

secticides (cyclodienes) is the GABA receptor. GABA is the inhibitory neurotransmitter released from the presynaptic terminal of the nervous system in insects and mammals. The GABA released into the synapse binds to the GABA receptor located on the postsynaptic membrane. The GABA receptors belong to the cysteine loop ligand-gated ion channel (LGIC) family, which is composed of five homologous subunits. In mammals, at least 19 homologous subunits have been reported.³⁾ The major receptor in the brain is a heteropentamer made of the combination of two $\alpha 1$, two $\beta 2$ and one $\gamma 2$ subunits. On the other hand most insect GABA receptors are reported to be homopentamers of $\alpha 1$ or $\alpha 2$.

Whereas γ -BHC and cyclodienes had been used extensively as the major insecticides for crop protection, environmental hygiene, and animal health, and thus majorly contributed to our well-being after the second World War, their use in agriculture was banned in Japan, US and Europe in the early 1970s, because of their persistency in the environment. It was unfortunate that, at the expiration of their registration as pesticides, they were assumed to be neuroactive agents, but neither their mode of action nor their target sites in insects and mammals were known at all. Moreover, the metabolic pathway of γ -BHC, and in particular the initial steps of the γ -BHC breakdown leading to aromatic metabolites, such as chlorophenols, chlorobenzenes and their derivatives, had not been elucidated by that time.

In 1970, just one year after the ban on BHC as a pesticide in Japan, I was involved in the team studying BHC at Kyoto University, in the labs of Profs. Nakajima and Kurihara. In the early 2000s, γ -BHC and cyclodienes were listed as persistent organic pollutants (POPs), and their production and use in agriculture were banned under the Stockholm Convention on Persistent Organic Pollutants.⁴⁾ Today, they are regarded as legacy insecticides. However, it is worth studying in detail the unsolved issues

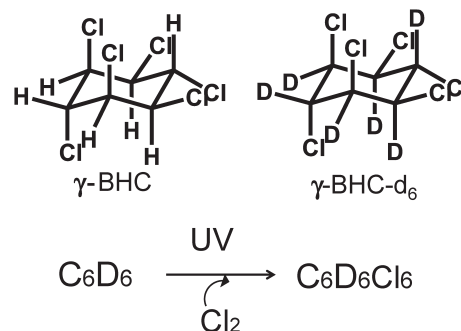


Fig. 1. γ -BHC and γ -BHC-d₆.

on their modes of action and their metabolism, using recent scientific knowledge and technologies. These studies would contribute to the development of the science of pesticides.

When I graduated from college (1972), the core aspects of the metabolism and the mode of action of γ -BHC and cyclodienes remained to be elucidated. I was absorbed in the study of the metabolism of γ -BHC since 1974 at Kyoto University, and the mode of action of γ -BHC and cyclodienes since 1981 at Michigan State University. The unsolved mystery to be tackled was “Why only γ -BHC, among the eight BHC isomers, is insecticidal?” This mystery has been investigated since 2012 at Kindai, Shimane and Nagoya Universities by studying the structure–activity relationship of γ -BHC analogues and related compounds. Starting in 1984, at the Sankyo Company, I had the chance to develop milbemycin, a chemical interacting with GABA- and glutamate-receptors chloride ion channel complexes, as an insecticide and a nematocide. For the development of the milbemycins as insecticide, their radiolabeled compounds were indispensable to study their metabolism and environmental fate.

Table 2. Insecticidal activities of γ -BHC and γ -BHC-d₆ (A) and their synergistic effect with piperonyl butoxide (B)

A. Insecticidal activity of γ -BHC and γ -BHC-d₆ at 25°C

	LD ₅₀ ($\pm 15\%$ 10 ⁻⁴ mmole/Insect)					
	Mos. ^{a)}	F. fly ^{b)}	H. fly ^{c)}	H. fly ^{d)}	G. cock. ^{e)}	A. cock. ^{f)}
γ -BHC (H)	1.3	6.53	816	>2200	16.0	200
γ -BHC-d ₆ (D)	0.32	2.06	68	86.2	1.95	24.5
H/D ^{g)}	4.06	3.17	11.76	>25	8.21	8.16

^{a)} *Culex pipens*. (3–5 days) female adult: Topical application. ^{b)} *Musca domestica* (S_{NAIDM} strain). (4 days) female adult: Topical application. ^{c)} *Musca domestica* (Toichi strain). (4 days) female adult: Topical application. ^{d)} *Musca domestica* (3rd-Yumenoshima strain). (4 days) female adult: Topical application. ^{e)} *Blattella germanica*. (14 days) male adult: Topical application. ^{f)} *Periplaneta Americana*. (60 days) male adult: Injection. ^{g)} H/D=LD₅₀ (γ -BHC/ γ -BHC-d₆).

B. Insecticidal activity and synergistic effect: LD₅₀^{h)} (10⁻⁴ μ mol/Insect)

	Mosquito			House fly		
	Alone	+p.b. ⁱ⁾	SR ^{j)}	Alone	+p.b. ⁱ⁾	SR ^{j)}
γ -BHC (H)	1.3	0.52	2.50	12.7	1.65	7.7
γ -BHC-d ₆ (D)	0.32	0.195	1.64	1.08	0.91	1.19
H/D ^{g)}	4.06	2.67		11.76	1.81	

^{h)} Topical LD₅₀. ⁱ⁾ Piperonyl butoxide (Mos.: 0.25 μ L of 0.1%, H. fly: 0.5 μ L of 0.5%). ^{j)} Synergistic ratio: LD₅₀ (alone)/(LD₅₀ (+p.b.)). ^{g)} H/D=LD₅₀ (γ -BHC/ γ -BHC-d₆).

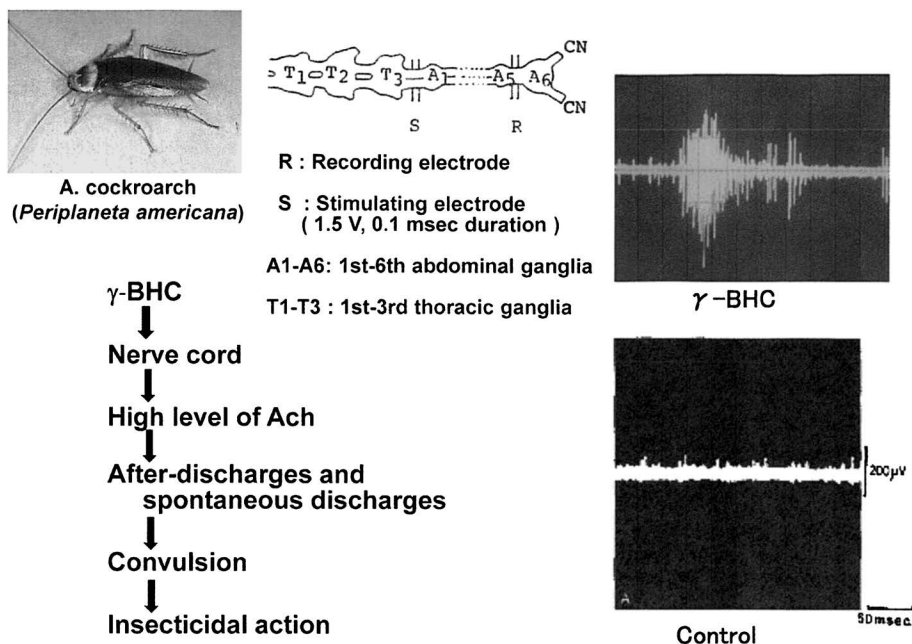


Fig. 2. Mode of action and excitatory symptoms of γ -BHC observed on the cholinergic synapse in the giant nerve cord of the American cockroach.

The preparation of two types of radiolabeled compounds, ^{14}C -labeled and ^3H -labeled milbemycins, were planned. In order to evaluate the fate of milbemycins, the studies targeted not only their parent molecules, but also their metabolites (degradation products). As milbemycins are relatively large and complex compounds, organic synthetic ^{14}C -labeling in their molecular skeletons is very hard. For the preparation of ^{14}C -labeled milbemycins, the biosynthetic approach using ^{14}C -propionate as a precursor was tried. In this paper, I will describe this attempt.

1. Deuterium Isotope Effect of γ -BHC- d_6 on Its Insecticidal Activity and Metabolic Rate⁵⁻⁷⁾

This study started from an interesting finding that hexadeuteriated γ -BHC (γ -BHC- d_6 , $\text{C}_6\text{D}_6\text{Cl}_6$, Fig. 1) is several times more insecticidal than "regular" γ -BHC ($\text{C}_6\text{H}_6\text{Cl}_6$) (Table 2). BHC is a simple compound of six carbon (C) atoms, six hydrogen (H) ones and six chlorine (Cl) ones. The possible metabolic breakdown of the BHC molecule at the first step must occur at any of the following bonds: C-C, C-H, and C-Cl. In order to study the metabolism of γ -BHC in detail, γ -BHC- d_6 was used. The deuterium isotope effect on the insecticidal and physiological activities, and the metabolic rate, must give useful information about not only its metabolism, but also its mode of action. This strategy is based on the fact that the physicochemical properties of the drug and its deuteriated version must be absolutely the same, except for their molecular weights and their C-H (D) bond strength. Indeed the penetration rates of γ -BHC and γ -BHC- d_6 inside the insect body were the same. If the C-H bond(s) cleavage of γ -BHC (e.g., dehydrochlorination and/or dehydrogenation) is the first and rate-limiting step of its degradation, the metabolic detoxification of γ -BHC- d_6 must be slower

than that of its counterpart, γ -BHC. Therefore, there must be some difference in their insecticidal activities. Highly hexadeuteriated γ -BHC- d_6 is easily prepared from the photochlorination of hexadeuteriated benzene- d_6 with chlorine gas in carbon tetrachloride as shown in Fig.1.

The after-discharge caused by γ -BHC in the central nervous system is the main cause of its convulsive action and lethality^{9,10)} (Fig. 2). γ -BHC and γ -BHC- d_6 exhibited equivalent neuroexcitatory activities in terms of minimum effective concentration causing after-discharge (MEC_{AD}), as shown in Table 3. In this experiment, isolated nerve cords were dipped in a saline solution containing γ -BHC or γ -BHC- d_6 , and measurements were made after 2 hr. Since the effect of metabolism in the nerve cord is negligible, these two compounds must be equipotent at the target of insecticidal action. This assumption was later confirmed¹¹⁾ by two following up experiments: The radio labelled ligand [^3H]EBOB (ethynylbicycloorthobenzoate, a GABA non-competitive antagonist) binding assay, and the membrane potential assay using a fluorescence probe (fluorescent membrane potential (FMP) assay).¹²⁾

The cockroaches injected with γ -BHC first showed uncoor-

Table 3. Neuroexcitatory, convulsive and insecticidal activities of γ -BHC and γ -BHC- d_6 against the American cockroach

	γ -BHC	γ -BHC- d_6	H/D
$-\log \text{LD}_{50}$ ($\mu\text{mol}/\text{insect}$) ^{a)}	1.7	2.6	8
$-\log \text{MEC}_{\text{Cac}}$ ($\mu\text{mol}/\text{insect}$) ^{b)}	2.3	2.6	2
$-\log \text{MEC}_{\text{AD}}$ (mol/L) ^{c)}	7.3	7.3	1

^{a)} LD_{50} : Insecticidal (Lethal) activity. ^{b)} MEC_{Cac} ($\mu\text{mol}/\text{insect}$): Convulsive activity. ^{c)} MEC_{AD} (mol/L): Neuroexcitatory activity.

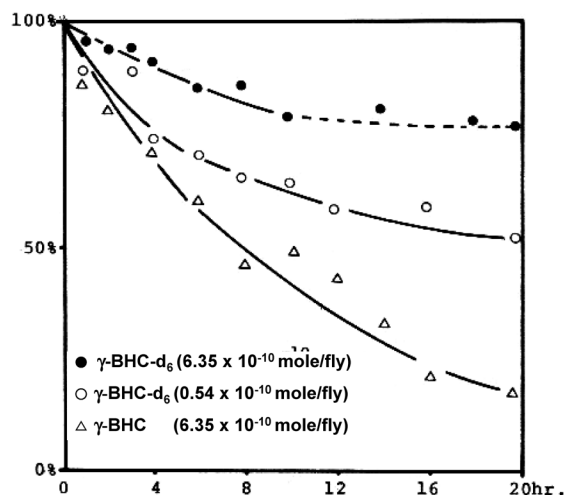


Fig. 3. Time courses of the disappearance of γ -BHC and γ -BHC- d_6 in the housefly (*in vivo*).

minated movements, then convulsions 3 hr after the treatment. Some later recovered, and others died. The recovery from these symptoms must be due to metabolic detoxification. Approximately a two-fold isotope effect was observed in the convulsive activity determined 3 hr after injection, while an eight-fold iso-

tope effect was found for LD_{50} values at 24 hr (Table 3).

When γ -BHC- d_6 was applied topically or by injection to the mosquito (*Culex pipiens pallens*), the housefly (*Musca domestica*), the German cockroach (*Blattella germanica*) and the American cockroach (*Periplaneta americana*), it was several times more toxic than γ -BHC against all the insects⁵⁾ (Table 2). γ -BHC was considerably synergized by piperonyl butoxide, but γ -BHC- d_6 was not (Table 2B). A large isotope effect was observed in the *in vivo* breakdown of γ -BHC- d_6 (Fig. 3). The difference in insecticidal activity must be due to a difference in biodegradation rate caused by the deuterium kinetic isotope effect.

In order to identify the key factor(s) causing the resistance to γ -BHC of the third Yumenoshima strain,¹³⁾ a strain of houseflies highly resistant to various insecticides, these insects were studied using γ -BHC- d_6 .¹⁴⁾ The LD_{50} ratio of γ -BHC to γ -BHC- d_6 in this strain, *i.e.*, the deuterium isotope effect on LD_{50} values, was much larger than that in the susceptible SNAIDM strain (Table 4A). The penetration rates of γ -BHC and γ -BHC- d_6 through the insect cuticle were about the same for both strains (Table 4B). Thus, differences in penetration rates do not cause resistance. The metabolic degradation *in vivo* of γ -BHC occurred in the resistant strain much faster than in the susceptible strain (Table 4C). This was also the case for the γ -BHC degradation processes *in vitro*, such as microsomal oxidation and glutathione conjuga-

Table 4. Third Yumenoshima strain: A. insecticidal activities of γ -BHC and γ -BHC- d_6 , and isotope effect; B, penetration of γ -BHC- d_6 in susceptible and 3rd-Yumenoshima strains, C: penetration and metabolism of γ -BHC and γ -BHC- d_6 in 3rd-Yumenoshima strain

A. Insecticidal activity and isotope effect on LD_{50} ^{a)} value

Compounds	Strain of H. fly: LD_{50} ^{a)} ($\times 10^{-10}$ mol/fly ^{b)})		Ratio (R/S)
	NAIDM (S)	3rd-Yumenoshima (R)	
γ -BHC (H)	6.53	>2200	>337
γ -BHC- d_6 (D)	2.06	86.2	41.8
H/D	3.17	>25	

^{a)} 24 hr mortality after topical application. The standard error of the listed mean value was less than 15% of each value. ^{b)} 4-day-old female adult.

B. Penetration of γ -BHC- d_6 in the H. fly strains: 60 min after topical application

Strains	Percentage recovery ($\pm 20\%$) ^{a), b)}			
	Insect surface	Insect internal ^{b)}	Container	Total amount recovered
3rd-Yumenoshima	24.0	60.5	5.0	89.5
NAIDM	22.5	66.5	5.5	94.5

^{a)} Topical application of γ -BHC- d_6 in acetone: 2×10^{-9} mol/0.5 μ L/fly. ^{b)} Average values of three experiments. Each experiment: 10 flies were used for each experiment: SE, $\pm 15\%$ of each value. ^{c)} Extracts of the insect body homogenate.

C. Penetration and metabolism in 3rd-Yumenoshima: 3 hr after topical application

Compounds	Percentage recovery ($\pm 20\%$) ^{a)}			
	Insect surface	Insect internal ^{b)}	Container	Total amount recovered
γ -BHC	13.7	17.4	4.7	35.8
γ -BHC- d_6	13.8	50.0	5.3	69.1

^{a)} Average values of three experiments. Each experiment: 5 female flies, applied with γ -BHC or γ -BHC- d_6 (5×10^{-9} mol/0.5 μ L of acetone solution/fly); SE, $\pm 15\%$ of each value. ^{b)} Extracts of the insect body homogenate.

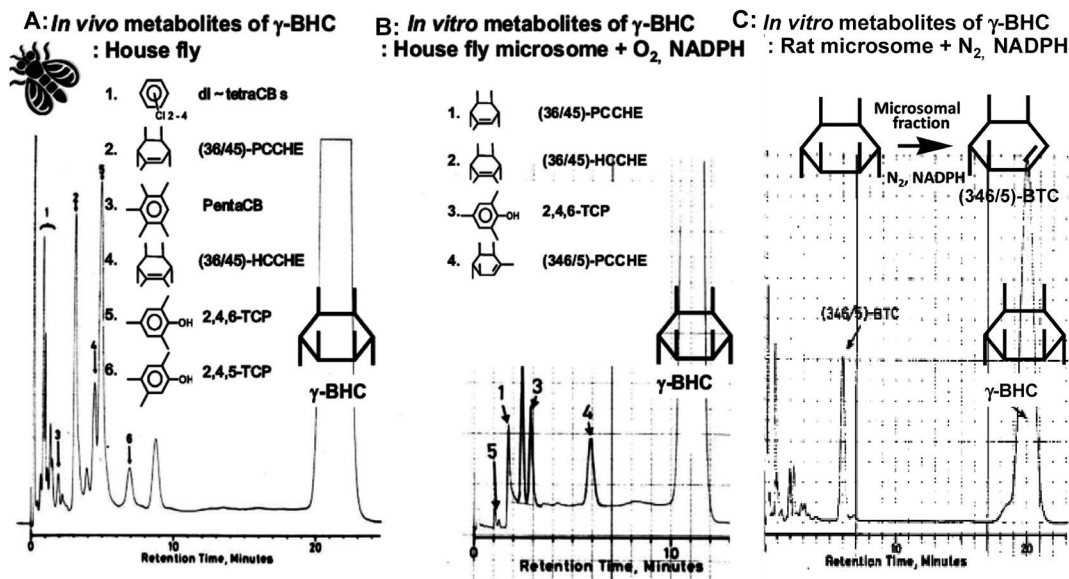


Fig. 4. Gas chromatograms of the hexane soluble metabolites in the housefly. A: *In vivo*; B: *in vitro* aerobic metabolism with the microsomal fraction of housefly abdomen; C: *in vitro* anaerobic metabolism with the microsomal fraction of rat liver.

tion. In both strains, significant isotope effects were observed in the degradation rates *in vitro* of γ -BHC- d_6 . Therefore, the principal biodegradation and detoxification pathways should include reactions cleaving the C–H bonds. When the much less biodegradable γ -BHC- d_6 was applied to both strains, the susceptible strain became much more intoxicated than the resistant one within 20 to 30 min. This indicates that both greater degradability and lower sensitivity at the action site, in combination, are the main factors underlying resistance in the third Yumenoshima strain. When this study was conducted, neither the action site of γ -BHC nor its mode of action were known at all. Later the point mutation of alanine moiety to serine on T2 membrane region of GABA receptor was found to result the insect to be resistant to GABA non-competitive antagonist type insecticides such as γ -BHC (see Ref. 43–45).

2. Metabolic Studies of BHC^{5–7,14–17,34,37}

In the 1970s a number of metabolic studies of γ -BHC were performed with mammals and various species of insects, which identified the terminal metabolites. However, at that time nothing definite was known about the metabolism of γ -BHC. In particular, the initial steps leading to its terminal metabolites, such as polychlorocyclohexenols, chlorophenols, chlorobenzenes and polychlorophenyl-gluthathiones (or -mercapturic acids), had not been well established, and had been the subject of long controversies. Some thought that (36/45)-PCCHE (1,3,4,5,6-pentachlorocyclohex-1-ene, γ -isomer of PCCHE, see Fig. 4 for the structural formulas) was first formed from γ -BHC by *trans*-dehydrochlorination and then underwent various metabolic pathways such as dehydrogenation, dehydrochlorination, oxygenation and glutathione conjugation to produce these terminal metabolites. An early investigation showed that PCCHE is formed from γ -BHC in houseflies.¹⁸⁾ Reed and Forgash re-

ported the presence of (36/45)-PCCHE and “iso-PCCHE” in the hexane-soluble metabolites of γ -BHC-treated houseflies.¹⁹⁾ However, Clark *et al.* questioned the role of PCCHE isomers as major intermediates of the aromatic products from γ -BHC in insects.²⁰⁾ In the presence of *p,p'*-tetra-methyldiaminodiphenylmethane, an inhibitor of the metabolism of PCCHE isomers, they observed a significant disappearance of γ -BHC. From the results of an isotope ($[^{14}\text{C}]$ γ -BHC) dilution technique, Bridges suggested that (36/45)-PCCHE is a minor metabolite and is not associated with the main metabolic pathway.²¹⁾

2.1. Oxidative metabolism^{6,7,15–17)}

Various mono- and polychlorophenols have been identified as urinary metabolites of γ -BHC and its isomers (α , β and δ -BHC) in mammals.^{22–28)} Among them, 2,4,6-TCP (trichlorophenol) is a common major metabolite of these BHCs.^{23–25)} As explained above, the initial metabolites leading to these terminal metabolites had long been the subject of controversy. PCCOL (2,3,4,5,6-pentachloro-2-cyclohexen-1-ol)^{*2} was as one of the metabolites of γ -BHC in rats, but it was reported to be hardly metabolized *in vitro* to chlorophenols.^{25,26)} Various mono- and polychlorobenzenes, which are also metabolites of γ -BHC and

*2 Chadwich *et al.*, named this compound based on IUPAC rule, in which the numbering of carbon atoms of cyclohexene starts from the one attached with OH group, and this nomenclature is scientifically correct. However this nomenclature has disadvantage for comparing a series of analogues or derivatives. The numbering of the carbon-position among analogues changes with the substituent such as OH whether it attaches or not on the cyclohexene ring. In this paper other rule is use, in which the carbon atom of the double bond in cyclohexene is 1-position. This compound will be named as 1,2,4,5,6-pentachloro-1-hexene-3-ol, PCC-3-OL.

Table 5. 2,4,6-TCP formation from BHC isomers by rat liver microsomes in the presence of NADPH and O₂

Substrates ^{a)} (400 × 10 ⁻¹⁰ mol)	2,4,6-TCP: Major phenolic metabolite (× 10 ⁻¹⁰ mol)
α-BHC(III)	10.6
β-BHC(II)	4.6
γ-BHC(I)	8.3
δ-BHC(IV)	127.5
ε-BHC(V)	45.6

^{a)} Incubation time: 15 min.

its isomers, have been considered precursors of various chlorophenols by many investigators.^{28,29)} Among the three TCB (trichlorobenzene) isomers, only 1,3,5-TCB²⁹⁾ gave exclusively 2,4,6-TCP as a metabolite. However 1,3,5-TCB was a quite minor metabolite of γ-BHC.¹⁹⁾ Based on these data, the existence of completely unknown metabolic routes or intermediates to 2,4,6-TCP was postulated for the metabolism of γ-BHC and its isomers.^{16,17)}

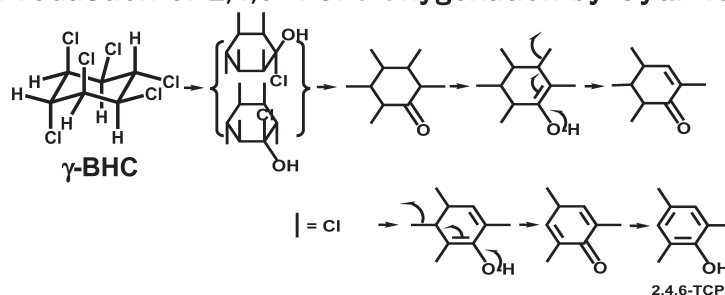
In γ-BHC-treated houseflies, a *cis*-dehydrogenated metabolite (36/45)-HCCHE (1,2,3,4,5,6-hexachlorocyclohex-1-ene), a *trans*-dehydrochlorinated one (36/45)-PCCHE, di-pentachlorobenzene, and two isomers of TCP (2,4,6- and 2,4,5-TCP) were identified by gas-liquid chromatography and mass spectrometry with their authentic chemicals (Fig. 4). The *in vitro* metabolism study showed that in the presence of NADPH and O₂ the microsomal fraction of housefly, rat and mouse livers converted γ-BHC to 2,4,6-TCP and three hexane-soluble metabolites. These three metabolites were identified as (36/45)-HCCHE, (36/45)- and (346/5)-PCCHE (Fig. 4). In the presence of NADPH (nicotinamide adenine dinucleotide phosphate) and molecular oxygen (O₂), rat liver and housefly microsomes metabolized γ-BHC and its isomers (α, β, δ and ε),

and 2,4,6-TCP was identified as a common major metabolite for all five BHC isomers (Table 5). The order of the reactivity in the isomers was δ > ε > α > γ > β. Beside 2,4,6-TCP, γ-BHC gave (36/45)-HCCHE and (36/45)-PCCHE. α- and δ-BHC gave (346/5) and (35/46)-PCCHE, respectively, but no detectable amount of dehydrogenated metabolites (HCCHE). β-BHC did not yield any PCCHE or HCCHE. In a similar reaction procedure, all of five PCCHE isomers and four HCCHE isomers gave 2,4,5-TCP and 2,3,4,6-TeCP (tetrachlorophenol), respectively, as major phenolic metabolites. These results show that 2,4,6-TCP, as a major metabolite of BHC isomers, is produced *via* neither PCCHE nor HCCHE. There should exist hitherto unknown intermediates, which degrade mainly to 2,4,6-TCP. We have disclosed new metabolic pathways from γ-BHC and its isomers, and HCCHE and PCCHE isomers to chlorophenols, which proceed through direct oxygenation of the cyclohexane or cyclohexene ring of these polychlorohydrocarbons.¹⁵⁻¹⁷⁾ The possible candidate intermediate of BHC metabolism is pentachlorocyclohexanone-*gem*-chlorohydrin, which is formed from the hexachlorocyclohexane molecule by direct hydroxylation with microsomes. The chlorohydrins seem to be so labile that they are easily converted to the corresponding cyclohexanones, which, in turn, undergo two-step dehydrochlorination *via* their enol forms to yield 2,4,6-TCP (Fig. 5). This mechanism, which is supported by the model chemical reaction described below, explains very well the fairly large isotope effect (H/D, about 10-11) observed in this phenol formation, and the predominant production of this phenol from all five BHC isomers, including the extremely stable β-BHC (Table 5).

The oxidative chemical reaction of four pentachlorocyclohexanol isomers with CrO₃, converting C-OH to C=O, produced only 2,4,6-TCP as the main product in good yields, instead of their pentachlorocyclohexanone isomers.¹⁷⁾

Pathways leading to other terminal metabolites such

Production of 2,4,6-TCP: Oxygenation by Cyt.P450



Production of 2,4,5-TCP and 2,3,4,6-TeCB

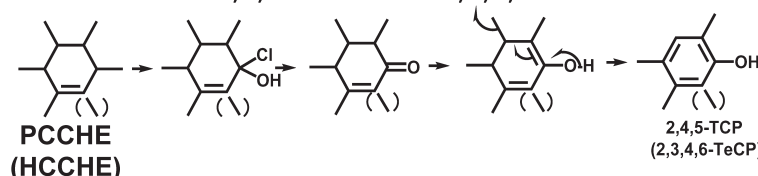


Fig. 5. Possible mechanisms of production of 2,4,6-TCP from BHC, and of 2,4,5-TCP and 2,3,4,6-TeCP from PCCHE and HCCHE, respectively.

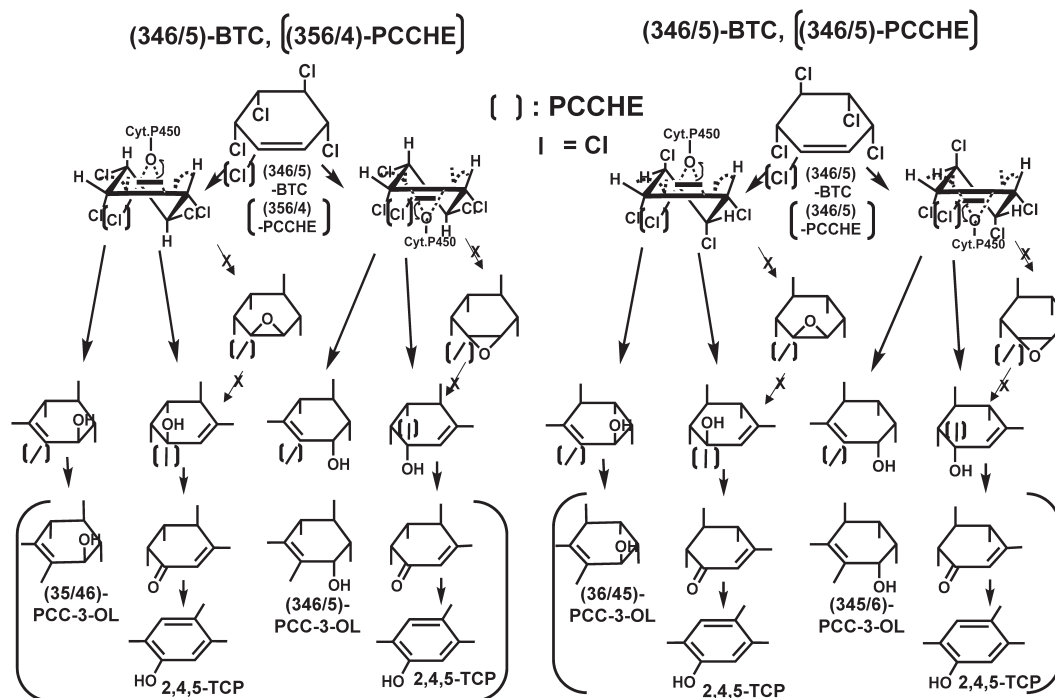


Fig. 6. Possible mechanisms of production of tetrachlorocyclohex-1-en-3-ol from (346/5)-BTC, and 2,4,5-TCP and pentachlorocyclohex-1-en-3-ol (PCC-3-OL) from (3,4,6/5)- and (3,5,6/4)-PCCHE.

as 1,2,4-trichlorobenzene, tetrachlorobenzene isomers, 2,4,5-trichlorophenol, and tetrachlorophenol isomers include the route through other initial metabolites, *i.e.*, PCCHE and HCCHE.

The fact that 2,4,6-TCP formation from BHC isomers is dependent on molecular oxygen and NADPH, being inhibited by carbon monoxide (32% of normal reaction in CO/O₂: 94/6), suggests that cytochrome P450, a terminal oxidase in microsomes, participates in this reaction. Under the same reaction conditions as above, the PCCHE isomers are metabolized more effectively than BHC isomers to give 2,4,5-TCP and one or two polar non-phenolic metabolites, which are identified as stereoisomers of PCCOL by gas chromatography-mass spectrometry (GC-MS). In (356/4)- and (346/5)-PCCHE, the four chlorine atoms attached to the *sp*³-carbons have a common configuration. (356/4)-PCCHE gives 2,4,5-TCP (53% based on the metabolized substrate) much more easily than (346/5)-PCCHE (6%). On the other hand, (346/5)-PCCHE produces the two PCCOL isomers much more than (356/4)-PCCHE. The amounts of PCCOL isomers produced from the (346/5)-isomer are estimated to be about fifty-fold greater, or more, than those from the (356/4)-isomer. The structures of the PCCOL isomers from (346/5)-PCCHE are shown to be (345/6)- and (36/45)-PCC-3-OL by comparing the chlorination (SO₂Cl₂ in pyridine) products of these PCCOL isomers with authentic HCCHE isomers by gas-chromatography. The (346/5)-PCCHE isomer is probably hydroxylated at the 2-position *via* an ene-like mechanism,³⁰ accompanied by double bond migration. With BTC isomers, similar ene-like hydroxylation is observed. (346/5)-BTC af-

fords mainly the (36/45)- and (345/6) isomers of tetrachlorocyclohexen-3-ol, showing selective oxygen attack on one of *sp*²-carbons. Such hydroxylation on (356/4)-PCCHE occurs similarly at position 1 to give the *gem*-chlorohydrins, which should be very labile, enough to undergo enone formation followed by further dehydrochlorination to TCP. The oxidation of (36/45)-4,5,6-trichlorocyclohexen-3-ol with chromic acid affords only 2,4-DCP (dichlorophenol), not 2,3-DCP. This result is in agreement with the fact that 2,4,5-TCP, and not 2,3,5-TCP, is obtained in the biochemical transformation of the 1,4,5,6-tetrachlorocyclohexen-3-one through the *gem*-chlorohydrins derived from PCCHE (Fig. 6), as described above. 2,3,4,6-TeCP is also produced from HCCHE isomers quite similarly.

In the presence of NADPH and O₂ microsomal fraction,

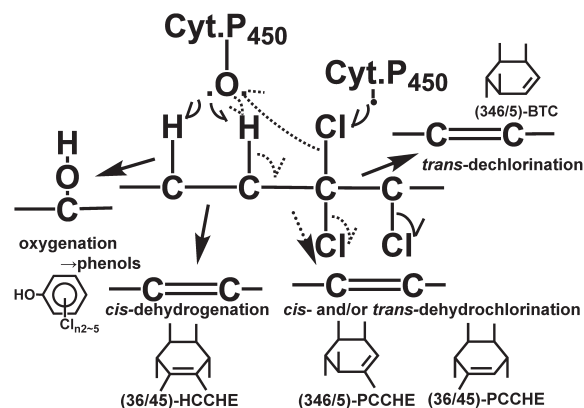


Fig. 7. Metabolism of BHC mediated by cytochrome P450.

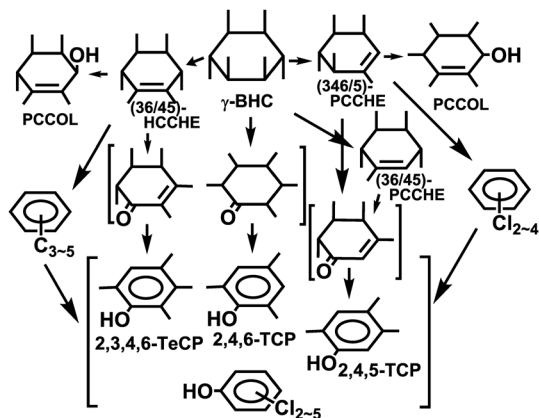


Fig. 8. Summary of the oxidative metabolism of γ -BHC.

cytochrome P450 mediates at least three metabolic reactions, namely *cis*-dehydrogenation to HCCHE, *cis*- and possibly *trans*-dehydrochlorination to PCCHE, and hydroxylation by direct insertion of oxygen into the C–H bond (Fig. 7). As a result, the phenolic metabolites of γ -BHC and other BHC isomers are produced through, at least, three pathways. One is the direct hydroxylation of γ -BHC, other BHC isomers, and their derivatives such as PCCHE and HCCHE. In the case of γ -BHC and other BHC isomers, the gem chlorohydrins thus formed are decom-

posed to labile pentachlorocyclohexanones, which easily degrade to 2,4,6-TCP *via* two-step dehydrochlorination of their tautomers. The second is the pathway through the PCCHE and HCCHE isomers, which undergo an ene-like reaction with activated oxygen on cytochrome P450, and not through their corresponding epoxides, to yield PCCOL and *gem*-chlorohydrins, which spontaneously afford the corresponding enones. The tautomeric form of the enone undergoes one-step dehydrochlorination to yield 2,4,5-TCP from PCCHE (and 2,3,4,6-TeCP from HCCHE) (Figs. 5 and 6). The direct hydroxylation of chlorobenzenes is the third pathway. From the results described above, the oxidative metabolism of γ -BHC in insects and mammals may be summarized as shown in Fig. 8.

Beside the oxidative metabolism, under anaerobic conditions, the *trans*-dechlorinated metabolite (346/5)-BTC was identified in the *in vitro* metabolism of γ -BHC with rat microsomes and NADPH¹⁷⁾ (Figs. 4C and 7).

2.2. Metabolism of glutathione conjugation^{5-7,34,37)}

γ -BHC was known to be broken down by an enzymatic reaction with the post-microsomal fraction of housefly homogenates in the presence of glutathione,³¹⁾ but the detailed mechanism of this reaction had not been determined. As shown in a previous section, a fairly large isotope effect (about 6.5) was observed in the *in vitro* breakdown of γ -BHC-*d*₆ with the post-microsomal

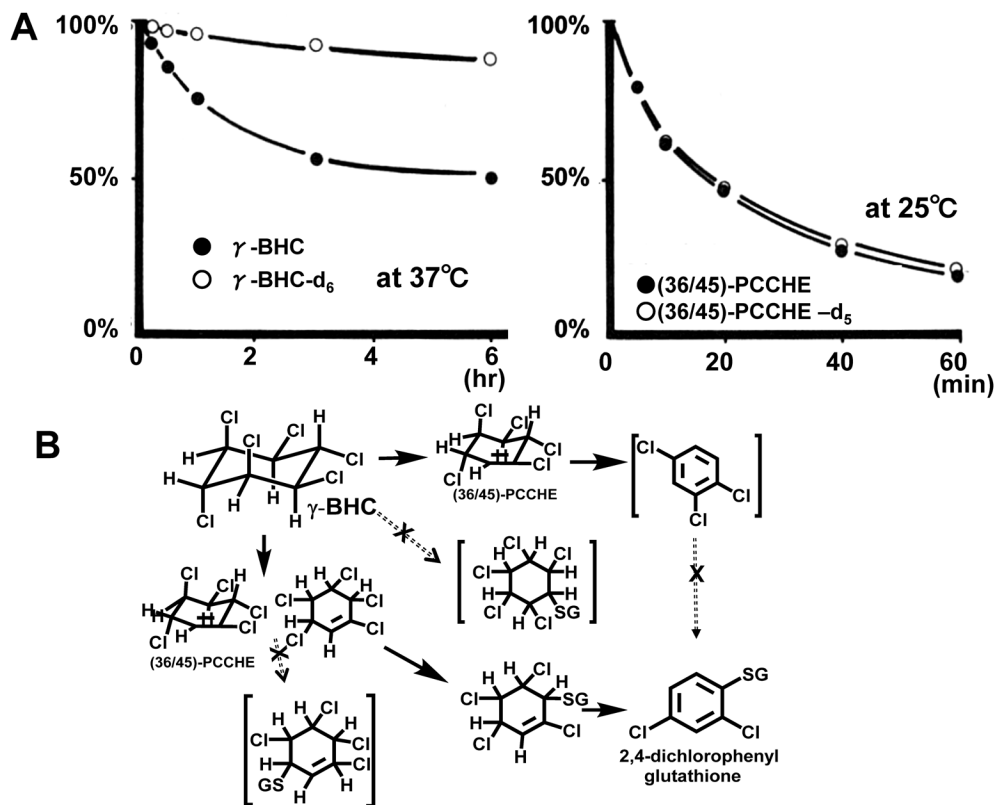


Fig. 9. Time courses of the *in vitro* breakdown of γ -BHC, its *trans*-dehydrochlorinated product (36/45)-PCCHE and their deuteriated counterparts (A) and possible metabolic routs (B).

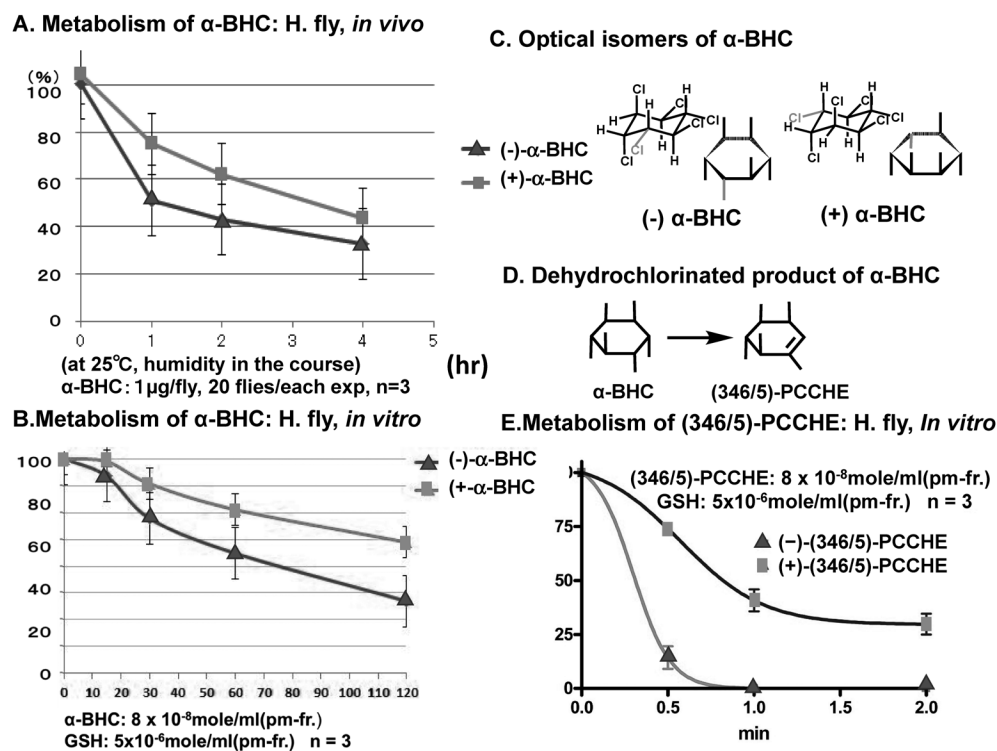


Fig. 10. *In vivo* and *in vitro* metabolism of enantiomers of α -BHC and *in vitro* metabolism of enantiomers of (346/5)-PCCHE.

fraction of the housefly.⁵⁾ This observed isotope effect suggests that the initial step in the reaction may be a dehydrochlorination. The direct substitution of glutathione on the γ -BHC molecule, as suggested by Clark *et al.*³²⁾ and Bradbury *et al.*,³³⁾ is not likely. Enzymatic conjugation with glutathione would take place at the stage of the PCCHE isomers, since no significant isotope effect was observed in the reaction using (36/45)-PCCHE-*d*₅ as the substrate (about 1.2, Fig. 9A). The very low isotope effect suggests that the conjugation with glutathione would occur nucleophilically on the very reactive allylic carbon of (36/45)-PCCHE. Since *S*-(2,4-dichlorophenyl)-glutathione was obtained as the conjugate, the conjugation should take place at position 6 of the molecule, for the product conjugating at position 3 could not give *S*-(2,4-dichlorophenyl)-glutathione by the following 1,2- or 1,4-dehydrochlorination (Fig. 9B).

α -BHC is also a good substrate of this enzymatic reaction with the post-microsomal fraction of housefly homogenates in the presence of glutathione.⁵⁾ α -BHC is the only chiral isomer among BHC ones, and was a major component in technical-grade BHC. The absolute configuration of (+) α -BHC was assigned by X-ray crystal structure analysis. The enantioselective metabolism of α -BHC racemate by the microbes and the eider duck in the marine ecosystem were reported.^{35,36)}

Each individual enantiomer of α -BHC was separated on the polysaccharide stereoselective HPLC column, and the comparative metabolism of the α -BHC enantiomers in the housefly (*in vivo* and *in vitro* systems using post-microsomal fraction in the presence of glutathione) was investigated (Fig. 10A, B).

(346/5)-PCCHE, the dehydrochlorinated product of α -BHC, is a putative intermediate of α -BHC metabolic degradation. This intermediate is also a chiral compound. The *in vivo* disappearance rate of α -BHC in the housefly was different between the enantiomers. The *in vitro* post-microsomal fraction in the presence of glutathione enantioselectively metabolized not only α -BHC but also (346/5)-PCCHE. The (–) enantiomer of α -BHC was metabolized faster than the (+) one both *in vivo* and *in vitro*. One of the (346/5)-PCCHE enantiomers, the (–) isomer, was

Table 6. Subcellular localization of [³H] α -dihydropicrotoxinin binding in American cockroach heads

Sucrose density gradient fraction (M) ^{a)}	Binding (dpm/mg/protein) ^{b)}	
	Total	Specific
0.8	13777 \pm 104	720 \pm 186
0.8–1.0	15862 \pm 359	720 \pm 102
1.0–1.2	16688 \pm 104	1783 \pm 66
1.2–1.5	12344 \pm 45	605 \pm 10
1.5–1.8	10072 \pm 630	553 \pm 417
1.8	11970 \pm 1061	— ^{c)}

^{a)} Fraction 0.8(M), cell membrane; 0.08–1.0, large pieces of cell membrane and small pieces of nerve ending particles; 1.0–1.2, pinched off nerve endings (synaptic vesicles) and a few mitochondria; 1.2–1.5, nerve ending particles (more electron dense nature) and mitochondria; 1.5–1.8, cell fragments and a few mitochondria; 1.8, nonneral tissue.
^{b)} [³H] α -Dihydropicrotoxinin binding (sp act, 30 Ci/mmol), 11.1×10^{-9} M. Data are expressed as means \pm S.E. of two of three experiments, each experiment involving three determinations. ^{c)} Not tested.

Table 7. Effect of cyclodienes and other agents on [³H]α-dihydropicrotoxinin binding

Agents ^{b)}	%Inhibition of [³ H]α-DHPTX specific binding	Agents ^{b)}	%Inhibition of [³ H]α-DHPTX specific binding
Dihydropicrotoxinin	100	α-BHC(III)	21.4±18.5
Aldrin	36.6±0.6	β-BHC(II)	15.3±15.4
Dieldrin(XX)	58.5±2.3	γ-BHC(I)	91.5±20.0
Photodieldrin	79.0±4.3	δ-BHC(VI)	55.0±16.0
Heptachlor	36.6±18.6	Allethrin	13.2±6.7
Heptachlorepoxyde	57.3±31.3	Cypermethrin	7.7±3.1
Isodrin	35.6±3.8	Decamethrin	-0.1±1.0
Endrin	62.3±12.4	Fenvarelate	-0.4±5.8
γ-Chlordane	56.2±2.0	Tetramethylene-disulfotetramine (TETS)	27.1±13.0
Oxychlordane	77.8±6.2	<i>t</i> -Butyl bicyclic phosphate	46.6±2.9
Hexchlorocyclo-pentadiene	0.3±2.2	Pentobarbital	21.1±8.1
Mirex	14.7±15.7	Benzodiazepam	45.1±10.8
Kepone	135.5±18.4	SQ-65396	21.5±5.7
Toxaphene	72.3±28.1	SQ-20009	-8.3±4.2
DDT	5.4±3.6		
Parathion	4.1±26.3		

^{a)} [³H]α-dihydropicrotoxinin, 11.1 nM; five American cockroach heads were used for one experiment, which involved three determinations. ^{b)} Dihydropicrotoxinin, 100 μM; others, 10 μM. ^{c)} Data are expressed as means±S.E. of two or three experiments, each experiment involving three determinations.

found to be metabolized faster than its counterpart (+) isomer³⁷⁾ (Fig. 10E).

3. Picrotoxinin Receptor on the GABA Receptor Chloride Ion Channel Complex, and the Mode of Action of γ-BHC and Cyclodiene Insecticides³⁷⁻⁴²⁾

As a result of toxicity tests, it was established that all cyclodiene-resistant strains of the German cockroach are also resistant to picrotoxinin. This cross-resistance pattern was specific to picrotoxinin and did not extend to other neuroexcitants such as bicuculline, β-bungarotoxin, DDT, and organophosphates. By using electrophysiological techniques, picrotoxinin was confirmed to act at the presynaptic area, stimulating the release of an excitatory transmitter. These electrophysiological symptoms caused by picrotoxinin were quite similar to those of γ-BHC and dieldrin.³⁸⁾ [³H]α-dihydropicrotoxinin binding experiments confirmed that the nerve membrane in the CNS of the American cockroach and in the rat brain contained a component with high affinity for picrotoxinin, and can be thus regarded as a picrotoxinin receptor. By using sucrose density centrifugation, it was determined that the fraction sedimented at the interphase

of 1.0 to 1.2M sucrose at 100,000g contained the highest level of specific binding sites (Table 6). The receptor showed sensitivity to several insecticidal cyclodienes, including photodieldrin, oxychlordane, endrin, heptachlor epoxide, γ-chlordane, dieldrin, aldrin, heptachlor, isodrin, toxaphene, and TBPS (*tert*-butylbicyclophosphorothionate). Among four BHC isomers, the γ-isomer showed the highest affinity to this receptor³⁸⁻⁴⁰⁾ (Table 7). Dieldrin resistant German cockroaches showed cross-resistance to the chemicals having binding affinity to the picrotoxinin receptor.⁴¹⁾ The nervous system of the resistant cockroach

Table 8. Time to onset of poisoning symptoms in the abdominal nerve cord of susceptible (CSMA) and resistant (LPP) German cockroaches^{a)}

Strain of G. cockroach	Minute to onset of poisoning symptoms ^{b)}
CSMA	26.5±3.7
LPP	74.0±6.7

^{a)} Dihydropicrotoxinin (10⁻⁵ M) with piperonyl butoxide (10⁻⁴ M).

^{b)} Data are expressed as means±S.E. of five experiments.

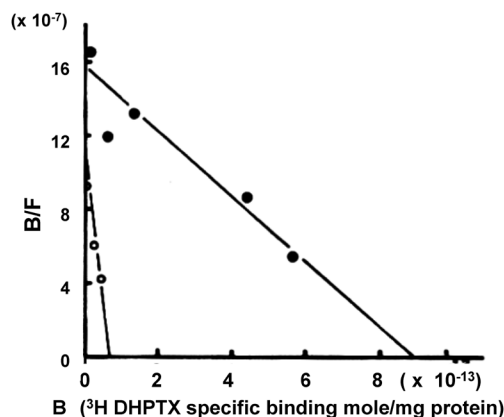


Fig. 11. Scatchard plot analysis of [³H]α-dihydropicrotoxinin binding to the brain membrane preparation from two German cockroach strains: the Dieldrin susceptible CSMA strain (●) and the resistant LPP strain (○). *B*_{max} receptor number (mole/mg protein) was CSMA, 9.0×10⁻¹³; LPP, 7.1×10⁻¹⁴. The dissociation constant *K*_d (M) was CSMA, 5.8×10⁻⁷; LPP, 6.45×10⁻⁸. Data are expressed as means of two independent experiments, each experiment involving three determinations.

Table 9. Comparison of the action of GABA, milbemycin and avermectin on $^{36}\text{Cl}^-$ uptake processes^{a)} in the leg muscles of the American cockroach

Compounds	<i>n</i>	$^{36}\text{Cl}^-$ influx (%)
Control	8	100.00 ± 4.32
Avermectin B1a (10^{-7}M)	8	115.87 ± 7.95
Milbemycin D (10^{-7}M)	8	116.63 ± 4.99
GABA (10^{-3}M)	8	104.18 ± 5.92
Avermectin B1a (10^{-7}M)+GABA (10^{-3}M)	8	116.30 ± 8.22

^{a)} The procedure of this experiment is shown in Fig. 12.

was found to be less sensitive to dihydropicrotoxinin (Table 8). Furthermore, it was determined that the nerve components from the resistant cockroaches had significantly lower binding capacity to [^3H] α -dihydropicrotoxinin (Fig. 11). The most likely explanation for this phenomenon is that these cockroaches have developed cyclodiene resistance by altering the nerve receptor for picrotoxinin. Later, a single A(alanine)2'S(serine) or A2'G(glutamate) mutation was found in the GABA receptor subunit of the resistant strains of *Drosophila*, housefly, German cockroach and other insects, which results in resistance to dieldrin.^{43–45)}

Milbemycin (see the structures in Fig. 14) and avermectin were found to stimulate Cl^- uptake by the leg muscles of the American cockroach within 4 min. at 10^{-7}M (Table 9, Fig. 12). This stimulatory action could be antagonized by picrotoxinin (10^{-4}M). It was concluded that the action of milbemycin and avermectin is to open the chloride channel on the plasma mem-

Cluster of leg muscles

- Equilibrate in insect saline
- Preincubate with or without avermectin (milbemycin) in insect saline,
- Transfer to saline solution containing $^{36}\text{Cl}^-$, [^3H]mannitol, and the same concentration of avermectin (milbemycin)
- Solubilize the muscles

Radioactivity ($^{36}\text{Cl}^-$, ^3H): measured by liquid scintillation counter

Fig. 12. Experiment of Cl^- uptake by the leg of muscles of the American cockroach.

brane. Their action does not seem to be mediated through the GABA (or picrotoxinin) receptor, and suggest that they directly open the chloride channel in both the central nervous and neuromuscular systems.^{39,42)}

4. Development of Milbemycin as Insecticide and Acaricide^{46–49)}

Milbemycin, discovered back in 1967, was first introduced into the Japanese market in 1990 as an agricultural pesticide. From the screening of the fermentation broths of an actinomycete, *Streptomyces hygroscopicus* ssp. *aureolacrimosus*, Mr. A. Aoki isolated sixteen-membered macrocyclic lactones with a spiroketal ring system consisting of two six-membered rings.⁵⁰⁾ Most of the milbemycins showed dramatically potent activity against various polyphagous mites and plant-parasitic nematodes.^{50,51)} Milbemectin, a mixture of milbemycins A_3 and A_4 (M. A_3 and M. A_4 , respectively), was developed as a pesticide for plant protection, commercialized as Milbeknock[®], Koromite[®] and Matsuguard[®]. Figure 13 shows a very effective preventive trial, in

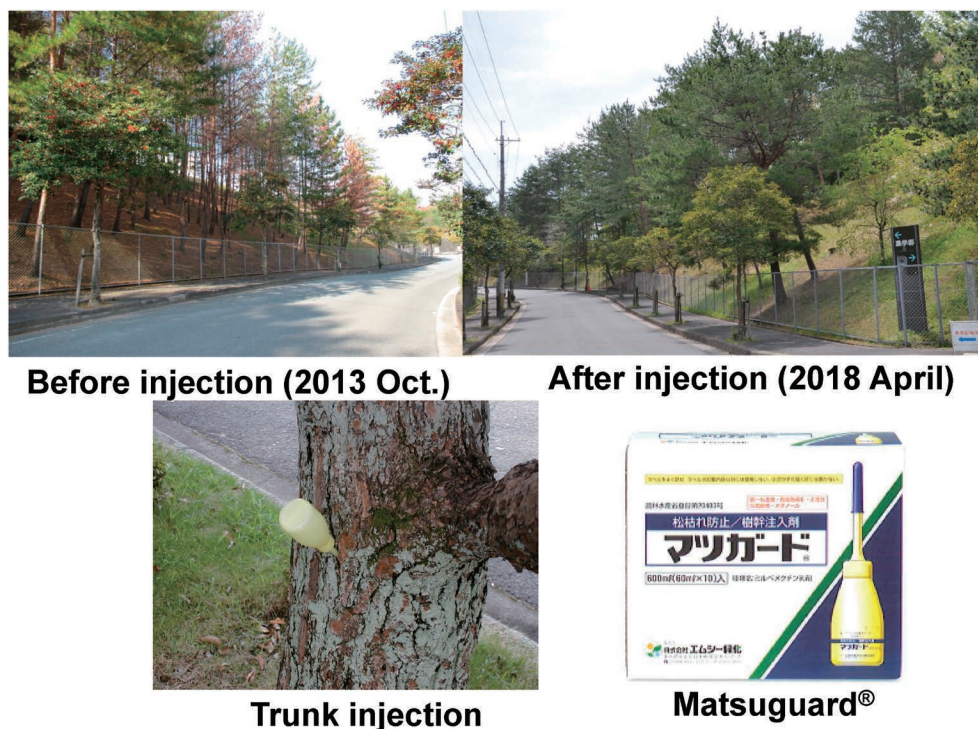


Fig. 13. Field trial of the trunk injection of Matsuguard[®] against pine wilt disease: Matsuguard[®] was injected on 2014 (Feb.).

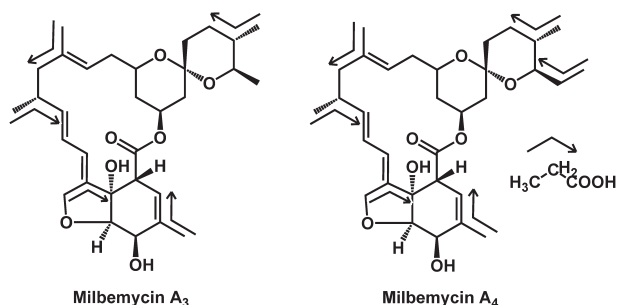


Fig. 14. Incorporation of propionate skeleton into the molecules of milbemycin A₃ and A₄.

which Matsugard[®] protected a heavily infested and seriously damaged area, the Nara campus of Kindai University, from pine wilt nematode infestation.

¹⁴C-Labeled M. A₃ and M. A₄ were prepared by the fermentation of *Streptomyces hygroscopicus* ssp. *aureolacrimosus* with ¹⁴C-labeled precursors. In order to choose an appropriate precursor for biosynthesis, comparative studies of the incorporation of ¹³C into milbemycin molecules from Na [¹³C] propionate and Na [¹³C] acetate were conducted. The incorporation rate of ¹³C into milbemycin molecules was higher with Na [¹³C] propionate than with Na [¹³C] acetate. Milbemycin A₃ and milbemycin A₄ isolated from the culture broth fed with Na [¹³C] propionate were composed of 5 and 6 propionate units, respectively (Fig. 14). The biosynthetic preparation of ¹⁴C-labeled milbemycins A₃ and A₄ was conducted by feeding Na [¹⁴C] propionate

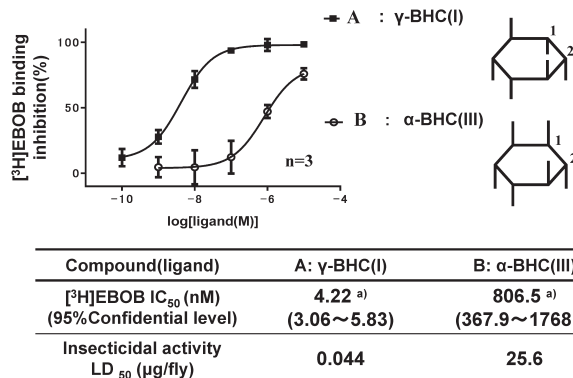


Fig. 15. Insecticidal and GABA-antagonist ([³H]EBOB binding inhibition) activities of α -BHC (III) and γ -BHC (I).

as a ¹⁴C-labeled precursor. ¹⁴C-labeled milbemycins A₃ and A₄ were extracted with ethyl acetate from the culture broth and isolated by several chromatogram purification procedures.⁴⁸⁾

Using these ¹⁴C-labeled M. A₃ and M. A₄, and ³H-synthetically labeled M. A₃ and M. A₄ at the 5-position of their molecules, the metabolic fate of milbemycin A₃ and A₄ was studied. When orally administered to male and female rats, the ³H was almost completely excreted in the urine and feces within 7 days after administration of ³H-M. A₃ or ³H-M. A₄. ³H levels remained relatively high in the fat and liver, while ³H in the tissues rapidly decreased to a very low level 7 days after administration. Concentrations of ³H and the parent compound in the tissues were lower in rats treated with ³H-M. A₃ than in those treated with

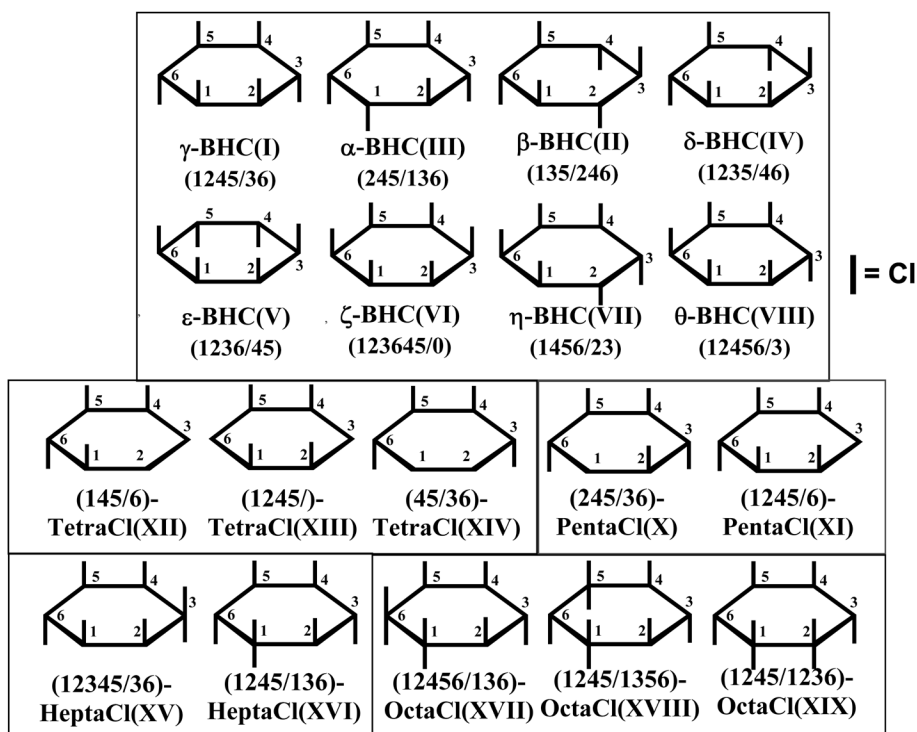


Fig. 16. Planar structures of isomers of BHC, TetraCl, PentaCl, HeptaCl, and OctaCl (see Table 12).

Table 10. Insecticidal activities against housefly (topical application LD₅₀) and GABA-antagonist activities (evaluated by [³H]EBOB binding and FMP assays) of various isomers of BHC, TetraCl, PentaCl, HeptaCl, and OctaCl

Compounds	LD ₅₀ (μg/fly)	[³ H]-EBOB binding inhibitory activity IC ₅₀ (μM) ^{a)}	FMP assay IC ₅₀ (μM) ^{b)}
γ-BHC (1245/36) (I)	0.044	0.004±0.001 (3)	0.04±0.003 (4)
α-BHC (245/136) (III)	25.6	0.81±0.26 (3)	>10 (2)
β-BHC (135/246) (II)	>10 (mortality 0% at 10 μg/fly)	>10 (3)	>10 (2)
δ-BHC (1235/46) (IV)	10.2	>10 (3)	>10 (2)
ε-BHC (1236/45) (V)	>10 (mortality 0% at 10 μg/fly)	>10 (3)	>10 (2)
<hr/>			
(145/6)-TetraCl (XII)	2.69	>10 (3)	Not tested
(1245/-)-TetraCl (XIII)	>10 (mortality 0% at 10 μg/fly)	>10 (3)	>10 (2)
(45/36)-TetraCl (XIV)	>10 (mortality 0% at 10 μg/fly)	>10 (3)	>10 (2)
<hr/>			
(245/36)-PentaCl (X)	0.50	0.232±0.098 (3)	0.431±0.009 (2)
(1245/6)-PentaCl (XI)	0.14	0.314±0.105 (3)	>10 (2)
<hr/>			
(12345/36)-HeptaCl (XV)	0.465	0.08±0.01 (3)	0.24±0.01 (2)
(1245/136)-HeptaCl (XVI)	11.2	0.7±0.08 (3)	>10 (2)
<hr/>			
(12456/136)-OctaCl (XVII)	18.8	>10 (3)	6.5±0.3 (2)
(1245/1356)-OctaCl (XVIII)	>10 (mortality 0% at 10 μg/fly)	>10 (3)	>10 (2)
(1245/1236)-OctaCl (XIX)	>10 (mortality 0% at 10 μg/fly)	>10 (3)	>10 (2)

^{a)} Data are expressed as means±SD of three experiments, each experiment involving three determinations. ^{b)} Data are expressed as means±SD of two or four experiments, each experiment involving three determinations.

³H-M. A₄, suggesting M. A₃ was metabolized faster than M. A₄.

Hydroxylation was the main metabolic pathway and various mono-, di-, and trihydroxy metabolites were formed. The main metabolites were 13-hydroxy-M. A₃ and -M. A₄. Some of these metabolites were glucuronidated. Most of these metabolites were mainly eliminated *via* bile, and a smaller amount was excreted *via* urine. Males had higher urine excretion than females, especially at low doses. There was rapid excretion in the first 24 hr followed by prolonged slow excretion, and the elimination was faster at low than at high doses. This result seemed to be reflected in the blood concentrations. No essential difference was observed between the metabolic fates of M. A₃ and M. A₄ in the rats.

5. “Why Only γ-BHC, among the Eight BHC Isomers, is Insecticidal?”: Structure–Activity Study of γ-BHC and Its Related Compounds^{52–56)}

Among the BHC isomers, γ-BHC (I) showed the most insecticidal activity against houseflies, followed by α-BHC (III) (25.6 μg/housefly) (Fig. 15). Why only γ-BHC (I), of all the BHC isomers, is insecticidal remains unknown. In the early 1970s a number of γ-BHC analogs were synthesized, in which some chlorine atoms were replaced by substitutes such as hydrogen, halogens other than chlorine, alkoxy groups, *etc.*⁵⁷⁾ Among these analogs, γ-BHC (I) was most insecticidal against the mosquito, the housefly and the German cockroach. Recently, we have focused on polychlorinated γ-BHC analogs having four to eight chlorine (Cl) atoms on the cyclohexane ring (Fig. 16), and stud-

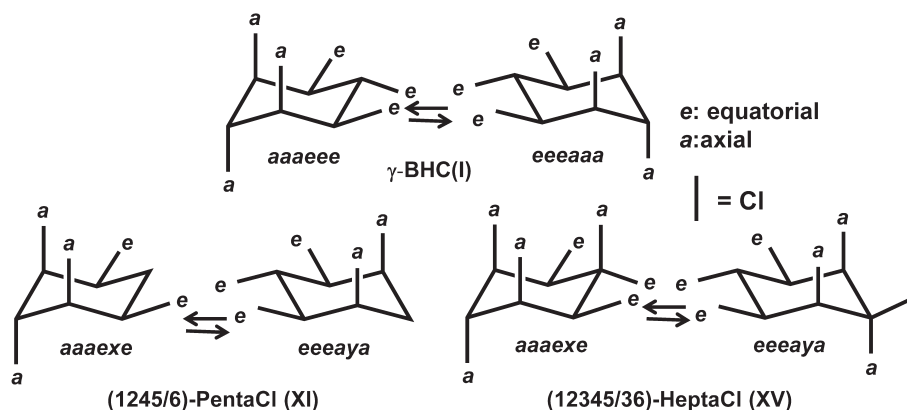


Fig. 17. Orientation (axial (a) or equatorial (e)) of chloride atoms on γ-BHC (I), (1245/6)-PentaCl (XI) and (12345/36)-HeptaCl (XV).

ied their activity not only as insecticides, but also as GABA antagonists, to elucidate the molecular requirements and the role of Cl atom(s) for γ -BHC (I) activity⁵⁶ (Table 10). Regarding the molecular requirements for activity, and in particular the structural and steric requirements on the chlorine atoms in the cyclohexane ring, this study clearly shows that 6- (or 3-) chlorine atoms substituted as three vicinal chlorine atoms in the axial conformation and three in the equatorial conformation are necessary for potent GABA-antagonistic activity. The change in the orientation of one chlorine atom from axial (γ -BHC (I)) to equatorial (α -BHC (III)) at the 1-position on the cyclohexane ring drastically decreased the insecticidal and GABA antagonistic activities. The intermediate compound of γ -BHC (I) and α -BHC (III), *i.e.*, (245/36)-PentaCl (X), was less insecticidal than γ -BHC (I) but more active than α -BHC (III). The GABA-antagonist activity of this compound (X) is also intermediate between those of γ -BHC (I) and α -BHC (III).

γ -BHC (I) and α -BHC (III) induced the excitation and eventual death of the houseflies and German cockroaches. The intoxication symptoms obtained with (12345/36)-HeptaCl (XV) were also essentially identical to those of γ -BHC (I), except for the onset time. (12345/36)-HeptaCl (XV) took more time to induce the symptoms than γ -BHC (I). The difference between those two compounds must depend on their physicochemical properties, such as their hydrophobicity (*i.e.*, lipophilicity, whose numerical value is expressed as the partition coefficients of the drugs between water and a relatively nonpolar organic solvent such as 1-octanol). The passive penetration of drugs to their site of action in insects and animals depends on their physicochemical properties.

Other BHC isomers were neither excitatory nor insecticidal at the highest dosage tested, and although δ -BHC (IV) showed a weak insecticidal activity (LD₅₀, 10.2 μ g/housefly), it was a depressant of the CNS, and did not cause an excitatory effect. δ -BHC (IV) might interact allosterically with sites other than the γ -BHC binding site on the GABA receptor and act as a depressant in houseflies.

γ -BHC (I) and α -BHC (III) could be regarded as GABA antagonists; other BHC isomers (β (II)-, δ (IV)-, ϵ (V)-, and η (VII)-isomers) are not GABA antagonists. It is unknown whether ζ -BHC (VI) and θ -BHC (VIII) have GABA antagonistic activity or not, because these two isomers have not been available. However, judging from the GABA antagonistic activity of (1245/6)-PentaCl (XI) and (12345/36)-HeptaCl (XV), as shown in Table 10 and Figs. 16, 17, these isomers must be active GABA antagonists, because they have five essential chlorine atoms, *i.e.*, *aaaexe* or *eeaya*: Specifically, the ζ -isomer (VI) has *aeaeae* and the θ -isomer (VIII) has *eeaeae* or *aaaeae*, similar to γ -BHC (I), (1245/6)-PentaCl (XI) and (12345/36)-HeptaCl (XV).

Among BHC isomers, only α -BHC (III) is a chiral molecule. The structural difference between γ -BHC (I) and α -BHC (III) is the orientation of one Cl atom, respectively axial and equatorial in γ -BHC (I) and α -BHC (III). This difference in orientation is crucial for their insecticidal and GABA-antagonist activities. The

α -BHC's insecticidal and GABA-antagonist activities are less potent than those of γ -BHC(I). Therefore, only one Cl atom orientation change on a BHC molecule results in such a large variation of GABA-antagonist activity. On the other hand, there is no difference in activity between the α -BHC (III) enantiomers. This result clearly implies that the pore of the chloride ion channel of the GABA receptor, which is the target site of α -BHC (III), does not recognize the chirality of its enantiomers. On the other hand, the metabolic enzyme (the supernatant fraction of housefly homogenate in the presence of glutathione) recognizes the chirality of α -BHC (III). The (–) enantiomers of α -BHC (III) and its dehydrochlorinated metabolite were metabolized enantioselectively compared to their (+) counterparts in *in vivo* and *in vitro*.³⁷

γ -BHC (I) exerts its insecticidal activity by binding to the insect GABA receptor in the nervous system. The GABA receptor is a member of LGIC family, which is present in the nervous system of insects and mammals.³ Insects can become resistant to γ -BHC and cyclodienes due to a single point mutation (alanine to serine) in the ion channel lining of the transmembrane 2 (TM2) region.^{43–45} This point mutation confers target-site insensitivity, and hypothetically could reveal the primary binding site in the channel pore. γ -BHC (I) could bind inside the channel pore in the TM2 region of the GABA receptor. From the insecticidal and GABA-antagonistic activities of γ -BHC (I) and its related compounds, γ -BHC (I) was confirmed to be the most insecticidal compound, with the most potent GABA-antagonistic activity. Judged from the structure–activity relation of γ -BHC (I) and α -BHC (III), the equatorial chlorine atom at the 1-position on the cyclohexane ring seems to cause steric hindrance preventing the binding on the inside pore of the chloride ion channel of the GABA receptor, and thus to reduce its GABA-antagonistic and insecticidal activities.

Among the BHC-related compounds tested, γ -BHC (I) has the molecular structure most suitable to fit inside the pore of the chloride ion channel of the GABA receptor of the housefly and rat brain. The internal structure of the pore must not be able to recognize the chirality of enantiomers.

Conclusion

Hexadeuterated γ -BHC (γ -BHC-*d*₆) is a powerful tool to study the metabolism and mode of action of γ -BHC. The first step in the metabolism of γ -BHC in mammals and insects is C–H bond cleavage. Though BHC isomers are listed as POPs they are relatively easily metabolized in mammals and insects to mainly 2,4,6-TCP. Even β -BHC, which is known to be extraordinarily stable under severe alkaline conditions, is found to be easily metabolized. It can be concluded from this study that the highly stereospecific recognition of γ -BHC by the target site could be the answer to the question, “Why only γ -BHC among the eight BHC isomers is insecticidal?”

Though γ -BHC and dieldrin are now legacy insecticides, it is worth studying in detail the unsolved issues of their modes of action and metabolism. The new findings thus obtained will contribute to the development of the science of pesticides.

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