Studies on the metabolism, mode of action, and development of insecticides acting on the GABA receptor

Keiji Tanaka*

Kindai University, Faculty of Agriculture, Naka-machi, Nara, Nara 631–8505, Japan

(Accepted December 9, 2018)

γ-BHC and dieldrin are legacy insecticides that were extensively used after the second World War. When they were banned, their modes of action and metabolism were not known. This article aims at providing a picture of the metabolism of γ-BHC and the modes of action of γ-BHC and dieldrin. γ-BHC is metabolized via two independent metabolic pathways. One is a glutathione conjugation pathway resulting in the formation of dichlorophenyl mercapturic acid and the other is an oxidative metabolism catalyzed by microsomes to mainly 2,4,6-trichlorophenol (TCP) and (36/45)-1,2,3,4,5,6-hexachlorocyclohex-1-ene (HCCHE). Other metabolites of this pathway are 2,4,5-TCP, 2,3,4,6-tetrachlorophenol (TeCP), (36/45)- and (346/5)-1,3,4,5,6-pentachlorocyclohex-1-enes (PCCHE). Nowadays, γ-BHC and dieldrin are very important reagents which are used to study the GABA receptor in insects and mammals. They were found to be noncompetitive GABA antagonists blocking the chloride ion selective pores in the GABA-gated chloride channels and leading to inhibition of chloride ion conductance. [3H]EBOB binding data showed that γ-BHC, its analogs, dieldrin, and other cyclodiene insecticides interact with the same site on GABA receptor as picrotoxinin. Only γ-BHC, among other BHC isomers, exhibits this binding characteristic. Milbemycin, currently widely used as an insecticide, acaricide and nematicide, has been found to open the GABA-gated chloride channel. © Pesticide Science Society of Japan

Keywords: GABA receptor chloride ion channel, deuterium isotope effect, γ-BHC, dieldrin, picrotoxinin, milbemycin.

Introduction

Four major neuroactive targets of insecticides are known: acetylcholine esterase, nicotinic acetylcholine receptor, γ-aminobutyric acid (GABA) receptor, and Na⁺ channel. Among them, the GABA receptor (GABA receptor chloride ion channel complex) is generally accepted to be one of the most important targets of insecticides and nematicides. The involvement of the GABA receptor as the site of insecticide action was first reported by Matsumura and colleagues, who observed the close cross-resistance of γ-BHC*1 (benzene hexachloride) and dieldrin resistant insects to picrotoxinin, a GABA-receptor non-competitive antagonist1,2) (Table 1). This observation encouraged to conclude that the primary target of γ-BHC and cyclodiene insecticides is the GABA receptor.

Table 1. Cross resistance of dieldrin, γ-BHC and picrotoxinin against susceptible strain (CSMA) and two resistant strains (FRPP and LPP) of the German cockroach

| Strain | Dieldrin | γ-BHC | Picrotoxinin |
|--------|---------|-------|-------------|
| CSMA   | 6.4     | 0.23  | 0.11        |
| FRPP   | 33.6    | 0.95  | 0.70        |
| LPP    | 120.0   | 2.17  | 5.11        |

* To whom correspondence should be addressed.

E-mail: tkeiji@nike.eonet.ne.jp

Published online January 24, 2019

© Pesticide Science Society of Japan

*1 BHC: BHC is a common name of a group of photochlorination products of benzene with chlorine gas under ultraviolet, e.g., UV light. BHC is the abbreviation of benzene hexachloride and it is called by various other names including 1,2,3,4,5,6-hexachlorocyclohexane, HCH and HCCH. In this article I use BHC. Theoretically, BHC has eight stereoisomers (see Fig. 16). Among seven isomers of the eight ones were isolated (including one racemate), only γ-BHC isomer was found to have a potent insecticidal activity. The following abbreviations are used. BHC: 1,2,3,4,5,6-hexachlorocyclohexane; TetraCl: 1,2,3,4- or 1,2,4,5-tetrachlorocyclohexane; PentaCl: 1,2,3,4,5-pentachlorocyclohexane; HeptaCl: heptachlorocyclohexane; OctaCl: octachlorocyclohexane; PCCHE: 1,3,4,5,6-pentachlorocyclohex-1-ene; HCCHE: 1,2,3,4,5,6-hexachlorocyclohex-1-ene; BTC: 3,4,5,6-tetrachlorocyclohex-1-ene; TCB: trichlorobenzene; TCP: trichlorophenol; TeCP: tetrachlorophenol

© Pesticide Science Society of Japan

* © Pesticide Science Society of Japan

Table 1. Cross resistance of dieldrin, γ-BHC and picrotoxinin against susceptible strain (CSMA) and two resistant strains (FRPP and LPP) of the German cockroach

| Strain | Dieldrin | γ-BHC | Picrotoxinin |
|--------|---------|-------|-------------|
| CSMA   | 6.4     | 0.23  | 0.11        |
| FRPP   | 33.6    | 0.95  | 0.70        |
| LPP    | 120.0   | 2.17  | 5.11        |

a) Median lethal time, film contact method. b) Median lethal dose, topical application method. c) Median lethal dose, injection method.
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. The major receptor in the brain is a heteropentamer made of the combination of two α1, two β2 and one γ2 subunits. On the other hand most insect GABA receptors are reported to be homopentamers of α1 or α2.

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 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 nematicide. 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

|                | LD50 (±15% 10⁻⁴ mmole/Insect) |            |            |            |            |            |
|----------------|--------------------------------|------------|------------|------------|------------|------------|
|                | Mos.¹                       | F. fly²     | H. fly³    | H. fly⁴    | G. cock⁵   | A. cock⁶   |
| γ-BHC (H)      | 1.3                          | 6.53       | 816        | >2200      | 16.0       | 200        |
| γ-BHC-d6 (D)   | 0.32                         | 2.06       | 68         | 86.2       | 1.95       | 24.5       |
| H/D⁸           | 4.06                         | 3.17       | 11.76      | >25        | 8.21       | 8.16       |

¹ Culex pipiens. (3–5 days) female adult: Topical application. ² Musca domestica (SNAIDM strain). (4 days) female adult: Topical application. ³ Musca domestica (Toichi strain). (4 days) female adult: Topical application. ⁴ Musca domestica (3rd-Yumenoshima strain). (4 days) female adult: Topical application. ⁵ Blattella germanica. (14 days) male adult: Topical application. ⁶ Periplaneta Americana. (60 days) male adult: Injection. ⁷ H/D=LD50 (γ-BHC/γ-BHC-d6).

B. Insecticidal activity and synergistic effect: LD50 (10⁻⁴μmol/Insect)

|                | LD50 (±15% 10⁻⁴ μmol/Insect) |            |            |            |            |            |
|----------------|--------------------------------|------------|------------|------------|------------|------------|
|                | Mos.                              | F. fly      | H. fly      | H. fly     | G. cock    | A. cock    |
| γ-BHC (H)      | 1.3                              | 0.52       | 2.50       | 12.7       | 1.65       | 7.7        |
| γ-BHC-d6 (D)   | 0.32                             | 0.195      | 1.64       | 1.08       | 0.91       | 1.19       |
| H/D⁸           | 4.06                             | 2.67       | 11.76      | 11.76      | 1.81       |

¹ Topical LD50. ² Piperonyl butoxide (Mos.: 0.25μL of 0.1%, H. fly: 0.5μL of 0.5%). ³ Synergistic ratio: LD50 (alone)/(LD50 (+p.b.)). ⁴ H/D=LD50 (γ-BHC/γ-BHC-d6).
The preparation of two types of radiolabeled compounds, \[^{14}\text{C}\]-labeled and \[^{3}\text{H}\]-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 \[^{14}\text{C}\]-labeling in their molecular skeletons is very hard. For the preparation of \[^{14}\text{C}\]-labeled milbemycins, the biosynthetic approach using \[^{14}\text{C}\]-propionate as a precursor was tried. In this paper, I will describe this attempt.

1. Deuterium Isotope Effect of \(\gamma\)-BHC-\(d_6\) on Its Insecticidal Activity and Metabolic Rate\(^6\)\(^-7\)

This study started from an interesting finding that hexadeuteriated \(\gamma\)-BHC (\(\gamma\)-BHC-\(d_6\), \(\text{C}_6\text{D}_6\text{Cl}_6\), Fig. 1) is several times more insecticidal than “regular” \(\gamma\)-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 \(\gamma\)-BHC in detail, \(\gamma\)-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 \(\gamma\)-BHC and \(\gamma\)-BHC-\(d_6\) inside the insect body were the same. If the C–H bond(s) cleavage of \(\gamma\)-BHC (e.g., dehydrochlorination and/or dehydrogenation) is the first and rate-limiting step of its degradation, the metabolic detoxification of \(\gamma\)-BHC-\(d_6\) must be slower than that of its counterpart, \(\gamma\)-BHC. Therefore, there must be some difference in their insecticidal activities. Highly hexadeuteriated \(\gamma\)-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 \(\gamma\)-BHC in the central nervous system is the main cause of its convulsive action and lethality\(^9\)\(^\)\(^10\)\)\) (Fig. 2). \(\gamma\)-BHC and \(\gamma\)-BHC-\(d_6\) exhibited equivalent neuroexcitatory activities in terms of minimum effective concentration causing after-discharge (MEC\(_{\text{AD}}\)), as shown in Table 3. In this experiment, isolated nerve cords were dipped in a saline solution containing \(\gamma\)-BHC or \(\gamma\)-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 confirmed\(^{11}\) by two following up experiments: The radio labelled ligand [\(^{3}\text{H}\)]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 \(\gamma\)-BHC first showed uncoor-
dinated 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 isotope 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 insects (5) (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, a strain of houseflies highly resistant to various insecticides, these insects were studied using γ-BHC-d_{6}. 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 conjugation.

**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

| Compounds          | Strain of H. fly: LD_{50} (×10^{-10} mol/fly) | 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         |          |

*24 hr mortality after topical application. The standard error of the listed mean value was less than 15% of each value. 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 (±20%)^{a, b} |
|-----------------------|----------------------------------|
|                       | Insect surface | Insect internal^{c} | Container | Total amount recovered |
| 3rd-Yumenoshima       | 24.0          | 60.5               | 5.0       | 89.5                 |
| NAIDM                 | 22.5          | 66.5               | 5.5       | 94.5                 |

*Topical application of γ-BHC-d_{6} in aceton: 2×10^{-6} mol/0.5 μL/fly. ^{b} Average values of three experiments. Each experiment: SE, ±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 (±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                  |

*Average values of three experiments. Each experiment: 5 female flies, applied with γ-B-HC or γ-BHC-d_{6} (5×10^{-6} mol/0.5 μL of acetone solution/fly): SE, ±15% of each value. ^{b} Extracts of the insect body homogenate.
tion. In both strains, significant isotope effects were observed in the degradation rates in vitro of γ-BHC-d6. Therefore, the principal biodegradation and detoxification pathways should include reactions cleaving the C–H bonds. When the much less biodegradable γ-BHC-d6 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

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-glutathiones (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-pentachlorocylohex-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. Reed and Forgash reported the presence of (36/45)-PCCHE and “iso-PCCHE” in the hexane-soluble metabolites of γ-BHC-treated houseflies. However, Clark et al. questioned the role of PCCHE isomers as major intermediates of the aromatic products from γ-BHC in insects. In the presence of p,p′-tetra-methyldiaminodiphenyl methane, an inhibitor of the metabolism of PCCHE isomers, they observed a significant disappearance of γ-BHC. From the results of an isotope ([14C]γ-BHC) dilution technique, Bridges suggested that (36/45)-PCCHE is a minor metabolite and is not associated with the main metabolic pathway.

2.1. Oxidative metabolism

Various mono- and polychlorophenols have been identified as urinary metabolites of γ-BHC and its isomers (α, β and δ-BHC) in mammals. Among them, 2,4,6-TCP (trichlorophenol) is a common major metabolite of these BHCs. 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) was as one of the metabolites of γ-BHC in rats, but it was reported to be hardly metabolized in vitro to chlorophenols. Various mono- and polychlorobenzenes, which are also metabolites of γ-BHC and

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

* 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.
its isomers, have been considered precursors of various chlo-
rophenols by many investigators.\textsuperscript{28,29) Among the three TCB (trichlorobenzene) isomers, only 1,3,5-TCB\textsuperscript{29) gave exclusive-
ly 2,4,6-TCP as a metabolite. However 1,3,5-TCB was a quite
minor metabolite of $\gamma$-BHC.\textsuperscript{19) Based on these data, the exis-
tence of completely unknown metabolic routes or intermediates
to 2,4,6-TCP was postulated for the metabolism of $\gamma$-BHC and
its isomers.\textsuperscript{16,17)}

In $\gamma$-BHC-treated houseflies, a cis-dehydrogenated me-
tabolite (36/45)-HCCHE (1,2,3,4,5,6-hexachlorocyclo-
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 $O_2$ the microsomal fraction of housefly, rat and
mouse livers converted $\gamma$-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 phos-
phate) and molecular oxygen ($O_2$), rat liver and housefly mi-
crosomes metabolized $\gamma$-BHC and its isomers ($\alpha$, $\beta$, $\delta$ and $\epsilon$),
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 $\delta > \epsilon > \alpha > \gamma > \beta$. Beside 2,4,6-TCP, $\gamma$-BHC
gave (36/45)-HCCHE and (36/45)-PCCHE. $\alpha$- and $\delta$-BHC
gave (346/5) and (35/46)-PCCHE, respectively, but no detect-
able amount of dehydrogenated metabolites (HCCHE). $\beta$-BHC
did not yield any PCCHE or HCCHE. In a similar reaction pro-
cedure, all of five PCCHE isomers and four HCCHE isomers
gave 2,4,5-TCP and 2,3,4,6-TeCP (tetrachlorophenol), respec-
tively, 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 $\gamma$-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.\textsuperscript{15–17) The
possible candidate intermediate of BHC metabolism is penta-
chlorocyclohexanone-$\gamma$-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, ex-
plains very well the fairly large isotope effect (H/D, about 10–11)
oberved in this phenol formation, and the predominant pro-
duction of this phenol from all five BHC isomers, including the
extremely stable $\beta$-BHC (Table 5).
The oxidative chemical reaction of four pentachlorocyclo-
hexanol isomers with CrO$_3$, converting C–OH to C=O, produced
only 2,4,6-TCP as the main product in good yields, instead of
their pentachlorocyclohexanone isomers.\textsuperscript{17) Pathways leading to other terminal metabolites such

![Production of 2,4,6-TCP: Oxygenation by Cyt.P450](image)

![Production of 2,4,5-TCP and 2,3,4,6-TeCB](image)

Table 5. 2,4,6-TCP formation from BHC isomers by rat liver micro-
somes in the presence of NADPH and $O_2$

| Substrates\textsuperscript{a)} (400×10$^{-10}$ mol) | 2,4,6-TCP: Major phenolic metabolite (×10$^{-10}$ mol) |
|----------------|-------------------------|
| $\alpha$-BHC(III) | 10.6 |
| $\beta$-BHC(II) | 4.6 |
| $\gamma$-BHC(I) | 8.3 |
| $\delta$-BHC(IV) | 127.5 |
| $\epsilon$-BHC(V) | 45.6 |

\textsuperscript{a)} Incubation time: 15 min.

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.
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 sp3-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, accompanied by double bond migration. With BTC isomers, similar ene-like hydroxylation is observed. (346/5)-BTC affords mainly the (36/45)- and (346/5) isomers of tetrachlorocyclohex-1-en-3-ol, showing selective oxygen attack on one of the sp2-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-trichlorocyclohex-1-en-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-1-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,
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 decomposed 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 NADPH (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-6 with the post-microsomal
fraction of the housefly.51 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-d5 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.51 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).

(36/45)-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 (36/45)-PCCHE. The (−) enantiomer of α-BHC was metabolized faster than the (+) one both in vivo and in vitro. One of the (36/45)-PCCHE enantiomers, the (−) isomer, was

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

| Sucrose density gradient fraction (M) | Binding (dpm/mg/protein) |
|-------------------------------------|--------------------------|
|                                     | Total                    | Specific                  |
| 0.8                                 | 13777±104                | 720±186                  |
| 0.8–1.0                             | 15862±359                | 720±102                  |
| 1.0–1.2                             | 16688±104                | 1783±66                  |
| 1.2–1.5                             | 12344±45                 | 605±10                   |
| 1.5–1.8                             | 10072±630                | 553±417                  |
| 1.8                                 | 11970±1061               | —                        |

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-Dihydropicrotoxin binding (sp act, 30 Ci/mmol), 11.1×10−4 M. Data are expressed as means±S.E. of two of three experiments, each experiment involving three determinations. c) Not tested.

Fig. 10. In vivo and in vitro metabolism of enantiomers of α-BHC and in vitro metabolism of enantiomers of (36/45)-PCCHE.
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) 

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

| Agents        | %Inhibition of [3H]α-DHPTX specific binding |
|---------------|---------------------------------------------|
| Dihydropicrotoxinin | 100                                        |
| Aldrin        | 36.6±0.6                                    |
| Dieldrin(XX)  | 58.3±2.3                                    |
| Photodieldrin | 79.0±4.3                                    |
| Heptachlor    | 36.6±18.6                                   |
| Heptachlorepoxide | 57.3±31.3                                  |
| Isodrin       | 35.6±3.8                                    |
| Endrin        | 62.3±12.4                                   |
| γ-Chlordane   | 56.2±2.0                                    |
| Oxychlordane  | 77.8±6.2                                    |
| Heichlorocylo-pentadiene | 0.3±2.2                                  |
| Mirex         | 14.7±15.7                                   |
| Kepone        | 135.5±18.4                                  |
| Toxaphene     | 72.3±28.1                                   |
| DDT           | 5.4±3.6                                     |
| Parathion     | 4.1±26.3                                    |
| α-BHC(III)    | 21.4±18.5                                   |
| β-BHC(II)     | 15.3±15.4                                   |
| γ-BHC(I)      | 91.5±20.0                                   |
| δ-BHC(VI)     | 55.0±16.0                                   |
| Allethrin     | 13.2±6.7                                    |
| Cypermethrin  | 7.7±3.1                                     |
| Decamethrin   | −0.1±1.0                                    |
| Fenvarelate   | −0.4±5.8                                    |
| Tetrathionate | 27.1±13.0                                   |
| t-Butylbicyclophosphorothionate | 27.1±13.0                    |

Table 8. Time to onset of poisoning symptoms in the abdominal nerve cord of susceptible (CSMA) and resistant (LPP) German cockroaches45)

| Strain of G. cockroach | Minute to onset of poisoning symptoms45) |
|------------------------|------------------------------------------|
| CSMA                   | 26.5±3.7                                 |
| LPP                    | 74.0±6.7                                 |

Table 8. (Continued)

| Agents        | %Inhibition of [3H]α-DHPTX specific binding |
|---------------|---------------------------------------------|
| α-BHC(III)    | 21.4±18.5                                   |
| β-BHC(II)     | 15.3±15.4                                   |
| γ-BHC(I)      | 91.5±20.0                                   |
| δ-BHC(VI)     | 55.0±16.0                                   |
| Allethrin     | 13.2±6.7                                    |
| Cypermethrin  | 7.7±3.1                                     |
| Decamethrin   | −0.1±1.0                                    |
| Fenvarelate   | −0.4±5.8                                    |
| Tetrathionate | 27.1±13.0                                   |
| t-Butylbicyclophosphorothionate | 27.1±13.0                    |

43) [3H]α-dihydropicrotoxinin, 11.1 nM; five American cockroach heads were used for one experiment, which involved three determinations. 44) Dihydropicrotoxinin, 100 μM; others, 10 μM. 45) Data are expressed as means±S.E. of two or three experiments, each experiment involving three determinations.

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 (○). Bmax, receptor number (mole/mg protein) was CSMA, 9.0×10−13; LPP, 7.1×10−14. The dissociation constant Kd (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.
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 \( ^{3}H \)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)2S(serine) or A2G(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 diel- drin.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-

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

| Compounds               | \( n \) | \(^{36}\)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\(^{-4}\) M)     | 8       | 104.18±5.92                   |
| Avermectin B1a (10\(^{-7}\) M)+GABA (10\(^{-4}\) M) | 8         | 116.30±8.22                   |

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

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

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

#### Fig. 12. Experiment of Cl\(^{-}\) uptake by the leg of muscles of the American cockroach.

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 spiro-ketal 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 Milbemeclock\(^{©}\), Koromite\(^{®}\) and Matsuguard\(^{®}\). Figure 13 shows a very effective preventive trial, in

---

**Fig. 12.** Experiment of Cl\(^{-}\) uptake by the leg muscles of the American cockroach.

**Fig. 13.** Field trial of the trunk injection of Matsuguard\(^{®}\) against pine wilt disease: Matsuguard\(^{®}\) was injected on 2014 (Feb.).
which Matsuguard® 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 [1-13C] propionate and Na [1-13C] acetate were conducted. The incorporation rate of 13C into milbemycin molecules was higher with Na [1-13C] propionate than with Na [1-13C] acetate. Milbemycin A3 and milbemycin A4 isolated from the culture broth fed with Na [1-13C] 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 [1-14C] propionate 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 administrated 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. 14.** Incorporation of propionate skeleton into the molecules of milbemycin A3 and A4.

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

---

| Compound (ligand) | A: γ-BHC (I) | B: α-BHC (III) |
|-------------------|--------------|----------------|
| [3H]EBOB IC50 (nM) | 4.22 ± 0.1 | 808.5 ± 400.0 |
| 95% confidential level | (3.06~5.83) | (367.9~1768.0) |

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

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. 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-
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) activity.30 (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 µg/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., aaeae or eceae. Specifically, the ζ-isomer (VI) has aaeae and the θ-isomer (VIII) has eceae or eaeae, 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-BHCs 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 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.41–45 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**

Hexadeuteriated γ-BHC (γ-BHC-d6) 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.
Acknowledgements

I express my sincere acknowledgement to Professors Minoru Nakajima, Norio Kurihara and Toshio Fujita of Kyoto University, Professor Fumio Matsumura of Michigan University (later University of California, Davis), Professor Kazuhiro Matsuda of Kindai University, Professor Yoshihisa Ozoe of Shimane University, Professor Arata Katayama of Nagoya University, Associate Professor Miki Akamatsu of Kyoto University, Mr. Shogo Takahashi and Dr. Mitsuo Ishida of Sankyo Co. Ltd., and Dr. Kiyoshi Arai of Mitsuil Chemical Agro Co. Ltd., for their various encouragements.

All of the studies introduced in this review were done at the following four laboratories, 1. RI Research Center (Professor N. Kurihara) and Graduate School of Agriculture (Professors, M. Nakajima and T. Fujita), Kyoto University, 2. Pesticide Research Center (Professor F. Matsumura), Michigan State University, 3. Agrochemical Research Laboratories (Directors Mr. S. Takahashi and Dr. M. Ishida), Sankyo Co. Ltd., and (Dr. K. Ari) Mitsuil Chemical Agro Co. Ltd., 4. Laboratory of Professor K. Matsuda, Faculty of Agriculture, Kindai University; Laboratory of Professor Y. Ozoe, Faculty of Life and Environmental Science, Shimane University; Laboratory of Professor A. Katayama, Institute of Materials Systems for Sustainability (iMaSS), Nagoya University. They have given me an opportunity and place to pursue my research.

References

1) F. Matsumura and S. M. Ghiasuddin: J. Environ. Sci. Health B 18, 1–14 (1983).
2) A. A. Kadous, S. M. Ghiasuddin, F. Matsumura, J. G. Scott and K. Tanaka: Pestic. Biochem. Physiol. 3, 77–86 (1973).
3) Y. Ozoe: Adv. Insect Physiol. 44, 211–286 (2013).
4) J. Vijgen, P. C. Abhilash, Y. F. Li, R. Lal, M. Forter, J. Torres, N. Singh, M. Yunus, C. Tian, A. Schaeffer and R. Weber: Environ. Sci. Pollut. Res. Int. 18, 152–162 (2011).
5) K. Tanaka, N. Kurihara and M. Nakajima: Pestic. Biochem. Physiol. 6, 386–391 (1976).
6) K. Tanaka, N. Kurihara and M. Nakajima: Pestic. Biochem. Physiol. 6, 392–399 (1976).
7) K. Tanaka: PhD. Thesis, Kyoto University (1979).
8) R. E. Slade: Chem. Ind. 40, 314–319 (1945).
9) M. Uchida, Y. Irie, N. Kurihara, T. Fujita and M. Nakajima: Pestic. Biochem. Physiol. 5, 258–264 (1975).
10) T. Yamasaki and T. Ishii: Bochu-Kagaku 19, 106–112 (1954).
11) K. Tanaka: Symposium “Fifty Years of Research and Mentoring: Symposium in Honor of the Life and Career of Professor Fumio Matsumura,” Inhibitory Chloride Channels as Targets for Lindane and its Analogs. Abstract No. Agro 18, ICPC (International Conference of Pesticide Chemistry) 2014 in SF (San Francisco).
12) T. Nakao, A. Naoi, N. Kawahara and K. Hirase: Pestic. Biochem. Physiol. 97, 262–266 (2010).
13) K. Yasutomi: Ipn. J. Sanit. Zool. 26, 257–258 (1975) (in Japanese).
14) K. Tanaka, N. Kurihara and M. Nakajima: Pestic. Biochem. Physiol. 16, 149–157 (1981).
15) K. Tanaka, N. Kurihara and M. Nakajima: Pestic. Biochem. Physiol. 10, 79–95 (1979).
16) K. Tanaka, N. Kurihara and M. Nakajima: Pestic. Biochem. Physiol. 10, 96–103 (1979).
17) K. Tanaka, N. Kurihara and M. Nakajima: Agric. Biol. Chem. 41, 723–725 (1977).
18) J. Sternburg and C. W. Kearns: J. Econ. Entomol. 49, 548–552 (1956).
19) W. T. Reed and A. J. Forgash: Science 160, 1232 (1968).
20) A. G. Clark, S. Murphy and J. N. Smith: Biochem. J. 113, 89–96 (1969).
21) G. Bridges: Nature 184(Suppl 17), 1337–1338 (1959).
22) J. J. Freal and R. W. Chadwick: J. Agric. Food Chem. 21, 424–427 (1973).
23) J. C. Karapally, J. G. Saha and Y. W. Lee: J. Agric. Food Chem. 21, 811–818 (1973).
24) N. Kurihara and M. Nakajima: Pestic. Biochem. Physiol. 4, 220–231 (1974).
25) R. W. Chadwick and J. J. Freal: Bull. Environ. Contam. Toxicol. 7, 137–146 (1972).
26) R. W. Chadwick, L. T. Chuang and K. Williams: Pestic. Biochem. Physiol. 5, 575–5 (1975).
27) P. L. Grover and P. Sims: Biochem. J. 96, 521–525 (1965).
28) G. T. Brooks: “Chlorinated Insecticides,” Vol. 2, CRC. Press, Cleaveland, Ohio, pp. 80–84, 1974.
29) W. R. Jondorf, D. V. Parke and R. T. Williams: Biochem. J. 61, 512–521 (1955).
30) K. Gollnick: Adv. Photochem. 6, 1–122 (1968).
31) M. Ishida and P. A. Dahm: J. Econ. Entomol. 58, 383–392 (1965).
32) A. G. Clark, M. Hitchcock and J. N. Smith: Nature 209, 103 (1966).
33) F. R. Bradbury and H. Standen: Nature 183, 983–984 (1959).
34) N. Kurihara, K. Tanaka and M. Nakajima: Pestic. Biochem. Physiol. 10, 137–150 (1979).
35) R. Kallenborn, H. Hühnnerfuss, W. Ilirjird and A. König: Angew. Chem. Int. Ed. Engl. 30, 320–321 (1991).
36) K. Moller, C. Bretzke, H. Hühnnerfuss, R. Kallenborn, J. N. Kinkel, J. Kopf and G. Rinkus: Angew. Chem. Int. Ed. Engl. 33, 882–884 (1994).
37) K. Tanaka: J. Pestic. Sci. 42, 49–54 (2017) (in Japanese).
38) K. Tanaka, J. G. Scott and F. Matsumura: Pestic. Biochem. Physiol. 22, 117–127 (1984).
39) F. Matsumura, K. Tanaka and Y. Ozoe: “Sites of Action for Neurotoxic Pesticides,” ACS Symposium Series 356, eds. by R. M. Hollingworth and M. B. Green, pp. 44–70, (1987).
40) F. Matsumura and K. Tanaka: “Cellular and molecular Neurotoxicology,” ed. by T. Narahashi, Raven Press, pp. 225–240, 1984.
41) K. Tanaka and F. Matsumura: “Membrane Receptors and Enzymes as Targets of Insecticidal Action,” eds. by J. M. Clark and F. Matsumura, Plenum Press, New York, pp. 33–49, 1986.
42) K. Tanaka and F. Matsumura: Pestic. Biochem. Physiol. 24, 124–135 (1985).
43) R. H. Ffrench-Constant, T. A. Rocheleau, J. C. Steichen and A. E. Chalmers: Nature 363, 449–451 (1993).
44) M. Tompson, J. C. Steichen and R. H. Ffrench-Constant: Insect Mol. Biol. 2, 149–154 (1993).
45) K. Kaku and F. Matsumura: Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 108, 367–376 (1994).
46) S. Sadakane, K. Tanaka, E. Muraoka and M. Ando: J. Pestic. Sci. 17, 147–154 (1992).
47) S. Sadakane, K. Tanaka, K. Sato and M. Ando: J. Pestic. Sci. 17, 199–203 (1992).
48) S. Sadakane, K. Tanaka and M. Ando: Annu. Rep. Snakyo Res. Lab. 48, 111–118 (1996).
49) K. Okuno and K. Tanaka: Agrochemicals Japan 78, 13–16 (2001).
50) A. Aoki, R. Fukuda, T. Nakayabu, K. Ishibashi, C. Takeichi and M. Ishida: JP 4750758 (1974).
51) J. Ide, T. Okazaki, M. Ono, A. Sato, K. Nakagawa, S. Naito, K. Sato, K. Tanaka, H. Yoshikawa, M. Ando, S. Katsumi, K. Matsumoto, T. Toyama, M. Shibano and M. Abe: Annu. Rep. Snakyo Res. Lab. 45, 1–98 (1993).
52) K. Tanaka: Pestic. Biochem. Physiol. 120, 91–100 (2015).
53) K. Tanaka, T. Tatsumi, K. Nagasaki, Y. Ozoe, K. Kuroda, K. Matsuda, N. Poster and N. Kurihara: ICPC 2014 in SF, Poster, Abstract No. Agro 685 (2014).
54) K. Tanaka, Y. Ozoe, K. Matsuda, M. Morimoto, N. Kurihara: ICPC 2014 in SF, Poster, Abstract No. Agro 686.
55) K. Nagasaki, K. Tanaka, T. Tatsumi, K. Matsuda, Y. Ozoe, N. Kurihara: ICPC 2014 in SF, Poster, Abstract No. Agro 687 (2014).
56) K. Tanaka, T. Sakamoto, T. Iwai, K. Kuroda, K. Nagasaki, Y. Ozoe, M. Akamatsu and K. Matsuda: “Ion Channels and G Protein-Coupled Receptors (GPCRs) as Targets for Pest Control Volume 2: GPCRs and Ion Channels” ACS Symposium Series 1265, eds. by A. Gross, Y. Ozoe and J. Coats, pp. 41–54 (2017).
57) M. Kiso, T. Fujita, N. Kurihara, M. Uchida, K. Tanaka and M. Nakajima: *Pestic. Biochem. Physiol.* 8, 33–43 (1978).