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Effects of Low Concentrations of Ethanol on the Fluidity of Spin-Labeled Erythrocyte and Brain Membranes

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SUMMARY

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The effects of ethanol on membrane fluidity at 37° have been assessed by a sensitive electron paramagnetic resonance technique. Erythrocyte and brain membranes from DBA/2J mice were spin-labeled with 5-doxylstearic acid (*N*-oxyl-4',4'-dimethyloxazolidine derivative of 5-ketostearic acid). The molecular motion of the spin label was measured from the EPR spectrum by determining the order parameter *S*, an index of membrane fluidity. The fluidity of both erythrocyte and synaptosomal membranes was greater than that of myelin but less than that of mitochondrial membranes. The addition of low concentrations (0.02 or 0.04 M) of ethanol *in vitro* increased fluidity in erythrocyte, mitochondrial, and synaptosomal membranes. This fluidizing effect of ethanol was dose-related up to 0.35 M in all the membranes except myelin. These data suggest that nonlethal concentrations of ethanol may increase membrane fluidity *in vivo*.

INTRODUCTION

The simplicity of the chemical structure of ethanol suggests that its effects on membranes are similar to those of a large group of structurally unrelated anesthetic agents. The correlation of anesthetic potency with lipid solubility among these latter compounds has led to the concept that anesthetic agents disrupt neuronal function by acting nonspecifically in hydrophobic regions of membranes rather than specifically at receptor sites. Changes in membrane fluidity affect biological functions such as growth, membrane permeability, transport, enzymatic activity, and fusion processes (1, 2). Anesthetic agents expand membranes (3) and increase the fluidity of both biological and model membrane systems (4-7).

The present experiments were undertaken to determine whether concentrations of ethanol that would be nonlethal *in vivo* could change the fluidity of biomembranes as measured by a fatty acid spin label and electron paramagnetic resonance techniques. Similar studies with much higher ethanol concentrations or with more potent alcohols have been reported by others (6, 8-10). In the present, more detailed study we used DBA/2J mice, an inbred strain that is particularly well suited for our studies of physical dependence on ethanol (11).

METHODS

Male DBA/2J mice weighing 20-25 g were obtained from Jackson Laboratories,

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Bar Harbor, Me. Cardiac blood from six to eight mice was combined for preparation of erythrocyte membranes, and the brains of the same groups of mice were pooled for preparation of brain membrane fractions. Erythrocyte membranes were prepared by a modification of the method used by Kury and McConnell (12). The cells were washed three times and brought to 50% hematocrit in isotonic buffer (13) (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 3.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 10 mM dextrose, pH 7). The cells were lysed by a 10-min incubation at 0° with 5 volumes of a 1:5 dilution of the buffer, a lysis technique that minimizes solubilization of membrane-bound proteins (14). The membranes were washed three times with buffer, suspended in buffer to a concentration of 67% particulate matter by volume, and stored under nitrogen at 4°. They were used within 24 hr of preparation.

Synaptosomal membranes, myelin, and mitochondria were isolated from mouse brains by the method of Jones and Matus (15), a combined flotation and sedimentation density gradient centrifugation technique. After separation from the sucrose gradient, both myelin and mitochondrial fractions were hypotonically lysed by addition of glass-distilled water and sonicated for three 1-min periods. All separated fractions were diluted with buffer and pelleted by centrifugation for 1 hr at 144,000 × g. Brain membrane fractions were suspended in the same buffer used for the erythrocytes, except that no dextrose was added, to make a final protein concentration (16) of 8 mg/ml for myelin, 15 mg/ml for synaptosomal membranes, and 18 mg/ml for mitochondrial membranes. Aliquots of the brain fractions were stored under nitrogen at −15° until used.

Membranes were spin-labeled with 5-doxylstearic acid (*N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid, Syva Company, Palo Alto, Ca.). Solutions of the spin label, 0.15 mg in ethanol, were evaporated on the sides of silanized conical tubes with nitrogen and placed under vacuum overnight to remove all traces of the solvent. A sample (600 μl) of freshly prepared or thawed membranes was sonicated for 5–15 sec, transferred to the coated tube containing the spin label fatty acid, and incubated at 37° for two 15-min periods, each followed by 1 min of Vortex mixing. The spin-labeled membranes were then removed to another clean tube to be stored on ice until analyzed. Approximately 80% of the spin label was incorporated into the membranes. From literature values of the protein to lipid ratio in the different membranes (17), the spin label concentration was calculated to be approximately 1% by weight of membrane lipid. The residual spin label in the coated tube was dissolved in absolute ethanol and was quantitatively determined from the amplitude of the low-field signal in the EPR isotropic spectrum. Prior to recording of EPR spectra, 5 μl of buffer or dilute ethanol were added to 50 μl of the spin-labeled membranes. The suspension was mixed on a Vortex apparatus for 1 min and then incubated for 10 min at 37°. The spin-labeled membrane dispersion (50 μl) was transferred to a disposable pipette with its tip sealed and the other end covered with Parafilm. The sample was then centered vertically in a microwave cavity thermostated at 37° ± 0.1° and was allowed to equilibrate. EPR spectra were measured on a modified Varian EM-500 spectrometer and digitized on line by a PDP 8/e computer. As previously described (18), the sensitivity and accuracy of our spectral measurements were enhanced by computer curve fitting and determination of the hyperfine splittings which were used in the calculation of the order parameter, S, by the method of Hubbell and McConnell (19). The order parameter, a commonly used index of membrane fluidity, can have values from zero to one. It is zero in a completely fluid substance and one in a rigid matrix. S was calculated from the equation

$$S = \frac{(T_{\parallel}' - T_{\perp}')}{(T_{zz} - 1/2(T_{xx} + T_{yy}))} \cdot \frac{a}{a'}$$

where $T_{zz} = 30.8$ G, $T_{xx} = T_{yy} = 5.8$ G, $a = 1/3(T_{zz} + T_{yy} + T_{xx})$, and $a' = 1/3(2T_{\perp}' + T_{\parallel}')$.

The spectrum from each sample was scanned two or three times to obtain aver-

age values of S. Samples with different concentrations of ethanol were studied in random order with control samples interspersed. Within the same experiment, replicate samples were analyzed when possible. The experimental results are the mean values of the change from baseline controls in each of five different experiments.

RESULTS

Membrane fluidity (Fig. 1) was different in the four types of membrane. Myelin was the most ordered, and mitochondrial membranes the most fluid.

An example of the spectral changes induced by low concentrations of ethanol is illustrated in Fig. 2. The very small decrease in the outer hyperfine splitting ($2T_{\parallel}'$) on addition of ethanol to 0.09 M is indicated by the shift in the inflection point of the high-field peak from *a* to *b*. Such small changes could be detected reliably only by use of a computer and by repeated measurements.

Membrane fluidity was increased by the addition of ethanol (0.01–0.35 M aqueous concentration) in all types of membranes (Figs. 3–6). In erythrocyte and synaptosomal membranes the decreases in the order parameter were statistically significant at

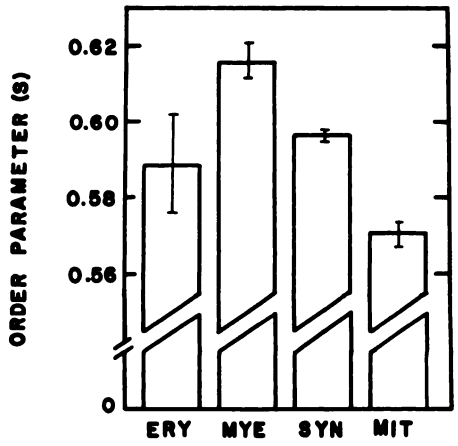

FIG. 1. *Membrane fluidity as measured by the order parameter S in erythrocyte (ERY), myelin (MYE), synaptosomal (SYN), and brain mitochondrial (MIT) membranes*

Each bar represents the mean of five membrane preparations, each from six to eight mice. Vertical bars indicate standard errors of the mean.

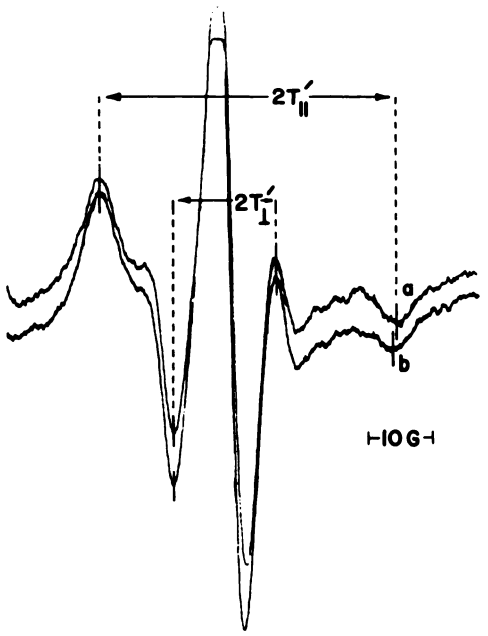

FIG. 2. *First derivative EPR spectra of 5-doxylstearic acid spin label in mouse synaptosomal membranes at 37°*

Lower curve, ethanol added to a final concentration of 0.09 M; upper curve, control (buffer added). The PDP 8/e computer digitized the data on line at 0.05-G intervals, identified inflection points by fitting parabolic curves to the peaks, marked these points with vertical lines as shown, and computed $2T_{\parallel}'$ and $2T_{\perp}'$ from the hyperfine splittings. The corrected isotropic nitrogen hyperfine coupling constant (a') and the order parameter (S) were then computed by the method of Hubbell and McConnell (19).

low concentrations of ethanol. The ethanol effect was generally concentration-related, and the slopes of the regression lines calculated from the changes in order parameter vs. concentration were significantly different from zero ($p < 0.01$ for erythrocyte and synaptosomal membranes; $p < 0.05$ for mitochondrial membranes). However, in mitochondrial membranes no significant change in fluidity was observed in 0.09 M ethanol (Fig. 5). At the highest concentration of ethanol (0.35 M) the order parameter was significantly less than control in all the membranes except myelin.

DISCUSSION

The decreases in the order parameter that we report here are very small. The

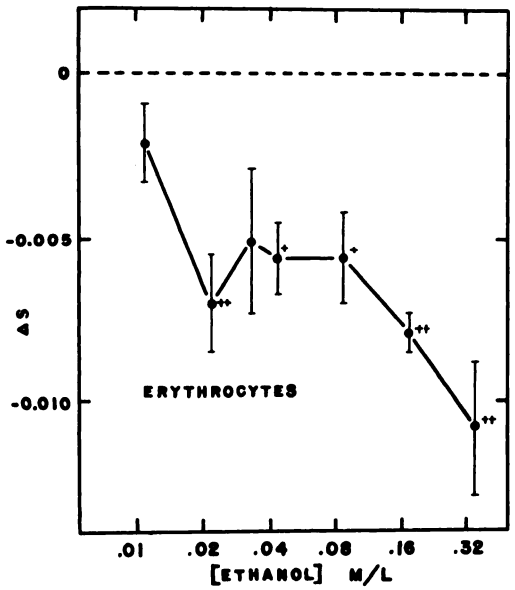

FIG. 3. *Change in order parameter* (ΔS) *of erythrocyte membranes with added ethanol*

Five preparations of erythrocyte membranes from DBA mice were examined at each of the ethanol concentrations shown on the abscissa. The ordinate represents changes in S from the baseline (without ethanol) in each preparation. Vertical bars indicate standard errors of the mean. The significance of differences from baseline order parameter is: +, $p < 0.05$; ++, $p < 0.01$.

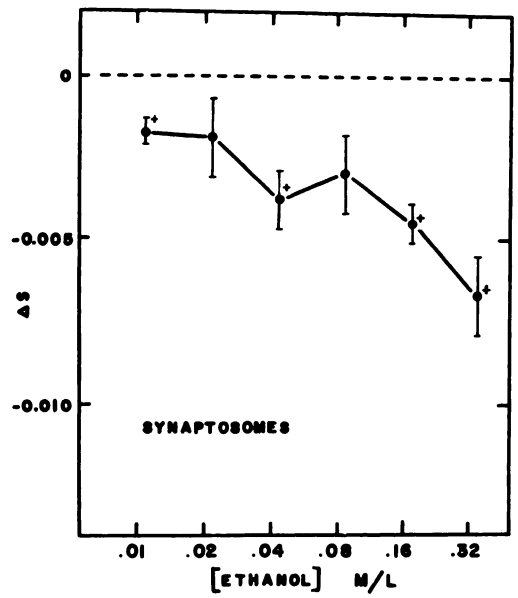

FIG. 4. *Changes in membrane fluidity* (ΔS) *with ethanol in synaptosomal membranes*

Symbols are the same as in Fig. 3.

use of the computer to increase the sensitivity and accuracy of our measurements allowed us to observe smaller changes than are detectable with the conventional manual analysis of spectra. We believe our results are meaningful because they show concentration-related membrane effects of ethanol in the expected direction and with a magnitude that would be predicted by extrapolation from the results of others, who used much higher concentrations of membrane-active drugs. We can compare our results with those of Trudell *et al.* (7) in the following way. Our midrange ethanol concentration, 0.09 M aqueous, is equivalent to about 0.02 mole of drug in the membrane per mole of lipid, assuming a membrane/buffer partition coefficient of 0.14 (3) and assuming that lipids with mol wt 800 make up approximately half the membrane volume. Trudell *et al.* (7) added halothane or methoxyflurane to phospholipid-cholesterol model membranes and observed a change in order parameter of about −0.025 for each 0.1 concentration unit, over the range 0.1–0.6 mole of drug per mole of lipid, for either drug. Extrapo-

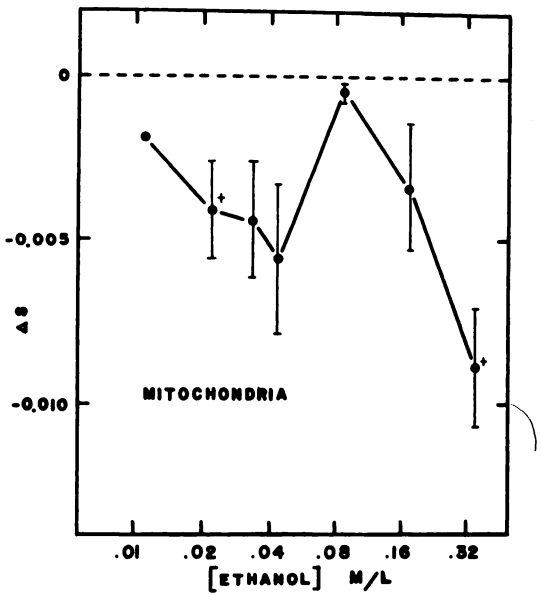

FIG. 5. *Changes in membrane fluidity* (ΔS) *with ethanol in mitochondrial membranes*

The increase in membrane fluidity at 0.03 and 0.04 M ethanol was significant at $p < 0.05$ in a one-tailed test. Symbols are the same as in Fig. 3.

lating downward to our concentration of 0.02 mole of ethanol per mole of lipid, we would expect $\Delta S = -0.005$, which is the value we observed.

Small and consistent effects might, however, be due to small systematic errors. We randomized the order of testing different concentrations of ethanol so that any progressive time-dependent changes would not give spurious results. Attachment of ethanol to the spin label probe itself, perhaps by hydrogen bonding, is another possibility. However, this should occur in all samples without regard for the type of membrane. Our failure to find an ethanol effect in myelin tends to rule out such an artifact. Temperature changes also affect the order parameter. We have calculated from the data of others (20, 21) that the temperature must be increased 0.65–0.85° in order to decrease the order parameter by 0.005 in synaptosomal membranes at 37°. Since our EPR cavity was thermostated to within ±0.1°, temperature fluctuations cannot account for the observed decreases in order parameter.

We believe, therefore, that we have measured a real ethanol effect on fluidity in membranes. It is another question entirely to inquire whether such small changes in fluidity could affect neuronal function. An analogy to the phenomenon of pressure reversal of anesthesia suggests that they could. Part of the argument that anesthetic agents act by disordering membrane structure rests on the observation that anesthetized animals will regain their righting reflex when helium pressure is applied, presumably because membranes are forced back into an ordered configuration. In accordance with this hypothesis, it has been shown that elevated pressure raises the order parameter in model membranes (18). Pressure of 34 atm, which is in the range for partial reversal of anesthesia (22, 23), changes the order parameter by +0.005. In our experiments, there is a change of −0.005 with ethanol at anesthetic concentrations (0.09 M). If an increase in S of 0.005 can wake an anesthetized animal, it is reasonable to suppose that a decrease in S of 0.005 could be related to depressant actions of ethanol.

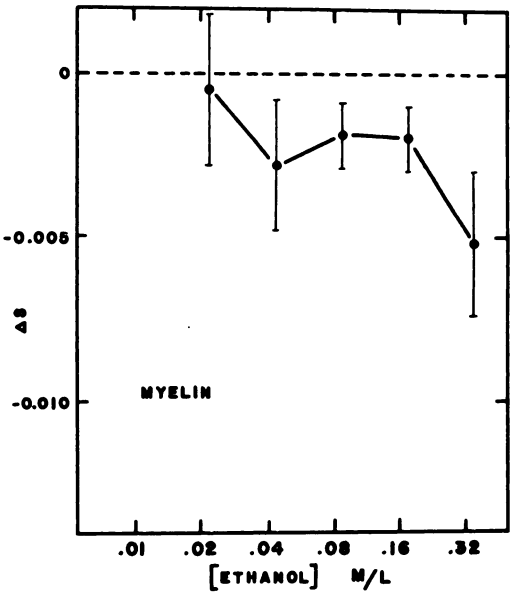

FIG. 6. *Change in membrane fluidity* (ΔS) *with ethanol in myelin*

Symbols are the same as in Fig. 3.

In contrast to our ethanol findings of increased membrane fluidity in the mouse, Rosenberg *et al.* (21) reported that low concentrations of halothane produced an ordering effect in synaptosomal membranes in the rat. In preliminary experiments we found that 28.5% (w/w) sucrose, which was used by these workers to suspend their membrane preparations, was too viscous for optimal EPR recordings. We thus routinely removed most of the sucrose by pelleting all membrane fractions in excess buffer and then resuspended them in isotonic buffer. Differences in the suspending medium may account for the discrepancy between the ethanol and halothane results at low concentrations, although species differences have not been ruled out.

Several previous studies have shown that alcohols increase fluidity in biological or model membrane systems as measured by nuclear magnetic resonance (5, 24), electron paramagnetic resonance (6, 8, 9, 10), optical methods (25), differential scanning calorimetry (26), or fluorescence probes (10, 27), but these membrane effects were observed only with much higher ethanol concentrations than used in our

experiments, or with the more potent lipid-soluble aromatic or longer-chain aliphatic alcohols. Data from different laboratories thus support our findings that ethanol increases membrane fluidity.

Our spin label results showing differences in fluidity in four types of mouse membranes (Fig. 1) confirm our preliminary studies in Swiss Webster mice (28) and are consistent with those obtained from spin-labeled rat membranes (20) and from microviscosity measurements by fluorescence probes (29, 30). The higher order parameter reported by Rosenberg *et al.* (21) for rat synaptosomal membrane may be due to the high concentration of sucrose in their suspending medium. The magnitude of the order parameter may reflect differences in chemical composition among the membrane types, such as the ratio of cholesterol to phospholipid, the degree of unsaturation of the fatty acids, the type of phospholipid, or the type of proteins. The order parameter was highest in myelin, which has the highest cholesterol to phospholipid ratio in its membrane (17) and less polyunsaturated fatty acids than synaptosomes (31). Myelin, which functions mainly as an insulator, was the least sensitive of the membranes to the effects of ethanol (Fig. 6) in our experiments. In contrast, the order parameter was lowest in mitochondrial membrane, which contains very little cholesterol but large amounts of unsaturated fatty acids and proteins that are involved in a variety of enzymatic activities. Our results, showing more significant slope changes in the concentration-related ethanol effects in both erythrocyte and synaptosomal membranes than in mitochondrial membranes, confirm the findings of Miller and Pang (32) in phospholipid model membranes that the effects of anesthetic agents on fluidity depend upon the chemical composition of the system.

The usefulness of spin label probes in studying biomembrane functions is well documented (33–35). The fatty acid spin label used in these experiments reflects the local changes in the hydrophobic region near the surface of the membrane. However, the present studies, using intact membranes, do not allow us to distinguish whether specific interactions occur between the spin label and the lipid or the protein. A variety of spin labels have been shown to be bound to both membrane lipids and proteins (8). Lenaz *et al.* (10) have shown that 1-butanol produced greater fluidizing effects in intact beef heart mitochondrial membranes than in phospholipid vesicles or lipid-depleted mitochondrial membranes. From these data the authors suggested that the 1-alkanols modify the long-range lipid-protein interaction in intact membranes without affecting the layer of strongly bound phospholipids around the proteins. The findings by Seeman (36) that ethanol produced larger volume increases in intact membranes than in cholesterol-phospholipid liposomes are consistent with an effect on the lipid-protein interaction.

The maintenance of an optimum level of fluidity in the membrane appears to be important for normal function of some membrane-bound proteins (1, 2). The ethanol-induced increased fluidity in the biomembranes used in our experiments may disturb normal lipid-protein interactions *in vivo* by the same mechanisms proposed for anesthetic agents (37, 38).

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