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Some Properties of Brain Specific Benzodiazepine Receptors: New Evidence for Multiple Receptors

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SQUIRES, R. F., D. I. BENSON, C. BRAESTRUP, J. COUPET, C. A. KLEPNER, V. MYERS AND B. BEER. *Some properties of brain specific benzodiazepine receptors: New evidence for multiple receptors.* PHARMAC. BIOCHEM. BEHAV. **10**(5) 825–830, 1979.—Several new lines of evidence suggest the existence of two or more distinct types of benzodiazepine receptors, in contrast to earlier results suggesting the presence of only one class of receptors. Appropriate thermoinactivation experiments indicate two receptors with different thermostabilities. Several triazolopyridazines, with some of the pharmacological properties of anxiolytics have recently been shown to displace ^{3}H-diazepam and ^{3}H-flunitrazepam with Ki values in the 6 to 100 nanomolar range. These new substances are active in conflict tests in rats and monkeys and prevent metrazol induced seizures *in vivo*, but strikingly lack the ataxia and sedative properties of the benzodiazepines. Hill analyses of dose-response curves for some of these substances yields Hill coefficients in the range of 0.4–0.6, suggesting that these compounds may be able to discriminate between several types of benzodiazepine receptors.

Benzodiazepine receptors Multiple receptors

BRAIN specific benzodiazepine receptors which selectively recognize pharmacologically and clinically active benzodiazepines (BDZ) have been extensively characterized in several vertebrate species using ^{3}H-diazepam and ^{3}H-flunitrazepam as ligands [1, 2, 4, 6, 12, 15–18, 22, 24, 25]. These two ligands bind to the receptors in rat brain with high affinity (K_D values for ^{3}H-flunitrazepam and ^{3}H-diazepam are about 1 nM and 4 nM, respectively) at 0°, and Scatchard analysis of equilibrium binding in several buffer systems indicates a single, homogenous class of binding sites [4–6, 12, 15, 16, 24, 25].

A large number of clinically and pharmacologically active BDZ's displace ^{3}H-diazepam and ^{3}H-flunitrazepam from the brain specific binding sites with Hill coefficients near unity (also indicating a single class of binding sites) and Ki values which correlate well with corresponding ED_{50} values in several pharmacological tests predictive of anxiolytic activity in man [5]. These correlations strongly indicate that the pharmacological and clinical effects of the BDZ's *in vivo* are mediated through the ^{3}H-diazepam and ^{3}H-flunitrazepam binding sites, although it is not yet known whether the BDZ's act as agonists or antagonists to the postulated endogenous ligand(s).

Binding of both ^{3}H-diazepam and ^{3}H-flunitrazepam are highly temperature dependent: the apparent affinity constants (K_D), as well as the apparent B_{max} values, are functions of temperature. At 37° the apparent K_D values for ^{3}H-diazepam (24 nM) and ^{3}H-flunitrazepam (6.6 nM) are 6–7 times greater than the values determined at 0° [6]. The apparent B_{max} for ^{3}H-diazepam (46 pmol/gm whole brain) is about 7 times higher at 0° than at 37° (6.9 pmol/gm), while the apparent B_{max} for ^{3}H-flunitrazepam (64 pmol/gm) at 0° is only about twice that at 37° (29 pmol/gm) [6]. The dissociation rates of both ^{3}H-diazepam and ^{3}H-flunitrazepam from brain specific binding sites are also highly temperature dependent. However, at 0° the dissociation of ^{3}H-diazepam is about 8 times more rapid than the dissociation of ^{3}H-flunitrazepam. This fact, together with the overall higher affinity of ^{3}H-flunitrazepam for the BDZ receptors, makes this substance the ligand of choice for BDZ receptor binding studies [6,24]. Several lines of evidence suggest that ^{3}H-diazepam and ^{3}H-flunitrazepam bind essentially to the same receptors [6]

BDZ receptors are unevenly distributed in brain, with the highest concentrations in all neocortical regions, intermediate levels in the limbic structures and cerebellum and lowest levels in pons, medulla oblongata and spinal cord. So far, the "brain-specific" BDZ receptors have not been detected in significant concentrations anywhere outside the CNS [4,26].

There are no detectable BDZ receptors on primary mouse astrocytes in tissue culture, indicating the absence of these receptors from glial cells in brain [3]. It has so far proved difficult to localize BDZ receptors in brain using a variety of lesions, probably indicating that the receptors are located on

both cell bodies and terminals of many different kinds of neurons [6]. The selective neurotoxin, kainic acid, particularly when injected twice into the same brain region, causes significant reductions in BDZ receptor concentrations in the substantia nigra [6], and in the cerebellum (C. Braestrup, M. Nielsen and R. F. Squires, unpublished data). There is also a significant decrease in the concentration of BDZ receptors in the cerebellum, but not frontal cortex, of "nervous" mutant mice, compared to normal litter-mate controls, indicating the presence of BDZ receptors on cerebellar Purkinje cells [9], a conclusion also supported by the ability of microiontophoretically applied flurazepam to inhibit the firing of these cells [10]. Recently, a reduction of BDZ receptors in the putamen of human patients dying with Huntingdons Chorea, a disease with known neuronal degeneration, has been reported [7,20].

Phylogenetic surveys indicate that brain-specific BDZ receptors are found in the brains of all vertebrate species, but are lacking in the nervous tissue of all invertebrate species [19]. Interestingly, BDZ receptors are not detectable in the brains of cyclostomes and elasmobranches, but occur in those ancestral fishes giving rise to the higher bony fishes [7]. Opiate receptors are found in the brains of cyclostomes and elasmobranches [7].

More than 200 non-benzodiazepines, representing 22 distinct pharmacological classes, as well as 14 presumed neurotransmitters in the CNS, and more than 100 peptides, exhibited low affinity (Ki>0.1 mM) for ^{3}H-diazepam binding sites and were, therefore, considered physiologically irrelevant for the BDZ's suggesting that the "endogenous ligand(s)" for the brain specific BDZ receptors has not yet been identified [1,5].

There have been several reports of substances isolated from mammalian brain with some affinity for ^{3}H-diazepam binding sites [8,14]. Two such substances have been identified as inosine and hypoxanthine [23]. A large number of purines including several adenosine and guanosine nucleotides also inhibit ^{3}H-diazepam and ^{3}H-flunitrazepam binding with Ki values close to those for inosine and hypoxanthine (C. Klepner and R. Squires, unpublished). The extremely low affinities of these substances (Ki values near 1 mM) and their lack of specificity make physiological roles for them improbable. The structural similarities between the purines, the active triazolopyridazines [10] and indole are striking. If the endogenous ligand(s) for the BDZ receptors were peptides, the receptor might be able to recognize some of its constituent amino acids individually at very high concentrations. All 20 naturally occurring amino acids were, therefore, tested for inhibition of ^{3}H-flunitrazepam binding to rat brain membranes.

L-tryptophan was clearly the strongest amino acid inhibitor of ^{3}H-flunitrazepam binding with an ED_{50} of 5 mM and D-tryptophan was about equipotent. Other amino acids with detectable activity at 20 mM were L-methionine, D- and L-phenylalanine, D- and L-proline, D- and L-glutamic acid, D- and L-cystine, D- and L-aspartic acid and L-arginine. In a second series of experiments some dipeptides containing the most active amino acids were tested. Of these, several (but not all) dipeptides containing L-tryptophan were more active than tryptophan itself, with IC_{50} values below 1 mM. By far the most potent of these is L-tryptophanyl-glycine with an IC_{50} near 80 μM, making it about 10 times more potent than inosine or hypoxanthine. Several peptides containing L-proline and L-aspartic acid were active at the 1 mM level ($\geq$30% inhibition). It is striking that an amino acid sequence occurring in β-lipotropin Trp-Gly-Ser-Pro-Pro-Lys-Asp

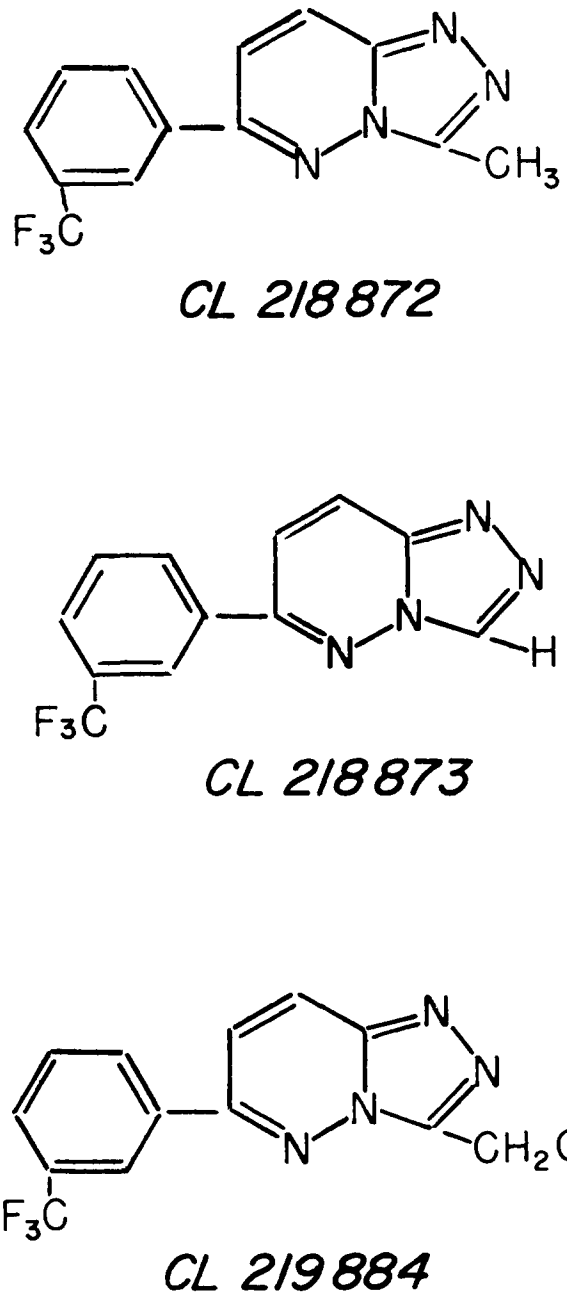

FIG. 1. Structural formulas for 3 triazolopyridazines with affinity for the benzodiazepine receptors.

(residues 52–58) contains both the active Trp-Gly sequence as well as 2 other amino acids with some activity in the binding (Pro and Asp).

The 51–52 Trp-Gly sequence in β-lipotropin is part of a near repeat of the ACTH sequence, which, curiously, also occurs intact in a large precursor protein (M.W.$\approx$30,000) together with β-lipotropin [13,21].

The first 12 amino acid residues of ACTH contain Trp-Gly, as well as several of the active amino acids (Arg, Glu, Phe and Pro). However, neither ACTH [5] nor β-MSH (S. Paul, personal communication) block ^{3}H-diazepam binding

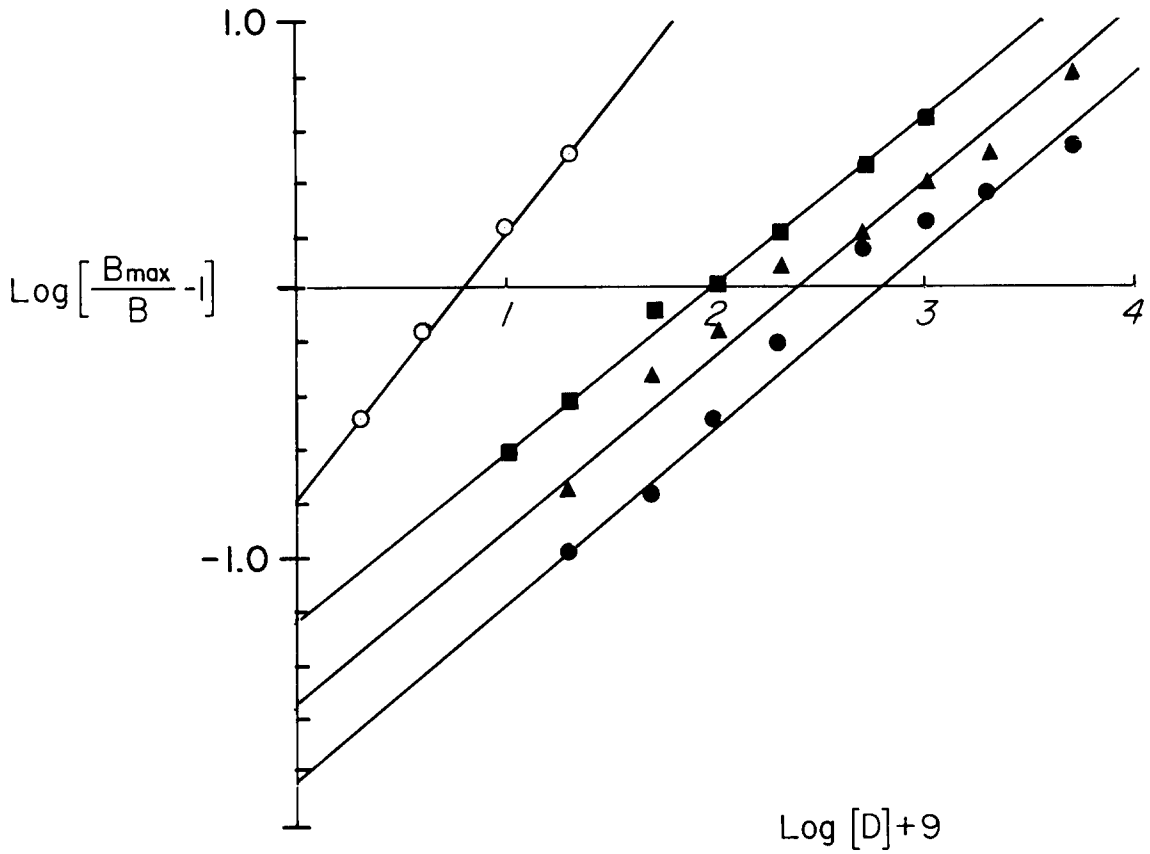

FIG. 2. Hill plots for displacement of ^{3}H-flunitrazepam from rat brain receptors by diazepam (○), CL 218,872 (■), CL 219,884 (▲), and CL 218,873 (●). Assay as described in Fig. 4.

to rat brain membrane receptors. It is tempting to speculate that the endogenous ligand for the BDZ receptor is a peptide with an amino acid sequence homologous to the ACTH/β-lipotropin sequences described above, perhaps arising during evolution by gene duplication and subsequent modification.

A new class of pharmacologically unique substances, several triazolopyridazines (TPZ's) synthesized at Lederle Laboratories (Fig. 1), exhibit activity in restoring behavior suppressed by punishment, and in antagonizing metrazol convulsions. In contrast to the BDZ's, the TPZ's do not produce ataxia (inclined screen test) or sedation, even at doses 5–10 times higher than those which are effective in the conflict and metrazol tests [10]. Several TPZ's displace ^{3}H-diazepam and ^{3}H-flunitrazepam with Ki values in the 100–1000 nM range. However, the dose-response curves for these substances are much "flatter" than corresponding curves for the benzodiazepines, and Hill coefficients for the TPZ's are consistently in the range of 0.5–0.7, in contrast to a large number of benzodiazepines, which yield Hill coefficients near unity (Fig. 2, Table 1). The Hill coefficients for all substances tested were similar in Tris·HCl and sodium phosphate buffer. Hill coefficients significantly less than one may indicate the presence of two (or more) binding sites for ^{3}H-diazepam and ^{3}H-flunitrazepam with different affinities for these TPZ's. This is supported by another experiment in which the rat brain P_2-fraction was pre-treated by heating for 60 minutes at 60° in 50 mM sodium phosphate buffer, pH 7.5, to selectively inactivate the thermolabile component. Dose response curves for CL 218,872 and diazepam, respectively, using this partially heat inactivated preparation, show that for CL 218,872 the Hill coefficient increases from 0.60 to 0.85 and its apparent IC_{50} value from 40 to 98 nM. In contrast, partial heat inactivation of the BDZ receptor preparation does not change either the Hill coefficient (near 1) nor the IC_{50} value for diazepam (Fig. 3).

Heat inactivation of ^{3}H-diazepam binding sites in rat brain membranes suspended in 50 mM Tris·HCl, pH 7.4, revealed a first-order disappearance with a half-life of about 10 minutes at 60° [4]. However, more recent experiments show that when heat inactivation is carried out in 50 mM sodium phosphate buffer, pH 7.5, ^{3}H-flunitrazepam binding sites disappear in an apparently bi-phasic fashion with half-lives of about 10 minutes and 70 minutes at 60° (see Fig. 4).

An investigation of BDZ receptors in the brains of 2 normal and 2 photoepileptic baboons (Papio papio) showed that the concentrations of ^{3}H-flunitrazepam binding sites in cerebral cortex, as well as the K_D values for ^{3}H-flunitrazepam binding were very similar for all four baboons [26]. However, heat inactivation of BDZ receptors in homogenates of cortex suspended in 50 mM sodium phosphate, pH 7.4, revealed that one animal out of the four, a spontaneously epileptic female baboon, exhibited a clearly less heat stable flunitrazepam binding site. In all four animals the disappearance of ^{3}H-flunitrazepam binding sites was apparently bi- (or poly-) phasic at 65° in 50 mM sodium phosphate buffer. Be-

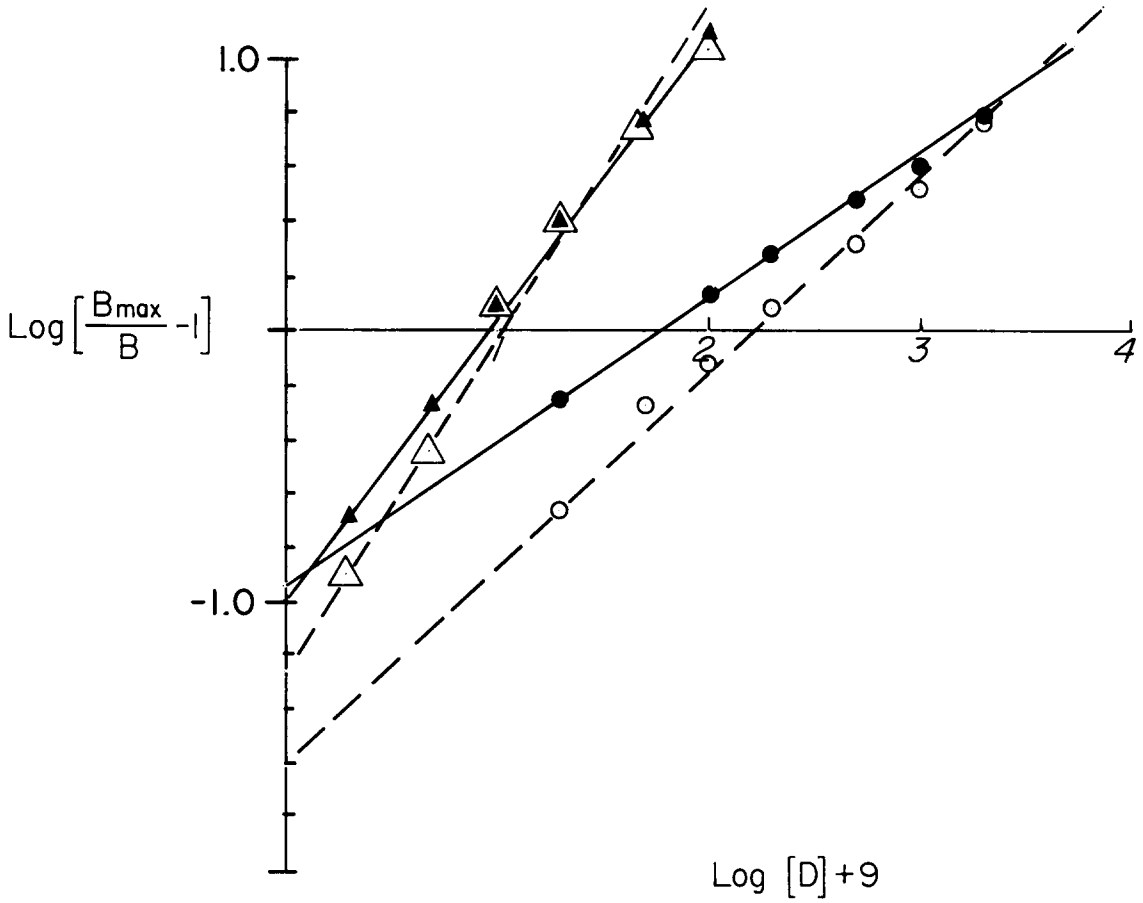

FIG. 3. Effect of pre-heating rat brain membranes for 60 minutes at 60° in 50 mM sodium phosphate buffer, pH 7.5, on Hill coefficients and Ki values for diazepam (△, ▲) and CL 218,872 (○, ●). Open symbols for pre-heated membranes, solid symbols for non-heated controls.

TABLE 1

INHIBITION OF SPECIFIC ^{3}H-DIAZEPAM BINDING (1.5 nM) OR ^{3}H-FLUNITRAZEPAM BINDING (1 nM) TO RAT BRAIN MEMBRANES BY TRIAZOLOPYRIDAZINES

Drug	^{3}H-Diazepam Hill Coefficient ± SD	*Ki ± SD	^{3}H-Flunitrazepam Hill Coefficient ± SD	*Ki ± SD
CL 218,872	0.69 ± 0.06	67 ± 20	0.65 ± 0.06	66 ± 30
CL 218,873	0.64 ± 0.09	341 ± 110	0.67 ± 0.05	407 ± 151
CL 219,884	0.60 ± 0.02	156 ± 49	0.65 ± 0.03	249 ± 56

$$*Ki = \frac{IC_{50}}{1 + [L]/K_d}$$

Where: IC_{50}=50% Inhibition of specific binding.
[L]=Concentration of Ligand (1.5 nM for Diazepam).
(1.0 nM for Flunitrazepam).
K_d=Affinity Constant (3.0 nM for Diazepam).
(1.0 nM for Flunitrazepam).

N=2 to 4 determinations in $NaPO_4$ buffer, 50 mM, pH 7.5.

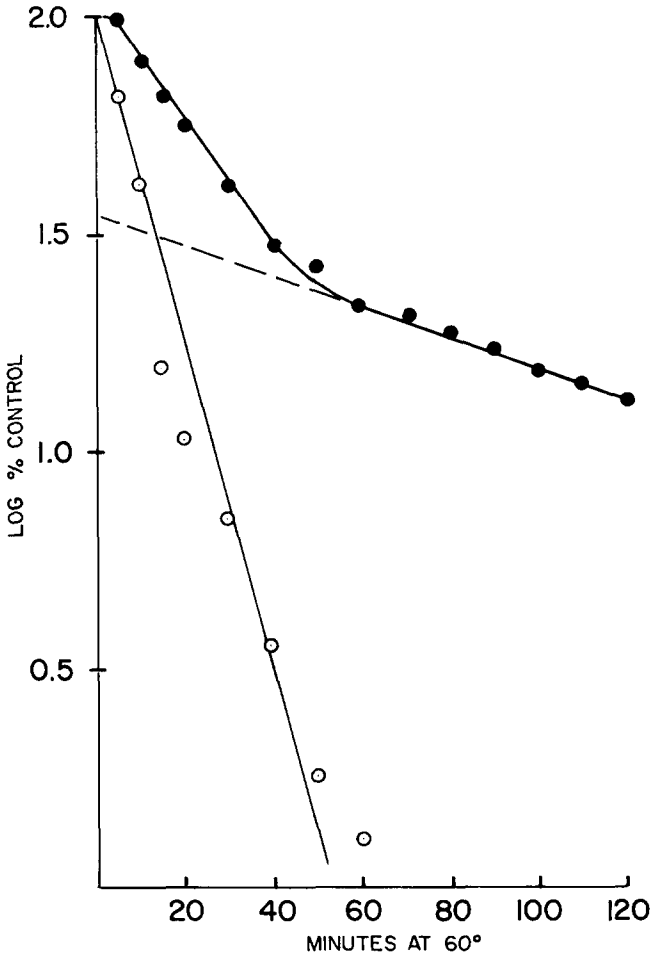

FIG. 4. Heat inactivation of ^{3}H-flunitrazepam binding sites in a P_2-synaptosomal fraction from rat brain. Tubes containing about 0.2 mg P_2 membrane protein in 1.8 ml 50 mM sodium phosphate (●) or Tris·HCl buffer (○), pH 7.5, were kept at 60° in a water bath for times ranging from 0 to 120 minutes. After subsequent cooling, 100 μl of either buffer or clonazepam (to give a final concentration of 1 μM) for total and non-specific binding, respectively, was added to each tube, followed by 100 μl of ^{3}H-flunitrazepam (NEN) to give a final concentration of 1 nM. The tubes were then incubated for 30 minutes at 37° followed by 30 minutes at 0°. The samples were filtered under vacuum through Whatman GF/C glass fiber filters. The filters were washed twice with 5 ml iced buffer and counted by conventional scintillation counting.

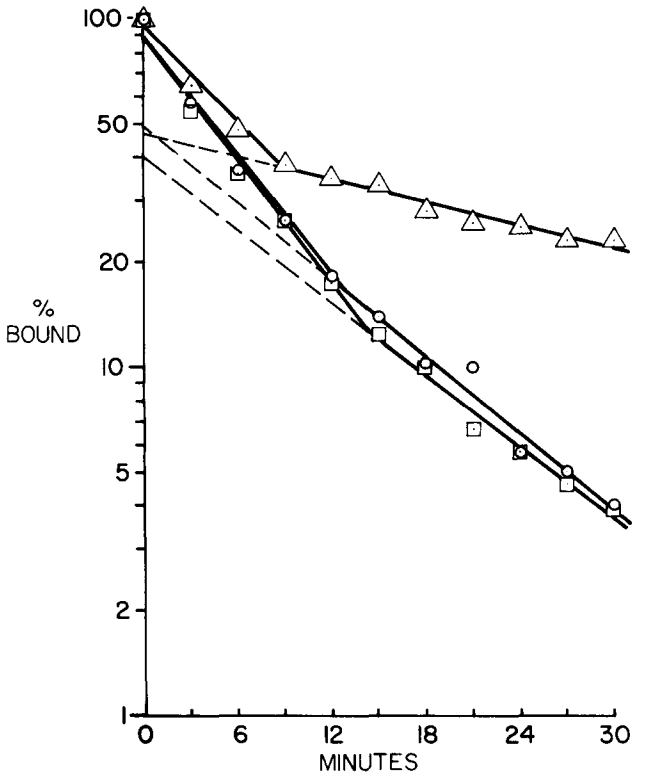

FIG. 5. Dissociation of ^{3}H-diazepam from binding sites in rat brain membranes in presence of 1.0 μM each of unlabeled diazepam (○), flunitrazepam (□) and CL 218,872 (△). Equilibrium binding of ^{3}H-diazepam was established as described in Fig. 4, before adding the unlabeled displacer. The dissociation was stopped at different times by rapid filtration.

cause of the great similarity of ^{3}H-flunitrazepam binding sites in the brains of all four baboons, it was speculated that the spontaneously epileptic baboon might lack a factor in its brain which normally stabilizes the BDZ receptors, for example, a normal chloride ionophore which might be coupled to the receptor [26].

More detailed investigations of ^{3}H-diazepam and ^{3}H-flunitrazepam dissociation from brain specific binding sites indicated the presence of two components at 0° in either 50 mM sodium phosphate or 50 mM Tris·HCl (both pH 7.5). The two components of ^{3}H-diazepam dissociation have half-lives of 2 and 9 minutes after addition of 1 μM of non-radioactive diazepam for flunitrazepam (Fig. 5). When CL 218,872 (1 μM) is used as the displacing substance, the fast component of ^{3}H-diazepam dissociation is unaffected, while the "slow component" becomes even slower (the apparent half-time increasing from 9 to 27 minutes). Suggesting that CL 218,872 may have significantly lower affinity for the "slow receptor" than for the "fast receptor."

The original conclusion that the brain specific BDZ receptor represented a single homogeneous class of binding sites was based on: linear Scatchard plots; monophasic thermal inactivation of the binding sites in Tris·HCl buffer; Hill coefficients near unity for 15 BDZ's with widely varying affinities for the receptors; near-linear dissociation curves ("off-rates") for ^{3}H-diazepam and ^{3}H-flunitrazepam; and, very similar Ki values for 14 BDZ's in human frontal cortex and cerebellum, respectively. However, since this early work was done a number of additional observations tended to suggest receptor multiplicity: (1) in codfish brain, ^{3}H-diazepam binding yields curved Scatchard plots [7,19]; (2) binding in three mammalian species (rat, mouse, baboon) exhibited bi- or poly-phasic thermal inactivation curves in 50 mM sodium phosphate, pH 7.5; (3) several new classes of non-benzodiazepines, including the triazolopyridazines described here, displace bound ^{3}H-diazepam and ^{3}H-flunitrazepam with Hill coefficients of 0.5 to 0.7. After selective removal of one binding component by heating, the Hill coefficient for a TPZ (CL 218,872) increases to near unity together with an increase in Ki; and (4) ^{3}H-diazepam dissociates from its binding sites in a bi- or poly-phasic fash-

ion, an effect which can be amplified by carrying out the dissociation in the presence of excess CL 218,872.

Taken together, these findings suggest the existence of two or more brain specific "benzodiazepine-receptors." It is important to note that it has so far not been possible to find a buffer system which yields a nonlinear Scatchard plot for ^{3}H-diazepam or ^{3}H-flunitrazepam in rat brain. Therefore, most benzodiazepines must bind to all types of receptors with almost identical affinities. On the other hand, evidence presented here indicates that the TPZ's bind to one type of BDZ receptor with considerably higher affinity than to the other type. This suggests that the postulated multiple receptors may be identical with respect to a region which recognizes benzodiazepines, but differ with respect to an adjacent functional structure, such as an ionophore or nucleotide cyclase, which also participate in binding TPZ's.

Several TPZ's have been found active in anti-conflict and anti-metrazol tests, while producing relatively little sedation, ataxia or potentiation of ethanol and barbiturates [10]. These findings indicate that the anti-conflict and anti-metrazol effects of the benzodiazepines might be mediated by one receptor type, while sedation, ataxia and ethanol potentiation may be mediated by other receptor types.

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