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# A DEUTERIUM NMR AND STEADY-STATE FLUORESCENCE ANISOTROPY STUDY OF THE EFFECTS OF CHOLESTEROL ON THE LIPID MEMBRANE-DISORDERING ACTIONS OF ETHANOL

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**Abstract**—We examined the effects of cholesterol on the membrane-disordering action of ethanol by using deuterium nuclear magnetic resonance ($^2$H-NMR) and fluorescence spectroscopy. Specifically, the effects of ethanol were measured on the $^2$H-NMR spectra of di(perdeuteropalmitoyl)phosphatidylcholine (DPPC-$d_{62}$) and on the steady-state emission anisotropy of diphenylhexatriene (DPH) incorporated into hydrated egg phosphatidylcholine (eggPC)/cholesterol dispersions. Analysis of the $^2$H-NMR spectra of DPPC-$d_{62}$ incorporated into eggPC liposomes showed that the addition of cholesterol up to 30 mol% enhanced the ability of ethanol to disorder methylene groups all along the phospholipid acyl chains. This effect was somewhat greater toward the terminal methyl groups. However, above 30 mol% cholesterol, the bilayer-disordering action of ethanol on both the upper and lower portions of the acyl chains decreased to an apparent constant change up to the highest cholesterol content examined (50 mol%). Analysis of the fluorescence anisotropy of DPH, on the other hand, suggested that cholesterol attenuated the ability of ethanol to disorder the bilayers, which is in agreement with a previous EPR study [Chin and Goldstein, *Mol Pharmacol* **19**: 425–431, 1981]. Re-analysis of our previous fluorescence anisotropy results with DPH incorporated into dispersions of brain-lipid extracts as a percent change [Johnson *et al.*, *Mol Pharmacol* **15**: 739–746, 1979] indicated that the chemical composition of the lipid bilayers also affects the apparent ability of cholesterol to modulate the membrane-disordering action of ethanol, because the addition of cholesterol to brain-lipid extracts had no significant effect on the membrane-disordering action of ethanol. Given the greater likelihood that the $^2$H-NMR probes accurately monitor bulk phospholipid properties, some caution is required in the analysis of the membrane-disordering actions of drugs using EPR and fluorescence spectroscopy.

Ethanol is a sedative-hypnotic. No consensus exists on the molecular basis of its action; however, both membrane protein [1] and lipid [2] sites of action of ethanol have been proposed. In lipid membranes, ethanol increases the rotational freedom of membrane probes partitioned into both natural [2, 3] and artificial [4] membranes. Brain plasma membranes from animals chronically exposed to ethanol are less sensitive to the membrane-perturbing effects of ethanol, indicating that tolerance develops to this effect [5]. Artificial membranes formed from the lipid extracts of these tolerant brain membranes are also less susceptible to the membrane-perturbing effects of ethanol, indicating that a lipid composition change is at least partially responsible for the expression of this form of tolerance [4]. While it is unclear what lipid composition change is responsible for this tolerance, the presence of membrane cholesterol is required [4], and some laboratories have detected elevated levels of cholesterol in the brain membranes of ethanol-tolerant animals [6].

Spectroscopic studies of the membrane-perturbing effects of ethanol have primarily utilized EPR or fluorescence spectroscopic methods [1–4]. While these methods have provided much useful information, they suffer from the limitations that they require the use of exogenous, bulky probes which by themselves perturb the lipid membrane and that they only provide information on the molecular motions of the probes, rather than the lipids studied. Deuterium nuclear magnetic resonance ($^2$H-NMR§) spectroscopic approaches, on the other hand, have the advantages that the required probes are not membrane perturbing and more detailed information on the membrane organization can be assessed [7, 8].

Several unresolved issues exist on the lipid membrane-perturbing actions of ethanol: (1) the regions of the lipid membrane that are perturbed, (2) the nature of the ethanol-induced perturbation (e.g. ordering, disordering) and (3) the effect of the cholesterol content on the ability of ethanol to perturb the lipid bilayer. Chin and Goldstein [5], using spin-labeled stearic acid positional isomers, observed that ethanol produces its greatest lipid bilayer-disordering effects toward the interior of egg phosphatidylcholine (eggPC) bilayers. Hitzemann *et al.* [9] examining the effects of [$^2$H]ethanol on the choline and methylene $^1$H-NMR spectra of neuronal lipids, found evidence suggesting that ethanol orders the membrane surface and disorders the membrane

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§ Abbreviations: $^2$H-NMR, deuterium nuclear magnetic resonance; eggPC, eggphosphatidylcholine; DPH, diphenylhexatriene; and DPPC-$d_{62}$, di(perdeuteropalmitoyl)phosphatidylcholine.

core. EPR studies of eggPC/cholesterol dispersions indicate that cholesterol attenuates the ethanol-induced bilayer perturbations [5], while fluorescence emission polarization studies of dispersions of neuronal-membrane lipid extracts suggest the opposite conclusions [4].

In this manuscript we re-examine the effects of cholesterol on the lipid membrane-disordering actions of ethanol by assessing the effects of ethanol on the $^2$H-NMR spectra of di(perdeuteropalmitoyl)phosphatidylcholine (DPPC-$d_{62}$) in hydrated eggPC/cholesterol dispersions. To assess the effect of phospholipid composition of lipid bilayers on the ethanol-modulatory actions of cholesterol and to compare $^2$H-NMR, EPR, and fluorescence approaches for monitoring the membrane-disordering actions of ethanol, we also examined the effects of ethanol on the steady-state emission anisotropy of diphenylhexatriene (DPH) incorporated into eggPC/cholesterol dispersions.

## MATERIALS AND METHODS

EggPC, cholesterol, and DPPC-$d_{62}$ were purchased from Avanti Polar Lipids (Birmingham, AL), and were found to be pure by thin-layer chromatography. Ethanol was obtained from the Gold Shield Chemical Co. (Hayward, CA). All other chemicals were reagent grade.

*$^2$H-NMR spectroscopy.* Multilamellar lipid dispersions were made by first dissolving eggPC/cholesterol/CPPC-$d_{62}$ mixtures in chloroform. The solvent was then evaporated with dry nitrogen and the sample was kept under vacuum (<1 mtorr) for at least 8 hr. The thin film thus formed was hydrated at 40° with 0.5 mL of an aqueous Tris–HCl buffer solution (25 mM, pH 7.4), prepared in $^2$H-depleted $H_2O$ (Sigma Chemical Co., St. Louis, MO). Ethanol was added with the buffer. The pH of the lipid suspensions was found not to be affected by ethanol. The concentration of total phospholipid was 0.2 M, and the eggPC:DPPC-$d_{62}$ ratio was 3:1 (w/w). The samples were typically 30% (w/w) in buffer.

$^2$H-NMR spectra were acquired at 11.74 telsa (corresponding to 500.13 MHz $^1$H-, and 76.78 MHz $^2$H-NMR frequencies) with a General Electric GN-500 spectrometer equipped with a high-power probe (Doty Scientific, Columbia, SC) using standard quadruple echo sequence [10]. The spectral width was 0.5 to 1 MHz, refocusing time 64 μsec, and 90° pulse of 3.5 μsec.

*Fluorescence spectroscopy.* The multilamellar dispersons were prepared basically as were done for the $^2$H-NMR measurements except that DPH (Aldrich, St. Louis, MO) was mixed with the lipid solutions in chloroform and then dried. When resuspended in the Tris–HCl buffer (25 mM, pH 7.4), the concentrations of eggPC and DPH were 250 and 0.62 μM, respectively. All samples containing DPH were protected from ambient light.

Fluorescence measurements were made before and after the addition of ethanol (0.6 M) with a Perkin–Elmer MPF-66 spectrofluorometer set at excitation and emission wavelengths of 355 and 430 nm, respectively. Rotatable Polaroid HNP'B (Norword, MA) and Melles Griot (Irvine, CA) dichroic film polarizers were placed in the paths of the excitation and emission beams, respectively. The temperature of all samples was held at 37° with a water-jacketed cuvette holder. The fluorescence anisotropy, $r$ was calculated according to the following equation:

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}} \quad (1)$$

where $I$ is the fluorescence intensity, and the first and second subscripts refer to vertical or horizontal orientation of polarization of excitation and emission beams, respectively. The factor $G$ corrects for inequivalent transmittance of polarized light and equals $I_{HV}/I_{HH}$.

## RESULTS

*$^2$H-NMR spectroscopy.* The $^2$H-NMR spectrum of the fully hydrated chain-perdeuterated phospholipid represents the superposition of axially averaged powder patterns. This spectrum arises from the different deuterons of the various $C^2H_2$ segments along the phospholipid acyl chains and of the terminal $C^2H_3$ segment. The C—$^2$H bond order parameters, $S^i_{C^2H}$, for each ($i$th) segment can be calculated from the observed peak-to-peak quadrupole splittings, $\Delta\nu^i$, which correspond to the splitting of the $i$th bond that would be obtained for a membrane oriented so that the normal to its surface is perpendicular to the external magnetic field, by using the expression:

$$\Delta\nu^i = (3/4)(e^2qQ/h)S^i_{C^2H} \quad (2)$$

where $(e^2qQ/h)$ equals 167 kHz and is the static quadrupole coupling constant of the deuteron in the C—$^2$H bond in an alkane [11]. The molecular order parameters for the $C^2H_2$ and $C^2H_3$ bonds are $2 \times S_{C^2H}$ and $6 \times S_{C^2H}$, respectively ([12], see also [13] for a discussion of the assumptions involved). $^2$H-NMR studies with lipids deuterated at specific positions in the phospholipid acyl chains, show an order parameter profile along the acyl chains of the lipid molecule in a bilayer state. The superposition of the quadrupole splittings of the first seven to eight $C^2H_2$ segments near the glycerol backbone forms the plateau (maximum splitting) of the $^2$H-NMR spectrum [7, 8–14]. Farther away from the glycerol backbone, the methylene groups are progressively more disordered, usually resulting in well-resolved peaks.

The $^2$H-NMR spectra of DPPC-$d_{62}$ in hydrated dispersions of eggPC at 37° in the presence and absence of ethanol (0.6 M) and/or 50 mol% cholesterol, shown in Fig. 1, are characteristic of a lipid bilayer in the liquid crystalline phase. The spectra in Fig. 1 are typical of lipids in a single phase, suggesting that all of the DPPC-$d_{62}$ was completely miscible with the eggPC/cholesterol. Differential scanning calorimetry measurements also confirmed the absence of unmixed DPPC-$d_{62}$ (data not shown). The presence of cholesterol dramatically increased all peak-to-peak quadrupole splittings (75–140%, Fig. 2, inset), which is consistent with the decreased segmental motion of the phospholipid acyl chains and is in agreement with other NMR studies

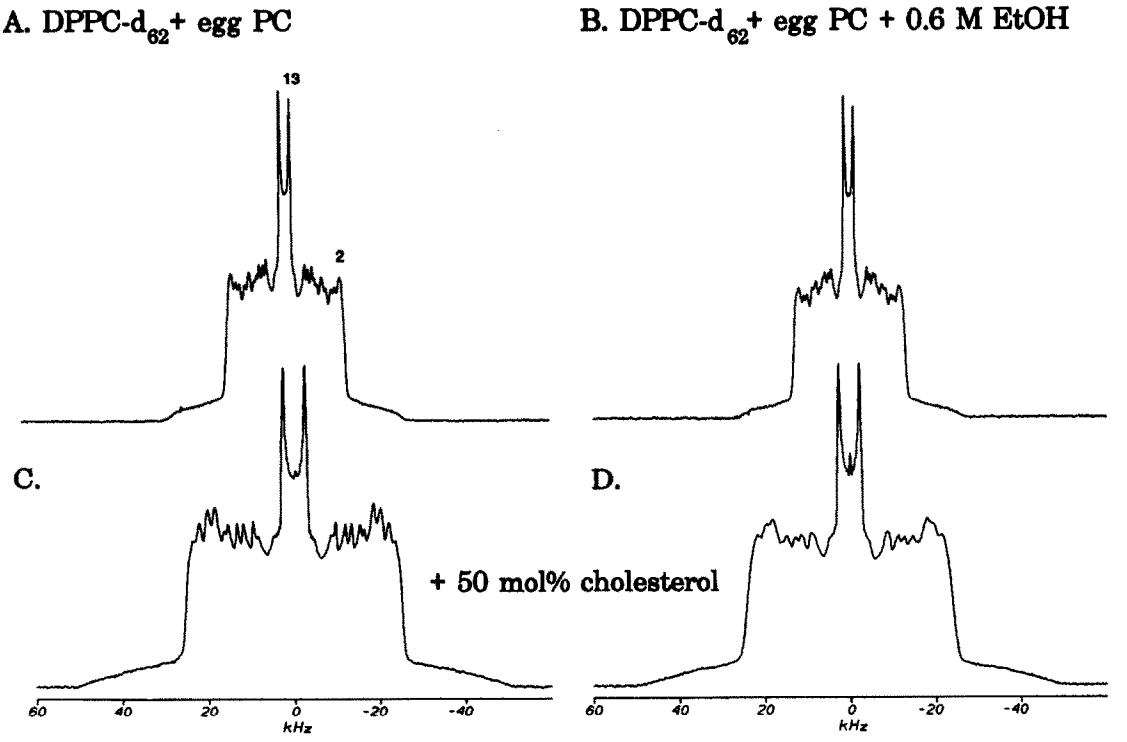


Fig. 1. Effects of cholesterol and ethanol on a $^2$H-NMR spectrum of DPPC-$d_{62}$ in eggPC liposomes (1:3 mol:mol) at 37°. Right (B, D): ethanol (0.6 M) was added. Bottom (C, D): 50 mol% cholesterol was added. Eleven peaks were usually resolved in the spectra at 37°. The reason the numbering of the peaks starts at 2 is that the peaks 1 and 3 are resolved from peak 2 only at a higher temperature.

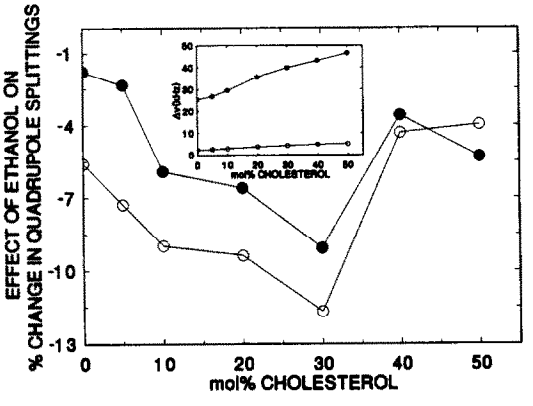


Fig. 2. Effect of cholesterol on ethanol-induced (0.6 M) changes in the peak-to-peak quadrupole splittings of peaks 2 (●) and 13 (○) of the $^2$H-NMR spectrum of DPPC-$d_{62}$ in eggPC liposomes at 37°. Inset: Quadrupole splittings of DPPC-$d_{62}$-eggPC *versus* mol% cholesterol: (●) peak 2 and (○) peak 13.

[15]. The effect of ethanol (0.6 M) on the lipid side-chain order parameters was complex. In the absence of cholesterol, ethanol had little or no significant effect (<2%) on the quadrupole splittings arising from the upper portion of the phospholipid side chains (peak 2) but did have an appreciable effect (~6%) on the terminal methyl groups (peak 13) (Fig. 2). In the presence of cholesterol, ethanol induced a significant *decrease* of the order parameters of all segments of the phospholipid side chains: the magnitude of the effect was a function of the cholesterol concentration. The percent changes in the quadrupole splittings of peaks 2 and 13 produced by ethanol are plotted in Fig. 2. The concentration dependence of cholesterol enhancement of the ethanol-induced perturbation of peaks 2 and 13 was almost U-shaped. Between 0 and 30 mol% addition of cholesterol incrementally enhanced the effects of ethanol from 2 to 9% for peak 2 and from 6 to 12% for peak 13. Above 30 mol% cholesterol, the ethanol effect was reduced to 4–5% and did not change significantly with further addition of cholesterol to 50 mol%.

It should also be noted that ethanol increased the width of the individual peaks in the presence of cholesterol (Fig. 1D); this may reflect a reduction in the membrane fluidity.

*Fluorescence anisotropy spectroscopy.* For comparison, the effect of cholesterol on the ethanol-induced perturbations of the emission anisotropy of DPH incorporated into eggPC/cholesterol dispersions at 37° was assessed. Plotted in Fig. 3 as a percent change in DPH anisotropy produced by ethanol, the results show an almost linear cholesterol concentration-dependent *attenuation* of the ethanol-induced perturbation of DPH anisotropy.

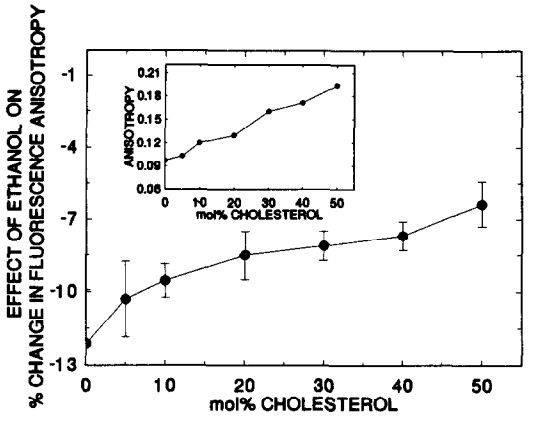


Fig. 3. Effect of cholesterol on ethanol-induced (0.6 M) changes in the fluorescence anisotropy of DPH incorporated into eggPC liposomes at 37°. Values are means ± SD, N = 3. Inset: Fluorescence anisotropy of DPH incorporated into eggPC dispersions *versus* mol% cholesterol in the absence of ethanol.

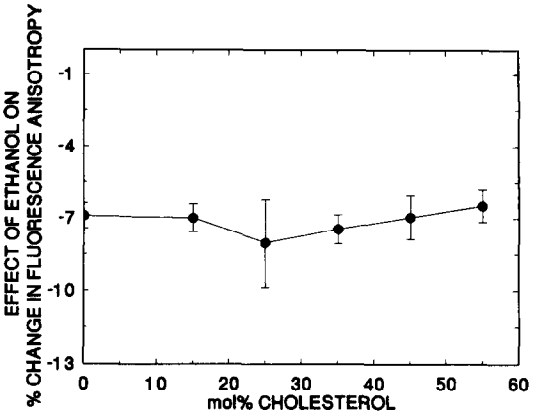


Fig. 4. Re-analysis of previously published data (Fig. 5B in [4]) on the effect of various mol% of cholesterol on ethanol-induced (0.35 M) changes in the fluorescence anisotropy of DPH incorporated into liposomes (100 μM with respect to lipid phosphorus) composed of lipid extracts from murine synaptosomal membranes. Values are means ± SD, N = 3.

## DISCUSSION

In view of the unresolved issues in the literature, we initiated this study to clarify the effects of cholesterol on the membrane-disordering action of ethanol. We used eggPC/cholesterol dispersions in order to compare our results with the findings from other laboratories and to have a relatively simple system with significant acyl chain unsaturation which might better model biological membranes. We found that the effects of ethanol on the mobility of phospholipid acyl chains were complex and depended on the spectroscopic method used. With $^2$H-NMR spectroscopy, the addition of cholesterol up to 30 mol% *enhanced* the ability of ethanol to disorder both the methylene groups toward the upper portion of the phospholipid chains and the terminal methyl groups (Fig. 2). The cholesterol enhancement of the effect of ethanol was slightly greater at the terminal methyl groups than toward the upper portion of the phospholipid chains. Above 30 mol% cholesterol, no significant difference was observed in the acyl chain-disordering action of ethanol either toward the glycerol backbone or the terminal methyl groups. Interestingly, the cholesterol enhancement of the chain-disordering action of ethanol increased almost linearly up to 30 mol% after which it decreased to an apparent constant 4–5% effect up to the highest cholesterol content examined (50 mol%). The physical basis for this complex interaction between ethanol and cholesterol on eggPC/DPPC-$d_{62}$ dispersions is unclear. The membrane actions of drugs can be complicated by several factors such as: (1) the complexity of the phase behavior of the membranes associated with the presence of cholesterol, i.e. a possible phase separation [15], or (2) a cholesterol-induced change in the ethanol/bilayer partition coefficient. Although we are unaware of any work directly assessing the effect of membrane cholesterol on the bilayer/water partition coefficient of ethanol, cholesterol decreases the bilayer/water partition coefficients of pentobarbitone [16] and a variety of gaseous anesthetics [17].

Importantly, our $^2$H-NMR results are in contrast to previous EPR [5] and fluorescence polarization [4] studies on the interaction of membrane-cholesterol and ethanol. Measured as ethanol-induced changes (%), the order parameters of both 5-doxyl- and 12-doxyl-stearic acid were incrementally attenuated by increasing the cholesterol content of bilayers composed of egg PC [5]. The ethanol-induced (0.35 M) percent reduction of the order parameter of the 5-doxyl probe went from ~1.7% to ~1% and the 12-doxyl order parameter went from ~5.8% to ~0.7% as the cholesterol content increased to 50 mol% [5]. When the previously reported fluorescence anisotropy data [4] are re-analyzed as a percent change, the ethanol-induced decrease in diphehylhexatriene anisotropy appears to be unaffected by the addition of cholesterol to bilayers composed of brain-lipid extracts (Fig. 4). Ethanol (0.35 M) produced a 6–8% decrease in the anisotropy of the DPH emission, independently of the cholesterol content of the bilayers.

Because the above-cited EPR and fluorescence studies utilized different phospholipids, eggPC and brain-lipid extracts, respectively, we examined the effect of cholesterol incorporation into eggPC bilayers by monitoring DPH anisotropy. When eggPC was used instead of brain-lipid extracts, the fluorescence spectroscopic approach gave qualitatively the same results as the EPR approach, namely, attenuation of ethanol-induced membrane disordering by cholesterol. This indicates that the membrane chemical composition can determine whether or not cholesterol attenuates the membrane-disordering actions of ethanol when measured by EPR or fluorescence spectroscopy.

Why the EPR and fluorescence spectroscopic

results differ from our $^2$H-NMR results is unclear. The differences between the various approaches cannot be explained by the intrinsic differences in the sampling time scale of the various techniques. The $^2$H-NMR order parameters have about the same magnitude as the EPR 5-doxyl- and 12-doxyl-stearic acid order parameters. Furthermore, sensitivities of both EPR and fluorescence to molecular reorientation correlation times are about the same ($\sim 10_s^{-9}$) [18]. Chemical compositional differences cannot explain the discrepancy, because the same lipids (eggPC) were used with all three spectroscopic approaches. The most likely explanation for the differences between the $^2$H-NMR and EPR and fluorescence results is that the spin and fluorescence probes produce a unique chemical environment for ethanol that differs from the native environment created by phospholipids. One must ultimately conclude that caution is required when utilizing bulky exogenous lipid probes to assess the membrane-disordering action of ethanol. This view has been expressed by others [19, 20].

Although we are unaware of other NMR studies on the effect of cholesterol on the lipid-membrane disordering actions of ethanol, it is possible to deduce the effect of cholesterol on the actions of two long chain *n*-alkanols, 1-octanol and 1-decanol, in eggPC bilayers, by combining the results of two NMR studies from the same laboratory [21, 22]. The effect of the long-chain *n*-alkanol in the presence of cholesterol (~33 mol%) depends on the region of the bilayer examined. The acyl chain-disordering actions of both 1-octanol and 1-decanol are attenuated near the upper portion of the phospholipid acyl chains but enhanced toward the terminal methyl groups. The fact that we observed a cholesterol-induced enhancement of the actions of ethanol on both the acyl groups near the upper portion of the phospholipic acyl chains and the terminal methyl groups may reflect a major difference in the action of short *versus* long chain *n*-alcohols. Westerman *et al.* [23] reported significant differences between short and long chain *n*-alkanols on dimyristoyl-phosphatidylcholine bilayers. 1-Octanol orders the upper portion of the phospholipid acyl chains, whereas 1-butanol disorders them.

In summary, we found that the effects of ethanol on the mobility of phospholipid acyl chains were complex and depended on the spectroscopic method used. Analysis of the $^2$H-NMR spectra of DPPC-$d_{62}$/eggPC dispersions showed that low mol% of cholesterol *enhanced* the ability of ethanol to disorder phospholipid acyl chains. High mol% of cholesterol (>30 mol%) attenuated the actions of ethanol. Analysis of the emission anisotropy of DPH incorporated into eggPC dispersions showed cholesterol to *attenuate* the DPH-disordering actions of ethanol at every mol% examined. Re-analysis of our previous fluorescence anisotropy results with DPH incorporated into dispersions of brain-lipid extracts indicated that the chemical composition of the lipid bilayers also affects the ability of cholesterol to modulate the membrane-disordering action of ethanol. The addition of cholesterol up to 55 mol% has *no measurable effect* on the ability of ethanol to disorder DPH in the bilayers. It should be possible to obtain a greater molecular understanding of the interaction between ethanol and cholesterol by further $^2$H-NMR studies examining such issues as: (1) concentration-dependence of the action of ethanol, (2) the phospholipid and fatty acyl chain composition dependence of this interaction, (3) the effect of cholesterol on the bilayer partitioning of ethanol, and (4) perturbations of the glycerol backbone and choline head group.

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