Microbial Cleavage of Various Organophosphorus Insecticides

ARTHUR ROSENBERG AND MARTIN ALEXANDER*

Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York 14853

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Bacteria able to utilize Aspon, Azodrin, Dasanit, diazinon, malathion, Orthene, parathion, Trithion, dimethoate, Dylox, methyl parathion, and Vapona as sole phosphorus sources were isolated from soil and sewage. Individual isolates used from 3 to 10 of these insecticides as sole phosphorus sources. The extent of growth of two *Pseudomonas* strains in media containing diazinon and malathion was in the range expected from the amount of insecticide supplied, and their proliferation resulted in disappearance of the chemical. Resting cells of the pseudomonads derived from cultures grown on diazinon or malathion but not orthophosphate caused extensive destruction of these two organophosphates in the presence or absence of chloramphenicol. Extracts of the two bacteria derived from organophosphate-grown cultures catalyzed the disappearance of Aspon, Azodrin, Dasanit, diazinon, malathion, Orthene, parathion, and Trithion but not dimethoate, Dylox, methyl parathion, and Vapona. Results from gas chromatographic analysis suggested that the extracts formed dimethyl phosphate from azodrin, dimethyl phosphorodithioate from malathion, diethyl phosphorodithioate from Trithion, and diethyl phosphorothioate from Dasanit, diazinon, and parathion. Dimethyl phosphate, dimethyl phosphorothioate, dimethyl phosphorodithioate, diethyl phosphate, and diethyl phosphorothioate were not used by the pseudomonads as sole phosphorus sources.

The chlorinated hydrocarbon insecticides are characteristically persistent in soils and waters, and their persistence as well as their susceptibility to biomagnification and subsequent toxicity to higher animals have resulted in the termination of use of many of these pesticides. The replacement chemicals for many agricultural pest control operations are the organophosphates, a group that includes a large number of compounds that usually are readily destroyed in soils and waters.

The microbial attack on organophosphate insecticides is well documented. For example, microorganisms bring about the destruction of Disyton (2), malathion (3), parathion (6), and diazinon (10). Some reports of microbial growth on these compounds are questionable, however, because the slight growth obtained may be attributable to contaminating orthophosphate in the medium, and even the demonstration of pesticide disappearance under such circumstances is not adequate evidence that the pesticide is serving as a phosphorus source for growth because many synthetic chemicals are subject to cometabolism. Furthermore, although most members of this class of pesticides have in common the presence of two methoxy or ethoxy groups linked to the phosphorus atom, only modest attention has been given to the specific-

ity of microbial enzymes or enzyme preparations (14).

The present study was undertaken to determine the specificity of enzyme preparations from microorganisms able to metabolize organophosphate insecticides and to establish the products of enzyme breakdown. Because of the frequent growth of bacteria on contaminating orthophosphate in media receiving synthetic organophosphates as sole added phosphorus sources, special care was taken to minimize the contribution of the contaminating nutrients to microbial growth.

MATERIALS AND METHODS

Materials. The sources of the chemicals were as follows: Aspon, methyl parathion, and Trithion were from Stauffer Chemical Co., Westpoint, Conn; Azodrin and dimethyl dichlorovinyl phosphate (Vapona) were from Shell Chemical Co., San Ramon, Calif.; Dasanit and Dylox were from Chemagro Agricultural Div., Kansas City, Mo.; diazinon was from Ciba-Geigy, Greensboro, N.C.; dimethoate, malathion, dimethyl phosphorothioate, and dimethyl phosphorodithioate (potassium salt) were from American Cyanamid Co., Princeton, N.J.; Orthene was from Chevron Chemical Corp., San Francisco, Calif.; ethyl parathion was from Monsanto Co., St. Louis, Mo.; chloramphenicol and sodium barbital were from Calbiochem, San Diego, Calif.; diethyl phosphate was from Eastman Kodak

Co., Rochester, N.Y.; diethyl phosphorothioate, diethyl phosphorodithioate, triethyl phosphorothioate, triethyl phosphate, dimethyl phosphate, trimethyl phosphorothioate, trimethyl phosphate, and tri-*n*-butyl phosphate were from Aldrich Chemical Co., Milwaukee, Wis.; and crystalline bovine serum albumin was from Nutritional Biochemicals Corp., Cleveland Ohio. All chemicals were of the highest purity available commercially.

Glassware. Glassware was cleaned by rinsing in water followed by a 24-h immersion in 20% (vol/vol) HNO₃. The nitric acid was removed by thorough washing in tap water followed by washing in distilled water.

Medium and cultural conditions. Medium free of inorganic phosphate was buffered with 10 mM sodium barbital (pH 7.2) and contained (per liter): KCl, 0.20 g; (NH₄)₂SO₄, 0.50 g; NaCl, 0.10 g; MgSO₄·7H₂O, 0.2 g; CaCl₂·H₂O, 50 mg; FeCl₃·6H₂O, 20 mg; and 0.2 g each of glucose, glycerol, and sodium succinate (1). The basal medium and the insecticides were sterilized by filtration through sterile Nalgene filter units (Nalge-Sybron Corp., Rochester, N.Y.) containing 0.2-μm membrane filters. Cultures in screw-cap tubes (16 by 100 mm) were incubated at 29°C without agitation. Growth curves and kinetics of substrate utilization were established with cultures incubated at 29°C in 125-ml baffled Erlenmeyer flasks on a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 150 rpm. The cells were harvested by centrifugation at 15,000 × *g* and 4°C for 30 min.

Isolation of bacteria. Enrichment cultures were used to obtain isolates able to utilize a given organophosphate as the sole source of phosphorus. When provided as the sole phosphorus source, the organophosphate was present at a concentration of 0.2 mM. The organophosphates were each prepared with 10 ml of 95% ethanol. The enrichment culture (final volume, 3.0 ml) received either 0.5 ml of municipal sewage collected from the primary settling tank and utilized within 30 min of sampling or 0.5 g of soil. When growth greater than that in organophosphate-free solutions was observed (usually after 1 to 3 days), the enrichments were subcultured into fresh medium. After three successive transfers, enrichments using organophosphates as the sole phosphorus sources were streaked on nutrient agar plates (Difco Laboratories, Detroit, Mich.). Isolates that were able to utilize the organophosphates were subsequently identified by their growth in selective liquid medium.

Preparation of resting cell suspensions. Cells grown to the early stationary phase were harvested, washed three times with sodium barbital buffer (10 mM, pH 7.2), and suspended in the same buffer to an optical density of 1.5 at 420 nm. To 5-ml portions of resting cell suspensions in 25-ml Erlenmeyer flasks was added 30 to 40 μg of diazinon or malathion per ml. Chloramphenicol (100 μg/ml) was used to inhibit protein synthesis. At intervals, 0.5-ml portions were removed for extraction and analysis.

Preparation of cell-free extracts. Cells were grown to the early stationary phase, harvested, and washed in the barbital buffer. The pellet was suspended to about 5 mg (wet weight) per ml of the buffer. Extracts were prepared by sonic disruption at 4°C by using the microtip of a model W185D cell

disruptor (Heat Systems-Ultrasonic, Inc., Plainview, N.Y.) at 70% power in four 30-s periods, separated by 30-s cooling periods in ice. Debris and whole cells were removed by centrifugation at 15,000 × *g* and 4°C for 30 min.

Enzyme assay. The reaction mixture was assayed at 29°C. The complete reaction mixture contained, in 5.0 ml: 10 mM sodium barbital buffer (pH 7.5); KCl, 50 μmol; NaCl, 10 μmol; MgSO₄·7H₂O, 25 μmol; CaCl₂·2H₂O, 5.0 μmol; FeCl₃·6H₂O, 1.5 μmol; organophosphate, 0.1 mM; and 1.0 ml of extract containing 700 to 1,000 μg of protein. At intervals, 0.5-ml portions were removed for extraction and analysis. Sterile controls and boiled cell-free extract were used to determine abiotic disappearance of the test compound. Activity is reported as nanomoles of organophosphate destroyed per minute per milligram of protein.

Analytical methods. Turbidity was measured at 420 nm in 1-cm cuvettes in a Bausch and Lomb Spectronic 20 spectrophotometer. Inorganic phosphate was assayed by the method of Dick and Tabatabai (7), and none of the organophosphates or organic buffer interfered.

Protein was assayed at 660 nm by the method of Lowry et al. (12). Crystalline bovine serum albumin was used as the standard. The organic buffer did not interfere in the assay.

Organophosphate disappearance was measured with a Perkin-Elmer model 3920B gas-liquid chromatograph equipped with a flame ionization detector and a flame photometric detector fitted with phosphorus and sulfur filters. The packing material was 3% OV-17 on 100/120-mesh Gas-Chrom Q in a Teflon-lined stainless steel column (1.83 m by 2-mm ID; Applied Science Laboratory, State College, Pa.). The operating temperatures were as follows: 140, 200, and 230°C for the column; 190 and 245°C for the injector; and 250 and 275°C for the interface (detector). For the flame photometric and flame ionization detectors, the helium carrier gas flow rate was 30 ml/min. The organophosphate concentration was determined from standard curves of each compound.

To extract the organophosphates, portions of the cultures were mixed with equal volumes of pesticide-grade ethyl acetate (Fisher Scientific Co., Rochester, N.Y.), and the organic layer was removed. The procedure was repeated twice, and the organic phases were dried with anhydrous Na₂SO₄. Based on a standard concentration of each chemical, the two extractions removed more than 95% of the chemical substrate from the sample. Portions of the extract were injected into the gas chromatograph.

To determine water-soluble products of organophosphate breakdown, samples were treated with diazomethane by the method of Daughton et al. (5). The derivatized products were extracted twice with ethyl acetate and dried with anhydrous Na₂SO₄ over glass wool before gas chromatography. Derivatives of dimethyl and diethyl phosphate and thiophosphates were prepared similarly.

RESULTS

Enrichment cultures able to use the 12 organophosphates as sole phosphorus sources were

readily obtained from sewage and soil samples. None of the organophosphates could serve as a sole carbon source; i.e., there was not the expected increase in turbidity in a medium with 10 to 20 mg-atoms of pesticide carbon per liter. To determine the capacity of the isolates to use a variety of organophosphates as phosphorus sources, the bacteria were first grown in media containing the organophosphate on which they were isolated. These cultures were then inoculated into tubes containing 0.2 mM organophosphate in the basal medium. Growth was measured turbidimetrically and compared with that in solutions with no added phosphorus. A summary of the results of a study to show the ability of 12 bacterial isolates to grow on a variety of organophosphorus insecticides is given in Table 1. Organisms isolated from diazinon and malathion enrichments were the most versatile, able to metabolize 10 and 9 compounds, respectively. Azodrin, Dasanit, malathion, and Orthene were the most widely used, whereas Trithion and Vapona were the least frequently used.

The two organisms (both from soil) obtained from diazinon and malathion enrichments were characterized by the procedures described by Skerman (15). The isolates corresponded to the description of species of *Pseudomonas* (4) in being strictly aerobic, gram-negative, motile rods that bore polar flagella, produced catalase, were oxidase positive, had a respiratory metabolism, and had simple growth requirements. The presence of fluorescent pigmentation and arginine

dihydrolase, lack of poly- β -hydroxybutyrate accumulation, and growth at 4 but not at 41°C suggested that the organism isolated from diazinon enrichments was *Pseudomonas putida*, but because extensive taxonomic tests were not performed, the bacterium was designated *Pseudomonas* 28. The organism isolated from malathion enrichments was designated *Pseudomonas* 7.

To determine the optimum phosphorus concentration for growth, *Pseudomonas* 28 was grown in basal medium with various concentrations of inorganic phosphate, diazinon, or malathion. The extent of growth, as measured by turbidity, was linearly related to the concentration of phosphorus, with maximum growth occurring at 0.15 mM (Fig. 1). At this concentration, the molar ratio of carbon to phosphorus in the medium was 15:1 and approximated the C:P molar ratio of bacteria (13). The specific growth rate, which varied with the phosphorus source, was highest for KH_2PO_4 . Similar growth responses were obtained at temperatures of 20 to 45°C and pH values of 6.5 to 8.5. The growth responses and C:P molar ratios in the medium were similar with *Pseudomonas* 7.

To confirm that *Pseudomonas* 28 was utilizing the insecticides as phosphorus sources and not growing on contaminating inorganic phosphate, the organism was grown in basal medium amended with 0.20 mM diazinon (61 $\mu\text{g}/\text{ml}$) or malathion (66 $\mu\text{g}/\text{ml}$) as the sole phosphorus source. As Fig. 2 shows, growth of *Pseudomonas*

TABLE 1. Organophosphorus compounds used as phosphorus sources

Source of bacterial isolate		Compounds used as sole P source ^a
Common name	Chemical structure	
Aspon	<i>O,O,O,O</i> -tetra- <i>n</i> -propyl dithiopyrophosphate	As, Az, Da, Di, Dm, Ma, O, P
Azodrin	<i>O,O</i> -dimethyl <i>O</i> -(2-methylcarbamoyl-1-methylvinyl) phosphate	As, Az, Da, Di, Dm, Ma, Me, O
Dasanit	<i>O,O</i> -diethyl <i>O</i> -[4-(methylsulfinyl)phenyl] phosphorothioate	Az, Da, Dm, Dy, Ma, Me, O, V
Diazinon	<i>O,O</i> -diethyl <i>O</i> -(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate	As, Az, Da, Di, Dm, Dy, Ma, Me, O, P
Dimethoate	<i>O,O</i> -dimethyl <i>S</i> -(<i>N</i> -methylcarbamoylmethyl) phosphorodithioate	Az, Da, Dm, Ma
Dylox	Dimethyl(2,2,2-trichloro-1-hydroxyethyl) phosphonate	Az, Da, Di, Dm, Dy, Ma, O
Malathion	<i>O,O</i> -dimethyl <i>S</i> -(1,2-dicarbethoxyethyl) phosphorodithioate	As, Az, Da, Dm, Ma, Me, O, P, V
Methyl parathion	<i>O,O</i> -dimethyl <i>O</i> - <i>p</i> -nitrophenyl phosphorothioate	Az, Da, Di, Ma, Me, P
Orthene	<i>O,S</i> -dimethyl acetylphosphoramidothioate	As, Az, Da, Di, Ma, O
Parathion	<i>O,O</i> -diethyl <i>O</i> - <i>p</i> -nitrophenyl phosphorothioate	Da, Di, Dm, Dy, Ma, O, P
Trithion	<i>S</i> -[(<i>p</i> -chlorophenylthio)methyl] <i>O,O</i> -diethyl phosphorodithioate	Da, Di, Dm, Ma, T
Vapona	2,2-Dichlorovinyl <i>O,O</i> -dimethyl phosphate	Da, Dm, V

^a Abbreviations: As, Aspon; Az, Azodrin; Da, Dasanit; Di, diazinon; Dm, dimethoate; Dy, Dylox; Ma, malathion; Me, methyl parathion; T, Trithion; V, Vapona; O, Orthene; and P, parathion. If a compound is not listed, no turbidity increase compared with P-free solutions was evident.

FIG. 1. Growth of *Pseudomonas* 28 after 48 h with various concentrations of K_2HPO_4 , malathion, or diazinon provided as the sole phosphorus source.

28 measured as protein was correlated with insecticide disappearance. More than 75% of both diazinon and malathion disappeared by 84 h. During the growth cycle, the amount of phosphorus utilized for growth agreed closely with the amount needed for cell protein, assuming that approximately 2% of the protein is phosphorus (13). The growth of *Pseudomonas* 28 at the expense of the insecticides and the extent of growth could not be attributed to contaminating inorganic phosphate from diazinon, malathion, or other medium ingredients. Furthermore, colorimetric analyses of the organophosphates indicated no detectable inorganic phosphate, the method being sensitive to less than $1.0 \mu\text{g}$ of phosphate per ml. Similar results were obtained with *Pseudomonas* 7.

Pseudomonas 28 was grown for 36 h in media containing K_2HPO_4 , diazinon, or malathion as the sole phosphorus source. Resting cells (optical density, 1.5) were prepared from these cultures, and these cells were incubated on a rotary shaker for 24 h at 29°C with 30 to $40 \mu\text{g}$ of diazinon or malathion per ml in the presence or absence of chloramphenicol ($100 \mu\text{g}/\text{ml}$). The amount of substrate metabolized was determined by gas chromatography. It is evident from the data in Table 2 that only resting cells derived

from cultures grown on the insecticides metabolized diazinon and malathion to a significant extent. Cells derived from cultures grown on one of the insecticides metabolized the other. The values reported are corrected for substrate disappearance in the presence of cell suspensions boiled for 10 min, the values for which ranged from 2.2 to $4.1 \mu\text{g}$ of diazinon or malathion per ml in 24 h. Except in one instance, the percentage of breakdown was unaffected by the presence of chloramphenicol. Similar results were obtained with *Pseudomonas* 7. The data thus indicate that diazinon and malathion were metabolized by an induced enzyme system.

To assess the specificities of the crude enzyme preparations for various organophosphates, cell-

FIG. 2. Growth of *Pseudomonas* 28 and disappearance of organophosphorus insecticides. D and M refer to protein content of cells grown on diazinon and malathion, respectively.

TABLE 2. Metabolism of diazinon and malathion by resting-cell suspensions of *Pseudomonas* 28

P source for growth	Chloramphenicol	Amt of substrate metabolized ($\mu\text{g}/\text{ml}$)	
		Diazinon	Malathion
KH_2PO_4	+	0.2	0.2
	—	1.0	1.0
Diazinon	+	19	36
	—	18	22
Malathion	+	25	19
	—	25	18

free extracts of both organisms were incubated at 29°C with each of the 12 organophosphorus compounds at a 0.1 M concentration. The bacteria were grown on either diazinon or malathion, and the extracts contained 700 to 1,000 μ g of protein per ml. Extracts from both diazinon- and malathion-grown cells of both isolates catalyzed initial phases of the degradation of 8 of the 12 organophosphates (Table 3), but extracts of neither organism catalyzed the breakdown of dimethoate, Dylox, methyl parathion, or Vapona. The maximum activities reported are corrected for substrate disappearance in the presence of extracts boiled for 10 min, the values for which ranged from 2.4 to 8.6 nmol/min per mg of protein.

To determine whether ionic dialkyl phosphorus esters were formed by *Pseudomonas* 7 and *Pseudomonas* 28, products formed by these crude enzyme preparations (after 10 h with 0.10 mM substrate) were extracted with ethyl acetate, derivatized with diazomethane, and analyzed by gas chromatography. On the basis of a comparison with retention times for the derivatized standards, the metabolites of six of the organophosphorus compounds were tentatively identified. Both *Pseudomonas* 7 and *Pseudomonas* 28 liberated the following: (i) dimethyl phosphate from Azodrin; (ii) diethyl phosphorothioate from Dasanit, diazinon, and parathion; (iii) dimethyl phosphorodithioate from malathion; and (iv) diethyl phosphorodithioate from Trithion. The liberation of products tentatively identified as ionic dialkyl phosphorus and thiophosphorus esters suggests that a major pathway for organophosphate degradation in these bacteria is hydrolytic attack by a phosphatase

or phosphotriesterase to liberate the dialkyl phosphoester. Peaks with areas less than 10% of the areas of the major dialkyl phosphate peaks were observed, but these were not identified.

Since *Pseudomonas* 7 and *Pseudomonas* 28 liberated products that appeared to be dialkyl phosphate and thiophosphates, an experiment was conducted to determine whether the organisms could utilize various phosphoesters as sole phosphorus sources. The basal medium was amended with 0.2 mM phosphorus (a nonlimiting concentration) as trimethyl phosphate, triethyl phosphate, tri-*n*-butyl phosphate, dimethyl phosphate, dimethyl phosphorothioate, dimethyl phosphorodithioate, trimethyl phosphorothioate, diethyl phosphate, diethyl phosphorothioate, or triethyl phosphorothioate. Growth of *Pseudomonas* 28 occurred only with trimethyl, triethyl, and tri-*n*-butyl phosphates (Fig. 3). The maximum optical densities were between 1.0 and 1.2. Similar results were obtained with *Pseudomonas* 7.

TABLE 3. Maximum activity of cell-free extracts of *Pseudomonas* 7 and *Pseudomonas* 28 on various insecticides

Substrate	Maximum activity (nmol of substrate destroyed per min per mg of protein)			
	<i>Pseudomonas</i> 7		<i>Pseudomonas</i> 28	
	Diazinon	Malathion	Diazinon	Malathion
Aspon	62 ^a	110 ^a	44 ^a	88 ^a
Azodrin	92 ^a	108 ^a	121 ^a	133 ^a
Dasanit	238 ^b	212 ^b	205 ^b	210 ^b
Diazinon	283 ^c	250 ^c	301 ^c	263 ^c
Malathion	222 ^c	275 ^c	257 ^c	288 ^c
Orthene	52 ^a	41 ^a	37 ^a	32 ^a
Parathion	189 ^c	200 ^c	259 ^c	212 ^c
Trithion	142 ^b	125 ^b	138 ^b	117 ^b

^a At 10.0 h.

^b At 7.0 h.

^c At 3.5 h.

FIG. 3. Growth of *Pseudomonas* 28 in media with trimethyl, triethyl, or tri-*n*-butyl phosphate as the sole phosphorus source.

DISCUSSION

The results show that the two pseudomonads used the insecticides as sole sources of phosphorus and were not utilizing contaminant orthophosphate as the phosphorus source. The anomalous increase in protein concentration compared with insecticide disappearance between 24 and 45 h may be a result of the conversion of the substrate to an intermediate phosphorus-containing compound that the organisms can use for growth. The data also demonstrate that the breakdown of such chemicals resulted from an induced enzyme or enzyme system. The broad substrate specificity of the enzyme systems in the cell-free extracts is in line with the versatility of the organisms in using various organophosphates as phosphorus sources for growth. Cleavage of the organophosphates by the enzyme preparations was not prevented by alkyl substituents since methyl, ethyl, and propyl esters were metabolized. Nevertheless, substituents on the phosphorus atom did affect the rate of enzymatic action, an observation in agreement with previous reports (8, 9).

The inability of the bacteria to utilize the dialkyl phosphates, phosphorothioates, and phosphorodithioate as phosphorus sources is surprising since they could obtain phosphorus for growth from the insecticides. A possible explanation is the penetration of the nonionic insecticides into the cells and their metabolism within the cell to the ionic alkyl phosphorus compounds but the lack of permeability of the bacteria to the exogenously supplied ionic alkyl phosphorus compounds. This view is supported by the finding that the nonionic trialkyl phosphorus compounds are utilized.

It is not surprising that a single enzyme preparation acts on several pesticides. For example, Munnecke (14) found that a crude extract from a mixed bacterial culture growing on parathion had hydrolase activity not only for parathion but also for eight other pesticides. Similarly, Kearney and Kaufman (11) reported that a purified enzyme from a pseudomonad grown on isopropyl-*N*-(3-chlorophenyl)carbamate hydrolyzed several similar phenyl carbamates, as well as two acylanilide herbicides. In the present instance, the tentative identification of the products of cleavage as dimethyl phosphate, dimethyl phosphorodithioate, diethyl phosphorothioate, and diethyl phosphorodithioate suggests that the enzyme acts by catalyzing hydrolysis of the aryl P-O bond, a view supported by other investigators (8, 9).

The organophosphorus pesticides examined were chosen because they contained several alkyl moieties and because they are among the

most commonly used insecticides in the United States (16). Thus, the crude enzyme preparations used in this study and in another study (14) can hydrolyze chemicals that represent an important portion of the marketed insecticidal compounds. Moreover, the reactions may be a key step in the detoxication of many biologically active organophosphorus compounds and thus a major mechanism for destroying significant environmental toxicants.

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