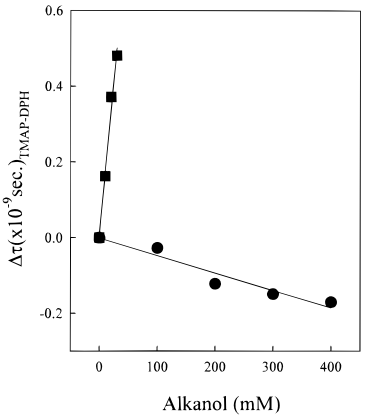

FIGURE 2: Effect of ethanol and hexanol on the fluorescence lifetime of TMAP-DPH on POPC bilayers: Ethanol (●); hexanol (■). Experimental details are as described under Materials and Methods.

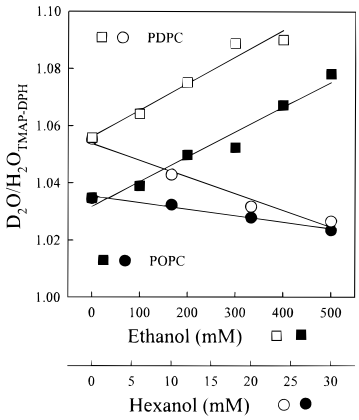

FIGURE 3: Effect of ethanol and hexanol on the deuterium isotope effect on TMAP-DPH POPC and PDPC bilayers. The fluorescence intensity ratio (D_2O/H_2O) of TMAP-DPH increases with ethanol but decreases with hexanol. The filled symbols are for POPC; the open symbols for PDPC; ethanol (circles); hexanol (squares). Experimental details are as described under Materials and Methods.

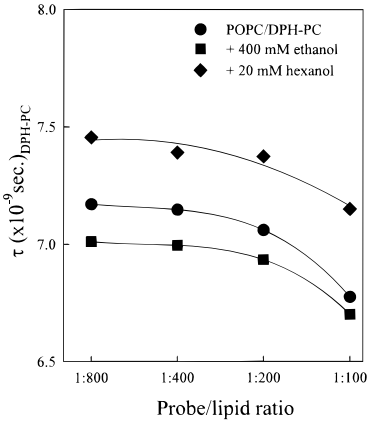

FIGURE 4: Effects of ethanol and hexanol on the fluorescence lifetime of DPH-PC for varying probe/lipid molar ratios. For probe/lipid molar ratio <1:200, the DPH-PC fluorescence lifetime and the effects of 400 mM ethanol and 20 mM hexanol were constant. Data are shown for POPC/DPH-PC alone (●), + 400 mM ethanol (■), or + 20 mM hexanol (◆). Experimental details are as described under Materials and Methods.

facts has to be considered. In Figure 4, the relationship between the probe:lipid ratio and the fluorescence lifetime of DPH-PC is shown. First, it can be seen that for probe: lipid ratios <1:200 the fluorescence lifetime is independent of the ratio. Second, the increase in lifetime induced by hexanol, and decrease induced by ethanol, is maintained to

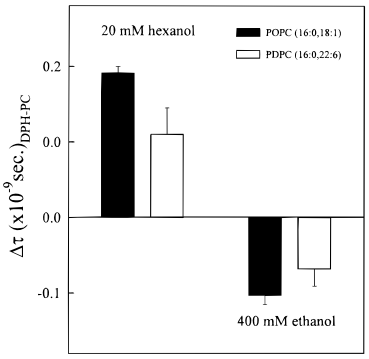

FIGURE 5: Effect of a short-chain *n*-alkanol (ethanol) as compared to a long-chain *n*-alkanol (hexanol) on the fluorescence lifetime of DPH-PC in POPC as compared to PDPC bilayers. Experimental details are as described under Materials and Methods.

lower and lower probe ratios; i.e., the effects are independent of the amount of fluorophore and therefore not induced by the fluorophore itself.

To test the effect of varied phospholipid unsaturation on *n*-alkanol effects on water in the acyl chain region, vesicles composed of monounsaturated POPC (one *cis*-double bond in the *sn*-2 chain) were compared with those composed of the polyunsaturated PDPC (six *cis*-double bonds in the *sn*-2 chain). Previously, we showed that the more unsaturated lipid harbored a great level of water in the acyl chain region (Ho et al., 1995). Upon comparing the effect of short- and long-chain *n*-alkanols, the effect on the fluorescence lifetime of DPH-PC was found to be smaller for the more unsaturated lipid, as shown in Figure 5. Multiple *cis*-double bonds introduce an inflexibility into the acyl chain, forcing it to adopt a helical and relatively inflexible configuration (Stubbs & Smith, 1984; Applegate & Glomset, 1986). As a result, acyl chain order of PDPC bilayers, as detected by DPH- type probes (which reflect the average motional properties of the entire acyl chain rather than individual segments), is lower than that of POPC bilayers (Stubbs et al., 1981). The inflexibility may offer a reduced ability to accommodate alcohols in the bilayer, seen as a reduced sensitivity to disordering (Slater et al., 1993b) in the more unsaturated system, offering a possible explanation of the reduced effect on the DPH-PC fluorescence lifetime by *n*-alkanols. By contrast, the effect of ethanol on the *rate* of phospholipid desorption (a function of head group hydrogen-bonding strength) is greater for PDPC than POPC (Slater et al., 1993b), supporting the idea that *n*-alkanols are accommodated less easily in the more unsaturated systems, which are therefore more perturbed by their presence. A similar argument has been proposed recently regarding the disordering effect of ethanol on DOPE/PC mixtures (Separovic & Gawrisch, 1996).

Effect of n-Alkanols on Acyl Chain Order. The effects on lipid acyl chain order (S_{DPH-PC}) calculated from the steady-state fluorescence anisotropy of DPH-PC in POPC bilayers (see Data analysis section under Materials and Methods for detail) are shown in Figure 6a. The data is also plotted in terms of the mole fraction of *n*-alkanol in the membrane (Figure 6b). While the *slopes* of the plots from Figure 6a are a linear function of *n*-alkanol chain length (Figure 6c), by contrast, the *potency*, (defined as the ΔS_{DPH-PC} for the *same* mole fraction of each alcohol in the membrane, set as the amount of ethanol that induces a ΔS_{DPH-PC} of 10%) is *greater* for short-chain *n*-alkanols. Overall, DPH-PC shows a similar alcohol-induced acyl chain

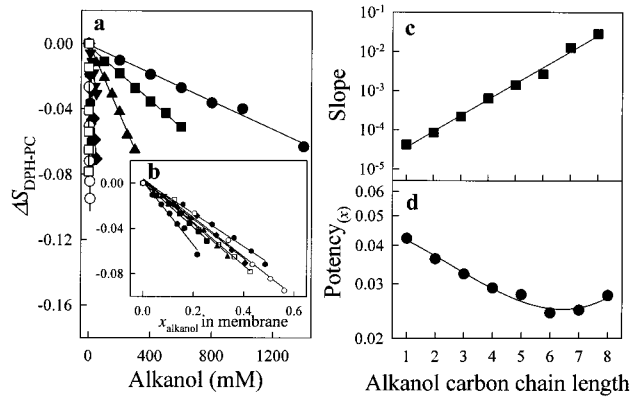

FIGURE 6: Effect of *n*-alkanols on acyl chain order. (a) Effects on acyl chain order (ΔS_{DPH-PC}) in POPC bilayers as a function of *n*-alkanol concentration, calculated from the fluorescence anisotropy of DPH-PC as described under Materials and Methods. Symbols are as for Figure 1. (b) Effect of *n*-alkanols, in terms of ΔS_{DPH-PC}, as a function of the mole fraction of *n*-alkanol in membrane. The $x_{alkanol}$ is defined as in Figure 1b. (c) Effect of *n*-alkanols on ΔS_{DPH-PC} in terms of the slopes of the effect for each *n*-alkanol (ΔS_{DPH-PC}/[*n*-alkanol]) from panel a. (d) Effect of *n*-alkanols on ΔS_{DPH-PC} in terms of potency, based on the ΔS_{DPH-PC} induced by the same mole fraction of each *n*-alkanol in membranes (the mole fraction of ethanol in the membrane that produces a 10% ΔS_{DPH-PC}).

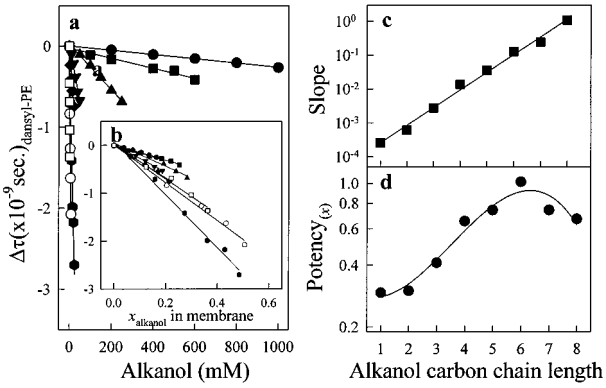

FIGURE 7: Effect of *n*-alkanols on the dansyl-PE fluorescence lifetime. (a) Effects on dansyl-PE fluorescence lifetime ($\Delta\tau_{dansyl-PE}$) in POPC bilayers as a function of *n*-alkanol concentration determined as described under Materials and Methods. Symbols are as for Figure 1. (b) Effect of *n*-alkanols in terms of $\Delta\tau$ as a function of the mole fraction of *n*-alkanol in membrane. The $x_{alkanol}$ is defined as in Figure 1b. (c) Effect of *n*-alkanols on $\Delta\tau$ in terms of the slopes of the effect for each *n*-alkanol ($\Delta\tau_{dansyl-PE}$ /[mM]) from Figure 7a. (d) Effect of *n*-alkanols on $\Delta\tau_{dansyl-PE}$ in terms of potency, based on the $\Delta\tau_{dansyl-PE}$ induced by the same mole fraction of each *n*-alkanol in membrane (the mole fraction of ethanol in the membrane that causes a 2% $\Delta\tau_{dansyl-PE}$). Experimental details are as described under Materials and Methods.

disordering, as reported by other fluorescent probes or different spectroscopic techniques (Pope & Dubro, 1986; Westerman et al., 1988; Miller et al., 1989), indicating that the effects on the fluorescence lifetime are unlikely to be due to fluorophore or bilayer artifacts. Thus it appears that there is no simple relationship between the effects of *n*-alkanols on the water content of the acyl chain region as reflected by DPH-PC or TMA-DPH fluorescence lifetime and acyl chain order, as reflected by S_{DPH-PC}. Thus the short-chain *n*-alkanols have the opposite effects to long-chain *n*-alkanols on the water content of the acyl chain region, yet both disorder the acyl chain region.

Effect of n-Alkanols on the Dansyl-PE Fluorescence Lifetime. The fluorescence lifetime of the dansyl fluorophore, attached to PE, was used to assess effects of *n*-alkanol perturbation within the head group region of POPC bilayers. While effects on the fluorescence lifetime also result from contributions of excited-state reactions, charge transfer and excimer formation, etc., these events are unlikely to be important in the system under investigation here. Other studies on lipid bilayers probed by dansyl fluorophores suggest that the lifetime of the excited state of the dansyl moiety is primarily sensitive to its local dielectric constant environment, to which water is the major contributing factor (Ghiggino et al., 1981; Kimura & Ikegami, 1985; Epand & Leon, 1992). Previous studies indicate that the location of the dansyl fluorophore is close to the lipid−water interface (Waggoner & Stryer, 1970; Stubbs et al., 1985; Kimura & Ikegami, 1985; Ohki & Arnold, 1990; Epand & Leon, 1992) . The effects of a homologous series of *n*-alkanols, from methanol to octanol (C1−C8), on the fluorescence lifetime (major lifetime center from bimodal distribution) of dansyl-PE in large unilamellar vesicles of POPC is shown in Figure 7a. A bimodal Lorentzian distributional analysis of the fluorescence decay data was chosen on the basis of the comparison of different fits shown in Table 1. It was found that for each *n*-alkanol the fluorescence lifetime of dansyl-PE decreased ($\Delta\tau_{dansyl-PE}$) with increasing alcohol concentration. From the data in Figure 7a, a plot of $\Delta\tau_{dansyl-PE}$ as a function of mole fraction of *n*-alkanol in the membrane was constructed, as shown in Figure 7b. Figure 7c shows the *slope* of the plots from Figure 7a as a function of *n*-alkanol chain length, while Figure 7d shows the effects in terms of the *potency* as before, that is, the $\Delta\tau_{dansyl-PE}$ for the same mole fraction of *n*-alkanol in the membrane (set as the amount of ethanol in the membrane to produce a $\Delta\tau_{dansyl-PE}$ of 2%). The potency increased with increased *n*-alkanol chain length, reached the maximum at hexanol, and leveled off or slightly reversed after hexanol. The increased potency for long-chain *n*-alkanols is not surprising since the increased hydrophobic interaction in the acyl chain region will increase the packing density in this region and induce increased lipid head group spacing. Further increases of the *n*-alkanol chain length (to C7−C8) will likely counteract the increased acyl chain packing density due to reduced acyl chain free volume. A decreased fluorescence lifetime of dansyl-PE is consistent with an increased water content around the dansyl moiety, probably due to the intercalation of alcohols within lipid bilayers causing an increase of interlipid head group spacing (Barry & Gawrisch, 1994, 1995), similar to the effect of cholesterol (Newman & Huang, 1975; Brown & Seelig, 1978; Ho et al., 1995).

The decrease in lifetime, while compatible with an increase in water, could also be induced by increased presence of the alcohol itself in the head group region. Using the D_2O/H_2O fluorescence intensity ratio approach is not useful here since the alcohol also has an exchangeable proton so that an increased ratio could also be induced by increased alcohol presence near the fluorophore. However, this point was examined in a previous study (Slater et al., 1993b), and from a plot of the fluorophore emission maxima against solvent dielectric constant (using a series of solvents with known values) the dielectric constant value for the fluorophore in the bilayer was estimated. For NBD-PE in POPC bilayers (before alcohol addition) the dielectric constant was 38, the value increasing to 44 upon addition of ethanol. Since the value for ethanol is 24, then on balance water rather than ethanol must have been entering the head group region where the fluorophore resided. This led us to conclude that, in the

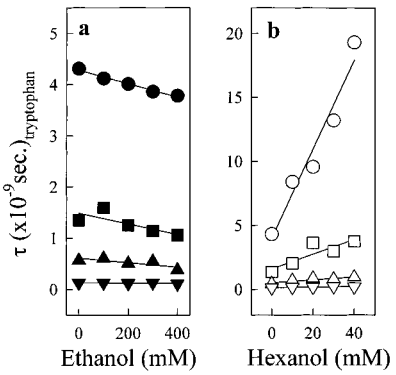

FIGURE 8: Effect of a short-chain *n*-alkanol (ethanol) as compared to a long-chain *n*-alkanol (hexanol) on the fluorescence lifetimes of gramicidin tryptophans. The fluorescence decay was analyzed as a four-exponential decay and the respective lifetimes are plotted as a function of alcohol concentration. Gramicidin (channel form) was incorporated into POPC vesicles as described under Materials and Methods. Ethanol (up to 400 mM) decreases, while hexanol (up to 40 mM) increases the fluorescence lifetime. Experimental details are as described under Materials and Methods.

head group region, water in the hydration shell has a reduced polarizability due to the interactions with lipid head group. When alcohol is added, it can replace the water molecules in the hydration shell and increase the head group spacing, allowing more water molecules to locate in the vicinity of the dansyl fluorophore, thus elevating local water polarizability.

Effect of n-Alkanols on Gramicidin Tryptophan Fluorescence Lifetime. To investigate further the contrasting effects of short- and long-chain *n*-alkanols on the presence of water in the acyl chain region, the effects on the intrinsic tryptophan fluorescence of gramicidin, incorporated into the bilayer in its transbilayer channel configuration, was studied. The tryptophans of gramicidin protrude into the acyl chain region of the lipid bilayer (Scarlata, 1988, 1991; Cornell, 1987) and it is therefore useful as a model membrane protein. In a previous study we found that the ratio of the fluorescence intensity of the gramicidin tryptophans in D_2O:H_2O was greater than 1, indicative of water residing within the excited-state solvent cage of the fluorophore, i.e., within the acyl chain region at the protein-lipid interface (Ho & Stubbs, 1992). POPC bilayers with 10 mol % of channel-form gramicidin were therefore prepared and the effects of a representative short- and long-chain *n*-alkanol on the fluorescence lifetime of gramicidin determined. To obtain an adequate χ^2_R and residuals, three or four lifetime components were required for the analysis (Table 2), in agreement with the literature (Masotti et al., 1986). For the purpose of comparison, a discrete model with four lifetime components was chosen.

The effects of ethanol and hexanol on the fluorescence decay of gramicidin tryptophans are displayed in Figure 8. The short-chain *n*-alkanol decreased the lifetime (all lifetime components), while the long-chain *n*-alkanol increased the lifetime. Thus, these results are in broad agreement with those on the DPH-PC lifetime and further reinforce the conclusion that the effects are probe-independent. The presence of water molecules at the gramicidin protein−lipid interface was previously inferred from D_2O/H_2O tryptophan fluorescence intensity ratio measurements of gramicidin−POPC (Ho & Stubbs, 1992). It is likely that water in the bulk acyl chain region and protein−lipid interfacial water rapidly exchange; however, near the protein surface water may potentially hydrogen-bond to amino acid side chains where it could potentially influence conformation.

In conclusion, evidence for the amount of water residing within the acyl chain region of lipid bilayers increasing upon the addition of short-chain *n*-alkanols and decreasing for long-chain *n*-alkanols was found. By contrast, the level of hydration within the head group region increased with a potency that increased with *n*-alkanol chain length. A similar effect was recently shown in the modulation of the formation of metarhodopsin II, which was increased by short-chain *n*-alkanols (ethanol, butanol, and hexanol) but inhibited by long-chain *n*-alkanols (octanol and decanol) (Mitchell et al., 1996). It was pointed out that a short-chain *n*-alkanol such as ethanol binding to lipid−water interface will increase head group spacing and the bilayer acyl chain packing free volume. By contrast, long-chain *n*-alkanols progressively occupy more space deeper than the head group region and reduce the gain in bilayer free volume associated with increased head group spacing. Overall the results indicate unusual divergence in the behavior between ethanol and general anesthetics/long-chain *n*-alkanols in having opposite effects (on acyl chain water), therefore not strictly following "Meyer−Overton".

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