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# Anxiolytic Cyclopyrrolone Drugs Allosterically Modulate the Binding of [$^{35}$S]*t*-Butylbicyclophosphorothionate to the Benzodiazepine/γ-Aminobutyric Acid-A Receptor/Chloride Anionophore Complex

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SUMMARY

The influence of a number of anxiolytic cyclopyrrolone drugs, which include zopiclone and suriclone, on the binding of [$^{35}$S]*t*-butylbicyclophosphorothionate (TBPS), to benzodiazepine/γ-aminobutyric acid-A receptor/chloride anionophore complexes has been characterized in rat brain. Suriclone and its metabolites RP35,489 and RP46,166 are the most potent ($IC_{50}$ ~ 3nM) inhibitors of [$^{35}$S]TBPS binding thus far described, about an order of magnitude more potent than TBPS itself. The pattern of inhibition of [$^{35}$S]TBPS binding by suriclone is distinctive; at ~10 nM there is approximately 50% inhibition of [$^{35}$S]TBPS binding and inhibition "plateaus" at this level until suriclone concentrations exceed 1 μM. RP35,489 and RP46,166 display patterns of inhibition similar to suriclone. In saturation studies of [$^{35}$S]TBPS binding, suriclone reduces the $B_{max}$ of [$^{35}$S]TBPS-binding sites, with little or no effect on $K_D$. Muscimol also displays a noncompetitive pattern of inhibition of [$^{35}$S]TBPS binding, whereas inhibition by picrotoxinin appears competitive. [$^{35}$S]TBPS dissociation is multiphasic and similar whether initiated by 10 μM TBPS or 10 μM picrotoxinin. By contrast, dissociation of [$^{35}$S]TBPS is much faster (and nearly monophasic) when initiated by 10 μM TBPS/100 nM suriclone, 10 μM TBPS/1 μM muscimol, or 10 μM TBPS/1 mM pentobarbital. These results suggest that suriclone influences [$^{35}$S]TBPS binding allosterically, at sites distinct from the TBPS/picrotoxinin recognition site. Inhibition of [$^{35}$S]TBPS binding by suriclone varies regionally with a "plateau" at ~20% inhibition in the cerebellum, ~50% in the cerebral cortex, hippocampus and brain stem, and ~65% in the striatum and midbrain; by contrast, inhibition of [$^{35}$S]TBPS by picrotoxinin, muscimol, and pentobarbital shows little regional variation. The inhibition of [$^{35}$S]TBPS binding by suriclone is reversed by bicuculline [$ED_{50}$ ~1 μM] in several brain regions examined. Bicuculline alone has little or no influence on [$^{35}$S]TBPS binding in the cerebral cortex, hippocampus, and cerebellum, but produces a dose-dependent enhancement of [$^{35}$S]TBPS binding in the striatum, midbrain, and hypothalamus. Regional differences in the effects of suriclone and bicuculline on [$^{35}$S]TBPS recognition sites suggest possible heterogeneity in the coupling of cyclopyrrolone and bicuculline recognition sites to [$^{35}$S]TBPS recognition sites in rat brain.

## INTRODUCTION

A variety of anxiolytic drugs which lack obvious structural similarity to classical benzodiazepines interact potently with benzodiazepine receptor binding sites. Examples of such agents include the quinoline derivatives PK8165 and PK9084 (1), the triazolopyridazine CL-218,872 (2), and the cyclopyrrolone drugs zopiclone and suriclone (3, 4). Initial studies by Blanchard *et al.* (3, 4) suggested that zopiclone and suriclone compete directly with benzodiazepines at receptor sites. However, despite their pharmacologic agonist profile, cyclopyrrolones behave like antagonists in some receptor binding paradigms (5). Recently, we showed that cyclopyrrolones inhibit [$^{3}$H]Ro-15-1788 binding noncompetitively, and that they regulate benzodiazepine receptor binding allosterically by acting at a unique recognition site (6).

Benzodiazepines facilitate GABA[1]-mediated neurotransmission which involves an enhancement of chloride ion conductance (7, 8). Convulsant drugs such as picrotoxin and cage convulsants such as TBPS and barbitu-

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[1] The abbreviations used are: GABA, γ-aminobutyric acid; TBPS, *tert*-butylbicyclophosphorothionate.

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rates are thought to prevent the opening of chloride channels (9, 10). GABA, benzodiazepines, sedatives, and convulsants may influence a large receptor complex associated with chloride channels. The barbiturate enhancement of benzodiazepine receptor binding is antagonized by convulsants such as picrotoxin (8).

Direct studies of receptors for picrotoxin-like convulsants have employed [$^3$H]dihydropicrotoxinin (8, 10–12) and more recently [$^{35}$S]TBPS (13). The binding of [$^{35}$S]TBPS is absolutely dependent upon chloride or other appropriate anions and is inhibited by barbiturates and various convulsant drugs. On the other hand, benzodiazepines exhibit low affinity for these sites and their relative activities do not parallel known influences at benzodiazepine receptors (13–15). In the present study, we describe a very potent allosteric modulation of [$^{35}$S]TBPS binding by the anxiolytic cyclopyrrolone drugs.

## MATERIALS AND METHODS

*Materials.* [$^{35}$S]TBPS (specific activity, 103.5 Ci/mmol at the start of the study, thereafter adjusted for the decay of $^{35}$S; $t_{½}$ = 87.2 days) was obtained from New England Nuclear Company (Boston, MA). The cyclopyrrolones suriclone, zopiclone, RP35,489, and RP46,166 were gifts from Dr. L. Julou, Rhone-Poulenc Recherches (Vitry-sur-Seine, France). The triazolopyridazine CL-218,872 was a gift from Dr. A. Lippa, Lederle Laboratories (Pearl River, NY). The drugs flunitrazepam, Ro-15-1788, and methyl β-carboline 3-carboxylate (Ro-22-7497), were kind gifts of Dr. P. Sorter, Hoffmann-LaRoche (Nutley, NJ). All other chemicals were obtained from commercial sources.

*Preparation of rat brain membranes.* Appropriate brain regions were dissected from male Sprague-Dawley rats. The tissue was homogenized in 20 volumes of ice-cold 0.32 M sucrose using a Teflon-glass homogenizer. Following centrifugation for 10 min × 3,500 × *g*, the supernatant was centrifuged 30 min × 150,000 × *g* in a preparative ultracentrifuge. The pellet was resuspended in 20 volumes of ice-cold distilled water with a Polytron (Brinkmann Instruments, Westbury, NY) and again centrifuged 30 min × 150,000 × *g*. The pellet was resuspended in 20 volumes of 50 mM Tris-citrate (pH 7.5 at 0°) with a Polytron and centrifuged 20 min × 48,000 × *g*. The final pellet was resuspended to 50 mg original wet weight/ml (approximately 2 mg of protein/ml) in ice-cold 50 mM Tris-citrate with a Polytron, and this suspension was used in binding studies. Tissue prepared in this way could be stored at −80° in either the pellet or suspension form for several months with little or no loss in [$^{35}$S]TBPS-binding activity; however, only freshly prepared tissue homogenates were utilized in the studies described here.

*[$^{35}$S]TBPS-binding assays.* To 100 μl of tissue homogenate prepared as described above were added 50 μl of [$^{35}$S]TBPS at the appropriate concentration (final assay concentration, 2 nM in experiments other than saturation analysis) in 1 M NaBr and 50 μl of 50 mM Tris-citrate buffer. After a 90-min incubation at room temperature (21°), samples were filtered over glass-fiber filter strips (Schleicher and Schuell No. 32) using a cell harvester filtration manifold (model M-24R, Brandel, Incorporated, Gaithersburg, MD) followed by three 3-ml washes with 0.9% NaCl (equilibrated to room temperature). Filters were then counted in 4 ml of Formula 947 (New England Nuclear) by liquid scintillation spectrometry to define total [$^{35}$S]TBPS binding. Nonspecific [$^{35}$S]TBPS was determined using 10 μM unlabeled TBPS in the medium. Specific [$^{35}$S]TBPS binding was defined as total binding less nonspecific binding. Under these conditions, the specific binding in cerebral cortex using [$^{35}$S]TBPS with an initial specific activity of 100 Ci/mmol was about 1300 cpm. and nonspecific binding about 250 cpm; the specific binding was thus ~80–90% of total binding, comparable to the results of others (13, 16) using different tissue preparations. Control experiments revealed that binding was linear with tissue concentration until at least 5 mg of protein/ml. The radioligand could be stored for at least 4 months at −20° with no apparent deterioration in specific binding; routinely, however, the [$^{35}$S]TBPS was obtained in small aliquots and utilized within 1 month after purchase. In competition experiments, the appropriate drug was added in place of buffer and assayed as previously described.

In experiments where the dissociation kinetics of [$^{35}$S]TBPS were studied, 100-μl aliquots of tissue homogenate were incubated as described above with 50 μl of [$^{35}$S]TBPS (final concentration, 2 nM). At the appropriate time, 50 μl of TBPS (10 μM final concentration) or a mixture of TBPS and various drugs were added. The incubation was terminated by filtration as previously described. Nonspecific [$^{35}$S]TBPS binding was determined separately only at the end of the assay (90 min after initiation of dissociation).

## RESULTS

*Inhibition of [$^{35}$S]TBPS binding by cyclopyrrolones.* As reported by others (13, 14), [$^{35}$S]TBPS binding is inhibited by muscimol, picrotoxin, and pentobarbital (Table 1). Also as reported previously (14), benzodiazepines and related agents such as methyl β-carboline-3-carboxylate compete for binding in micromolar concentrations. In agreement with the report of Ramanjaneyulu and Ticku (14), these weak effects of benzodiazepines are not correlated with their potency at influencing benzodiazepine receptor binding. Thus, Ro-15-1788, whose $K_D$ for benzodiazepine receptors is about 0.8 nM (17), fails to influence [$^{35}$S]TBPS binding in concentrations as high as 100 μM.

The cyclopyrrolones suriclone and zopiclone and the suriclone metabolites RP35,489 and RP46,166 display a pattern quite different from that of the benzodiazepines. These drugs affect [$^{35}$S]TBPS binding in a biphasic fashion (Fig. 1). Cyclopyrrolones produce considerable inhibition of [$^{35}$S]TBPS binding at low nanomolar concentrations, but inhibition then plateaus until concentrations above 1 μM are reached. We can thus readily define "suriclone-sensitive" and "suriclone-insensitive"

TABLE 1

*Inhibition of [$^{35}$S]TBPS binding to rat cerebral cortex by various drugs*

Inhibition of [$^{35}$S]TBPS binding by various drugs was determined as described in Materials and Methods. Values are from a representative experiment replicated at least two times with less than 20% variation. Each inhibition curve was determined using at least six concentrations of the appropriate drug.

| Drug | $IC_{50}$ |
|---|---|
| | μM |
| Suriclone | 0.003, 3* |
| RP35,489 | 0.003, 3* |
| RP46,166 | 0.003, 3* |
| Zopiclone | 0.01, 3* |
| TBPS | 0.07 |
| Picrotoxinin | 0.21 |
| Muscimol | 0.49 |
| GABA | 3.0 |
| CL-218,872 | 4.5 |
| Flunitrazepam | 5.8 |
| β-CCM† | 6.2 |
| Pentobarbital | 83.2 |
| Ro-15-1788 | >100 |

* These $IC_{50}$ values refer to the inhibition of the suriclone-sensitive (lower values) and -insensitive (higher values) components of [$^{35}$S]TBPS binding (see text).

† β-CCM, methyl β-carboline 3-carboxylate.

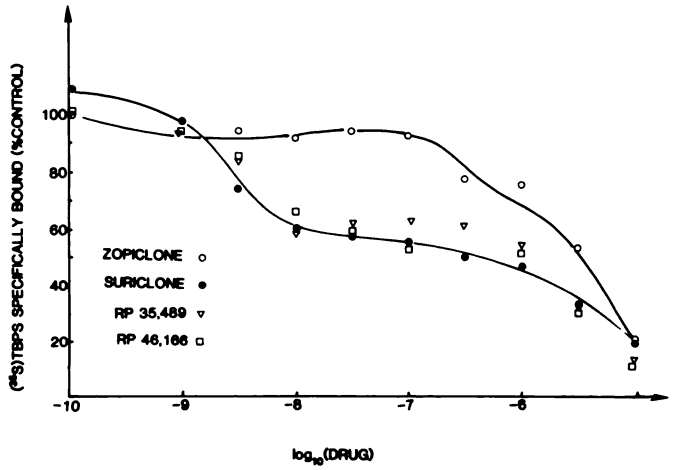


FIG. 1. *Inhibition of specific [$^{35}$S]TBPS binding to rat cerebral cortical membranes by various cyclopyrrolones*

Inhibition curves were determined as described in Materials and Methods. The data presented are from a representative experiment which has been replicated (at least) three times with very similar results. Individual points are the mean of duplicate determinations. Lines are drawn through the experimental data for zopiclone and suriclone. The total specific binding of [$^{35}$S]TBPS with no drug added was about 1300 cpm (or about 65 fmol/mg of protein).

components of [$^{35}$S]TBPS binding as binding abolished or remaining, respectively, in the presence of 100 nM suriclone. The suriclone-sensitive component amounts to about 47% of total [$^{35}$S]TBPS binding in rat cerebral cortex. This suriclone-sensitive component of [$^{35}$S]TBPS binding is inhibited 50% by about 3 nM suriclone, RP35,489, or RP46,166. The high affinity component of zopiclone inhibition is less prominent, comprising only about 10% of [$^{35}$S]TBPS binding but with effects also exerted at about 4–8 nM.

To further investigate the nature of cyclopyrrolone effects on [$^{35}$S]TBPS binding, we explored a wide range of [$^{35}$S]TBPS concentrations in the presence of various concentrations of suriclone as well as muscimol and picrotoxinin (Fig. 2). As noted elsewhere (10), picrotoxinin is a competitive inhibitor of [$^{35}$S]TBPS binding, increasing the $K_D$ value for the ligand without altering its $B_{max}$ (Fig. 2A). Muscimol appears noncompetitive, reducing numbers of binding sites with little change in $K_D$ (Fig. 2A). With a variety of concentrations of suriclone, we consistently observe apparently noncompetitive inhibition, with a progressive reduction in the $B_{max}$ for [$^{35}$S]TBPS binding and little or no change in $K_D$. The effect of suriclone on the $B_{max}$ of [$^{35}$S]TBPS-binding plateaus at about 10 nM (Fig. 2B).

*Cyclopyrrolone influences on [$^{35}$S]TBPS-binding kinetics.* If suriclone were allosterically regulating [$^{35}$S]TBPS binding rather than competing directly, then one would anticipate an influence of suriclone upon [$^{35}$S]TBPS dissociation. Accordingly, we explored the dissociation of [$^{35}$S]TBPS in cerebral cortical membranes incubated to equilibrium with [$^{35}$S]TBPS (Fig. 3). Dissociation is biphasic when initiated by 10 μM TBPS, as reported previously (13). A very similar dissociation rate is observed when dissociation is initiated by 10 μM picrotoxinin, consistent with the suggestion that picrotoxinin is a competitive inhibitor of [$^{35}$S]TBPS binding.

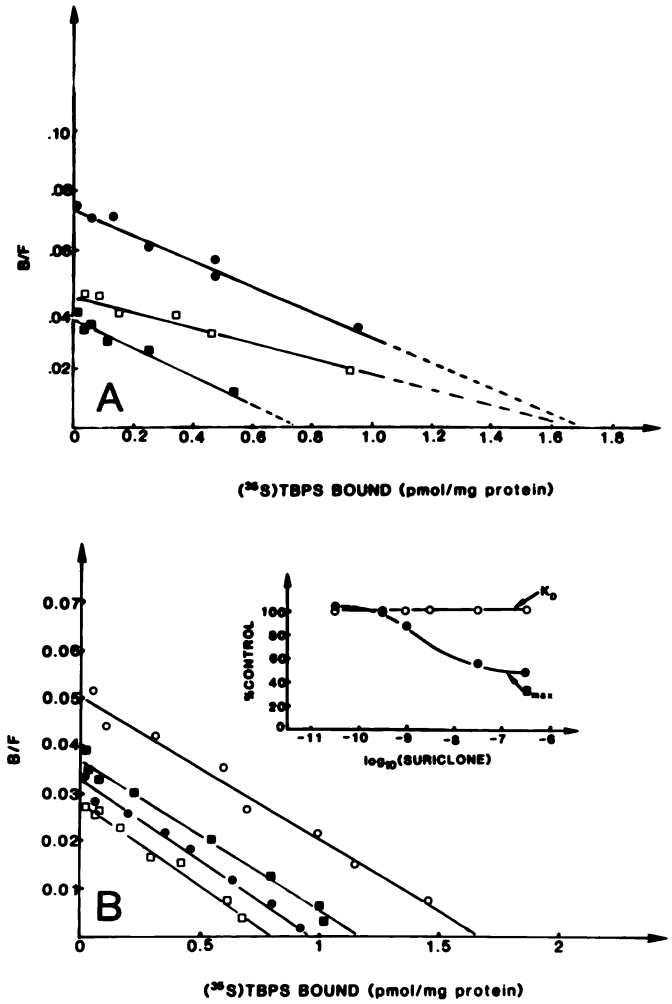


FIG. 2. *Scatchard analyses of [$^{35}$S]TBPS binding to rat cerebral cortical membranes in the presence of various drugs at 21° in 0.25 M NaBr*

All Scatchard plots are transformations of saturation experiments utilizing six to eight concentrations of [$^{35}$S]TBPS (ranging from a final assay concentration of 0.5 to 50 nM), as described in Materials and Methods. All data are from a representative experiment that has been replicated, with very similar results, at least twice. Lines drawn are unweighted least squares fit through the experimental data. A, [$^{35}$S]TBPS saturation in the presence of no added drug (●), 0.3 μM picrotoxinin (□) or 0.3 μM muscimol (■). For control, $K_D$ = 17 nM, $B_{max}$ = 1.7 pmol/mg protein; with 0.3 μM picrotoxinin added, $K_D$ = 29 nM, $B_{max}$ = 1.7 pmol/mg protein; with 0.3 μM muscimol added, $K_D$ = 17 nM, $B_{max}$ = 0.75 pmol/mg protein. B, [$^{35}$S]TBPS saturation in the presence of no added drug (○), suriclone at 3 nM (■), 30 nM (●), and 0.3 μM (□); for clarity, data for 0.3 nM, 30 pM, and 1 nM suriclone have been omitted. With no suriclone added, $B_{max}$ = 1.65 pmol/mg protein, $K_D$ = 20 nM. Inset: the per cent change in the apparent $K_D$ and $B_{max}$ for [$^{35}$S]TBPS binding (relative to values in the presence of no added drug) are plotted as a function of suriclone concentration.

In marked contrast to the effects of picrotoxinin, suriclone (0.1 μM) markedly accelerates the dissociation of [$^{35}$S]TBPS and converts it into a monophasic dissociation; very similar results were obtained if 10 μM TBPS/100 nM suriclone is used to initiate dissociation. Muscimol (10 μM) or 10 μM muscimol/10 μM TBPS and pen-

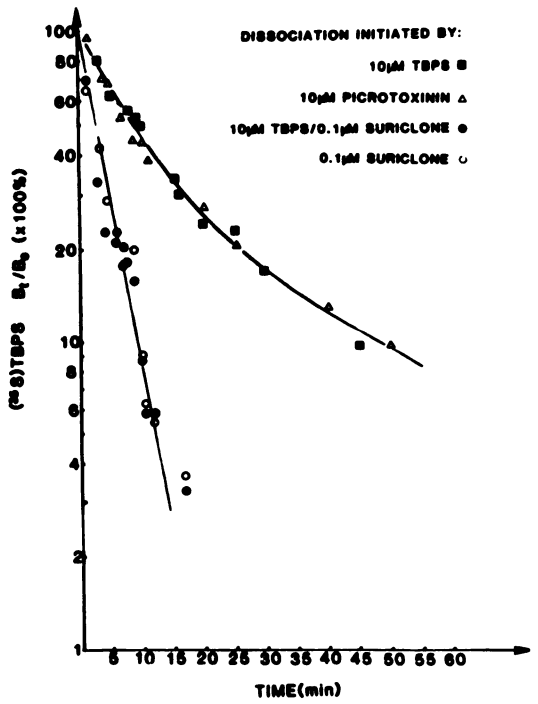


FIG. 3. *Dissociation of [$^{35}$S]TBPS from rat cerebral cortical membranes at 21° in 0.25 M NaBr*

Dissociation kinetics were determined as described in Materials and Methods. Data are from a representative experiment replicated twice with very similar results. Data for similar experiments with 10 μM TBPS/10 μM muscimol and 10 μM TBPS/10 mM pentobarbital are not shown as in these cases dissociation was essentially completed in less than 30 sec. Lines drawn are weighted least squares fits, for 10 μM TBPS and 10 μM TBPS/0.1 μM suriclone, respectively.

tobarbital (1 mM) or 1 mM pentobarbital/10 μM TBPS also accelerates the dissociation of [$^{35}$S]TBPS. The effects of muscimol and pentobarbital are so marked that dissociation in their presence appears complete within 1 min. These results suggest that the picrotoxinin and TBPS recognition sites partially or completely overlap and suriclone recognition sites are distinct from TBPS recognition sites.

*Regional differences in effects of suriclone on [$^{35}$]TBPS binding.* The pattern of inhibition of [$^{35}$S]TBPS binding by suriclone varies regionally in rat brain (Fig. 4A). The suriclone-sensitive component of binding is most prominent in the corpus striatum and midbrain, where it constitutes about 65% of total [$^{35}$S]TBPS binding. The suriclone-sensitive component of [$^{35}$S]TBPS binding is about 50% of total binding in the cerebral cortex, hippocampus, and brain stem. By contrast, in the cerebellum, only about 20% of [$^{35}$S]TBPS binding is suriclone sensitive. While the amount of the suriclone-sensitive component varies regionally, the $IC_{50}$ for suriclone inhibition of the suriclone-sensitive component (3 nM) is similar in all regions examined.

Muscimol, picrotoxinin, and pentobarbital do not display marked regional differences in potencies at influencing [$^{35}$S]TBPS binding in rat brain (Fig. 4B–D).

*Interactions between suriclone and bicuculline in influencing [$^{35}$S]TBPS binding.* Squires *et al.* (13, 18) reported that inhibition of [$^{35}$S]TBPS binding by GABA-mimetic and sedative-anticonvulsant drugs was reversed by the GABA antagonist bicuculline or a potent bicuculline analogue R5135. Suriclone inhibition of [$^{35}$S]TBPS binding is also reversed by bicuculline (Fig. 5). Maximal reversal is apparent with 1 μM bicuculline, which restores [$^{35}$S]TBPS binding to 80–95% of control values. The $ED_{50}$ for bicuculline at reversing the inhibition of [$^{35}$S]TBPS binding produced by 100 nM suriclone is about 0.3 μM (data not shown).

Bicuculline alone does not affect [$^{35}$S]TBPS binding in the cerebral cortex, but does stimulate binding in other brain regions (Fig. 6). The greatest stimulation is apparent in the hypothalamus, reaching levels double control values. In the midbrain and corpus striatum, maximal enhancements of [$^{35}$S]TBPS binding are 80 and 40% above control values, respectively. Negligible stimulation occurs in the cerebellum, hippocampus, or cerebral cortex even though bicuculline reverses suriclone inhibition of [$^{35}$S]TBPS binding in these areas. The $ED_{50}$ for bicuculline enhancement of [$^{35}$S]TBPS binding (approximately 1 μM) is similar to its $ED_{50}$ at reversing inhibition of [$^{35}$S]TBPS binding by suriclone, muscimol, or pentobarbital.

## DISCUSSION

The major finding of the present study is that cyclopyrrolone anxiolytics are very potent inhibitors of [$^{35}$S]TBPS binding to benzodiazepine/GABA-A receptor/chloride anionophore complexes in rat brain. The pattern of inhibition of [$^{35}$S]TBPS binding by cyclopyrrolones is distinctive, exhibiting a high affinity (suriclone-sensitive) component for which suriclone and structurally related compounds RP35,489 and RP46,166 display an $IC_{50}$ of approximately 3 nM, some 10 times more potent than any of the drugs examined in previous studies (13, 14). Strikingly, however, there is no further inhibition of [$^{35}$S]TBPS binding by cyclopyrrolones in the concentration range 10 nM–1 μM, and concentrations above 1 μM are required to inhibit residual (suriclone-insensitive) [$^{35}$S]TBPS binding.

The influence of suriclone on the suriclone-sensitive component of [$^{35}$S]TBPS binding appears to be allosteric for several reasons. First, the inhibition of [$^{35}$S]TBPS binding by suriclone appears noncompetitive, involving a reduction in the apparent $B_{max}$ for [$^{35}$S]TBPS binding with little change in $K_D$. Moreover, the dissociation of [$^{35}$S]TBPS from rat cortex is much faster when initiated by 10 μM TBPS/0.1 μM suriclone than when initiated by 10 μM TPBS alone, or by 10 μM picrotoxinin. Furthermore, TBPS fails to influence [$^{3}$H]suriclone binding in concentrations up to 100 μM (data not shown). As shown here and by others (14), muscimol also produce a noncompetitive pattern of inhibition of [$^{35}$S]TBPS binding. Also, dissociation of [$^{35}$S]TBPS is much faster when initiated by 10 μM TBPS in the presence of 10 μM muscimol or 0.1–1.0 mM pentobarbital than when initiated by 10 μM TBPS alone. This suggests that suriclone, muscimol, and pentobarbital all influence [$^{35}$S]TBPS binding allosterically. It had been previously believed that barbiturates act at a site closely related to the recognition site for picrotoxin and cage convulsants such

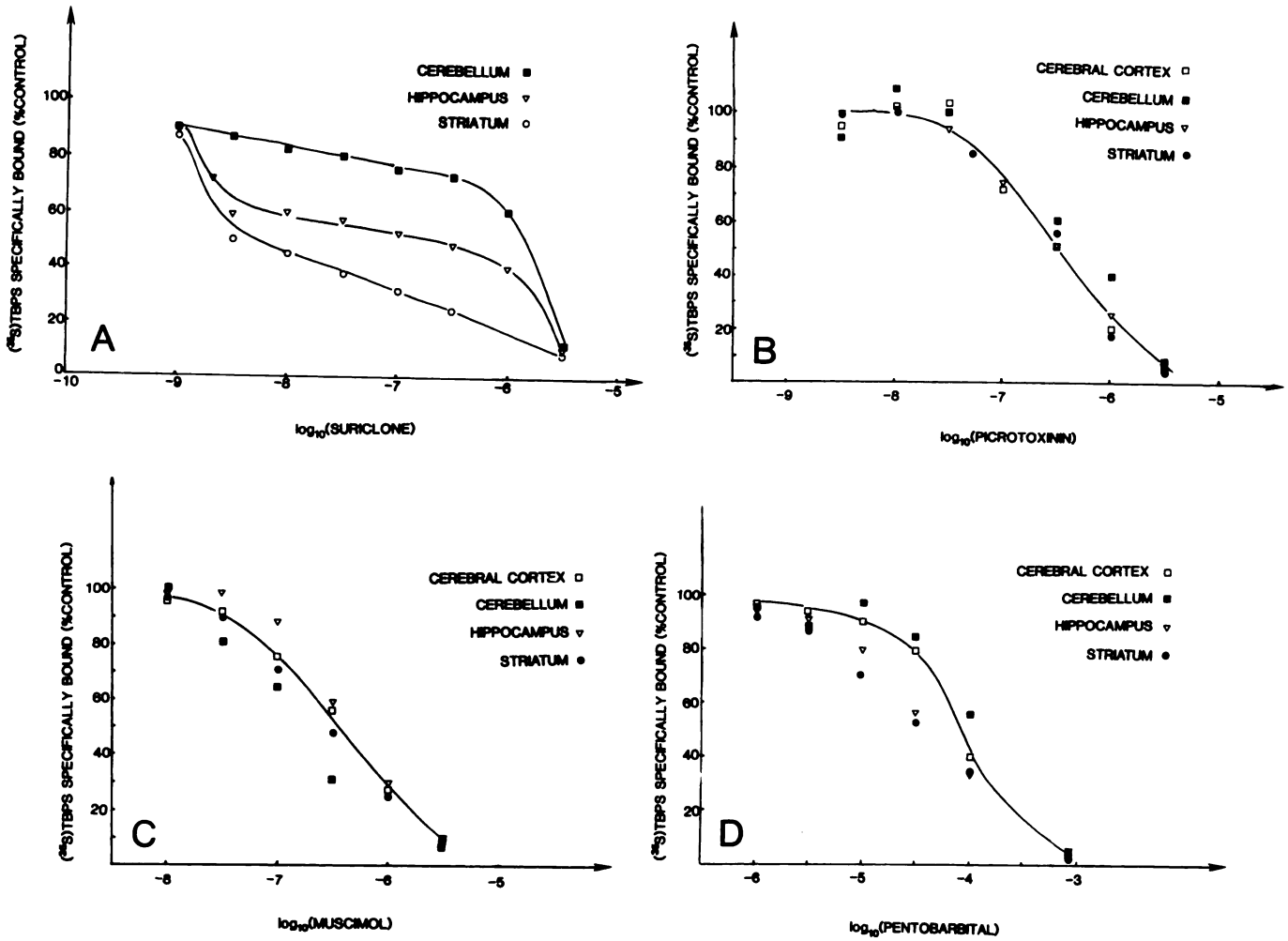


FIG. 4. *Regional variations in inhibition of [$^{35}$S]TBPS binding to rat brain by (A) suriclone, (B) picrotoxinin, (C) muscimol, and (D) pentobarbital*
Membranes from the stated brain regions were prepared according to Materials and Methods. Data are from a representative experiment replicated (at least) two further times with very similar results. Similar data for membranes prepared from the cerebral cortex, brainstem, and midbrain/diencephalon are omitted for clarity. The lines drawn are the weighted least square fit through the experimental data. In all regions, $K_D$ = 17 nM for controls. Control $B_{max}$ values (in picomoles/mg of protein) were 1.7 (cerebral cortex), 1.4 (cerebellum), 1.5 (hippocampus), 1.3 (striatum), 1.4 (hypothalamus), and 0.8 (medulla-diencephalon).

as TBPS (8, 10, 19). It now seems possible that pentobarbital and perhaps other barbiturates act at a site that is distinct from the recognition site for picrotoxin and TBPS. We had previously shown that cyclopyrrolones label a site on the benzodiazepine receptor complex distinct from the recognition sites for GABA and pentobarbital (6); abundant evidence (8) indicates that GABA and barbiturates act at distinct sites. Thus, there appear to be at least three mutually distinct sites on the benzodiazepine receptor complex through which [$^{35}$S]TBPS binding can be allosterically influenced.

Cyclopyrrolone anxiolytics such as zopiclone and suriclone display a pharmacologic profile in animals and man which resembles that of classical benzodiazepine agonists, such as diazepam (3, 4). Using *in vitro* radioligand binding techniques, we recently provided evidence that cyclopyrrolones bind to a site on the benzodiazepine receptor complex distinct from benzodiazepines (6). The present findings support our previous conclusions. Whereas cyclopyrrolones are the most potent agents at influencing [$^{35}$S]TBPS binding, benzodiazepines and alkyl $\beta$-carboline 3-carboxylates are weak inhibitors of [$^{35}$S]TBPS binding (refs. 13 and 14 and this study). It is difficult to understand how cyclopyrrolones and benzodiazepines could have such widely differing effects on [$^{35}$S]TBPS binding if they acted at a common recognition site. It should be noted, however, that a recent report (20) has suggested that benzodiazepine agonists, antagonists, and inverse agonists differentially modulate TBPS binding.

The influence of cyclopyrrolones on [$^{35}$S]TBPS binding appears to be noncompetitive, involving a progressive reduction in $B_{max}$ with little change of $K_D$. This pattern of inhibition could reflect an irreversible influence of cyclopyrrolones on [$^{35}$S]TBPS binding. However, bicuculline can fully reverse suriclone inhibition of [$^{35}$S]TBPS binding. In previous studies, suriclone was somewhat anomalous in that, while displaying a anxiolytic pharmacologic profile, binding of this drug to benzodiazepine receptors was not regulated by GABA (4, 6), $Cl^-$,

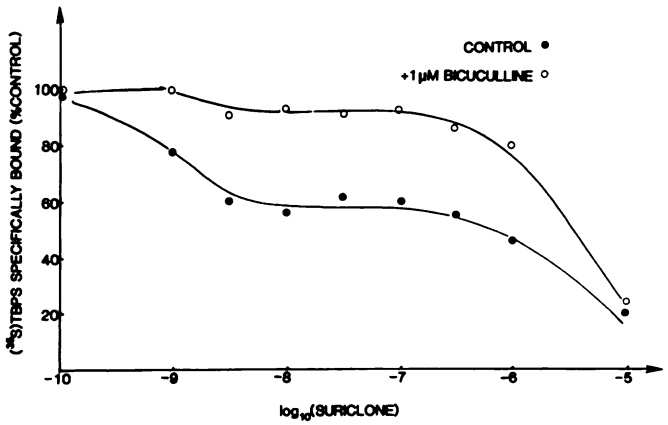


FIG. 5. *Reversal of inhibition of [$^{35}$S]TBPS binding to rat cerebral cortical membranes by bicuculline*

Inhibition of [$^{35}$S]TBPS binding by suriclone in control membranes (●) and in the presence of 1 μM bicuculline (○). Rat cerebral cortical membranes were prepared according to Materials and Methods. To 100 μl of membranes, 50 μl of [$^{35}$S]TBPS (10 nM in 1.25 M NaBr) was added along with 50 μl of suriclone to give the appropriate final concentration and 50 μl of bicuculline methiodide to give final concentration stated. The mixture was then processed as described in Materials and Methods. Data are from a representative experiment replicated at least two times with very similar results. Lines drawn are weighted least squares fit through the experimental data. The total specific binding of [$^{35}$S]TBPS with no drug added was about 1300 cpm (or about 65 fmol/mg of protein).

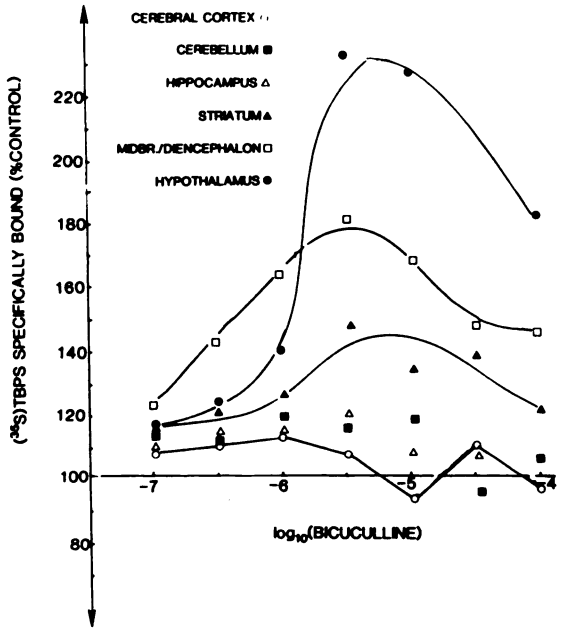


FIG. 6. *Regional variations in the influence of bicuculline on baseline [$^{35}$S]TBPS binding to rat brain*

Membranes were prepared as described in Materials and Methods for the various brain regions. To 100 μl of membranes, 50 μl of [$^{35}$S]TBPS was added (8 nM in 1 M NaBr) along with 50 μl of bicuculline methiodide to give the appropriate final concentration. Data are from a representative experiment replicated twice. Each point is the mean of duplicate determinations. Lines drawn are the weighted least squares fit to the experimental data. For clarity, no lines are drawn through the experimental data for the cerebellum and hippocampus. Control $K_D$ and $B_{max}$ values are as stated in the legend to Fig. 4.

barbiturates, or pyrazolopyridines (6). Furthermore, the potency of suriclone at inhibiting the binding of [$^{3}$H]Ro-15-1788 is little affected by exhaustive flunitrazepam photoaffinity labelling (5, 6). Both GABA enhancement of binding and affinity changes after exhaustive flunitrazepam photoaffinity labeling ("photoshift") have been proposed as criteria for placing benzodiazepine-like drugs on an agonist-antagonist continuum (21). By either of these criteria, one would (incorrectly) fail to predict that suriclone is an anxiolytic drug. Squires *et al.* (13, 18) demonstrated that inhibition of [$^{35}$S]TBPS binding by a wide variety of anticonvulsants/anxiolytics/hypnotics is reversible by bicuculline and a potent bicuculline analogue R5135, whereas inhibition of [$^{35}$S]TBPS binding by convulsants is not affected by the bicuculline analogue. Thus, the criterion of Squires *et al.* would correctly identify suriclone as an anxiolytic drug. Conceivably, bicuculline (or R5135) reversal of drug effects on [$^{35}$S]TBPS binding will be a valuable supplement to existing criteria for characterizing drugs on a anxiolytic/anticonvulsant-proconvulsant continuum using *in vitro* radioligand-binding techniques.

An interesting feature of cyclopyrrolone influences on [$^{35}$S]TBPS binding is the regional variation in size of the suriclone-sensitive component of [$^{35}$S]TBPS binding. There are modest variations in the size of this component (~50–70%) in most brain regions, with the exception of the cerebellum where only about 20% of [$^{35}$S]TBPS-binding sites are suriclone-sensitive. The size of the suriclone-sensitive component is correlated somewhat with the fraction of putative type II benzodiazepine receptors, which follow the order striatum > hippocampus > cerebral cortex > cerebellum (22), although the correlation is not perfect. For example, the midbrain-diencephalon section contains the thalamus and substantia nigra, regions rich in type I benzodiazepine receptors (22) and yet this region appears to have one of the largest proportions of suriclone-sensitive [$^{35}$S]TBPS binding. Autoradiographic studies may be helpful in a more detailed examination of the correlation between suriclone-sensitive and insensitive [$^{35}$S]TBPS components and type I/type II benzodiazepine receptors as conventionally defined (22, 23).

Another property of [$^{35}$S]TBPS binding which seems to vary regionally is the influence of bicuculline on baseline binding. In some brain regions (hypothalamus, striatum, midbrain-diencephalon), bicuculline produces a dose-dependent stimulation of [$^{35}$S]TBPS binding; this is particularly dramatic in the hypothalamus, where levels reach twice control values. In other regions, such as the cerebral cortex, hippocampus, and cerebellum, there is much less bicuculline stimulation. However, in all of these regions, bicuculline can reverse inhibition of [$^{35}$S]TBPS binding produced by suriclone. Regions which contain larger fractions of suriclone-sensitive [$^{35}$S]TBPS binding tend to be those in which bicuculline has its greatest effects on baseline [$^{35}$S]TBPS binding although, again, the correlation is not perfect. The degree of bicuculline sensitivity for (reversal of) pentobarbital enhancement of [$^{3}$H]diazepam binding also varies regionally (24) but these regional variations (cortex > hippo-

campus > thalamus-midbrain~striatum > medulla-pons~cerebellum) differ considerably from regional variations in either the size of the suriclone-sensitive component of [$^{35}$S]TBPS or influences of bicuculline on baseline [$^{35}$S]TBPS binding.

The results reported here suggest regional heterogeneity of [$^{35}$S]TBPS-binding sites with regard to their coupling to cyclopyrrolone and bicuculline recognition sites, which might be due to structural differences in the binding sites or other factors, such as multiple coupling states. However, the heterogeneity does not fit neatly into the framework of type I/type II benzodiazepine receptors (23) or the framework discussed by Leeb-Lundberg *et al.* (24). This suggests that receptor populations defined by each of these two schemes might themselves be heterogeneous, which leaves one with an overall impression that there is extensive, but subtle, heterogeneity in brain benzodiazepine/GABA-A receptor/chloride anionophore complexes. Ultimately, detailed investigation of these issues will require purification of intact receptor complexes from various regions of brain.

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