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Phosphorus-Containing Pesticide Breakdown Products: Quantitative Utilization as Phosphorus Sources by Bacteria

ALASDAIR M. COOK, CHRISTIAN G. DAUGHTON, AND MARTIN ALEXANDER*
Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York 14853

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Bacteria were isolated that could utilize representatives of the following ionic phosphorus-containing breakdown products of organophosphorus pesticides as sole phosphorus sources: dialkyl phosphates, dialkyl phosphorothioates, dialkyl phosphorodithioates, alkyl arylphosphonates, alkyl arylphosphonothioates, and alkyl alkylphosphonates. Utilization of each organophosphorus compound, which was complete for 7 of 12 compounds studied, was confirmed by determination of protein yield from the amount of phosphorus source consumed. This is the first report of the utilization of an ionic dialkyl thiophosphate or dithiophosphate by microorganisms.

The environmental fate of the initial products of pesticide breakdown is largely unknown because research has been devoted principally to studying the initial transformations that a pesticide will undergo. The organophosphorus pesticides are a major class of biocides that are used in massive quantities worldwide. They generally exhibit very high acute toxicity and sometimes delayed neurotoxicity, and they are widely regarded as "nonpersistent." These organophosphorus pesticides offer a unique opportunity for a simplified study of the potential fate of their breakdown products since all organophosphorus pesticides are susceptible to hydrolysis, resulting in the release of an ionic phosphorus-containing moiety. This moiety is almost invariably an ionic dialkyl phosphate or alkyl- or arylphosphonate, or a sulfur analog thereof. Consequently, a few ionic phosphorus compounds can serve as representative models for the entire spectrum of phosphorus-containing breakdown products from these pesticides. These ionic compounds are also released to soils or natural waters because of their extensive use in industry, e.g., as ore flotation promoters in mining, additives to lubricants, flame retardants, and wastes from pesticide manufacture. The limited literature on the metabolism and toxicology of these products of pesticide hydrolysis is inconclusive and contradictory and has been reviewed recently by several authors (4, 5, 7; C. G. Daughton, Ph.D. thesis, University of California, Davis, 1976).

Definitive evidence for microbial utilization of an ionic dialkyl phosphorus compound has been obtained only for dimethyl hydrogen phosphate (9), and indirect evidence for the bacterial degradation of diethyl hydrogen phosphate has been presented (2). Several organophosphonates have been reported to serve as phosphorus sources for bacteria (1, 4, 11, 16, 22), whereas the failure to obtain enrichments on phosphoryl-thio analogs has been reported (7; J. M. Tiedje, M. S. thesis, Cornell University, Ithaca, N.Y., 1966).

The present study was designed to determine whether the exceedingly unreactive (12) ionic organophosphorus products of pesticide hydrolysis are susceptible to degradation by microorganisms. Twelve representative compounds were examined. We report the first definitive evidence for the existence of microorganisms freshly isolated from natural ecosystems able to utilize ionic dialkyl phosphorothioates, dialkyl phosphorodithioates, and methyl phenylphosphonate as sole phosphorus sources.

MATERIALS AND METHODS

Materials. The organophosphorus compounds used and their sources are described in Table 1. Abbreviations used in this paper are given in Table 1. The mole fraction of inorganic orthophosphate (P_i) in the organophosphorus compounds was less than 0.0005, except for DMP (0.041) and MPn (0.026), and the theoretical phosphorus content was observed in all instances (A. M. Cook, C. G. Daughton, and M. Alexander, Anal. Chem., in press). All other chemicals were of the highest purity available commercially.

Glassware. The glassware was washed in 5 M nitric acid to remove contaminative phosphate (4).

Media. The growth media were buffered at pH 7.4 with either 50 mM tris(hydroxymethyl)methylamine or 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] and contained 2.7 mM KCl, 0.8 mM $MgSO_4$, 40 mM NH_4Cl, and trace elements (18). This portion of the medium, together with a stable carbon source, could be autoclaved without formation of a precipitate. When required, carbon and phosphorus sources were added aseptically. Organophosphorus compounds, glucose, and glycerol were sterilized by

TABLE 1. *Ionic organophosphorus compounds*

Abbreviation	Chemical name	Chemical formula
DMP	*O,O*-dimethyl hydrogen phosphate[a]	$(CH_3O)_2P(O)OH$
DEP	*O,O*-diethyl hydrogen phosphate[b]	$(C_2H_5O)_2P(O)OH$
DMTP	*O,O*-dimethyl potassium phosphorothioate[c]	$(CH_3O)_2P(S)OK$
DETP	*O,O*-diethyl potassium phosphorothioate[c]	$(C_2H_5O)_2P(S)OK$
DMDTP	*O,O*-dimethyl potassium phosphorodithioate[c]	$(CH_3O)_2P(S)SK$
DEDTP	*O,O*-diethyl potassium phosphorodithioate[c]	$(C_2H_5O)_2P(S)SK$
ϕPn	Dihydrogen phenylphosphonate[d]	$C_6H_5P(O)(OH)_2$
MϕPn	*O*-methyl hydrogen phenylphosphonate[d]	$(CH_3O)C_6H_5P(O)OH$
MϕTPn	*O*-methyl potassium phenylphosphonothioate[d]	$(CH_3O)C_6H_5P(S)OK$
MPn	Dihydrogen methylphosphonate[e]	$CH_3P(O)(OH)_2$
IMPn	*O*-isopropyl hydrogen methylphosphonate[e]	$[(CH_3)_2CHO]CH_3P(O)OH$
PMPn	*O*-pinacolyl hydrogen methylphosphonate[e]	$[(CH_3)_3CCH(CH_3)O]CH_3P(O)OH$

[a] Pfaltz & Bauer, Stamford, Conn.
[b] Eastman Organic Chemical Div., Rochester, N.Y.
[c] American Cyanamid, Princeton, N.J.
[d] Velsicol Chemical Corp., Chicago, Ill.
[e] Chemical Systems Laboratory, Aberdeen Proving Ground, Md.

passage through a membrane filter (4).

Enrichment and isolation of bacteria. Sewage from a primary settling tank was centrifuged (20,000 × *g* for 10 min at 4°C), and the supernatant fluid was discarded to reduce the quantity of extraneous phosphorus sources. The pellet was suspended in buffered salts solution and used as the inoculum for growth media containing 0.1 mM phosphorus source as previously described (4). The resulting enirchment cultures were streaked on nutrient agar, and each colony that gave rise to growth in the selective liquid medium (i.e., homologous with the enrichment medium) was subcultured again in selective liquid media before restreaking on nutrient agar. This procedure was repeated three times to yield colonies of homogeneous characteristics. Strain A was stored on nutrient agar slants. Most strains lost their activity on the phosphorus compounds when maintained on nutrient agar and thus were maintained by subculturing in selective liquid media at intervals of 2 to 4 weeks. In addition to the freshly isolated bacteria, *Pseudomonas testosteroni* was used; this bacterium was previously designated as strain 11 (4).

Quantification of growth and substrate utilization. Cultures (30 ml) contained in 50-ml Erlenmeyer flasks and closed with cotton plugs were incubated at 29°C on an orbital shaker (170 rpm, 3-cm eccentricity). Utilization experiments were done in media with 0.1 mM of the phosphorus source and 5 mM *p*-hydroxybenzoate as carbon source, and they were inoculated (3%, vol/vol) with a culture grown with limiting organophosphorus (0.1 mM). The data for turbidity and protein at zero time were calculated from the corresponding values in the culture used for inoculation; P_i and total phosphorus in the extracellular fluid were assayed after passage through a membrane filter (0.2-μm pore diameter). After growth ceased, samples were assayed for turbidity, protein, and extracellular P_i and total phosphorus. A control culture with P_i as phosphorus source was grown for each strain. Sterile controls for each phosphorus source served to confirm that there was no nonbiological breakdown to P_i.

Analytical methods. Protein (5-ml samples) and turbidity were measured by the methods previously described (4). A turbidity of 1.0 at 500, 420, and 650 nm represented 100, 85, and 170 μg of protein per ml, respectively. The assay for P_i and the wet ashing procedure for total phosphorus will be published elsewhere (Cook et al., Anal. Chem., in press). The concentration of organophosphorus compound in the extracellular solution was calculated as the difference between total phosphorus and P_i concentrations.

RESULTS AND DISCUSSION

Isolation of strains. The use of pure phosphorus compounds is of utmost importance in studying phosphorus-limited growth. Contamination of the organic phosphorus compounds with small amounts of P_i (2 to 4%) caused no interference during enrichment when the media were limiting in phosphorus, but 10 of 11 isolates enriched and selected after growing in media with commercially available and unpurified phosphorodithioates and hydrolyzed chlorophosphorothioates (DMDTP, DEDTP, DMTP, and DETP) were unable to grow in media with the respective pure compound. Many strains lost the ability to utilize organophosphorus compounds when repeatedly transferred on nutrient agar; as a result, each isolate was purified by alternate growth in selective liquid medium (containing the appropriate phosphorus source) and nutrient agar. Selective solid media could not be prepared because of the quantity of contaminative P_i (4).

Four pure cultures (*P. testosteroni* and strains A, D, and K) were able to utilize 11 of the 12 organophosphorus compounds tested (Table 2), and a mixed culture (J) grew slowly with MøTPn as the sole phosphorus source. In no instance was growth on the test chemical attributable to its spontaneous decomposition to P_i, because

TABLE 2. *Growth of bacteria and utilization of phosphorus sources*

Strain	Days of incubation	Phosphorus source	Increase in protein (μg/ml)	Concn (μM)[a]				Δ Protein/−Δ phosphorus (kg/mol)
				Organic phosphorus		P_i		
				Initial	Final	Initial	Final	
A	2	P_i	105	0	1	114	4	0.96
	2	DMP	106	83	4	21	2	1.08
	2	DEP	100	97	3	4	4	1.06
D	4	P_i	115	0	0	106	24	1.40
	4	DMTP	117	91	0	3	22	1.63
	4	DETP	123	98	0	3	33	1.81
	4	DMDTP	59	99	76	2	1	2.46
	4	DEDTP	111	97	0	2	31	1.63
K	4	P_i	104	0	1	105	1	1.01
	4	φPn	74	104	26	2	1	0.94
	4	MφPn	57	100	61	2	1	1.43
J[b]	2	P_i	125	0	1	110	4	1.19
	12	MφTPn	20	90	56	6	6	0.59
P. testosteroni	2	P_i	95	0	3	114	11	0.95
	2	MPn	95	94	3	5	10	1.10
	2	IMPn	96	101	2	2	10	1.05
	10	PMPn		109	33	1	1	
Sterile	2	P_i	0	0	0	118	120	

[a] Concentration in extracellular media.
[b] Mixed culture.

such decomposition did not happen in the sterile controls incubated under identical conditions.

Quantification of organophosphorus utilization. Strain A grew rapidly with P_i, DMP, or DEP as the phosphorus source (Table 2). The organism used 96% of the P_i and produced about 1 kg of protein per mol of phosphorus consumed. The two alkyl phosphates were used as efficiently as P_i. Since the residual P_i and organophosphate concentrations were about equal when growth ceased, the organism presumably has uptake systems of similar K_m values for the three phosphorus sources. Strain A did not grow on the sulfur analogs of the two alkyl phosphates, and these analogs did not inhibit growth on DMP.

Strain D utilized P_i, DMTP, DETP, and DEDTP and produced about 1.6 kg of protein per mol of phosphorus consumed. This is the first report of an organism that can utilize the sulfur analogs of dialkyl phosphates. In each instance, no organophosphate remained at the end of growth, but 20 to 30% of the added phosphorus source was released to the extracellular solution as P_i, thus confirming the conversion of DMTP, DETP, and DEDTP to P_i. The bacterium may have an inefficient P_i uptake system, but the high protein yield per mole of phosphorus probably reflects the exhaustion of the carbon source because the medium only had 350 g-atoms of C per g-atom of P (see below). In contrast to the other thiophosphates, DMDTP was incompletely utilized by this isolate, a fact confirmed by the smaller amount of protein synthesized. Presumably, at least one enzyme has a much lower affinity for DMDTP than for DEDTP. The organism could utilize both DMP and DEP as phosphorus sources.

Strain K utilized øPn and MøPn, albeit incompletely (Table 2). Longer incubation did not increase the yield of bacteria, so the lack of complete utilization may have resulted from poor affinity of an enzyme for the substrates or, for MøPn, an inability to use one of the two optical isomers. Alam and Bishop (1) reported practically no utilization of øPn by a strain of *Escherichia coli*. The mixed culture designated J synthesized a small amount of protein while degrading nearly 40% of the MøTPn provided.

P. testosteroni grew equally well with P_i, MPn, or IMPn as phosphorus source, producing, like strain A, about 1 kg of protein per mol of phosphorus. PMPn, which has four optical isomers, supported less growth. *P. testosteroni* did not utilize øPn as a phosphorus source, and strain K did not utilize MPn.

TABLE 3. *Comparison of published growth media and growth yields per mole of phosphorus for several organophosphorus compounds*

Phosphorus source	Medium composition (g-atoms/g-atom of P)		P concn (mM)	Apparent growth yield[a] (kg of protein/mol of P)	Reference
	C	N			
P_i or 7 separate phosphorus compounds	350	400	0.1	1–1.6[b]	This study
P_i or DMP	3,300	190	0.1	2.6[c]	21
P_i or dihydrogen 2-aminoethylphosphonate	1,700	95	0.2	0.9[d]	11
P_i or dihydrogen 2-aminoethylphosphonate	1,000	60	0.3	0.4[c]	1
Dihydrogen 2-aminoethylphosphonate	295	76	2.5	0.38	19
Dihydrogen 2-aminoethylphosphonate	370	15	1.2	0.09[d]	20
Dihydrogen ethylphosphonate	0.9	0.2	82	0.0008[e]	22
MPn	1.3	0.3	61	0.0003[e]	16
MPn	3.0	1.1	40	—[f]	14
Diazinon	1.7	0.4	49	0.0008[e]	10

[a] Estimated in this laboratory from the change in culture density and the moles of phosphorus supplied.
[b] Real growth yield calculated from protein produced per mole of phosphorus consumed.
[c] Assuming a turbidity of 1.0 at 650 nm equivalent to 170 μg of protein per ml.
[d] Assuming a turbidity of 1.0 at 420 nm equivalent to 85 μg of protein per ml.
[e] Assuming 1 Klett unit is equivalent to 0.3 μg of protein per ml.
[f] Units on axes of graph not given.

Significance of organophosphorus utilization. Most of the compounds tested (11 of 12) were extensively degraded as sole phosphorus sources by bacteria newly isolated from sewage. This observation does not prove that these compounds will be degraded in natural environments, however, especially in the presence of available P_i. By use of these isolates, it may be possible to develop procedures using cells or enzymatic methods for decontaminating wastes or spills, complementary to those of Daughton and Hsieh (6) and Munnecke (17).

We observed that 1.0 to 1.6 kg of protein is formed per mol of phosphorus consumed, roughly consistent with data in a review (15), which quotes 0.5 to 1.6 kg of bacterial dry weight per mol of phosphorus in exponentially growing cells. Growth medium must contain about 300 g-atoms of carbon source and 25 g-atoms of nitrogen source to enable utilization of 1 g-atom of phosphorus (4); only five investigations appear to have met these criteria (Table 3). Although variations in the turbidity-protein ratio are expected from different spectrophotometers (13), the apparent growth yields we calculated (Table 3) indicate that only four reports (1, 11, 19, 21) conclusively describe organisms capable of using the respective organophosphorus compound (i.e., 2-aminoethylphosphonate or DMP). In contrast, the growth yields calculated in Table 3 suggest that the data given in several published reports are inadequate to confirm the claim of utilization of the organophosphorus compound. It is possible in these latter studies that the test organisms grew on P_i that contaminated the organophosphorus sources, as a contamination level of 0.01% would support the growth observed. The difficulty in establishing quantitative growth and substrate utilization applies also to work on pesticide utilization by fungi (3), where mycelial dry weight may be in large part chitin and lipid, and the pesticide may dissolve in the lipid rather than being utilized for growth. Similar caution should be observed when considering papers (8) claiming enzymatic cleavage of dialkyl phosphorodithioates, where no activity was observed at substrate concentrations below about 50 mM.

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LITERATURE CITED

1. **Alam, A. U., and S. H. Bishop.** 1969. Growth of *Escherichia coli* on some organophosphonic acids. Can. J. Microbiol. **15**:1043–1046.
2. **Berry, W. L., E. Bartnicki-Garcia, and J. Kumamoto.** 1967. Diethyl phosphate as a phosphate nutrient source. BioScience **17**:817–818.
3. **Bhaskaran, R., D. Kandasamy, G. Oblisami, and T. R. Subramaniam.** 1973. Utilization of disyston as carbon and phosphorus sources by soil microflora. Curr. Sci. **42**:835–836.
4. **Cook, A. M., C. G. Daughton, and M. Alexander.** 1978. Phosphonate utilization by bacteria. J. Bacteriol. **133**:85–90.
5. **Daughton, C. G., D. G. Crosby, R. L. Garnas, and D. P. H. Hsieh.** 1976. Analysis of phosphorus-containing hydrolytic products of organophosphorus insecticides in water. J. Agric. Food Chem. **24**:236–241.

6. **Daughton, C. G., and D. P. H. Hsieh.** 1977. Accelerated parathion degradation in soil by inoculation with parathion-utilizing bacteria. Bull. Environ. Contam. Toxicol. **18**:48–56.
7. **Daughton, C. G., and D. P. H. Hsieh.** 1977. Parathion utilization by bacterial symbionts in a chemostat. Appl. Environ. Microbiol. **34**:175–184.
8. **Forrest, I. S.** 1955. Enzymatic hydrolysis of *O,O*-diisopropyl thionothiolphosphate. Arch. Biochem. Biophys. **58**:178–180.
9. **Gerlt, J. A., and G. J. R. Whitman.** 1975. Purification and properties of a phosphohydrolase from *Enterobacter aerogenes*. J. Biol. Chem. **250**:5053–5058.
10. **Gunner, H. B., B. M. Zuckerman, R. W. Walker, C. W. Miller, K. H. Deubert, and R. E. Longley.** 1966. The distribution and persistence of Diazinon applied to plant and soil and its influence on rhizosphere and soil microflora. Plant Soil **25**:249–264.
11. **Harkness, D. R.** 1966. Bacterial growth on aminoalkylphosphonic acids. J. Bacteriol. **92**:623–627.
12. **Kirby, A. J., and M. Younas.** 1970. The reactivity of phosphate esters. Diester hydrolysis. J. Chem. Soc. B **1970**:510–513.
13. **Koch, A. L.** 1970. Turbidity measurements of bacterial cultures in some available commercial instruments. Anal. Biochem. **38**:252–259.
14. **Labeyrie, S., and E. Neuzil.** 1977. Influence des ions zinc et cobalt sur la chromogenèse de *Pseudomonas aeruginosa* en présence d'acide méthylphosphonique. C. R. Seances Soc. Biol. Paris **171**:755–759.
15. **Luria, S. E.** 1960. The bacterial protoplasm: composition and organization, p. 1–34. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 1. Academic Press Inc., New York.
16. **Mastalerz, P., Z. Wieczorek, and M. Kochman.** 1965. Utilization of carbon-bound phosphorus by microorganisms. Acta Biochim. Pol. **12**:151–156.
17. **Munnecke, D. M.** 1977. Properties of an immobilized, pesticide-hydrolyzing enzyme. Appl. Environ. Microbiol. **33**:503–507.
18. **Pfennig, N., and K. D. Lippert.** 1966. Über das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. **55**:245–256.
19. **Rosenberg, H., and J. M. La Nauze.** 1967. The metabolism of phosphonates by microorganisms. The transport of aminoethylphosphonic acid in *Bacillus cereus*. Biochim. Biophys. Acta **141**:79–90.
20. **Tamari, M., M. Horiguchi, and M. Kandatsu.** 1977. Growth of *Escherichia coli* TM-1 on natural phosphonic acids and their related compounds. J. Gen. Appl. Microbiol. **23**:49–52.
21. **Wolfenden, R., and G. Spence.** 1967. Derepression of phosphomonoesterase and phosphodiesterase activities in *Aerobacter aerogenes*. Biochim. Biophys. Acta **146**:296–298.
22. **Zeleznick, L. D., T. C. Myers, and E. B. Titchener.** 1963. Growth of *Escherichia coli* on methyl- and ethylphosphonic acids. Biochim. Biophys. Acta **78**:546–547.