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CONTRACTOR REPORT ARCSL-CR-78013

INDUCTION OF MICROBIAL METABOLISM OF ORGANOPHOSPHORUS COMPOUNDS

FINAL REPORT

by

M. Alexander

A. M. Cook

C. G. Daughton

12

December 1977

CORNELL UNIVERSITY

Ithaca, New York 14853

Contract No. DAAA15-76-C-0137

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER ARCSL-CR-78013	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER rept.
4. TITLE (and Subtitle) INDUCTION OF MICROBIAL METABOLISM OF ORGANO- PHOSPHORUS COMPOUNDS.	5. AUTHOR(s) M. Alexander, A. M. Cook C. G. Daughton	6. TYPE OF REPORT & PERIOD COVERED FINAL Oct 1976 - Sept 1977
9. PERFORMING ORGANIZATION NAME AND ADDRESS Cornell University Ithaca, NY 14853		6. PERFORMING ORG. REPORT NUMBER
11. CONTROLLING OFFICE NAME AND ADDRESS Director, Chemical Systems Laboratory Attn: DRDAR-CLJ-R Aberdeen Proving Ground, MD 21010		8. CONTRACT OR GRANT NUMBER(s) DAAA15-76-C-0137 NEW
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) Director, Chemical Systems Laboratory Attn: DRDAR-CLB-CA Aberdeen Proving Ground, MD 21010 (CPO-Johnnie Albizo 671-3850)		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 1T061102A71A02
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited		12. REPORT DATE Dec 1977
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		13. NUMBER OF PAGES 34
18. SUPPLEMENTARY NOTES		15. SECURITY CLASS. (of this report) UNCLASSIFIED
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) P-C bond cleavage Phosphonate degradation Pseudomonas putida Pseudomonas testosteroni Phosphonate analysis Dihydrogen methylphosphonate O-Isopropyl hydrogen methylphosphonate O-Pinacolyl hydrogen methylphosphonate Dihydrogen (2-aminoethyl)phosphonate Phosphonate analysis		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE NA
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Bacterial cleavage of the carbon-phosphorus bond was investigated. Bacteria were isolated that were capable of growing with one or more organophosphonates as sole sources of phosphorus. Cleavage of the C-P bond was shown for Pseudomonas putida growing with 2-aminoethylphosphonate and for Pseudomonas testosteroni growing with isopropyl hydrogen methylphosphonate, pinacolyl hydrogen methylphosphonate or dihydrogen methylphosphonate as sole phosphorus sources. Various metabolites were identified by gas chromatography and mass spectrometry.		

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19. Keywords

Phosphonates
Alkylphosphonates
Isopropanol
Pinacolyl alcohol
Alcohol analysis
Methane

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PREFACE

The work described in this report was authorized by project IT061102A-71A02, Life Sciences Basic Research in Support of Material. It was performed under contract DAAA15-76-C-0137, Induction of Microbial Metabolism of Organophosphorus Compounds. Work was carried out from October 1976 to September 1977.

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SUMMARY

This investigation was conducted to obtain induced bacterial isolates capable of degrading alkylphosphonates.

Bacteria able to use at least one of 13 ionic alkylphosphonates or O-alkyl or O,O-dialkyl alkylphosphonates as phosphorus source 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.

Methane was produced in stoichiometric yield from the cleavage of the C-P bond of methylphosphonates by Pseudomonas testosteroni, an aerobic bacterium utilizing methylphosphonates as sole phosphorus sources. This is the first direct evidence of enzymatic cleavage of the C-P bond of simple aliphatic alkylphosphonates. A new mechanism for aerobic methane formation is indicated.

O-Isopropyl hydrogen methylphosphonate (IMP) and O-pinacolyl hydrogen methylphosphonate (PMP) in aqueous solution were derivatized by extractive methylation using diazomethane and quantified by gas chromatography. Isopropanol, pinacolyl alcohol and methane were quantified by gas chromatography.

Pseudomonas testosteroni degraded PMP to yield pinacolyl alcohol, which was identified by combined gas chromatography-mass spectrometry. Isopropanol was tentatively identified as a product of the metabolism of IMP. It was concluded that bacteria can be easily obtained from sewage that are capable of cleaving the carbon-phosphorus bond of ionic organophosphonates. Enrichments for phosphonate-phosphorus-utilizing bacteria must be designed considering a maximum phosphorus concentration of 0.1 mM and a carbon to phosphorus molar ratio of about 300:1. Pseudomonas putida grew with hydrogen 2-aminoethylphosphonate as a sole carbon, nitrogen and phosphorus source, releasing excess phosphorus as orthophosphate. Pseudomonas testosteroni cleaved the carbon-phosphorus bond of methylphosphonates with the concomitant release of equimolar amounts of methane. Pinacolyl alcohol was also identified as a metabolite of pinacolyl hydrogen methylphosphonate.

It was recommended that future work on bacterial utilization of organophosphonates as sole phosphorus sources must consider the proper concentration of phosphonate to assure that the phosphorus is limiting, that the substrate concentration is not toxic and that growth is not at the expense of contaminating phosphate.

Analytical methods need to be improved for the detection of potential phosphonate metabolites: alcohols, phosphonic acid and ionic alkylphospho-

ates. These methods will allow for the total delineation of the metabolic pathway for phosphonates.

Purification of the methane-releasing phosphonatase and the alcohol releasing esterase from Pseudomonas testosteroni and characterization of their properties should be immediately initiated to evaluate their potential as highly specific assays to distinguish GB acid (IMP) and GD acid (PMP).

An investigation should be initiated to determine the feasibility of using phosphonate-utilizing bacteria or the purified phosphonatase for detoxication of phosphonate residues or the use of continuous culture for demilitarization of nerve gases.

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INDUCTION OF MICROBIAL METABOLISM OF ORGANOPHOSPHORUS COMPOUNDS

I. INTRODUCTION

The investigation of the bacterial metabolism of alkylphosphonates concentrated on (a) the breakdown products of soman and sarin: ionic isopropyl methylphosphonate, $[(CH_3)_2CHO](CH_3)P(O)OH$ (IMP), ionic pinacolyl methylphosphonate, $[(CH_3)_3CCH(CH_3)O](CH_3)P(O)OH$ (PMP) and ionic methylphosphonate, $CH_3PO(OH)_2$ (MP), and (b) the naturally occurring 2-aminoethylphosphonic acid (AEP).

The study was initiated by conducting enrichments for and isolation of bacteria from soil and sewage that were able to utilize these phosphonates and others as phosphorus sources. Subsequent work involved two of the original isolates, now identified as strains of Pseudomonas putida and P. testosteroni. P. putida was found to be capable of utilizing AEP as a sole source of C, N and P. This trait allowed for a more definitive description of AEP metabolism than hitherto reported. This work is described in Part II of this report.

Among the metabolic capabilities of P. testosteroni was the ability to utilize MP, IMP and PMP as sole sources of phosphorus. The investigation focused on the metabolic fates of the phosphonyl methyl group (P-CH₃) and on the alkoxy substituents. The results provide the first unequivocal identification of methane as the metabolite from the phosphonyl methyl moiety. This work is fully described in Part III of this report.

The alkoxy groups are probably removed by hydrolytic cleavage. Pinacolyl alcohol has been positively identified as a metabolite of PMP by combined gas chromatography-mass spectrometry. Isopropyl alcohol was tentatively identified as a metabolite of IMP.

The procedures for identification and quantification of these alcohols and for MP, IMP and PMP are described in the Appendix (Part IV). Analysis for MP will hopefully elucidate the order of removal of the phosphonomethyl and alkoxy groups from IMP and PMP. The mechanism of the C-P bond cleavage is probably hydrolytic or reductive. A reductive cleavage would yield phosphonic acid, $HP(O)(OH)_2$, as the initial metabolite of MP, and a tentative procedure for the analysis of phosphite is also given in the Appendix. Using these procedures in the forthcoming period, we hope to fully describe the metabolism of MP, IMP and PMP.

II. MICROBIAL UTILIZATION OF PHOSPHONATES

INTRODUCTION

Alkylphosphonates, compounds with a C-P bond, occur widely in nature either free or combined in lipids¹, polysaccharides², or proteins³. 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^{4,5}.

With certain exceptions, the C-P bond is highly resistant to chemical hydrolysis⁶, thermal decomposition⁶ and photolysis⁷; 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 microorganisms^{8,9}, some of which use phosphonates solely as phosphorus sources or sometimes as nitrogen sources^{10,11}. Evidence for cleavage of the C-P bond rarely has been obtained, but one phosphonate (2-phosphonoacetaldehyde phosphohydrolase EC 3.11.1.1) has been described in detail^{11,12}, and preliminary evidence for another has been published¹³.

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 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-piperazineethane sulfonic acid] were obtained from Calbiochem, San Diego, Calif. Isopropyl methylphosphonate (Na salt), pinacolyl methylphosphonate (Na salt) (60% pure; the contaminants were inorganic ions, mainly Na^+ and F^-), and methylphosphonic acid were obtained from Edgewood Arsenal, Aberdeen Proving Ground, Md. Aldrich Chemical Co. (Milwaukee, Wisc.) 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-LaRoche, 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-hr immersion in 20% (v/v) HNO_3 . Nitric acid was removed by thorough rinsing in tap 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-hydroxymethylpropane-1,3-diol) (pH 7.4) or 15 mM Hepes (pH 7.2) and contained (per liter): KCl, 0.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $(\text{NH}_4)_2\text{SO}_4$, 0.5 g; ferric ammonium citrate, 1.0 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 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 urethane 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 bars¹⁴. The cells were harvested by centrifugation at 20,000 X 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 at carbon sources. When serving as the potential carbon source, the phosphonates were present at 0.025 g-atom 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 and Lomb spectrophotometer, model Spectronic 88. Inorganic phosphate was assayed by the method of Dick and Tabatabail¹⁵, and none of the phosphonates and neither of the organic buffers interfered. The assay of Weatherburn¹⁶ 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 Fewson¹⁷. The protein was separated from the organic buffers by precipitation with 0.45 M trichloroacetic acid (final concentration) followed by centrifugation at 20,000 X g at 4°C. The precipitate was resuspended and washed with 0.45 M trichloroacetic acid.

No specific assay exists for AEP⁸. The Fluram assay for amines¹⁸ was unaffected by ammonium ion, but the standard curve from 0 to 10 nmol 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 buffer¹², 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, NY) 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 X 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 Rosenberg¹¹. The complete reaction mixture contained, in 3 ml: Tris buffer (pH 8.5) 300 µmol; MgCl₂, 15 µmol; ethylenediamine tetraacetate (K 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 hr, 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 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 μ g bacitracin/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 to 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 to that on equimolar phosphate, it appeared that the *O,O*-di-alkyl 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 phosphonates).

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.¹⁹.

Strain 11, when grown in 0.1 mM phosphate or phosphonate and excess quantity of carbon source, gave a cell yield (by turbidimetric assay) equal to that obtained in 10 mM gluconate and excess phosphate. This indicates that the carbon demand is 600 times the phosphorus demand. Phosphonate concentrations of

0.1 mM seem to be advantageous in enrichments for phosphonate-phosphorus utilizers because the levels are high enough to reduce the possibility of isolating organisms using phosphate contaminating the enrichment yet low enough to minimize toxicity effects.

Growth of *P. putida* with AEP as carbon, nitrogen and phosphorus source. Strain 5 was chosen for further work, as it clumped less during growth than 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 wall 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°C or 41°C; oxidase positive; arginine dihydrolase positive; ortho ring cleavage of protocatechuate²¹; no growth on geraniol, inositol, galactose, maltose, or trehalose as carbon sources; 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.). Growth continued until about 5.5 hr, as shown by increases in protein concentration. The anomalous increase in turbidity at 5.5 hr 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 ($\mu = 0.55 \text{ hr}^{-1}$). 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 following 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 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*²².

Enzymic cleavage of the C-P bond. Cell-free extracts were prepared from cells of *P. putida* which 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/mg 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 Bacillus cereus¹¹.

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 Rosenberg¹¹ and La Nauze et al.¹², transamination of AEP preceding cleavage of the C-P bond. The latter reaction is inhibited by phosphite.

La Nauze and Rosenberg¹¹ predicted that organisms other than Bacillus cereus would use the system they elucidated. It is, however, likely that at least one more system exists because Cassaigne et al.¹³ 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 Rosenberg¹¹ and La Nauze et al.¹².

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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.

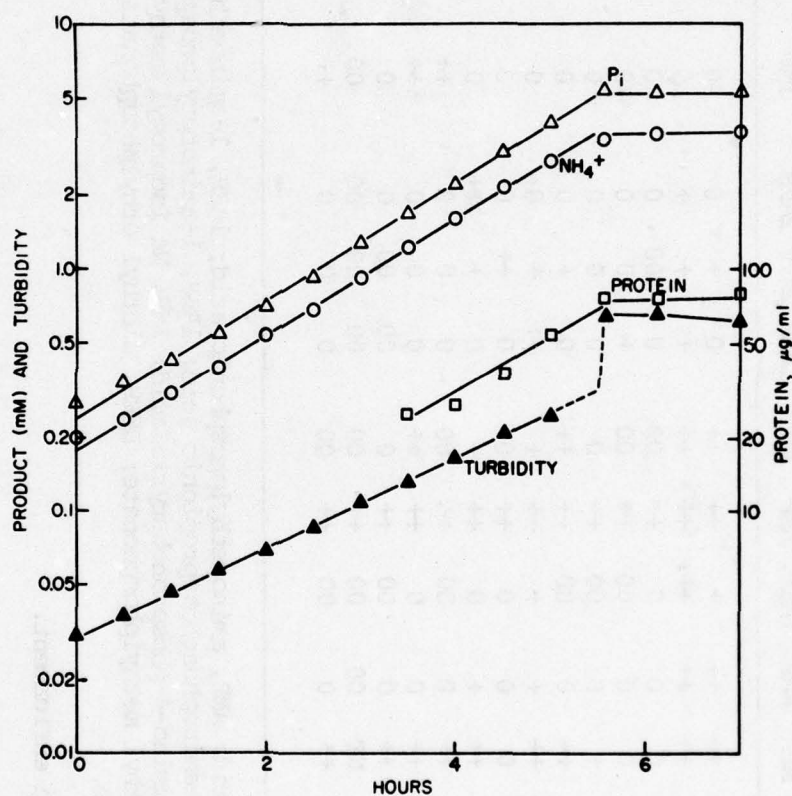


Figure. Growth of and Product Formation by *P. putida* in Medium with AEP as the Sole Source of Carbon, Nitrogen, and Phosphorus.

Table 1. Ability of Isolates to Use Phosphonates as Phosphorus Sources^a

Strain	Phosphorus source in enrichment	Alkylphosphonates ^a								O-Alkyl- phosphonates ^a		O,O-Dialkyl- alkylphosphonates ^a			
		MPA	AMP	LAEP	AEP	3APP	LABP	2APP	2APB	IMP	PMP	DMP	DEEP	DAAP	DEVP
1	AMP	++	++			++	+	0	0	0	0	0	0	0	0
2	LAEP	++	++			++	+	0	0	0	0	0	0	0	0
3	AEP ^b	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	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	LABP	++	+	+	++	+	++	+	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
10	IMP	++	0	00	++	00	0	0	++	0	0	00	+	+	00
11	PMP	++	0	0	++	++	0	0	++	+	0	0	+	+	0
12	DWMP	++	0	00	++	0	00	00	0	00	+	+	+	0	0
13	DEEP	NT	00	00	++	00	00	00	00	00	0	+	+	00	0
14	DAAP	++	0	00	++	00	0	0	++	0	+	+	+	+	0

^aMPA, methylphosphonic acid; AMP, aminomethylphosphonic acid; LAEP, 1-aminoethylphosphonic acid; AEP, 2-aminoethylphosphonic acid; 3APP, 3-aminophosphonopropionic acid; LABP, 1-aminobutylphosphonic acid; 2APP, 2-amino-3-phosphonopropionic acid; 2APB, 2-amino-4-phosphonobutyric acid; IMP, Na isopropyl methylphosphonate; PMP, Na pinacolyl methylphosphonate; DWMP, dimethyl methylphosphonate; DEEP, diethyl ethylphosphonate; DAAP, diallyl allylphosphonate; DEVP, diethyl vinylphosphonate.

^bUsed as carbon source in enrichment.

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.

III. FORMATION OF METHANE FROM METHYLPHOSPHONATES

INTRODUCTION

Organophosphorus pesticides are generally regarded as nonpersistent because of the susceptibility of phosphoesters to hydrolysis, even though the fate of the phosphorus-containing breakdown products in nature has rarely been studied. These products, however, are possibly persistent and toxic¹. We have, therefore, examined the microbial metabolism of a particularly refractory type of phosphorus-containing breakdown product, the ionic alkylphosphonates. The C-P bond in unsubstituted alkylphosphonates is highly resistant to chemical hydrolysis and photolysis, a property that is of environmental significance because compounds containing the C-P bond, such as methylphosphonic acid, have been found as terminal residues in plants and animals that were exposed to certain organophosphonate pesticides and nerve gases^{2,3,4}.

We present here the first direct evidence for the enzymatic cleavage of the C-P bond of synthetic unsubstituted organophosphonates. Previous evidence for cleavage of the C-P bond of simple aliphatic ionic alkylphosphonates has been only indirect; i.e., the observation that some bacteria are capable of using alkylphosphonates as sole phosphorus sources⁵, which presumably indicates utilization of the phosphorus after it is released as orthophosphate. Furthermore, the present data reveal the existence of a new biochemical pathway for the formation of methane, namely, via the aerobic cleavage of the C-P bond of methylphosphonates.

METHODS

A bacterium capable of growth with ionic methylphosphonate, isopropyl methylphosphonate, or pinacolyl methylphosphonate⁶ as sole phosphorus source was isolated from the primary settling tank of a municipal sewage treatment plant. The organism was identified as a strain of Pseudomonas testosteroni^{9,10}.

Methylphosphonic acid and isopropyl methylphosphonic acid were prepared by Chemical Systems Laboratory, Aberdeen Proving Ground, Edgewood, Md. Pinacolyl hydrogen methylphosphonate was prepared from a crude salt mixture⁶ and the resultant organic extract was purified by evaporative bulb-to-bulb distillation. The infrared spectra for isopropyl hydrogen methylphosphonate and dihydrogen methylphosphonate agreed with those published⁷, and the spectrum for pinacolyl hydrogen methylphosphonate agreed with published data⁸. The structures of the methylphosphonates were confirmed by mass spectrometry.

Identification of methane in headspace samples was by coinjection with authentic methane (99.9 percent) on a Perkin Elmer 3920B gas chromatograph equipped with an FID detector and a subambient IN₂ accessory. Samples containing methane gave a single peak which cochromatographed with authentic methane on a 1 m SS column (2 mm ID) packed with 60/80 mesh Durapak (phenylisocyanate on poracil C) (Applied Science Laboratories) at 2°C and -90°C and on a 1.8 m SS column (2 mm ID) packed with 60/80 mesh 5A molecular sieve (Linde, Keasbey, NJ) at 20°C. The identity of the peak was confirmed by using the molecular sieve column interfaced with a Finnigan 3300 quadrupole mass spectrometer (19 eV ionization energy). Quantification of the methane in 200 µl headspace samples was by injection on the Durapak column at 2°C and comparison of peak heights with those obtained from methane standards.

To detect O₂, samples of headspace at the end of the incubation were injected into an anaerobic atmosphere over colorless solutions of methylene blue, and a blue coloration appeared. Injections of N₂ caused no color change.

P. testosteroni was grown in 30 ml of liquid medium on an orbital shaker at 29°C in 250 ml screw-cap glass bottles. The phosphorus-limited growth medium (pH 7.4) contained 50 mM tris buffer, 2.7 mM KCl, 0.8 mM MgSO₄, 3.8 mM (NH₄)₂SO₄, 14 μM CaCl₂, 1 mg/l ferric ammonium citrate, 120 mg-atom/l of the carbon source, and 0.01 to 0.10 mM of the phosphorus source in distilled water. The buffered salts solution was autoclaved, and the sterile carbon and phosphorus sources and the iron and calcium salts were added aseptically. The cultures were sealed with Mininert Teflon sampling valves (Applied Science Laboratories), and samples of the headspace were analyzed for methane until the concentration had stabilized for several days.

RESULTS

P. testosteroni grew rapidly with orthophosphate, ionic methylphosphonate or ionic isopropyl methylphosphonate as the phosphorus source in a gluconate-salts medium. Growth terminated within 24 or 48 hours, depending on the phosphorus concentration. During its growth, the pseudomonad catalyzed the aerobic cleavage of the C-P bond, resulting in release of the phosphorus-bound methyl group as methane (table). Cultures grown with phosphate as the phosphorus source and either gluconate or β-hydroxybutyrate as carbon source generated no methane. Methane was released in amounts equimolar with the supplied phosphorus source in cultures containing ionic methylphosphonate or ionic isopropyl methylphosphonate and the concentration of the methane then remained constant for at least 10 days. The yield of methane from cultures supplied with 3 μmol of ionic isopropyl methylphosphonate was the same whether the carbon source was gluconate, β-hydroxybutyrate, or p-hydroxybenzoate, and the cultures were still aerobic at the end of growth. No methane was produced in uninoculated media containing 3.0 μmol of the phosphorus sources.

Methane was also released when *P. testosteroni* grew with ionic pinacolyl methylphosphonate as phosphorus source in a gluconate-salts medium, but the yield was only about three-fourths of theoretical (table). The incomplete recovery of the methyl group as methane may result from the organism's inability to use all optical isomers of ionic pinacolyl methylphosphonate.

DISCUSSION

Methanogenesis is generally considered as a process of strictly anaerobic bacteria, with the carbon usually coming from CO₂ but sometimes from the methyl group of acetate¹¹. The present study shows that methane can be derived from the cleavage of the C-P bond by an aerobic bacterium. This cleavage process may be either hydrolytic or reductive. To date, the only characterized phosphonatase is a hydrolase from *Bacillus cereus* which cleaves 2-oxoethylphosphonic acid to acetaldehyde and orthophosphate^{12,13,14}. The only previous report of the release of an alkane or arene as a result of the cleavage of a heteroatom-carbon bond by an aerobe is the reductive cleavage of alkyl- and aryl-mercury compounds^{15,16}. Microorganisms are also known to act on the carbon-arsenic bond of methylarsonic acid, a chemical analog of methylphosphonic acid, but in this instance the product released was CO₂¹⁷.

The cleavage of the C-P bond by P. testosteroni represents a newly reported catabolic pathway for methane formation in nature. Moreover, the existence of such a reaction in vitro suggests that the alkylphosphonates may not accumulate in soils or waters which are exposed to synthetic organophosphonates.

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Table. Production of Methane from Methylphosphonates by

Pseudomonas testosteroni

Phosphorus source	Phosphorus supplied (μmol)	Methane formed (μmol)
Orthophosphate	3.0	0.0
Ionic methylphosphonate	0.30	0.29
	1.0	1.0
	3.0	3.2
Ionic isopropyl methylphosphonate	0.30	0.30
	1.0	0.97
	3.0	3.0
Ionic pinacolyl methylphosphonate	2.0	1.6
	3.0	2.3

IV. APPENDIX

ANALYSIS OF IONIC ALKYLPHOSPHONATES

I. Esterified ionic alkylphosphonates

IMP and PMP were analyzed by extractive/derivatization with diazomethane (Daughton, C. G., D. G. Crosby, R. L. Garnas and D. P. H. Hsieh, J. Agric. Food Chem. 24:236, 1976) followed by gas chromatography using a flame photometric detector (GC-FPD).

A. Standards

Methylated standards of isopropyl and pinacolyl hydrogen methylphosphonates were prepared by adding appropriate volumes of 1.0 mg/ml stock standards in ethyl acetate to 5 ml volumetric flasks. Several drops of methanol were added followed by the dropwise addition of ethereal-ethanolic diazomethane. After 10 min, the excess diazomethane was consumed by the dropwise addition of 1% formic acid/ethyl acetate, and methanol was added to volume.

Diazomethane was synthesized from Diazald (N-methyl-N-nitroso-p-toluene-sulfonamide) (Aldrich) using an Aldrich Diazald kit (cat. No. Z10,025-0), following the directions of the manufacturer except that the distillate was collected at -70°C (Caution: diazomethane is highly toxic and explosive). The diazomethane was stable for several months when stored at -20°C in an amber glass bottle with poly-seal screwcap.

B. Samples

To analyze bacterial cultures and supernatant fluids for IMP and PMP, 2 ml samples were added to 12 ml glass centrifuge tubes with Teflon-lined screw caps. NaCl was added in excess of saturation followed by 2 drops of 12.5 N HCl; nitric acid may be better for this phase (see Blair, D. and H. R. Roderick, J. Agric. Food Chem. 24:1221, 1976). Two milliliters of ethyl acetate/methyl-ethylketone (1:1) was added and mixed by vortexing. Diazomethane solution was added (ca. 0.5 ml) and carefully vortexed. With a yellow color persisting in the organic phase, the organic phase was removed with a Pasteur pipet and passed over Na₂SO₄ (anhydrous) into a 5 ml volumetric flask. The extraction/derivatization was repeated, and the organic phases were pooled. Methanol was added to volume.

C. Quantification

A 2 µl syringe was used to inject derivatized samples on one of two columns: (a) 6 ft x 2 mm id glass (treated with 5% DMCS in toluene) packed with 5% OV-210, 80/100 Gas Chrom Q and (b) 6 ft x 2 mm id glass (treated with 5% DMCS in toluene) packed with 5% Carbowax 20M, 80/100 Gas Chrom Q. To reduce tailing, both columns were vapor-phase deposited with 10% Carbowax 20M, 60/80 Chromosorb W, using the technique of N. F. Ives and L. Giuffrida, J. Assoc. Anal. Chem. 53:973, 1970; this involved disconnecting the column from the detector, adding a 2-3 inch packing of Carbowax 20M/Chromosorb W to the silylated glass injector liner and conditioning overnight (He, 10 ml/min; injector, 230°C; column, 225°C).

A Perkin Elmer 3920B gas chromatograph equipped with a flame photometric detector (FPD) (phosphorus filter) was used. The operating parameters were: column, 130-140°C; injector, 200°C; detector, 250°C; He, 30 ml/min; air, 109 ml/min; H₂, 70 ml/min.

D. Results

Recoveries for solutions fortified to final PMP concentrations of 2, 4, 6, 8 and 10 µM were 92, 92, 91, 95 and 99%, respectively. Recoveries for solutions fortified to final IMP concentrations of 2.5, 5.0, 7.5, 10 and 12.5 µM were 84, 85, 89, 88 and 86%, respectively. The lowest analyzable concentration was about 0.15 µM. Neither orthophosphate nor MP can be analyzed by this technique of extractive/methylation.

Using the OV-210 column (operation at 110° for 1 min and then programmed at 4°/min to 135°) gave relative retention times for dimethyl methylphosphonate, trimethylphosphate, O-isopropyl O-methyl methylphosphonate, O,O-diethyl ethylphosphonate and O-pinacolyl O-methyl methylphosphonate of 0.83, 1.0, 1.13, 1.68 and (3.05, 3.23), respectively; the methyl ester of pinacolyl hydrogen methylphosphonate gives two peaks (i.e., 3.05 and 3.23) with this program, probably the result of its optical isomers.

Using the Carbowax 20 M column (operation at 125° for 1 min and the programmed at 4°/min to 145°) gave relative retention times for dimethyl methylphosphonate, O-isopropyl O-methyl methylphosphonate, trimethylphosphate, O,O-diethyl ethylphosphonate and O-pinacolyl O-methyl methylphosphonate of 0.74, 0.74, 1.0, 1.0 and 1.58, respectively.

II. Ionic methylphosphonate

MP was analyzed by a tentative procedure of derivatization of dried samples, forming the benzyl esters, followed by GC-FPD.

A. Derivatizing reagent

3-Benzyl-1-p-tolyltriazene (Aldrich) (BTT) reagent was prepared as a 0.1 M solution in diethyl ether and stored at -20°C (Caution: BTT is a carcinogen). A precipitate forms during storage, but this can be ignored.

B. Derivatization

The sample was dried by either lyophilization or heat (since MP is stable) in a screw cap 2-dram glass vial. To the dry residue, 0.15 ml BTT reagent was added, and the vial was then sealed with a Teflon-lined screw cap and the mixture refluxed at 70°C for 2.75 h. One drop of 6.25 N HCl was added to destroy excess BTT and then 2 ml of NaCl saturated water and 2 ml of ethyl acetate were added. The mixture was shaken well for 30 s, and organic phase over Na₂SO₄ (anhyd.) was pipetted into a 10 ml volumetric flask. The extraction was repeated, and the organic phases were pooled. The solution was brought to volume with ethyl acetate and injected on GC-FPD using the OV-210 column.

C. Results

Using the OV-210 column and operation at 225°C (injector, 250°C), the relative retention times for O-isopropyl O-benzyl methylphosphonate, O-pinacolyl

O-benzyl methylphosphonate and O,O-dibenzyl methylphosphate were 1.0, 1.81 and 4.97, respectively. Orthophosphate did not yield a peak. The reagent blank gave a peak (3 x baseline) with about the same retention time as the benzyl ester of IMP. The derivatization process was apparently independent of MP being protonated.

ANALYSIS OF ALCOHOLS

I. Isopropanol

Two techniques were used for detection of isopropanol (IP): (a) detection by GC-flame ionization (GC-FID) in direct aqueous injections and (b) detection of the nitrous acid ester derivative in carbon disulfide extracts.

A. Direct aqueous injection

Two columns gave promising results: (a) 6 ft SS, 2 mm id (Teflon-lined) packed with 100/120 Chromosorb 101 and vapor-deposited with Carbowax 20 M, and (b) 6 ft SS, 2 mm id (Teflon-lined) packed with 80/100 Porapak QS.

The first column (operated at 70°C for 1 min and then programmed at 32°/min to 170°C) gave retention times for methanol, ethanol and isopropanol of 3.35, 4.35 and 5.0 min, respectively (injector, 100°C; detector, 200°C; He, 35-60 ml/min). The limit of detectability for IP was less than 0.1 mM.

The second column, when operated isothermally at 170-200°C, separated methanol, ethanol and IP (retention time, 2 min) (injector, 230°C; detector, 250°C; He, 58 ml/min).

When culture filtrates were injected onto the columns, interfering peaks were encountered. In contrast to the first column, pinacolyl alcohol could be chromatographed (250°C) and a retention time of 3 min was obtained on the second column. This column was less efficient than the first column.

B. Derivatization

The nitrous acid ester of IP, isopropyl nitrite (IP-N), was formed by the following procedure. To a 1.0-5.0 ml sample of growth medium in a 15 ml glass Sorvall centrifuge tube were added 5 ml of 4.0 M NaNO₂ and 1 ml of 2 N H₂SO₄. The tube was sealed with a polyethylene cap and vortexed. After 5 min, 1 ml of CS₂ (spec. grade, Aldrich) was added, the tube was capped and extracted for 1 min. The organic phase was taken off into a 2 ml volumetric flask. The extraction was repeated, and the pooled organic phase was brought to volume with CS₂. With some samples rich in protein, centrifugation may be necessary to separate the emulsion. Using a 2 µl syringe, injections were made on GC-FID using a 36 ft Teflon (FEP) column, 2 mm id, packed with 12% OS-124 (polyphenyl-ether, 5 ring), 0.5% phosphoric acid, 40/60 Chromosorb T (Note: The Teflon ferrules used for this column will withstand carrier gas delivery pressures only less than 50 psi and flow rates less than 45-50 ml/min.). The operating parameters were: column, 150°C; injector, 150°C; detector, 200°C; N₂, 40 ml/min.

The retention times were 1.8 min for IP-N and 3.2-14 min for CS₂ and impurities. A control containing IMP gave no IP-N peak after derivatization. IP was tentatively identified as a metabolite of IMP and a concentration of approximately 0.4 mM IP was found in a culture fully grown on 0.5 mM IMP. The detectable limit for IP-N was less than 0.1 mM.

II. Pinacolyl alcohol (3,3-dimethyl-2-butanol)

The determination of pinacolyl alcohol (PA) via formation of its nitrous acid ester was not successful. Because of its lower volatility and higher molecular weight, PA was also not amenable to analysis by direct aqueous injection. In contrast to IP, PA has a favorable partition coefficient from water into CS₂; consequently, PA was analyzed by extraction with CS₂ and detection with GC-FID.

A. Analysis

The GC column used was 12 ft SS (2 mm id) packed with 5% DEGS (Stabilized), 80/100 Chromosorb W-HP. Operation at 80°C gave a retention time of 3.5 min for PA and retention times of 1.0-2.5 min for various components of CS₂. The operating conditions were: injector, 160°C; detector, 160°C; He, 44.5 ml/min.

Samples (5 ml) of supernatant fluids from cultures of *P. testosteroni* grown on various concentrations of PMP or orthophosphate as phosphorus source and adipate as carbon source were introduced into 12 ml glass Sorvall centrifuge tubes; 2.5 ml of CS₂ was added to each tube, and the tubes were then sealed with polyethylene caps and shaken for 30 s. The resultant emulsion was separated by centrifugation at 18,000 rpm for 20 min. The organic phase was transferred to a 5 ml volumetric flask. The extraction was repeated, the organic phases were pooled and CS₂ was added to volume.

For quantification, peak heights from unknowns were compared with peak heights from culture media fortified to various concentrations with PA. For GC-MS, the CS₂ extracts were concentrated under nitrogen.

B. Results

The standard curve from PA-fortified samples (0.1-0.4 mM) was linear. The limit of detectability (without preconcentration under N₂) was 0.05 mM. The CS₂ extract of all PMP-grown cultures gave a peak which cochromatographed with PA; no peak was evident in orthophosphate-grown controls. Approximately 0.05 mM PA was detected in a culture grown with 0.1 mM PMP; this represents 50% release of the pinacolyl moiety from the PMP, and these data agree with the results obtained in studies of methane release from PMP (see above). In addition, when PMP-grown cultures were analyzed for residual PMP, approximately 40-60% remained. These results are evidence that the four optical isomers of PMP are attacked at different rates.

The peaks from samples of PMP-grown cells were confirmed to be PA by GC-MS. The DEGS column was interfaced with a Finnigan 3300 quadrupole mass spectrometer and System's Industries data system (column, 80°C; He, 15 ml/min). The mass spectrum of PA (*m/e* values and relative intensities >8%): 39 (19.2), 41 (71.2), 43 (19.9), 45 (60.4), 56 (55.2), 57 (100), 69 (74.3), 84 (12.8), 87 (34.6), *M* = 102 (2.4). These results also show that 2,3-dimethyl-2-butanol was not present. The mass spectrum for the metabolically-formed PA agreed with that of authentic PA (Aldrich) and with the spectrum published in the Registry of Mass Spectral Data (p. 97).

METABOLISM OF THE PHOSPHONYL MOIETY OF METHYLPHOSPHONATES

The mechanism of release of the methyl group from ionic methylphosphonate is presumably either reductive or hydrolytic. On the one hand, hydrolytic

cleavage would yield either metaphosphate or orthophosphate from the enzyme surface. Metaphosphate would spontaneously hydrate to yield orthophosphate. On the other hand, reductive cleavage would produce phosphonic acid (phosphite).

A tentative procedure was examined for determination of phosphite by forming phosphonic acid followed by reduction to phosphine and analysis by GC-FPD. For the analysis of phosphite, a 4.5 ml sample was added to a 20 ml culture tube, and the pH was adjusted to 0.7 with conc H_2SO_4 . Zinc dust was added, the tube (in the hood) was immediately sealed with a serum stopper and the mixture was vortexed (Caution: phosphine is highly toxic). After 60 min, the headspace was sampled with a gas-tight syringe (with sampling valve), and 100-800 μl samples were injected on GC-FPD (phosphorus filter) equipped with a 6 ft SS Teflon-lined (2 mm id) column packed with 100/120 Chromosorb 101 (column, 80°C ; detector, 200°C ; injector, 100°C ; He, 40 ml/min). Samples with standard $\text{Na}_2\text{HPO}_3 \cdot 5\text{H}_2\text{O}$ gave peaks which chromatographed with standard phosphine (generated from magnesium phosphide). The retention time was 1.0 min. A second peak which occurs at 1.5 min disappeared as the reaction progressed. The limit of detectability using a 4.5 ml sample with 15 ml headspace (800 μl injection) was 0.1 mM.

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