Structure–Activity Correlations for Interactions of Bicyclophosphorus Esters and Some Polychlorocycloalkane and Pyrethroid Insecticides with the Brain-Specific \(t\)-Butylbicyclophosphorothionate Receptor

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\[^{[35S]}t\text{-Butylbicyclophosphorothionate or }[^{[35S]}\text{TBPS is an improved radioligand for the picrotoxinin binding site in rat brain synaptic membranes. The toxic isomers of the hexachlorocyclohexanes, polychlorobornanes, and chlorinated cyclodienes displace }[^{[35S]}\text{TBPS with a stereospecificity and potency generally correlated with their mammalian toxicity. In a few cases this correlation is improved by correction for metabolic activation or detoxification on using a coupled brain receptor/liver microsomal oxidase system. The }\alpha\text{-cyano-3-phenoxyl benzyl pyrethroids, although less potent, inhibit }[^{[35S]}\text{TBPS binding in a stereospecific manner correlated with their toxicity. Scatchard analyses indicate that these three classes of polychlorocycloalkane insecticides act at the TBPS binding site within the }\gamma\text{-aminobutyric acid (GABA) receptor–ionophore complex whereas the }\alpha\text{-cyano pyrethroids interact with a closely associated site. These insecticides and TBPS analogs may serve as useful probes further to elucidate the topography of the TBPS binding site and its relationship to the chloride channel.}\]

Introduction

The safe and effective use of bioactive chemicals requires adequate knowledge of their structure–activity relationships, metabolic activation or detoxification, and mode of action at an organismal and enzyme or receptor level, in both target and nontarget species. This ideal has been largely achieved with the organophosphorus and methylcarbamate insecticides which inhibit acetylcholinesterase at synapses and with some of the first chlorinated hydrocarbon and pyrethroid insecticides, such as DDT and allethrin, which disrupt sodium channels on neuron or muscle cell membranes. Our recent studies indicate that bicyclophosphorus esters (BPs) and some polychlorocycloalkane and newer pyrethroid insecticides act within the \(\gamma\)-aminobutyric acid (GABA) receptor–ionophore complex and that this coupled system and its inhibitors are conveniently examined with a simple radioligand assay using \[^{[35S]}t\text{-Butylbicyclophosphorothionate (}\[^{[35S]}\text{TBPS) and rat brain synaptic membranes.}\]

This review considers the development and properties of \[^{[35S]}\text{TBPS as an improved radioligand for the picrotoxinin (PTX) receptor within the GABA receptor–ionophore complex and the interaction of diverse insecticides and other neuroactive agents with the TBPS binding site.}\]

Picrotoxinin (PTX) and \(t\)-Butylbicyclophosphorothionate (TBPS)

PTX is a polycyclic epoxylactone (Fig. 1) from seeds of \textit{Anamirta cocculus} \(L\). It inhibits GABA-stimulated chloride permeability by acting as a noncompetitive GABA antagonist (\(I\)–\(\delta\)). Structure–toxicity relationships for PTX [mouse intraperitoneal (IP) \(LD_{50} = 9.0\) mg/kg (\(\delta\))] and its analogs have been used to define critical features for convulsant action (\(\delta\)) and to char-
acterize the topography of the chloride channel (3). The PTX receptor has been extensively studied with the radioligand analog α-[8,10-[3H]dihydropicrotoxinin ([3H]DHP) (Fig. 1) (6–8) (NET-606 of New England Nuclear Corp., Boston, MA). [3H]DHP is less than satisfactory as a radioligand because of its very low affinity and almost unacceptably high nonspecific binding (6–8). Many types of convulsants inhibit [3H]DHP binding to rat brain synaptic membranes but unfortunately with the BPs there is a relatively poor correlation between inhibitory potency and toxicity (8).

The BPs are xenobiotics, and certain analogs such as TBPS (Fig. 1) are potent convulsants (4,9,10). Electronic reactions such as phosphorylation and alkylation are probably not involved in their primary mode of action (10,11). Findings on the relation of structure to mammalian toxicity may be directly applicable to neuroreceptor potency since the BPs act quickly relative to their rate of detoxification (9,12). Optimal IP mouse toxicity requires a completely symmetrical cage (9), implying a channel- or pore-type receptor(s) (4,18). It also depends on the hydrophobicity and bulkiness of branching of the extra cyclic R substituent (Fig. 2), possibly due to steric interaction with the neuroreceptor which is hydrophobic in nature (4,9,10). The opposite end of the cage, bearing a high concentration of negative charge, is likely to be associated with a polar medium, i.e., protein (4).

TBPS and some other BPs are noncompetitive GABA antagonists of much higher potency than PTX but acting in the same way (14–16), almost certainly at the chloride channel. An attempt to use n-propylicbiclophosphosphate [mouse IP LD50 = 0.38 mg/kg (4)] as a [3H] radioligand with rat brain membranes was partially successful but the replacement potency of various BPs did not correlate with their toxicity (17). The most toxic BPs are TBPS and its phosphate and phosphate analogs (LD50 = 0.04 mg/kg (4)). TBPS is synthesized by addition of sulfur to the phosphate (4) and the use of [35S]S gives [35S]TBPS of high specific activity (> 60 Ci/mmmole) (NEG-049 of New England Nuclear). [35S]TBPS appears to be the best BP radioligand since it is easily synthesized at adequate specific activity and in binding assays it is 4-fold more potent than its oxygen analog (Fig. 3) (18). There are some differences but many similarieties in the binding characteristics of [35S]TBPS and [3H]DHP (18) and PTX competitively inhibits [35S]TBPS binding (19). In general, [35S]TBPS is preferred over [3H]DHP as a radioligand for the PTX site because of much greater affinity and signal-to-noise ratio (18). In addition, the relative potencies of six BPs in displacing [35S]TBPS from its binding site are correlated with their mouse IP toxicity (Fig. 3). It is therefore appropriate to detail optimal conditions for [35S]TBPS receptor preparation and assay and to consider interactions of other neuroactive agents with this site.

**[35S]TBPS Receptor Binding Assays**

**Preparation of Rat Brain Synaptic Membranes**

The P2 (mitochondrial) fraction from rat brain homogenate is an appropriate source of synaptic mem-
Table 1. Characteristics of [35S]TBPS binding to rat brain synaptic membranes prepared by two techniques.

| Characteristic | Fresh EDTA/water-dialyzed |
|---------------|---------------------------|
| Protein yield, mg/g brain wt | 13 ± 2 | 2.5-4.3 |
| [35S]TBPS specific binding parameters | | |
| Specific binding, % of totala | 84-89 | 85-90 |
| Bmax, pmole/mg protein | 1.7-2.5 | 5.8-7.3 |
| Kd, nM | 61-80 | 61-74 |
| IC50 of inhibitors, μMb | TBPS 0.078 | 0.062 |
| | PTX 0.76 | 0.34 |
| | α-Endosulfan 0.10 | 0.11 |
| | GABA ~10 | 0.88 |
| | Diazepam ~10 | ~10 |
| Effect of 10 nM R 51355 | Stimulation of binding Yes Yesa |
| | Reverses inhibition by: No Noa |
| | Noncompetitive inhibitors Yes Yesa |
| | Competitive inhibitors No Noa |
| Stable to freezing No Yes |

a Standard assays in 5 mM Na phosphate/200 mM NaCl (pH 7) buffer for fresh membranes (19) and in 5 mM Tris-HCl/1 mM EDTA/200 mM KBr (pH 7.5) buffer for dialyzed membranes (22), both at 37°C. Assays of the latter preparations at 25°C gave Kd ~20 nM and IC50 values of 0.017, 0.19, and 0.34 μM for TBPS, PTX, and GABA, respectively (18).
b Determined at 2 pmole [35S]TBPS assay.

Brines for [35S]TBPS binding assays. Two different general methods used for membrane preparation are detailed here. One utilizes fresh P2 membranes in Na phosphate/NaCl buffer (19-21) and the other involves treatment of the P2 membranes with ethylenediaminetetraacetic acid (EDTA) and extensive dialysis against distilled water (18, 22). The characteristics of the preparations are compared in Table 1.

**Fresh Membrane Preparations.** Whole brains (~1.7 g) from male albino rats (~175 g; sacrificed by decapitation) are quickly placed in 0.32 M sucrose at 4°C and then gently homogenized (six passes at ~2000 rpm) in 10 volumes of fresh sucrose solution using a 55-mL Teflon-glass tissue grinder. The supernatant from centrifugation (10 min, 1000g, 4°C) is carefully removed with a cold pipette and centrifuged a second time (20 min, 9000g) to sediment the P2 fraction. All of this supernatant is carefully removed as above and the P2 pellet is immediately resuspended in about five volumes of 5 mM Na phosphate/200 mM NaCl, pH 7.0, buffer (6) at 4°C with homogenization (four gentle passes) in a 10-mL tissue grinder with care to avoid aerating the suspension. The protein content is then quantitated (22) and adjusted to 2 mg/mL by addition of the same buffer. For homogeneity, the tube is inverted several times prior to the removal of aliquots. This fresh membrane preparation is held on ice and must be used within a few hours due to gradual loss of TBPS binding capacity; the activity is not retained on freezing and thawing.

**EDTA/Water-Dialyzed Membrane Preparations.** Rat brains are homogenized in 50 volumes of 1 mM EDTA at 4°C and the homogenate is centrifuged as above to obtain the P2 fraction which is resuspended in ice-cold 1 mM EDTA (50 times the original wet tissue weight). The P2 suspension is dialyzed three successive times (1-2 hr each) against 20 volumes of distilled water at 4°C and then centrifuged (30 min, 25,000g) to obtain a pellet that is either suspended in assay buffer for immediate use or frozen for storage at -80°C. The frozen pellets are resuspended as above but in 5 mM Tris-HCl/200 mM KBr (pH 7.5) buffer at 4°C, and the protein content is determined and adjusted to 0.5 mg/mL. The frozen pellets retain their TBPS binding capacity for several months but when thawed their assay suspension has similar instability to that of the fresh membrane preparation.

**Assay of [35S]TBPS Binding and Displacement by Direct Inhibitors**

The assay involves a simple, rapid and highly reproducible filtration technique (19, 22). A stock solution of radioiodinated [35S]TBPS (40 pmole in 1-5 μL ethanol) to 10 mL of 5 mM Na phosphate/200 mM NaCl (pH 7) buffer (for fresh membrane assay) or of 5 mM Tris-HCl/200 mM KBr (pH 7.5) buffer (for dialyzed membrane assay). An 0.5-mL aliquot containing 2 pmole [35S]TBPS is then added to a glass incubation vial (20-mL) followed by addition of dimethyl sulfoxide (DMSO) (5 μL; an amount which has no effect on [35S]TBPS binding) alone or containing a candidate inhibitor. After 2 or 3 min at 37°C for temperature equilibration, the assays involve addition of 0.5 mL cold membrane preparation (1 or 0.25 mg protein for fresh or EDTA/water-dialyzed preparation, respectively) and shaking at medium speed for 30 min at 37°C in a Dubnoff Metabolic Shaking Incubator (GCA Corporation, Chicago, IL). A variation of this procedure uses EDTA/water-dialyzed membranes in 5 mM Tris-HCl/200 mM KBr (pH 7.5) buffer and incubation in 2 mL total volume for 90-100 min at 25°C (18).

Bound [35S]TBPS is determined by quickly diluting each sample with 5 mL ice-cold buffer and filtration through prewetted glass microfiber filters (2.4 cm diameter, GF/C, Whatman Inc., Clifton, NJ) with a filtration manifold (VFM1, Amicon Corp., Danvers, MA) and an aspirator pump and regulator (K-7048, Cole-Parmer Instrument Co., Chicago, IL) operating at constant vacuum (63.5 cm Hg). The filters are rapidly rinsed (5-7 sec total time) with two additional 5-mL portions of ice-cold buffer, then transferred to glass scintillation vials containing 10 mL of 2.5-diphenyloxazole (0.55% w/v) in toluene—methylcellulose (2:1) mixture for direct liquid scintillation counting; it is not necessary to use a tissue solubilizer. Total and nonspecific [35S]TBPS binding are determined in the absence and presence of 2 n mole unlabeled TBPS, respectively, added initially in 5 μL DMSO. Specific binding is the difference in total and nonspecific binding.
Median inhibitory concentrations (IC\textsubscript{50} values) are taken directly from Hill plots (24), log (B\textsubscript{i}/B – 1) versus log [inhibitor], where B\textsubscript{i} and B are specific \[^{35}\text{S}]\text{TBPS}\) binding in the absence and in the presence, respectively, of a particular concentration of inhibitor. Confidence limits for IC\textsubscript{50} values are calculated by computer-assisted linear regression analysis of Hill plot data. TBPS binding parameters (K\textsubscript{d} and B\textsubscript{max}) are determined by Scatchard analysis (25) of binding at different ligand concentrations (generally between 2 and 250 nM). The \[^{35}\text{S}]\text{TBPS}\) concentration is varied by dilution with unlabeled TBPS.

**Coupled Microsomal Metabolism/ \[^{35}\text{S}]\text{TBPS\) Binding Assay**

Fortification of the standard TBPS binding assay with rat liver microsomes and reduced nicotinamide–adenine dinucleotide phosphate (NADPH) allows determination of the effect of microsomal metabolism on the potency of inhibitors in displacing \[^{35}\text{S}]\text{TBPS\) binding (20,21). The standard pre-incubation mixture consists of 2 pmole \[^{35}\text{S}]\text{TBPS\) in 0.2 mL 5 mM Na phosphate/200 mM NaCl (pH 7) buffer, 0.1 mL of the same buffer containing 0 or 2 \(\mu\) mole NADPH (control and oxidase systems, respectively), rat liver microsomes (0.1 mg protein) in 0.2 mL 50 mM Na phosphate (pH 7.4) buffer, and 5 \(\mu\)L DMSO with or without inhibitor. The assay is initiated by addition of 0.5 mL fresh membrane suspension and performed exactly as described above. Samples containing microsomes and NADPH have about 15% higher overall binding compared to microsomes without NADPH. The absolute increase in total and in nonspecific binding are equivalent; specific binding is not significantly altered by the oxidase system. Appropriate corrections for the NADPH-dependent increased nonspecific binding are made with suitable controls, i.e., total and nonspecific binding determined in both the presence and absence of added NADPH. The NADPH-enhanced nonspecific binding probably results from TBPS desulfuration yielding a protein-bound hydrodiisulfide (28) (Fig. 4). This is a fortuitous radioligand for this type of activation assay, since it undergoes specific binding in brain but not liver (18), and bicyclophosphorothionates are unusually resistant to microsomal oxidative desulfuration (9).

**Assay of in Vivo Inhibition of \[^{35}\text{S}]\text{TBPS\) Binding Site**

Rats as above are treated intravenously with DMSO (50 \(\mu\)L) alone (control) or containing a toxicant or drug. At an appropriate stage of poisoning or time after treatment, the rats are decapitated, their brains are rapidly excised, and fresh membrane preparations are assayed as previously described for specific \[^{35}\text{S}]\text{TBPS\) binding, comparing the treated and control animals for the level of specific binding. The fresh membrane preparation technique is ideally suited for this type of assay.

**Polychlorocycloalkane Insecticides**

Lindane, toxaphene and the chlorinated cyclodienes (see Figs. 5–7 for type structures) have a synaptic site of action in the central nervous system of mammals and cause hypersensitivity and tonic and clonic convulsions. These signs of poisoning are distinct from those observed for DDT (27,28).

**Hexachlorocyclohexanes and Polychlorobornanes**

Hexachlorocyclohexane insecticide (also known as benzene hexachloride or BHC) consists of four major isomers (\(\alpha\), \(\beta\), \(\gamma\), and \(\delta\)), of which only the \(\gamma\) isomer or

![FIGURE 4. Possible oxidative metabolism of \[^{35}\text{S}]\text{TBPS\) to generate \[^{35}\text{S}\) bound microsomal cytochrome P-450.](image)

![FIGURE 5. Synthesis and composition of toxaphene (30,31).](image)

![FIGURE 6. Relation of inhibitory potency at TBPS receptor to mammalian toxicity of hexachlorocyclohexanes and polychlorobornanes. \[^{35}\text{S}]\text{TBPS\) receptor binding data are based on fresh rat brain membranes and toxicity values on IP treated mice (20). The toxaphene components are assayed in mice pretreated with piperonyl butoxide (31).](image)
lindane is highly insecticidal; this isomer is responsible for the acute mammalian toxicity of the technical mixture (29). Lindane is also the only isomer effective in displacing \(^{35}\text{S}\)TBPS binding (20).

Toxaphene, from chlorination of camphene, is a complex mixture consisting mostly of isomeric polychlorobornanes (Fig. 5) of which heptachloroborne B, 8-Cl-B and particularly 9-Cl-B account for most of the toxicity to mice, goldfish and houseflies (30,31). These three hepta- and octachloroboranes also account for the potency of toxaphene as an inhibitor of \(^{35}\text{S}\)TBPS binding (20). This finding provides strong evidence for the toxicological relevance of the \(^{35}\text{S}\)TBPS binding assay since it recognizes B and 9-Cl-B as the most potent and toxic of at least 29 heptachloroboranes and 64 octachloroboranes, respectively.

The hexachlorocyclohexanes and polychloroboranes follow the same trend in correlating potency for \(^{35}\text{S}\)TBPS receptor inhibition with mouse IP toxicity (Fig. 6). The data used for the polychloroboranes are from mice pretreated with the synergist piperonyl butoxide. This cytochrome P-450 inhibitor increases the toxicity of B by 8-fold without changing the toxicity of the five other polychloroboranes or mixtures examined (31). Interestingly, B is also the only one of these materials detoxified in the coupled receptor/microsome system (Table 2) (20,21).

**Chlorinated Cyclodienes**

There are six major cyclodiene insecticides of current or historical interest, i.e., aldrin, chlordane, dieldrin, endosulfan, endrin (Fig. 7) and heptachlor. The structure–toxicity relationships, photochemistry and metabolic fate of the cyclodienes are well defined (27,32,33). Some of them undergo metabolism to form epoxides and photolysis to yield epoxides and bridged photoisomers, e.g., heptachlor is converted to heptachlor epoxide and each in turn undergoes a photobridging reaction (27) to photoheptachlor and photoheptachlor epoxide, respectively. Some of the parent cyclodienes and metabolites, e.g., isobenzan and 12-ketoendrin, are highly toxic convulsants. Heptachlor epoxide and lindane inhibit both GABA-induced chloride permeability in cockroach coxal muscle and \(^{3}H\)DH specific binding to rat brain synaptosomes (34,35).

The isomer specificity for inhibitor potency at the TBPS receptor is the same as that for acute mammalian toxicity. This finding is based on the following eight pairs of compounds (the least potent isomer is given first): aldrin vs. isodrin, dieldrin vs. endrin, \(\beta\)- vs. \(\alpha\)-endosulfan, trans- vs. cis-chlordane, photo-trans- vs. photo-cis-chlordane, heptachlor vs. photoheptachlor, heptachlor epoxide vs. photoheptachlor epoxide, and anti-12- vs. syn-12-hydroxyendrin. There is also general agreement between inhibitory potency and toxicity when the cyclodienes are subdivided on the basis of elemental composition, i.e., those containing carbon, hydrogen, and chlorine but no oxygen, and the chlorinated hydrocarbons with oxygen or with both oxygen and sulfur (Table 3); attempts at more precise corre-

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**Table 2. Effect of microsomal metabolism on inhibitory potency of polychlorocycloalkane insecticides at TBPS receptor of fresh rat brain membranes.**

| Compound        | IC\(_{50}\), \(\mu\)M | Presumed microsomal metabolism product | IC\(_{50}\), \(\mu\)M | Microsome/receptor assay, % inhibition with substrate\(^{b}\) |
|-----------------|---------------------|----------------------------------------|---------------------|-----------------------------------------------|
| Aldrin          | 8.7                 | Dieldrin                               | 1.4                 | 26 ± 3                                         |
| trans-Chlordane | 26                  | Oxychlordane                           | 0.98                | 26 ± 2                                         |
| Heptachlor      | 7.5                 | Heptachlor epoxide                     | 0.98                | 30 ± 9                                         |
| Isodrin         | 1.4                 | Endrin                                | 0.22                | 30 ± 2                                         |
| anti-12-Hydroxyendrin | 4.4         | 12-Ketoendrin                          | 0.036               | 22 ± 5                                         |
| \(\beta\)-Endosulfan | 1.5         | Endosulfan sulfate                     | 0.15                | 27 ± 4                                         |
| Heptachloroborne n | 0.17               | Oxidized                               | —                   | 40 ± 2                                         |
| Endosulfan sulfate | 0.15               | Hydrolyzed                             | —                   | 20 ± 4                                         |

\(^{a}\)Other compounds indicated in Fig. 6 and Table 3 are neither activated nor detoxified under these assay conditions except for toxaphene which undergoes marginal activation.

\(^{b}\)Assays without added NADPH (control) or in the presence of 2 \(\mu\)mole NADPH (oxidase). Means and standard errors based on six determinations in at least two experiments.
lations are not appropriate since the mammalian toxicity data used are literature values from a variety of studies and seven of the cyclodiene undergo metabolic activation or detoxification as described below (20).

Four cyclodiene lacking oxygen undergo in vivo epoxidation to products of enhanced potency in inhibiting the binding of [35S]TBPS (Table 2). The known metabolic conversions of anti-12-hydroxyendrin and β-endosulfan to 12-ketoendrin and endosulfan sulfate, respectively, are metabolic activation reactions relative to inhibitory potency at the [35S]TBPS receptor. These six cyclodiene, and only these six of 22 studied, are converted to more potent inhibitors (by a factor of 2- to 5-fold) in the microsome/NADPH system, verifying the importance of epoxidation in activating the first four compounds and oxidation for the latter two cyclodiene. Endosulfan sulfate, on the other hand, is detoxified in the coupled receptor/microsome system (Table 2).

Comparative data on in vitro inhibitory potency and in vivo toxicity help define which reactions in a metabolic pathway are critical bioactivation steps, as illustrated with isodrin and endrin in Figure 7. The toxicity data in themselves are insufficient to define whether or not the epoxidation of endrin to endrin is an activation step (27,36), but with the receptor data (20) this point becomes clear. Hydroxylation of endrin at the 12-position is either an activation or a detoxification step depending on whether the syn or anti metabolite is formed, respectively. Final oxidation of the anti-12-hydroxy compound is slow but a distinct activation step whereas oxidation of syn-12-hydroxyendrin provides only a small toxicity increase.

Dieldrin has sufficient affinity to remain at the specific site in mammalian brain during membrane preparation

and assay. Thus, specific TBPS binding is reduced in a dose-dependent manner for preparations from rats receiving acutely toxic intravenous doses of dieldrin (Table 4) (20,21).

Three other types of chlorinated hydrocarbon insecticides, DDT, mirex and toxaphene, act by different mechanisms than the cyclodiene, lindane and toxaphene and their IC50 values in the [35S]TBPS assay are > 10 μM alone or with the microsome-NADPH system.

Pyrethroid Insecticides

The pyrethroids include the most potent and most selective insecticides. Those of the highest potency are esters of α-cyano-3-phenoxycinnamyl alcohol, e.g., cypermethrin (Fig. 8), deltamethrin (the dibrom analog of

Table 3. Relation of inhibitory potency at TBPS receptor to mammalian toxicity of chlorinated cyclodiene. [35S]TBPS receptor binding data are based on fresh rat brain membranes and toxicity values primarily on orally treated rats with supplemental studies involving orally or IP-treated mice.

| Compounds | IC50, μM | LD50, mg/kg |
|-----------|---------|------------|
| Containing no oxygen | | |
| Chlordene, trans-chlordane | | |
| photo-trans-chlordane | | |
| Aldrin, cis-chlordane | | |
| heptachlor | | |
| Isoxazole, photo-cis-chlordane, photoheptachlor | | |
| Containing oxygen | | |
| Dieldrin, heptachlor epoxide, oxychloride, aldrin-trans-diol, anti-12-hydroxyendrin | | |
| Endrin, isobenzan, photoheptachlor epoxide, 12-ketoendrin, syn-12-hydroxyendrin | | |
| Containing oxygen and sulfur | | |
| β-Endosulfan | 1.5 | 240 |
| α-Endosulfan, endosulfan sulfate | 0.1-0.2 | 76 |

Table 4. TBPS receptor inhibition by intravenously administered dieldrin in rats.

| Dieldrin | Convulsion* | [35S]TBPS specific binding |
|---------|------------|--------------------------|
| mg/kg   | fmo/mg protein | % of control |
| 0       | None        | 53 ± 3 100 |
| 10      | Moderate    | 35 ± 6 67 |
| 25      | Severe      | 12 ± 2 22 |

*Rats sacrificed 7 min after administration of DMSO only or dieldrin in DMSO.

N = 5 for control and N = 3 for each dieldrin dose.

![Figure 8](image-url)
cypermethrin) and fenvalerate (Fig. 8). The cyano substituent not only increases the potency but apparently also changes the mode of action, referred to as type I for the early or noncyano compounds and type II for the cyano pyrethroids (37). The primary type II poisoning action appears to be in the central nervous system of mammals whereas the type I action has a greater peripheral component (37). Deltamethrin but not its nontoxic aR epimer inhibits the binding of [3H]DHP to rat brain synaptosomes (6). These findings suggest that, in contrast to the early pyrethroids which disrupt sodium channels, the α-cyano pyrethroids may act in part within the GABA receptor-ionophore complex.

Studies with 37 pyrethroids, 16 giving the type I and 21 the type II poisoning syndrome, establish an absolute agreement, i.e., no false positives or negatives, between the potency in inhibiting [35S]TBPS binding and the mouse intracerebral toxicity (19). Three relationships are of particular interest. First, all toxic cyano compounds but none of their nontoxic stereoisomers are inhibitors of [35S]TBPS binding (Fig. 8). Second, the cis-cyclopropanecarboxylates are more potent than the trans-cyclopropanecarboxylates as both toxicants and inhibitors, establishing that this specificity occurs at least in part at the receptor level (Fig. 8). Third, all noncyano pyrethroids are much less potent or inactive, indicating some differences in their mode of action, in agreement with their symptomology. The types I and II actions have the same stereochemical requirements for the acid moieties (Fig. 8), yet different target sites appear to be involved at least in the mammalian brain, suggesting that the relevant types I and II receptors have some common topographic features.

### Other Neuroactive Compounds

GABA and several GABA-mimetics inhibit TBPS binding with muscimol being the most potent (18) (Table 5). The interaction of GABA-mimetics with the TBPS receptor is fundamentally different from that of the cage convulsants since they facilitate rather than inhibit GA-

**Table 5. Inhibitory potency of GABA-mimetics, t-butylbicycloorthocarboxylates and other neuroactive compounds at TBPS receptor of rat brain EDTA/water-dialyzed membranes.***

| Compound                                      | IC50 μM |
|-----------------------------------------------|---------|
| GABA-mimetics                                 |         |
| Muscimol                                      | 0.059   |
| 3-Aminopropanesulfonic acid                   | 0.24    |
| Dihydmuscimol                                 | 0.26    |
| GABA                                          | 0.34    |
| trans-4-Aminocrotonic acid                    | 0.73    |
| Isoguvacine                                   | 0.95    |
| t-Butylbicycloorthocarboxylates, (CH3)3CC(CH20)3CR |         |
| R = phenyl                                    | 0.035   |
| R = butyl                                     | 0.059   |
| R = hydrogen                                  | 8.5     |
| Other convulsants                             |         |
| Anisatin                                      | 0.074   |
| PTX                                           | 0.19    |
| TETS                                          | 0.82    |
| Pyrazolopyridines, barbiturates and benzodiazepines |         |
| Cartazolate                                   | 0.19    |
| Etazolate                                     | 0.48    |
| G (+-)Etomidate                               | 2.8     |
| Ro 5-3636 and 5-4566                          | ~10     |

*The IC50 for ethanol is 212 mM.

**[35S]TBPS as Neuropharmacological Probe for the GABA Receptor–Ionophore Complex**

Compounds from every class of known GABAergic mechanisms inhibit or enhance the binding of [35S]TBPS, making this radioligand an excellent probe for studies on mode of action and structure–activity relationships, and more generally the interactions between the various components of the GABA receptor–ionophore complex. Specific binding of [35S]TBPS or [3H]n-propylbicyclophosphophate is enhanced by the GABA receptor antagonists bicuculline and R 5135 (17,21) and by the convulsant benzodiazepine Ro 5-4864 (21) which apparently interacts with a novel class of BDZ receptors (40). The remaining compounds, which inhibit TBPS binding with IC50 values ranging from ~20 nM to >10 μM, can be divided into two classes—those which are noncompetitive or indirect inhibitors and those which are competitive or direct inhibitors. The nature of the inhibition

**Table 6. Properties of various inhibitors in displacement of specific [35S]TBPS binding to fresh rat brain membranes.***

| Inhibitor                  | Type of inhibition       | R 5135 reversal |
|----------------------------|-------------------------|----------------|
| GABA                       | Noncompetitive          | Complete       |
| [1R,cis,αS]-Cypermethrin   | Noncompetitive or mixed | Partial        |
| PTX                        | Competitive              | None           |
| Lindane                    | Competitive              | None           |
| 8-Cl-B                     | Competitive              | None           |
| 12-Ketoendrin              | Competitive              | None           |

*Table 4. Inhibitory potency of GABA-mimetics, t-butylbicycloorthocarboxylates and other neuroactive compounds at TBPS receptor of rat brain EDTA/water-dialyzed membranes.***

| Compound                                      | IC50 μM |
|-----------------------------------------------|---------|
| GABA-mimetics                                 |         |
| Muscimol                                      | 0.059   |
| 3-Aminopropanesulfonic acid                   | 0.24    |
| Dihydmuscimol                                 | 0.26    |
| GABA                                          | 0.34    |
| trans-4-Aminocrotonic acid                    | 0.73    |
| Isoguvacine                                   | 0.95    |
| t-Butylbicycloorthocarboxylates, (CH3)3CC(CH20)3CR |         |
| R = phenyl                                    | 0.035   |
| R = butyl                                     | 0.059   |
| R = hydrogen                                  | 8.5     |
| Other convulsants                             |         |
| Anisatin                                      | 0.074   |
| PTX                                           | 0.19    |
| TETS                                          | 0.82    |
| Pyrazolopyridines, barbiturates and benzodiazepines |         |
| Cartazolate                                   | 0.19    |
| Etazolate                                     | 0.48    |
| G (+-)Etomidate                               | 2.8     |
| Ro 5-3636 and 5-4566                          | ~10     |

*The IC50 for ethanol is 212 mM.
is defined by Scatchard analyses with supplemental information based on the reversibility of the inhibition by R 5135 (Table 6).

Noncompetitive or Indirect Inhibitors

GABA shows purely noncompetitive behavior upon Scatchard analysis (21), and the inhibition of TBPS binding by GABA and GABA-mimetics is completely reversed by 10 nM R 5135 (18). It therefore appears that GABA and GABA-mimetics inhibit TBPS binding through an opening (stimulating) the chloride channel and maintaining it in a configuration which distorts the TBPS binding site. This is consistent with the R 5135 effect whereby its displacement of GABA (41) returns the chloride channel to its closed (unstimulated) state, thus restoring the TBPS binding site.

Most BDZs and barbiturates enhance GABAergic synaptic transmission per se (42), and their inhibition of TBPS binding is reversed by R 5135 (18). These compounds appear to be noncompetitive inhibitors acting in a manner similar to GABA-mimetics, but at a distinct binding site, e.g., the BDZ receptors for the BDZs.

The cyanophenoxybenzyl pyrethroids inhibit [35S]TBPS binding maximally by 60 to 70% (Fig. 9) (19,21) and appear to be indirect inhibitors, since they give noncompetitive or mixed-type Scatchard plots (Fig. 10) and only partial reversal of the inhibition by R 5135 (Table 6) (19). Unlike GABA, however, these compounds block rather than enhance GABA-stimulated chloride permeability (16), making it likely that their inhibition of TBPS binding is through an interaction with a closely associated yet distinct binding site, possibly involving an allosteric mechanism.

Competitive or Direct Inhibitors

PTX, lindane, a mixture of 8- and 9-Cl-B and 12-ketoendrin give 100% maximum inhibition of [35S]TBPS binding (Fig. 9) (20,21). Scatchard analyses indicate that heptachlor epoxide (Fig. 10) (21) and lindane, 8-Cl-B and 12-ketoendrin (Table 6) (20) are competitive inhibitors based on increasing the $K_a$ without changing the $B_{max}$ for [35S]TBPS binding (20,21). Inhibition by these polychlorocycloalkanes is not reversed by R 5135 (Table 6) (20,21). PTX, lindane and heptachlor epoxide inhibit chloride permeability (1,35), so their actions probably result from direct interaction with the chloride ionophore-associated TBPS receptor (20,21) or with an allosteric hydrophobic regulatory site (7).

Many cage convulsants with defined three-dimensional structures are potent inhibitors of TBPS binding, e.g., the PTX analogs, bicycloposphorus and bicycloothcarboxylic acid esters, and polychlorocycloalkane insecticides. These compounds are excellent probes for characterization of the properties and topography of the TBPS binding site and its relationship to the chloride ionophore.

Health and Environmental Relevance

The [35S]TBPS receptor assay has helped to elucidate or verify the mode of action of several natural products and xenobiotics including a variety of toxicants and important insecticides. Ethylbicyclophtate is the toxic principle (43) in the smoke produced on burning non-commercial fire-retarded polyurethane foams based on

Figure 9. Displacement of specific [35S]TBPS binding to rat brain membranes by picROTOXIN and a representative compound from each of four classes of insecticidal inhibitors (20,21). Structures for the inhibitors are given in Figs. 1 and 6-8.

Figure 10. Scatchard plots of specific [35S]TBPS binding to rat brain membranes in the presence of (A) 0.5 μM Picrotoxin, (B) 1 μM heptachlor epoxide, and (C) 5 μM (1R,cis αS)-cypermethrin (19–21). B and F are bound and free [35S]TBPS. $K_a$ is given as nM and $B_{max}$ as pmole/mg protein.
a trimethylolpropane-initiated short-chain polyol and a phosphate flame retardant (44). Almost three billion pounds of hexachlorocyclohexanes, polychlorobornanes, and chlorinated cyclodienes have been applied to crops and soils over the past 40 years (20, 27). The cyanophenylbenzyl pyrethroids are the major new insecticides of the past decade and are much more potent than earlier analogs (45). Our initial investigation on bicyclophephorus compounds (43) served as a basis for new ways to examine polychlorocycloalkanes, α-cyano pyrethroids, and other diverse groups of neuroactive agents of health and environmental relevance. "When we try to pick out anything by itself, we find it hitches to everything else in the universe" (46).

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