

Review

Biodegradation of Lindane (γ -BHC) and Its Isomers by Mammals and Insects

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Abbreviations used: BHC: benzene hexachloride i.e. hexachlorocyclohexane; PCCH: pentachlorocyclohexene; HCCH: hexachlorocyclohexene; BTC: benzene tetrachloride i.e. tetrachlorocyclohexene; GC-MS: gas chromatography-mass spectrometer; glc: gas liquid chromatograph(y); ecd: electron capture detector.

KEY WORDS: BHC/ Oxidative metabolism/ Glutathione conjugation/
Isotope effects/

INTRODUCTION

Lindane is the most potent insecticidal diastereomer among the isomers of BHC (benzene hexachloride, i.e. 1,2,3,4,5,6-hexachlorocyclohexane). Both the purified isomer and the isomeric mixture were being widely used for about 25 years as an effective insecticide in agriculture and public health service. However, they have not been used in Japan since 1970, because of its environmental contamination, and of its possible chronic toxic effects.

Metabolic fates of these compounds are interesting and important problems in xenobiotic biochemistry, and have long been studied by many entomologists, toxicologists and biochemists. Reviews on these problems have appeared.¹⁾

In this review, we do not attempt to extensively survey the literatures on metabolism studies of lindane and related compounds. Instead, we will focus the attention on the following questions:

- (1) What kind of reactions are involved in metabolic transformations of lindane in mammals and insects?
- (2) What biological systems (enzyme etc.) participate in the metabolic reactions in mammals and insects?
- (3) What chemical mechanisms operate in these metabolic reactions?

Metabolic conversions of pesticides are usually directed toward "detoxication." Although examples of "toxication" or toxicity enhancement are not rare, including classical examples of parathion to paraoxon, and aldrin to dieldrin,^{2,3)} metabolites from a pesticide in major cases show lower acute toxicity than the original compound. In this sense, lindane is a typical pesticide. Not only water-soluble glucuronides, sulfates and mercapturic acids, but also highly chlorinated and still hydrophobic metabolites such as pentachlorocyclohexenes and trichlorophenols have lower in-

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secticidal activity and lower mammalian acute toxicity than lindane. (Pentachlorophenol, one of the metabolites, is more toxic than lindane against mammals, though.)^{4,5)}

Metabolic transformation of pesticides usually converts lipophilic or hydrophobic compounds to hydrophilic ones. Elimination of chlorines from the molecule of chlorinated compounds generally makes the compounds less hydrophobic. Introduction of oxygens into the molecule to generate hydroxyl groups also converts the compounds less hydrophobic or more hydrophilic. Conjugation reactions with various carbohydrates, amino acids, oligopeptides and sulfates also convert the compounds hydrophilic. Hydrophilic compounds are easily excreted. The formation of the easily excretable metabolites is another means, by which mammals and insects can get rid of toxic hydrophobic compounds. Glutathione conjugation, chlorophenol formation, its glucuronidation and sulfate formation in the metabolic routes of BHC isomers are all such reactions.

In this review, oxygenation and its preceding steps, and glutathione conjugation will occupy most of the description. Dehydrogenation, dehydrochlorination and dechlorination as a primary step of the above reactions will be included. Toxicity change by initial reactions in the metabolism will also be dealt with. We don't precisely describe glucuronidation and sulfate formation.

Nomenclature and numbering system in this review are according to the literature.⁶⁾

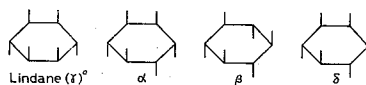
IDENTIFICATION OF METABOLITES

§ Historical

Table I shows the principal metabolites identified in the experiments using mammals and insects. In this table,

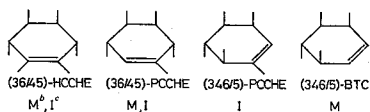
1. Both *in vivo* and *in vitro* metabolites are listed.
2. Phenols are listed as free forms, though some of them have been identified as conjugates. (There is an example in which most phenolic metabolites are identified as glucuronides and sulfates. See also Table II.)
3. Major positional isomers are indicated in parentheses.
4. The metabolites are from lindane. From other BHC isomers, similar metabolites are produced, but stereoisomeric distributions are different, and positional isomeric distribution is generally lacking in variety.

For identification and structure determination of these metabolites, we can nowadays utilize numerous means of instrumental analyses. In 1950's, when lindane metabolism study started, however, all scientists faced many difficulties in separating and identifying microquantities of metabolites. They had to use a colorimetric method of limited specificity, and to rely on data from paper chromatography and electrophoresis of unsatisfactory separation power for diastereoisomeric mixture. But, a very sensitive analytical method, isotope dilution technique (carrier-addition method), was being employed,⁷⁾ and later gradually new devices have appeared. Among them, gas chromatograph with an electron capture detector is one of the

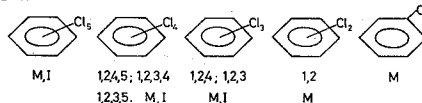
Table I. Structures of substrates and metabolites^aSUBSTRATE
BHC

METABOLITE

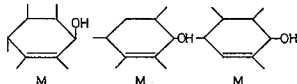
Polychlorocyclohexene



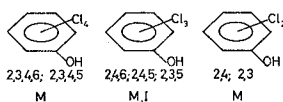
Polychlorobenzene



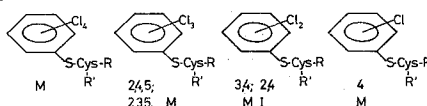
Polychlorocyclohexenol



Polychlorophenol



Polychlorophenylmercapturic acid (S-Polychlorophenyl-L-glutathione)



a A short line attaching to cyclohexane (—ene) ring represents a C-Cl bond orientation.

b M=mammals: mouse, rat, and rabbit.

c I=insects: house fly, blow fly, locust.

Table II. Major *in vivo* metabolites of lindane found in mammal urine^a

	Rabbit ^b (p.o. multiple dose)	Rat ^c (p.o. single dose)	Mouse ^d (i.p. single dose)
246-TCP ^e	4.1 %	3.8 %	14.8 %
245-TCP	4.9	1.0	+
235-TCP	4.61	0.6	—
2346-TeCP ^f	0.2	1.8	—
2345-TeCP	1.92	0.3	—
PCCOL ^g	—	+	—
23-DCP ^h	1.54	—	—
24-DCP	1.41	—	2.85
12-DCB ⁱ	1.92	—	—

a) Percentages of administered dose are shown.

b) Ref. 29. Metabolites listed here are free forms and ether-extractable.

c) Ref. 30.

d) Ref. 31. Metabolites listed here are mostly excreted as conjugates: sulfates and glucuronides.

e) TCP=trichlorophenol (e.g. 246-TCP=2,4,6-trichlorophenol)

f) TeCP=tetrachlorophenol

g) PCCOL=pentachlorocyclohexenol

h) DCP=dichlorophenol

i) DCB=dichlorobenzene

most powerful analytical instruments for organochlorine compounds. With its very high sensitivity and its versatility, this instrument has exhibited the power for identification of many metabolites such as tri-, tetra- and pentachlorobenzene, and for quantification of pentachlorocyclohexene.^{8,9,10} Development and popularization of GC-MS and related sophisticated techniques have given us much more reliable, facile and rapid identification procedure.

Some of the important metabolites in Table I have been identified with the progress of analytical methods after many arguments. We will describe some examples below.

§ (36/45)-Pentachlorocyclohexene ((36/45)-PCCHE)

Sternburg and Kearns¹¹ assumed that lindane undergoes dehydrochlorination to afford (36/45)-PCCHE, according to the discovery of the monodehydrochlorination reaction in DDT metabolism,^{12,13} and of an enzyme DDT-dehydrochlorinase¹⁴ which converts DDT to DDE. They actually reported¹¹ the presence of pentachlorocyclohexene as a lindane metabolite in house fly, based on a spectrophotometric method on colored complexes prepared by Schechter-Hornstein procedure as well as paper chromatography of the derivatives obtained by a similar procedure. The result was followed up by many scientists. Bradbury and Standen,¹⁵ and Bridges¹⁶ showed unambiguously the presence of (36/45)-PCCHE as a metabolite of [¹⁴C]-lindane in house fly by trapping the labeled metabolite with a large excess of added cold PCCHE. But, the amount of this metabolite seemed very small and many people suspected^{15,16} its importance as a lindane metabolite. Clark *et al.*¹⁷ employed blowflies and grass grubs and tested the effects of various metabolic inhibitors on lindane metabolism. They concluded according to their experimental results that (36/45)-PCCHE was not a major intermediary metabolite in the metabolic biodegradation of lindane at least in these species of insects. But, in house fly, Reed and Forgash^{8,10} later proved the presence of (36/45)-PCCHE as a lindane metabolite by means of glc-ecd and GC-MS, and reported the importance of this PCCHE as an intermediary metabolite. Further details and newer results including ours are described in later sections.

§ Glutathione conjugates

In house fly, S-dichlorophenyl-glutathione isomers were first suggested by Bradbury and Standen⁷ as metabolites of α - and γ -BHC (lindane) according to the identification of dichlorothiophenol isomers as the alkaline hydrolytic products of the lindane metabolites. They mixed the [¹⁴C]-metabolites with pure unlabeled dichlorothiophenol and treated the mixture with an aqueous alkali. Each of the dichlorothiophenol isomers was treated in this way. The product in each case was derivatized appropriately, and the derivative was purified by repeated recrystallization. By this procedure, they quantitated all isomers of dichlorothiophenol, and suggested that the metabolites were S-dichlorophenyl-glutathione isomers. Clark, Smith and their collaborators purified glutathione conjugates from the metabolite mixture in ticks, house flies and locusts (*in vivo* and *in vitro*), and identified a major

metabolite with S-2,4-dichlorophenyl-glutathione chromatographically.¹⁸⁾

House fly enzymes that catalyze glutathione conjugation of lindane and its isomers were studied by Ishida and Dahm.^{19,20)} Corresponding rat enzymes were also studied.²⁰⁾ They showed that the house fly enzyme metabolized pentachlorocyclohexene isomers to S-dichlorophenyl-glutathiones.²¹⁾ Some of the conjugates were crystallized and their compositions were determined by elemental analyses.²¹⁾

In the rat urine, 2,4-dichlorophenylmercapturic acid was excreted as one of the metabolites of (36/45)-PCCHE. Grover and Sims²²⁾ converted the mercapturic acid into a crystalline derivative and identified. They suggested that this isomer of mercapturic acid was also a principal metabolite of lindane based on its paper chromatographic behavior.

In these studies described above, separation and identification of the compounds were mostly performed by means of recrystallization, paper chromatography and paper electrophoresis. These techniques are very useful in general but are not very satisfactory as separation- and identification-tools for isomers of very similar properties. Actually, S-dichlorophenyl-glutathione (and -cysteine) isomers show very similar R_f values in their paper and thin layer chromatography.^{17,21)}

When acid-hydrolyzed, the glutathione conjugates and mercapturic acids give S-aryl-cysteines. The latter can be derivatized to volatile compounds which are analyzed by gas chromatography.²³⁾ Dichlorothiophenols and their derivatives, which are the products of alkaline hydrolysis of glutathione conjugates (and mercapturic acids), can also be analyzed by gas chromatography.^{23,24,25)} Gas chromatography shows a much higher resolution than usual thin layer-, paper- and liquid-chromatography, and has recently been used for identifying the sulfur containing BHC metabolites. Various glutathione conjugates are now identified by these means. On this subject, we describe later in detail.

§ Chlorophenols

Investigation with rat by Grover and Sims²²⁾ clearly proved the presence of 2,4,5-trichlorophenol as a metabolite of (36/45)-PCCHE. 2,3,5-Trichlorophenol was also suggested as a metabolite. But the phenolic metabolites from lindane were examined only by paper chromatography, and further studies were expected. By using rabbit liver microsomes, Nakatsugawa and his collaborators²⁶⁾ demonstrated the metabolism of (36/45)- and (35/46)-PCCHE, and the reaction was suggested as an oxidative one. Later, such *in vitro* studies became an important clue to solve the chlorophenol formation route from lindane.

Phenolic metabolites in house flies were suggested by Bradbury and Standen²⁷⁾ in 1958, but newer reports on this type of metabolites in insects have begun to appear in 1970's.²⁸⁾ These subjects are described in the chapter "Oxidative Metabolism."

OXIDATIVE METABOLISM

§ Construction of metabolic pathways

As shown in Table I, chlorophenols are one of the most important metabolite

groups. Experiments using mammals have shown that various chlorophenols other than the previously expected 2,4,5-isomer are also excreted in urine when lindane is administered.

A major portion of the results obtained in rabbit,²⁹⁾ rat³⁰⁾ and mouse³¹⁾ is summarized in Table II. There are several difficulties when we try to construct the metabolic pathway, especially that leading to the following compounds.

- (1) 2,4,6-Trichlorophenol
 - (2) 2,3,4,6-(and 2,3,4,5)-Tetrachlorophenol
 - (3) Dichlorophenols and dichlorobenzenes
- (1) The 2,4,5- and 2,3,5-isomer of trichlorophenol can be derived from 1,2,4-trichlorobenzene by oxidative metabolism.³²⁾ And 1,2,4-trichlorobenzene is the most abundant product of alkaline degradation of lindane and other BHC isomers.³³⁾ Thus, metabolic dehydrochlorination of lindane probably by glutathione participation and/or by microsomal fraction also can yield 1,2,4-trichlorobenzene as a major metabolite. Though, of course, we must prove this assumption experimentally, the 2,4,5- and 2,3,5-isomer of trichlorophenol are not unexpected metabolites. For producing 2,4,6-trichlorophenol by a similar pathway, 1,3,5-trichlorobenzene was once assumed as the most probable candidate, but the formation of this trichlorobenzene from lindane is very unlikely. How can we explain, then, the abundant formation of 2,4,6-trichlorophenol?
- (2) Are tetrachlorophenols formed from tetrachlorobenzenes? Are there other pathways? Dehydrogenation reaction is presumed to occur on the way to these metabolites from lindane: On what compound and by what enzyme system does the dehydrogenation occur?
- (3) Formation of dichlorophenol and dichlorobenzene suggests that the dechlorination reaction is involved in the pathway. By what enzyme system is this reaction catalyzed?

Before discussing these problems, we will describe biological isotope effects in insects. The isotope effects were found to be a very powerful tool for solving the oxidative metabolic pathway of lindane and related compounds.

§ *Isotope effect on the insecticidal activity and on the metabolic rate*^{34,35)}

Highly deuterated benzene, D-content of which is over 99.5 %, is now readily available. After photochlorination of this compound, recrystallization and chromatographic purification of the product mixture afford each [d₆]-BHC isomer in its pure state. The gamma-isomer, namely [d₆]-lindane shows an insecticidal activity several times higher than that of undeuterated lindane. Examples are listed in Table III.^{34,35,36)}

Lindane exhibits its insecticidal activity by the neuroexcitatory action. On electrophysiological examination of the central nervous system from American cockroach, after-discharge is observed by lindane treatment.^{37,38)} This phenomenon is due to the excessive release of acetylcholine from the presynaptic membrane of the nervous system.³⁹⁾ When deuterated lindane is compared with normal lindane in its neuroexcitatory activity, there is no difference at all. Thus, their intrinsic

Table III. Insecticidal activity^a of lindane (normal and deuterated)

	Mos. ^b	House fly ^c			G. cockroach ^d	Am. cockroach ^e
		SNAIDM	Toichi	3rd-Yumenoshima		
Lindane	1.3	6.53	816	>2200	16.0	200
Lindane-[d ₆]	0.32	2.06	68	86.2	1.95	24.5
H/D	4.06	3.17	11.76	>25	8.21	8.16

a) Topical (unless otherwise noted) LD₅₀ values (10⁻¹⁰ mol/insect) are shown.

b) Mosquito: *Culex pipiens pallens* (3–5 days) female adult.

c) House fly: *Musca domestica* (4 days) female adult.

d) German cockroach: *Blattella germanica* (14 days) male adult.

e) American cockroach: *Periplaneta americana* (60 days) male adult (Injection LD₅₀ values).

[Reproduced with permission from Ref.³⁴) and K. Tanaka³⁵]

activity at the site of action is identical.³⁴⁾

The above physiological examination was performed during 2 hours-period on the isolated nerve cord. At the whole insect level, we can observe violent quiverings of the body after treating with lindane, and can determine the minimum concentration to produce this poisoning symptom within 3 hours after injection of chemicals. On this poisoning activity—we call it convulsive activity—, approximately a two-fold isotope effect is observed between [d₆]-lindane and normal lindane, when American cockroach is used.³⁴⁾ Thus a definite isotope effect is observed at the whole body level, although smaller than that on lethal activity (H/D of LD₅₀=approx. 8 for American cockroach). This suggests that the isotope effect on lethal activity is due to that on metabolic detoxication rates: the unlabeled toxicant is presumed to be metabolized and detoxified more rapidly than the deuterated one. The LD₅₀ value of normal lindane is four-fold its minimum effective concentration for convulsive activity, whereas these values for the deuterated counterpart are identical. This indicates the effective detoxication of lindane during convulsion, but not of the deuterated toxicant.

When we examine the *in vitro* metabolic rate of this pair of compounds, the disappearance of lindane is much faster than [d₆]-lindane, *e.g.* by the action of house fly soluble fraction and glutathione (Fig. 1 left).³⁴⁾

Experiments with house fly *in vivo* also show the same trend. One to one mixture of D and H-insecticide was applied on each fly and the remaining normal and deuterated lindane were individually and simultaneously analyzed by means of mass fragmentography (Fig. 2).³⁵⁾

On the biodegradation of (36/45)-PCCHE, primary isotope effect such as above on lindane metabolism is not observed. The ratio of H-D is only slightly larger than one⁴⁰⁾ (Fig. 2 and 1 right).

Isotope effects on lethal activity and metabolic disappearance rates of lindane (and other BHC isomers) show the values significant enough to suggest the involvement of cleavage of C-H(D) bond(s) at the rate-determining step and most probably at the first step in the detoxication process, since any transformation of lindane results in detoxication.

Biodegradation of Lindane and Isomers

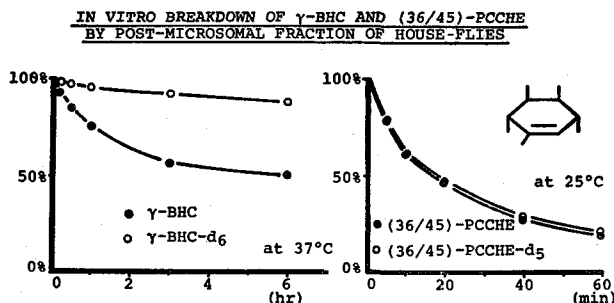


Fig. 1. Deuterium isotope effects in the *in vitro* breakdown of lindane and (36/45)-PCCHE by 105,000 $\times$ g supernatant of house flies. The reaction mixture contained in a final volume of 1 ml, 50 mM potassium phosphate (pH 7.0), supernatant (20 mg of protein), 5 mM glutathione, and one of the following substrates: 4 μ M [h_6]-lindane, 4 μ M [d_6]-lindane, 8 μ M [h_5]-PCCHE, and 8 μ M [d_5]-PCCHE. [Reproduced with permission from Ref.^{34,40}]

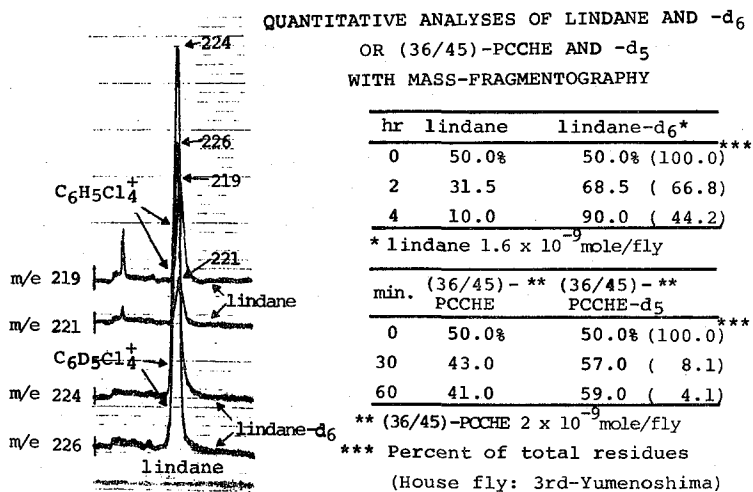


Fig. 2. Quantitative analyses of the remaining substrates in house fly (3rd-Yumenoshima: a highly resistant strain to pesticides). One to one mixture of perdeuterated chemical and nondeuterated counterpart was topically applied. Hexane extract was purified with a florasil column and analyzed by means of mass fragmentography. [Reproduced with permission from K. Tanaka.³⁵]

We can postulate dehydrochlorination, dehydrogenation and oxygenation, for such important steps. Among primary metabolites presumed to be formed by these reactions, dehydrochlorination product, (36/45)-PCCHE had only been identified in insects treated with lindane. Reexamination was necessary for iso-PCCHE reported by Reed and Forgash,⁸) and research was required for other possible metabolites that would be consistent with the isotope effect studies.

§ Oxidative metabolism in house fly

Hexane extract of house flies administered with lindane was examined by gas

chromatograph equipped with ecd. Many peaks of metabolites were observed. With the aid of mass spectrometry, various important metabolites were identified.⁴⁰⁾

One of them is (36/45)-HCCHE, the cis-dehydrogenated product of lindane. Structure was confirmed by GC-MS, and stereoisomerism was determined by comparison of its retention time of glc with those of authentic samples.⁴⁰⁾ This compound is also identified almost at the same time as one of the lindane metabolites by rat liver microsomes by Chadwick and his collaborators.³⁰⁾ iso-PCCHE reported earlier is considered as identical with this HCCHE from various properties of these two reported compounds.

Metabolites from house flies include trichlorophenol isomers, which were identified also by GC-MS and other means (Table I).^{28,35)}

Reactions which produce these metabolites such as HCCHE and trichlorophenols are oxidative reactions. Therefore, experiments using microsomes, which are believed to concern majority of the oxidative metabolism of xenobiotics, became necessary. Aerobic metabolism study using house fly abdomen microsomes fortified with NADPH revealed the formation of (36/45)-PCCHE, (36/45)-HCCHE and 2,4,6-trichlorophenol from lindane, and also showed the presence of (346/5)-PCCHE which should be a cis-dehydrochlorination product.⁴⁰⁾

These metabolites give us many suggestions on the metabolic pathway. The active reagent that affords (36/45)-HCCHE is probably oxygen molecule activated by microsomes. The first identification of trichlorophenols as metabolites of lindane in insects shows the existence of oxygenation reaction during lindane metabolism in insects. These primary reactions, oxygenation, dehydrogenation as well as dehydrochlorination are consistent with the biological isotope effects described in the previous section. Also in German and American cockroach which show the isotope effect in their lethality, oxidative detoxication metabolism is presumed to take place.

The reactions to produce above metabolites in house fly are inhibited by carbon monoxide and SKF-525A (β -diethylaminoethyl diphenylpropylacetate HCl), but not by CN^- . They require NADPH as the most effective cofactor. Thus, the enzyme system which participates in the reactions is supposed to be cytochrome P-450 dependent. Reaction mechanisms should be similar to those of oxidative metabolism in rats, and will be discussed both together in the following section.

§ Oxidative metabolism in mammals and reaction mechanism in oxidative metabolism

(1) Desaturation

Among the rat *in vitro* metabolites identified by Chadwick *et al.*, there is (36/45)-HCCHE. Pentachlorocyclohexenol is identified as one of the metabolites also in rat, *in vivo* and *in vitro*,^{30,41)} and is one of the metabolic products of (36/45)-HCCHE. Thus, the formation mode of this HCCHE is one of the important problems in lindane metabolism.

(36/45)-HCCHE is produced by the oxygen attack on lindane, and the oxygen is believed to be activated by microsomal cytochrome P-450.^{30,40)} There are various discussions and hypotheses on the active form of oxygen, and one of the most probable forms is oxenoid.⁴²⁾ The oxenoid mechanism assumes the attack of the rea-

gent that can release a sextet oxygen, and should be an electrophilic mechanism in the singlet state of the sextet oxygen just like carbene and nitrene, although they often behave as radicals in their triplet state. This electrophilic oxenoid mechanism has been supported by the epoxidation of double bond, especially of aromatic ring, as the first step of hydroxylation.⁴³⁾ Insertion of oxygen atom into C-H bond is also conveniently explained by this mechanism from analogy with carbene insertion reactions.^{42,43)}

According to Hamilton,⁴²⁾ properties of many desaturases are similar to those of oxygenases, and dehydrogenation reactions like the above presumably proceed by the oxenoid mechanism. Thus, in this reaction, oxenoid abstracts hydrogens to produce double bond instead of inserting oxygen into C-H bonds.

Formation of (36/45)-HCCHE from lindane, namely cis-dehydrogenation, in rat and house fly is considered to be one of the good examples of the oxenoid reaction.

(2) Oxygenation

Alcoholic and phenolic metabolites such as pentachlorocyclohexenol, trichlorophenols and tetrachlorophenols are known to arise from polychlorocyclohexenes in the lindane metabolic pathway (Table I and Fig. 3).^{22,44,45)} To clarify the mechanism of these reactions is one of the major points in lindane metabolism study.

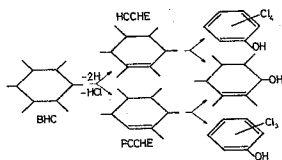


Fig. 3. Formation scheme of tetrachloropheno', pentachlorocyclohexenol, and trichlorophenol from BHC isomers via hexa- and pentachlorocyclohexene isomers.

HCCHE and PCCHE isomers have vinylic chlorine(s). As a simpler model of polychlorocyclohexenes, tetrachlorocyclohexene (BTC) isomers which have no vinylic chlorines were metabolized. BTC isomers themselves may be possible intermediary metabolites of BHC isomers. The glc of the oxidative metabolites by microsomes are illustrated in Fig. 4 along with their structures.⁴⁴⁾

First, we describe some of the structure elucidation procedures.

An identical pair of tetrachlorocyclohexenols is obtained as metabolites of both (36/45)- and (345/6)-BTC. One of the alcohols, the compound-(2) was identified by comparison with the authentic sample that was synthesized via an unambiguous pathway. Then, the other one should have the structure named (1), since it has a common configuration in three C-Cl bonds with those of (36/45)- and (345/6)-BTC. In the mass spectra, both compounds-(1) and -(2) show a characteristic pattern at m/e 138, 140 and 142 of the intensity ratio 9:6:1 due to retro-Diels-Alder cleavage fragment ion: $[C_4H_3OCl_2]^+$.

Most compounds thus obtained from other BTC isomers also show the same characteristic pattern in their mass spectra, but the compounds-(4) and -(7) have a different cluster of peaks at m/e 156, 158, 160 and 162 of the ratio 27:27:9:1, which

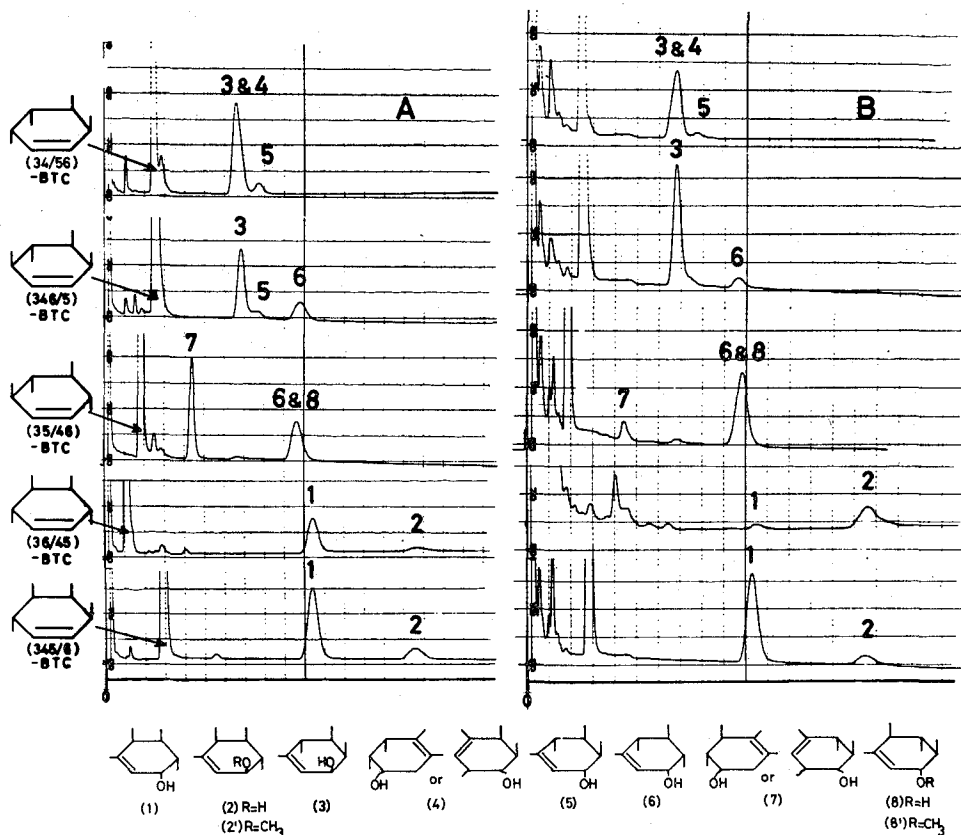


Fig. 4. Gas chromatograms of the hexane-soluble metabolites of five BTC isomers with microsomes of house fly abdomen (A), and rat liver (B). The incubation mixture contained in a final volume of 1 ml, 0.2 M potassium phosphate (pH 7.4), microsomal suspension (1.7 to 2.3 mg, and 0.8 to 1.2 mg, respectively, for rat liver and house fly abdomen), 1 mM NADPH, and 40 μ M substrate. The reaction was conducted at 32°C for 30 min. Gas chromatographic conditions were: 5 % DEGS on Gas-Chrom Q, 1.5 m, 180°C, N₂ flow rate: 25 ml/min. Peaks designated by the numerals are metabolites tetrachlorocyclohexenols, the structure of which are illustrated under the chromatogram. [Reproduced with permission from Ref.⁴⁴⁾]

corresponds to the fragment ion $[C_4H_3Cl_3]^+$. The formation of this ion indicates that the parent molecule is the further rearranged product in its double bond and chlorine (or hydrogen) substituent. (See the structures in Fig. 4). Structures of all other metabolites have been similarly determined or presumed.

What is the most plausible mechanism for these reactions? We consider it to be an ene-like reaction of oxygen molecule, as illustrated in Fig. 5 for the formation of compound-(1) and -(2) from (36/45)-BTC; and of (4) from (34/56)-BTC with a shift of chlorine or hydrogen.⁴⁴⁾

In the formation of (1) and (2), π -electrons are withdrawn by an active oxygen; the neighboring hydrogen is removed as a proton; a new double bond forms; and the C-Cl bond is converted from sp^3 hybrid to sp^2 hybrid. In this sequence, the leaving of the proton is assisted by an independent base such as the solvent, by the

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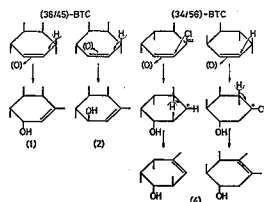


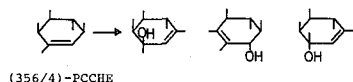
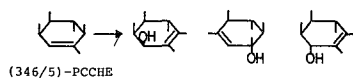
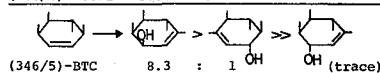
Fig. 5. Reaction mechanism of the tetrachlorocyclohexenol formation from (36/45)-BTC and (34/56)-BTC. (O) is the attacking oxygen that is activated in microsomes, is probably making a ternary complex with enzyme and substrate, and is electrophilic.

tail of the oxygen molecule, or by a certain group in the enzyme. In the above cases, the concerted attack by a base seems an adequate mechanism for the production of (1), while an appropriate mechanism for the production of (2) may be a cyclic participation of oxygen molecule or of its complex with enzyme. When the neighboring hydride or chloride ion shifts to one of the vinylic carbon concomitantly with the oxygen-attack, it would produce a rearranged alcohol as shown in Fig. 5 for the formation of (4) from (34/56)-BTC. The pathway that includes the shift of pseudoaxial chlorine seems more probable. Tetrachlorocyclohexenols are also found among the *in vivo* metabolites of lindane: Recently, Chadwick and his collaborators reported⁴⁶⁾ the isolation and identification from the rat urine of two positional isomers of tetrachlorocyclohexenols, both of which yield, on dehydrogenation, only 2,3,4,6-tetrachlorophenol. Based on this result and mass spectral examination, they concluded that the metabolites are 2,3,4,6-tetrachloro-2-cyclohexen-1-ol and 2,4,5,6-tetrachloro-2-cyclohexen-1-ol. Neither one is *in vitro* metabolite of BTC isomers we examined. Thus, the tetrachlorocyclohexenols as *in vivo* metabolites seem to give a clue to reveal another novel metabolic pathway of lindane.

(3) Chlorophenol and pentachlorocyclohexenol formation from polychlorocyclohexenes.

Table IV. Production of 2,4,5-trichlorophenol and pentachlorocyclohexenol from (346/5)- and (356/4)-PCCHE
[Reproduced with permission from Ref.⁴⁴⁾]

Substrate	Metabolites	
	2,4,5-TCP (mole)	PCCOL ^{a)}
(346/5)-PCCHE	6.0×10^{-10}	26
(356/4)-PCCHE	37.6×10^{-10}	1



a) Relative peak area on glc-ecd.

It is natural for PCCHE and HCCHE isomers also to undergo ene-like reactions such as those observed for BTC isomers. In Table IV, the principal products from (346/5)- and (356/4)-PCCHE by the action of rat liver microsomes in the presence of NADPH are shown.⁴⁴⁾

The former isomer of PCCHE produced more pentachlorocyclohexenol than the latter, and, with this, the production of 2,4,5-trichlorophenol seems to be compensated. To the explanation of this product distribution, the pathway that affords the compound-(3) from (346/5)-BTC by oxidative metabolism can be referred. Stereochemically corresponding site of oxygen-attack for the (346/5)- and (356/4)-PCCHE is the carbon-2 and carbon-1, respectively. From (346/5)-isomer, pentachlorocyclohexenol is thus produced, but (356/4)-PCCHE would give gem-chlorohydrin which is presumed to give a ketone spontaneously. The α,β -unsaturated ketone can easily enolize, and be further dehydrochlorinated. 2,4,5-Trichlorophenol is the most reasonable end-product.

On the other PCCHE isomers also, oxygen-attack at the vinylic carbon bearing chlorine would result in the formation of 2,4,5-trichlorophenol. This seems the principal pathway of 2,4,5-trichlorophenol formation from PCCHE isomers (Fig. 6), although there are possible minor pathways (see below).

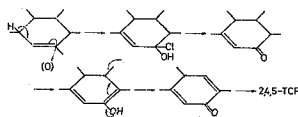


Fig. 6. Ene-like reaction mechanism in the formation of 2,4,5-trichlorophenol from pentachlorocyclohexene isomers. TCP=trichlorophenol.

A similar discussion on HCCHE isomers gives a clear explanation of 2,3,4,6-tetrachlorophenol formation.⁴⁴⁾

(4) Active oxygen in the ene-like reaction

By what type of oxygen the ene-like reactions described above are conducted? It might be either an electron-deficient form such as oxenoid or a very reactive species "singlet oxygen molecule." Action of various inhibitors suggests⁴⁴⁾ the possibility of the participation of singlet oxygen molecule, but does not definitely indicate it.

There is a study on the stereochemistry of the singlet oxygen ene-like reaction with 2-methyl-3-hexene isomers to produce 2-methyl-4-hexen-3-ol⁴⁷⁾ (Fig. 6a). In this

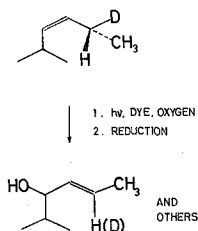


Fig. 6a. Ene-like oxygenation of 2-methyl-3-hexene. 5-Deuterated and undeuterated substrates are examined.⁴⁷⁾

reaction, the abstraction of the proton (or deuteron), carbon-oxygen bond formation, and double bond migration concomitantly occur. Kinetic deuterium isotope effect is not observed with allyl-deuterated substrate; and the present biochemical ene-like reaction by oxygen is accompanied by a significant deuterium isotope effect. Thus, the rate determining steps seem different between these two reactions. The active form of oxygen of the latter reaction might also be different from that of the former. But, when the solvent is methanol in the reaction with 2-methyl-3-hexene a kinetic deuterium isotope effect is observed.⁴⁷⁾ In this case, abstraction of proton by the hydroxyl group in the methanol molecule becomes rate-determining. Therefore, this organic reaction in hydroxylic solvent is similar to the biochemical ene-like reaction to a certain extent. Anyway, it is too early to conclude our discussion on the active form for oxygen in this biochemical reaction.

(5) Nucleophilic oxygenation

One of the HCCHE isomer, (36/45)-HCCHE is the cis-dehydrogenated metabolite of lindane as described already. This isomer afforded, by rat liver microsomes in the presence of NADPH, pentachlorocyclohexenol, 1,2,4-trichlorobenzene and 2,3,4,6-tetrachlorophenol; the last of which was already described.⁴⁴⁾ On 1,2,4-trichlorobenzene formation, we will discuss in a later chapter "reductive metabolism". Here we consider the formation mechanism of pentachlorocyclohexenol from HCCHE.

The conformation of the pentachlorocyclohexenol is shown in Table I: one of the allylic chlorine in (36/45)-HCCHE molecule is substituted by OH-group of reverse configuration. For understanding the formation of this alcohol it is most appropriate to assume a nucleophilic attack of a certain oxygen species substituting the chlorine group.⁴⁴⁾ A candidate of the nucleophilic form of oxygen is superoxide anion, O_2^- .^{42,48)}

Tetrachlorocyclohexenols which were found in the rat urine as metabolites of lindane⁴⁵⁾ might also be produced by nucleophilic attack of oxygen to some polychlorocyclohexenes. 2,4,5,6-Tetrachloro-2-cyclohexen-1-ol may result from the substitution of one of the allylic chlorines in PCCHE by a hydroxyl group. In a similar way, 2,3,4,6-tetrachloro-2-cyclohexen-1-ol can be produced from 1,2,3,4,6-pentachlorocyclohexene which, in turn, may result from reductive dechlorination (substitution by hydrogen) of an HCCHE isomer at one of the homoallylic positions. Experimental evidences for the formation of these tetrachlorocyclohexenols *in vitro* are not available yet, though.

There are several enzymatic oxygenations in which superoxide anion may be involved: the reaction by the intestinal tryptophane 2,3-dioxygenase⁴⁹⁾ and some reactions by cytochrome P-450.⁵⁰⁾ In the present pentachlorocyclohexenol-formation, the peroxide that would primarily form by O_2^- attack to HCCHE will easily become alcohol by a coexisting peroxidase activity.⁵¹⁾ Thus, the participation of superoxide anion is a plausible mechanism of this alcohol formation from HCCHE. Experiments⁴⁴⁾ using inhibitors including superoxide dismutase^{48,52)} seem to support the above mechanism, but are not conclusive.

(6) Oxygen insertion into C-H bonds

It is well known that the oxygen activated in microsomes especially by P-450 undergoes insertion reaction into various C-H bonds. The formation of 2,4,5-trichlorophenol and 2,3,4,6-tetrachlorophenol from PCCHE and HCCHE, respectively, can also be explained by this type of reaction,⁴⁵⁾ although the main pathway to these phenols is that involving ene-like reaction⁴⁴⁾ as described in section-(3). A minor phenolic metabolite from PCCHE, 2,4,6-trichlorophenol, can be produced by an oxygen insertion mechanism that yields an enone group. It is illustrated in Fig. 7 along with 2,4,5-trichlorophenol formation from PCCHE by the same

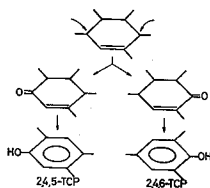


Fig. 7. Oxygen-insertion mechanism in the biodegradation of pentachlorocyclohexenes. Bent arrows in the top structural formula indicate the oxygen-attacking sites. TCP=trichlorophenol.

mechanism as suggested by Stein, Portig *et al.*⁴⁵⁾ The formation of 2,4,6-trichlorophenol from PCCHE may also be explained by an ene-like oxygen attack with a shift of an allylic chlorine, which is similar to the compound-(4)-formation pathway from (34/56)-BTC illustrated in Fig. 5.

As a metabolite of BHC isomers including lindane, the 2,4,6-isomer of trichlorophenol is one of the major one. In some cases such as γ - and β -BHC metabolism in mouse,³¹⁾ 2,4,6-trichlorophenol is the most abundant phenolic metabolite. The corresponding *in vitro* reaction is observed in the presence of rat liver microsomes and NADPH. All BHC isomers yield 2,4,6-trichlorophenol as the most dominant metabolite in this system.^{28,53)} We need, therefore, a proper explanation for the major formation route of 2,4,6-trichlorophenol. It should not be the above route going through PCCHE, because β -BHC hardly gives PCCHE, and because 2,4,6-trichlorophenol formation is only a minor pathway in PCCHE biodegradation.

Direct oxygenation of BHC molecule was postulated independently by Stein *et al.*⁴⁵⁾ and by us.²⁸⁾ Both groups have conducted model chemical reactions for BHC oxygenation, and proved the correctness of this postulate. In both model reactions as illustrated in Fig. 8, pentachlorocyclohexanone, an assumed intermediate, undergoes further dehydrochlorinations to afford 2,4,6-trichlorophenol as the major product.

What kind of activated oxygen participates in this biochemical reaction? Again, the most probable form seems an oxenoid. An oxenoid reagent in its singlet state is considered to insert oxygen by an ionic mechanism and in a concerted manner (Fig. 9).

In vitro reactivity of BHC isomers in the 2,4,6-trichlorophenol formation decreases in the order of: δ -BHC > ϵ -BHC > α -BHC > lindane > β -BHC. There are

Biodegradation of Lindane and Isomers

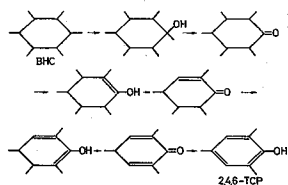


Fig. 8. Reaction mechanism of the 2,4,6-trichlorophenol formation from BHC isomers: oxygen insertion into one of the C-H bonds.

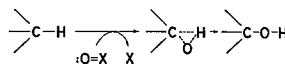


Fig. 9. Oxygen insertion by an oxenoid reagent that enables us to explain the facile biodegradation of saturated polychloro-compounds.

some possible factors modifying the reactivity. The major factors are considered to be the affinity of the substrate to enzyme and/or to the reaction site, or the reactivity toward the active oxygen of C-H bonds of various molecular environment influenced by steric effects of neighboring C-Cl bonds.

REDUCTIVE METABOLISM

We have discussed various oxidative metabolic reactions that are mainly conducted by the microsomal fraction, namely endoplasmic reticulum in the cell, in mammals and insects. By microsomes, (36/45)-HCCHE, one of the important primary metabolites of lindane, yields 1,2,4-trichlorobenzene as well as various oxidative metabolic products such as tetrachlorophenols (see Table V).⁴⁴⁾ In order to produce 1,2,4-trichlorobenzene, the HCCHE must undergo dechlorination reaction in one of its degradation steps. When this HCCHE was incubated with rat liver microsomes and NADPH under anaerobic condition, the product consists of 1,2,4-trichlorobenzene mainly,⁵⁴⁾ accompanied by a small amount of 1,2-dichlorobenzene. The rate of substrate consumption in this condition is much higher than in the aerobic condition, the usual microsomal oxidative reaction condition. Thus,

Table V. Comparative *in vitro* metabolism of (36/45)-HCCHE under aerobic and anaerobic condition^a

[Relative amount is shown (1,2,4-trichlorobenzene=100).]

	DCB ^b	124-TCB ^c	TeCB ^d	PentaCB ^e	TCP ^f		TeCP ^g		PCCOL ^h
					246-	235 & 245	2346	2345	
Aerobic ⁱ	—	100	17	8	7	17	44	16	+++
Anaerobic ^j	5	100	—	—	—	—	+	—	+

a) Reaction mixture contained 0.2 M potassium phosphate (pH 7.4), rat liver microsomes (1.7 to 2.3 mg/ml in aerobic, and 0.5 to 1.5 mg/ml in anaerobic reaction), 3 mM NADPH, and 40 μ M substrate.

b) DCB=dichlorobenzene. c) TCB=trichlorobenzene. d) TeCB=tetrachlorobenzene. e) PentaCB=pentachlorobenzene. f) TCP=trichlorophenol. g) TeCP=tetrachlorophenol.

h) PCCOL=pentachlorocyclohexenol.

i) From Ref. 44. Under the air. Substrate disappearance rate at the above condition: 0.95 ± 0.05 nmol/min/mg protein.

j) From Ref. 55. Under the nitrogen gas. Substrate disappearance rate at the above condition: 14.1 ± 1.8 nmol/min/mg protein.

one can say that the formation of 1,2,4-trichlorobenzene from (36/45)-HCCHE is inhibited by oxygen. Similar anaerobic reaction by rat liver microsomes with lindane as substrate affords (346/5)-BTC exclusively as a primary metabolite,^{30,44, 46-54} although other BHC isomers do not react in a similar condition. (346/5)-BTC formation is the result of 1,2-trans-dechlorination of lindane. We have presumed a similar reaction above in the formation route to 1,2,4-trichlorobenzene from (36/45)-HCCHE. Thus, we can depict this route as the first 1,2-dechlorination followed by spontaneous dehydrochlorination to give the aromatic product.⁵⁴

All the isomers of HCCHE, PCCHE, and BTC that we have tested are metabolized rapidly by the similar reaction condition. HCCHE isomers and PCCHE isomers produced 1,2,4-trichlorobenzene and 1,4-dichlorobenzene, respectively as a main product.⁵⁵

These reactions are again dependent on microsomal cytochrome P-450, and are inhibited by carbon monoxide. When this pigment production is induced by pretreatment of rat with phenobarbital, the reaction rate is proportional to the amount of P-450, but not to the total microsomal protein.^{54,55}

Cytochrome P-450 usually transports electrons to oxygen molecule in its ternary complex including substrate, and catalyzes the oxygenation of the substrate. But, when the oxygen molecule is absent, and the substrate is sufficiently of high redox potential, the substrate can directly accept the electrons from P-450. Thus, our polychlorocyclohexenes undergo reductive dechlorination. Examples of reductive metabolism by cytochrome P-450 have been reported: nitroreductase⁵⁶ and amine N-oxide reductases.⁵⁷ By house fly microsomes, the reductive dechlorination of lindane is hardly observed. There are serious differences between rat liver microsomes and house fly abdomen microsomes in this sense.

As a model reaction for the metabolic reductive dechlorination, a typical electrochemical reduction — polarographic study has been undertaken on the above polychlorocyclohexenes. Compounds of more positive half-wave potential are found to be more reactive in the above microsomal reductive dechlorination. The microsomal reaction should require a hydrophobic site. When the log(partition coefficient) values are selected as a measure of hydrophobicity, a linear combination of the values and the half-wave potentials shows a good positive correlation to the log(rate) values, in which the rate is the substrate disappearance rate in the microsomal reductive metabolism (Fig. 10.)⁵⁵ The result is one of the basis for the mechanism of the metabolic dechlorination which is assumed to be the reduction by electrons at a certain hydrophobic site.

There are some reports^{58,59} on the dechlorination of lindane and on the further dechlorination of the metabolites by soil anaerobic bacteria, in which lindane affords monochlorobenzene as a final product.⁵⁹ The intermediary metabolite is (346/5)-BTC like that in the reaction by liver microsomes, which would, in turn, give dichlorocyclohexadiene on one molar dechlorination. The cyclohexadiene should be so unstable as to undergo spontaneous dehydrochlorination to yield monochlorobenzene.

The next problem is: How significant are these dechlorination reactions in mammals *in vivo*? Probably, dichlorophenols and dichlorobenzenes found in the

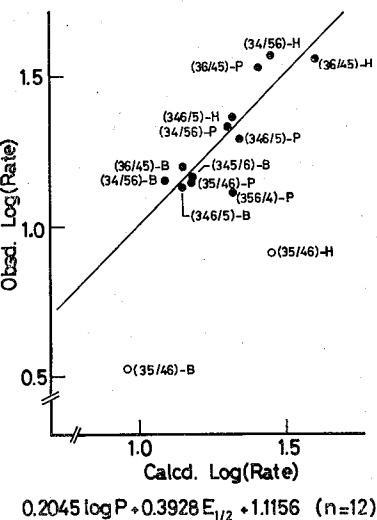


Fig. 10. Observed and calculated $\log(\text{Rate})$ values according to the equation shown here. In the graph, the following symbols and abbreviations are used: $\bigcirc$ = compounds not included in the correlation, H = hexachlorocyclohexene, P = pentachlorocyclohexene, B = benzene tetrachloride, i.e. tetrachlorocyclohexene. In the equation, P and $E_{1/2}$ are partition coefficient and half-wave potential, respectively. [Ref.⁵⁵]

urinary metabolites in the rabbit (Table II) should be a result of the metabolic pathway that includes such reductive dechlorination reaction. Another example that demonstrates the significance of the reaction will be mentioned in the following section on the mercapturic acid formation pathways.

GLUTATHIONE CONJUGATION

§ Substrates for glutathione conjugation

Many lindane metabolites in the rat urine can be explained only by oxidative metabolic pathways. 2,4,6-Trichlorophenol and 2,3,4,6-tetrachlorophenol are the examples, though the involvement of a spontaneous dehydrochlorination is assumed. Among the other important types of metabolites in mammals and insects, there are compounds that have cysteine moiety in the molecule: mercapturic acids found in mammalian urine and glutathione conjugates in various insects (see Table I). In the pathway to produce these metabolites, glutathione-attacking step has been one of the subjects of controversy. Among various arguments, the hypothesis that includes direct attack of glutathione on lindane molecule was once prevailing based on the undiscovery of appropriate intermediates and on the studies using various inhibitors.¹⁷⁾ But, at present, we consider that glutathione conjugation mainly occurs at the polychlorocyclohexene stages on the basis of the recently available experimental data.^{23,25,34,40)}

Enzymes that catalyze glutathione conjugation are glutathione-S-transferases. They are completely or partially purified from rat liver,^{60,61)} sheep liver,⁶²⁾ monkey

liver,^{62a)} human liver⁶³⁾ and insects such as American cockroach,⁶⁴⁾ house fly^{20,65)} and a kind of moth.⁶⁶⁾ The transferases from each source are composed of several enzymes that have various substrate specificity. They commonly catalyze the nucleophilic reactions: substitution, addition-elimination and addition by sulfur atom in the glutathione molecule.

As described already, the identification of the isomeric glutathione conjugate was accompanied by many difficulties before 1960's. But recent developments of chromatographic technique, especially glc with a capillary column enables us to achieve a high resolution of the components, and various group-specific detectors also make the identification procedure much more facile than before. Here we show the typical results^{7,23,67)} of the analysis of glutathione conjugates and mercapturic acids (Tables VI-VIII, Fig. 11). The *in vitro* results listed here are based on the experiments using 105,000×g supernatant fraction in the presence of excess amount of reduced glutathione.

On the results in house fly experiments⁶⁷⁾ (Table VI), we can say that *in vivo* metabolites reported by Bradbury and Standen⁷⁾ and *in vitro* metabolites listed here are similar in the composition, although some minor components found *in vivo* are not detected in the *in vitro* experiments. The similar metabolite distribution in the *in vivo* and *in vitro* experiments, and that in the BHC and corresponding PCCHE experiments, support the view that each BHC isomer is dehydrochlorinated in house

Table VI. Percent composition of S-dichlorophenyl-glutathione from BHC and PCCHE isomers in house fly *in vivo* and *in vitro*. [Ref.⁷⁾ and Ref.⁶⁷⁾.]

Substrate			S-Dichlorophenyl-glutathione					
			2, 3-	2, 4-	2, 5-	2, 6-	3, 4-	3, 5-
α -BHC	vivo (R) ^a		3	29	29	7	30	<3
	vitro (R)		—	27	29	t ^b	44	—
	(S) ^c		—	21	23	—	56	—
(346/5)-PCCHE	vitro (R)		—	34	19	—	47	—
Lindane	vivo (R)		<2	50	25	14	6	<3
	vitro (R)		—	26	74	—	—	—
	(R)		—	26	68	6	—	—
	(S)		—	37	52	11	—	—
(36/45)-PCCHE	vitro (R)		—	34	66	t	—	—
	(R)		—	38	54	8	—	—
	(S)		—	33	45	22	—	—
δ -BHC	vitro (R)		—	87	t	—	13	—
	(R)		—	70	—	—	30	—
(35/46)-PCCHE	vitro (R)		—	77	—	—	23	—
	(R)		—	68	t	—	32	—

a) R=resistant strain

b) t=trace (<1%)

c) S=susceptible strain

fly by glutathione participation to the corresponding PCCHE, followed by nucleophilic substitution by glutathione. Alicyclic glutathione conjugates thus formed can easily undergo dehydrochlorinations to produce dichlorophenyl-glutathione isomers. Deuterium isotope effect in the reaction rate is significant on the glutathione conjugate formation from lindane, whereas it is not from PCCHE as described already^{34,40)} (see Figs. 1 and 2). This result also supports the above sequence. Thus, in house fly, at least two roles are played by the glutathione-dependent enzyme system in glutathione conjugate formation from lindane and BHC isomers: dehydrochlorination of BHC and glutathione transfer to PCCHE. The 105,000×g supernatant glutathione-dependent system should contain several types of glutathione S-transferases,^{19,20)} and is believed that dehydrochlorination of BHC to PCCHE is also catalyzed by the glutathione-dependent system.^{34,40)} Glutathione molecule might play as a base in the dehydrochlorination, whereas the molecule is a nucleophile in the next step. Glutathione dependent dehydrochlorination deserves further enzymatic, especially mechanistic, studies.

In the rat, the pathway for glutathione conjugate formation is considerably different from that in house fly: there is a larger variation in the rat than in the fly

Table VII. Composition of the chlorophenylmercapturic acids formed from lindane and related compounds *in vivo*^a [Reproduced with permission from Ref.³³⁾]

Substrate	Dose (μ mol)	CPMA ^b			DCPMA ^c				TCPMA ^d	
		2-	3-	4-	2, 3-	2, 4-	2, 5-	3, 4-	2, 3, 5-	2, 4, 5-
Lindane	34.4(BR) ^e	t ^f	t	15	4	9	6	33	17	16
	17.2 (AR) ^g	t	t	10	t	9	2	36	21	23
(36/45)-HCCHE	34.6 (BR)	t	0 ^h	3	4	16	5	24	22	26
	17.3 (O) ⁱ	t	0	t	t	12	t	36	24	28
	10.4 (O)	t	0	0	t	14	1	34	25	27
(36/45)-PCCHE	39.2 (BR)	0	0	t	0	97	2	0	t	t
	39.2 (O)	0	0	t	0	95	3	0	t	t
(346/5)-PCCHE	11.8 (BR)	0	0	20	0	7	16	57	t	t
	5.5 (AR)	t	0	1	0	8	6	85	0	0
	1.0 (AR)	0	0	t	0	20	24	56	t	t
(346/5)-BTC	45.4 (AR)	0	0	100	0	0	0	0	0	0
	4.5 (AR)	0	0	100	0	0	0	0	0	0

^a Intraperitoneally administered to rat. Percentages in the mercapturic acid fraction are shown. The standard error in the glc determination was estimated as less than 5% of each value.

^b (CPMA) Chlorophenyl-mercapturic acid.

^c (DCPMA) Dichlorophenyl-mercapturic acid.

^d (TCPMA) Trichlorophenyl-mercapturic acid.

^e (BR) Administered as an emulsion in Ringer's solution made from benzene solution.

^f (t) Trace amount; less than 1%.

^g (AR) Administered as an emulsion in Ringer's solution made from acetone solution.

^h (0) Not detectable.

ⁱ (O) Administered as an olive oil solution.

Table VIII. Composition of the S-chlorophenyl-glutathione formed from polychlorocyclohexenes *in vitro*^a [Reproduced with permission from Ref.²³]

PCCHE	No. of experiments	DCPG ^b formed (sum=100)			
		2,4-	2,5-	2,6-	3,4-
(36/45)	10	95±2 ^c	0	3±1	3±1
(35/46)	4	67±3	0	0	33±1
(356/4)	1	44	2	20	34
(346/5)	4	14±2	13±2	2	73±5
(34/56)	5	33±3	60±3	2±1	5±2

Substrate	No. of experiments	Product
(35/46)-BTC	2	4-CPG ^d (almost exclusively)
(346/5)-BTC	2	4-CPG (almost exclusively)
(36/45)-BTC	2	4-CPG (almost exclusively)
(34/5)-1,3,4,5-Tetrachlorocyclohexene	1	3-CPG (almost exclusively)
(36/45)-HCCHE	4	2,4,6-TCPG ^e and others
(35/46)-HCCHE	1	One unknown (2,3,6- or 3,4,5-TCPG) > 2,3,4-TCPG
(346/5)-HCCHE	1	2,3,4-TCPG > 2,3,5-TCPG > others

^a Temperature: 37°C. See the list under the title for abbreviations of substrates.

^b Neither the 2,3- nor the 3,5-isomer was produced from any of the PCCHE isomers tested. (DCPG) *S*-Dichlorophenyl-glutathione.

^c Average value ± SE.

^d (CPG) *S*-Chlorophenyl-glutathione,

^e (TCPG) *S*-Trichlorophenyl-glutathione.

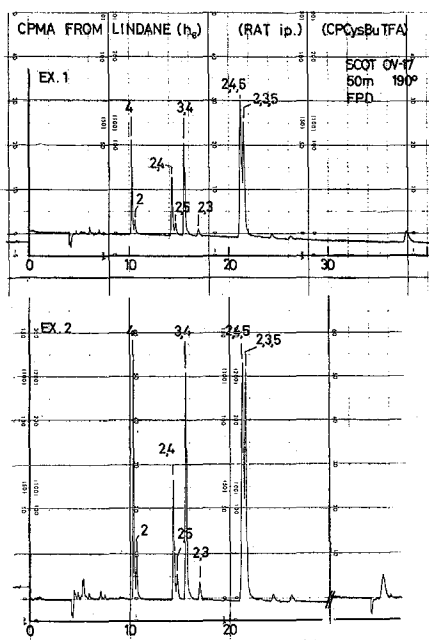


Fig. 11. Examples of gas chromatograms of N-trifluoroacetyl-S-chlorophenylcysteine butyl ester mixture. Typical results of the analysis of mercapturic acids found in the rat urine. Lindane was administered intraperitoneally once, and the urine was collected for 48 hours. Glc. conditions: glass capillary SCOT OV-17 50 m, 190°, detector FPD.

of the number of chlorines in the conjugate molecules and of the isomeric composition of metabolites (Table VII). From lindane, 3,4- and 2,5-dichlorophenyl derivatives as well as the 2,4-isomer are produced. The latter is the major mercapturic acid from (36/45)-PCCHE, which is the trans-dehydrochlorination product of lindane. Besides, various monochloro- and trichlorophenylmercapturic acids are also found as metabolites of lindane. These are the great differences from the results in house fly. As described above, the route: BHC→(dehydrochlorination)→PCCHE→(glutathione conjugation)→S-dichlorophenyl-glutathione is the principal pathway in house fly. In rat, various important pathways to glutathione conjugates seem to coexist.

It is natural to consider that each of the trichloro-, dichloro- and monochlorophenylmercapturic acids has its respective precursor which undergoes glutathione conjugation (Table VIII). For trichlorophenyl-derivatives, several precursors can be assumed. HCCHE isomers, trichlorobenzene epoxide or other intermediates are conceivable. When (36/45)-HCCHE is administered to rat, both 2,3,5- and 2,4,5-trichlorophenylmercapturic acid are abundantly excreted in the urine (see Table VII). These acids are the principal mercapturic acids also from lindane, and (36/45)-HCCHE is one of the principal primary metabolites. Therefore, (36/45)-HCCHE is the most probable precursor for these two mercapturic acids. But, *in vivo*, this HCCHE must not directly undergo glutathione conjugation, since *in vitro* glutathione conjugation reaction of this compound using rat liver cytosol (105,000×g supernatant), where a major portion of glutathione S-transferase activity exists, gives entirely different isomeric composition of S-trichlorophenyl-glutathione (Table VIII). The major product in this *in vitro* reaction is 2,4,6-trichlorophenyl-derivative, the formation of which is readily anticipated on the basis of chemical mechanism, if glutathione molecule initially attacks at one of the vinylic positions and displaces the allylic chlorine. The abundant formation of 2,3,5- and 2,4,5-isomer from this HCCHE and from lindane in the living rat should be explained by a different sequence of the reaction. When (36/45)-HCCHE forms from lindane in the liver cell endoplasmic reticulum, we can assume that the metabolite is hardly solubilized into cytosol, different from other primary metabolites. This assumption is based on the finding that (36/45)-HCCHE is more hydrophobic than lindane⁵⁵⁾ whereas the other primary metabolites, polychlorocyclohexenes, are less hydrophobic, on the scale of partition coefficient between n-octanol/water.⁵⁵⁾ It is then reasonable that (36/45)-HCCHE staying for a long time at the hydrophobic endoplasmic reticulum is subject to further transformation there before undergoing glutathione conjugation in the cytosol (Fig. 12).

Thus, the formation pathway of 2,3,5- and 2,4,5-trichlorophenylmercapturic acid *in vivo* can be depicted as illustrated in Fig. 13 (top), though an intermediate, 1,4-diene, can react with glutathione to form the products (Fig. 12).

Dichlorophenylmercapturic acids should come from PCCHE isomers, and especially 2,4-dichloro-isomer from (36/45)-PCCHE. Then, what is the precursor of 3,4-dichlorophenylmercapturic acid? It might again be (36/45)-HCCHE as depicted in Fig. 13 (bottom), since the HCCHE gives the 3,4-acid in the *in vivo* experi-

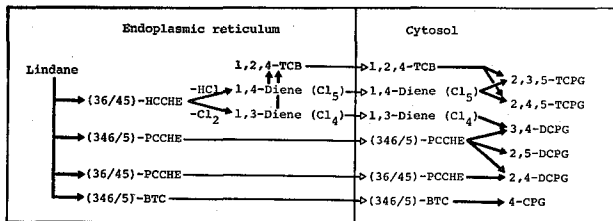


Fig. 12. Pathways for the formation of glutathione conjugates from lindane in the rat, as the primary step of mercapturic acid formation. $\blacktriangleright$ = biochemical transformation, $\rightarrow$ = transport, TCB = trichlorobenzene and/or trichlorobenzene epoxide, CPG, DCPG, and TCPG = S-chloro-, S-dichloro-, and S-trichlorophenyl-glutathione, respectively. For abbreviations of other compounds, see the list under the title. The structure of dienes: 1,4-diene (Cl_5) is 1,2,3,4,6-pentachlorocyclohexadiene-1,4, and 1,3-diene (Cl_4) is 1,2,5,6-tetrachlorocyclohexadiene-1,3. Both of them are tentatively assumed intermediary metabolites. [Reproduced with permission from Ref.²³]

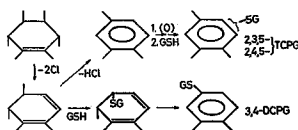


Fig. 13. *In vivo* pathway for the glutathione conjugate formation from (36/45)-HCCHE. (O)=active oxygen that epoxidizes the aromatic nucleus, GSH=glutathione, TCPG and DCPG=S-trichloro- and S-dichlorophenyl-glutathione, respectively.

ments. Another probable precursor is (346/5)-PCCHE. This isomer of PCCHE is one of the metabolites in house fly, but not in the rat. It can be produced by cis-dehydrochlorination of lindane, and is a possible primary metabolite also in rat. This possibility deserves further experimental examinations.

As illustrated in Fig. 12, major reaction sites in mammals to form polychlorocyclohexenes, which are vulnerable against glutathione-attack, are considered to exist in endoplasmic reticulum. But a recent purification of a BHC-dechlorinating enzyme from rat liver cytosol⁽⁶⁸⁾ may modify the general picture on these pathways. The enzyme catalyzes dehydrochlorination of BHC and the subsequent glutathione conjugation, although slowly. It seems a kind of glutathione S-transferase, but appears different from any other reported transferases.

Very recently, an important report has appeared, which states the significant contribution to BHC biodegradation of glutathione S-transferases bound to microsomes and mitochondria.²⁵⁾ These enzymes are studied especially with α -BHC as a substrate, and are proved to catalyze the dehydrochlorination of BHC to (346/5)-PCCHE (just like the above enzyme in liver cytosol), and the subsequent glutathione conjugation. When (346/5)-PCCHE is metabolized, the products are S-dichlorophenylglutathione isomers, and their isomeric composition is somewhat different from that by supernatant (cytosol) glutathione S-transferases. The authors of the study have estimated that the glutathione-dependent conversion of α -BHC by isolated particle fractions is roughly one third of the total activity detected in

the homogenate.²⁵⁾ Thus, the contribution of particulate transferases to the overall production of S-dichlorophenyl-glutathiones from BHC isomers in rat liver may not be negligible. The particulate glutathione transferases might also play a role in producing trichlorophenylmercapturic acids from lindane, and metabolism study of (36/45)-HCCHE by this system seems required to further elucidate the details of lindane metabolism.

One of the monochlorophenylmercapturic acids, the 4-isomer, is an important metabolite of lindane. As a candidate of intermediate to produce this mercapturic acid, we can easily appoint (346/5)-BTC, because this BTC gives the S-4-chlorophenyl-cysteine derivative both *in vivo* and *in vitro* (Fig. 14).²³⁾ Besides, (346/5)-BTC is

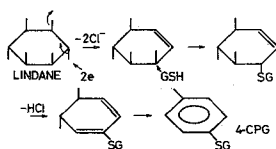


Fig. 14. S-4-Chlorophenyl-glutathione formation from lindane. GSH = glutathione, CPG = S-chlorophenyl-glutathione.

an *in vitro* metabolite of lindane under anaerobic condition as stated before.^{30,44,46,54)} We don't know to what extent the rat liver cell is in reductive or anaerobic condition, but this apparent anaerobic dechlorination of lindane presumably occurs in the living rat. This view is supported by a deuterium isotope effect study described below.

§ Isotope effects on mercapturic acid formation

When 1:1 mixture of lindane and [d₆]-lindane is given to a rat, and the mercapturic acid fraction in the urine is to be analyzed, we can simultaneously determine the individual quantity of various undeuterated and deuterated mercapturic acids, after appropriate derivatization, by mass fragmentographic technique. The peak height ratio of unlabeled and the corresponding D-labeled compound indicates the quantity ratio of a certain metabolite from normal lindane and from the deuterated counterpart. Results are shown in Table IX.

Significant isotope effects are associated with the formation of trichloro- and dichlorophenylmercapturic acids, though some deviations are observed depending on the individual rat. Each value is the ratio of quantity excreted during 48 hours, and reflects, but is not, the kinetic isotope effect itself. The true value for the kinetic effect would be larger than that in the table; since about 65 % of unlabeled lindane has been metabolized at this period, and the disappearance rate of the unlabeled substrate becomes much slower at this period than at the beginning when the disappearance obeys a (pseudo)-first order kinetic, whereas the metabolism rate of deuterated substrate does not decrease so much.

These significant isotope effects *in vivo* support the already proposed pathway (Fig. 12): namely the dehydrogenation and dehydrochlorination of lindane occurs before glutathione-conjugation that leads to tri- and dichlorophenylmercapturic acids.

Table IX. Deuterium isotope effects on the *in vivo* formation of mercapturic acids from a 1:1 mixture of normal and hexadeuterated lindane^a

Excreted compound	Duration of urine collection	k_H/k_D
Substrate	0-48 hr ^b	(0.21) ^c
(lindane)	48-96 ^d	(0.09) ^c
MonoCPMA ^e (mostly 4-)	0-48 hr	1.31 ± 0.17 (4)
DiCPMA ^e (mostly 2,4- and 2,5-)	0-48 hr	2.36 ± 0.34 (2)
	48-96	1.50
	0-48 hr	3.50 ± 0.59 (2)
	48-96	2.38
(mostly 3,4-)		
TriCPMA ^e (mostly 2,4,5- and 2,3,5-)	0-48 hr	2.67 ± 0.38 (2)

^a The k_H/k_D values are means and standard deviations with the number of experiments in parentheses or are results of single experiments.

^b The first 48 hours after administration.

^c The ratio of the remaining normal and hexadeuterated lindane.

^d The second 48 hours after administration.

^e Mono-, Di- and TriCPMA = mono-, di- and trichlorophenyl- mercapturic acid, respectively.

These C-H bond-cleaving reactions are accompanied by a significant isotope effect when examined in the *in vitro* systems.⁶⁷⁾

The ratio of H-D in case of 4-chlorophenyl-derivative is close to unity. Deuterium isotope effect is not significant on the formation of this mercapturic acid. As mentioned in the preceding section, (346/5)-BTC is the most probable precursor for 4-chlorophenylmercapturic acid. Besides, there is no significant (primary) isotope effect on the *in vitro* dechlorination of lindane to (346/5)-BTC as expected.

These results combined together strongly suggest that the primary step of 4-chlorophenylmercapturic acid formation is the dechlorination of lindane to (346/5)-BTC. The major site of the dechlorination is probably the liver, although anaerobic microbes in the rat might partly contribute to the conversion.

CONCLUDING REMARKS

We have discussed major metabolic pathways and reactions of lindane and related compounds. Among them, dehydrogenation, dehydrochlorination and oxygenation are included. In the oxygenation reactions, several mechanisms are operating: ene-like reaction, oxygen insertion into C-H bond, and nucleophilic oxygen attack. There is another type of metabolic reaction: conjugation reactions, among which we described only glutathione conjugation. This reaction generally occurs by a nucleophilic attack of glutathione molecule towards one of the allylic carbons in the substrate. Reductive metabolism causing dechlorination was also mentioned. We tried to discuss some mechanistic features of these metabolic reactions.

The formation route of some metabolites remains unexplained: tetrachlorocyclohexenol isomers⁴⁶⁾ and tetrachlorophenylmercapturic acid.⁴⁵⁾ Some explanations for

reaction mechanisms are still rather speculative.

We believe that most part of the problems on the lindane metabolism pathways are solved now, although smaller portions of them still remain unsolved. The remaining problems deserve further investigations. Though these problems appear not very important at present, they might present general big problems in xenobiotic biochemistry in the future. Further progress is expected in this field.

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