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[^{35}S]*t*-Butylbicyclophosphorothionate Binds with High Affinity to Brain-Specific Sites Coupled to γ-Aminobutyric Acid-A and Ion Recognition Sites

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SUMMARY

[^{35}S]*t*-Butylbicyclophosphorothionate (TBPS) binds to specific sites on EDTA/water-dialyzed rat brain P_2 membranes, with a dissociation constant (K_d) near 17 nM, in 200 mM KBr at 25° and pH 7.5. Nonspecific binding is about 33% of total binding using a filtration assay. [^{35}S]TBPS binding is entirely dependent on appropriate salts, with the anion playing the predominant role. The optimal temperature for [^{35}S]TBPS specific binding is near 21°, with none at 0°. The pH optimum for binding is 7.5–8.5. At 25° in 200 mM KBr, [^{35}S]TBPS (2 nM) associates with a major binding site with a half-time near 11 min, and dissociates in a polyphasic way. The slow component of dissociation has a half-time near 29 min and constitutes about 70% of the total specific binding. NaCl alone can almost completely protect specific [^{35}S]TBPS binding sites against heat inactivation (30 min, 60°) with 50% of maximal protection near 200 mM and a Hill number near 1.6. In whole rat forebrain [^{35}S]TBPS receptor density is near 50 pmoles/g of wet tissue. Receptor density is higher in cerebral cortex, cerebellum, and hippocampus (50–60 pmoles/g) than in hypothalamus, striatum, and pons-medulla (in decreasing order). There is negligible specific binding in liver, kidney, and lung. The affinites of Eccles anions (chloride, bromide, iodide, and thiocyanate) for the binding-enhancement site are selectively reduced by γ-aminobutyric acid (GABA) and all GABA-A receptor agonists tested, but not by baclofen. The affinities of non-Eccles anions (fluoride, sulfate, phosphate, and bicarbonate) for the anion site are either unaffected or increased by GABA. The inhibitory effects of GABA-A receptor agonists are potently reversed by the bicuculline-like GABA antagonist R 5135. Specifically bound [^{35}S]TBPS is potently displaced (IC_{50} values < 1 μM) by all picrotoxin-like ("cage") convulsants tested with the exception of *p*-chlorophenylsilatrane. TBPS, dihydropicrotoxinin, and benzodiazepine binding sites have similar densities and distributions. Specific [^{35}S]TBPS binding is inhibited by several barbiturates with IC_{50} values in the 30–60 μM range as well as the barbiturate-like substances (+)-etomidate (2.8 μM) and methaqualone (43 μM). The pyrazolopyridines etazolate and cartazolate are highly potent displacers of [^{35}S]TBPS binding (IC_{50} values < 1 μM). The inhibitions of [^{35}S]TBPS binding by barbiturates, etomidate, methaqualone, pyrazolopyridines, ethanol, and meprobamate are all potently, but noncompetitively, reversed by R 5135 (10 nM) whereas the inhibitions by most of the convulsants tested are potentiated or unaffected. TBPS probably binds to the same sites as dihydropicrotoxinin but has the advantages of higher affinity and a better signal-to-noise ratio. Many convulsants, anticonvulsants, sedatives, hypnotics, and anxiolytics seem to exert their characteristic effects by acting on or near TBPS (picrotoxin) sites, in benzodiazepine/ion/GABA/picrotoxin receptor complexes.

INTRODUCTION

The convulsant picrotoxin[3] is a non-competitive inhibitor of GABA[4] neurotransmission which binds to an independent site in GABA/benzodiazepine/pictrotoxinin/anion/cation receptor complexes (1–6). Several other convulsants, including bemegride (7), pentylenetetrazole

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(7), anisatin (8, 9), the bicyclophosphates (10, 11) and TETS (12, 13) also block GABA neurotransmission. Some of these convulsants potently displace [^{3}H]DHP from specific binding sites on brain membranes (14–16). Picrotoxin and several related convulsants specifically antagonize the chloride-dependent enhancing effects of barbiturates (3) and pyrazolopyridines (4) on [^{3}H]diazepam and [^{3}H]FLU binding to brain-specific sites, as well as inhibit the binding of [^{3}H]muscimol to brain-specific sites in a chloride ion-dependent way (17); they also enhance the ability of 200 mM NaCl to protect brain-specific [^{3}H]FLU binding sites against heat inactivation (5, 6).

The unusually high toxicities of *t*-butylbicyclophosphite, -phosphate, and -phosphorothionate (LD_{50} values near 50 μg/kg in mice) (11, 18) suggested to us that one of these substances might prove to be a better ligand for the "picrotoxin" receptor than [^{3}H]DHP, which has a relatively low affinity (K_d near 1 μM) and gives almost unacceptable (70–80%) nonspecific binding (14–16, 19–22). [^{35}S]*t*-Butylbicyclophosphorothionate [^{35}S]TBPS, (Fig. 1) was selected since it is easily synthesized in a single step by treating *t*-butylbicyclophosphite with elemental ^{35}S (18). The characteristics of [^{35}S]TBPS binding to brain-specific sites are described here.

MATERIALS AND METHODS

Preparation of rat brain P_2 membranes. Whole rat forebrain (or brain regions) were stored frozen at −20°. After thawing, the tissue was homogenized in 50 volumes of ice-cold 1 mM EDTA using a Teflon-glass homogenizer, and the P_2 fraction was prepared by conventional differential centrifugation. This fraction was resuspended in 50 times the original tissue weight of ice-cold 1 mM EDTA and dialyzed against three successive portions of ice-cold, double ion-exchanged water for 1–2 hr each.[5] The volume of water outside the dialysis bag was at least 20 times the volume of the P_2 suspension inside the bag. After dialysis the membranes were pelleted once more by centrifugation (about 25,000 × *g* for 30 min); the supernatant was discarded and the pellets were stored frozen at −20° in the plastic centrifuge tubes. Stored frozen in this way the [^{35}S]TBPS binding sites were stable for several weeks.

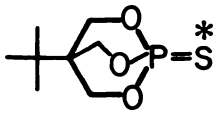

FIG. 1. *Structure of t-butylbicyclophosphorothionate*

Chemicals. [^{35}S]TBPS (specific activity about 30 Ci/mmole at the start of the investigation), was synthesized at the New England Nuclear Corporation (Boston Mass.) by treating *t*-butylbicyclophosphite with elemental ^{35}S (18); its radiochemical purity was 97% based on thin-layer chromatography. Because of the relatively short half-life of ^{35}S (about 90 days) the specific activity of our [^{35}S]TBPS declined to about 8 Ci/mmole at the end of the investigation in August 1982, 6 months after synthesis of the ligand. However, its binding and displacement properties appeared to remain constant over this period. Syntheses or sources of the bicyclophosphites, -phosphates, and -phosphorothionates, the bicyclo ortho esters, and *p*-chlorophenylsilatrane were described previously (18, 23). Deltamethrin, a pyrethroid insecticide, was provided by Roussel-Uclaf (Paris, France). SQ 20,009 (etazolate) and SQ 65,396 (cartazolate) were obtained from J. Lucania, Squibb Institute for Medical Research (Princeton, N. J.). Tracazolate (ICI 136,753) was provided by David H. McCurdy, Stuart Pharmaceuticals (Wilmington, Del.). The test substances were dissolved in water if possible or otherwise in ethanol. Ethanol is a moderately potent inhibitor of [^{35}S]TBPS binding (IC_{50} = 1.3% = 212 mM), and 0.1% ethanol, which inhibits about 2%, was the maximal concentration used.

Sources for other chemicals were: GABA mimetics from Povl Krogsgaard-Larsen, Royal Danish School of Pharmacy (Copenhagen, Denmark); (+)-etomidate (ZR 16659) and (−)-etomidate (ZR 36932) from W. Van Becer, Janssen Pharmaceutica (Beerse, Belgium); anisatin from Kiyoyuki Yamada, Nagoya University (Nagoya, Japan); picrotoxin, picrotoxinin, picrotin, and pentylenetetrazole (Metrazole, Leptazol) from Sigma Chemical Company (St. Louis, Mo.) and bemegride from Aldrich Chemical Company (Milwaukee, Wisc.); trazodone from Keith W. Wheeler, Mead Johnson (Evansville, Ind.); benzodiazepines from W. E. Scott, Hoffmann-La Roche, Inc. (Nutley, N. J.); cyproheptadine from Merck & Company (West Point, Pa.); R 5135 from Peter Hunt, Roussel Uclaf (Romainville, France); (±)-dimethylbutylbarbiturate (DMBB) (Lilly) from Richard W. Olsen, University of California, (Riverside, Calif.). The other barbiturates, meprobamate, and methaqualone were obtained from Applied Science Division, Milton Roy Laboratories Group (State College, Pa.) and from Research Triangle Institute (Research Triangle Park, N. C.).

Standard [^{35}S]TBPS binding procedure. EDTA/water-dialyzed rat brain P_2 membranes, stored frozen at −20° in pellet form, were diluted in water, and aliquots containing the equivalent of about 20 mg of fresh brain tissue were added to test tubes (140 × 13 mm) containing appropriate concentrations of test drugs together with 200 mM KBr, 5 mM Tris-HCl (pH 7.5), 1 mM EDTA,[6] and

[3] Picrotoxin, a non-nitrogenous natural product from the climbing shrub *Anamirta cocculus*, is an equimolar mixture of picrotoxinin and the less active picrotin.

[4] The abbrevations used are: chemicals other than bicyclophosphorus compounds and ortho esters: GABA, γ-aminobutyric acid; APSA, 3-aminopropanesulfonic acid; BGPA, 3-guanidinopropionic acid; BZ, benzodiazepine; DHP, dihydropicrotoxin; DMBB, dimethylbutylbarbiturate; Eccles anions, anions which can substitute for chloride in producing an inhibitory postsynaptic potential [J. C. Eccles, *Science* (*Wash. D. C.*) **145**:1140–1147 (1964); FLU, flunitrazepam; IGV, isoguvacine; ImAA, imidazoleacetic acid; P4S, piperidine-4-sulfonate; *t*-4-ACA, *trans*-4-aminocrotonic acid; TETS, tetramethylenedisulfotetramine; THIP, 4,5,6,7-tetrahydro-isoxazolo-(5,4*c*)-pyridine-3-ol; bicyclophosphorus compounds and ortho esters R_4-$C(CH_2O)_3R_1$: EPO, ethyl, P=O; EPS, ethyl, P=S; IPPO, isopropyl, P=O; IPPS, isopropyl, P=S; PPO, propyl, P=O; TBOA, *t*-butyl, C—CH_3; TBOB, *t*-butyl, C—C_6H_5; TBOF, *t*-butyl, C—H; TBOP, *t*-butyl, $C(CH_2)_3CH_3$; TBPO, *t*-butyl, P=O; TBPS, *t*-butyl, P=S.

[5] This EDTA treatment removes about 90% of the GABA firmly bound to rat brain P_2 membranes which cannot be removed by dialysis against water alone (5, 6).

[6] EDTA can be omitted without affecting [^{35}S]TBPS binding.

2 nM [^{35}S]TBPS in a final volume of 2.0 ml. The samples were incubated for 90–100 min at 25°, filtered through Whatman GF/A microfiber filter discs (2.4 cm diameter) on a Millipore 12-sample filtration manifold using slight vacuum, and washed twice with 5.0-ml portions of 200 mM KBr containing 5 mM Tris-HCl (pH 7.5) and 1 mM EDTA at 25°. The filter discs were then placed in polypropylene scintillation vials (W. Sarstedt, Princeton, N. J.) together with 5 ml of Liquiscint scintillation cocktail (National Diagnostics, Sommerville, N. J.) containing 8% water, and counted by conventional liquid scintillation counting.

Nonspecific [^{35}S]TBPS binding is defined as that obtained in the presence of 10 μM *t*-butylbicyclophosphate (TBPO) and constitutes about 33% of the total [^{35}S]TBPS binding under the standard conditions described above. Specific [^{35}S]TBPS binding was determined as a function of membrane concentration, and was found to be linear up to the concentration routinely used (corresponding to about 20 mg of original wet brain tissue per 2.0-ml assay).

TABLE 1

Effects of various salts on [^{35}S]TBPS binding in the absence or presence of 500 nM GABA

Specific binding of [^{35}S]TBPS (2 nM) to EDTA/water-dialyzed rat forebrain P_2 membranes was determined as described in the text. Six or seven concentrations of the salts indicated, with or without GABA, were incubated with aliquots of EDTA/water-dialyzed rat brain P_2 membranes, corresponding to 20 mg of fresh brain tissue, together with 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 nM [^{35}S]TBPS in a final volume of 2.0 ml. for 100 min at 25°, then filtered through Whatman GF/A microfiber filter discs using slight vacuum, washed twice with 5.0-ml portions of 200 mM KBr containing 5 mM Tris-HCL (pH 7.5) and 1 mM EDTA at 25°. The filters were then transferred to polypropylene scintillation vials and counted by conventional liquid scintillation counting. In the presence of 5 mM Tris-HCl (pH 7.5) without additional salts, there was no detectable specific [^{35}S]TBPS binding. B_{max} values were estimated from double-reciprocal plots, $1/B$ versus 1/[salt], where B is the specific binding of [^{35}S]TBPS, at 2 nM, in the presence of a given concentration of salt. EC_{50} values and Hill numbers were then estimated from Hill plots, log $(B_{max}/B - 1)$ versus log[salt]. An EC_{50} value corresponds to the salt concentration giving 50% of maximal binding based on six or seven concentrations used in each experiment. Usually there were about equal numbers of points above and below the EC_{50} value. The results are the averages of n experiments ± standard deviation.

Salt varied	n	B_{max}	EC_{50}	Hill no.
		fmoles	*mM*	
KBr	6	120 ± 21	82 ± 26	0.99 ± 0.16
+GABA (0.5 μM)	5	150 ± 18	710 ± 260	1.0 ± 0.059
NaBr	4	120 ± 13	79 ± 10	1.0 ± 0.21
+GABA (0.5 μM)	4	140 ± 27	530 ± 160	1.0 ± 0.10
KCl	4	130 ± 22	110 ± 8.1	0.89 ± 0.098
+GABA (0.5 μM)	4	130 ± 30	310 ± 83	1.1 ± 0.082
NaCl	4	160 ± 21	110 ± 14	0.86 ± 0.060
+GABA (0.5 μM)	3	180 ± 27	420 ± 19	1.0 ± 0.099
Na_2SO_4	2	120 ± 7.8	460 ± 130	0.58 ± 0.066
+GABA (0.5 μM)	2	190 ± 13	410 ± 42	0.97 ± 0.083
$NaPO_4$	2	54 ± 13	32 ± 6.4	0.94 ± 0.030
+GABA (0.5 μM)	2	50 ± 0	31 ± 4.3	0.87 ± 0.29
NaF	2	60 ± 5.6	110 ± 3.5	0.90 ± 0.095
+GABA (0.5 μM)	2	56 ± 2.8	93 ± 33	1.2 ± 0.056
$NaHCO_3$	2	83 ± 13	66 ± 43	1.1 ± 0.13
+GABA (0.5 μM)	2	34 ± 6.4	27 ± 11	0.92 ± 0.28

TABLE 2

Specific [^{35}S]TBPS binding as a function of KBr concentration alone and in the presence of 200, 500, and 1000 nM GABA

For conditions and terms, see the text and Table 1.

Addition	n	EC_{50} for KBr	B_{max}	Hill no.
		mM	*fmoles/assay*	
None	6	82 ± 26	120 ± 21	0.99 ± 0.16
GABA				
200 nM	2	280 ± 48	130 ± 2.1	1.1 ± 0.042
500 nM	5	710 ± 260	150 ± 18	1.0 ± 0.059
1000 nM	2	1400 ± 250	200 ± 20	1.0 ± 0.021

RESULTS

Influence of salts and GABA on binding. Preliminary results indicated that specific [^{35}S]TBPS binding was ion-dependent, had a temperature optimum near 20°, and was potently inhibited by GABA in the presence of 200 mM KBr. There was little specific [^{35}S]TBPS binding to membranes in whole brain homogenates or conventional P_2 preparations, presumably due to the inhibitory effect of endogenous GABA, which could be reversed by addition of the bicuculline-like GABA antagonist R 5135 (24). Similarly, dialysis of rat brain P_2 preparations against 1 mM EDTA, then water, a procedure known to remove substantial amounts of tightly bound GABA which cannot be removed by dialysis against water alone (5, 6), resulted in a large increase in specific [^{35}S]TBPS binding. The addition of R 5135 to EDTA/water-dialyzed P_2 membranes produced only small (10–20%) increases in specific [^{35}S]TBPS binding. As with [^{3}H]FLU (6, 25), the specific binding of [^{35}S]TBPS was entirely dependent on appropriate ions. At 200 mM, all salts tested promoted some specific binding, with KBr among the most effective, and NaF among the least effective (Table 1). The concentration of KBr providing 50% of maximal binding was near 82 mM, and this value was increased to 280, 710, and 1400 mM by GABA at concentrations of 200, 500, and 1,000 nM, respectively (Fig. 2A; Table 2). Conversely, GABA inhibited [^{35}S]TBPS binding with IC_{50} values of 340, 640, and 1500 nM in 200, 500, and 1,000 mM KBr, respectively (Fig. 2B; Table 3), suggesting competitive, or pseudo-competitive, antagonism between KBr and GABA.

TABLE 3

Inhibition of specific [^{35}S]TBPS binding by GABA in the presence of 200, 500, and 1000 mM KBr

Six or seven concentrations of GABA were used in each experiment, usually with about equal numbers of points above and below the IC_{50} values. Assay conditions as described in the text and Table 1. The IC_{50} values and Hill numbers were taken directly from Hill plots, where log $(B_0/B_i - 1)$ was plotted against log[GABA]. B_0 is the specific binding at each KBr concentration without GABA, using 2 nM [^{35}S]TBPS, and B_i is the binding in the presence of a given concentration of GABA. The values are the means of n separate experiments ± standard deviation.

[KBr]	n	IC_{50} for GABA	Hill no.
mM		*nM*	
200	6	340 ± 68	1.1 ± 0.071
500	2	640 ± 85	1.1 ± 0.028
1000	2	1500 ± 220	1.1 ± 0.00

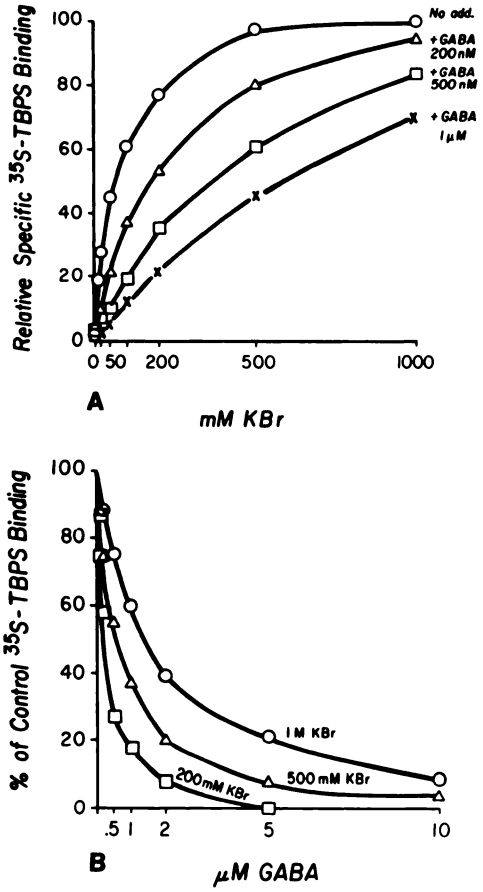

FIG. 2. *Opposite effects of KBr and GABA on binding of [^{35}S] TBPS*

A. Specific [^{35}S]TBPS binding to EDTA/water-dialyzed rat forebrain P_2 membranes as a function of KBr concentration alone (○) and in the presence of 200 nM (△), 500 nM (□), and 1 μM (×) GABA, respectively. All assays contained 5 mM Tris-HCl (pH 7.5)/1 mM EDTA and were incubated for 100 min at 25°.

B. Inhibition of specific [^{35}S]TBPS binding by GABA in the presence of 200, 500, and 1000 mM KBr. Binding procedure as described in Table 1.

A comparison of concentration-response curves for several salts revealed that anions play a predominant role in promoting and modulating [^{35}S]TBPS binding (Table 1). KBr and NaBr produced similar effects: at saturating concentrations both salts yielded almost identical B_{max} values (120–140 fmoles/assay) which were not significantly affected by GABA (0.5 μM). However, the same concentration of GABA (0.5 μM) increased by a factor of 8–10 the concentrations of these salts required to give 50% of maximal [^{35}S]TBPS binding (EC_{50} values). NaCl and KCl also behaved similarly: both salts in saturating concentrations promoted large maximal specific [^{35}S]TBPS binding (170 and 130 fmoles/assay, respectively) which was not significantly affected by GABA (0.5 μM). The same concentration of GABA increased the EC_{50} values by a factor of 3–4, significantly smaller effects than those produced by KBr and NaBr. In striking contrast, the same concentration of GABA (0.5 μM) had no effect on, or reduced, the EC_{50} values for sulfate, phosphate, fluoride, and bicarbonate. Saturating concentrations of phosphate, fluoride, and bicarbonate gave much smaller B_{max} values (one-third to one-half) than those produced by chloride, bromide, and sulfate ions. Phosphate and bicarbonate ions were more potent than the others in promoting half-maximal binding. GABA (0.5 μM) greatly reduced the B_{max} value for bicarbonate ions (Table 1). Iodide and thiocyanate, both Eccles anions, increased [^{35}S]TBPS binding at low (≤100 mM) concentrations, but inhibited at higher concentrations. GABA (0.5 μM) strongly inhibited binding in the presence of both iodide and thiocyanate (100 mM) (data not shown), suggesting effects similar to those obtained with bromide and chloride ions, and also Eccles anions. Thus, GABA may be able to differentiate between Eccles anions and non-Eccles anions by selectively reducing the affinities of Eccles anions in promoting [^{35}S]TBPS binding.

Influence of temperature, pH, preloading, ligand concentration, and brain region. Specific binding of [^{35}S] TBPS (2 nM) to EDTA/water-dialyzed membranes from whole rat forebrain was measured after 2 hr of incubation at several temperatures in 200 mM KBr containing 5 mM Tris-HCl (pH 7.5). There was almost no specific binding at 0° under these conditions, and specific binding increased with increasing temperature to about 21°, then decreased at higher temperatures (Fig. 3). The pH optimum for specific binding was 7.5–8.5 (Fig. 4). Under standard conditions (25°, pH 7.5, 200 mM KBr, 2 nM [^{35}S] TBPS), [^{35}S]TBPS associates with 85–90% of its binding sites with a half-time near 11 min (Fig. 5A).

After preloading the specific binding sites by incubating with 2 nM [^{35}S]TBPS for 100 min under standard conditions, the rate of dissociation was measured after adding 5 μM unlabeled TBPO at time zero. Dissociation of specifically bound [^{35}S]TBPS is bi- or polyphasic with a rapid component having a half-life near 10 min and a slower component with a half-life near 29 min and constituting about 70% of the total binding (Fig. 5B).

Specific binding was examined as a function of [^{35}S] TBPS concentration. The results were analyzed using double-reciprocal, Scatchard, and Hill Plots, which revealed a single binding component with a dissociation

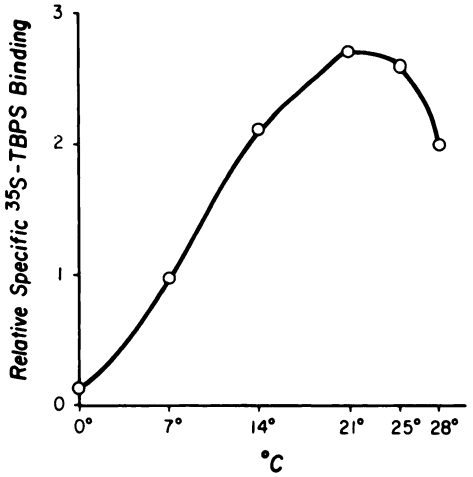

FIG. 3. *Effect of incubation temperature on specific [^{35}S]TBPS binding to EDTA/water-dialyzed rat forebrain P_2 membranes*

The incubation medium contained 200 mM KBr, 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 nM [^{35}S]TBPS. The incubation time was 2 hr at six temperatures followed by filtration and washing with 2 × 5.0 ml of 200 mM KBr/5 mM Tris-HCl (pH 7.5)/1 mM EDTA at the indicated temperature.

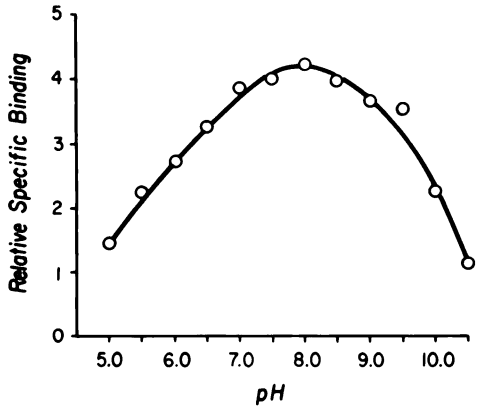

FIG. 4. *Effect of pH on specific [^{35}S]TBPS binding at 25° in 200 mM KBr + 1 mM EDTA*

The [^{35}S]TBPS concentration was 2 nM and the incubation time was 100 min. The buffers used were Tris-acetate (pH 5.0, 5.5); $NaPO_4$ (6.0, 6.5, 7.0, 7.5), Tris-HCl (7.5, 8.0, 8.5, 9.0, 9.5), and sodium glycinate (10, 10.5), all at 10 mM.

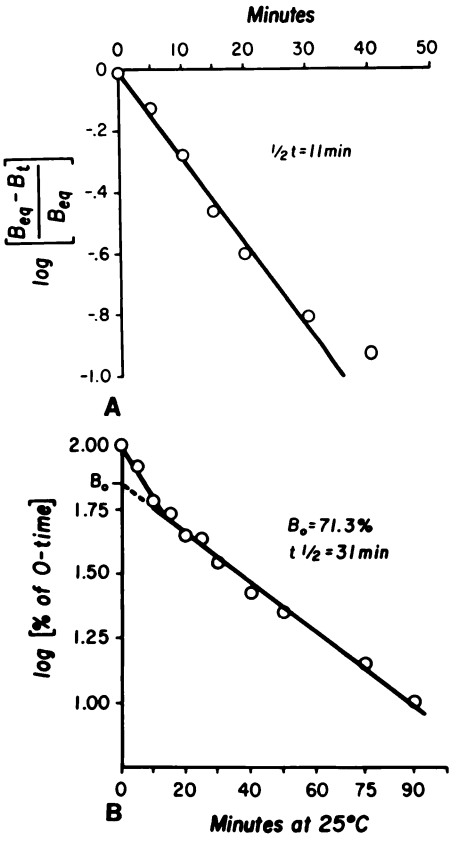

FIG. 5. *Time course of [^{35}S]TBPS association and dissociation*

A. Time course of [^{35}S]TBPS association with brain-specific receptors (logarithmic transformation). [^{35}S]TBPS was incubated at a final concentration of 2 nM with EDTA/water-dialyzed rat forebrain P_2 membranes at 25° in 200 mM KBr containing 5 mM Tris-HCl (pH 7.5) plus 1 mM EDTA and filtered at the indicated times. The membrane concentration corresponded to 10 mg of original wet brain tissue per milliliter. Under these conditions [^{35}S]TBPS associated with a major specific binding site, corresponding to 85–90% of the total binding sites, with a half-time near 11 min.

B. Time course of [^{35}S]TBPS dissociation from brain-specific sites. EDTA/water-dialyzed rat brain P_2 membranes were preincubated for 100 min at 25° in 200 mM KBr containing 5 mM Tris-HCl (pH 7.5) plus 1 mM EDTA and 2 nM [^{35}S]TBPS. *t*-Butylbicyclophosphate (final concentration 5 μM) was added at time zero, and aliquots were filtered at the times indicated. The slow component of dissociation has a half-time near 31 min and constitutes 71% of the total specific [^{35}S]TBPS binding.

constant (K_d) near 16 ± 2 nM (n = 2) at 25° in 200 mM KBr (pH 7.5) (Fig. 6). The B_{max} value corresponds to about 48 ± 3.5 (n = 2) pmoles/g of wet brain tissue, which underestimates the true receptor density due to loss during centrifugation and dialysis. It is 2–3 times greater than early reports of [^{3}H]diazepam receptor density in rat brain (26, 27), close to a later value reported for [^{3}H]FLU receptor density in rat brain (28), but less than one-half of a DHP binding site density estimate (14).

[^{35}S]TBPS binding sites are unevenly distributed in the rat brain, with highest densities in cerebral cortex, cerebellum, and hippocampus and lower densities in striatum, hypothalamus, and pons-medulla (Table 4). Binding site densities are 4–5 times higher in cerebral cortex than in pons-medulla. Nonspecific [^{35}S]TBPS binding is about 20% of total binding in cortex and cerebellum. There appeared to be small but measurable specific [^{35}S]TBPS binding in liver and lung, which was about 7% and 2%, respectively, of the binding in cerebral cortex. There was no detectable specific binding in kidney. Thus the distribution of TBPS binding sites in the rat resembles those of BZ (26, 27) and DHP (14, 19) binding sites: all three binding sites are essentially restricted to the brain, where high concentrations are found in cortex, hippocampus, and cerebellum and lower concentrations in corpus striatum, hypothalamus, pons-medulla, and spinal cord.

Heat inactivation of [^{35}S]TBPS binding sites. Like BZ receptors (5, 6, 29), [^{35}S]TBPS binding sites are completely destroyed by heating for 30 min at 60° in 2 mM Tris-HCl (pH 7.5), and NaCl alone can almost completely protect TBPS sites against such destruction, with 50% of the maximal protection near 190 mM NaCl. In contrast to [^{3}H]FLU binding sites, GABA has a biphasic effect on the NaCl concentration-response curve, potentiating the protective effects of low (<100 mM) NaCl concentration against heat inactivation (30 min at 60°), while reducing the protective effects of higher (>200 mM) concentrations (data not shown). Time courses of heat inactivation of [^{35}S]TBPS binding sites at 60° in 200 mM NaCl/2 mM Tris-HCl (pH 7.5) were bi- or polyphasic with a slow component ($t_{1/2}$ = 79 ± 8.5 min, n = 2) constituting about 60 ± 8% of all the binding sites (Fig. 7).

Effect of GABA mimetics and R 5135 on [^{35}S]TBPS binding. The inhibition of specific [^{35}S]TBPS binding by GABA in 200 mM KBr is completely and competitively reversed by the potent bicuculline-like GABA antagonist R 5135 (24) (Table 5; Fig. 8).[7] At concentrations of 5, 10, and 20 μM, GABA inhibits [^{35}S]TBPS binding more than

[7] The inhibitory effect of GABA is also effectively reversed by bicuculline (10 μM) or strychnine (100 μM).

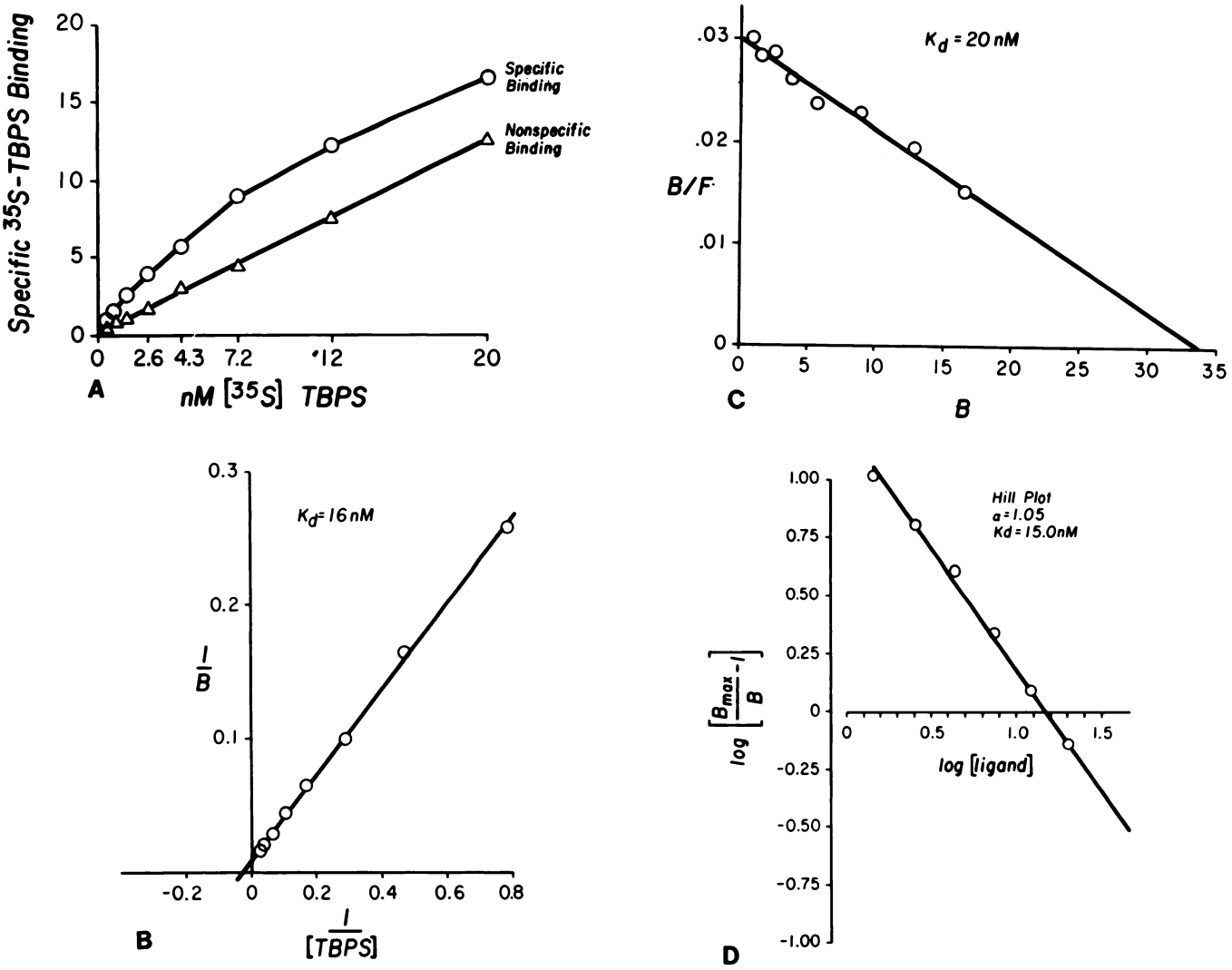

FIG. 6. *Effect of varying [^{35}S]TBPS concentration on its binding to specific sites in EDTA/water-dialyzed rat forebrain P_2 membranes*

A. Linear plot showing nonspecific binding (binding in the presence of 10 μM *t*-butylbiclophosphate) and specific binding (total binding, not shown, minus nonspecific binding) as a function of [^{35}S]TBPS concentration.

B. Double-reciprocal plot: (specific binding)$^{-1}$ against (TBPS concentration)$^{-1}$ to obtain B_{max} value (46 pmoles/g wet brain).

C. Scatchard transformation of the same data.

D. Hill plot of the same data.

90% but is 50% reversed by 8.5, 17, and 40 nM R 5135, respectively (Fig. 8A; Table 5). Conversely, at 10, 20, and 50 nM R 5135, the concentrations of GABA required to inhibit [^{35}S]TBPS binding 50% are 4.7, 11, and 30 μM, respectively (Fig. 8B; Table 6). All other GABA-A receptor agonists tested also inhibited [^{35}S]TBPS binding, with muscimol being the most potent (IC_{50} = 59 nM) and (+)-methylmuscimol (IC_{50} = 99 μM) the least potent (Table 7). R 5135 (10 nM) completely reversed the inhibitory effects of muscimol (100 nM), APSA (500 nM), *t*-4-ACA (1 μM) and P4S (2 μM) (Table 8). The GABA-B receptor agonist baclofen did not inhibit binding at 100 μM.

It is striking that the inhibition of [^{35}S]TBPS binding by GABA-A receptor agonists, as well as by all drugs tested which may act by potentiating GABA neurotransmission [including barbiturates, BZs, (+)-etomidate, methaqualone, SQ 65,396, meprobamate, and ethanol] are potently reversed by R 5135 (Table 8).

Effect of convulsants and pyrazolopyridines on [^{35}S] TBPS binding. Specific [^{35}S]TBPS binding is also potently blocked by most picrotoxin-like convulsants, the most potent of which is TBPS itself (IC_{50} = 17 nM). Picrotoxin and picrotoxinin are equipotent (IC_{50} near 190 nM) while picrotin is much less potent (IC_{50} = 12 μM). In general, the bicyclophosphorothionates are 2–4 times more potent than the corresponding bicyclophosphates (Table 7). There seems to be a reasonably good correlation between the affinities of many convulsants for the TBPS binding site *in vitro* and their LD_{50} values *in vivo*. Bemegride and pentylenetetrazole, while having much lower affinities (IC_{50} values 130 and 560 μM, respectively) for the TBPS site than the bicyclophosphates, appear to have LD_{50} and EC_{50} values *in vivo* (7) consistent with

TABLE 4

Densities of specific [^{35}S]TBPS binding sites in several regions of rat brain

Rat brain tissues (stored frozen) were homogenized in 50 volumes of ice-cold water and centrifuged for 5 min at about 3000 × *g*. One milliliter of the supernatant was incubated together with 1 M KBr, 1 mM EDTA, 5 mM Tris-HCl (pH 7.5), 500 nM R 5135, and 2 nM [^{35}S]TBPS in a final volume of 2.0 ml for 100 min at 25° before filtering. R 5135 supresses the inhibitory action of endogenous GABA. In the absence of R 5135 there was no detectable specific [^{35}S]TBPS binding. Rat liver and lung tissue, treated in the same way, exhibited small but significant specific [^{35}S]TBPS binding, corresponding to about 7% and 2%, respectively, of the binding in cerebral cortex. There was no detectable specific binding in rat kidney. $B_{max} = B(K_d/[TBPS] + 1)$, where B is the specific binding using a given concentration of ligand [^{35}S]TBPS.

Brain region	*n*	B_{max}	Nonspecific binding
		pmoles/g	*as % of total binding*
Cerebral cortex	3	65 ± 7.1	18 ± 0.6
Hippocampus	3	57 ± 10	19 ± 2
Cerebellum	3	52 ± 8.4	20 ± 0.6
Hypothalamus	3	44 ± 2.6	29 ± 5
Striatum	3	43 ± 13	23 ± 4
Pons-medulla	3	14 ± 1.3	48 ± 2

their affinities *in vitro* (see *Merck Index*). R 5135 has no effect on, or potentiates, the inhibitory actions of all of the convulsants tested with the exception of anisatin (Table 8).

The pyrazolopyridines cartazolate and etazolate also proved to be surprisingly potent blockers of [^{35}S]TBPS binding (Table 7), in agreement with the reported ability of picrotoxin and IPPO to block the chloride ion-dependent enhancement of [^{3}H]FLU binding by these substances (4). The related pyrazolopyridine phosphodiesterase inhibitor SQ 20,006, which lacks anticonflict activity in animals, also fails to block [^{35}S]TBPS binding at 10 μM. Inhibition of [^{35}S]TBPS binding by SQ 65,396 is

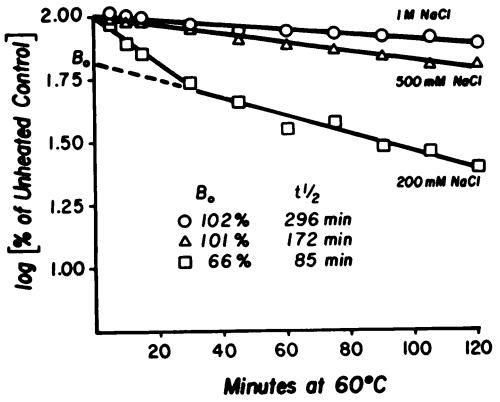

FIG. 7. *Time course of heat inactivation of specific [^{35}S]TBPS sites at 60° in the presence of 200, 500, and 1000 mM NaCl*

EDTA/water-dialyzed rat forebrain P_2 membranes were heated for various times at 60° with the indicated NaCl concentrations containing 2 mM Tris-HCl (pH 7.5). One-milliliter samples were withdrawn from the 60° bath at the indicated times and placed in an ice-bath. When the time course was completed at 120 min, NaCl was adjusted to 500 mM in all samples and [^{35}S]TBPS was added to give a final concentration of 2 nM in a final volume of 2.0 ml. The samples were then incubated for 100 min at 25° before filtering as usual.

TABLE 5

Reversal of GABA inhibition of specific [^{35}S]TBPS binding by R 5135 in 200 mM KBr

Determination of specific [^{35}S]TBPS binding is described in the text and Table 1. All three concentrations of GABA inhibited specific [^{35}S]TBPS binding more than 90%. Seven or eight concentrations of R 5135 were used to construct concentration-response curves, and there were usually almost equal numbers of points above and below 50% reversal of GABA inhibition. There was complete reversal by R 5135 at all three GABA concentrations. Maximal reversal was estimated by double-reciprocal plots or taken directly from the plateaus of linear concentration-response curves. The EC_{50} values (the concentrations of R 5135 required to reverse 50% of the inhibition by GABA) and Hill numbers were taken directly from a Hill plot.

[GABA]	*n*	EC_{50} for R 5135	Hill no.	ΔB_{max}
μM		*nM*		*as % of control binding*
5	2	8.5 ± 0.82	1.0 ± 0.095	120 ± 6.4
10	2	17 ± 3.1	1.0 ± 0.047	120 ± 2.1
20	2	40 ± 1.4	1.0 ± 0.080	120 ± 5.6

partially reversed by R 5135, but, in contrast to the inhibition produced by GABA, the reversal of SQ 65,396 by R 5135 is noncompetitive, and R 5135 can reverse only about 11% (maximally) of the inhibition produced by 10 μM SQ 65,396 (data not shown). The anxiolytics meprobamate and ethanol both inhibited specific [^{35}S]TBPS binding, with IC_{50} values near 1.4 mM and 212 mM (1.3%), respectively, in an R 5135-reversible way (Table 8).

Effect of barbiturates and other compounds on [^{35}S]TBPS binding. The inhibition of specific [^{35}S]TBPS binding by barbiturates was comparable to their inhibition of [^{3}H]DHP binding (20, 21), with IC_{50} values ranging from 31 μM for (−)-secobarbital to 110 μM for (+)-pentobarbital (Table 7). Phenobarbital was essentially inactive at 100 μM (data not shown). (+)-Etomidate, a highly potent substance with a sedative barbiturate pharmacological profile (30), reportedly enhances the binding of [^{3}H]diazepam to sites in rat forebrain, but not cerebellar, membranes (31), and displaces [^{35}S]TBPS from its brain-specific binding sites more potently than any barbiturate tested (Table 7). (−)-Etomidate has a 10-fold lower affinity for the TBPS binding site than does (+)-etomidate, demonstrating the stereospecificity of the site (Table 7). Similarly, the (−)-isomers of secobarbital and pentobarbital are more potent than their (+)-isomers in inhibiting [^{35}S]TBPS binding (Table 7), in agreement with the results of Ticku (21) using ^{3}H-DHP. Methaqualone, which resembles pharmacologically both BZs and barbiturates (32), displaces [^{35}S]TBPS with an IC_{50} value (43 μM) similar to those for (−)-secobarbital and (−)-pentobarbital (Table 7).

The convulsant BZ Ro 5-3663 was reported to displace [^{3}H]DHP binding potently (IC_{50} near 100 nM) (16, 22), in contrast to a very low affinity for [^{35}S]TBPS binding sites in the present study (IC_{50} near 20 μM). BZs in general are weak inhibitors of [^{35}S]TBPS binding, with only four (Ro 5-3636, Ro 5-3663, Ro 11-6896(S), and Ro 5-4556) producing 50% inhibition at concentrations near 10 μM, in contrast to the greater potencies of several BZs in displacing [^{3}H]DHP from specific binding sites (22) at 0°. There is clearly no correlation between the affinities

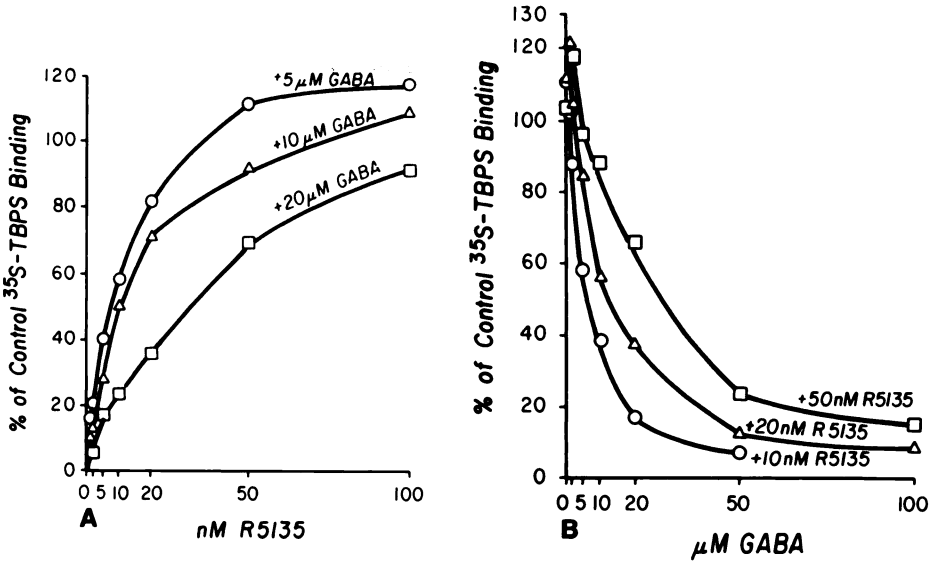

FIG. 8. *Competitive antagonism between R 5135 and GABA on [^{35}S]TBPS binding*

A. Reversal by R 5135 of specific [^{35}S]TBPS binding inhibition by GABA at 5 μM (○), 10 μM (△), and 20 μM (□). All three GABA concentrations inhibited [^{35}S]TBPS binding by more than 90%. Binding assay conditions were as described in Table 1.

B. Inhibition of specific [^{35}S]TBPS binding as a function of GABA concentration in the presence of 10, 20, and 50 nM R 5135. Binding assay conditions are described in Table 1.

of BZs for [^{3}H]diazepam and [^{3}H]FLU binding sites and their potencies in displacing either [^{3}H]DHP (22) or [^{35}S]TBPS.

DISCUSSION

There is ample evidence that picrotoxin antagonizes GABA neurotransmission indirectly by acting on a binding site coupled to independent binding sites for GABA, anions, cations, and BZs (1–7). Hitherto, [^{3}H]DHP has been the only ligand available for the direct characterization of the "picrotoxin" binding site, and its use is hampered by relatively low affinity and almost unacceptably large nonspecific binding (14, 15, 19–22). Since several bicyclophosphates are potent picrotoxin-like GABA antagonists (4, 10, 11) with much greater toxicity than picrotoxin, they might serve as useful ligands. Ozoe (33), using [^{3}H]PPO as the ligand, found that binding, with a temperature optimum near 20°, was strongly dependent on NaCl and enhanced by bicuculline methiodide. We also labeled a bicyclophosphorus compound in an attempt to develop a new ligand for the picrotoxin binding site lacking the above-mentioned disadvantages of [^{3}H]DHP. This proved successful, since [^{35}S]TBPS binds with high affinity (K_d = 17 nM) to brain-specific sites with properties similar to, but not identical with, [^{3}H]DHP binding sites. [^{35}S]TBPS gives comparably little nonspecific binding (about 20% in cerebral cortex). The relatively low rates at which [^{35}S]TBPS dissociates from its binding sites, even at 25°, permits the reliable use of the more convenient filtration binding assay.

There are three striking properties of brain-specific [^{35}S]TBPS binding which have not been described for [^{3}H]DHP binding: (a) the optimal temperature for [^{35}S]TBPS binding is near 21° (there is a negligible specific binding at 0° even after prolonged incubations in 200 mM KBr); (b) binding is entirely dependent on appropriate ions; and (c) in the presence of Eccles anions at 200 mM it is potently inhibited by GABA and all GABA-A receptor agonists tested. It may be of interest to investigate the effects of temperature, GABA mimetics, and ions on [^{3}H]DHP binding. The complete dependence of specific [^{35}S]TBPS binding on ions and its complete inhibition by GABA strongly suggest that all TBPS binding sites are coupled to independent recognition sites for anions and/or cations as well as for GABA. The competitive reversal of the inhibitory effect of GABA on [^{35}S]TBPS binding by the bicuculline-like GABA antagonist R 5135 (24) demonstrates that GABA exerts its inhibitory effect by acting on a receptor different from the [^{35}S]TBPS binding site. This GABA receptor is clearly a "classical" stereospecific GABA-A receptor.

There are many similarities between [^{35}S]TBPS and [^{3}H]DHP binding sites and several important differences relative to displacement of binding by convulsants, barbiturates, and other compounds. Some of the differences may be due to variations in ionic environment, as well as time and temperature of incubation. Both [^{3}H]DHP (15,

TABLE 6

Inhibition of specific [^{35}S]TBPS binding by GABA in 200 mM KBr alone and in the presence of 10, 20, and 50 nM R 5135

Specific [^{35}S]TBPS binding was determined as described in the text and Table 1. Seven or eight concentrations of GABA were used in each experiment. Usually there were about equal numbers of points above and below 50% inhibition. The IC_{50} values and Hill numbers were taken directly from Hill plots, log $(B_0/B_i - 1)$ against log[GABA], where B_0 is the specific [^{35}S]TBPS binding at 2 nM and B_i is specific binding in the presence of a given concentration of GABA.

Addition	*n*	IC_{50} for GABA	Hill no.
		μM	
None	6	0.34 ± 0.068	1.1 ± 0.071
R 5135			
10 nM	3	4.7 ± 0.94	1.2 ± 0.098
20 nM	2	11 ± 0.92	1.2 ± 0.056
50 nM	2	30 ± 3.5	1.4 ± 0.028

TABLE 7

Displacement of specific [^{35}S]TBPS binding to rat brain membranes by various substances

The following substances inhibited specific [^{35}S]TBPS binding less than 20% at 100 μM (or another concentration given in parentheses): acetyl salicylate, adamantanamine (10 μM), adenosine, *d,l*-2-amino-7-phosphonoheptanoate, 4-aminopyridine (10 μM), antipyrine, baclofen, bicuculline, 8-bromoadenosine, calycanthine (10 μM), carbromal, CGS 8216 (1 μM), CGS 9896 (1 μM), 2-chloroadenosine, chlorphenphiramine (1 mM), cloperidone (500 μM), clozapine, colchicine, *N,N*-diethylnicotinamide (10 mM), diphenylhydantoin, EMD 28422 (10 μM), ethosuximide (1 mM), Fenobam, gelsemine, glutethimide, guanosine, 3-isobutyl-1-methylxanthine, levamisole, mephenesine, meprobamate, methyl-β-carboline-3-carboxylate (100 nM), 1-methyl-isoguanosine, nalidixic acid, nipecotic acid, oxolinic acid (1 mM), phenacetin, phenobarbital, phensuximide (1 mM), 4-phenylpyridine, pipemidic acid, piracetam, piromidic acid (1 mM), Ro 15-1788 (100 nM), SQ 20,006 (10 μM), strychnine, thalidomide, theobromide, theophylline, tolazoline, urethane, and valproate (1 mM). Concentration-inhibition curves were made using six or seven concentrations of inhibitor with roughly equal numbers of concentrations on either side of 50% inhibition (1, 2, 5, 10 dilution series). The IC_{50} values and Hill numbers were taken directly from a Hill plot, log $(B_0/B_i - 1)$ against log[inhibitor], where B_0 is the specific binding in the presence of 2 nM [^{35}S]TBPS and B_i the specific binding in a given concentration of inhibitor. Binding assay conditions were as described in the text and Table 1.

Compound	*n*	IC_{50}	Hill no.	Compound	*n*	IC_{50}	Hill no.
		nM				*nM*	
GABA-mimetics				IPPO	3	320 ± 11	0.87 ± 0.02
Muscimol	2	59 ± 7.4	0.92 ± 0.30	TETS	3	820 ± 250	0.88 ± 0.07
APSA	2	240 ± 90	0.93 ± 0.12	EPS	3	1,600 ± 180	0.94 ± 0.05
Dihydromuscimol	2	260 ± 16	0.98 ± 0.11	EPO	3	3,400 ± 550	0.91 ± 0.04
GABA	6	340 ± 68	1.1 ± 0.07	TBOF	2	8,500 ± 1,100	0.89 ± 0.10
t-4-ACA	2	730 ± 57	1.0 ± 0.01	Picrotin	3	12,000 ± 1,600	0.90 ± 0.04
IGV	2	930 ± 98	0.94 ± 0.03	Bemegride	2	130,000 ± 13,000	0.83 ± 0.09
P4S	2	1,100 ± 120	1.0 ± 0.13	Pentylenetetrazole	2	560,000 ± 49,000	0.90 ± 0.02
(+)-3-OH-GABA	2	2,000 ± 64	0.96 ± 0.00	Pyrazolopyridines			
BGPA	2	2,600 ± 420	0.99 ± 0.09	SQ 65,396 (cartazolate)	2	190 ± 20	0.92 ± 0.23
(±)-Homo-β-proline	2	3,500 ± 260	1.0 ± 0.01	SQ 20,009 (etazolate)	2	480 ± 12	1.0 ± 0.07
(−)-3-OH-GABA	2	4,000 ± 630	0.96 ± 0.09	Tracazolate	2	1,700 ± 28	1.4 ± 0.07
THIP	2	5,000 ± 740	0.91 ± 0.07	Barbiturates and related compounds			
ImAA	2	9,200 ± 120	0.95 ± 0.01	(+)-Etomidate	3	2,800 ± 970	0.81 ± 0.08
(−)-Methylmuscimol	2	22,000 ± 490	0.99 ± 0.05	(−)-Secobarbital	2	31,000 ± 490	1.2 ± 0.07
3-Pyroline carboxylate	2	35,000 ± 5,500	1.0 ± 0.07	(−)-Etomidate	2	32,000 ± 6,400	0.94 ± 0.08
(+)-Methylmuscimol	2	99,000 ± 28,000	1.1 ± 0.11	(±)-DMBB	2	41,000 ± 2,700	1.3 ± 0.06
Convulsants				Methaqualone	2	43,000 ± 10,000	0.97 ± 0.17
TBPS	3	17 ± 5.2	0.83 ± 0.14	(−)-Pentobarbital	2	58,000 ± 13,000	1.2 ± 0.13
TBOB	2	35 ± 1.2	0.94 ± 0.04	(+)-Secobarbital	2	61,000 ± 20,000	1.1 ± 0.10
TBOP	2	59 ± 3.8	0.86 ± 0.01	(+)-Pentobarbital	2	110,000 ± 18,000	1.1 ± 0.10
TBPO	3	73 ± 20	0.96 ± 0.07	Others			
Anisatin	3	74 ± 20	0.95 ± 0.10	Deltamethrin[a]	2	5,600 ± 350	1.1 ± 0.07
IPPS	3	180 ± 23	0.97 ± 0.18	Cyproheptadine	2	59,000 ± 1,400	1.2 ± 0.20
Picrotoxin	3	180 ± 36	0.91 ± 0.05	Trazodone	2	160,000 ± 18,000	1.3 ± 0.04
Picrotoxinin	3	190 ± 21	0.87 ± 0.03				

[a] Deltamethrin produces plateau inhibition (83 ± 4.4% maximal inhibition).

16), and [^{35}S]TBPS are displaced from specific binding sites by most cage convulsants, as well as by certain barbiturates (16, 20, 21) and by the pyrazolopyridine SQ 20,009 (16). However, in general, the cage convulsants are more potent, and the barbiturates (especially (±)-DMBB) less potent in displacing [^{35}S]TBPS than in displacing [^{3}H]DHP. Several BZs, including the convulsant BZ Ro 5-3663, which were reported to be rather potent displacers of [^{3}H]DHP (16, 22) were much weaker displacers of [^{35}S]TBPS, the three most potent BZs having IC_{50} values near 10 μM. The pyrazolopyridines, especially SQ 65,396, were found to be more potent displacers of [^{35}S]TBPS than of [^{3}H]DHP. Diphenylhydantoin was reported to displace [^{3}H]DHP with an IC_{50} value near 100 μM, but displaced [^{35}S]TBPS less than 20% at this concentration. Deltamethrin inhibited [^{3}H]DHP and [^{35}S]TBPS binding with IC_{50} values of 0.1 and 5.6 μM, respectively.

The reversal by R 5135 of some compounds inhibiting [^{35}S]TBPS binding possibly provides a means to discriminate *in vitro* between anticonvulsants and convulsants acting at picrotoxin (TBPS) sites. Thus, R 5135 reverses the inhibitory effects of all anticonvulsant, anxiolytic, and/or sedative-hypnotic substances (including barbiturates, pyrazolopyridines, (+)-etomidate, BZs, cyproheptadine, meprobamate, and ethanol) while having no effect on, or potentiating, the inhibitory effects of all convulsants tested with the notable exception of anisatin. The noncompetitive reversal of SQ 65,396 inhibition of [^{35}S]TBPS binding by R 5135 (in contrast to the competitive reversal of GABA inhibition) suggests that the occupation of GABA receptors by R 5135 induces conformational changes in adjacent picrotoxin (TBPS) receptors such that the affinities for most convulsant ligands are increased (or unchanged) while the affinities for anticonvulsant/anxiolytic ligands are decreased.

The apparent competitive antagonism between Eccles anions and GABA on [^{35}S]TBPS binding provides further evidence for the direct modulation of ion recognition sites by GABA receptors. It was previously reported that three piperidine-derived GABA mimetics (THIP, P4S, and IGV) reduce the affinities of ions required for [^{3}H]FLU binding to brain-specific sites in a GABA reversible way (6, 25). However, these inhibitory effects of THIP, P4S, and IGV on [^{3}H]FLU binding are not strictly competitive with ions, since they formed plateaus and did not

TABLE 8

Effect of R 5135 (10 nM) on inhibition of [^{35}S]TBPS binding by various substances

All substances at the indicated concentrations were tested at least three times for effects on specific [^{35}S]TBPS binding in 200 mM KBr, with or without R 5135 (10 nM), under standard conditions, with similar results.

Substance (concentration)	% Control [^{35}S]TBPS binding		
	Alone	R 5135	% Change
Convulsants			
IPPO (500 nM)	48	32	−16
IPPS (200 nM)	41	32	−9
TBOB (50 nM)	28	19	−8
TBOP (100 nM)	47	34	−13
TBOF (10 μM)	42	30	−12
TBOA (10 μM)	57	52	−5
EPO (5 μM)	42	31	−11
EPS (2 μM)	46	35	−11
TETS (500 μM)	67	48	−19
Picrotoxinin (300 nM)	52	50	−2
Picrotin (20 μM)	46	36	−10
Anisatin (100 nM)	44	70	+26
Bemegride (200 μM)	45	37	−8
Pentylenetetrazole (1 mM)	42	36	−6
Ro 5-3663 (10 μM)	82	82	0
p-Chlorophenylsilatrane (100 μM)	93	96	+3
GABA mimetics			
GABA (500 nM)	51	100	+49
Muscimol (100 nM)	44	110	+64
APSA (500 nM)	52	110	+58
t-4-ACA (1 μM)	61	110	+49
P4S (2 μM)	51	110	+49
Anticonvulsants/anxiolytics/hypnotics			
SQ 65,396 (250 nM)	32	82	+50
(+)-Etomidate (5 μM)	48	88	+40
Methaqualone (50 μM)	52	92	+40
(−)-Secobarbital (50 μM)	54	97	+43
(−)-Pentobarbital (100 μM)	36	90	+54
Ro 5-3636 (10 μM)	64	96	+32
Ro 5-4556 (10 μM)	41	92	+51
Meprobamate (1 mM)	61	89	+28
Ethanol (2%)	43	60	+17
Others			
(±)-DMBB (50 μM)	57	97	+40
Trazodone (200 μM)	51	89	+38
Deltamethrin (10 μM)	34	75	+41
Cyproheptadine (50 μM)	61	78	+17

inhibit [^{3}H]FLU binding completely (25), in contrast to the complete inhibition of [^{35}S]TBPS binding by all GABA-A receptor agonists tested. NaCl alone protects both [^{3}H]FLU (5, 6, 29) and [^{35}S]TBPS sites against thermal inactivation with 50% of maximal protection near 200 mM and Hill numbers near 2. GABA potentiated the ability of NaCl to protect [^{3}H]FLU binding sites against thermal inactivation at 60° and shifted the entire NaCl concentration-response curve to the left, reducing the EC_{50} value for NaCl from about 240 mM to near 30 mM (5, 6, 29). In contrast, GABA potentiates the protective effects of low (≤50 mM), but not high (≥100 mM) NaCl concentrations when [^{35}S]TBPS is used as the ligand (data not shown). The concentration-response curve for NaCl in the presence of GABA suggests two populations of [^{35}S]TBPS sites, one which is protected by NaCl alone and not potentiated by GABA, and another which is protected by NaCl in a way which can be potentiated by GABA. In the absence of ions, GABA protects neither [^{35}S]TBPS nor [^{3}H]FLU binding sites against thermal inactivation.

There are other similarities between BZ and TBPS binding sites: both exhibit bi- or polyphasic time courses of heat inactivation at 60° in 200 mM NaCl; both are essentially restricted to the central nervous system, where they have similar distributions and densities (26, 28, 34); and both are linked to the same type of GABA receptors and ion recognition sites (5, 6, 29).

Interactions of several other compounds with TBPS binding are of special interest. Methaqualone, a substance with pharmacological properties similar to both barbiturates and BZs (32), displaces [^{35}S]TBPS binding with an IC_{50} value near 43 μM, making it more potent than (−)-pentobarbital and several other barbiturates. Since methaqualone displaces [^{3}H]diazepam with an IC_{50} value near 150 μM (35), it appears that the site at which methaqualone exerts its pharmacological effects may be closer to the TBPS (picrotoxin) than to the BZ binding site. Both meprobamate and ethanol inhibit [^{35}S]TBPS binding (IC_{50} values 1.4 and 212 mM, respectively) in ways which can be reversed by R 5135, suggesting that these two anxiolytics may act at physiologically relevant sites close to the TBPS (picrotoxin) binding site. Davis and Ticku (36) have reported that ethanol enhanced [^{3}H]diazepam binding to Lubrol-solubilized BZ receptors, with a half-maximal effect at 30 mM, in a way which was antagonized by picrotoxinin and (+)-bicuculline. Ethanol also inhibited [^{3}H]DHP binding by 22% at a concentration of 100 mM, similar to the inhibition of [^{35}S]TBPS binding reported here. Ethanol (100 mM) had no effect on [^{3}H]muscimol binding (36). Cyproheptadine also inhibits [^{35}S]TBPS binding more potently than most of the barbiturates tested (IC_{50} = 58 μM). This substance is of special interest since it has been shown to produce BZ-like anticonflict effects (Geller conflict test) in the rat and 5–20 mg/kg dose range (37). Cyproheptadine has also been reported to reduce the affinity of [^{3}H]FLU for its binding site through an allosteric mechanism with an IC_{50} value near 60 μM (29).

Two or more types of TBPS binding sites are suggested by the polyphasic dissociation of [^{35}S]TBPS from its receptor, polyphasic heat inactivation of the receptors and the differential action of GABA in potentiating the protective effect of low, but not high, NaCl concentrations against heat inactivation. The multiplicity of TBPS sites is not surprising in view of the demonstrated multiplicity of BZ receptors (38).

This investigation provides further evidence that several groups of psychotropic drugs, including picrotoxin-like convulsants, barbiturates, and related substances as well as the pyrazolopyridines, exert their behavioral effects by acting on TBPS (picrotoxin) binding sites, independent sites in BZ/ion/GABA/picrotoxin receptor complexes.

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