MOLECULAR PHARMACOLOGY, 26:228–240

Kinetic Differences between Type I and Type II Benzodiazepine Receptors

ROSARIO R. TRIFILETTI,[1] MATHEW M. S. LO, AND SOLOMON H. SNYDER[2]

Departments of Neuroscience, Pharmacology and Experimental Therapeutics, and Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received November 1, 1983; Accepted April 16, 1984

SUMMARY

The kinetic properties of [^{3}H]flunitrazepam ([^{3}H]FNZ) binding to central benzodiazepine receptors of bovine brain membranes at 0° have been studied. Both dissociation and association (under pseudo-first order conditions) kinetics are biphasic, enabling definition of "fast" and "slow" compartments for both processes. In four brain regions examined, the proportion of receptor in the rapidly associating and rapidly dissociating compartments correlates with the proportion of Type I benzodiazepine receptor as determined from equilibrium radioligand binding studies in the four brain regions examined. Preincubation with the Type I-selective drugs CL-218,872 or methyl-β-carboline-3-carboxylate (Ro 22-7497) reduces the size of the fast but not the slow kinetic compartments. Potencies of the drugs in eliciting these alterations in [^{3}H]FNZ kinetics correlate with their potencies in displacing [^{3}H]FNZ from Type I benzodiazepine receptors. Thus, in membrane preparations the more rapidly associating and dissociating site appears to represent the pharmacologically defined Type I benzodiazepine receptor, whereas the Type II receptors display slower association and dissociation kinetics. Soluble Type I receptors also display more rapid dissociation and association kinetics than soluble Type II receptors.

INTRODUCTION

A variety of evidence suggests the existence of at least two distinct benzodiazepine receptor subtypes. Drugs such as the triazolopyridazine CL-218,872 and several β-carbolines differ in their potencies in various brain regions and thus are thought to have selectively higher affinity for Type I than Type II receptors (1–5). There appear to be differences in heat sensitivitiy (3, 6) and apparent molecular weights (7) between the two apparent receptor subtypes. We have obtained direct evidence for two physically distinct receptors based on differential solubility from brain membranes, with Type I receptors being more resistant to detergent solubilization (8, 9). Light microscopic autoradiography shows that the differential distribution of detergent-resistant and -sensitive receptors corresponds closely to the respective variations in distribution of pharmacologically defined Type I and Type II receptors (10, 11). Moreover, striatonigral lesions markedly reduce numbers of apparent nigral Type II receptors while augmenting numbers of Type I sites 2- to 3-fold (12).

Saturation analysis of benzodiazepine binding to brain membranes generally indicates a single population of binding sites (13, 14), suggesting that benzodiazepines may have similar affinities for Type I and Type II receptors (15). In numerous studies, the dissociation of [^{3}H]diazepam or [^{3}H]FNZ[3] from binding sites appears biphasic (3, 16–18). Some kinetic studies suggest that these biphasic dissociation curves reflect two interconvertible receptor states (16). In the present study, we present evidence that biphasic association and dissociation rates of [^{3}H]FNZ to bovine brain benzodiazepine receptors reflect a composite of the kinetic behavior of Type I and Type II benzodiazepine receptors. Type I receptors display more rapid association and dissociation rates than do Type II receptors.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]FNZ (72.4 Ci/mmol) was obtained from New England Nuclear Corporation (Boston, Mass.). Unlabeled β-CCM (Ro 22-7497), FNZ, and diazepam were kind gifts of Dr. W. Scott, Hoffmann-La Roche, Inc. (Nutley, N. J.). CL-218,872 was a gift from Lederle Labo-

This work was supported by United States Public Health Service Grants MH-18501, DA-00266, and NS-16375, and a grant from the McKnight Foundation.

[1] Recipient of United States Public Health Service Training Grant GM-073090.

[2] Recipient of United States Public Health Service Research Scientist Award DA-00074.

[3] The abbreviations used are: FNZ, flunitrazepam; β-CCM, methyl β-carboline 3-carboxylate; GABA, γ-aminobutyric acid; PEI, polyethyleneimine.

0026-895X/84/050228-13$02.00/0

ratories (Pearl River, N. Y.). All other materials were obtained from commercial sources.

Preparation of brain membranes. Fresh calf brain was obtained locally, dissected, and either frozen immediately at −70° or used in experiments. Tissues were homogenized in 10 volumes of ice-cold 50 mM Tris-citrate (pH 7.2 at 0°) by Polytron (Brinkmann Instruments, Westbury, N. Y.) and centrifuged at 48,000 × *g* for 15 min. Pellets were then subjected to five freeze/thaw/wash cycles, with resuspension in 50 mM Tris-citrate (pH 7.2) throughout. As shown by others (19, 20), this washing procedure reduces endogenous GABA to negligible levels. The extensively washed pellet was resuspended to 2 mg of original tissue (wet weight) per milliliter in the homogenization buffer and used immediately, or stored at −20°.

Preparation and physical separation of soluble benzodiazepine receptors. Bovine cortex membranes, prepared as described above, were solubilized according to the procedure of Lo *et al.* (8, 9) with 1% (w/v) Triton X-100 as the detergent. Both detergent and detergent plus salt extracts were dialyzed overnight at 4° against 50 volumes of 0.1% Triton X-100 in 50 mM Tris-citrate (pH 7.2), as the stability of soluble benzodiazepine binding activity improves considerably if the detergent concentration is reduced, in agreement with the findings of Stephenson *et al.* (21). Soluble extracts were used in experiments immediately after preparation.

Association kinetics of [^{3}H]FNZ to bovine brain membranes. Association of [^{3}H]FNZ with receptors was examined under pseudo-first order conditions at 0°. Brain membranes were prepared as described, [^{3}H]FNZ (1 nM final concentration) was added, and triplicate 1.0-ml aliquots were rapidly removed at various times and filtered over prewashed Whatman GF/B filters followed by three washes with 5 ml of ice-cold buffer. Filters were counted in 10 ml of Formula 947 (New England Nuclear Corporation) by liquid scintillation spectrometry, at 46% counting efficiency. Specific binding was defined as the binding observed less binding in the presence of 10 μM FNZ. Unless otherwise noted, nonspecific binding was determined at the end of the assay ($t \geq 30$ min), as control experiments revealed that nonspecific binding was constant in less than 1 min of incubation.

Association data were fit by unweighted least squares to the integrated rate equation appropriate for pseudo-first order association into *n* independent compartments (see Appendix A for derivation).

$$\frac{B_e - B_t}{B_e} = \sum_{j=1}^{N} f_j \exp\{-(k_j[L] + k_{-j})t\} \quad (1)$$

subject to the constraint

$$\sum_{j=1}^{N} f_j = 1 \quad (2)$$

where B_e and B_t are the amounts of specifically bound [^{3}H]FNZ at equilibrium and time *t*, respectively, k_j and k_{-j} are forward and reverse rate constants for the *j*th compartment, f_j is the fraction of the total specifically bound ligand bound specifically to the *j*th kinetic compartment at equilibrium, and $[L]$ is the free [^{3}H]FNZ concentration. In all association experiments, the specifically bound [^{3}H]FNZ concentration was always less than 5% of the total [^{3}H]FNZ concentration. This confirmed that pseudo-first order conditions obtained and enabled approximation of the free [^{3}H]FNZ concentration by the total [^{3}H]FNZ concentration with negligible error.

Dissociation kinetics of [^{3}H]FNZ to bovine brain membranes. Dissociation of [^{3}H]FNZ from bovine brain membranes was examined at 0° under conditions where negligible reassociation of dissociated label occurs. Lack of reassociation was confirmed (data not shown) according to standard procedures (22, 23). In experiments where dissociation was initiated by excess unlabeled drug, bovine membranes [2 mg (wet weight) per milliliter] were incubated with [^{3}H]FNZ (1 nM final concentration) for 60 min at 0°, unless otherwise noted, in the presence or absence of various drugs. Dissociation was initiated by the addition of unlabeled FNZ (10 μM final concentration), and 1-ml aliquots were filtered and counted as before. In other experiments where dissociation was initiated by limiting dilution, bovine brain membranes [200 mg (wet weight) per milliliter] were incubated as above and diluted 100-fold into ice-cold 50 mM Tris-citrate (pH 7.2) or 50 mM Tris-citrate (pH 7.2) containing various drugs at specified concentrations (22, 23). In each case, nonspecific binding was defined as the binding remaining 60 min after the initiation of dissociation, and was determined separately only at the end of the experiment, as others have done (16).

Dissociation data were fit to an equation of the form (see Appendix A for derivation)

$$\frac{B_t}{B_0} = \sum_{j=1}^{N} f_j \exp\{-k_j t\} \quad (3)$$

in a manner similar to association data. B_0 and B_t represent the amount of specific [^{3}H]FNZ bound at time zero and time *t*, respectively, k_{-j} is the dissociation rate constant for the *j*th kinetic compartment, and f_j is the fraction of total specifically bound ligand specifically bound to the *j*th kinetic compartment prior to initiation of dissociation. During all dissociation experiments, the specifically bound [^{3}H]FNZ concentration was always less than 5% of the total [^{3}H]FNZ concentration, indicating that "ligand excess" conditions apply (see Appendix A).

Kinetic studies on physically separated soluble benzodiazepine receptors. Recently, Bruns *et al.* (24) reported a convenient binding assay for a variety of soluble receptors, including soluble benzodiazepine receptors, based on the ability of PEI-coated filters to retain soluble receptor. Briefly stated, after incubation with [^{3}H]FNZ, 1.0-ml aliquots of soluble extracts (approximately 0.5 mg of protein per milliliter) were filtered over Whatman GF/B filters that had been presoaked for at least 3 hr with an aqueous solution of 0.3% (v/v) PEI made to pH 7.2 with HCl. Filters were then washed, dried, and counted as previously described. For soluble benzodiazepine receptors, we have confirmed (data not shown) the finding (24) that the PEI-coated filter method gives results comparable to the more tedious polyethylene glycol/γ-globulin precipitation assay for soluble receptor (25).

RESULTS

General kinetic and equilibrium properties of [^{3}H]FNZ binding to bovine cerebral cortex membranes. Previous studies have generally found a single apparent population of [^{3}H]FNZ binding sites in rat (13–15) and bovine (26 27) cerebral cortex. Kinetic studies, on the other hand, indicate a biphasic dissociation (Fig. 1A). Biphasic dissociation curves may reflect dissociation of the ^{3}H-ligand from two (possibly interconvertible) binding sites or negative cooperative interactions at a single receptor. In the case of negative cooperativity, dissociation in the presence of substances eliciting negative cooperativity should be more rapid than dissociation estimated by infinite dilution techniques (22, 23). We find no difference between the dissociation of [^{3}H]FNZ whether measured by infinite dilution into buffer alone or into buffer containing β-CCM, CL-218,872, or FNZ at 1 μM concentration (Fig. 1B); in three separate replications, data for each of these conditions do not differ significantly (by Student's two-tailed *t*-test) for each time point considered. As reported by others earlier (16), GABA (at 10 μM) slows the dissociation rate.

Regional differences in [^{3}H]FNZ binding kinetics and equilibrium interactions. Other studies have suggested that the relative amounts of Type I and Type II benzodiazepine receptors vary among different regions: the cerebellum contains almost exclusively Type I receptors, whereas the corpus striatum has a high proportion of

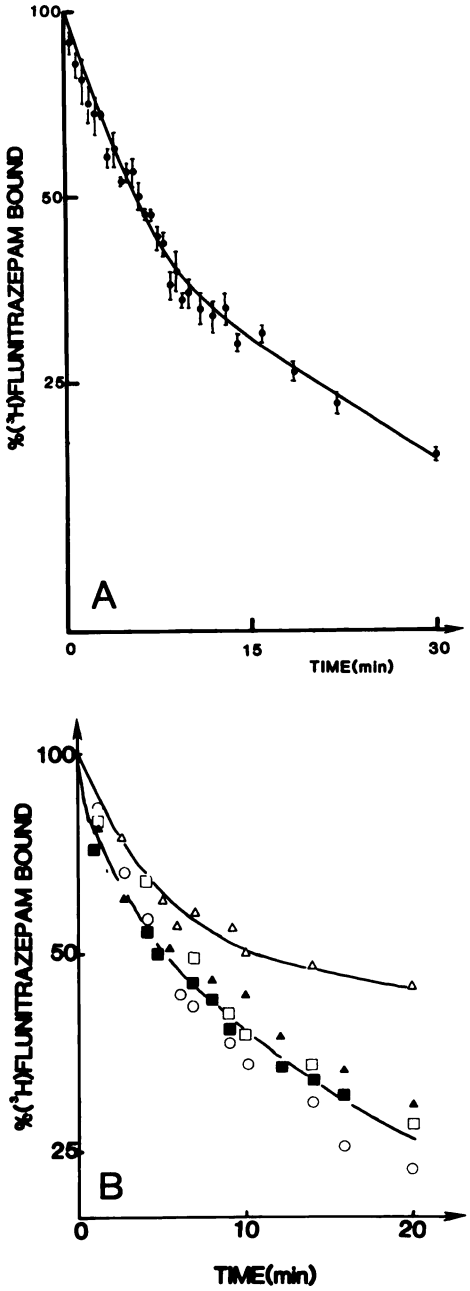

FIG. 1. *Dissociation of [³H]FNZ from bovine cortical membranes*

A. Dissociation of [³H]FNZ from bovine cerebral cortical membranes at 0° by drug displacement. Bovine cerebral cortical membranes were prepared, and dissociation kinetics of [³H]FNZ was determined (initiated by the addition of unlabeled FNZ to a final concentration of 10 μM) according to Experimental Procedures. All data points represent the mean of three separate determinations; error bars show the standard error of the mean of these determinations. In a typical experiment, total specific binding at time zero was approximately 6000 cpm and nonspecific binding approximately 800 cpm.

B. Dissociation of [³H]FNZ from bovine cerebral cortical membrane at 0° initiated by limiting dilution into various media. Bovine cortical membranes (prepared according to Experimental Procedures) were resuspended to a final concentration of 200 mg (original wet weight) per milliliter. To 5.0 ml of concentrated homogenate was added [³H] FNZ to a final concentration of 1 nM. After a 60-min incubation at 0°, the concentrated membranes were added (with constant stirring) to 495 ml of ice-cold 50 mM Tris-citrate (pH 7.2 at 0°) buffer (■) or an equal amount of buffer containing 1 μM β-CCM (□), 1 μM CL-218,872 (▲), 1 μM FNZ (○), or 10 μM GABA (△), and dissociation was permitted to proceed at 0°. At various times, triplicate 5.0-ml aliquots were withdrawn and collected over Whatman GF/B filters, followed by three 5-ml washes with ice-cold 50 mM Tris-citrate (pH 7.2). Filters were counted for tritium as described under Experimental Procedures. Data are from a representative experiment replicated three times. Lines displayed represent the unweighted linear least-squares fit to the data for limiting dilution into buffer (*lower curve*) and buffer containing 10 μM GABA (*upper curve*). In a typical experiment, the total specific binding at time zero was approximately 3000 cpm and nonspecific binding approximately 400 cpm.

Note that the ordinates for A and B are logarithmic scales.

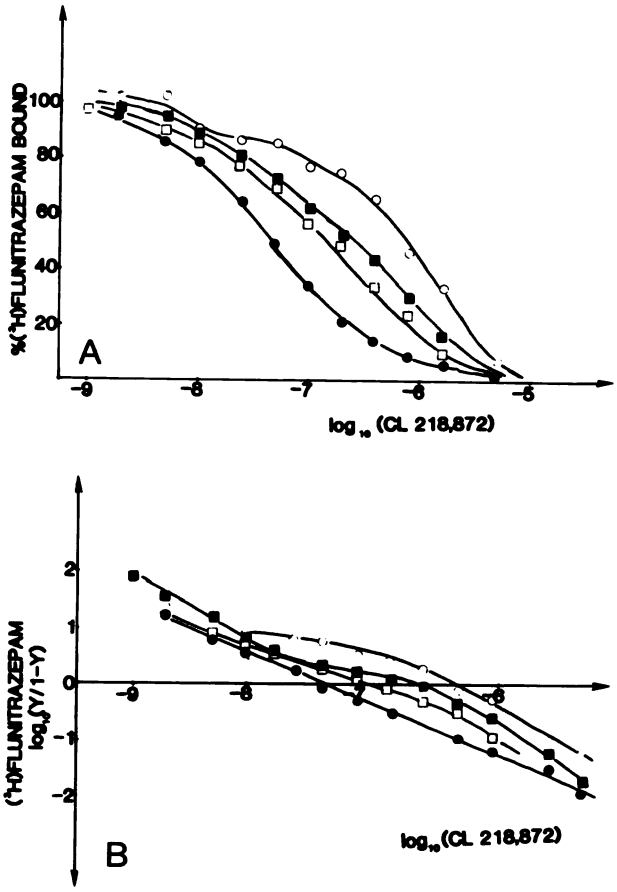

FIG. 2. *Competition by CL-218,872 for [³H]FNZ binding*

a. Competition by CL-218,872 for [³H]FNZ binding to membranes from various bovine brain regions at 0°. Membranes from bovine cerebellum (●), cerebral cortex (□), hippocampus (■), and striatum (○) were prepared as described under Experimental Procedures. Each assay consisted of 750 μl of membrane homogenate from the appropriate brain region, 100 μl of [³H]FNZ (final concentration 1.0 nM), 50 μl of 50 mM Tris-citrate (pH 7.2), and 100 μl of CL-218,872 at various concentrations. After a 60-min incubation at 0°, assays were terminated as described in the legend to Fig. 1. Nonspecific binding was determined by adding 100 μl of unlabeled FNZ to a final concentration of 10 μM. Data are from a representative experiment replicated three times. In a typical experiment, the total specific binding was approximately 6000 cpm and nonspecific binding was approximately 800 cpm.

B. Hill plot transformation of data of A. Symbols are the same as for A.

Type II sites (3, 11, 28). To evaluate the possibility that the two kinetic components of [³H]FNZ binding might reflect different kinetic properties of Type I and Type II receptors, we examined equilibrium binding and associ-

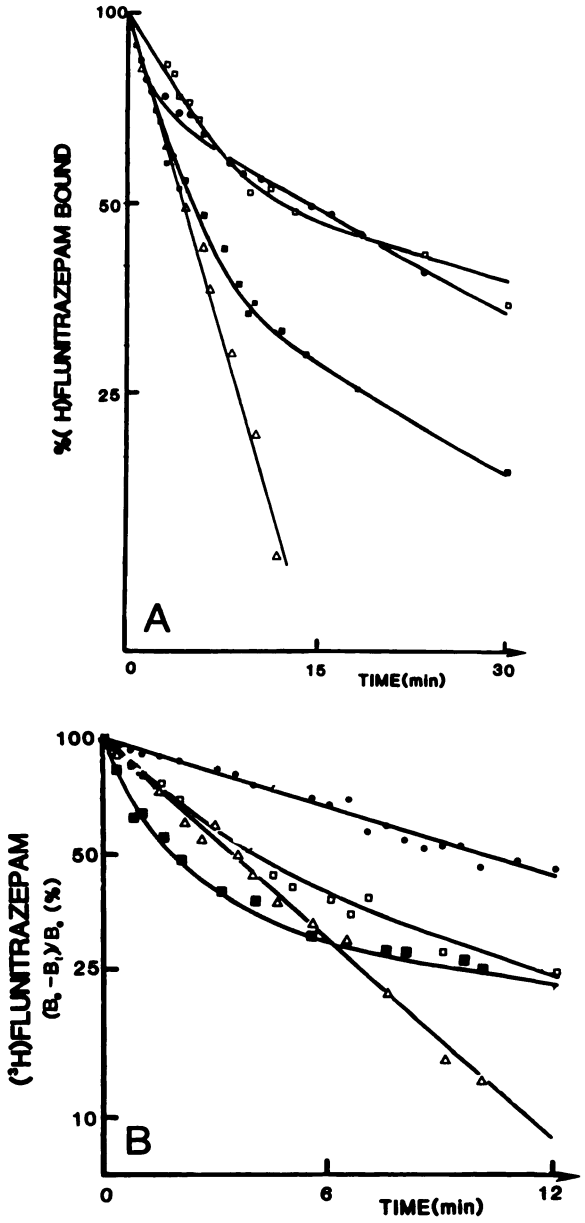

FIG. 3. *Dissociation and association of [³H]FNZ in various brain regions*

A. Dissociation of [³H]FNZ from membranes prepared from various bovine brain regions initiated by drug displacement of 0°. Membranes from bovine cerebellum (△), cerebral cortex (■), hippocampus (□), and striatum (●) were prepared, and dissociation kinetics of [³H]FNZ was determined (initiated by the addition of [³H]FNZ to a final concentration of 10 μM) as described under Experimental Procedures. Data are from a representative experiment replicated three times. In a typical experiment, specific binding values at time zero were approximately 5200, 6000, 3600, and 3200 cpm in the cerebellum, cerebral cortex, hippocampus, and striatum, respectively; nonspecific binding was 500–1000 cpm. Lines are the unweighted least-squares fit to the experimental data.

B. Association of [³H]FNZ to membranes prepared from various bovine brain regions of 0°. Membranes from bovine cerebellum (△), cerebral cortex (■), hippocampus (□), and striatum (●) were prepared, and association kinetics was determined as described under Experimental Procedures. Data are from a representative experiment performed in triplicate. The plot presented is a transformation of the association data appropriate for pseudo-first order conditions (see Eq. 1 of text or Appendix A). Lines represent the unweighted least-squares fit to the experimental data. In a typical experiment, specific binding values at equilibrium were approximately 4500, 5500, 3000, and 2800 cpm in the cerebellum, cerebral cortex, hippocampus, and striatum, respectively; nonspecific binding was 500–1000 cpm.

Note that the ordinates for A and B are logarithmic scales.

ation and dissociation rates of [³H]FNZ in bovine cerebellum, corpus striatum, hippocampus, and cerebral cortex (Figs. 2 and 3; Tables 1 and 2). As reported previously (2, 29), CL-218,872 has different potencies in various brain regions (Fig. 2A). It is most potent in displacing binding in the cerebellum, with an IC_{50} of about 50 nM; least potent in the corpus striatum, where the IC_{50} is 1000 nM; and intermediate in potency in the hippocampus and cerebral cortex. Asano *et al.* (27) have recently observed a similar regional variation in potency in benzodiazepine receptors from bovine brain. Although the displacement of [³H]FNZ binding by CL-218,872 follows mass action in the cerebellum, the displacement is more shallow (Fig. 2B) in the other brain regions, as others have noted (2, 27). Hill plot analysis (Fig. 2B) of CL-218,872 displacement of [³H]FNZ binding to bovine brain membranes indicates a linear Hill plot in the case of the cerebellum ($n_H = 0.95 \pm 0.03$). Hill plots for cortex and hippocampus appear to be nonlinear, with the lowest slope of the Hill plot near logit ([³H]FNZ bound) = 0. Such behavior would be expected (see Appendix B) if the cortex and hippocampus have a mixture of Type I and Type II receptors with different affinities for CL-218,872, as has been suggested in the rat (2, 11, 29) (reviewed in ref. 15).

Accordingly, we have attempted to fit competition data for [³H]FNZ binding by CL-218,872 to a two-site model as discussed in Appendix B. For all brain regions, the data suggest a model with $(K_i)_1 = (25 \pm 5)$ nM and $(K_i)_2 = (500 \pm 50)$ nM, where $(K_i)_1$ and $(K_i)_2$ are the respective dissociation constants for CL-218,872 at Type I and Type II receptors. This result is in keeping with findings in the rat suggesting that regional differences in CL-218,872 potency are due to regional variations in density of two distinct receptor populations: CL-218,872 displays differing affinities for the two subtypes and for a given subtype, and the affinity of CL-218,872 does not vary regionally (30). The calculated densities of Type I receptors are displayed in Table 1, along with summed squares of residuals for the observed fraction of zero inhibitor-specific [³H]FNZ binding relative to the given model. A value of the test statistic F is calculated according to standard procedures (20). For the cerebellum and striatum, data do not fit a two-site model significantly better than a one-site model of all Type I and all Type II receptors, respectively. In the cerebral cortex and hippocampus, however, the data fit a two-compartment model significantly better than a one-compartment model.

Both association and dissociation of [³H]FNZ vary among the four brain regions (Fig. 3). The most rapid dissociation of [³H]FNZ occurs in the cerebellum, where it is monophasic. For the other three brain regions, dissociation displays two phases with the relative proportions differing in the various brain regions. We have calculated the relative proportion of fast and slow dis-

sociating components in the four brain regions as discussed under Experimental Procedures.

The cerebellum also displays the most rapid and monophasic association whereas the corpus striatum has the slowest association rate, which is also monophasic. Association in the hippocampus and cerebral cortex is biphasic (Fig. 3B). We have also computed the relative proportions of rapidly and slowly associating components of [^{3}H]FNZ for the different brain areas (Table 2).

Whether calculated from equilibrium experiments, from association kinetics, or from dissociation kinetics, we obtain essentially the same values for the proportion of Type I and Type II receptors in the various brain areas (Table 2). The cerebellum appears to contain exclusively Type I receptors by drug displacement and exclusively rapidly associating and dissociating components by kinetic analysis. The corpus striatum possesses only about 20% of Type I receptors by drug displacement experiments and 20% of rapidly dissociating receptors. We fail to detect any rapidly associating binding sites in the corpus striatum. The cerebral cortex and hippocampus are intermediate between the corpus striatum and cerebellum. In both cerebral cortex and hippocampus, the proportions of Type I and Type II receptors defined pharmacologically correspond to the relative proportions of rapidly and slowly associating and dissociating components, respectively. Interestingly, calculation of the dissociation constants of the rapid and slow kinetic binding sites indicates that they display similar affinities for [^{3}H]FNZ, which fits with a single component of [^{3}H]FNZ binding apparent on Scatchard plots.

Differential effects of CL-218,872 on the two kinetic

TABLE 1

Estimation of fraction Type I benzodiazepine receptor content from CL-218,872 displacement of [^{3}H]FNZ in bovine brain

For the cerebral cortex and hippocampus, best-fit one-site models gave $K_i = 70 \pm 5$ and 133 ± 8 for displacement by CL-218,872, respectively. F calculations for these models were 17.8 and 11.4 relative to the best-fit two-site model for cerebral cortex and hippocampus, respectively. Displacement data better fit a two-site model than a one-site model in the cerebral cortex ($p < 0.025$) and hippocampus ($p < 0.05$).

Region	Best-fit two-site,[a] % Type I	δ^2,[b] Best-fit two-site (A)	δ^2, All Type I (B)	δ^2, All Type II (C)	F calc.[c]	
					A vs. B	A vs. C
Cerebellum	98 ± 2	0.0239	0.0243	1.76	0.025 (NS)	110.8 ($p < 0.01$)
Cerebral cortex	55 ± 5	0.00867	0.472	0.440	80.16 ($p < 0.001$)	74.62 ($p < 0.01$)
Hippocampus	50 ± 5	0.00638	0.549	0.452	127.5 ($p < 0.01$)	104.7 ($p < 0.01$)
Striatum	20 ± 3	0.0189	1.70	0.079	133.4 ($p < 0.01$)	4.76 (NS)

[a] Values represent means ± standard error of the mean for three separate experiments, calculated according to Experimental Procedures.

[b] δ^2 = Sum over data points of squared residuals; theoretical model generated according to Appendix B. A smaller value of δ^2 implies a better fit.

[c] Calculated test statistic for the "extra sum-of-squares test," or F test, as detailed in ref. 20. NS, Not significant.

TABLE 2

Regional variation of kinetic parameters for [^{3}H]FNZ-benzodiazepine receptor interactions in bovine brain at 0°

Values presented are derived from unweighted least-squares analysis of the results of Fig. 3A and B. Each value is the mean ± standard error of the mean of three separate experiments.

Region	Type I receptor[a]	Kinetics in fast compartment		k_{-1}	
		Association	Dissociation	Fast component	Slow component
	%	%		10^{-3} sec^{-1}	
Cerebellum	98–100	100	100	2.5 ± 0.5	—
Cerebral cortex	55 ± 5	57 ± 5	53 ± 5	3.5 ± 0.5	0.40 ± 0.06
Hippocampus	50 ± 5	42 ± 5	40 ± 5	2.1 ± 0.5	0.40 ± 0.04
Striatum	0–20	0	20 ± 5	3.1 ± 0.3	0.42 ± 0.03

Region	k_1[b]		Calculated K_D[c]	
	Fast component	Slow component	Fast component	Slow component
	10^6 M^{-1} sec^{-1}		nM	
Cerebellum	0.9 ± 0.3	—	2.7 ± 1.4	—
Cerebral cortex	3.3 ± 0.5	0.42 ± 0.05	1.1 ± 0.3	0.9 ± 0.2
Hippocampus	3.1 ± 0.5	0.9 ± 0.2	0.7 ± 0.3	0.4 ± 0.2
Striatum	—	0.7 ± 0.1	—	0.6 ± 0.2

[a] From Table 1. A range is indicated if the data do not fit a multisite model better than a one-site model.

[b] Calculated from $m = k_1(L) + k_{-1}$, where m is slope of pseudo-first order association plot (see Fig. 3B).

[c] Calculated from $K_D = k_{-1}/k_1$.

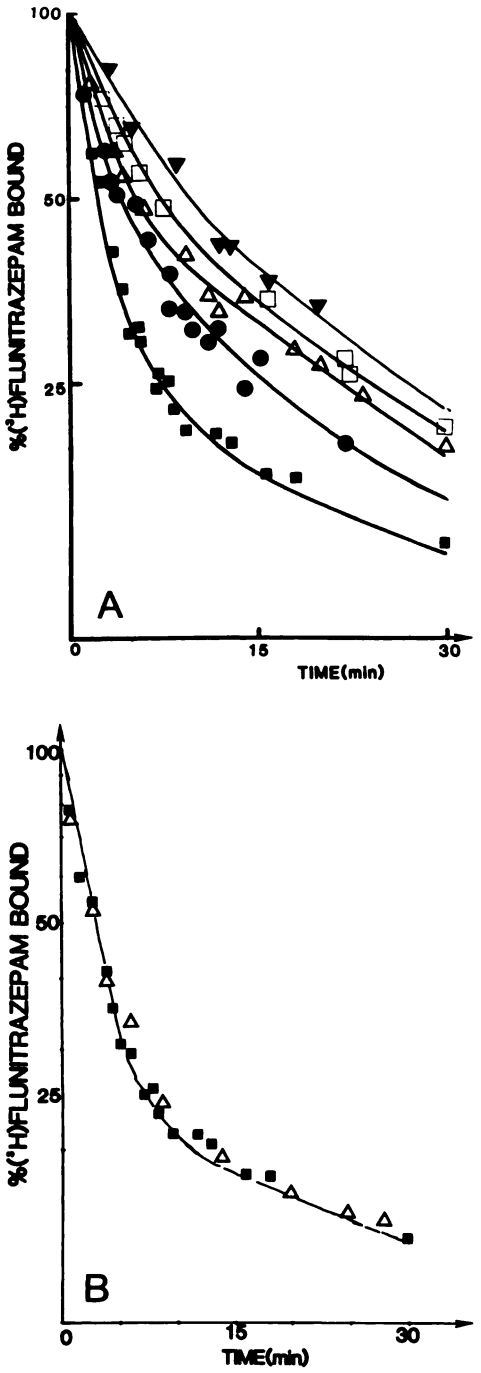

FIG. 4. *Dissociation of [³H]FNZ after preincubation with CL-218,872 and diazepam*

A. Dissociation of [³H]FNZ from bovine cerebral cortex membranes after preincubation with CL-218,872 at various concentrations at 0°. Bovine cerebral cortex membranes were prepared according to Experimental Procedures. To sixty milliliters of membranes [2 mg (wet weight) per milliliter] was added CL-218,872 to 0 nM (■), 20 nM (●), 100 nM (△), 200 nM (□), and 400 nM (▼) final concentration and [³H]FNZ (1.0 nM final concentration), and incubated for 60 min at 0°. Dissociation was then initiated by the addition of unlabeled FNZ to a concentration of 10 μM as described under Experimental Procedures. Data are from a representative experiment replicated three times. Lines are the unweighted least-squares fit to the experimental data. Typical values for the amount of [³H]FNZ specifically bound at time zero are displayed in Table 3. The nonspecific binding was typically 500–1000 cpm. Data for [CL-218,812] = 1000 nM are not shown but are displayed in Table 3.

components of [³H]FNZ binding. If the rapidly dissociating [³H]FNZ labels Type I receptors and the slowly dissociating radioligand is associated with Type II sites, then one would expect a Type I receptor-selective drug such as CL-218,872 to have higher affinity for the more rapidly dissociating [³H]FNZ binding sites. To examine this possibility, we incubated [³H]FNZ binding to equilibrium with bovine cerebral cortex membranes in the presence of several concentrations of CL-218,872. We then monitored the dissociation of the bound [³H]FNZ, initiating the displacement with 10 μM unlabeled FNZ (Fig. 4A). Receptors labeled with [³H]FNZ in the absence of CL-218,872 display biphasic dissociation kinetics similar to those described above. With increasing concentrations of CL-218,872, there is a progressive loss of the rapid component with no apparent loss of the slow component of dissociation.

From these data we could calculate the proportions of rapidly and slowly dissociating [³H]FNZ binding sites in the presence of different concentrations of CL-218,872 (Table 3). We then computed the extent to which CL-218,872 alters the amount of [³H]FNZ bound to the rapidly and slowly dissociating components, enabling us to plot displacement curves of CL-218,872 influencing [³H]FNZ binding to the two kinetic compartments (Fig. 5). The displacement of total [³H]FNZ binding by CL-218,872 is shallow, as observed previously. On the other hand, displacement by CL-218,872 or [³H]FNZ bound to the rapidly dissociating compartment is monophasic, with a Hill coefficient of 1.0 and a K_i value of about 20 nM. This value corresponds well to the potency of CL-218,872 at Type I receptors estimated here and in other studies (29). CL-218,872 appears to be much weaker in displacing [³H]FNZ binding from the slowly dissociating compartment, which accords with its low potency in inhibiting binding to Type II benzodiazepine receptors. However, at [CL-218,872] = 1000 μM, approximately 50% inhibition of binding to the slow kinetic compartment is observed; this is in agreement with what is expected accordig to Appendix B, with a K_D of 1.2 nM for [³H]FNZ.

We also estimated the relative potencies of β-CCM, another putative Type I-selective drug (28), at the two kinetic compartments of [³H]FNZ binding (Fig. 5). The results are quite similar to those obtained with CL-218,872. β-CCM displays a shallow displacement curve for total [³H]FNZ binding (n_H = 0.7 ± 0.1). When the effect of β-CCM is examined only on the rapid component of [³H]FNZ displacement, a Hill coefficient of 1.0 is observed with a K_i value of about 0.3 nM. β-CCM is much weaker in competing for [³H]FNZ binding to the slowly dissociating component with a K_i of less than 20 nM. These findings fit well with the higher affinity of β-CCM for Type I than Type II receptors (5, 28).

B. Dissociation of [³H]FNZ from bovine cerebral cortex membranes after preincubation with diazepam at 0°. Experimental details are the same as for A, with preincubation with diazepam at 0 nM (■) and 25 nM (△). Data are from a representative experiment replicated two times. Lines are the unweighted least-squares fit to the experimental data.

Note that the ordinates for A and B are logarithmic scales.

TABLE 3

Effect of preincubation with CL-218,872 upon dissociation kinetics of [^{3}H]FNZ (at 1.0 nM) from bovine cortex at 0°

Using the data displayed in Fig. 4A, the fraction of [^{3}H]FNZ bound to the apparent fast kinetic compartment was computed according to unweighted least-squares analysis and is displayed below. This fraction, along with the total [^{3}H]FNZ bound at a given concentration of CL-218,872, allows computation of the amount of [^{3}H]FNZ bound to the fast and slow kinetic compartments at a given concentration of CL-218,872. Looking at [^{3}H]FNZ bound to fast and slow kinetic compartments individually, one may construct "competition curves" for CL-218,872 at both of these kinetic compartments. These data are displayed in the rightmost columns.

CL-218,872	Total specific [^{3}H]FNZ bound	Fraction of total [^{3}H]FNZ bound to fast compartment	[^{3}H]FNZ specifically bound to fast compartment	[^{3}H]FNZ specifically bound	
				Fast compartment	Slow compartment
nM	*cpm*		*cpm*	%	
0	5772 ± 129	0.75 ± 0.06	4329 ± 443	100	100
20	4392 ± 106	0.57 ± 0.05	2503 ± 279	57.8 ± 8.7	130 ± 57
40	3018 ± 90	0.57 ± 0.03	1720 ± 141	39.7 ± 5.2	90 ± 39
100	2608 ± 84	0.45 ± 0.06	1174 ± 194	27.1 ± 5.3	99 ± 44
200	2354 ± 72	0.37 ± 0.05	871 ± 144	20.1 ± 3.9	102 ± 43
400	1991 ± 95	0.28 ± 0.04	558 ± 106	12.8 ± 2.7	99 ± 42
1000	708 ± 91	0.06 ± 0.03	42 ± 26	0.9 ± 0.56	46 ± 19

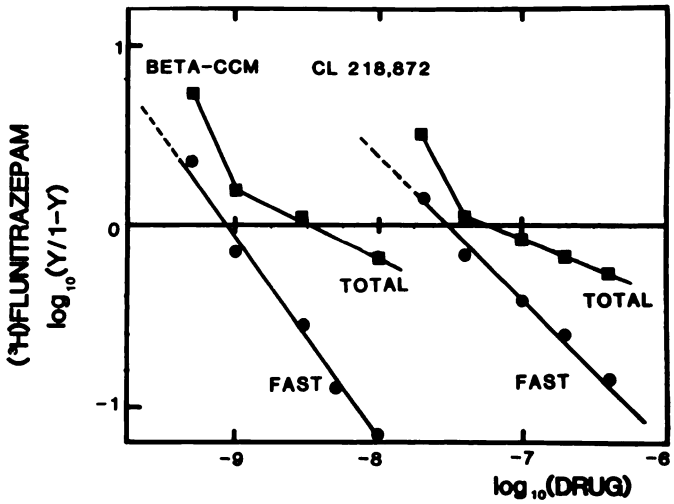

FIG. 5. *Hill replots for the displacement of [^{3}H]FNZ from the rapidly associating kinetic compartment in bovine cerebral cortex membranes at 0°.*

The percentage of total [^{3}H]FNZ bound to the fast kinetic compartment deduced from the data in Fig. 4A, as indicated in Table 3, was computed for various concentrations of CL-218,872. This was then transformed to a Hill plot, shown above. An experiment similar to that shown in Fig. 4A was also performed for the putative Type I selective ligand β-CCM (data not shown), and the corresponding displacement for the fast kinetic compartment was calculated. Displacement of [^{3}H]FNZ to whole bovine cortical membranes (total) determined by standard equilibrium binding was also determined for each drug in the same experiment. For CL-218,872, one finds apparent K_i values of 20 and 60 nM (based on one-compartment analysis) for displacement of [^{3}H]FNZ from the fast kinetic compartment and whole membranes, respectively. For β-CCM, one finds apparent K_i values of 0.46 and 1.6 nM for the fast kinetic compartment and whole membranes. Displacement by either drug from the fast kinetic compartment is monophasic with a Hill coefficient of unity, within experimental error.

To determine whether the change in the biphasic nature of [^{3}H]FNZ dissociation by CL-218,872 is related to the differential effect of the drug on Type I and Type II receptors or merely due to alterations of [^{3}H]FNZ occupancy, we conducted similar experiments using diazepam, which has the same affinities for Type I and Type II receptors (15). As has been reported by others (3), the biphasic character of [^{3}H]FNZ dissociation does not change when receptors are labeled in the presence of diazepam (Fig. 4B).

Dissociation of [^{3}H]FNZ binding after partial association. The similarity in properties of the rapidly associating, rapidly dissociating, and pharmacologically defined Type I receptors suggests that they reflect the same sites, whereas slowly dissociating, slowly associating, and pharmacologically defined Type II receptors appear to be the same. If the rapidly associating receptors are the same as the rapidly dissociating receptors, then the dissociation of [^{3}H]FNZ should be more rapid from receptors labeled with briefer incubation periods (Fig. 6). Indeed, we find more rapid and nearly monophasic dissociation of [^{3}H]FNZ when bovine cortex membranes are labeled with [^{3}H]FNZ for only 30 sec. At 30 sec of incubation time, we find 12% of the equilibrium occupancy and over 90% of the bound [^{3}H]FNZ dissociating rapidly from the receptor with a calculated k_{-1} of (4.0 ± 0.5) 10^{-3} sec^{-1} for the fast compartment. This value is in excellent agreement with the value presented in Table 2. Furthermore, if Type I receptors are rapidly associating and dissociating, the fraction of ligand bound to the rapidly dissociating compartment, u_1, after a time t of association ought to be given by (see Appendix A):

$$u_1 = \frac{f_1[1 - \exp\{-(k_1L + k_{-1})t\}]}{f_1(1 - \exp\{-(k_1L + k_{-1})t\}) + (1 - f_1)(1 - \exp\{-(k_2L + k_{-2})t\}} \quad (4)$$

where f_1 is the fraction of total receptor that is Type 1, k_1 and k_2 are the on-rate constants for [^{3}H]FNZ to the two apparent kinetic compartments, respectively, k_{-1} and k_{-2} are the corresponding off-rate constants, and L is the free [^{3}H]FNZ concentration. In this experiment, $L = 1.0$ nM and $t = 30$ sec; using the values from Table 2 for bovine cortex and Eq. 3, one computes $f_{fast} = 0.90$. This agrees quite well with the experimental findings, and thus supports the notion of kinetic differentiation of benzodiazepine receptors by [^{3}H]FNZ.

Kinetic differentiation between soluble Type I and Type

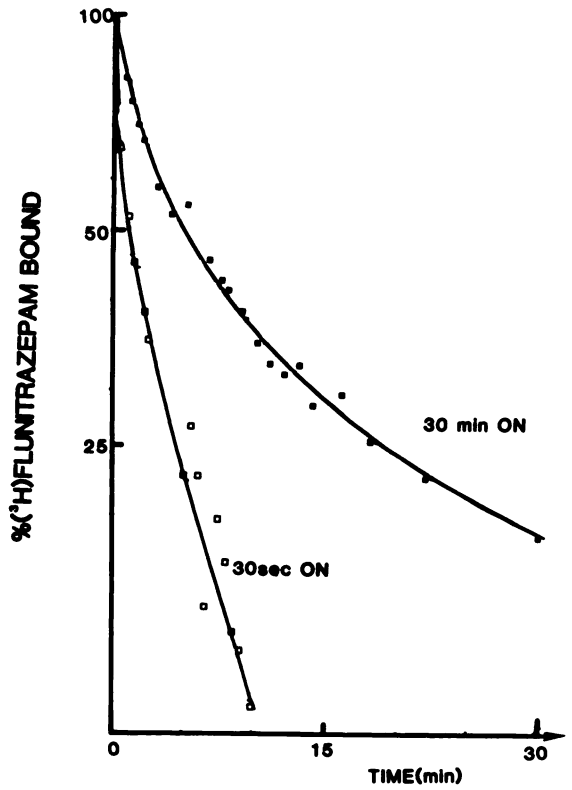

FIG. 6. *Dissociation of [³H]FNZ from bovine cerebral cortical membranes after partial association at 0°C*

Bovine cerebral cortical membranes were prepared according to Experimental Procedures. To 60 ml of these membranes at a final concentration of 2 mg (wet weight) per milliliter, [³H]FNZ was added to a final concentration of 1.0 nM and permitted to associate for 30 sec (□) or 30 min (■) at 0°. Dissociation kinetics was determined (with dissociation initiated by the addition of unlabeled flunitrazepam to a concentration of 10 μM) as indicated under Experimental Procedures. Data are from a representative experiment replicated three times. Lines shown are the unweighted least-squares fit to the experimental data. Note that the ordinate is a logarithmic scale.

II receptors. Apparent Type I and Type II benzodiazepine receptors can be separated physically by differential detergent solubilization (8–10). To test the prediction that Type I and Type II receptors have fast and slow kinetics, respectively, we evaluated the association and dissociation rates of [³H]FNZ at solubilized Type I and Type II receptors (Fig. 7). Both the association and dissociation rates of solubilized Type I and Type II receptors are monophasic. Both the association and dissociation kinetics are more rapid for Type I than for Type II receptors. The calculated association and dissociation rate constants for the solubilized Type I and Type II receptors correspond closely to what is obtained for the two kinetic components of binding in membranes (Table 4). As in the membrane state, the association and dissociation rates are 10 times more rapid for Type I than for Type II sites, but the resultant K_D is the same for the two receptor subtypes.

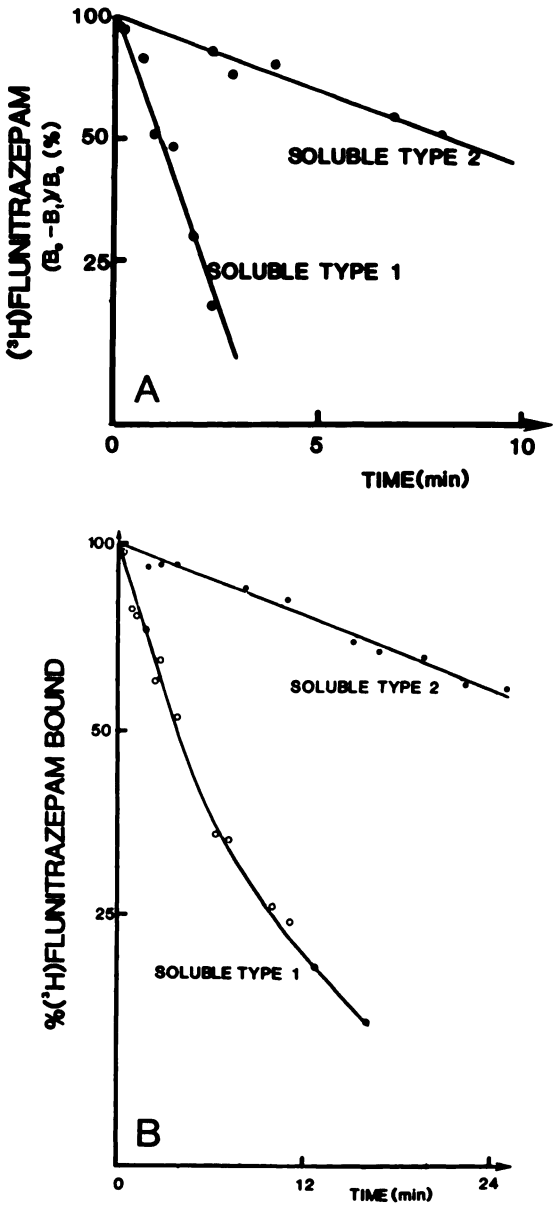

FIG. 7. *Association and dissociation of [³H]FNZ at solubilized Type I and Type II receptors*

A. Association of [³H]FNZ to differentially solubilized benzodiazepine receptors from bovine cerebral cortex at 0°. Benzodiazepine receptors were differentially solubilized from bovine cerebral cortex, and association kinetics to the respective soluble fractions was determined as described under Experimental Procedures. Data for 1% Triton X-100 soluble (soluble Type 2) and 1% Triton X-100/1 M NaCl (soluble Type 1) fractions are represented as a transformation appropriate for pseudo-first order conditions. Data are from a representative experiment replicated three times.

B. Dissociation of [³H]FNZ from differentially solubilized benzodiazepine receptors from bovine cerebral cortex at 0°C. Dissociation kinetics from respective soluble fractions was determined as described under Experimental Procedures. Data for 1% Triton X-100 soluble (soluble Type 2) and 1% Triton X-100/1 M NaCl (soluble Type 1) fractions are presented.

Note that the ordinates in A and B are logarithmic scales.

DISCUSSION

The major finding of the present study is that biphasic association and dissociation kinetics of [³H]FNZ binding to bovine brain benzodiazepine receptors reflect the different kinetic properties of Type I and Type II benzodiazepine receptors. [³H]FNZ associates and dissociates more rapidly at Type I than at Type II receptors. The resultant affinity of [³H]FNZ at the two receptors, how-

TABLE 4

Kinetic properties of [^{3}H]FNZ binding to physically separated soluble benzodiazepine receptors from bovine cortex at 0°

Values presented are derived from unweighted least-squares analysis of the results of Fig. 7A and B. In computing ratio of parameters, the appropriate results from Table 2 have been utilized. Each value is the mean ± standard error of the mean of three separate experiments.

	k_{-1}	k_1	Calculated K_D
	10^{-3} sec^{-1}	10^6 M^{-1} sec^{-1}	nM
Type I soluble receptor	4.3 ± 0.3	3.9 ± 0.5	1.1 ± 0.2
Fast kinetic phase (membrane state)	3.5 ± 0.5	3.3 ± 0.5	1.1 ± 0.3
Type II soluble receptor	0.41 ± 0.08	0.8 ± 0.2	0.5 ± 0.2
Slow kinetic phase (membrane state)	0.40 ± 0.06	0.42 ± 0.05	0.9 ± 0.2
Ratio of parameters			
Type I soluble/ Type II soluble	11 ± 3	5 ± 2	2.2 ± 1.2[a]
Fast kinetic phase (membrane)/ Slow kinetic phase (membrane)	8.8 ± 2.6	7.9 ± 2.1	1.2 ± 0.6[a]

[a] Ratio not significantly different from unity (two-tailed Student's *t*-test).

ever, is similar. The evidence for these conclusions is as follows.

1. The regional variations in size of the fast and slow compartments of either [^{3}H]FNZ association or dissociation correspond to regional variations in pharmacologically defined Type I and Type II receptors, respectively. It is unlikely that the regional differences are due to regional variations in residual endogenous GABA, as membranes were prepared by a procedure (19, 20) designed to reduce GABA to negligible levels.

2. The differential potencies of CL-218,872 and β-CCM in displacing [^{3}H]FNZ from rapidly and slowly dissociating compartments, respectively, correspond to the differential potencies of these drugs at Type I and Type II receptors.

3. Receptors labeled with [^{3}H]FNZ for briefer incubation periods display a more rapid dissociation of the ^{3}H-ligand than do receptors labeled for longer incubation periods.

4. The kinetics of interactions of [^{3}H]FNZ with solubilized and physically separated Type I and Type II receptors is as predicted from membrane experiments. Specifically, solubilized Type I receptors display more rapid association and dissociation kinetics than do solubilized Type II receptors.

Several other workers have studied benzodiazepine receptor binding kinetics (3, 16–18, 20). In one of the earliest studies suggesting the existence of multiple benzodiazepine receptors, Squires *et al.* (3) detected a partially biphasic dissociation rate for [^{3}H]diazepam in rat brain membranes which was slowed by CL-218,872 but not by unlabeled diazepam, suggesting that CL-218,872 might have preferential affinity for more rapidly dissociating receptors, similar to our present results. However, one possibilty that must be considered when multiphasic dissociation and association curves are observed is homotropic cooperative interaction. Although Doble and collaborators (17, 18) have suggested that such cooperative benzodiazepine-receptor interaction may exist, neither we (Fig. 1B) nor Squires *et al.* (3) have found evidence to support this notion. Furthermore, Scatchard plots, which are thought to be sensitive indicators of cooperative interaction (22) in receptor systems, are monophasic for [^{3}H]benzodiazepines (13, 14, 20).

Chiu and co-workers (16) observed both biphasic association and dissociation for [^{3}H]FNZ in rat brain membranes, similar to our present results in bovine brain. These authors also observed that more rapidly associating [^{3}H]FNZ dissociates more rapidly than does slowly associating [^{3}H]FNZ. Chiu *et al.* (16) interpreted their kinetic results as reflecting two different affinity states of a single benzodiazepine receptor that can interconvert. This interpretation could be consistent with an apparent monophasic Scatchard plot. However, our finding of striking parallels between the fractional Type I receptor content (as defined pharmacologically) and size of rapidly associating and dissociating kinetic compartments in four bovine brain regions (Table 2) is difficult to explain in the context of an interconverting receptor state scheme (16) involving only one physically distinct receptor, unless it is proposed that interconversion properties vary regionally. Chiu *et al.* (16) found that GABA could influence the distribution of receptor between the various affinity states proposed by these authors. As noted previously, it is unlikely that regional variations in GABA levels are responsible for the regional differences in kinetic properties observed here. If a regionally varying factor acting on a homogeneous population of interconvertible receptors accounts for the regional variations in the kinetic properties of [^{3}H]FNZ binding observed here, that factor must persist after the extensive washing procedure utilized here in the preparation of membranes. Furthermore, as this hypothetical factor is very likely not GABA, there is little to suggest what it might be.

Another way of accounting for the kinetic data presented here is to propose that Type I and Type II receptor populations are distinct, noninterconvertible populations with different kinetic properties. One should expect a multiphasic Scatchard plot for [^{3}H]FNZ in this case, and this is generally not observed. In this report, however, we have demonstrated that the dissociation constant for [^{3}H]FNZ binding to the rapidly associating/dissociating and slowly associating/dissociating compartments does not differ appreciably at 0°; thus it is not surprising that saturation analyses for [^{3}H]FNZ binding are consistently observed to be monophasic (20). Several other observations also support the notion that Type I and Type II populations might be physically distinct and noninterconvertible (15). Most recently, this laboratory has demonstrated that the two putative benzodiazepine receptor subtypes are differentially localized in the substantia nigra (12). Type I receptors are localized predominantly postsynaptically whereas Type II receptors have a presynaptic localization. Once again, these results are difficult to explain in terms of an interconverting receptor

state scheme unless a factor (which is highly compartmentalized and thus far unidentified) is available to "freeze" the otherwise freely interconvertible states into one or another conformer.

One group has reported that CL-218,872 appears to discriminate receptor subtypes less at 37° than at 0°, an observation which could be consistent with the interconvertibility of the putative subtypes (31). It is possible that this finding could be due to differential thermodynamic properties of benzodiazepine-receptor and triazolopyridazine-receptor interactions. As Martin *et al.* have noted (32), definitive resolution of the issue of whether or not the two pharmacologically defined receptors are interconvertible will require structural information about the components of the apparent benzodiazepine receptor subtypes. Recently, conditions for obtaining highly reproducible partial proteolytic maps of [^{3}H]FNZ photoaffinity-labeled receptors were described (33); such maps could prove useful in searching for possible primary sequence differences in the benzodiazepine binding subunit of the putative receptor subtypes.

In conclusion, the present study has demonstrated that two very basic properties of benzodiazepine-receptor interactions, monophasic saturation isotherms and multiphasic dissociation kinetics, can be reconciled by the existence of two populations of benzodiazepine receptors with different kinetic properties. The two kinetically defined receptor populations can be directly related to the two pharmacologically defined receptor subtypes (Type I and Type II), thus further supporting the existence of multiple benzodiazepine receptor populations. It is not yet entirely clear whether the apparent multiple benzodiazepine receptor populations are a reflection of multiple interconvertible conformers of a homogeneous receptor population or of multiple noninterconverting receptors, although the present results would appear to strengthen the latter possibility.

ACKNOWLEDGMENTS

The authors wish to thank Arnold Lippa for helpful discussions, and Dawn C. Dodson for manuscript preparation.

APPENDIX A

Derivation of Kinetic Formulae

We consider n independent receptor compartments, each of which is assumed to interact with ligand according to the scheme (written for the jth compartment)

$$R_j + L \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} R_jL \tag{A1}$$

For notational convenience, we call $R_jL = X_j$, denote derivates with respect to time by a superscript dot, and omit concentration brackets. Now, by mass action,

$$\dot{X}_j = k_jLR_j - k_{-j}X_j \tag{A2}$$

But from conservation of receptor and ligand

$$L = L_0 - \sum_{r=1}^{n} X_r \tag{A3}$$

$$R_j = (R_j)_0 - X_j \tag{A4}$$

where subscript zero denotes initial concentration. Using Eqs. A3 and A4 in Eq. A2, one obtains

$$\begin{aligned}\dot{X}_j &= k_j[(R_j)_0 - X_j][L_0 - \sum_{r=1}^{n} X_r] - k_{-j}X_j \\ &= k_jL_0[(R_j)_0 - X_j](1 - \lambda) - k_{-j}X_j\end{aligned} \tag{A5}$$

where

$$\lambda = \left(\sum_{r=1}^{n} X_r\right) \Big/ L_0$$

Equation A5 indicates that the kinetics of the system will be described by a set of n coupled nonlinear first-order differential equations. The mathematical analysis is greatly simplified if experimental conditions of *ligand excess* are employed, i.e., the domain

$$L_0 \gg \sum_{r=1}^{n} X_r \quad \text{or} \quad \lambda \approx 0 \tag{A6}$$

In practice, $\lambda = 0$ can never obtain. Notice that λ, which depends on all of the X_r, will in general be a complicated function of time. For purposes of discussion, let us *assume* that λ takes on a constant value R_0/L_0 throughout the experiment. This is the maximal value that λ can assume, since $\sum_{r=1}^{n} X_r \leq R_0$; thus, this approach will give a "worst possible case" estimate of the effect of finite receptor concentration on the results. From Eq. A5, we can see that the *effective* dissociation rate constant is still k_{-j}; for the model of multiple independent compartments (i.e., no cooperativity), receptor concentration will not influence dissociation rate constant determinations. Equation A5 shows that the *effective* association rate constant, however, is now $k_j(1 - \lambda)$ for *each* compartment. Thus, the apparent association rate constant determined will be *less* than the "true" association rate constant by (no worse than) a factor of $(1 - \lambda)$. In the experiments described here, we thought it reasonable to fix assay conditions so that $R_0/L_0 < 0.05$. This ensures that the "true" association rate constant differs by no more than 5% from the determined "apparent" association rate constant, commensurate with the experimental error inherent in determining this value. Since λ is actually not a constant but a (possibly complicated) function of time, a simple correction in the apparent association rate would have no theoretical basis.

In the ligand excess domain Eq. A5 becomes

$$\dot{X}_j = k_jL_0[(R_j)_0 - X_j] - k_{-j}X_j \tag{A7}$$

Thus, in the ligand excess domain, association to all compartments is pseudo-first order and the kinetic equations are simultaneously linearized and uncoupled. For notational convenience, denote

$$\alpha_j = k_jL_0(R_j)_0 \tag{A8}$$

$$\beta_j = k_jL_0 + k_{-j} \tag{A9}$$

Then Eq. A7 becomes

$$\dot{X}_j = \alpha_j - \beta_jX_j \tag{A10}$$

We consider α_j and β_j as constants for fixed L_0 and $(R_j)_0$, as we find no experimental evidence for cooperative

phenomena; De Lean and Rodbard (22) discussed a more general case for a one-compartment model. Equation A10 integrates to

$$\alpha_j - \beta_j X_j = [\alpha_j - \beta_j (X_j)_0]\exp\{-\beta_j t\} \quad \text{(A11)}$$

which rearranges to

$$X_j = (X_j)_0\exp\{-\beta_j t\} + \frac{\alpha_j}{\beta_j}[1 - \exp\{-\beta_j t\}] \quad \text{(A12)}$$

But

$$\frac{\alpha_j}{\beta_j} = \frac{k_j (R_j)_0 L_0}{k_j L_0 + k_{-j}} = (R_j)_0\left(\frac{L_0}{L_0 + K_j}\right) \quad \text{(A13)}$$

where K_j is the dissociation constant of L in the jth compartment. Thus,

$$\frac{\alpha_j}{\beta_j} = (X_j)_{eq} \quad \text{(A14)}$$

so that the integrated rate equation for each compartment becomes a "state switching" equation.

$$X_j = (X_j)_0\exp\{-\beta_j t\} + (X_j)_{eq}[1 - \exp\{-\beta_j t\}] \quad \text{(A15)}$$

Now if B_t denotes the total receptor bound at time t

$$B_t = \sum_{j=1}^{n} X_j \quad \text{(A16)}$$

then

$$B_t = \sum_{j=1}^{n} [(X_j)_0\exp\{-\beta_t t\} + (X_j)_{eq}[1 - \exp\{-\beta_j t\}]] \quad \text{(A17)}$$

Equation A17 can be used to treat association experiments and dissociation experiments following either complete or partial association.

Case 1: association. Here $(X_j)_0 = 0$, so that Eq. A17 reduces to

$$\beta_t = \sum_{j=1}^{n} (X_j)_{eq}[1 - \exp\{-\beta_j t\}] \quad \text{(A18)}$$

We define the fraction of *total* bound ligand in compartment j by

$$f_j = \frac{(X_j)_{eq}}{(B_t)_{eq}} \quad \text{(A19)}$$

so that dividing Eq. A17 by $B_e = (B_t)_{eq}$

$$\frac{B_t}{B_e} = \sum_{j=1}^{n} f_j[1 - \exp\{-\beta_j t\}] \quad \text{(A20)}$$

but upon using Eq. A16 in Eq. A19

$$\sum_{j=1}^{n} f_j = 1 \quad \text{(A21)}$$

using Eq. A9 and Eq. A21 in Eq. A20, rearranging, we obtain Eq. 1 of the text

$$\frac{B_e - B_t}{B_e} = \sum_{j=1}^{n} f_j\exp\{-(k_j L + k_{-j})t\} \quad \text{(A22)}$$

subject to constraint Eq. A21, which is identical with Eq. 2.

Case 2: dissociation. In the case of experimental conditions employed here, dissociation proceeds with negligible reassociation and to negligible final specific binding. Thus

$$(X_j)_{eq} = 0 \quad \text{and} \quad \beta_j \approx k_{-1} \quad \text{(A23)}$$

using Eq. A21 in Eq. A16, one obtains

$$B_t = \sum_{j=1}^{n} (X_j)_0\exp\{-k_{-j}t\} \quad \text{(A24)}$$

In this case, the fraction of total bound ligand initially bound to the jth compartment is

$$f_j = \frac{(X_j)_0}{B_0} \quad \text{(A25)}$$

Upon using Eq. A16 in Eq. A25, one obtains constraint Eq. A21; upon dividing Eq. A25 by B_0, one obtains Eq. 3 of the text:

$$\frac{B_t}{B_0} = \sum_{j=1}^{n} f_j\exp\{-k_{-j}t\} \quad \text{(A26)}$$

Case 3: dissociation after partial association. In the case of an n compartment model, the total label bound after an association time t_0 will be given by Eq. A22. The total amount of label bound to the j compartment will be

$$B_j = f_j[1 - \exp\{-\beta_j t_0\}] \quad \text{(A27)}$$

Upon dividing Eq. A24 by Eq. A19 and using Eq. A20, one obtains the fraction of label bound at time t_0 that is bound to the j kinetic compartment, u_j

$$u_j = \frac{(f_r)_{eq}[1 - \exp\{-\beta_j t_0\}]}{\sum_{r=1}^{n} (f_r)_{eq}[1 - \exp\{-\beta_r t_0\}]} \quad \text{(A28)}$$

where $(f_r)_{eq}$ denotes the fraction of label bound to the rth kinetic compartment had the association been allowed to proceed to equilibrium. If Eq. A28 is written for the specific case $n = 2$, Eq. 4 of the text is obtained. By Eq. 3, the expression for the dissociation of bound ligand after a partial association of time t_0 ought to be given by:

$$\frac{B_t}{B_0} = \sum_{j=1}^{n} \frac{(f_r)_{eq}(1 - \exp\{-\beta_j t_0\})\exp\{-k_{-j}t\}}{\sum_{r=1}^{n} (f_r)_{eq}[1 - \exp\{-\beta_r t_0\}]} \quad \text{(A29)}$$

where t denotes the time after which dissociation was initiated.

It should be emphasized that all of the derived equations (with the exception of Eq. A16 and Eq. A21) assume interactions of a single ligand with n distinct (noninterconvertible) receptor compartments in the restricted situation where there are no cooperative ligand-receptor interactions either within compartments (homotropic cooperativity) or between compartments (heterotropic cooperativity). In addition (with the exception of Eq. A16 and Eq. A21), Eq. A7 to Eq. A29 assume that "ligand excess conditions" obtain.

APPENDIX B

Calculation of Fraction Type I Receptor from CL-218,872 Displacement Curve Data

Assuming that CL-218,872 competes for [^{3}H]FNZ binding according to the scheme, as supported by Experiment 3

$$\begin{array}{ccc} R_1 + L^* \overset{K_L}{\rightleftharpoons} R_1L^* & & R_2 + L^* \overset{K_L}{\rightleftharpoons} R_2L^* \\ + & & + \\ I & & I \\ (K_I)_1 \downarrow & & (K_I)_2 \downarrow \\ R_1I & & R_2I \end{array} \tag{B1}$$

The apparent fractional occupancy $(h_{sat})_I$ by [^{3}H]FNZ in the presence of a concentration (I) of CL-218,872 will be given by

$$(h_{sat})_I = \frac{h_1 L}{L + \left[1 + \frac{(I)}{(K_I)_1}\right]K_L} + \frac{(1 - h_1)L}{L + \left[1 + \frac{(I)}{(K_I)_2}\right]K_L} \tag{B2}$$

where L is the free concentration of [^{3}H]FNZ, K_L is the dissociation constant of [^{3}H]FNZ, h_1 is the fraction of total benzodiazepine receptors that are Type I, and $(K_I)_1$ and $(K_I)_2$ are the dissociation constants for C1-218872 at Type I and II receptors, respectively. If one sets $(I) = 0$ in the above equation, then one obtains

$$(h_{sat})_{I=0} = \frac{L}{L + K_L} \tag{B3}$$

The fractional inhibition θ, will be

$$\theta = \frac{(h_{sat})_I}{(h_{sat})_{I=0}} \tag{B4}$$

$$= (L + K_L)\left[\frac{h_1}{L + \left[1 + \frac{(I)}{(K_I)_1}\right]K_L} + \frac{(1 - h_1)}{L + \left[1 + \frac{(I)}{(K_I)_2}\right]K_L}\right]$$

Using the functional form (Eq. B4), the least-squares (unweighted) best fit values of $(K_I)_1$, $(K_I)_2$, and h_1 were determined.

Hill plot. Define for notational convenience

$$\mu_1 = 1 + \frac{(I)}{(K_I)_1} \quad \mu_2 = 1 + \frac{(I)}{(K_I)_2} \tag{B5}$$

Upon combining terms in Eq. B4, one may write

$$\theta = \frac{(L + K_L)(L + h_1(\mu_2 - \mu_1)K_L + \mu_1 K_L)}{(L + \mu_1 K_L)(L + \mu_2 K_L)} \tag{B6}$$

Hence

$$\text{logit}_{10}\,\theta = \log_{10}\frac{\theta}{1 - \theta} = log_{10}\left[\frac{(L + \mu_1 K_L)(L + \mu_2 K_L)}{(L + K_L)(L + h_1(\mu_2 - \mu_1)K_L + \mu_1 K_L)} - 1\right] \tag{B7}$$

Note that unless h_1 approaches 0 or 1 (i.e., a one-site model), logit_{10} will not be a linear function of $\log_{10}(I)$. Hence a nonlinear Hill plot for Cl-218872 displacement of [^{3}H]FNZ should be expected if CL-218,872 distinguishes multiple benzodiazepine receptors (Fig. 2B).

REFERENCES

1. Lippa, A. S., L. R. Meyerson, and B. Beer. Molecular substrates of anxiety: clues from the heterogeneity of benzodiazepine receptors. *Life Sci.* **31**:1409–1417 (1982).
2. Klepner, C. A., A. S. Lippa, D. I. Benson, M. C. Sano, and B. Beer. Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors. *Pharmacol. Biochem. Behav.* **11**:457–462 (1979).
3. 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. *Pharmacol. Biochem. Behav.* **10**:825–830 (1979).
4. Nielsen, M., and C. Braestrup. Ethyl β-carboline-3-carboxylate shows differential benzodiazepine receptor interaction. *Nature (Lond.)* **286**:606–607 (1980).
5. Ehlert, F. J., W. R. Roeske, and H. I. Yamamura. Multiple benzodiazepine receptors and their regulation by γ-aminobutyric acid. *Life Sci.* **29**:235–248 (1981).
6. Squires, R. F., and E. Saederup. γ-Aminobutyric acid receptors modulate cation binding sites coupled to independent benzodiazepine, picrotoxin, and anion binding sites. *Mol. Pharmacol.* **22**:327–334 (1982).
7. Sieghart, W., and M Karobath. Molecular heterogeneity of benzodiazepine receptors. *Nature (Lond.)* **286**:285–287 (1980).
8. Lo, M. M. S., S. M. Strittmatter, and S. H. Snyder. Physical separation and characterization of two types of benzodiazepine receptors. *Proc. Natl. Acad. Sci. U. S. A.* **79**:680–684 (1982).
9. Lo, M. M. S., R. R. Trifiletti, and S. H. Snyder. Physical separation and characterization of two central benzodiazepine receptors, in *Pharmacology of Benzodiazepines* (E. Usdin, P. Skolnick, J. F. Tallman, D. Greenblatt, and S. M. Paul, eds). Macmillan Press, London, 165–174 (1982).
10. Lo, M. M. S., D. L. Niehoff, M. J. Kuhar, and S. H. Snyder. Autoradiographic differentiation of multiple benzodiazepine receptors by detergent solubilization and pharmacology specificity. *Neurosci. Lett.* **39**:37–44 (1983).
11. Young, W. S., III, D. Niehoff, M. J. Kuhar, B. Beer, and A. S. Lippa. Multiple benzodiazepine receptor localization by light microscopic radiohistochemistry. *J. Pharmacol. Exp. Ther.* **221**:670–675 (1982).
12. Lo, M. M. S., D. L. Niehoff, M. J. Kuhar, and S. H. Snyder. Differential localization of type I and type II benzodiazepine binding sites in substantia nigra. *Nature (Lond.)*, in press (1983).
13. Squires, R. F., and C. Braestrup. Benzodiazepine receptors in rat brain. *Nature (Lond.)* **266**:732–734 (1977).
14. Mohler, H., and T. Okada. Properties of [^{3}H]diazepam binding to benzodiazepine receptors in rat cerebral cortex. *Life Sci.* **20**:2101–2110 (1977).
15. Lippa, A. S., L. R. Meyerson, and B. Beer. Molecular substrates of anxiety: clues from the heterogeneity of benzodiazepine receptors. *Life Sci.* **31**:1409–1417 (1982).
16. Chiu, T. H., D. M. Dryden, and H. C. Rosenberg. Kinetics of [^{3}H]flunitrazepam binding to membrane-bound benzodiazepine receptors. *Mol. Pharmacol.* **21**:57–65 (1982).
17. Doble, A., L. L. Iversen, and I. L. Martin. The benzodiazepine binding site: one receptor or two? *Br. J. Pharmacol.* **75**:42p (1982).
18. Doble, A. GABA abolishes cooperativity between benzodiazepine receptors. *Eur. J. Pharmacol.* **83**:313–316 (1982).
19. Gardner, C. R., J. Klein, and J. Grove. Endogenous GABA determines the characteristics of [^{3}H]GABA binding. *Eur. J. Pharmacol.* **75**:83–92 (1981).
20. Quast, U., and H. Mahlmann. Interaction of [^{3}H]flunitrazepam with the benzodiazepine receptor: evidence for a ligand induced conformational change. *Biochem. Pharmacol.* **31**:2761–2768 (1982).
21. Stephenson, F. A., A. E. Watkins, and R. W. Olsen. Physiological characterization of detergent solubilized γ-aminobutyric acid and benzodiazepine receptor proteins from bovine brain. *Eur. J. Biochem.* **123**:291–298 (1982).
22. De Lean, A. and D. Rodbard. Kinetics of cooperative binding, in *The Receptors: A Comprehensive Treatise* (R. D. O'Brien, ed.), Vol. 1. Plenum Press, New York (1979).
23. DeMeyts, P., J. Roth, D. M. Neville, J. R. Gavin III, and M. A. Lesniak,

Insulin interaction with its receptors: experimental evidence for negative cooperativity. *Biochem. Biophys. Res. Commun.* **55**:154–161 (1973).
24. Bruns, R. F., K. Lawson-Wendling, and T. A. Pugsley. A rapid filtration assay for soluble receptors using polyethyleneimine-treated filters. *Anal. Biochem.* **132**:74–81 (1983).
25. Gavish, M., and S. H. Snyder. Soluble benzodiazepine receptors: GABAergic regulation. *Life Sci.* **26**:579–582 (1980).
26. Fehske, C. J., I. Zube, H. O. Borbe, U. Wollert, and W. E. Muller. β-carboline binding indicates the presence of benzodiazepine receptor subclasses in the bovine central nervous system. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **319**:172–177 (1982).
27. Asano, T., Y. Yamada, and N. Ogasawara. Charcterization of the solubilized GABA and benzodiazepine receptors from various regions of bovine brain. *J. Neurochem.* **40**:209–214 (1983).
28. Braestrup, C., and M. Nielsen. [^{3}H]Propyl-β-carboline-3-carboxylate as a selective radioligand for the BZ_1 benzodiazepine receptor subclass. *J. Neurochem.* **37**:333–341 (1981).
29. Niehoff, D. L., R. D. Mashal, W. D. Horst, R. A. O'Brien, J. M. Palacios, and M. J. Kuhar. Binding of a radiolabeled triazaolopyridazine to a subtype of benzodiazepine receptor in the rat cerebellum. *J. Pharmacol. Exp. Ther.* **221**:670–675 (1982).
30. Lippa, A. S., C. A. Klepner, D. I. Benson, D. J. Critchett, M. C. Sano, and B. Beer. The role of GABA in mediating the anti-convulsant property of benzodiazepines. *Br. Res. Bull.* **5**:861–865 (1980).
31. Gee, K. W., and H. I. Yamamura. Regional heterogeneity of benzodiazepine receptors at 37°C: an *in vitro* study in various regions of the rat brain. *Life Sci.* **31**:1939–1945 (1982).
32. Martin, I. L., C. L. Brown, and A. Doble. Multiple benzodiazepine receptors: structures in the brain or structures in the mind? A critical review. *Life Sci.* **32**:1925–1933 (1983).
33. Trifiletti, R. R., and Snyder, S. H. Partial proteolytic mapping of the benzodiazepine binding subunit of central benzodiazepine receptors. *Soc. Neurosci. Abstr.* **9**:404 (1983).

Send reprint requests to: Dr. Solomon H. Snyder, Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD. 21205.