Direct NMR Evidence for Ethanol Binding to the Lipid–Water Interface of Phospholipid Bilayers

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ABSTRACT: The mechanisms behind the membrane-mediated effects of ethanol were examined via the interaction of ethanol with phospholipid bilayers at hydration levels of 10–12 water molecules per lipid. ^2H and ^{31}P nuclear magnetic resonance (NMR) spectroscopy was used to monitor deuterated water and ethanol and the headgroups and acyl chains of neutral phospholipids. Ethanol was found to interact strongly with both phosphatidylcholine (PC) and phosphatidylethanolamine (PE) bilayers, giving ^2H NMR quadrupolar splittings for CH_3CD_2OH between 6.3 and 9.4 kHz. The quadrupolar splittings for ethanol in gel-phase lipids remained well resolved and were not significantly larger than those in the L_α phase, suggesting that little or no ethanol was bound in the hydrocarbon interior of the bilayer. Ethanol binding significantly altered the orientation of the lipid headgroups, as shown with headgroup-deuterated PC bilayers. The entire lengths of the acyl chains were significantly disordered by the ethanol interaction, evidenced by significant reductions in the ^2H NMR order parameters of the chains. The disordering corresponds to an increase in the area per lipid by an estimated 6% with one ethanol molecule per lipid, and a total of 18% with a second ethanol per lipid. This pronounced area increase is presumably caused by the disruption of lipid packing in the rigid region of the glycerol backbone rather than in the acyl chains, since the order of hydrocarbon chains is not affected to a significant degree by incorporation of alkanes and long-chain alcohols into the hydrocarbon interior. From these data it was concluded that ethanol interacts with phospholipid bilayers at the lipid–water interface (consisting of the headgroup, glycerol backbone, and uppermost chain methylene groups) rather than in the hydrocarbon interior. An interfacial binding of ethanol that is also capable of disordering the entire length of the acyl chains could explain the small ethanol-induced fluidization of membrane lipids that has been reported frequently in the literature.

A large body of evidence exists to suggest that the acute and chronic effects of ethanol involve the interaction of ethanol with membrane processes, but the specific mechanisms of membrane-mediated action are unknown. This question and the theories proposed to answer it have been the subject of a number of reviews (Taraschi & Rubin, 1985; Hoek et al., 1988; Tabakoff et al., 1988; Klemm, 1990). One hypothesis is that ethanol acts by binding to membrane proteins at specific sites in the same site-saturable manner as ligand–receptor binding interactions (Franks & Lieb, 1987; Covarrubias & Rubin, 1993). There are a number of examples in the literature of altered protein function at physiological concentrations of ethanol. For instance, ethanol affects the phospholipid-dependent signal transduction mechanisms which control the activities of phospholipases C and D (Hoek & Rubin, 1990). The function of many proteins in central nervous system membranes have been shown with patch–clamp experiments to be modified by ethanol, including γ-aminobutyric acid- and *N*-methyl-D-aspartate-activated ion channels (Lovinger et al., 1989; Aguayo, 1990; White et al., 1990), nicotinic acetylcholine receptors (Bradley et al., 1980), the serotonin-activated 5-hydroxytryptamine$_3$ channel (Lovinger, 1991; Lovinger & White, 1991), and ATP-gated ion channels (Weight, 1992). One drawback of the site-specific protein hypothesis is that ethanol appears to act in a nonspecific manner: it affects the function of many different proteins and produces similar acute changes both *in vitro* and *in vivo* in many different cells and organs. Also, the doses of ethanol required for action are higher than those expected for receptor binding.

The apparent lack of specificity in the action of ethanol suggested the possibility that a less discriminating interaction may occur between ethanol and membranes in general. A nonspecific interaction is consistent with the characteristics of ethanol intoxication in that large numbers of ethanol molecules are required to produce an effect, and increasing numbers increase the effect over the physiologically relevant range. Such an interaction potentially could involve either the entire membrane (both the hydrocarbon interior and the membrane–water interface), or it may be limited primarily to just one of these regions. It is well established that ethanol decreases the order in biological membranes and model lipid bilayers (Chin & Goldstein, 1977; Harris et al., 1984; Polokoff et al., 1985; Johnson et al., 1992). These observations lead to the hypothesis that ethanol disorders the lipid chains by partitioning between them in the hydrocarbon interior, which in turn could affect membrane protein function. However, the disordering produced by intoxicating doses of ethanol is equivalent to only a 1 °C or 2 °C change in temperature (Franks & Lieb, 1987), leading to questions as to whether increased bulk fluidity alone could be responsible for the action of ethanol.

The membrane–water interface is also a potential site for nonspecific interactions with ethanol. Ethanol is a small, polar molecule with a hydroxy group capable of hydrogen bonding with polar groups at the membrane–water interface such as phosphates, carbonyls, carboxyls, and basic amino groups. At the same time, the hydrophobic ethyl group can interact with

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hydrophobic regions of macromolecules. There is some evidence in the literature to suggest that ethanol binds to molecular groups in the membrane–water interface region. One of the most compelling is the observation that ethanol increases the phase transition temperature (T_h)[1] from the L_α phase to the inverted hexagonal (H_{II}) phase (Hornby & Cullis, 1981; Veiro et al., 1989). Also, a radio tracer study measuring the partitioning of ethanol between a membrane pellet and aqueous supernatant found that mouse brain synaptosomes had a partition coefficient 60 times higher than that for L_α phase dipalmitoylphosphatidylcholine (DPPC) bilayers (Sarasua et al., 1989), suggesting that in synaptosomes ethanol may be able to bind to a variety of polar groups, including those of proteins and carbohydrate groups. Fourier transform infrared (FTIR) studies of inverted micelles of DPPC and gangliosides (water in carbon tetrachloride) suggest that ethanol displaces some bound water at the micellar surface (Chiou et al., 1992; Yurttas et al., 1992). Data by Klemm and co-workers [reviewed in Klemm (1990)] suggest that the acute effects of ethanol in the brain could be mediated by changes in the hydrogen or electrostatic bonding between gangliosidic sialic acid and receptor proteins, due to the replacement of water with ethanol. Slater et al. (1993) hypothesized that ethanol reduces lipid–lipid interactions by binding in the lipid–water interface, based on the faster desorption of phospholipid fluorescent probes from small unilamellar vesicles in the presence of 200 mM ethanol. Finally, the interdigitated gel phase induced by high concentrations of ethanol ($\sim$1 M) is presumably brought about by the binding of ethanol molecules in the interfacial region of lipid bilayers (Simon & McIntosh, 1984; Nambi et al., 1988).

What has not yet been provided in support of the interface interaction hypothesis is direct evidence for either a binding interaction between ethanol and lipid bilayers or the location of this interaction on or within the bilayer. The goal of this work was to demonstrate directly a lipid–ethanol interaction and to identify whether the region of interaction is in the hydrocarbon interior, at the lipid–water interface, or both. For purposes of the current work, the term "lipid–water interface" encompasses the region of the bilayer containing and strongly affected by water and polar lipid groups. This includes the phosphate headgroup, glycerol backbone, and the uppermost methylene groups of the acyl chains. The term "hydrocarbon interior" used here refers to the solely hydrophobic portion of the bilayer.

We have investigated phospholipid bilayers at low hydration levels (10–12 water molecules per lipid) in the presence of ethanol, using ^2H and ^{31}P nuclear magnetic resonance (NMR) spectroscopy to monitor deuterated water, ethanol, and the headgroups and acyl chains of phosphatidylcholines (PC) and a phosphatidylethanolamine (PE). It was necessary to work with low amounts of water for two reasons. Firstly, lipids that are either fully hydrated or in excess water form small particles. The small molecules investigated in this work, water and ethanol, diffuse very rapidly over such surfaces, averaging out any quadrupolar splittings that may exist. With lower hydration levels of about 10 water molecules per lipid, however, large, flat bilayers are formed, between which all water in the system lies (Finer & Darke, 1974; Gawrisch et al., 1985). Secondly, the low hydration conditions enhance the interaction between ethanol and lipids by trapping the ethanol between the bilayers because there is no pool of bulk water into which it can partition. Under these conditions, we were able to observe directly that ethanol bound strongly to the lipid bilayer at the lipid–water interface, altered the orientation of the lipid headgroups, and through the interfacial interaction caused significant disordering along the entire length of the hydrocarbon chains.

To maintain our conditions of restricted water, we adjusted the ethanol/lipid ratio rather than the ethanol concentration in water. It is likely that our experimental conditions enhance binding of ethanol to lipids. However, properties of the bilayer such as phase transition temperatures and area per lipid at 10–12 H_2O/lipid are very close to those in fully hydrated bilayers (Chapman et al., 1967; Gawrisch et al., 1985). Therefore, the conclusion that ethanol interacts with phospholipid bilayers in the lipid–water interface region and very little or not at all in the interior of the bilayer will not be altered by the presence of physiological concentrations of water.

EXPERIMENTAL PROCEDURES

The 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), *Escherichia coli* phosphatidylethanolamine (*E. coli* PE), 1,2-perdeuteriodimyristoyl-*sn*-glycero-3-phosphocholine (DMPC-d_{54}), and α,β-methylenecholine-deuterated DMPC (DMPC-d_5) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). (One methyl proton in the choline group of the DMPC-d_5 was also deuterated.) The lipids were dried in an evacuated desiccator over phosphorus pentoxide (P_2O_5) for at least 12 h. The deuterated water (D_2O) and 1,1-ethanol-d_2 (CH_3CD_2OH) were purchased from Cambridge Isotope Labs (Woburn, MA). The nondeuterated solvents were deuterium-depleted H_2O (Isotec, Inc., Miamisburg, OH) and anhydrous ethanol (CH_3CH_2OH, Spectrum Chemical Manufacturing Corp., New Brunswick, NJ).

The following mixtures were investigated in the L_α phase: DOPC/CH_3CD_2OH/H_2O, DOPC/CH_3CH_2OH/D_2O, DMPC-d_5/CH_3CH_2OH/H_2O, DMPC-d_{54}/CH_3CH_2-OH/H_2O, and *E. coli* PE/CH_3CD_2OH/H_2O. Mixtures of DPPC/CH_3CD_2OH/H_2O were examined in the gel phase as well as in the L_α phase. A total of 10 solvent molecules (water plus ethanol) were used in the DOPC and DPPC samples, 12 for all DMPC samples, and 9.6 for the *E. coli* PE mixture. The samples contained 0, 0.2, 1, or 2 ethanol molecules per lipid. In order to avoid additional water uptake by the dried lipids from the air humidity, all sample preparation was performed in an argon-filled glovebag with ultrapure argon (<1 molar ppm H_2O) passed through a tube of $CaSO_4$ before entering the glovebag. The samples were prepared in 5-mm-o.d. Pyrex tubes about 10 mm long attached to a ground glass connector sealed with a ground glass stopper. Typically 25-30 mg of dried lipid was placed in the tube, except for the DMPC-d_5, of which about 6 mg was used. It was assumed that two water molecules per lipid were present before solvent was added. Appropriate amounts of water and ethanol were added to the lipid gravimetrically with microliter syringes. After the tubes were sealed with a Teflon-greased ground glass stopper and parafilm, the samples were mixed by centrifugation. The tubes were placed in O-ring sealed cryovials at room temperature for at least 24 h before the NMR experiment was run.

[1] Abbreviations: CSA, chemical shift anisotropy; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; *E. coli* PE, *Escherichia coli* phosphatidylethanolamine; FID, free induction decay; FTIR, Fourier transform infrared; H_{II}, inverted hexagonal; L_α, lamellar liquid-crystalline; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; T_h, L_α–H_{II} phase transition temperature; T_m, gel–L_α phase transition (or chain-melting) temperature.

Table 1: ^2H and ^{31}P NMR Spectral Parameters for DOPC/Ethanol/Water Mixtures[a]

ethanol/DOPC	water/DOPC	water/ethanol	$\Delta\nu_Q$ D_2O (kHz, ±0.03)	$\Delta\nu_Q$ CH_3CD_2OH (kHz, ±0.07)	CSA (ppm, ±0.3)
0	10.3 ± 0.4	∞	1.25		-43.4
0.18 ± 0.02	9.7 ± 0.3	54 ± 3		9.44	-44.1
1.0 ± 0.1	9.3 ± 0.3	10 ± 1	1.18		-41.3
1.0 ± 0.1	9.1 ± 0.3	9 ± 1		8.05	-42.0
2.0 ± 0.1	8.2 ± 0.2	4.1 ± 0.4	1.06		-39.5
2.0 ± 0.1	8.0 ± 0.2	4.0 ± 0.4		6.81	-39.4

[a] Molar ratios.

Most NMR spectra were acquired on a Bruker MSL 300 spectrometer (Karlsruhe, Germany) at a magnetic field strength of 7 T. A high-power, double-resonance probe was used with a 5-mm solenoid sample coil tuned to 46.07 or 121.51 MHz for ^2H or ^{31}P, respectively, and 300.13 MHz for ^1H NMR. The ^2H NMR experiments were performed using a phase-cycled quadrupolar echo sequence (Davis et al., 1976). L_α phase spectra were typically acquired with a 3.4-μs 90° pulse, a 100 μs pulse separation, a 62.5 kHz sweep width, 8192 data points, and a 500 ms recycle delay. Gel phase DPPC spectra were collected at 22 °C with a 250 kHz sweep width, a 30 μs interpulse delay, and 4096 data points. Proton-decoupled ^{31}P NMR spectra were observed with a Hahn echo pulse sequence with phase cycling (Rance & Byrd, 1983) using a 1.75 μs 90° pulse, a 100 μs delay between the 90° and 180° pulses, a 20 kHz sweep width, 4096 data points, and a 1 s recycle delay. The ^{31}P NMR free induction decays (FIDs) typically consisted of between 2000 and 3500 scans, with a 100 Hz line broadening applied before Fourier transformation. ^2H NMR spectra for the DMPC-d_{54} and DMPC-d_5 samples were acquired on a Bruker AMX 360 NMR spectrometer operating at 55.3 MHz with a phase-cycled quadrupolar echo pulse sequence. The pulse parameters used were a 5 μs pulse width, a 500 kHz sweep width, a 36 μs delay between pulses, and a recycle delay of 500 ms; 4096 data points were collected, with 100 Hz line broadening applied to the DMPC-d_{54} FIDs and 10 Hz to DMPC-d_5. For all spectra, the sample temperature was controlled to ±0.1 °C with the spectrometer temperature control unit, and the actual temperature was determined using a digital thermometer with the probe placed immediately above the sample.

^2H NMR spectra of deuterated lipids were transferred to an IBM-compatible personal computer, where they were dePaked using the procedure of Bloom et al. (1981). The dePaked spectra were then integrated and the positions of the integrated peaks used to calculate the quadrupolar splittings. The carbon–deuterium bond order parameter, $|S_{CD}|$, was calculated from the relation (Davis, 1983):

$$(\Delta\nu_Q)_\perp = {}^3/_4\left(\frac{e^2qQ}{h}\right)|S_{CD}| \qquad (1)$$

where $(\Delta\nu_Q)_\perp$ is the quadrupolar splitting between peaks due to $\theta = 90°$ orientation of the lipids with respect to the magnetic field and (e^2qQ/h) is the static quadrupolar coupling constant, equal to ~168 kHz for a methylene segment (Burnett & Müller, 1971).

RESULTS

Ethanol Binding. Table 1 summarizes the ^2H and ^{31}P NMR results for deuterated ethanol and water interacting with DOPC bilayers in the presence of varying amounts of ethanol. Under conditions of low hydration (10–12 water molecules per lipid), deuterated ethanol interacted strongly with DOPC to produce a large quadrupolar splitting, the magnitude of which decreased as the amount of ethanol per lipid was increased (cf. Figure 1). The absence of an isotropic ^2H NMR peak due to free ethanol indicates either that no unbound ethanol exists or that it is in fast exchange with the bound ethanol. Increasing the water content caused the quadrupolar splittings to decrease and the line shapes to broaden, making comparisons between samples difficult. When excess water was added above one of the samples and the sample was allowed to equilibrate undisturbed for 1 week, most of the ethanol diffused into the bulk water phase, resulting in a ^2H NMR spectrum that consisted of a single isotropic signal.

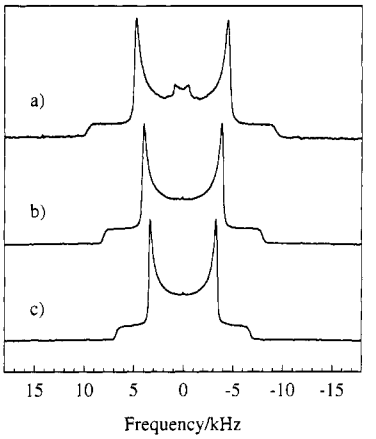

FIGURE 1: ^2H NMR spectra of deuterated ethanol (CH_3CD_2OH) mixed with L_α phase DOPC and water at 22 °C. Ten solvent molecules (water plus ethanol) are present per lipid, and the molar CH_3CD_2OH/DOPC ratios are (a) 0.2, (b) 1.0, and (c) 2.0. A small signal from D_2O is present in spectrum a. The quadrupolar splittings (given in Table 1) are similar in magnitude to those from the methylene groups in phosphocholine (Figure 4), indicating that ethanol binds strongly to the lipid bilayer.

To compare the interaction of ethanol with the gel and L_α phases, ^2H NMR spectra were acquired on two DPPC/CH_3CD_2OH/H_2O mixtures at 22 °C and 52 °C. The ethanol content of the samples was 0.85 and 2.0 ethanol molecules per lipid; spectra for the sample with the lower ethanol content are shown in Figure 2. Comparable to the DOPC samples, large L_α phase $\Delta\nu_Q$ values of 7.20 and 6.33 kHz were observed for 1 and 2 ethanol/DPPC, respectively. In the gel phase, the line shapes were equally well resolved, with slightly larger values for $\Delta\nu_Q$ of 7.79 and 7.99 kHz, respectively.

To examine the interaction of ethanol with phosphatidylethanolamines, *E. coli* PE was chosen because it is in the L_α phase at room temperature. In an *E. coli* PE mixture with 0.9 CH_3CD_2OH molecules per lipid, a $\Delta\nu_Q$ value of 7.20 kHz was observed (data not shown), demonstrating that a significant binding interaction also occurs between ethanol and PE bilayers.

Influence of Ethanol on Water of Hydration. In the absence of ethanol, a quadrupolar splitting of 1.25 kHz for D_2O bound to the lipid bilayer was observed (Figure 3 and Table 1), as reported previously for similar systems (Finer & Darke, 1974;

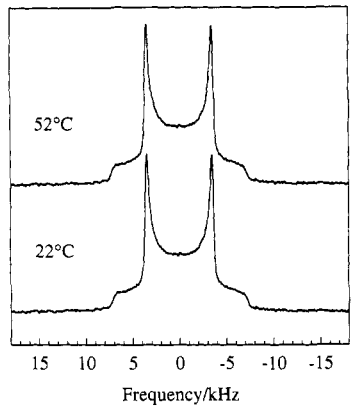

FIGURE 2: ^2H NMR spectra of CH_3CD_2OH in mixtures of 10:0.85:1 H_2O:CH_3CD_2OH:DPPC (molar), in L_α phase (52 °C) and gel phase (22 °C) DPPC. The fact that the gel phase spectrum is nearly identical to that of the L_α phase indicates that ethanol was not located to a detectable degree among the acyl chains (see text).

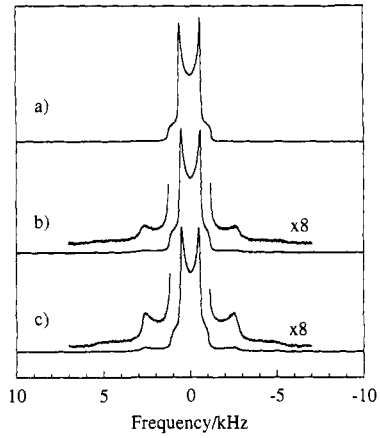

FIGURE 3: ^2H NMR spectra deuterated water mixed with DOPC and ethanol at 22 °C, with a total of 10 solvent molecules per lipid. The molar ratios of ethanol per lipid are (a) 0, (b) 1.0, and (c) 2.0. The spectra from samples with ethanol contain an additional low-intensity signal due to CH_3CH_2OD, from exchange between D_2O and CH_3CH_2OH. Quadropolar splittings for bound D_2O are given in Table 1.

Gawrisch et al., 1985). Evidence for the exchange of the alcohol proton with D_2O can be seen in Figure 3 as additional low-intensity powder patterns with $\Delta\nu_Q \sim 5.5$ kHz due to bound CH_3CH_2OD. The small $\Delta\nu_Q$ for D_2O is believed to be due to rapid rotation of the bound molecule about its C_{2v} symmetry axis, causing extensive averaging of the quadropolar interaction (Gawrisch et al., 1992). As with bound ethanol, the $\Delta\nu_Q$ of bound D_2O also decreased with increasing amounts of ethanol.

Influence of Ethanol on the Headgroup Region. The chemical shift anisotropies (CSA) of the DOPC ^{31}P NMR spectra show a significant decrease with increasing quantities of ethanol (Table 1), evidence for either a change in headgroup conformation and/or an increase in headgroup mobility (Smith & Ekiel, 1984). (The small variations in CSA between samples with similar ethanol content are largely due to experimental variations in the ratio of water to DOPC). Additional information on the effects of ethanol on the headgroups was obtained from ^2H NMR spectra of headgroup-deuterated DMPC (DMPC-d_5) (Figure 4). The quadrupolar splittings from deuterated choline methylene groups have been shown to be very sensitive to changes in the orientation of the headgroup [for example, see Scherer and Seelig (1989)]. The splittings cannot describe specifically the orientation, but any change in a splitting indicates that the headgroup conformation

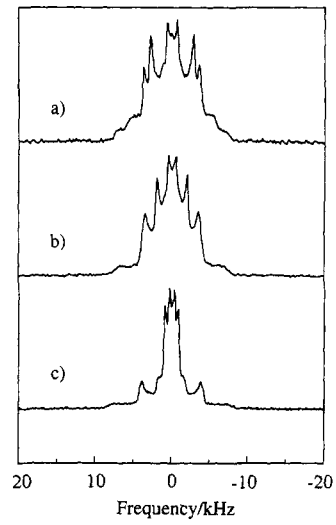

FIGURE 4: ^2H NMR spectra of headgroup-deuterated DMPC (DMPC-d_5) in the L_α phase (30 °C), showing the influence of (a) 0, (b) 1.0, and (c) 2.0 ethanol molecules per lipid. Each sample contained 12 solvent molecules per lipid. The peak assignments (Scherer & Seelig, 1989) go as $\Delta\nu_Q(\alpha) > \Delta\nu_Q(\beta) > \Delta\nu_Q$(methyl); only one choline methyl proton is deuterated.

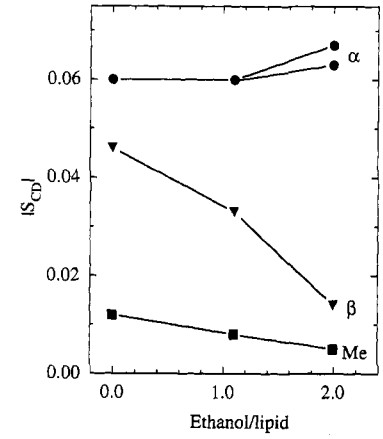

FIGURE 5: Change in the carbon–deuterium bond order parameter, S_{CD}, as a function of ethanol content in headgroup-deuterated DMPC (DMPC-d_5). The values for S_{CD} were calculated from the dePaked spectra according to eq 1 as described in the text; errors in S_{CD} are of the same order as the symbol size. The ethanol-induced changes in the quadrupolar splittings demonstrate that ethanol binding has altered the conformation of the phosphocholine headgroup. Note that the order parameters of the two α-methylene protons are not equal in the presence of ethanol.

has been altered. The changes in splittings and order parameters for DMPC-d_5, shown in Figures 4 and 5, demonstrate that ethanol binding alters the orientation of the headgroup. Note that because quadrupolar splittings depend on the orientation of the deuterium-containing bond with respect to the magnetic field, the particularly pronounced effect of ethanol binding on the $\Delta\nu_Q$ for the β-methylene position does not necessarily reflect a greater change in conformation for this segment over those for the α-methylene and methyl segments.

Influence of Ethanol on the Acyl Chains. The widths of the ^2H NMR spectra of chain-perdeuterated DMPC (DMPC-d_{54}) in Figure 6 are substantially reduced as a result of ethanol binding. The carbon–deuterium bond order parameters calculated from the dePaked spectra are plotted in Figure 7, which shows that the entire length of the chains experienced a decrease in order, for both 1 and 2 ethanol molecules per lipid. Reduced order for hydrocarbon chains in lipid bilayers

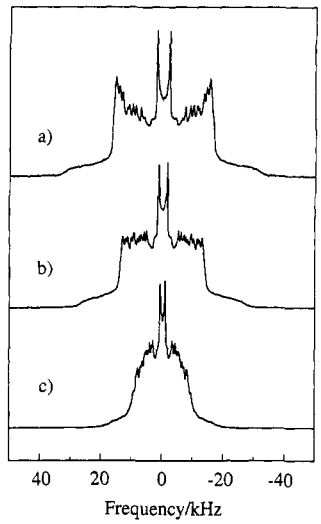

FIGURE 6: Influence of ethanol on the hydrocarbon chains of L_α phase DMPC (30 °C), as shown by ^2H NMR spectra of DMPC-d_{54} with (a) 0, (b) 1.0, and (c) 2.0 CH_3CH_2OH/lipid.

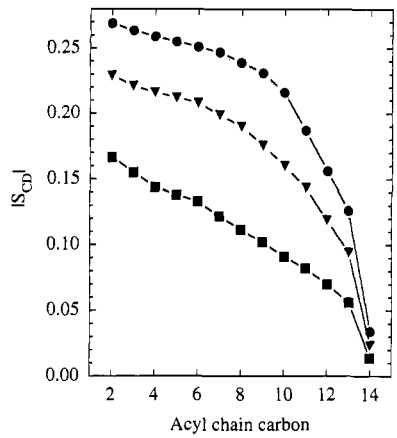

FIGURE 7: Order parameter profiles for the hydrocarbon chains of DMPC-d_{54}, showing the effects of ethanol on acyl chain order for no ethanol (●), 1 ethanol/lipid (▼), and 2 ethanol/lipid (■). The values were calculated from the dePaked spectra of DMPC-d_{54} in Figure 6; errors in the values are of the same order as the symbol size. The pronounced decrease in order corresponds to an increase in the area available to each lipid as a result of ethanol binding. The area increase was estimated to be about 6% with 1 ethanol/DMPC and a total of 18% when 2 ethanol molecules were present per lipid (see text).

corresponds to a shortening of the chains and an increase in the area per lipid (Lafleur et al., 1989).

DISCUSSION

Binding of Ethanol and Water. The large quadrupolar splittings observed for CH_3CD_2OH in DOPC, *E. coli* PE, and DPPC demonstrate that ethanol interacts with phospholipid. Quadrupolar splittings are a function of both the motion of the deuteron and its orientation with respect to the magnetic field. Therefore, it is not possible to separate the effects on $\Delta\nu_Q$ due to strength of binding versus the orientation of the ethanol molecule in the bound state. However, the range of quadrupolar splittings observed for the methylene group of bound CH_3CD_2OH is very similar to that for the choline α- and β-methylene groups. The large splitting for CH_3CD_2OH indicates that a significant binding occurs directly between the ethanol and the lipid. Ethanol could also conceivably interact with the bilayer with only a hydrogen bond to the hydroxy group and the ethyl moiety extending out into the interfacial water. However, the great motional freedom of the ethanol molecule under such conditions would presumably lead to considerable motional averaging of the splitting.

^2H NMR studies on D_2O in lipid–water dispersions have shown that the headgroup of PC in the L_α phase is surrounded by an inner hydration shell of partly ordered water. High-pressure FTIR studies of fully hydrated disaturated PCs and PEs have pinpointed water hydrogen-bound to PO_2^- and to the *sn*-2 C=O group but not to the *sn*-1 C=O (Wong & Mantsch, 1988). It was also shown that water undergoes polar interactions with $(CH_3)_3N^+$. The binding of ethanol at the lipid–water interface may perturb the inner hydration shell around the lipid headgroup, based on the release of bound water observed with FTIR (Chiou et al., 1992; Yurttas et al., 1992) and the decreased $\Delta\nu_Q$ for bound D_2O in the current work. At the same time, however, it is possible that the increase in the area per lipid caused by ethanol binding (discussed below) may lead to an overall *increase* in the amount of partly ordered water associated with the lipid–water interface. The current data neither contradict nor support the hypothesis that binding of ethanol in the membrane–water interface results in a replacement of structured water there.

Ethanol–Lipid Binding Site. The magnitude of the quadrupolar splitting does not determine the location of ethanol on or within the lipid bilayer. However, three lines of evidence taken together indicate that ethanol binds primarily at the lipid–water interface rather than interacting with the hydrocarbon interior.

(a) Effect of Ethanol on Headgroup Orientation. One line of evidence is the significant change in the ^2H NMR quadrupolar splittings for the α- and β-methylene groups of DMPC-d_5 (Figures 4 and 5), clearly showing that ethanol binding changes headgroup conformation. Longer chain *n*-alcohols in DMPC [$CH_3(CH_2)_nOH$, n = 3, 7, 11, 13] have also been observed to alter the orientation of the choline α- and β-CD_2 groups (Westerman et al., 1988). These authors propose that *n*-alcohols are anchored in the bilayer, with the hydroxy group near the lipid–water interface and the alcohol chain intercalated among the lipid chains. Ethanol may differ from the longer chain members of the series only in that the ethyl moiety is too short to extend into the hydrocarbon interior of the bilayer.

(b) Binding of Ethanol to the Gel Phase. Other evidence for an interfacial interaction is that CH_3CD_2OH in gel phase DPPC samples (1 or 2 CH_3CD_2OH molecules per lipid) exhibited very sharp quadrupolar splittings which were not significantly larger than those in the L_α phase. If the ethanol had been residing in the hydrocarbon portion of the L_α phase bilayer, there are two possible fates for ethanol in the gel phase. First, it is possible that most or all of the ethanol would have been squeezed out of the bilayer when the chains were cooled into the more tightly packed gel phase. The result would be either an isotropic NMR signal due to free ethanol or a decreased quadrupolar splitting due to partially excluded ethanol. This exclusion of solute molecules from the gel phase was reported for alkanes and butanol in various PC bilayers (Jacobs & White, 1984a,b; Pope & Dubro, 1986; Gawrisch & Janz, 1991). Second, it is possible that if ethanol were intercalated among the acyl chains, it would remain there and be substantially immobilized, producing a line shape much wider than that in the L_α phase. The ^2H NMR spectra of decanol, octanol, and dodecane in gel phase DPPC or DMPC, for example, were extensively broadened relative to the L_α phase (Pope & Dubro, 1986; Thewalt et al., 1986). The absence in the gel phase of an isotropic signal, a reduced splitting, or a broadened signal demonstrates that little if any

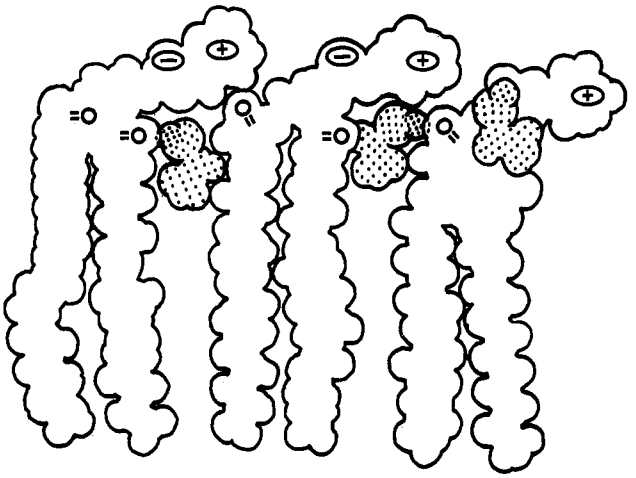

FIGURE 8: Schematic drawing of a phosphatidylcholine bilayer with ethanol bound in the region of the lipid–water interface. The ethanol molecules are denoted by stippling, and the double-bonded oxygen atoms denote the carbonyl groups of the lipid glycerol backbone. For sake of illustration, binding is shown here as a hydrophobic interaction of the ethyl moiety in the vicinity of the glycerol backbone (and just below) and a hydrophilic (presumably hydrogen-bonding) interaction between the hydroxyl group and a carbonyl or phosphate oxygen.

ethanol is located in the hydrocarbon interior of the bilayer. The results, however, do not preclude the possiblity that small amounts of ethanol may interact with the hydrocarbon interior.

(c) Effect of Ethanol Binding on Hydrocarbon Interior. The third line of evidence for an interfacial interaction is that ethanol binding induced a marked disordering of the acyl chains, most likely resulting from an increased area per lipid. Using the method of Nagle (1993), the increase in area was estimated to be 6.3% with one ethanol molecule per lipid, and a total of over 18% with the second ethanol per lipid. Such a pronounced increase in area would almost certainly arise from the intercalation of ethanol between lipids in the tightly packed glycerol backbone region of the lipid–water interface. It has been shown with ^2H NMR order profiles of L_α phase bilayers that the incorporation of molecules into the hydrocarbon interior of the bilayer has very little or no effect on bilayer order. This has been observed over a wide range of molecular weights for both alkanes and alcohols, including 25 mol % of the longer chain alcohols octanol and decanol in DPPC (Thewalt et al., 1985), 26 mol % of 1-hexane in DMPC (Jacobs & White, 1984a), 50 mol % of 1-hexane in DOPC (Jacobs & White, 1984b), and 5 mol % in DPPC of pristane, a bulky 19-carbon alkane (Gawrisch & Janz, 1991). The ability of the hydrocarbon region to accommodate these molecules comes from the high degree of flexibility in the acyl chains. In the vicinity of the glycerol backbone, however, lipid packing is at its tightest, and any incorporation of molecules into this region should disrupt lipid packing much more than incorporation into the chains. Figure 8 illustrates an example of the sort of interaction that may occur between ethanol and the lipid–water interface, in which the hydrophobic ethyl moiety intercalates into the less hydrophilic region near the glycerol backbone and the hydroxy group forms a hydrogen bond with a carbonyl or phosphate oxygen.

An interfacial location for ethanol is also strongly supported by the fact that ethanol stabilizes the lamellar phase relative to the H_{II} phase (Hornby & Cullis, 1981; Veiro et al., 1989). The H_{II} phase is favored relative to the L_α phase by smaller headgroup area and/or greater chain volume [see Figure 16 of Cullis et al. (1985)]. Therefore, the increase in the L_α-to-H_{II} transition temperature supports a location for ethanol in the lipid–water interface that forces the lipids apart and creates an increase in the effective area per headgroup. If ethanol was located primarily among the acyl chains, the H_{II} phase would have been *promoted* by the resulting increase in chain volume, as observed for longer chain alcohols (≥C6) (Hornby & Cullis, 1981). Like ethanol, the short-chain alcohols methanol, propanol, and butanol also increase the L_α-to-H_{II} phase transition temperature (Hornby & Cullis, 1981; Veiro et al., 1989). Interfacial binding is also supported by the observation that fully hydrated gel phase PCs become *interdigitated* in the presence of high concentrations of ethanol, presumably due to the binding of ethanol between lipid headgroups (Simon & McIntosh, 1984; Nambi et al., 1988). We have not investigated gel phase structure in the current study, so it is not known if gel phase DPPC is interdigitated at reduced levels of hydration.

CONCLUSIONS

The interaction of ethanol with phospholipid bilayers was investigated with ^2H and ^{31}P NMR at low hydration levels (10–12 waters/lipid). A significant interaction between ethanol and lipid was demonstrated by large quadrupolar splittings for deuterated ethanol. There was little or no ethanol binding in the hydrocarbon interior of the bilayer, based on several lines of evidence. Gel phase spectra of bound CH_3-CD_2OH were very similar to the corresponding L_α phase spectra, which previous studies (see Discussion) have shown is not the case for longer chain alkanols and alkanes residing among L_α phase chains. Also, the binding of ethanol significantly disordered the entire length of the hydrocarbon chains. This effect is most likely caused by the intercalation of ethanol into the tightly packed glycerol backbone region, increasing the spacing between lipids and therefore the area available to each lipid molecule. It was shown by earlier studies on a variety of molecules that incorporation of molecules into the hydrocarbon interior has no significant effect on bilayer order (Jacobs & White, 1984a; Thewalt et al., 1985; Gawrisch & Janz, 1991). Interfacial ethanol binding is also supported by past evidence that the addition of ethanol raises the lipid transition temperature from the L_α to the H_{II} phase (Hornby & Cullis, 1981; Veiro et al., 1989). Lastly, binding caused significant alterations in the orientation of the lipid headgroups, as determined by ^2H NMR of deuterated headgroups. Based on the combined data from the current and previous work, it was concluded that ethanol binds strongly to the bilayers in the region of the lipid–water interface with little or no intercalation into the hydrocarbon interior. Although there was little if any direct interaction of ethanol with the acyl chains in their entire length, the interfacial binding described here could explain the small ethanol-induced disordering of biological and model membranes reported in the literature.

We anticipate that the lipid systems studied here can be used as a model for similar interactions in the hydrophilic–hydrophobic interfaces of all biological membrane components, including lipids, carbohydrates, and proteins. Note that lipid–protein interfaces, receptor surfaces, and internal pockets of water in proteins are also potential sites for ethanol interactions that could alter membrane function. The marked influence of interfacial interactions on chain order, together with the fact that membrane lipids are known to participate in the function of a number of membrane proteins, reminds us that the membrane structure is balanced by a variety of interactions. Therefore, we would expect that perturbations can be easily transferred from the membrane–water interface to both the membrane interior and the protein function. We suggest that

the altered biophysical properties of hydrophobic–hydrophilic interfaces as a result of ethanol binding may be associated with the acute and chronic effects of ethanol on membrane-bound processes.

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