In Vitro Metabolism of Desmethyl Tetrachlorvinphos by Soluble Fraction (105 000g) from Chicken Liver Homogenates

M. Humayoun Akhtar

The metabolism of desmethyl tetrachlorvinphos [2-chloro-1-(2,4,5-trichlorophenyl)[^{14}C]vinyl methyl hydrogen phosphate] was investigated in vitro with the soluble fraction (105 000g) from chicken liver homogenates. The primary step in the metabolism was associated with the enzymatic hydrolysis to yield 2,4,5-trichlorophenacyl chloride. The hydrolytic metabolite was rapidly metabolized to 2,4,5-trichloroacetophenone, 1-(2,4,5-trichlorophenyl)ethanol, and 2-chloro-1-(2,4,5-trichlorophenyl)ethanol. The soluble fraction was shown to contain hydrolase, reductase, and GSH-dependent reductive dechlorinase.

It has been shown that soluble enzyme fractions (100 000–105 000g) from mammalian (mouse, rat, rabbit, and pig) and avian (chicken) liver contain a reduced glutathione GSH-dependent enzyme system which metabolizes an organophosphorus triester into two products, namely *S*-methylglutathione and the monodesmethyl derivative (a diester) of the corresponding insecticide (Hodgson and Casida, 1962; Fukami and Shishido, 1966; Morello et al., 1968; Stenerson, 1969; Hollingworth, 1970; Hutson et al., 1972; Akhtar and Foster, 1977). This enzyme system has been classified as glutathione *S*-alkyltransferase.

Tetrachlorvinphos, a triester and an important member of the vinyl organophosphates, is widely used in both crop and animal production. Akintonwa and Hutson (1967) investigated the metabolism of the insecticide in rat and dog and observed a distinct species difference in the mode of degradation. Thus, during in vivo metabolism tetrachlorvinphos [2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate] underwent (1) P–O–CH_3 and (2) P–O–vinyl cleavage in dog and rat, respectively. The first produced polar monodesmethyl tetrachlorvinphos (a diester), whereas the second generated lipophilic 2,4,5-trichlorophenacyl chloride. In vitro demethylation of tetrachlorvinphos by the soluble fraction from mammalian liver (mouse, rat, rabbit, and pig), in the presence of GSH, proceeded with similar activity (Hutson et al., 1972). On the other hand, Donninger et al. (1972) demonstrated that demethylation by the microsomal fraction from the liver of these species required molecular oxygen and NADPH as a cofactor and noted a species difference. The degradation proceeded in the order dog > rabbit > mouse > rat. Donninger et al. (1971) had also reported that mammalian (rat and pig) liver contained an enzyme which catalyzed the hydrolysis of desmethyl tetrachlorvinphos to 2,4,5-trichlorophenacyl chloride.

Recently, Akhtar and Foster (1977) found that the soluble fraction from chicken liver also contained an enzyme system capable of desmethylating the insecticide in the presence of GSH and observed that the polar fraction was further metabolized by other enzyme systems to benzene-extractable metabolites. The present investigation was undertaken to clarify the nature of the enzyme(s) and the reaction mechanism(s) responsible for the degradation of desmethyl tetrachlorvinphos into various organosoluble metabolites.

EXPERIMENTAL SECTION

Materials. Pesticide and AR grade solvents were used. [^{14}C]vinyl-labeled desmethyl tetrachlorvinphos (I), 2,2,-4,5-tetrachloroacetophenone (II), 2,4,5-trichloroacetophenone (III), 1-(2,4,5-trichlorophenyl)ethanol (IV), and 2-chloro-1-(2,4,5-trichlorophenyl)ethanol (V) were synthesized according to the procedures described earlier (Akhtar and Foster, 1977).

Enzyme Preparation and in Vitro Incubations. Soluble fraction (105 000g) from chicken liver homogenates was obtained as described earlier (Akhtar and Foster, 1977). It was incubated with I, II, III, IV, and V and appropriate amounts of freshly prepared reduced glutathione (GSH) in separate experiments. Incubations were carried out in glass-stoppered Erlenmeyer flasks (50 mL). A typical incubation mixture contained enzyme preparation (4.5 mL), reduced glutathione (0.5 mL), and desmethyl [*vinyl*-^{14}C]tetrachlorvinphos (110 μg, 3.1×10^{-7} mol). Reduced glutathione (GSH) was prepared just prior to addition by mixing 0.0027 g (8.7×10^{-6} mol) of glutathione with 0.5 N NaOH (0.05 mL) and neutralizing with 0.134 M phosphate buffer, pH 7.4 (0.45 mL). The flask was degassed with dry N_2, stoppered, and incubated at 37.5 °C for specific time intervals. These incubations were carried through in duplicate and on occasion in triplicate. The reactions were terminated by addition of excess acetone.

Extraction of Metabolites. The incubation mixture was filtered and the flask rinsed with acetone (3 × 10 mL). The residues were washed thoroughly with acetone and the combined extracts (120–130 mL) were evaporated to 5–7 mL. The concentrate was transferred to a centrifuge tube (15 mL), the flask washed with acetone (2 × 3 mL), and the combined solution reduced in volume (3–4 mL) under a gentle stream of air. The final volume of the solution was adjusted to 5 mL. The solution was extracted with benzene (3 × 5 mL) and separated into two phases by centrifugation. The combined benzene extracts were dried over anhydrous Na_2SO_4 and reduced in volume (2–3 mL) on a rotary evaporator. The final volume of the extract was adjusted to 5 mL and the radioactivity was measured.

Assay of Radioactivity. The radioactivity of the extracts was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3320, using an external standard and correcting the data for quenching. Benzene extracts were assayed in a scintillation solution containing PPO and POPOP in toluene (5 g, 50 mg, 1000 mL) whereas the radioactivity in aqueous fractions was measured in Aquasol (New England Nuclear), a xylene-based scintillation cocktail.

Gas Chromatography (GC). Benzene extracts were analyzed on a Packard-Becker 420 gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD). The column was a 1.83 m × 4 mm (i.d.) glass tube packed with 3% (w/w) SE-30 on 80–100 mesh Chromosorb WHP.

Animal Research Institute, Research Branch, Agriculture Canada, Ottawa, Ontario, K1A 0C6.

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Table I. Comparative Metabolism of Desmethyl Tetrachlorvinphos (I) by the Soluble Fraction (105 000g) from Chicken Liver Homogenates

Time, min	% I (unrecovered) Soluble	Soluble and GSH	Soluble (dial.)	Soluble (boiled)
30	35-40	32-38[a]		
75	59-60	63-64.5[a] 67-69[b] 66-71[c]		
180	83.5-84.5	84.5-86.5[a]	79-82[d] 75-81[e]	8-10[f]
285	90-92	93.5-94.5[a]		

[a] GSH concentration, 8.7×10^{-6} mol. [b] GSH concentration, 2.18×10^{-5} mol. [c] GSH concentration 3.5×10^{-5} mol. [d] Dialyzed against 0.134 M phosphate buffer, pH 7.4 at 4 °C for 4 h. [e] Dialyzed for 14 h. [f] Heated in boiling water for 5 min.

The operating temperatures for injector, column, and detector were 135, 130, and 295 °C, respectively; 5% methane–argon (flow through column 28 mL/min, purge 15 mL/min) was the carrier gas. Under the GC conditions described, the relative retention times for II, III, IV, and V were 1.00, 0.51, 0.74, and 1.26, respectively.

Thin-Layer Chromatography (TLC). Silica gel OF plates (New England Nuclear) were developed in an ethyl acetate–hexane (3:7, v/v) system. Compounds were located by spraying with silver nitrate–2 phenoxyethanol solution and viewing under UV light (254 nm). The R_f values for II, III, IV, and V were 0.63, 0.58, 0.44, and 0.49, respectively. Silica gel G plates (Fisher Scientific) were used for the analyses of water-soluble metabolites. Compound I had an R_f value of 0.42 in methanol–isopropyl alcohol–acetone (1:1:8, v/v/v).

Gas Chromatography–Mass Spectrometry (GC–MS) Analyses. The GC–MS analysis was accomplished in a Finigan Model 3100 mass spectrometer connected to a Finnigan Model 9500 gas chromatograph by means of a jet separator. The mass spectrometer was interfaced with a Model 6100 computer-controlled data acquisition system. A 1.52 m × 4 mm (i.d.) glass column packed with 3% SE-30 on 80–100 mesh Chromosorb WHP was used for gas chromatographic separation. The column was run at 175 °C and the helium flow rate was 35 mL/min. The mass spectra were recorded at 70 eV.

RESULTS

The data on incubation of substrate (I) with soluble fraction under various conditions are listed in Table I. The substrate remained unmetabolized (90–92% recovery) when incubated with boiled enzyme preparation for 3 h, but was efficiently metabolized when incubated with freshly prepared soluble fraction. Incubation of I with dialyzed soluble fraction did not lower the rate of metabolism; similarly the addition of GSH to the incubating media had no effect on the rate of metabolism.

Soluble fraction (105 000g) from chicken liver preparation rapidly metabolized I to organoextractable products. Figure 1, a plot percent original radioactivity in the two phases (aqueous and benzene) vs. time shows a gradual decrease in the radioactivity in the aqueous phase accompanied by an increase in the benzene extract. However, the decrease in radioactivity in the aqueous phase did not produce an identical increase with that in the benzene extract. The most striking changes were observed from 0 to 180 min, where the difference was most pronounced. For example, a 85% decrease in the aqueous phase of 180 min resulted in only a 65% increase in radioactivity in benzene extracts. This suggests the involvement of a complex reaction. It was observed that 74% of I had been metabolized to benzene extractable products at 285 min.

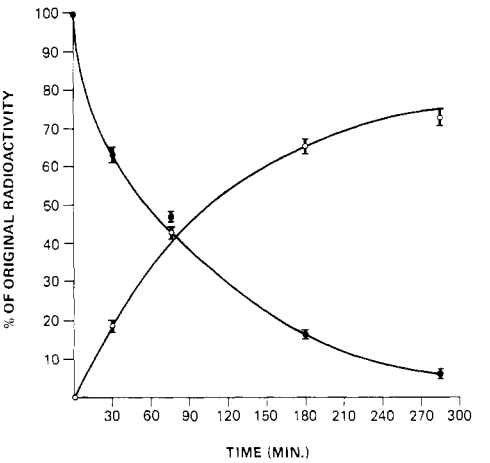

Figure 1. Percentage of the original radioactivity in the aqueous phase (●) and the benzene phase (○) of incubation medium at various intervals during an in vitro incubation.

Incubation of I with soluble fraction produced II, III, IV, and V. Metabolites IV and V were present in the benzene extracts of all the incubation mixtures. The ratio of these metabolites remained constant throughout the entire reaction. The benzene extracts of 30 and 75 min also contained II and III. A maximum concentration of II (8.5%) and III (11.2%) was found in the incubation mixture at 30 min. Both metabolites II and III could not be detected at 180 min. The identity of the metabolites II, III, IV, and V was established by cochromatography with authentic standards and finally by GC–MS analyses. For example, II, III, IV, and V exhibited molecular ion peaks at 256, 207, 209, and 258, respectively. Their mass spectra were identical with those recorded for standards.

Incubation of I with enzyme and GSH (8.7×10^{-6} mol) also produced all four metabolites. However, the amount of IV was higher than that recorded without additional GSH. Incubation of I with GSH (3.5×10^{-5} mol) and enzyme preparation for 180 min yielded IV as the only organoextractable product. On the other hand, incubation of I with a 14-h dialyzed soluble fraction contained both IV and V. The amount of V was much higher than that previously recorded.

It was shown by TLC that the aqueous phase of 0 to 285 min contained only the unmetabolized I, R_f 0.42. The identify of this product was established by treating the dried aqueous phase with diazomethane to yield tetrachlorvinphos.

To investigate the nature of unextractable radioactivity (not soluble in benzene and water), the residues (inorganic salts, denatured protein) of the incubation mixtures was refluxed with 6 N HCl for 4 h. The benzene extract accounted for 20–25% of the unextractable radioactivity. The radioactivity was associated with II, IV, and V. Careful neutralization of the dried acidic aqueous phase, ether extraction, and methylation with diazomethane yielded tetrachlorvinphos, 15–27%. Analyses of the aqueous phase on TLC in 1-butanol–acetic acid–water (11:4:5, v/v/v) exhibited ninhydrin positive spots. One of the spots was also sensitive to $AgNO_3$–2-phenoxyethanol system, which indicates a chlorinated amino acid.

The incubation of residues with soluble fraction produced II, III, IV, and V. Again, the addition of GSH to the incubation media produced a higher amount of IV. On

Table II. Effect of Reduced Glutathione (GSH) on Metabolism of 2,2,4,5-Tetrachloroacetophenone (III) to 1-(2,4,5-Trichlorophenyl)ethanol (IV) and 2-Chloro-1-(2,4,5-trichlorophenyl)ethanol (V)

Enzyme system[a]	% IV	% V
Not dialyzed	62.8	31.7
Not dialyzed + GSH (8.7×10^{-6} mol)	90.7	9.3
Dialyzed[b]	48.2	51.8
Dialyzed[c]	37.9	62.1
Dialyzed[d]	28.6	71.4
Dialyzed[d] + GSH (3.5×10^{-5} mol)	91.6	8.4
Boiled[e]	<2	
Boiled + GSH	<2	

[a] Reaction conditions were the same as recorded in the text, 102 μg (3.9×10^{-7} mol) of III, time 1 h. [b] Dialyzed against 0.134 M phosphate buffer, pH 7.4 at 4 °C, 4 h. [c] Dialyzed for 6 h. [d] Dialyzed for 14 h. [e] Heated in boiling water for 5 min.

the other hand, the dialyzed enzyme preparation was not very effective in releasing the radioactivity.

To investigate the method of degradation and mechanism of dechlorination, II was incubated with the enzyme preparation. Compound II was metabolized very rapidly and yielded products similar to those obtained from incubation of I.

The results of incubation of II with the enzyme preparation are listed in Table II. Boiled enzyme preparation did not convert II into III, IV, V; but II was not free to be extracted with benzene. Changing the GSH concentration of the incubation media produced varying amounts of IV and V. Increasing the GSH concentration increased the rate of formation of IV. On the other hand, incubation of II with dialyzed enzyme preparation increased the formation of V. Metabolite III was very rapidly metabolized to IV by the enzyme preparation.

To ascertain whether metabolite V was converted enzymatically into metabolite IV, V was incubated with enzyme preparation and varied amounts of GSH. No degradation of V by the enzyme preparation was observed. However, 10–12% of V remained unextractable with benzene.

DISCUSSION

The metabolic pathway of desmethyl tetrachlorvinphos (I) by the soluble fraction of chicken liver homogenates, as deduced from the *in vitro* studies reported above, is shown in Figure 2. Compound I, a dealkylated and primary metabolite of tetrachlorvinphos, was rapidly metabolized after incubation with the enzyme preparation. Changing the GSH concentration, either by removal by dialysis or by addition, of the incubation media did not affect the rate of metabolism of I (Table I). Thus, the first step in the metabolism of I is by a route which is not dependent on GSH concentration. This step may be identified as an enzyme-catayzed hydrolysis to yield II. Supporting evidence is found in the work of Donninger et al. (1971) who have previously observed a similar enzyme system in rat and pig liver which catalyzed the hydrolysis of I to II.

Incubation of I with the enzyme preparation produced benzene-soluble metabolites II, III, IV, and V. Metabolites II and III were not detected at 75 min. At the end of 180 min, the benzene extract contained metabolites IV and V in the ratios of 77:23. Changes in GSH concentration of the incubation media also changed the ratio of IV and V. A higher concentration (by addition) of GSH completely supressed the formation of V. Similarly, removal of GSH by dialysis increased the amount of IV produced. These

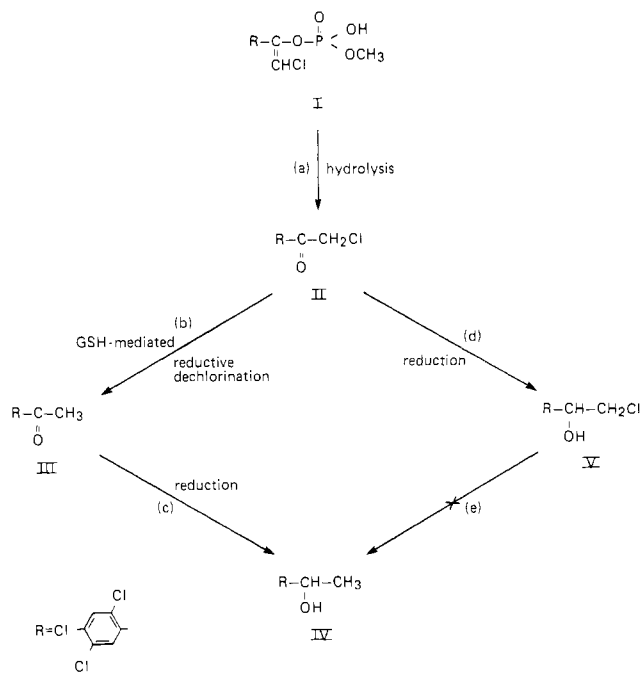

Figure 2. Metabolic pathways of desmethyl tetrachlorvinphos in chicken based on in vitro studies.

observations strongly suggest that the formation of V is dependent on GSH concentration in the incubation media.

The incubation of II with various enzyme systems produced products identical with those obtained by incubation of I with these systems. This established that metabolite II lies in the center of the metabolic pathway (Figure 2).

Enzymatic conversion of II to IV and V may occur by routes b,c and d, respectively (Figure 2). Since III was readily reduced to IV, the ratio of IV to V at any reaction time would be an estimate of the contribution due to each step. The data in Table II show the effect of GSH concentration on the in vitro production of IV and V. It can be seen that GSH concentration has a great effect on the ratio of IV and V. The higher the concentration of GSH in the incubation media, the greater the rate of formation of IV. Incubation of II and boiled enzyme with or without GSH did not produce III, IV, and V. These data indicate routes b,d are enzymatic in nature; and also point that dechlorination is an enzyme-catalyzed GSH-dependent route. However, these data do not permit deduction of the exact role of GSH in dechlorination. A reaction (enzymatic and chemical) between II and GSH to form a glutathione conjugate followed by subsequent enzymatic action to produce III appears to be a likely mode of conversion. Since this investigation was completed, an interesting study on the dechlorination of 2,4-dichlorophenacyl chloride, an intermediate arising from chlorfenvinphos, was reported by Hutson et al. (1976). These workers showed that the in vitro conversion of the chloride to 1-(2,4-dichlorophenyl)ethanol was a three-stage sequence which involved (1) reaction of the chloride with GSH to yield the phenacyl glutathione, (2) an enzyme-catalyzed reaction of a further molecule of GSH with the conjugate to form the ketone, and finally (3) the enzymatic reduction of the ketone to the alcohol. The present study is in agreement with the facts that dechlorination of II involves a GSH-dependent enzyme-catalyzed step and that the reduction of III to IV is an enzyme catalyzed reductive step. In view of the report of Hutson et al. (1976), it would be desirable to synthesize *S*-(2,4,5-trichlorophenacyl)glutathione and show that further conversion is catalyzed by a GSH-de-

pendent enzyme system. This aspect is currently being investigated in detail.

It might be expected that, under favorable conditions, V could be converted to IV via a route (e) (Figure 2). However, V remained unmetabolized with enzyme preparation and varied amounts of GSH. This indicates that V was not a substrate for IV.

Detection of I, IV, and V after acid treatment of the residues demonstrates that these compounds are bound to proteins. The nature of this binding was not further elucidated. Detection of a chlorinated amino acid (sensitive to both ninhydrin and $AgNO_3$–2-phenoxyethanol) is noteworthy. The identity of this compound could not be established because of insufficient material and lack of conjugated standard. However, the nature of the compound can be postulated. In view of the work of Hutson et al. (1976), II, a good alkylating agent, would readily react with GSH, a good nucleophile, to yield *S*-(2,4,5-trichlorophenacyl)glutathione. Treatment of this product with 6 N HCl at reflux would probably result in *S*-(2,-4,5-trichlorophenacyl)cysteine, glutamic acid, and glycine (Fukanaga et al., 1969). All these compounds would be ninhydrin positive, but only the phenacyl cysteine would also be detected by $AgNO_3$–2-phenoxyethanol.

The present studies indicate that the soluble fraction from chicken liver contains enzyme systems capable of performing hydrolysis, reduction, and reductive dechlorination. The study is being continued with enzyme preparations of liver homogenates of other species, such as goose, pig, sheep, etc. An in vivo study with laying hens will test the metabolic pathways hypothesized from in vitro studies.

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Accumulation and Depletion of Some Organochlorine Pesticides in High-Producing Laying Hens

Cornelis A. Kan* and Jenny C. Jonker-den Rooyen

Addition of low concentrations of organochlorine pesticides to the feed of high-producing laying hens for 16 weeks had no influence on feed consumption, body weight, egg production, egg weight, and deformation of the egg. Accumulation ratios (concentration of the pesticide in the egg or fat to its concentration in feed) on fat basis were: hexachlorobenzene in egg 11, fat 13; α-hexachlorocyclohexane in egg 2, fat 2; β-hexachlorocyclohexane in egg 13, fat 15; γ-hexachlorocyclohexane (lindane) in egg 2, fat 2; heptachlor(epoxide) in egg 5, fat 7; DDT (total) in egg 10, fat 12, dieldrin in egg 11, fat 14. Up to 80% of the pesticides ingested were excreted via eggs and feces. Half-value times of depletion of residues (with uncontaminated feed for 12 weeks) were 1.5–2 weeks for α- and γ-HCH. The other pesticides have half-value times of about 6–8 weeks. Correlations between concentrations of pesticides in abdominal fat and in egg fat within hens are generally very high ($r > +0.9$). The same holds for correlations between concentrations in abdominal fat and fat of the thigh muscle, breast muscle, liver, and egg.

The effect of egg production on accumulation of organochlorine pesticides in laying hens has been clearly demonstrated (Cecil et al., 1973; Kan and Tuinstra, 1976b; Kan and Jonker-den Rooyen, 1978b). The accumulation ratios in high-producing (laying percentage >90%) hens could, however, not be predicted from those experiments.

After reaching a plateau in residues in eggs, differences in residues between samples of different dates in these experiments still occur (Kan and Jonker-den Rooyen, 1978b; Waldron and Naber, 1974). It has not been established whether this variation is due to day-to-day differences in the analytical procedures, to changes for one hen, or to differences between hens, as generally not all eggs laid are used for analysis.

Descending half-value times of several pesticides in laying hens have been reported (e.g., Cummings et al., 1966, 1967; Wesley et al., 1966, 1969). The same holds for several procedures which accelerate depletion such as charcoal (e.g., Waibel et al., 1972), phenobarbital (e.g., Mick et al., 1973) or starvation (e.g., Wesley et al., 1969). However, ascending half-value times have not been reported.

In a previous study with heavy, low-producing broiler breeder hens (Kan and Jonker-den Rooyen, 1978b), a good correlation existed between residues in eggs and abdominal and intramuscular fat within hens, whereas between hens

Spelderholt Institute for Poultry Research, Ministry of Agriculture and Fisheries, 7361 DA Beekbergen, Netherlands.

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