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Ethanol and the Physical Properties of Brain Membranes

Fluorescence Studies

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SUMMARY

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Synaptic plasma membranes and myelin were prepared from mouse brain and their physical properties were evaluated by using the fluorescent probe molecules 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-aminopyrene. The absorption-corrected fluorescence output and fluorescence polarization of DPH (a probe of the membrane core) were decreased when synaptic plasma membranes were exposed *in vitro* to low concentrations (10–20 mM) of ethanol. Fluorescence of 1-aminopyrene (a probe of the membrane surface) was affected only by high ethanol concentrations. Ethanol did not alter the excitation or emission maxima of DPH in synaptic plasma membranes, which indicates that it did not alter polarity in the vicinity of the probe. Compared with the intact synaptic plasma membranes, DPH fluorescence in myelin membranes and in lipids extracted from myelin or synaptic plasma membranes was less sensitive to the effects of ethanol. Analysis of fluorescence polarization at various temperatures indicated that ethanol altered the phase behavior of membrane lipids. These results indicate that low, physiologically relevant concentrations of ethanol selectively fluidize the hydrophobic core of synaptic membranes. The sensitivity of intact synaptic membranes is discussed in terms of lipid-protein interactions disrupted by ethanol.

INTRODUCTION

Recent studies regarding the mechanism of action of ethanol have focused on its effects on biomembranes. Ethanol and related alkanols decrease the temperature of the gel-to-liquid-crystalline phase transition of model membranes (1, 2), expand membranes (3), and alter the surface charge of membrane lipids (4). However, these effects have been obtained with ethanol concentrations in the range of 500–1500 mM, whereas serum concentrations above 200 mM are usually lethal in humans and laboratory animals. Also, purified lipids or lipid extracts of biological membranes, rather than intact plasma membranes, have been used in most of the reported studies. One study (5) addressed the effects of low concentrations of ethanol on intact brain membranes. These investigators reported that *in vitro* exposure to ethanol (10–350 mM) produces small but significant decreases in the order parameter of a spin-labeled fatty acid incorporated into synaptic membranes. This finding suggests that physiologically relevant concentrations of ethanol may increase nerve membrane fluidity. A number of membrane-bound enzymes and membrane transport processes are affected by membrane fluidity (6, 7), suggesting that this ethanol effect might lead to alterations in neuronal function. Thus, knowledge of the effects of ethanol on the physical properties of brain membranes may be crucial if the molecular mechanisms involved in alcohol intoxication, tolerance, and dependence are to be understood. In recent years, fluorescent probe molecules have been used to study the physical properties of synthetic and biological membranes (8). These probes offer several distinct advantages over other techniques: (a) they yield information about the polarity and microviscosity of their environment, (b) different probes are available to monitor specific membrane regions, and (c) they can be used at low concentrations, which minimizes membrane perturbation by the probe (2, 8, 9).

In this report, we describe the use of specific fluorescent probes in conjunction with a computer-centered spectrofluorimeter for evaluating the effects of ethanol on various regions of synaptosomal and myelin membranes from mouse brain. In addition, we compare the

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effects of ethanol on lipid and phospholipid extracts to its effects on intact membranes.

METHODS

Membrane isolation. Male Swiss-Webster mice (Charles River Laboratories, Portage, Mich.) were used in all experiments. By using Ficoll and sucrose density gradient centrifugation (10), myelin and two fractions of synaptosomal plasma membranes (SPM-1 and SPM-2)[1] were prepared from brain homogenates. These membrane fractions were previously characterized in our laboratory (11). Membranes were suspended in PBS without Ca^{++} or Mg^{++} at pH 7.4 (12), at a concentration of 3–4 mg of protein/ml, and kept at 4° prior to analysis.

Lipid extraction. Membranes were suspended as described above, and aliquots were taken for protein analysis (13) and fluorescence studies of intact membranes (see below). The remainder of the suspension was extracted by the method described by Ames (14) to yield a "total lipid" extract. A portion of this extract was chromatographed on a silicic acid column (Unisil; Clarkson Chemical Company, Williamsport, Penn.) for separation of neutral lipids and phospholipids (12). The phospholipids and total lipid extracts were dried under N_2 and dissolved in $CHCl_3$ at a volume equal to that of the aliquot of PBS used for the initial extraction. All organic solvents were glass-distilled, and all glassware was washed with sulfuric acid-dichromate before being used.

Fluorescence probe incorporation. A suspension containing 50 μg of protein per milliliter of PBS was used for studies of intact membranes. For total lipid and phospholipid extracts, an aliquot of the $CHCl_3$ solution equivalent to 100 μg of protein was dried under N_2 and resuspended by Vortexing with 2 ml of PBS. The fluorescence probe molecule, DPH (Aldrich Chemical Company, Inc., Milwaukee, Wis.), was incorporated into membrane suspensions at a concentration of 0.5 μg/ml. The probe was dissolved in THF, and a volume of 0.5 μl of THF/ml of PBS was either added directly to the membrane suspension in PBS or was dried onto the tube prior to adding PBS and membranes. Results of these procedures were equivalent. The suspension was incubated in the dark at 37° and was Vortexed frequently until maximal fluorescence was obtained (30–60 min). Also, 1-AP (Aldrich Chemical Company, Inc.) was dissolved in THF and incorporated in the same way as DPH. The final concentration of 1-AP was 0.1 μg/ml. Unlike DPH, 1-AP not associated with membranes displayed fluorescence that required removal of unbound 1-AP from membrane-bound probe. This was accomplished by column chromatography with Sephadex G-50.

After incorporation of probe, the membrane suspensions were cooled to room temperature and placed in quartz cuvettes. Fluorescence was then determined as described below. After control levels of fluorescence were determined, an aliquot of absolute ethanol (1–80 μl) was added to the cuvette and fluorescence was again determined. In some experiments, in order to assure that the changes produced by ethanol addition were not systemic artifacts, distilled water rather than ethanol was added to the cuvette. To study the temperature dependence of fluorescence, cuvettes were placed in the thermostated sample compartment and cooled to 10°. After equilibration, the temperature of the membrane suspension was increased at a rate of 1°/min and fluorescence was recorded at 1-min intervals.

Fluorescence instrumentation. The computer-centered spectrofluorimeter designed by Holland *et al.* (15) was used to measure AB, CO (corrected for the inner filter effect), RFE (directly proportional to quantum yield), CFE, light scattering, and corrected excitation and emission spectra. These procedures are described in earlier reports (16). P was determined with an Aminco-Bowman spectrophotofluorimeter modified to contain movable polarization filters in the excitation and emission beams (16). Polarization was determined as described elsewhere (16), by using a grating correction factor to compensate for the polarization artifacts of the optical system. Serial dilutions were used to correct polarization for membrane absorbance. Twenty determinations of each parameter were averaged for each membrane preparation. The sample was stirred continuously with an MKII Microstirrer (Lawrence Instruments, London; Ontario, Canada), and temperature was monitored by a thermistor in the cuvette. DPH excitation and emission wavelengths were 355 and 430 nm, respectively; for 1-AP, they were 360 and 425 nm.

Statistical analysis. Values obtained before and after adding ethanol were compared by Student's *t*-test for paired observations. When multiple comparisons were made with a single control, Dunnett's tables (17) were used to determine significance levels. For concentration-dependent parameters (e.g., CO and AB), the change in volume produced by adding ethanol was taken into account using Beer's law. The validity of this approach was verified by using distilled water as a diluent.

RESULTS

DPH fluorescence in intact membranes. The intensity of CO and P of DPH incorporated into intact membranes followed the order: myelin > SPM-1 > SPM-2 (Table 1); membrane absorbance followed the same order. The presence of more lipid is known to increase CO and AB, whereas more rigid lipid (more cholesterol) increases CO and P. Thus, the values in Table 1 likely reflect the higher lipid/protein and cholesterol/phospholipid ratios of myelin and the possible contamination of SPM-1 by myelin (11). DPH dissolved in ethanol displayed a high degree of fluorescence, with negligible polarization of fluorescence, indicating a high degree of rotational mobility (Table 1). Fluorescence was negligible when DPH was added to PBS that contained no membranes (data not shown).

Effects of ethanol on corrected fluorescence output (CO) of DPH in intact membranes. CO has been shown to be sensitive to both the viscosity and the polarity of the probe environment (18, 19). When ethanol was added

[1] The abbreviations used are: SPM, synaptosomal plasma membranes; PBS, phosphate-buffered saline; DPH, 1,6-diphenyl-1,3,5-hexatriene; THF, tetrahydrofuran; 1-AP, 1-aminopyrene; AB, absorbance; CO, absorbance-corrected fluorescence; RFE, relative fluorescence efficiency; CFE, corrected fluorescence emission; P, fluorescence polarization; DPPC, dipalmitoyl phosphatidylcholine.

TABLE 1

Fluorescence parameters of DPH dissolved in brain membranes or ethanol

Solution[a]	Corrected output	Polarization	Absorbance
Myelin/PBS (n = 3)	69 ± 5[b]	0.39 ± 0.03	0.39 ± 0.01
SPM-1/PBS (n = 8)	61 ± 3	0.34 ± 0.01	0.27 ± 0.01
SPM-2/PBS (n = 8)	31 ± 2	0.32 ± 0.01	0.22 ± 0.01
Absolute ethanol (n = 3)	249 ± 8	0.002[c]	0.25 ± 0.01

[a] DPH (1 μg) was added to myelin or synaptic plasma membranes (SPM-1, SPM-2) suspended in 2 ml of PBS or was added to 2 ml of absolute ethanol. The absorption-corrected fluorescence, polarization of fluorescence, and absorbance were determined at 25° as described under Methods.

[b] Values represent mean ± standard error of the mean.

[c] n = 1 for this determination.

to brain membrane suspensions, the corrected fluorescence of DPH was decreased (Fig. 1). SPM-2 was most sensitive to this ethanol effect, and a concentration of 10 mM produced a significant decrease in CO. SPM-1 was less sensitive than SPM-2, and myelin was clearly the least sensitive, since 800 mM ethanol produced only a small decrease in CO. The effects of ethanol on CO of DPH in SPM tended to be biphasic, with low (10–25 mM) ethanol concentrations producing a decrease that was not exceeded until very high concentrations were used (Fig. 1). The addition of ethanol produced a CO decrease within 1 min, which was stable for at least 30 min (Fig. 2).

The corrected excitation, emission, and absorbance spectra of DPH incorporated into SPM-2 are shown in

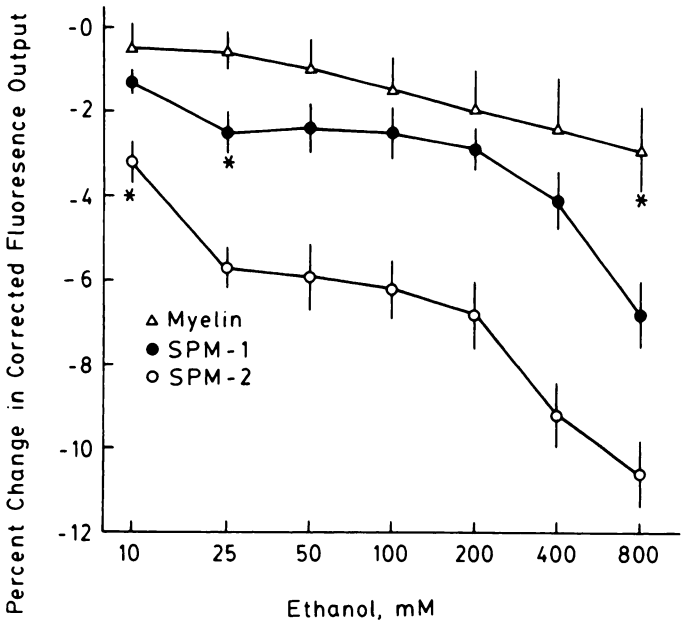

FIG. 1. *Effects of ethanol on the CO of DPH incorporated into myelin, SPM-1, and SPM-2 from mouse brain*

Ethanol was added *in vitro* at the concentration indicated on the *abscissa*. CO was determined at 25°. *Vertical bars* represent ± standard error of the mean. For myelin, n = 4; for SPM-1 and SPM-2, n = 8. *Asterisks* signify the lowest concentration of ethanol which produced a significant ($p < 0.05$) change in CO.

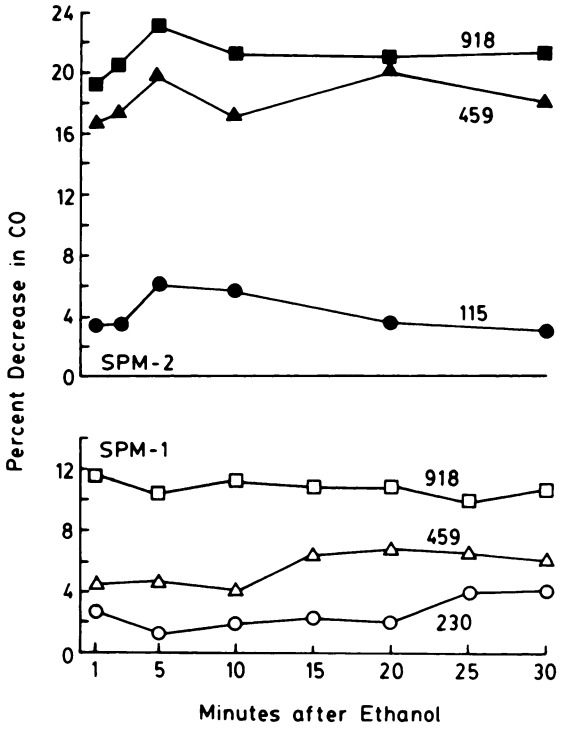

FIG. 2. *Time course of the reduction in corrected fluorescence produced by ethanol*

DPH was incorporated into SPM-2 (*upper panel*) or SPM-1 (*lower panel*) and fluorescence was determined at 25°. The percentage decrease in corrected fluorescence output is given on the *ordinate* and the time after ethanol addition is given on the *abscissa*. The final ethanol concentration (millimolar) is indicated either above or below each line. Each point is the mean obtained from two membrane preparations.

Fig. 3 (upper panels). The scans of CO, RFE, and CFE demonstrated the three peaks characteristic of DPH. In the absence of DPH, the emission intensities were negligible (Fig. 3, lower panels). In this membrane preparation, ethanol apparently decreased the height of all peaks to the same extent without altering their maxima. This observation was confirmed by quantitating the spectra obtained with six different preparations of SPM-2 (Table 2). Even when very high (900 mM) ethanol concentrations were added, there was no alteration of the maximum wavelengths of any of the peaks or of their relative heights. Similar results were obtained with SPM-1 and myelin (not shown). In contrast, when DPH was dissolved in ethanol, the wavelengths of the peaks of excitation (CO) and CFE intensity were reduced by 5 to 20 nm, compared with SPM. The shape of the emission spectrum of DPH was also different in ethanol than in membranes; the left peak was diminished and the right peak was enhanced in ethanol as compared with membranes (Table 2). These spectral changes indicate that DPH incorporated into brain membranes resides in a hydrophobic environment (18, 19).

Effects of ethanol on fluorescence polarization of DPH in intact membranes and lipid extracts. Fluorescence polarization reflects the rotational mobility of the probe molecule and is influenced by the microviscosity of the membrane environment of the probe (8). Ethanol

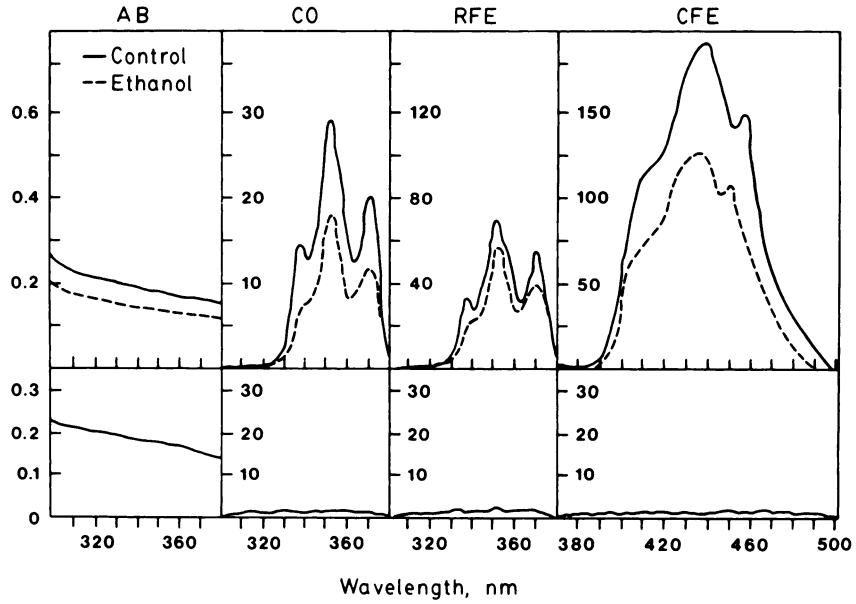

FIG. 3. *Fluorescence and absorbance spectra of SPM-2 and SPM-2 containing DPH*

Upper panel, (——); spectra of SPM-2 containing DPH; *upper panel,* (- - -): same as *solid lines,* but obtained after addition of 900 mM ethanol. *Lower panels:* spectra of SPM-2 without addition of DPH. From *left* to *right,* the *panels* present *AB, CO, RFE,* and *CFE.* Values on the *ordinate* represent absorbance units for AB, whereas CO, RFE, and CFE are given in arbitrary units. Spectra were obtained at 25°.

produced dose-dependent decreases in the polarization of fluorescence of DPH in brain membranes (Fig. 4). For SPM-2, polarization was significantly decreased by 20 mM ethanol, whereas with myelin, 330 mM was necessary to significantly decrease polarization.

Lipid extracts of myelin and SPM-2 were also studied. DPH polarization in intact membranes, total membrane lipid, and membrane phospholipid is presented in Table 3. Polarization of DPH followed the order: intact > total lipid > phospholipid. This indicates that both membrane proteins and neutral lipids (such as cholesterol) exert an ordering effect upon the phospholipids. Fluorescence polarization is considerably greater for lipids from myelin membranes than for those from SPM-2. It should be noted that the values shown in Table 3 for intact membranes were measured at 37° and therefore were lower than the values measured at 25° (Table 1). Membrane lipid sensitivity to ethanol differed from that of the intact membranes. Fluorescence polarization of DPH in total lipids from SPM-2 was decreased by 333 mM ethanol, but not by lower concentrations (Fig. 5). In phospholipids from SPM-2, the polarization of DPH was not altered by 333 mM ethanol. Likewise, ethanol failed to change the CO of DPH in lipids from SPM-2 (data not shown). Compared with the SPM lipids, myelin lipids were more sensitive to ethanol. An ethanol concentration of 85 mM produced significant, though small, decreases in the polarization of DPH in myelin lipids and phospholipids

TABLE 2

Effects of ethanol on the excitation and emission spectra of DPH in brain membranes[a]

Sample[b]	Spectral parameter	Maximum wavelengths						Relative peak heights			
		Control			Ethanol[c]			Left/middle		Right/middle	
								Control	Ethanol	Control	Ethanol
		nm									
SPM-2											
	CO	340 ± 1	355 ± 1	373 ± 1	340 ± 1	354 ± 1	372 ± 1	0.63 ± 0.07	0.63 ± 0.06	0.71 ± 0.02	0.71 ± 0.03
	RFE	340 ± 1	355 ± 1	373 ± 1	340 ± 1	354 ± 1	371 ± 1	0.57 ± 0.07	0.59 ± 0.07	0.81 ± 0.04	0.81 ± 0.05
	CFE	406 ± 2	429 ± 2	488 ± 2	408 ± 2	430 ± 2	449 ± 2	0.63 ± 0.03	0.64 ± 0.02	0.86 ± 0.05	0.89 ± 0.03
Absolute ethanol											
	CO	335 ± 2	351 ± 2	369 ± 1	—	—	—	0.80 ± 0.01	—	0.76 ± 0.03	—
	AB	335 ± 1	350 ± 1	368 ± 1	—	—	—	0.79 ± 0.02	—	0.78 ± 0.01	—
	CFE	402 ± 2	427 ± 2	446 ± 2	—	—	—	0.55 ± 0.01	—	1.11 ± 0.02	—

[a] Values represent mean ± standard error of the mean. For SPM-2, $n = 6$; for absolute ethanol, $n = 4$.

[b] DPH (1 μg) was incorporated into brain membranes or absolute ethanol (no membranes or buffer) and the fluorescence spectra were determined as described under Methods.

[c] Ethanol was added to the cuvette after the control spectra were obtained to give a final concentration of 900 mM.

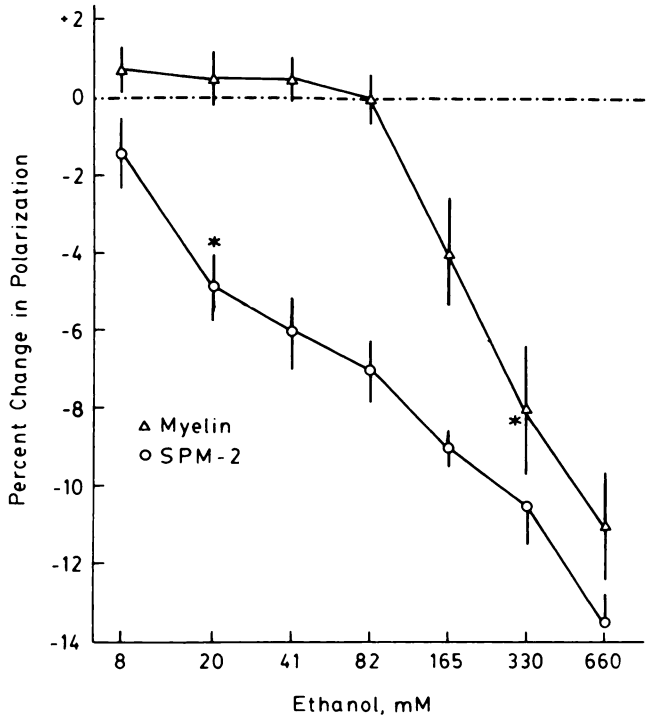

FIG. 4. *Effects of ethanol on the fluorescence polarization of DPH incorporated into brain membranes*

Ethanol was added *in vitro* to give the concentration indicated on the *abscissa*. *Vertical bars* represent ± standard error of the mean. For myelin, $n = 4$; for SPM-2, $n = 8$. Fluorescence was determined at 25°. Asterisks indicate the lowest concentration of ethanol which produced a significant ($p < 0.05$) change in fluorescence polarization.

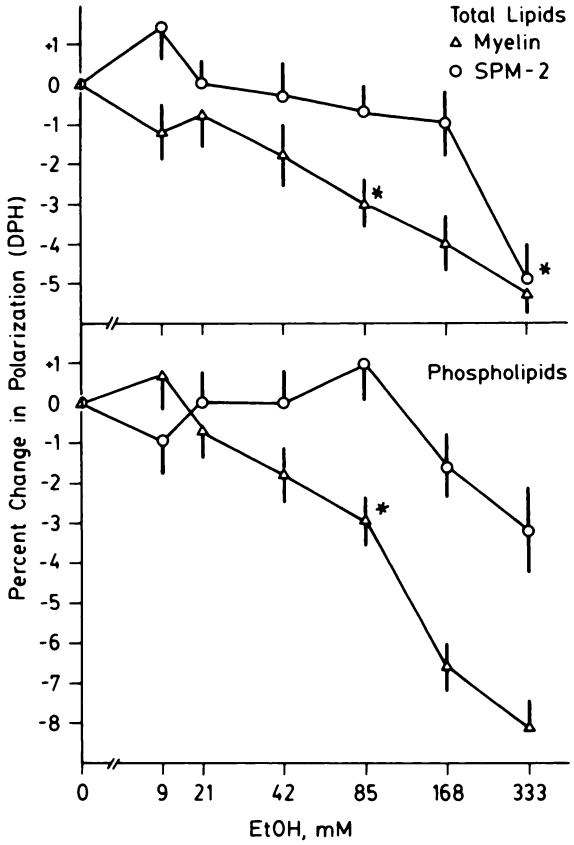

FIG. 5. *Effects of ethanol on the fluorescence polarization of DPH incorporated into lipids extracted from brain membranes*

Lipid vesicles were prepared from total lipids (*upper panel*) and phospholipids (*lower panel*) extracted from myelin or SPM-2. Ethanol was added *in vitro* to the concentration indicated on the *abscissa*. *Vertical bars* represent ± standard error of the mean, $n = 6$. Fluorescence was determined at 37°. Asterisks denote the lowest concentration of ethanol which produced a significant ($p < 0.05$) change in fluorescence polarization.

(Fig. 5). Thus, the lipids from SPM-2 were less sensitive to ethanol than were the intact membranes, whereas the opposite was true for myelin.

Influence of temperature on fluorescence polarization. The effects of temperature and ethanol on fluorescence polarization of DPH in intact SPM-2, total lipids, and phospholipids are shown in the form of Arrhenius plots (Figs. 6–8). For intact membranes, ethanol decreased fluorescence polarization at all temperatures. An increase in temperature also decreased polarization (Fig. 6). Two discontinuities, or "breaks," were noted in the Arrhenius plots of intact membranes. These occurred at about 23 and 31° under control conditions and were lowered 2–5° after ethanol was added (Fig. 6). The parallel shift of the curve indicates that ethanol increased the entropy of the membrane without altering the flow activation energy (8).

In total lipid from SPM-2, an increase in temperature also produced a decrease in fluorescence polarization; the decrease was biphasic, with a discontinuity at about 25° (Fig. 7). Polarization decreased at all temperatures when ethanol was added. Ethanol also reduced the temperature of the discontinuity to about 23°. Phospholipids from SPM-2 showed a major break in the Arrhenius plot at 31° and a smaller change in slope at about 26° (Fig. 8). With these lipids, only at the lower temperatures did ethanol decrease fluorescence polarization. The discontinuity at 31° was not altered by ethanol, but the break at 26° was shifted to 21°. These results again demonstrated that SPM phospholipids are less sensitive to the effects of ethanol than are total lipids from SPM.

Fluorescence of 1-AP in SPM. The fluorescent mole-

TABLE 3

Polarization of fluorescence of DPH and 1-AP in membranes and membrane lipids

Membrane	Polarization[a]		
	DPH	1-AP	
		pH 4.8	pH 7.4
SPM-2			
Intact	0.30 ± 0.01	0.35 ± 0.01	0.32 ± 0.01
Total lipid	0.27 ± 0.01	0.29 ± 0.01	
Phospholipid	0.18 ± 0.01		
Myelin			
Intact	0.35 ± 0.01[b]		
Total lipid	0.32 ± 0.01		
Phospholipid	0.23 ± 0.01		

[a] Polarization was determined at 37°. Membranes or lipids were suspended in PBS at pH 7.4 for DPH and pH 4.8 for 1-AP. See Methods for details.

[b] Values represent mean ± standard error of the mean, $n = 6$–8.

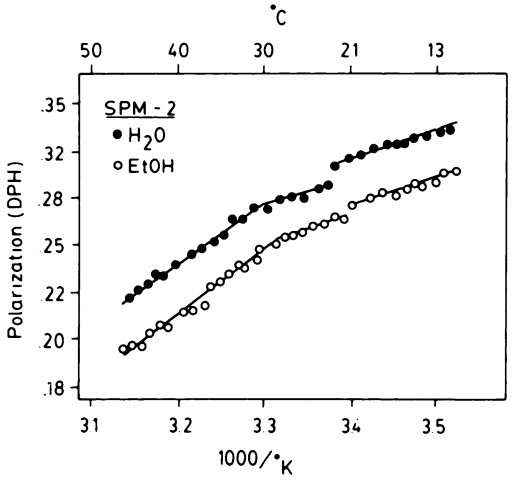

FIG. 6. *Arrhenius analysis of the effects of temperature and ethanol on the fluorescence polarization of DPH in SPM-2*

Fluorescence polarization is indicated on the *ordinate* (log scale), whereas temperature is shown on the *abscissa* as 1000/°K (lower scale) or °C (upper scale). The ethanol concentration was 333 mM. Each point represents the mean from three membrane preparations.

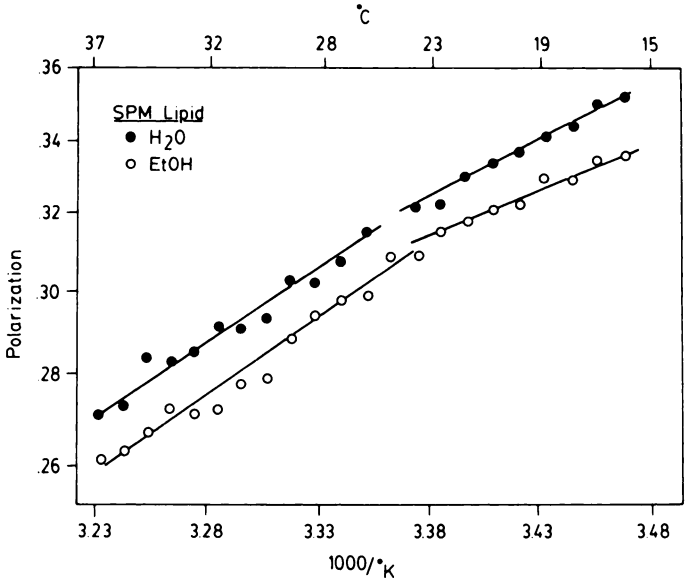

FIG. 7. *Arrhenius analysis of the effects of temperature and ethanol on the fluorescence polarization of DPH in total lipid extracted from SPM-2*

Fluorescence polarization is indicated on the *ordinate* (log scale), whereas temperature is shown on the *abscissa* as 1000/°K (lower scale) or °C (upper scale). The ethanol concentration was 333 mM. Each point represents the mean from two membrane preparations.

cule 1-AP was tested as a probe of hydrophobic regions near the membrane-buffer interface (20). In some experiments, a pH of 4.8 was used to protonate the amino group of 1-AP, thus assuring that the probe did not penetrate into the membrane core (20). The fluorescence polarization of 1-AP in SPM-2 was higher than that of DPH, indicating a more rigid environment (Table 3). Studies of other biological membranes have also noted that the membrane surface is more rigid than the membrane core (21). Addition of ethanol had little effect on the fluorescence of 1-AP in intact membranes or membrane lipid. An ethanol concentration of 333 mM produced only a 4% decrease in fluorescence polarization of 1-AP in intact SPM-2 or total lipid (Fig. 9). Ethanol concentrations ranging from 10–333 mM did not significantly alter the corrected fluorescence output of 1-AP in intact SPM-2 or total lipids from SPM-2 (data not shown).

Effects of ethanol on membrane absorbance. In order to correct for absorbance artifacts, the computer-centered fluorimeter continuously monitored the sample absorbance. In initial experiments, we noted that ethanol

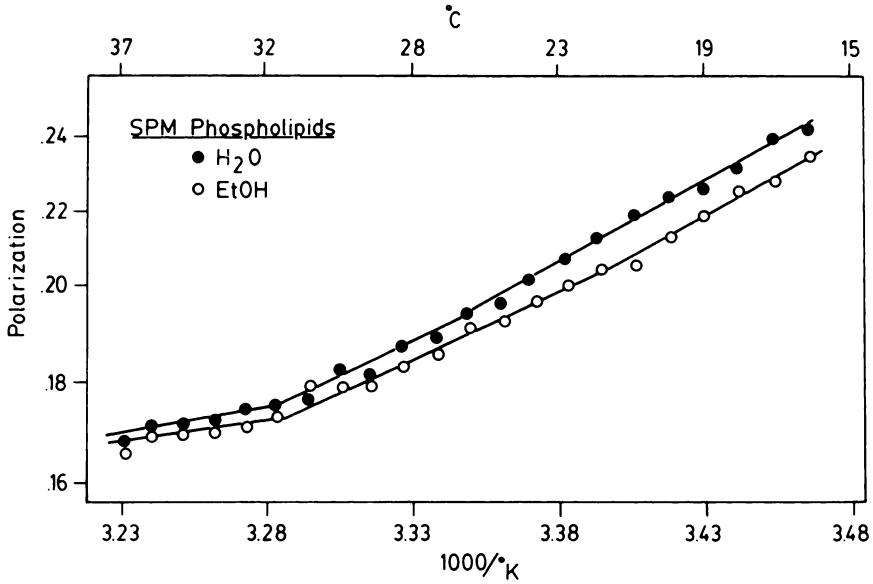

FIG. 8. *Arrhenius analysis of the effects of temperature and ethanol on the fluorescence polarization of DPH in phospholipids extracted from SPM-2*

Fluorescence polarization is indicated on the *ordinate* (log scale), whereas temperature is shown on the *abscissa* as 1000/°K (lower scale) or °C (upper scale). The ethanol concentration was 333 mM. Each point represents the mean from two membrane preparations.

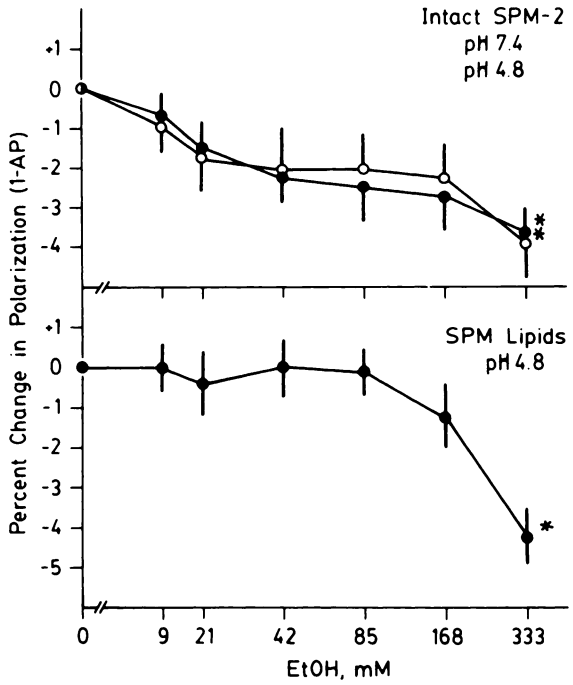

FIG. 9. *Effects of ethanol on the fluorescence polarization of 1-AP incorporated in SPM-2 (upper panel) or a total lipid extract from SPM-2 (lower panel)*

Ethanol was added *in vitro* to give the concentrations given on the *abscissa*. Data was obtained at pH 7.4, (○); and at pH 4.8, (●). Fluorescence was determined at 37°. *Vertical bars* signify ± standard error of the mean, $n = 4$. *Asterisks* denote the lowest concentration of ethanol producing a significant ($p < 0.05$) change in fluorescence polarization.

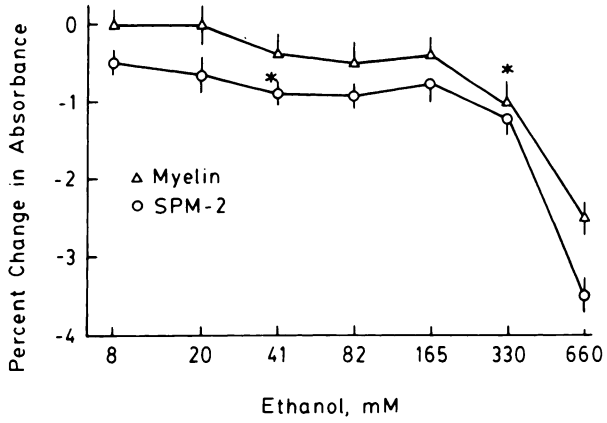

FIG. 10. *Effects of ethanol on the absorbance of brain membranes*

Ethanol was added *in vitro* to give the concentration indicated on the *abscissa* and the membrane absorbance was measured at 355 nm. *Vertical bars* represent ± standard error of the mean. For myelin, $n = 3$; for SPM-2, $n = 5$. *Asterisks* denote the lowest concentration of ethanol producing a significant ($p < 0.05$) change in absorbance.

decreased the absorbance of membranes containing DPH. This absorbance decrease was similar at all wavelengths from 300–380 nm (Fig. 3). Membranes not containing DPH were also evaluated, and ethanol was found to decrease absorbance in a dose-dependent manner (Fig. 10). The SPM-2 fraction was more sensitive to this ethanol effect than was myelin. Ethanol concentrations as low as 20 mM produced significant decreases in SPM-2 absorbance, whereas a concentration of 330 mM was necessary in order to reduce the absorbance of myelin. These absorbance changes were small (1–4%), but statistically significant.

Temperature effects on membrane absorbance were also investigated and are presented in the form of Arrhenius plots (Fig. 11). The absorbance of SPM-2 changed little when measured at temperatures ranging from 10–46°. Ethanol decreased absorbance at all temperatures, and this effect was enhanced at the lower temperatures (Fig. 11).

DISCUSSION

In this study, physiologically relevant concentrations of ethanol decreased the fluorescence output and fluorescence polarization of DPH in synaptic membranes from mouse brain. Interpretation of these findings is facilitated by other studies that have shown that both fluorescence output and polarization are related to the viscosity of the microenvironment surrounding the probe molecule (8, 18, 19). In addition, it has been shown that DPH selectively partitions into the hydrophobic lipid core of the membrane bilayer (8, 9) and selectively accumulates at the interface between lipid domains (22). Because ethanol and other short-chain alcohols are hydrophilic, they would be expected to localize at the membrane-water interface and to perturb the membrane surface. Our results, however, indicate that ethanol fluidizes

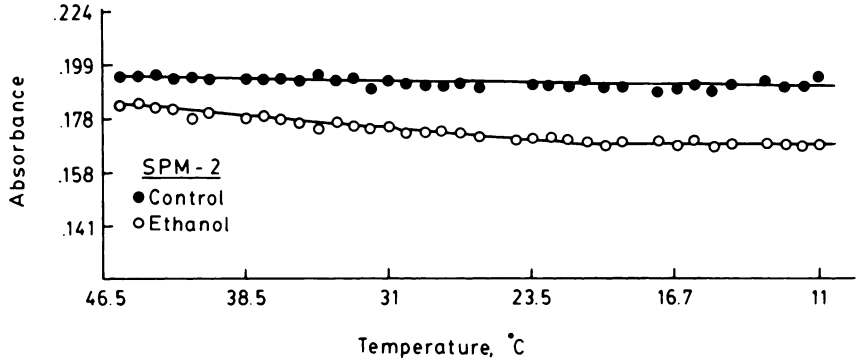

FIG. 11. *Arrhenius analysis of the effects of temperature and ethanol on the absorbance of SPM-2*

Absorbance is presented on the *ordinate* (log scale), whereas temperature is shown on the *abscissa* (reciprocal scale). Absorbance was measured at 355 nm. The ethanol concentration was 450 mM. Each point is the mean of two membrane preparations.

the inner core of the bilayer. We observed that the fluorescence of 1-AP, a probe of more superficial membrane regions (20), was not affected by low concentrations of ethanol; likewise, the fluorescence of toluidylnaphthalene sulfonate, a probe of the membrane surface, is not affected by *n*-butyl alcohol (23), whereas the fluidity of the membrane core is increased by *n*-butyl alcohol (24, 25). Studies with spin-label probes also indicate a selective effect of ethanol on the membrane core (26). Thus, our results, and those of others (23–27), indicate that ethanol, *n*-butyl alcohol, and *t*-butyl alcohol affect the inner core of the membrane, whereas little evidence has been obtained for an effect of these drugs on the membrane surface. Vanderkooi (20) studied the fluorescence of 1-AP bound to dimyristoyl phosphatidyl choline vesicles and reported that low concentrations of ethanol perturbed the surface of these vesicles. In our study, we were unable to obtain analogous results by using 1-AP bound to intact SPM or lipids from SPM.

Compared with the intact membranes, extracted lipids from synaptic membranes were less sensitive to ethanol. For example, an ethanol concentration of 20 mM produced a 5% decrease in polarization of DPH fluorescence in intact SPM, but a concentration of 333 mM was necessary to produce a similar change in SPM lipids. Our values are consistent with the report that 700 mM ethanol produces a 7% decrease in fluorescence polarization of DPH in lipids extracted from brain membranes (27). Phospholipids were even less sensitive to ethanol than was the total lipid extract. Again, this is consistent with the data of Johnson *et al.* (27), who reported that adding cholesterol to phospholipid vesicles enhanced the effects of ethanol. The effect of butanol and other anesthetics on spin-labeled probes is also greater in intact membranes than in lipid extracts (24), and Seeman (3) reported that the membrane-expanding effect of ethanol is greater in intact membranes than in cholesterol-phospholipid vesicles. The loss of ethanol sensitivity following extraction of membrane lipids may be due to removal of membrane proteins or destruction of the asymmetric lipid distribution found in the intact membranes. The first possibility implies that ethanol-induced effects result from disruption of lipid-protein interactions or protein-DPH interactions. It is not clear if DPH partitions exclusively into the lipid portions of nerve membranes or if it also binds to membrane proteins. Tryptophan quenching experiments suggest that DPH is not associated with protein in biological membranes (22), whereas DPH has been shown to bind to hydrophobic regions of proteins in aqueous media (28). The second possibility implies that the asymmetric composition (11) and physical properties (29) of the intact bilayer increase membrane sensitivity to ethanol. For myelin, the intact membrane was much less sensitive to ethanol than SPM, but extracted lipids from myelin were as sensitive to ethanol as the intact membrane. Two factors may be responsible for these observations. First, there is less lipid-protein interaction in myelin than in SPM (due to the lower ratio of protein to lipid) and many myelin proteins are different from SPM proteins (30). Thus, ethanol may not affect the lipid-protein interactions in myelin as it does in SPM. Second, the lipid composition of myelin is different from that of SPM. In the intact membrane, the lipids may be arranged such that they are resistant to ethanol, whereas the reconstituted lipid bilayers lack this order and are more sensitive to ethanol. As discussed elsewhere (31), the physical properties of the lipids, as well as lipid-protein interactions, may influence the sensitivity of membranes to ethanol.

In addition to decreasing the fluorescence polarization of DPH, ethanol also decreased the absorbance-corrected fluorescence output. The decrease in fluorescence output produced by ethanol is consistent with a decrease in membrane viscosity, but fluorescence intensity is also sensitive to the polarity or dielectric of the probe environment (18, 19). The possibility that ethanol could indirectly alter the membrane polarity or directly interact with DPH was evaluated by analyzing the corrected excitation and emission spectra of DPH in ethanol and in membranes exposed to ethanol. The excitation and emission maxima and the ratios of the peak heights are sensitive to the polarity of the probe environment (18, 19). As would be expected, the spectra of DPH dissolved in ethanol was considerably different from the spectra of DPH in brain membranes. Addition of ethanol to synaptic or myelin membranes containing DPH, however, did not alter the fluorescence spectra, which indicated that ethanol did not produce a detectable change in membrane dielectric in the vicinity of the probe and did not directly interact with the probe.

Arrhenius analysis of the effects of temperature and ethanol on synaptic membranes and membrane lipids suggested that ethanol, in addition to fluidizing the lipids, altered their phase behavior. In intact membranes, total lipids, and phospholipids, a discontinuity in the Arrhenius plot occurred at from 23–26°. This discontinuity has been noted in other studies of the physical properties of membranes (32, 33) and in studies of the activity of membrane-bound brain Na,K-ATPase (34). These discontinuities have been attributed to phase transitions (gel-to-liquid-crystalline) and to lateral phase separations of different lipid species (6, 8, 33). One study indicates that these discontinuities are due to a change in the fluorescence lifetime of DPH (32). In our study, ethanol exposure shifted the discontinuities in the Arrhenius plots of fluorescence polarization to lower temperatures. In agreement with this finding, 500 mM ethanol has been shown to decrease the discontinuity temperature of brain (Na,K)-ATPase from 24.4–18.8° (34). These results indicate that ethanol facilitates either a phase transition or a lateral phase separation of synaptic lipids. Most brain lipids (i.e., polyunsaturates and sterols) do not display transitions in this range; DPPC, however, is a rigid lipid that has recently been reported as a major constituent of brain membranes (35). Thus, DPPC may be involved in the observed discontinuities. Ethanol has been shown to decrease the phase transition temperature of DPPC (2).

Membrane absorbance has proved to be useful when investigating membrane physical properties (36). In our study, we observed that ethanol decreased the absorbance of brain membranes and that synaptic membranes were more sensitive than myelin to this effect. The decrease in absorbance is likely due to membrane expansion and the resultant changes in particle size and bilayer

thickness produced by ethanol (3, 36). A change in membrane refractive index also affects absorbance (36), and this has not been ruled out as a possible action of ethanol. During phase transitions, there is a change in lipid absorbance due to a change in lipid refractive index (36). Analysis of intact SPM absorbance did not reveal any clear temperature dependence, although in the presence of ethanol a discontinuity in the Arrhenius plot was seen at about 20°. In contrast, Arrhenius analysis of fluorescence polarization demonstrated two discontinuities. These differences likely involve distinctions between the methods. Absorbance reflects the bulk properties of the membrane, whereas fluorescence polarization reflects the properties of discrete areas of the membrane surrounding the probe molecule. In addition to giving information about the physical properties of membranes, the effects of ethanol on membrane absorbance are important because they may lead to artifactual results. For example, fluorescence intensity is increased when absorbance is decreased (15). Although we found that ethanol decreased the absorption-corrected fluorescence output of DPH, this change would be obscured by the absorbance artifact if uncorrected values of fluorescence intensity were obtained. Fluorescence polarization is also dependent upon absorbance (33, 37). In addition to these fluorescence artifacts, the membrane absorbance in some colorimetric assays of intact membranes may be greater than the chromophore absorbance (e.g., measurement of membrane sulfhydryl groups by Ellman's reagent) (38). In such experiments, the effects of ethanol on membrane absorbance could lead to erroneous conclusions if proper control experiments were not carried out.

The results presented here, and those of Chin and Goldstein (5, 26), suggest that low concentrations of ethanol increase the fluidity of synaptic membranes. We must now consider whether this change is sufficient to alter synaptic function. The magnitude of the ethanol effect is not large; Chin and Goldstein (5) reported that 160 mM ethanol decreases the order parameter of a spin-labeled fatty acid by about 0.8%. In our study, this concentration of ethanol decreased the fluorescence polarization by 6–9%. This change in polarization may, however, reflect changes in microviscosity that are physiologically significant. In systems of known viscosity, the relationship between fluorescence polarization of DPH and viscosity is not linear. Thus, in paraffin oil, a 7% change in P (e.g., 0.30 to 0.28) corresponds to a viscosity change of about 20% (8). Assuming that the relationship between P and microviscosity is similar in biological membranes, the observed alcohol effects may be sufficient to alter the activity of viscosity-dependent processes such as ion transport and enzyme activities. This concept is supported by observations that a 30% decrease in the apparent microviscosity of the erythrocyte membrane produces a 20-fold increase in the rate constant of adenylate cyclase (39), and that a 5% decrease in the apparent microviscosity of phospholipid vesicles produces a 50% increase in ion permeability (40). Thus, membrane viscosity and membrane function can be coupled, and small changes in membrane viscosity may result in significant functional changes.

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