Environmental Health Perspectives
Vol. 61, pp. 123-132, 1985

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

by John E. Casida* and Lowell J. Lawrence*†

[^{35}S]*t*-Butylbicyclophosphorothionate or [^{35}S]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 [^{35}S]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 α-cyano-3-phenoxybenzyl pyrethroids, although less potent, inhibit [^{35}S]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 γ-aminobutyric acid (GABA) receptor–ionophore complex whereas the α-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 γ-aminobutyric acid (GABA) receptor–ionophore complex and that this coupled system and its inhibitors are conveniently examined with a simple radioligand assay using [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) and rat brain synaptic membranes.

This review considers the development and properties of [^{35}S]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 *Anamirta cocculus* L. It inhibits GABA-stimulated chloride permeability by acting as a noncompetitive GABA antagonist (*1–3*). Structure–toxicity relationships for PTX [mouse intraperitoneal (IP) LD_{50} = 9.0 mg/kg (*4*)] and its analogs have been used to define critical features for convulsant action (*5*) and to char-

*Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, CA 94720.
†Current address: Pharmaceutical Research Laboratory, Lexington, KY 40509.

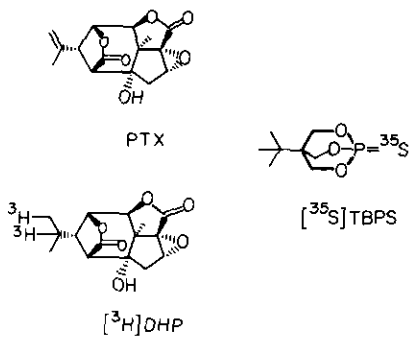

FIGURE 1. Structures of picrotoxinin, [^{3}H]dihydropicrotoxinin, and [^{35}S]*t*-butylbicyclophosphorothionate.

acterize the topography of the chloride channel (*3*). The PTX receptor has been extensively studied with the radioligand analog α-[8,10-^{3}H]dihydropicrotoxinin ([^{3}H]DHP) (Fig. 1) (*6–8*) (NET-606 of New England Nuclear Corp., Boston, MA). [^{3}H]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 [^{3}H]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) (*4,13*). It also depends on the hydrophobicity and bulkiness or branching of the extracyclic 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*-propylbicyclophosphate [mouse IP LD_{50} = 0.38 mg/kg (*4*)] as a ^{3}H radioligand with rat brain membranes was partially successful but the displacement potency of various BPs did not correlate with their toxicity (*17*). The most toxic BPs are TBPS and its phosphite and phosphate analogs (LD_{50} ≈ 0.04 mg/kg) (*4*). TBPS is synthesized by addition of sulfur to the phosphite (*4*) and the use of ^{35}S gives [^{35}S]TBPS of high specific activity (> 60 Ci/mmole) (NEG-049 of New England Nuclear). [^{35}S]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 similarities in the binding characteristics of [^{35}S]TBPS and [^{3}H]DHP (*18*) and PTX competitively inhibits [^{35}S]TBPS binding (*19*). In general, [^{35}S]TBPS is preferred over [^{3}H]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 [^{35}S]TBPS from its binding site are correlated with their mouse IP toxicity (Fig. 3). It is therefore appropriate to detail optimal conditions for [^{35}S]TBPS receptor preparation and assay and to consider interactions of other neuroactive agents with this site.

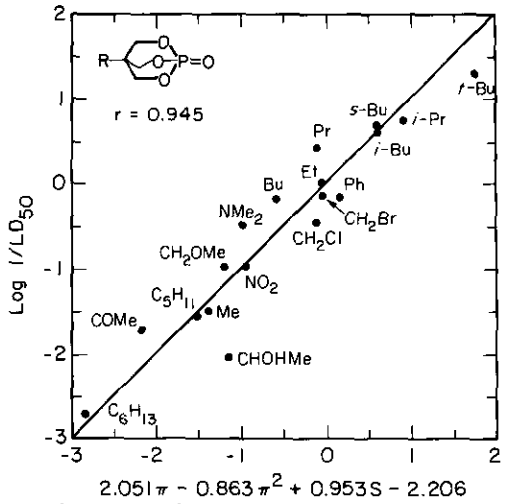

FIGURE 2. Relation of hydrophobicity and steric factors of the R-substituent to mammalian toxicity of bicyclophosphates. π is the hydrophobicity parameter and S the number of substituents at the α- or β-atom of the exocyclic R-substituent (*10*).

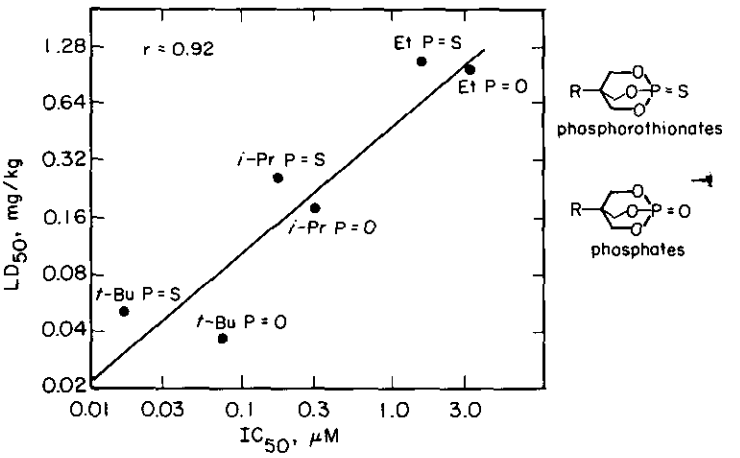

FIGURE 3. Relation of inhibitory potency at TBPS receptor to mammalian toxicity of bicyclophosphorus esters. [^{35}S]TBPS receptor binding data are based on rat brain EDTA/water-dialyzed membranes (*18*) and toxicity values on IP treated mice (*4*).

[^{35}S]TBPS Receptor Binding Assays

Preparation of Rat Brain Synaptic Membranes

The P_2 (mitochondrial) fraction from rat brain homogenate is an appropriate source of synaptic mem-

Table 1. Characteristics of [^{35}S]TBPS binding to rat brain synaptic membranes prepared by two techniques.

Characteristic	Type of preparation	
	Fresh	EDTA/water-dialyzed
Protein yield, mg/g brain wt	13 ± 2	2.5–4.3
[^{35}S]TBPS specific binding parameters[a]		
Specific binding, % of total[b]	84–89	85–90
B_{max}, pmole/mg protein	1.7–2.5	5.6–7.3
K_d, nM	61–80	61–74
IC_{50} of inhibitors, μM[b]		
TBPS	0.078	0.062
PTX	0.76	0.34
α-Endosulfan	0.10	0.11
GABA	~ 10	0.89
Diazepam	~10	~10
Effect of 10 nM R 5135[b]		
Stimulation of binding	Yes	Yes[c]
Reverses inhibition by:		
Noncompetitive inhibitors	Yes	Yes[c]
Competitive inhibitors	No	No[c]
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 K_d ~20 nM and IC_{50} values of 0.017, 0.19, and 0.34 μM for TBPS, PTX, and GABA, respectively (*18*).

[b] Determined at 2 pmole [^{35}S]TBPS assay.

[c] See Squires et al. (*18*).

branes for [^{35}S]TBPS binding assays. Two of several general methods used for membrane preparation are detailed here. One utilizes fresh P_2 membranes in Na phosphate/NaCl buffer (*19–21*) and the other involves treatment of the P_2 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, 1000*g*, 4°C) is carefully removed with a cold pipette and centrifuged a second time (20 min, 9000*g*) to sediment the P_2 fraction. All of this supernatant is carefully removed as above and the P_2 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 (*23*) 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 P_2 fraction which is resuspended in ice-cold 1 mM EDTA (50 times the original wet tissue weight). The P_2 suspension is dialyzed three successive times (1–2 hr each) against 20 volumes of distilled water at 4°C and then centrifuged (30 min, 25,000*g*) 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 [^{35}S]TBPS Binding and Displacement by Direct Inhibitors

The assay involves a simple, rapid and highly reproducible filtration technique (*19,22*). A stock solution of radioligand is freshly prepared for each experiment by adding [^{35}S]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 [^{35}S]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 [^{35}S]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 [^{35}S]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–methylcellosolve (2:1) mixture for direct liquid scintillation counting; it is not necessary to use a tissue solubilizer. Total and nonspecific [^{35}S]TBPS binding are determined in the absence and presence of 2 nmole unlabeled TBPS, respectively, added initially in 5 μL DMSO. Specific binding is the difference in total and nonspecific binding.

Median inhibitory concentrations (IC_{50} values) are taken directly from Hill plots (*24*), log ($B_0/B_i - 1$) versus log [inhibitor], where B_0 and B_i are specific [^{35}S]TBPS binding in the absence and in the presence, respectively, of a particular concentration of inhibitor. Confidence limits for IC_{50} values are calculated by computer-assisted linear regression analysis of Hill plot data. TBPS binding parameters (K_d and B_{max}) are determined by Scatchard analysis (*25*) of binding at different ligand concentrations (generally between 2 and 250 nM). The [^{35}S]TBPS concentration is varied by dilution with unlabeled TBPS.

Coupled Microsomal Metabolism/ [^{35}S]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}S]TBPS binding (*20,21*). The standard pre-incubation mixture consists of 2 pmole [^{35}S]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 μmole 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 μL 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 hydrodisulfide (*26*) (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}S]TBPS Binding Site

Rats as above are treated intravenously with DMSO (50 μL) 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}S]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 (α, β, γ, and δ), of which only the γ isomer or

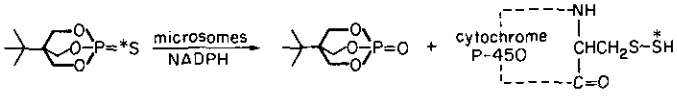

FIGURE 4. Possible oxidative metabolism of [^{35}S]TBPS to generate ^{35}S-bound microsomal cytochrome P-450.

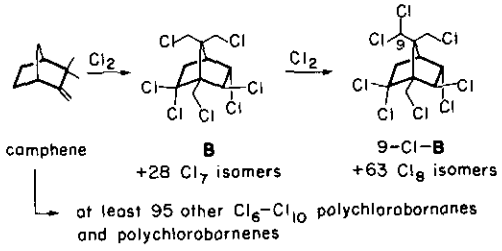

FIGURE 5. Synthesis and composition of toxaphene (*30,31*).

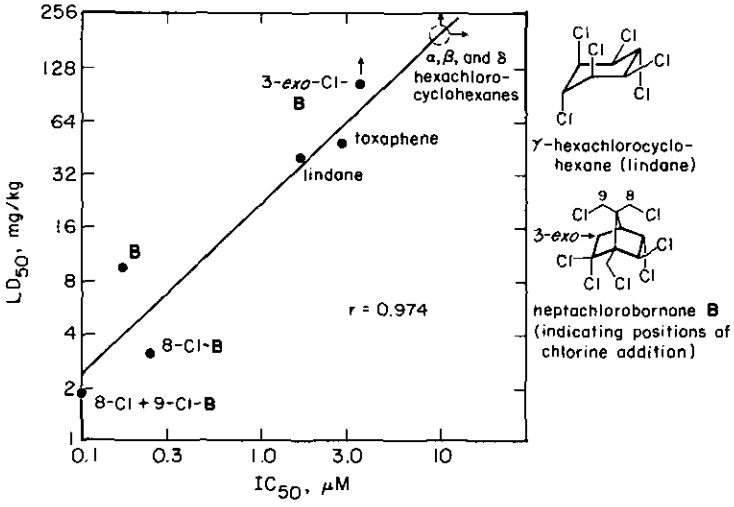

FIGURE 6. Relation of inhibitory potency at TBPS receptor to mammalian toxicity of hexachlorocyclohexanes and polychlorobornanes. [^{35}S]TBPS receptor binding data are based on fresh rat brain membranes and toxicity values on IP treated mice (*20*). The toxaphene components are assayed in mice pretreated with piperonyl butoxide (*31*).

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}S]TBPS binding (*20*).

Toxaphene, from chlorination of camphene, is a complex mixture consisting mostly of isomeric polychlorobornanes (Fig. 5) of which heptachlorobornane **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 octachlorobornanes also account for the potency of toxaphene as an inhibitor of [^{35}S]TBPS binding (*20*). This finding provides strong evidence for the toxicological relevance of the [^{35}S]TBPS binding assay since it recognizes **B** and 9-Cl-**B** as the most potent and toxic of at least 29 heptachlorobornanes and 64 octachlorobornanes, respectively.

The hexachlorocyclohexanes and polychlorobornanes follow the same trend in correlating potency for [^{35}S]TBPS receptor inhibition with mouse IP toxicity (Fig. 6). The data used for the polychlorobornanes are

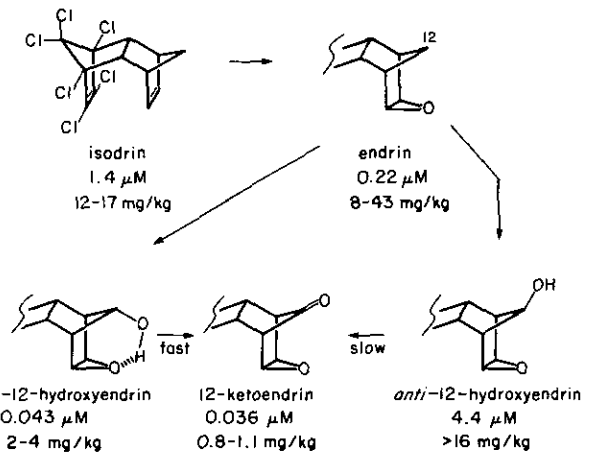

FIGURE 7. Oxidative bioactivation of isodrin and endrin. [^{35}S]TBPS receptor binding data (IC_{50}, μM) are based on fresh rat brain membranes and toxicity values (LD_{50}, mg/kg) on orally treated rats (*20,36*).

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 polychlorobornanes 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]DHP 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, β- vs. α-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-

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

Polychlorocycloalkane[a]		Presumed microsomal metabolism product		Microsome/receptor assay, % inhibition with substrate[b]	
Compound	IC_{50}, μM	Compound	IC_{50}, μM	Control	Oxidase
		Activation			
Aldrin	8.7	Dieldrin	1.4	26 ± 3	53 ± 4
trans-Chlordane	26	Oxychlordane	0.93	26 ± 2	46 ± 4
Heptachlor	7.5	Heptachlor epoxide	0.98	30 ± 9	52 ± 12
Isodrin	1.4	Endrin	0.22	30 ± 2	69 ± 2
anti-12-Hydroxyendrin	4.4	12-Ketoendrin	0.036	22 ± 5	31 ± 6
β-Endosulfan	1.5	Endosulfan sulfate	0.15	27 ± 4	64 ± 5
		Detoxification			
Heptachlorobornane B	0.17	Oxidized	—	40 ± 2	9 ± 5
Endosulfan sulfate	0.15	Hydrolyzed	—	20 ± 4	< 5

[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 μmole 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 cyclodienes undergo metabolic activation or detoxification as described below (*20*).

Four cyclodienes lacking oxygen undergo *in vivo* epoxidation to products of enhanced potency in inhibiting the binding of [^{35}S]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 [^{35}S]TBPS receptor. These six cyclodienes, 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 cyclodienes. 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 isodrin 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 kepone, act by different mechanisms than the cyclodienes, lindane and toxaphene and their IC_{50} values in the [^{35}S]TBPS assay are > 10 μM alone or with the microsome-NADPH system.

Table 3. Relation of inhibitory potency at TBPS receptor to mammalian toxicity of chlorinated cyclodienes. [^{35}S]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	IC_{50}, μM	LD_{50}, mg/kg
Containing no oxygen		
Chlordene, *trans*-chlordane, photo-*trans*-chlordane	>10	>500
Aldrin, *cis*-chlordane, heptaclor	2–9	38–600
Isodrin, photo-*cis*-chlordane, photoheptachlor	0.2–1.4	12–20
Containing oxygen		
Dieldrin, heptachlor epoxide, oxychlordane, aldrin-*trans*-diol, *anti*-12-hydroxyendrin	1–>10	>16–1250
Endrin, isobenzan, photoheptachlor epoxide, 12-ketoendrin, *syn*-12-hydroxyendrin	0.04–0.3	1–≈20
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, mg/kg	Convulsions[a]	[^{35}S]TBPS specific binding: fmole/mg protein[b]	[^{35}S]TBPS specific binding: % of control
0	None	53 ± 3	100
10	Moderate	35 ± 6	67
25	Severe	12 ± 2	22

[a]Rats sacrificed 7 min after administration of DMSO only or dieldrin in DMSO.
[b]N = 5 for control and N = 3 for each dieldrin dose.

Pyrethroid Insecticides

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

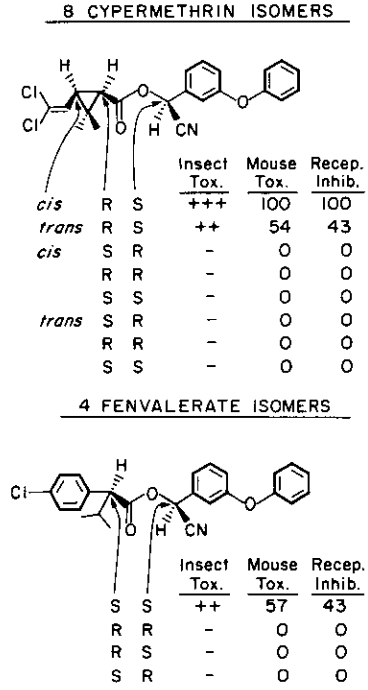

FIGURE 8. Relation of inhibitory potency at TBPS receptor to mammalian toxicity of α-cyano-3-phenoxybenzyl pyrethroids. [^{35}S]TBPS receptor binding data are based on fresh rat brain membranes and toxicity values on intracerebrally treated mice. The potency of [1R,*cis*,αS]cypermethrin is normalized to 100 (*19*). Structures of the most potent isomers are shown. Other tabulated isomers involve inversion of configuration at the carbon atoms designated by arrows.

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*). Diazepam is more effective in delaying the type II than the type I poisoning signs (*38,39*). There are some similarities between the cyano pyrethroids and PTX in their poisoning signs and diazepam effects in cockroaches and intracerebrally treated mice but not in frogs (*37–39*). Deltamethrin but not its nontoxic αR epimer inhibits the binding of [^{3}H]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 [^{35}S]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 [^{35}S]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.

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

Compound[a]	IC_{50}, μM
GABA-mimetics	
Muscimol	0.059
3-Aminopropanesulfonic acid	0.24
Dihydromuscimol	0.26
GABA	0.34
trans-4-Aminocrotonic acid	0.73
Isoguvacine	0.93
t-Butylbicycloorthocarboxylates, $(CH_3)_3CC(CH_2O)_3CR$	
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
(+)-Etomidate	2.8
Ro 5-3636 and 5-4556	~10

[a]The IC_{50} for ethanol is 212 mM.

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 GABAergic transmission.

Other convulsants displacing specific [^{35}S]TBPS binding are as follows: *t*-butylbicycloorthocarboxylates (*18*) (Table 5), a series which has the same optimal 4-substituents as in the phosphate series (*4*); the candidate rodenticides tetramethylenedisulfotetramine (TETS) and *p*-chlorophenylsilatrane (*18,21,22*); the insecticidal natural products pipercide (an isobutylamide) and Ivermectin (an avermectin) (*21,22*); the polycyclic bislactone anisatin from seeds of *Illicium anisatum* L. (*18,22*).

Some pyrazolopyridines, barbiturates, benzodiazepines (BDZs), and ethanol also inhibit TBPS binding (*18*) (Table 5) but are generally less potent than the GABA-mimetics and convulsants. The *in vitro* potencies of the BDZs in displacing TBPS are generally not directly related to their *in vivo* pharmacological effects.

[^{35}S]TBPS as Neuropharmacological Probe for the GABA Receptor–Ionophore Complex

Compounds from every class of known GABAergic mechanisms inhibit or enhance the binding of [^{35}S]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 [^{35}S]TBPS or [^{3}H]*n*-propylbicyclophosphate 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 IC_{50} 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 [^{35}S]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-C1-B	Competitive	None
12-Ketoendrin	Competitive	None

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 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 [^{35}S]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.

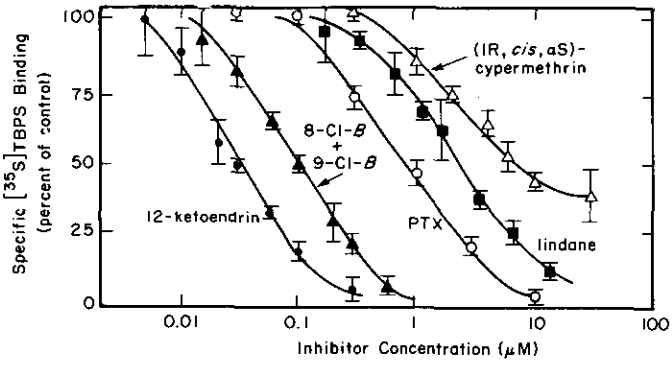

FIGURE 9. Displacement of specific [^{35}S]TBPS binding to rat brain membranes by picrotoxinin and a representative compound from each of four classes of insecticidal inhibitors (*20,21*). Structures for the inhibitors are given in Figs. 1 and 6–8.

Competitive or Direct Inhibitors

PTX, lindane, a mixture of 8- and 9-Cl-**B** and 12-ketoendrin give 100% maximum inhibition of [^{35}S]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_d without changing the B_{max} for [^{35}S]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, bicyclophosphorus and bicycloorthocarboxylic 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 [^{35}S]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. Ethylbicyclophosphate is the toxic principle (*43*) in the smoke produced on burning noncommercial fire-retarded polyurethane foams based on

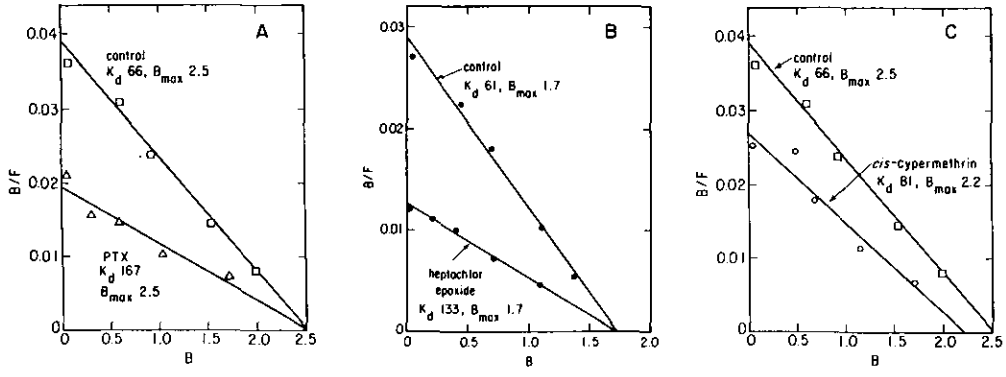

FIGURE 10. Scatchard plots of specific [^{35}S]TBPS binding to rat brain membranes in the presence of (*A*) 0.5 μM picrotoxinin, (*B*) 1 μM heptachlor epoxide, and (*C*) 5 μM (1R,*cis* αS)-cypermethrin (*19–21*). B and F are bound and free [^{35}S]TBPS. K_d is given as nM and B_{max} as pmole/mg protein.

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 cyanophenoxybenzyl pyrethroids are the major new insecticides of the past decade and are much more potent than earlier analogs (*45*). Our initial investigation on bicyclophosphorus 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 hitched to everything else in the universe" (*46*).

Portions of this research were supported by National Institute of Environmental Health Sciences Grant 5P01 ES00049. The authors thank Loretta Cole, Josef Seifert and Ephraim Cohen of this laboratory for helpful discussions. Presented in part by L.J.L. on August 30, 1983 at the 186th American Chemical Society National Meeting, Division of Pesticide Chemistry (Abstract 24), Washington, DC, and by J.E.C. on November 7, 1983 at the Fourth Annual Meeting of the Society of Environmental Toxicology and Chemistry, Arlington, VA.

REFERENCES

1. Takeuchi, A., and Takeuchi, N. A study of the action of picrotoxin on the inhibitory neuromuscular junction of the crayfish. J. Physiol. 205: 377–391 (1969).
2. Woodbury, D. M. Convulsant drugs: mechanisms of action. In: Antiepileptic Drugs: Mechanisms of Action (G. H. Glaser, J. K. Penry and D. M. Woodbury, Eds.), Raven Press, New York, 1980, pp. 249–304.
3. Klunk, W. E., Kalman, B. L., Ferrendelli, J. A., and Covey, D. F. Computer-assisted modeling of the picrotoxinin and γ-butyrolactone receptor site. Mol. Pharmacol. 23: 511–518 (1983).
4. Milbrath, D. S., Engel, J. L., Verkade, J. G., and Casida, J. E. Structure-toxicity relationships of 1-substituted-4-alkyl-2,6,7-trioxabicyclo[2.2.2]octanes. Toxicol. Appl. Pharmacol. 47: 287–293 (1979).
5. Jarboe, C. H., Porter, L. A., and Buckler, R. T. Structural aspects of picrotoxinin action. J. Med. Chem. 11: 729–731 (1968).
6. Leeb-Lundberg, F., and Olsen, R. W. Picrotoxinin binding as a probe of the GABA postsynaptic membrane receptor-ionophore complex. In: Psychopharmacology and Biochemistry of Neurotransmitter Receptors (H. I. Yamamura, R. W. Olsen and E. Usdin, Eds.), Elsevier, New York, 1980, pp. 593–606.
7. Ticku, M. K., and Maksay, G. Convulsant/depressant site of action at the allosteric benzodiazepine-GABA receptor-ionophore complex. Life Sci. 33: 2363–2375 (1983).
8. Ticku, M. K., and Olsen, R. W. Cage convulsants inhibit picrotoxinin binding. Neuropharmacology 18: 315–318 (1976).
9. Casida, J. E., Eto, M., Moscioni, A. D., Engel, J. L., Milbrath, D. S., and Verkade, J. G. Structure-toxicity relationships of 2,6,7-trioxabicyclo[2.2.2]octanes and related compounds. Toxicol. Appl. Pharmacol. 36: 261–279 (1976).
10. Eto, M., Ozoe, Y., Fujita, T., and Casida, J. E. Significance of branched bridge-head substituent in toxicity of bicyclic phosphate esters. Agric. Biol. Chem. 40: 2113–2115 (1976).
11. Ozoe, Y., Mochida, K., and Eto, M. Reaction of toxic bicyclic phosphates with acetylcholinesterases and α-chymotrypsin. Agric. Biol. Chem. 46: 2527–2531 (1982).
12. Milbrath, D. S., Eto, M., and Casida, J. E. Distribution and metabolic fate in mammals of the potent convulsant and GABA antagonist *t*-butyl-bicyclophosphate and its methyl analog. Toxicol. Appl. Pharmacol. 46: 411–420 (1978).
13. Ozoe, Y., Mochida, K., and Eto, M. Bicyclic phosphate binding to cyclodextrin: a receptor model. Agric. Biol. Chem. 45: 2623–2625 (1981).
14. Bowery, N. G., Collins, J. F., and Hill, R. G. Bicyclic phosphorus esters that are potent convulsants and GABA antagonists. Nature 261: 601–603 (1976).
15. Bowery, N. G., Collins, J. F., Hill, R. G., and Pearson, S. *t*-Butyl bicyclophosphate: a convulsant and GABA antagonist more potent than bicuculline. Brit. J. Pharmacol. 60: 275P–276P (1977).
16. Gammon, D., and Casida, J. E. Pyrethroids of the most potent class antagonize GABA action at the crayfish neuromuscular junction. Neurosci. Letters 40: 163–168 (1983).
17. Ozoe, Y., Mochida, K., and Eto, M. Binding of toxic bicyclic phosphates to rat brain synaptic membrane fractions. Agric. Biol. Chem. 46: 2521–2526 (1982).
18. Squires, R. F., Casida, J. E., Richardson, M., and Saederup, E. [^{35}S]*t*-Butyl-bicyclophosphorothionate binds with high affinity to brain-specific sites coupled to γ-aminobutyric acid-A and ion-recognition sites. Mol. Pharmacol. 23: 326–336 (1983).
19. Lawrence, L. J., and Casida, J. E. Stereospecific action of pyrethroid insecticides on the γ-aminobutyric acid receptor–ionophore complex. Science 221: 1399–1401 (1983).
20. Lawrence, L. J., and Casida, J. E. Interactions of lindane, toxaphene and cyclodienes with brain-specific *t*-butylbicyclophosphorothionate receptor. Life Sci. 35: 171–178 (1984).
21. Lawrence, L. J. Toxicology of Pyrethroid and Chlorinated Hydrocarbon Insecticides: Stereospecific Interactions with Brain Specific *t*-Butylbicyclophosphorothionate Receptor. Ph.D. dissertation. Univ. Calif. Berkeley, 1983, 102 pp.
22. Cole, L. M., Lawrence, L. J., and Casida, J. E. Similar properties of [^{35}S]*t*-butylbicyclophosphorothionate receptor and coupled components of the GABA-receptor-ionophore complex in brains of human, cow, rat, chicken and fish. Life Sci. 35: 1755– 1762 (1984).
23. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254 (1976).
24. Hill, A. V. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. J. Physiol. 40: iv–vii (1910).
25. Scatchard, G. The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51: 660–672 (1949).
26. Neal, R. A. Chemical mechanisms of the cytochrome P-450 monooxygenase-catalyzed metabolism of phosphorothionate triesters. In: Sulfur in Pesticide Action and Metabolism (ACS Symposium Series, No. 158), American Chemical Society, Las Vegas, Nevada, 1981, pp. 19–34.
27. Brooks, G. T. Chlorinated Insecticides. CRC Press Inc., Cleveland, Ohio, 2 Vol., 1974.
28. Joy, R. M. Mode of action of lindane, dieldrin and related insecticides in the central nervous system. Neurobehav. Toxicol. Teratol. 4: 813–823 (1982).
29. Negherbon, W. O. Handbook of Toxicology, Vol. 3. W. B. Saunders Co., Philadelphia, 1959.
30. Saleh, M. A. and Casida, J. E. Toxaphene composition, structure-toxicity relations and metabolism. In: Advances in Pesticide Science (H. Geissbühler, Ed.), Pergamon, New York, 1979, pp. 562–566.
31. Turner, W. V., Engel, J. L., and Casida, J. E. Toxaphene components and related compounds: preparation and toxicity of some hepta-, octa-, and nonachlorobornanes, hexa- and heptachlorobornenes, and a hexachlorobornadiene. J. Agr. Food Chem. 25: 1394–1401 (1977).
32. Soloway, S. B. Correlation between biological activity and molecular structure of the cyclodiene insecticides. Adv. Pest. Control Res. 6: 85–126 (1965).
33. Brooks, G. T. Progress in metabolic studies of the cyclodiene insecticides and its relevance to structure-activity correlations. World Rev. Pest Control 5: 62–84 (1966).
34. Matsumura, F., and Ghiasuddin, S. M. Evidence for similarities between cyclodiene type insecticides and picrotoxinin in their action mechanisms. J. Environ. Sci. Health B18: 1–14 (1983).
35. Ghiasuddin, S. M., and Matsumura, F. Inhibition of gamma-ami-

nobutyric acid (GABA)-induced chloride uptake by gamma-BHC and heptachlor epoxide. Comp. Biochem. Physiol. 73C: 141–144 (1982).
36. Bedford, C. T., Hutson, D. H., and Natoff, I. L. The acute toxicity of endrin and its metabolites to rats. Toxicol. Appl. Pharmacol. 33: 115–121 (1975).
37. Casida, J. E., Gammon, D. W., Glickman, A. H., and Lawrence, L. J. Mechanisms of selective action of pyrethroid insecticides. Ann. Rev. Pharmacol. Toxicol. 23: 413–438 (1983).
38. Gammon, D. W., Lawrence, L. J., and Casida, J. E. Pyrethroid toxicology: protective effects of diazepam and phenobarbital in the mouse and the cockroach. Toxicol. Appl. Pharmacol. 66: 290–296 (1982).
39. Cole, L. M., and Casida, J. E. Pyrethroid toxicology in the frog. Pestic. Biochem. Physiol. 20: 217–224 (1983).
40. Schoemaker, H., Boles, R. G., Horst, W. D., and Yamamura, H. I. Specific high-affinity binding sites for [^{3}H]Ro 5 4864 in rat brain and kidney. J. Pharmacol. Exptl. Therap. 225: 61–69 (1982).
41. Hunt, P., and Clements-Jewery, S. A steroid derivative, R 5135, antagonizes the GABA/benzodiazepine receptor interaction. Neuropharmacol. 20: 357–361 (1981).
42. Haefely, W. E. GABA and the anticonvulsant action of benzodiazepines and barbiturates. Brain Res. Bull. 5 (Suppl. 2): 873–878 (1980).
43. Bellet, E. M., and Casida, J. E. Bicyclic phosphorus esters: high toxicity without cholinesterase inhibition. Science 182: 1135–1136 (1973).
44. Petajan, J. H., Voorhees, K. J., Packham, S. C., Baldwin, R. C., Einhorn, I. N., Grunnet, M. L., Dinger, B. G., and Birky, M. M. Extreme toxicity from combustion products of a fire-retarded polyurethane foam. Science 187: 742–744 (1975).
45. Elliott, M. and Janes, N. F. Synthetic pyrethroids—a new class of insecticides. Chem. Soc. Revs. 7: 473–505 (1978).
46. Muir, J. My First Summer in the Sierra. Houghton Mifflin Co., Boston, Mass., 1916, p. 157.