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Histidine modification with diethyl pyrocarbonate shows heterogeneity of benzodiazepine receptors

(diazepam/γ-aminobutyric acid receptor/chloride–ionophore/barbiturates)

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ABSTRACT **The effect of diethyl pyrocarbonate modification of histidine on the specific binding of [^{3}H]diazepam and its enhancement with muscimol and (±)-pentobarbital was investigated. Diethyl pyrocarbonate treatment produced a dose-related inhibition of specific [^{3}H]diazepam binding to rat brain membranes with a maximal inhibition of ≈40% at 1 mM. Scatchard analysis of the binding data showed that diethyl pyrocarbonate, while having no effect on the affinity (K_d), decreased the binding capacity (B_{max}) of diazepam from a control value of 1543 ± 116 fmol/mg of protein to 789 ± 79 fmol/mg of protein (mean ± SD; $P < 0.005$; $n = 4$). Under conditions in which ≈40% of the diazepam binding sites were modified by diethyl pyrocarbonate treatment, the ability of muscimol and pentobarbital to enhance diazepam binding was not altered. These results suggest that a histidine residue is critical for a part (≈40%) of the benzodiazepine binding sites and that there may exist a heterogeneity of benzodiazepine binding sites. Furthermore, these results indicate that perhaps only a portion of the benzodiazepine binding sites are functionally coupled to the γ-aminobutyric acid receptor–ionophore complex.**

Benzodiazepine binding sites in the mammalian central nervous system have been characterized (1, 2). Neurophysiological and biochemical (3–5) studies have shown that benzodiazepine binding sites are associated with the inhibitory neurotransmitter γ-aminobutyric acid (GABA) receptor–ionophore complex. Binding of GABA to its receptor sites (6–8) and of α-dihydropicrotoxinin to possible GABA receptor-linked ionophores has also been characterized (9, 10). The physiological postsynaptic response to GABA, both in mammalian motoneurons (11) and in crayfish muscle (12), involves an increase in anion conductance that has a selectivity sequence of $Br^- > Cl^- > I^-$. Benzodiazepines have been reported to potentiate the Cl^--dependent GABA-mediated response (13).

GABA agonists (3, 14), barbiturates (14, 15), and ethanol (16) have been reported to enhance [^{3}H]diazepam binding to rat brain membranes. The ability of picrotoxin and bicuculline to prevent this enhancement suggests that the GABA receptor and ionophore components are involved in these actions (3, 14, 15). Furthermore, anions, which permeate the ion channel at the spinal cord inhibitory synapse (17), increase both basal diazepam binding (18) and the pentobarbital-induced increase (15). These results suggest that the chloride–ionophore complex is critically associated with the benzodiazepine–GABA–receptor complex.

To understand the molecular pharmacology of benzodiazepines, it is essential not only to characterize the ligand binding but also to determine the functional groups that may directly or indirectly participate in the physiologic and pharmacologic responses. We have investigated the effect of modification of histidine by diethyl pyrocarbonate ($Et_2C_2O_5$) on diazepam binding and on the muscimol- and pentobarbital-induced enhancement of diazepam binding to rat brain membranes.

MATERIALS AND METHODS

[^{3}H]Diazepam (76.8 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from New England Nuclear, muscimol was from Research Organics (Cleveland, OH), and other chemicals were from Sigma. Diazepam was a gift from W. A. Scott (Hoffman–La Roche).

Tissue Preparation. Tissue for [^{3}H]diazepam binding studies was prepared as described (14, 15). Briefly, male Sprague–Dawley rats (150–200 g) were decapitated, and their brains (minus pons-medulla) were rapidly removed and placed in buffer A (0.2 M sodium chloride/10 mM sodium phosphate, pH 7.0). The tissue was homogenized with a Brinkman Polytron twice, with 5-sec bursts. The homogenate was centrifuged at 1000 × *g* for 10 min, and the supernatant was then centrifuged at 140,000 × *g* for 45 min to obtain the mitochondrial plus microsomal ($P_2 + P_3$) fraction. The pellet was washed five times in buffer A by suspension and centrifugation and frozen overnight. On the following day, the pellet was thawed at room temperature, repelleted, washed once, and resuspended at a protein concentration of 0.5–0.8 mg/ml. Extensive washing and freeze-thaw procedures are necessary to remove endogenous GABA and other inhibitory substances (7). All procedures were done at 0–4°C. Protein was estimated by the method of Lowry *et al.* (19).

Histidine Modification. Extensively washed and freeze-thawed $P_2 + P_3$ fraction was suspended in buffer B (0.2 M NaCl/10 mM sodium phosphate, pH 6.0) and treated with 1 mM $Et_2C_2O_5$ for 20 min at 0°C. Under these conditions, $Et_2C_2O_5$ reacts primarily with histidine residues (20–22). Control tissue was treated in an identical manner with buffer B and without $Et_2C_2O_5$. After this treatment, the tissue was pelleted to remove excess $Et_2C_2O_5$ and resuspended in buffer A for binding studies. Control tissue was processed in the same manner.

Binding Studies. [^{3}H]Diazepam binding was studied as described (14, 15). Routinely, 0.7 ml of control or $Et_2C_2O_5$-treated membrane suspension was incubated with 1 nM [^{3}H]diazepam for 30 min at 0°C with or without other ligands in a total incubation volume of 1 ml. After incubation, triplicate 250-μl aliquots were rapidly filtered under reduced pressure on Whatman GF/B filters. The filters were washed twice with 3-ml portions of ice-cold buffer, dried, and assayed in 5 ml of toluene/0.2% BBS (Beckman) containing 5 g of 2,5-diphenyloxazole per liter. The counting efficiency, determined by [^{3}H]toluene, was

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Abbreviations: GABA, γ-aminobutyric acid; $Et_2C_2O_5$, diethyl pyrocarbonate; B_{max}, maximum binding capacity; EC_{50}, concentration that produces 50% of maximal enhancement.

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Table 1. Effect of $Et_2C_2O_5$ on specific [^{3}H]diazepam binding

	Specific binding, fmol/mg of protein	Inhibition, %
Control	129.25 ± 14	—
$Et_2C_2O_5$ treated	72.71 ± 7.5	39.6 ± 3.7

Extensively washed and freeze-thawed $P_2 + P_3$ rat brain membrane fraction was incubated with buffer B (control) or with 1 mM $Et_2C_2O_5$/buffer B for 20 min at 0–4°C. The samples were centrifuged at 140,000 × *g* for 30 min, and the pellets were then suspended in buffer A. Aliquots of control or $Et_2C_2O_5$-treated homogenate were incubated with 1 nM [^{3}H]diazepam with or without 10 μM unlabeled diazepam for 30 min at 0–4°C. Binding was studied as described in *Materials and Methods*. Background obtained in the presence of 10 μM diazepam was subtracted from total binding to obtain specific binding. Results are mean ± SD of eight experiments each done in triplicate.

44 ± 2%. The background was determined in the presence of 10 μM diazepam. The specific binding usually represented 78 ± 10% of the total binding activity. For Scatchard plots, the concentration of [^{3}H]diazepam was 0.2–25 nM.

RESULTS

The mean results of eight experiments in which the effect of 1 mM $Et_2C_2O_5$ was investigated on specific diazepam binding are given in Table 1. $Et_2C_2O_5$ had no effect on the background (data not shown) but produced a 39.6 ± 3.7% decrease in specific diazepam binding. The effect of various concentrations of $Et_2C_2O_5$ on specific diazepam binding are shown in Fig. 1. $Et_2C_2O_5$ inhibited specific diazepam binding in a dose-related fashion with a maximal inhibition of 40% at 1 mM. At 2 mM, $Et_2C_2O_5$ produced a similar (but no larger) decrease in specific diazepam binding. Higher concentrations of $Et_2C_2O_5$ were not tried, as they have been reported to alter functional groups other than histidine (20–22).

Scatchard plots of diazepam binding in control and 1 mM $Et_2C_2O_5$-treated preparations are shown in Fig. 2. $Et_2C_2O_5$ treatment decreased the maximum binding capacity (B_{max}) from a control value of 1533 fmol/mg of protein to 736 fmol/mg of protein. The results of four Scatchard analyses of the effects of $Et_2C_2O_5$ treatment on the kinetic constants of diazepam binding are summarized in Table 2. $Et_2C_2O_5$ treatment does not appear to alter the K_d of diazepam binding significantly. However, it produced a decrease in B_{max} from a control value of 1543 ± 116 fmol/mg of protein to 789 ± 79 fmol/mg of protein.

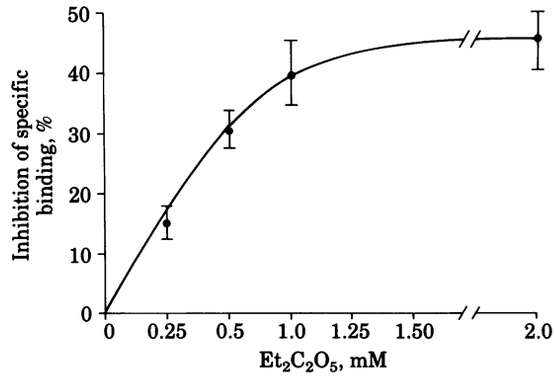

FIG. 1. Concentration-dependent inhibition of specific [^{3}H]diazepam binding by $Et_2C_2O_5$. The extensively washed and freeze-thawed membrane suspension was incubated with buffer B (control) or various concentrations of $Et_2C_2O_5$/buffer B for 20 min at 0°C. After incubation, tissue was pelleted and suspended in buffer A. Results are mean ± SD of six to nine determinations.

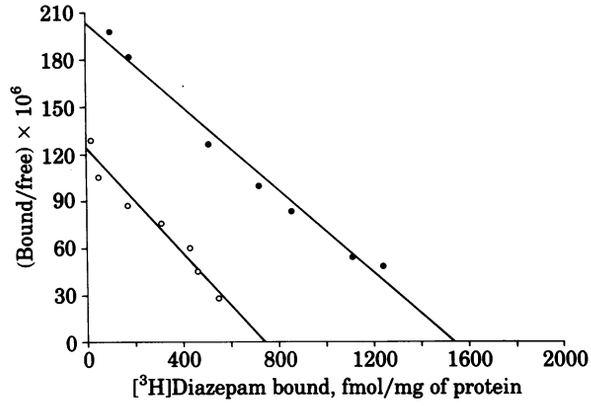

FIG. 2. Scatchard plots of [^{3}H]diazepam (0.25–25 nM) binding to control (●) and $Et_2C_2O_5$-treated (○) rat brain membranes. Results are means of one experiment (in triplicate), which was repeated four times with similar results. The K_d and B_{max} were obtained by linear regression of the binding data.

GABA agonists have been reported to enhance diazepam binding by increasing the affinity of diazepam to its binding sites (3). This enhancement is blocked by bicuculline (3, 14) and apparently occurs at the level of the GABA recognition site. To determine whether $Et_2C_2O_5$ treatment altered the ability of muscimol to enhance diazepam binding, we investigated the effects of muscimol on diazepam binding in control and $Et_2C_2O_5$-treated preparations.

The effect of $Et_2C_2O_5$ on the muscimol (0.1 μM) enhancement of diazepam binding is summarized in Table 3. $Et_2C_2O_5$ treatment decreased the basal diazepam binding from a control value of 172.6 ± 5.3 fmol/mg of protein to 117.4 ± 3.9 fmol/mg of protein (32% decrease) but did not alter the ability of muscimol to enhance diazepam binding. Muscimol (0.1 μM) increased specific diazepam binding by 69.9 fmol in control and by 73.4 fmol in $Et_2C_2O_5$-treated preparations and produced a dose-dependent enhancement of diazepam binding in both control and $Et_2C_2O_5$-treated preparations. The EC_{50} (i.e., the concentration that produces 50% of the maximal enhancement) of muscimol in control was 50 ± 10 nM and in $Et_2C_2O_5$-treated preparations was 60 ± 8 nM ($n = 3$). We also analyzed the effect of muscimol on the kinetic constants of diazepam binding in control and $Et_2C_2O_5$-treated preparations. Muscimol (10 μM) changed the K_d of diazepam in control from 7.3 ± 0.32 nM to 2.1 ± 0.32 nM ($n = 2$) without significantly altering B_{max} (Fig. 3A); diazepam Scatchard plots obtained in the presence of 10 μM muscimol in control and in $Et_2C_2O_5$-treated preparations are shown in Fig. 3B. The K_d of diazepam in muscimol-treated control was 2.1 ± 0.32 nM and in $Et_2C_2O_5$-treated preparations was 2.3 ± 0.22 nM ($n = 2$). The B_{max} in $Et_2C_2O_5$-treated preparations was 65 ± 4% of the corresponding control value. Mus-

Table 2. Effect of $Et_2C_2O_5$ on kinetic constants of [^{3}H]diazepam binding

	K_d, nM	B_{max}, fmol/mg of protein
Control	7.21 ± 0.23	1543 ± 116
$Et_2C_2O_5$ treated	6.67 ± 0.92	789 ± 79*

Control and $Et_2C_2O_5$-treated tissue was prepared and assayed as described in the legend to Table 1 and *Materials and Methods*. [^{3}H]Diazepam was 0.2–25 nM. The binding data were analyzed by Scatchard analysis (Fig. 2). The K_d and B_{max} values were obtained by linear regression of the Scatchard data. Values represent mean ± SD of four experiments each done in triplicate.
* $P < 0.005$ when compared with control.

Table 3. Effect of $Et_2C_2O_5$ on muscimol enhancement of [^{3}H]diazepam binding

	Specific binding, fmol/mg of protein	Enhancement, fmol
Buffer		
Control	172.6 ± 5.3	—
+ 0.1 μM muscimol	242.5 ± 4.7	69.9
$Et_2C_2O_5$ treated		
Control	117.4 ± 3.9	—
+ 0.1 μM muscimol	190.8 ± 6.5	73.4

Control of $Et_2C_2O_5$-treated tissue was incubated with 1 nM [^{3}H]diazepam alone and in the presence of 0.1 μM muscimol. Values represent mean ± SD of one experiment done in triplicate. These experiments were replicated five times with similar results.

Table 4. Effect of $Et_2C_2O_5$ on (±)-pentobarbital enhancement of [^{3}H]diazepam binding

	Specific binding, fmol/mg of protein	Enhancement, fmol
Buffer		
Control	152.5 ± 5.3	—
+ 200 μM (±)-pentobarbital	227.6 ± 6.9	75.1
+ 1000 μM (±)-pentobarbital	278.4 ± 6.1	125.9
$Et_2C_2O_5$ treated		
Control	99.6 ± 3.7	—
+ 200 μM (±)-pentobarbital	179.5 ± 4.2	79.9
+ 1000 μM (±)-pentobarbital	216.9 ± 5.3	117.3

Control of $Et_2C_2O_5$-treated tissue was incubated with 1 nM [^{3}H]diazepam alone and in the presence of 100 μM (±)-pentobarbital. Values represent mean ± SD of one experiment done in triplicate. These experiments were replicated five times with similar results.

cimol, by itself, did not alter the B_{max} of diazepam binding significantly.

Like muscimol, pentobarbital also enhances diazepam binding by increasing the affinity of diazepam (14, 15). The effect of pentobarbital on diazepam binding is summarized in Table 4. Thus, 200 μM and 1000 μM pentobarbital produced enhancement of diazepam binding, in both control and $Et_2C_2O_5$-treated preparations. The EC_{50} for pentobarbital enhancement of diazepam binding in the control was 88 ± 20 μM and that in $Et_2C_2O_5$-treated preparation was 102 ± 15 μM (n = 4). Fig. 4*A* shows the effect of 500 μM pentobarbital on diazepam binding in control, and Fig. 4*B* shows the Scatchard plots obtained in the presence of 500 μM pentobarbital in control and in $Et_2C_2O_5$-treated preparation. Pentobarbital changed the K_d from 7.3 ± 0.23 nM to 3.65 ± 0.41 nM in the control and from 6.7 ± 0.92 nM to 4.01 ± 0.37 nM in the $Et_2C_2O_5$-treated preparation (n = 2).

DISCUSSION

Benzodiazepines produce a variety of effects in the central nervous system, including antianxiety, anticonvulsant, muscle relaxant, and sedative hypnotic. Benzodiazepines could mediate these effects by acting through a single or multiple receptor(s). Recent studies have shown that benzodiazepine binding sites are intimately associated with the GABA receptor–ionophore system (3–5, 18). Benzodiazepines have been reported to potentiate GABA-mediated responses (4, 23), and these effects are blocked by picrotoxin and bicuculline (4, 23). Furthermore, GABA agonists (3, 14), pentobarbital, (14, 15), and ethanol (16) have been reported to increase the affinity of diazepam to its binding sites. The ability of anions (but not cations, including Na^+) to enhance diazepam binding has led to the speculation that benzodiazepine sites are closely associated with the GABA receptor-regulated chloride ionophores (18).

We have investigated the effect of histidine modification on diazepam binding and its enhancement by muscimol and pentobarbital. Histidine modification was carried out with $Et_2C_2O_5$ at pH 6.0 and 0°C. Although $Et_2C_2O_5$ reacts with many nucleophiles at pH >7.0, it is reasonably specific for the imidazole ring of histidine at pH 6.0, resulting in the formation of *N*-ethyloxyformyl-histidine (20–22).

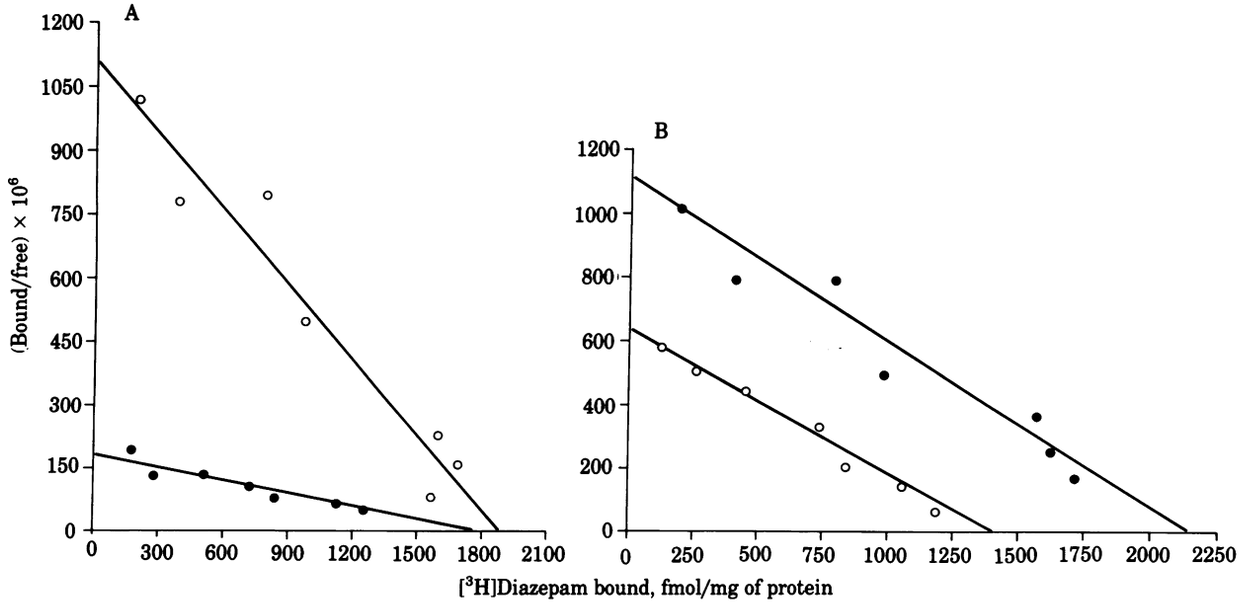

FIG. 3. (*A*) Scatchard plot of [^{3}H]diazepam (0.25–25 nM) binding to rat brain membranes in control (●) and in the presence of 10 μM muscimol (○). The K_d for control was 9.1 nM and that in the presence of 10 μM muscimol was 2.1 nM. (*B*) Scatchard plot of [^{3}H]diazepam binding in the presence of 10 μM muscimol in control (●) and in 1 mM $Et_2C_2O_5$-treated (○) rat brain membranes. The K_d in muscimol-treated control was 2.1 nM and that in the presence of muscimol in $Et_2C_2O_5$-treated membranes was 2.3 nM. The B_{max} in the $Et_2C_2O_5$-treated preparation was 67% of the control value.

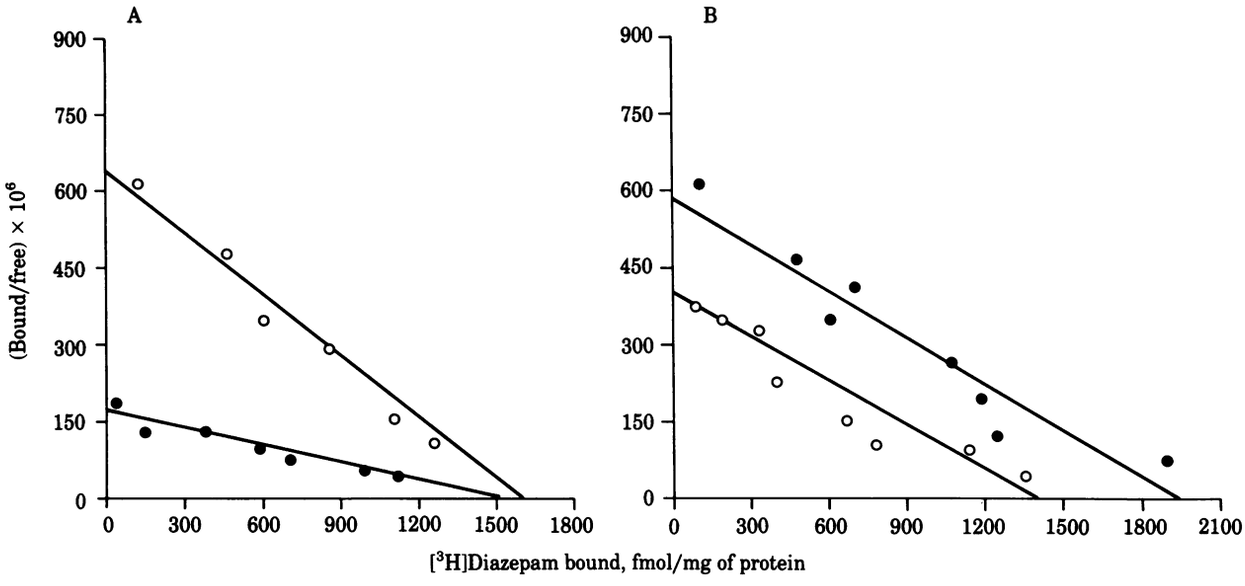

FIG. 4. (*A*) Scatchard plot of [^{3}H]diazepam binding in control (●) and in the presence of 500 μM pentobarbital (○). The K_d in control was 9.4 nM and that in the presence of 500 μM pentobarbital was 3.4 nM. B_{max} was not significantly different in the two groups. (*B*) Scatchard plot of [^{3}H]diazepam binding in the presence of 500 μM pentobarbital in control (●) and in 1 mM $Et_2C_2O_5$-treated preparation (○). The K_d in the pentobarbital-treated control was 3.4 nM and that in the presence of pentobarbital in the $Et_2C_2O_5$-treated preparation was 4.1 nM. The B_{max} in the $Et_2C_2O_5$-treated group was 70% of the control value.

Our results indicate that histidine modification with $Et_2C_2O_5$ results in a dose-dependent decrease in specific diazepam binding (Fig. 1). This decrease is due to a reduction in the number of diazepam binding sites (Fig. 2). It is interesting that, although 1 mM $Et_2C_2O_5$ produced a 40 ± 5% decrease, higher concentrations did not produce any further decrease. The results suggest that a histidine residue is closely associated with the benzodiazepine binding sites. However, inability of $Et_2C_2O_5$ to block all the diazepam binding sites suggests that histidine function may be critical for only a part (40–45%) of the diazepam binding sites. This is an interesting finding, as heterogeneity of benzodiazepine binding sites has been demonstrated (24).

GABA agonists and barbiturates such as pentobarbital (3, 14, 15) produce a dose-related increase in diazepam binding and appear to act at different sites at the benzodiazepine–GABA-receptor–ionophore complex. To further characterize the involvement of histidine containing diazepam receptor sites at this complex, we investigated the effect of $Et_2C_2O_5$ treatment on the ability of muscimol and pentobarbital to enhance diazepam binding. We found that even when 40% of the diazepam binding sites were blocked, the ability of muscimol (Table 3 and Fig. 3) and pentobarbital (Table 4 and Fig. 4) to enhance diazepam binding was not altered. The ED_{50} values of muscimol and pentobarbital in control and in $Et_2C_2O_5$-treated preparations were similar. Furthermore, these ligands increased the affinity (without altering the B_{max}) of diazepam to the same extent in control and in $Et_2C_2O_5$-treated preparations. These results suggest that the histidine residue is not at the level of the GABA recognition site or the pentobarbital site. Our previous studies have shown that barbiturates, including pentobarbital, inhibit potently the binding of the GABA antagonist α-dihydropicrotoxinin to rat brain membranes (25, 26) and to solubilized receptors (27, 28). Furthermore, picrotoxinin prevents pentobarbital enhancement of diazepam binding (14, 15). $Et_2C_2O_5$-treatment has no effect on the binding of α-dihydropicrotoxinin (29). Inability of $Et_2C_2O_5$ treatment to alter pentobarbital enhancement of diazepam binding or to inhibit α-dihydropicrotoxinin binding is consistent with the finding that pentobarbital may act at the level of the picrotoxinin site at the benzodiazepine–GABA-receptor–ionophore complex. Neurophysiological observations also support this hypothesis (30).

In summary, these results suggest that not all benzodiazepine receptors are functionally coupled to the GABA receptor–ionophore complex. At least two classes of benzodiazepine binding sites are suggested—one for which a histidine residue appears critical that is not coupled to the GABA receptor system and a second that is not modified by $Et_2C_2O_5$ but is coupled.

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