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Heterogeneity of Benzodiazepine Receptor Interactions with γ-Aminobutyric Acid and Barbiturate Receptor Sites

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SUMMARY

Benzodiazepine receptor binding heterogeneity evident from differential affinities of some ligands was compared with that suggested by differential interactions with γ-aminobutyric acid (GABA)/bicuculline and pyrazolopyridine/barbiturate receptor sites. The GABA receptor antagonist bicuculline only partially reverses pentobarbital enhancement of [^{3}H]diazepam binding in rat brain membranes, while totally blocking both GABA and etazolate enhancement. The degree of bicuculline sensitivity varies with brain region (cortex > hippocampus > thalamus-midbrain = striatum > medulla-pons = cerebellum) in a manner which does not correlate with over-all barbiturate enhancement nor with baseline [^{3}H]diazepam or [^{3}H]GABA binding; it does correlate instead with the degree of barbiturate-enhanced [^{3}H]GABA binding, which varies similarly with brain region. The bicuculline-insensitive barbiturate enhancement of [^{3}H]diazepam binding in cortex is blocked by low concentrations of ethyl β-carboline-3-carboxylate sufficient to inhibit primarily its high-affinity sites. [^{3}H]Diazepam binding remaining in the presence of nanomolar β-carbolines shows a similar degree of barbiturate enhancement, which is now totally blocked by bicuculline; an augmentation of the maximal enhancement by GABA is also observed. Furthermore, the residual [^{3}H]diazepam binding unoccupied by nanomolar β-carbolines shows an increased relative enhancement by pyrazolopyridines, which appears to reflect a selective reversal of the inhibitory portion of the biphasic dose-response curve seen with these compounds. Etazolate, cartazolate, and certain barbiturates, which also show enhancement of [^{3}H]diazepam binding at low concentrations but reversal of this effect at high concentrations, give essentially only the enhancement effect in the presence of low concentrations of β-carbolines. The independent nature of the enhancement and inhibition phases of the biphasic dose-response curves for these compounds was confirmed by selective blockade of only the enhancement phase by the GABA chloride channel antagonist picrotoxinin and by the GABA receptor antagonist bicuculline. The distinction by β-carbolines between bicuculline-sensitive and -insensitive barbiturate enhancement and pyrazolopyridine-enhanced and -inhibited [^{3}H]diazepam binding sites defines two classes of benzodiazepine receptors. Discrimination between these subtypes was also observed with the benzodiazepine antagonist Ro 15-1788, but not with the pyrazoloquinoline antagonist CGS 8216. Neither were the two classes of sites differentiated by depressant benzodiazepines such as diazepam and lorazepam, nor by the anxiolytic triazolopyridazine CL 218,872. Since the two subpopulations differentiated by ethyl β-carboline-3-carboxylate do not correspond *in toto* with the two populations showing different affinities for CL 218,872, a third class of sites is defined. The results, taken together, suggest that the heterogeneous subpopulations of [^{3}H]diazepam binding sites defined by the interactions with bicuculline/GABA and barbiturate/pyrazolopyridine receptors cannot yet be correlated in any simple way with agonist/antagonist conformational states of the benzodiazepine receptor nor with subpopulations defined by heterogeneous binding affinities for some ligands. A speculative model suggests that multiple coupling states of a single type of GABA and benzodiazepine receptor might account for at least part of the heterogeneity. Ultimate resolution will await further biochemical evidence on subtypes and physiological studies on the functional relevance of this heterogeneity observed *in vitro*.

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INTRODUCTION

Benzodiazepines are drugs with considerable clinical use as anxiolytics, anticonvulsants, muscle relaxants, and sedatives. Many of these actions appear to involve an enhancement of inhibitory synaptic transmission mediated by GABA[1] (1–3). Benzodiazepines have been shown to enhance the postsynaptic nerve membrane response to GABA of activating chloride ion channel conductance (3, 4). Other depressant drugs such as barbiturates and related substances also enhance or mimic GABA responses (3–5), whereas the convulsants bicuculline and picrotoxinin block GABA responses (5).

Consistent with the GABA hypothesis for the action of these drugs (2, 3), considerable *in vitro* evidence for interactions of these drugs with GABA receptor-ionophores has accumulated. Distinct, pharmacologically relevant receptor sites for GABA/bicuculline (5–8), benzodiazepines (1, 9–11), and barbiturate/picrotoxinin-like drugs (5, 12–14) have been thoroughly characterized in mammalian brain membranes. Reciprocal *in vitro* chloride ion-sensitive interactions between the various receptors suggest their close association in the postsynaptic membrane. These interactions appear to represent physiologically relevant processes within a functional GABA receptor-ionophore complex. The interactions include the ability of GABA agonists to enhance the affinity for benzodiazepine receptor binding in a chloride-stimulated and bicuculline-sensitive manner, an effect presumed to be mediated via a postsynaptic GABA receptor (1, 5). Barbiturates (pentobarbital) (8, 12–17) and pyrazolopyridines (etazolate) (13, 17–19) enhance the affinity for both benzodiazepine binding and GABA binding in a fashion strictly dependent on chloride ions and sensitive to both picrotoxinin and bicuculline. The enhancement of benzodiazepine binding by barbiturates and pyrazolopyridines is distinct from that of GABA agonists, and is mediated via barbiturate/picrotoxinin binding sites (5, 12–14).

Benzodiazepine receptor heterogeneity is apparent from the low Hill numbers for displacement of [^{3}H]diazepam or [^{3}H]flunitrazepam by certain nonbenzodiazepine ligands, including anxiolytic triazolopyridazines (CL 218,872) (20) and β-carbolines (β-CCE) (9). Binding studies are consistent with two or more subpopulations of binding sites which can be distinguished by different affinities of these ligands, even though the benzodiazepines themselves bind with an apparent homogeneous affinity (9, 20–22). It is not known whether the heterogeneity represents distinct receptor proteins or different states (conformational or coupling) of a single receptor. A differential brain distribution, ontogenetic development, protein subunit molecular weight, and susceptibility to detergent solubilization support but do not prove the multiple separate receptor hypothesis (9, 20–23). Differential sensitivity of benzodiazepine receptor ligands to allosteric perturbation by GABA ligands (10, 11, 22) has been interpreted to represent either a difference between pharmacological agonists and antagonists, or subpopulations of distinct receptor sites.

Heterogeneity is also apparent in the benzodiazepine ligand binding kinetics, susceptibility to affinity labels, protein reagents, and heat inactivation (5, 20–25), and in interactions with drugs acting at barbiturate/picrotoxinin receptor sites in the GABA receptor-ionophore complex (13, 14, 17). Pentobarbital enhancement of benzodiazepine receptor binding shows a heterogeneity of susceptibility to reversal by the GABA antagonist bicuculline (13). The current study compares the heterogeneity in ligand affinity for β-carbolines and triazolopyridazines with the heterogeneity apparent in agonist and antagonist categories of ligands, as well as with the heterogeneity seen in interactions with GABA/bicuculline and barbiturate/pyrazoloypridine/picrotoxinin receptors. The data support even more strongly the heterogeneity of benzodiazepine receptor sites, and suggest some possible explanations.

MATERIALS AND METHODS

[*N-methyl*-^{3}H]Diazepam (76.8 or 83.5 Ci/mmole) and [^{3}H]toluene were purchased from New England Nuclear Corporation. 4-Amino-*n*-[2,3-^{3}H]butyric acid ([^{3}H]GABA; 57 Ci/mmole) was purchased from Amersham. β-CCE was a gift of C. Braestrup, A/S Ferrosan, Denmark. Diazepam, lorazepam, Ro 15-1788, and CL 218,872 were gifts of R. O'Brien, Hoffmann-La Roche. CGS 8216 was a gift of Ciba-Geigy, Inc. The amidine steroid, R 5135, was a gift of Hoechst-Roussel. Etazolate and cartazolate were gifts of E. R. Squibb and Sons, Inc. (−)-Mephobarbital, (+)-hexobarbital, and (−)-MPPB were gifts of J. Knabe, University of Saärlandes. THIP was a gift of P. Krogsgaard-Larsen, Copenhagen, Denmark. All other drugs and materials were obtained from commercial sources.

White Sprague-Dawley rats (200–300 g) were decapitated and their brains were removed rapidly and bathed in ice-cold 0.32 M sucrose. The appropriate brain region was then dissected out, removed, and homogenized (12 passes at 400 rpm) in 20 volumes of 0.32 M sucrose in a glass homogenizer fitted with a Teflon pestle (Type C; Arthur H. Thomas Company, Philadelphia, Pa.). The homogenate was centrifuged at 2000 rpm (1000 × *g*) for 10 min at 0°–4° (Beckman JA 17 rotor). The pellet (P_1) was discarded and the supernatant fraction was centrifuged at 45,000 rpm (140,000 × *g*) for 45 min (Spinco rotor 60 Ti). The pellet (P_2 and P_3) was then resuspended [Ultra-Turrax (Cincinnati, Ohio), 25 sec at setting 40%] in 25 volumes of ice-cold double-distilled water (osmotic shock) and centrifuged at 45,000 rpm for 45 min. The resulting pellet was then treated in two different ways depending on the binding assay to be done. Membranes for [^{3}H]diazepam binding were washed once more in ice-cold buffer [0.2 M KCl or NaCl/20 mM potassium phosphate (pH 7.0)]. The pellet was resuspended to a final protein concentration of 0.2–1.0 mg/ml in buffer. Membranes for [^{3}H]GABA binding were again resuspended in water (same conditions) and stored overnight at −18°.

[1] The abbreviations used are: GABA, γ-aminobutyric acid; CL 218,872, 3-methyl-6-[3-trifluoromethyl-phenyl]-1,2,4-triazolo-[4,3-*b*]pyridazine; β-CCE, ethyl β-carboline-3-carboxylate; Ro 15-1788, ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*]-[1,4]benzodiazepine-3-carboxylate; CGS 8216, 2-phenylpyrazolo[4,3-*c*]quinolon-3(5*H*)-one; R 5135, 3α-hydroxy-16-imino-5β-17-aza-androstan-11-one; MPPB, (−)-1-methyl,5-phenyl,5-propyl barbituric acid; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol.

The sample was then thawed and washed once more in water; the pellet was resuspended in 50 mM Tris-citrate (sodium-free) buffer (pH 7.1) and frozen again. On the day of the assay, the sample was thawed, centrifuged, and washed once more in buffer [0.2 M KCl/20 mM potassium phosphate (pH 7.0)]. The resulting pellet was resuspended to a final concentration of protein of 0.5–1.0 mg/ml in buffer.

[^{3}H]Diazepam binding (12–14). Aliquots of the membrane suspension were incubated in triplicate for 60 min at 0° with 0.5 nM [^{3}H]diazepam with and without drugs in a total volume of 1 ml. The buffer was 0.2 M NaCl/20 mM potassium phosphate (pH 7.0) except in the experiments of Table 2 and Fig. 1 comparing [^{3}H]diazepam binding to [^{3}H]GABA binding, where the buffer was instead 0.2 M KCl/20 mM potassium phosphate (pH 7.0). At the end of the incubation, the membranes were rapidly trapped on Whatman GF/B filters. An additional 2 ml of 0.2 M NaCl were added twice to the incubation vial and poured onto the filter. The filters were placed into plastic vials and counted in 5 ml of CytoScint/toluene (2:1, v/v) (WestChem Products) in a Beckman model LS 3155T scintillation counter. Efficiency (45%) was routinely determined with [^{3}H]toluene. Background was determined in the presence of 10 μM nonradioactive diazepam and usually accounted for 10% of the total radioactivity.

[^{3}H]GABA binding (6–8). Aliquots of the membrane suspension were incubated in triplicate for 20 min at 0° with 4 nM [^{3}H]GABA with and without drugs in a total volume of 1 ml of 0.2 M KCl/20 mM potassium phosphate (pH 7.0). At the end of the incubation, the membranes were pelleted rapidly by centrifugation at 20,000 rpm (50,000 × *g*) for 15 min in a JA 20.1 or JA 14 rotor. After the supernatant was discarded, the vials were superficially rinsed twice with ice-cold assay buffer without disturbing the membrane pellet. The membranes were solubilized overnight in 0.3 ml of Soluene 350/toluene (1:1, v/v) (Packard). Toluene (3 ml) containing 5% diphenyloxazole was then added and radioactivity was measured, efficiency 30%. Nonspecific binding was estimated with 0.1 mM nonradioactive GABA and contributed 30–50% of the total radioactivity.

RESULTS

Specific [^{3}H]diazepam binding to fresh rat brain membranes was enhanced by GABA and GABA receptor agonists, anxiolytic pyrazolopyridines such as etazolate, and by numerous depressant barbiturates such as pentobarbital (Table 1). The enhancement of benzodiazepine binding by each set of compounds has previously been reported to be due to an effect on the binding affinity (K_D), rather than an effect on the maximal number of binding sites (B_{max}) (1, 12–19). Under our conditions, no significant difference was observed between the maximal level of enhancement (E_{max}) for GABA (89 ± 6%) and etazolate (84 ± 1%) in cerebral cortex. This was true both in comparing brain regions in an individual experiment or in average values from different experiments. Pentobarbital, on the other hand, generally enhanced benzodiazepine binding equally to or more than the former two compounds in the same brain region (114 ± 7% in cortex; $p < 0.001$ versus either other enhancing agent). The E_{max} for pentobarbital was, however, lower in cerebellum than in other brain regions. In a typical experiment comparing the six brain regions listed in Table 1, the E_{max} for pentobarbital was 100–120% for cortex, hippocampus, medulla-pons, and striatum, 93% in thalamus-midbrain, and 65% in cerebellum. Average values for several experiments showed a significantly lower E_{max} in cerebellum than in cortex (Table 1). The E_{max} for etazolate and GABA did not coincide in all brain regions; e.g., etazolate gave less enhancement in hippocampus than other areas and showed a lower E_{max} than GABA in this region, in agreement with a previous report (17). The E_{max} values for GABA were similar in all areas tested. Comparison of the potencies of the three drugs showed that the EC_{50} values for pentobarbital and etazolate were similar in all brain regions, but the value for GABA varied somewhat, from 0.54 μM in cerebellum to 4.5 μM in hippocampus. The two extreme values were significantly ($p < 0.005$)

TABLE 1

Brain regional variation in enhancement of benzodiazepine receptor binding by various agents

[^{3}H]Diazepam binding was measured as described under Materials and Methods, without and with varying concentrations of the three enhancing agents. The results are presented as maximal enhancements (E_{max}) in percentage over control and as the concentration necessary to produce 50% of maximal enhancement (EC_{50}). Each assay was done in triplicate, and the results are averages ± standard deviations of the values of (*n*) experiments.

Brain region	[^{3}H]Diazepam binding					
	+ Pentobarbital		+ Etazolate		+ GABA	
	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}
	mM	%	μM	%	μM	%
Cerebral cortex	0.14 ± 0.02	114 ± 7 (10)[a]	0.72 ± 0.14	84 ± 1 (5)[b]	1.6 ± 0.5	89 ± 6 (8)
Cerebellum	0.17 ± 0.01	62 ± 2 (3)[b]	0.41 ± 0.07	57 ± 1 (3)[b]	0.54 ± 0.44[c]	83 ± 3 (3)
Hippocampus	0.22 ± 0.12	117 ± 10 (2)	0.69 ± 0.17	32 ± 2 (2)[b]	4.5 ± 0.4	82 ± 11 (3)
Striatum	0.20 ± 0.03	106 ± 4 (2)	0.84 ± 0.07	82 ± 4 (2)[b]	1.3 ± 0.1	100 ± 5 (3)
Thalamus-midbrain	0.15 ± 0.01	86 ± 1 (2)	0.46 ± 0.06	64 ± 4 (2)[b]	3.5 ± 0.6	73 ± 6 (3)
Medulla-pons	0.13 ± 0.01	108 ± 2 (2)	0.60 ± 0.04	86 ± 5 (2)[b]	2.4 ± 0.5	99 ± 4 (3)

[a] Significantly different from the E_{max} value in cerebellum ($p < 0.001$); significantly different from the E_{max} value of etazolate and GABA ($p < 0.001$) in the same brain region (*t*-test).

[b] Inhibition of [^{3}H]diazepam binding at higher concentrations.

[c] Significantly different from the EC_{50} value in hippocampus ($p < 0.005$).

TABLE 2

Brain regional distribution of GABA-barbiturate receptor interactions

[^{3}H]GABA was measured as described under Materials and Methods without or with varying concentrations of pentobarbital or etazolate. EC_{50} and E_{max} are defined as in Table 1. [^{3}H]Diazepam binding was measured as described in Fig. 1, with 100 μM pentobarbital and varying concentrations of bicuculline to determine the maximal plateau inhibition of enhancement. The buffer in both cases was 0.2 M KCl/20 mM potassium phosphate (pH 7.0).

Brain region	[^{3}H]GABA binding				[^{3}H]Diazepam binding
	+ Pentobarbital		+ Etazolate		
	EC_{50}	E_{max}	EC_{50}	E_{max}	Fraction of pentobarbital enhancement reversed by 10 μM bicuculline
	mM	%	μM	%	
Cerebral cortex	0.43 ± 0.17	170 ± 20 (4)	4.6 ± 0.6	163 ± 13 (3)	64 ± 7 (7)
Cerebellum	0.22 ± 0.11	35 ± 11 (4)[a]	7.0 ± 6.2	29 ± 7 (3)[a]	4 ± 8 (3)[b]
Hippocampus	0.57 ± 0.09	158 ± 20 (2)	4.3 ± 1.1	122 ± 7 (3)	48 ± 5 (3)
Striatum	0.46 ± 0.013	91 ± 14 (2)	5.3 ± 4.3	52 ± 11 (2)	41 ± 5 (3)
Thalamus-midbrain	0.36 ± 0.01	95 ± 14 (2)	4.1 ± 2.0	79 ± 10 (2)	42 ± 4 (3)
Medulla-pons	0.44 ± 0.29	35 ± 34 (3)	5.2 ± 4.9	21 ± 8 (3)	17 ± 8 (3)

[a] Significantly lower than cortex or hippocampus ($p < 0.001$).

[b] Significantly lower than cortex or hippocampus ($p < 0.001$).

different from each other, but not from the other brain regions.

Barbiturates and pyrazolopyridines also enhanced specific Na^+-independent [^{3}H]GABA binding (Table 2). In contrast to the effect on benzodiazepine binding, E_{max} values for pentobarbital and etazolate on GABA binding were not significantly different from each other, and agreed closely throughout several brain regions. As previously observed (8, 17), the relative level of enhancement of GABA binding by pentobarbital and etazolate varied considerably with brain regions (cerebral cortex > hippocampus > thalamus-midbrain = striatum > medulla-pons = cerebellum; Table 2). Again, these results were apparent both in individual experiments comparing all brain regions and in the average values obtained from several experiments done on different preparations and at different times. The E_{max} for both drugs in cerebellum and medulla-pons were significantly lower than cortex and hippocampus ($p < 0.001$). Similar results were observed in dialyzed fresh (unfrozen) membranes. This regional variation in the enhancement of GABA binding by pentobarbital and etazolate was not seen for the enhancement of benzodiazepine binding by the same drugs (Table 1). Neither did the regional variation in E_{max} correlate with over-all sodium-independent GABA binding, previously shown to be highest in cerebellum (6, 7, 26), nor with over-all benzodiazepine binding (9, 20, 26) nor with over-all [^{3}H]dihydropicrotoxinin binding (26), both of which showed cerebellum levels roughly equal to cortex. The EC_{50} values for either etazolate or pentobarbital were similar in all brain regions.

The specific enhancement of benzodiazepine binding by GABA, etazolate, and pentobarbital was reversed by the GABA antagonist bicuculline (IC_{50} = 35 ± 10 nM, n = 3 for each enhancer), and also by high concentrations of strychnine (IC_{50} = 1.2 ± 0.5 μM, n = 2) and low concentrations of the novel steroid derivative R 5135 (27) (IC_{50} = 5 ± 2 nM, n = 2). This shows that the etazolate- and barbiturate-enhanced benzodiazepine sites are also associated with bicuculline/GABA receptors. Whereas GABA and etazolate enhancement were completely reversed by bicuculline, pentobarbital enhancement was reversed only partially, reaching a maximum of 67 ± 7% in cortex (13) and lower elsewhere. This plateau effect is shown in Fig. 1 for cerebellum (10% bicuculline-sensitive in this experiment) and for hippocampus (50% block) and in Fig. 2 for cortex (70% block). A similar flattening (plateau) of the dose-response curve was observed at high concentrations of strychnine and R 5135 (data not shown).

The maximal extent of inhibition by bicuculline of

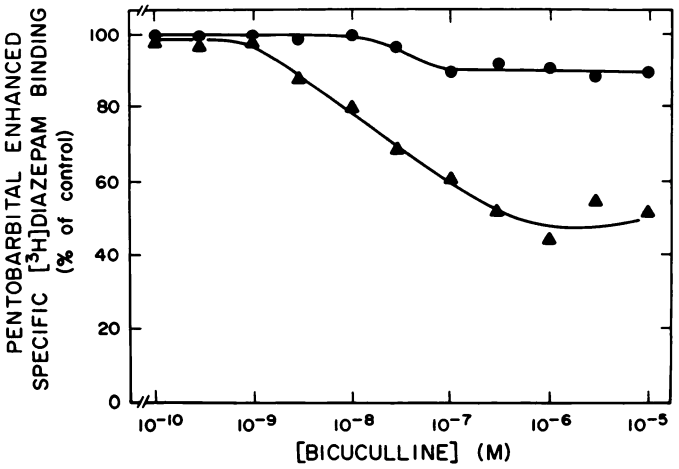

FIG. 1. *Inhibition by bicuculline of pentobarbital-enhanced [^{3}H] diazepam binding in cerebellum and hippocampus*

Fresh rat brain membranes were prepared and assayed as described under Materials and Methods. Control enhancement by pentobarbital (100 μM) was normalized to 100%. Bicuculline was added at varying concentrations at the time of incubation from a stock solution of 5 mM kept at pH 3. The results are typical of three experiments with mean values given in Table 2. Bicuculline did not inhibit baseline [^{3}H]diazepam binding, which was typically 3718 ± 78 minus 413 ± 19 (background) = 3305 ± 80 cpm (mean ± standard error of triplicates) without pentobarbital, 4474 ± 55 cpm (44% enhancement, with pentobarbital). ●, Cerebellum; ▲, hippocampus.

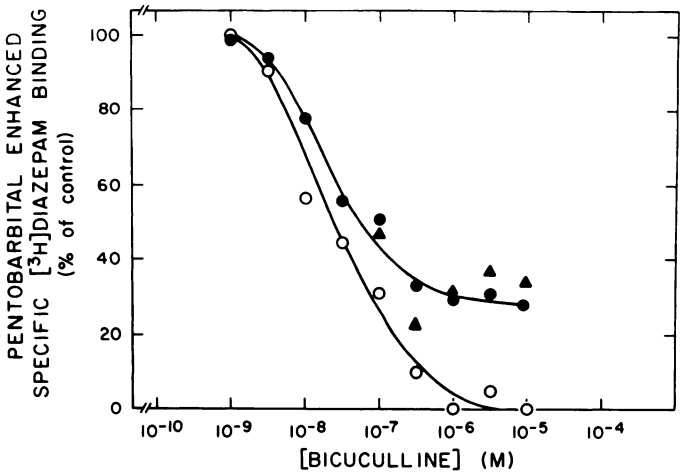

FIG. 2. *Inhibition by bicuculline of pentobarbital-enhanced [^{3}H] diazepam binding in cortex in the absence and presence of 50% blockade of binding by β-CCE or CL 218,872*

Membranes were prepared as described under Materials and Methods. The bicuculline concentration was varied in the absence (●) or presence of 2 nM β-CCE (○) or 200 nM CL 218,872 (▲). Control enhancement of [^{3}H]diazepam binding by pentobarbital (100 μM) was 45% above baseline and normalized to 100%. The plateau values at bicuculline >0.1 μM showed a variation of 7%; the points at 0.3, 1, 3, and 10 μM bicuculline in the absence of both β-CCE and CL 218,872 (control) or in the presence of CL 218,872 were not significantly different from each other by *t*-test; both of these were significantly different from those obtained in the presence of β-CCE ($p < 0.05$). The results were typical of three observations.

pentobarbital enhancement of [^{3}H]diazepam binding varied with brain region (Table 2), with the greatest effect in cerebral cortex and the lowest in cerebellum ($p < 0.001$ versus cortex). Interestingly, this regional distribution disagreed sharply with the relative enhancement of benzodiazepine binding, but agreed roughly with the relative maximal level of enhancement of *GABA* binding by pentobarbital (or etazolate). That is, bicuculline-sensitive pentobarbital enhancement showed a distribution similar to that of pentobarbital-enhanced GABA binding. It should be noted, however, that although the membranes used in the two types of assay were in some cases prepared from the same samples of rat brain regions, those used for the GABA binding assays were frozen and thawed and more repeatedly washed than those used for benzodiazepine binding (see Discussion).

β-CCE and the anxiolytic triazolopyridazine CL 218,-872 are competitive benzodiazepine receptor ligands which can distinguish two different affinity subpopulations or states (9, 20–23). We attempted to determine whether these subpopulations might be related to the bicuculline-insensitive fraction of pentobarbital-enhanced [^{3}H]diazepam binding sites. Inclusion of a low concentration (2 nM) of β-CCE sufficient to block 50% of the [^{3}H]diazepam bound (at 0.5 nM) and to occupy most of the high-affinity β-CCE sites and few of the low-affinity subpopulation (see Discussion) totally reversed the bicuculline insensitivity. That is, pentobarbital enhancement of the residual [^{3}H]diazepam binding was 100% blocked by low concentrations (≥1 μM) of bicuculline (Fig. 2). This suggests that the bicuculline-insensitive sites have a high affinity for β-CCE; when these sites are occupied by β-CCE, they do not bind [^{3}H]diazepam. The remaining [^{3}H]diazepam sites which have a lower affinity for β-CCE are those which are sensitive to bicuculline. On the other hand, CL 218,872 at low concentrations (200 nM) sufficient to block only its high-affinity sites, did not alter this bicuculline-insensitive pentobarbital enhancement (Fig. 2). Thus bicuculline-insensitive sites have no selective affinity for CL 218,872.

Figure 3 shows dose-response curves for enhancement of [^{3}H]diazepam binding in cortex for GABA, etazolate, and pentobarbital. As mentioned, the E_{max} levels for etazolate and GABA were similar, whereas that for pentobarbital was higher. Low levels of β-CCE and CL 218,872 also had differential effects on GABA and pyrazolopyridine interactions with benzodiazepine receptors. Occupation of high-affinity β-CCE sites resulted in a greatly improved enhancement of the residual [^{3}H]diazepam sites by GABA (Fig. 3A, □, ■), suggesting that the lower-affinity β-CCE sites (unblocked by 2 nM) are more sensitive to GABA, as well as being more sensitive to bicuculline, than are the high-affinity β-CCE sites. However, 200 nM CL 218,872 did not improve but *reduced* GABA enhancement (Fig. 3B). Thus CL 218,872 does not distinguish the same populations that β-CCE does in regard to bicuculline and GABA interactions. "Classical" benzodiazepines such as diazepam and lorazepam at IC_{50} concentrations (1–10 nM) gave no effect or a slight *reduction* in both GABA and etazolate enhancemnent (data not shown).

Furthermore, different effects of β-CCE and CL 218,-872 were also observed regarding pyrazolopyridine, but not barbiturate interactions. Etazolate enhancement of benzodiazepine binding follows a biphasic dose-response curve, with inhibition seen at higher concentrations (Fig. 3A and B, △, ▲). This feature was shared by cartazolate (Fig. 4A). CL 218,872 at 200 nM had no significant effect on the etazolate dose-response curve (Fig. 3B). However, β-CCE, at 2 nM, reversed the inhibitory phase of the etazolate and cartazolate effects on benzodiazepine binding, while enhancement seen at lower concentrations of the drugs remained, and was even augmented. In the absence of β-CCE, concentrations of 30–100 μM etazolate gave very little enhancement, whereas in the presence of 2 nM β-CCE, they enhanced by over 100% that fraction of [^{3}H]diazepam binding which remained unblocked by the β-CCE. Scatchard plot analysis (not shown) confirmed that 50 μM etazolate had almost no effect on the [^{3}H]diazepam binding curve in the absence of β-CCE (K_D = 3 nM with or without etazolate), but reversed the competitive inhibition (2-fold increase in apparent K_D to 7 nM) seen with 2 nM β-CCE (back to 4 nM with β-CCE plus etazolate). This effect of β-CCE on the dose-response curves of the pyrazolopyridines was dose-dependent (Fig. 4A), increasing the E_{max} for these drugs to approximately 130% (almost up to the level of pentobarbital).

The concentration dependence for β-CCE to displace [^{3}H]diazepam binding was compared with that for improving etazolate enhancement, with etazolate present at a concentration (40 μM) on the inhibitory phase of the curve. Figure 5A shows that as the concentration of β-CCE was raised and began to displace [^{3}H]diazepam

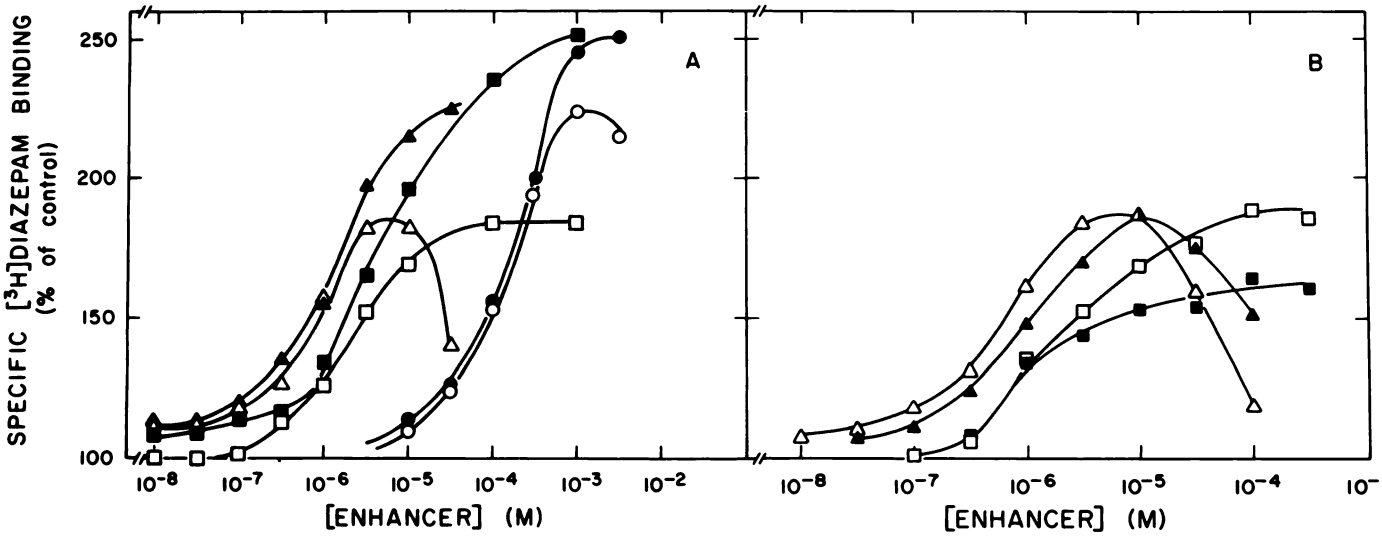

FIG. 3. *Enhancement of [^{3}H]diazepam binding by various agents in the absence and presence of 50% blockade of binding by β-CCE or CL 218,872*

Rat cortex membranes were prepared and assayed as described under Materials and Methods for [^{3}H]diazepam binding. Percentage enhancement over baseline was determined for varying concentrations of GABA (□, ■) etazolate (△, ▲), and pentobarbital (○, ●) in the presence (*closed symbols*) and absence (*open symbols*) of (A) 2 nM β-CCE or (B) 200 nM CL 218,872. In A, GABA and etazolate, but not pentobarbital E_{max} is significantly different under the two conditions ($p < 0.05$). In B, GABA, but not etazolate, E_{max} is significantly different under the two conditions ($p < 0.05$). The results are an average of three experiments which differed by ≤10%.

binding, the percentage enhancement of the residual [^{3}H] diazepam binding sites by the 40 μM etazolate was greatly augmented, apparently due to a reversal of the inhibitory phase of the etazolate dose-response curve (Fig. 3A). The reversal of etazolate inhibition occurred at concentrations of β-CCE significantly below its IC_{50} value on benzodiazepine binding, confirming that the high-affinity β-CCE sites are more associated with the inhibitory phase of etazolate effects. Figure 5A also shows that the IC_{50} for β-CCE was 1.5 nM in the absence of etazolate and reproducibly higher (4 nM) in the presence of etazolate (although this could be due to the increased affinity for [^{3}H]diazepam as compared with β-CCE).

The benzodiazepine Ro 15-1788, a high-affinity benzodiazepine receptor antagonist but unable to distinguish subpopulations of different affinity (11, 24), behaved in a manner similar to β-CCE, dose-dependently and saturably reversing the inhibitory phase of the etazolate dose-response curve (Figs. 4B and 5B). A concentration of 3 nM Ro 15-1788 gave the maximal increase in etazolate enhancement, with higher concentrations such as 10 nM beginning to fall back down to control levels. In the case of both β-CCE and Ro 15-1788, the enhancement by etazolate gradually diminished as the [^{3}H]diazepam sites became fully occupied by the displacer. By contrast, the depressant benzodiazepine, diazepam itself, did not augment etazolate enhancement at any concentration of nonradioactive diazepam (Fig. 5C), although the dimi-

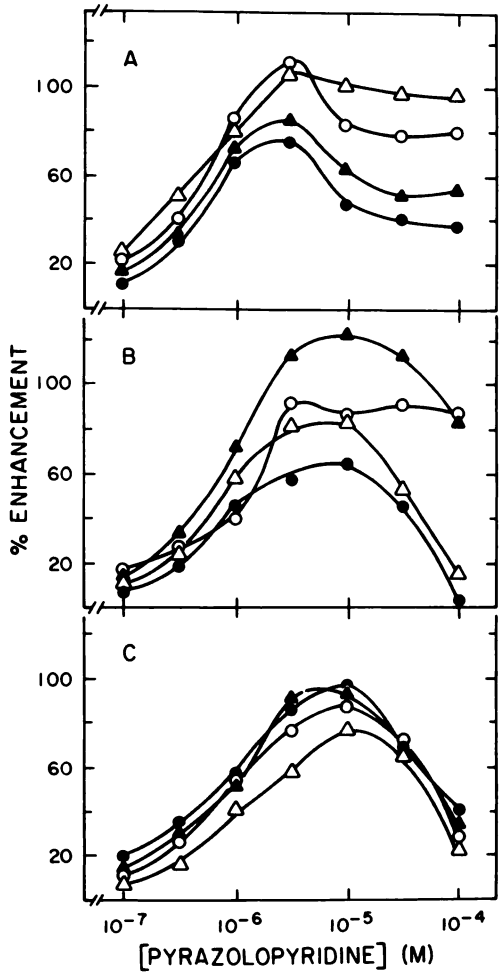

FIG. 4. *Effect of partial occupancy of [^{3}H]diazepam binding sites by various ligands on dose-response curves for pyrazolopyridine enhancement.*

Rat cortex membranes were prepared and assayed as described under Materials and Methods, measuring enhancement over baseline at various concentrations of pyrazolopyridines in the presence of various fixed concentrations of various benzodiazepine receptor ligands. A, Cartazolate enhancement in the presence of (●) 0, (▲) 1 nM, (○) 3 nM, and (△) 10 nM β-CCE. B, Etazolate enhancement in the presence of (●) 0, (△) 0.3 nM, (▲) 3 nM, and (○) 10 nM Ro 15-1788. C, Etazolate enhancement in the presence of (●) 0, (○) 0.03 nM, (▲) 0.1 nM, and (▲) 0.3 nM CGS 8216. The results are an average of three experiments which differed by ≤10%.

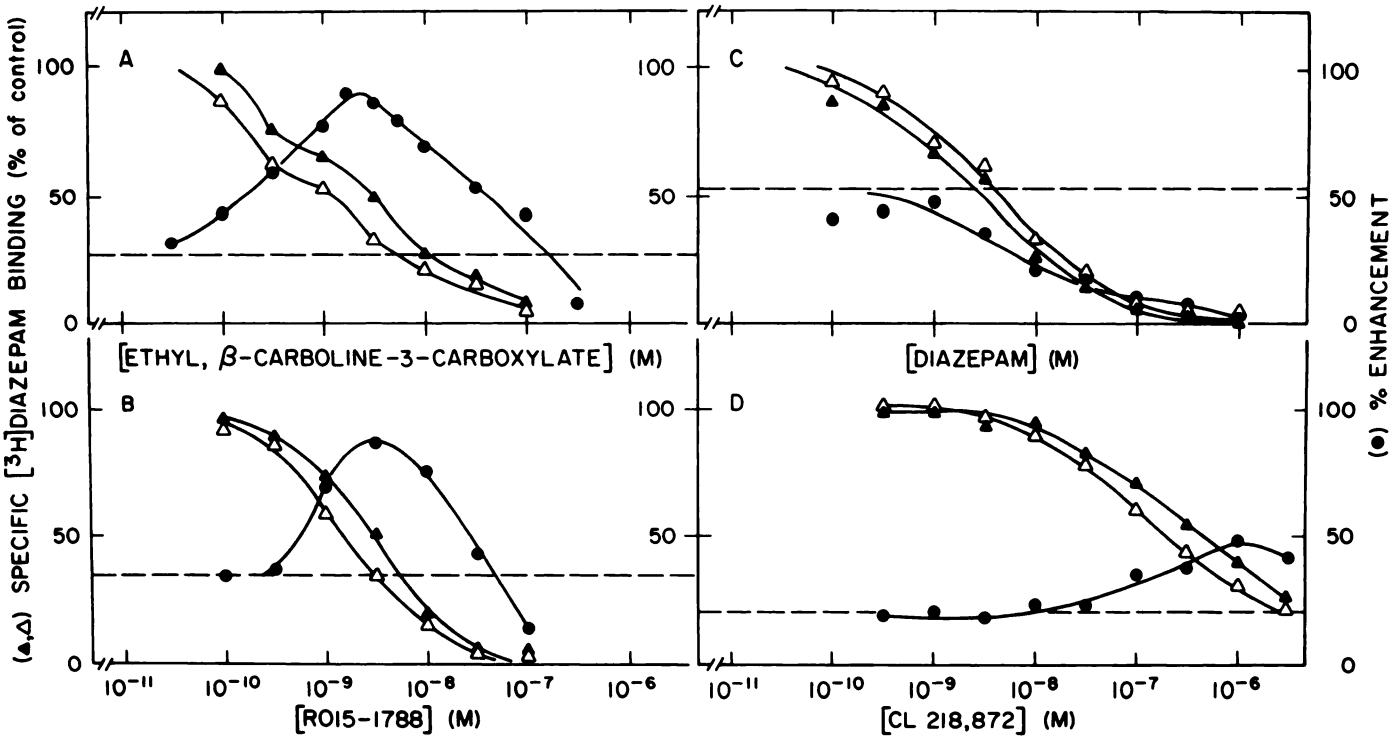

FIG. 5. *Effect of various benzodiazepine receptor ligands on [^{3}H]diazepam binding and enhancement by etazolate*

Rat cortex membranes were prepared and assayed for [^{3}H]diazepam binding as described under Materials and Methods. The displacement of [^{3}H]diazepam binding was determined in the absence (△) or presence (▲) of 40 μM etazolate for (A) β-CCE, (B) Ro 15-1788, (C) diazepam, and (D) CL 218,872. At the same time the percentage enhancement by this amount of etazolate on the [^{3}H]diazepam binding remaining at that concentration of displacing agent was determined (●). *Broken lines* represent the control level of enhancement by etazolate in the absence of displacer. The results are the average of three experiments which differed by ≤10%.

nution with increasing concentrations of displacing agent was still present. An identical pattern was observed with lorazepam (data not shown). As previously mentioned, the maximal enhancement by etazolate was also not improved by the anxiolytic CL 218,872 (Fig. 3B). However, in this case there was some relative improvement at concentrations of CL 218,872 *above* its IC_{50} to displace [^{3}H]diazepam (Fig. 5D), rather than the diminution seen with the previously described three agents. This resulted from a small rightward (rather than upward) shift by CL 218,872 of the etazolate curve (Fig. 3B). However, the improvement of etazolate enhancement was not observed (Fig. 4C) with the potent pyrazoloquinoline antagonist ligand, CGS 8216 (28).

The independent nature of the two phases of the etazolate dose-response curve was further demonstrated by the retention of the inhibitory aspect alone when the enhancement was completely prevented by inclusion of saturating levels of 100 μM picrotoxinin (Fig. 6). Likewise 10 μM bicuculline completely blocked etazolate enhancement (11) but revealed some inhibition of baseline [^{3}H] diazepam binding by etazolate (≃30% at 30 μM). The summation of these two independent enhancement and inhibition effects by etazolate appears to explain the "partial agonist" character of certain barbiturates such as (−)-mephobarbital, (−)-MPPB, and (+)-hexobarbital, which showed biphasic dose-response curves on [^{3}H]diazepam binding (14). As with etazolate, the enhancement by (−)-MPPB and (−)-mephobarbital became greatly augmented, especially in the inhibitory phase of the curve, in the presence of low levels of β-CCE (Fig. 7). It appears that removal of the inhibition by β-CCE revealed an enhancement more resembling the effects of a "full agonist" (14). Likewise, the enhancement by (−)-MPPB (maximally 30%), (−)-mephobarbital (maximally 60%),

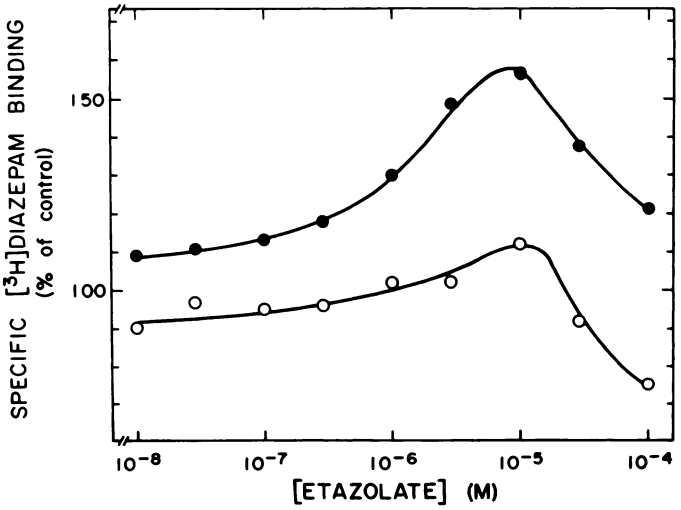

FIG. 6. *Dose-response curve for etazolate enhancement of [^{3}H]diazepam binding in the absence and presence of picrotoxinin*

Rat cortex membranes were prepared and assayed for [^{3}H]diazepam binding as described under Materials and Methods. Varying concentrations of etazolate were included in the absence (●) or presence (○) of 100 μM picrotoxinin to determine the percentage enhancement over control. The results are typical of three observations with picrotoxinin and 10 experiments without picrotoxinin (see, for example, Figs. 3A and B and 4B and C, and refs. 13–19).

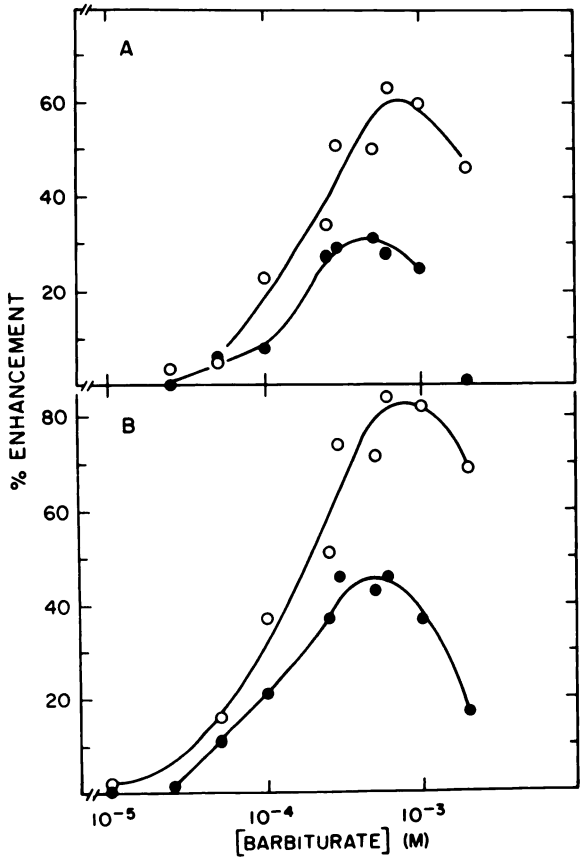

FIG. 7. *Dose-response curves for "partial agonist" barbiturates to enhance [^{3}H]diazepam binding in the absence and presence of 50% blockade of binding by β-CCE*

Rat cortex membranes were prepared and assayed as described. Varying concentrations of (A) (−)-MPPB and (B) (−)-mephobarbital were included to determine enhancement over control in the presence (○) or absence (●) of 2 nM β-CCE. The results are the average of two experiments which differed by ≤10%.

(+)-hexobarbital (maximally 70%), and phenobarbital (maximally 20%) at 0.5 mM barbiturate was virtually eliminated by 10 μM bicuculline, leaving only an inhibition of [^{3}H]diazepam binding evident at high concentrations of these barbiturates (data not shown).

The nature of the bicuculline-insensitive fraction of the pentobarbital enhancement of [^{3}H]diazepam binding was further investigated. Preliminary observations indicate that the enhancement remaining in the presence of >1 μM bicuculline involves an increase in affinity, which is blocked by picrotoxinin, favored by high concentrations of chloride ions, and stereospecific for the (−)-isomer of pentobarbital and related barbiturates.

DISCUSSION

A number of benzodiazepine receptor ligands were examined for interactions with GABA/bicuculline and picrotoxinin/pyrazolopyridine/barbiturate receptor ligands. These included (a) the classical benzodiazepine depressants (diazepam); (b) ligands which distinguish multiple-affinity subpopulations or states of benzodiazepine receptor sites, both "agonist" anxiolytic triazolopyridazines (CL 218,872) and "antagonist" β-carbolines (β-CCE); and (c) "antagonists" which did not distinguish multiple affinities, such as the benzodiazepine Ro 15-1788 and the pyrazoloquinoline CGS 8216.

Four types of allosteric interactions within the GABA/benzodiazepine receptor complex were analyzed with respect to differential effects of the various categories of benzodiazepine-receptor ligands. These included the partial block by the GABA receptor antagonist bicuculline of the barbiturate (pentobarbital) enhancement of [^{3}H]diazepam binding (13); the biphasic dose-response curve for pyrazolopyridines (etazolate) (13, 17–19) and some barbiturates (14) to enhance [^{3}H]diazepam binding; the basic maximal enhancement by GABA of [^{3}H]diazepam binding; and the basic maximal enhancement by barbiturates of [^{3}H]GABA receptor binding.

The plateau effect of bicuculline to inhibit pentobarbital enhancement of benzodiazepine binding indicates that there are two types of barbiturate-benzodiazepine receptor interactions, one more sensitive to bicuculline and one less sensitive. This could be due to either two different *actions* of barbiturates on a single benzodiazepine receptor—one sensitive to bicuculline and one not—or two different *kinds* of benzodiazepine-barbiturate receptor complexes—one sensitive to bicuculline and one not.

Several observations suggest that the latter interpretation is more likely. First, the observation of a similar brain regional distribution between the extent of bicuculline-sensitive pentobarbital-enhanced benzodiazepine binding and the E_{max} for pentobarbital enhancement of GABA binding would be consistent with a population of GABA/bicuculline receptor-linked barbiturate receptor sites, because it is reasonable to assume that the extent of these two effects is proportional to the quantity of receptors showing the phenomenon. Additionally, these data suggest the existence of a population of GABA/bicuculline receptors not linked to barbiturate receptors or linked to them in a different manner (those not enhanced by pentobarbital) and a population of barbiturate receptors (associated with benzodiazepine receptors) which are not linked to GABA/bicuculline receptors or linked to them in a different manner (those not blocked by bicuculline).

The correspondence in brain regional variation between pentobarbital effects on GABA binding and bicuculline effects on pentobarbital enhancement of benzodiazepine binding are unlikely to be coincidental. Although the GABA binding was done with frozen-thawed membranes and the benzodiazepine binding with fresh membranes, these variables are unlikely to have important effects on the observations reported here, although we cannot exclude an effect of freezing on the conformational flexibility of a membrane protein. With respect to brain regional variation in pentobarbital/etazolate enhancement of GABA binding, this has been observed both in frozen and thawed [GABA-depleted (5–7)] or fresh membranes. With respect to diazepam binding, it is possible that the membrane preparation used would leave endogenous GABA in levels which varied with brain region and therefore might affect differentially the pentobarbital enhancement and the bicuculline reversal thereof. However, the variable bicuculline effect is not

seen when reversing the enhancement by GABA or etazolate. Second, our membrane preparations (osmotic shock, Tissuemizer homogenization) contain little endogenous GABA as indicated by the lack of inhibition by bicuculline or imidazole-acetic acid of baseline benzodiazepine binding (13). Most important, there is some evidence that the pentobarbital enhancement of benzodiazepine binding, while sensitive to ligands at the GABA receptor site, does not involve the presence of endogenous GABA. This is suggested by the failure of imidazole acetic acid or THIP to reverse the pentobarbital enhancement (using the same membrane preparations employed in the current study) (13), despite their occupation of GABA receptor sites under conditions where they themselves do not enhance benzodiazepine binding. Also, the benzodiazepine enhancement by barbiturates is preserved in detergent-solubilized preparations from which any GABA present had been removed by size separation column chromatography (29). The bicuculline plateau effect is further unlikely to reflect variable regional levels of endogenous receptor-bound GABA since medulla-pons (low GABA levels) shows a smaller bicuculline sensitivity than striatum and hippocampus (high GABA levels).

Barbiturates appear to increase GABA binding (8) to both high- and low-affinity subpopulations (7), some of which are probably not associated with benzodiazepine receptors (5). Under the assay conditions employed here, GABA would be bound to a mixture of high- and low-affinity sites and the *number* of both populations is *apparently* increased by maximal concentrations of pentobarbital. We have shown this to be due to an increase in *affinity* of low-affinity ($K_D \simeq 1$ μM)GABA sites by barbiturates (8). The variation with brain region for the maximal pentobarbital enhancement reflects a secondary degree of heterogeneity in GABA receptor sites, i.e., barbiturate sensitivity. The heterogeneity of benzodiazepine receptors observed with respect to bicuculline (GABA) interactions suggests the possibility that barbiturate enhancement of GABA sites is limited to that population of GABA sites which is capable of interaction with benzodiazepine receptors. These GABA sites would appear to be a very low-affinity population.

A second point supporting the interpretation of heterogeneity in benzodiazepine receptors is that the bicuculline-insensitive fraction of pentobarbital-enhanced benzodiazepine sites can be eliminated by low levels of β-CCE sufficient to block only its high-affinity sites. Displacement of [^{3}H]diazepam binding by nonradioactive benzodiazepine receptor ligands was analyzed by a nonlinear regression computer program as described by Yamamura and colleagues (22). Data such as those shown in Fig. 5 confirmed the literature (9, 20–23) reports of two IC_{50} values for β-CCE (≃1 nM and 15 nM) and for CL 218,872 (70 nM and 3 μM), whereas one affinity was most suitable to describe the binding of diazepam (3 nM) and Ro 15-1788 (1.5 nM). Therefore including 2 nM β-CCE or 200 nM CL 218,872 to inhibit about 50% of the binding blocks primarily high-affinity sites and leaves [^{3}H]diazepam bound primarily to low-affinity sites.

At the same time, low concentrations of β-CCE lead to an increase in the maximal extent of GABA enhancement of the remaining benzodiazepine sites. Furthermore, low concentrations of β-CCE lead to an augmented enhancement of benzodiazepine binding by pyrazolopyridines and certain barbiturates which normally show biphasic dose-response curves for the enhancement effect. Low concentrations of β-CCE appear to block a subset of benzodiazepine binding sites which are selectively not enhanced but subject to inhibition seen at high concentrations of the pyrazolopyridines and "partial agonist" barbiturates (14); the low-affinity β-CCE sites remaining are revealed to show a more "full agonist"-like enhancement at lower concentrations of these enhancing agents. Bicuculline, on the other hand, blocks the enhancement of benzodiazepine binding by these agents, leaving only an inhibitory action seen at high concentrations. Picrotoxinin also inhibits enhancement completely, while not affecting the inhibitory aspect. A report in the literature (30) describes bicuculline-sensitive and bicuculline-insensitive enhancement of [^{3}H]diazepam binding by avermectins, whereas avermectin enhancement of [^{3}H]β-CCE is completely insensitive to bicuculline. This is consistent with our observations of bicuculline-insensitive high-affinity sites for β-CCE.

Bicuculline-insensitive pentobarbital-enhancement of benzodiazepine binding is not removed by low concentrations of CL 218,872 sufficient to block only its high-affinity sites. Likewise this compound does not improve GABA enhancement of benzodiazepine binding but rather decreases it. This would be consistent with a low affinity of CL 218,872 for the bicuculline- and GABA-insensitive population, accompanied by GABA sensitivity for high affinity CL 218,872 sites [GABA enhances binding of [^{3}H]CL 218,872 (22)]. The decrease in GABA enhancement might be due to a greater fraction of [^{3}H]diazepam bound to GABA-insensitive sites in the presence of 200 nM CL 218,872. Furthermore, CL 218,872 does *not* improve the maximal enhancement of benzodiazepine binding by etazolate (does not discriminate between the inhibitory and enhancement phases of the etazolate dose-response curve). Thus the bicuculline-sensitive barbiturate-enhanced and etazolate-enhanced sites also have low affinity for CL 218,872. The GABA-sensitive high-affinity sites for CL 218,872 must therefore represent yet a third class of sites. This type may be insensitive to pyrazolopyridines and probably has high affinity for β-carbolines.

All of these observations support the existence of separate bicuculline-sensitive and bicuculline-insensitive benzodiazepine/barbiturate receptors (subpopulations or states). High-affinity β-CCE sites (blocked by low concentrations) correspond to relatively lower bicuculline sensitivity and lower GABA sensitivity than low-affinity β-CCE sites. High-affinity β-CCE sites also correspond to the inhibitory phase of the biphasic dose-response curves for pyrazolopyridine and "partial agonist" barbiturate enhancement of benzodiazepine binding, while low-affinity β-CCE sites correspond to the enhancement phase. Thus the bicuculline-insensitive pentobarbital-enhanced benzodiazepine receptor sites appear to be a distinct subpopulation or state having a high affinity for β-CCE, a low affinity for CL 218,872, poor or no enhancement by GABA, and showing only inhibition by pyrazolopyridines and "partial agonist" barbiturates. The bi-

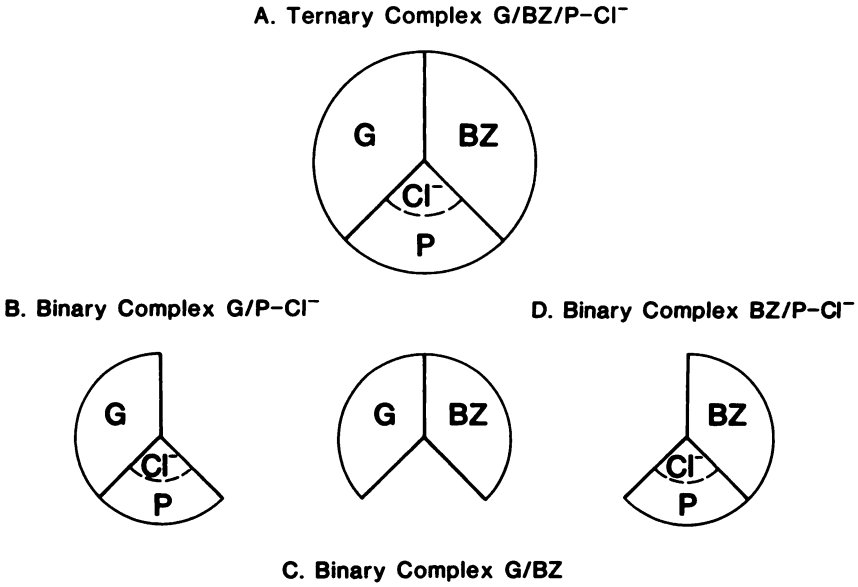

FIG. 8. *Hypothetical multiple-coupling state model for GABA-benzodiazepine receptor complex*

G, GABA receptor; *BZ*, benzodiazepine receptor; *P*, picrotoxin/pentobarbital/pyrazolopyridine receptor, including Cl^- (chloride ion channel). The relationship of each of the four complexes to heterogeneity of benzodiazepine receptor interactions with GABA and barbiturate receptors is described in the text. A, Three-receptor complex (*G/BZ/P*-Cl^-); B, binary complex (*G/P*-Cl^-); C, binary complex (*G/BZ*); D, binary complex (*BZ/P*-Cl^-).

cuculline-sensitive pentobarbital-enhanced benzodiazepine receptor sites have a low affinity for β-CCE, a low affinity for CL 218,872, are well enhanced by GABA, and show monophasic enhancement by pyrazolopyridines and "partial agonist" barbiturates.

Finally, the bicuculline-insensitive fraction of pentobarbital enhancement of benzodiazepine binding showed a similar but not identical profile of barbiturate interactions. The barbiturate enhancement was picrotoxinin-sensitive and chloride-dependent, and was stereospecific [(−)-pentobarbital > (+)-pentobarbital], but it appeared to be limited to "full agonists" only, since the enhancement by pyrazolopyridines and "partial agonist" barbiturates was completely or nearly completely blocked by bicuculline. Thus the bicuculline-insensitive barbiturate/benzodiazepine receptors do not seem to be identical with the bicuculline-sensitive barbiturate/benzodiazepine receptors, although the presence or absence of the GABA/bicuculline receptor component of the complex might be responsible for the difference. It is not clear at this time what, if any, pharmacological actions of barbiturates might involve this bicuculline-insensitive barbiturate enhancement of benzodiazepine sites.

None of these observations show conclusively whether the two types of benzodiazepine receptor subtypes defined by bicuculline sensitivity (and the third type defined by high affinity for CL 218,872) correspond to distinct receptor protein subpopulations or to different conformational or coupling states of a single receptor protein. If multiple states are involved, the myriad interactions do not seem to be explained simply by differences in agonists/antagonists. Although partial block of benzodiazepine receptors by low levels of β-CCE and the benzodiazepine antagonist Ro 15-1788 both improve etazolate and GABA enhancement, while the agonists diazepam and CL 218,872 do not, neither does the antagonist CGS 8216. Also, the improvement of GABA and etazolate enhancement of benzodiazepine binding by β-CCE and Ro 15-1788 cannot be due to a relative increase in affinity of the [^{3}H]diazepam versus that of the nonradioactive displacer (due to GABA or etazolate). This is true since CGS 8216 does not improve GABA and etazolate enhancement even though CGS 8216, like β-CCE and Ro 15-1788, is not itself enhanced in affinity by GABA (28) or etazolate.[2] However, if the unique class of compounds represented by CGS 8216 should turn out to be either a partial agonist/antagonist, or to bind with differential affinity to subpopulations of benzodiazepine receptors, then the effects of β-CCE and Ro 15-1788 could be due to their antagonist character. The effects of β-CCE and Ro 15-1788, not seen with diazepam, CL 218,872, or CGS 8216, on benzodiazepine receptor interactions with GABA/bicuculline and barbiturate/pyrazolopyridine ligands could be due to either stabilization of an antagonist-preferring conformation of a two-state oligomeric receptor, or to selective occupation of an antagonist-preferring subpopulation of receptor sites, as suggested also by others (11, 22, 24, 25).

The differential effects of β-CCE and CL 218,872 on bicuculline/GABA and pyrazolopyridine/barbiturate interactions with benzodiazepine receptors clearly are not due simply to different populations or states showing differential affinities for certain drugs. It appears that the high-affinity β-CCE sites do not correspond *in toto* to the high-affinity CL 218,872 sites, although they may overlap. In addition, Ro 15-1788 has effects similar to β-CCE even though it does not distinguish multiple affinities of benzodiazepine sites.

While it is evident that benzodiazepine receptor sites

[2] A. M. Snowman, L. M. F. Leeb-Lundberg, and R. W. Olsen, unpublished observations.

can exist in more than one conformational state, probably related to agonist/antagonist-preferred binding affinities, the heterogeneity apparent in these and other studies would require at least three conformational states and possibly even greater complexity superimposed upon the multiple conformational states. We propose (Fig. 8) that a reasonable model involves different coupling states for the benzodiazepine and GABA receptor sites in which they may be found associated with each other and the barbiturate/picrotoxinin receptors and associated chloride ion channels in a ternary complex, or in binary complexes involving any two of the receptors, and possibly as single receptor entities. The ternary complex (*A*) would involve benzodiazepine sites which are enhanced by GABA, pentobarbital, and etazolate and have low affinity for β-CCE. The ternary complex has been solubilized in high yield from cortex (29). This form would have to exhibit at least two conformational states required by the allosteric enhancement, and probably has a low affinity for GABA, at least in membranes. The barbiturate/benzodiazepine receptor dimer lacking a GABA receptor component (*D*) would appear to be a good candidate for the bicuculline-insensitive sites which are inhibited but not enhanced by etazolate, and have high affinity for β-CCE and probably low affinity for CL 218,872. One might also speculate that the GABA/benzodiazepine receptor dimer lacking the pyrazolopyridine/pentobarbital/picrotoxinin receptor-Cl^- channel (*C*) could represent high-affinity sites for CL 218,872. The GABA/P-Cl^- channel dimer lacking the benzodiazepine site (*B*) could represent high-affinity, anion-sensitive GABA sites which show a brain regional distribution, especially in cerebellum, different from benzodiazepine receptors. Alternatively, the high-affinity GABA sites might not be coupled to benzodiazepine or barbiturate receptor (5). Proof of such a model would require isolation of the various species, which has not been reported thus far. It should also be noted that these multiple forms of benzodiazepine receptors, whatever the theoretical explanation in molecular terms, have no physiological or pharmacological correlates at this time, and whether or not they represent homogenization artifacts awaits further information. Nevertheless, heterogeneity in GABA and benzodiazepine receptor function is a reasonable possibility, and the heterogeneity in binding studies may turn out to be relevant.

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