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# Phosphonate Utilization 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 able to use at least one of 13 ionic alkylphosphonates or *O*-alkyl or *O,O*-dialkyl alkylphosphonates as phosphorus sources were isolated from sewage and soil. Four of these isolates used 2-aminoethylphosphonic acid (AEP) as a sole carbon, nitrogen, and phosphorus source. None of the other phosphonates served as a carbon source for the organisms. One isolate, identified as *Pseudomonas putida*, grew with AEP as its sole carbon, nitrogen, and phosphorus source and released nearly all of the organic phosphorus as orthophosphate and 72% of the AEP nitrogen as ammonium. This is the first demonstration of utilization of a phosphonoalkyl moiety as a sole carbon source. Cell-free extracts of *P. putida* contained an inducible enzyme system that required pyruvate and pyridoxal phosphate to release orthophosphate from AEP; acetaldehyde was tentatively identified as a second product. Phosphite inhibited the enzyme system.

Alkylphosphonates, compounds with a C-P bond, occur widely in nature either free or combined in lipids (18), polysaccharides (11), or proteins (6). Many synthetic organophosphonates are being added in large quantities to natural ecosystems in the form of insecticides, herbicides, and flame retardants. The potential fates of phosphonates are poorly understood. However, some evidence exists that ionic nonesterified alkylphosphonates may be persistent (9, 21).

With certain exceptions, the C-P bond is highly resistant to chemical hydrolysis (4), thermal decomposition (4), and photolysis (17); hence, biological cleavage of the C-P bond in natural habitats assumes importance to prevent the accumulation of phosphonates. Cleavage of the C-P bond is apparently limited to certain microorganisms (8, 16). Although these organisms use phosphonates solely as phosphorus sources or sometimes as nitrogen sources (13, 18), evidence for cleavage of the C-P bond rarely has been obtained. Only one phosphonatase, 2-phosphonoacetaldehyde phosphohydrolase (EC 3.11.1.1), has been described in detail (14, 15), and tentative evidence for another has been published (1).

The present report is part of a study of the potential for metabolism of phosphonates in natural environments. Bacteria capable of using different alkylphosphonates were isolated from sewage and soil. By use of an organism able to utilize 2-aminoethylphosphonic acid (AEP) as its sole source of carbon, nitrogen, and phosphorus, the cleavage in vivo of the C-P bond of this widely occurring phosphonate has been demonstrated.

## MATERIALS AND METHODS

**Materials.** Aminomethylphosphonic acid, 1-aminoethylphosphonic acid, AEP, 3-aminopropylphosphonic acid, 1-aminobutylphosphonic acid, alcohol dehydrogenase (yeast), and reduced nicotinamide adenine dinucleotide (NADH) were purchased from Sigma Chemical Co., St. Louis, Mo. 2-Amino-3-phosphonopropionic acid, 2-amino-4-phosphonobutyric acid, and HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] were obtained from Calbiochem, San Diego, Calif. Isopropyl methylphosphonate (sodium salt), pinacolyl methylphosphonate (sodium salt) (60% pure; the contaminants were inorganic, mainly NaF, ferric and carbonate ions, and NaOH), and methylphosphonic acid were obtained from Edgewood Arsenal, Aberdeen Proving Ground, Md. Aldrich Chemical Co. (Milwaukee, Wis.) provided dimethyl methylphosphonate and diethyl vinylphosphonate. Diethyl ethylphosphonate and diallyl allylphosphonate were from Pfaltz and Bauer, Stamford, Conn. Fluram (fluorescamine) was a product of Hoffman-La Roche, Nutley, N.J. Pyridoxal phosphate and dithiothreitol were from Nutritional Biochemicals Corp., Cleveland, Ohio. All other chemicals were of the highest purity available commercially.

**Glassware.** Glassware was cleaned by steeping in water followed by a 12-h immersion in 20% (vol/vol) $HNO_3$. Nitric acid was removed by thorough washing in tap water followed by distilled water. Apparatus sensitive to acid and organic solvents was rinsed thoroughly in distilled water. By these means, the residual phosphate level was reduced to a level that supported negligible growth in cultures free of added phosphorus (turbidity of less than 0.01 at 500 nm).

**Media and culture conditions.** Unless otherwise indicated, media free of inorganic phosphate were buffered with 50 mM Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] (pH 7.4) or 15 mM HEPES (pH 7.2) and contained (per liter): KCl, 0.2 g;

$MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 0.5 g; ferric ammonium citrate, 1.0 mg; $CaCl_2 \cdot 2H_2O$, 2.0 mg; and a carbon source. On occasion, the organic buffer and KCl were replaced with 6 mM potassium orthophosphate buffer (pH 7.4). Basal media were autoclaved, after which a sterile solution containing the carbon source and another containing the iron and calcium salts were added aseptically. All phosphonates and carbon sources were sterilized by filtration through sterile 0.2-μm membrane filters. Cultures in tubes were closed with polyurethane foam plugs and incubated at 30°C without agitation. Growth curves and kinetics of substrate utilization were studied at 30°C in cultures aerated by magnetically driven stirring bars (5). The cells were harvested by centrifugation at 20,000 × *g* at 4°C.

**Isolation of bacteria.** Enrichment cultures were used to obtain isolates able to utilize a given phosphonate as a sole source of phosphorus and/or carbon. When provided as sole phosphorus sources, the phosphonates were present at 0.5 mM, and 0.3 g each of glucose, glycerol, and sodium succinate per liter were present as carbon sources. When serving as the potential carbon source, the phosphonates were present at 0.025 g-atom of carbon per liter. The enrichment cultures (3.0 ml, final volume) received 0.5 ml of sewage or about 0.2 g of soil, and when growth greater than that in phosphonate-free solutions was observed (usually 1 to 3 days), the enrichment was subcultured into fresh medium. After three successive transfers, enrichments using phosphonates as phosphorus sources were streaked on nutrient agar plates. Isolates that were able to utilize phosphonates were subsequently recognized by their growth in selective liquid media. Bacteria using phosphonates as carbon sources were picked from plates containing 10 mM substrate in mineral agar.

**Analytical methods.** Turbidity was measured at 500 nm in 1-cm cuvettes in a Bausch & Lomb spectrophotometer, model Spectronic 88. Inorganic phosphate was assayed by the method of Dick and Tabatabai (2), and none of the phosphonates and neither of the organic buffers interfered. The assay of Weatherburn (22) was used for ammonium ion determinations; HEPES did not interfere in this method, but the iron source and AEP gave low absorbance values, which were subtracted from the assay values.

Protein was assayed as described by Kennedy and Fewson (10). The protein was separated from the organic buffers by precipitation with 0.45 M trichloroacetic acid (final concentration) followed by centrifugation at 20,000 × *g* at 4°C. The precipitate was resuspended and washed with 0.45 M trichloroacetic acid.

No specific assay exists for AEP (8). The Fluram assay for amines (3) was unaffected by ammonium ion, but the standard curve from 0 to 10 nmol of AEP ran from 50 ± 10 to 70 ± 10 fluorescence units. Consequently, AEP utilization could not be measured accurately by this assay.

**Preparation of cell-free extracts.** Cells early in the stationary phase were harvested and washed in extraction buffer (15), and the resulting pellet was stored at −20°C. The thawed cell pellet was resuspended to about 5 mg (wet weight)/ml in extraction buffer, and extracts were prepared by sonic disruption at 4°C, using the microtip of the model W185D cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) at 70% power in four 30-s periods, each separated with 30-s cooling periods. Debris and whole cells were removed by centrifugation at 40,000 × *g* for 15 min at 4°C.

**Enzyme assay.** The coupled reaction of AEP transamination and 2-phosphonoacetaldehyde phosphohydrolase (E.C. 3.11.1.1) was assayed at 30°C essentially as described by La Nauze and Rosenberg (14). The complete reaction mixture contained, in 3 ml: Tris buffer (pH 8.5), 300 μmol; $MgCl_2$, 15 μmol; ethylenediaminetetraacetate (potassium salt), 1.5 μmol; dithiothreitol, 1.5 μmol; pyruvate, 15 μmol; pyridoxal phosphate, 1.5 μmol; AEP, 15 μmol; and protein, 0.2 to 0.8 mg. At intervals, 0.5-ml portions were treated with 0.1 ml of 3.0 M trichloroacetic acid, the protein was removed by centrifugation, and a portion of the supernatant fluid was analyzed for inorganic phosphate. The reaction was linear for at least 2 h, and the rate was proportional to the amount of protein present.

Portions of the supernatant fluid from the enzyme assays were tested for the presence of aldehyde, presumably acetaldehyde, by the oxidation of NADH in the presence of alcohol dehydrogenase. The small quantity of trichloroacetic acid in the sample did not affect the controls with authentic acetaldehyde as substrate for the enzyme.

**Electron microscopy.** Negative stains were prepared by using suspensions in distilled water of cells grown on nutrient agar. The cells were supported on carbon-Formvar-coated copper grids and stained with an aqueous solution containing 10 mg of phosphotungstic acid (pH adjusted to 7.4 with KOH) and 50 μg of bacitracin per ml. The stained grids were observed in a Philips EM 300 transmission electron microscope at 60 kV.

## RESULTS AND DISCUSSION

**Isolation of strains.** Enrichment cultures able to use phosphonates as sole phosphorus sources were readily obtained. Two sewage samples each yielded enrichments on all but one (diethyl vinylphosphonate) of the 13 phosphonates tested as phosphorus sources, whereas three soil samples yielded only enrichments utilizing AEP. The only phosphonate used as a carbon source was AEP, and enrichments were obtained from all soil and sewage samples.

To determine the capacity of the isolates to use a variety of phosphonates as phosphorus sources, the bacteria were first grown in media containing the phosphonate on which they were isolated. These cultures were then used to inoculate tubes containing 0.5 mM phosphonate, the glucose-glycerol-succinate mixture as carbon source, and inorganic salts. Growth was measured turbidimetrically and compared with that in solutions receiving no supplemental phosphorus (controls); in the latter tubes, only sparse

growth was evident, probably a result of some transfer of phosphorus together with the inoculum. A summary of a study to show the ability of 14 bacterial isolates to grow on a variety of phosphonates is given in Table 1. Each of the strains, except for 4 and 5, was isolated when the phosphonate was supplied as a phosphorus source. All isolates used AEP as a phosphorus source, and except for strain 3, all were able to use more than one of the phosphonates. In general, the alkylphosphonates and *O*-alkyl alkylphosphonates appeared to serve as good phosphorus sources because the extent of growth was similar to that with equimolar phosphate. By comparing growth with that on equimolar phosphate, it appeared that the *O,O*-dialkyl alkylphosphonates were incompletely attacked. Strains isolated on one class of alkylphosphonates (e.g., ionic nonesterified phosphonates) usually had little activity on another class (e.g., *O*-alkyl alkylphosphonates).

Except for strains 3, 4, 5, and 8, each of which could use AEP as a carbon source, no isolate used any of the phosphonates as carbon sources. It is noteworthy that the presence of one phosphonate could inhibit or even abolish growth on another phosphonate. For example, growth of strain 5 in a medium containing 5 mM AEP as a carbon source was abolished in the presence of equimolar 1-aminoethylphosphonic acid. A similar effect has been observed by Lacoste et al. (12).

Strain 11 was grown in media with limiting levels of phosphate, ammonium, or gluconate. The extent of growth, as measured by turbidity, was linearly related to the levels of phosphate, ammonium, and gluconate for concentration ranges of at least up to 0.1, 4.0, and 10.0 mM, respectively. Growth in the salts solution containing 5.0 mM gluconate was equivalent to growth in 0.1 mM phosphate; i.e., the ratio of gram-atoms of carbon utilized per gram-atom of phosphorus was about 300:1, although this value varied with the carbon source. Correspondingly, the molar ratio for ammonium/phosphate was about 25:1. The data indicate that high concentrations of carbon and nitrogen are required when doing yield studies with phosphonates and also show that enrichments can be performed satisfactorily at 0.1 mM phosphonate to minimize toxicity effects. The high cell yield on phosphate explains the need for scrupulously clean glassware and why agar, which always contains phosphate, cannot be used as a solidifying agent in phosphorus-limited media.

**Growth of *P. putida* with AEP as carbon, nitrogen, and phosphorus source.** Strain 5 was chosen for further work, since it clumped less during growth than did strains 3 and 4. Strain 5 was an aerobic, motile rod which was difficult to stain by the Gram reaction, but electron micrographs of the bacterium showed a wrinkled cell surface, clearly indicating a gram-negative organism. Polar multitrichous flagellation was observed. On the basis of the following tests, the isolate was identified as a strain of *Pseudomonas putida* (20): no polyhydroxybutyrate accumulated; positive fluorescent pigment; negative phenazine pigment; no methionine requirement; no denitrification; no growth at 4 or 41°C; oxidase positive; arginine dihydrolase positive; *ortho* ring cleavage of protocatechuate (7); no growth on geraniol, inositol, galactose, maltose, or trehalose as carbon source; and growth on phenylacetate, glycine, sarcosine, glucose, xylose, and citrate.

In a preliminary experiment, the cell yield of *P. putida* was directly proportional to the AEP concentration in the range 0 to 5 mM when AEP was the only source of carbon, nitrogen, and phosphorus. The HEPES buffer was neither a carbon nor a nitrogen source; moreover, orthophosphate was not present in the buffer, salts, or AEP, and the level of nitrogen in the AEP-free medium was insufficient to support significant growth when measured by turbidimetric means.

To study the products of growth on AEP, cells of *P. putida* derived from a culture grown on this phosphonate were inoculated into fresh medium containing 5 mM AEP. Under these conditions, the turbidity increased exponentially, and a lag phase was not detectable (Fig. 1). Growth continued until about 5.5 h, as shown by increases in protein concentration. The anomalous increase in turbidity at 5.5 h was not indicative of growth because a similar increase in protein was not observed; this sudden rise in turbidity presumably resulted from the breaking up of the small flocs of cells which were observed. The release of ammonium and phosphate into the medium was exponential and concomitant with growth. The specific growth rate (indicated by protein concentration) was about the same as the specific exponential rate of ammonium or phosphate release ($\mu = 0.55$/h). The increase in the extracellular phosphate concentration was 5 mM, a level equal to the amount of AEP present initially. This quantitative release of AEP-phosphorus as orthophosphate was expected since only a low percentage of phosphorus would be incorporated into the cells after cleavage of the C–P bond. The fluorimetric assay for AEP was imprecise but confirmed disappearance of AEP during growth.

Growth of *P. putida* on AEP was abolished in a medium containing 5 mM phosphite at a pH of 7.2. In contrast, phosphite had no effect

TABLE 1. *Ability of isolates to use phosphonates as phosphorus sources*[a]

| Strain | Phosphorus source in enrichment | Alkylphosphonates | | | | | | | | *O*-alkyl alkylphosphonates | | *O,O*-dialkyl alkylphosphonates | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MPA | AMP | 1AEP | AEP | 3APP | 1ABP | 2APP | 2APB | IMP | PMP | DMMP | DEEP | DAAP | DEVP |
| 1 | AMP | ++ | ++ | + | ++ | ++ | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 1AEP | ++ | ++ | ++ | ++ | ++ | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | AEP | 0 | 0 | 0 | ++ | 00 | 0 | 00 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | AEP[b] | 0 | 0 | 00 | ++ | 00 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | AEP[b] | + | 0 | 00 | ++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 3APP | ++ | 0 | 00 | ++ | ++ | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 1ABP | ++ | + | + | ++ | + | ++ | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 2APP | 0 | 0 | 0 | ++ | 0 | 0 | ++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 2APB | ++ | + | 0 | ++ | + | 0 | + | ++ | 0 | 0 | + | 0 | + | 0 |
| 10 | IMP | ++ | 0 | 00 | ++ | 00 | 0 | 0 | 0 | ++ | 0 | + | 00 | + | 00 |
| 11 | PMP | ++ | 0 | 0 | ++ | ++ | 0 | 0 | 0 | ++ | ++ | + | 0 | + | 0 |
| 12 | DMMP | ++ | 0 | 00 | ++ | 0 | 00 | 00 | 0 | 0 | 0 | + | + | + | 0 |
| 13 | DEEP | NT | 00 | 00 | ++ | 00 | 00 | 00 | 00 | 00 | 00 | 0 | + | 00 | 0 |
| 14 | DAAP | ++ | 0 | 00 | ++ | 00 | 0 | 0 | 0 | ++ | 0 | + | 0 | + | 0 |

[a] Abbreviations: MPA, methylphosphonic acid; AMP, aminomethylphosphonic acid; 1AEP, 1-aminoethylphosphonic acid; AEP, 2-aminoethylphosphonic acid; 3APP, 3-aminophosphonopropionic acid; 1ABP, 1-aminobutylphosphonic acid; 2APP, 2-amino-3-phosphonopropionic acid; 2APB, 2-amino-4-phosphonobutyric acid; IMP, sodium isopropyl methylphosphonate; PMP, sodium pinacolyl methylphosphonate; DMMP, dimethyl methylphosphonate; DEEP, diethyl ethylphosphonate; DAAP, diallyl allylphosphonate; DEVP, diethyl vinylphosphonate. Symbols: ++, heavy growth; +, some growth above that of control; 0, no growth above that of control; 00, growth less than that of control. NT, Not tested. Controls: Solutions with no added phosphorus compound.

[b] Used as carbon source in enrichment.

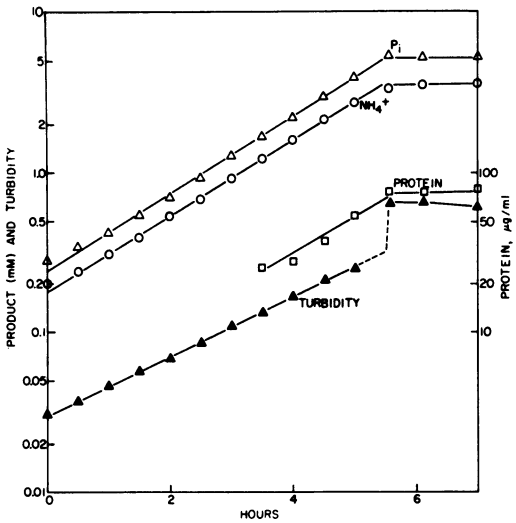


FIG. 1. *Growth of and product formation by P. putida in medium with AEP as the sole source of carbon, nitrogen, and phosphorus. A culture of P. putida was grown to stationary phase in HEPES medium containing 5 mM AEP as sole added source of carbon, nitrogen, and phosphorus, and it was subcultured into fresh homologous medium. Samples for ammonium and phosphate were analyzed in duplicate.*

on growth with acetate as the carbon source in a medium where 2 mM phosphate was the phosphorus source. Similar results have been obtained with *Bacillus cereus* (19).

**Enzymatic cleavage of the C-P bond.** Cell-free extracts were prepared from cells of *P. putida* that had been grown in media containing either 5 mM AEP as sole source of carbon, nitrogen, and phosphorus or in 5 mM acetate-salts medium. Extracts from AEP-grown cells catalyzed the release of phosphate from AEP, the specific activity being 180 to 230 nmol/min per mg of protein. In contrast, acetate-grown cells had no activity (<1% of the level of AEP-grown bacteria). This phosphonatase system is thus inducible, as observed in *B. cereus* (14). When the rate of orthophosphate release fell to zero, the molar yield of phosphate equaled the amount of AEP originally added.

Assays for phosphonatase involved measurement of phosphate release, but a second product was also found. When this product was incubated with alcohol dehydrogenase in the presence of NADH, the coenzyme was oxidized. Hence, the second substance was presumably acetaldehyde, the product of phosphonoacetaldehyde phosphonatase. When pyruvate and pyridoxal phosphate were omitted in the assay for the phosphonatase, no phosphate was released. Phosphite (5 mM) totally inhibited phosphate release from AEP. This enzyme system is apparently identical to that of La Nauze and Rosenberg (14) and La Nauze et al. (15), transamination of AEP preceding cleavage of the C-P bond. The latter reaction is inhibited by phosphite.

La Nauze and Rosenberg (14) predicted that organisms other than *B. cereus* would use the system they elucidated. It is, however, likely that at least one more system exists, because Cassaigne et al. (1) reported cleavage of the C-P bond of 3-aminopropylphosphonic acid without prior removal of the amino group. Furthermore, our strain 11 utilizes ionic isopropyl methylphosphonate as a phosphorus source, but this process is not abolished by phosphite, in contrast with the system of La Nauze and Rosenberg (14) and La Nauze et al. (15).

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