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Fate of Trichlorfon in Buffer and Soluble Fraction (105000*g*) from Cow and Chicken Liver Homogenates

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The fate of trichlorfon in pH 7.4 buffer and the soluble fractions from cow and chicken liver homogenates prepared in the same buffer was studied. In pH 7.4 buffer, trichlorfon was readily converted into dichlorvos. With the enzyme systems, the insecticide was metabolized into both dichlorvos and desmethyltrichlorfon. The rate of degradation of trichlorfon in enzyme systems was greater than that in buffer. Cow liver soluble fraction metabolized trichlorfon at a slightly higher rate than chicken liver preparation. A modified analytical technique for the detection of important metabolites is also described.

Trichlorfon, (2,2,2-trichloro-1-hydroxyethyl)phosphonic acid dimethyl ester, is registered under different trade names, Neguvon, Dipterex, Dylox, etc., as an insecticide for the control of both endo- and ectoparasites of domestic animals.

It is well documented that trichlorfon rearranges via dehydrochlorination in slightly acidic, neutral, or alkaline media to yield a highly potent insecticide, dichlorvos (DDVP) (Barthel et al., 1955; Lorenz et al., 1955; Mattson et al., 1955). The rate of dehydrochlorination is pH dependent—i.e., the higher the pH, the greater the rate of formation of dichlorvos (Miyamoto, 1959; Metcalf et al., 1959; Aksnes and Yuksekisik, 1974).

Arthur and Casida (1957) and Hassan et al. (1965) suggested that trichlorfon is a direct inhibitor of acetylcholinesterase, while Metcalf et al. (1959) and Miyamoto (1959) held the view that the compound is itself a poor inhibitor and owes its insecticidal properties to the formation of the strong inhibitor dichlorvos. Miyamoto (1959) also concluded that the formation of dichlorvos in different biological systems was not necessarily the result of dehydrochlorinating enzyme(s) but could occur spontaneously under normal physiological conditions.

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The present studies were undertaken to obtain information on the in vitro metabolism of trichlorfon in poultry and farm animals. This report records the data on the effects of buffer and chicken and cow liver soluble enzymes on the degradation of trichlorfon. The paper also provides a modified method for the detection of important trichlorfon metabolites in biological samples.

EXPERIMENTAL SECTION

Materials. Glass-distilled pesticide-grade solvents were used as received. Trichlorfon (I) and dichlorvos (II) were prepared by following published procedures (Barthel et al., 1955; Lorenz et al., 1955). *O*-Desmethyltrichlorfon (III) and desmethyldichlorvos (IV) were obtained by refluxing the parent compound with equimolar amounts of sodium iodide in methyl ethyl ketone (Schneider and Fischer, 1977). Diazomethane was prepared by treating *N*-nitroso-*N*-methylurea with cold aqueous 50% potassium hydroxide (Schultz et al., 1971). Silylating agents Tri-Sil and Tri-Sil Concentrate were purchased from Chromatographic Specialties, Ltd., Brockville, Ontario, Canada.

Enzyme Preparation. The soluble fraction (105000*g*) from livers of chicken and cow were prepared in ice-cold 0.134 M phosphate buffer (pH 7.4) in a glass–Teflon homogenizer as described earlier (Akhtar and Foster, 1977).

Rate of Degradation. The rate of degradation studies for trichlorfon were carried out at 37.5 °C for specified

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times under N_2. A stock solution of trichlorfon was prepared fresh in distilled water just prior to use. A typical incubation flask contained trichlorfon (100 μL, ≃102–103 μg), enzyme preparation (4.5 mL), and buffer (0.5 mL). In the case of control flasks and when buffer alone was studied, the amount of buffer used was 5.0 mL. Both the chemical and the biochemical reactions were arrested by the addition of 2 mL of 0.8% aqueous HCl.

The reaction mixture was filtered into a 50-mL centrifuge tube and washed with 5–6 mL of distilled water, and the final volume of the filtrate was adjusted to 15 mL. The aqueous phase was shaken with 15 mL of hexane. After centrifugation, the hexane layer was pipetted into a 50-mL volumetric flask, and the aqueous phase was extracted 3 times with 10 mL of hexane. The volume of hexane layers was adjusted to 50 mL, and sufficient anhydrous Na_2SO_4 was added to remove traces of water. This extract, after appropriate dilution where necessary, was analyzed for dichlorvos.

Traces of hexane were removed from the aqueous layer by means of a stream of dry air or nitrogen, and the final volume of this phase was adjusted to 15 mL with distilled water. The aqueous phase was shaken with 15 mL of chloroform and centrifuged, and the chloroform layer was carefully removed by pipet. This process was repeated 5 times. The combined chloroform layer was dried over anhydrous Na_2SO_4, filtered, and concentrated on a rotary evaporator (≃2–3 mL) at 35–40 °C. The residual liquid was transferred to a 15-mL centrifuge tube. The flask was washed twice with 3 mL of chloroform. The combined chloroform extract was evaporated to dryness under a slow stream of nitrogen. Tri-Sil Concentrate (100 μL) was added, the flask was stoppered, and the mixture was mixed thoroughly on a vortex mixer. The reaction mixture was allowed to stand for 4 h. The mixture was evaporated to dryness under slow stream of nitrogen, diluted with benzene, and analyzed by gas chromatography.

Aliquots (100 μL), in duplicate, of the stock solution of trichlorfon were placed in centrifuge tubes, evaporated to dryness, and silylated with Tri-Sil Concentrate. This served as the reference standard for trichlorfon and represented the concentration of trichlorfon at zero time (i.e., at the start of the reaction).

The disappearance of trichlorfon from incubation media was followed by noting the decrease in the peak height of the silylated compound of the chloroform extracts, as compared to that of the reference standard above.

Gas Chromatography (GC). Analyses of hexane and benzene extracts were carried out with a Perkin-Elmer Sigma 1 gas chromatograph equipped with a ^{63}Ni electron capture detector. The column was a glass tube [1 m × 4 mm (i.d.)] packed with 3% SE-30 on 80–100-mesh Chromosorb WHP. The operating temperatures for the injector, column, and detector were 175, 150, and 400 °C, respectively; 5% methane–argon at the rate of 35 mL/min was the carrier gas. The retention times for dichlorvos and trimethylsilyltrichlorfon were 2.1 and 5.15 min, respectively.

A typical gas chromatogram of the mixture of dichlorvos and silylated trichlorfon is shown in Figure 1. Under the GC conditions detailed above, dichlorvos and trichlorfon gave a half-scale deflection ($^1/_2$ fsd) for 0.45 and 0.17 ng, respectively. The response for dichlorvos was linear between 0.15 and 0.6 ng while for trichlorfon it was linear between 0.025 and 0.25 ng.

Gas Chromatography–Mass Spectrometry (GC–MS). The GC–MS analyses were performed on a Finnigan Model 3100 mass spectrometer connected to a Finnigan

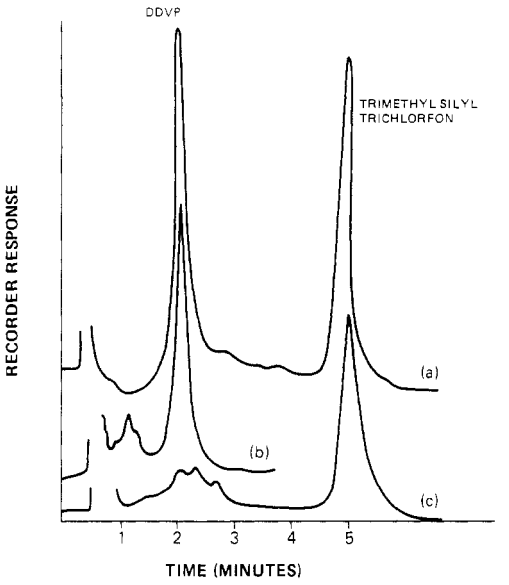

Figure 1. Gas chromatograms of the reference standards and the extracts: (a) mixture of standard dichlorvos and trimethylsilyl derivative of trichlorfon; (b) hexane extracts; (c) trimethylsilylated solution of chloroform extracts.

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 operated at 150 °C, and the helium flow rate was 35 mL/min. The mass spectra were recorded at 70 eV.

RESULTS AND DISCUSSION

Methods. Gas chromatographic techniques for the determination of trichlorfon residues in animal tissues, plants, and water systems have been reported. These involved thermal decomposition of trichlorfon in injector block and quantitation via the decomposed products (El-Refai and Giuffrida, 1965; Anderson et al., 1966; Pieper and Richmond, 1976). Alkaline conversion of trichlorfon to dichlorvos has also been used as a technique for its detection and estimation (Zitko and Sergeant, 1977). However, all these methods have their limitations. Anderson et al. (1966) also made use of an acylation technique which is not a clean reaction (Look and White 1981) and found that the acyl derivative did not decompose when passed through a hot injector block.

When the present study was underway, Zitko and Sergeant (1977) reported the determination of trichlorfon residues in water as the trimethylsilyl derivative. These workers also examined the possibility of using a methylation technique as an alternative. However, methylation of trichlorfon, using diazomethane, yielded a complex mixture.

Sergeant and Zitko (1979) used Tri-Sil as the silylating agent in which pyridine was the solvent. In the present study, depending upon the length of reaction time, when Tri-Sil was used as the silylating agent, 10–15% dichlorvos was also produced. So that the formation of dichlorvos during the silylation step could be avoided, Tri-Sil Concentrate was used as the silylating agent.

The recovery data of trichlorfon, dichlorvos, desmethyltrichlorfon, and desmethyldichlorvos are given in Table I.

Incubation and Degradation. In general, an enzymatic reaction can be terminated by the addition of acetone (Akhtar and Foster, 1977). For termination of both the

Table I. Recovery of Trichlorfon, Dichlorvos, Desmethyltrichlorfon, and Desmethyldichlorvos from Incubation Media

amount, μg[a] added	amount, μg[a] recovered[b]	%
	Trichlorfon (I)[c]	
102.5	99.5	97.0
51.2	54.3	106.0
25.6	23.2	90.6
10.2	9.7	95.1
5.1	4.3	84.3
	Dichlorvos (II)	
103.3	97.5	94.4
51.6	50.8	98.5
25.8	23.3	90.3
10.3	8.8	85.4
	Desmethyltrichlorfon (III)	
98.7	75.3	76.3
49.3	37.1	75.3
24.7	18.3	74.1
9.9	7.3	73.7
	Desmethyldichlorvos (IV)	
97.1	71.5	73.6
48.5	33.5	68.1
24.1	15.6	64.7
9.7	5.1	52.6

[a] Samples were added to buffer and shaken for 30 s, and 2 mL of 0.8% HCl was added. Extraction procedures were the same as detailed in the text. [b] Average of duplicate runs. [c] See Figure 3 for structures.

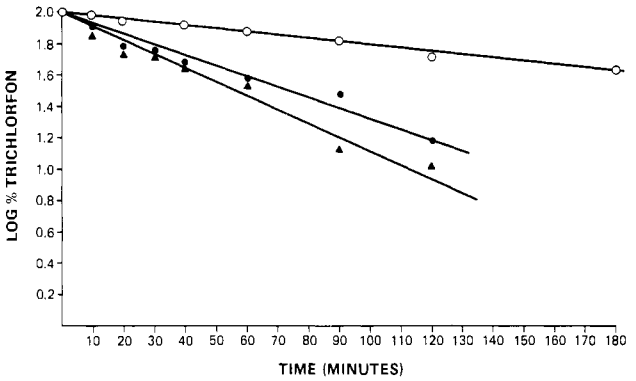

Figure 2. Effect of pH and the enzymes on the decomposition of trichlorfon at 37.5 °C: (▲) cow liver preparation, (●) chicken liver preparation, and (○) buffer; each point represents the average of duplicate determinations.

chemical and the biochemical processes, in the present experiments, simultaneously, 2 mL of 0.8% aqueous HCl was added to the incubation mixture. This addition of HCl precipitated enzyme and at the same time lowered the pH of the incubation media to 5.6 at which the degradation of trichlorfon is very slow.

Dichlorvos is a very volatile compound. Incorporation of an evaporation step resulted in a 35–65% loss of the standard dichlorvos. The problem was overcome by removing the evaporation step whenever analysis and quantitation of dichlorvos were involved.

Incubation of trichlorfon with buffer and enzyme preparations in buffer resulted in degradation of the compound. Plots of the logarithm of the concentration of trichlorfon (i.e., residual percentage of trichlorfon) vs. time in various media are shown in Figure 2. A good linear relationship was obtained for the degradation of trichlorfon in buffer which indicates a pseudo-first-order reaction. Pseudo-first-order reaction rates have been observed in buffer by previous workers (Aksnes and Yuksekisik, 1974; Metcalf et al., 1959; Miyamoto, 1959). In enzyme systems, a good linear relationship was not observed for the degradation of trichlorfon. However, a linear plot was constructed primarily to estimate the half-life of trichlorfon in these media. From these plots, the half-life in buffer, chicken, and cow enzyme preparations was estimated to be 148, 46, and 34 min, respectively.

Table II. Amounts of Dichlorvos (II), Desmethyltrichlorfon (III), and Desmethyldichlorvos (IV) Found in Incubation Mixtures[a] at Various Time Intervals

time, min	amount, μg buffer II	buffer III	buffer IV	cow II	cow III	cow IV	chicken II	chicken III	chicken IV
10	2.8			1.2	10.7	tr	0.9	3.5	0.7
20	5.2			2.1	22.5	1.5	1.9	8.2	1.2
30				3.2	36.5	2.7	2.3	15.6	4.1
40	8.4			3.9	43.2	2.9	4.7	13.8	5.2
60	13.8			4.4	46.2	1.8	5.8	21.7	6.2
90	16.7			4.9	44.7	5.2	7.8	36.5	4.9
120	25.7			5.7	57.3	4.6	6.2	33.8	7.3
180	32.8			5.3			6.9		

[a] Incubation mixtures consisted of the following: buffer, 5.0 mL, pH 7.4, and 104.6 μg of substrate; cow, 103.6 μg of substrate, 4.5 mL of soluble fraction, and 0.5 mL of buffer; chicken, 102.7 μg of substrate, 4.5 mL of soluble fraction, and 0.5 mL of buffer.

The significant difference between the half-life in buffer alone and that in the enzyme preparations in buffer strongly suggests simultaneous participation of two independent reactions; one can be identified as chemical transformation while the other is due to enzymatic reactions.

Identification of Compounds. Dichlorvos is readily degraded in both buffer and enzymatic systems. Dichlorvos was the only compound detected and identified in the hexane extract of the incubation mixtures. Its identity was established by comparing the GC–MS data with those for standard dichlorvos, m/e 220.

The amount of dichlorvos produced under both chemical and enzymatic conditions is recorded in Table II. Dichlorvos was produced at a faster rate in the buffer system than in the enzyme system even though trichlorfon is degraded at a faster rate in the enzyme system than in the buffer system (see Figure 2). This suggests that a route, other than dehydrochlorination to produce dichlorvos, may also be operating in the enzyme system. If enzyme(s) was (were) involved in the production of dichlorvos, a higher concentration would have been observed in the enzymatic systems. However, this was not the case. Thus, it appears that formation of dichlorvos in enzyme systems was mainly due to buffer present in the incubation media.

Chloroform extracts of the aqueous phase contained trichlorfon (identified as the silyl derivative) as the major product. The MS of the trimethylsilyl derivative of the extract exhibited peaks at m/e 313 (weak), 182 (medium), and 167 (base peak) which were identical with those obtained for authentic trimethylsilyl derivation of trichlorfon. A similar pattern has been reported by Zitko and Sergeant (1977).

Evaporation of aqueous phase to dryness and treatment with freshly prepared diazomethane (low concentration) yielded dichlorvos, methyltrichlorfon [(1-methoxy-2,2,2-trichloroethyl)phosphonic acid dimethyl ester], and traces of other unidentified products. Methyltrichlorfon was the major product.

When standard trichlorfon was treated with diazomethane, other minor products were also produced in addition to methyltrichlorfon. The reaction of trichlorfon

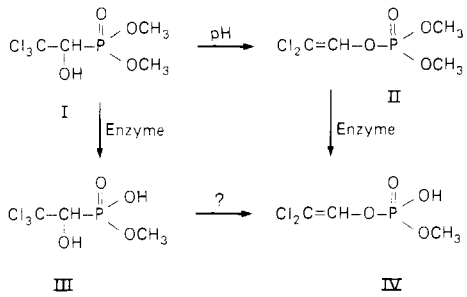

Figure 3. Possible metabolic route of trichlorfon in liver soluble fractions of chicken and cow prepared in pH 7.4 buffer.

with diazomethane was irreproducible and required a longer reaction time (10–14 h) for completion. Zitko and Sargeant (1977) also encountered difficulties when using diazomethane as a methylating agent for trichlorfon. Methylation of desmethyltrichlorfon by means of diazomethane to yield trichlorfon was very consistent and required only a few minutes for completion.

Since methylation of dried aqueous aliquots produced interfering peaks, other possibilities for the quantitation of desmethyltrichlorfon were examined. Sequential methylation and silylation of dried aqueous aliquots provided a comparatively cleaner solution for GC analyses. Desmethyldichlorvos concentration was estimated as dichlorvos from the resultant methylation extracts. Recovery of desmethyldichlorvos ranged between 53 and 74%. The concentration of desmethyltrichlorfon was calculated as trimethylsilyltrichlorfon concentration. Recovery of desmethyltrichlorfon was between 73 and 76%.

Table II records the concentrations of desmethyldichlorvos and desmethyltrichlorfon. It can be seen that the concentration of desmethyltrichlorfon increased with an increase in incubation time. The concentration of desmethyldichlorvos is always lower than that of desmethyltrichlorfon, suggesting a slower rate of formation. This is to be expected since production of desmethyldichlorvos involves two steps, i.e., the formation of dichlorvos followed by enzymatic dealkylation.

A sum of the concentrations of dichlorvos and desmethyldichlorvos at any given time is the contribution by buffer to trichlorfon degradation. However, the concentration of desmethyltrichlorfon is the result of enzymatic action(s) on trichlorfon. Since the total concentration of dichlorvos and desmethyldichlorvos under the conditions used in the current study is lower than that of desmethyltrichlorfon, dealkylation (O-demethylation) is suggested as the main degradation route in chicken and cow livers. The data in Table II suggest that desmethyl trichlorfon is not easily degraded. Thus, it appears that if trichlorfon enters the blood stream of cows and chickens after topical application or by ingestion, it would be effectively eliminated from the system as water-soluble desmethyltrichlorfon. Supporting evidence is found in the data of Robbins et al. (1956), who observed that 65% of [^{32}P]trichlorfon administered to cows was excreted in urine in 12 h, of which only 17% was associated with dimethyl phosphate, a further degradation product.

Figure 3 gives the initial steps in the degradation of trichlorfon based on the data presented here. The scheme shows that the conversion of trichlorfon to dichlorvos is mainly due to the chemical transformation action of buffering media. However, a small contribution due to enzymatic reaction may not be ruled out. Soluble enzymes in chicken and cow liver effectively metabolize trichlorfon into water-soluble desmethyltrichlorfon. Similarly, enzymes also convert dichlorvos into desmethyldichlorvos, which is in agreement with previously published data (Hodgson and Casida, 1962). Both chemical conversion and enzymatic conversion of desmethyltrichlorfon to desmethyldichlorvos are possible. However, this aspect was not examined. No desmethyldichlorvos was produced when dichlorvos was incubated with buffer (pH 7.4).

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