wash fraction. By incorporating the ethoxylation step, the 3-hydroxycarbofuran was converted to 3-ethoxycarbofuran and was then totally separable from the phenols.

The formation of the 2,4-dinitrophenyl ether derivatives with the phenols of interest was optimum over a pH range of 8 to 9. Apparent release of acidic components during the derivatization reaction step lowered the initial phosphate buffer pH from 11.0 to the optimum range. A borax buffer of pH 9.0 was also found to give equivalent results. The derivatization was also enhanced by the addition of acetone. The addition of acetone produced a homogeneous solution which allowed greater contact between the water-soluble phenols and organo-soluble 1-fluoro-2,4-dinitrobenzene. The DNPE derivative standards are stable in a pure form and may be stored intact under refrigeration for indefinite periods of time. Storage of stock solvent solutions are also known to be stable for periods exceeding 3 months when stored under refrigeration. Room temperature storage of very dilute solutions in hexane and ethyl acetate are stable for at least 2 to 3 months. The derivatives are also stable under reflux conditions for at least 1 h.

The chromatographic characteristics of the nonpolar derivatives required little prior conditioning of the gas chromatographic column. Overnight heating at 275 °C with a constant carrier gas flow was usually sufficient for conditioning.

The methods described here can be applied to the detection of carbofuran phenols in a wide variety of commodities. Applicability of the technique to other systems requiring phenol analysis should also be readily attainable.

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Metabolism of Tetrachlorvinphos by the Soluble Fraction (105 000*g*) from Chicken Liver Homogenates

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The soluble fraction (105 000*g*) from chicken liver homogenates contains an enzyme(s) which metabolizes tetrachlorvinphos. Studies which employed the [^{14}C]vinyl-labeled insecticide indicated that the reaction was glutathione dependent. The primary step was demethylation to desmethyl tetrachlorvinphos, a water-soluble metabolite. It was shown that GSH acts as an acceptor for the transferred methyl group to form *S*-methylglutathione. The soluble fraction contains enzymes other than glutathione *S*-alkyltransferase. The polar fraction was further metabolized to benzene extractable compounds which were identified as 2,4,5-trichloroacetophenone, 1-(2,4,5-trichlorophenyl)ethanol and 2-chloro-1-(2,4,5-trichlorophenyl)ethanol.

Tetrachlorvinphos (2-chloro-1-(2,4,5-trichlorophenyl)-vinyl dimethyl phosphate) is an important member of the vinyl phosphate insecticide family. It is used on a wide range of crops including fruit, vegetables, corn etc. for the control of corn earworm, fall armyworm, codling moth, gypsy moth etc. In the cattle industry, it is used to control external parasites such as ticks, lice, and flies and is used to control mites and lice on and around chickens.

The metabolism of tetrachlorvinphos has been investigated in the rat and dog (Akintonwa and Hutson, 1967) and in the dairy cow (Gutenmann et al., 1971). Residue studies of the insecticide in tissues and eggs of chicken have also been reported (Ivey et al., 1969; Wasti and Shaw, 1971). Residues of the insecticide were detected in the omental fat and muscle of sprayed cattle (Ivey et al., 1968). Studies have also been reported on the residues of the insecticide in milk after dermal application (Oehler et al., 1969) and after feeding trials (Miller and Gordon, 1973).

The metabolic fate of tetrachlorvinphos in supernatant (100 000*g*) from mammalian (mouse, rat, rabbit, and pig) liver has been investigated (Hutson et al., 1972). The oxidative dealkylation of the insecticide by microsomes from liver homogenates has also been studied (Donninger et al., 1972). The present studies were undertaken to obtain information on the metabolism of tetrachlorvinphos by the soluble fraction (105 000*g*) from chicken liver homogenates.

The possible reactions involved in the metabolism of tetrachlorvinphos (I) in laying hens are summarized in Figure 1. The metabolites which might be encountered

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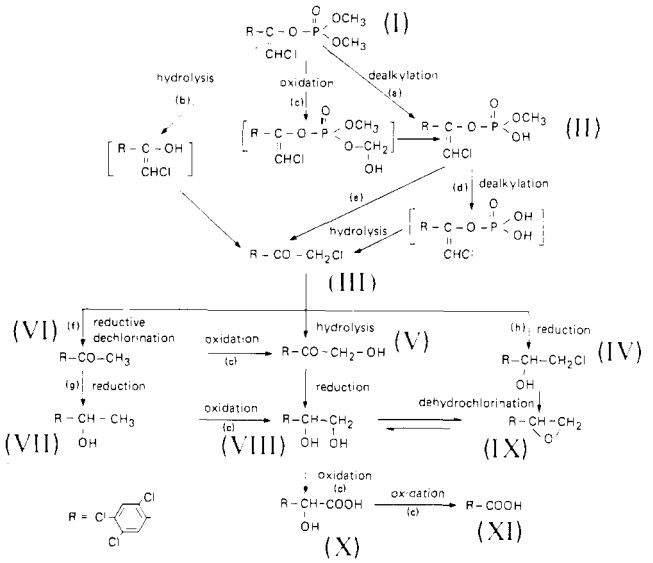

Figure 1. Possible metabolism of tetrachlorvinphos.

during the study are: desmethyl tetrachlorvinphos (II), 2,2,4,5-tetrachloroacetophenone (III), 2-chloro-1-(2,4,5-trichlorophenyl)ethanol (IV), 2,4,5-trichlorophenacyl alcohol (V), 2,4,5-trichloroacetophenone (VI), 1-(2,4,5-trichlorophenyl)ethanol (VII), 2,4,5-trichlorophenylethanediol (VIII), 2,4,5-trichlorophenyloxirane (IX), 2,4,5-trichloromandelic acid (X), and 2,4,5-trichlorobenzoic acid (XI).

EXPERIMENTAL SECTION

Chemicals. Pesticide and reagent grade solvents were used. Glutathione was obtained from Sigma Chemicals. Some possible metabolites and ^{14}C-labeled tetrachlorvinphos and desmethyl [^{14}C]tetrachlorvinphos were synthesized as follows:

2,2,2,4,5-Pentachloro[1,2-^{14}C]acetophenone. This compound was prepared by the condensation of dichloro[1,2-^{14}C]acetyl chloride (New England Nuclear, 95% purity) with 1,2,4-trichlorobenzene in the presence of anhydrous aluminum chloride at 110 °C for 5 h (Whetstone et al., 1966).

2-Chloro-1-(2,4,5-Trichlorophenyl)[^{14}C]vinyl Dimethyl Phosphate. [^{14}C]Tetrachlorvinphos was synthesized by the reaction of trimethyl phosphite with the pentachloro[^{14}C]acetophenone in toluene at reflux temperature for 1 h. The resultant labeled compound (4.1×10^4 dpm/105 μg) was found to be 99% pure by thin-layer chromatography (TLC) on silica gel G developed in chloroform–acetic acid (2:98, v/v), mp 96–96.5 °C (Whetstone et al., 1966, mp 97–98 °C).

2-Chloro-1-(2,4,5-Trichlorophenyl)[^{14}C]vinyl Methyl Hydrogen Phosphate. The sodium salt of desmethyl-[^{14}C]tetrachlorvinphos was obtained by reacting equimolar amounts of [^{14}C]tetrachlorvinphos and NaI in acetone at room temperature for 6 h. Free desmethyl [^{14}C]tetrachlorvinphos was liberated by treatment with a few drops of 1 N HCl, mp 135–136 °C, mixed melting point with nonlabeled standard was not depressed; 3.8×10^4 dpm/96 μg (Whetstone et al., 1966, mp 136–137 °C).

2,4,5-Trichloromandelic Acid. 2,2,2,4,5-Pentachloroacetophenone (5 g) was stirred constantly for 45 min with 12% (w/v) NaOH (25 mL). The mixture was treated with 6 N HCl until precipitation was complete. The precipitate was collected, washed with distilled water, and air dried. The yield was 4.03 g (92%), mp 142–149 °C. The compound was recrystallized from hot benzene as analytically pure 2,4,5-trichloromandelic acid, mp 148–149.5 °C (Akintonwa and Hutson, 1967, mp 120–133 °C).

Methyl 2,4,5-Trichloromandelate. 2,4,5-Trichloromandelic acid (3 g) in methanol (20 mL) was treated with an excess of freshly prepared diazomethane (from *N*-nitroso-*N*-methylurea treated with 50% aqueous KOH at −5 °C) to yield 2.8 g (88%) of this methyl ester; white needles, mp 117–118 °C. The structure of the compound was confirmed by IR and NMR spectra.

2,4,5-Trichlorophenylethanediol. A solution of methyl mandalate (1.35 g) in anhydrous ether (50 mL) was added dropwise to a suspension of lithium aluminum hydride (500 mg) in anhydrous ether (100 mL) over a period of 30 min. The reaction mixture was stirred at room temperature overnight and treated with aqueous 10% sodium–potassium tartrate. The precipitate was removed by filtration and washed with ether, and the combined ethereal fractions were dried over anhydrous Na_2SO_4. Removal of the solvent resulted in flakes of 2,4,5-trichlorophenylethanediol, 1.12 g (91%), mp 84–85 °C (Akintonwa and Hutson, 1967, mp 90–91 °C). The IR and NMR spectra agreed with the assigned structure.

2,4,5-Trichlorophenacyl Chloride. The chloride was prepared by refluxing tetrachlorvinphos with aqueous 40% (v/v) H_2SO_4 for 24 h. The product was extracted with dichloromethane and crystallized from hexane, mp 64–65 °C (Akintonwa and Hutson, 1967, mp 66–67 °C).

2,4,5-Trichloroacetophenone. Sodium iodide (1.8 g) was gradually dissolved in a cold (0–5 °C) solution of 2,2,4,5-tetrachloroacetophenone (2.6 g) in dry acetone (25 mL) and the mixture stored in a refrigerator for 10 h. The precipitate was removed by filtration and washed twice with cold dry acetone. The combined acetone washes were evaporated to dryness in vacuo and the orange-brown solid, presumably 2,4,5-trichlorophenacyl iodide, was dissolved in methanol (50 mL) and cooled to −15 °C. Solid sodium borohydride (400 mg) was added to this cold solution in portions over a period of 45 min. The mixture was allowed to stand at that temperature for a further 60 min. The solvent was removed in vacuo, the residue partitioned between ether and water and the ether layer dried over anhydrous Na_2SO_4. Removal of the solvent yielded a yellow oil which was shown by TLC on silica gel G developed in hexane–benzene (1:1, v/v) to consist mainly of one component, 2,4,5-trichloroacetophenone. The oil was chromatographed on neutral alumina (British Drug Houses) by elution with hexane–benzene (4:1, v/v). Evaporation of the solvent in vacuo yielded 2,4,5-trichloroacetophenone as an oil which on trituration with petroleum ether (bp 40–60 °C) and on cooling resulted in a solid, mp 40–41 °C (Baker and Tweed, 1942, mp 47 °C).

1-(2,4,5-Trichlorophenyl)ethanol. Sodium borohydride (100 mg) was added portionwise to a methanolic solution (20 mL) of 2,4,5-trichloroacetophenone (500 mg), and after 30 min, the concentrated solution was partitioned between water and ether. The ether-soluble fraction was crystallized from hexane–benzene (3:1, v/v); yield, 389 mg (78%), mp 85–86 °C (Akintonwa and Hutson, 1967, mp 87–89 °C).

2-Chloro-1-(2,4,5-trichlorophenyl)ethanol. Sodium borohydride reduction of a methanolic solution of 2,2,4,5-tetrachloroacetophenone resulted in the desired compound which was crystallized from hexane–benzene (3:1, v/v), mp 73–74.5 °C. The IR and NMR spectra were in agreement with the structure.

2,4,5-Trichlorobenzoic Acid. Pyridine (1 mL) was added to 2,4,5-trichlorophenacyl chloride (2.56 g) in benzene (10 mL). The reaction vessel was stoppered and

allowed to stand at room temperature for 2 days during which time granules of 2,4,5-trichlorophenacylpyridinium chloride precipitated and yielded 1.52 g (44%), mp 225–228 °C (decomposed).

Aqueous 50% NaOH (2 mL) was added to a suspension of the pyridinium salt (1 g) in cold water (20 mL). The reaction mixture was allowed to stand at room temperature for a further 2 h. Careful neutralization of the solution with 6 N HCl precipitated the free acid as a white solid. The solid was collected by filtration, washed with cold water, dried, and yielded 507 mg (73%), mp 161–163 °C; when recrystallized from hot benzene, mp 165–166 °C (Baker and Tweed, 1942, mp 166 °C).

***S*-Methylglutathione.** This compound was prepared by the reaction of methyl iodide and reduced glutathione (GSH) in alkaline media for 2 h, mp 204–207 °C (decomposed) (Hutson et al., 1972, mp 200–204 °C).

Gas Chromatography (GC). Benzene extracts were analyzed for I and its metabolites on a Packard Becker 420 gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD). Two columns were used. Column 1 was a 1.83 m × 4 mm (i.d.) glass column packed with 3% (w/w) SE-30 on 80–100 mesh Chromosorb WHP. The operating temperatures for injector, column, and detector were 175, 170, and 295 °C, respectively; nitrogen (flow through column 40 mL/min, purge 30 mL/min) was the carrier gas. On this column, the relative retention times for I, III, IV, VI, and VII were 1.0, 0.29, 0.35, 0.18, and 0.22, respectively. Column 2 was a glass tube 1.83 m × 4 mm (i.d.) packed with 10% (w/w) DC-200 on 80–100 mesh Gas-Chrom Q. The injector, column, and detector temperatures were 260, 200, and 295 °C, respectively. The nitrogen carrier gas flow rate was 60 mL/min. The relative retention times for III, IV, VI, and VII were 1.0, 1.36, 0.55, and 0.74, respectively.

Thin-Layer Chromatography (TLC). Tetrachlorvinphos and metabolites were also analyzed on precoated (250 μm) TLC plates. Benzene-soluble metabolites were analyzed on silica gel OF (New England Nuclear) in an ethyl acetate–hexane (3:7, v/v) solvent system, air dried, and examined under UV light (254 nm). The R_f values for I, III, IV, VI, aand VII were 0.15, 0.63, 0.49, 0.58, and 0.44, respectively. The aqueous phase was analyzed on silica gel G (Fisher Scientific Co.) precoated TLC plates in methanol–isopropanol–acetone, (1:1:8, v/v/v). The R_f value for II was 0.43. *S*-Methylglutathione was identified by developing a silica gel G plate in butanol–isopropanol–water (11:4:5, v/v/v). The plate was then sprayed with 0.1% ninhydrin solution in acetone and air dried. The glutathione derivative was visible after standing in light as a purple spot with an R_f value of 0.48.

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

Other Analyses. Melting points were determined on a Fisher-Jones apparatus and are reported uncorrected. IR and NMR spectra were recorded on a Beckman IR 12 spectrophotometer and a Varian A-60 spectrometer, respectively. The gas chromatography-mass spectrometry (GC–MS) was accomplished in a Finnigan 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.

Enzyme Preparation. Single Comb White Leghorn hens, approximately 1.5 years old at 70% production, were killed by cervical dislocation. The livers were removed, washed in distilled water, and placed on crushed ice. A homogenate was prepared in ice-cold 0.134 M phosphate buffer, pH 7.4, (8 g/40 mL) in a glass–Teflon homogenizer. The homogenate was centrifuged at 2500 rpm for 5 min at 4 °C. The precipitate was discarded and the supernatant centrifuged at 10 000 rpm for 10 min at 4 °C. The precipitate (mitochondria) was discarded and the supernatant centrifuged at 40 000 rpm (105 000*g*) for 30 min at 4 °C. The precipitate (microsomes) was discarded and the clear supernatant or soluble fraction was the enzyme preparation. Normally a fresh preparation was made on the day each series of incubations was carried out; however, there appeared to be no significant loss in activity in preparations which had been stored at −20 °C for up to 10 days.

In Vitro Incubations. 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 [*vinyl*-^{14}C]-tetrachlorvinphos (105 μg). Reduced glutathione (GSH) was prepared just prior to addition by mixing 0.0027 g 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 flasks were gassed with dry N_2, stoppered, and incubated at 37.5 °C for specified time intervals. The reaction was terminated by addition of excess acetone.

Extraction of Metabolites. The incubation mixture was filtered and the flask was rinsed with acetone (3 × 10 mL). The residues were washed thoroughly with acetone and the combined aqueous–acetone extracts (120–130 mL) were concentrated to 5–7 mL on a rotary evaporator at room temperature. The concentrate was transferred to a centrifuge tube (15 mL), the flask washed with acetone (2 × 2 mL), and the combined solution concentrated to 3–4 mL under a gentle stream of dry air. The final volume of the solution was adjusted to 5 mL. Benzene (5 mL) was added and the tube was shaken vigorously for 30 s. After centrifugation, the benzene layer was pipetted into a flask and the aqueous phase was re-extracted with benzene (2 × 5 mL). The combined benzene extracts were dried over anhydrous Na_2SO_4, concentrated as above, and the final volume adjusted to 5 mL. Traces of benzene were removed from the aqueous layer by means of a stream of dry air and the final volume of this phase was adjusted to 5 mL. The radioactivity in each fraction was determined in duplicate aliquots (1 mL) as described above.

Isolation of Metabolites. The benzene extracts were analyzed by TLC and GC on column 1. The individual metabolites in the benzene extracts were isolated by TLC on silica gel OF in hexane–ethyl acetate (7:3). The plates were viewed under UV light (254 nm), and the different zones were scraped off the plate and extracted with methanol. The methanol was evaporated and the residue partitioned between benzene and distilled water. The benzene layer was dried over anhydrous Na_2SO_4, concentrated, and analyzed by GC and TLC as well as for radioactivity.

The aqueous phase was continuously extracted with ether for 18 h. The ether extract was reduced in volume

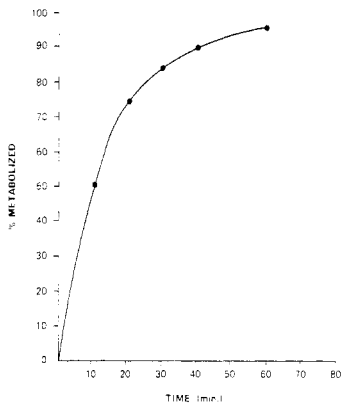

Figure 2. Rate of metabolism of tetrachlorvinphos by the soluble fraction (105 000*g*) of chicken liver homogenates in vitro.

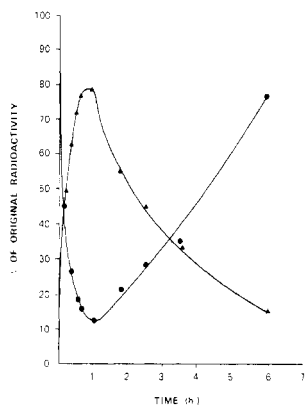

Figure 3. Percentage of the original radioactivity in the benzene extract (●) and the aqueous phase (▲) of incubation medium at various time intervals during an in vitro incubation.

on a rotary evaporator and taken to dryness with a stream of dry air. The residue was redissolved in ether and analyzed by TLC on silica gel G in methanol–isopropanol–acetone (1:1:8, v/v/v). The zone between R_f 0.4 and 0.5 (R_f for desmethyl tetrachlorvinphos = 0.43) was scraped off the plate and extracted with ether. The ether was removed, the residue redissolved in distilled water, and the radioactivity determined as indicated above.

RESULTS

In in vitro incubations, the soluble fraction (105 000*g*) from chicken liver homogenates, rapidly metabolizes tetrachlorvinphos. Figure 2 indicates that 95% of the tetrachlorvinphos has been metabolized in 1 h. The percentage metabolism was calculated from the ratio of the peak area obtained by GC analysis at the indicated time to that at zero time.

It was shown by TLC analyses that the aqueous phase contained a single compound which had an R_f value and a relative retention time similar to those for desmethyl tetrachlorvinphos. In Figure 3, a plot of percent radioactivity in the two phases (benzene and aqueous) against time, indicates that up to 1 h the main metabolic pathway was demethylation to the water soluble metabolite. From 1 to 6 h there was a steady decrease in the radioactivity in the aqueous phase accompanied by a concomitant increase in activity in the benzene extract. This suggests that desmethyl tetrachlorvinphos was being metabolized further to benzene soluble metabolite(s) by the same or some other enzyme systems.

The data in Table I indicate that the enzyme reaction is glutathione dependent. The enzyme preparation contained sufficient GSH for 53% of the tetrachlorvinphos to be metabolized in 1 h; however, addition of GSH to the

Table I. Role of Reduced Glutathione (GSH) in the Metabolism of Tetrachlorvinphos

Treatment of enzyme preparation	% tetrachlorvinphos metabolized[a]
Not dialyzed	53
Not dialyzed + GSH (0.0027 g)	89
Dialyzed[b]	38
Dialyzed[c]	16
Dialyzed[c] + GSH (0.0027 g)	27
Dialyzed[c] + GSH (0.0054 g)	60
Dialyzed[c] + GSH (0.0135 g)	91

[a] The incubation mixture contained 4.5 mL of enzyme preparation, 105 μg of [^{14}C]tetrachlorvinphos and either 0.5 mL of 0.134 M phosphate buffer, pH 7.4 or 0.5 mL of GSH solution. The incubation was carried out under N_2 at 37.5 °C for 1 h. [b] Dialyzed against 0.134 M phosphate buffer, pH 7.4, at 4 °C for 3 h. [c] Dialyzed against 0.134 M phosphate buffer, pH 7.4, at 4 °C for 18 h.

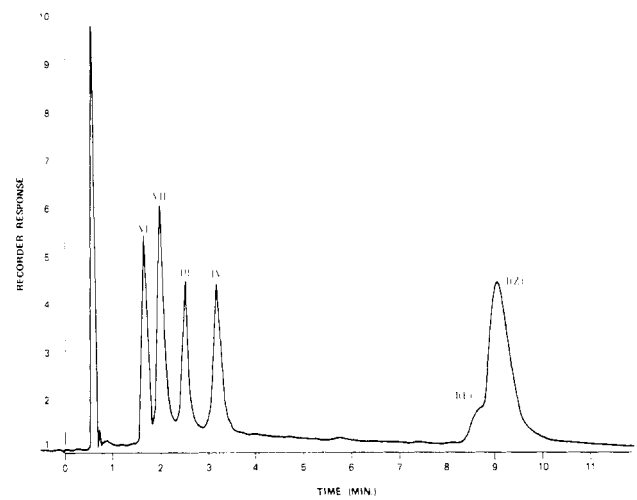

Figure 4. Gas chromatogram of a synthetic mixture of (I) the *E* and *Z* isomers of tetrachlorvinphos (950 pg), (III) 2,4,5-trichlorophenacyl chloride (52 pg), (IV) 2-chloro-1-(2,4,5-trichlorophenyl)ethanol (48 pg), (VI) 2,4,5-trichloroacetophenone (38 pg), and (VIII) 1-(2,4,5-trichlorophenyl)ethanol (42 pg). GC conditions: glass column 1.83 m × 4 mm i.d. packed with 3% SE-30 on Chromosorb WHP; injector, column, and detector temperatures, 175, 170, and 295 °C, respectively; ^{63}Ni ECD; carrier gas (nitrogen) flow rate, 40 mL/min.

incubation medium increased metabolism to 89% in this time period. When the enzyme preparation was dialyzed against 0.134 M phosphate buffer, pH 7.4 for 3 h, considerably less metabolism occurred, 38%; after 18 h dialysis, the metabolism of tetrachlorvinphos had decreased to 16%. However, the activity of the enzyme preparation could be restored by addition of GSH and, in the experiment reported, was completely recovered by addition of 4.4 × 10^{-5} mol GSH.

The gas chromatogram of a synthetic mixture of I, III, IV, VI, and VII is shown in Figure 4. The column separated all the compounds, with the exception of the *E* and *Z* isomers of I, with good resolution. Under the GC conditions described, the compounds give a 50% full-scale deflection in the 38–52 pg range with the exception of I (950 pg).

Tetrachlorvinphos and its metabolites in the benzene extracts of incubation mixtures over a period of 10 min to 6 h are shown by chromatograms 1 to 7 in Figure 5. The retention times of the peaks were identical with those of the reference standards in Figure 4. It can be seen that

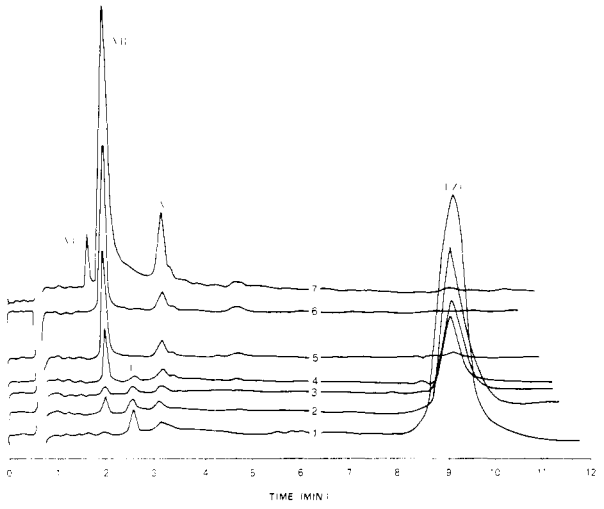

Figure 5. Gas chromatograms of benzene extracts of incubation media carried out in vitro for the following time intervals: (1) 10, (2) 20, (3) 30, (4) 60, (5) 105, (6) 210, and (7) 360 min. GC conditions: the same as recorded in Figure 4.

after 10-min incubation, there were small but demonstrable amounts of metabolites III and IV in the benzene extract. After 20 min, metabolites III, IV, and VII were detected. Tetrachlorvinphos (I) and III which were present at 60 min were not detectable at 105 min while the amounts of IV and VII continued to increase until they appeared maximal at 6 h. At this time, VI was also detected.

The identity of each of the compounds responsible for the peaks were confirmed by cochromatography with authentic standards and finally by GC–MS analyses. Thus, a GC–MS of peak VI (Figure 5), the metabolite with a retention time of 1.7 min, showed a molecular ion at m/e 222 $[M]^{+\cdot}$ and had an isotope pattern for a molecule containing three chlorine atoms. Other major ions were recorded at m/e 207 $[M - CH_3]^{+\cdot}$ and m/e 179 $[M - COCH_3]^{+\cdot}$. This spectrum was identical with that for the standard 2,4,5-trichloroacetophenone.

Similarly, a GC–MS of peak VII (Figure 5), the metabolite with a retention time of 2.0 min, exhibited a molecular ion peak at m/e 224 $[M]^{+\cdot}$ with a distinctive pattern for three chlorine atoms. In addition, the mass spectra also had peaks at m/e 209 $[M - CH_3]^{+\cdot}$ and m/e 181 $[C_6H_4Cl_3]^{+\cdot}$ with the three chlorine atoms pattern; m/e 145 $[C_6H_3Cl_2]^{+\cdot}$ and 109 $[C_6H_2Cl]^{+\cdot}$. The spectrum was identical with that recorded for 1-(2,4,5-trichlorophenyl)ethanol.

In addition, GC–MS analysis of peak IV (Figure 5), which had a retention time of 3.2 min, revealed a molecular ion peak at m/e 258 $[M]^{+\cdot}$ and exhibited patterns associated with a four chlorine atom system. Furthermore, the spectra also recorded peaks at m/e 209 $[M - CH_2Cl]^{+\cdot}$, m/e 181 $[C_6H_4Cl_3]^{+\cdot}$, m/e 145 $[C_6H_2Cl_2]^{+\cdot}$, and m/e 109 $[C_6H_2Cl]^{+\cdot}$. The spectra was consistent with that of the reference compound 2-chloro-1-(2,4,5-trichlorophenyl)-ethanol.

The ether extract of the aqueous phase was taken to dryness and methylated by treatment with freshly prepared diazomethane. Aliquots were analyzed by GC on column 1 and by TLC in benzene–chloroform (2:98, v/v) and ethyl acetate–hexane (3:7, v/v). In all three systems, the compound was identical with tetrachlorvinphos. The GC–MS analysis of the methylated compound with a retention time of 9.1 min showed a weak molecular ion peak at m/e 364 $[M]^{+\cdot}$ with a base peak of 109 $[OP(OCH_3)_2]^{+\cdot}$. Other peaks were observed at m/e 329 $[M - Cl]^{+\cdot}$, 238 $[M - HO\text{-}P(OCH_3)_2]^{+\cdot}$ and 204 $[M - Cl - O_2P(OCH_3)_2]^{+\cdot}$. This spectrum was identical with that for standard tetrachlorvinphos. Thus, the desmethyl tetrachlorvinphos had been methylated to the original insecticide.

Aliquots of the aqueous phase which had been extracted by benzene and ether, were analyzed by TLC in 1-butanol–acetic acid–water (11:4:5, v/v/v). The TLC plates were air dried and sprayed with 0.1% ninhydrin in acetone. After standing for 15 min, dark purple spots were visible with R_f 0.48 to 0.50 which was identical with that for *S*-methylglutathione. Other spots with lower intensities at R_f 0.15 and 0.43 were identical with oxidized and reduced glutathione, respectively.

DISCUSSION

The data reported above are evidence for the metabolic pathway of tetrachlorvinphos in in vitro incubations with the soluble fraction of chicken liver homogenates. Hutson et al. (1972) have described a GSH-dependent soluble enzyme system in mouse, rat, rabbit, and pig liver homogenates which catalyzed the demethylation of tetrachlorvinphos. A similar GSH-dependent enzyme system from rat liver had earlier been shown to be responsible for the degradation of methyl iodide (Johnson, 1966) and for methyl parathion (Fukami and Shishido, 1966).

The data in Figure 3 indicate that tetrachlorvinphos is first rapidly metabolized to a water-soluble metabolite which was identified as desmethyl tetrachlorvinphos. The data in Table I indicate that the enzyme in the soluble fraction is GSH dependent. The activity which remained after the enzyme preparation had been dialyzed for 18 h was possibly due to protein bound glutathione. It is concluded that a soluble enzyme, probably reduced glutathione *S*-alkyltransferase, is present in the soluble fraction (105 000*g*) of chicken liver homogenates which, in in vitro incubations, is responsible for the metabolism of tetrachlorvinphos to the water soluble metabolite II via route a in Figure 1. This is further substantiated by the identification of *S*-methylglutathione in the aqueous phase. The formation of II is the result of demethylation, i.e., the cleavage of P–O–CH_3. If cleavage had occurred at P–O–vinyl or via route b in Figure 1, the metabolite dimethyl phosphate would result. This metabolite was not detected in the present studies; however, a partial involvement of such a pathway may not be eliminated.

The dealkylation of organophosphorus compounds has generally been accepted as a detoxification mechanism since nonpolar, lipophilic compounds are thereby converted to polar, water-soluble compounds which can be excreted. Akintonwa and Hutson (1967) reported that in the dog, metabolism of I proceeded mainly via demethylation, yielding II which was rapidly cleared by the kidneys into the urine, whereas, in the rat, the urine contained mainly glucuronides of VII and X, which indicated that metabolism of I was proceeding via fission of P–O–vinyl. However, the production of III via a single- or two-step hydrolysis of II should not be excluded.

The data in Figure 3 indicate that, after 60 min of incubation, the water-soluble metabolite II was further metabolized to benzene-soluble metabolites which is further substantiated by the data in Figure 5. At the end of the 6-h incubation, the benzene extract contained metabolites IV, VI, and VII in ratios of 7:3:89%, based on measurement of radioactivity of each component.

The metabolic pathway for the metabolism of II to yield benzene-soluble metabolites is not clear. Figure 1 depicts two possible routes, d and e, by which III may result from II. It would be expected that the extent of the formation of other metabolites such as IV, VI, and VII would depend

on the concentration of III. However, as can be seen in Figure 5, the concentration of III was never extremely high, although from a maximum of 10 min, it continued to decrease until it could not be detected at 105 min. This tends to indicate that as quickly as metabolite III is formed, it is further metabolized very rapidly.

In the metabolic pathway indicated in Figure 1, route f which results in metabolite VI must be considered a possibility since VI is an acceptable transformation product of III. Biological conversion of VI to VII via route g would be expected to be the major process since the transformation of acetophenone to methylphenylcarbinol has been well documented (Smith et al., 1954). However, the formation of VII from IV is also quite possible.

In the present studies with the soluble fraction (105 000g) as the enzyme preparation, metabolites VIII, X, and XI were not detected. It seems unlikely that the oxidation steps in Figure 1, route c, take place. The study continues with an investigation of the enzymes in the mitochondrial and microsomal fractions of chicken liver homogenates. These should permit postulation of a metabolic pathway for the metabolism of tetrachlorvinphos in the laying hen which can be tested by in vivo studies.

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Mammalian Metabolism of Chlordimeform. Formation of Metabolites Containing the Urea Moiety

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The metabolism of chlordimeform-^{14}C following intraperitoneal treatment of mice and oral treatment of rats and following incubation with rat liver microsomes was examined. In addition to chlordimeform and other previously identified metabolites such as N'-(4-chloro-o-tolyl)-N-methylformamidine, 4′-chloro-o-formotoluidide, 4-chloro-o-toluidine, N-formyl-5-chloroanthranilic acid, and 5-chloroanthranilic acid, the following novel metabolites were present: N'-(4-chloro-o-tolyl)formamidine, 1,1-dimethyl-3-(4-chloro-o-tolyl)urea, 1-methyl-3-(4-chloro-o-tolyl)urea, and 3-(4-chloro-o-tolyl)urea. The N'-(4-chloro-o-tolyl)formamidine was formed by sequential N-demethylation of chlordimeform. It was suggested that at least one of the urea metabolites was formed by hydroxylation of the amidine carbon of one of the formamidines, followed by a shift to the keto tautomer. Apparently N-demethylation also was involved.

The metabolic fate of chlordimeform pesticide has been studied in dogs and goats (Sen Gupta and Knowles, 1970) and in rats in vivo (Knowles and Sen Gupta, 1970; Morikawa et al., 1975) and in vitro (Ahmad and Knowles, 1971a; Morikawa et al., 1975). Organosoluble metabolites identified included N'-(4-chloro-o-tolyl)-N-methylformamidine or demethylchlordimeform, 4′-chloro-o-formotoluidide, 4-chloro-o-toluidine, N-formyl-5-chloroanthranilic acid, and 5-chloroanthranilic acid (Ahmad and Knowles, 1971a; Knowles and Sen Gupta, 1970; Sen Gupta and Knowles, 1970). Benezet and Knowles (1976), studying the metabolism of demethylchlordimeform-^{14}C in rats, reported the isolation and tentative identification of a new formamidine metabolite, N'-(4-chloro-o-tolyl)-formamidine or didemethylchlordimeform. Examination of autoradiographs of thin-layer chromatograms from urine of rats treated orally with chlordimeform (Knowles and Sen Gupta, 1970) and of autoradiographs of thin-layer chromatograms from in vitro studies of chlordimeform metabolism by rat liver subcellular fractions (Ahmad and Knowles, 1971a) indicated that an unknown metabolite with R_f coincident with that of didemethylchlordimeform was present. Subsequent to these observations it was learned that didemethylchlordimeform was toxic to rats (Benezet et al., 1977). Therefore, it was deemed necessary to reinvestigate the metabolism of chlordimeform in rats to confirm the identity of didemethylchlordimeform and to gain data relative to its concentration in rat urine. During the course of these experiments, three metabolites containing the urea moiety were identified. This paper

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