Phosphate and Soil Binding: Factors Limiting Bacterial Degradation of Ionic Phosphorus-Containing Pesticide Metabolites

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Soils that had a high binding capacity for inorganic orthophosphate (P_i) had reduced capacities to bind ionic alkyl phosphorus compounds. Only ionic methylphosphonate (MPn) and ionic phenylphosphonate exhibited moderate binding. *Pseudomonas testosteroni* used either MPn or P_i as a sole phosphorus source and exhibited diauxic utilization of MPn and P_i. The utilization of MPn was suppressed in the presence of P_i. This suppression was abolished by a P_i-binding soil. The soil did not have a significant effect on the maximum rate of degradation of either MPn or the poorly bound ionic *O*-isopropyl methylphosphonate, whereas the amount of MPn (but not the amount of *O*-isopropyl methylphosphonate) metabolized was reduced in the presence of soil.

The microbial degradation of synthetic chemicals of environmental importance is frequently studied in solution under conditions far different from those in nature. However, abiotic factors in natural ecosystems may have a marked influence on the rate and extent of degradation and sometimes even the products and pathways of decomposition. These factors may be of considerable importance to the microbial decomposition of ionic alkyl phosphorus compounds, which are potentially significant in natural environments because of their widespread use, possible toxicity, and stability to physical and chemical modification (3, 6, 7).

Recent studies have demonstrated that phosphorus-containing products generated from phosphonate toxicants, although chemically stable, are metabolized by bacteria in culture, and the C-P bond of the molecules is thereby cleaved (3; C. G. Daughton, A. M. Cook, and M. Alexander, FEMS Microbiol. Lett., in press). Environmental factors that may affect this process include the retention of the substrate by soil constituents (5, 10–12); nevertheless, some alkyl phosphates apparently bind poorly to soil (14). The presence of inorganic orthophosphate (P_i) in soils or waters may also affect the microbial transformation because P_i is reported to suppress the metabolism of dihydrogen 2-aminoethylphosphonate (15, 16) and to delay the onset of mineralization of biological phosphate esters in soil (5).

The present investigation was designed to measure the retention of ionic alkyl phosphorus compounds by soil, to study the influence of such retention on the utilization of these compounds, and to establish the effect of P_i on their metabolism.

MATERIALS AND METHODS

Materials. The alkyl phosphorus compounds used and their sources and abbreviations are listed in Table 1. All other chemicals were of the highest purity available commercially. Also given in Table 1 are representative pesticides and other commercially important chemicals that, after initial hydrolysis, could serve as sources for the alkyl phosphorus compounds studied.

The soils were air-dried and screened to pass a 2-mm sieve. The surface 5- to 13-cm portion of an Adirondack Spodosol from Blue Mountain, N.Y., that was used contained 35% organic matter and had a pH value of 3.9. The Nipe (0 to 20 cm), Humatas (130 to 150 cm), and Mabi (30 to 90 cm) tropical clays have been described (17). Lima-Honeoye silt loam (0 to 15 cm in depth) from Aurora, N.Y., contained 5.5% organic matter and had a pH value of 6.5. The bacterium used was isolate no. 11 (3), which was identified as a strain of *Pseudomonas testosteroni*.

All glassware was washed in 5 M nitric acid to remove contaminative phosphorus compounds (3).

Binding of phosphorus-containing compounds by soil. Each phosphorus compound in 5.0 ml of distilled water (unless otherwise indicated) was added to soil (1.0 g, dry wt) contained in a 30-ml screw-cap glass vial, and the mixture was agitated for 2 h on a wrist-action shaker. Total free phosphorus, free P_i, or both were determined for filtrates of samples (passed through 0.2-μm-pore-diameter membranes) obtained before addition of each phosphorus-containing compound to soil and after the 2-h equilibration with soil. The amount of phosphorus compound bound was determined as the difference between the initial and

TABLE 1. *Phosphorus-containing breakdown products and representative potential sources*

Abbreviation	Compound	Representative potential source
DETP	O,O-diethyl hydrogen thiophosphate[a]	Parathion, disulfoton, Diazinon, Dursban, Thimet
DMTP	O,O-dimethyl hydrogen thiophosphate[a]	Methyl parathion, malathion, dimethoate, Guthion
DEDTP	O,O-diethyl hydrogen dithiophosphate[a]	Thimet, disulfoton
DMDTP	O,O-dimethyl hydrogen dithiophosphate[a]	Malathion, dimethoate, Guthion
DEP	O,O-diethyl hydrogen phosphate[b]	Parathion, TEPP
MϕTPn	O-methyl hydrogen phenylphosphonothioate[c]	Phosvel
PMPn	O-pinacolyl hydrogen methylphosphonate[d]	Soman
IMPn	O-isopropyl hydrogen methylphosphonate[d]	Sarin
MϕPn	O-methyl hydrogen phenylphosphonate[e]	Phosvel
ϕPn	Dihydrogen phenylphosphonate[e]	Cyanophenphos, Phosvel, EPN, EPBP, Inezin
MPn	Dihydrogen methylphosphonate[d]	Sarin, soman, Colep, flame retardants (Antiblaze 19, Fyrol 76)

[a] Potassium salt. From American Cyanamid Co., Princeton, N.J.
[b] Eastman Organic Chemical Division, Rochester, N.Y.
[c] Potassium salt. From Velsicol Chemical Corp., Chicago, Ill.
[d] Chemical Systems Laboratory, Aberdeen Proving Ground, Md.
[e] Velsicol Chemical Corp.

final nonbound values, minus a soil blank value (for example, equivalent to 121 nmol of extractable phosphorus per g of Spodosol). In no instance was P_i found to be liberated from the soils during equilibration.

Quantification of phosphorus utilization and concomitant growth. Cultures were grown on a shaker at 29°C in 30 ml of medium contained in 250-ml Erlenmeyer flasks. The medium contained a limiting concentration (0.2 mM) of the phosphorus source, 15 mM p-hydroxybenzoate, 2.7 mM KCl, 0.80 mM $MgSO_4$, 40 mM NH_4Cl, trace elements (13), and it was buffered at pH 7.4 with 50 mM Tris [tris(hydroxymethyl)methylamine]. In preparing the medium, the sterile phosphorus source was added aseptically to the autoclaved phosphorus-free medium. These cultures were then inoculated into 300 or 600 ml of homologous medium contained in 1.0-liter side-arm flasks, and these larger volumes were incubated at 30°C, aerated at 50 ml/min, and mixed by magnetically driven stirring bars (9). Cells in the late logarithmic phase were collected by centrifugation at 20,000 × g at 4°C in sterile tubes, and cells suspended in sterile buffered salts medium were used as inocula for growth experiments. Samples were taken at intervals for turbidity and protein measurement (3) and,

after passage of the liquid through 0.2-μm-pore-diameter membrane filters, for extracellular P_i and total phosphorus measurement (2).

Effect of soil on MPn utilization. The stoichiometric release of the phosphonyl-methyl group of MPn and IMPn as methane by *P. testosteroni* has been described (Daughton et al., in press). The utilization of MPn and IMPn was followed by quantifying the yield of methane in the headspace of 250-ml screw-cap bottles sealed with Mininert valves. The inoculum was prepared as described above. Each bottle contained 30 ml of p-hydroxybenzoate–salts medium buffered with 50 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) and one of the following treatment combinations: inoculum or no inoculum of P_i-grown *P. testosteroni* (0.06 mg of protein), MPn and/or P_i, or IMPn (0.1 mM unless otherwise indicated) and Spodosol (0, 0.6, 1.2, or 2.0 g). HEPES was substituted for Tris because of the acidity of the Spodosol. The suspensions were shaken at 30°C, and 200-μl headspace samples were periodically withdrawn for determination of methane by gas chromatography using a flame ionization detector (Daughton et al., in press).

RESULTS

Isolation and identification of phosphonate-utilizing bacterium. By using the methods described and cited by Cook et al. (3), a bacterium was isolated from sewage that was capable of using alkylphosphonates as sole phosphorus sources. This organism was a gram-negative motile rod with polar multitrichous flagellation. It accumulated poly-β-hydroxybutyrate, was oxidase positive, and effected a *meta* cleavage of diphenols. It had no fluorescent or phenazine pigments, methionine requirement, arginine dihydrolase, or capacity for denitrification or growth at 4 or 41°C. The bacterium grew with adipate, suberate, azelaate, sebacate, m-hydroxybenzoate, benzoylformate, benzoate, and testosterone but not L-arabinose, D-glucose, D-galactose, pelargonate, putrescine, D-fructose, mannitol, malonate, maleate, quinate, phenylacetate, DL-tryptophan, β-alanine, DL-α-aminobutyrate, γ-aminobutyrate, and acetamide as sole carbon sources. Based on these findings, the organism was identified as *P. testosteroni*. This is the only organism for which the utilization of alkylphosphonates has been demonstrated (4).

Binding of alkyl phosphorus acids to soils. The Spodosol had a large capacity to bind P_i (Table 2). At low concentrations (0.51 to 5.2 μmol in 5 ml per g of soil), P_i was removed from solution almost completely. At the high concentration (280 μmol in 15 ml per g of soil), 34% of the P_i was removed from solution, representing 95 μmol per g of soil. In contrast, none of the dialkoxy compounds was retained by the soil, and except for ϕPn and MPn, the binding of the

TABLE 2. *Binding of P_i and alkyl phosphorus compounds to a Spodosol*

Compound	P initially present in aqueous phase (μmol)	P not bound (%)
P_i	0.51	0.6
P_i	1.6	0.6
P_i	5.2	0.6
P_i	15.0[a]	5.6
P_i	280[a]	66
DETP	4.9	103
DMTP	4.5	101
DEDTP	4.5	97
DMDTP	4.9	94
DEP	4.9	92
MϕTPn	4.5	89
PMPn	5.3	68
IMPn	4.8	58
MϕPn	5.0	54
ϕPn	4.8	8.6
MPn	4.2	4.6

[a] 15 ml of water.

alkyl phosphorus acids was almost nil or much less than that of P_i.

Three tropical soils and a calcareous New York soil (Lima-Honeoye silt loam) were tested for their abilities to bind P_i, MPn, and DEDTP (Table 3). The P_i that was not bound by the tropical soils varied from 0.3% for Humatas clay to 4.5% for Mabi clay. The calcareous Lima-Honeoye soil showed a much lower phosphate retention capacity and a parallel inability to bind MPn. In comparison, the amount of unbound MPn in the tropical soils was 6- to 10-fold greater than the amount of unbound P_i (Table 3), a ratio similar to that found previously for the Spodosol. DEDTP was not bound to a detectable or significant degree by any soil tested.

Since MPn was one of only two phosphorus compounds extensively bound by the Spodosol, it was chosen as a model substrate to determine the effect of the Spodosol on the utilization of a bound phosphorus compound by *P. testosteroni*. IMPn was not retained to the same extent, and, as an analog of MPn, it was used as a control that, by comparison, would indicate an effect of the Spodosol on MPn utilization caused by the binding.

Effect of P_i on MPn utilization. To determine the effect of P_i on the utilization of MPn by MPn-induced *P. testosteroni*, cells were inoculated into Tris-buffered salts medium (as described in Materials and Methods) containing P_i and/or MPn as sole phosphorus source. The cells grew rapidly when inoculated into a medium containing MPn as sole phosphorus source (Fig. 1). The specific growth rate on MPn rose to 0.47 h^{-1} and was maintained until about 5.5

h, at which time the MPn was exhausted (as determined by assay of culture filtrates) and the rate of increase in turbidity fell. During the first 5 h, the increase in turbidity and protein level paralleled MPn utilization. Throughout the experiment, protein concentration was a linear function of turbidity, indicating that a carbon-containing storage polymer was not accumulated.

The growth pattern of *P. testosteroni* with P_i as phosphorus source was similar to that on MPn, with a maximum specific growth rate of 0.54 h^{-1} (Fig. 1). During exponential growth,

TABLE 3. *Retention of three phosphorus compounds by various soils*

Soil	P not bound (%)[a]		
	P_i	MPn	DEDTP
Humatas clay	0.3	2.9	96
Spodosol	0.6	4.6	97
Nipe clay	0.8	ND[b]	105
Mabi clay	4.5	28	107
Lima-Honeoye silt loam	77	89	100

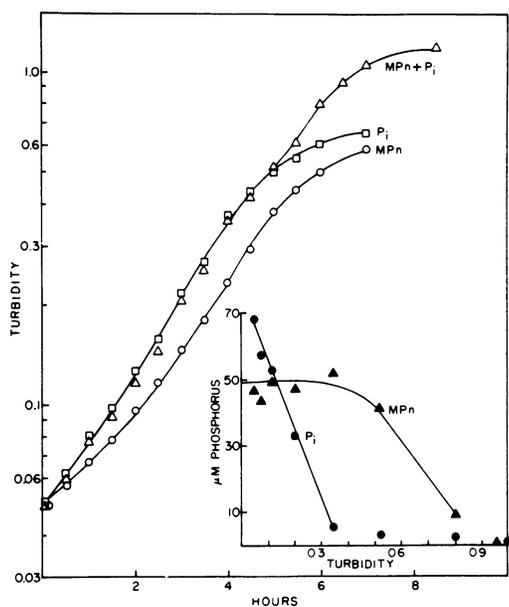[a] 5.0 μmol of the phosphorus compound added to the soil suspension.

[b] Not determined.

FIG. 1. *Growth of P. testosteroni and utilization of limiting phosphorus source. The medium contained 50 μM MPn, 50 μM P_i, or a mixture of 50 μM P_i and 50 μM MPn. Inset shows P_i and MPn concentrations in the culture filtrate corresponding to growth curve when the cells were provided both compounds. Turbidity was determined at 500 nm.*

phosphorus utilization was a linear function of turbidity, the decrease in P_i concentration in the culture filtrate being 216 times the change in turbidity measured as absorbance at 500 nm.

When P_i and MPn were supplied together as sources of phosphorus, the initial phase of logarithmic growth (4 h) was at the expense of P_i (Fig. 1, inset), and the growth curve was identical to that in the culture lacking MPn (Fig. 1); the utilization of phosphate was the same linear function of turbidity (206 times the change in turbidity) as in the control without MPn. These data indicate little or no utilization of MPn while P_i was present. The failure of MPn-induced cells to utilize MPn until the P_i concentration fell markedly was confirmed by direct assay of P_i and MPn in culture filtrates during growth (Fig. 1, inset). After exhaustion of P_i, the bacteria initiated MPn utilization and continued growing, as indicated by the further increase in turbidity. When *P. testosteroni* was preinduced to use P_i as a phosphorus source for growth, the results were almost indistinguishable from those in Fig. 1.

Effect of Spodosol on MPn utilization. Since the Spodosol could bind much P_i, less MPn, and still less IMPn, this soil was used to examine two possible effects: (i) elimination of diauxic growth on P_i and MPn by binding the P_i and making it inaccessible to the cells, and (ii) reduction in the rate or amount of degradation of MPn compared with IMPn because of the partial binding of MPn. Utilization of MPn and

IMPn was quantified by determination of the methane released from the phosphonates because turbidity, protein, and cell mass could not be analyzed in the presence of the soil.

The effect of the Spodosol on diauxic growth of *P. testosteroni* is shown in Fig. 2A. When P_i and MPn were present together as phosphorus sources, no methane was liberated until 5 h after inoculation. This time corresponded to the exhaustion of P_i. Methane was not released in an amount stoichiometric to the initial amount of MPn, since carbon became limiting. In sharp contrast, the release of methane from the culture containing P_i, MPn, and soil began as if P_i had not been present; in the presence of the P_i-binding Spodosol, no diauxic effect was observed.

The possible influence of soil on the availability of MPn and IMPn for bacterial growth was assessed by using 0.6, 1.2, and 2.0 g of soil per 30 ml of medium. The maximum rate and extent of release of methane from IMPn were almost the same in the presence or absence of soil (Fig. 2B). The only significant effect of the Spodosol was a shortening of the time for the onset of the maximum rate of methane evolution.

Figure 2C depicts the release of methane from MPn. When compared with release from liquid culture without soil, the maximum rate of production was unaffected by the soil. In sharp contrast to IMPn, however, the total amount of methane released from MPn was decreased with increasing amounts of soil. The time required for the initiation of rapid MPn utilization was
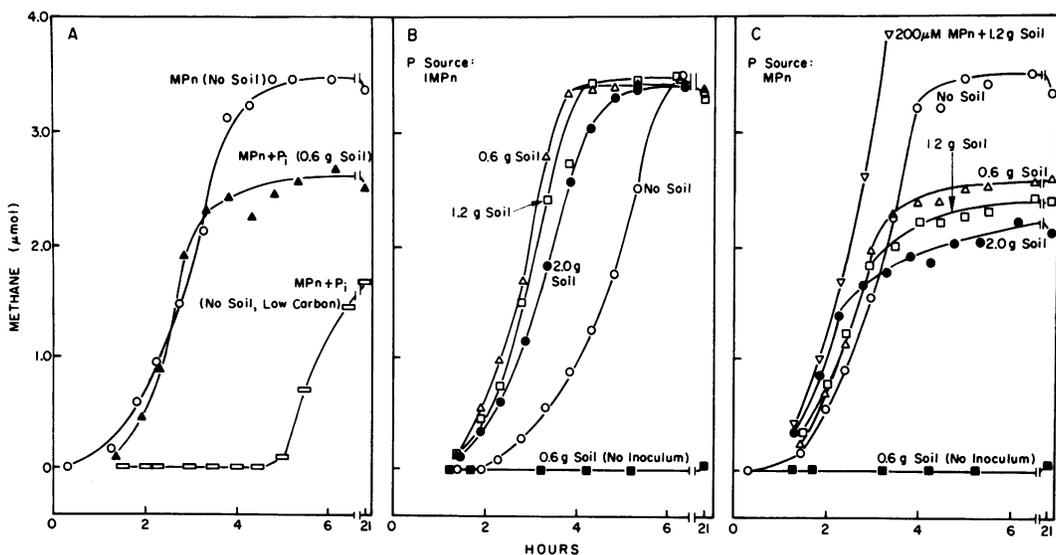

FIG. 2. *Effect of a P_i-binding Spodosol and P_i on the release of methane by P. testosteroni from MPn and IMPn. P. testosteroni was induced to grow on 0.5 mM P_i as phosphorus source, harvested in late logarithmic phase, suspended in HEPES-buffered salts medium, and, unless otherwise indicated, inoculated into HEPES-buffered salts medium containing 100 µM of each phosphorus source shown, 15 mM p-hydroxybenzoate (low concentration, 3 mM) as carbon source, and 0 to 2 g of soil.*

shortened by the soil (Fig. 2B).

Uninoculated medium containing soil and MPn, IMPn, or P_i produced no methane. Inoculated medium containing soil and 0.2 mM MPn (twice the usual concentration) released twice as much methane, confirming that the effect of the soil was related to the availability of the substrate and not to a toxicity to *P. testosteroni*. If the inoculum was grown on MPn or IMPn and these cells were introduced into media with P_i and MPn in the absence of soil, an initial phase of slow arithmetic release of methane from MPn or IMPn occurred during utilization of P_i. This is characteristic of repression of an induced enzyme that is not subject to marked inhibition (1).

DISCUSSION

Most of the ionic phosphorus-containing compounds that were herein studied, as well as monoalkyl phosphates (14), were found not to bind to soils that had a high capacity for P_i retention. The lack of retention of these compounds strongly contrasts with that of the parent pesticide (10) and with the naturally occurring organophosphates, which are not normally found free in the soil solution (11). The data shown here indicate that binding occurs only with the nonesterified alkyl- and arylphosphonates (e.g., MPn and ϕPn). The trend observed, from the nonretention to the partial binding of the other nine alkyl phosphorus compounds, is that binding increases as alkyl chain length, sulfur content, and degree of esterification decrease and as the electrophilicity of the phosphorus increases. This trend is the reverse for that of the sorptive characteristics for this class of compounds that was observed by using the macroreticular nonionic adsorbent resin XAD-4 (6). It therefore may be presumed that these products of pesticide breakdown will generally be accessible to microorganisms in soils and sediments, since such products are exceedingly water soluble and generally not susceptible to sorption; these properties contrast with the low water solubility of organophosphorus pesticides and their retention by soil (10).

The presence of available P_i in natural environments could be a major determinant of biodegradation of phosphonates, and little degradation of these compounds might be evident in environments where the supply of available P_i would repress synthesis of the degradative enzymes. In waters of low nutrient concentration, as considered by Deevey (8), degradation of phosphorus-containing synthetic compounds could be limited because of the high carbon and nitrogen requirement for complete utilization of these phosphorus sources (3). In contrast, the greater binding of P_i than of the alkyl phosphorus compounds in soil might allow for attack of the alkyl phosphorus, as herein demonstrated by the abolition of diauxic growth by a P_i-binding Spodosol.

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