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Metabolism of Hexachlorocyclohexane to Chlorophenols and Effect of Isomer Pretreatment on Lindane Metabolism in Rat

Joseph J. Freal* and Robert W. Chadwick

The role of γ-2,3,4,5,6-pentachlorocyclohex-1-ene (γ-PCCH) in the metabolism of lindane (γ-HCH) and the effect of pretreatment with various HCH isomers on lindane metabolism was evaluated in this study. Four groups of rats were treated perorally daily with one of four configurational isomers of hexachlorocyclohexane (α-, β-, γ-, or δ-HCH). One group of animals received γ-PCCH. After 1 week of pretreatment, all animals received an oral dose of γ-HCH. The daily urinary excretion of free chlorophenols was analyzed by glc. While β-HCH was metabolized to 2,4,6-trichlorophenol and γ-PCCH was metabolized to 2,4,5-trichlorophenol, the α- and δ-HCH were metabolized to both 2,4,5-, and 2,4,6-trichlorophenol. Rats treated with lindane, however, excreted 2,4,6-, 2,3,5-, and 2,4,5-trichlorophenol, 2,3,4,6- and 2,3,4,5-tetrachlorophenol, and 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol. If γ-PCCH is an intermediate, it is a minor one, and more efficient pathways are involved in the metabolism of lindane. This study also indicates that pretreatment with the isomers of HCH alters the metabolism of lindane in rats.

Grover and Sims (1965) reported the urinary excretion of the metabolites 2,4,5- and 2,3,5-trichlorophenol (2,4,5- and 2,3,5-TCP) in rats treated with γ-hexachlorocyclohexane (γ-HCH, lindane). They also administered γ-2,3,4,5,6-pentachlorocyclohex-1-ene (γ-PCCH) intraperitoneally and again detected these two trichlorophenols. Because this metabolic pattern resembled the degradation of 1,2,4-trichlorobenzene in rabbits, these investigators speculated that the metabolism of lindane in rats proceeds *via* dehydrochlorination through γ-PCCH to 1,2,4-trichlorobenzene and then to the chlorophenols. However, it has recently been reported that in addition to the chlorophenols identified by Grover and Sims, rats pretreated with lindane also excreted 2,4,6-trichlorophenol (2,4,6-TCP), 2,3,4,5- and 2,3,4,6-tetrachlorophenol (2,3,4,5- and 2,3,4,6-TTCP), and 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol (PCCOL) (Chadwick and Freal, 1972a). These newly identified lindane metabolites are all excreted in greater quantities than either 2,3,5- or 2,4,5-trichlorophenol. If lindane is metabolized exclusively through γ-PCCH, then administration of this intermediate to rats should also result in the excretion of all the known lindane metabolites. However, Reed and Forgash (1968) have reported finding a second PCCH isomer from the exposure of houseflies to lindane. This isomer, referred to as iso-PCCH, is metabolized to a greater extent than γ-PCCH in the fly. Therefore, it is reasonable to assume that lindane metabolism may proceed through more than one intermediate. Furthermore, the metabolite excretion patterns resulting from the biodegradation of other hexachlorocyclohexane isomers might yield information on the type of pentachlorocyclohexene intermediates involved. Finally, pretreatment with other isomers of hexachlorocyclohexane could significantly alter the metabolism of a single oral dose of lindane.

The present study was undertaken to determine the type and quantity of metabolites excreted by rats treated with γ-PCCH and α-, β-, γ-, and δ-HCH. In addition, after 1 week of pretreatment, lindane was administered to all rats and the urine samples were analyzed to determine the comparative excretion of lindane-derived metabolites.

APPARATUS

The gas chromatograph employed was a Micro-Tek 220 using a Coulson electrolytic conductivity detector operated in the oxidative mode. A 6 ft × ¼ in. U-tube glass column containing 5% DEGS on 80/100 mesh Gas Chrom Q and isothermally maintained at 165° was used in the analysis. The temperatures of the combustion furnace, the transfer valves, and the inlet were 800, 225, and 200°, respectively. The nitrogen carrier gas flow was regulated at 90 ml/min.

REAGENTS

The isomers of HCH were obtained from the Primate and Pesticide Effects Laboratory Repository and were 99+% pure. The γ-PCCH was obtained from 98+% pure lindane, with mild NaOH dehydrochlorination (Nakajima *et al.*, 1949) and purified using a silicic acid column (Reed and Forgash, 1970). Technical grade chlorophenol standards (Aldrich Chemical Company) were purified by recrystallization.

PROCEDURE

Weanling female Sprague–Dawley rats were maintained on a purified vitamin A test diet (Nutritional Biochemicals Corporation, Cleveland, Ohio). The rats were kept in individual metabolism cages so that urine and feces could

Environmental Protection Agency, Primate and Pesticide Effects Laboratory, Perrine, Florida 33157.

be collected quantitatively. A vitamin supplement (Vi-Penta Multivitamin drops, Hoffman-LaRoche, Inc., Nutley, N. J.) was administered in their water.

Four groups of four rats each were randomly selected and treated with either α-, β-, γ-, or δ-HCH. One group of two rats was randomly assigned and received γ-PCCH. The doses of all compounds were 2 mg/rat per day, administered orally in 0.1 ml of peanut oil. A control group of four randomly selected rats received 0.1 ml of peanut oil orally each day.

After 7 days of pretreatment, all animals were administered 2 mg/rat of lindane. Twenty-four-hour urine samples were collected under toluene. Samples that could not be extracted and analyzed immediately were stored frozen.

Urine samples were acidified with acetic acid to pH 4 and extracted three times with equal volumes of benzene. After separation of the two phases, the chlorophenols were partitioned from the benzene into 10 ml of 0.1 *N* NaOH. The 0.1 *N* NaOH was then acidified and extracted with 2 ml of benzene. The benzene extract, diluted when necessary, was then analyzed on the gas chromatograph. The PCCOL content of the urine was determined from the glc analysis of the initial benzene fraction after partitioning the chlorophenols into NaOH.

Duncan's multiple range test (Duncan, 1955) and the Students "*t*" test (Snedecor, 1956) were used as aids in interpreting the data.

RESULTS AND DISCUSSION

Of the five compounds administered, two are metabolized to two trichlorophenols and lindane is metabolized to at least six chlorophenols (Figure 1). γ-PCCH is metabolized primarily to 2,4,5-TCP, with only a trace of 2,3,5-TCP being detected. β-HCH is metabolized to 2,4,6-TCP. α- and δ-HCH are metabolized to both 2,4,5- and 2,4,6-TCP. In contrast, lindane (γ-HCH) is metabolized to 2,4,6-, 2,3,5-, and 2,4,5-TCP, and 2,3,4,6- and 2,3,4,5-TTCP, in addition to a configurational isomer of PCCOL. It will be noted that the metabolite 2,4,6-TCP, which had been previously detected in the urine of rats administered α-HCH (Koransky and Portig, 1962), is also excreted by the rats treated with β-, γ-, and δ-HCH.

Figure 2 summarizes the quantitative excretion of the chlorophenols observed during the 7-day dosing period. δ-HCH pretreated animals excrete significantly more 2,4,6-TCP than the α-, β-, or γ-HCH pretreated rats. After the second day of pretreatment, α-HCH pretreated rats excrete significantly more 2,4,5-TCP than the other HCH pretreated animals. γ-PCCH is degraded to 2,4,5-TCP much more efficiently than any of the other compounds used. Only γ-HCH pretreated rats excrete measurable quantities of 2,3,5-TCP.

After 1 week of pretreatment with the individual HCH isomers, the total amount of chlorophenol excreted increased in the order $\delta > \gamma > \alpha \gg \beta$. The solubility of the isomers in organic solvents (Slade, 1945) follows the same sequence. Since the availability of the microsomal enzymes to a substrate is dependent upon the lipid solubility of the substrate, the total chlorophenol excretion could represent the relative accessibility of the respective HCH isomers to these drug metabolizing enzymes.

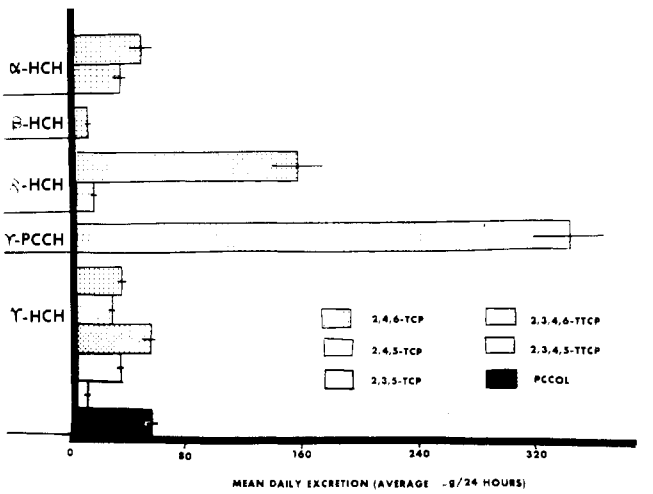

Figure 2. Comparative mean daily excretion of chlorophenols by rats pretreated for 1 week with either one of four HCH isomers or γ-PCCH. For the HCH pretreatment, each bar represents the mean μg/24 hr excretion of four animals ± S.E. For the PCCH pretreatment, the bar represents the mean μg/24 hr excretion of two animals ± S.E.

However, the order of storage of the HCH isomers in fat, as determined by Davidow and Frawley (1951), is $\beta \gg \alpha > \delta > \gamma$. Therefore the order of metabolism and/or urinary excretion of the HCH isomers may be reasonably assumed to be $\gamma > \delta > \alpha \gg \beta$. Comparing this sequence with the order of elimination of the HCH isomers *via* the chlorophenols suggests that γ-HCH is not eliminated entirely in the form of chlorophenols. In fact, lindane must be metabolized by routes not exclusively dependent on dehydrochlorination, as indicated by the significant excretion of 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol, which can not result from dehydrochlorination. For example, on day 7 the γ-HCH pretreated rats excreted 4.6% (83 μg) of the daily dose of γ-HCH as PCCOL compared with 13.7% (248 μg) as total free chlorophenols. Preliminary data also indicate that PCCOL is not an intermediate metabolite in the pathway to the chlorophenols.

Since β-HCH is metabolized to 2,4,6-TCP and to no other chlorophenols (Figure 1), it appears that *cis*-dehydrochlorination may lead exclusively to this metabolite. On the basis of this conclusion it may be further hypothesized that *trans*-dehydrochlorination results in the formation of the 2,4,5-TCP metabolite, since this compound was produced from those isomers where the orientation of adjacent hydrogen and chlorine alone permits a *trans*-dehydrochlorination to occur. Accordingly, twice as much 2,4,6-TCP should be excreted by the rats pretreated with α- and γ-HCH, since both compounds have twice as many adjacent *cis*- as *trans*-hydrogen and chlorine atoms available for dehydrochlorination (see Figure 3). The degradation of δ-HCH, on the other hand, should lead to a preponderance of 2,4,6-TCP elimination. After pretreatment with δ-HCH, the excretion of 2,4,6-TCP is nine times greater than that of 2,4,5-TCP, as expected. However, in the case of α-HCH metabolism, the excretion ratio of 2,4,6- to 2,4,5-TCP is only 1.3:1 and not the expected 2:1. The breakdown of γ-HCH leads to about the same excretion ratio of 2,4,6- to 2,4,5-TCP as the α isomer, but is further complicated by additional metabolic pathways,

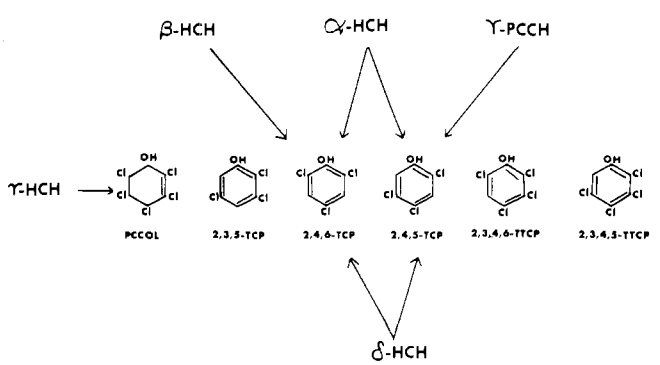

Figure 1. Chlorophenol excretion in rats pretreated with either one of four HCH isomers or γ-PCCH. Only γ-HCH produces all the chlorophenols shown.

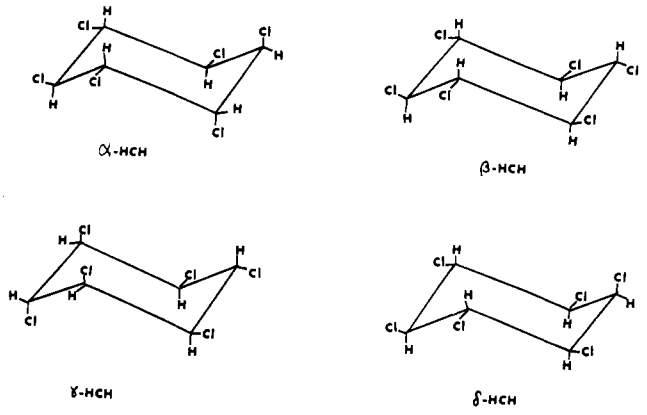

Figure 3. The structural configurations of the four hexachlorocyclohexanes used in this study. Note that β-HCH has all equatorial chlorines.

resulting in the formation of tetrachlorophenols and other metabolites, including PCCOL.

Thus the spatial orientation of hydrogen and chlorine atoms within the molecule is apparently not the only factor governing the metabolism of these compounds. The specific stereochemical configuration of the isomers, however, presumably controls the preferred reaction sequence and the ultimate excretion ratio of the chlorophenols.

The metabolism of γ-PCCH to 2,4,5-TCP is approximately 14 times faster than the metabolism of γ-HCH to 2,4,5-TCP. It appears that the first dehydrochlorination is the rate-limiting step. These observations confirm the speculation of Grover and Sims (1965) that dehydrochlorination is the rate-limiting step for the metabolism of lindane to the trichlorophenols.

Figure 4 summarizes the chlorophenol and PCCOL excretion pattern of the 24-hr urine samples following lindane administration to all rats on the last day. The data are presented as percent of control values. Although pretreatment of rats with either α- or δ-HCH produces a significantly higher excretion of total trichlorophenols than that of the control animals, only pretreatment with the α-isomer results in a significantly greater elimination of each individual trichlorophenol. The γ-HCH pretreated animals excreted significantly more tetrachlorophenols than the controls or the animals pretreated with the other HCH isomers.

The effect of pretreatment with the HCH isomers upon the metabolism of lindane to chlorophenols decreases in the order $\alpha > \delta > \gamma \gg \beta$, as measured by the total excreted chlorophenols. Only α-HCH pretreatment results in the excretion of significantly more 2,3,5-TCP than the controls. Since this trichlorophenol is not derived from stored α-HCH, it can be taken as an index of the stimulation of the enzymes responsible for the metabolism of lindane to the trichlorophenols. γ-HCH pretreatment stimulates the excretion of significantly greater amounts of the tetrachlorophenols than all other pretreatments. In a previous study, after pretreating a group of rats with lindane for 2 weeks, these animals plus an equal number of controls were administered [^{14}C]lindane (Chadwick *et al.*, 1971). The animals pretreated with lindane excreted significantly more radioactivity in the urine than the controls. Later work confirmed this result and further demonstrated that the lindane-pretreated rats were excreting significantly more radioactivity than the controls in the urinary extract containing the chlorophenols (Chadwick and Freal, 1972b). Thus, though stored γ-HCH is probably contributing some tetrachlorophenol to that excreted by the γ-HCH pretreated rats, there is also a significant increase which can only be attributed to induced enzyme activity. Furthermore, in the present study, the proportion of tetrachlorophenols to total excreted chlorophenols from the γ-HCH pretreated rats is significantly higher than that from the controls. Thus, it would appear that pretreatment with this isomer stimulates the specific enzymes responsible for the metabolic reaction leading to the formation of tetrachlorophenols. PCCOL excretion is not significantly affected by the various pretreatments.

Figure 5 summarizes the most recent data regarding the metabolism of lindane in animals. The portion of the proposed scheme from lindane through γ-PCCH and iso-PCCH to the chlorobenzenes is from the work by Reed and Forgash on houseflies (Reed and Forgash, 1969). Pen-

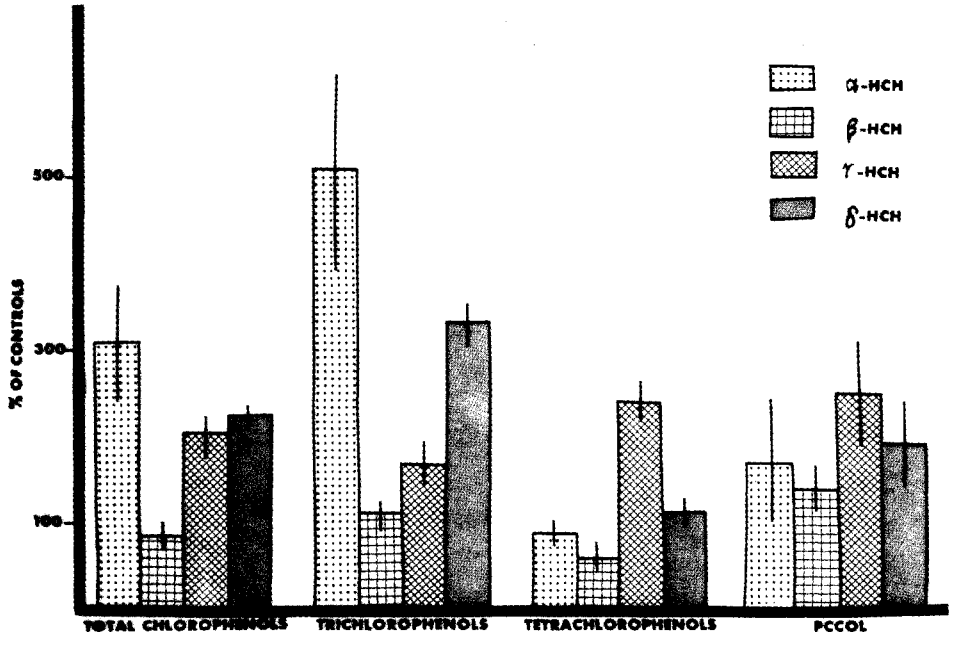

Figure 4. Influence of HCH isomer pretreatment on the excretion of chlorophenols and PCCOL after a single dose of lindane. The bars represent the mean values of the treatment groups relative to that of the controls and are plotted as percent ± S.E. (treatment mean/control mean × 100). Each bar represents the mean value of four animals. Control value means (μg excreted) ± S.E. for: 2,4,5-TCP, 43 ± 7.4; 2,3,5-TCP, 4 ± 0.5; 2,4,5-TCP, 17 ± 2.2; 2,3,4,6-TTCP, 44 ± 7.5; 2,3,4,5-TTCP, 14 ± 3.6; and PCCOL, 41 ± 11.5.

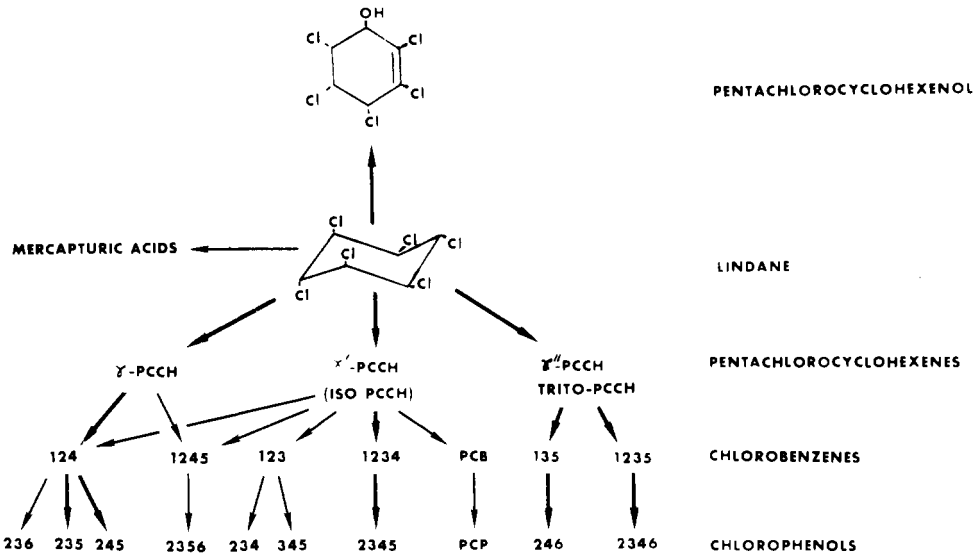

Figure 5. Summary of the identified lindane metabolites and a proposed pathway for the formation of the chlorophenols from lindane. The heavy arrows signify major pathways in the rat.

tachlorophenol has been detected as a metabolite in rabbits (Karapally *et al.*, 1971) but not in rats (Chadwick and Freal, 1972a). Furthermore, guinea pigs treated with DDT + lindane excrete measurable amounts of 2,3,5,6-TTCP (Chadwick *et al.*, 1973). Lindane is probably metabolized to mercapturic acid derivatives through chlorobenzenes, since mercapturic acids were excreted when chlorobenzenes were administered to rabbits (Jondorf *et al.*, 1955). If pentachlorocyclohexene isomers are intermediates in the metabolism of lindane to the chlorophenols in rats, as they are in flies, then at least one more remains to be identified (referred to as "trito-PCCH in Figure 5), since neither γ-PCCH nor iso-PCCH could account for the formation of 2,4,6-TCP or 2,3,4,6-TTCP (Reed and Forgash, 1969). However, although γ-PCCH has been identified in houseflies, plants, and microorganisms, there is no evidence that mammals produce this intermediate. Furthermore, the formation of PCCOL would be difficult to account for by an initial step involving dehydrochlorination.

Thus it would appear that the metabolism of lindane in mammals involves additional, more efficient pathways than dehydrochlorination through γ-PCCH to the trichlorophenols. While the α isomer appears to stimulate the dehydrochlorination route, pretreatment with γ-HCH stimulates the enzymes of the more efficient pathway leading to formation of the tetrachlorophenols.

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