

# Determining Ethanol Distribution in Phospholipid Multilayers with MAS–NOESY Spectra

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**ABSTRACT:** The location of an ethanol molecule within a membrane, an issue of considerable controversy, was investigated directly by NMR with two-dimensional NOESY. Lipid and ethanol <sup>1</sup>H NMR resonances of multilamellar liposomes were resolved by magic-angle spinning (MAS). We observed strong proton lipid–ethanol crosspeaks in dispersions of saturated dimyristoylphosphatidylcholine and monounsaturated stearyl-oleoylphosphatidylcholine and in polyunsaturated stearyl-docosahexaenoylphosphatidylcholine. Crosspeak intensity has been interpreted in terms of an ethanol distribution function over the lipid bilayer. Ethanol resides with the highest probability at the lipid water interface near the lipid glycerol backbone and upper methylene segments of lipid hydrocarbon chains. Chain unsaturation has only a minor influence on the ethanol distribution function. In all cases, the ethanol concentration in the bilayer core is significantly lower. At ambient temperature all lipid–ethanol crosspeaks are positive. Crosspeak intensity decreases with increasing water content and increasing temperature most likely because of shorter correlation times of lipid and ethanol reorientation. This suggests a lifetime for specific lipid–ethanol contacts of about 1 ns. Lipid–ethanol and lipid–lipid crosspeaks reflect the high degree of motional disorder of lipids and incorporated ethanol in membranes and the rather arbitrary nature of the location of the lipid–water interface.

The partitioning of ethanol into biological and model membranes at physiological and higher ethanol concentrations has been established (Katz & Diamond, 1974; McCreery & Hunt, 1978; Kreishman et al., 1985; Sarasua et al., 1989). It is not agreed upon *where* ethanol locates within a membrane or *how* its presence instigates an anesthetic effect in acute doses and more deleterious and complicated effects in chronic doses. Since the location of ethanol within a membrane may ultimately offer insight to its mechanism of action, this question has been the focus of many studies. *In vitro* addition of alcohol to biological or model membranes causes a disordering of lipid hydrocarbon chains (Chin & Goldstein, 1977; Schroeder et al., 1988; Schueler et al., 1989) and shifts the phase transition between liquid-crystalline and gel (Rowe, 1982, 1983). Since these properties are mainly associated with packing of the acyl chains, it became widely accepted that ethanol (and anesthetics) locates in the membrane hydrophobic core.

When specific regions within a membrane were considered, it became clear that the lipid–water interface would be a more likely location for ethanol and the membrane surface became the target for discussions about ethanol location. Indeed, a growing body of evidence suggests a nonspecific binding of ethanol to the hydrophobic–hydrophilic interface of biological membranes (Vanderkooi, 1979; Harris et al., 1984; Klemm & Williams, 1996). The most direct evidence of interfacial binding is from Barry and Gawrisch (1994) that showed only a small change in the quadrupolar splitting of ethanol in dipalmitoylphosphatidylcholine membranes between the gel and liquid-crystalline phases. Gangliosides, which locate in the outer leaflet of

synaptosomes, enhance the surface binding of ethanol in model and synaptosomal membranes, presumably caused by the hydrophilic sugar residues located in the ganglioside headgroup (Harris et al., 1984; Sarasua et al., 1989; Schueler et al., 1989; Barry & Gawrisch, 1995).

The amphiphilic nature of the ethanol molecule makes interfacial binding seem probable, but evidence for an interior location for ethanol has persisted. An NMR<sup>1</sup> study by Kreishman et al. (1985) suggests that two distinct membrane regions (membrane surface and bilayer inner core) may exist for ethanol interaction. Data obtained with [<sup>14</sup>C]ethanol that are consistent with this model suggest that ethanol can undergo both bulk phase hydrophobic partitioning into the bilayer core and nonspecific binding to the bilayer surface, with surface binding indicated at higher ethanol concentrations (Sarasua et al., 1989). Hitzemann et al. (1986) observed that above 24 °C ethanol concentrations of 0.1 and 0.2% (v/v) *ordered* the membrane surface but that higher concentrations of 0.4 and 1.0% ethanol were needed before the membrane interior was affected and that effect was one of *disorder*. A model was proposed in which ethanol located in both regions.

Location of ethanol in membranes may also depend on lipid species, in particular, on acyl chain unsaturation. Colles et al. (1995) suggest that the penetration depth of water and ethanol into the membrane hydrophobic core could be modulated by lipid chain unsaturation.

<sup>1</sup> Abbreviations: NMR, nuclear magnetic resonance; MAS, magic-angle spinning; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; COSY, correlated spectroscopy; DMPC-*d*<sub>27</sub>, 1-myristoyl-*d*<sub>27</sub>-2-myristoyl-*sn*-glycero-3-phosphocholine; SOPC-*d*<sub>35</sub>, 1-stearoyl-*d*<sub>35</sub>-2-oleoyl-*sn*-glycero-3-phosphocholine; SDPC-*d*<sub>35</sub>, 1-stearoyl-*d*<sub>35</sub>-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

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Many of the conclusions regarding the location of ethanol drawn by previous studies were based on indirect measurements involving the use of probe molecules or ethanol-induced changes in lipid order or phase transition. Alternatively, determinations of ethanol location have relied heavily on interpretation of order parameters and motion of ethanol in a particular environment. No spacial information for ethanol location within membranes exists. We have chosen solid-state NMR to investigate the location of ethanol in model, saturated and unsaturated, phosphatidylcholine membranes using two-dimensional NOESY. This method can be used to estimate distances in space, or close approach, between pairs of protons that are separated by a distance of 2–5 Å (Jeener et al., 1979; Wagner & Wüthrich, 1982). NOESY experiments have been used to investigate location of anesthetics and hydrophobic ions in sonicated liposomes (Ellena et al., 1987; Yokono et al., 1989; Peng et al., 1995). Partitioning of molecules into sonicated liposomes may be perturbed by their high curvature. In the present study we prepared large multilamellar liposomes and used magic-angle spinning (MAS) to achieve high-resolution spectra, a technique similar to that used by Volke and Pampel (1995) to study lipid–water interaction. Our results on lipid–ethanol interaction indicate that the highest ethanol density occurs in the lipid glycerol backbone region and the upper parts of the acyl chains with progressively lower density in either direction outward from the backbone toward the bilayer interior or the outer regions of the headgroup.

## EXPERIMENTAL PROCEDURES

**Sample Preparation.** 1-Myristoyl-*d*<sub>27</sub>-2-myristoyl-*sn*-glycero-3-phosphocholine (DMPC-*d*<sub>27</sub>), 1-stearoyl-*d*<sub>35</sub>-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC-*d*<sub>35</sub>), and 1-stearoyl-*d*<sub>35</sub>-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (SDPC-*d*<sub>35</sub>) were obtained from Avanti Polar Lipids, Alabaster, AL. DMPC-*d*<sub>27</sub> was received as a dry powder and SOPC-*d*<sub>35</sub> and SDPC-*d*<sub>35</sub> in methylene chloride with the antioxidant BHT added. Lipid samples containing unsaturation were transferred from sealed ampules to 3 mL glass tubes, and the organic solvent was removed with a stream of argon. The lipid film was redispersed in 1 mL of degassed cyclohexane, frozen with dry ice, and subjected to a vacuum of 50 μm of Hg overnight. When lyophilization was complete, the vacuum manifold was back-filled with argon and the sample removed and transferred to an argon-filled glovebox (Manostat, New York, NY). The weight of the dry lipid, added to a preweighed sample tube, was determined gravimetrically. Multilamellar vesicles were prepared by adding a mixture of ethanol in D<sub>2</sub>O to the dry lipid. Ethanol concentration was 1 ethanol/lipid or 0.1 ethanol/lipid, and water (D<sub>2</sub>O) concentration was 6 waters/lipid or 12 waters/lipid. Before acquiring data, the sample was spun at 1 kHz in the MAS rotor for 30 min at a temperature that was 10–20 °C above the phase transition to ensure complete mixing of the lipid and solvent. Only the liquid-crystalline phase was investigated.

**NMR Measurements.** NMR experiments were performed at 500.13 MHz on a Bruker DMX500 widebore spectrometer. Sample spinning at 6 kHz was accomplished in a Bruker double gas bearing MAS probehead for 4 mm rotors. Nitrogen gas or dry, compressed air was passed through a cooling bath to maintain the bearing air supply at 25 °C for DMPC-*d*<sub>27</sub>, or at 11 °C for SOPC-*d*<sub>35</sub> and SDPC-*d*<sub>35</sub>. Inserts made of Kel-F containing an 8 μL internal volume were used

to keep the samples well-centered within the 4 mm zirconia MAS rotors.

Two-dimensional NOESY was carried out in the phase-sensitive mode. The pulse sequence (90°–*t*<sub>1</sub>–90°–*τ*<sub>m</sub>–90°–acquire [*t*<sub>2</sub>])<sub>*n*</sub> was employed with 512 *t*<sub>1</sub> values, mixing times (*τ*<sub>m</sub>) of 50–1000 ms, and *n* = 8. A 6.2 μs 90° pulse and 6.7 kHz spectral width were used. No provisions were made for locking the field frequency. Data were analyzed with XWIN-NMR software (Bruker Instruments, Billerica, MA).

Crosspeak intensities in two-dimensional spectra were calculated with AURELIA software (Bruker Instruments, Billerica, MA) by a process of iterative segmentation. The segmentation starts at the top of each peak and advances down recursively until data points of other peaks or the baseline level are reached. To correct for the number of lipid protons contributing to the intensity of a lipid–ethanol crosspeak, the crosspeak volume was divided by the diagonal peak volume of the corresponding lipid protons. These ratios were normalized by dividing the individual ratio by the sum of all ratios.

## RESULTS

**Spectra and Assignments.** Obtaining well-resolved <sup>1</sup>H NMR MAS spectra of multilamellar liposomes is facilitated by the rapid axial diffusion of the entire lipid molecule and high-frequency *gauche-trans* isomerization of fatty acid chains. The one-dimensional proton NMR spectrum of the phosphatidylcholines DMPC-*d*<sub>35</sub>, SOPC-*d*<sub>35</sub>, and SDPC-*d*<sub>35</sub> with 1:1 mol/mol ethanol is shown in Figure 1. Chemical shift assignments are given in Table 1. The assignments are based on reported chemical shifts together with results from two-dimensional correlated NMR spectroscopy (COSY) experiments which we performed on these lipids. In fully protonated lipids, a strong peak appears at 1.29 ppm that is due to the acyl chain methylenes, (CH<sub>2</sub>)<sub>*n*</sub>. The perdeuterated *sn*-1 chains present in all lipids used in this investigation attenuate this peak, allowing better resolution of the methyl peak in ethanol as well as reducing the dynamic range of peak intensities. Notable peaks are those labeled e and f from Figure 1, which are due to protons on the first methylenes from *both* chains. In the *sn*-2 chain, these protons (f) are inequivalent and separate resonances are observed as has been reported previously (Hauser et al., 1976). The intensity of the peak appearing at 2.29 ppm indicates that some exchange of deuterons for protons in the first methylene of the perdeuterated *sn*-1 chain (e) has occurred, likely during lipid synthesis. No other unexpected resonances from the deuterated *sn*-1 chain occur and assignment of the remaining lipid proton resonances is straightforward as listed in Table 1.

Two-dimensional NOESY spectra for the lipid–ethanol dispersions were collected at several mixing times. NOESY spectra of SOPC-*d*<sub>35</sub>/ethanol (1:1 mol/mol) and 6 D<sub>2</sub>O/per lipid at a mixing time of 300 ms are shown in Figure 2. The assignments for the diagonal peaks are the same as in Figure 1 and Table 1. The crosspeaks in the F2 dimension outlined with a box are intermolecular, occurring between lipid and the methyl group of ethanol. The methylene peak of ethanol is broader and has less intensity due to one fewer proton compared to the methyl proton peak. This could provide an explanation for the lower intensity of the crosspeaks that occur between lipid and ethanol methylene protons.

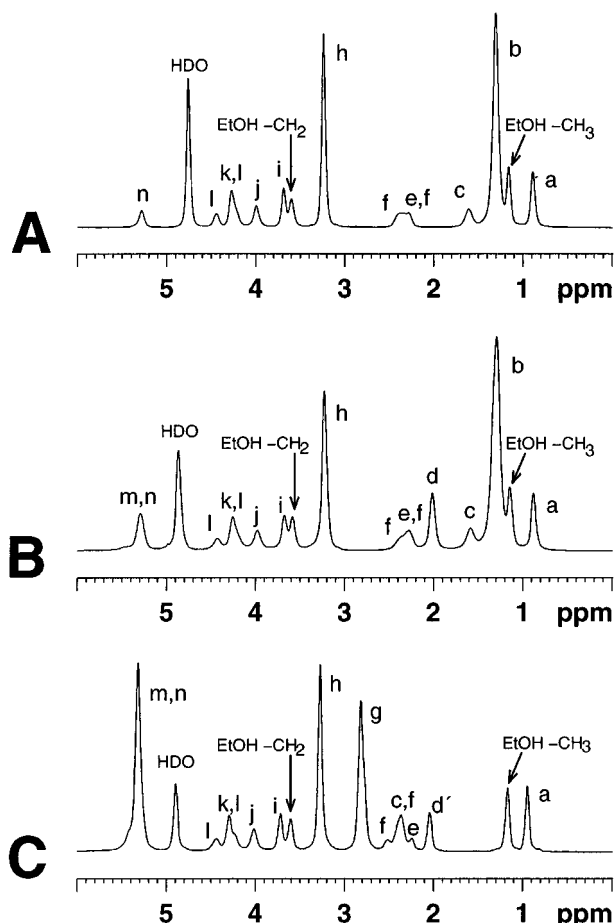


FIGURE 1: 500 MHz  $^1\text{H}$  magic-angle spinning NMR spectra of (A) DMPC- $d_{27}$ /ethanol/ $\text{D}_2\text{O}$  at 25  $^\circ\text{C}$ , (B) SOPC- $d_{35}$ /ethanol/ $\text{D}_2\text{O}$  at 11  $^\circ\text{C}$ , and (C) SDPC- $d_{35}$ /ethanol/ $\text{D}_2\text{O}$  (1:1:6) at 11  $^\circ\text{C}$ . All samples contained 1 mol of ethanol and 6 mol of  $\text{D}_2\text{O}$  per lipid and used a spinning rate of 6 kHz. Lettered peaks are identified in Table 1.

Table 1: Chemical Shift Assignment of the  $^1\text{H}$  Resonances of Multilamellar Phosphatidylcholine Dispersions in  $\text{D}_2\text{O}$

peak id	proton(s)	chemical shift, ppm		
		DMPC- $d_{27}$	SOPC- $d_{35}$	SDPC- $d_{35}$
a	$\text{CH}_3$ ( <i>sn</i> -2 chain)	0.885 <sup>a</sup>	0.885 <sup>a</sup>	0.950 <sup>a</sup>
b	$(\text{CH}_2)_n$ ( <i>sn</i> -2)	$1.30 \pm 0.005^b$	1.30	N/A
c	$\text{CH}_2-\text{CH}_2-\text{CO}$ ( <i>sn</i> -2)	1.60	1.59	2.37
d	$\text{CH}_2-\text{C}=\text{C}-\text{CH}_2$	N/A	2.02	N/A
d'	$\text{CH}_3-\text{CH}_2-\text{C}=\text{C}$	N/A	N/A	2.04
e	$\text{CH}_2-\text{CO}$ ( <i>sn</i> -1)	2.28	2.29	2.25
f	$\text{CH}_2-\text{CO}$ ( <i>sn</i> -2)	2.28	2.29	2.37
		2.37	2.40	2.52
g	$=\text{C}-\text{CH}_2-\text{C}=\text{C}$	N/A	N/A	2.82
h	$\text{N}-(\text{CH}_2)_3$	3.23	3.22	3.28
i	$\text{CH}_2-\text{N}$	3.68	3.68	3.72
j	$\text{CH}_2-\text{OP}$ (glycerol)	3.99	3.99	4.02
k	$\text{PO}-\text{CH}_2$ (choline)	4.27	4.26	4.30
l	$\text{OCO}-\text{CH}_2$ (glycerol)	4.21	4.20	4.24
		4.44	4.43	4.44
m	$\text{HC}=\text{CH}$	N/A	5.30	5.32
n	$\text{OCO}-\text{CH}$ (glycerol)	5.28	5.28 <sup>c</sup>	5.28 <sup>c</sup>

<sup>a</sup> The chemical shift of the *sn*-2 chain terminal methyl group was taken from Frost and Gunstone (1975) and was used to reference the spectrum. <sup>b</sup> An error of  $\pm 0.005$  ppm in the assignment of chemical shift is expected due to limitations in digital resolution. However, larger deviations of  $\pm 0.03$  ppm are not unlikely for protons whose resonances may be shifted under slightly different conditions of hydration. <sup>c</sup> The chemical shift for the proton on the second glycerol carbon was taken from the spectrum of DMPC.

**Water Concentration and Temperature Influence Lipid-Ethanol Crosspeak Intensity.** Mixtures of lipid, ethanol, and  $\text{D}_2\text{O}$  were prepared with molar ratios of 1 or 0.1 ethanol/lipid and 6 or 12 water ( $\text{D}_2\text{O}$ )/lipid. Reduction in ethanol

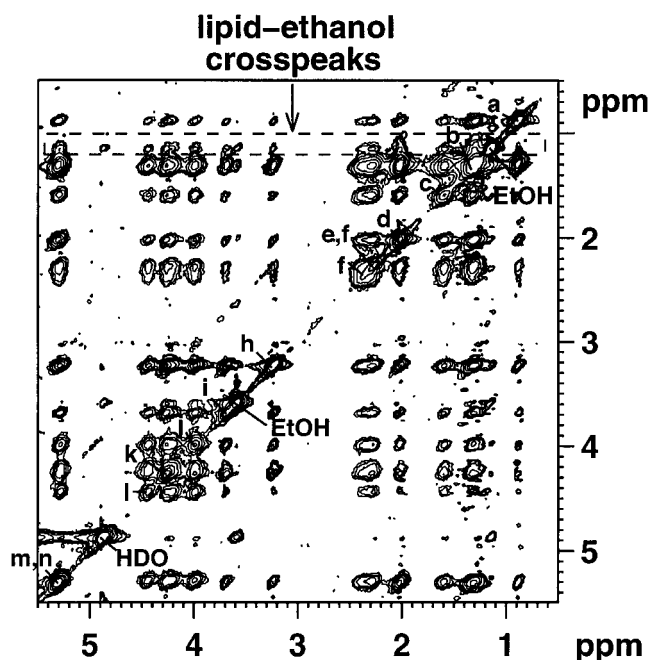


FIGURE 2: NOESY contour plot of SOPC- $d_{35}$ /ethanol/ $\text{D}_2\text{O}$  (1:1:6, mol/mol/mol), obtained at 11  $^\circ\text{C}$ , spinning rate 6 kHz, and  $\tau_M = 300$  ms. Volumes for highlighted crosspeaks in the F2 dimension, between lipid protons and the methyl group protons of ethanol, were calculated and used to determine ethanol distribution in bilayers.

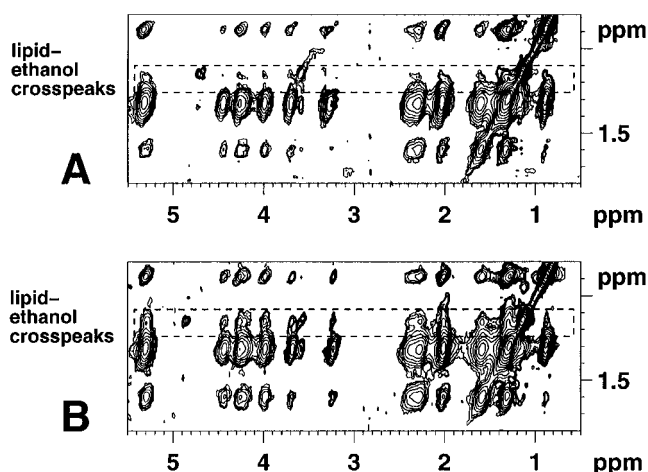


FIGURE 3: NOESY contour plots of lipid-ethanol crosspeaks for SOPC- $d_{35}$ /ethanol/ $\text{D}_2\text{O}$  (1:1:6, mol/mol/mol) at different temperatures: (A)  $T = 30$   $^\circ\text{C}$ , (B)  $T = 11$   $^\circ\text{C}$ . The lipid was liquid-crystalline at both temperatures, but the lower temperature clearly enhances crosspeak intensity.

concentration resulted in a proportional decrease in lipid-ethanol crosspeak intensity. At constant ratio of lipid to ethanol, crosspeak intensity was higher at the lower water concentration.

Crosspeak intensities could be enhanced by reducing the sample temperature. At 11  $^\circ\text{C}$  the intensities of SOPC- $d_{35}$ /ethanol crosspeaks increased compared to 30  $^\circ\text{C}$ , shown in Figure 3, while still maintaining the liquid-crystalline lipid phase. The differences in crosspeak intensities are most likely related to differences in motional correlation times of ethanol and lipids. The best conditions for producing strong crosspeaks were those which increased correlation times, i.e., less water and lower temperatures, leading to the following optimized conditions for the NOESY measurement: lipid:ethanol: $\text{D}_2\text{O}$  ratios of 1:1:6 (mol/mol/mol), and a sample temperature of 11  $^\circ\text{C}$  for unsaturated lipids.



throughout the bilayer suggests that the motions describing lipid-ethanol interactions are not widely different and crosspeak intensities are relatively unbiased with respect to correlation time for this system.

The relay of spin polarization through a network of protons that have strong dipolar couplings, called spin diffusion, can lead to anomalous NOESY crosspeaks. In lipid molecules the acyl chain methylene protons near the glycerol are particularly strongly coupled and magnetization could be transferred along the chain until it reaches the terminal methyl group (Feigenson & Chan, 1974; Xu & Cafiso, 1986; Chen & Stark, 1996). This would present the possibility that the weak crosspeaks we observe between ethanol and protons from the lipid bilayer core have been enhanced by spin diffusion. However, the protons in the lipid molecule that exhibit the strongest crosspeaks with ethanol are in a region where a spin diffusion pathway is interrupted by the phosphate and ester groups. The perdeuteration in the *sn*-1 acyl chain eliminates a possible spin diffusion pathway, and magic-angle spinning also suppresses spin diffusion. Measurements of lipid-ethanol crosspeak intensities as a function of mixing time did not show any signs of anomalous NOE buildup which could be attributed to spin diffusion. NOE mixing times were chosen to be as short as possible, while still allowing discernable lipid-ethanol crosspeaks.

**Lifetime of Lipid-Ethanol Contacts.** We may develop insight to the lifetime of ethanol residence at a specific location from the temperature dependence of the crosspeak intensities with the arguments that follow. NOESY crosspeaks have negative intensities for very short motional correlation times of the vector connecting lipid and ethanol protons. With increasing correlation times the crosspeak intensity approaches zero and then changes to positive. At a resonance frequency of 500 MHz and for isotropic motions, this change in sign occurs at correlation times of 0.5 ns. However, motion of lipid-ethanol complexes is anisotropic, and this has some influence on data analysis. It is probably correct to assume that the small ethanol molecule moves more rapidly around its own axis and up and down the bilayer normal compared to the lipids. Under these conditions, lipid-ethanol interactions become comparable to the complete water proton exchange in and out of a hydration site, investigated by Otting et al. (1991). Their study showed that 600 MHz  $^1\text{H}$  NOESY crosspeaks between solvent and matrix have zero intensity at bound state water lifetimes of 0.5 ns and positive intensities at longer lifetimes. The disappearance of lipid-ethanol crosspeaks with increasing temperature indicates that the system is near lifetimes for which crosspeak intensity is zero. The positive sign of crosspeaks at ambient temperature indicates that our lifetimes are slightly longer than 0.5 ns, resulting in an order of magnitude of 1 ns for specific lipid-ethanol contacts.

## DISCUSSION

**The Nature of the Ethanol Binding Site Is Amphiphilic.** The strong crosspeaks in NOESY spectra between ethanol and the lipid glycerol backbone and upper parts of the acyl chains offer the most direct evidence yet of an interfacial location for ethanol in phospholipid bilayers. We are not implying, however, that ethanol molecules do not intercalate into the membrane core. The interfacial location of ethanol means that the probability of finding ethanol molecules is highest at the interface. Locating at the interface would offer

the opportunity for the hydroxyl group of ethanol to hydrogen bond with the acyl chain carbonyl, phosphate group, and water and keep the hydrophobic ethyl portion of ethanol inserted into the lipid chains, thus satisfying the amphiphilic needs of ethanol.

The spectra indicate that crosspeaks with a wide range of intensities occur between the methyl group of ethanol and nearly every lipid proton resonance and also between nearly all lipid resonances. This underscores the ability of ethanol to partition into the membrane in the liquid-crystalline phase where lipids adopt an ensemble of conformations allowing contact to occur between and within lipid molecules. The distribution of crosspeaks between lipid and ethanol indicates that no one unique location for ethanol exists; rather the intensity of a crosspeak indicates the probability for a particular location. The strongest lipid-ethanol crosspeaks were observed with lipid protons from the glycerol and upper chain segments, which lends strong support for the hypothesis of an interfacial location for ethanol in membranes as suggested by previous studies and is also in good agreement with data that show a lower partition coefficient for ethanol into the membrane interior.

We compared ethanol distribution between saturated, monounsaturated, and polyunsaturated phosphatidylcholine membranes and found only minor differences. Polyunsaturation makes the hydrocarbon core of membranes slightly more polar, which may enhance partitioning of ethanol into the hydrocarbon core. In contrast to expectation, ethanol has slightly more intense crosspeaks to upper chain segments in the saturated than the unsaturated lipids. Obviously, the polarity profile of the lipid-water interface appears to be more important for determining lipid-ethanol interaction than minor differences in polarity of the membrane core.

In the present study, the experimental conditions were optimized in a number of ways, but we do not believe the presence of more physiological conditions would alter the results; rather it would make them more difficult to observe. The partially dehydrated membranes we employed may enhance ethanol partitioning into the membrane, but it is unlikely that the distribution function of ethanol in bilayers would be much altered by higher water concentrations. Current  $^2\text{H}$  NMR investigations in our laboratory have shown that the perturbation of lipid structure caused by partial dehydration is modest for phosphatidylcholines containing unsaturation. The amount of ethanol in the membrane is given as a lipid:ethanol molar ratio because at restricted water no bulk solution exists for which to calculate ethanol concentration. We assume that lipid, ethanol, and water form one phase.

Decreasing the amount of ethanol to 0.1 ethanol/lipid (mol/mol) resulted in the strongest crosspeaks occurring between ethanol and the upper parts of the acyl chain and somewhat weaker interactions with the lipid backbone and headgroup. While these experiments were compromised by poor signal-to-noise, this may reflect the hypothesis that low concentrations of ethanol tend to partition more into the hydrophobic portion of the membrane near the interface. The ethanol content in the investigated membranes is likely to exceed ethanol concentration under conditions of physiological intoxication. However, we feel we are still in a range of ethanol concentration where the membrane location for ethanol is independent of the quantity used. From 0.1 to 1 ethanol/lipid almost no change in order for deuterated ethanol was observed (Barry & Gawrisch, 1994), indicating that

ethanol is in a similar environment at either ethanol concentration. The present work showed that ethanol locates with the highest probability near the upper portion of the acyl chains and glycerol backbone at levels of either 0.1 or 1 ethanol/lipid.

**Ethanol Distribution in Bilayers.** Describing ethanol location in bilayers as a distribution emphasizes properties of the membrane environment that are important for understanding any membrane phenomenon. Terms for ethanol location such as "binding site" or "pool" may give the impression that a fixed membrane position exists for the location of ethanol, whether it be at the surface or in the hydrophobic core. Lipid molecules in biological membranes are continually fluctuating via *trans-gauche* isomerization, axial rotation, lateral diffusion, and vertical motion (surface roughing), all occurring on a time scale of micro- to picoseconds. These motions are well-known but often forgotten when models are derived. Without them, the picture of membranes would be far too static. The plethora of lipid-lipid crosspeaks in a 2-D NOESY NMR spectrum reminds us of the multiconformational nature of lipid molecules. The dynamic environment of a lipid membrane allows contact to occur between different parts of a lipid molecule through either *inter-* or *intramolecular* interactions. These motions ensure that the boundary between hydrophobicity and hydrophilicity is constantly blurred and what at one moment might be the correct combination of amphiphilicity for ethanol is in the next instance unfavorable because a lipid molecule has shifted position. Ethanol is a small molecule encompassing both hydrophobic and hydrophilic moieties that allow it to diffuse across a lipid bilayer and thus encounter all the environments a lipid has to offer. No region is excluded from visitation by ethanol; some are simply more likely to satisfy its requirements for solvation, and this is where at a given moment, ethanol is most likely to reside. Ethanol location in membranes may best be described by a distribution. The temperature dependence of lipid-ethanol crosspeak intensity suggests that the average lifetime for a specific lipid-ethanol contact is on the order of 1 ns.

**What Are the Consequences of an Interfacial Location Regarding the Action of Ethanol?** The diverse nature of ethanol's actions, indicated by the amassed literature on the subject, suggests that it involves a multifaceted mechanism. There is no real agreement on the location of the site(s) of action: lipid, protein, or both; which is primary or which secondary. It is likely that no explanation taken in isolation is satisfactory. A common thread linking many studies may be that ethanol and water compete for identical binding sites, therefore allowing ethanol to dehydrate membranes (Klemm, 1990; Slater et al., 1993; Isobe et al., 1994). Ethanol replacement of surface water may change the interfacial dipole potential (Simon & McIntosh, 1989; Gawrisch et al., 1992; Qin et al., 1996) and induce a conformational change in lipid headgroup structure (Barry & Gawrisch, 1994). Permeability studies that show decreased water diffusion across phospholipid membranes in the presence of ethanol are in agreement with a model whereby ethanol blocks the entry of water into membranes (Huster et al., 1996). Alternatively, an interfacial location may potentiate ethanol's effect via an alteration of the lipid bilayer lateral tension profile (Gruner & Shyamsunder, 1991).

In this study the interaction of ethanol with a phospholipid bilayer has been investigated, but we consider the general

features of lipid-ethanol interactions also valid for protein-ethanol interactions [e.g., Franks and Lieb (1987)]. In particular, the frequently proposed hypothesis that alcohols bind in protein hydrophobic pockets via hydrophobic interactions may need reconsideration. The amphiphilic nature of alcohols clearly favors an interfacial location, and interactions are driven by both the opportunity for hydrogen bonding and hydrophobic interactions as has been shown by this study.

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