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CNS Benzodiazepine Receptors: Physiological Studies and Putative Endogenous Ligands

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SKOLNICK, P., P. J. MARANGOS, P. SYAPIN, F. K. GOODWIN AND S. M. PAUL. *CNS benzodiazepine receptors: Physiological studies and putative endogenous ligands*. PHARMAC. BIOCHEM. BEHAV. **10**(5) 815–823, 1979.—The recent demonstration of benzodiazepine receptors in the mammalian CNS has provided new information on the mechanism of action of this important class of drugs. In addition, the presence of these receptors has prompted studies on their physiological significance, including attempts at isolating an endogenous ligand. The isolation of a number of substances from bovine brain that competitively inhibit (^{3}H)-diazepam binding to synaptosomal membrane suggests the presence of an endogenous ligand. Two of these substances have been identified as the purines inosine and hypoxanthine. Pharmacological studies of these purines suggest that they may have diazepam-like effects *in vivo*. The possibility that the brain may contain its own benzodiazepine-like compound is currently being studied.

Benzodiazepine receptors Physiological studies Seizures Endogenous ligands Purines

DESPITE the widespread use of the benzodiazepines and their established efficacy in the treatment of anxiety states, seizure disorders, and insomnia, little is known about their underlying mechanism of action. The recent identification and characterization of specific benzodiazepine receptors in the central nervous system (CNS) of both laboratory animals [11, 16, 17] and man [2] has provided new clues as to the possible biochemical mechanisms mediating the pharmacological and behavioral effects of these drugs. In addition, these may have a physiological role in the various behavioral states responsive to treatment with the benzodiazepines, thus providing new approaches to the study of seizure disorders and anxiety states. The presence of CNS benzodiazepine receptors also implies the existence of a substance(s) which binds to the receptor and functions as a physiological (i.e., endogenous) ligand. Such a compound(s) would undoubtedly have important central actions as either a neurotransmitter or neuromodulatory agent.

During the past year, our laboratory has studied the effects of various physiological and pharmacological conditions on CNS benzodiazepine receptors in an attempt to understand the neurophysiological significance of these receptors. In addition, attempts at isolating an endogenous ligand for the benzodiazepine receptor have yielded substance(s) with some of the pharmacological properties of the benzodiazepines themselves. This report will review these studies and present data suggesting an important role for the benzodiazepine receptor in the regulation of seizure activity.

IDENTIFICATION OF BENZODIAZEPINE RECEPTORS IN VIVO

The initial reports on the presence of CNS benzodiazepine receptors utilized lysed membrane (synaptosomal) preparations of brain [2, 11, 16]. However, the specific binding of ^{3}H-diazepam to crude synaptosomal membranes at physiological temperatures *in vitro* was less than 5% of the binding obtained at 0–4°C. In addition, kinetic studies suggested a possible conformational change in the receptor at 18°C [3]. The failure to demonstrate specific binding under physiological conditions raised serious questions as to whether receptor binding occurs *in vivo*. For these reasons and because the labelling of benzodiazepine receptors *in vivo* would provide a valuable tool for studying these receptors under physiological conditions, we studied the binding of (^{3}H)-diazepam *in vivo* [17,18].

Adult male Sprague-Dawley rats were administered (^{3}H)-diazepam via the lateral tail vein. Animals were sacrificed approximately 1–2 minutes after injection, since peak brain levels occur at this time. Furthermore, virtually no metabolism of the (^{3}H)-diazepam would take place during this time. This is especially important since the radioactive label of (^{3}H)-diazepam is located on the methyl group most susceptible to metabolic removal. Furthermore, the major metabolite (n-desmethyldiazepam) is pharmacologically active, and it would therefore compete with the unmetabolized diazepam for receptor sites.

Following dissection, tissues were homogenized in 2:

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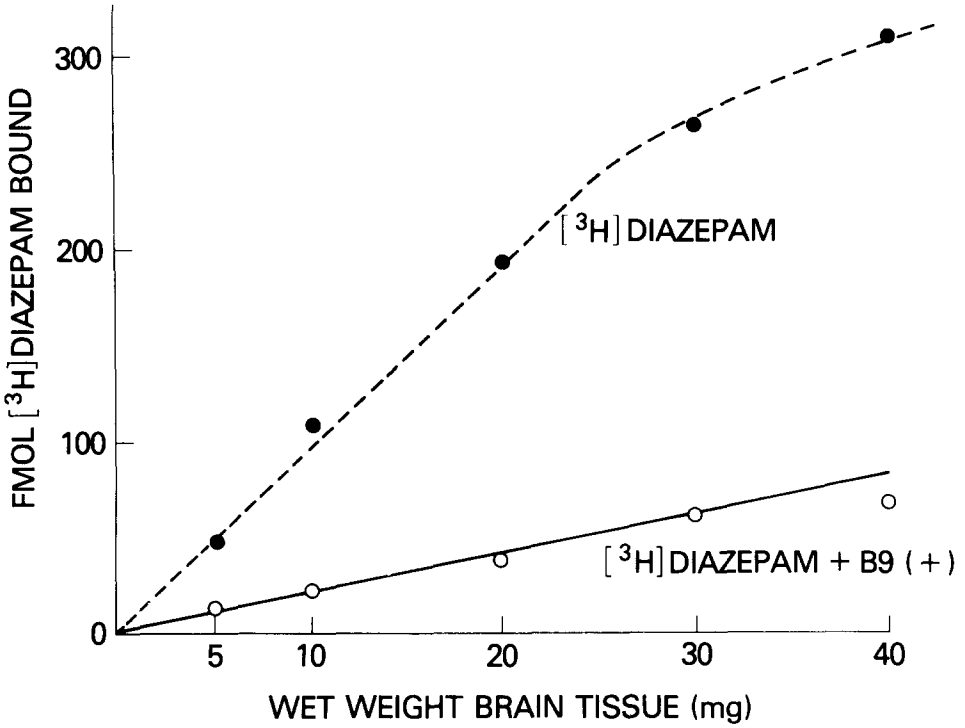

FIG. 1. Effects of tissue concentration on (^{3}H)-diazepam binding. Rats were injected with 50 μC (0.34 μg) of (^{3}H)-diazepam and brain homogenates (whole brain less cerebellum and pons-medulla) were prepared as described in ref. Aliquots of 5 to 40 mg of tissue were assayed in triplicate 10 minutes after homogenization. These data are compared to a parallel experiment in which 700 μg/kg B9 (+) was co-injected with 50 μC (^{3}H)-diazepam. Total forebrain concentrations of (^{3}H)-diazepam in these experiments was 22.7 ± 1 pmol/g tissue (wet weight) (n=4).

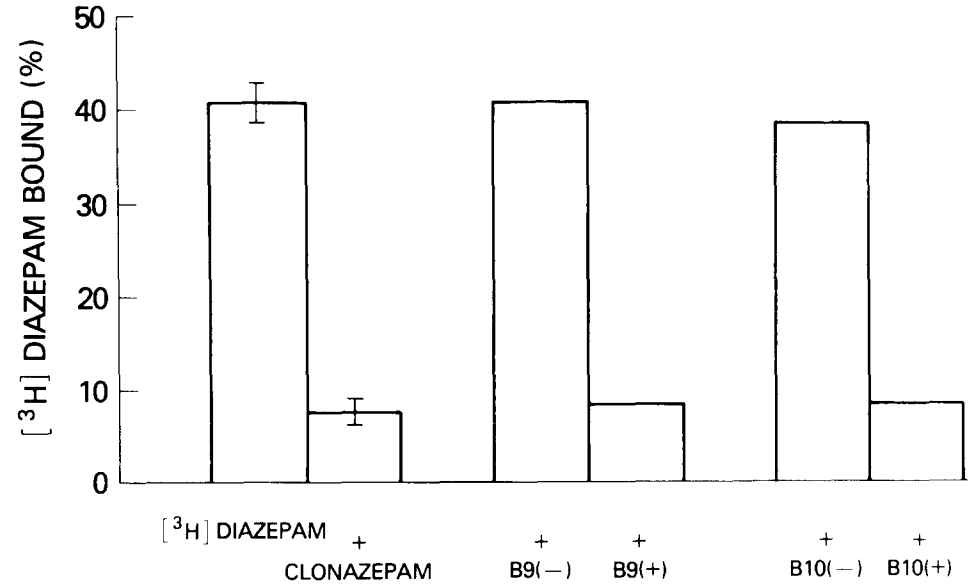

FIG. 2. Effects of clonazepam and the benzodiazepine enantiomers of B9 and B10 on *in vivo* (^{3}H)-diazepam binding. Binding is markedly decreased by pharmacologically active benzodiazepines (clonazepam, B9 (+), B10 (+)), while inactive compounds (B9 (-), B10 (-)) have no effect.

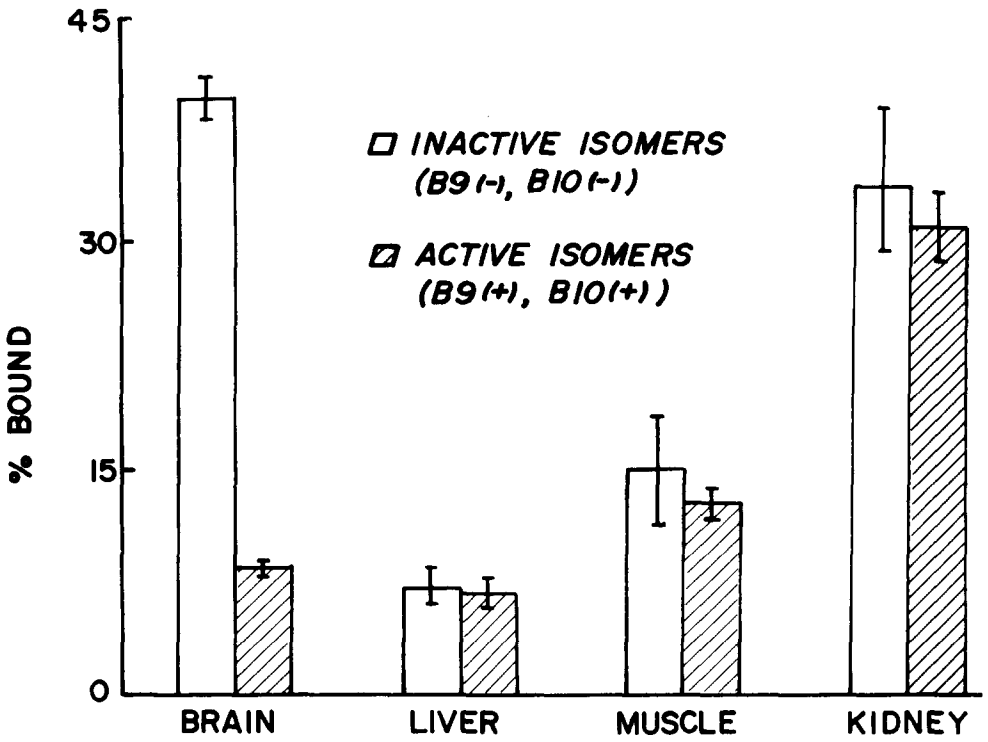

FIG. 3. Stereospecific inhibition of ^{3}H-diazepam binding *in vivo*. Rats were injected with 50 μCi ^{3}H-diazepam in combination with the enantiomers of B9 or B10 (700 $\mu g/kg^{-1}$). Values represent mean ± SEM in brain (n=8), liver (n=6) and other tissues (n=2). Percent of ^{3}H-diazepam bound is the amount of ^{3}H-diazepam in the homogenate retained by the GF/B filter divided by the amount of ^{3}H-diazepam in an equal volume of homogenate=100. ▨, Active isomers (B9(+), B10(+)): □, inactive isomers (B9(−), B10(−)).

volumes of buffer and filtered under vacuum through Whatman GF/B filters as previously described [17]. The amount of radioactivity retained on the filter is measured and expressed as the percent of the total radioactivity present in the homogenate. This calculation corrects for any variability in the levels of (^{3}H)-diazepam in the brain. Non-specific binding was determined by pre-injection or co-injection with a large excess of another pharmacologically active benzodiazepine.

Results of the intravenous injection of (^{3}H)-diazepam with and without a pharmacologically active benzodiazepine are shown in Fig. 1. Specific binding is linear with protein concentration and the total binding is markedly reduced in the presence of an active benzodiazepine. To determine whether the binding of (^{3}H)-diazepam had occurred *in vivo* and was not an artifact of the homogenization procedure, a post-homogenization time-course was carried out. The binding of (^{3}H)-diazepam to rat forebrain was maximal as early as 30 seconds following homogenization and remained constant for at least 60 minutes. Since the binding of (^{3}H)-diazepam reaches equilibrium within 10–15 minutes *in vitro*, this data strongly suggests that the observed binding had occurred *in vivo*.

The effects of co-injection of two benzodiazepine enantiomers on (^{3}H)-diazepam binding *in vivo* revealed that the binding to brain was also stereo-specific (Fig. 2). In contrast, while binding of (^{3}H)-diazepam occurs readily in peripheral tissues such as liver, kidney and muscle, it is not displaced from these tissues in a stereospecific fashion (Fig. 3). These findings confirm the marked localization and specificity of benzodiazepine receptors in the CNS. Finally, the regional distribution of (^{3}H)-diazepam binding to brain *in vivo* was determined. Specific binding was highest in the cerebral cortex and lowest in the pons-medulla. The rank order concentration of benzodiazepine receptors in various cortical and subcortical areas was identical to that observed in the *in vitro* assay. The demonstration of benzodiazepine receptors under physiological conditions circumvents concerns about methodological artifacts of the *in vitro* studies. Furthermore, this technique permits further experiments on receptor changes during various pharmacological and/or physiological states and may help in visualizing the receptor by autoradiography.

CELLULAR LOCALIZATION OF CNS BENZODIAZEPINE RECEPTORS

Although the gross anatomic and subcellular distribution of CNS benzodiazepine receptors has been described, little is known about the cellular localization of these sites. In addition, no direct functional link between benzodiazepine receptors and their neurophysiological actions has yet been demonstrated. Since benzodiazepines have profound neurochemical [4] and neurophysiological effects [5] on cerebellar Purkinje cells, it was hypothesized that these neurons might contain a high concentration of ben-

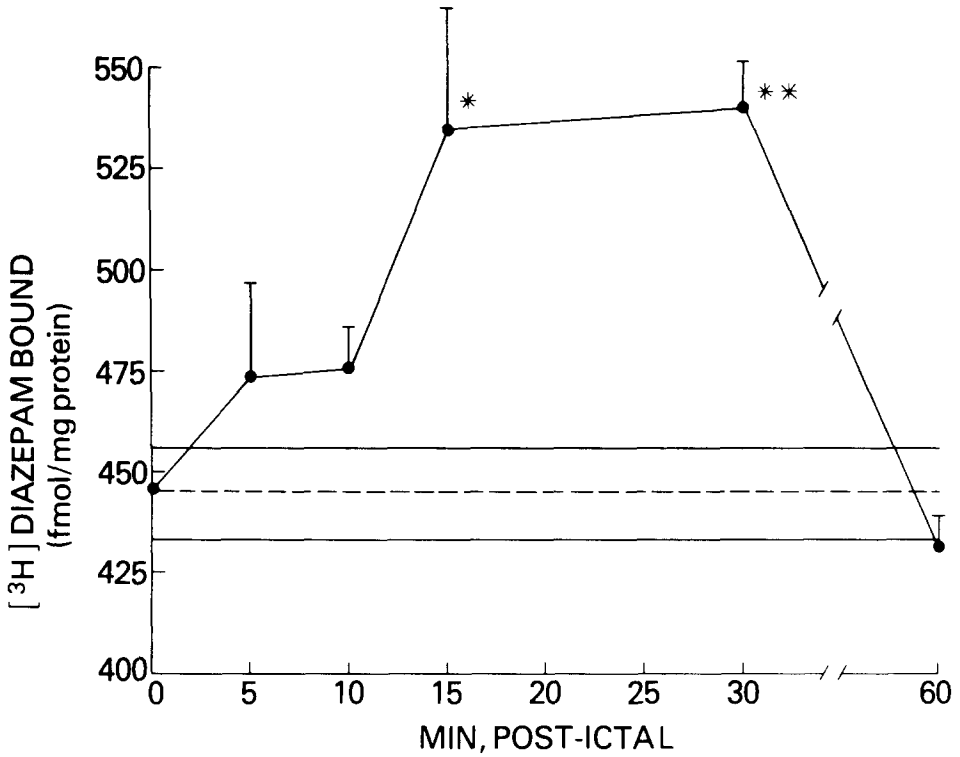

FIG. 4. Temporal changes in (^{3}H)-diazepam binding in rats subjected to electrically induced seizures. Values represent means (± standard error) of four animals per time point. *$p<0.005$; **$p<0.001$ compared with sham-shocked controls. The binding of (^{3}H)-diazepam to synaptosomal membranes from sham-shocked rats was 446 ± 12 fmole per milligram of protein (N=8) at a diazepam concentration of 1.87 nM. The dashed line at the bottom of the figure represents the mean binding of sham-shocked controls; solid lines represent ± standard error. Control animals were killed from 5 to 60 minutes after a sham shock.

zodiazepine receptors. To test this hypothesis we determined the number of benzodiazepine receptors in the brains of mutant mice which have undergone a selective loss of cerebellar Purkinje cells. These mice, the so-called "nervous" mutant, have been previously shown to be unresponsive to some of the pharmacological effects of the benzodiazepines [9]. Their littermate controls are phenotypically normal and have no evidence of Purkinje cell degeneration.

"Nervous" mutant mice have a marked decrease in the number of cerebellar benzodiazepine receptors when compared to either littermate controls or normal mice [15]. No changes in either the number of receptors or their affinity constants were observed in the forebrains of either the mutant or control mice.

The dramatic reduction in benzodiazepine receptors observed in the cerebella of "nervous" mice, which suffer a selective loss of Purkinje cells, clearly identify these cells as containing a large number and high density of receptors since Purkinje cells represent only a small fraction of the total cerebellar neurons. In addition, "Weaver" mutant mice which undergo a selective loss of cerebellar granule cells have been found to have no reduction in cerebellar benzodiazepine receptors (Chang and Snyder, personal communication). These obsevations provide strong evidence for both the neuronal localization of benzodiazepine receptors and a direct functional association between benzodiazepine receptors and the pharmacological actions of these drugs.

EFFECTS OF EXPERIMENTAL SEIZURES ON BENZODIAZEPINE RECEPTORS

Since competition for the benzodiazepine receptor by other benzodiazepines closely parallels their potency as anticonvulsants, it has been suggested that these receptors may normally be involved in the regulation or pathogenesis of seizure activity [12]. To test this hypothesis, we induced generalized seizures in rats by either electroshock or by administering the convulsant pentylenetetrazole, and examined their effects on cortical benzodiazepine receptors. Electroconvulsive shock was chosen because it is a reliable and drug-free method of producing seizures. Seizures were induced using a Medicraft electroconvulsive therapy unit (150 V, 1 sec AC) through ear clips attached to each pinnae. Control rats received either sub-convulsive electroshock (70 V, 0.4 sec AC) or sham-shock. Following development of a generalized tonic-clonic seizure animals were sacrificed and crude synaptosomal membranes of the cerebral cortex were prepared. Benzodiazepine receptors were measured by incubating the crude synaptosomal membranes with (^{3}H)-diazepam of high specific activity as previously described [12].

The amount of (^{3}H)-diazepam specifically bound to cere-

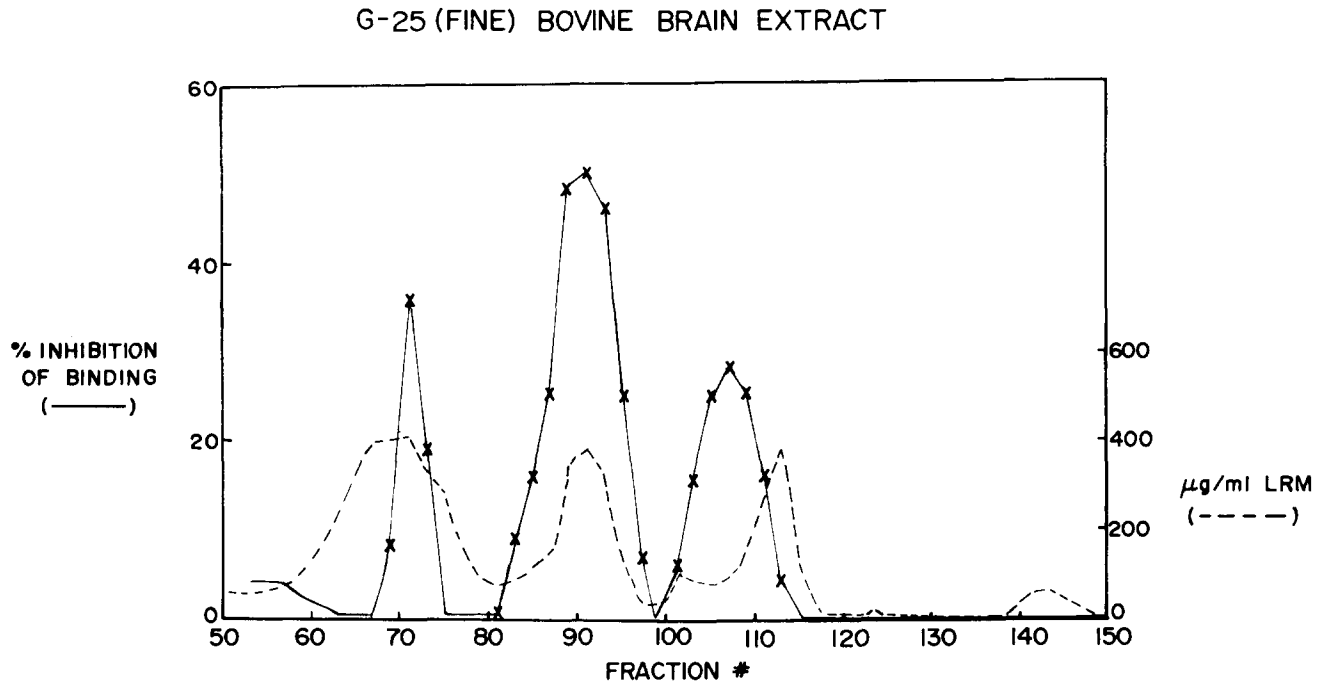

FIG. 5. Sephadex G-25 chromatography of bovine brain extract. Fifteen ml of bovine brain extract resulting from the preparation of 800 grams of tissue was applied on a 2.5×100 cm Sephadex G-25 column equilibrated in 1 mM Tris-HCl buffer pH 7.4. Fractions were collected (2.5 ml) and assayed for both inhibition of (^{3}H)-diazepam binding (400 μl) and Lowry reactive material. The void volume of the column was at tube 42.

bral cortex membranes increased rapidly following the development of generalized seizures. Specific binding increased by 21% at 15 minutes post-ictal and returned to preseizure levels by 60 minutes (Fig. 4). Scatchard analysis on data derived from both electroshock and sham-shocked controls revealed that the post-ictal enhancement of (^{3}H)-diazepam binding is due to a rapid increase in the number of receptors rather than a change in receptor affinity. Furthermore, subconvulsive electroshock was without effect on (^{3}H)-diazepam binding, suggesting that a generalized seizure is a requisite for the post-ictal increase in benzodiazepine receptors. Significantly, pentylenetetrazole-induced seizures resulted in a comparable increase in benzodiazepine receptors (21% $p<0.05$) at 30 min post-ictal, suggesting that the enhancement of (^{3}H)-diazepam binding may be a general phenomenon. In order to rule out artifactual changes due to inter-ictal or post-ictal hypoxia, the effects of hypoxia on cortical benzodiazepine receptors was examined. No significant changes in diazepam binding were observed in rats rendered hypoxic by inhalation of argon gas when compared to controls.

The rapid changes in cortical benzodiazepine receptors following seizures is reminiscent of receptor supersensitivity previously observed in a variety of central and peripheral neurotransmitter systems. Correspondingly, the enhanced binding may help explain the marked potency and rapid therapeutic effects of the benzodiazepines in the treatment of seizure disorders such as status epilepticus.

Furthermore, since the anti-convulsant effects of the benzodiazepine(s) result from their marked inhibitory action on neuronal activity, a post-ictal increase in diazepam receptors may enhance the physiological effect of a normally occurring inhibitory ligand (see below).

DIRECT CORRELATION BETWEEN BENZODIAZEPINE RECEPTORS AND ANTI-CONVULSANT EFFECT

In order to assess the significance of any change in benzodiazepine receptor number (such as those observed after seizures) it is important to know the number of receptors necessary to mediate the pharmacological effect(s) of the drug. To determine this, groups of mice were injected with diazepam (4 mg/kg) and the number of benzodiazepine receptors occupied by the drug, as well as its anti-convulsant effects, were measured over time. One-half of the mice in each group were sacrificed for determining the apparent number of CNS benzodiazepine receptors and the other half were challenged with pentylenetetrazole (80 mg/kg) to determine the present protected against seizures.

As in previous studies, this dose of diazepam protected against pentylenetetrazole-induced seizures as long as 48 hr following administration of the drug. In addition, both the number of benzodiazepine receptors occupied by diazepam and the percentage of animal(s) protected against seizures varied over time. An excellent correlation ($r=0.98, p<0.001$) was obtained between the number of receptors occupied and protection against seizures. Furthermore, these studies also demonstrated that only a small percentage (approximately 20%) of benzodiazepine receptors need be occupied to afford complete protection against pentylenetetrazole-induced seizures. These results provide the first direct correlation between the occupation of a central receptor site and the clini-

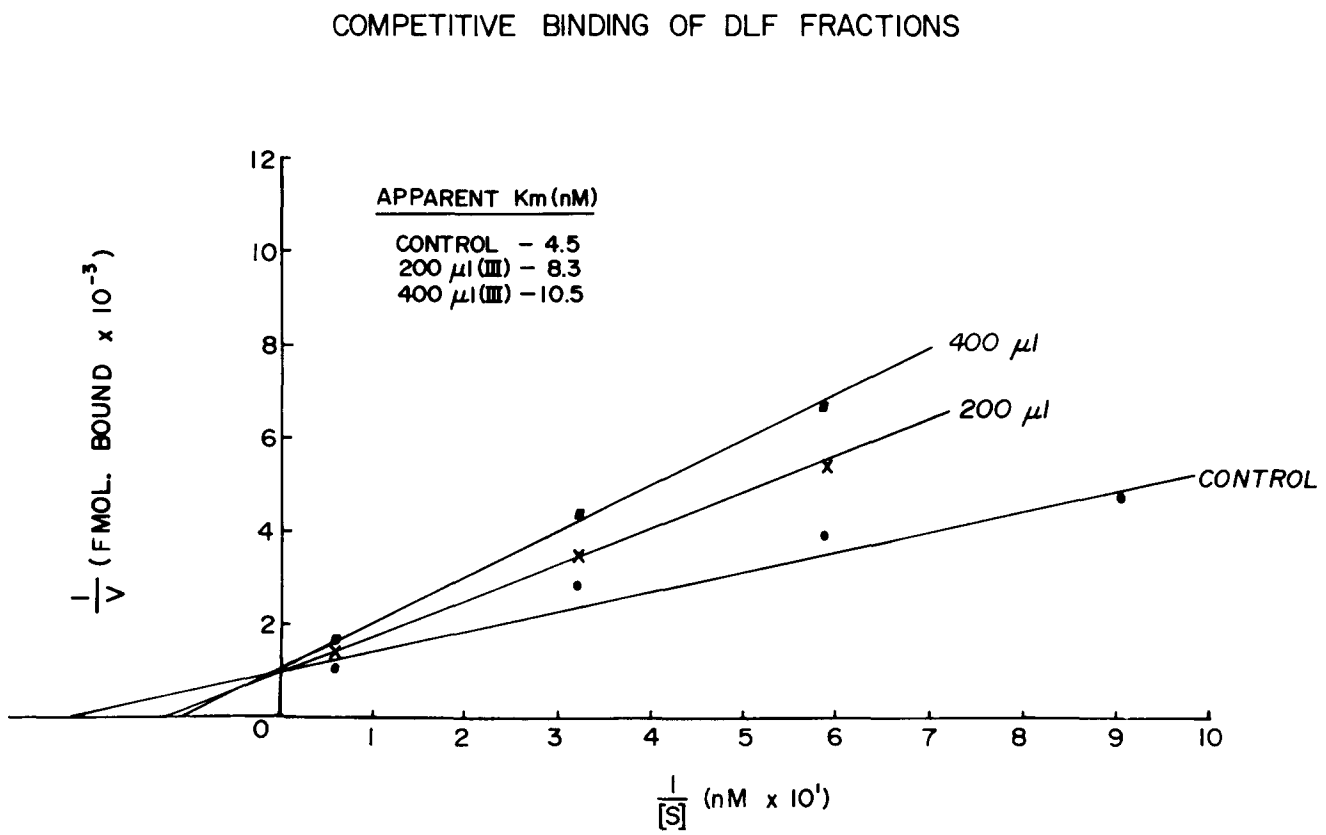

FIG. 6. Double reciprocal analysis of Peak III inhibitory activity. The synaptosomal membrane preparation was assayed for specific binding with increasing amounts of (^{3}H)-diazepam in the absence and presence of 200 μl and 400 μl of the Sephadex Peak III fraction. Ordinate: B is the amount of specifically bound (^{3}H)-diazepam (fmol/mg protein) as defined in Methods.

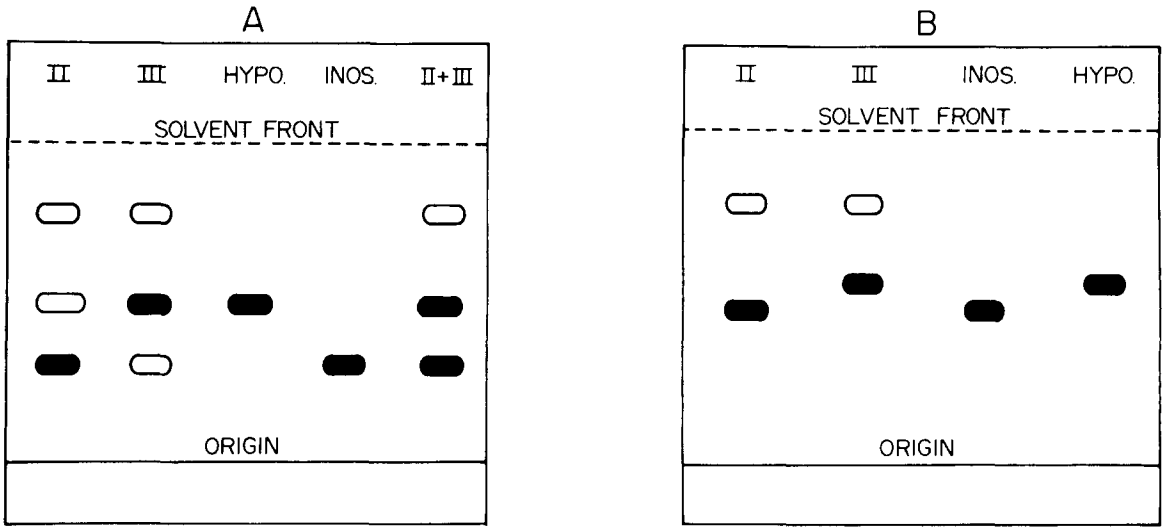

FIG. 7. TLC of Sephadex G-10 eluates: pooled peak fractions from Sephadex G-10 were spotted on TLC plates (150 μl). The composition of solvent systems A and B appear in Methods. Intense areas of μv absorbing material are indicated by dark bands; faint areas of μv absorbance are indicated by unshaded bands.

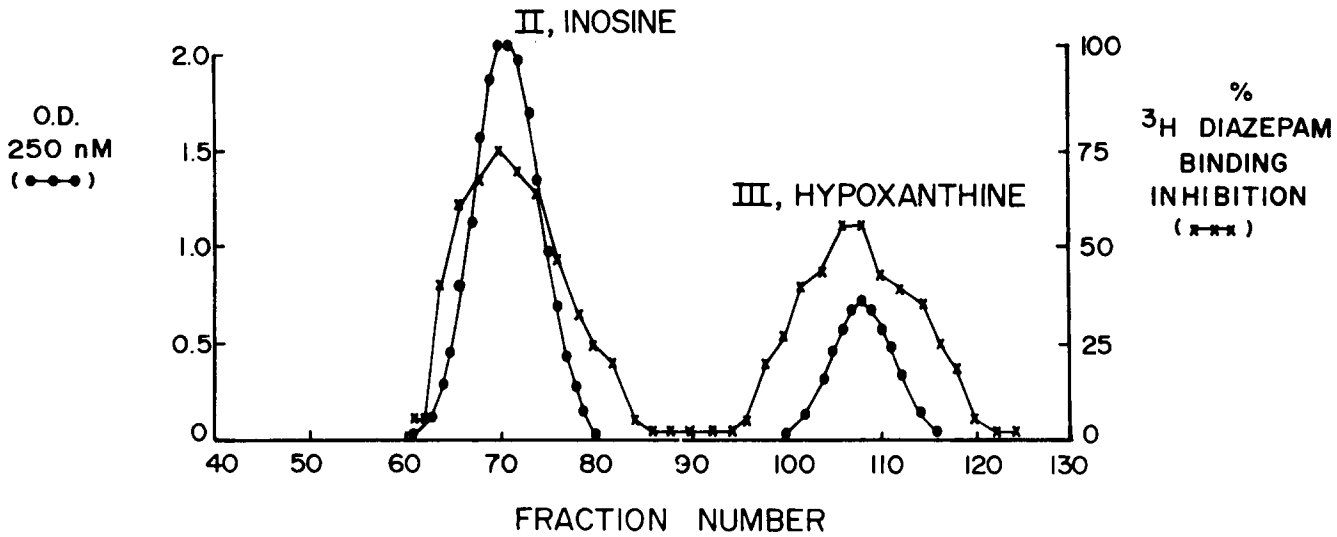

FIG. 8. Sephadex G-10 chromatography of Peak II, Peak III, inosine and hypoxanthine. Peak II and Peak III fractions from a Sephadex G-25 column were mixed, chromatographed and the eluate assayed for (^{3}H)-diazepam binding inhibitory activity. A mixture of inosine and hypoxanthine was chromatographed on the same column and the eluate assayed for absorbance at 250 nm.

cal effects of a drug. In addition, they suggest that "spare" receptors may exist for the benzodiazepines, a phenomenon already shown for many peripheral receptor system.

ISOLATION AND CHARACTERIZATION OF ENDOGENOUS INHIBITORS OF (^{3}H) DIAZEPAM BINDING

Initial attempts to isolate an endogenous ligand(s) of the benzodiazepine receptor from the mammalian CNS have utilized brain extracts obtained by homogenization of tissue in acidified acetone or methanol [1, 6, 10, 13]. These techniques were employed to minimize proteolytic degradation and to remove macro-molecules, since it was presumed that an endogenous ligand(s) would be a small molecule such as a peptide. Further purification of this homogenate by filtration, centrifugation and lipid extraction resulted in a "crude extract" which markedly inhibited (^{3}H)-diazepam binding to rat synaptosomal membranes. The specific activity of this crude extract (based on activity/mg protein) was approximately 10-fold greater than a crude extract prepared from liver, and 5-fold more active than a crude extract obtained from brain which was incubated at 37°C for 16 hours prior to preparation. Characterization of the crude brain extract revealed that inhibitory activity was: (a) heat stable (not destroyed by boiling for 10 min) (b) dialyzable (molecular weight presumably less than 5000 daltons) (c) non-lipid (no loss of activity following extraction with diethyl ether) (d) non-protein (i.e., resistant to degradation by trypsin, peptidase and protease). Although the observation that the crude extract resisted proteolytic degradation suggests that the inhibitory substance(s) was not protein in nature, a small or uniquely structured peptide could also be resistant to degradation.

Further purification of the crude extract from bovine brain using gel filtration chromatography yielded three discreet peaks of inhibitory activity, designated Peaks I, II and III according to their order of elution from a Sephadex G-25 column (Fig. 5). Calculation of the specific activities (inhibition per unit of protein) of the pooled peak fractions revealed the specific activity of Peak I to be lower than the crude extract, while Peaks II and III were approximately 2.5 and 12-fold higher, respectively.

The specificity of these inhibitory fractions was examined by determining their inhibitory activity in other receptor systems [10]. Peak I significantly inhibited the binding of both (^{3}H)-dihydroalprenolol to beta adrenergic receptors and (^{3}H)-D-ala-met-enkephalin to opiate receptors more potently than it inhibited (^{3}H)-diazepam binding. In contrast, both Peaks II and III minimally inhibited (^{3}H)-dihydroalprenolol and (^{3}H)-D-ala-met-enkephalin binding, while significantly inhibiting (^{3}H)-diazepam binding. Recently, Asano and Spector [1] have confirmed and extended these observations, demonstrating no significant inhibition by Peaks II and III on either muscarinic cholinergic or GABA receptor binding. Furthermore, these investigators demonstrated that the same active fractions had no effect on (^{3}H)-diazepam binding to peripheral tissues such as liver and kidney, which is consistent with their specificity for the brain benzodiazepine receptor.

The competitive nature of the inhibition by Peaks II and III was also observed (Fig. 6). Competitive inhibition of (^{3}H)-diazepam binding is an important criteria for establishing the physiological relevance, if any, of these compounds since neurotransmitters usually bind to receptors in a competitive fashion.

Further purification of the active inhibitory fractions was achieved by multiple chromatographic techniques. Thin layer chromatography of the pooled Sephadex G-25 peaks demonstrated several discrete bands of intense ultraviolet absorption for Peaks II and III, while Peak I yielded a diffuse region of ultraviolet absorption. No inhibitory activity was observed in the thin layer eluates from Peak I, while Peaks II and III had discrete bands of inhibitory activity which comigrated with ultraviolet absorbing material. An ultraviolet spectrum of the bands of inhibitory activity from Peaks II and III obtained by thin layer chromatography revealed a distinct absorption maximum at approximately 250 nm. This absorption maximum is characteristic of the purines. Tentative identification of Peaks II and III as purines was consistent with the other physicochemical data obtained (i.e., low

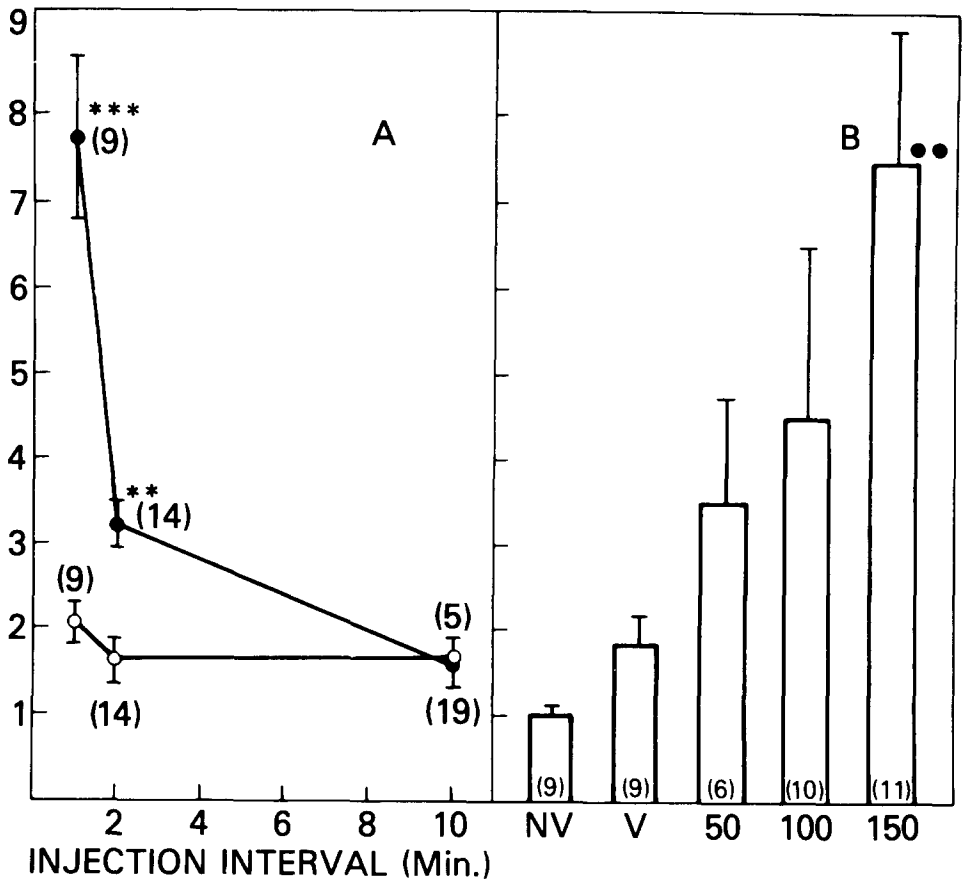

FIG. 9. The effects of intraventricularly administered inosine on pentylenetetrazole-induced seizure latency. (A) Effects of the time interval between inosine injection and pentylenetetrazole on seizure latency, *** $p<0.01$, ** $p<0.01$ compared to saline controls. (B) Effects of various doses of inosine (50 μg, 100 μg, 150 μg) on seizure latency, $p<0.01$ compared to vehicle alone.

molecular weight as indicated by Sephadex chromatography and dialysis, heat stability, non-lipid nature, and resistance to proteolytic degradation).

Identification of Peaks II and III as the purines inosine and hypoxanthine was provided by thin-layer chromatography in a solvent system giving excellent resolution for various purines (Fig. 7), gel filtration chromatography (Fig. 8), and by co-elution using high-pressure liquid chromatography [13]. In addition, the inhibition of (^{3}H)-diazepam binding by Peaks II and III corresponds to that observed with similar concentrations of authentic inosine and hypoxanthine as calculated by their extinction coefficients.

PHARMACOLOGIC EVALUATION OF INOSINE AS AN ANTICONVULSANT

Identification of inosine and hypoxanthine as endogenous inhibitors of (^{3}H)-diazepam binding *in vitro* suggests that the compounds may serve as ligands to the benzodiazepine receptor *in vivo*. However, binding studies *in vitro* cannot distinguish between an agonist/antagonist action of these compounds, nor do they establish pharmacologic (or physiologic) activity. If inosine or hypoxanthine interact with the benzodiazepine receptor *in vivo*, it is likely they will either mimic or antagonize the pharmacologic effects of the benzodiazepines. Therefore, we have examined the effects of intraventricularly (IV) administered inosine on pentylenetetrazole (PTZ)-induced seizures [14]. This experimental paradigm was employed because of the well-described techniques for assessing the convulsant/anti-convulsant properties of drugs, and the fact that the anti-convulsant effects of the benzodiazepines as well correlated with the anxiolytic potencies of these compounds [7]. Furthermore, a pharmacologic action antagonistic to the benzodiazepines (i.e., seizure production) would also be readily detected using these techniques.

Male mice of the C3H/HEN and NIH general purpose strains were used in these studies. The animals were lightly anesthetized with diethyl ether and an incision made in the scalp to expose the bregma. The mice were placed in a holding cage and allowed 20 min to recover from the ether prior to testing. The mice were immobilized and 10 μl of test compound or vehicle (phosphate buffered saline, pH 7.2) was injected into the right lateral ventricle. The mice were then transferred to individual plastic cages where PTZ was injected intraperitoneally at specified intervals and the mice observed for the onset of tonic-clonic seizures.

Intraventricularly administered inosine caused a time and dose-dependent increase in the interval between injection of PTZ and the onset of generalized seizures (which we term seizure latency). Maximal increases in seizure latency (3–4 fold increase compared to vehicle alone) were observed one

minute after injection of inosine (Fig. 9). Statistically significant (2-fold) increases were also observed with a two minute interval between inosine and PTZ. a 10 minute interval between IV injection of inosine and intraperitoneal administration of PTZ caused no increase in seizure latency (Fig. 9).

The effect of various inosine derivatives on their ability to inhibit (^{3}H)-diazepam binding *in vitro* and in altering PTZ-evoked seizure latency *in vivo* was examined. 2-Deoxyinosine, which is approximately three times more potent than inosine *in vitro*, was twice as potent in increasing seizure latency. In contrast, 7-methylinosine and thymidine, which were inactive *in vitro* had no effect on seizure latency. It, therefore, appears that within the limited series of compounds examined, there is good agreement between the *in vitro* and *in vivo* activities of these compounds.

The observation that inosine and 2-deoxyinosine increase the latency to seizures produced by PTZ in a dose and time-dependent fashion suggests a partial antagonism of the effects of PTZ, rather than a complete blockade of seizures routinely observed with pharmacologic doses of most benzodiazepines. This increase in seizure latency might be envisioned to be similar to that of a very short-acting benzodiazepine. The rapid loss of protection against PTZ could be explained by the relatively rapid loss of intraventricularly administered inosine from the CNS (more than 80% gone within two minutes of injection) as well as the metabolic transformation of inosine to inactive purines, or its translocation to sites distant from the receptor. In contrast, administration of diazepam results in significant amounts of this drug bound to receptors for many hours following a single dose (see earlier portion of chapter).

Nevertheless, since the affinities of inosine and hypoxanthine for the benzodiazepine receptor *in vitro* are low when compared to benzodiazepines such as diazepam [13] it is difficult to reconcile their role as endogenous modulators of the benzodiazepine receptor. However, recent studies from our laboratory have clearly shown that only a small fraction of benzodiazepine receptors need be occupied to fully protect an animal from PTZ-evoked seizures. This hypothesis is further supported by the recent report of Lippa *et al.* [8], who demonstrated that only 10–20% of benzodiazepine receptors need be occupied to fully manifest an anti-anxiety response in a standard conflict test for anxiolytic activity. These data suggest that the IC50 of these purines may be irrelevant to the physiologic effects of these compounds *in vivo*.

Further studies are now in progress to examine the physiological and pharmacological effects of these purines and their relationship to benzodiazepine receptors.

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