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Kinetics of [^{3}H]Flunitrazepam Binding to Membrane-Bound Benzodiazepine Receptors

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SUMMARY

The kinetics of [^{3}H]flunitrazepam binding to synaptosomal (P_2) membranes from rat cerebral cortex was studied. The pseudo-first order association at 0° was curvilinear and the association rate was increased with increasing concentrations of ligand. The association curve was resolved best as two linear exponentials, representing a fast and a slow component. The percentage of each association component varied with the ligand concentration. Association was slightly decreased in the presence of γ-aminobutyric acid. Dissociation at 0°, whether equilibrium binding had occurred at 0° or 37°, was also biphasic. The dissociation of the complexes was more rapid at higher concentrations of ligand. Dissociation was slower in the presence of γ-aminobutyric acid, and this effect was more pronounced at higher concentrations of ligand. Prolonged preincubation at 37° either in the presence or in the absence of [^{3}H]flunitrazepam also decreased the dissociation rate. Dissociation was faster before equilibrium than after equilibrium had been reached. These results are discussed in terms of one homogeneous type of benzodiazepine receptor with two interconvertible conformations.

INTRODUCTION

Stereospecific, high-affinity, and saturable binding sites for benzodiazepines have been demonstrated in mammalian central nervous system (1–3). The rank order of potency of a large number of benzodiazepines in displacing [^{3}H]diazepam or [^{3}H]FNP[2] from these binding sites correlates very well with their potencies in clinical and animal studies as anxiolytics, anticonvulsants, and muscle relaxants (1, 3–6), suggesting that these binding sites may mediate the pharmacological actions of benzodiazepines. Furthermore, correlation between receptor occupation and behavioral and anticonvulsant effects of diazepam in rats has been reported (7, 8).

Scatchard analysis of the saturation binding studies of [^{3}H]diazepam and [^{3}H]FNP revealed a single class of binding sites (1–3, 6, 9). Consistent with this observation was the finding that many benzodiazepines displaced [^{3}H]diazepam binding with a Hill coefficient of near unity (4). However, recent studies have suggested the existence of more than one type of benzodiazepine binding site. For example, several triazolopyridazines, a new class of compounds effective in conflict and pentylenetetrazole convulsion tests, displaced [^{3}H]FNP binding with Hill coefficients below unity, indicating multiple binding sites (10). However, these results could also be explained by negative cooperative binding of the triazolopyridazines. Inactivation of [^{3}H]FNP binding sites at high temperature in 50 mM sodium phosphate buffer demonstrated two inactivation processes (10). Multiple components of the dissociation curve for [^{3}H]FNP binding have also been reported (10, 11). Heterogeneity of benzodiazepine receptors as well as regional differences in the proportions of these receptors within the central nervous system have been characterized by competition studies with an alkylating benzodiazepine (12). In addition, photoaffinity labeling of benzodiazepine receptors with [^{3}H]FNP and subsequent sodium dodecyl sulfate electrophoresis of the complex indicated the existence of more than one molecular species in rat hippocampus (13). More recently, ethyl β-carboline-3-carboxylate has been shown to inhibit differentially the binding of [^{3}H]FNP to different regions of rat brain (14). Although these results strongly support the existence of more than one distinct class of benzodiazepine receptor in the central nervous system, some of these observations could be adequately explained by assuming one distinct type of binding site with more than one conformation.

In order to assess whether there is more than one type and/or more than one conformation of benzodiazepine receptor, the kinetics of [^{3}H]FNP binding to rat cerebral cortices was studied. The results suggest that there are two interconvertible conformations of benzodiazepine re-

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[2] The abbreviations used are: [^{3}H]FNP, [^{3}H]flunitrazepam; GABA, γ-aminobutyric acid.

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ceptors, one with a relatively low affinity and the other with a relatively high affinity for benzodiazepines. Binding of ligands to the high-affinity sites forms a readily reversible complex which rearranges to the more stable complex. GABA enhances benzodiazepine binding primarily by decreasing the rate of dissociation of the reversible complex. A model is presented to describe the kinetic data.

MATERIALS AND METHODS

Preparation of synaptosomal membranes. Triple-washed crude synaptosomal membranes (P_2) were prepared from cerebral cortices (including hippocampus and corpus striatum) of male Sprague-Dawley rats (250–350 g) as previously described (15) except that 50 mM sodium phosphate buffer (pH 7.4) was used throughout the experiments. The whole procedure was carried out at 4° unless otherwise indicated. No differences were observed in the characteristics of [^{3}H]FNP binding to membranes prepared from fresh tissues or tissues frozen at −20° for 3 weeks. The triple-washed preparation had been shown to contain a very low amount of endogenous GABA (about 0.06 nmole/mg of protein), and a reasonable response to exogenous GABA can be obtained (16).

Association kinetics of [^{3}H]FNP binding. Although both [^{3}H]diazepam and [^{3}H]FNP bind to the same benzodiazepine receptors, the latter was used in the present study because it possesses a higher affinity, dissociates more slowly from the binding sites, and has less nonspecific binding (10, 17). It therefore allows more accurate measurement of the binding.

Control experiments had shown that equilibrium was reached after 2–3 min incubation at 37°. Therefore, for measuring the association kinetics, the binding was started by adding [^{3}H]FNP to synaptosomal preparations maintained at 0°. At various time intervals after the addition of [^{3}H]FNP, aliquots of 0.5 ml of the reaction mixture were removed and filtered immediately through glass-fiber filters (Whatman GF/B, which were washed with ice-cold buffer immediately before sample filtration) positioned over filtration manifolds (Amicon, Model VFM1) under suction. The filters were washed twice with 5-ml aliquots of ice-cold (0 ± 0.5°) buffer. The entire procedure was completed within 10 sec. The filters were dried and counted as before (15). Nonspecific binding was measured in the presence of 1×10^{-5} M diazepam. Control experiments showed that nonspecific binding reached the steady-state level after 1 min of incubation, and remained constant throughout the experiment. Therefore, nonspecific binding was determined separately only at the end of the assay. Specific binding is the difference between binding in the absence and in the presence of 1×10^{-5} M diazepam.

To analyze the association kinetics, the data obtained were plotted using the integrated rate equation for bimolecular reaction (18):

$$\ln \frac{B_e - B_t}{B_e} = -(k_1[L] + k_{-1}) \cdot t \qquad (1)$$

where B_e and B_t are the concentrations of bound receptor at equilibrium and at time t, k_1 and k_{-1} are the rate constants for the forward and backward reactions, and $[L]$ is the free radiolabeled ligand concentration at steady state. The concentrations of [^{3}H]FNP used were 0.5, 2, and 20 nM. The protein concentration of the synaptosomal membrane preparation used was 0.1 mg/ml. The B_{max} (maximal binding capacity) and K_D (dissociation constant) of [^{3}H]FNP binding from four membrane preparations were 2.21 ± 0.26 (SEM) pmoles/mg of protein and 1.06 ± 0.21 (SEM) nM, respectively, when the initial binding was performed at 37°. Under such conditions, the amount of initial free ligand bound with 0.5 nM [^{3}H]FNP was less than 10% (data not shown). The amount of initial free ligand bound was further decreased when binding was at 0°. The percentage of ligand bound was much less (<2%) with higher concentrations of ligand whether binding was at 0° or 37°. Therefore, the free ligand concentration, $[L]$, in Eq. 1 can be considered constant throughout the reaction. Under such pseudo-first order conditions, data plotted according to Eq. 1 should yield a single component if the reaction is bimolecular. In some experiments, the effects of GABA on the association kinetics were studied by adding 5×10^{-5} M GABA at the same time as the [^{3}H]FNP.

Dissociation kinetics of [^{3}H]FNP binding. Synaptosomal membranes with a protein concentration of 0.5 mg/ml were used. This higher protein concentration as compared with that used in association studies was employed to ascertain that measurable bound activity remained during dissociation measurements. Preliminary studies indicated that dissociation was virtually complete within ~2 min at 37°. Such fast dissociation at high temperature has been reported (19). Therefore, membranes were incubated with 0.1, 2, or 20 nM [^{3}H]FNP at 37° for 30 min, followed by cooling at 0° for 30 min before initiating dissociation. Aliquots were then removed to determine the bound [^{3}H]FNP at equilibrium, which was defined as the total binding at time zero. Dissociation was initiated by adding 1×10^{-5} M diazepam to the steady-state incubation mixture. In a few experiments, 1×10^{-5} M flurazepam was used to displace the radioligand. At various time intervals after the addition of excess diazepam, aliquots of 0.5 ml were removed and filtered as described above, and the bound radioactivity, which was the total binding at time t, was determined. Nonspecific binding in the presence of 1×10^{-5} M diazepam was started at the same time as that of total binding and was determined separately only at the end of the assay. Specific binding was the difference between total binding and nonspecific binding. The specific binding was fully reversible over a period of 2 hr. However, the dissociation was routinely measured only for 68 min, by which time more than 80% of the complex had dissociated. Moreover, results (in Table 2) indicated that the two dissociation components, obtained from the least-squares analysis of the data, could account for all of the binding species. The data obtained were evaluated according to the following equation derived from a simple bimolecular reaction:

$$\ln \frac{B_t}{B_e} = -k_{-1} \cdot t \qquad (2)$$

where B_e is the specific binding at equilibrium or at time zero, B_t is the specific binding at time t, and k_{-1} is the

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dissociation rate constant. The effects of GABA on dissociation were also studied by including 5×10^{-5} M GABA in the incubation. In one set of experiments, membranes were incubated with 2 nM [^{3}H]FNP at 0° for 60 min before initiating the dissociation. In other experiments, membranes alone were incubated at 37° for 30 min, then at 0° for 30 min, followed by binding at 0° for 30 min before dissociation studies.

Protein concentration was determined by the method of Lowry *et al.* (20). Statistical significance was analyzed according to Student's *t*-test, using two tails. [^{3}H]FNP (72 Ci/mmole) was obtained from Amersham Corporation (Chicago, Ill.). GABA was obtained from Sigma Chemical Company (St. Louis, Mo.). Diazepam and flurazepam were the generous gift of Hoffmann-La Roche, Inc. (Nutley, N. J.).

RESULTS

Association kinetics. The forward reaction between [^{3}H]FNP and benzodiazepine receptors was studied under pseudo-first order conditions. Three concentrations of [^{3}H]FNP (0.5, 2, and 20 nM, representing low, nearly half-saturation, and nearly saturation concentration) were incubated with synaptosomal membranes (0.1 mg/ml) at 0° and the rate of the complex formation was measured. An example of the time course of association is illustrated in Fig. 1. The binding reactions proceeded without a lag with all three concentrations tested. With 20 nM [^{3}H]FNP, binding reached the steady-state level within 3–5 min after the reaction was initiated. As the ligand concentration was decreased, the time required to achieve the equilibrium level was prolonged. It reached 50% saturation at 5 and 2 min with 0.5 and 2 nM [^{3}H]FNP, respectively. The kinetic analysis, according to Eq. 1, of the association rate is shown in Fig. 2. It is clear that, under pseudo-first order conditions, association was not linear for all three concentrations, although the association for 20 nM [^{3}H]FNP seemed to approach linearity. The association can be described as consisting of a fast and a slow component. Since the data do not fall on straight lines, the simple bimolecular association mechanism can be discarded.

The slow association components for 0.5 and 2 nM [^{3}H]FNP can be approximated by fitting straight lines to the data points after 8 min of incubation. The fraction of binding sites reacting in the slow exponential phase of the reaction at a specific [^{3}H]FNP concentration can therefore be given by extrapolating the straight lines to $t = 0$. Inspection of the association kinetics in Fig. 2 reveals that the fraction of receptor bound in the slow phase of the reaction decreased from 70% to 40% as the concentration of [^{3}H]FNP was increased from 0.5 to 2 nM. The pseudo-first order association rates (i.e., $k_1[L] + k_{-1}$, calculated from the slope of the linear exponentials $\times$ 2.303) estimated by least-squares fit (similar to that shown for Fig. 4) of the data points for 2 nM [^{3}H]FNP were 0.4691/min and 0.1271/min for the fast and slow component, respectively. Addition of 5×10^{-5} M GABA to the incubation mixture slightly decreased the association rate (results not shown).

Dissociation kinetics after equilibrium binding at 37°. After equilibrium binding at 37° for 30 min and cooling at 0° for 30 min, the dissociation was started by the addition of 1×10^{-5} M diazepam to the reaction mixture. An example of the dissociation kinetics, plotted according to Eq. 2, is shown in Fig. 3. All of the dissociation curves were curvilinear at the three [^{3}H]FNP concentrations tested (0.1, 2, and 20 nM) and consisted of two components. Similar biphasic curves were obtained when dissociation was initiated by adding 1×10^{-5} M flurazepam. The over-all rate of dissociation was concentration-dependent. The higher the ligand concentration, the faster the dissociation rate. To compare the relative time course of dissociation, the time required to dissociate 50% and 80% of the steady-state level of the complex was computed, and the data are presented in Table 1. It is obvious that the lower the ligand concentration, the longer the time needed for dissociation of the complex. However, further decrease of [^{3}H]FNP to 0.05 nM did not significantly prolong the dissociation time (data not shown). The $t_{1/2}$ and 80% dissociation time of the control experiments for 0.2, 2, and 20 nM [^{3}H]FNP were significantly different from one another (Table 1A). Addition of 5×10^{-5} M GABA to the incubation mixture decreased the rate of dissociation (Fig. 4). This effect was more pronounced at higher [^{3}H]FNP concentrations (Table 1A). In the presence of 5×10^{-5} M GABA, the $t_{1/2}$ of dissociation was increased by 39, 35, and 26% for 20, 2, and 0.1 nM [^{3}H]FNP, respectively.

The rate of dissociation during the slow exponential phase was evaluated by a least-squares fit of the data obtained after 40 min. The rate of the fast component was then measured after subtraction of the slow exponential phase. The rate constants of two linear exponentials can then be calculated from the slopes of the curves. Intercepts on the ordinate of the extrapolated curves

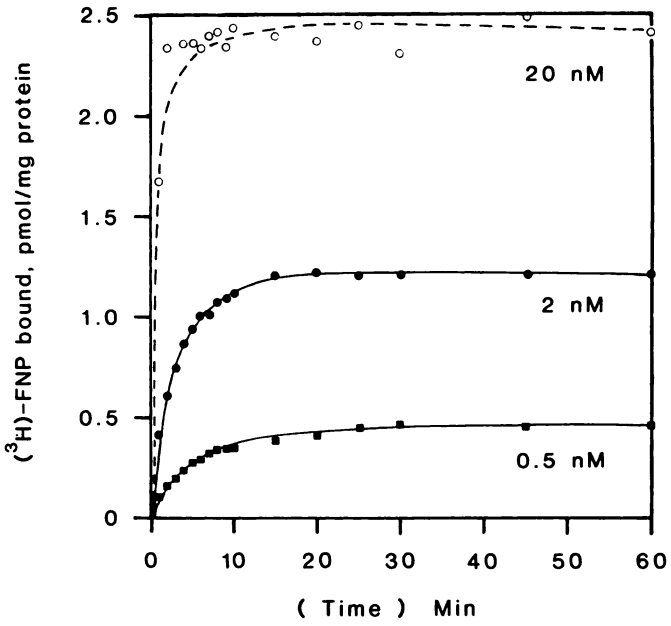

FIG. 1. *Time dependence of [^{3}H]FNP binding*

A homogenate of rat cortical synaptosomal membranes (0.1 mg of protein per milliliter, pre-equilibrated at 0°) was incubated with 0.5 nM (■), 2 nM (●), or 20 nM (○) [^{3}H]FNP at time zero. Aliquots were removed and filtered at the indicated time, and the specifically bound [^{3}H]FNP was determined as described under Materials and Methods.

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represent the steady-state percentage of the two linear exponentials. An example of such an analysis is illustrated in Fig. 4 for 2 nM [^{3}H]FNP. The compiled results are shown in Table 2A. The combined percentage of the two exponentials can account for all of the [^{3}H]FNP-bound complex. For all three [^{3}H]FNP concentrations tested, whether alone or in the presence of 5×10^{-5} M GABA, the fast exponential dissociated 3.5–4.3 times faster than the slow exponential.

The rate constants of both slow and fast phases seemed to increase with increasing [^{3}H]FNP concentrations, although there were no significant differences between the same exponential rate constant for 0.1 and 2 nM [^{3}H]FNP (Table 2A). However, both rate constants for 20 nM [^{3}H]FNP were significantly higher than the corresponding rate constants for either 2 or 0.1 nM [^{3}H]FNP. With a

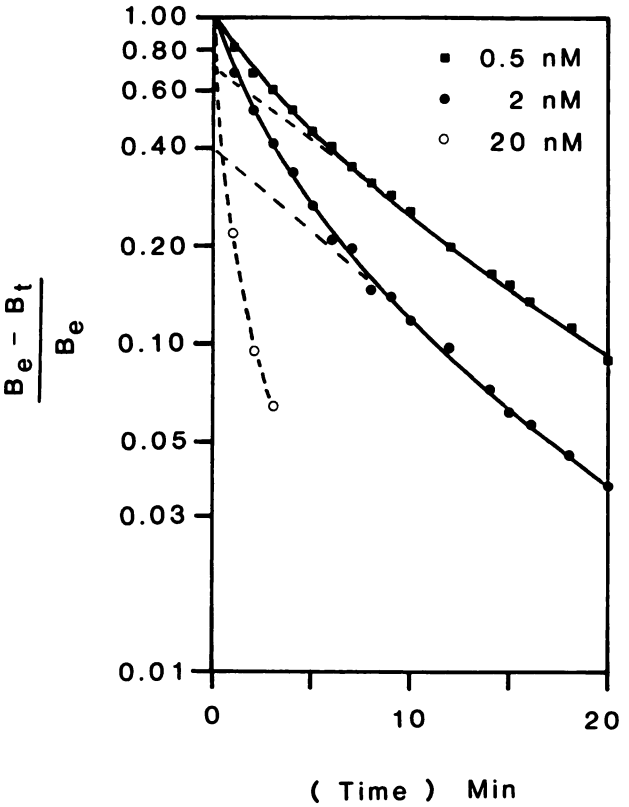

FIG. 2. *Rate of [^{3}H]FNP binding at different [^{3}H]FNP concentrations*

Rat cortical synaptosomal membranes at 0° were incubated with 0.5 nM (■), 2 nM (●), or 20 nM (○) [^{3}H]FNP for the indicated time, and bound [^{3}H]FNP was determined as described under Materials and Methods. The data for each ligand concentration (average of four to seven experiments similar to that shown in Fig. 1) were plotted according to Eq. 1, described under Materials and Methods. The slopes of the curves are equal to $(k_1[L] + k_{-1})/2.303$.

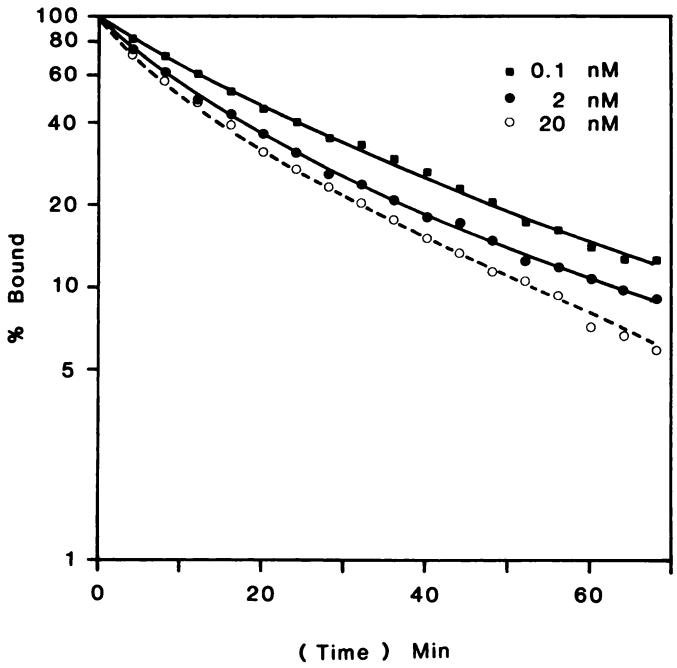

FIG. 3. *Time course of dissociation of [^{3}H]FNP complex*

Rat cortical synaptosomal membranes (0.5 mg/ml) were incubated with 0.1 nM (■), 2 nM (●), or 20 nM (○) [^{3}H]FNP at 37° for 30 min and at 0° for 30 min. Dissociation was started by the addition of 1×10^{-5} M diazepam at time zero. The percentage of the remaining specifically bound [^{3}H]FNP after various time intervals was plotted according to Eq. 2 under Materials and Methods. At time zero, there were 0.074, 1.23, and 2.25 pmoles of [^{3}H]FNP bound per milligram of protein for 0.1, 2, and 20 nM [^{3}H]FNP, respectively.

TABLE 1

Concentration-dependent dissociation of [^{3}H]FNP binding to rat cortical synaptosomal membranes

Dissociation with 1×10^{-5} M diazepam was started after binding with synaptosomal membranes (0.5 mg of protein per milliliter) either at 37° for 30 min and then an additional 30 min at 0°, or at 0° for 60 min.

[^{3}H]FNP (nM)	Dissociation time (min)			
	50% Dissociation[a]		80% Dissociation[a]	
	Control	GABA (5×10^{-5} M)	Control	GABA (5×10^{-5} M)
	A. After binding at 37° for 30 min + 0° for 30 min[b]			
0.1	17.1 ± 1.1 (5)	21.5 ± 0.9 (5)	49.9 ± 0.4 (5)	61.2 ± 1.0 (5)
2	13.6 ± 0.6 (10)	18.4 ± 0.7 (4)	39.3 ± 1.1 (10)	52.0 ± 0.4 (4)
20	10.4 ± 0.9 (4)	14.4 ± 0.6 (4)	31.0 ± 1.9 (4)	40.9 ± 0.8 (4)
	B. After binding at 0° for 60 min[b]			
2	10.0 ± 0.6[c] (7)	14.8 ± 1.0[c] (4)	28.2 ± 1.4[c] (7)	42.9 ± 1.7[c] (4)

[a] Shown are the mean ± standard error of the mean of each group. Figures in parentheses indicate number of experiments

[b] Under all conditions, GABA significantly increased the time required for dissociation ($p < 0.05$ by paired and by group *t*-test). Dissociation time was significantly decreased by each increment in [^{3}H]FNP concentration ($p < 0.05$ by *t*-test).

[c] $p < 0.05$ versus the dissociation time after initial 2 nM [^{3}H]FNP binding at 37°, using the two-tailed test.

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low concentration of ligand, e.g., 0.1 nM [^{3}H]FNP, the steady-state ratio ($t = 0$) of the slow to fast dissociation complex was 70.7:30.7%. This ratio was 52.9:45.7% when [^{3}H]FNP was increased to 2 nM. No further change in the ratio was observed when the concentration of [^{3}H]FNP was increased to 20 nM. A concentration of 5×10^{-5} M GABA decreased the rate constants for both the fast and slow dissociation phases. This was most apparent with higher concentrations of ligand, and was significant only for the fast-rate constant at 2 nM [^{3}H]FNP, and for both rate constants at 20 nM [^{3}H]FNP.

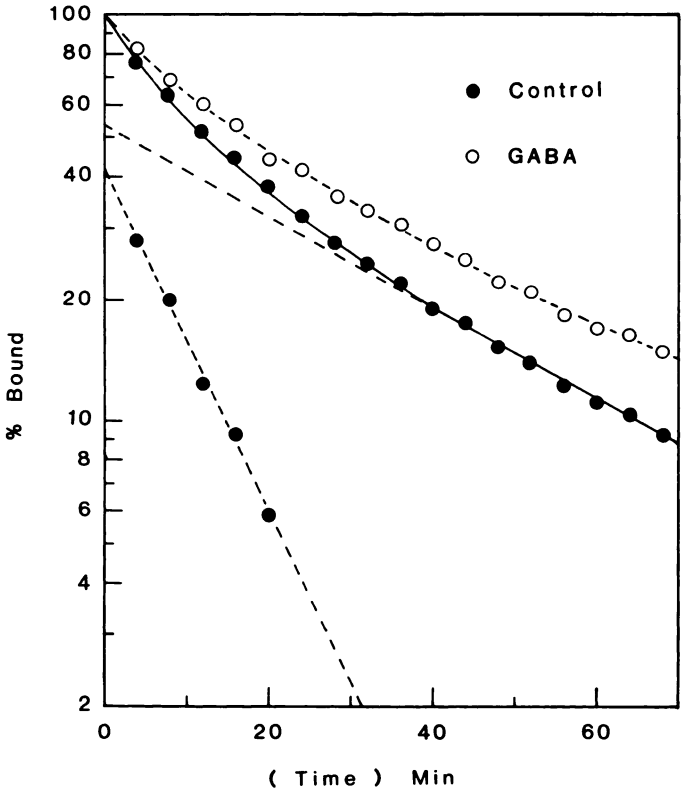

FIG. 4. *Effect of GABA on the rate of dissociation of [^{3}H]FNP*

GABA, 5×10^{-5} M, and [^{3}H]FNP, 2 nM, were incubated with the rat synaptosomal membranes (0.5 mg/ml) under the same conditions as described in Fig. 3. At time zero, there were 0.89 and 1.01 pmoles of [^{3}H]FNP bound per milligram of protein for control (●) and in the presence of 5×10^{-5} M GABA (○). Least-squares fit analysis of the two exponentials (●– – –●) is shown for the control experiment.

In one experiment, the synaptosomal membrane preparation (0.5 mg of protein per milliliter) was incubated at 37° for 30 min and then equilibrated in a 0° bath for 30 min in the absence of [^{3}H]FNP. This was followed by binding at 0° in the presence of 2 nM [^{3}H]FNP for 30 min before initiating dissociation. In a parallel control experiment, synaptosomal membrane was incubated with 2 nM [^{3}H]FNP at 37° for 30 min, which was followed by cooling for 30 min and then dissociation at 0°. The 50% and 80% dissociation times were 14.4 min and 41.5 min, respectively, for the control, and 15.6 min and 44.2 min, respectively, for the preincubation at 37° in the absence of the ligand. The slow- and fast-rate constants for the control and for preincubation in the absence of [^{3}H]FNP were 0.0236/min and 0.0868/min, and 0.0253/min and 0.0958/min, respectively. These values were comparable to those found from the mean of 10 experiments after binding at 37° (Tables 1A and 2A). The dissociation rate constants were significantly decreased after binding at 37° when compared with those observed after binding and dissociation at 0° (Table 2B).

Dissociation kinetics after equilibrium binding at 0°. Similar to those observed in the preceding experiments, a relatively fast and a slow dissociation phase were found when equilibrium binding and dissociation were carried out at 0°. However, the dissociation rate using membranes preincubated at 0° was comparatively faster than that using membranes preincubated at 37°. Table 1B shows that the 50% and 80% dissociation times with membranes preincubated at 0° were significantly less than those with membranes preincubated at 37° with the same ligand concentration. The dissociation rate con-

TABLE 2

Dissociation rate constants of [^{3}H]FNP binding to rat cortical synaptosomal membranes

Dissociation was initiated after equilibrium binding with synaptosomal membrane (0.5 mg of protein per milliliter) either at 37° for 30 min and an additional 30 min at 0°, or at 0° for 60 min. Dissociation occurred in the presence of 1×10^{-5} M diazepam. Values are mean ± standard error of the mean of each group.

[^{3}H]FNP (nM)	Fast exponential		Slow exponential	
	%	Rate constant (min^{-1})	%	Rate constant (min^{-1})
	A. After binding at 37° for 30 min + 0° for 30 min			
0.1 (5)[a]	30.7 ± 3.9[b]	0.0924 ± 0.0051	70.7 ± 3.9[b]	0.0253 ± 0.0010
0.1 + GABA[c] (5)	28.2 ± 2.9	0.0908 ± 0.0044	72.7 ± 3.4	0.0238 ± 0.006
2 (10)	45.7 ± 2.7[d]	0.0970 ± 0.0041[d,e]	52.9 ± 1.9[d]	0.0260 ± 0.0012[e]
2 + GABA (4)	30.6 ± 1.8	0.0810 ± 0.0032	70.6 ± 1.9	0.0237 ± 0.0006
20 (4)	39.8 ± 4.5	0.1405 ± 0.0093[d]	56.9 ± 3.7	0.0324 ± 0.0012[d]
20 + GABA (4)	39.3 ± 3.9	0.0910 ± 0.0076	57.8 ± 5.1	0.0250 ± 0.0010
	B. After binding at 0° for 60 min			
2 (7)	56.7 ± 3.0[d,f]	0.1115 ± 0.0066	43.1 ± 2.2[d,f]	0.0303 ± 0.0010[d,f]
2 + GABA (7)	45.3 ± 2.5[f]	0.0921 ± 0.0077	54.0 ± 2.3[f]	0.0231 ± 0.0010

[a] Number in parentheses indicates number of experiments.
[b] $p < 0.01$ versus 2 nM [^{3}H]FNP.
[c] The concentration of GABA was 5×10^{-5} M.
[d] $p < 0.05$ versus in the presence of GABA.
[e] $p < 0.05$ versus 20 nM [^{3}H]FNP.
[f] $p < 0.05$ versus 2nM [^{3}H]FNP binding at 37°.

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stants at 0° for 2 nM [^{3}H]FNP are presented in Table 2B. The rate constants for both exponentials were larger after 0° preincubation than after 37° preincubation, indicating that binding at 37° decreased the dissociation rates of the complexes. Furthermore, the proportion of binding in the fast dissociation phase at 0° was significantly larger than that of membranes treated at 37° (Table 2B).

Addition of 5×10^{-5} M GABA to the incubation mixture prolonged the dissociation time (Table 1B) and reduced the dissociation rate constants of both fast and slow phases (Table 2B). GABA reduced the ratio of the fast to slow phase more in membranes treated at 37° than in those maintained at 0°.

Dissociation kinetics after prolonged binding at 37°. The effects of prolonged binding on dissociation kinetics were studied by incubating synaptosomal membrane (0.5 mg of protein per milliliter) with 2 nM [^{3}H]FNP at 37° for 2 hr, followed by cooling the incubation mixture at 0° for 30 min. Dissociation at 0° was then started by the addition of 1×10^{-5} M diazepam. Figure 5 demonstrates that dissociation of the complex was slower after prolonged incubation at 37° for 2 hr. This was due to an increase of the percentage of the slow exponential from around 50% to around 75% when the incubation time was increased from 30 to 120 min. The rate constants after 2 hr of incubation at 37° were 0.0235 ± 0.0013 min^{-1} (mean ± SEM, $n = 3$) and 0.1018 ± 0.0020 min^{-1} (mean ± SEM, $n = 3$) for slow and fast exponentials, respectively, which were very similar to those obtained after incubation at 37° for 30 min (Table 2). The amount of [^{3}H]FNP bound (picomoles per milligram of protein) increased significantly from 0.989 ± 0.040 (mean ± SEM, $n = 10$) after 30 min of incubation to 1.150 ± 0.043 (mean ± SEM, $n = 3$) after 120 min of incubation at 37°. Inclusion of 5×10^{-5} M GABA in the incubation mixture further increased the percentage of the slow phase to about 90%, leaving the rate constant of the slow exponential unaffected. Similar results, i.e., an increase in the percentage of the slow-exponential phase, were obtained after incubating the synaptosomal membrane with 20 nM [^{3}H]FNP at 37° for 120 min. In a typical experiment, the slow phase increased from around 55% to 80% when the incubation time was increased from 30 to 120 min. The rate constants were not changed significantly after prolonged incubation. In other experiments, the synaptosomal membrane was prepared from fresh cortices with all of the solutions maintained at 37°. The crude P_2 fraction was prepared in 0.32 M sucrose containing proteolytic enzyme inhibitors, phenylmethyl sulfonyl fluoride (75 μg/ml), pepstatin A (5 μg/ml), and leupeptin (1 μg/ml). Synaptosomal membrane was prepared and kept at 37° for a total of about 4 hr before the binding assay. Binding of membrane (0.5 mg of protein per milliliter) with 20 nM [^{3}H]FNP was carried out at 37° for 30 min, followed by equilibration at 0° for 30 min before dissociation. The membrane thus prepared demonstrated dissociation kinetics similar to those found for 20 nM [^{3}H]FNP after a 2-hr incubation at 37°. The ratio of slow to fast phase was about 80:20%.

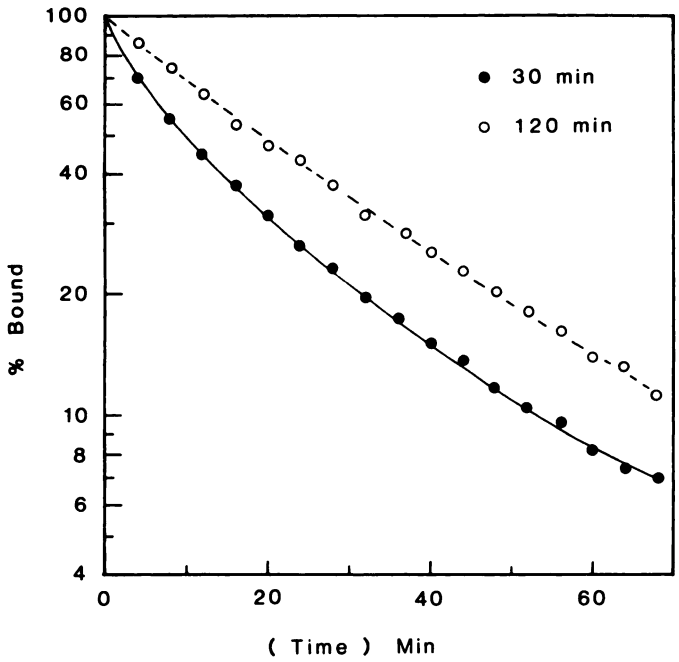

FIG. 5. *Effect of incubation duration on dissociation*
Synaptosomal membrane (0.5 mg of protein per milliliter) from rat cortex was incubated with 2 nM [^{3}H]FNP at 37° for either 30 min (●) or 120 min (○) and then at 0° for 30 min. Dissociation was the same as that shown in Fig. 3.

Dissociation kinetics at pre-equilibrium state. In order to characterize further the dissociation kinetics of [^{3}H]FNP binding, the dissociation of the complex was compared under pre-equilibrium and equilibrium conditions. The dissociation with diazepam was started after 3 min or after 30 min of incubation at 0°. These two time points represent the pre-equilibrium and near-equilibrium state of the complex. As shown in Fig. 6A, the dissociation rate of the complex was faster at pre-equilibrium than that at the equilibrium condition. This is more clearly demonstrated in Fig. 6B, where the dissociation was plotted according to Eq. 2. Two distinct dissociation components were observed whether at pre-equilibrium or the equilibrium condition. The rate of dissociation of the slower-dissociating complex was faster for the pre-equilibrium condition. The ratio of fast to slow dissociating complex was greater at pre-equilibrium than at the equilibrium condition.

DISCUSSION

Earlier saturation binding studies, using equilibrium binding of either [^{3}H]diazepam (1–3) or [^{3}H]FNP (6, 9) demonstrated only a single type of benzodiazepine receptor in mammalian brain tissues. This was further substantiated by the observation that many benzodiazepines displace [^{3}H]diazepam binding with a Hill coefficient of near unity (4). Several triazolopyridazines, a new class of anxiolytics that cause no sedation in animals, have been found to displace [^{3}H]FNP binding with Hill coefficients of less than unity (10). These compounds have also been found to protect one of the two heat-inactivation processes of [^{3}H]FNP binding sites (10). From these results, together with the report of multicomponent dissociation

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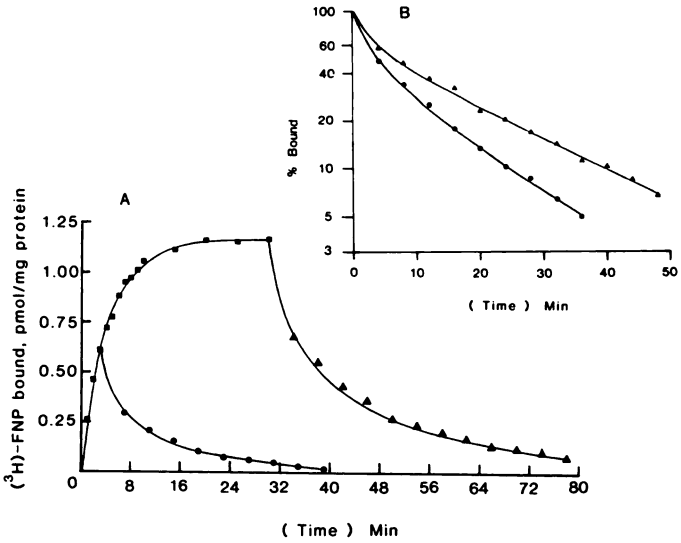


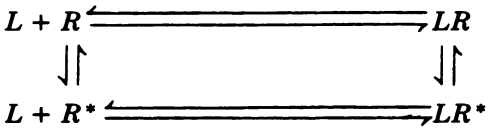
FIG. 6. *Rate of [^{3}H]FNP dissociation before and at nearly steady-state level of binding*

A. Homogenate of rat cortical synaptosomal membranes (0.5 mg of protein per milliliter) was incubated with 2 nM [^{3}H]FNP at 0°. After 3 min (●) and 30 min (▲) of incubation, 1×10^{-5} M diazepam was added to portions of the reaction mixture, and specific [^{3}H]FNP remaining bound was determined at the indicated times. The rate of [^{3}H]FNP association was also measured (■).

B. Rates of [^{3}H]FNP dissociation after the addition of 1×10^{-5} M diazepam at 3 min (●) and 30 min (▲) were plotted according to Eq. 2.

curves (10), it was suggested that two types of benzodiazepine receptors exist in the central nervous system (10). However, it is equally plausible that these results could be interpreted by assuming one type of benzodiazepine receptor with two interconvertible conformations or functional states, each having different kinetic properties. The kinetic experiments reported in this study were designed to characterize further whether there are multiple types or multiple conformational (or functional) states of benzodiazepine receptors.

To investigate the association kinetics, the binding was carried out under pseudo-first order conditions; i.e., the concentration of ligand was much larger than the concentration of binding sites. It was found that the pseudo-first order association curves are biphasic and the percentage of the fast binding component increases with increasing ligand concentrations. These results do not support a simple bimolecular reaction mechanism between benzodiazepines and their receptors. Similar association phenomena have been observed in the muscarinic acetylcholine receptor system from mouse brain (21) and developing chick heart (22). A model consisting of an initial reversible complex which then rearranges or isomerizes to a more stable complex has been proposed from such kinetic data by these investigators (21, 22). However, it is assumed that the ligand dissociates only from the initial reversible complex, and not from both forms of complex. In the present study, there was no evidence to limit the dissociation of the ligand from the more stable complex. Therefore, the following cyclic model of binding is proposed from the association kinetic studies.



In this model, benzodiazepine receptors are perceived as one distinct type of receptor with two interconvertible binding conformations or binding states, R and R^*, with the latter possessing higher affinity for the ligand. The binding of ligand to the lower-affinity R conformation forms an initial, readily reversible, complex which isomerizes to the more stable LR^* complex. The latter can also be obtained when the ligand binds to the high-affinity R^* state. Implicit in this model is that the measured bound radioactivity must come from LR and LR^*, and the rate of formation of LR must be faster than its isomerization to LR^* under the conditions used in these experiments.

The percentage of either fast or slow dissociating complexes at equilibrium, which can be determined from the intercepts on the ordinate of the two dissociation phases, is influenced by the temperature and the duration of binding and by GABA. The dissociation of the ligand-receptor complexes was faster and the ratio of fast to slow dissociation phase was larger when equilibrium binding was carried out at 0° instead of 37°, indicating that there is a higher percentage of the receptor complexes existing in the lower-affinity state at low temperature. Prolonged incubation at 37° increases the percentage of the complexes that dissociate slowly. These results suggest that incubation at 37° favors the formation of more stable complexes. In addition, our observation that the dissociation rates were very similar whether incubation at 37° was carried out in the presence or in the absence of [^{3}H]FNP strongly suggests the conversion of R to R^*. This finding also indicates that the slowly-dissociating complexes are formed primarily between [^{3}H]FNP and the high-affinity binding conformation of benzodiazepine receptors. The isomerization of the initial readily reversible complex LR to LR^*, which is required to complete the proposed cyclic model, was substantiated by the finding that dissociation was faster at pre-equilibrium than at the equilibrium state (Fig. 6).

An alternative explanation of the observation of two association and two dissociation phases is the existence of two distinct types of receptors, one binding with high affinity and the other with low affinity. Since [^{3}H]FNP binds to the benzodiazepine receptors without any lag and proceeds to equilibrium with reasonably fast association kinetics, it would be expected that it binds preferentially to the high-affinity sites (i.e., sites characterized by a small dissociation rate constant and a large association rate constant) at the beginning of the association. As the reaction continues, [^{3}H]FNP will bind more to the low-affinity sites. Consequently, it is expected that the dissociation of the complexes would be slower at pre-equilibrium than that at the equilibrium state. Exactly the opposite result was found in the present study, which argues against the simple two-site binding model. Furthermore, the increase in the proportion of the slow dissociation phase with no change in its dissociation rate

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constant after prolonged binding at 37° indicates a time-dependent conformational change of low-affinity binding sites, low-affinity complexes, or both. Taken together, these results do not support a simple model of two independently binding sites. In addition, a model with two distinct, noninteracting binding sites would predict a nonlinear Scatchard plot in the equilibrium binding studies. However, this has not been reported for rat brain tissues.

The concentration-dependent dissociation can be adequately explained by the proposed cyclic model. At low concentrations of ligand, LR^* will be the predominant species, and a slower dissociation rate is seen. The faster dissociation observed with high concentrations of ligand is likely to be due to the higher proportion of LR, since its formation is faster than its isomerization to LR^*. The effect of GABA in decreasing the dissociation rates depends on the ligand concentration; the higher the ligand concentration, the larger the GABA effect. Therefore, it seems likely that GABA decreases the dissociation primarily by stabilizing LR, the ligand-low affinity conformation complex. Possibly, GABA might also have an effect on the isomerization step between LR and LR^*. Incubation at 37° appears to favor the formation of R^*, since this incubation increased the fraction of slowly dissociating complex whether or not ligand was present during the incubation. This conclusion was substantiated by a similar experiment which demonstrated that preincubation of tissue at 37° increases the binding affinity of [^{3}H]FNP without altering the maximal binding capacity (23). It has been demonstrated that some of the GABA present in brain tissues is very difficult to remove (24). Therefore, it is conceivable that the conversion of R to R^* observed after prolonged incubation at 37° may be partially influenced by GABA being slowly released during incubation. The presence of ligand does not appear to have an effect on the conversion of LR to LR^*, although such an action might have been masked by the effect of incubation at 37°.

It is possible to estimate the dissociation constants of the binding of [^{3}H]FNP to the two conformational states of benzodiazepine receptors from the kinetic parameters obtained in the present study. The pseudo-first order association rates (equivalent to $k_1[L] + k_{-1}$) for 2 nM [^{3}H]FNP at 0° were estimated to be 0.4691/min and 0.1271/min for the fast and slow components, respectively. The corresponding dissociation constants for the fast and slow association components after binding at 0° with 2 nM [^{3}H]FNP were 0.0303/min and 0.115/min, respectively. (Note that the results discussed above indicate that the conformational state that binds [^{3}H]FNP more easily i.e., with higher affinity, is very likely to dissociate from the ligand less easily.) From these kinetic values, the dissociation constants for the high- and low-affinity conformational states were found to be 0.14 nM and 14.3 nM, respectively. A difference of 100-fold in affinities would have been demonstrated as two components in the Scatchard analysis if the two binding sites are distinct, separate entities. However, our kinetic data support the interconversion of two conformations or functional states. The dissociation constant obtained from four saturation binding experiments in the present study was 1.22 ± 0.15 nM (mean ± SEM) at 0°, which is between the two dissociation constants for the two binding sites obtained from kinetic studies and, therefore, compatible with the interconversion model. A similar model was recently presented to account for the binding of acetylcholine to nicotinic receptors (25). In that study, kinetic data also suggested the existence of two interconvertible functional states of the receptor, with a 400-fold difference in dissociation constants for the two states. As in the present study, equilibrium binding experiments yielded an intermediate dissociation constant and a linear Scatchard plot. These results underscore the inability of Scatchard analysis of equilibrium binding studies to demonstrate interconvertible forms of receptors, and is analogous to the problem of analysis of so-called hysteretic enzymes, as outlined by Neet and Ainslie (26).

Binding of 20 nM [^{3}H]FNP, a nearly saturating concentration, to membranes prepared at 37° could permit us to estimate the relative percentage of the slow and fast dissociation phase, and hence the relative percentage of the two receptor conformations. From this study, an upper limit of 80% was found for the high-affinity conformation of benzodiazepine receptors from rat cortex. Although the results presented in this study can be interpreted by the cyclic model, additional experiments are needed to support or modify the reaction schemes. In this regard, it is important to note that two or more conformations of some classes of benzodiazepine receptors have been proposed primarily from the influences of GABA and GABA analogues and heat treatment of [^{3}H]diazepam binding in washed rat forebrain tissue (27). Results of the heat treatment, which increased and then decreased [^{3}H]diazepam binding, strongly suggest conformational changes of benzodiazepine receptors. However, the effects of GABA and its analogues on the binding did not differentiate whether they change the conformation of benzodiazepine receptors or merely stabilize the ligand-receptor complexes, as shown in the present experiment.

Further work is necessary to establish which of these conformations mediates all or some of the pharmacological effects of benzodiazepines. Also, it will be important to determine whether these different conformations of benzodiazepine receptors can account for the differential binding of triazolopyridazines (10) or β-carbolines (14). The exact nature of the conformational change also remains to be elucidated. Since these studies were conducted with membrane-bound receptors, the two states may represent the receptor with or without another interacting molecule affecting its function. Examples of such a molecule might be the GABA receptor (16, 28) or a membrane-bound regulator (29). Studies using solubilized receptors may help to answer these questions. One recent exciting development is the discovery of specific benzodiazepine antagonists (30). These compounds may have special utility in refining the reaction mechanisms proposed in this study.

In conclusion, kinetic data provided in this study suggest that benzodiazepine receptors exist as one homogeneous population with two interconvertible conformational states, both of which bind benzodiazepines. The conversion of low-affinity to high-affinity states is facili-

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tated by incubating the synaptosomal membranes at 37° for a prolonged period of time. Binding of the ligand to the low-affinity conformation forms a more easily reversible complex which isomerizes to the more stable complex. GABA decreases the dissociation rate primarily by reducing the dissociation of the less tightly-bound reversible complex. The functional significance of these conformations remains to be elucidated.

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