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Kinetic Regulation of Convulsant (TBPS) Binding by GABAergic Agents

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SUMMARY

The kinetics of specific [^{35}S]-*t*-butylbicyclophosphorothionate (TBPS) binding was studied in rat brain synaptosomal membrane preparations. The effects of a representative depressant barbiturate, *R*-(−)-*N*-(1)-Me-5-Phe-5-Pr-barbituric acid [*R*(−)MPPB, and γ-aminobutyric acid (GABA) were biphasic on TBPS binding, but the enhancements of binding by low concentrations of *R*(−)MPPB or GABA disappeared when binding equilibrium was reached. The slope factors of TBPS displacement and the IC_{50} values of *R*(−)MPPB and GABA decreased when TBPS binding approached equilibrium. Up to 70 min, binding in the presence of 300 μM *R*(−)MPPB exceeded, then remained below, the level of the control. *R*(−)MPPB decreased the apparent association half-life of TBPS binding from 41.5 min to 11.9 min. The GABA agonist, muscimol, in the concentration range of 20 and 200 nM, progressively accelerated the rate of TBPS dissociation. This effect was completely reversed by 20 μM bicuculline methochloride. GABA antagonists, bicuculline methochloride (20 μM) and R 5135 (20 nM), alone decelerated the rate of TBPS dissociation. Dissociation of TBPS was also initiated by dilution in the presence of various salts. Replacement of Cl^- by Br^- ions (0.5 molar) accelerated the rate of dissociation, whereas replacement of K^+ by Na^+ ions had no effect. This indicates the role of Eccles anions, not of cations, in TBPS binding and the possible involvement of the chloride ionophore. A kinetic model is discussed for the allosteric modulation of TBPS binding by various GABAergic agents. Interconvertible populations of TBPS sites are proposed with rapid and slow kinetics. Model calculations involving modulation in the same direction of the on and off rates of binding can reproduce observed phenomena. The model predicts that GABA agonists, barbiturate and pyrazolopyridine depressants, as well as 1,4-benzodiazepine agonists, would allosterically increase the proportion of a rapid kinetic population of TBPS sites. In contrast, the model predicts that a decrease in the contribution of the rapid phase might be brought about by GABA antagonists and β-carboline inverse agonists. The slow and rapid kinetic populations of TBPS sites might represent the closed and open states of the chloride ionophores, respectively.

[^{35}S]TBPS labels a convulsant site of $GABA_A$ receptor-regulated chloride ionophores (1–4). Several convulsants such as tetrazoles (5), picrotoxin-like cage compounds, and β-alkyl-substituted γ-butyrolactones (6) bind to these sites in an apparently competitive manner as suggested by the structural similarities of these compounds (2, 7) and their kinetic ability to elicit identical rates of TBPS dissociation (8, 9). Some GABAergic depressants such as barbiturates and the pyrazolopyridine etazolate, etomidate, and also ethanol appear to interact allosterically with these convulsant sites since they accelerate the dissociation of TBPS (9–11). Furthermore, these compounds were reported to display a biphasic effect on TBPS binding, i.e., enhancement at low concentrations of the agents and displacement at higher concentrations (3). The effects of GABA agonists (1, 12, 13) and BZ receptor ligands (3, 14–16) on TBPS binding are obviously allosteric. However, it is difficult to understand, from the pharmacological point of view, how the above agents with anticonvulsant activity can enhance the specific binding of the convulsant TBPS (3, 14, 16–18). It also seems anomalous that convulsant β-carbolines decrease the specific binding of TBPS (3, 14–16). We have recently demonstrated that the effects of the BZ receptor ligands on TBPS binding can be attributed to nonequilibrium conditions of TBPS binding (19). Kinetic analysis of binding supported by model calculations reveals, in this paper, that GABAergic agents regulate allosterically and simultaneously both association and dissociation rates of TBPS binding. A model for interconvertible rapid and slow kinetic populations of convulsant sites is assumed which is in agreement with kinetic measurements and may explain some pharmacological effects of GABAergic agents via the chloride ionophore.

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ABBREVIATIONS: TBPS, *t*-butylbicyclophosphorothionate; BZ, 1,4-benzodiazepine; BCM, bicuculline methochloride; DMCM, 6,7-dimethoxy-4-ethyl-β-carboline-3′-carboxylate methyl ester; GABA; γ-aminobutyric acid; *R*(−)MPPB, *R*-(−)-*N*(1)-methyl-5-phenyl-5-propyl-barbituric acid; R 5135, 3α-hydroxy-16-imino-5β-17-aza-androstane-11-one.

Experimental Procedures

Materials. ^{35}S-labeled TBPS (specific radioactivity: 30–100 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and stored at −20°. R 5135 was kindly donated by Dr. P. Hunt (Roussel-Uclaf, France) and *R*(−)MPPB was donated by Prof. J. Knabe (FRG). Picrotoxin was obtained from Sigma Chemical Co. (St. Louis, MO), muscimol from Serva (FRG), and BCM from Pierce Chemical Co. (Rockford, IL).

Membrane preparation. Synaptosomal membranes of cerebral cortex were prepared with slight modifications of the method described by Maksay and Ticku (9). Brains of male Wistar rats (120–200 g) were rapidly dissected; then, the cortex was homogenized in 20 volumes of 0.32 M sucrose with a Teflon-glass homogenizer and centrifuged at 1,000×*g* for 10 min. The supernatant was centrifuged at 48,000×*g* for 30 min. The pellet was homogenized in cold, double-distilled water and treated with Ultra-Turrax for 20 sec. It was centrifuged at 48,000×*g* for 30 min. The pellet was washed again by resuspension and centrifugation in distilled water. Then, the pellet was homogenized in 5 mM Tris-HCl buffer (pH = 7.4) containing 200 mM KCl, similarly centrifuged, and frozen in the same buffer. The next day it was thawed, centrifuged at 48,000×*g* for 30 min, washed twice in the same buffer with centrifugations, and refrozen. On the day of the assay, it was thawed and washed by a similar centrifugation, and resuspended in 5 mM Tris-HCl buffer containing the appropriate salt concentration for the binding study.

To detect the biphasic effect of depressants on TBPS binding, synaptosomal membranes of rat forebrains were prepared by the modified method of Supavilai and Karobath (3). Forebrains of male Wistar rats were homogenized in 0.32 M sucrose and centrifuged at 1,000×*g* for 10 min. The supernatant was centrifuged at 48,000×*g* for 30 min, and the pellet was homogenized in 5 mM Tris-HCl buffer (pH = 7.4), similarly centrifuged, then washed twice in 50 mM Tris-HCl buffer and frozen. On the day of the assay, it was thawed and centrifuged in 50 mM Tris-HCl.

[^{35}S]TBPS binding. Biphasic effects of GABA and *R*(−)MPPB on TBPS binding were studied in the forebrain membrane preparation. A membrane suspension (1–2 mg of protein/ml) in 50 mM Tris-HCl containing 300 mM NaCl was incubated with 1.3 nM [^{35}S]TBPS at 22° in the presence or absence of different concentrations of GABA or *R*(−)MPPB. At 30, 60, and 180 min of incubation, aliquots of 0.7 ml were filtered on Whatman GF/B filters under vacuum in duplicate and washed twice with 3 ml of cold buffer. Radioactivity on the filters was determined in a benzyl alcohol-based scintillation cocktail with an efficiency of about 85%.

The rate of TBPS association was studied under conditions identical to those above (forebrain membranes, in the presence or absence of 300 μM *R*(−)MPPB). Specific binding determined in duplicate at various times (B_t) was related to equilibrium TBPS binding (B_e, at 180 min). For the determination of the apparent association rate constant (k_{app}), $\ln \frac{B_e}{B_e - B_t}$ values were plotted against time. The slope of linear regression resulted in the k_{app} value.

All other binding studies were conducted with the extensively washed cortical membrane preparation. For the dissociation of [^{35}S]TBPS by dilution, the cortical membrane suspension (5–6 mg of protein/ml) was incubated with 10 nM [^{35}S]TBPS at 22° for 140 min in 5 mM Tris-HCl buffer containing 0.5 M KCl. Dissociation was initiated by 100-fold dilution with 5 mM Tris-HCl buffer containing 0.5 M KCl, NaCl, or KBr. Aliquots of 5 ml were filtered as above.

To study the effect of GABA agonists and antagonists on TBPS dissociation, the cortical membrane suspension (1–2 mg of protein/ml) was incubated with 3 nM [^{35}S]TBPS in 5 mM Tris buffer containing 200 mM KBr for 100 min at 22° in the absence or presence of 20 nM R 5135 or 20 μM BCM. Dissociation was initiated by the addition of 40 μM picrotoxin which gives dissociation plots identical to that of unlabeled TBPS (8, 9). Aliquots of 0.5 ml were filtered at various times and processed as above. Nonspecific binding was determined in the presence of 40 μM picrotoxin. The logarithm of the B_t/B_o ratio (specific binding at time *t* related to that at *t*=0, i.e., at the initiation of dissociation) was plotted against time. The half-life of dissociation was determined from the slope of a linear fit to the data. When the dissociation was biphasic, two phases were separated by computer-assisted nonlinear regression. Significance of the difference of two dissociation plots was determined by a pairwise comparison of the data using the Student's *t* test. Dissociation plots were considered to be equivalent if the data pairs were not significantly different ($p < 0.01$).

Model calculations for binding kinetics. Representative curves were computed from the general kinetic solution for reversible association-dissociation (20) by a Hewlett-Packard HP-41C calculator. Input parameters applied for control curves (*a*) were: K_D = 17 nM, receptor concentration = 60 pM; [^{35}S]TBPS concentration = 3 nM; [^{35}S]TPBS bound at 180 min was taken at 95% of the equilibrium value and is expressed on the ordinate relative to the equilibrium binding. For modulated kinetic curves the values of the association (k_1) and dissociation rate constants (k_{-1}) of the control were multiplied by the factors indicated in the legends of the figures.

Results

The effect of monovalent ions on TBPS dissociation. The specific binding of TBPS requires the presence of high concentrations of certain salts (1, 4, 13). The kinetics of TBPS dissociation were examined by dilution which allowed the systematic replacement of monovalent anions and cations. Control dissociation was initiated by 100-fold dilution by the same buffer containing 0.5 M KCl. This slow, apparently monophasic dissociation (Fig. 1) was characterized by a half-life of 94 min. When TBPS dissociation was initiated by dilution with Tris buffer containing 0.5 M KBr (Fig. 1), it resulted in the appearance of a rapid first phase of dissociation with a half-life of 17 min. In contrast, when dissociation was initiated in the presence of 0.5 M NaCl, its rate was not significantly different from that with KCl (Fig. 1). These data indicate that the rate of TBPS dissociation is sensitive for monovalent anions, but not for the cations.

The biphasic effect of GABA agonists and depressants on TBPS binding. We reproduced the cortical membrane preparation of Supavilai and Karobath (3) and the experimen-

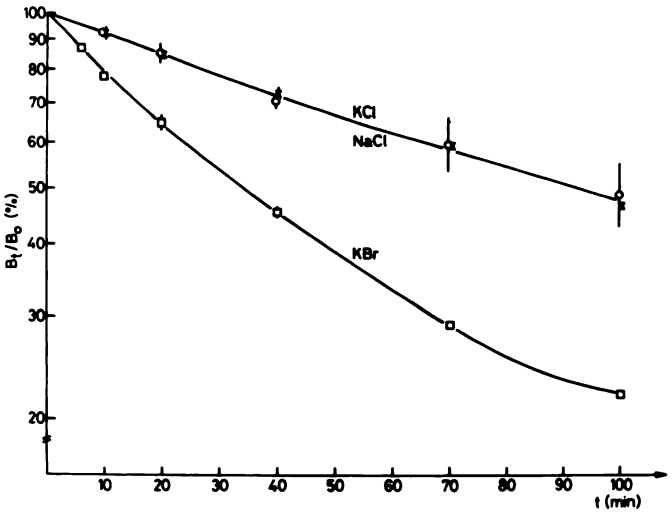


Fig. 1. The effect of monovalent cations and anions on TBPS dissociation. The cortical membrane suspension (5–6 mg of protein/ml) was incubated with 10 nM [^{35}S]TBPS at 22° for 140 min in 5 mM Tris-HCl buffer containing 0.5 M KCl. Dissociation was initiated by 100-fold dilution with 5 mM Tris buffer containing 0.5 M KCl (×), NaCl (○), or KBr (□). *Points* are averages (±SD) of three experiments determined in duplicate. Dissociations in the presence of KCl and NaCl are not significantly different (apparently monophasic dissociation with a half-life of 94 min). In the presence of KBr, the phases of dissociation had equal distribution and half-lives of 17 and 79 min.

tal conditions for which biphasic effects of muscimol, etazolate, and pentobarbital were found on TBPS binding. However, for the concentrations of these agents which were reported to cause maximal enhancements of TBPS binding (3), we failed to detect any enhancement in equilibrium incubation (180 min, data not shown). In contrast, at 90 min of incubation applied in that study, control TBPS binding reached only 70% of its equilibrium value. When such nonequilibrium binding data were regarded as control, the above modulatory agents exerted a transient enhancing effect on TBPS binding (data not shown). Furthermore, we examined how the above effects on TBPS binding depended on time. A forebrain membrane preparation and a combination of the experimental conditions were used for which pharmacologically anomalous effects of GABAergic agents have been reported (3, 14–19). The *R*-(−)-enantiomer of MPPB was chosen as a representative barbiturate depressant which has been shown to accelerate the dissociation of TBPS most extensively (9). Fig. 2 shows a regular displacement by *R*(−)MPPB when binding approached its equilibrium (180 min). However, the displacement curves became biphasic when shorter incubation times (30 or 60 min) were applied (Fig. 2). Such enhancement at small concentrations has been called a "low dose hook" (21). The further the binding was from equilibrium, the more pronounced was the low dose hook for *R*(−)MPPB. The displacing potency of *R*(−)MPPB apparently increased with time as demonstrated by the IC_{50} values reported in Table 1. The slope factors of the displacement curves simultaneously decreased in time (Table 1), but the equilibrium slope factor remained significantly higher than unity. Fig. 3 shows that the displacement curve by GABA was also of regular shape under equilibrium conditions. Before equilibrium (60 min), it resulted in a low dose hook, also. Table 1 also contains the IC_{50} values of GABA and the slope factors of its TBPS displacement. These values decrease in time, such as those for *R*(−)MPPB.

Association rate of TBPS binding. The rate of TBPS association was studied under the same conditions as the biphasic effects. The effect of 300 μM *R*(−)MPPB was examined. Fig. 4a shows a characteristic pair of kinetic curves. In the presence of *R*(−)MPPB-modulated TBPS, binding approaches equilibrium more rapidly than in control. Consequently, at the first stage control binding remains below the modulated one, whereas the former surpasses the latter at the later stage closer to equilibrium. The apparent association rate constants can be determined from the slopes of Fig. 4b. The apparent half-lives of association were 41.5 ± 5.7 min in control and 11.9 ± 0.7 min in the presence of *R*(−)MPPB (mean ± SD of three experiments).

The effect of GABA antagonists on TBPS dissociation. A cortical membrane preparation was equilibrated with [^{35}S]TBPS in the presence of 20 nM R 5135 or 20 μM BCM, and dissociation of TBPS was initiated by an excess (40 μM) of picrotoxin. Fig. 5 shows that both antagonists significantly decelerated the rate of dissociation. Since long incubations at 22° might not be advantageous for the stability of the membrane preparation, the medium contained 0.2 M KBr instead of KCl; thus, the kinetics became more rapid. Polyphasic dissociation of the control was not significantly different from that initiated by dilution in the presence of KBr (Fig. 1) and could be characterized by half-lives of 11 and 74 min, and the contri-

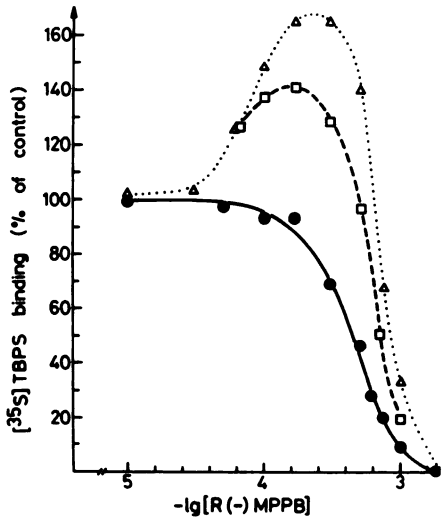


Fig. 2. The effect of *R*(−)MPPB on [^{35}S]TBPS binding with different times of incubations. The forebrain membrane preparation was incubated for 30 (△), 60 (□), and 180 min (●) at 22° with 1.3 nM [^{35}S]TBPS and varying concentrations of *R*(−)MPPB in 50 mM Tris buffer containing 300 mM NaCl. Data are expressed as percentage of control specific binding [without R(−)MPPB]. Specific [^{35}S]TBPS binding of the control, related to that in equilibrium (180 min), reached 31 ± 1% at 30 min and 52 ± 2% at 60 min (not shown). *Points* are averages of three experiments determined in duplicate which varied by less than 7%.

TABLE 1

The effect of incubation time on the displacing potencies of GABA and *R*(−)MPPB on [^{35}S]TBPS

The specific binding of 1.3 nM [^{35}S]TBPS was examined in a synaptosomal membrane preparation of rat forebrains in 50 mM Tris-HCl buffer containing 300 mM NaCl at 22°. Data are averages (±SE) of three experiments. IC_{50} data represent 50% of untreated control binding. For the formal calculation of slope factors of shorter incubation times (30 and 60 min), displacement data were related to the maximum of the low dose hook. Slope factors are all significantly ($p < 0.01$) greater than unity. If the displacement data are related to untreated control binding, slope factors become even greater: e.g., for *R*(−)MPPB at 60 min: 3.1 ± 0.1 increases to 4.2 ± 0.1.

Time	*R*(−)MPPB		GABA	
	IC_{50}	Slope factor	IC_{50}	Slope factor
min	*μM*		*μM*	
30	870 ± 28	4.1 ± 0.3		
60	716 ± 24	3.1 ± 0.1	1.96 ± 0.39	1.86 ± 0.05
180	420 ± 46	2.4 ± 0.2	0.81 ± 0.12	1.35 ± 0.15

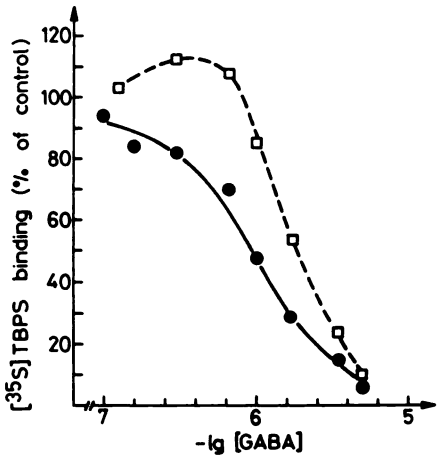


Fig. 3. The effect of GABA on [^{35}S]TBPS binding with different times of incubation. The incubation times were 60 min (□) and 180 min (●). Other experimental conditions were the same as in Fig. 2. *Points* are averages of three experiments determined in duplicate which varied by less than 7%.

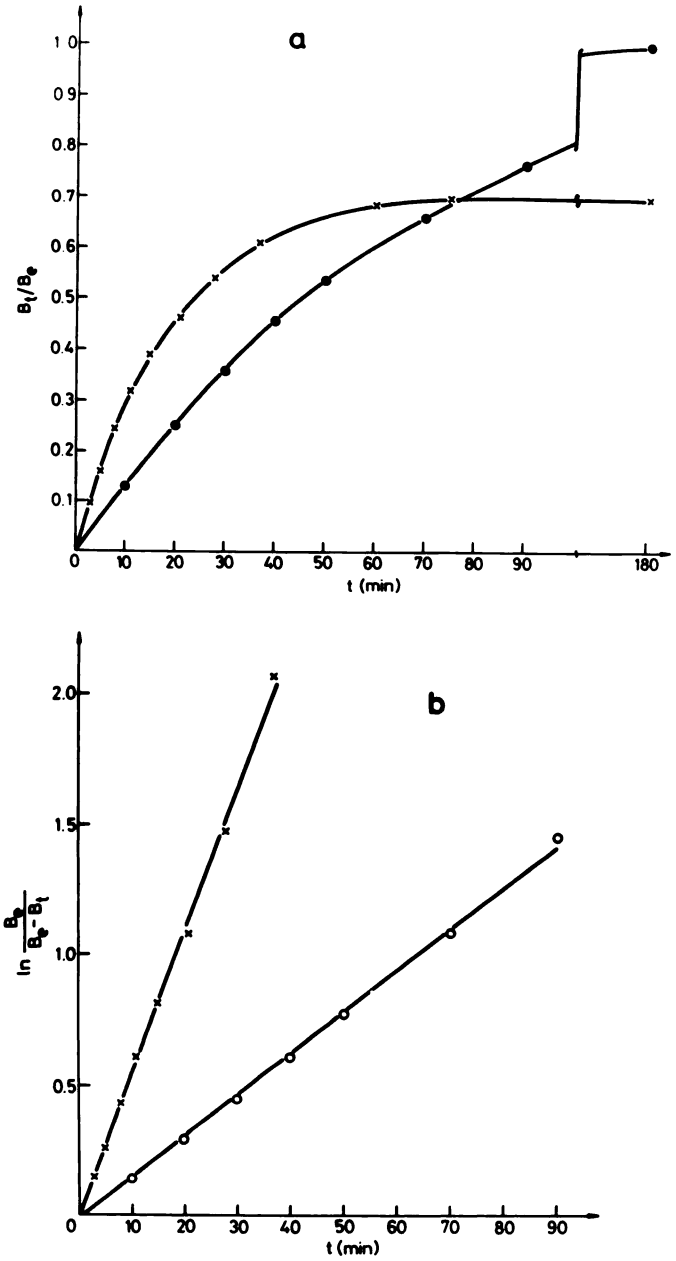


Fig. 4. The effect of *R*(−)MPPB on the association rate of [^{35}S]TBPS binding. The forebrain membrane preparation was incubated with 1.3 nM [^{35}S]TBPS in 50 mM Tris-HCl buffer containing 300 nM NaCl at 22° in the presence (×) or absence (●) of 300 μM *R*(−)MPPB. a. Time dependence of TBPS binding. Specific binding (B_t) is related to equilibrium binding (B_e at 180 min) of control. The *curves* are representatives of three experiments. *Points* are averages of duplicate determinations which varied by less than 2%. A 300 μM concentration of *R*(−)MPPB decreased TBPS binding to 66.0 ± 3.7% (mean ± SD of three experiments) in equilibrium. b. Determination of the apparent association rate constant (k_{app}) of TBPS binding. The *points* were calculated from the corresponding data of a. The average k_{app} values (±SD) of three experiments were 0.0167 ± 0.0023 min^{-1} in control and 0.0583 ± 0.0035 min^{-1} in the presence of *R*(−)MPPB.

bution of the fast phase was 46%. Both R 5135 and BCM decreased the contribution of the fast phase to 13%, whereas the slope of the slower phases remained essentially unaffected (half-lives of 70 and 96 min, respectively). Data in the legend of Fig. 5 show that binding in equilibrium was not significantly affected by BCM and was slightly decreased by R 5135.

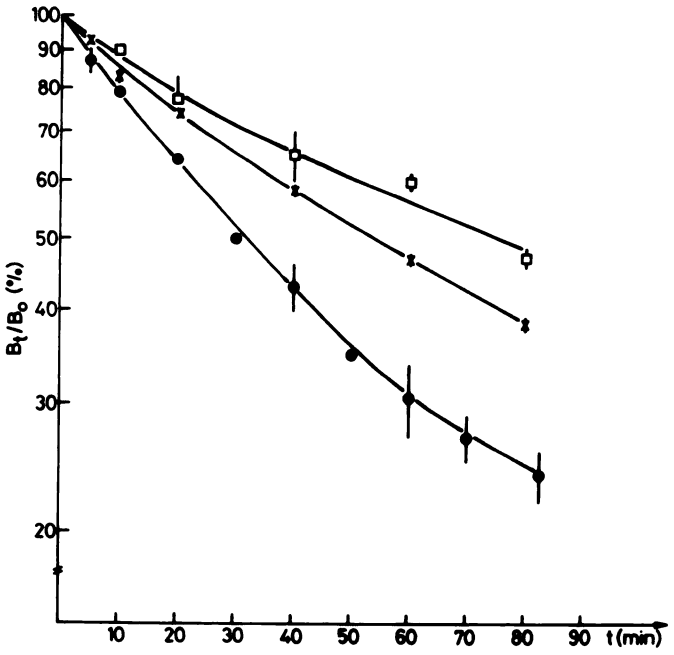


Fig. 5. The effect of GABA antagonists on TBPS dissociation. The cortical membrane preparation was incubated with 3 nM [^{35}S]TBPS in the presence of 20 nM R 5135 (×) or 20 μM BCM (□), in 5 mM Tris buffer containing 200 mM KBr for 100 min at 22°. ●, incubation without GABA antagonists (control). Specific TBPS binding in equilibrium, expressed as percentage of control, was 89±1% for R 5135 and 103±4% for BCM (not shown). Dissociation was initiated by the addition of 40 μM picrotoxin. The contribution of the first rapid phase of dissociation was 46% in control and 13% for R 5135 and BCM. *Points* are averages (±SD) of three experiments determined in duplicate.

The effect of different muscimol concentrations was also measured on TBPS dissociation. Fig. 6 shows that muscimol concentrations of 20, 60, and 200 nM progressively accelerated the rate of dissociation. This appears to be an allosteric effect via GABA receptors of rather low affinity. Since muscimol was co-incubated with [^{35}S]TBPS, it also decreased the equilibrium TBPS binding (B_o). In the presence of 200 nM muscimol, B_o decreased to 8% of control. This greatly decreased the accuracy of the determination of subsequent dissociation (Fig. 6). Fig. 6 also shows that the effect of muscimol was completely antagonized by 20 μM BCM regardless of the muscimol concentration. Dissociation kinetics of TBPS in the presence of BCM were not significantly different for various muscimol concentrations. Moreover, it was not significantly different from dissociation kinetics in the presence of BCM alone (Fig. 5), either. The half-life of the second phase of dissociation was 87 min and its contribution was 85%.

Discussion

The effect of anions. The binding site for the convulsant TBPS has been considered to be most intimately coupled to GABA receptor-regulated chloride ionophores (1–4). This view is supported by findings such as: 1) that TBPS binding requires the presence of certain anions, and 2) that the TBPS displacing potency of GABA can be reduced by Eccles anions (1). The kinetics of TBPS binding might thus serve as an *in vitro* model to characterize indirectly the ionophore. The rates of TBPS dissociation have been reported to be either rapid and polyphasic or slow and monophasic (1, 8–10). The differences can

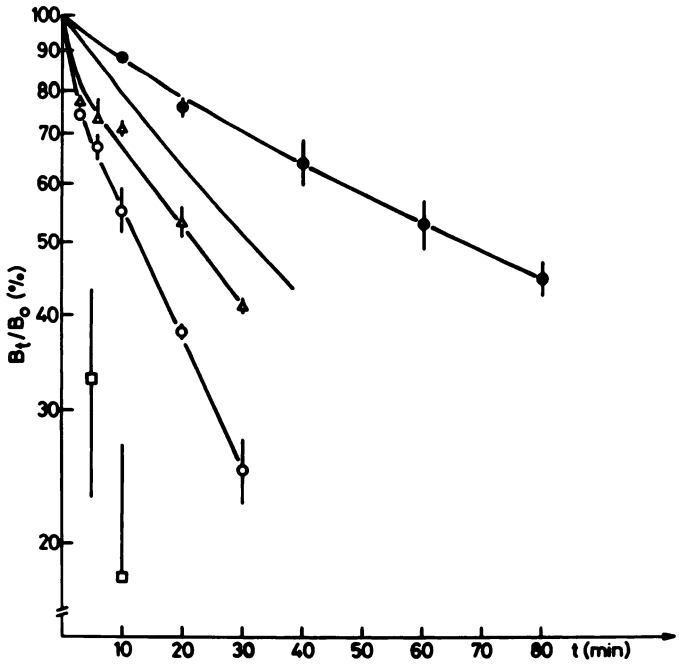


Fig. 6. The effect of muscimol on TBPS dissociation and antagonism by BCM. Muscimol concentrations of 20 nM (△), 60 nM (○), and 200 nM (□) were co-incubated with [^{35}S]TBPS. When 20 μM BCM was simultaneously applied, all muscimol concentrations resulted in data not significantly different from each other; therefore, they were combined (●) to a single dissociation curve. Other experimental conditions were the same as in Fig. 5. The fitted line for control dissociation in Fig. 5 was included for comparison. Specific TBPS binding in equilibrium, expressed as percentage of control, was 91% for 20 nM muscimol, 44% for 60 nM muscimol, and 8% for 200 nM muscimol, whereas in the presence of 20 μM BCM, it was 108, 95, and 78%, respectively (not shown). Half-lives of the second phase of TBPS dissociation in the presence of 20 nM and 60 nM muscimol were 28 min and 18 min, respectively. *Points* are averages (±SD) of three experiments in duplicate. The *points* for BCM plus muscimol (●) were not significantly different from those for BCM alone (□ in Fig. 5).

be attributed to different GABA contents of the membrane preparations (11, 12), but they might be partly due to the application of different monovalent ions in different studies. Furthermore, the biphasic effects of muscimol and depressants on TBPS binding could be demonstrated in the presence of Cl^- but not of Br^- ions (3). This study compared systematically the effect of the most commonly used anions and cations on TBPS dissociation. The large difference in the hydrated size of the cations (Na^+ and K^+) did not significantly affect the rate of dissociation (Fig. 1). In contrast, replacement of Cl^- by Br^-, with hardly smaller hydrated size (22), did accelerate the dissociation. Modulation of TBPS kinetics by these Eccles anions but not by cations supports the involvement of the chloride ionophore. In addition, the finding that TBPS kinetics are slower for Cl^- than for Br^- ions explains that biphasic effects of GABAergic facilitatory agents could be demonstrated only for Cl^- ions (3). For Br^- ions the incubation time must have been enough to reach equilibrium of TBPS binding, whereas nonequilibrium conditions for Cl^- could result in a low dose hook.

Consequences of nonequilibrium binding. The association rate of TBPS binding is rather slow, e.g., the half-life of association was 41.5 min for the forebrain membranes in Fig. 4. GABAergic facilitatory agents accelerate the approach to equilibrium; e.g., the anxiolytic BZ, flunitrazepam (1 μM), accelerated it by a factor of about 1.8 (19) and 300 μM *R*(−)MPPB by 3.5 (Fig. 4). Transient enhancements of TBPS binding by these agents can be found if the time of incubation is sufficient to reach equilibrium in the presence of the accelerating agent while equilibrium is not yet reached in control (e.g., up to 1 hr in Fig. 4a).

We stress that the low dose hooks in Figs. 2 and 3 are artifacts, due to the deliberate application of nonequilibrium conditions of binding. Biphasic displacement curves with a low dose hook have been observed in various binding studies and are frequently interpreted to indicate positive cooperativity of the binding sites (3, 21, 23–26). In almost all of these cases binding was slow and incubations of hours or days would have been required to reach equilibrium. Hence, in some of these cases the low dose hook might be due to the nonequilibrium conditions applied. For TBPS binding, kinetic studies failed to detect homotrope cooperativity in binding (11).

Slope factors of displacement curves have been frequently used to suggest a heterogeneity of binding sites. Our data in Table 1 indicate that the slope factors and IC_{50} values of the displacement curves are rather sensitive for the incubation time. This can explain the discrepancies of the slope factors reported previously for barbiturate depressants. Shorter incubations, especially in the presence of Cl^- ions, resulted in slope factors between 2 and 4 (3, 6), probably due to nonequilibrium incubations. Conversely, for longer incubations, in the presence of Br^- and/or endogenous GABA which both accelerate the kinetics of binding, the slope factors were close to unity (10, 27, 28). Although the slope factors for GABA and *R*(−)MPPB declined in time (Table 1), they remained significantly greater than 1 even in equilibrium. This also supports the view that GABA and depressant barbiturates bind to sites different from that of TBPS (10, 12, 28, 29).

This study emphasized the importance of equilibrium conditions for allosteric binding interactions. For competitive displacements, the consequences of nonequilibrium conditions have been discussed in more detail (30, 31).

The effect of GABA agonists and antagonists. Fig. 6 demonstrates that the dissociation of TBPS can be accelerated progressively by 20, 60, and 200 nM muscimol. These concentrations are beyond the K_D values of the high affinity sites for muscimol (32). Furthermore, the accleration of TBPS dissociation requires micromolar concentrations of GABA (11, 12). These data suggest that low rather than high affinity GABA receptors are involved in the allosteric regulation of the convulsant sites. Since the acceleration can be completely antagonized by bicuculline derivatives (Fig. 6), $GABA_A$ receptors are involved. The methochloride derivative of bicuculline was used, which is more stable and less potent than bicuculline itself (33). Still, 20 μM BCM per se decelerated the dissociation (Fig. 5) to the same extent as in the presence of the highest concentration of muscimol applied (Fig. 6). Therefore, the corresponding GABA sites appear to possess rather high affinity for GABA antagonists.

Both GABA antagonists BCM and R 5135 per se decreased the contribution of the fast phase of TBPS dissociation (Fig. 5). This deceleration cannot be attributed to the elimination of the effect of endogenous GABA since: 1) we used an extensively washed, freeze-thawed membrane preparation which was suitable to detect high affinity nM GABA binding, and 2) acceleration of TBPS dissociation required μM concentrations of

GABA (11). Equilibrium binding of TBPS was not significantly affected by BCM and it was slightly decreased by R 5135 (see the legend of Fig. 5). Therefore, if the rate of TBPS dissociation decreased, but binding in equilibrium did not increase, then the rate of association must have decreased, also.

Model calculations for the kinetic modulation of binding. We have recently studied the effect of BZ receptor ligands on TBPS binding (19). It has been demonstrated that the enhancing effect of a BZ agonist and the decreasing effect of a β-carboline inverse agonist disappears when TBPS binding approaches its equilibrium. The association and dissociation rates of TBPS are modulated by BZ receptor ligands to the same extent so that binding equilibrium remains unaffected. For the sake of simple kinetic calculations we can assume a homogeneous set of binding sites for the reversible binding of TBPS. BZ receptor ligands have been supposed to modulate allosterically *on* and *off* rate constants of TBPS binding (19). The extent of modulation can be expressed by an accelerating factor (19) that is >1 for BZ agonists and <1 for inverse agonists. This is illustrated by the calculated curves in Fig. 7. Curve b mimics the time dependence of TBPS binding modulated by a BZ agonist so that the *on* and *off* rate constants are equally doubled. Curve b displays increased binding as compared to the control curve a but approaches the same equilibrium value. Curve c represents the modulation by β-carboline inverse agonists so that both *on* and *off* rate constants are halved. Fig. 8 depicts the time dependence of the ratio modulated/control binding. Curves b and c approach unity at equilibrium and can be extrapolated to the value of the accelerating factor at $t=0$. These curves represented well our experimental data for the effect of flunitrazepam and the convulsant DMCM on TBPS binding (19).

We assumed that such a kinetic model could be extended for the allosteric effect of GABA agonists and depressants. This model could explain the transient biphasic effect of these agents on TBPS binding. GABA agonists and depressants were supposed to accelerate the kinetics of TBPS binding so that the

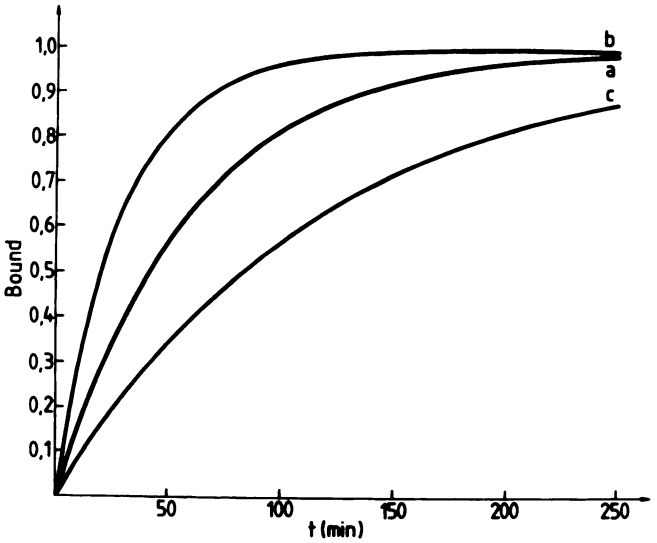


Fig. 7. Kinetics of binding: identically modulated *on* and *off* rate constants. Calculated specific binding on the *ordinate* is related to equilibrium binding in control (curve a). Primed k values refer to modulated rate constants. Curve b: $k_1' = 2k_1$ and $k'_{-1} = 2k_{-1}$. Curve c: $k'_1 = 0.5k_1$ and $k'_{-1} = 0.5\ k_{-1}$. Note that each curve approaches the same equilibrium binding (i.e., unity).

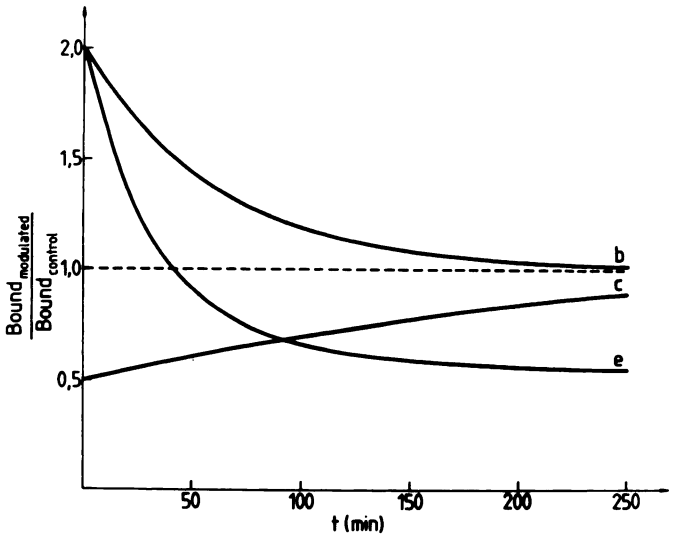


Fig. 8. Time dependence of the ratio modulated/control binding. Curves b and c in Fig. 7 and curve e in Fig. 9 were devided by their control (curve a).

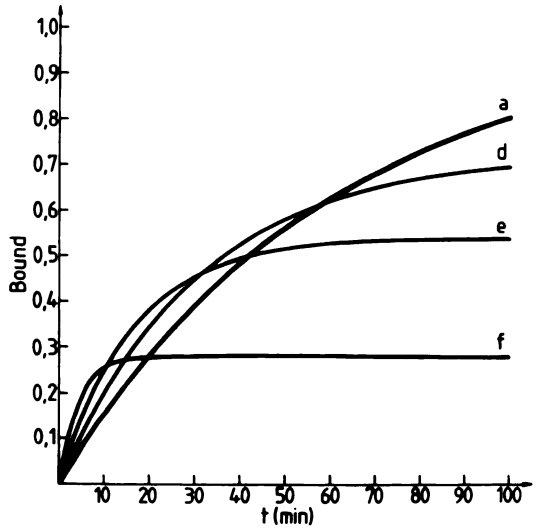


Fig. 9. Kinetics of binding: differently modulated *on* and *off* rate constants. Calculated specific binding is related to equilibrium binding in control (curve a). Curve d: $k'_1 = 1.4k_1$ and $k'_{-1} = 2k_{-1}$; curve e: $k'_1 = 2k_1$ and $k'_{-1} = 4k_{-1} = 4k_{-1}$; curve f: $k'_1 = 4k_1$ and $k'_{-1} = 16k_{-1}$.

extent of enhancement of the dissociation constant exceeds that of the association rate constant. There was a progressive increase in the dissociation rate constant of TBPS due to the occupation of GABA sites by muscimol (Fig. 6). But this is not enough to cause any enhancement of binding such as that demonstrated in Figs. 2–4. There should be a parallel, although less pronounced, increase in the association rate constant of TBPS, as well. Fig. 9 illustrates the time dependence for such binding. Curves d, e, and f were generated by multiplying the control association rate constant with 1.4, 2, and 4, while the increasing factors for the corresponding dissociation rate constants were 2, 4, and 16, respectively. These values mimicked the accelerating effect of increasing concentrations of GABA agonists or depressants. The greater the acceleration was, the more the initial phase of the curves exceeded the control curve a and the less binding the curves reached in equilibrium. The calculated curves a and e in Fig. 9 excellently mimic the

TABLE 2

The effect of GABAergic agents on the specific binding of [^{35}S]TBPS

The arrows indicate an increase (↑) or decrease (↓) in specific TBPS binding or in its rate constants. The size of the arrows demonstrates the extent of the effect. The numbers in parentheses indicate the references.

	Before equilibrium	In equilibrium	Kinetics *On/Off*
Benzodiazepine agonists (anticonvulsants)	↑ (3, 14–16)	—[a] (19)	↑/↑ (19)
β-Carboline inverse agonists (convulsants)	↓ (3, 14–16)	— (19)	↓/↓ (19)
GABA agonists	↑ + ↓ (3)	↓ (1, 12, 13, 27)	↑/↑ (9, 11, 12)
GABA antagonists (BCM, R 5135)	↓	—	↓/↓
Depressants (barbiturates, pyrazolopyridines, etomidate)	↑ + ↓ (3, 17, 18)	↓ (10, 12, 27)	↑/↑ (9–11)
Cage convulsants, tetrazoles	↓	↓ (5)	↓/— (8, 9)[a]

[a] —, no effect.

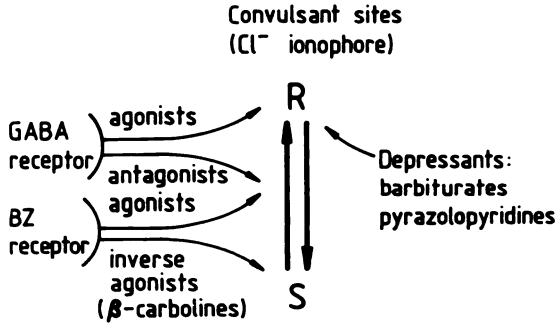
experimental curves for the effect of *R*(−)MPPB in Fig. 4a. When the ratio modulated/control binding was depicted (curve e in Fig. 8), the time dependence curve started with enhancement and ended with displacement of binding. This can explain why the low dose hooks for the depressant *R*(−)MPPB and GABA (Figs. 2 and 3) turned into displacement when binding was approaching equilibrium.

In conclusion, the time-dependent low dose hooks for GABA agonists and depressants can be explained by an allosteric kinetic modulation of TBPS binding which involves a simultaneous but unequal acceleration of both association and dissociation rate constants. Table 2 summarized the up- and down-regulatory effects of various GABAergic agents on TBPS binding. It demonstrates that almost all of these classes of agents affect TBPS binding differently before and in equilibrium. The first column shows that the nonequilibrium effects of allosteric GABAergic agents on TBPS binding cannot be reconciled with the convulsant activity of TBPS: anticonvulsant and GABAergic facilitatory agents appear to enhance TBPS binding, whereas β-carboline convulsants appear to decrease it. The kinetics of TBPS binding explain this anomaly. The third column demonstrates the effects on the association and dissociation rates concluded from this and previous studies. It can be seen that all agents acting allosterically regulate the *on* and *off* rates of TBPS binding in the same direction. GABAergic facilitatory agents, such as BZ and GABA agonists and depressants, accelerate the kinetics of binding, whereas GABAergic inhibitory agents, such as β-carboline inverse agonists and GABA antagonists, decelerate the kinetics.

It can also be concluded from the last column of Table 2 that the insufficient removal of endogenous GABA greatly accelerates the approach to equilibrium of TBPS binding (11). Different efficiencies of various membrane preparation procedures to remove endogenous GABA and the choice of monovalent anions (Cl^- or Br^-) are ultimate factors which determine the kinetics of TBPS binding.

Kinetic model of TBPS binding. All equilibrium studies on TBPS binding suggested a homogeneous population of sites (1, 8, 10, 12, 13, 27) except for recent reports on curvilinear Scatchard curves of binding by one group (34, 35). However, all kinetic studies demonstrated the polyphasic dissociation of TBPS, thus suggesting some kind of heterogeneity (1, 9–12). The presence of GABA (11, 12), muscimol (Fig. 6), or GABAergic depressants (9, 11) resulted in the appearance of a rapid kinetic phase of dissociation. The accelerating effects are large: dissociations elicited by displacing concentrations of these agents can be accelerated by more than 2 orders of magnitude. The half-life of dissociation can be decreased from 70 min (control dissociation elicited by unlabeled TBPS), e.g. by *R*(−)MPPB to less than 1 min (9). This example also demonstrates that the acceleration appears to be immediate. This rapidity does not contradict the receptor binding of the effectors, since low affinity binding of GABA was shown to be unmeasurably fast even at lower temperature (36, 37). The accelerating effect appears to be reversible because, for barbiturates, it could be eliminated by washing of the membranes (3, 10). The ligands of BZ receptors exert only minor effects on the kinetics of TBPS binding (19). Saturation of BZ receptors by a 1 μM concentration of their ligands such as flunitrazepam or DMCM can affect the rate of TBPS binding only by a factor of 2 (3, 19). This is in agreement with the pharmacological view that BZs can modulate GABAergic neurotransmission only via GABA receptors but TBPS binding can be studied in preparations whose GABA content has been reduced (13).

The kinetic model in Scheme 1 can account for all available data on the kinetics of TBPS binding. We assumed the existence of two interconvertible states with rapid and slow (*R* and *S*) kinetics for the TBPS sites. Similar kinetic observations for a binding site of the central nucleoside transport system have been interpreted analogously (38). GABAergic facilitatory agents act allosterically to tune up the kinetics of TBPS binding, whereas GABAergic inhibitory agents act in the opposite direction. The rapid and slow kinetic phases might correspond to different conductance states of the chloride ionophore (34, 39). The rapid phase of TBPS binding might represent an open state of the ionophore involving either a steric exposure of the convulsant (TBPS) sites or an easier transport of the radioligand. Conversely, the slow phase of TBPS kinetics might reflect a closed state of the ionophore with a steric or transport barrier



SCHEME 1. A kinetic model of the convulsant (TBPS) binding

Interconvertible populations of TBPS sites (*R* and *S*) refer to rapid and slow kinetic phases. Agents bound to GABA or BZ receptors can allosterically shift the proportion of the kinetic phases. Anticonvulsant/hypnotic agents such as barbiturates, pyrazolopyridines, etomidate, and ethanol bound to different sites enhance the proportion of the rapid kinetic phase of TBPS binding.

for the convulsant sites. High concentrations of barbiturates and GABA agonists which exert the greatest shift toward a rapid kinetic population of TBPS sites can open the chloride ionophores (40, 41). In contrast, GABAergic inhibitory agents, which result in the predominance of the slow phase of TBPS kinetics, enhance the blockade of the chloride ionophores (40).

In conclusion, this kinetic study demonstrated some pitfalls of nonequilibrium studies for TBPS binding. The concluding kinetic model offered a framework to explain several contradicting findings for TBPS binding. The model is also in agreement with the pharmacological relationship of several GABAergic anticonvulsants and picrotoxin-like convulsants. Studies on the rate constants of TBPS binding can thus contribute to the distinction of GABAergic facilitatory and inhibitory agents. The relationship of the model to the chloride ionophore is rather speculative and would require a direct study of the ionophore.

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