Pharmacology Biochemistry & Behavior, Vol. 10, pp. 809–813. Printed in the U.S.A.

Modulation of Benzodiazepine Binding Site Sensitivity

JOHN F. TALLMAN AND DOROTHY W. GALLAGER

Section on Biochemistry and Pharmacology, Biological Psychiatry Branch
National Institute of Mental Health
Bethesda, MD 20014

TALLMAN, J. F. AND D. W. GALLAGER. *Modulation of benzodiazepine binding site sensitivity.* PHARMAC. BIOCHEM. BEHAV. **10**(5) 809–813, 1979.—Recent studies on agents which alter benzodiazepine binding site sensitivity in brain are described. GABAergic agonists enhance and antagonists inhibit binding to the brain specific benzodiazepine binding site, and the binding can be correlated with effects on neuronal cell firing in the dorsal raphe nucleus. Anions such as chloride, iodide and nitrite also enhance (^{3}H)diazepam binding and this enhancement is consistent with their role in postsynaptic inhibition. Pretreatment of animals with the anticonvulsant, diphenylhydantoin, enhances both diazepam binding and the electrophysiological response to diazepam suggesting one possible locus for the anticonvulsant action of diphenylhydantoin in brain. Taken together, these results suggest the existence of a GABA/Cl^- ionophore/BZ binding complex in brain. Preliminary results on the purification of the BZ component of this complex and fluorescent probes for its study are described.

Diazepam Diphenylhydantoin GABA Muscimol Benzodiazepine

BENZODIAZEPINES (BZ) are a class of drugs which possess a number of valuable properties. They are potent muscle relaxants, anticonvulsants, and anxiolytic agents. While extensively used in clinical medicine, the biochemical mechanisms underlying these diverse properties remain obscure. Diazepam, the most well known of the benzodiazepines, modifies the activities of various neuronal systems, and effects of diazepam on the turnover of monoamines and acetylcholine have been extensively studied [7]. None of these studies have provided a convincing hypothesis to explain the action of the benzodiazepines and many of the effects described may be secondary to diazepam's action at another site.

ELECTROPHYSIOLOGICAL STUDIES

Diazepam has been known to potentiate presynaptic inhibition in the cat spinal cord [21], and γ-aminobutyric acid (GABA) has been implicated as the transmitter at these axo-axonal synapses. This led to the speculation that diazepam modulated in a general way the synaptic transmission mediated by GABA [15]. Although it now seems clear that GABA and the benzodiazepines interact, the exact mechanism remains in doubt, as facilitation of GABAergic transmission [9], potentiation of GABAergic inhibition [6,17], direct activation of GABA receptors [10], and antagonism of GABA-mediated inhibition [12] have all been observed.

In studies from this laboratory on the dorsal raphe nucleus [13], one of us has consistently observed a synergistic effect between GABA and the BZ electrophysiologically (Fig. 1). Figure 1A shows the lack of effect of the diazepam vehicle and sequential intravenous doses of diazepam on the spontaneous firing rate of these cells. Lysergic acid diethylamide (LSD), a serotonin-like agonist, possessed its usual inhibitory properties. In Fig. 1B, animals were pretreated for 1 hr with amino-oxyacetic acid (AOAA), an agent which raises brain GABA levels by inhibting GABA catabolism. In these animals a single low dose of diazepam significantly inhibited spontaneous firing of these cells. The GABA receptor antagonist, picrotoxin, reversed this inhibition; LSD still was able to inhibit cell firing indicating that its site of action is separate from GABA and BZ. In Fig. 1C, the direct iontophoresis of BZ potentiated the inhibition due to GABA but not that due to serotonin, indicating a specific interaction between GABA and BZ but not between serotonin and BZ in this nucleus.

PHARMACOLOGICAL STUDIES

GABA-Benzodiazepine Interaction

Pharmacological and biochemical studies on the mechanism of action of the BZ have been made possible by the use of (^{3}H)-diazepam and (^{3}H)-flunitrazepam of high specific activity (see the review of Squires and associates, this volume) [2,20]. Using these ligands and receptor binding techniques (successful in identifying other brain receptors [16]), a high affinity binding site has been demonstrated in rat brain which possesses the pharmacology expected for the site of action of the BZ [2,20]. Although initially it was believed that this site did not have any relationship to a known transmitter, careful studies at low ligand concentrations indicated an intimate interaction of the high affinity BZ binding site with GABA.

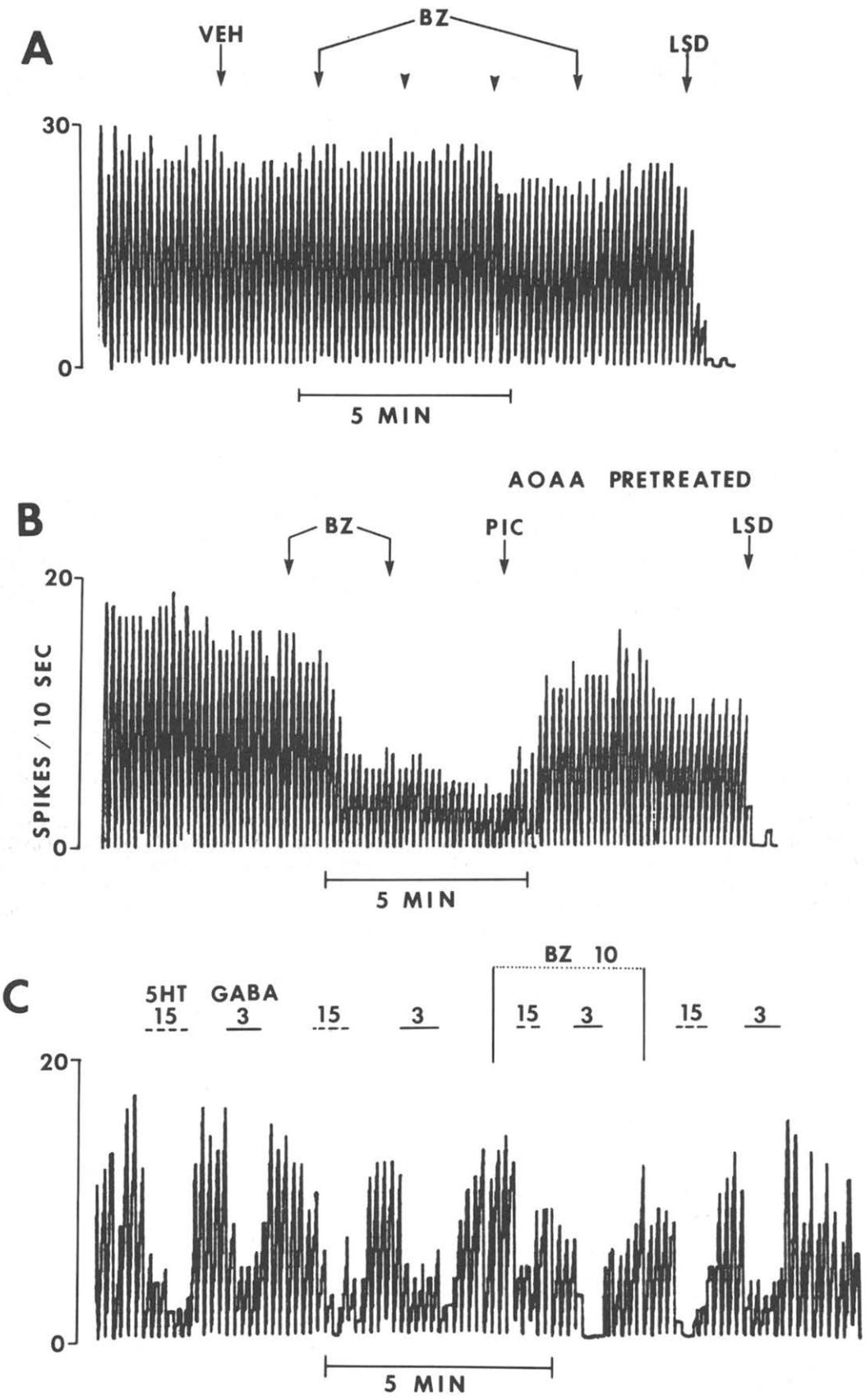
A
VEH
BZ
LSD
30
0
5 MIN
AOAA PRETREATED
B
BZ
PIC
LSD
20
SPIKES / 10 SEC
0
5 MIN
BZ 10
C
5HT GABA
15 3 15 3 15 3 15 3
20
0
5 MIN

FIG. 1

FIG. 1(A) Top trace is a frequency histogram showing the lack of effect of either the diazepam solvent (VEH) or a cumulative IV dose 6 mg/kg diazepam (BZ) (1 mg/kg, 1 mg/kg, 2 mg/kg and 2 mg/kg at arrows) on the spontaneous firing rate of a neuron in the dorsal raphe nucleus. LSD (10 μg/kg, IV) produced its usual depressant effect in this neuron.
(B) Middle trace is the spontaneous firing rate of a dorsal raphe neuron following pretreatment of the animal with a 40 mg/kg IP dose of amino-oxyacetic acid (AOAA) for 60 min prior to the recording. Within 2 min following the IV administration of 1 mg/kg diazepam the rate decreased to approximately one-third of the original firing rate. A subsequent dose of 1 mg/kg diazepam had little further effect while a 0.6 mg/kg IV dose of picrotoxin (PIC) reversed the depression almost back to prediazepam firing rates. This cell was also inhibited by an IV dose of 10 μg/kg of LSD.
(C) Bottom trace is a rate histogram showing the response of a dorsal raphe neuron to the microiontophoretic application of 5-HT, GABA and the benzodiazepine (BZ), flurazepam. A submaximal inhibition of firing rate was produced by the ejection of 5-HT (0.05 M, pH 4.0) using a current of 15 nA for 60 sec (dashed line) and the ejection of GABA (0.02 M in 0.2 M sodium chloride, pH 4.0) using a current of 3 nA for 60 sec (solid line). The simultaneous iontophoresis of the benzodiazepine (0.1 M flurazepam, pH 4.0) for 240 sec (dotted line and bracket) potentiated the response to GABA (3 nA) but not to 5-HT (15 nA). This potentiation of GABA did not last beyond the ejection of BZ.

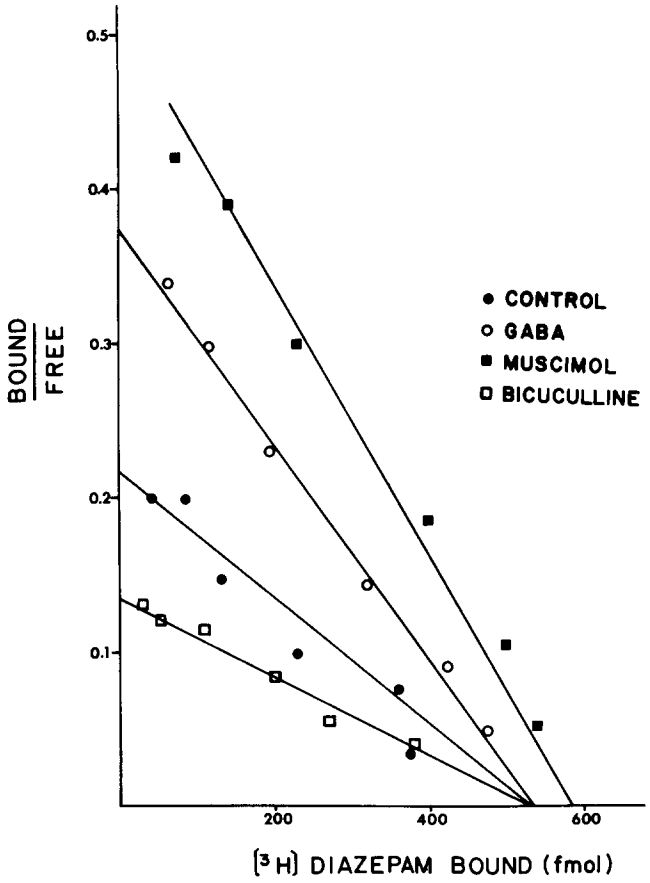

FIG. 2. Scatchard analysis of (^{3}H)-diazepam binding *in vitro*. Washed cortical membranes were obtained by homogenizing cortex in 100 vol (w/v) 50 mM Tris-HCl, pH 7.4, centrifuging at 20,000×G for 10 min, then resuspending and pelleting two additional times. Binding of (^{3}H)-diazepam was assayed as described [22] and the concentration of (^{3}H) was varied. Control (●); GABA (1×10^{-5} M) (○); muscimol (1×10^{-5} M) (■); (+)bicuculline methiodide (1×10^{-4} M) (□).

Using cortical membranes *in vitro* [22], GABA and its analogue muscimol were shown to increase the affinity of BZ for its binding site leading to enhanced binding of (^{3}H)-diazepam (Fig. 2). This effect was specific, as other putative amino acid transmitters including glycine, glutamate and aspartate did not show this increase in the affinity of the BZ binding site. In further support of this association of GABA and BZ, blockade of the GABA enhancement of BZ binding was obtained with the GABA antagonist (+)bicuculline (Fig. 1). It should be noted that the change in the apparent equilibrium constant was due to an increase in the association rate constant without alteration of the dissociation rate.

TABLE 1

EFFECT OF SYSTEMICALLY ADMINISTERED GABAERGIC DRUGS ON (^{3}H) DIAZEPAM BINDING

Treatment*	(^{3}H) Diazepam Bound (fmol/10 mg tissue)
Saline	123.7 ± 6.7
Muscimol (3.5 mg/kg)	143.7 ± 3.0†
Lioresal (20 mg/kg)	152.2 ± 2.6‡
AOAA (40 mg/kg)	151.7 ± 5.2†
GBL (450 mg/kg)	150.9 ± 7.9‡
Pentobarbital (25 mg/kg)	132.4 ± 7.6
Thiosemicarbazide (10 mg/kg)	122.8 ± 2.6

*Animals (n=6) received IP injections 60 min prior to sacrifice. (^{3}H) Diazepam binding was determined as described [14].
†$p<0.02$ compared to saline controls.
‡$p<0.005$ compared to saline controls.

The relevance of this *in vitro* study to *in vivo* pharmacological mechanisms was demonstrated in two studies [14,23] in which animals were pretreated with either muscimol or AOAA. Both of these agents increased the binding of (^{3}H)-diazepam to the high affinity site not only when the brains were removed and binding studied *in vitro* (Table 1), but also when *in vivo* binding to brain following intravenous administration of (^{3}H)-diazepam was studied (Table 2). Similarly, other agents thought to be GABA analogues, lioresal and γ-butyrolactone also enhanced binding while an anesthetic barbiturate, pentobarbital, showed only a small effect (Table 1). Attempts to lower GABA levels by inhibiting synthesis with thiosemicarbazide failed to alter BZ binding although it should be noted that this agent has only weak effects on GABA levels and perhaps a more chronic or complete depletion of GABA is required.

TABLE 2
EFFECT OF AOAA AND MUSCIMOL ON (^{3}H) DIAZEPAM BINDING *IN VIVO*

Treatment* (No. of Animals)	Diazepam Bound (fmol/2 ml) Total	Specific
Control (5)	1663 ± 124	980 ± 94
Muscimol (4)	2116 ± 111†	1423 ± 94†
AOAA (5)	4391 ± 378‡	2606 ± 206‡

*Animals were injected with AOAA, 40 mg/kg (IP) or muscimol, 5 mg/kg (IP), 1 hr prior to IV injection of (^{3}H) Diazepam (200 μCi/rat, total dose of 60 μg/kg) in a total volume of 1 ml saline representing a 1:20 dilution of diazepam. Animals were maintained at 37° and were killed 5 min after injection. Brain homogenates were treated as described in [23]. Each binding value represents the mean of quintuplicate samples.
†$p<0.05$.
‡$p<0.01$.

Benzodiazepine-Chloride Interactions

Additional evidence about the function of the BZ binding site has come from other *in vitro* binding studies. A selective anion effect on binding of (^{3}H)-diazepam has been observed. The halides (iodide, bromide and chloride, but not fluoride), nitrite, and thiocyanate enhanced the binding of (^{3}H)-diazepam by altering the affinity of the BZ site for its ligand [8]. The increase in affinity observed in these studies parallel the ability of these anions to penetrate the activated inhibitory postsynaptic membrane of cat motoneurons as measured electrophysiologically [11]. In this case, the enhanced affinity of the BZ binding site is due to a decreased dissociation rate constant and no change in the association rate.

The prediction may be made from studies of GABA and Cl^- that the effect of both these agents on enhancement of (^{3}H)-diazepam binding will be at least additive, possibly synergistic, since each affects a separate kinetic parameter; indeed, this is the case and a synergistic relation is found. This data, taken with electrophysiological evidence of Cl^- involvement in GABA function [18,19], support the speculation that in at least some regions of brain, GABA and BZ binding sites are functionally coupled to a chloride ion conductance mechanism.

Benzodiazepine-Diphenylhydantoin Interaction

Another set of pharmacological studies has been directed toward an understanding of the mechanism of action of diphenylhydantoin (DPH). DPH and diazepam are both said to possess anticonvulsant properties and some reports have indicated a common site of action for these two drugs [4]. Using electrophysiological studies similar to those described in Fig. 1, we have demonstrated that pretreatment with DPH alone does not lead to inhibition of the spontaneous firing of cells in the dorsal raphe nucleus (Gallager and Tallman, submitted). However, in animals pretreated with DPH, the BZ are effective inhibitors of cell firing. Thus, DPH pretreatment is quite similar to AOAA. However, the effects of DPH do not seem to be mediated through GABA as there is no potentiation of the GABA response. Additionally, the effects of DPH on sensitivity to BZ are a result of enhancement of BZ binding at low concentrations, producing an increased affinity of the receptor for (^{3}H)-diazepam. This effect seems to be due to an alteration in the membrane itself as extensive washing of membrane fractions did not lead to a reversal of the enhancement. Taken with the single unit recording studies, this would indicate that a soluble mediator such as GABA is not involved. The site of action of DPH appears to be distal to the GABA receptor as the addition of GABA to brain membranes from DPH pretreated animals did not lead to enhanced binding. Our current hypothesis is that DPH may work directly on the BZ/Cl^- ionophore in an allosteric fashion. Another possibility is that DPH may require time to dissociate an endogenous modulator from the receptor. A metabolite of DPH does not seem to be involved as inhibition of DPH metabolism by SKF-525A did not block the effects of DPH [1].

STUDIES ON THE SOLUBLE BENZODIAZEPINE RECEPTOR

In an attempt to understand the above interactions, we have begun to solubilize each of the components in the GABA/BZ/ionophore complex (Yousufi and Tallman, in preparation). Using washed membranes from cerebral cortex, solubilization of about 50% of the (^{3}H)-diazepam binding sites was obtained when membranes were treated with 0.5% Lubrol for 1/2 hr. The solubilized receptor remains in the supernatant when centrifuged for several hours at 100,000×G, has a molecular weight of about 200,000 daltons, and possesses an apparent equilibrium K_D of about 8 nM. The order of potency of several BZ in displacing binding from the membrane-bound receptor is maintained in the solubilized receptor indicating a relatively intact active site.

The temperature dependence of binding of the solubilized receptor is the same as the membrane-bound protein; binding is high at 4°C, declining to about 30% of maximum at 37°. Since in the solubilized receptor many of the problems inherent in interpreting data from physical studies of membrane-bound proteins are absent, we hope to be able to use fluorescent probes (see below) to characterize the active site. For example, since the temperature curve indicates significant hydrophobic interactions at the active site, using the solubilized receptor, we may be able to determine whether the binding is "entropy driven."

Purification of the receptor is underway using a BZ bound to Sepharose beads (Fig. 3) and preliminary evidence indicates that substantial purification over the soluble receptor (>100 fold) can be obtained by adsorbing solubilized receptor to the column, then using substrate elution to specifically displace the receptor. A major problem has been recovery and stability of the eluted protein, and we are presently at this point in the purification.

BENZODIAZEPINE ANALOGUES

In addition to radioactive BZ, fluorescent analogues and photoaffinity ligands (Thomas and Tallman, submitted) could be of value in the localization of receptors in brain regions and on cell surfaces. Since some controversy exists as to the cellular distribution of the BZ binding sites in brain, the ability to visualize these receptors on neuronal [3] and/or

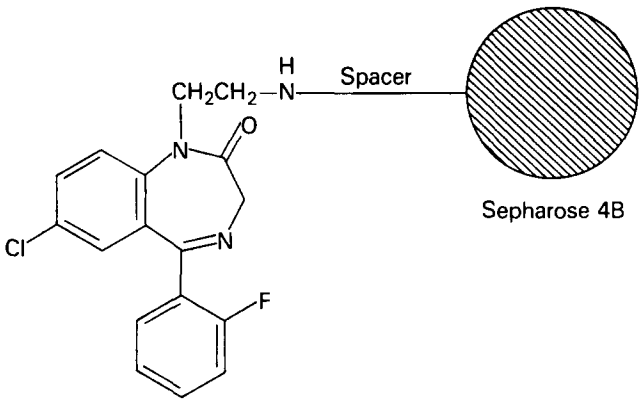

FIG. 3. Affinity column for the isolation of diazepam binding site. A flurazepam analogue (RO7-1986/1) was coupled to activated CH-Sepharose 4B (Pharmacia, Piscataway NJ) using the procedure described in their handbook on Affinity Chromatography. Unreacted RO7-1986/1 was removed by extensive washing of the gel and the gel was then stored at 4°C until use.

glial [5] elements would provide valuable information about their localization. We have prepared two fluorescent analogues by coupling fluoroscein isothiocyanate and dansylchloride to the flurazepam derivative used for the column (Fig. 3). Both fluoroscein flurazepam and dansylflurazepam are high affinity, competitive inhibitors of (^{3}H)-diazepam binding ($K_i \sim 30$ nM). Initial studies with an astrocytomia cell line with 3×10^5 high affinity (^{3}H)-diazepam binding sites per cell (Mallorga and Tallman, unpublished) indicate that it may be possible to visualize receptors on the cell using fluoroscine flurazepam and block the fluorescence with an appropriate receptor antagonist (RO5-4864) [3]. Present investigations are underway to extend this finding to brain tissue.

Dansylated flurazepam will be used in the fluorescence polarization studies described above for investigating the nature of the active site of the BZ binding site. Finally, we are preparing a radioactive photoaffinity agent with a similar structure which may assist in receptor purification studies.

In summary, we have demonstrated prominent electrophysiological and biochemical interactions between the BZ and the neurotransmitter GABA, and we conclude that the actions of these substances are functionally linked in brain. We are currently investigating the importance of this interaction as related to the various properties of the BZ and are attempting to determine the anatomical and biochemical properties of the BZ binding site.

REFERENCES

1. Axelrod, J., J. Reichenthal and B. Brodie. Mechanism of the potentiating action of β-diethylaminoethyldiphenylproplacetate. *J. Pharmac. exp. Ther.* **112:** 49–54, 1954.
2. Braestrup, C., R. Albrechtsen and R. F. Squires. High densities of benzodiazepine receptors in human cortical areas. *Nature* **269:** 702–704, 1977.
3. Braestrup, C. and R. Squires. Brain specific benzodiazepine receptors. *Br. J. Psychiat.* **133:** 249–260, 1978.
4. Camerman, A. and N. Camerman. Diphenylhydantoin and diazepam: molecular structure similarities and steric basis of anticonvulsant activity. *Science* **168:** 1457–1458, 1970.
5. Chang, R. S. L., V. T. Tran, S. E. Poduslo and S. H. Snyder. Glial localization of benzodiazepine receptors in mammalian brain. *Neurosci. Abst.* **IV:** 1632, 1978.
6. Chio, D. T., D. H. Farb and G. D. Fischbach. Chlordiazepoxide selectively augments GABA action in spinal cord cultures. *Nature* **269:** 342–344, 1977.
7. Costa, E. and P. Greengard. *Mechanism of Action of Benzodiazepines, Advances in Biochemical Psychopharmacology,* Vol. 14. New York: Raven Press, 1975.
8. Costa, T., D. Rodbard and C. B. Pert. Is the benzodiazepine receptor coupled to a chloride anion channel? *Nature,* in press.
9. Curtis, D. R., C. J. A. Game and D. Lodge. *In vivo* inactivation of GABA and other inhibitory amino acids in the cat nervous system. *Expl Brain Res.* **25:** 413–428, 1976.
10. Dray, A. and D. W. Straughan. Benzodiazepines: GABA and glycine receptors on single neurons in the rat medulla. *J. Pharm. Pharmac.* **28:** 314–315, 1976.
11. Eccles, J. C. The ionic mechanisms of excitatory and inhibitory synaptic action. *Ann. N.Y. Acad. Sci.* **137:** 473–495, 1966.
12. Gähwiler, B. H. Diazepam and chlordiazepoxide: powerful GABA antagonists on explants of rat cerebellum. *Brain Res.* **107:** 176–179, 1976.
13. Gallager, D. W. Benzodiazepines: potentiation of a GABA inhibitory response on the dorsal raphe. *Eur. J. Pharmac.* **49:** 133–143, 1978.
14. Gallager, D. W., J. W. Thomas and J. F. Tallman. Effect of GABAergic drugs on benzodiazepine binding site sensitivity in rat cerebral cortex. *Biochem. Pharmac.,* in press.
15. Haefely, W. E. Central actions of benzodiazepines: general introduction. *Br. J. Psychiat.* **133:** 231–238, 1978.
16. Hollenberg, M. D. and P. Cuatrecasas. Biochemical identification of membrane receptors: principles and techniques. In: *Handbook of Psychopharmacology,* Vol. 2, edited by L. L. Iversen, S. D. Iversen and S. H. Snyder. New York: Plenum Press, 1975, pp. 129–179.
17. Kozhechkin, S. H. and R. U. Ostrovskaya. Are benzodiazepines GABA antagonists? *Nature* **269:** 72–73, 1977.
18. Krnjevic, K. Glutamate and γ-aminobutyric acid in brain. *Nature* **228:** 119–214, 1970.
19. MacDonald, R. L. and J. L. Barker. Specific antagonism of GABA-mediated post-synaptic inhibition in cultured mammalian spinal cord neurons: a common mode of convulsant action. *Neurology* **28:** 325–330, 1978.
20. Möhler, H. and T. Okada. Benzodiazepine receptor: demonstration in the central nervous system. *Science* **198:** 849–851, 1977.
21. Schmidt, R. F., E. Vogel and M. Zimmerman. Die wirkung von diazepam auf de prasynaptische hemmung und andere ruckensnarksreflexe. *Naunyn Schmiedebergs Arch. Pharmac.* **258:** 69–82, 1967.
22. Tallman, J. F., J. W. Thomas and D. W. Gallager. GABAergic modulation of benzodiazepine binding site sensitivity. *Nature* **274:** 383–385, 1978.
23. Tallman, J. F., J. W. Thomas and D. W. Gallager. Identification of diazepam binding in intact animals. *Life Sci.* **24:** 873–880, 1979.