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Biotransformation of Lindane in the Rat

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The metabolism and toxicity of benzene hexachloride (BHC), especially of the γ-isomer (lindane), in various living organisms has been periodically reviewed (Matsumura 1976, Engst et al. 1979). On the basis of the nature of urinary metabolites isolated from rats fed BHC, Grover and Sims (1965) proposed a pathway involving mainly dehydrochlorination reactions yielding chlorinated aromatic compounds. This postulated scheme of detoxification of the pesticide has formed the basis of several subsequent studies to characterise the metabolites (Freal and Chadwick 1973 Engst et al. 1979). Whereas there is general agreement that a series of chlorinated phenols and benzenes are the ultimate excretory products, either in the free form or as conjugated water-soluble compounds, opinions on the nature of the early transformations of lindane are divergent. Based on their extensive investigations, Engst et al. (1976) have suggested that γ-pentachlorocyclohexene (γ-PCCHE) is the first intermediate arising from lindane and that it has a central position in the mammalian degradation of lindane. On the other hand, Chadwick and his collaborators were unable to detect γ-PCCHE (Chadwick and Freal 1972, Freal and Chadwick 1973) and have suggested (Chadwick et al. 1975) a novel dehydrogenation reaction of lindane, resulting in the formation of γ-hexachlorocyclohexene (γ-HCCHE). The existence of this important oxidative metabolite, HCCHE from lindane has been proved by other investigators recently (Stein et al. 1977, Tanaka et al. 1979).

The major emphasis in the investigations reported in this paper has been on *in vitro* enzymatic capabilities of rat liver, although some studies with the whole animal have also been conducted. These investigations have provided conclusive evidence for the aromatisation of lindane to hexachlorobenzene.

MATERIALS AND METHODS

Analytical standards of γ-hexachlorocyclohexane (lindane), and some metabolites were from Environmental Protection Agency, Research Triangle Park, North Carolina, USA; Hexachlorobenzene (OAS grade) from BDH Chemicals, Poole, UK; Glucose-6-phosphate, Glucose-6-phosphate dehydrogenase, NADP, nicotinamide, Trishydroxymeth-ylamine methane, Triton X-100, Florisil (60-100 mesh, PR grade), 2,5-diphenyloxazol (PPO) and 1,4-bis-2-(5-phenyl-oxazolyl)-benzene (POPOP), from Sigma Chemical Co., St. Louis, USA; U-14C-lindane, (sp. act. 46 mCi/mmole) from the Radiochemical Centre, Amersham, UK; The antibiotic 'Keflin' from Eli Lilly & Co., Indianapolis, Indiana, USA; SKF 525-A from Smith, Kline & French Laboratories, Philadelphia; Silica gel G from E. Merck, Darmstadt, Germany. All other chemicals and solvents were of 'Analar' grade obtained from BDH Ltd., Poole, England. Aroclor 1254 was obtained as a gift from Monsanto Chemical Co., St. Louis, Missouri, USA.

Male albino rats of the Wistar strain, weighing between 100-150 g and reared on a nutritionally adequate laboratory stock diet, were used. For studies on induction of microsomal enzymes, the rats were administered a single dose of Aroclor 1254 (50 mg/kg body wt), by stomach tube 48 h before sacrifice.

Animals were administered orally, 40 mg/kg body wt of lindane in groundnut oil on days 1,3 and 5. Urine and faeces were collected daily up to day 7. The metabolites were extracted and fractionated as described (Crist et al. 1975).

The liver slices (0.5 mm thick, 1.0 g) suspended in 4 ml of Krebs-Ringer phosphate buffer containing 1 μCi of U-14C-lindane and 10 mg of a broad spectrum cephalosporin antibiotic (Keflin), were incubated for 60 min at 37°C in Warburg flasks. The incubation was terminated by the addition of 1.0 ml 2 N HCl . The liver slices as well as the incubation medium were homogenised and extracted with benzene (Chadwick et al. 1975). The extracts were dried under vacuum.

Livers were suspended in ice-cold 0.1 M Tris-HCl buffer, pH 7.5 and made into 20% homogenate in the same medium. The subcellular fractions were prepared by the method described by (Chadwick et al. 1975). Protein concentration was determined by the Lowry method (Lowry et al. 1951).

The *in vitro* incubation mixture of total 3 ml consisted of 1 ml of 9000 g supernatant or microsomes or cyto-

sol (corresponding to 0.2 g wet liver) plus 2 ml of an aqueous mixture containing Tris-HCl buffer (pH7.5, 160 μmoles), nicotinamide (60 μmoles), NADP (2.05 μmoles), Glucose-6-phosphate (10 μmoles), Glucose-6-phosphate dehydrogenase (0.5 units), and lindane (0.34 μmoles dissolved in 0.1 ml of acetone). The heat inactivated enzyme preparation (100°C, 2 min) served as a blank to monitor non-enzymatic conversion. The mixture, in a 10 ml Erlenmeyer flask, was incubated for 2 h in a Dubnoff metabolic shaking incubator at 37°C. The reaction was terminated by the addition of 1.5 ml of 1 N HCl to the incubation mixture. The incubation mixture was extracted with benzene (Chadwick et al. 1975). The extracts were analysed by gas-liquid chromatography.

The metabolites obtained in the in vivo and the in vitro studies were separated by thin layer chromatography on silica gel GF-254, first with benzene as solvent and then with chloroform as solvent (Yang et al. 1975). The radioactive samples of hexachlorobenzene, were diluted with the unlabelled compound and repeatedly crystallised from cyclohexane.

The metabolites were separated by gas-liquid chromatography using Toshniwal Model RL-04 unit (Toshniwal Instruments, Bombay) fitted with an electron capture detector (3H source). The columns used for analysis consisted of 6 ft x 0.25 inch spiral glass tubes packed with 1.5% OV-17 + 1.95% QF-1 on chromosorb W DMCS and were maintained at 165°C. The injection port temperature was 210°C, the detector temperature was 185°C and the carrier gas was nitrogen at a flow rate of 80 ml/min.

The metabolites were also analysed by high pressure liquid chromatography using a Waters Assoc. Model ALC/GPC 244 Chromatograph, equipped with a model 6000 A solvent-delivery system, U6K injector and Model 440 UV Absorbance detector (254 nm). A μ-Bondapak C_{18} column (stainless steel, 300x3.9 mm I.D) with particle size 10 mm was used. Acetonitrile-water (60:40) at a flow rate of 2.5 ml/min. was used as the solvent.

The samples were taken into the counting vials to which 10 ml of scintillation fluid (0.3% of PPO and 0.01% of dimethyl-POPOP in toluene) was added. Counting was done in a Beckman LS-100 liquid scintillation spectrometer.

RESULTS AND DISCUSSION

The data on the incorporation of radioactivity from lindane into hexachlorobenzene (HCB), is summarised

in Table 1. A significant amount of radioactivity is found to be incorporated into HCB, which is found to be enhanced by prior treatment of the animal with Aroclor 1254.

Table 1. Incorporation of radioactivity from U-^{14}C-lindane into metabolites by rat liver slices*

Group	Radioactivity incorporated into Hexachlorobenzene (DPM x 10^{-3})
Control	35.00 ± 0.29
Aroclor administered	80.00 ± 0.41

* The results are given as mean ± SEM of four experiments

The time course of in vitro conversion of lindane to HCB has been investigated, and the data is presented in Figure 1. It is seen that the peak corresponding to HCB shows a time-dependent increase. Thus, while this peak is barely discernible at 30 min, it is quite marked at 120 min. It is observed however, that even at 120 min, the amount of HCB formed is relatively small, as seen by the compartive peak sizes. When the enzymes system was heat inactivated, prior to incubation, the conversion to HCB was totally abolished.

Figure 2 shows the gas chromatographic evidence for the oxidative transformation of lindane with microsomes from rat liver. It is observed from Fig. 2 that rat liver microsomes, in the presence of the NADPH generating system, metabolised lindane into HCB. Incubation of lindane with the 105,000 g supernatant fraction (cytosol) of rat liver homogenates, however, did not reveal any formation of HCB.

The effect of SKF 525-A, a known inhibitor of the mixed function oxidase of microsomes and especially of microsomol cytochrome P 450-catalysed oxidation, on the coversion of lindane to HCB has been studied and the results are shown in Figure 3. It is observed that there are gross changes in the pattern of metabolites in the presence and absence of SKF 525-A. Among the notable alterations is the total abolition of the peak due to HCB, when the incubation is carried out in the presence of SKF 525-A.

Figure 4 illustrates the results of the high pressure liquid chromatographic separation of the lindane meta-

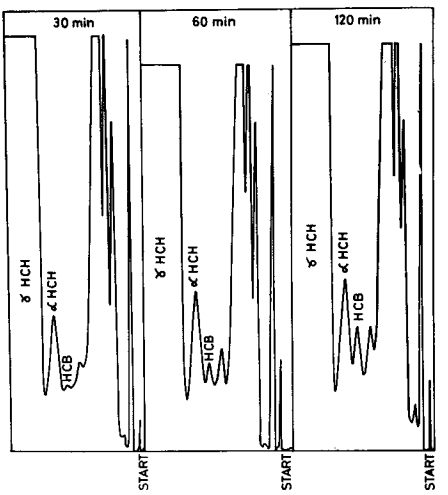

Figure 1 Time course of in vitro formation of HCB from lindane. Following incubation of 9000 g liver supernatant, metabolites have been fractionated by GLC.

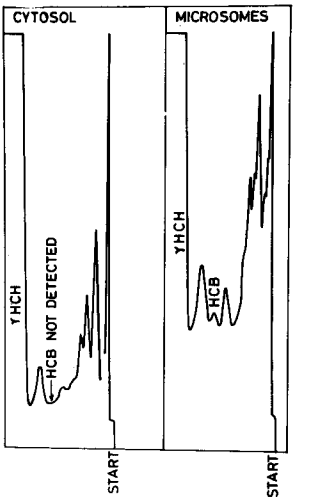

Figure 2. Microsomal formation of HCB from lindane. Following incubation of microsomes or cytosol, metabolites have been fractionated by GLC.

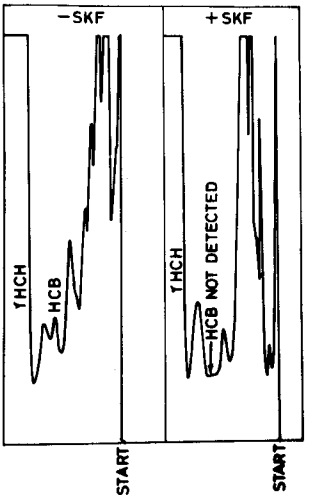

Figure 3. Inhibition of in vitro formation of HCB by SKF 525-A Following incubation of 9000 g liver supernatant, metabolites have been fractionated by GLC.

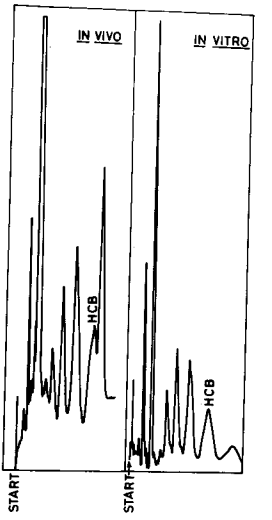

Figure 4. Detection of HCB in (i) faeces of rats fed lindane and (ii) liver 9000 g supernatant following incubation with lindane. The metabolites have been fractionated by HPLC.

bolites obtained from the in vivo and in vitro studies. The retention time of HCB under the experimental conditions was observed to be 8 min. In vivo experiments showed the presence of HCB only in the extracts from the faeces and not in the urine of rats administered lindane. The HCB peak was also present in the metabolites from the in vitro studies.

The large number of studies that have been carried out earlier on the identification of metabolites, especially in the urine, following the oral administration of lindane in the whole animal, have been helpful in elucidating the overall detoxification of the pesticide. Such efforts do not, however, yield unequivocal data on the mammalian capability for specific metabolic transformations, as they do not delineate the influence of the intestinal micro-organisms. The possibility also exists that the characterisation of urinary metabolites, comprising the ultimate water-soluble excretory products, may not generate comprehensive information on the metabolic pathway, since the tissue may contain metabolites that are either very poorly or not at all degraded and may therefore, tend to accumulate. In view of these considerations, the present studies, have concentrated mainly on in vitro systems involving the liver tissue, although some attention has also been paid to whole animal studies, to assess their relevance.

Among the most important observations emanating from the present studies is the conversion of lindane to HCB. This metabolite has been shown to arise in vitro as well as in vivo. The inability in the present investigations to detect HCB in urine may have its explanation in the findings of others that this compound is essentially excreted through the faecal route, both in monkeys (Yang et al. 1978) and in rats(Koss and Koransky 1975). Its identification has been established beyond doubt using both gas liquid chromatography and high pressure liquid chromatography, as well as by crystallisation to radio purity. The enzymatic nature of its formation is further confirmed by the time course of its formation and its absence as a metabolite, when the enzymes are heat inactivated. The formation of HCB from lindane would represent an aromatisation process involving the sequential removal of 6 hydrogen atoms, but ;with all the chlorine atoms being retained in the molecule. This is not a novel biological reaction, as HCB has been identified as a metabolite of lindane in both microorganisms and plants (Engst et al. 1977). The presence of this compound has not, however, been established in animal tissues, although its possible existence has been referred to one in investiga-

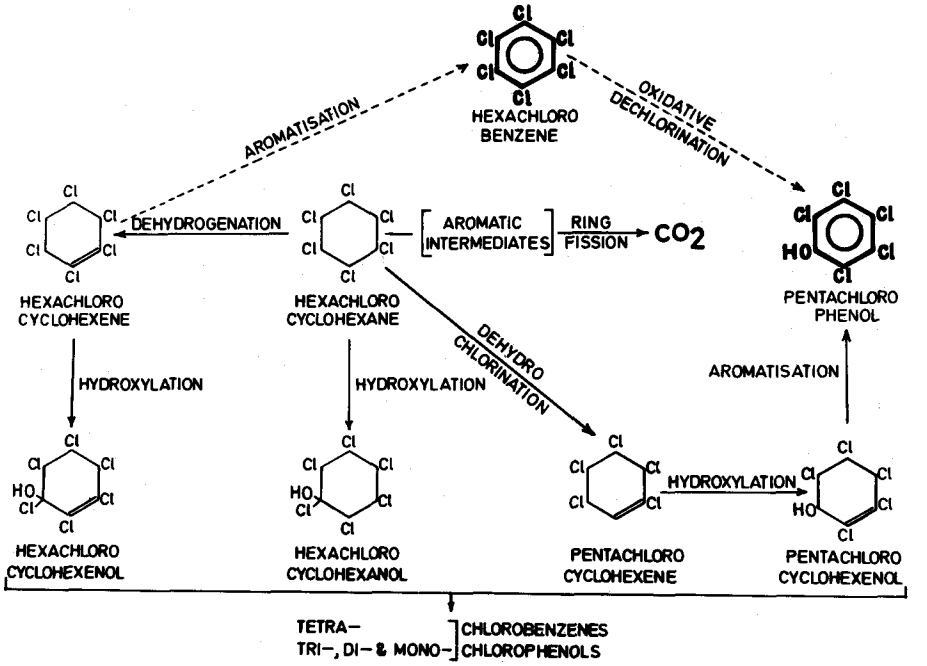

Figure 5. Mammalian biotransformation of lindane The metabolites identified in the present studies are shown in bold; the broken lines represent pathways that have been proposed for the first time.

tion (Seidler et al. 1975). Of considerable interest is the recent report that the whole blood of persons exposed to hexachlorocyclohexane contained HCB and other metabolites in substantial quantities (Engst et al. 1978).

Although the precise reaction mechanism is unknown at the present time, it is likely that the formation of HCB may involve the partially dehydrogenated metabolite HCCHE, as an intermediate. The formation of HCCHE has been shown to be catalysed by microsomal enzymes (Chadwick et al. 1975), and it has been proposed that it may take place by the oxenoid mechanism. The microsomal nature of the conversion of lindane to HCB in the present studies may be inferred from:)(i) liver cell fractionation studies, where the activity is localised in the post-mitochondrial fraction, (ii) enhancem-ent of the reaction by administration of Aroclor, an inducer of microsomal enzymes, and (iii) inhibition of the transformation by SKF 525-A which is known to interfere with the microsomal mixed function oxidase. The dehydrogenation iof lindane to HCB has a parallel

in the conversion of several hydro-aromatic precursors to benzenoid derivatives (Aiyar et al. 1971), and may be a more widespread reaction mechanism in mammalian organisms than is generally believed.

The findings presented in this paper provide conclusive evidence for the formation of HCB from lindane and demonstrates the existence of some important and novel routes of mammalian degradation of the pesticide not hitherto known. The presence of hexachlorobenzene in the faeces of rats dosed with lindane, may point to the in vivo relevanc e of the liver enzyme studies.

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