Effects of Ethanol on Lipid Bilayers Containing Cholesterol, Gangliosides, and Sphingomyelin

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ABSTRACT: The influence of lipid composition on the response of bilayers to ethanol binding was investigated with ^2H NMR spectroscopy. The bilayers were composed of various combinations of the lipids most often found in neural cell membranes: phosphatidylcholines (PCs), gangliosides, sphingomyelin, and cholesterol. The PCs, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), were chain-perdeuterated to allow the response of bilayer order to ethanol to be monitored at all positions through the depth of the bilayer interior. All bilayers were investigated in the lamellar liquid-crystalline (L_α) phase. The results from the de-Paked NMR spectra demonstrate that ethanol binding in the lipid−water interface [Barry, J. A., & Gawrisch, K. (1994) *Biochemistry 33*, 8082−8088] alters order parameter profiles in the bilayer interior differently for the various lipid mixtures. The presence of 10 mol % brain gangliosides enhanced the disordering effect of ethanol and altered the response of the order profile along the PC chains. This effect was apparently caused by sugar−ethanol interactions in the oligosaccharide head group. The impact of the ceramide moiety of brain sphingomyelin (50 mol % in DMPC) was negligible. In bilayers containing cholesterol, the binding of ethanol and its effects on the hydrocarbon interior were found to reflect the phase transition to the liquid-ordered phase at about 25 mol % cholesterol [Thewalt, J. L., & Bloom, M. (1992) *Biophys. J. 63*, 1176−1181]. Results from the quadrupolar splittings for deuterated ethanol (CH_3CD_2OH) bound to cholesterol-containing bilayers showed that ethanol binding decreased with increasing amounts of cholesterol. This suggests that the carbonyl groups in the PC glycerol backbone are favored hydrogen bonding sites for ethanol. High cholesterol levels of ≥30 mol % rendered PC bilayers significantly less susceptible to disordering by ethanol, but lower concentrations (<22 mol %) *enhanced* the effect of ethanol. This correlation between cholesterol content and ethanol-induced disordering is very similar to the correlation between cholesterol and water permeability, indicating that ethanol and water interact similarly with lipids. This serves as a reminder that the hydrophilic as well as the hydrophobic nature of ethanol plays a role in the interactions between ethanol and membrane lipids and proteins. The results demonstrate that cholesterol and gangliosides in bilayers alter the response of the hydrocarbon interior to ethanol binding in the lipid−water interface.

The physiological impact of ethanol encompasses both the acute effects of intoxication and a host of longer term compositional and functional changes in the body due to chronic alcohol consumption. Although the mechanisms for the acute and chronic effects of ethanol are not known, it is generally accepted that these physiological changes are facilitated at least in part by effects on the cell membrane. It has recently been shown that the interaction of ethanol with the lipid−water interface (head group and glycerol backbone region) of a completely saturated phosphatidylcholine (PC)[1] bilayer causes a disordering of the hydrocarbon interior (Barry & Gawrisch, 1994). The purpose of this study was to monitor the ways in which cholesterol, gangliosides, and sphingomyelin modify the effects of ethanol on saturated PC bilayers. Gangliosides are most highly concentrated in the nervous system, accounting for up to 10 mol % of all lipids in synaptosomal membranes (Thomas & Brewer, 1990). Cholesterol is a prominent component of some biological membranes. The outer leaflet of the synaptic cell membrane contains most of the PCs, sphingomyelins, and gangliosides in the membrane, and normally only about 12 mol % of the total cholesterol (Wood & Schroeder, 1992). Chronic alcohol consumption, however, was found to increase the amount of cholesterol in the outer leaflet of mouse synaptic plasma membranes by a factor of 2, to about 28 mol % of the total cholesterol (Wood & Schroeder, 1992).

The three classes of non-steroid lipids offer the possibility for correlating responses of the bilayer to ethanol binding to structural features of the lipids. Phosphatidylcholines consist of a phosphocholine head group, glycerol backbone, and two fatty acid chains. Sphingomyelin contains the same head group, but ceramide replaces the diacylglycerol moiety. The chains of ceramide are usually 18 or 20 carbons long, with one chain being a monounsaturated aliphatic amine with a *trans* double bond at the fourth carbon, and the other a amide-linked acyl chain (often saturated) at carbon 2. Like

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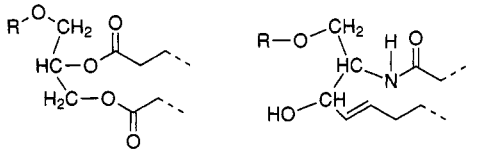
[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EPR, electron paramagnetic resonance; G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)galactosylglucosylceramide; L_α, lamellar liquid-crystalline; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; T_m, lamellar gel−liquid-crystalline phase transition temperature.

sphingomyelin, the hydrophobic moiety of gangliosides is also a ceramide, but the head group is an acidic oligosaccharide. The polar regions connecting the hydrophilic head group to the hydrophobic chains are shown schematically (**1**) for phosphoglyceride (left) and ceramide, where R denotes a phosphocholine (or other phosphate head group) for the phosphoglyceride, and either a phosphocholine or an oligosaccharide for the ceramide.



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It was shown previously with DPH fluorescence that 10 mol % of various types of gangliosides in DMPC enhanced the disordering effects of ethanol (Harris & Groh, 1985; Harris et al., 1984). Similar experiments were done on cholesterol-containing bilayers with fluorescence, EPR, and ^{2}H NMR spectroscopies, but inconsistent results were obtained (Chin & Goldstein, 1981, 1984; Harris et al., 1984; Johnson et al., 1992). In the current work, the influence of ethanol on membranes of varying lipid composition was revisited, using ^{2}H NMR spectroscopy of chain-perdeuterated lipids in order to monitor changes at all positions along the chain. In this way, we could more completely evaluate the influence of lipid composition on the way the hydrocarbon interior is altered by ethanol binding in the lipid–water interface. The response of bound ethanol to changes in bilayer composition was also studied, through ^{2}H NMR measurements of CH_3CD_2OH.

In the case of ethanol binding, it is reasonable to regard the lipid–water interface as a model for a general hydrophilic–hydrophobic interface, such that ethanol binding can be expected to occur with proteins as well as lipids (including glycoproteins and glycolipids). As suggested earlier by Brockerhoff (1982) and Klemm (1990), such interactions in the interfacial region of membrane lipids and/or proteins may influence neuronal membrane function, leading to some of the physiological effects of ethanol.

EXPERIMENTAL PROCEDURES

The chain-perdeuterated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC-d_{54}) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC-d_{62}), α,β-choline-deuterated DMPC (DMPC-d_4), pig brain sphingomyelin, and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Gangliosides (bovine brain, >98%) were supplied by ICN Biochemicals (Cleveland, OH). Deuterated solvents (D_2O and CH_3CD_2OH) were obtained from Cambridge Isotope Labs (Woburn, MA), deuterium-depleted H_2O from Isotec, Inc. (Miamisburg, OH), and anhydrous ethanol from Spectrum Chemical Manufacturing Corp. (New Brunswick, NJ).

Preparation of NMR Samples with Deuterated Lipids. The mixtures studied with deuterated lipids were as follows: head-group-deuterated DMPC (DMPC-d_4) with cholesterol (0, 12.5, and 29.8 mol %); DMPC-d_{54} with cholesterol (0, 12.5, 24.6, 30.3, and 49.4 mol %); DMPC-d_{54} with sphingomyelin (50 mol %); and DPPC-d_{62}/gangliosides/cholesterol (90:10:0, 76:10:14, and 60:10:30 mol %). The most common chain length in gangliosides is 18 carbons, followed by 20 carbons (Masserini & Freire, 1986; Masserini et al., 1988). DPPC, with 16-carbon chains, was chosen for the ganglioside mixtures over the shorter DMPC because there is evidence that chain length differences contribute to lateral phase separations in mixtures of DMPC and gangliosides (Terzaghi et al., 1993).

All lipids were dried in a vacuum desiccator over P_2O_5; when the lipids were transferred directly from an ultra cold freezer, it was found that 2 h of drying in this manner was sufficient to reach constant weight. The lipids were quickly weighed after being removed under argon from the desiccator, and then combined for lyophilization (or hydrated immediately in the case of pure DMPC). For calculations with molecular weight, it was assumed that during weighing two water molecules per lipid were attached to dried DMPC, DPPC, sphingomyelin, and gangliosides, and one on cholesterol; the number of water molecules remaining bound to gangliosides after drying may be greater than two due to the oligosaccharide head group. The average molecular weight of brain gangliosides was estimated to be 1850 based on the ganglioside composition of human and rat brain synaptosomes (Svennerholm, 1980), and assuming both fatty acid chains are 18 carbons long.

To ensure the homogeneus combination of lipids in the mixtures, the weighed lipids were codissolved in an appropriate solvent, and the solvent was evaporated with a stream of argon. Then the film was taken up in another solvent, thoroughly frozen in a dry ice/ethanol bath, and put under high vacuum to evaporate the solvent. The DMPC/cholesterol mixtures were dissolved in CH_2Cl_2 with a small amount of cyclohexane, and then lyophilized from cyclohexane. For the sphingomyelin mixture, $CHCl_3$ was used to combine the lipids, and double-distilled water was used for lyophilization. The ganglioside mixtures were combined using warm 1:1 $CHCl_3$/methanol, and lyophilized from water. All organic solvents were high-purity spectroscopic grade. Following lyophilization, the mixture was removed from vacuum under argon, 10–30 mg of lipid was weighed into a 5 mm o.d. glass tube, and then the lipid was hydrated gravimetrically with deuterium-depleted water. Where added, anhydrous ethanol was combined with the water to reach a level of one ethanol molecule per lipid. The bilayers were hydrated to a level of 27 H_2O/lipid in the absence of gangliosides, and 66 H_2O/lipid in the presence of gangliosides. The concentrations of water and ethanol are discussed in more detail under Results.

The sample tubes had ground-glass joints at one end, enabling them to be sealed with ground-glass stoppers. Following hydration, the tubes were capped without grease and centrifuged, and the lipids were stirred vigorously but very briefly with a fine needle. The sample was then recapped, centrifuged again, and capped for the last time using a very thin coat of Teflon grease, and the cap was wrapped with parafilm. The samples were kept above T_m during this process with a warm water bath, using O-ring sealed cryovials to protect the samples from the water.

Preparation of NMR Samples with Deuterated Ethanol. Samples containing methylene-deuterated ethanol, CH_3CD_2OH, consisted of DMPC combined with cholesterol in amounts of 0, 12.5, 24.9, 33.0, 42.3, and 50.1 mol %. The lipids were hydrated to a submaximal level of 9:1:1 ^1H_2O/CH_3CD_2OH/lipid (molar ratios), in order to allow the

quadrupolar splitting of the ethanol to be observed (Barry & Gawrisch, 1994). Sample preparation followed the procedure described above, except that extra care was taken to keep the lipids under a dry atmosphere of argon as much as possible throughout the procedure.

NMR Spectroscopy. The ^{2}H NMR spectra were acquired on a Bruker MSL 300 spectrometer (Billerica, MA) at a frequency of 46.07 MHz, equipped with a high-power, double-tuned probe with a 5-mm solenoid sample coil. A phase-cycled quadrupolar echo pulse sequence was used (Davis et al., 1976) with a 3.0 μs 90° excitation pulse, a 30 μs delay between pulses, and a relaxation delay of 500 ms. A data set of 4096 points was collected for each scan, over a spectral range of 250 kHz for the chain-perdeuterated lipids, and 62.5 kHz for deuterated ethanol. Line broadening of between 5 and 100 Hz was applied before Fourier transformation.

The temperature of the samples was controlled to ±0.5 °C with the temperature control unit in the spectrometer, which was periodically calibrated with a digital thermometer. All samples were investigated in the L_α phase: DMPC-d_{54} and DMPC-d_4 at 30 °C, DPPC-d_{62}/gangliosides at 47 °C, and DMPC/CH_3CD_2OH at 30 and 40 °C.

The chain-perdeuterated spectra were dePaked on an IBM-compatible Compaq computer (Houston, TX) with a 486 processor, using the algorithm of Sternin et al. (1983). Due to overlap of some peaks, the dePaked spectra were integrated and divided into steps corresponding to the intensities of the methylene groups. Each step in the integral corresponds to a quadrupolar splitting, $\Delta\nu_Q$, for the methylene groups, assuming that order decreases steadily from the glycerol backbone to the terminal methyl group. Acyl chain order is expressed in terms of order parameters, S_{CD}, for the individual carbon−deuterium bonds along the chain, which are calculated from the quadrupolar splittings according to (Davis, 1983):

$$(\Delta\nu_Q)_\perp = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)|S_{CD}| \quad (1)$$

The quadrupolar splitting $(\Delta\nu_Q)_\perp$ is measured between the peaks corresponding to lipids oriented at a 90° angle with respect to the magnetic field; e^2qQ/h is the static quadrupolar coupling constant of ~168 kHz for a methylene segment (Burnett & Müller, 1971).

RESULTS

Hydration Levels. For the deuterium-labeled lipids (without gangliosides), hydration levels were chosen such that only a small excess of bulk water would be present beyond the maximum amount accommodated between the bilayers. This ensured that fully hydrated systems were studied, but avoided the spontaneous formation of small vesicles that occurs with increasing amounts of excess water, and enhanced the partitioning of ethanol into the lipid. For pure DMPC-d_{54}, it was found that, at a constant ethanol:lipid ratio of 1:1, the effect of ethanol on bilayer order was equal in the presence of between 15 and 25 water molecules per lipid, and then the effect was slightly diminished with 27 H_2O/lipid (data not shown). Therefore, to create systems with a slight excess of water, a hydration level of 27 H_2O/lipid (~40−45 wt % water) was chosen for all DMPC-d_{54} mixtures; the corresponding ethanol concentration with one ethanol molecule per lipid is 2.2 M. With the ganglioside mixtures, a hydration level of 66 water molecules per lipid was used, corresponding to about 60 wt % H_2O and an ethanol concentration of 800 mM (1:1 molar ethanol/lipid). A larger quantity of water was used for the ganglioside mixtures for two reasons. First, significantly more water is required to fully hydrate ganglioside-containing bilayers due to the large oligosaccharide head group. For example, about 60 molecules of water are required to fully hydrate one ganglioside molecule in the hexagonal structures formed by gangliosides extracted from brain (Thompson & Tillack, 1985). Second, gangliosides make the bilayer more sensitive to ethanol (Harris & Groh, 1985; Harris et al., 1984), so lower ethanol concentrations can be used to produce effects similar in magnitude to those in the absence of gangliosides.

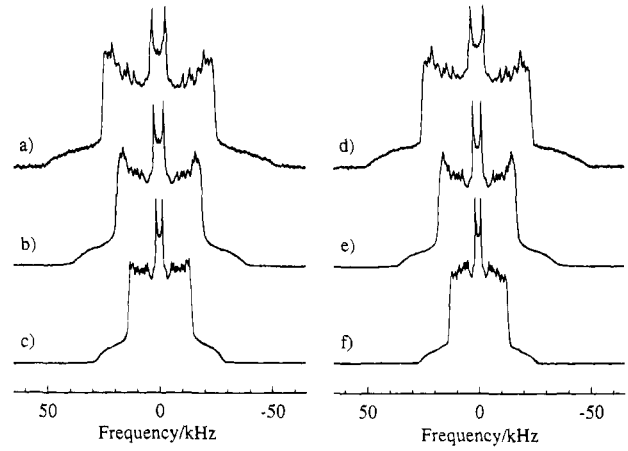

FIGURE 1: ^{2}H NMR spectra of DPPC-d_{62} mixed with 10 mol % bovine brain gangliosides and varying amounts of cholesterol, at 47 °C (~10 °C above the T_m for pure DPPC-d_{62}). The spectra in the left panel have no ethanol and contain cholesterol in amounts of (a) 30%, (b) 14%, and (c) 0%. The spectra in the right panel are for the same mixtures but with 800 mM ethanol. All mixtures were hydrated to a level of 66 water molecules per lipid, or ~60 wt % water.

The Response of Cholesterol-Containing Bilayers to Ethanol. ^{2}H NMR spectra for mixtures of chain-perdeuterated DPPC, gangliosides, and cholesterol are shown in Figure 1, in the presence and absence of ethanol. These spectra illustrate that cholesterol increases bilayer order (broadens the spectra), while ethanol decreases order. It has been shown that although ethanol interacts with PC bilayers in the lipid−water interface (including the head group, glycerol backbone, and perhaps the uppermost methylene groups of the chains), the effect of ethanol is largely manifested as diminished order in the hydrophobic core (Barry & Gawrisch, 1994).

The competing effects of cholesterol and ethanol can be examined in detail in Figures 2, 3, and 4. Figure 2 shows the order parameter profiles of DMPC-d_{54} under the influence of ethanol and varying amounts of cholesterol. The information contained in these order profiles is evaluated in two different ways in Figures 3 and 4. The changes induced by ethanol are expressed in this work (in Figures 3, 4, 6, and 7) as the percent decrease in S_{CD} due to ethanol, or $\Delta S/S_0$, where S_0 is the order parameter in the absence of ethanol. The effect of cholesterol and ethanol on overall bilayer order is given in Figure 3, in terms of the average order parameter for both chains. The same plots of S_{CD} for the individual methylene groups showed that the trends occurring in the average order parameter were similar to those for the

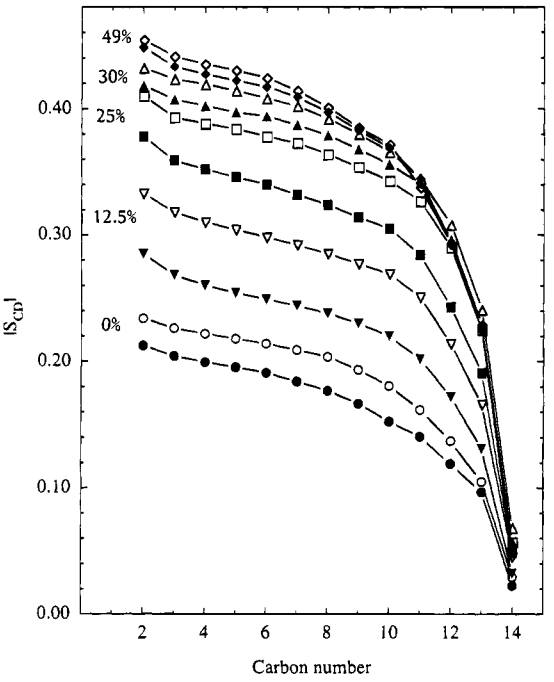

FIGURE 2: Order parameter profiles for DMPC-d_{54} mixed with cholesterol in the presence and absence of ethanol (filled and open symbols, respectively), illustrating the competing effects of ethanol and cholesterol on bilayer order. The cholesterol concentrations (in mol %) are indicated on the graph adjacent to each pair of curves. The mixtures contained a slight excess of water beyond maximal hydration (27 H_2O/lipid or ~40 wt %), and one ethanol molecule per lipid. The spectra were acquired at 30 °C, 10 °C above the phase transition for pure DMPC-d_{54}. S_{CD} is defined in eq 1 in the text.

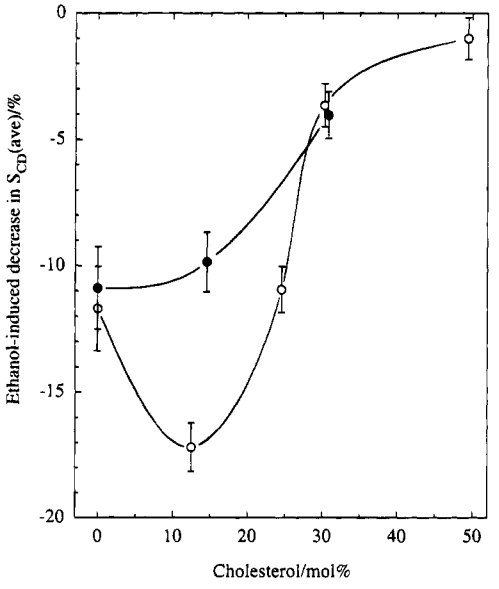

FIGURE 3: Ethanol-induced decrease in the average order parameter for mixtures of DMPC-d_{54}/cholesterol (open symbols) and DPPC-d_{62}/ganglioside/cholesterol (filled symbols). The mixtures are described in detail in Figures 1 and 2, and in the text. Although cholesterol concentrations >25 mol % markedly diminish the effect of ethanol on DMPC-d_{54} bilayers, concentrations below about 22 mol % *enhance* the effect of ethanol compared to bilayers lacking cholesterol.

individual positions along the chain (not shown). The effect of ethanol on order in DMPC bilayers depended on cholesterol in a complex manner: cholesterol concentrations of less than about 22 mol % significantly enhanced the disordering

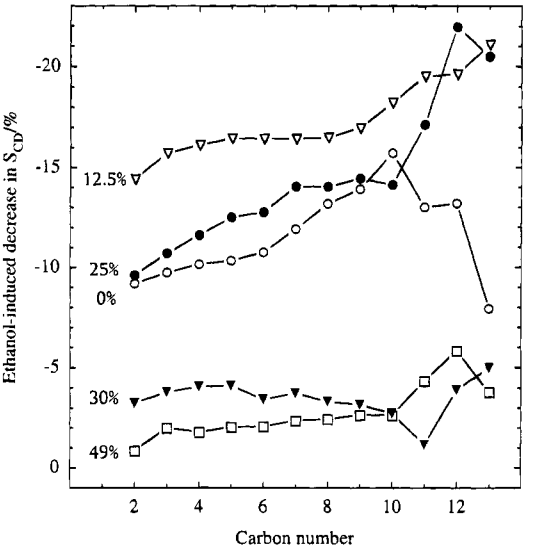

FIGURE 4: Ethanol-induced decrease in the order parameter S_{CD} for each methylene group along the chain in DMPC-d_{54}/cholesterol mixtures, calculated from the order profiles in Figure 2. The cholesterol content (in mol %) is given in the figure. The changes in the order parameter profiles reveal that bilayers with 0% and 25% cholesterol are affected differently by ethanol even though the average values for bilayer order (Figure 3) are equal. The figure also reveals an abrupt change in bilayer organization between 25% and 30% cholesterol, in which bilayers containing ≥30% cholesterol are influenced little by ethanol.

effect of ethanol; 30% and higher sharply attenuated ethanol-induced disordering; and levels of roughly 25% caused a response similar to that with no cholesterol.

Rather than an average order, Figure 4 illustrates the response of the different mixtures to ethanol along the entire length of the acyl chains. In the bilayer without cholesterol, the chains are most affected by ethanol in the region between methylene carbons 7 through 12, inclusive, with the maximum effect occurring at carbon 10. That is, the most disordered section lies between the most highly ordered "plateau" (approximately consisting of carbons 2 through 6) and the very end of the chain. This pattern changes in the presence of cholesterol. At lower cholesterol concentrations, which either enhance the overall effect of ethanol (12.5%) or change it very little (25%), ethanol binding causes the chain to become progressively less ordered all the way down the chain. This difference is particularly apparent when comparing the curves with 0 and 25% cholesterol. The bilayers respond very similarly up to carbon 10, both gradually becoming more disordered down the chain. At carbon 11, however, order in the cholesterol-containing bilayer continues to decrease along the remainder of the chain, while in the cholesterol-free bilayer the relative decrease in order in the last three methylene groups became smaller. Interestingly, between 25 and 30% cholesterol, there is a abrupt change in the response of the bilayer to ethanol. This difference of only 5% cholesterol caused the bilayer to become nearly resistant to the effects of ethanol. Also, the ethanol-induced disordering was nearly evenly propagated down the chain. At a cholesterol level of 49%, the bilayer responded even less to ethanol, and again the disordering was nearly uniform along the chain.

The quadrupolar splittings for deuterated choline (DMPC-d_4) varied between 5.4 and 6.6 kHz for the α-methylene group, and between 4.4 and 5.3 kHz for β-methylene. The

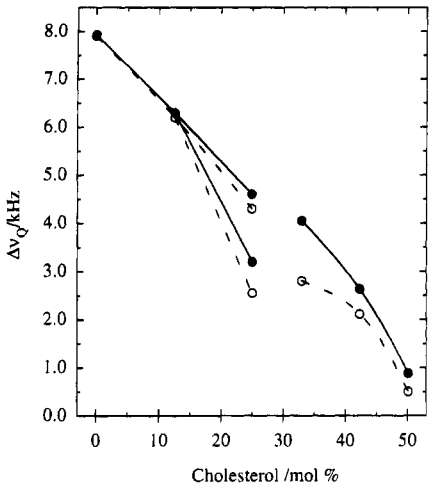

FIGURE 5: Influence of cholesterol on the quadrupolar splitting ($\Delta\nu_Q$) of CH_3CD_2OH bound to DMPC-d_{54}/cholesterol bilayers at 30 (○) and 40 °C (●). Since the data appear to display a discontinuity in the region of 25 and 30 mol % cholesterol, lines were drawn with cubic spline functions through groups of points which may represent distinct differences in the binding of ethanol to cholesterol-containing lipids. Since cholesterol is known to bind to carbonyl groups in the interface, the decreased splittings with increased cholesterol suggest that cholesterol displaces ethanol from hydrogen bonding interactions with these same sites.

changes in $\Delta\nu_Q$ due to either ethanol or cholesterol were not significant, varying between 0 and 1 kHz (data not shown), indicating that cholesterol and ethanol had very little effect on the head group. A conformational change in the head group would have produced more substantial changes in the α- and β-splittings in opposite directions (Bechinger & Seelig, 1991).

The quadrupolar splittings of methylene-deuterated ethanol (CH_3CD_2OH) are given in Figure 5 as a function of cholesterol concentration. The splittings are a function both of the motion of the ethanol (related to binding strength) and of the angle it makes with respect to the magnetic field (related to its orientation). In general, the splittings $\Delta\nu_Q$ decreased as the proportion of cholesterol increased. Also, $\Delta\nu_Q$ was greater at 40 °C than at 30 °C, except there was no temperature difference within experimental error at 0 and 12.5% cholesterol. However, the data appear to display a discontinuity from 25 to 30% cholesterol. At both temperatures, the splittings decreased linearly from 0 to 25% cholesterol, but at 25% a second smaller splitting appeared. Then the spectra with ≥30% cholesterol displayed only one splitting, but the splittings appear to follow a different trend from that occurring at lower cholesterol concentrations. The lines were drawn in Figure 5 to reflect this apparent discontinuity.

The Effect of an Oligosaccharide Head Group and Ceramide. Sphingomyelin increased the order of the DMPC-d_{54} chains and, at the concentration of 50 mol % studied here, raised the gel-to-L_α phase transition from 19.5 °C (Barry et al., 1991) to about 29 °C. The influence of ceramide on ethanol-induced disordering of DMPC-d_{54} is shown by the comparison of pure DMPC and 50:50 DMPC/sphingomyelin in Figure 6. DMPC combined with sphingomyelin responded very similarly to pure DMPC in the top eight methylene groups, yet the decrease in order caused by ethanol was much more pronounced in the last three methylene groups.

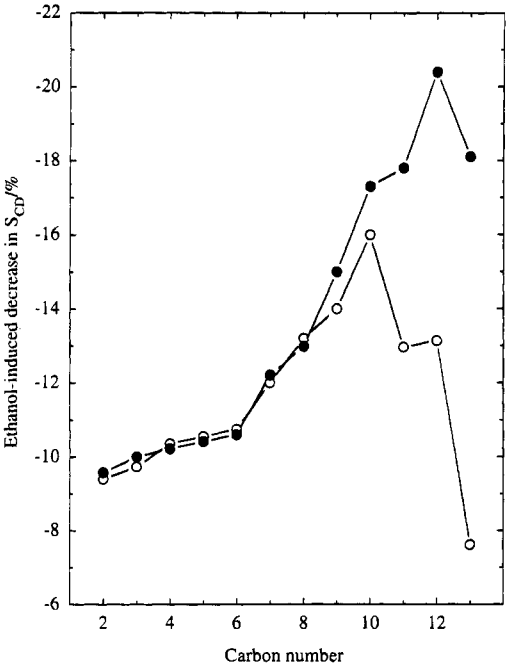

FIGURE 6: Effect of sphingomyelin on ethanol-induced disordering in DMPC-d_{54}/sphingomyelin bilayers (filled symbols; 1:1 molar) as compared to pure DMPC-d_{54} (open symbols). The values are given as the percent decrease in S_{CD} values at each methylene position along the chain in DMPC-d_{54}. The sphingomyelin bilayers (T_m ~29 °C) were studied at 37 °C and the pure DMPC bilayers (T_m = 19.5 °C) at 30 °C; both bilayers were hydrated to 27 solvent molecules per lipid (~40 wt % water), including one ethanol/lipid where present. The results indicate that ceramide and glycerol backbones respond very similarly to ethanol; the pronounced difference in the influence of ethanol in the ends of the chains is presumably due to the large chain length difference between DMPC and sphingomyelin.

Figure 3 shows the response of the average bilayer order to ethanol in the presence of 10 mol % gangliosides. The ethanol concentration in the DPPC/ganglioside bilayers (800 mM) was much lower than that in the DMPC system (2.2 M), yet the response of these two systems to ethanol was essentially equal in magnitude. This demonstrates that gangliosides enhance the response of a bilayer to the disordering effects of ethanol, as shown by Harris and coworkers (Harris & Groh, 1985; Harris et al., 1984). Also in the presence of gangliosides, low and intermediate amounts of cholesterol attenuate rather than enhance the effects of ethanol on bilayer order.

Figure 7 shows the results of adding both gangliosides (composed of ceramide and an oligosaccharide head group) and cholesterol on the order parameter profiles of DPPC-d_{62}. In the absence of cholesterol, the DPPC/ganglioside bilayer responds to ethanol differently than DMPC/sphingomyelin, indicating that the oligosaccharide head group contributes to the effects of ethanol experienced by the hydrocarbon interior. Notice also the corresponding curves in Figures 4 and 7 for DMPC and DPPC/ganglioside bilayers containing 30 mol % cholesterol. Although ethanol substantially reduced the average order in both systems, the shapes of the curves were quite different. In the absence of gangliosides, the influence of ethanol on bilayer order was nearly uniform throughout the bilayer; in the presence of gangliosides, different regions of the chain were affected differently by ethanol.

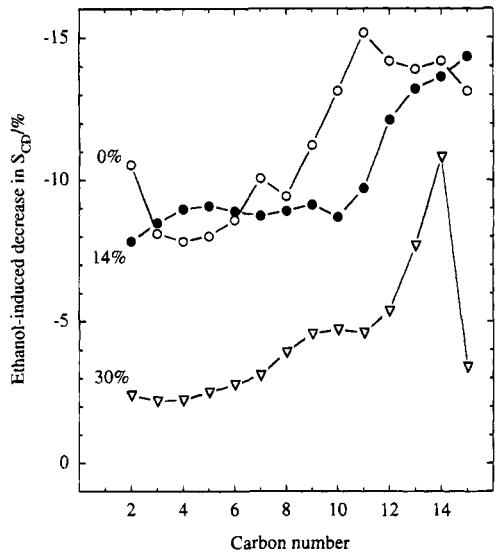

FIGURE 7: Influence of ethanol on order at all methylene positions along the chain in DPPC-d_{62}/ganglioside mixtures with 0, 14, and 30% cholesterol, calculated from the spectra in Figure 1. The difference between the curve for 0% cholesterol and the curve in Figure 6 for DMPC-d_{54}/sphingomyelin demonstrates that sugar–ethanol interactions in the oligosaccharide head group influence order in the bilayer interior.

DISCUSSION

Lipids from structurally distinct classes—cholesterol, gangliosides, and sphingomyelin—were added to saturated PC bilayers to evaluate how they influence the effect of ethanol on lipid bilayers. The use of chain-perdeuterated DMPC and DPPC allowed the response of all carbon positions along the chains to be monitored with ^{2}H NMR. This information provides insight on both lipid–ethanol interactions and lipid–lipid interactions (i.e., lipid packing and the organization of mixtures).

In order to obtain clearly measurable results with NMR, the ethanol concentrations used in our model membranes were much higher than those required in the bloodstream for intoxication. The lower limit for intoxication in individuals who do not chronically abuse alcohol is 22 mM, with death occurring at about 100 mM. Chronic abusers, on the other hand, can appear sober with blood levels of 100 mM ethanol, and can survive levels in excess of 300 mM (Diamond, 1992). At present, it is not known what order of magnitude change induced by ethanol in acyl chain order is biologically significant. It is possible that changes in membrane function are related to very small changes in lipid order.

Further, it is important to emphasize the distinction between the pure water used in our experiments and either blood or other fluids in tissue. The large concentration of proteins, electrolytes, and miscellaneous organic molecules in the interstitial spaces and cytosol causes a large decrease in the activity of water. The effective concentration (or activity) of ethanol at membrane surfaces in tissues is not known. We can say only that the large quantities of organic and inorganic matter in the aqueous phase will cause the effective ethanol concentration to increase relative to the simple blood alcohol levels given as amount of ethanol per unit volume of blood.

It is also important to emphasize that the components of a biological membrane should make the membrane much more responsive to ethanol as compared to a pure phospholipid bilayer. In addition to lipids, membrane proteins and carbohydrates should also interact with ethanol, which may lead to modified membrane properties and function. For example, in the ganglioside-containing bilayers in this work, 800 mM ethanol in the presence of 10 mol % gangliosides had the same magnitude effect as 2.2 mM ethanol on pure PC bilayers. The addition of just this one component to a PC bilayer, in amounts physiologically relevant for synaptosomal membranes, produced a bilayer that was significantly disordered (by 11%) by 800 mM ethanol (Figure 3). This decrease in order is far in excess of what we would expect to occur *in vivo*, yet the concentration is only about 10 times greater than that required in the blood for intoxication.

The Influence of Cholesterol on Acyl Chain Response to Ethanol. The influence of ethanol on cholesterol-containing bilayers was found to depend on the quantity of cholesterol present (Figure 3). Although ≥30 mol % cholesterol significantly curbed ethanol-induced disordering in DMPC/cholesterol bilayers, amounts below about 22% cholesterol had the opposite effect. In the presence of 10 mol % gangliosides, 30 mol % cholesterol also sharply attenuated the effect of ethanol, while 14% cholesterol made very little difference from 0% cholesterol. The different susceptibility of bilayers to ethanol at high and lower cholesterol concentrations may be related to the increase in cholesterol content in the development of tolerance with chronic ethanol consumption (Wood & Schroeder, 1992).

The impact of cholesterol on a bilayer's response to ethanol has been reported before in the literature, with varying conclusions. Chin and Goldstein (1981) used the 5-doxyl- and 12-doxylstearic acid spin-labels to monitor the response of egg PC bilayers to 350 mM ethanol. At 10 and 15 °C, which are comparable relative temperatures to that used in the current work, the trend for the 5-doxyl probe was similar to that found here (Figure 3): 10 mol % cholesterol enhanced the ethanol-induced disordering, 20% responded much like 0%, and then the disordering was progressively attenuated from 30 to 50%. With the 12-doxyl label, however, even low amounts of cholesterol diminished the effect of ethanol. This disagrees with our results for DMPC in that a low concentration of cholesterol (such as 12.5%) enhanced the disordering effect of ethanol at *all* positions along the chain (discussed below). Their results do concur with ours, however, in that the response of ethanol was more pronounced deeper in the bilayer than close to the interface. These same authors (Chin & Goldstein, 1984) also found that erythrocyte membranes from cholesterol-fed quail were less sensitive to disordering by ethanol (87.5–700 mM). Harris et al. (1984) used the fluorescent probe DPH to compare the response of DMPC bilayers to 280 mM ethanol in the presence and absence of 33 mol % cholesterol. However, only the same small decrease in order was observed in both bilayers, presumably due to the low concentration of ethanol.

Recently, ^{2}H NMR results for a mixture of egg PC and DPPC-d_{62} (3:1 molar) were compared to fluorescence anisotropy results for pure egg PC (Johnson et al., 1992). The fluorescence data for the effects of 600 mM ethanol agreed with the results of Chin and Goldstein (1981) for egg PC. The NMR results, however, differed markedly from both the fluorescence data and other reported work, including the current study. The ethanol-induced disordering reached a

maximum at 30 mol % cholesterol, and even bilayers with 40 and 50% cholesterol were *more* disordered by ethanol than those with no cholesterol. The authors proposed that the difference was due to the "bulky exogenous lipid probes" used by fluorescence and EPR spectroscopy, and that the NMR results represented the accurate response of these bilayers to ethanol. In light of the NMR results of the present work, however, this conclusion is unlikely. Our ^{2}H NMR results for DMPC are in good qualitative agreement with both the EPR and fluorescence data for egg PC, given the differences in lipid composition, temperature, and ethanol concentration. The reason for the anomalous NMR results of Johnson et al. (1992) may be due to differences in sample preparation. For example, the mixture of egg PC and DPPC was hydrated from an evaporated film, rather than being lyophilized to a powder before hydration. This may lead to an inhomogeneous mixture due to differing solubilities for the two lipids in the organic solvent and/or water.

The effect of ethanol on chain order is not uniform along the hydrocarbon chains, as seen from a comparison of order parameter profiles. Bilayers with 0 and 25 mol % cholesterol, for example, have the same average order (within experimental error), but the ethanol-induced changes in the order profiles show that these bilayers respond very differently to ethanol at the ends of the chains. The response to ethanol of pure DMPC bilayers demonstrated a different pattern within the bilayer interior than mixtures containing either cholesterol or gangliosides: the portion of the chains most strongly disordered by ethanol lay in between the highly ordered plateau and the far ends of the chains. Ethanol binding in the lipid−water interface forces the lipids apart, presumably by interfering with the tight packing in the glycerol backbone region, and decreases order all the way along the chains (Barry & Gawrisch, 1994). However, the fact that the effect is less pronounced in the plateau region may be because order here is more strongly influenced by the anchoring of the chain to the glycerol backbone than by the increased effective area per molecule. The very tail end of the chains presumably showed less of a change due to ethanol because the very high mobility in this region diminished the effects of ethanol.

With 12.5 and 25 mol % cholesterol, however, the tail end of the chains is the region *most* affected by ethanol. It would follow, then, that one effect of cholesterol is to remove the very high freedom of motion normally present in the center of the bilayer. Then the disordering induced by ethanol, which before was overshadowed by the high degree of mobility in the center of the bilayer, would now be seen. Plots of the cholesterol-induced increase in S_{CD} for DMPC-d_{54} (not shown) support this interpretation: at a given cholesterol concentration, all of the plateau methylene groups (up to carbon 8) are ordered by cholesterol to a similar degree, and then cholesterol increased order progressively toward the end of the chain. For example, 25 mol % cholesterol enhanced order in the plateau region by about 43 and 44%, but in the last four methylene groups the increase in order jumped from 47% to over 53%. In bilayers with 12.5% cholesterol, the plateau was ordered by cholesterol by about 29%, while the terminal three methylene groups had increased in order by ~37%.

Similarities between Ethanol−Lipid and Water−Lipid Interactions. The results from the current work relating cholesterol content with ethanol−lipid interactions very closely match past results relating cholesterol content to water−lipid interactions. It was found over 2 decades ago that lower concentrations of cholesterol (below 25 mol %) rendered egg PC bilayers more permeable to water than no cholesterol, while concentrations above about 33 mol % *reduced* water permeability (Jain et al., 1973). Also, studies on vesicle hydration by Newman and Huang (1975) suggest that the quantity of bound water increased in egg PC/cholesterol mixtures up to 22 mol % cholesterol, and then abruptly decreased at the higher cholesterol contents examined of 29 and 32 mol %. These trends for water were reflected in the current work with ethanol, where less than about 22 mol % cholesterol rendered DMPC bilayers more disordered by ethanol than either higher quantities of cholesterol (>25 mol %) or none at all (Figure 3). This suggests that cholesterol-induced packing changes affect ethanol−bilayer interactions in much the same way as water−bilayer interactions. Such a correction is to be expected given that the interaction of both water and ethanol with lipid bilayers includes hydrogen bonds to polar moieties such as phosphate and glycerol backbone carbonyl groups (Blume et al., 1988; Wong & Mantsch, 1988; Chiou et al., 1991, 1992). These observations underscore that interactions between ethanol and the polar moieties of biological macromolecules mimic those of water in some respects. Therefore, the hydrophilic as well as the hydrophobic nature of ethanol should be borne in mind when considering sites of interaction of ethanol with membrane lipids and proteins.

Correlation to the PC/Cholesterol Phase Diagram. It has been shown that PC/cholesterol bilayers undergo a phase transition to the fluid yet highly ordered "liquid ordered" phase at approximately 25 mol % cholesterol, both for DPPC (Vist & Davis, 1990) and for two mono-*cis*-unsaturated PCs (Thewalt & Bloom, 1992). Evidence from both the influence of cholesterol on the disordering effect of ethanol and the influence of cholesterol on the quadrupolar splitting of bound CH_3CD_2OH reflects this phase transition in the DMPC-d_{54}/cholesterol bilayers. From the average order displayed in Figure 3, DMPC bilayers with ≤22% cholesterol enhanced ethanol-induced disordering, while those with >25% attenuated it. The order parameter profiles (Figure 4) show that bilayers with 12.5 and 25 mol % cholesterol were substantially disordered by ethanol, and that the effect increased with depth in the bilayer until the ends of the chains were the most highly disordered. The addition of only 5% more cholesterol not only sharply attenuated the effect of ethanol, but the effect was fairly uniform throughout the bilayer. In ^{2}H NMR spectra of DPPC-d_{62} mixed with cholesterol, the terminal methyl signal split into two signals of equal area above 20% cholesterol, probably because the *sn*-1 and *sn*-2 methyl groups occupy slightly different positions in the bilayer (Vist & Davis, 1990). Also, from 20% to 25% cholesterol, the individual peaks in the NMR spectra went from broad to sharp. Both the split methyl peaks and the sharpening of the spectrum can also be seen here in Figure 1 by comparing the spectra at 14 and 30% cholesterol. Also reflecting the transition at ~25% cholesterol is the discontinuity in the quadrupolar splittings of deuterated ethanol at this point (Figure 5; discussed in more detail below). These results for PC/cholesterol mixtures demonstrate that ethanol−bilayer interactions occurring in the lipid−water interface are correlated with the phase behavior of cholesterol-containing systems.

The Effect of Cholesterol on CH_3CD_2OH Binding. Since cholesterol interacts with the PC interface at the carbonyl groups in the glycerol backbone (Franks, 1976; Worcester & Franks, 1976), the decreased CH_3CD_2OH splitting due to increased cholesterol indicates that cholesterol competes for ethanol's favored binding sites. The progressive decrease in $\Delta\nu_Q$ for CH_3CD_2OH may also reflect the condensing of the bilayer. Although cholesterol increases the spacing between PC head groups (Yeagle et al., 1977; McIntosh et al., 1989), it causes an increased packing density of the cholesterol and PC (evidenced, for example, by the enhanced bilayer order due to cholesterol). The two splittings observed at 25 mol % cholesterol, at the transition to the liquid-ordered phase, indicate that ethanol interacts differently with the two PC/cholesterol phases.

The Influence of Sphingomyelin. The presence of sphingomyelin had no appreciable effect on the response to ethanol of the top two-thirds of the DMPC chains (Figure 6). The similarity in the way the upper portion of the ceramide and diacylglycerol chains responded to ethanol suggests that ethanol binds in a similar manner to the ceramide and glycerol backbones. If it is assumed that interfacial ethanol binding involves hydrogen bonding, it would follow that the *sn*-2 carbonyl oxygen in PC is the most important hydrogen bonding group for ethanol in the backbone region of the interface, and that the amide carbonyl in sphingomyelin (**1**) serves this same role. The *sn*-2 carbonyl has been shown with infrared spectroscopy to be a major hydrogen bonding site for water in phospholipids (Blume et al., 1988; Wong & Mantsch, 1988).

The only difference in the response of the sphingomyelin-containing bilayer occurred in the last three methylene groups of the DMPC-d_{54} chains. This is presumably due to the large chain length difference between DMPC and sphingomyelin (usually four carbons), rather than differences between the ceramide and glycerol backbones near the lipid–water interface. The last few carbons at the ends of the chains in pure DMPC-d_{54} were less disordered by ethanol than carbons higher up in the chain. This region probably appeared to be less affected by ethanol because the center of the bilayer is characterized by a very high degree of mobility even in the absence of ethanol. In an equimolar mixture of DMPC-d_{54} and sphingomyelin, however, the ethanol-induced disordering increased with depth in the bilayer, even in the last quarter of the DMPC-d_{54} chains. In this case, adjacent to the longer chained sphingomyelins, the ends of the DMPC chains are no longer located at the highly disordered center of the bilayer.

The Influence of Gangliosides. The response to ethanol of order parameter profiles for bilayers containing gangliosides (Figure 7) was distinct from those containing sphingomyelin. Since both gangliosides and sphingomyelin have ceramide hydrophobic moieties, but gangliosides possess a large oligosaccharide head group, the differing response of these two types of bilayers must be due in large part to the oligosaccharide moiety. There are two possible mechanisms by which ethanol alters order in the interior of ganglioside-containing bilayers: (1) through ethanol binding to the carbohydrate head group; and/or (2) through enhanced interactions with PC head groups and the glycerol/ceramide backbone region. First, considering the latter option, the results with sphingomyelin-containing bilayers show that the response of the ceramide moiety to ethanol is not appreciably different from that of the glycerol backbone. However, it is also conceivable that the large amount of water binding to the *carbohydrate* head groups could reduce the number of water molecules near the *PC* head groups, and thereby effectively increase the local ethanol concentration in the lipid–water interface (PC head groups and the glycerol/ceramide backbone region). However, this scenario would require that the amount of water available for hydration be limited, which is not the case under the conditions of 66 H_2O/lipid used in this study. After fully hydrating the gangliosides (about 60 H_2O per ganglioside) (Thompson & Tillack, 1985), this hydration level leaves about 60 water molecules available to each DPPC molecule. This amount is far in excess of the 27 H_2O/PC used in the samples without gangliosides, yet the ganglioside bilayers with 66 H_2O/lipid were about equally affected by ethanol as those which lacked gangliosides and had much less water. Also, previous experiments showing an enhanced response to ethanol in the presence of gangliosides (Harris et al., 1984) were carried out in an extreme excess of water (~60 μM lipid). It can be concluded, then, that the altered effects of ethanol on the hydrocarbon core in ganglioside-containing bilayers are largely due to ethanol–sugar interactions in the carbohydrate head group.

The question remains as to how ethanol–sugar interactions alter the carbohydrate head group, and how this effect is translated from the aqueous environment to the hydrophobic interior. One possible mechanism is through steric and/or electrostatic interactions between gangliosides in the same monolayer, or by interactions between sugars and PC head groups. Such interactions would require that ethanol binding induce the oligosaccharides to bend over into a configuration more parallel to the bilayer surface. If this were the case, inter-ganglioside interactions are conceivable considering the relative sizes of the lipids. The ganglioside G_{M1}, when mixed with PC in fully hydrated bilayers, has a cross sectional area of about 100 Å, with a pentasaccharide head group that projects straight out from the bilayer surface at least 12 Å beyond the edge of the PC head group, or about 22 Å from the glycerol backbone (McIntosh & Simon, 1994). The other common gangliosides in the brain are even larger, containing additional, branched sialic acid groups. Since a PC molecule is only ~10 Å across (McIntosh & Simon, 1986), gangliosides in a 10 mol % dispersion may be able to interact with one another given sufficient ethanol-induced configurational changes in the oligosaccharides.

CONCLUDING REMARKS

It was found that the relations described here between cholesterol content and *ethanol*–lipid interactions have some parallels with those reported in the literature between cholesterol content and *water*–lipid interactions (Jain et al., 1973; Newman & Huang, 1975). This is consistent with the interfacial binding of ethanol in the same region as bound water, since ethanol is a small, polar molecule having hydrophilic as well as hydrophobic properties. We suggest, therefore, that sites of interaction between ethanol and biological membranes should most often occur at hydrated hydrophilic–hydrophobic interfaces of membrane lipids, carbohydrates, and proteins.

The results presented in the current work demonstrate that lipid composition can influence the effect of ethanol on

bilayer properties. This in turn may alter lipid−protein interactions and therefore membrane protein function. An example recently appeared in the literature in which ethanol altered protein function by way of the membrane lipids. Mitchell and Litman (1994) found that the function of the retinal protein rhodopsin was potentiated by ethanol, and that the effect was enhanced by polyunsaturated lipids; strong evidence was presented that the effect was mediated by the acyl chains rather than a direct binding of ethanol to rhodopsin.

REFERENCES

Barry, J. A., & Gawrisch, K. (1994) *Biochemistry 33*, 8082−8088.

Barry, J. A., Trouard, T. P., Salmon, A., & Brown, M. F. (1991) *Biochemistry 30*, 8386−8394.

Bechinger, B., & Seelig, J. (1991) *Chem. Phys. Lipids 58*, 1−5.

Blume, A., Hübner, W., & Messner, G. (1988) *Biochemistry 27*, 8239−8249.

Brockerhoff, H. (1982) *Lipids 17*, 1001−1003.

Burnett, L. J., & Müller, B. H. (1971) *J. Chem. Phys. 55*, 5829−5831.

Chin, J. H., & Goldstein, D. B. (1981) *Mol. Pharmacol. 19*, 425−431.

Chin, J. H., & Goldstein, D. B. (1984) *Lipids 19*, 929−935.

Chiou, J.-S., Kuo, C.-C., Lin, S. H., Kamaya, H., & Ueda, I. (1991) *Alcohol 8*, 143−150.

Chiou, J.-S., Krishna, P. R., Kamaya, H., & Ueda, I. (1992) *Biochim. Biophys. Acta 1110*, 225−233.

Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117−171.

Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390−394.

Diamond, I. (1992) *Cecil Textbook of Medicine* (Wyngaarden, J. B., Smith, L. H., & Bennett, J. C., Eds.) Vol. 1, pp 44−47, W. B. Saunders, Philadelphia, PA.

Franks, N. P. (1976) *J. Mol. Biol. 100*, 345−358.

Harris, R. A., & Groh, G. I. (1985) *Anesthesiology, 62*, 115−119.

Harris, R. A., Groh, G. I., Baxter, D. M., & Hitzemann, R. J. (1984) *Mol. Pharmacol. 25*, 410−417.

Jain, M., Touissaint, D. G., & Cordes, E. H. (1973) *J. Membr. Biol. 14*, 1−16.

Johnson, D. A., Valenzuela, C. F., & Zidovetzki, R. (1992) *Biochem. Pharmacol. 44*, 769−774.

Klemm, W. R. (1990) *Alcohol 7*, 49−59.

Masserini, M., & Freire, E. (1986) *Biochemistry 25*, 1043−1049.

Masserini, M., Palestini, P., Venerando, B., Fiorilli, A., Acquotti, D., & Tettamanti, G. (1988) *Biochemistry 27*, 7973−7978.

McIntosh, T. J., & Simon, S. A. (1986) *BIochemistry 25*, 4058−4066.

McIntosh, T. J., & Simon, S. A. (1994) *Biochemistry 33*, 10477−10486.

McIntosh, T. J., Magid, A. D., & Simon, S. A. (1989) *Biochemistry 28*, 17−25.

Mehlhorn, I. E., Parraga, G., Barber, K. R., & Grant, C. W. M. (1986) *Biochim. Biophys. Acta 863*, 139−155.

Mitchell, D. C., & Litman, B. J. (1994) *Biochemistry 33*, 12752−12756.

Newman, G. C., & Huang, C. (1975) *Biochemistry 14*, 3363−3369.

Smith, T. L. (1985) *J. Pharmacol. Exp. Ther. 232*, 702−707.

Sternin, E., Bloom, M., & MacKay, A. L. (1983) *J. Magn. Reson. 55*, 274−282.

Svennerholm, L. (1980) *Adv. Exp. Med. Biol. 125*, 533−544.

Terzaghi, A., Tettamanti, G., & Masserini, M. (1993) *Biochemistry 32*, 9722−9725.

Thewalt, J. L., & Bloom, M. (1992) *Biophys. J. 63*, 1176−1181.

Thomas, P. D., & Brewer, G. J. (1990) *Biochim. Biophys. Acta 1031*, 277−289.

Thompson, T. E., & Tillack, T. W. (1985) *Annu. Rev. Biophys. Chem. 14*, 361−386.

Vist, M. R., & Davis, J. H. (1990) *Biochemistry 29*, 451−464.

Wong, P. T. T., & Mantsch, H. H. (1988) *Chem. Phys. Lipids 46*, 213−224.

Wood, W. G., & Schroeder, F. (1992) *Alcohol and Neurobiology: Receptors, Membranes and Channels* (Watson, R. R., Ed.) pp 101−184, CRC Press, Boca Raton, FL.

Worcester, D. L., & Franks, N. P. (1976) *J. Mol. Biol. 100*, 359−378.

Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267−287.

Yeagle, P. L., Hutton, W. C., Huang, C., & Martin, R. B. (1977) *Biochemistry 16*, 4344−4349.

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