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# Group-Selective Reagent Modification of the Benzodiazepine-γ-Aminobutyric Acid Receptor-Ionophore Complex Reveals that Low-Affinity γ-Aminobutyric Acid Receptors Stimulate Benzodiazepine Binding

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SUMMARY

The modification of membrane proteins with diethylpyrocarbonate (DEP) and diazotized sulfanilate was investigated on the binding of three benzodiazepine radioligands in three brain regions. Both of these reagents produced a dose-dependent inactivation of [$^3$H]diazepam, [$^3$H]flunitrazepam, and [$^3$H]propyl β-carboline-3-carboxylate binding to cortex, cerebellum, and hippocampus. Both DEP and diazotized sulfanilate decrease the $B_{max}$ of the benzodiazepine binding sites without altering the $K_D$. The ability of muscimol and pentobarbital to enhance [$^3$H]diazepam binding was not altered by DEP pretreatment in any of the three regions. Scatchard analysis indicated that, following the inactivation of 40–50% of [$^3$H]diazepam binding by 1 mM DEP, pentobarbital and muscimol were still able to increase the affinity of [$^3$H]diazepam binding in cortex, cerebellum, and hippocampus. In contrast, diazotized sulfanilate pretreatment abolishes the ability of muscimol and pentobarbital to enhance [$^3$H]diazepam binding in these three regions. The effects of these reagents on [$^3$H]γ-aminobutyric acid (GABA) binding revealed that sulfanilate but not DEP eliminates the low-affinity GABA receptor sites in cortex and cerebellum. Thus, while both DEP and sulfanilate inactivate benzodiazepine binding sites, only sulfanilate abolishes the low-affinity GABA binding sites and the ability of the GABA agonists to enhance [$^3$H]diazepam binding. These results suggest that the stimulation of benzodiazepine binding appears to be mediated by the low-affinity GABA receptors.

## INTRODUCTION

Specific, high-affinity binding sites for radiolabeled benzodiazepine ligands have provided useful information for the identification and characterization of benzodiazepine binding sites in the mammalian central nervous system (1–4). Several lines of evidence obtained by electrophysiological (5, 6) and pharmacological (3, 4, 7) experiments strongly suggest that benzodiazepines appear to mediate most, if not all, effects by GABAergic[3] mechanisms. The benzodiazepine-GABA receptor-ionophore complex appears to be an oligomeric structure with multiple interacting components. Three components of this complex have been well characterized by using [$^3$H]GABA agonists (8, 9), [$^3$H]benzodiazepine ligands (1, 2), and [$^3$H]DHP; (10, 11) as probes. Furthermore, the interactions between benzodiazepines and GABA (12), GABA and benzodiazepines (3, 7), and barbiturates and benzodiazepines (13–15) and GABA (16, 17) have been demonstrated. In addition, several other ligands appear to modulate one or more of these sites *in vitro* (e.g., ref. 4). GABA is known to bind to a high- and a low-affinity site (4, 8, 9). Recent studies have indicated that the GABA enhancement of benzodiazepine binding occurs at concentrations which may correlate most closely to its binding to the low-affinity site (3, 7, 18). However, interactions between benzodiazepines and the high-affinity GABA site have also been demonstrated (12).

To characterize further the benzodiazepine binding site and its relationship to GABA receptors, we have investigated the effect of two protein-modifying reagents (diethyl pyrocarbonate and diazotized sulfanilate) on the binding of three benzodiazepine ligands in three brain regions. DEP is not a highly selective reagent, but under appropriate conditions, it reacts primarily with histidine groups, resulting in the formation of an *N*-carbethoxy-histidyl derivative (19–24). Diazotized sulfanilic acid is

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[3] The abbreviations used are: GABA, γ-aminobutyric acid; DHP, α-dihydropicrotoxinin; DEP, diethylpyrocarbonate; PrCC, β-carboline-3-carboxylate.

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known to react with histidine and tyrosine residues of proteins and is a less selective reagent than DEP (21, 22). Furthermore, to understand better the interaction of GABA and pentobarbital sites with the benzodiazepine binding sites, we have also investigated the effect of these reagents on GABA and pentobarbital enhancement of [$^3$H]diazepam binding and on [$^3$H]GABA binding. We have previously reported that DEP (up to 2 mM) partially inactivated (40–50%) [$^3$H]diazepam binding to whole brain membranes, and speculated about the heterogeneity of benzodiazepine binding sites (25).

## MATERIALS AND METHODS

[$^3$H]Diazepam, [$^3$H]flunitrazepam, [$^3$H]PrCC, and [$^3$H]GABA were obtained from New England Nuclear Corporation (Boston, Mass.). Diazotized sulfanilate was obtained from Calbiochem (La Jolla, Calif.). Diethyl pyrocarbonate and other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.).

*Tissue preparation.* Male Sprague-Dawley rats (125–200 g) were decapitated and their brains were rapidly removed and placed in 0.32 M sucrose. Cerebral cortex, cerebellum, and hippocampus were dissected and pooled when necessary. For benzodiazepine binding studies, these regions were homogenized in 30 volumes of 0.32 M sucrose, using a Teflon-glass homogenizer, and centrifuged at 1,000 × *g* for 10 min. The supernatant was centrifuged at 140,000 × *g* for 30 min to obtain the $P_2$ + $P_3$ fraction. The pellet ($P_2$ + $P_3$) was disrupted in 30 volumes of ice-cold double-distilled water with a Brinkmann Polytron with two 5-sec bursts, 10 sec apart, at half-setting. The homogenate was centrifuged at 140,000 × *g* for 30 min. The pellet was resuspended in 30 volumes of buffer containing 0.2 M NaCl/10 mM sodium phosphate (pH 7.0) and pelleted. The tissue was washed four times by resuspension and centrifugation in the above buffer. Following extensive washing, the tissue was resuspended at a protein concentration of 0.2–0.5 mg/ml for the binding studies. All experiments (except hydroxylamine reversal) were conducted on freshly prepared tissue.

For GABA binding studies, tissue was prepared by a modification of the previously described method (26). Briefly stated, the mitochondrial plus microsomal ($P_2$ + $P_3$) fraction was subjected to osmotic shock, using the Polytron, and centrifuged at 140,000 × *g* for 30 min. The pellet was homogenized in ice-cold water, using a glass homogenizer, and the homogenate was pelleted. The pellet was resuspended in 0.05 M Tris-HCl buffer (pH 7.1) and frozen overnight. The pellet was thawed at room temperature, centrifuged, washed twice with the buffer, and frozen. On the day of the assay, the tissue was thawed, pelleted, washed twice by resuspension and centrifugation, and resuspended in the Tris-HCl buffer at a protein concentration of 0.6–0.8 mg/ml for binding assays. Prior to the binding assays, 0.05 M KCl was added to the tissue homogenate.

*DEP treatment.* DEP was stored at 4° and a stock solution was made in the buffer by rapidly vortexing just prior to the experiment. Care was exercised while using DEP since it can hydrolyze rapidly (20). Initial experiments revealed that preparing stock solutions of DEP just prior to its addition to incubation medium in either ethanol or buffer gave similar results.

For benzodiazepine binding studies, fresh, extensively washed membranes were resuspended in pH 6.0 buffer (200 mM NaCl/10 mM sodium phosphate) and incubated with various concentrations of DEP (0.25–10 mM) for 20 min at 0°. DEP modification of the membrane was carried out at pH 6.0, since carbethoxylation of histidine appears to be specific and stable at this pH (19–22). Furthermore, buffers which can act as nucleophiles (e.g., Tris) were avoided for DEP modification experiments (20). Following the incubation with DEP, the tissue was pelleted by centrifugation to remove excess DEP. The pellet was resuspended in the pH 7.0 buffer (200 mM NaCl/10 mM sodium phosphate) and used for the binding studies, as described below. Control tissue was treated in an identical manner with pH 6.0 buffer, but without DEP. For DEP treatment of GABA binding, freeze-thawed and extensively washed tissue was resuspended in Tris-free buffer (0.05 M KCl/10 mM potassium phosphate, pH 6.0) and treated with various concentrations of DEP as described above for the benzodiazepine binding. Following the DEP pretreatment, the tissue was resuspended in 0.05 M Tris-HCl (pH 7.1)/0.05 M KCl and used for the binding studies.

*Diazotized sulfanilic acid treatment.* Membrane suspensions in pH 7.0 buffer were incubated with various concentrations (0.25–10 mM) of diazotized sulfanilate for 30 min at 37° in the dark. The membrane suspension was then centrifuged to remove excess sulfanilate, and the pellet was resuspended in the pH 7.0 buffer at the appropriate protein concentration for the binding studies. Diazotized sulfanilate solution was made just prior to its addition to the tissue homogenate.

*Reversal of carbethoxylation by hydroxylamine.* Control and 1 mM DEP-treated membranes were placed in washed dialysis sacs and dialyzed for various times against 2.0 M hydroxylamine in pH 7.0 buffer at 0–4° prior to the binding studies.

*Binding studies.* Routinely, aliquots of control and pretreated membrane preparations were incubated with [$^3$H]diazepam (1 nM; 30 min), [$^3$H]flunitrazepam (0.3 nM; 90 min), or [$^3$H]PrCC (0.3 nM; 60 min) in a total incubation volume of 1.0 ml. The nonspecific binding was determined in the presence of $10^{-5}$ M nonradioactive diazepam. Following incubation, triplicate 250-μl aliquots were filtered over GF/B filters under vacuum and twice washed rapidly with ice-cold buffer (5 ml each time). The filters were dried and the radioactivity was determined in toluene/2,5-diphenyloxazole/BBS (Bio-Solv 3; Beckman) scintillation cocktail, as described (14, 25). The counting efficiency, determined with [$^3$H]toluene, was 40% ± 2%.

For Scatchard analysis, the concentration of [$^3$H]diazepam was varied between 0.2 nM and 20 nM. The $K_D$ and $B_{max}$ values were obtained by the linear regression of the Scatchard data. The $EC_{50}$ values (i.e., the concentration that produces 50% enhancement over the basal value) for muscimol and pentobarbital were determined in control and DEP-treated membranes. Six concentrations of muscimol ($10^{-9}$–$10^{-4}$ M) and pentobarbital ($10^{-6}$–$10^{-3}$ M) were incubated with 1 nM [$^3$H]diazepam for 30 min to deter-

mine the $EC_{50}$ value. The effect of sulfanilate on benzodiazepine binding and its enhancement was determined routinely using 1 nM [$^3$H]diazepam.

[$^3$H]GABA binding was measured by a centrifugation assay, as described previously (26). Briefly stated, aliquots of control and pretreated membrane suspension were incubated with [$^3$H]GABA (4 nM) in the presence and absence of excess nonradioactive GABA (100 μM) in a total incubation volume of 1.0 ml for 10 min. Following incubation, the vials were centrifuged at 48,000 × *g* for 10 min. The vials were washed once the contents were solubilized, and the radioactivity was determined as described (26). For Scatchard plots, the concentrations of [$^3$H]GABA were varied up to 4 nM; concentrations between 4 nM and 1004 nM were subjected to radioisotopic dilution. Routinely, 13 concentrations of [$^3$H]GABA were utilized for a Scatchard plot. Nonspecific binding obtained in the presence of 100 μM GABA was subtracted from the total binding to obtain the specific binding. Protein was estimated by the method of Lowry *et al.* (27). The statistical significance of the results was calculated by using Student's *t*-test.

## RESULTS

*Inactivation of benzodiazepine binding by DEP and diazotized sulfanilate.* The effect of pretreatment with various concentrations of DEP and diazotized sulfanilate on the binding of ligands which bind to benzodiazepine binding sites was investigated in cortex, cerebellum, and hippocampus. Besides [$^3$H]diazepam and [$^3$H]flunitrazepam, we also used [$^3$H]PrCC, since, under certain conditions, this radioligand has been reported to bind to a subclass of benzodiazepine receptors (28).

Figure 1A and B shows that increasing concentrations of diazotized sulfanilate and DEP ranging from 0.25 mM to 10 mM produce a dose-dependent inhibition of [$^3$H]diazepam (●), [$^3$H]flunitrazepam (○), and [$^3$H]PrCC (△) binding to cerebral cortex (Fig. 1A) and cerebellum (Fig. 1B). Both of these reagents were able to cause complete inactivation of all three radioligands in these regions. DEP at concentrations of 1–1.5 mM produced inactivation of 50% of the binding sites for three ligands in cortex and cerebellum. Diazotized sulfanilate was slightly more potent than DEP, producing 50% inhibition of the binding at 0.5–1.0 mM. Similar results were obtained in hippocampus (data not shown).

*Effect of muscimol and pentobarbital on [$^3$H]diazepam binding in control and DEP-pretreated regions.*

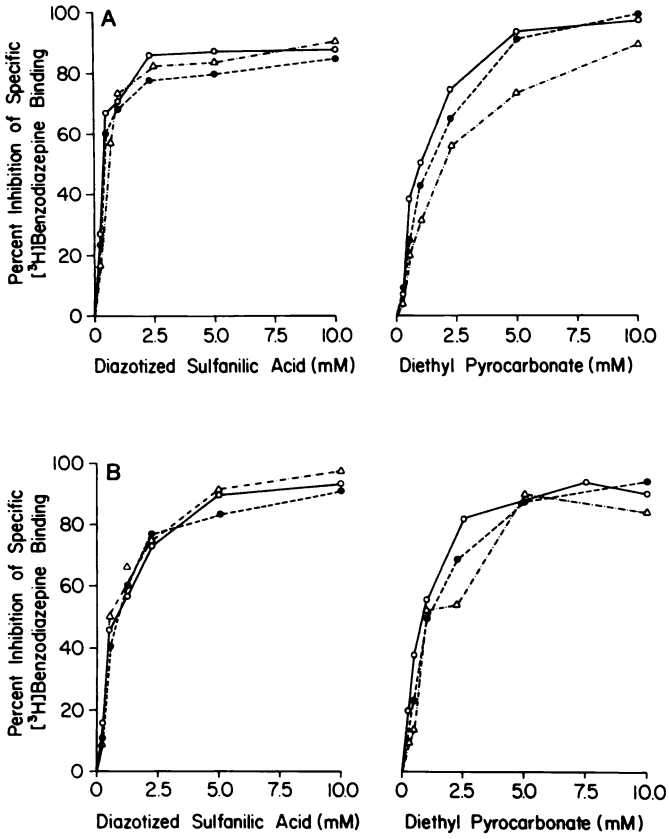


FIG. 1. *Effect of diazotized sulfanilate and DEP on benzodiazepine binding to cerebral cortex (A) and cerebellum (B)*

The membrane suspensions were pretreated with various concentrations of sulfanilate (pH 7.0) or DEP (pH 6.0), as described under Materials and Methods. The excess reagent was removed by centrifugation and resuspension in pH 7.0 buffer. Aliquots of control or DEP- or sulfanilate-pretreated membrane homogenates were incubated with 1 nM [$^3$H]diazepam for 30 min (●), 0.3 nM [$^3$H]flunitrazepam for 90 min (○), or 0.3 nM [$^3$H]PrCC for 60 min (△). Nonspecific binding obtained in the presence of $10^{-5}$ diazepam was subtracted from the total binding. Each point represents the mean value of at least three experiments, each performed in triplicate.

Table 1 shows the effect of the GABA agonists muscimol ($10^{-5}$ M) and pentobarbital ($10^{-3}$ M) that produce maximal enhancement of [$^3$H]diazepam binding (14) in control and DEP-pretreated brain regions. Both muscimol ($10^{-5}$ M) and pentobarbital ($10^{-3}$ M) produced relatively greater enhancement of [$^3$H]diazepam binding in control cortex and hippocampus, relative to cerebellum. Table 1 also

TABLE 1

*Muscimol and pentobarbital enhancement of [$^3$H]diazepam binding in control and DEP-pretreated regions*

The homogenates of brain regions were incubated with 200 mM NaCl, 10 mM sodium phosphate (pH 6.0), or the same buffer plus 1 mM DEP for 20 min at 0–4°. Following the treatment, the homogenates were pelleted and resuspended in pH 7.0 buffer for the binding studies. For binding studies, the control and treated membranes were incubated with 1 nM [$^3$H]diazepam for 30 min 0–4° in the absence and in the presence of muscimol or pentobarbital. The values are the percentage enhancement over the basal binding. DEP (1 mM) decreased the basal binding in these regions by 40–60%. Results are the mean ± standard deviation of the number of experiments indicated in parentheses, each performed in triplicate.

| | % Enhancement over basal binding | | | | | |
|---|---|---|---|---|---|---|
| | Cortex | | Cerebellum | | Hippocampus | |
| | Control | DEP-pretreated | Control | DEP-pretreated | Control | DEP-pretreated |
| Muscimol, $10^{-5}$ M | 99 ± 12.5 (8) | 122.0 ± 35.5 (5) | 64.0 ± 10.3 (8) | 58 ± 8.0 (3) | 100.8 ± 14.7 (8) | 110 ± 19.8 (6) |
| Pentobarbital, $10^{-3}$ M | 70 ± 12.6 (7) | 66.2 ± 8.5 (6) | 40.1 ± 6.3 (7) | 52 ± 5.6 (3) | 61.3 ± 9.3 (5) | 63 ± 8.5 (3) |

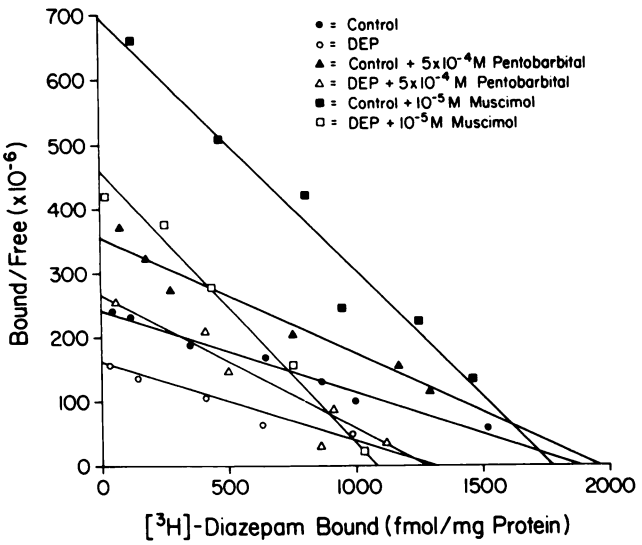


FIG. 2. *Typical Scatchard plots of [³H]diazepam binding in control and DEP-pretreated (1 mM) cortical membranes in the absence and in the presence of $10^{-5}$ M muscimol or $5 \times 10^{-4}$ M pentobarbital*
The results are summarized in Table 2.

shows that, following the treatment of these regions with 1 mM DEP (which inactivates about 40–50% of the binding sites), muscimol and pentobarbital were still able to enhance [³H]diazepam binding to the same extent as the controls. Furthermore, partial inactivation of cerebral cortex, cerebellum, or hippocampus membrane with DEP (1 mM) did not alter the $EC_{50}$ values of either muscimol or pentobarbital in any of the three regions (data not shown).

*Effect of DEP on the binding constants of [³H]diazepam binding.* Figure 2 shows the effect of muscimol ($10^{-5}$ M) and pentobarbital ($5 \times 10^{-4}$ M) on the [³H]diazepam Scatchard plots in control and DEP-pretreated cerebral cortex. It is apparent from Fig. 2 that DEP pretreatment decreased the $B_{max}$ of [³H]diazepam binding and did not alter the $K_D$ values. Similar results were obtained using cerebellum and hippocampus membranes (data not shown). Pentobarbital and muscimol increased the affinity of [³H]diazepam binding in both control and DEP-pretreated regions.

Table 2 summarizes the binding constants of [³H]diazepam in control and DEP-pretreated cerebral cortex and cerebellum and the effect of muscimol and pentobarbital on these constants. DEP (1 mM) decreased the $B_{max}$ of [³H]diazepam in cortex by 49% and in cerebellum by 46% without altering the $K_D$ of diazepam (Table 2). In both regions, pentobarbital ($5 \times 10^{-4}$ M) and muscimol ($10^{-5}$ M) increased the affinity of [³H]diazepam (decreasing the $K_D$ values) in the control. Although the $B_{max}$ of [³H]diazepam was lower by ~50% following the DEP pretreatment, pentobarbital and muscimol were still able to increase the affinity of diazepam in DEP-pretreated brain regions. These results indicate that the sites that are not modified by the DEP pretreatment are still able to interact with the GABA receptor and pentobarbital-sensitive sites of the benzodiazepine-GABA receptor-ionophore complex. Similar results were obtained in hippocampus (data not shown). It is apparent that, in all three regions, muscimol and pentobarbital increase the affinity of [³H]diazepam for its receptor sites. Similar results were obtained using [³H]flunitrazepam as the radioligand (data not shown).

*Reversal of carboxymethylation by hydroxylamine.* To determine whether DEP inhibition of benzodiazepine binding is due to carboxymethylation, we investigated the hydroxylamine reversal of this interaction. Control and DEP-pretreated cortical membranes were dialyzed against buffer (pH 7.0) containing 2.0 M hydroxylamine for various times. Following the hydroxylamine treatment, the tissue was pelleted and used for [³H]diazepam binding. Figure 3 shows that partially inhibited [³H]diazepam binding was reversed by dialyzing against hydroxylamine for 2 hr.

*Effect of diazotized sulfanilate on benzodiazepine binding and its enhancement.* Figure 4 shows typical Scatchard plots obtained in cerebral cortex following the pretreatment with sulfanilate. Like DEP, sulfanilate produced a dose-related decrease in the $B_{max}$ of [³H]diazepam binding without altering the $K_D$.

TABLE 2

*Effects of DEP pretreatment on the binding constants of [³H]diazepam to cerebral cortex and cerebellum*

Scatchard plots of [³H]diazepam binding to cerebral cortex in control and DEP-pretreated membranes were determined in the absence and in the presence of pentobarbital or muscimol. $K_D$ and $B_{max}$ values were obtained by the linear regression of the binding isotherms. Binding assays were performed as described in Table 1, using [³H]diazepam concentrations between 0.2 and 20 nM. The values represent the mean ± standard deviation of the number of experiments (each performed in triplicate) indicated in parentheses. Typical [³H] diazepam Schatchard plots obtained are described in Fig. 2.

| | Specific [³H]diazepam binding | | | |
|---|---|---|---|---|
| | Cortex | | Cerebellum | |
| | $K_D$ | $B_{max}$ | $K_D$ | $B_{max}$ |
| | *nM* | *fmoles/mg protein* | *nM* | *fmoles/mg protein* |
| Control | 9.22 ± 1.60 (4) | 1802 ± 188 | 9.53 ± 1.12 (2) | 788 ± 104 |
| + 1 mM DEP | 8.96 ± 0.72 (3) | 929 ± 028[a] | 10.40 ± 1.56 (2) | 445 ± 96[b] |
| + $5 \times 10^{-4}$ M pentobarbital | 5.11 ± 0.34[a] (3) | 1826 ± 103 | 3.04 ± 0.62[a] (3) | 736 ± 98 |
| + $10^{-5}$ M muscimol | 2.78 ± 0.61[a] (2) | 1702 ± 45 | 2.93 ± 0.77[a] (2) | 653 ± 95 |
| + 1 mM DEP + $5 \times 10^{-4}$ M pentobarbital | 4.97 ± 0.44[c] (3) | 1299 ± 125 | 2.40 ± 0.42[c] (2) | 369 ± 78 |
| + 1 mM DEP + $10^{-5}$ M muscimol | 3.50 ± 0.56[c] (2) | 1066 ± 225 | 2.66 ± 0.89[c] (2) | 455 ± 64 |

[a] $p > 0.005$ as compared with control.
[b] $p < 0.002$ as compared with control.
[c] $p > 0.005$ as compared with DEP control.

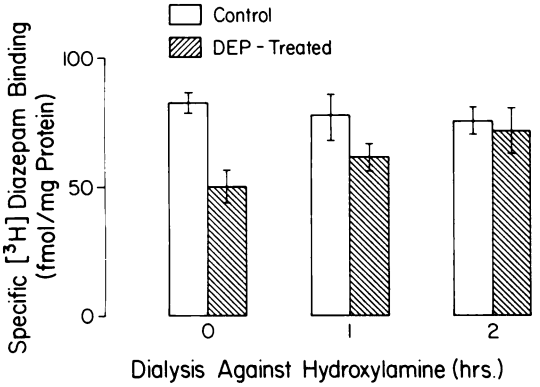


FIG. 3. *Hydroxylamine reversal of DEP inactivation of [$^3$H]diazepam binding to cortex*

Control or DEP-pretreated (1 mM) cortical membranes were placed in washed dialysis sacs and dialyzed against 2 M hydroxylamine in pH 7.0 buffer for various times. The binding of [$^3$H]diazepam (1 nM) was measured as described under Materials and Methods at 0, 1, and 2 hr.

Table 3 summarizes the effect of sulfanilate on the binding constants of [$^3$H]diazepam. Thus, pretreatment of cerebral cortex and cerebellar membranes with 1 mM sulfanilate decreased the $B_{max}$ of [$^3$H]diazepam binding by 63% in cortex and 68% in cerebellum. Similar results were obtained using hippocampal membranes (data not shown).

Table 4 shows the effect of sulfanilate on the ability of muscimol and pentobarbital to enhance [$^3$H]diazepam binding in cortex and cerebellum. Following pretreatment of these membranes with 1 mM sulfanilate, muscimol ($10^{-5}$ M) and pentobarbital ($10^{-3}$ M) were unable to enhance [$^3$H]diazepam binding in these regions (Table 4). These results are in contrast to those obtained with DEP treatment. Thus, although both DEP and sulfanilate inhibit in a dose-related fashion the binding of ligands to the benzodiazepine binding site, only sulfanilate abolishes the muscimol and pentobarbital enhancing effect.

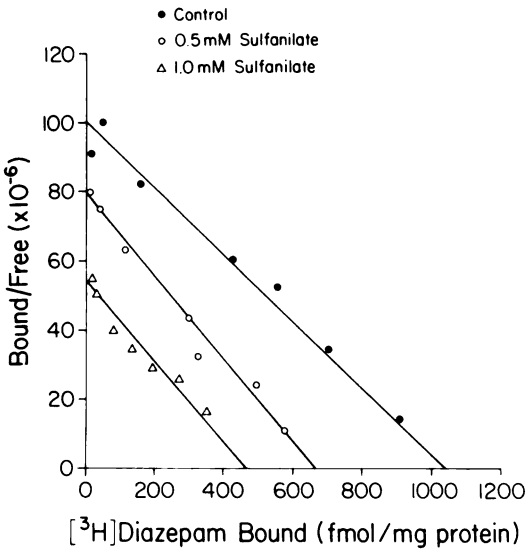


FIG. 4. *Typical Scatchard plot of [$^3$H]diazepam binding to cortex in control and following pretreatment with diazotized sulfanilate*

The experimental conditions used are described under Materials and Methods. Similar results were replicated three times.

*Effect of DEP and diazotized sulfanilate on [$^3$H] GABA binding.* Extensively washed and freeze-thawed membranes were incubated with DEP (pH 6.0) or sulfanilate (pH 7.0) and centrifuged to remove the excess reagent prior to its binding studies, as described under Materials and Methods. Using a single-point analysis (4 nM [$^3$H]GABA) and using cortical membranes, 0.5 mM sulfanilate reduced the specific [$^3$H]GABA binding from a control value of 440 ± 43.2 fmoles/mg of protein ($n$ = 4) to 234 ± 14.6 fmoles/mg of protein ($n$ = 3). At 1 mM sulfanilate, the specific binding was decreased to 125 ± 21.6 fmoles/mg of protein ($n$ = 4).

Table 5 summarizes the effect of sulfanilate on [$^3$H] GABA binding to cortex and cerebellar membranes. Sulfanilate produced a dose-related inhibition of [$^3$H]GABA binding to both cortex and cerebellum. However, 2 and 5 mM sulfanilate inhibited [$^3$H]GABA binding to the same extent. In contrast, DEP concentrations of 1–5 mM gave variable enhancement of [$^3$H]GABA binding which was not dose-related. In six experiments, we always observed either no effect or enhancement of [$^3$H]GABA binding in both cortex and cerebellum at 1–5 mM (data not shown). However, at 10 mM DEP, a slight (10–20%) inhibition of [$^3$H]GABA binding was observed.

To characterize further the effect of sulfanilate on GABA binding, we determined the effect of 1 mM sulfanilate on the binding constants of [$^3$H]GABA by Scatchard analysis. Figure 5 shows a typical Scatchard plot of [$^3$H] GABA in control cerebral cortex (●) and sulfanilate-pretreated (○) cortex. Control cerebral cortex bound [$^3$H] GABA to a high- and a low-affinity site; however, following the pretreatment with 1 mM sulfanilate, only the high-affinity [$^3$H]GABA site could be detected. Thus, 1 mM sulfanilate, which abolishes the muscimol enhancement of [$^3$H]diazepam binding, also eliminates the low-affinity [$^3$H]GABA binding site. Table 6 summarizes the effect of 1 mM sulfanilate on the binding constants of [$^3$H]GABA to cortex and cerebellum. Similar results were obtained using hippocampal membranes (data not shown).

## DISCUSSION

Our results indicate that modification of cortex, cerebellar, and hippocampal membranes with either DEP or diazotized sulfanilate results in complete inactivation of benzodiazepine binding sites. This interaction is dose-related and appears to be due to a decrease in the $B_{max}$ of the benzodiazepine binding sites, without any changes in the apparent $K_D$. In our study, besides using [$^3$H] diazepam and [$^3$H]flunitrazepam, we also used [$^3$H]PrCC for studying benzodiazepine binding. [$^3$H]PrCC was chosen since it appears to exhibit selectivity (under certain conditions) toward a subpopulation of the benzodiazepine receptors, especially in cerebellum (28). However, DEP and sulfanilate did not exhibit any selectivity in inactivating the benzodiazepine binding sites as indicated by the findings that the binding of all three radioligands was abolished in the three regions (Figs. 1 and 2). [$^3$H] PrCC binding in the three regions was measured under conditions which have been reported to show selectivity in cerebellum (28). Although we have previously reported that DEP (up to 1–2 mM) inactivated only a portion of

TABLE 3

*Effects of diazotized sulfanilate on specific [$^3$H]diazepam binding*

Membrane homogenates were incubated without or with 1 mM diazotized sulfanilate in pH 7.0 buffer at 37° for 30 min, followed by centrifugation to remove excess reagent. The pellets were resuspended in pH 7.0 buffer and assays were performed at 0–4. [$^3$H]Diazepam concentrations were varied between 0.2 and 20 nM to determine the Scatchard plots. The $K_D$ and $B_{max}$ values were obtained by the linear regression of the binding data. The results are the mean ± standard deviation of the number of experiments indicated in parentheses, each performed in triplicate. Typical Scatchard plots obtained are shown in Fig. 4.

| | Specific [$^3$H]diazepam binding | | | |
|---|---|---|---|---|
| | Cortex | | Cerebellum | |
| | $K_D$ | $B_{max}$ | $K_D$ | $B_{max}$ |
| | *nM* | *pmoles/mg protein* | *nM* | *pmoles/mg protein* |
| Control | 9.6 ± 1.9 (5) | 1.513 ± 0.33 | 10.4 ± 2.2 (3) | 1.946 ± 0.36 |
| + 1 mM sulfanilate | 8.5 ± 1.5 (3) | 0.559 ± 0.14[a] | 8.3 ± 2.5 (2) | 0.621 ± 0.22[a] |

[a] $p < 0.005$ as compared with corresponding controls.

the [$^3$H]diazepam binding sites in whole brain (25), in that study we did not use higher concentrations of DEP since it had been reported in the literature that DEP, at concentrations >1 mM, may modify groups other than histidine (23, 24). However, a thorough literature survey indicated that, in many cases, DEP at concentrations up to 10 mM was used to modify histidine function selectively (19–21). On this basis we reinvestigated the effect of DEP (up to 10 mM) on the [$^3$H]benzodiazepine binding. Furthermore, in the present study we also have used another reagent, diazotized sulfanilate, which can modify histidine and tyrosine residues. The present study shows that our earlier conclusion (25) that histidine modification reveals heterogeneity of benzodiazepine receptors is not justified.

The ability of DEP to inactivate all three benzodiazepine ligand binding sites in the three regions indicates a similar mechanism for inactivation. Thus, we were unable to distinguish the PrCC binding sites from diazepam or flunitrazepam binding sites in any of the three regions. Furthermore, the ability of hydroxylamine to reverse DEP inactivation would suggest that a critical histidine group is present at the benzodiazepine receptor protein. Several other studies have used hydroxylamine reversal of DEP inactivation to characterize the histidine groups in proteins (19, 20, 23).

It is widely accepted that the benzodiazepine-GABA receptor-ionophore complex is composed of multiple interacting components (4, 9). However, the coupling mechanisms and functional groups that may be critical for these interactions have yet to be defined. Several laboratories have demonstrated that GABA agonists (3, 4, 7, 14, 18, 30) and barbiturates (13–15, 25) enhance [$^3$H]benzodiazepine binding to the brain membranes. The second aim of the present study was to determine whether we can selectively modify, with DEP or sulfanilate, the coupling of GABA or pentobarbital binding sites with the benzodiazepine binding sites. Partial inactivation of [$^3$H]diazepam binding by 1 mM DEP did not alter the ability of either muscimol or pentobarbital to enhance [$^3$H]diazepam binding. Scatchard analysis indicated that 1 mM DEP decreases the $B_{max}$ of [$^3$H]diazepam by 40–50%; however, muscimol and pentobarbital were able to increase the affinity of [$^3$H]diazepam binding in both control and DEP-pretreated regions to the same extent as controls. These results indicate that the sites which are not modified by DEP are able to interact with the GABA and pentobarbital binding sites. Furthermore, neither the percentage enhancement nor the $EC_{50}$ values of muscimol or pentobarbital were altered by DEP pretreatment.

Like DEP, sulfanilate did not alter the apparent $K_D$

TABLE 4

*Effect of diazotized sulfanilic acid on [$^3$H]diazepam binding*

Control and diazotized sulfanilate-pretreated membranes were incubated with 1 nM [$^3$H]diazepam for 30 min at 0–4° in the presence and in the absence of muscimol ($10^{-5}$ M) or pentobarbital ($10^{-3}$ M). Basal values obtained in absence of these ligands are represented as 100%. In a typical experiment, 1 mM sulfanilate pretreatment decreased the basal [$^3$H]diazepam binding by 60% ± 12%. The results are the mean ± standard deviation of the number of experiments indicated in parentheses.

| | Specific [$^3$H]diazepam binding | |
|---|---|---|
| | Cortex | Cerebellum |
| | *% of control* | |
| Control | 100 | 100 |
| + $10^{-5}$ M muscimol (4) | 185 ± 10 | 214 ± 20 |
| + $10^{-3}$ M pentobarbital (4) | 158 ± 9 | 145 ± 9 |
| Diazotized sulfanilate (1 mM) | 100 | 100 |
| + $10^{-5}$ M muscimol (5) | 104 ± 8 | 102 ± 6 |
| + $10^{-3}$ M pentobarbital (4) | 88 ± 10 | 90 ± 6 |

TABLE 5

*Inhibition of specific [$^3$H]GABA binding by diazotized sulfanilate*

Freeze-thawed and extensively washed membranes were incubated without or with various concentrations of diazotized sulfanilate for 30 min at 37°, followed by centrifugation. [$^3$H]GABA binding was performed at 0–4° using 4 nM [$^3$H]GABA. The results are the mean ± standard deviation of the number of experiments indicated in parentheses, each performed in triplicate.

| Sulfanilate | Inhibition of specific [$^3$H]GABA binding | |
|---|---|---|
| | Cortex | Cerebellum |
| *mM* | % | % |
| 0.25 | 38 ± 6 (2) | 47 ± 7 (2) |
| 1.00 | 73 ± 11 (3) | 68 ± 15 (3) |
| 2.00 | 79 ± 15 (4) | 83 ± 8 (3) |
| 5.0 | 87 ± 9 (3) | 85 ± 5 (4) |

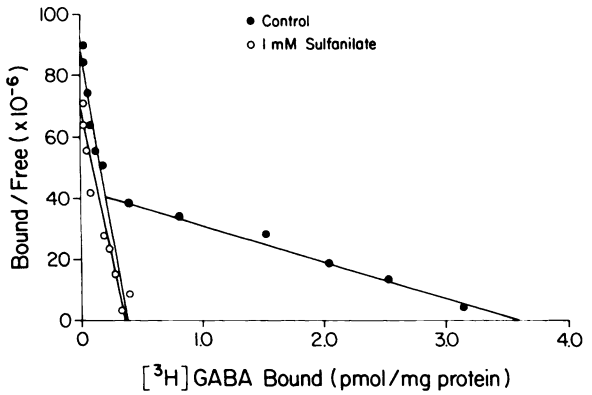


FIG. 5. *Typical Scatchard plots of [³H]GABA binding to control cerebral cortex (●) and following pretreatment with 1 mM diazotized sulfanilate (○)*

[³H]GABA was varied between 0.25 and 1004 nM, as described under Materials and Methods. The $K_D$ and $B_{max}$ values are summarized in Table 6.

but decreased the $B_{max}$ of benzodiazepine binding sites. However, sulfanilate pretreatment of the three brain regions differed from the DEP pretreatment since, following partial inactivation with sulfanilate, muscimol and pentobarbital failed to enhance the [³H]diazepam binding in any of the three regions. Thus, although both DEP and sulfanilate pretreatments inactivated [³H]benzodiazepine binding, only sulfanilate prevents the ability of muscimol and pentobarbital to interact with the [³H] diazepam binding sites. Similar results were obtained using [³H]flunitrazepam as the radioligand (data not shown). Since sulfanilate did not change the apparent conformation of benzodiazepine binding sites (i.e., no change in the $K_D$ values), it either (a) alters the coupling mechanism or (b) interferes with the GABA and pentobarbital binding sites. Since the exact coupling mechanism(s) between GABA and benzodiazepine sites have yet to be identified and defined, we investigated the effect of sulfanilate on [³H]GABA binding. It must be recognized that GABA and benzodiazepine binding experiments were not carried out with identical tissue preparations. Our studies indicate that pretreatment with sulfanilate (1 mM), which prevents the muscimol enhancement of [³H]diazepam binding, also inhibits [³H] GABA binding. Scatchard analysis indicated that sulfanilate (1 mM) pretreatment selectively eliminates the low-affinity [³H]GABA binding sites in both cortex and cerebellum. Elimination of the low-affinity GABA binding sites by sulfanilate and the inability of GABA agonists such as muscimol to enhance [³H]diazepam binding following sulfanilate pretreatment strongly suggest that the interaction of benzodiazepine binding sites with GABA occurs via the low-affinity GABA receptors. This would also be consistent with the concentrations of muscimol that produce 50% enhancement of [³H]diazepam binding (3, 7, 14, 18). These results are also in agreement with recent studies with thiocyanate (31), sulfhydryl reagents (18), protection and heat inactivation experiments (32), and diazepam enhancement of low-affinity GABA receptors (33), which also support the notion that the low-affinity GABA receptors stimulate benzodiazepine binding.

Since sulfanilate pretreatment also abolishes pentobarbital and GABA enhancement of [³H]diazepam binding, it is possible that it may also modify the [³H]DHP binding sites, since pentobarbital apparently acts via the DHP-sensitive site of the benzodiazepine-GABA receptor-ionophore complex (34, 35). The possibility that sulfanilate modification of low-affinity GABA binding sites also produces perturbation of the barbiturate/DHP site cannot be ruled out. However, since sulfanilate pretreatment abolishes both the muscimol and the pentobarbital enhancing effect, it is feasible that sulfanilate affects the coupling of GABA and barbiturate receptors to the benzodiazepine receptor. Furthermore, since sulfanilate, but not DEP, inhibits GABA binding and muscimol enhancement of diazepam binding, it is apparent that sulfanilate may modify some other functional groups besides histidine (e.g., tyrosine), or it may simply interact with different histidine residues. Further studies on the effect of sulfanilate and other group-selective reagents on the interaction of various components of the benzodiazepine-GABA receptor-ionophore complex should provide useful information about the molecular nature of this oligomeric complex.

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TABLE 6

*Effect of diazotized sulfanilate on [³H]GABA binding*

Membranes were treated as described under Materials and Methods. GABA Scatchard plots were determined using 12 concentrations of [³H] GABA (0.25–1004 nM). The $K_D$ and $B_{max}$ for the control group were determined by the Feldman analysis (29), assuming one ligand binding to two noninteracting sites. [³H]GABA Scatchard plots in the sulfanilate-treated group exhibited a single site (see Fig. 5). The values represent the mean ± standard deviation of four control and three treatment experiments for cortex and three control and three treatment experiments for cerebellum.

| Treatment | Specific [³H]GABA binding | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cortex | | | | Cerebellum | | | |
| | $K_D$ | | $B_{max}$ | | $K_D$ | | $B_{max}$ | |
| | $K_D^1$ | $K_D^2$ | $B_{max}^1$ | $B_{max}^2$ | $K_D^1$ | $K_D^2$ | $B_{max}^1$ | $B_{max}^2$ |
| | *nM* | | *pmoles/mg protein* | | *nM* | | *pmoles/mg protein* | |
| None | 8.3 ± 2.8 | 154 ± 28 | 0.437 ± 0.11 | 4.883 ± 0.74 | 7.6 ± 1.7 | 40 ± 4.7 | 0.869 ± 0.22 | 3.091 ± 0.46 |
| Sulfanilate, 1 mM | 17.0 ± 4.8 | ND[a] | 0.328 ± 0.12 | ND | 10.2 ± 2.5 | ND | 0.585 ± 0.26 | ND |

[a] ND, not detectable.

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