Isolation of an Enzyme Inhibitor, 2-*sec*-Butoxy-6,6-dichloro-1,4-dioxolan-5-one, from a Formulation of Trichlorfon

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The isolation of an enzyme-inhibiting substance from a commerical formulated product of trichlorfon using chromatographic techniques is described. The structure of this material is shown to be 2-*sec*-butoxy-6,6-dichloro-1,4-dioxolan-5-one by spectroscopic methods. A synthetic approach toward the formation of the enzyme inhibitor involving the adjuvants and impurities is proposed.

Trichlorfon, *O,O*-dimethyl-(2,2,2-trichloro-1-hydroxyethyl) phosphonate, although first patented by Farbenfabriken Bayer A.G. (Belgian Patent 1962), was approved for veterinary use under the Food, Drug and Cosmetic Act (Federal Register, 1964, 1965) in the United States. In Canada this phosphonate ester is registered under the Pest Control Products Act as an insecticide for the control of ectoparasitic anthropods of domestic animals under different trade names—Neguvon, Dipterex, Dylox, etc.

Recently in Southern Ontario, there was a report of death among cattle a few days after the percutaneous application of a formulated product of trichlorfon. Other sporadic incidences have occurred on the surrounding farms where similar formulations were topically applied on domestic animals. The veterinarians suspected that the insecticide might be the causative factor for these outbreaks (Morrow, 1974).

The formulated product was labeled to contain 8% trichlorfon along with 89.9% Dowanol (DPM, dipropylene glycol methyl ether), 2.1% of Tween 20, complex aliphatic esters, ester–ethers obtained from sorbitol, alkene oxides, and fatty acids. Besides, the label also indicated the presence of alkyl phosphites, salt, and trichloroacetic acid as impurities. Quantitative analysis of the formulated product for trichlorfon confirmed the labeled content of the active ingredient. Moreover, no dichlorvos, the major, highly toxic metabolite of trichlorfon, was detected. Furthermore, the literature survey for toxicity studies on trichlorfon (Adkins and Arant, 1957; Robbins et al., 1956; Arthur and Casida, 1957; Dorsey, 1962; Adkins, 1966; Graham and Drummond, 1967; Hagen, 1967; and Loomis et al., 1970) indicated that trichlorfon was readily absorbed, rapidly metabolized, and eliminated; only trace amounts of the insecticide residue were found in all the organs and tissues except in omental fat in which a maximum of 9.2 ppm was detected in a few samples up to the seventh day postadministration. On the basis of the foregoing evidence, it was concluded that there might be other exogenous toxic component(s) which was (were) attributed to the deaths of cattle. This article reports the isolation and characterization of an enzyme-inhibiting and possibly toxic substance found in the formulated product of trichlorfon. It is also hypothesized that this new exogenous substance might have been derived from the adjuvants and impurities present in the trichlorfon formulation. Whether the newly isolated enzyme inhibitor was the cause of the observed deaths of cattle in Southern Ontario is not certain.

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EXPERIMENTAL SECTION

Materials and Methods. Thin-layer chromatography was carried out on 20 × 20 cm plates coated with 250 μm layer of (E. Merck) silica gel GF_{254}. Infrared spectrum was obtained using Beckman Model IR-7. Gas–liquid chromatographic analyses were performed with a Hewlett-Packard Model 5710 equipped with flame ionization, nickel-63 electron-capture detectors, and Tracor MT 160 with flame photometric detector. Mass spectral analyses were conducted on a Finnigan 1015 C quadrupole spectrometer interfaced with HP 5710 FID gas chromatograph. 5-Bromoindoxyl acetate was obtained from Sigma Chemical Co., St. Louis, Mo.; 15 mg of the acetate dissolved in 15 mL of absolute ethanol was used as a spray reagent. Anhydrous sodium sulfate (granular) (Fisher Scientific Co.) was incinerated at 700 °C for 18 h, cooled to 200 °C, and kept in a desiccator at room temperature.

Preparation of Bovine Liver Enzymes. Fresh liver enzymes were prepared according to the modified method of Winterlin et al. (1968). Fresh liver (ca. 20 g) was macerated in 50 mL of salt solution (8.12 g of manganous chloride and 8.77 g of sodium chloride per liter) in a precooled container using a Brinkmann Polytron homogenizer. The liver homogenate was then transferred to two 50-mL polypropylene centrifuge tubes with two rinsings of 10-mL aliquots of the salt solution. After centrifugation for 10 min at 10 000 rpm at −10 °C in a Beckman high-speed centrifuge Model J-21, the supernate was decanted into 10-mL vials and immediately frozen using dry ice–acetone bath and stored at −10 °C.

Determination of Trichlorfon in the Formulated Product. A suspension of about 150 mL of the formulated product in 150 mL of water was partitioned with chloroform (5 × 100 mL) to free the active principles from highly polar excipients and adjuvants. During the first two extractions, the mixture was gently shaken with the organic solvent to prevent formation of an intractable emulsion, then shaken vigorously thrice. After evaporating the solvent at 10–14 Torr/35 °C, the residual reddish oil was redissolved in about 50 mL of chloroform and reextracted with 3 × 75 mL portions of water. The chloroform layer was allowed to percolate through a bed of anhydrous sodium sulfate which was then rinsed with 20 mL of chloroform. Evaporation of the solvent left a pale-yellow oil.

The oil was chromatographed according to the method of Hamilton (1966) with simplified solvent elution system, and the trichlorfon was quantitatively separated from other organic components. The thick residual oil (0.72 g) was topped on a wet-packed (hexane) hydrated silicic acid (10 g; 100 mesh; column length 20 × 1.5 cm) and eluted with 200 mL of hexane (fraction I) and then washed with 250 mL of chloroform (fraction II). The chloroform fraction

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on evaporation left a residue which, after making to 10-mL volume in acetone, was analyzed to contain 7.9% trichlorfon both by flame ionization and flame photometric gas chromatographic techniques using 6 ft × 0.125 in. glass column packed with 3% OV-1 coated on HMDS-treated chromosorb W. The column and the detectors were maintained at 125 and 250 °C, respectively. The nitrogen gas flow was at 40 mL/min. No detectable levels of dichlorvos were observed above 2 ng.

Detection of an Enzyme-Inhibiting Compound in Fraction (I). The hexane fraction (I) was concentrated to about 1 mL, and a volume of 10 μL of this concentrate was spotted on an activated silica gel thin-layer chromatoplate and developed with 6% ether in benzene solvent system. The air-dried plate, when viewed under short-wave UV light, showed one major spot with R_f 0.16 and three minor spots with R_f's 0.23, 0.39, and 0.68, respectively.

The same plate was then sprayed with liver enzyme solution (ca. 15 mL) followed sequentially by incubation at 38 °C for 4 h and spraying with 5-bromoindoxyl acetate solution (Ackermann, 1966; Mendoza et al., 1968). The spot with the R_f 0.16 appeared as a pale-white region against a blue background. A moderate enzyme activity was observed at the origin.

An aliquot (2 μL) of the concentrate was qualitatively analyzed using an HP gas chromatograph equipped with a flame ionization detector under the same conditions as described above except the temperature of the column was programmed from 125 to 250 °C at 4 °C/min and the nitrogen carrier gas flow was maintained at 20 mL/min. At least eight major peaks were observed. However, under identical conditions, except that the temperature of the column was kept at 140 °C on a Tracor gas chromatograph equipped with a flame photometric detector, the sample showed no peak, indicating none of these eight components contained phosphorus. Also, the oil showed the absence of nitrogen by sodium fusion test, and this was confirmed by analyzing the sample on a gas chromatograph equipped with nitrogen-specific Coulson detector. However, the sample showed the presence of chlorine (sodium fusion test) which was confirmed by obtaining a single peak (retention time 4.0 min) on an HP gas chromatograph equipped with a ^{63}Ni electron-capture detector under the gas chromatographic parameters as described for the flame photometric analysis.

Attempted Isolation of the Chlorinated Component from the Concentrate by Column Chromatography. The crude concentrate was chromatographed on a hydrated silicic acid column (35 g; Mallinckrodt Chemical Co., 100 mesh; wet-packed in hexane). The column was then eluted with 400 mL of "nanograde" hexane to collect 80 × 5 mL fractions. Every fifth fraction was concentrated to approximately 0.1 mL and analyzed by GC–FID as described before. Fractions 1–50 contained no detectable levels of residue. Fractions 51–59 gave a residue which was found to be phthalate esters of C_4 and C_5 alcohols. The remaining fractions 60–80 showed minor quantities of phthalate esters, ester of fatty acids, and an unknown component containing chlorine as observed from its mass spectrum (vide infra).

Gas Chromatographic–Mass Spectrometric (GC–MS) Analysis. Mass spectral data of the various components of fractions (60–80) obtained from the silicic acid column were determined. The operating conditions for the mass spectral scans were: ionization voltage, 70 eV at 3 × 10^{-5} Torr and ion source temperature maintained at 300 °C.

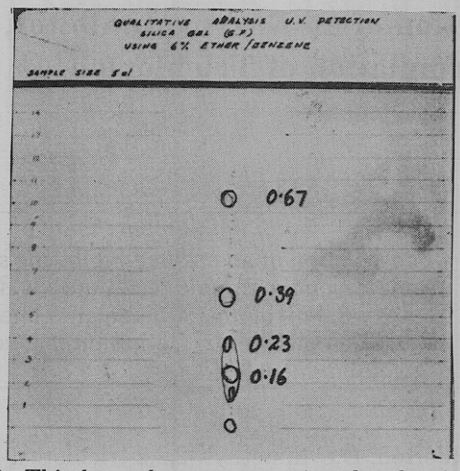


Figure 1. Thin-layer chromatogram viewed under short-wave UV light before spraying.

Infrared (IR) Spectral Analysis. The fractions (60–80) were concentrated to ca. 0.5 mL and the components separated using an HP gas chromatograph equipped with an EC detector. The fraction having the retention time 3.2 min was trapped from the exit port of the chromatograph by condensation in a dry ice cooled 100 × 3 mm borosilicate tube. The purity of this component was established by performing analytical gas chromatography of the collected residue on an identical column using an FID detector by observing a single peak (retention time ca. 4.0 min). Further, this component showed a strong liver enzyme inhibition property. The IR spectrum of this sample was immediately obtained as a liquid film sandwiched between two NaCl plates.

RESULTS AND DISCUSSION

A literature survey of the toxicological studies on trichlorfon and the absence of its highly toxic metabolite, dichlorvos, prompted us to suspect the labeled content of the active ingredient and other possible toxic contaminants in the formulated product. After a silicic acid column cleanup the material in the chloroform fraction was found to contain 7.9% of trichlorfon (observed as the only major peak along with two negligibly small peaks). It was thus apparent that the hexane fraction of the formulated products might possibly contain the enzyme-inhibiting impurity. Therefore, the hexane fraction was subjected to further investigation to isolate and characterize the impurities, if present.

Concentration of the hexane fraction of the formulated product left a residual oil which on a thin-layer chromatogram (Figure 1) separated into four spots, the major of which had an R_f 0.16. Enzymatic inhibition test (Figure 2) revealed that the major component was shown to strongly inhibit the enzyme. This deactivation of the enzyme is probably due to the intense toxicity of the component(s) or to their relatively high proportion in the hexane fraction. Further, qualitative analysis of the oil indicated the absence of phosphorus and nitrogen but gave a positive test for chlorine.

An attempt was made to fractionate the crude concentrate of the hexane fraction using a very large excess of hydrated silicic acid and collecting 5-mL volume fractions. The last 21 fractions (60–80) on analysis have been shown to contain at least eight components, indicating that these substances are fairly nonpolar and inseparable under these experimental conditions.

The fractions 60–80 were pooled and evaporated to leave an oily residue which was subjected to gas chromatographic analysis under careful conditions. Figure 3 shows the

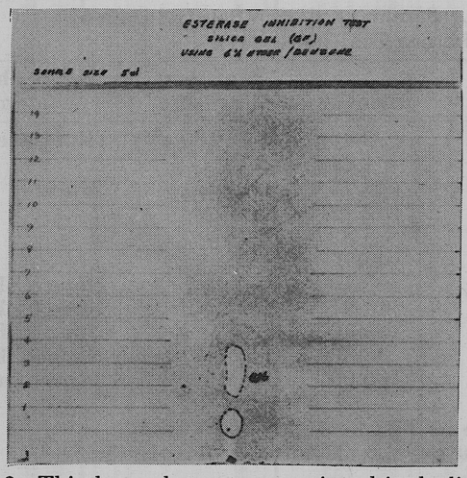
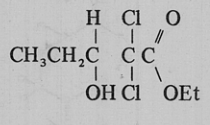


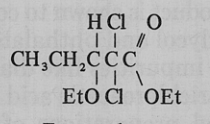
Figure 2. Thin-layer chromatogram viewed in daylight after spraying.

major peaks with retention time (4.0, 6.0, 8.2, 11.5, 12.5, 16.5, 20.5, 24.3, and 27.8 min) of which the first peak having the retention time 4.0 min is the major component in this residue (attenuation X1024; in the original hexane extract this constituent is present to the extent of 3.74% relative to other components). The crude concentrate was then fractionated by mini-scale preparative gas chromatography and the major component with the retention time (182 s; peak 1) was collected using a dry ice cooled trap at the port. From several injections through the gas chromatograph, a quantity of 2.7 mg of a colorless oil was collected.

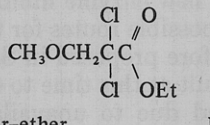
The IR spectrum of a neat, thin film of the oil showed strong absorption bands at 920, 1023, 1112, 1240, and 1767 cm^{-1} and no hydroxyl bands were observed. The very strong carbonyl absorption at 1767 cm^{-1} indicates that it is a saturated halogenated ester of a halogenated δ-lactone. Villieras and Castro (1968) reported that the ester carbonyl absorption of α,α-dichloro fatty acid esters (a to c) had an absorption band between 1760 and 1765 cm^{-1} whereas the α,α-unsubstituted esters absorbed between 1735 and 1750 cm^{-1}. Since limited spectral data are available in the literature regarding α-halogenated δ-lactones, an analogy is drawn from α-halogenated cyclic ketones and α-halogenated γ-butyrolactones.



Esters	Ester–ether	Ether
ν = C–O	ν = –C–O–C	ν = –C–O–C
1760	1250	



Esters	Ester–ether	Ether
1761	1250–1230	1082



Ester	Ester–ether	Ether
1765	1240	1130 and 1095–1070

Jones et al. (1952) reported that the carbonyl absorption of dibromocyclohexanone) was observed at 1737 cm^{-1} and the equatorial monobromocyclohexanone had an absorption at 1734 cm^{-1}, indicating that the second halogen atom has very little effect on the ketone carbonyl frequency.

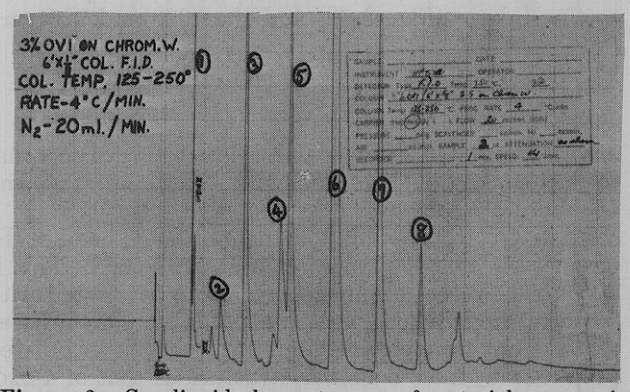


Figure 3. Gas–liquid chromatogram of material present in fractions 60–80 from silicic acid column chromatography.

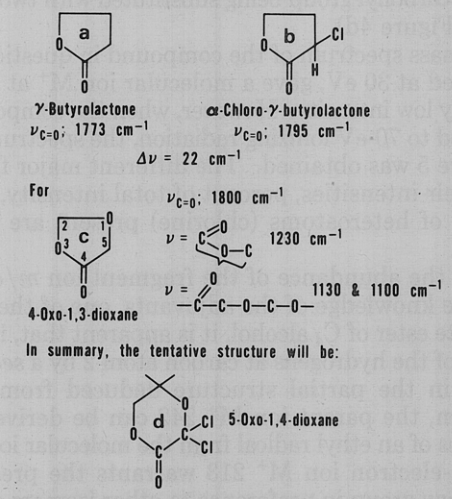
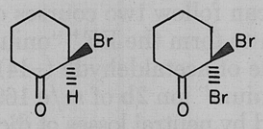


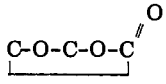
Figure 4. Infrared spectral data of substituted γ- and δ-lactones.



Brugel et al. (1956) observed that α-chloro-γ-butyrolactone had an absorption maximum at 1795 cm^{-1} for the lactone carbonyl group, whereas γ-butyrolactone itself was known to absorb at 1773 cm^{-1} (Storm et al., 1972) with a shift of $\Delta\nu$ = +22 cm^{-1} higher than the parent γ-butyrolactone (Figure 4a,b). If the same difference of +22 cm^{-1} along with the contribution of $\Delta\nu$ = +3 cm^{-1} due to dihalogen substitution is added to the carbonyl absorption of δ-valerolactone which has a frequency at 1737 cm^{-1} (Rasmussen and Brattain, 1949; Jones et al., 1959), the carbonyl absorption of α,α-dichloro-δ-lactone is calculated to be 1762 cm^{-1}, somewhat lower than 1767 cm^{-1} as was found for the suspected enzyme-inhibiting substance. This increased shift may be ascribed to the chlorine atoms which are known to absorb at higher frequencies than carbonyl compounds containing bromine.

The IR spectrum of the unknown component also showed absorptions at 1240 cm^{-1} for lactonic ether oxygen –C(=O)–O–C–, and other absorptions at 1112 and 1023 cm^{-1} suggest the –C–O–C stretching of an ether linkage. Farines and Soulier (1970) reported that 4-oxo-1,3-dioxane and its homologues had absorption bands at 1230, 1100, 1030, and 920 cm^{-1} apart from the lactonic absorption at 1800 cm^{-1}. These authors ascribed the 1230 and 1100 cm^{-1}

to the linkage (Figure 4c) and the 1030 and 920 cm^{-1}



absorptions to C–O–C bonds. It is interesting to note that the lactonic carbonyl absorptions of 4-oxo-1,3-dioxane and of γ-butyrolactone were observed at 1800 and 1773 cm^{-1}, respectively, whereas the δ-lactonic carbonyl band in the impurity in question was found at 1767 cm^{-1}. This is explained by the minimum ring strain associated with six-membered cyclic ether lactone. It is therefore inferred that the unknown enzyme inhibitor from the formulated product may have a similar structure as that of 4-oxo-1,3-dioxane except that to account for the δ-lactone it might be 5-oxo-1,4-dioxane with the α position to the lactonic carbonyl group being substituted with two chlorine atoms (Figure 4d).

The mass spectrum of the compound in question, when irradiated at 30 eV, gave a molecular ion M^+ at m/e 242 with very low intensity. However, when the compound was subjected to 70-eV ionizing radiation, the spectrum shown in Figure 5 was obtained. The different major fragment ions, their intensities, percent of total intensity, and the number of heteroatoms (chlorine) present are given in Table I.

From the abundance of the fragment ion m/e 73 and from the knowledge of the adjuvants, one of them being phthalate ester of C_4 alcohol, it is apparent that, in Figure 4d, one of the hydrogens at carbon atom 2 by a *sec*-butoxy moiety in the partial structure deduced from the IR spectrum, the parent ion M^+ 242 can be derived. The facile loss of an ethyl radical from the molecular ion to give an even-electron ion M^+ 213 warrants the presence of *sec*-butoxy group in preference to other isomers of the C_4 alcohol (Figure 6). Further, making use of this structural formula, the formation of all fragment ions shown in Table I can be explained according to Scheme I.

The loss of an ethyl radical from the molecular ion **1** (M^+ 242) gives rise to even-electron ion (EE^+) **2** of m/e 213. The EE^+ ion **2** can follow two courses of fragmentation. It can lead to **2a** to form the EE^+ "onium" ion which, on losing a molecule of acetaldehyde (–44), will give rise to another EE^+ "onium" ion **2b** of m/e 169. On heterolytic cleavage followed by neutral losses of dichlorocarbene and carbon dioxide **2b** leads to **2e** having an m/e 43.

By the second route, the EE^+ ion **2** can undergo 1,4 hydrogen rearrangement to give another EE^+ ion **3** which leads to the formation of an EE^+ "onium" ion **4**. Heterolytic cleavage of the C–O bond gives rise to the species **5**. The even-electron ion **5** can also take two fragmentation pathways through a neutral loss of carbon monoxide followed by 1,5 hydrogen rearrangement and loss of a molecule of acetaldehyde, the ion **5** will lead another EE^+ ion **6b** of m/e 141. Further, **6b** may experience a neutral loss of glyoxal (–58) leading to another EE^+ fragment ion **6c** of m/e 83.

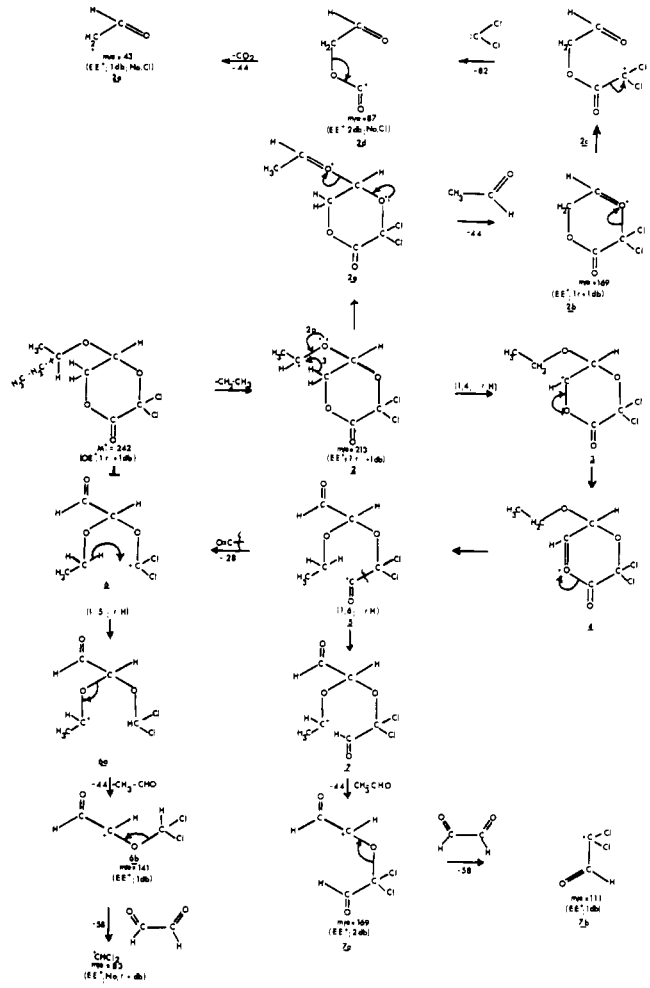
The fragment ion **5** can also undergo 1,6 hydrogen rearrangement leading to an EE^+ ion **7** from which **7a** can be formed due to loss of acetaldehyde. Finally, **7a** can further experience a neutral loss of glyoxal to give rise to the EE^+ ion **7b** of m/e 111. It would seem established that this enzyme inhibitor present in the formulated product of trichlorfon is tentatively identified as 2-*sec*-butoxy-6,6-dichloro-1,4-dioxolan-5-one (Figure 6).

It is known that the rate of formation of trichlorfon from trialkyl phosphite and chloral hydrate was accelerated by ethylene oxide to give higher yields and purer product of the organophosphate (Bliznyuk et al., 1966). Moreover,

Table I. Summary of Mass Spectral Data of the Unknown Toxicant

m/e	Intensity	% of total intensity	No. of chlorine atoms
242 (M^+)	2.43	0.15 (30 eV)	(Not discernible)
213	1.34	0.18	2
169	33.74	4.68	2
141	2.12	0.29	2
111	5.32	0.73	2
83	48.14	6.68	2
73	93.70	13.00	None ($C_4H_9O^+$)
59	44.97	6.24	None ($C_2H_3O_2^+$)
45	99.46	13.80	None ($C_2H_5O^+$)
43	38.36	5.32	None ($C_2H_3O^+$)
41	100.00	13.88	1 (irrelevant)

Scheme I. Fragmentation Pathways of 2-*sec*-Butoxy-6,6-dichloro-1,4-dioxolan-5-one



the formulated product is shown to contain adjuvants such as polyethylene glycol and phthalate ester of C_4 alcohol. Furthermore, the impurities like dialkyl phosphite, alkyl phosphates, and trichloroacetic acid are frequently found in the commerical preparations of trichlorfon. These impurities and adjuvants were considered as probable precursors of the new enzyme inhibitor to be formed in situ. One of the possible routes for the formation of this impurity is therefore proposed in Scheme II.

It is very difficult at this time to evaluate the toxicity of this compound due to unavailability of sufficient quantities of material. It is interesting to note that Kamel and Sebac (1970) reported that the effectiveness of trichlorfon was increased by surfactants whereas the adjuvants potentiated the toxicity of DDT and Sevin. However, a literature survey (Matsubara, 1968; Tosta-

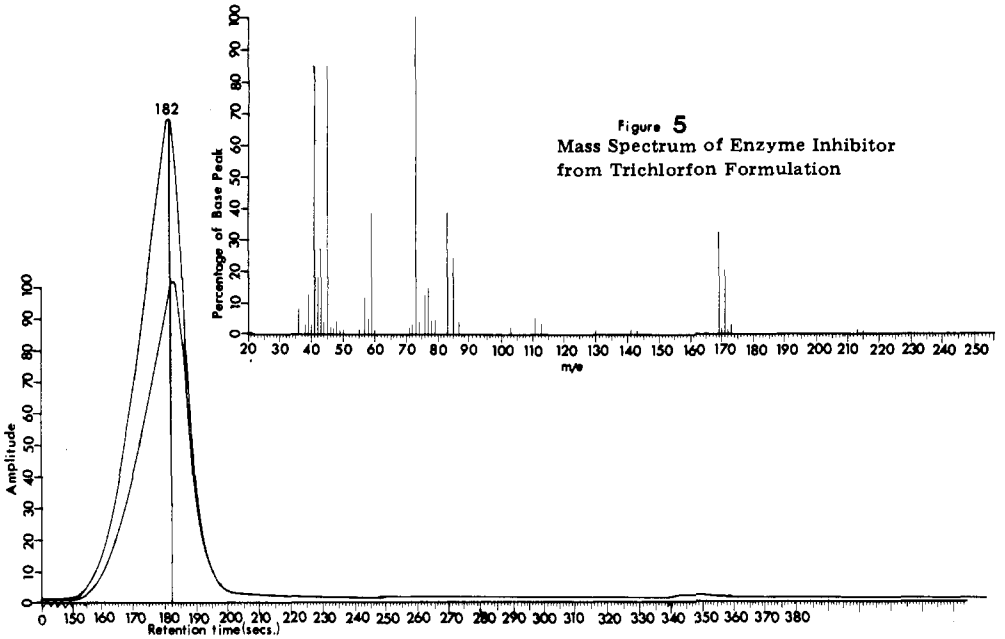
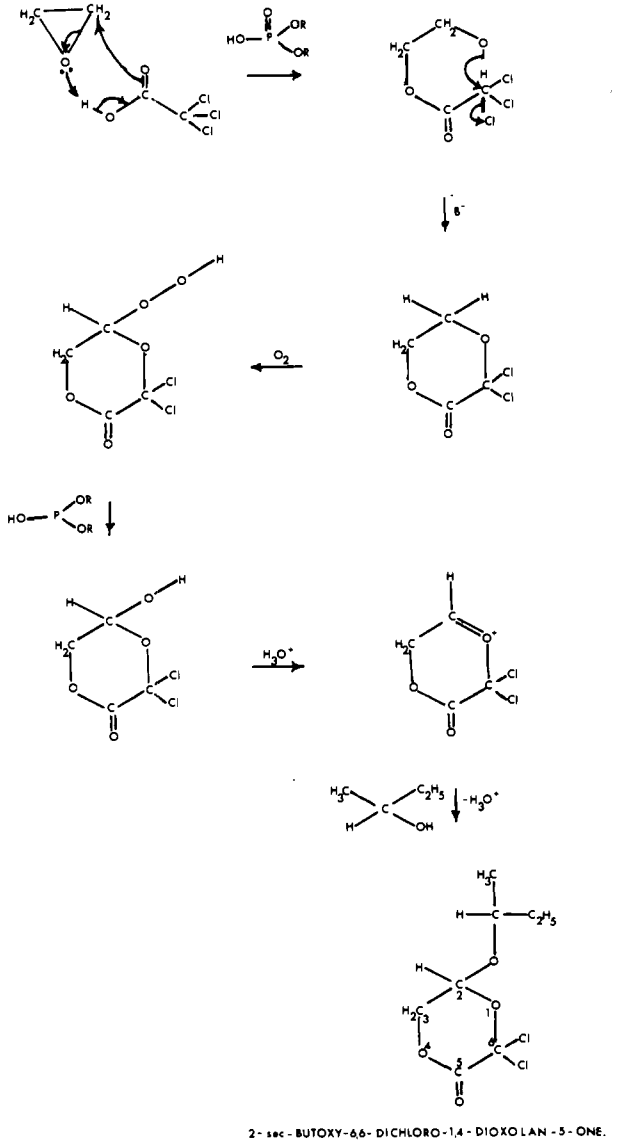


Figure 5
Mass Spectrum of Enzyme Inhibitor from Trichlorfon Formulation

Figure 5. Mass spectrum of the enzyme inhibitor from trichlorfon formulation.

Scheme II. Proposed Formation of the Enzyme Inhibitor Found in the Formulation of Trichlorfon



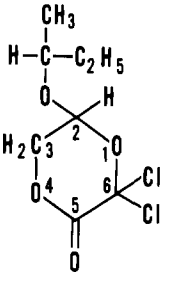


Figure 6. Proposed structure of the enzyme inhibitor from trichlorfon formulation.

novskaya et al., 1966; Goszczynska, 1966; and Tomov et al., 1966) indicates that the chlorinated hydrocarbon insecticides such as DDT, lindane, dieldrin, toxaphene, etc., act synergistically with trichlorfon. While it is possible that the toxicity of trichlorfon was potentiated by 2-*sec*-butoxy-6,6-dichloro-1,4-dioxolan-5-one, this impurity cannot be incriminated for the observed deaths of cattle in Southern Ontario.

ACKNOWLEDGMENT

The author wishes to thank O. Tannahill for his skilled technical assistance, W. Strachan for the mass spectrum, M. Schnitzer and F. A. Vandenheuvel for reviewing the manuscript. My sincere gratitude to O. E. Edwards (NRC, Canada) for helpful discussions and suggestions and J. P. Barrette for his continued encouragement.

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Received for review November 12, 1975. Resubmitted October 6, 1977. Accepted October 19, 1977.

Conversion of Parathion to Paraoxon in Foliar Residues: Effects of Dust Level and Ozone Concentration

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Dwarf Eureka lemon trees were treated with parathion and placed in an environmental chamber for 4 days with the object of determining the effect of foliar dust level and atmospheric ozone concentration on the production of paraoxon in the dislodgeable foliar residues. At low levels of foliar dust little paraoxon was produced regardless of ozone concentration. Compared to production at low dust levels, paraoxon production was increased by a factor of 4 at high dust levels without ozone and by nearly a factor of 30 at high dust levels in the presence of 300 ppb of ozone.

In California there have been at least 29 separate poisoning incidents reported among agricultural fieldworkers exposed to organophosphate pesticide residues since the introduction of these compounds in 1948 (Spear et al., 1975a). Parathion has been implicated in the majority of these incidents, and there is now strong evidence that its oxygen analogue, paraoxon, is the principal toxic constituent of the weathered residue on both foliage and in the soil of orange groves in Central California (Spear et al., 1977a,b). Although paraoxon has been detected in foliar residues in other regions of the United States (Utah Biological Test Laboratory Report, 1976) (UBTL), it appears that the environment of the Central Valley of California is particularly conducive to the formation and persistence of this highly toxic metabolite of parathion.

The sporadic occurrence of the known incidents and the findings of an investigation into a poisoning incident in June 1975 (Spear et al., 1977b) led us to inquire into the nature of unusual environmental circumstances which might lead to an abnormal production of paraoxon in foliar and soil residues. In an attempt to identify these factors, parathion-treated dwarf Eureka lemon trees were exposed to various experimental conditions in an environmental chamber. This report deals with the effects of foliar dust and of atmospheric oxidant concentrations on paraoxon residue levels observed in these chamber experiments. Complementary investigations were carried out with soil residues and will be reported subsequently.

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EQUIPMENT AND METHODS

The experiments described below were carried out in an environmental chamber constructed of enamelled plywood whose interior dimensions are 7.5 ft high by 7.8 ft wide by 14 ft in length. The chamber has both temperature and humidity control and is equipped with Atlas Electric RM-65 xenon arc lamps whose spectrum is quite similar to that of sunlight. The intensity of these lamps in the critical region from 280 to 450 nm results in approximately 75% of the radiant flux at summer noontime in the Central Valley of California.

Ozone was introduced into the chamber from a Wellsbach T-23 laboratory ozonator. Ozone levels were continuously monitored with a Dasibi 1003-AH UV photometer and frequently with a Mast Model 724 Oxidant Monitor. When ozone levels were within the operating ranges for both instruments, no differences were observed in their readings. Although hydrocarbons emanating from the chamber and/or from the trees in confined circumstances might lead to molecular species not normally found in the field, we believe the uniformity of ozone levels indicated by the Mast and Dasibi instruments suggest ozone to have been the only significant oxidizing species present in the chamber and that it was an adequate model oxidant for the purposes of this study.

Prior to pesticide application, the foliage of the trees was washed with a solution of Sur-Ten wetting agent in water to remove the preexisting dust load. The trees were then throughly rinsed with distilled water. This procedure was required in order to control the foliar dust level for ex-

0021-8561/78/1426-0434$01.00/0