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Gangliosides Enhance the Membrane Actions of Ethanol and Pentobarbital

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SUMMARY

The physical properties of vesicles containing phosphatidylcholine (PC) and gangliosides were evaluated by the fluorescent probes 1,6-diphenyl-1,3,5-hexatriene, *trans*-parinarate (probes of the lower acyl regions), and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (a probe of the glycerol backbone and upper acyl regions). Exposure of PC vesicles to ethanol produced a decrease in polarization of fluorescence of these probes, but large concentrations (200–600 mM) were required. Incorporation of 10 mole% rat brain synaptic gangliosides into the PC vesicles enhanced the effect of ethanol by several-fold. Synaptic gangliosides also enhanced the effect of ethanol on vesicles containing cholesterol in addition to PC. Comparison of mono-, di-, tri-, and asialogangliosides, sphingosine, and sphingomyelin indicated the importance of the oligosaccharide moiety, but not the sialic acid residues, in the enhancement of ethanol action by gangliosides. Variation of assay temperature and the acyl composition of the PC demonstrated that gangliosides increased the effects of ethanol only if the lipids were near the phase transition temperature. Gangliosides also produced a marked enhancement of the effect of pentobarbital on fluorescence polarization of probes in PC vesicles. The membrane partioning of pentobarbital was increased by gangliosides, but it was not clear that this increase in drug concentration in the membrane was sufficient to account for the large change in fluorescence polarization. These results suggest that ganglioside content may be an important determinant of the effects of alcohols and barbiturates on biomembranes. In particular, the high drug sensitivity of neuronal membranes may be related to the enrichment of gangliosides in these membranes.

INTRODUCTION

Alcohols and barbiturates disorder brain synaptic membranes (1–3). These effects occur at drug concentrations which are associated with intoxication and anesthesia *in vivo* and may be relevant to the pharmacological actions of the agents (1–3). Although intact synaptic membranes are perturbed by anesthetic concentrations of ethanol or pentobarbital, vesicles composed of phospholipids and neutral lipids from these membranes are relatively insensitive to effects of the drugs (1, 2). These results suggest that some constituent of synaptic membranes can increase the sensitivity of phospholipids to the disordering effects of alcohols and barbiturates. Gangliosides are found in high concentrations in brain and represent an important component of synaptic membranes (4, 5). It is important to note that in the most common procedures for extraction of membrane lipids (6, 7), gangliosides are lost into the aqueous phase[4] (8) and are not included in most studies of membrane lipids. Gangliosides have been implicated as binding sites for calcium, neurotransmitters, cholera toxin, and tetanus toxin (9–11), and may regulate the activity of brain adenylate cyclase (12). There are few studies of the effects of gangliosides on membrane physical properties, but they have been shown to broaden the phase transition of DMPC[5] and to increase the rigidity of liquid

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[5] The abbreviations used are: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; G_{D1a}, disialoganglioside; G_{M1}, monosialoganglioside; G_{T1b}, trisialoganglioside; PBS, phosphate-buffered saline; PC, phosphatidylcholine; POPC, palmitoleoylphosphatidylcholine; THF, tetrahydrofuran; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

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crystalline phospholipids (13). The experiments reported here were designed to elucidate the effects of gangliosides on the drug sensitivity of phospholipid vesicles. The lipid order, or "fluidity," was determined from the polarization of fluorescence of probe molecules incorporated into the vesicles. Most of the studies used DPH, a fluorescent probe of the terminal portions of the lipid acyl groups (14, 15). This probe has proven to be a sensitive indicator of the effects of ethanol and pentobarbital (1, 2). Two other fluorescent probes, TMA-DPH, which is localized near the membrane surface (16), and *trans*-parinarate, which is sensitive to the middle portions of the acyl chains (17, 18), were also tested. Gangliosides are localized in the outer half of the synaptic bilayer (19). The other major lipid constituents of this leaflet are PC and cholesterol (20). Accordingly, we chose PC and PC/cholesterol mixtures as the lipid matrix for evaluation of the effects of gangliosides. Most of the studies reported here used a mixture of gangliosides extracted from rat brain synaptic membranes, although some studies compared the effects of different species of brain gangliosides. The experiments demonstrated that gangliosides markedly enhance the sensitivity of phospholipid vesicles to the effects of ethanol and pentobarbital. To our knowledge, this is the only membrane component which produces such an effect. Therefore, this study represents the first demonstration of synthetic vesicles which retain the sensitivity of intact synaptic membranes to these drugs.

METHODS

Materials. Sphingomyelin (bovine brain), DMPC, DOPC, DPPC, and DSPC were obtained from Sigma Chemical Company (St. Louis, Mo.). No impurities were detected by thin-layer chromatography (silica gel H; $CHCl_3/CH_3OH/NH_4OH$; 14:7:1) and were used without purification. Asialoganglioside (asialo G_{M1}), monosialoganglioside (G_{M1}), disialoganglioside (G_{D1a}), and sphingosine were obtained from Supelco, Inc. (Bellefonte, Pa.). DPH, TMA-DPH, and *trans*-parinarate were from Molecular Probes, Inc. (Junction City, Ore.). 1-Palmitoyl-2-arachidonyl-[*arachidonyl*-1-^{14}C]phosphatidylcholine, 5-[*ring*-2-^{14}C]pentobarbital, and ^3H_2O were obtained from New England Nuclear Corporation (Boston, Mass.). Synaptic gangliosides were extracted from synaptic plasma membranes from adult rat brain (21) and purified essentially by the method of Ledeen and Yu (8). The ganglioside mixture consisted of 2.0% G_{Q1}, 22.7% G_{T1b}, 10.7% G_{D1b}, 2.45% $G_{D2/T1a}$, 28.1% G_{D1a}, 2.6% G_3, 19.4% G_{M1}, 3.0% G_{M2}, 8.6% G_{M3} + G_{M4}, and 1.0% unknown.

Preparation of lipid vesicles. Phospholipid (60 nmoles, dissolved in ethanol) was mixed with various amounts of sphingolipids (0–10 moles%; see Results for details) dissolved in $CHCl_3/CH_3OH$ (2:1) and 0.5 nmole of fluorescent probe dissolved in THF (DPH), THF/H_2O (1:1) (TMA-DPH), or ethanol (*trans*-parinarate). All solutions were stored under argon at −20°. The mixtures of lipids and probe were dried to a thin film by a stream of nitrogen and stored overnight under vacuum. One milliliter of PBS (NaCl, 8 g/liter; KCl, 0.2 g/liter; KH_2PO_4, 0.2 g/liter; $Na_2HPO_4 \cdot 7H_2O$, 1.15 g/liter; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.48 g/liter; pH 7.4) was added to each sample, and the tubes were purged with argon and incubated in the dark with occasional vortexing at about 10° above the phase transition temperature of the phospholipid. The samples were then placed in a bath-type sonicator for 1 min. (In one experiment, vigorous vortexing was compared with sonication, and both methods of vesicle preparation gave similar results.) These procedures give large multilamellar vesicles containing ganglioside and phospholipid (13). Vesicles of pure ganglioside were not formed because the concentration was well below the critical micellar concentration for gangliosides (0.2 mg/ml). After vigorous vortexing, the vesicle suspension was transferred to a quartz cuvette (10 × 4 mm) for fluorescence measurements.

Fluorescence measurements. An HH-1 T-format polarization spectrofluorimeter (BHL Associates, Burlingame, Calif.) with fixed excitation and emission polarization filters was used to measure fluorescence intensity parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the polarization plane of the exciting light (2). Polarization of fluorescence $[(I_\parallel - I_\perp)/(I_\parallel + I_\perp)]$ and intensity of fluorescence ($I_\parallel + 2I_\perp$) were calculated by an online microprocessor. Fluorescence lifetime and rotational relaxation time were determined with an SLM 4800 spectrofluorimeter as described previously (2). For DPH and TMA-DPH, the excitation wavelength was 362 nm, and a 03FCG001 filter (band pass of 310–390 nm) (Melles Griot, Irvine, Calif.) was used in the excitation beam and 03FIR045 filters (band pass of 424–444 nm) were used for the emitted light. For *trans*-parinarate, the excitation wavelength was 324 nm, and 03FIV113 (band pass of 380–440 nm) filters were used for the emission. Cuvette temperature was maintained by a circulating water bath and monitored continuously by a thermister inserted into the cuvette to a level just above the light beam. After determination of the baseline fluorescence polarization, aliquots (1–40 μl) of ethanol or pentobarbital solution were added and polarization was determined 2–4 min later (1, 2).

Lipid partitioning of pentobarbital. To determine whether gangliosides altered the membrane partitioning of pentobarbital, vesicles of DMPC were prepared with or without G_{M1}. Vesicles were prepared as described above except that 1500 nmoles of DMPC and 150 nmoles of G_{M1} were suspended in 1 ml of PBS. The vesicles were incubated with 3 mM pentobarbital containing 0.5 μCi of [^{14}C]pentobarbital and 2 μCi of ^3H_2O for 20 min at 30°. The vesicles were pelleted (100,000 × *g* for 90 min), the supernatant was removed, and the amount of ^{3}H and ^{14}C in the pellet was determined by liquid scintillation counting for dual label (Beckman LS9000 with data reduction program 951956). These data were used to determine the amount of water trapped in the pellet, the amount of pentobarbital in the trapped water, and the amount of pentobarbital in the lipid (22). The amount of phospholipid recovered in the pellet was determined with separate samples which contained [^{14}C]PC and did not contain [^{14}C]pentobarbital.

Statistical analysis. The effects of drug addition on fluorescence polarization were evaluated by a Student's *t*-test for paired samples. Comparisons between groups used a *t*-test for unpaired samples.

RESULTS

Effects of gangliosides on ethanol-induced membrane perturbation. Fluorescent probes were used to assess the membrane-disordering effects of ethanol. DPH was used as a probe of the hydrophobic core of the membrane (14, 15). The fluorescence polarization of DPH incorporated into DMPC vesicles was decreased by *in vitro* exposure to ethanol, suggesting an increase in probe mobility (15). However, these vesicles were rather insensitive to ethanol, as a concentration of 200–400 mM was required to produce a clear decrease in polarization (Fig. 1). Incorporation of 1–10 mole% synaptic gangliosides into the DMPC vesicles markedly enhanced the effect of ethanol. With 10% gangliosides, a significant ($p < 0.05$) decrease in polarization was obtained with the lowest concentration of ethanol tested (56 mM). In other experiments, TMA-DPH was used as a probe of the outer portions of the membranes (16), and *trans*-parinarate was used as probe of the lower region of the acyl chains (17, 18). *trans*-Parinarate also partitions into the more rigid (gel phase) domains of the membrane (18). With vesicles of DMPC alone, an ethanol concentration of about 400 mM was required to produce a decrease in the fluorescence polarization of either TMA-DPH or *trans*-parinarate

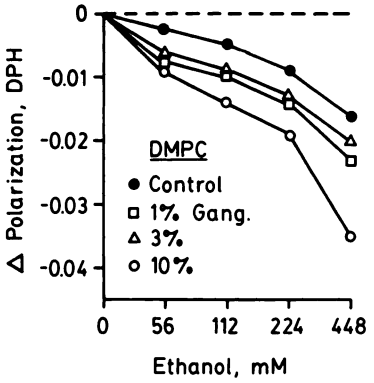

FIG. 1. *Effects of ethanol on the fluorescence polarization of DPH incorporated into vesicles of DMPC or DMPC and synaptic gangliosides*

Vesicles were exposed to the concentration of ethanol indicated on the abscissa; the change in fluorescence polarization is shown on the ordinate. ●, Vesicles of DMPC; □, DMPC plus 1 mole% gangliosides; △, DMPC plus 3 mole% gangliosides; ○, DMPC plus 10 mole% gangliosides. Assay temperature was 30°. Values represent the mean from three to five preparations. For each value, the standard error of the mean was ≤0.002. Values for baseline polarization are given in Table 3.

(Fig. 2). Addition of 10 mole% of synaptic gangliosides greatly increased the sensitivity of the membranes to ethanol. For all three probes, 10 mole% gangliosides enhanced the action of ethanol by about 5-fold (Figs. 1 and 2). However, DPH was more sensitive than TMA-DPH or *trans*-parinarate to the effects of ethanol with both DMPC and DMPC-ganglioside vesicles.

A decrease in fluorescence polarization usually reflects an increase in the mobility of the probe, but could result from an increase in fluorescence lifetime without a change in probe mobility (15, 23, 24). To distinguish between these possibilities, the fluorescence lifetime of DPH in vesicles of DMPC and DMPC plus ganglioside was determined in the absence and presence of ethanol. In agreement with studies of synaptic membranes (2), ethanol did not alter fluorescence lifetime; ganglioside also failed to affect lifetime (Table 1). Once fluorescence lifetime is known, changes in probe mobility can be quantified by calculating the rotational relaxation time (15). Ethanol produced a larger decrease in rotational relaxation time of DPH in vesicles of DMPC plus ganglioside than in vesicles of DMPC alone (Table 1). Thus, the observed changes in polarization reflect changes in probe mobility. Based on the literature in this area, we will consider an increase in probe mobility indicative of an increase in membrane fluidity or disorder (1, 2, 14–18, 23, 24). This interpretation is strengthened by the observation that similar results were obtained from three probes with different molecular configurations, different membrane locations and different types of motion within the membrane (14, 16, 18, 23, 24).

The importance of the acyl composition of the PC in the effects of gangliosides was studied by comparing the ethanol sensitivity of vesicles of DPPC, DSPC, and DOPC with and without the addition of 10 mole% synaptic gangliosides. DPH was used as the fluorescent probe in these studies. At 30° (the temperature used in the experiments with DMPC described above), DPPC

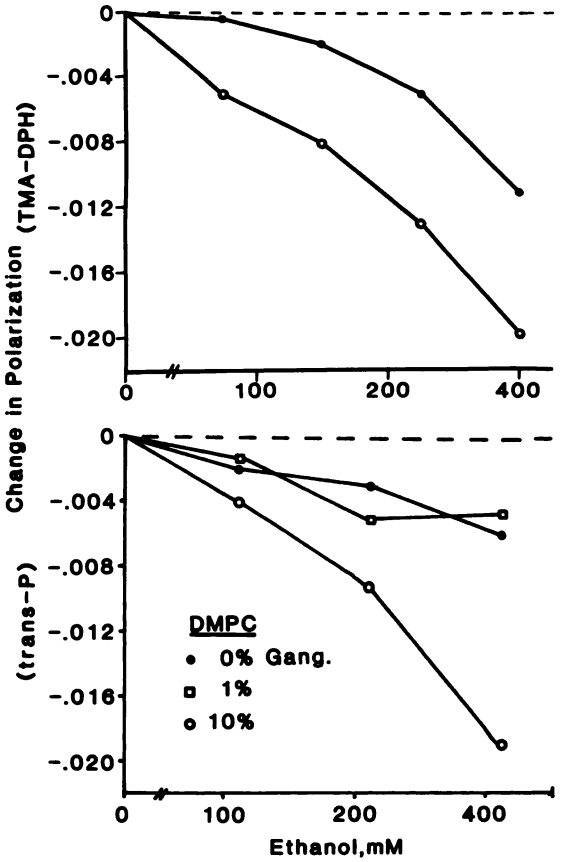

FIG. 2. *Effects of ethanol on the fluorescence polarization of TMA-DPH (upper panel) or trans-parinarate (lower panel) incorporated into vesicles of DMPC or DMPC and synaptic gangliosides*

Vesicles were exposed to the concentration of ethanol indicated on the abscissa; the change in fluorescence polarization is shown on the ordinate. ●, Vesicles of DMPC; □, DMPC plus 1 mole% gangliosides; ○, DMPC plus 10 mole% ganglioside. Assay temperature was 30°. Values represent the mean from three to four preparations. For each value, the standard error of the mean was ≤0.002. Values for baseline polarization are given in Table 3.

showed little effect of ethanol even at a concentration of 560 mM (Fig. 3, left panel). Addition of gangliosides produced only a slight enhancement of ethanol action. Because DPPC exists in a gel phase at 30° whereas DMPC is in a liquid crystalline phase at that temperature (25), we postulated that the effects of gangliosides may

TABLE 1

Effects of ethanol on the fluorescence lifetime (τ) and rotational relaxation time ($\bar{\rho}$) of DPH in vesicles of DMPC or DMPC + G_{M1}

Vesicles were prepared of DMPC or DMPC plus 10 mole% G_{M1}; DPH fluorescence was determined at 30° before and after addition of 170 mM ethanol.

Preparation	τ[a]	$\bar{\rho}$[a]	$\Delta\bar{\rho}$[a]
	nsec	*nsec*	*nsec*
DMPC	8.8 ± 0.1	10.5 ± 0.2	
+ ethanol	8.5 ± 0.2	9.7 ± 0.2[b]	0.8 ± 0.1
DMPC + G_{M1}	9.0 ± 0.2	14.1 ± 0.2[c]	
+ ethanol	8.8 ± 0.2	11.6 ± 0.2[b]	2.5 ± 0.2[c]

[a] Values are means ± standard error of the mean, $n = 3$.
[b] Significant effect of ethanol ($p < 0.01$).
[c] Significant effect of ganglioside ($p < 0.01$).

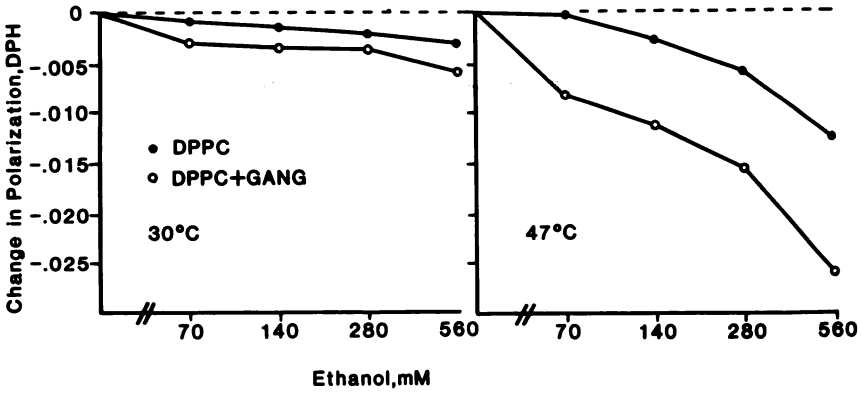

FIG. 3. *Effects of ethanol on the fluorescence polarization of DPH in vesicles of DPPC or DPPC and synaptic gangliosides above or below the phase transition temperature*

Vesicles were exposed to the concentration of ethanol indicated on the abscissa; the change in fluorescence polarization is shown on the ordinate. ●, Vesicles of DPPC; ○, DPPC plus 10 mole% gangliosides. Data shown in the left panel were obtained at 30°, those in the right panel at 47°. Values represent the mean from three to four preparations. The standard error of the mean was ≤0.002. Values for baseline polarization are given in Table 2.

require the presence of fluid phases. At 47°, gangliosides augmented the membrane-disordering effects of ethanol on DPPC vesicles to an extent similar to that observed with DMPC at 30° (Fig. 3). In addition, gangliosides enhanced the effects of ethanol on DSPC vesicles at 65° (Fig. 4), a temperature 6° above the phase transition temperature of DSPC. Gangliosides did not alter the action of ethanol below the phase transition of DSPC (data not shown). These results suggest that more fluid lipids may be more affected by inclusion of gangliosides. This possibility was evaluated by monitoring the effects of ethanol on DMPC and DMPC/ganglioside vesicles cooled through the phase transition temperature (Fig. 5). Ganglioside (10 mole%) elevated the phase transition by about 2° and enhanced the effect of ethanol at temperatures near the phase transition.

Cholesterol is a major component of synaptic membranes, and it was of interest to determine the effects of gangliosides on vesicles containing DMPC and 33 mole% cholesterol. As noted by others, cholesterol increased the

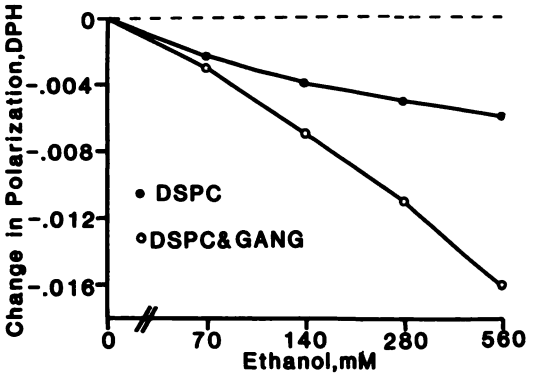

FIG. 4. *Effects of ethanol on the fluorescence polarization of DPH in vesicles of DSPC or DSPC and synaptic gangliosides*

Vesicles were exposed to the concentration of ethanol indicated on the abscissa; the change in fluorescence polarization is shown on the ordinate. ●, Vesicles of DSPC; ○, DSPC plus 10 mole% gangliosides. Assay temperature was 65°. Values represent the mean from three to four preparations. The standard error of the mean was ≤0.002. Values for baseline polarization are given in Table 2.

sensitivity of DMPC to a high concentration of ethanol (26) but did not alter the effects of low concentrations (Fig. 6). Synaptic gangliosides markedly enhanced the sensitivity of DMPC/cholesterol vesicles to all concentrations of ethanol. This increased sensitivity to ethanol was more pronounced at 47° than at 30° (Table 2).

All of the experiments presented above were carried out with mixed synaptic gangliosides. To determine the structure-activity relationship of the gangliosides, several sphingolipids were incorporated (10 mole%) into DMPC vesicles, and the ethanol sensitivity was determined (Fig. 7; Table 2). The largest enhancement of ethanol action was obtained with asialo G_{M1}, followed by G_{M1}, G_{D1a}, and G_{T1b}. Sphingosine and sphingomyelin produced little enhancement.

Effects of gangliosides on lipid rigidity. In addition to altering the drug sensitivity of DMPC vesicles, gangliosides also alter the baseline polarization of fluorescent probes in the lipid. Addition of synaptic gangliosides to DMPC increased the fluorescence polarization of DPH, TMA-DPH, and *trans*-parinarate (Table 3). The baseline polarization of DPH was increased by sphingolipids in the order following: asialo G_{M1} > G_{M1} > G_{D1a} > G_{T1b} > sphingosine > sphingomyelin (Table 2).

Effects of gangliosides on the sensitivity of DMPC to pentobarbital. The membrane-disordering effect of pentobarbital was studied with vesicles of DMPC and DMPC plus 10 mole% G_{D1a}. Fluorescence polarization of DPH was again used as a measure of membrane order. Vesicles of DMPC were not affected by *in vitro* exposure to concentrations of pentobarbital of 0.2–1.6 mM (Fig. 8). DMPC vesicles containing ganglioside, however, were quite sensitive to these concentrations of pentobarbital.

Effects of gangliosides on the membrane partitioning of pentobarbital. Because the membrane-disordering effects of ethanol and pentobarbital are dependent on their membrane concentrations (2, 20), it was of interest to determine whether gangliosides increased the amount of drug in the lipid phase. It is difficult to measure the membrane concentration of ethanol because of its low lipid solubility, but the partioning of [^{14}C]pentobarbital

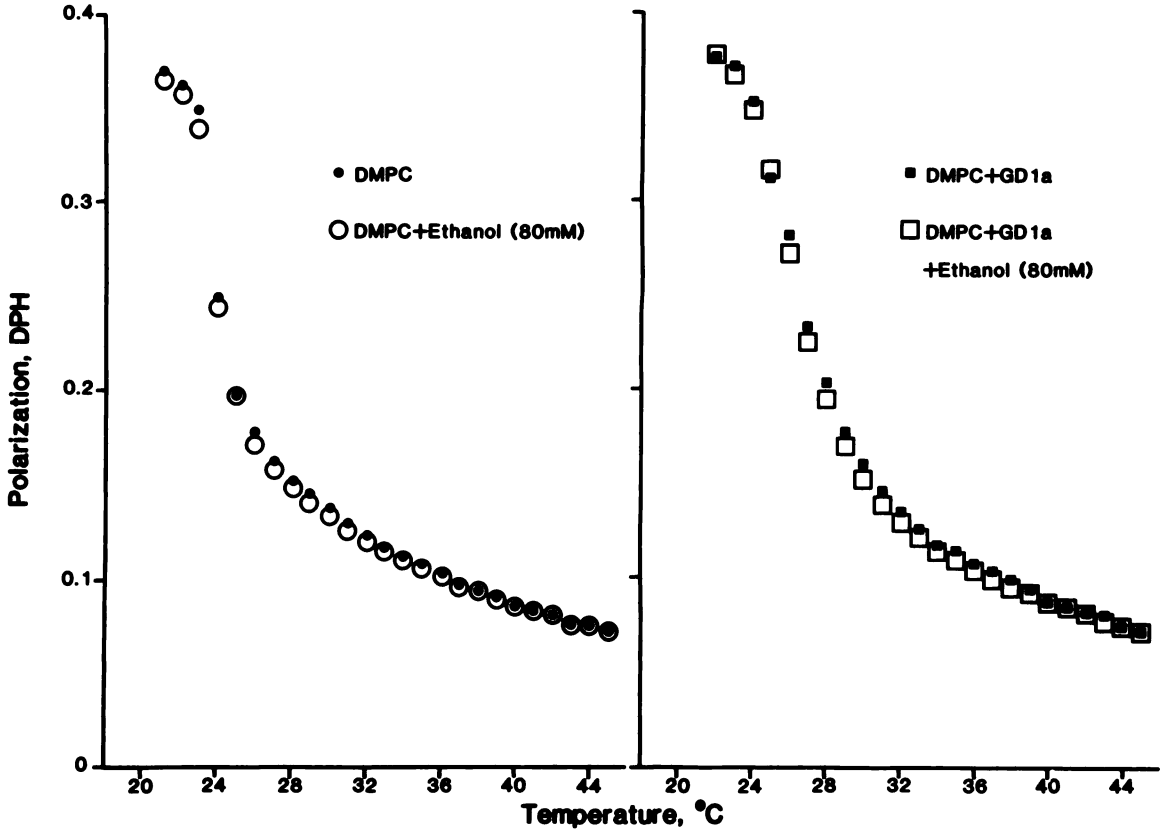

FIG. 5. *Effects of ganglioside and ethanol (80 mM) on the phase transition of DMPC*

DPH was incorporated into DMPC (left panel) or DMPC plus 10 mole% G_{D1a} (right panel). Fluorescence polarization was determined in the absence (●, ■) and presence (○, □) of 80 mM ethanol. Temperature was decreased from 45° to 22° at a rate of 0.5°/min. Values are the means from temperature scans obtained with three different membrane preparations. For each point, the standard error of the mean is smaller than the size of the symbols.

may be quantitated. Using an aqueous concentration of 3 mM pentobarbital, vesicles of DMPC contained 0.022 ± 0.001 moles of pentobarbital/mol lipid whereas vesicles of DMPC plus 10 mole% G_{D1a} contained 0.040 ± 0.003 mole of pentobarbital/mole of lipid (mean ± standard error of the mean, $n = 3$). Thus, ganglioside increased the membrane solubility of pentobarbital by 77% ($p < 0.01$).

DISCUSSION

The present results imply that gangliosides enhance the membrane-disordering effects of ethanol and of pentobarbital. The increased sensitivity to ethanol was observed with phosphatidylcholine vesicles containing gangliosides extracted from brain synaptic membranes as well as purified gangliosides. These results may be relevant to the actions of alcohols and barbiturates on synaptic membranes. Brain gangliosides are enriched in neuronal membranes, where they account for 5–15% of the lipid (4, 5, 27). Because gangliosides are specifically localized in the outer half of the synaptic bilayer, their concentration in that leaflet is 10–30% (19, 27). Phosphatidylcholine is also localized in the external face of synaptic membranes (20). Thus, a mixture of gangliosides and PC or gangliosides, PC, and cholesterol (another major synaptic lipid) represents a reasonable model of the external half of the synaptic membrane bilayer. Other studies have shown that intact synaptic membranes are more sensitive to the membrane-perturbing action of ethanol and pentobarbital than are vesicles formed from lipids (phospholipids or phospholipids plus neutral lipids) extracted from the membranes (1, 2). Thus, the sensitivity of synaptic membranes to the disordering effects of intoxicant-anesthetic drugs may be due, at least in part, to the presence of gangliosides in the membranes. This role of gangliosides has important consequences for the "disordered lipid" hypothesis of intoxication and anesthesia. One criticism of this hypothesis is that pharmacologically relevant concentrations of anesthetics have little or no effect on the order of phospholipid vesicles (28). The concentration of ethanol in the aqueous phase of mouse brain at the time of regaining righting reflex is 115 mM (calculated from the data in ref. 29 by assuming that brain tissue is 77% water and the membrane-water partition coefficient of ethanol is 0.1). The present study confirmed that concentrations of ethanol greater than 200 mM are required to perturb PC or PC/cholesterol vesicles, yet PC/ganglioside or PC/cholesterol/ganglioside vesicles were much more sensitive to ethanol and were affected by concentrations of ethanol (50–120 mM) associated with intoxication and anesthesia *in vivo*. A separate issue is

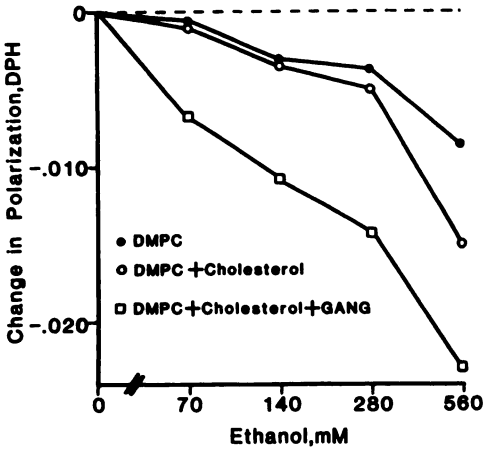

FIG. 6. *Effects of ethanol on the fluorescence polarization of DPH in vesicles of DMPC, cholesterol, and synaptic gangliosides*

Vesicles were exposed to the concentration of ethanol indicated on the abscissa; the change in polarization is shown on the ordinate. ●, Vesicles of DMPC; ○, vesicles of DMPC plus 33 mole% cholesterol; □, vesicles of DMPC, cholesterol (33 mole%), and gangliosides (10 mole%). Assay temperature was 47°. Values represent the mean from three preparations. The standard error of the mean was ≤0.002. Values for baseline polarization are given in Table 2.

TABLE 2

Comparison of baseline polarization of DPH fluorescence with the decrease in polarization produced by ethanol for different lipids

Lipid mixture	Assay temperature	Baseline polarization[a]	Ethanol-induced decrease in polarization[b]
Sphingolipid effects			
1. DMPC	30	0.140	0.005
2. DMPC + SG[c]	30	0.187	0.016
3. DMPC + Asialo G_{M1}	30	0.188	0.018
4. DMPC + G_{M1}	30	0.177	0.012
5. DMPC + G_{D1a}	30	0.175	0.013
6. DMPC + G_{T1b}	30	0.168	0.011
7. DMPC + sphingomyelin	30	0.153	0.008
8. DMPC + sphingosine	30	0.165	0.010
Cholesterol effects			
9. DMPC + Chol[d]	30	0.294	0.005
10. DMPC + Chol + SG	30	0.260	0.008
11. DMPC	47	0.073	0.004
12. DMPC + Chol	47	0.171	0.005
13. DMPC + Chol + SG	47	0.161	0.015
Acyl group and temperature effects			
14. DMPC	65	0.040	0.002
15. DMPC + SG	65	0.051	0.006
16. DPPC	30	0.391	0.002
17. DPPC + SG	30	0.344	0.004
18. DPPC	47	0.124	0.006
19. DPPC + SG	47	0.131	0.016
20. DSPC	65	0.068	0.005
21. DSPC + SG	65	0.067	0.011
22. DOPC	30	0.090	0.006
23. DOPC + SG	30	0.091	0.005
24. DOPC/DPPC (2:3) + SG	47	0.121	0.013
25. DOPC/DPPC (3:2) + SG	47	0.109	0.011

[a] Values are the means of three to five determinations. For the baseline polarization, variation among replicates was usually less than 0.005.

[b] Represents the decrease in polarization produced by *in vivo* exposure to 280 mM ethanol. Values are the means of three to five determinations; variation among replicates was usually less than 0.003.

[c] SG, synaptic gangliosides. The ganglioside (or sphingolipid) concentration was 10 mole% in all cases.

[d] Chol, cholesterol. The concentration was 33 mole%.

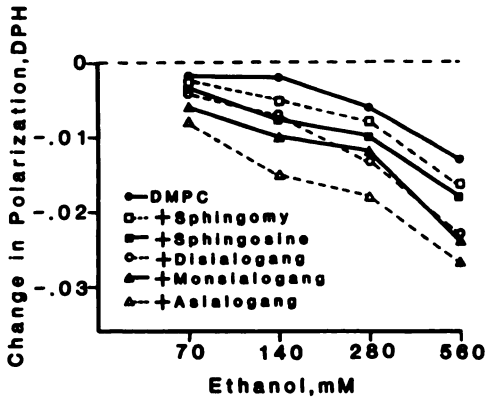

FIG. 7. *Effects of ethanol on the fluorescence polarization of DPH in vesicles of DMPC or DMPC with sphingolipids*

Vesicles were exposed to the concentration of ethanol indicated on the abscissa; the change in polarization is shown on the ordinate. ●——●, DMPC; □---□, DMPC plus sphingomyelin; ■——■, DMPC plus sphingosine; ○---○, G_{D1a}; ▲——▲, G_{M1}; △---△, asialo G_{M1}. The concentration of sphingolipid was 10 mole% in all cases. Assay temperature was 30°. Values are the mean from three to four preparations. The standard error of the mean was ≥0.002. Values for baseline polarization are given in Table 2.

TABLE 3

Effects of synaptic gangliosides on baseline fluorescence polarization of probe molecules in DMPC vesicles

Mole% gangliosides	Polarization of fluorescence[a]		
	DPH	TMA-DPH	*trans*-Parinarate
0	0.143 ± 0.002	0.230 ± 0.002	0.152 ± 0.001
1	0.152 ± 0.002[b]	0.235 ± 0.003	0.152 ± 0.003
3	0.164 ± 0.002[b]	0.250 ± 0.002[b]	0.158 ± 0.002[c]
10	0.189 ± 0.001[b]	0.262 ± 0.002[b]	0.167 ± 0.004[b]

[a] Values represent means ± standard error of the mean, n = 3–5. Fluorescence was determined at 30°.

[b] Significant effect of ganglioside ($p < 0.01$).

[c] Significant effect of ganglioside ($p < 0.05$).

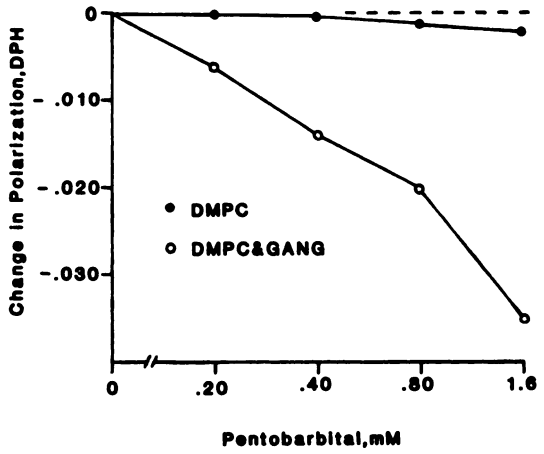

FIG. 8. *Effects of pentobarbital on the fluorescence polarization of DPH in vesicles of DMPC or DMPC and ganglioside*

Vesicles were exposed to the concentration of pentobarbital shown on the abscissa; the change in fluorescence polarization is shown on the ordinate. ●, Vesicles of DMPC; ○, DMPC plus 10 mole% G_{D1a}. Assay temperature was 30°. Values are the mean of three preparations. The standard error of the mean was ≥0.002.

whether the changes in membrane order produced by pharmacologically relevant concentrations of ethanol are sufficient to alter membrane function. There is evidence that relatively small changes in fluorescence polarization may result in significant functional changes (see refs. 1 and 30), but a definitive answer to this crucial question will require further studies.

The enhancement of alcohol action by gangliosides required that the PC be slightly above the phase transition temperature. This finding may be related to the observations of Tillack *et al.* (13) that asialo G_{M1} and DMPC form different types of mixtures depending upon the temperature. In the gel phase, the ganglioside appears to be segregated between ridges of PC, but above the phase transition the ganglioside exists as single molecules or small clusters. In a more fluid lipid (POPC at room temperature), the ganglioside forms large clusters. Thus, enhancement of ethanol action may occur only when gangliosides are able to mix with the PC and interact with most of the PC molecules. If the temperature (or fluidity) is either too low or too high, the gangliosides separate laterally into a separate phase which may have little influence on the properties of the PC phase.

One possible mechanism for the enhancement of ethanol and barbiturate effects by gangliosides is an increased penetration of the drugs in the lipid phase. Studies of the partitioning of pentobarbital demonstrated that gangliosides did increase the membrane concentration of the drug. However, the increase in drug concentration was only 77%, and even an 8-fold increase in pentobarbital concentration produced only a slight decrease in the polarization of DPH in pure DMPC, whereas ganglioside resulted in a 10-fold enhancement of pentobarbital action. It is possible that gangliosides do not affect all portions of the membrane equally and produce a large increase in drug concentration in specific regions. For example, if gangliosides increase the drug partitioning into fluid-phase lipids, but not into gel-phase lipids, then drug effects would not be enhanced below the phase transition temperature (31).

A comparison of several sphingolipids indicated the importance of the oligosaccharide moieties of the gangliosides for the enhancement of ethanol action. Sphingomyelin and sphingosine produced only a slight increase in alcohol effect, indicating that the acyl portion of gangliosides was not of primary importance in their action. The most potent compound was asialo G_{M1}, demonstrating the importance of the oligosaccharide constituents and the lack of importance of the sialic acid groups. The latter is also indicated by the similar effects of G_{M1}, G_{D1a}, and G_{T1b}. Sialic acid allows gangliosides to bind calcium and to interact with PC (9, 10, 27). Apparently neither of these factors was important for the enhancement of alcohol actions. The lack of a role for calcium was also noted in experiments where the effect of synaptic gangliosides on alcohol sensitivity was not altered by the addition of 0.1 mM $CaCl_2$ or ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid to the assay medium.[6]

[6] D. M. Baxter and R. A. Harris, unpublished data.

These results suggest that the ganglioside content of biological membranes is one factor that determines the sensitivity of the membranes to ethanol and other intoxicant-anesthetic drugs. Several reports indicate that alcohols stimulate synaptic neuraminidase activity (32) and that chronic ethanol ingestion changes the ganglioside and sialoglycoprotein content of mammalian membranes (33, 34). Although we failed to detect any effect of alcohol treatment on the ganglioside content of synaptic membranes (29), it is tempting to speculate that some of the changes in the physical properties of symnaptic membranes produced by chronic alcohol exposure may be due to subtle alterations in ganglioside arrangement or metabolism.

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REFERENCES

1. Harris, R. A., and F. Schroeder. Ethanol and the physical properties of brain membranes: fluorescence studies. *Mol. Pharmacol.* **20**:128–137 (1981).
2. Harris, R. A., and F. Schroeder. Effects of barbiturates and ethanol on the physical properties of brain membranes. *J. Pharmacol. Exp. Ther.* **223**:424–431 (1982).
3. 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).
4. Wiegandt, H. The subcellular localization of gangliosides in the brain. *J. Neurochem.* **14**:671–674 (1967).
5. Skrivanek, J. A., R. W. Ledeen, R. U. Margolis, and R. K. Margolis. Gangliosides associated with microsomal subfractions of brain: comparison with synaptic plasma membranes. *J. Neurobiol.* **13**:95–106 (1982).
6. Bligh, E. G., and W. J. Dyer. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**:911–917 (1959).
7. Folch, J., M. D. Lees, and G. H. Sloan-Stanley. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**:497–509 (1957).
8. Ledeen, R. W., and R. K. Yu. Methods for isolation and analysis of gangliosides, in *Research Methods in Neurochemistry*, Vol. 4 (N. Marks and R. Rodnight, eds.). Plenum Press, New York, 371–410 (1978).
9. Maggio, B., F. A. Cumar, and R. Caputto. Molecular behavior of glycosphingolipids in interfaces: possible participation in some properties of nerve membranes. *Biochim. Biophys. Acta* **650**:69–87 (1981).
10. Leskawa, K. C., and A. Rosenberg. Displacement of Ca^{2+} bound to synaptosomal membrane sialoglycoconjugates by serotonin and serotonergic drugs and the effect on endogenous sialidase (neuraminidase) activity. *Biochem. Pharmacol.* **31**:1787–1790 (1982).
11. Fishman, P. H. Role of membrane gangliosides in the binding and action of bacterial toxins. *J. Membr. Biol.* **69**:85–97 (1982).
12. Partington, C. R., and J. W. Daly. Effect of gangliosides on adenylate cyclase activity in rat cerebral cortical membranes. *Mol. Pharmacol.* **15**:484–491 (1978).
13. Tillack, T. W., M. Wong, M. Allietta, and T. E. Thompson. Organization of the glycospingolipid asialo-G_{M1} in phosphatidylcholine bilayers. *Biochim. Biophys. Acta* **691**:261–273 (1982).
14. Van Blitterswijk, W. J., R. P. Van Hoeven, and B. W. Van Der Meer. Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements. *Biochim. Biophys. Acta* **644**:323–332 (1981).
15. Shinitzky, M., and Y. Barenholz. Fluidity parameters of lipid regions determined by fluorescence polarization. *Biochim. Biophys. Acta* **515**:367–394 (1978).
16. Prendergast, F. G., R. P. Haugland, and P. J. Callahan. 1-[4-(Trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene: synthesis, fluorescence properties, and use as a fluorescence probe of lipid bilayers. *Biochemistry* **20**:7333–7338 (1981).
17. Schroeder, F. Fluorescence probes as monitors of surface membrane fluidity gradients in murine fibroblasts. *Eur. J. Biochem.* **112**:293–307 (1980).
18. Sklar, L. A., B. S. Hudson, and R. D. Simoni. Conjugated polyene fatty acids as fluorescent probes: synthetic phospholipid membrane studies. *Biochemistry* **16**:819–828 (1977).
19. Lehninger, A. L. The neuronal membrane. *Proc. Natl. Acad. Sci. U. S. A.* **60**:1069–1080 (1968).
20. Fontaine, R. N., R. A. Harris, and F. Schroeder. Aminophospholipid asym-

metry in murine synaptosomal plasma membrane. *J. Neurochem.* **34**:269–277 (1980).
21. Hitzemann, R. J. Developmental changes in the fatty acids of synaptic membrane phospholipids: effect of protein and malnutrition. *Neurochem. Res.* **6**:935–947 (1981).
22. Rottenberg, H., A. Waring, and E. Rubin. Tolerance and cross-tolerance in chronic alcoholics: reduced membrane binding of ethanol and other drugs. *Science (Wash. D. C.)* **213**:583–585 (1981).
23. Kinosita, K., R. Kataoka, Y. Kimura, O. Gotoh, and A. Ikegami. Dynamic structure of biological membranes as probed by 1,6-diphenyl-1,3,5-hexatriene: a nanosecond fluorescence depolarization study. *Biochemistry* **20**:4270–4277 (1981).
24. Lakowicz, J. R., F. G. Prendergast, and D. Hogen. Differential polarized phase fluorimetric investigations of diphenylhexatriene in lipid bilayers: quantitation of hindered depolarizing rotations. *Biochemistry* **18**:508–519 (1979).
25. Jain, M. K., and R. C. Wagner. *Introduction to Biological Membranes.* Wiley-Interscience, New York, 95 (1980).
26. 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).
27. Svennerholm, L. Gangliosides and synaptic transmission. *Adv. Exp. Med. Biol.* **125**:533–544 (1980).
28. Franks, N. P., and W. R. Lieb. Molecular mechanisms of general anaesthesia. *Nature (Lond.)* **300**:487–493 (1982).
29. Harris, R. A., D. M. Baxter, M. A. Mitchell, and R. J. Hitzemann. Physical properties and lipid composition of brain membranes from ethanol tolerant-dependent mice. *Mol. Pharmacol.* **25**:401–409 (1984).
30. Yamamoto, H.-A., and R. A. Harris. Effects of ethanol and barbiturates on Ca^{2+}-ATPase activity of erythrocyte and brain membranes. *Biochem. Pharmacol.* **32**:2787–2791 (1983).
31. Rowe, E. S. Lipid chain length and temperature dependence of ethanol-phosphatidylcholine interactions. *Biochemistry* **22**:3299–3304 (1983).
32. Scheel, G., E. Acevedo, E. Conzelmann, H. Nehrokorn, and K. Sandhoff. Model for the interaction of membrane-bound substrates and enzymes: hydrolysis of ganglioside G_{D1a} by sialidase of neuronal membranes isolated from calf brain. *Eur. J. Biochem.* **127**:245–253 (1982).
33. Stibler, H., E. Burns, T. Keuckeberg, P. Gaetano, E. Cervén, S. Borg, and B. Tabakoff. Effect of ethanol on synaptosomal sialic acid metabolism in the developing rat brain. *J. Neurol. Sci.* **59**:21–35 (1983).
34. Goldstein, D. B., B. L. Hungund, and R. C. Lyon. Increased surface glycoconjugates of synaptic membranes in mice during chronic ethanol treatment. *Br. J. Pharmacol.* **78**:8–10 (1983).

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