Microbial and Nonenzymatic Steps in the Decomposition of Dichlorvos (2,2-Dichlorovinyl *O,O*-Dimethyl Phosphate)

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Dichlorvos (2,2-dichlorovinyl *O,O*-dimethyl phosphate) was converted to dichloroethanol, dichloroacetic acid, and ethyl dichloroacetate by a microbial enrichment derived from sewage. These products were identified by combined gas chromatography–mass spectrometry. The compounds were not formed in the absence of cells. Inorganic phosphate was also generated from the insecticide in the presence of the microorganisms, but dimethyl phosphate was produced in the presence or absence of microbial cells. On the basis of these findings, a pathway is proposed for the transformation of dichlorvos.

Knowledge of the fate of pesticides is important in soils and fresh or marine waters because they are frequently present in these environments. The organophosphate insecticides are widely used because of their effectiveness in insect control and their relatively short persistence. The cleavage of alkyl and aryl phosphate esters has been shown to occur in mixed and axenic cultures of microorganisms as well as in enzyme preparations derived from microorganisms (Sethunathan et al., 1977; Rosenberg and Alexander, 1979; Adhya et al., 1981). The portions of the molecules that are cleaved may be transformed subsequently by enzymatic or nonenzymatic mechanisms (El Beit et al., 1978; Faust and Gomaa, 1972; Kearney and Helling, 1969).

Although microorganisms have been identified that can cause disappearance of dichlorvos (2,2-dichlorovinyl *O,O*-dimethyl phosphate, DDVP, Vapona) (Lamoreaux and Newland, 1978; Boush and Matsumura, 1967), the pathway by which it is metabolized by microorganisms has not been established. Hence, an investigation was undertaken to identify the products formed during the decomposition.

EXPERIMENTAL SECTION

Microbial Enrichment. A microbial enrichment capable of metabolizing dichlorvos was obtained from sewage by using the BOD (biological oxygen demand) test method (American Public Health Association, 1975) to measure oxygen depletion in the BOD inorganic salts solution that had been amended with 100 μg of dichlorvos/mL (99%, Shell Chemical Co., San Ramon, CA). This enrichment caused the depletion of more than 85% of the dissolved O_2 in 5–7 days at 23–25 °C. The enrichment was then transferred to a defined medium, in which it depleted more than 95% of the dissolved O_2 in 4 days. This medium contained 1.6 g of KH_2PO_4, 0.4 g of KNO_3, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NH_4NO_3, 25 mg of $CaSO_4 \cdot 2H_2O$, 2.5 mg of NaCl, 2.5 mg of $FeSO_4 \cdot 7H_2O$, 20 μg each of biotin and folic acid, 100 μg of pyridoxine hydrochloride, 50 μg each of riboflavin, thiamin hydrochloride, nicotinic acid, pantothenic acid, *p*-aminobenzoic acid, and thioctic acid, 1 μg of vitamin B12, and 250 mg of dichlorvos per L of distilled water. The final pH after autoclaving was 7.1. The vitamin supplement provided only 0.22 μg of carbon/mL, which was insufficient to account for the amount of O_2 depleted.

Pseudomonas aeruginosa and an unclassified species of *Pseudomonas*, which were identified by using the Analytical Profile Index (Analytab Products, Plainview, NY), and a motile, Gram-positive, spore-forming rod were isolated from the enrichment. Although each individually caused O_2 depletion in the BOD solution containing the vitamin supplement and 250 μg of dichlorvos/mL, their activity was less than that of the enrichment. For this reason, the enrichment culture was used in experimental procedures.

Experimental Design. The number of cells in the enrichment culture was increased by amending the dichlorvos medium with 0.1% each of disodium succinate and glucose and incubating at 29 ± 1 °C with shaking. After 7 days, the cells were collected by centrifugation at 4 °C for 15 min at 11700*g* and washed 3 times. In this manner, an initial cell count of 10^7–10^8 cells/mL was obtained as determined by plate counts on dichlorvos-containing agar. Three treatments were prepared in duplicte in 1.0-L Erlenmeyer flasks containing 500 mL of the inorganic salt solution supplemented with vitamins: (i) 250 μg/mL dichlorvos plus cells, (ii) dichlorvos alone, and (iii) microbial cells alone.

After a 7-day incubation period at 29 ± 1 °C on a rotary shaker, the cells were removed as described above. To all treatments, 2% NaCl was added first, and then an amount of anhydrous ethyl ether was added equal to 25–50% of the total volume; the liquid was mixed vigorously for 2 min, and the upper ether layer was collected. This procedure was carried out 3 times, and the ether fractions were pooled and designated the "neutral" fraction. The remaining aqueous phase was acidified to pH 2 with H_2SO_4 and extracted as above. This fraction was called the "acidic" fraction. The ether fractions were concentrated at 25 °C with a rotary evaporator and dried over anhydrous Na_2SO_4.

In an identical experiment, the neutral fraction was removed, and the remaining aqueous phase was concentrated 100-fold at 30 °C with a rotary evaporator until no free liquid was present. Two grams of the moist precipitate was then extracted with two 5-mL portions of acetone. The combined acetone extract was concentrated by a stream of air, and 200-μL quantities were treated with 200 μL of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (Regis Chemical Co., Morton Grove, IL) for 2 h at 80 °C to form the *tert*-butyldimethylsilyl derivatives used for gas chromatographic and mass spectrometric analysis.

Gas Chromatography and Mass Spectrometry. A Perkin-Elmer Model 3920B gas chromatograph fitted with a 183-cm glass column (2-mm i.d.) packed with 3% Silar 10C on Gas-Chrom Q (100–120 mesh) (Applied Science Laboratories, State College, PA) was used to detect ether-soluble metabolites. The temperature of the column was maintained at 70 °C for 4 min and then increased 8 °C/min to 160 °C, at which point the temperature was maintained for 4 min. The injector was at 200 °C, and the

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flame ionization detector was at 250 °C. N_2 at a flow rate of 55 mL/min was the carrier gas.

Metabolites in the aqueous phase were detected with a 183-cm stainless steel column (2 mm i.d.) packed with 10% OV-17–2% QF-1 in Chromosorb W (100–200 mesh, AW, DMCS, Supelco, Inc., Bellefonte, PA). The temperature of the column was maintained at 100 °C for 2 min and then increased 32 °C/min to 190 °C, at which point the temperature was maintained for 4 min. Both injector and flame ionization detector were at 250 °C. N_2 at a flow rate of 55 mL/min was the carrier gas.

The products separated by gas chromatography were subjected to electron impact and chemical ionization analysis on a Finnigan 3300 quadrupole mass spectrometer with Systems Industries 150 data system. For electron impact, the system was operated at 70 eV. Chemical ionization was performed by charging with CH_4.

Authentic 2,2-dichloroethanol and dichloroacetic acid were obtained from Eastman Organic Chemicals (Rochester, NY), and dimethyl phosphate was provided by American Cyanamid Co. (Princeton, NJ), respectively. Mass spectra were compared with standards, with published spectra (Stenhagen et al., 1974), or with a reference collection available through the Probability Based Matching computer package (Office of Computer Services, Cornell University, Ithaca, NY).

Phosphate Determination. Inorganic phosphate was detected in a P-free medium containing (per liter of water): 0.3 g of KCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NH_4NO_3, 0.025 g of $CaCl_2 \cdot 2H_2O$, 2.5 mg of $FeSO_4 \cdot 7H_2O$, 2.42 g of Tris–base (Sigma Chemical Co., St. Louis, MO), and 0.2 M NaOH to a final pH of 7.17. The water was purified with a Milli-Q water system (Millipore Corp., Bedford, MA). All glassware was washed in 10% nitric acid prior to use. Treatments with only 250 μg of dichlorvos/mL, 10^7 washed cells/mL alone, or both were prepared in 26 mL of salt solution in Tris buffer in 50-mL Erlenmeyer flasks. Triplicate samples taken on day 0 were immediately frozen; the remaining flasks were incubated at 29 ± 1 °C on a rotary shaker. Samples taken at 2 and 4 days were also frozen. At the time of analysis, the samples were thawed; if the liquid contained cells, they were removed by centrifugation. The inorganic phosphorus content of the supernatant liquid was determined by the phosphomolybdate complex method (Strickland and Parsons, 1972).

RESULTS AND DISCUSSION

Dichlorvos was still present after 7 days of incubation of the insecticide in the presence or absence of microbial cells. The mass spectrum of the presumed dichlorvos in the liquid was identical with that of authentic dichlorvos and the reference spectrum. Considering the high level of dichlorvos used, it is not unexpected that dichlorvos remained after 1 week. The hydrolysis rate of saturated water solutions of dichlorvos is 3%/day (Shell Chemical Co., 1973). If these kinetics are assumed for a concentration of 250 μg/mL, approximately 200 μg/mL would remain after 7 days.

Dimethyl phosphate appeared in the aqueous phase of samples incubated with dichlorvos with or without cells. The mass spectrum of the *tert*-butyldimethylsilyl derivative [$(CH_3O)_2P(O)OSi(CH_3)_2C(CH_3)_3$] is shown in Figure 1. Although the molecular ion (M^+, m/z 240) was not observed, the characteristic loss of methyl (m/z 225) and loss of *tert*-butyl (m/z 183) were noted. A subsequent loss of the methyl groups linked to silicon yielded a fragment with m/z 153 [$(CH_3O)_2P(O)OSi$]. A silicon rearrangement fragment at m/z 105 ($CH_3OSi(CH_3)_2O$) was also noted. Chemical ionization analysis of the samples and the authentic chemical confirmed the identification.

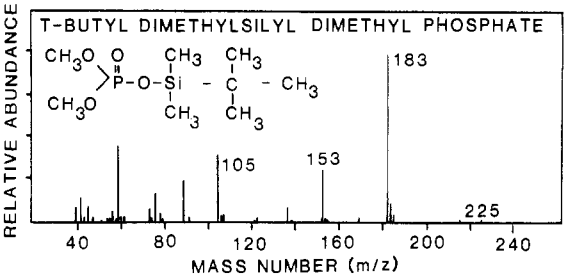

Figure 1. Mass spectrum of the *tert*-butyldimethylsilyl derivative of dimethyl phosphate.

Table I. Formation of Inorganic Phosphate in the Breakdown of Dichlorvos

day	inorganic phosphate, ng of P/mL: dichlorvos + inoculum	inorganic phosphate, ng of P/mL: inoculum alone
0	24.8 ± 0.3	4.6 ± 0.4
2	85.4 ± 3.4	16.8 ± 1.0
4	152 ± 12	31.1 ± 3.6

When the microbial enrichment was incubated for 4 days at 29 °C in a Tris-buffered P-free medium containing 250 μg of dichlorvos/mL, a 6-fold increase in inorganic phosphate was observed (Table I). The phosphate level increased somewhat in a suspension of the cells alone, presumably because of its release from the cells. The extent of inorganic phosphate formation was less than 0.5% of the dichlorovos P present. No inorganic phosphate was formed if dichlorvos was incubated without the cells.

Further evidence of a microbial role in the decomposition of the insecticide was obtained by analysis of the ether and aqueous fractions of the treatments with dichlorvos. Dichloroethanol and dichloroacetate were found if the insecticide was incubated with the cells but not if cells were absent. The mass spectra of both compounds showed typical P/(P + 2) mass clusters, indicating chlorinated molecules (Figure 2). The dichloroethanol in the ether fraction showed a prominent molecular ion (m/z 114) atypical of alcohols, but the spectrum of the authentic chemical had the same molecular ion. The fragments included m/z 83 (Cl_2CH), 79 ($ClCHCH_2OH$), 49 ($ClCH_2$), and 43 (C_2H_3O). Dichloroacetate was identified in the aqueous fraction following derivatization, which yielded the *tert*-butyldimethylsilyl derivative of dichloroacetate [$Cl_2CHC(O)OSi(CH_3)_2C(CH_3)_3$, m/z 242]. The mass spectrum of this molecule showed neither the M^+ nor $(M - 15)^+$ peaks, but $(M - 57)^+$ at m/z 185 from the loss of *tert*-butyl and mass fragments at m/z 157 [$Cl_2CHC(O)OSiH_2$], 115 (*tert*-butyldimethylsilyl), 93 [$ClCH_2C(O)O$], and 57 (*tert*-butyl) were evident. Chemical ionization and a comparison with the mass spectrum of the authentic chemical confirmed the identity of this molecule.

Ethyl acetate and ethyl dichloroacetate were also only found by gas chromatographic–mass spectrometric analysis of the acidified ether fraction derived from the treatment containing dichlorvos and cells and not in the treatment with no cells. This indicates that these products are biologically formed. The mass spectrum of ethyl dichloroacetate is shown in Figure 2. The fragments included m/z 141 (loss of methyl), 113 [$Cl_2CHC(O)H_2$], 77 (ClCHCOH), 48 (ClCH), and 43 ($OCCH_3$). The analysis of m/z 113 is complicated by a probable additive effect of a fragment or fragments at m/z 111. Chemical ionization analysis substantiated m/z 156 as the molecular weight of this

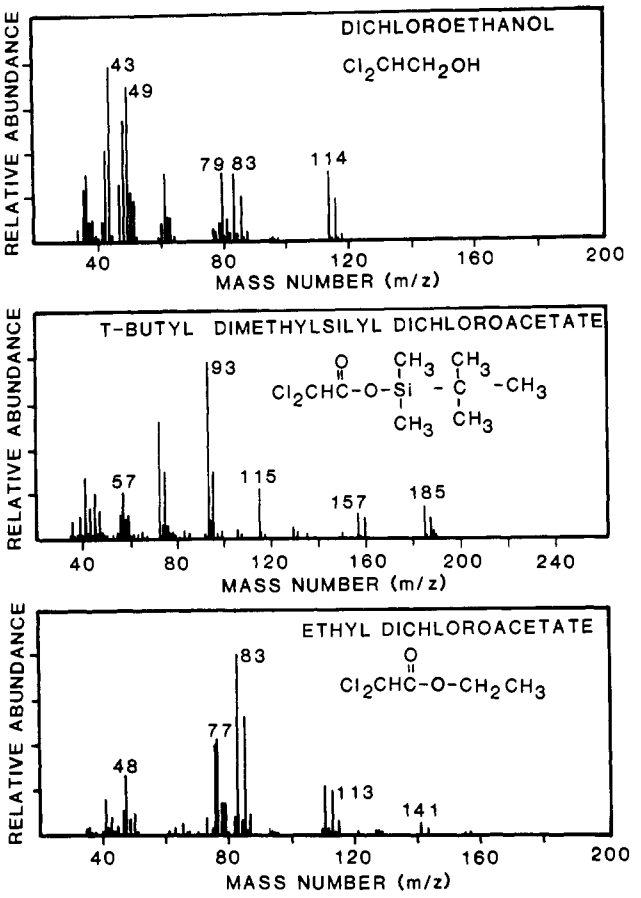

Figure 2. Mass spectra of products identified as dichloroethanol (top), *tert*-butyldimethylsilyl derivative of dichloroacetate (middle), and ethyl dichloroacetate (bottom).

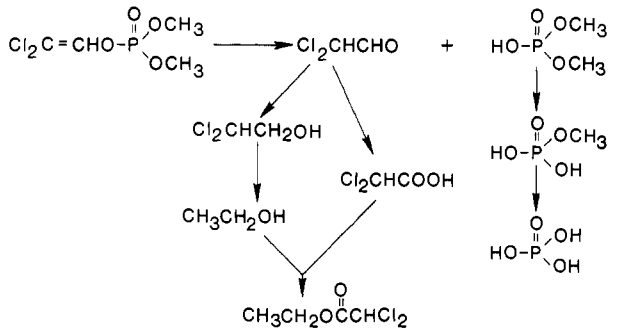

Figure 3. Proposed pathway for the breakdown of dichlorvos.

molecule. Confirmation of the identity of this molecule as ethyl dichloroacetate was obtained by using the Probability Based Matching computerized search. The spectrum of ethyl acetate was identical with published values (Stenhagen et al., 1974).

A possible pathway for dichlorvos breakdown is shown in Figure 3. Nonenzymatic hydrolytic cleavage of the parent molecule presumably gives rise to dichloroacetaldehyde and dimethyl phosphate. The latter is converted to inorganic phosphate with monomethyl phosphate being a likely intermediate. The aldehyde then is converted enzymatically to either dichloroethanol or dichloroacetic acid. Schultz et al. (1971) and Wright et al. (1979) proposed that these were products formed from dichlorvos by mammalian tissue. Eto (1974) suggested that dichloroacetaldehyde was formed nonenzymatically from dichlorvos. Ethyl dichloroacetate presumably arose by esterification of dichloroacetic acid with ethanol, the latter possibly being generated as a consequence of the dehalogenation of dichloroethanol.

Registry No. Dichlorvos, 62-73-7; 2,2-dichloroethanol, 598-38-9; dichloroacetic acid, 79-43-6; ethyl dichloroacetate, 535-15-9; dimethyl phosphate, 813-78-5.

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