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Ethanol Enhances [^{3}H]Diazepam Binding at the Benzodiazepine-γ-Aminobutyric Acid Receptor-Ionophore Complex

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SUMMARY

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Ethanol, which has a pharmacological profile similar to those of barbiturates and benzodiazepines, enhances [^{3}H]diazepam binding to the crude Lubrol-solubilized fraction. This fraction had specific binding sites for [^{3}H]diazepam, [^{3}H]muscimol, and [α-^{3}H]dihydropicrotoxinin (DHP). Ethanol enhanced [^{3}H]diazepam to this fraction in a dose-dependent manner with a maximal enhancement of 90 ± 8.5% occurring at 100 mM and a half-maximal effect occurring at 30 mM. Ethanol (100 mM) changed the K_D from a control value of 9.36 ± 126 to 4.40 ± 0.33 nM ($p > 0.005$). The B_{max} of [^{3}H]diazepam binding was not significantly altered. The enhancing effect of ethanol was blocked by picrotoxinin and (+)-bicuculline. Ethanol, while enhancing [^{3}H]diazepam binding to the crude Lubrol fraction, inhibited partially the binding of [^{3}H]DHP and had no effect on [^{3}H]muscimol binding. The rank order of enhancement of [^{3}H]diazepam binding with various alcohols (ethanol > methanol > isopropyl alcohol > propanol-1 = *t*-butyl alcohol = butanol-1) did not agree with their partition coefficients. These results suggest that ethanol, like pentobarbital, enhances [^{3}H]diazepam binding at the benzodiazepine-γ-aminobutyric acid (GABA) receptor-ionophore complex. This interaction will result in facilitation of GABAergic transmission and may be responsible for some of the central effects of alcohol, such as antianxiety, muscle relaxant, and sedative. We also provide evidence that ethanol does not enhance [^{3}H]diazepam directly at the benzodiazepine binding site, but, rather, indirectly via the picrotoxinin-sensitive site of the benzodiazepine-GABA receptor-ionophore complex.

INTRODUCTION

The molecular mechanisms by which alcohol (ethanol) produces its CNS[1] effects have yet to be elucidated. Ethanol and other general anesthetics are known to cause perturbation of biological membranes. Interaction between ethanol and membrane components, especially lipids, has been demonstrated (1-3). The ability of ethanol to produce fluidization of membranes, which is similar to that observed by measuring the phase transitions of lipids as a function of increasing temperature, has been reported (4). Furthermore, cross-tolerance between ethanol and increases in temperature have also been observed by Li and Hahn (4).

In spite of these observations, it is still a matter of debate as to whether ethanol produces its CNS effects by altering the lipids or by affecting the function of a particular synaptic pathway. Ethanol has a pharmacological profile very similar to those for barbiturates and benzodiazepines. Furthermore, ethanol (5-7), barbiturates (8, 9), and benzodiazepines (10) have been reported to facilitate GABA-mediated inhibitory transmission in the CNS. Benzodiazepine binding sites in the CNS have been characterized (11, 12). Recent studies have demonstrated that barbiturates enhance [^{3}H]diazepam binding to rat brain membranes (13-15) and a crude Lubrol-solubilized fraction (16). Benzodiazepine binding sites are intimately associated with the GABA receptor-ionophore complex (17, 18). On the basis of these observations, we have investigated the interaction between ethanol and diazepam. However, since it is widely accepted that the effects of ethanol in the CNS are due to its ability to alter membrane structure and function (1-4), we have examined the interaction of ethanol with [^{3}H]diazepam bind-

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[1] The abbreviations used are: CNS, central nervous system; GABA, γ-aminobutyric acid; DHP, α-dihydropicrotoxinin; PEG, polyethylene glycol.

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ing in a crude Lubrol-solubilized fraction and the purified fraction.

MATERIALS AND METHODS

[^{3}H]Diazepam (76.8 Ci/mmole), [^{3}H]muscimol (7.13 Ci/mmole), and [^{3}H]DHP (29.6 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). Picrotoxinin was purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wisc.), and other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.). Nonradioactive benzodiazepines were gifts from Dr. W. Scott (Hoffmann-La Roche Inc., Nutley, N. J.).

Tissue preparation. Male Sprague-Dawley rats (150–200 g) were decapitated and their brains were rapidly removed and placed in buffer containing 0.2 M sodium chloride and 10 mM sodium phosphate, pH 7.0, at 0–4°. The mitochondrial plus microsomal (P_2 + P_3) fraction was prepared as described previously (14). Briefly stated, the tissue was homogenized twice with a Brinkman Polytron with 5-sec bursts 15 sec apart and centrifuged at 580 × *g* for 10 min. The supernatant was centrifuged at 140,000 × *g* for 45 min to obtain the P_2 + P_3 fraction. The pellet was resuspended in the buffer (0.2 M NaCl and 10 mM sodium phosphate, pH 7.0) and centrifuged at 140,000 × *g* for 30 min. The pellet was washed five times to remove endogenous GABA and other inhibitory substances by resuspension in the same buffer and centrifugation. The pellet was then resuspended in the buffer and used for binding assays (fresh tissue) or frozen overnight. On the following day, the pellet was thawed at room temperature, pelleted, washed once, and resuspended in a protein concentration of ~0.6 mg/ml and used for binding assays (frozen tissue). Protein was estimated by the method of Lowry *et al.* (19).

Solubilization procedure. The P_2 + P_3 pellet (without extensive washing) was subjected to solubilization procedures as described for [^{3}H]DHP (20, 21). Briefly stated, the P_2 + P_3 pellet was resuspended in 20 volumes of buffer, mixed with 1% Lubrol-Wx, subjected to 10 strokes of homogenization with a Teflon pestle, and kept on ice for 30 min. The supernatant was then centrifuged at 100,000 × *g* for 45 min. The yellowish supernatant was removed and the pellet was discarded. The extract was dialyzed in a vacuum flask against 250 ml of buffer for 4 hr, at 0–4°, and is referred to as the crude Lubrol-solubilized fraction. This fraction was used as such for the binding studies or was subjected to gel filtration on equilibrated columns of Sephadex G-200 (1.5 × 84 cm). Gel filtration of this extract gave two major protein peaks with apparent molecular weights of 185,000 (Peak I, picrotoxinin-binding fraction) and 61,000 (Peak II, benzodiazepine-binding protein).[2]

Binding to membrane receptors. [^{3}H]Diazepam binding to membranes was studied by a filtration assay as described previously (14). Aliquots of membrane suspension (0.5–0.8 mg of protein) were incubated with 1 nM [^{3}H]diazepam for 30 min, at 0°, with or without ethanol or other drugs, in a total incubation volume of 1 ml. Following incubation, triplicate 250-μl aliquots were rapidly filtered on Whatman GF/B filters. The filters were washed twice with 3 ml of ice-cold buffer, dried, and counted in 5 ml of toluene containing 0.2% Beckman BioSolve (Beckman Instruments, Inc., Irvine, Calif.) and 5 g of 2,5-diphenyloxazole per liter. The counting efficiency was 44 ± 2%. The background was determined in presence of 10 μM flurazepam. The specific binding represented 85 ± 6% of the total binding activity.

Binding to solubilized receptors. The binding of [^{3}H]diazepam, [^{3}H]DHP, and [^{3}H]muscimol to the crude Lubrol-solubilized fraction and Peak I and II fractions was studied by a centrifugation assay as described previously (20, 21). Routinely, 0.7 ml of these fractions, in triplicate, was incubated with 1 nM [^{3}H]diazepam with or without ethanol or other drugs in a total incubation volume of 1 ml for 30 min at 0°. Following incubation, 0.5 ml of 1% (w/v) bovine γ-globulin and 0.5 ml of 24% PEG 6000 were added to the vials. The mixture was rapidly Vortexed and centrifuged at 48,000 × *g* for 10 min. The pellet was rapidly washed once with 5 ml of cold buffer, solubilized overnight with 0.3 ml of soluene (Packard Instrument Company, Downers Grove, Ill.) and counted in 3 ml of scintillation fluid containing 5 g of 2,5-diphenyloxazole per liter of toluene. Background was determined in the presence of 10 μM flurazepam. The specific binding, using the PEG-centrifugation assay, usually represented 50 ± 3% (n = 5) of the total pelleted radioactivity. The counting efficiency was 44 ± 2%. For Scatchard plots, the concentration of [^{3}H]diazepam was varied from 0.25 to 25 nM.

The binding of [^{3}H]DHP (20, 21) and [^{3}H]muscimol to the crude Lubrol-solubilized fraction and Peak I and Peak II fractions was studied by a PEG-centrifugation method analogous to that described for [^{3}H]diazepam. The specific binding for [^{3}H]DHP was 38 ± 4%, and for [^{3}H]muscimol it was 40 ± 4% of the total binding activity (in the crude Lubrol-solubilized fraction).

RESULTS

Effects of ethanol on [^{3}H]diazepam binding to membranes. Table 1 shows the effect of 20 and 100 mM ethanol on specific [^{3}H]diazepam binding to fresh and frozen rat brain membranes. Ethanol produced a relatively higher increase in diazepam binding to frozen membranes. The maximal enhancing effect of 38 ± 10% was observed at 50–100 mM ethanol, with half-maximal enhancement occurring at 30 mM ethanol. However, the

TABLE 1

Effect of ethanol on [^{3}H]diazepam binding to fresh and frozen membranes

[^{3}H]Diazepam binding to fresh and frozen rat brain membranes was studied by a filtration assay as described under Materials and Methods. Aliquots of membrane homogenate were incubated for 30 min at 0–4° in triplicate with 1 nM [^{3}H]diazepam in the presence and absence of ethanol. The specific binding was 85 ± 6% of the total binding. Values represent the mean ± standard deviation of four experiments.

Ethanol	% Enhancement of specific [^{3}H]diazepam binding	
	Fresh membrane	Frozen membrane
20 mM	7 ± 3	12 ± 6
100 mM	26 ± 5	38 ± 10

[2] W. C. Davis and M. K. Ticku, manuscript submitted for publication.

ability of ethanol to enhance diazepam binding to membranes was not reproducible and varied with batches of membrane preparations. In order to overcome this problem, we initiated studies to investigate the effects of ethanol on soluble diazepam receptors.

Ligand-binding sites in the crude Lubrol-solubilized fraction. With the PEG-centrifugation assay as described under Materials and Methods, the binding of [^{3}H]diazepam in the crude Lubrol-solubilized fraction was specific and saturable. A typical experiment, in triplicate, using 1 nM [^{3}H]diazepam and 0.53 mg of protein, gave a total binding of 9244 ± 211 cpm and a background of 4543 ± 195 cpm. Thus, the specific binding was 4701 cpm. The diazepam binding to the Lubrol extract was inhibited by benzodiazepines such as clonazepam (IC_{50} = 12 nM) and flurazepam (IC_{50} = 16 nM). These values are similar to those reported by others for the soluble diazepam-binding sites (22, 23). Figure 1 shows the Scatchard plots of diazepam binding to the membrane (○) as studied by a filtration assay and to the crude Lubrol-solubilized (●) fractions as studied by a centrifugation assay. Table 2 compares the binding constants of diazepam binding to membranes and to the crude Lubrol-solubilized fractions. The apparent affinities of [^{3}H]diazepam were similar in these two preparations; however, the B_{max} was 37% lower in the Lubrol-solubilized fraction. These results indicate that the affinity and the ligand specificity of diazepam is not altered by Lubrol solubilization, and that the PEG-centrifugation method can be used to measure [^{3}H]diazepam binding to the soluble fraction.

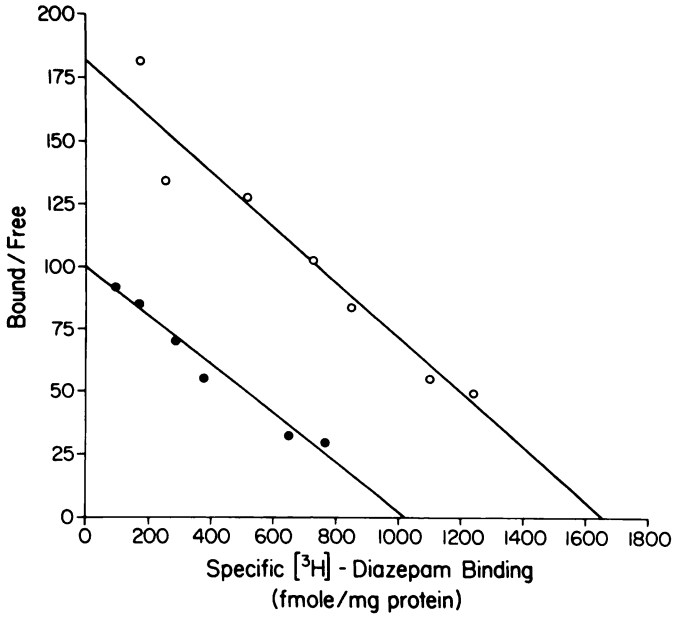

FIG. 1. *Scatchard plot of [^{3}H]diazepam binding to rat brain membrane* (○) *and the crude Lubrol-solubilized fraction* (●)

The concentrations of [^{3}H]diazepam were varied between 0.2 and 20 nM. The binding to membrane receptors was measured by filtration assay. The K_D and B_{max} values were obtained by computer-drawn linear regression of the binding data. For membrane receptors, the K_D was 9.12 nM and the B_{max} was 1652 fmoles/mg of protein. The correlation coefficient (r) was 0.95. The Lubrol-solubilized fraction had a K_D of 10.4 nM and the B_{max} was 1028 fmoles/mg of protein. The r value was 0.97 for the regression analysis.

TABLE 2

Binding constants of [^{3}H]diazepam binding to membrane and the crude Lubrol-solubilized fraction

[^{3}H]Diazepam binding to the membrane fraction was measured by a filtration assay and to the Lubrol-solubilized fraction by the PEG-centrifugation method as described in the text. [^{3}H]Diazepam was varied from 0.2 to 25 nM. The specific binding for membrane studies was 85%, and for soluble studies it was 50% of the total binding. The K_D and B_{max} values were obtained by computer-drawn linear regression of the binding data. Values represent the mean ± standard deviation. Numbers in parentheses are numbers of experiments, each performed in triplicate.

	K_D	B_{max}
	nM	*fmoles/mg protein*
Membrane fraction	9.48 ± 0.22 (4)	1722 ± 255 (4)
Crude Lubrol-solubilized fraction	9.34 ± 1.26 (3)	1082 ± 189 (3)

However, besides [^{3}H]diazepam binding, specific binding of [^{3}H]muscimol and [^{3}H]DHP occurred in the Lubrol-solubilized fraction (Table 3; 20, 21). The binding of [^{3}H]muscimol and [^{3}H]DHP (21) to the Lubrol extract has properties similar to those reported for membrane preparations. However, it may be noted that [^{3}H]DHP bound to two sites in the Lubrol fraction (20, 21), in contrast to only one site observed in membranes (24). Results in Table 3 indicate that diazepam, muscimol, and picrotoxinin sites are present in the crude Lubrol-solubilized fraction, and suggest that, under the conditions used in this study (see Materials and Methods), Lubrol has solubilized the benzodiazepine-GABA receptor-ionophore-complex. Table 3 shows that ethanol, pentobarbital, and muscimol enhance [^{3}H]diazepam binding to the crude Lubrol-solubilized fraction. Ethanol was more potent than *t*-butyl alcohol in enhancing [^{3}H]diazepam binding. Furthermore, ethanol and pentobarbital, while having no effect on [^{3}H]muscimol binding, inhibited the binding of [^{3}H]DHP to the crude Lubrol-solubilized fraction. In contrast, muscimol (GABA agonist) had no effect on [^{3}H]DHP binding; however, it displaced [^{3}H]muscimol binding. These results clearly indicate that, under the conditions of this study (see Materials and Methods), components of benzodiazepine-GABA receptor-ionophore complex interact in the crude Lubrol-solubilized fraction in a manner analogous to that described for membrane studies (13–15; 17).

Effect of various alcohols on specific [^{3}H]diazepam binding to benzodiazepine-GABA receptor-ionophore complex. Table 4 shows the dose-dependent effects of ethanol and *t*-butyl alcohol on [^{3}H]diazepam binding to the crude Lubrol-solubilized fraction. *t*-Butyl alcohol was chosen for comparison, since it has a partition coefficient 5 times greater than that of ethanol, and it has been reported to be 4–5 times more potent than ethanol in producing alcohol dependence (25). Ethanol concentrations of 10–100 mM produced a dose-dependent enhancement of specific diazepam binding, whereas ethanol concentrations >100 mM resulted in a gradual decrease of diazepam binding toward baseline levels (Table 4; Fig. 2). *t*-Butyl alcohol also produced a dose-related increase in diazepam binding up to concentrations of 100 mM. A concentration of *t*-butyl alcohol greater than 100 mM

TABLE 3

Effect of ethanol on the binding of [^{3}H]diazepam, [^{3}H]muscimol, and [^{3}H]DHP to the crude Lubrol-solubilized fraction

Aliquots of the Lubrol-solubilized fraction were incubated with 1 nM [^{3}H]diazepam (30 min), 10 nM [^{3}H]muscimol (15 min), or 34 nM[^{3}H]DHP (15 min) in the presence or absence of other ligands. Backgrounds were obtained in the presence of 10 μM flurazepam, 100 μM GABA, and 100 μM DHP, respectively. The specific binding as measured by the PEG-centrifugation presented 50 ± 4% for [^{3}H]diazepam, 40 ± 4% for [^{3}H] muscimol, and 38 ± 4% for [^{3}H]DHP. Values represent the mean ± standard deviation of a typical experiment (in triplicate). Similar results were replicated three times. NT, Not tested; NS, not significant.

Treatment	Specific binding					
	[^{3}H]Diazepam		[^{3}H]Muscimol		[^{3}H]DHP	
	cpm/assay	%Δ	cpm/assay	%Δ	cpm/assay	%Δ
Control (100%)	4,281 ± 315	——	3,635 ± 196	——	6,833 ± 317	——
+ 50 mM Ethanol	6,129 ± 129	+43	3,650 ± 106	NS	5,716 ± 95	−14
+ 100 mM Ethanol	8,500 ± 110	+98	3,545 ± 106	NS	5,316 ± 139	−22
+ 100 mM *t*-Butyl alcohol	5,623 ± 219	+31	NT	——	6,123 ± 439	−10
+ 100 μM Pentobarbital	5,351 ± 96	+25	3,675 ± 43	NS	1,582 ± 69	−77
+ 10 μM Muscimol	13,819 ± 910	+222	690 ± 54	−81	6,711 ± 110	NS

TABLE 4

Effects of ethanol and t-butyl alcohol on [^{3}H]diazepam binding to the crude Lubrol-solubilized fraction

Aliquots of the Lubrol-solubilized fraction were incubated with 1 nM [^{3}H]diazepam and various concentrations of ethanol or *t*-butyl alcohol for 30 min at 0–4°. The binding was measured by PEG-centrifugation method, as described under Materials and Methods. Specific binding represented 52% of the total binding. The results are the mean ± standard deviation of a typical experiment (in triplicate) which was replicated four times with similar results.

Alcohol concentration	Specific [^{3}H]diazepam binding			
	Ethanol		*t*-Butyl alcohol	
	fmoles/mg protein	% Enhancement	fmoles/mg protein	% Enhancement
Control	139.1 ± 4.6	——	139.1 ± 4.6	——
10 mM	143.8 ± 3.5	3.4	147.9 ± 6.9	6
20 mM	165.4 ± 4.4	19.0	156.5 ± 5.2	12
50 mM	184.5 ± 5.9	33.0	175.5 ± 4.8	26
100 mM	242.9 ± 6.5	75.0	176.7 ± 7.4	27
200 mM	220.6 ± 8.3	59.0	127.7 ± 9.2	↓ 8
500 mM	152.9 ± 7.4	9.9	119.4 ± 8.5	↓15

produced a decrease below the baseline levels. Although *t*-butyl alcohol produced a dose-dependent effect, it gave a maximal enhancement of only 23.0 ± 6.7% at 100 mM ($n = 6$) relative to 90.8 ± 8.5 for ethanol (Table 5, Column 3). Table 5 compares the ability of various alcohols (at 100 mM) with increasing chain lengths to enhance diazepam binding to the Lubrol fraction. The rank order of enhancement was ethanol > methanol > isopropyl alcohol > propanol-1 = *t*-butyl alcohol = butanol-1 (Table 5, Column 4). Ethanol was the most potent in enhancing diazepam binding, and long-chain alcohols had relatively weak activity. Table 5 also compares the partition coefficients of these alcohols. It is interesting that the rank order of enhancement of these alcohols did not correlate with their partition coefficients.

Effect of ethanol on specific [^{3}H]diazepam binding to the benzodiazepine-GABA receptor-ionophore complex. In contrast to membrane studies, ethanol enhancement of diazepam binding to the crude Lubrol-solubilized fraction was highy reproducible. Figure 2 shows the results on dose-related enhancement of diazepam binding from six different experiments. Ethanol produced a dose-dependent enhancement of diazepam binding with a maximal enhancement of 90.5 ± 8.5% occurring at 100 mM, and the half-maximal effect occurring at 30 mM. Concentrations greater than 100 mM ethanol produced a decrease toward the baseline values. Figure 3 shows the Scatchard plots of diazepam in control (●) and in presence of 100 mM ethanol (○). The K_D value in control was 10.36 nM (correlation coefficient = 0.98), and in ethanol-treated preparations the K_D was 4.09 (correlation coefficient = 0.93). The B_{max} in control was 1035 fmoles/mg of protein, and in ethanol-treated preparations it was 948.3 fmoles/mg of protein. Table 6 compares the K_D and B_{max} values of diazepam binding in control and ethanol groups. Ethanol (100 mM) changed the K_D of diazepam binding in control and ethanol groups. Ethanol (100 mM) changed the K_D of diazepam from a control value of 9.36 ± 1.26 to 4.40 ± 0.33 nM ($p < 0.005$) without significantly altering the B_{max}. Ethanol enhancement of diazepam binding was blocked by picrotoxinin in a dose-dependent manner with an IC_{50} value of 2 μM. Bicuculline also inhibited the enhancing effect of ethanol (Table 7).

Effect of ethanol on [^{3}H]diazepam and [^{3}H]DHP binding to 61,000 and 185,000 mol wt fractions. We have recently separated the [^{3}H]diazepam- and [^{3}H]DHP-

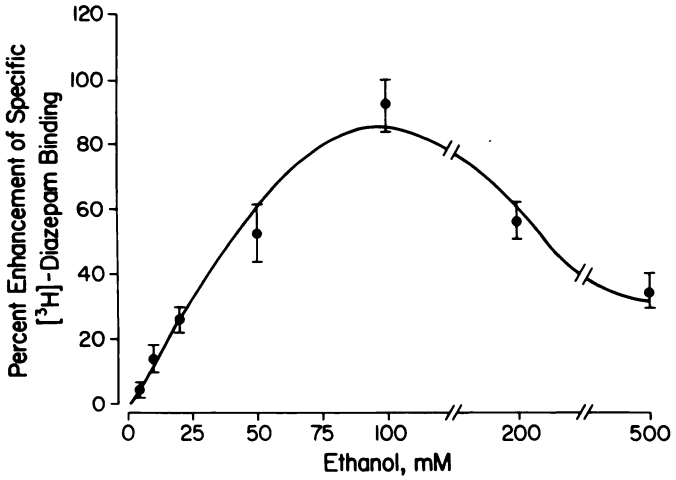

FIG. 2. *Effect of in vitro ethanol on the specific binding of [^{3}H]diazepam to the crude Lubrol-solubilized fraction*

Various concentrations of ethanol were incubated with aliquots of the Lubrol-solubilized fraction and 1 nM [^{3}H]diazepam for 30 min at 0–4° and assayed by the PEG-centrifugation method. A typical control experiment, in triplicate, using 0.48 mg of protein, gave a total binding of 8628 ± 228 cpm and a background of 4156 ± 59 cpm. In the presence of 100 mM ethanol, the total binding was increased to 12846 ± 148 cpm. This represents an enhancement of 94% over the basal level. Each *point* represents the mean ± standard deviation of six experiments, each performed in triplicate.

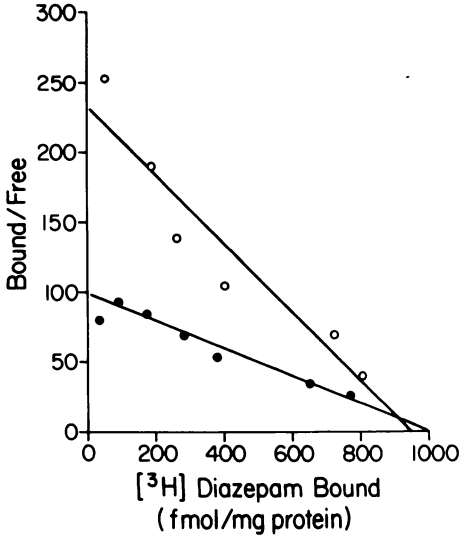

FIG. 3. *Typical Scatchard plot of [^{3}H]diazepam binding to the crude Lubrol-solubilized fraction in control (●) and in presence of 100 mM ethanol* (○)

The K_D for control was 10.3 nM and the B_{max} was 1000 fmoles/mg of protein (r = 0.98). For the ethanol group, the K_D was 4.1 nM and the B_{max} was 948 fmoles/mg of protein (r = 0.93). The average K_D and B_{max} values for the two groups are presented in Table 6.

binding proteins from the crude Lubrol-solubilized fraction by gel filtration.[2] [^{3}H]Diazepam and [^{3}H]DHP bind to two distinct proteins with apparent molecular weights of 61,000 and 185,000 (data not shown). Table 8 shows that ethanol and *t*-butyl alcohol did not enhance the binding of [^{3}H]diazepam to the 61,000 mol wt fraction (i.e., benzodiazepine receptor). However, ethanol, in a dose-dependent manner, inhibited partially the binding of [^{3}H]DHP to the 185,000 mol wt fraction (i.e., picrotoxinin receptor). Furthermore, *t*-butyl alcohol gave a weak inhibition of ~8% at concentrations of 50–500 mM.

TABLE 6

Effect of ethanol on the kinetic constants of [^{3}H]diazepam binding to crude Lubrol-solubilized fraction

[^{3}H]Diazepam binding was measured as described under Materials and Methods and in the legend to Table 2. The K_D and B_{max} values were obtained by computer-drawn linear regression of the binding data. Numbers in the parentheses are numbers of experiments, each performed in triplicate.

	K_D	B_{max}
	nM	*fmoles/mg protein*
Control	9.36 ± 1.26 (3)	1082 ± 189 (3)
100 mM Ethanol	4.40 ± 0.33 (4)[a]	995 ± 167 (4)

[a] $p < 0.005$ as compared with control.

DISCUSSION

Ethanol, barbiturates, and benzodiazepines are capable of producing similar CNS-depressing effects in a dose-related manner. All of these classes of drugs are muscle relaxants, anxiolytics, and anticonvulsants, and produce ataxia. It has been suggested that ethanol, like other sedative-hypnotics and anesthetics, produces its effects

TABLE 5

Effect of various alcohols on specific [^{3}H]diazepam binding to the crude Lubrol-solubilized fraction

Aliquots of the Lubrol extract were incubated with 1 nM [^{3}H]diazepam with or without 100 mM concentration of various alcohols for 30 min at 0–4° and assayed as described under Materials and Methods.

Alcohol (100 mM)	Specific [^{3}H]diazepam binding		Rank order	Partition coefficient[c]
	fmoles/mg protein[a]	% Enhancement[b]		
Control	139.1 ± 4.6	—	—	—
Methanol	204.9 ± 5.8	48.6 ± 8.9 (5)	2	0.036
Ethanol	242.9 ± 6.5	90.8 ± 8.5 (6)	1	0.096
Isopropyl alcohol	183.9 ± 7.3	39.2 ± 7.5 (5)	3	0.264
Propanol-1	174.7 ± 6.6	16.5 ± 10.3 (4)	4	0.438
Butanol-1	149.6 ± 5.4	19.8 ± 10.4 (5)	4	1.520
t-Butyl alcohol	176.7 ± 7.4	23.0 ± 6.7 (6)	4	0.469

[a] Values for specific [^{3}H]diazepam binding represent the mean ± standard deviation of one experiment performed in triplicate.
[b] Values for percentage enhancement are the means ± standard deviation of the number of experiments as indicated in the parentheses.
[c] Partition coefficient values are from McCreery and Hunt (26).

TABLE 7

Effect of antagonists on ethanol enhancement of specific [^{3}H] diazepam binding to crude Lubrol-solubilized fraction

The values represent the mean ± standard deviation of a typical experiment, performed in triplicate. Similar results were replicated three times. Picrotoxinin and (+)-bicuculline at the concentrations listed did not alter the baseline [^{3}H]diazepam binding.

Treatment	Specific [^{3}H]diazepam binding	% Inhibition
	fmoles/mg protein	
None (baseline)	159.3 ± 5.3	——
100 mM Ethanol	302.3 ± 10.6	——
100 mM Ethanol		
+ 1 μM Picrotoxinin	254.3 ± 8.4	33
+ 10 μM Picrotoxinin	177.5 ± 9.3	87
+ 100 μM Picrotoxinin	149.4 ± 7.6	100
+ 10 μM (+)-Bicuculline	238.3 ± 6.8	45

TABLE 8

Effect of ethanol on [^{3}H]diazepam and [^{3}H]DHP binding to 61,000 and 185,000 mol wt fractions

The binding was studied by a centrifugation assay as described under Materials and Methods, using 1 nM [^{3}H]diazepam and 34 nM [^{3}H]DHP. Total binding for [^{3}H]diazepam was 5467 cpm and the background obtained in the presence of 10 μM flurazepam was 989 cpm. For [^{3}H]DHP, the total binding was 5516 cpm and background obtained in the presence of 100 μM DHP represented 1014. The 61,000 and 185,000 mol wt fractions were obtained by gel filtration of the crude Lubrol-solubilized extract on equilibrated Sephadex G-200 (1.5 × 84 cm). Gel filtration revealed two protein peaks with apparent molecular weights of 185,000 (Peak I) and 61,000 (Peak II). Binding studies revealed that specific [^{3}H]DHP binding was associated with 185,000 fraction, and [^{3}H]diazepam with the 61,000 fraction. The binding properties of these fractions indicate that they are receptors for diazepam (61,000 mol wt fraction) and picrotoxinin (185,000 mol wt fraction).[2] Values represent the mean ± standard deviation of one experiment (in triplicate). Similar results were replicated three times. NT, Not tested.

Treatment	Specific binding			
	[^{3}H]Diazepam binding (61,000 mol wt fraction)		[^{3}H]DHP binding (185,000 mol wt fraction)	
	cpm/assay	%Δ	cpm/assay	%Δ
Control (100%)	4,478 ± 64	——	4,502 ± 85	——
+ 20 mM Ethanol	4,498 ± 210	+0	4,225 ± 110	−6
+ 50 mM Ethanol	4,368 ± 14	−2	3,853 ± 134	−14
+ 100 mM Ethanol	4,302 ± 133	−4	3,687 ± 179	−18
+ 50 mM *t*-Butyl alcohol	4,528 ± 110	+1	4,144 ± 202	−8
+ 100 mM *t*-Butyl alcohol	4,409 ± 98	−2	4,148 ± 238	−8
+ 500 mM *t*-Butyl alcohol	NT	——	4,159 ± 92	−8

by altering the properties of membrane components (1–4, 27). Thus, it has been speculated that ethanol, by altering the membrane fluidity, will produce changes in the conformation of membrane proteins resulting in the change of function of these proteins. Similarly, it has been speculated that barbiturates may act nonspecifically with the membrane components (1, 2). However, recent neurophysiological studies have demonstrated that ethanol (5–7), barbiturates (8, 9), and benzodiazepines (10) enhance GABA-mediated inhibitory transmission in the CNS. Ethanol was reported to be selective in this effect, since it did not alter the responses to glycine, serotonin, and dopamine (7). We have previously demonstrated that acute ethanol treatment increases the density of GABA receptor sites, and during chronic ethanol treatment, a tolerance to this effect was observed (28). GABA mimetics have been reported to increase the behavioral effects of ethanol, whereas GABA antagonists such as bicuculline decrease behavioral manifestations during ethanol intoxication (29, 30). Furthermore, GABA mimetics have been reported to reduce ethanol withdrawal symptoms, whereas the GABA antagonist picrotoxinin produces symptoms similar to those seen during ethanol withdrawal (30). Recent studies have demonstrated that the benzodiazepine binding component is associated with the GABA receptor-ionophore complex (13–18). Benzodiazepine (22, 23), GABA (31), and picrotoxinin (20, 21) binding sites have been solubilized. Furthermore, it has been shown that GABA agonists (17) and pentobarbital (13–15) enhance [^{3}H]diazepam binding to membranes and a crude Lubrol-solubilized fraction (16; this study). This enhancement is due to an increase in the affinity of diazepam for the benzodiazepine receptors and was blocked by picrotoxinin and bicuculline.

Ethanol also enhances [^{3}H]diazepam binding to membranes (13; this study). However, this enhancing effect was not reproducible and varied with different batches of membrane preparation. To determine whether ethanol affects the benzodiazepine binding site directly or indirectly, we investigated this interaction in the crude Lubrol-solubilized fraction and the purified 61,000 mol wt (benzodiazepine receptor) fraction. The crude Lubrol-solubilized fraction used in this study had the specific binding sites for ligands ([^{3}H]diazepam, [^{3}H]muscimol, and [^{3}H]DHP) which bind at three distinct sites at the benzodiazepine-GABA receptor-ionophore complex. Furthermore, GABA agonists such as muscimol, ethanol, and pentobarbital enhance [^{3}H]diazepam binding to the crude Lubrol-solubilized fraction (Table 3). These results indicate that Lubrol, under the experimental conditions (see Materials and Methods) of this study, solubilizes the benzodiazepine-GABA receptor-ionophore complex.

In the present study we found that ethanol, like barbiturates, produces a dose-related enhancement of [^{3}H]diazepam binding to the crude Lubrol-solubilized fraction. Ethanol enhancement of diazepam binding in this fraction was much greater and highly reproducible, relative to the membrane fraction. The reason for this is not clear. Since ethanol is known to interact with membrane components, especially cholesterol (1–4), it is feasible that lipids prevent the ethanol effect in the membranes. Although we did not measure the total lipid content, the cholesterol content in the crude Lubrol-solubilized fraction, as measured by the method of Allain *et al.* (32), was only 4 mg/100 ml. The possibility that Lubrol removes some endogenous ligand (or component) which prevents the ethanol effect in membranes cannot be ruled out.

Ethanol enhancement of diazepam binding to the crude Lubrol fraction was dose-dependent, with the maximal effect occurring at 30 mM. This enhancement was

due to an increase in the affinity of diazepam for its receptor sites. Although a variety of alcohols enhanced diazepam binding to the crude Lubrol fraction, none of them produced an effect equal to or larger than that of ethanol. The rank order of enhancement was ethanol > methanol > isopropyl alcohol > propanol-1 = *t*-butyl alcohol = butanol-1. This order of potency neither correlated with the partition coefficients of these alcohols nor with pharmacological potency in producing physical dependence (25–27).

The interaction of ethanol with [^{3}H]diazepam binding sites in the crude Lubrol fraction appears to be specific for the following reasons: (a) under identical conditions of tissue preparation, solubilization, incubation, and assay conditions, ethanol enhanced [^{3}H]diazepam binding but did not alter [^{3}H]muscimol binding (Table 3); (b) ethanol and pentobarbital inhibited [^{3}H]DHP binding (Table 3); (c) ethanol enhancement of [^{3}H]diazepam binding was prevented by picrotoxinin and (+)-bicuculline; (d) ethanol did not enhance [^{3}H]diazepam binding to the 61,000 mol wt fraction (benzodiazepine receptor), whereas it inhibited the binding of [^{3}H]DHP to the 185,000 mol wt fraction (picrotoxinin receptor) (Table 8).[2] These results suggest that the ethanol effect appears to be specific and may not be due to the detergent or lipids. The IC_{50} value of picrotoxinin (2 μM) for inhibition of ethanol enhancement is similar to the apparent K_D of DHP (24). Likewise, picrotoxinin blocks pentobarbital enhancement of [^{3}H]diazepam binding to the membranes (13–15) and crude Lubrol-solubilized (16) fractions. The ability of ethanol and pentobarbital to interact with the 185,000 mol wt fraction suggests that these drugs may enhance [^{3}H]diazepam binding allosterically by acting at the picrotoxinin-sensitive site of the benzodiazepine-GABA receptor-ionophore complex. The inability of ethanol to enhance [^{3}H]diazepam binding following gel filtration to the 61,000 mol wt fraction also supports this hypothesis, but the possibility that gel filtration removes some lipid component or a coupling factor from the benzodiazepine receptor cannot be ruled out.

However, bicuculline (which binds at the GABA receptor level) also blocked ethanol (this study) and pentobarbital enhancement of diazepam binding to membranes (13, 14) and the crude Lubrol fraction (16). Similarly, the enhancement of diazepam binding by a purine derivative (EMD 28422), which does not bind at the GABA receptor level, was blocked by bicuculline (33). These results indicate that GABA receptors that are intimately associated with the benzodiazepine-ionophore complex may be involved in modulating the physiological function of the benzodiazepine receptors. Thus, it is feasible that bicuculline, by binding at the GABA receptors, prevents the conformational changes induced by ligands such as ethanol, pentobarbital, and EMD 28422, which binds at other sites.

The EC_{50} for ethanol enhancement of [^{3}H]diazepam binding was 30 mM, and maximal enhancement occurred at ~100 mM. Although these concentrations appear to be high, they are physiologically relevant. Thus, following a single dose of ethanol (4 g/kg), which produced loss of the righting reflex in rats, blood ethanol concentrations of 75.6 mM were detected (34). Concentrations of ethanol ranging from 32.6 to 97.6 mM have been reported to facilitate presynaptic inhibition (GABA-mediated) (6). Furthermore, recent studies have indicated that only a fraction of benzodiazepine receptors (20–30%) need to be occupied for diazepam to produce anxiolytic and anticonvulsant effects (see ref. 35). Thus, ethanol activation of all of the [^{3}H]diazepam receptors may not be a prerequisite, and only fractional enhancement (which will occur at lower concentrations) may result in desired CNS therapeutic effects.

On the basis of all of these observations, it may be speculated that small-chain alcohols have a specific effect on the benzodiazepine-binding component of the benzodiazepine-GABA receptor-ionophore complex. This effect appears to be mediated not directly at the benzodiazepine receptor, but indirectly via the picrotoxinin site. Enhancement of [^{3}H]diazepam binding will result in facilitation of GABA-mediated inhibitory transmission. Neurophysiological studies also support this hypothesis (5–7). This interaction may be responsible for some of the central effects of alcohol: antianxiety, muscle relaxant, and sedative. Besides these effects, all alcohols may alter membrane structure nonspecifically in accordance with their partition coefficients (1–4, 27). Our results may also explain the synergistic effects observed with alcohol and diazepam *in vivo* and the use of antianxiety drugs as drugs of choice during alcohol withdrawal. However, further *in vivo* experimental evidence must be obtained prior to justifying such conclusions.

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REFERENCES

1. Seeman, P. The membrane action of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**:583–655 (1972).
2. Hill, M. W., and A. D. Bangham. General depressant drug dependency: a biophysical hypothesis. *Adv. Exp. Biol. Med.* **59**:1–9 (1975).
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. Li, G. C., and G. M. Hahn. Ethanol-induced tolerance to heat and adriamycin. *Nature* (*Lond.*) **274**:699–701 (1978).
5. Banna, N. R. Potentiation of cutaneous inhibition by alcohol. *Experientia* (*Basel*) **25**:619–620 (1969).
6. Davidoff, R. S. Alcohol and presynaptic inhibition in an isolated spinal cord preparation. *Arch. Neurol* **28**:60–63 (1973).
7. Nestoros, J. N. Ethanol specifically potentiates GABA-mediated neurotransmission in feline cerebral cortex. *Science* (*Wash. D. C.*) **209**:708–710 (1980).
8. Nicoll, R. A. Pentobarbital action on frog motoneurons. *Brain Res.* **96**:119–123 (1975).
9. Nicoll, R. A., and J. M. Wjotowicz. The effects of pentobarbital and related compounds on frog motoneurons. *Brain res.* **191**:225–237 (1980).
10. Choi, D. W., D. H. Farb, and G. D. Fischbach. Chlordiazepoxide selectively augments GABA action in spinal cord cell cultures. *Nature* (*Lond.*) **269**:342–344 (1977).
11. Mohler, H., and T. Okada. Benzodiazepine receptor: demonstration in the central nervous system. *Science* (*Wash. D. C.*) **198**:849–851 (1977).
12. Squires, R. F., and C. Braestrup. Benzodiazepine receptors in rat brain. *Nature* (*Lond.*) **266**:732–734 (1977).
13. Ticku, M. K., I. Chen, and W. C. Davis. Ethanol and barbiturates enhance [^{3}H]-diazepam binding at the benzodiazepine-GABA-receptor-ionophore complex in membrane and solubilized forms. *Fed. Proc.* **40**:310 (1981).
14. Ticku, M. K. Interaction of depressant, convulsant and anticonvulsant barbiturates with [^{3}H]diazepam binding at the benzodiazepine-GABA receptor-ionophore complex. *Biochem. Pharmacol.* **29**:411 (1981).
15. Leeb-Lundburg, F., A. Snowman, and R. W. Olsen. Barbiturate receptor sites

are coupled to benzodiazepine receptors. *Proc. Natl. Acad. Sci. U. S. A.* **77:** 7468–7472 (1980).
16. Davis, W. C., and M. K. Ticku. Pentobarbital enhances [^{3}H]diazepam binding to soluble receptors at the benzodiazepine-GABA receptor-ionophore complex. *Neurosci. Lett.* **23:**209–213 (1981).
17. Tallman, J. F., J. W. Thomas, and D. W. Gallager. GABA-ergic modulation of benzodiazepine binding site sensitivity. *Nature (Lond.)* **274:**383–384 (1978).
18. Costa, T., D. Rodbard, and C. B. Pert. Is the benzodiazepine receptor coupled to a chloride ion channel. *Nature (Lond.)* **277:**315–317 (1979).
19. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275 (1951).
20. Davis, W. C., and M. K. Ticku. Solubilization of the dihydropicrotoxinin binding sites. *Eur. J. Pharmacol* **68:**397–399 (1980).
21. Davis, W. C., and M. K. Ticku. Solubilization of the picrotoxinin binding receptor from mammalian brain. *J. Neurochem.* **36:**1572–1579 (1981).
22. Yousufi, M. A. K., J. W. Thomas, and J. F. Tallman. Solubilization of benzodiazepine binding site from rat cortex. *Life Sci.* **25:**463–470 (1979).
23. Gavish, M., R. S. L. Chang, and S. H. Snyder. Solubilization of histamine H-1, GABA and benzodiazepine receptors. *Life Sci.* **25:**783–790 (1979).
24. Ticku, M. K., M. Ban, and R. W. Olsen. Binding of dihydropicrotoxinin, a gamma-aminobutyric acid synaptic antagonist, to rat brain membranes. *Mol. Pharmacol.* **14:**391–402 (1978).
25. McComb, J. A., and D. B. Goldstein. Quantitative comparison of physical dependence on tertiary butanol and ethanol in mice: correlation with lipid solubility. *J. Pharmacol. Exp. Ther.* **208:**113–117 (1979).
26. McCreery, M. J., and W. A. Hunt. Physiochemical correlates of alcohol intoxication. *Neuropharmacology* **17:**451–461 (1978).
27. McComb, J. A., and D. Goldstein. Additive physical dependence: evidence for a common mechanism in alcohol dependence. *J. Pharmacol. Exp. Ther.* **210:**87–90 (1979).
28. Ticku, M. K. The effects of acute and chronic ethanol administration and its withdrawal on γ-aminobutyric acid receptor binding in rat brain. *Br. J. Pharmacol.* **70:**403–410 (1980).
29. Hanniken, H. N., and E. J. Kulonen. Ethanol intoxication and γ-aminobutyric acid. *J. Neurochem.* **27:**631–633 (1976).
30. Goldstein, D. B. Alcohol withdrawal reactions in mice: effect of drugs that modify neurotransmission. *J. Pharmacol. Exp. Ther.* **186:**1–9 (1973).
31. Greenlee, D. V., and R. W. Olsen. Solubilization of GABA receptor protein from mammalian brain. *Biochem. Biophys. Res. Commun.* **88:**380–387 (1979).
32. Allain, C. C., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:**470–475 (1974).
33. Skolonick, P., K. L. Lock, S. M. Paul, P. J. Marangus, R. Jones, and K. Irmscher. Increased benzodiazepine receptor number elicited *in vitro* by a novel purine, EMD 28422. *Eur. J. Pharmacol.* **67:**179–186 (1980).
34. Kiianmaa, K., and L. M. J. Attila. Alcohol intake, ethanol-induced narcosis and intoxication in rats following neonatal 6-hydroxydopamine or 5,7-dihydroxytryptamine treatment. *Naunyn-Schmiedberg's Arch. Pharmacol.* **308:** 165–170 (1979).
35. Tallman, J. F., S. M. Paul, P. Skolnick, and D. W. Gallager. Receptors for the age of anxiety: pharmacology of the benzodiazepines. *Science (Wash. D. C.)* **207:**274–281 (1980).

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