Inhibitory Effect of Parathion on the Bacterial Degradation of EPTC†

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Effects of organophosphorus insecticides on the degradation of EPTC (*S*-ethyl dipropylthiocarbamate) by *Rhodococcus* TE1 isolated from herbicide-exposed soil were studied. Among the insecticides tested, parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate) was found to inhibit EPTC degradation greatly. This effect was mainly due to the inhibition of growth of the bacterial strain TE1 by parathion. Parathion also inhibited the growth of other *Rhodococcus* strains irrespective of their ability to degrade EPTC. *Rhodococcus* TE1 was also shown to degrade parathion, releasing its inhibitory effect on growth and EPTC degradation. Parathion degradation by TE1 resulted in the formation of paraoxon and *p*-nitrophenol. Paraoxon also inhibited the growth of TE1 and EPTC degradation. Parathion addition to soil treated with EPTC and butylate (*S*-ethyl diisobutylthiocarbamate) inhibited the degradation of the two herbicides.

INTRODUCTION

Degradation by microorganisms is considered to be a major factor determining the persistence of most pesticides in soil (McRae, 1989). Under certain conditions of repeated applications, soil-applied pesticides are rapidly degraded by microorganisms. This accelerated degradation of a pesticide in soil, generally referred to as "enhanced biodegradation", is due to microbial adaptation to the pesticide and may ultimately result in the loss of pesticide efficacy (Wilson, 1984; Gray and Joo, 1985; Kaufman et al., 1985; Roeth, 1986; Harvey et al., 1987).

The use of microbial or enzyme inhibitors to control accelerated biodegradation of a pesticide, thereby effectively extending soil persistence of the chemical, has been reviewed (Kaufman, 1987; MacRae, 1989). For example, dietholate (*O,O*-diethyl *O*-phenyl phosphorothioate) has been used as an extender for thiocarbamate herbicides in soil (Roeth et al., 1989). It has been suggested that dietholate exerts this effect through inhibition of soil microbiological activity (Skipper et al., 1986). Structurally, dietholate resembles several organophosphorus (OP) insecticides that are often simultaneously or successively applied with certain herbicides in agricultural practices. It is likely that soil persistence of a herbicide can be altered when it is applied in combination with certain insecticides (Tu and Miles, 1976; Reddy et al., 1984; Felsot, 1989).

In a previous study we demonstrated the inhibitory effect of dietholate on the degradation of certain thiocarbamate herbicides by an EPTC (*S*-ethyl dipropylthiocarbamate)-degrading *Rhodococcus* bacterial strain, TE1 (Tam et al., 1988). The present study is a continuation of our investigations and reports on the inhibitory effects of certain OP insecticides on the degradation of EPTC by *Rhodococcus* TE1 reported previously (Tam et al., 1987).

MATERIALS AND METHODS

Bacterial Strains. The following bacteria were used: EPTC-degrading *Rhodococcus* TE1 isolated from EPTC-exposed soil and an EPTC degradation deficient mutant of TE1, designated TE3 as described earlier (Tam et al., 1987). They were grown in a basal salts medium, BMN (Behki and Khan, 1986), supplemented with 1 mg/mL glycerol. *Escherichia coli* K-12, *Pseudomonas putida* strain PAW1, *Rhodococcus erythropolis* (ATCC 15963), and *Arthrobacter oxydans* were grown as reported previously (Tam et al., 1988). A *Flavobacterium* strain was grown on glutamate in a mineral salts medium as described by Topp et al. (1988).

Chemicals. The thiocarbamate herbicides EPTC and butylate (*S*-ethyl diisobutylthiocarbamate) were supplied by Stauffer Chemicals, Richmond, CA, and were 95+% pure. Analytical grade OP insecticides included parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate), malathion [(diethyl [(dimethoxyphosphinothioyl)thio]succinate)], phorate (*O,O*-diethyl *S*-ethylthiomethyl phosphorodithioate), fonofos [*O*-ethyl *S*-phenyl (*R,S*)-ethylphosphonodithioate], and isofenphos [isopropyl *O*-[[ethoxy (isopropylamino)]phosphinothioyl]salicylate]. Stock solutions (40 mM) of each of the OP insecticides were made in methanol, filter-sterilized, and stored in the cold. Paraoxon and *p*-nitrophenol were obtained from Aldrich Chemical Co.

Soil. The soil used in this study was a clay loam soil from a corn field (Brandon, MB) exposed to four successive annual applications of the herbicide EPTC and was the source for isolate TE1 as described in an earlier publication (Tam et al., 1987). The soil has a neutral pH and organic carbon content of 1.8%.

Effect of OP Insecticides on the Degradation of EPTC by *Rhodococcus* TE1. Ten-milliliter cultures of *Rhodococcus* TE1 in BMN–glycerol as described earlier (Tam et al., 1987) were incubated with shaking at 29 °C with 200 μM EPTC in the presence of 40 μM of the test OP insecticides. The controls consisted of 10 mL of uninoculated BMN–glycerol medium containing EPTC and the OP insecticide and were incubated simultaneously under the same experimental conditions. Aliquots (10 mL) of TE1 incubated with EPTC alone contained methanol equivalents which were used as carrier for adding OP insecticides in the sample. At various intervals during the incubation period, 2-mL samples were withdrawn and centrifuged, and 1 mL of the cell-free supernatant was extracted with 2 mL of hexane. The hexane extracts were analyzed by gas chromatography (GC) as described later.

Effect of Parathion on the Growth of Various Bacterial Species. The bacterial cultures were adjusted to an initial O.D. (600 nm) of 0.07–0.11. Aliquots (10 mL) of these cultures were incubated with shaking in a gyrotory shaker at 29 °C with EPTC and/or parathion as described above. The O.D. of the cultures was monitored spectrophotometrically after 24 and 48 h of incubation. Viable cell counts were determined by serial dilution of the bacterial cultures and plating in triplicate on nutrient agar medium. Colonies were counted after incubation for 30 h at 29 °C.

Degradation of Parathion by TE1 Cells. Aliquots of bacterial culture (10 mL) were incubated with shaking at 29 °C with parathion (40 μM). The control consisted of uninoculated basal salt medium (10 mL) containing parathion and was incubated simultaneously under the same conditions. Samples were withdrawn at different time intervals and centrifuged, and

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Table I. Effect of Various Organophosphorus Insecticides on EPTC Degradation by *Rhodococcus* TE1[a]

addition	EPTC degraded,[b] %	
	24 h	48 h
control[c]	100	
parathion	9.7	15.8
fonofos	10.8	59.8
isofenphos	16.5	83.5
malathion	100	
phorate	100	

[a] Optical density at 0 time = 0.1 (600 nm). [b] Average of duplicate samples. [c] Control contained EPTC (200 μM) plus methanol equal to that used as carrier with the insecticides.

the cell-free supernatant culture medium was extracted with hexane and analyzed by GC as described below. A portion of the extract in hexane was evaporated to just dryness, dissolved in methanol, and derivatized with diazomethane (Yip and Howard, 1968) before analysis by GC.

Gas Chromatography (GC). A Varian Model 3700 gas chromatograph equipped with a thermionic specific detector was used. A 15-m × 0.5-mm silica Megabore column coated with DB-5 (1.5 μm) was operated at 120 °C. Detector and injector port temperatures were maintained at 275 and 170 °C, respectively. The nitrogen carrier flow was 20 mL/min. Under these conditions, EPTC, butylate, fonofos, parathion, paraoxon, and methylated *p*-nitrophenol gave peaks with retention times of 3.8, 5.6, 13.8, 18.2, 16.8, and 4.3 min, respectively. The identity of the compounds was confirmed by comparison of retention times with those of authentic samples, by cochromatography, and finally by gas chromatography–mass spectrometry. A mass spectrometer Model VGZAB-2F connected to a Varian Model 3700 GC was used. The mass spectra were recorded at 70 eV.

RESULTS AND DISCUSSION

Effect of OP Insecticides on the Degradation of EPTC by TE1. Parathion, fonofos, and isofenphos inhibited EPTC degradation by TE1 initially as shown in Table I. However, the inhibitory effect by fonofos and isofenphos was greatly diminished after 24 h. Addition of malathion and phorate had apparently no inhibitory effect on EPTC degradation, the degradation being similar to that in the control. Parathion was the most potent inhibitor of EPTC degradation by TE1, and its inhibitory effect persisted even after 48 h.

It became of interest to determine whether parathion could also inhibit degradation of other thiocarbamate herbicides. It was observed that the herbicide butylate (200 μM) was completely degraded by TEI in 24 h under the experimental conditions used in this study. Addition of parathion (40 μM) to the incubation mixture containing butylate (200 μM) resulted in severe inhibition as indicated by 7.8%, 15.5%, and 23.9% degradation of the herbicide after 24, 48, and 72 h, respectively.

In a separate experiment degradation of EPTC and butylate was monitored in soil suspensions containing 200 μM EPTC or butylate with and without the addition of 40 μM parathion. The data (Table II) show considerable inhibition of EPTC and butylate degradation in the presence of parathion. These observations further confirm the inhibitory effect of parathion on the degradation of the two thiocarbamate herbicides.

It was observed during the incubation experiments that TE1 cultures containing parathion, fonofos, and isofenphos did not show a visible increase in turbidity for 24 h. However, at the end of 48 h cultures containing fonofos and isofenphos had grown substantially, while very little growth was observed in the culture containing parathion (Figure 1). This indicated that the inhibition of EPTC degradation by parathion may result from inhibition of

Table II. Degradation of EPTC and Butylate by EPTC-Adapted Soil with and without the Addition of Parathion

additions, μg/mL	% degradation[a]	
	48 h	120 h
EPTC	38.5	100
EPTC + PAR	23.2	43.0
butylate	62.1	100
butylate + PAR	27.6	59.0
EPTC control (no soil)	4.7	8.9
butylate control (no soil)	3.7	8.1

[a] Two grams of soil suspended in 10 mL of BMN medium was incubated with 200 μM herbicides ± 40 μM parathion. Average of duplicate soil samples.

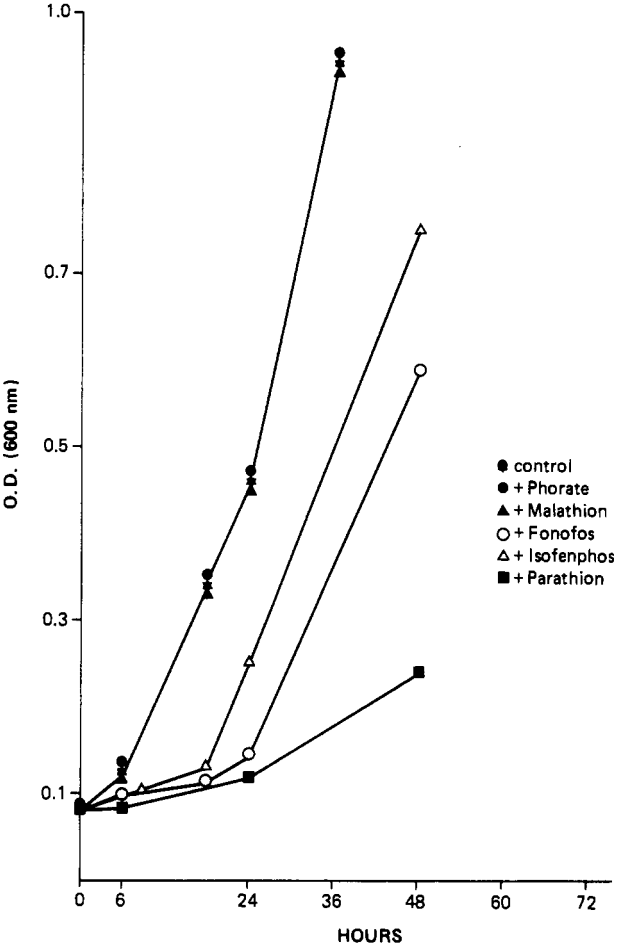

Figure 1. Effects of organophosphorus insecticides (40 μM) on the growth of TE1.

cell growth similar to our earlier findings with dietholate (Tam et al., 1988).

The effects of parathion concentration and time of incubation on TE1 growth and EPTC degradation were further investigated. It was observed that the effects of parathion on growth of TE1 and degradation of EPTC were dependent upon parathion concentration (Figure 2). Thus, at a concentration of 20 μM, the inhibitory effect of parathion was small. However, at higher concentrations, 40 or 60 μM, the inhibition of cell growth and EPTC degradation was severe and persisted for an extended period (Figure 2). The cultures containing 40 and 60 μM parathion resumed normal growth after about 110 and 140 h, respectively. At this time, degradation of EPTC resumed at the rate observed for samples containing no parathion (Figure 2).

It was observed that the inhibitory effects of parathion on growth of TE1 and degradation of EPTC were almost

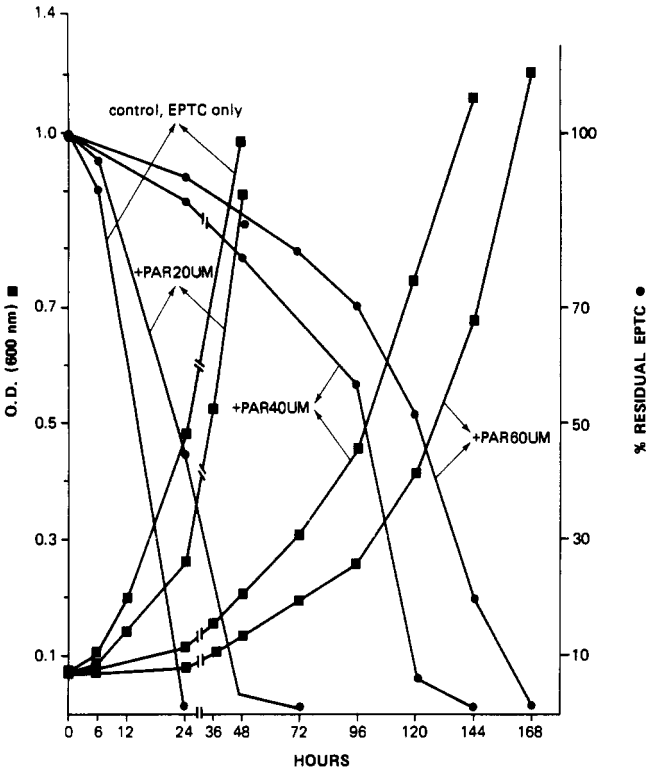

Figure 2. Effects of 20, 40, and 60 μM parathion (PAR) on the growth of TE1 and degradation of EPTC (200 μM).

Table III. Effect of Parathion (40 μM) on the Growth of Various Bacterial Species

	O.D. (600 nm) at time				
		24 h		48 h	
bacterial cultures[a]	0 h	-PAR	+PAR	-PAR	+PAR
P. putida	0.08	1.39	1.32	ND[b]	ND
Flavobacterium strain	0.07	0.48	0.49	1.26	1.25
E. coli K12	0.11	2.1	2.2	ND	ND
Rhodococcus TE1	0.08	0.44	0.16	1.51	0.24
Rhodococcus TE3	0.10	0.37	0.15	1.21	0.25
A. oxydans	0.10	1.53	1.39	ND	ND
R. erythropolis	0.11	0.87	0.18	1.84	0.32

[a] The cultures without parathion contained methanol equal to that in parathion cultures. [b] ND, not determined.

immediate. When parathion (40 μM) was added to TE1 cultures incubated with 200 μM EPTC at 0, 3, and 6 h, very little increase in O.D. (600 nm) of the cultures was noted. Furthermore, the amount of EPTC degraded during the 6-h period after parathion addition was only one-third to one-fourth of that degraded in cultures without parathion. The inhibitory effects of parathion also appeared to be reversible. It was observed that the removal of parathion from the incubation mixture after centrifugation resulted in EPTC degradation and growth of TE1 cells at a normal rate by the resuspended cells.

Effect of Parathion on the Growth of Various Bacterial Species. Parathion (40 μM) reduced the viable cell numbers in TE1 culture by about 10% during the initial 6 h. This was followed by a slow increase in viable cell count after 16 h, indicating that parathion exerted a bacteriostatic effect on TE1.

Although parathion markedly inhibited the growth of TE1 and the inhibition persisted for an extended period, parathion was not found to inhibit growth of *P. putida*, *E. coli*, *Flavobacterium*, or *A. oxydans* (Table III). However, parathion severely inhibited the growth of TE1, TE3, and *R. erythropolis*. The latter two strains do not degrade EPTC. The inhibitory effect of parathion on bacterial growth, therefore, appears to be species-specific but not related to the ability of the strains to degrade EPTC.

Table IV. Degradation of Parathion to Paraoxon by TE1 Cells[a]

	amount, % in the incubation mixture	
time, days	parathion	paraoxon[b]
0	100	0
1	93.2	1.7
2	81.6	4.9
3	70.2	15.0
5	31.7	22.2
7	7.4	16.3

[a] TE1 cells (O.D. of 0.4 at 600 nm) were incubated with parathion (40 μM). [b] Calculations are based on the assumption that an equimolar amount of paraoxon was formed from parathion.

Degradation of Parathion by TE1 Cells. The inhibition of TE1 growth and EPTC degradation by parathion was slowly diminished with time. At a cell density of 0.5 (600 nm) the severe inhibition lasted for about 50–60 h, whereas it lasted for about 110 h at an initial O.D. of 0.1. These suggested the possibility that parathion was being degraded by TE1 cells. To confirm these observations, the degradation of 40 μM parathion by cells of both TE1 and TE3 at an initial cell O.D. of 0.5 in BMN-glycerol medium was investigated. There was little degradation of parathion (8–10%) during the first 2 days by either culture. Subsequently, parathion was degraded by TEI and TE3 at a faster rate. Parathion degradation by TE1 averaged 27%, 46%, and 66% by the end of third, fifth, and seventh days, respectively. Similar results were obtained with TE3 cells with 21%, 35%, and 52% degradation after 3, 5, and 7 days, respectively. The results suggest that the 50.5 MDa plasmid present in TE1 and shown to be associated with EPTC degradation (Tam et al., 1987) was not involved in parathion degradation. This plasmid was missing in the mutant TE3 (Tam et al., 1987).

The degradation of parathion in soil and by bacteria has been extensively studied (Barik, 1984; Racke and Coates, 1987; MacRae, 1989). Microbial metabolism of parathion primarily involves hydrolysis to diethylthiophosphoric acid and *p*-nitrophenol. A secondary pathway involves the oxidation of parathion to paraoxon. Paraoxon has been reported to be short-lived in the environment and is hydrolyzed to diethylphosphoric acid and *p*-nitrophenol (Fuhremann and Lichtenstein, 1980). Microbial degradation of paraoxon and *p*-nitrophenol has also been reported (Munnecke and Hsieh, 1976; Adhya et al., 1981). To determine the pathway of parathion degradation by TE1, samples withdrawn at various time intervals from a culture of the bacteria incubated with 40 μM parathion were analyzed by GC as described earlier. A peak at a retention time (Rt) of 16.8 min was observed which was identical with that of authentic paraoxon. The peak height increased with a decrease in the amount of parathion in the culture. The GC–Ms of this peak (Rt = 16.8 min) showed a molecular ion at *m/z* 275 and a base peak at *m/z* 109. The spectrum also showed other ions at *m/z* 127, 99, and 81 which were relatively weak. The mass spectrum of this compound and fragmentation pattern were analogous to those of authentic paraoxon. The data in Table IV show the production of paraoxon from parathion by TEI cells. The progressive accumulation of paraoxon in the culture did not correspond quantitatively to the decrease in the amount of parathion, thereby indicating that paraoxon formed was being further degraded. The methylated extracts of the samples described above showed

Table V. Effect of Paraoxon and *p*-Nitrophenol on Growth and EPTC Degradation by TE1

additions	O.D. (600 nm) 0 h	8 h	24 h	% EPTC degraded 24 h
EPTC, 200 μM	0.11	0.34	1.21	100
EPTC + paraoxon, 40 μM	0.11	0.20	0.66	38
EPTC + paraoxon, 80 μM	0.11	0.16	0.30	20
EPTC + *p*-nitrophenol, 40 μM	0.11	0.24	1.32	100
EPTC + *p*-nitrophenol, 80 μM	0.11	0.20	1.27	100

a major peak in the gas chromatograms at Rt of 4.3 min, which was identical with the Rt of methoxy derivative of *p*-nitrophenol. The identity of this compound was further established by GC–MS. The mass spectrum represented by GC peak at Rt = 4.3 min exhibited a molecular ion at *m/z* 153, which decomposed to give ions at *m/z* 137, 123, 107, and 92 with further fragmentation analogous to that observed with the authentic methoxy derivative of *p*-nitrophenol. Thus, it is evident from the results that TEI cells degrade parathion to paraoxon and then to *p*-nitrophenol.

When cultures of TEI were incubated separately with paraoxon and *p*-nitrophenol at two concentrations, paraoxon but not *p*-nitrophenol inhibited the growth and degradation of EPTC by TEI (Table V). It appears that, in addition to the inhibitory effect of parathion, one of the degradation products, paraoxon, also contributed to the inhibition of the growth and EPTC degradation by TEI (Tables III and IV).

This study shows that parathion and one of its metabolites, paraoxon, inhibited EPTC degradation by *Rhodococcus* isolate TE1. The inhibitory effect lasted until the compounds were degraded to very low concentrations by TEI. The inhibitory effect of parathion on the bacterial growth was found to be species-specific irrespective of the capability of the bacteria to degrade EPTC. Current work in our laboratory has shown that fonofos and isofenphos also inhibited EPTC degradation by the bacteria, but the effect was relatively short-lived because of the rapid rate of degradation of these insecticides by TEI. Our results suggest that the persistence of EPTC and butylate could be substantially increased when they are used in combination with parathion. The significance of these results may have implications in preventing or at least slowing down biodegradation of thiocarbamate herbicides in the field. Thus, application of a proper combination of certain pesticides may show considerable promise for controlling residual activity of biodegradable pesticides.

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Registry No. EPTC, 759-94-4; parathion, 56-38-2; fonofos, 944-22-9; isofenphos, 25311-71-1; butylate, 2008-41-5; paraoxon, 311-45-5; *p*-nitrophenol, 100-02-7.