MOLECULAR PHARMACOLOGY, 19:425–431

Membrane-Disordering Action of Ethanol

Variation with Membrane Cholesterol Content and Depth of the Spin Label Probe

J. H. CHIN AND D. B. GOLDSTEIN

Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

Received August 4, 1980; Accepted December 2, 1980

SUMMARY

CHIN, J. H., AND D. B. GOLDSTEIN. Membrane-disordering action of ethanol: variation with membrane cholesterol content and depth of the spin label probe. *Mol. Pharmacol.* **19**:425–431 (1981).

With the use of 5-doxylstearic acid as the spin probe, we previously found that low concentrations of ethanol *in vitro* reduced the order of mouse biomembranes and that similar membranes isolated from mice after 8 days of ethanol treatment had an elevated cholesterol content and were resistant to the *in vitro* disordering effect of ethanol. To localize the effects of ethanol, compare ethanol effects with temperature effects, and explore the relationship between cholesterol and ethanol, we have now measured order parameters of mouse synaptosomal plasma membranes and of multilayer vesicles composed of egg phosphatidylcholine and cholesterol, spin-labeled with 5-doxyl-, 12-doxyl-, or 16-doxylstearic acid. The bilayer was more ordered at the region monitored by the 5-doxyl than the 12- or 16-doxyl label, and was made less ordered by warming or by the addition of ethanol (0.35 M). The disordering effect of ethanol (decrease in order parameter) was greater at high temperatures than at low temperatures and was progressively reduced by the addition of cholesterol. In brain membranes, ethanol had a greater effect with 12- and 16-doxylstearic acid than with the 5-doxyl label. In phospholipid model membranes, incorporation of cholesterol increased the order parameter of 12-doxylstearic acid more than that of the 5-doxyl label. In general, the effect of ethanol was greater at high temperatures, low cholesterol concentrations, or in the interior of the membrane between the surface and the core. Ethanol may thus act most strongly in relatively disordered domains of neuronal membranes.

INTRODUCTION

Ethanol is among the drugs that fit the Meyer-Overton concept: its anesthetic potency can be predicted from its lipid solubility (1). Previous studies have shown that ethanol and similar anesthetic drugs increase motion in membranes as measured by EPR (2–5), proton (6) or deuterium (7) NMR, or fluorescence techniques (5). Similarly, studies of phase transitions have shown that such drugs increase the proportion of lipid that is fluid (8). Considerable fluidity normally exists in excitable membranes (9), and a controlled degree of fluidity is apparently necessary for function. Thus, it seems possible that nonspecific "disordering" drug action could cause intoxication by disrupting the packing of phospholipids and altering the interaction of lipids and proteins.

Our previous work with biomembranes has shown that ethanol increases mobility of 5-doxylstearic acid, a probe that reflects the order near the surface of membranes (2). The question arose whether the effect of ethanol is confined to the surface of the bilayer. Here we report studies with probes that sample the membrane order at different depths. In addition to the 5-doxylstearic acid spin label, we used 12- and 16-doxylstearic acid probes, and observed that the effect of ethanol varied with depth. Data for mouse brain synaptosomal plasma membranes and for model membranes composed of egg phosphatidylcholine and cholesterol are reported here. The disordering action of ethanol was greater deep in the membrane than near the surface. The effects of ethanol were also compared with those of temperature, in order to investigate whether ethanol acts entirely nonspecifically (physically rather than chemically). If so, its effects should be closely similar to those of an increase in temperature. Ethanol and temperature effects were similar but not identical.

We have also previously shown that tolerance develops to the *in vitro* disordering effect of ethanol in erythrocyte

This research was supported in part by United States Public Health Service Grants AA 01066 and DA 00322.

0026-895X/81/030425-07$02.00/0

and synaptosomal plasma membranes after 8 days of treatment with ethanol *in vivo* (10), and that the ethanol-resistant biomembranes contain more cholesterol than do controls (11). Our present study, therefore, was designed to investigate whether extra cholesterol could cause the reduced membrane response to ethanol. We found that cholesterol attenuates the disordering effect of ethanol. Thus, the magnitude of ethanol's disrupting effect on membranes varies with depth in the bilayer and with the chemical composition of the membrane lipids. Since cholesterol may be distributed unevenly in membranes, a possible mechanism exists for localization of the action of ethanol.

METHODS

Membrane Preparations

Brain membranes. Synaptosomal plasma membranes were prepared as previously described (2), using whole brains pooled from two to four male Swiss Webster mice. They were stored in liquid nitrogen until used. For each experiment, portions of a single membrane preparation were spin-labeled with 5-, 12-, or 16-doxylstearic acid (Syva, Palo Alto, Calif.) by incubation for 30 min at 37° (2) to a probe concentration of about 12 μg/mg of protein. To 20- or 25 μl-samples of spin-labeled membranes, 4 or 5 μl of buffer or diluted ethanol was added. In the final suspension, the ethanol concentration (when used) was 0.35 M and the protein concentration was 20 mg/ml.

Phospholipid model membranes. Egg phosphatidylcholine (Calbiochem, La Jolla, Calif.), cholesterol (Calbiochem) and either 5-doxyl- or 12-doxylstearic acid were combined as follows. Egg phosphatidylcholine, 100 mg/ml in chloroform-methanol (3:2 v/v), was placed in a round or pear-shaped silanized flask. Cholesterol was added in varying amounts, followed by the spin label, which was diluted to 1 part in 250 of lipid by weight. The solvent was removed on a rotary evaporator and the preparation was placed under vacuum overnight. The dried lipid was weighed, hydrated with buffer (145 mM NaCl, 5 mM KCl, 3.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 1 mM $MgSO_4$, pH 7.0) to attain a lipid concentration of 24%, and was then vigorously mixed on a Vortex agitator for 5 min. Either ethanol (final concentration 0.35 M) or buffer was added to samples of these stock preparations. The calculated concentration of ethanol in the lipid, assuming a membrane-buffer partition coefficient of 0.14 (1), was 3–4 mole per cent, depending on the proportion of cholesterol. The final lipid concentration was 20%.

Samples from either brain or phospholipid membranes were mixed on a Vortex for 1 min with ethanol or buffer and allowed to stand at 37° for 10 min. The preparations were stored overnight at 4° with ethanol or buffer before use.

Determination of Order Parameters

The membrane suspension (20–30 μl) was transferred to a heat-sealed disposable micropipette and placed in the microwave cavity of the EPR spectrometer. The temperature of the cavity was maintained within 0.1° by regulating the flow of heated or cooled nitrogen gas passing through a Dewar that formed the lining of the cavity. The process was controlled by a Varian E-257 variable-temperature device, and was monitored by a Newport digital pyrometer. Spectra were obtained with the Varian E104A 9.5 GHz spectrometer over a 100-gauss range at about 3200 gauss, with 5 mW microwave power, 1-gauss modulation amplitude, and scan time of 8 min. The spectra were digitized on line at 0.04-gauss intervals by a PDP-11/03 minicomputer and the relevant maxima and minima were displayed on a VT-55 Decscope. Cursors were positioned to indicate the portion of the curve to be fitted by a smoothed third-order curve from which the locations of the inflection points were determined for estimation of the hyperfine splittings. The order parameter (S) and the isotropic nitrogen hyperfine coupling constant, a'_n, were calculated according to the method of Hubbell and McConnell (12), with correction of the $T_\perp$ value by the method of Gaffney (13). Reference values were those of crystalline 5-doxylpalmitic acid (13). Each sample was scanned three times and the order parameters from each spectrum were averaged to give a single estimate for that sample. Data presented here are means of two to four samples run under each condition. For the brain membranes the experimental results are the mean values for four to nine preparations from different groups of animals. The order parameters could not be calculated at higher temperatures with the 12-doxyl and 16-doxyl labels, since the outer peaks were not well resolved. The inner hyperfine splittings were resolved at all temperatures used.

RESULTS

Brain membranes. Table 1 shows that the order parameters and a'_n values for mouse synaptosomal plasma

TABLE 1

EPR spectral parameters of motion and of polarity at different depths and temperatures in mouse synaptosomal plasma membranes

Membrane fractions were isolated from whole-brain homogenates pooled from two to four mice. A portion of each preparation was spin-labeled with 5-, 12-, or 16-doxylstearic acid and analyzed by EPR to determine the order parameter (S) and the isotropic nitrogen hyperfine coupling constant (a'_n) at different temperatures. Data are expressed as mean ± standard error of mean for the number of preparations shown in parentheses.

	Temperature	5-doxyl label	12-doxyl label	16-doxyl label
Order parameter (S)	10.5°	0.770 ± 0.002 (6)	0.601 ± 0.003 (8)	0.268 ± 0.001 (4)
	19	0.707 ± 0.002 (5)	0.517 ± 0.006 (5)	0.228 ± 0.002 (4)
	28	0.643 ± 0.001 (7)	0.442 ± 0.003 (9)	—
a'_n values (gauss)	10.5	15.46 ± 0.03 (6)	14.68 ± 0.02 (8)	14.11 ± 0.02 (4)
	19	15.26 ± 0.02 (5)	14.31 ± 0.02 (5)	14.18 ± 0.01 (4)
	28	15.19 ± 0.01 (7)	14.16 ± 0.01 (9)	—

membranes were lowest with 16-doxylstearic acid, intermediate with the 12-doxyl label, and highest with the 5-doxyl probe, reflecting the known spin label gradient of increasing acyl chain mobility (12, 14) and decreasing polarity (15) toward the center of the bilayer. Increasing the temperature decreased the order parameter for each spin label, but not to the same extent. The decrease in order parameter on warming the membrane from 10.5 to 19° was 0.084 with 12-doxylstearic acid, 0.063 with the 5-doxyl label, and only 0.040 at the carbon-16 position, when the data are expressed in absolute terms. On a relative basis, the percentage decrease in order parameter going from 10.5 to 19° was 14.9% with 16-doxyl, 14.0% with 12-doxyl, and 8.2% with 5-doxyl label.

For each spin label, ethanol decreased the order parameter, more so as the temperature was increased (Fig. 1). The ethanol effect varied with depth of recording. When the data with 5- and 12-doxylstearic acid at the different temperatures were combined, the magnitude of the ethanol effect was highly correlated with the initial baseline order parameter regardless of whether the ethanol effect was expressed as absolute change ($r = 0.94$, $p < 0.001$, 38 df) or percentage change ($r = 0.94$, $p < 0.001$, 38 df). However, when data with 16-doxylstearic acid were also included in the analyses, the correlation was higher on a percentage basis ($r = 0.81$, $p < 0.001$, 46 df) than on an absolute basis ($r = 0.49$, $p < 0.001$, 46 df). The scatter plot of the data (not shown) indicated that ethanol produced smaller absolute changes in order parameter with 16-doxylstearic acid than with the 12-doxyl label, suggesting that there is a limit to the absolute magnitude of disordering that can be elicited in regions that are already highly disordered (low order parameter). The rank order of sensitivity to ethanol with depth was similar to that of temperature on percentage change basis (16-doxyl > 12-doxyl > 5-doxyl; Fig. 1), but the absolute values led to a different ranking (12-doxyl > 16-doxyl > 5-doxyl). As indicated by the magnitude of the slope (Fig. 1), temperature modulation of the ethanol effect was greater with 16-doxylstearic acid and least with the 5-doxyl probe. Ethanol produced no significant changes in the polarity of the environment of the three spin labels (a'_n values, data not shown).

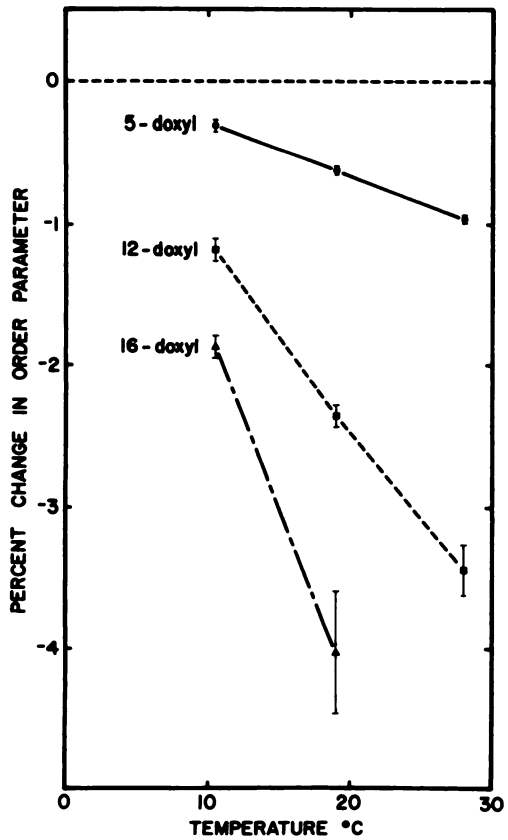

FIG. 1. *Effect of temperature on the membrane response to ethanol in mouse synaptosomal plasma membranes spin-labeled with 5-doxyl-, 12-doxyl-, or 16-doxylstearic acid*

The ordinate is the percentage change in order parameter produced by the addition of ethanol to a final concentration of 0.35 M. Vertical bars indicated the standard error of the mean for four to nine different preparations.

Phospholipid model membranes. Figure 2 shows order parameters of pure lipid vesicles as a function of cholesterol concentration at different temperatures. As in the brain membranes, the order parameters were higher with the 5-doxyl than with the 12-doxyl label. Increasing the temperature decreased the order parameter at all cholesterol concentrations and with both labels. Addition of cholesterol increased the order parameter; the effect was greatest when the concentration was increased from 20 to 30 mole per cent and there was little further effect above 40 mole per cent. This ordering effect of cholesterol was greater when measured with the 12-doxyl than with the 5-doxyl spin label as shown by the steeper slopes in Fig. 2B than in Fig. 2A.

In the lipid model membrane system, ethanol decreased the order parameter under almost all conditions. As in the brain membranes, the magnitude of the disordering effect was progressively decreased as the baseline order parameter was increased ($r = 0.70$, $p < 0.001$, 40 df for absolute changes; $r = 0.78$, $p < 0.001$, 40 df for relative

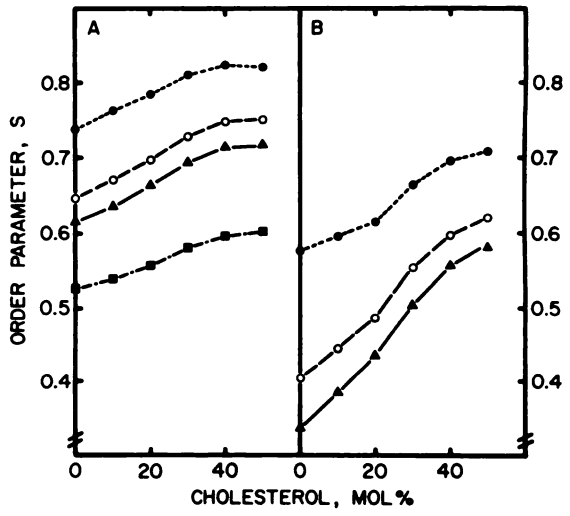

FIG. 2. *Effect of cholesterol on order parameter of spin-labeled egg phosphatidylcholine vesicles*

A, 5-Doxylstearic acid; B, 12-doxylstearic acid. In each part, the *top curve* represents samples analyzed at 0° (●), and the next two curves represent analysis at 10.5° (○) and 15° (▲). In A, data collected at 37° are also shown (■). Points are means of two to four samples. The replicate values of S were close enough together so that all fell within the boundaries of the symbols drawn in the figure.

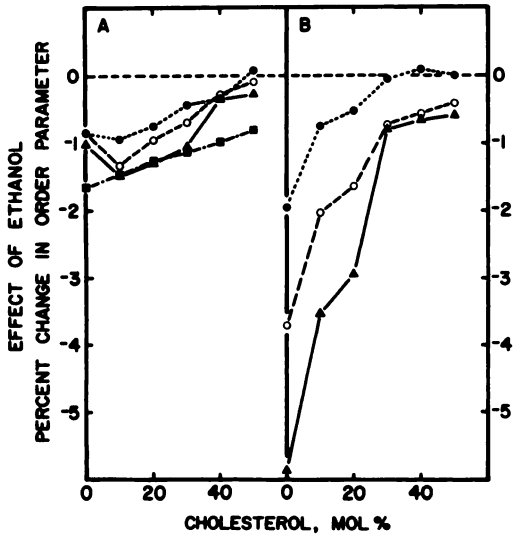

FIG. 3. *Effect of cholesterol on the membrane response to ethanol in egg phosphatidylcholine vesicles spin-labeled with 5-doxyl- (A) or 12-doxylstearic acid (B)*

The ordinate is the percentage change in order parameter produced by addition of ethanol to a final concentration of 0.35 M. Symbols designating temperature for each curve are the same as in Fig. 2.

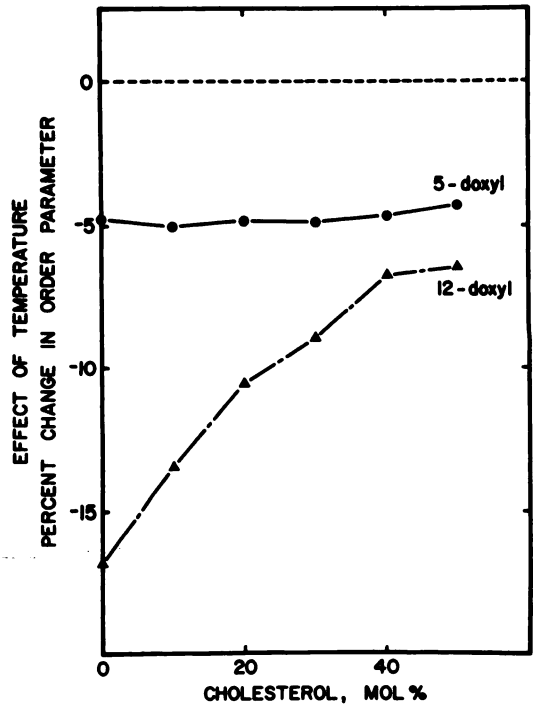

FIG. 4. *Effect of cholesterol on the membrane response to temperature in egg phosphatidylcholine vesicles spin-labeled with 5-doxyl- or 12-doxylstearic acid*

The ordinate is the percentage change in order parameter produced by warming the membrane from 10.5 to 15°.

changes). The extent of the disordering effect depended on the temperature, the cholesterol concentration, and the spin label, as Fig. 3 shows. There was no measurable ethanol effect at 0° with high cholesterol. With the 5-doxyl label (Fig. 3A) the ethanol effect became progressively smaller as cholesterol was increased, and ethanol had more disordering action at high temperature than at low temperature. The percentage changes in order parameters produced by ethanol at the different temperatures and cholesterol contents were subjected to a two-way analysis of variance. Either cholesterol ($F(5,15) = 24.4, p < 0.001$) or temperature ($F(3,15) = 20.7, p < 0.001$) significantly altered the ethanol response at the carbon-5 position. With the 12-doxyl label, reduction of the effect of ethanol by cholesterol was striking (Fig. 3B). In the absence of cholesterol, ethanol decreased the order parameter by about 6% (at 15°), but there was little or no effect of ethanol in the presence of cholesterol, 30–50 mole per cent. [A two-way analysis of variance confirmed the significance of the temperature and cholesterol modulation of the ethanol response with the 12-doxyl label; for ethanol effect versus temperature, $F(2,10) = 10.7, p < 0.01$; for ethanol effect versus cholesterol concentrations, $F(5,10) = 11.6, p < 0.001$.] At low cholesterol levels, the disordering effect of ethanol was larger at the 12-doxyl position than at the 5-position. Over-all, ethanol was most effective in the interior of the membrane, at relatively high temperatures, and in cholesterol-poor membranes (Fig. 3). A three-way analysis of variance demonstrated the statistical significance of each of the factors modulating the effects of ethanol [for spin label, $F(1,10) = 20.7, p < 0.001$; for temperature, $F(2,10) = 14.8, p < 0.001$; and for cholesterol, $F(5,10) = 16.9, p < 0.001$].

The magnitude of the disorder produced by warming lipid vesicles from 10.5 to 15° is shown in Fig. 4 as a function of cholesterol concentration and of depth in the membrane. Cholesterol reduced the response to warming with the 12-doxyl label, but had little effect at the 5-doxyl position. Similar effects of cholesterol were observed over other temperature ranges (0–10.5°; 15–37°). The greatest temperature effects were observed with low cholesterol, in the membrane interior.

DISCUSSION

We undertook these studies in part to determine whether the effect of ethanol was limited to the outer region of the membrane, since it seemed likely that ethanol would be held in this neighborhood by hydrogen bonding. We have confirmed the previously reported disordering action of ethanol (2) and we now find that the effect in brain membranes is stronger at the carbon-12 position on the acyl chains than near the surface. If absolute change in order parameter was measured, the maximal ethanol effect was at position 12, but when percentage change in order parameter was measured, it was most pronounced with 16-doxylstearic acid. Similarly, Lenaz *et al.* (16) reported that 1-butanol, halothane, and ketamine had more effect with the 16-doxyl than the 5-doxyl label in synaptic membranes. Our EPR data agree with NMR studies with specifically deuterated dimyristoylphosphatidylcholine; figure 1c of Turner and Oldfield (7) indicates that high concentrations of benzyl alcohol produced the greatest decrease in quadrupole splitting at carbon-8 rather than nearer the polar head group. We observed larger ethanol effects with 12-doxylstearic acid than with the 5-doxyl label not only in brain membranes but also in low-cholesterol phospholipid vesicles. Our results with high-cholesterol model

membranes, on the other hand, agree with those of Trudell *et al.* (4), who reported that the disordering effect of halothane and methoxyflurane in phospholipid model membranes containing cholesterol, 40 mole per cent, was the same when measured with phospholipids spin-labeled with 6- and 10-doxylstearic acid. Trudell *et al.* (4) suggested that these anesthetics act equally well at different depths in the membranes.

Our data also indicate that cholesterol greatly affects motion in the interior of lipid bilayers, and damps the effect of ethanol and temperature, confirming earlier studies which show that cholesterol modulates the actions of membrane perturbers (17, 18). Although the cholesterol steroid nucleus occupies the outer portion of the membrane leaflet and interacts with the outer 9 or 10 carbon atoms of the phospholipid acyl chains (19), it affects mobility of the 12-doxyl more than the 5-doxyl label (Fig. 2). The greater effect of cholesterol at greater depth in spin-labeled membranes has been previously reported (14, 20). Deuterium NMR data indicate that cholesterol produces almost uniform ordering of the acyl chain in this region when absolute changes are measured (21), but cholesterol has its greatest effect near the middle of the acyl chains if percentage change is measured (21, 22). NMR data also showed that cholesterol orders the terminal methyls least (21, 23), with little or opposite change in mobility of head groups (24). The greater ordering effect of cholesterol at the deeper location may tend to equalize the order at positions 5 and 12, flattening the "order profile" along the acyl chains. The slope of this profile may be important for protein conformation.

The effect of cholesterol on the 12-doxyl label apparently does not require the presence of the rigid steroid nucleus at that position. In support of this view are the NMR experiments of Oldfield *et al.* (25), who found that the change in deuterium order parameter profile produced by a heavy metal that binds to the polar head group was similar to that of cholesterol. By analogy, we assume that the maximal ethanol action at position 12 or 16 gives us no clear indication of the actual location of the drug molecules. Our results do not rule out the possibility that ethanol resides near the surface of the membrane but exerts its effect most strongly in the interior.

Cholesterol modulates both the disordering and ordering effects of membrane perturbers. It blocked the disordering action of ethanol in phospholipid vesicles in the present experiment and that of hexanol in an earlier report (26). In contrast, it also reduced the ordering effect of chlorpromazine and pentobarbital in model (18) or natural (17) membranes with low cholesterol, but in membranes with high cholesterol concentrations it reversed ordering to a net disordering (17, 18). Octanol, however, disordered membranes with low or high cholesterol content (18). Cholesterol might act in our experiments by reducing the partitioning of ethanol into the membrane, as shown for pentobarbital (27). Alternatively, if ethanol indeed enters the cholesterol-rich membrane, its disordering effect may somehow be blocked. The distribution of cholesterol may not be uniform in the plane of the bilayer, depending upon the membrane's chemical composition and structural organization. Membranes may contain cholesterol-rich and cholesterol-poor domains. If so, ethanol may act most strongly in low-cholesterol regions. This gives a possibility of preferential disruption of membrane function at different anatomical locations or in the vicinity of specific proteins. Such molecular segregation may account for the higher ethanol sensitivity of biomembranes than lipid membranes in our study, even when both contained cholesterol, 40 mole per cent.

Besides cholesterol, other conditions that ordered the membrane were also associated with a reduced response to ethanol. Lowering the temperature in brain or phospholipid membranes as in our study, or raising the pressure of spin-labeled isolated nerves treated with ethanol (28) partially reversed the disordering effect of ethanol. These results suggest that it is the inherent order of the membrane itself, rather than the specific perturber, which determines the magnitude of the ethanol response.

Mice that have been treated for 8 days with ethanol have cell membranes that are relatively unresponsive to the disordering effect of ethanol (10), and these membranes have 10–15% more cholesterol than controls (11). Our present data suggest that the extra cholesterol could account for some of the tolerance. The cholesterol synthesis inhibitor, diazacholesterol, given chronically to young mice, inhibited the subsequent development of functional tolerance to ethanol, also suggesting that cholesterol may mediate tolerance to ethanol (29). However, the observed change in membrane cholesterol would not be enough to explain the degree of tolerance *in vitro*, if the cholesterol effect were the same in biomembranes as in liposomes. There may be other lipid changes in tolerant membranes, such as the increased ratio of saturated to polyunsaturated fatty acids reported by Littleton and John (30). Johnson *et al.* (31) found that membranes from the crude P2 fraction of brain from tolerant mice had a slight (not statistically significant with $N = 3$) increase in cholesterol, but the difference in ethanol sensitivity between membrane lipids from tolerant and normal mice persisted even when the cholesterol content of the lipids was made equal *in vitro*. Nutritionally controlled studies in rats showed that chronic ethanol treatment produced some regional specificity of brain lipid changes (32); cholesterol and phosphatidylserine were significantly increased in microsomes, but cholesterol was decreased in myelin with no change in phosphatidylserine.

We used temperature as a variable here in order to compare its purely physical effects with those of chemical agents (cholesterol or ethanol) that might be localized in some region of the bilayer. We observed that the effects of ethanol and temperature were similar but not identical. In phospholipid model membranes we observed a larger temperature effect with the 12-doxyl than with the 5-doxyl label (Fig. 4), confirming results obtained with oriented multibilayers (33). Thus, temperature, like ethanol, does not affect order parameters equally in all regions of the membrane. Our observation that temperature effects with the 12-doxyl label were reduced by the addition of cholesterol to the bilayer (Fig. 4) agrees with NMR data of Darke *et al.* (23). The buffering of temperature effects by cholesterol apparently takes place fairly

deep in the membrane because, with the 5-doxyl label, cholesterol did not affect the temperature response (Fig. 4).

Recently Taylor and Smith (34) have raised the possibility that order parameters determined with doxyl spin labels do not faithfully represent the effects of external agents. The variation of the order parameter with position along the acyl chain produces a profile that is characteristic of EPR with doxyl spin labels but differs from that obtained by NMR with deuterated probes. The discrepancies between the techniques have still not been resolved but have generally been attributed to perturbation of the bilayer by the doxyl group or to the different time scales of EPR and NMR (35). Taylor and Smith (34) suggest that doxyl spin labels produce large perturbations in membranes and give inaccurate responses to the addition of cholesterol. They measured the quadrupole splitting of deuterated stearic acid with and without an added 5-doxyl group in egg phosphatidylcholine; the attachment of the doxyl group directly to the fatty acid between two deuterated carbon atoms reversed the usual ordering effect of cholesterol. However, they showed in the same paper that cholesterol increases EPR order parameters when the doxyl probe is used alone, as it does by NMR when only the deuterium probe is used. It seems to be the doubly labeled probe rather than the doxyl group itself that distorts the results.

Although data obtained with any extrinsic probe must be viewed with caution (since it is the property of the probe that is being measured) and different techniques may measure different aspects of the physical situation, our present EPR data, as already discussed, agree with those from deuterium NMR in many respects. By both techniques membranes are disordered by increasing temperature (22) or by anesthetic agents (7) and are ordered by cholesterol (21, 22). Also, cholesterol has its greatest ordering effect near the middle of acyl chains when data are expressed as relative changes (21, 22), and alcohols disorder membranes more in the interior than near the surface (7). In light of the similarities of EPR results to NMR results, we consider that doxyl spin labels are sensitive and informative monitors of membrane organization and its modulation by external agents.

In general, ethanol, cholesterol, and temperature acted most strongly in the middle region of the acyl chain. Ethanol's disordering effect was greatest at high temperatures, at low cholesterol concentrations, and at some position relatively distant from the membrane surface. A mechanism for localization of the action of ethanol both vertically and horizontally in membranes can now be envisioned.

ACKNOWLEDGMENTS

We wish to thank Ken Dill, James Trudell, and Robbe Lyon for helpful discussions.

REFERENCES

1. Seeman, P. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**:583–655 (1972).
2. Chin, J. H., and D. B. Goldstein. Effects of low concentrations of ethanol on the fluidity of spin-labeled erythrocyte and brain membranes. *Mol. Pharmacol.* **13**:435–441 (1977).
3. Paterson, S. J., K. W. Butler, P. Huang, J. Labelle, I. C. P. Smith, and H. Schneider. The effects of alcohols on lipid bilayers: a spin label study. *Biochim. Biophys. Acta* **266**:597–602 (1972).
4. Trudell, J. R., W. L. Hubbell, and E. N. Cohen. The effect of two inhalation anesthetics on the order of spin-labeled phospholipid vesicles. *Biochim. Biophys. Acta* **291**:321–327 (1973).
5. Lenaz, G., E. Bertoli, G. Curatola, L. Mazzanti, and A. Bigi. Lipid protein interactions in mitochondria: spin and fluorescence probe studies on the effect of *n*-alkanols on phospholipid vesicles and mitochondrial membranes. *Arch. Biochem. Biophys.* **172**:278–288 (1976).
6. Metcalfe, J. C., P. Seeman, and A. S. V. Burgen. The protion relaxation of benzyl alcohol in erythrocyte membranes. *Mol. Pharmacol.* **4**:87–95 (1968).
7. Turner, G. L., and E. Oldfield. Effect of a local anaesthetic on hydrocarbon chain order in membranes. *Nature (Lond.)* **277**:669–670 (1979).
8. Jain, M. K., and N. M. Wu. Effect of small molecules on the dipalmitoyl lecithin liposomal bilayer. III. Phase transition in lipid bilayer. *J. Membr. Biol.* **34**:157–201 (1977).
9. Hubbell, W. L., and H. M. McConnell. Spin-label studies of the excitable membranes of nerve and muscle. *Proc. Natl. Acad. Sci. U. S. A.* **61**:12–16 (1968).
10. Chin, J. H., and D. B. Goldstein. Drug tolerance in biomembranes: a spin label study of the effects of ethanol. *Science (Wash. D. C.)* **196**:684–685 (1977).
11. Chin, J. H., L. M. Parsons, and D. B. Goldstein. Increased cholesterol content of erythrocyte and brain membranes in ethanol-tolerant mice. *Biochim. Biophys. Acta* **513**:358–363 (1978).
12. Hubbell, W. L., and H. M. McConnell. Molecular motion in spin-labeled phospholipids and membranes. *J. Am. Chem. Soc.* **93**:314–326 (1971).
13. Gaffney, B. J. Practical considerations for the calculation of order parameters for fatty acid or phospholipid spin labels in membranes, in *Spin Labeling. Theory and Applications* (L. J. Berliner, ed.). Academic Press, New York, 567–571 (1976).
14. McConnell, H. M., and B. G. McFarland. The flexibility gradient in biological membranes. *Ann. N. Y. Acad. Sci.* **195**:207–217 (1972).
15. Griffith, O. H., P. J. Dehlinger, and S. P. Van. Shape of the hydrophobic barrier of phospholipid bilayers (evidence for water penetration in biological membranes). *J. Memb. Biol.* **15**:159–192 (1974).
16. Lenaz, G., G. Curatola, L. Mazzanti, E. Bertoli, and A. Pastuszko. Spin label studies on the effect of anesthetics in synaptic membranes. *J. Neurochem.* **32**:1689–1695 (1979).
17. Neal, M. J., K. W. Butler, C. F. Polnaszek, and I. C. P. Smith. The influence of anesthetics and cholesterol on the degree of molecular organization and mobility of ox brain white matter. Lipids in multibilayer membranes: a spin probe study using spectral simulation by the stochastic method. *Mol. Pharmacol.* **12**:144–155 (1976).
18. Pang, K.-Y. Y., and K. W. Miller. Cholesterol modulates the effects of membrane perturbers in phospholipid vesicles and biomembranes. *Biochim. Biophys. Acta* **511**:1–9 (1978).
19. Huang, C.-H. A structural model for the cholesterol-phosphatidylcholine complexes in bilayer membranes. *Lipid* **12**:348–356 (1977).
20. Schreier-Muccillo, S., D. Marsh, H. Dugas, H. Schneider, and I. C. P. Smith. A spin probe study of the influence of cholesterol on motion and orientation of phospholipids in oriented multibilayers and vesicles. *Chem. Phys. Lipids* **10**:11–27 (1973).
21. Stockton, G. W., and I. C. P. Smith. A deuterium nuclear magnetic resonance study of the condensing effect of cholesterol on egg phosphatidylcholine bilayer membranes. I. Perdeuterated fatty acid probes. *Chem. Phys. Lipids* **17**:251–263 (1976).
22. Oldfield, E., M. Meadows, D. Rice, and R. Jacobs. Spectroscopic studies of specifically deuterium labeled membrane systems. Nuclear magnetic resonance investigation of the effects of cholesterol in model systems. *Biochemistry* **17**:2727–2740 (1978).
23. Dare, A., E. G. Finer, A. G. Flook, and M. C. Phillips. Nuclear magnetic resonance study of lecithin-cholesterol interactions. *J. Mol. Biol.* **63**:265–279 (1972).
24. Brown, M. F., and J. Seelig. Influence of cholesterol on the polar region of phosphatidylcholine and phosphatidylethanolamine bilayers. *Biochemistry* **17**:381–384 (1978).
25. Oldfield, E., M. Glaser, R. Griffin, R. Haberkorn, R. Jacobs, M. Meadows, D. Rice, R. Skarjune, and D. Worcester. Spectroscopic studies of specifically deuterium labelled membrane systems, in *Biomolecular Structure and Function* (P. F. Agris, R. N. Loeppky, and B. D. Sykes , eds.). Academic Press, New York, 55–64 (1978).
26. Jain, M. K., D. G. Touissaint, and E. H. Cordes. Kinetics of water penetration into unsonicated liposomes: effects of *n*-alkanols and cholesterol. *J. Membr. Biol.* **14**:1–16 (1973).
27. Miller, K. W., and S.-C. T. Yu. The dependence of the lipid bilayer membrane: buffer partition coefficient of pentobarbitone on pH and lipid composition. *Br J. Pharmacol.* **61**:57–63 (1977).
28. Mastrangelo, C. J., J. J. Kendig, J. R. Trudell, and E. N. Cohen. Nerve membrane lipid fluidity: opposing effects of high pressure and ethanol. *Undersea Biomed. Res.* **6**:47–53 (1979).
29. Grieve, S. J., J. M. Littleton, P. Jones, and G. R. John. Functional tolerance to ethanol in mice: relationship to lipid metabolism. *J. Pharm. Pharmacol.* **31**:737–742 (1979).

30. Littleton, J. M., and G. John. Synaptosomal membrane lipids of mice during continuous exposure to ethanol. *J. Pharm. Pharmacol.* **29**:579–580 (1977).
31. Johnson, D. A., N. M. Lee, R. Cooke, and H. H. Loh. Ethanol-induced fluidization of brain lipid bilayers: required presence of cholesterol in membranes for the expression of tolerance. *Mol. Pharmacol.* **15**:739–746 (1979).
32. Moscatelli, E. A., and P. Demediuk. Effects of chronic consumption of ethanol and low-thiamin, low-protein diets on the lipid composition of rat whole brain and brain membranes. *Biochim. Biophys. Acta* **596**:331–337 (1980).
33. Marsh, D. Intermolecular steric interactions and lipid chain fluidity in phospholipid multibilayers: a spin label study. *Can. J. Biochem.* **52**:631–636 (1974).
34. Taylor, M. G., and I. C. P. Smith. The fidelity of response by nitroxide spin probes to changes in membrane organization. The condensing effect of cholesterol. *Biochim. Biophys. Acta* **599**:140–149 (1980).
35. Schreier, S., C. P. Polnaszek, and I. C. P. Smith. Spin labels in membranes. Problems in practice. *Biochim. Biophys. Acta* **515**:375–436 (1978).

Send reprint requests to: Dr. J. H. Chin, Department of Pharmacology, Stanford University School of Medicine, Stanford, Calif. 94305.