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# Effects of Cations and Anions on the Binding of $^3$H-Diazepam to Rat Brain (40211)

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$^3$H-Diazepam binds in a specific manner to homogenates of rat brain, and the potency of inhibition of this binding by analogous benzodiazepines is positively correlated with in vivo pharmacological activity (1–3). Binding is highest in cerebral cortex, hippocampus, cerebellum and midbrain, intermediate in medulla oblongata/pons and corpus striatum, and lowest in spinal cord. This differential binding is correlated, in a nonlinear fashion (3), with $^3$H-GABA binding (4) in the same brain areas. In general, $^3$H-diazepam binding meets the biochemical criteria that are indicative of binding to a receptor site (5).

The effect of ions on $^3$H-diazepam binding has not been extensively studied although it has been established that binding is not greatly affected by sodium ions and that binding is similar in Krebs–Tris, Tris–HCl and sodium phosphate buffers (6). The present report describes the effects of various ions on $^3$H-diazepam binding to subcellular fractions of whole rat brain.

*Materials and methods.* [$^3$H-methyl] Diazepam was prepared by *N*-methylation of desmethyl–diazepam with [$^3$H] methyl bromide (7) and purified as before (3).

Male rats of the CR-CD strain (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing between 200 and 300 g were used for all experiments. The rats were fed Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and tap water until they were killed by decapitation. Whole brain, including the attached segment of spinal cord, was quickly removed, weighed, and homogenized in 9 vol of cold (4°) 0.32 *M* sucrose using a Potter-Elvehjem homogenizer equipped with a teflon pestle. The homogenate was centrifuged at 1000*g* for 10 min, the pellet was discarded, and the supernatant centrifuged at 30,000*g* for 10 min. The resultant pellet was resuspended in the original volume of distilled water, and frozen for at least 18 hr at −76°. After thawing, the suspension was centrifuged at 30,000*g* for 10 min, the supernatant was discarded, and the pellet was resuspended in one-half the original volume of Tris–buffer (0.05 *M* or 0.1 *M*) or 0.32 *M* sucrose. A biuret procedure (8) was used for determining protein concentrations; when tris buffer was used, readings were corrected for interference.

Binding reactions were started by adding aliquots of brain homogenate (4°) to media (23°) that contained all other constituents (final volume 2 ml). The reaction mixtures were then incubated in a shaking water bath at 30° for 10 min. After incubation, the tubes were cooled to 4° and reactions were terminated by filtration through Whatman GF-C filter discs followed by a single wash with 8 ml of 0.15 *M* NaCl (4°) (3). Radioactivity that was bound to the tissue residue and the filter discs was determined by liquid scintillation counting as before (9–11). Specific $^3$H-diazepam binding was obtained by subtracting nonspecific binding, that is radioactivity detected in the presence of 4 $\mu$m diazepam, from total binding obtained in the absence of the unlabeled diazepam.

$^3$H-Diazepam, NCS tissue solubilizer, and PCS solubilizer were obtained from Amersham/Searle Corp., Arlington Heights, IL. Diazepam was a gift from Hoffmann-La Roche Inc., Nutley, NJ.

*Results.* The effects of chloride salts of $Li^+$, $Na^+$, $K^+$, $Cs^+$ and $NH_4^+$ on the specific binding of $^3$H-diazepam to hypotonically shocked, frozen and thawed $P_2$ fractions of whole rat brain are presented in Table I. All of the cations, with the exception of $Cs^+$, produced very slight enhancements of total and specific $^3$H-diazepam binding as the concentrations were raised from 3.5 to 350 m*M*; the largest increase was only 13% at 350 m*M* $Na^+$. A similar insensitivity to the presence of monovalent cations was found in freshly prepared $P_2$ pellets that were not frozen or osmotically shocked. Thus, unlike the binding of $^3$H-GABA (4) and $^3$H-opiates (12), $^3$H-diazepam does not interact with a receptor in a manner



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that is modulated by monovalent cations.

However several divalent cations produced marked stimulations of total and specific $^3$H-diazepam binding (Table II). Unfortunately, the salts could not be tested across the same concentration range because the highest testable concentration for each salt was determined by its solubility in the reaction mixture at pH 7.1. $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ concentrations were varied between 3.5 and 100 m*M*. Over this range, $Mg^{2+}$ did not have any effect and $Ca^{2+}$ caused only a very slight enhancement (13%) which was maximal at 35 m*M*; however, $Mn^{2+}$ caused a concentration-dependent enhancement which reached 32% at 100 m*M*. $Hg^{2+}$, $Ba^{2+}$, $Zn^{2+}$ and $Co^{2+}$ were tested in the range of 0.01 m*M* to 1.0 m*M*. Over this range, $Ba^{2+}$ did not have any effect but $Hg^{2+}$, $Zn^{2+}$, and $Co^{2+}$ produced enhancements which were maximal at 0.03 m*M* (36%), 0.29 m*M* (20%), and 1.0 m*M* (27%), respectively. $Cu^{2+}$, tested over the range of 0.001–0.1 m*M*, caused a concentration-dependent enhancement of binding which reached 21% at 0.1 m*M*. $Ni^{2+}$ caused the largest increase; over the range of 0.05–5.0 m*M* this cation produced a concentration-dependent enhancement that reached 86% at

TABLE I. INFLUENCE OF MONOVALENT CATIONS ON BINDING OF $^3$H-DIAZEPAM.[a]

| Ion concentration | Ion | | | | |
|---|---|---|---|---|---|
| | $Li^+$ | $Na^+$ | $K^+$ | $Cs^+$ | $NH_4^+$ |
| (m*M*) | (% of control binding) | | | | |
| 0 | 100 ± 2 | 100 ± 2 | 100 ± 2 | 100 ± 2 | 100 ± 2 |
| 3.5 | 105 ± 3 | 95 ± 4 | 105 ± 3 | 98 ± 2 | 105 ± 3 |
| 10 | 109 ± 3* | 105 ± 2 | 104 ± 2 | 104 ± 2 | 105 ± 2 |
| 35 | 107 ± 3 | 99 ± 3 | 111 ± 4* | 105 ± 4 | 108 ± 2* |
| 100 | 113 ± 2* | 104 ± 3 | 112 ± 2* | 102 ± 4 | 111 ± 4 |
| 350 | 109 ± 1* | 113 ± 2* | 107 ± 4 | 92 ± 3 | 94 ± 2 |

[a] Synaptosomal membranes were resuspended in 0.32 *M* sucrose. Reaction mixture (2 ml) was 0.13 mg synaptosomal protein/ml, 40 m*M* Tris·HCl (pH 7.1), 3.4 n*M* $^3$H-diazepam (for determining total binding) or 3.4 n*M* $^3$H-diazepam + 4 μ*M* diazepam (for determining nonspecific binding), and various concentrations of monovalent cations as their chloride salts. Control binding (no cations added): total, 633 dpm; nonspecific, 249 dpm; specific, 384 dpm. Values with added cations are means ± SEM for five replicate determinations.

* Significantly different from control, $P < 0.05$.

TABLE II. INFLUENCE OF DIVALENT CATIONS ON BINDING OF $^3$H-DIAZEPAM.[a]

| Ion concentration | Ion | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $Mg^{2+}$ | $Ca^{2+}$ | $Mn^{2+}$ | $Co^{2+}$ | $Ni^{2+}$ | $Hg^{2+}$ | $Cu^{2+}$ | $Zn^{2+}$ | $Ba^{2+}$ |
| (m*M*) | (% of control binding) | | | | | | | | |
| 0 | 100 ± 2 | 100 ± 2 | 100 ± 2 | 100 ± 2 | 100 ± 2 | 100 ± 2 | 100 ± 2 | 100 ± 2 | 100 ± 2 |
| 0.001 | | | | | | | 103 ± 5 | | |
| 0.0029 | | | | | | | 105 ± 6 | | |
| 0.01 | | | | 100 ± 2 | | 129 ± 4* | 116 ± 2* | 110 ± 8 | 96 ± 1 |
| 0.029 | | | | 114 ± 4* | | 136 ± 3* | 114 ± 2* | 116 ± 6 | 96 ± 3 |
| 0.05 | | | | | 127 ± 3* | | | | |
| 0.1 | | | | 109 ± 6 | | 127 ± 1* | 121 ± 2* | 118 ± 2* | 92 ± 2 |
| 0.145 | | | | | 136 ± 4* | | | | |
| 0.29 | | | | 109 ± 2* | | 115 ± 3* | | 120 ± 6* | 93 ± 4 |
| 0.5 | | | | | 159 ± 7* | | | | |
| 1.0 | | | | 127 ± 3* | | 112 ± 3* | | 118 ± 1* | 91 ± 3 |
| 1.45 | | | | | 172 ± 5* | | | | |
| 3.0 | | | | | | | | | |
| 3.5 | 99 ± 2 | 109 ± 7 | 109 ± 4 | | | | | | |
| 5.0 | | | | | 186 ± 7* | | | | |
| 10.0 | 105 ± 2 | 107 ± 3 | 117 ± 4* | | | | | | |
| 35.0 | 103 ± 3 | 113 ± 4* | 128 ± 3* | | | | | | |
| 100.0 | 99 ± 3 | 101 ± 2 | 132 ± 3* | | | | | | |

[a] Synaptosomal membranes were suspended in 0.1 *M* TRIS·HCl (pH 7.1). Reaction mixture (2 ml) was 0.25 mg protein/ml, 48 m*M* TRIS·HCl (pH 7.1), 3.4 n*M* $^3$H-diazepam (for determining total binding) or 3.4 n*M* $^3$H-diazepam + 4 μ*M* diazepam (for determining nonspecific binding), and various concentrations of divalent cations as their chloride salts. Control binding (no cations added): total, 1045 dpm; nonspecific, 292 dpm; specific, 753 dpm. Values with added cations are means ± SEM for five replicate determinations.

* Significantly different from control, $P < 0.05$.

5.0 m*M*. The rank order for the potency of binding enhancement at 0.1 m*M* cation concentration was $Ni^{2+}$, $Hg^{2+}$ > $Cu^{2+}$, $Zn^{2+}$ > $Co^{2+}$ > $Ba^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$.

In order to determine whether the divalent cations enhanced binding by increasing the number of sites or by increasing the affinity for the existing binding sites, saturation curves (Fig. 1a) were obtained for specific $^3$H-diazepam binding in the presence and absence of 5 m*M* $Ni^{2+}$. This cation was chosen because it produced the largest enhancement of binding. At low $^3$H-diazepam concentrations, binding was greatly enhanced by the $Ni^{2+}$ but as the concentration was raised the enhancement became progressively smaller. A double-reciprocal plot of the data (Fig. 1b) shows that both lines intersect at the same point on the *y*-axis, indicating that $Ni^{2+}$ enhanced the affinity of $^3$H-diazepam for its binding site but did not alter the total number of sites. A Scatchard plot (not shown) also led to the same conclusion. In the presence and absence of $Ni^{2+}$, the Hill coefficient was 1.0 indicating that the binding was noncooperative in nature.

The stimulatory effect of $Ni^{2+}$ was specific for the binding of $^3$H-diazepam since, as shown in Fig. 2, $Ni^{2+}$ enhanced the binding of $^3$H-diazepam but did not affect the binding of $^3$H-strychnine to glycine receptors or of $^3$H-quinuclidinyl benzilate (QNB) to muscarinic cholinergic receptors. In contrast, $Ni^{2+}$ markedly inhibited the binding of $^3$H-naloxone to opiate receptors.

Monovalent anions affected $^3$H-diazepam binding in a differential manner (Table III). Over the range of 3–300 m*M*, the sodium salts of $Cl^-$ and $Br^-$ produced very slight, negligible enhancements of total and specific binding whereas the salts of $I^-$ and $F^-$ were inhibitory. $F^-$ was a more potent inhibitor of binding than was $I^-$; at 300 m*M*, inhibitions by the two ions were 73% and 40%, respectively.

*Discussion.* The primary finding of this study is that divalent cations enhance the specific binding of $^3$H-diazepam to sites in the CNS whereas monovalent cations and anions do not. The effect is caused by increasing the affinity of the binding site for $^3$H-diazepam but the precise molecular mechanism is unknown. A possibility is that a divalent cation of high localized concentration is an actual part of the binding site complex and that its presence permits a better fit between the $^3$H-diazepam and its binding site. However, other experiments (unpublished) showed that 1–10 m*M* concentrations of EDTA did not inhibit binding of $^3$H-diaze-

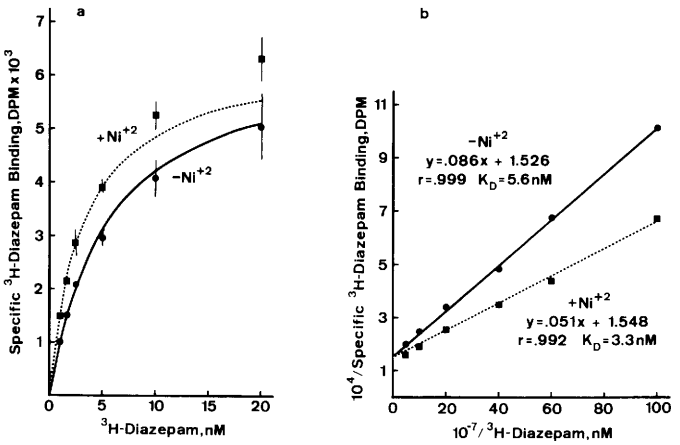


FIG. 1. The binding of $^3$H-diazepam in the presence and absence of 5 m*M* $NiCl_2$. Synaptosomal membranes were resuspended in 0.1 *M* Tris·HCl (pH 7.1). Reaction mixture (2 ml) was 0.25 mg synaptosomal protein/ml, 50 m*M* Tris HCl (pH 7.1), 1–20 n*M* $^3$H-diazepam with and without unlabeled diazepam at a 1000-fold greater concentration, and with and without 5 m*M* $NiCl_2$. Samples were incubated for 10 min at 30°, then cooled to 4°; the reaction was terminated by the filtration technique described in Methods. (a) Specific binding in the presence (■) and absence (●) of 5 m*M* $NiCl_2$. Values are means ± SEM of triplicate determinations. (b) Double reciprocal plot of the data of Fig. 1a.

pam by either fresh or osmotically-shocked $P_2$ preparations in the absence of added cation. If the cation was an integral part of the binding site complex, one might have expected binding to be inhibited. In this regard, Pasternak *et al.* (16) found that EDTA inhibited the binding of $^3$H-dihydromorphine to opiate receptor sites in the rat CNS, presumably by removing some divalent cation from the receptor site. We are now investigating other mechanisms by which cations may activate $^3$H-diazepam binding.

*Summary.* The effects of anions and cations on the specific binding of $^3$H-diazepam to rat brain were determined. Crude synaptic membranes from whole rat brain were prepared by differential centrifugation followed by hypo-osmotic shock and freezing. The monovalent cations (as their chloride salts) $Li^+$, $Na^+$, $K^+$, and $NH_4^+$ produced very slight enhancements of binding whereas $Cs^+$ did not have any effect. The monovalent anions (as their sodium salts) $F^-$ and $I^-$ were inhibitory whereas $Br^-$ produced a very slight enhancement. Divalent cations (as their chloride salts) produced marked enhancements of binding. $Ni^{2+}$ caused the greatest increase which reached 86% at 5 m*M*. This increased binding was the result of enhanced affinity for the binding site; the total number of sites was not altered. The rank order of potency for stimulating binding at 0.1 mM cation concentration was $Ni^{2+}$, $Hg^{2+} > Cu^{2+}$, $Zn^{2+} > Co^{2+} > Ba^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$. $Ni^{2+}$ did not affect the specific binding to brain homogenates of $^3$H-strychnine or of $^3$H-quinuclidinyl benzilate, but inhibited binding of $^3$H-naloxone.

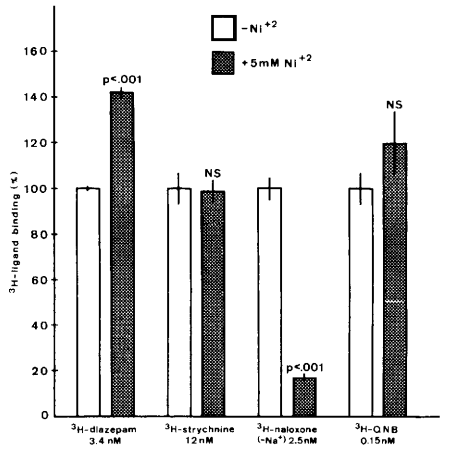


FIG. 2. Effect of 5 m*M* $NiCl_2$ on binding of several $^3$H-ligands to brain homogenates. The binding measurements were performed according to published methods: $^3$H-strychnine (13), $^3$H-naloxone (14), and $^3$H-quinuclidinyl benzilate (QNB) (15). $^3$H-Diazepam binding was determined as in Fig. 1. Ligand concentrations were: 12 n*M* $^3$H-strychnine with and without 50 $\mu M$ strychnine; 2.5 n*M* $^3$H-naloxone with and without 0.1 $\mu M$ levorphanol; 0.15 n*M* $^3$H-QNB with and without 1.0 $\mu M$ atropine; and 3.4 n*M* $^3$H-diazepam with and without 4 $\mu M$ diazepam. Half of the samples contained 5 m*M* $NiCl_2$. Values are means ± SEM of five replicate determinations.

TABLE III. EFFECTS OF MONOVALENT ANIONS ON BINDING OF $^3$H-DIAZEPAM.[a]

| Ion concentration | Ion | | | |
|---|---|---|---|---|
| | $F^-$ | $Cl^-$[b] | $Br^-$ | $I^-$ |
| (m*M*) | (% of specific control binding) | | | |
| 0 | 100 ± 4 | 100 ± 2 | 100 ± 4 | 100 ± 4 |
| 3 | 102 ± 12 | | 102 ± 1 | 102 ± 5 |
| 3.5 | | 95 ± 4 | | |
| 10 | 89 ± 2* | 105 ± 2 | 114 ± 5 | 113 ± 6 |
| 30 | 92 ± 13 | | 124 ± 10 | 113 ± 5 |
| 35 | | 99 ± 3 | | |
| 100 | 64 ± 6* | 104 ± 3 | 111 ± 11 | 95 ± 6 |
| 300 | 27 ± 2* | | 104 ± 10 | 61 ± 2* |
| 350 | | 113 ± 2* | | |

[a] Synaptosomal membranes were resuspended in 0.32 *M* sucrose. Reaction mixture (2 ml) was 0.25 mg synaptosomal protein/ml, 80 m*M* sucrose, 35 m*M* sodium potassium phosphate (pH 7.1), 3.4 n*M* $^3$H-diazepam (for determining total binding) or 3.4 n*M* $^3$H-diazepam + 4 $\mu M$ diazepam (for determining nonspecific binding), and various concentrations of monovalent anions as their sodium salts. Control binding (no anions added): total, 930 dpm; nonspecific, 324 dpm; specific, 606 dpm. Values with added anions are means ± SEM for three replicate determinations.

[b] Data from Table I.

* Significantly different from control, $P < 0.05$.

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