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Bacterial Conversion of Alkylphosphonates to Natural Products via Carbon–Phosphorus Bond Cleavage

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The phosphorus-containing breakdown products of *O*-alkyl alkylphosphonate toxicants, which are particularly resistant to cleavage at the C–P bond, were fully degraded to natural products by *Pseudomonas testosteroni*. When present as a sole and limiting phosphorus source, an *O*-alkyl alkylphosphonate was attacked aerobically via release of the alkoxy group as the alcohol, followed by cleavage of the alkyl–phosphorus bond (methyl, ethyl, or propyl) to produce the respective alkane and an inorganic phosphorus compound that was detected as inorganic orthophosphate. The bacterium could not cleave the bonds of other carbon–heteroatoms (e.g., arsonates, sulfonates, and mercurials). This is the first report of the metabolism of simple, aliphatic alkylphosphonates and the first pathway described for an organophosphorus toxicant to yield exclusively natural products (i.e., alcohols, alkanes, and phosphate).

The carbon–phosphorus bond is an exceedingly nonreactive constituent of unsubstituted alkyl- and arylphosphonate insecticides, herbicides, fungicides, nerve gases, flame retardants, and several other economically important categories of chemicals. Reviews of the metabolism of phosphonates have considered the terminal phosphorus-containing residues in animals and plants and emphasize that the existing evidence indicates that the C–P bond of methyl-, ethyl-, and phenylphosphonates resists cleavage by higher organisms (Menn, 1971; Menn and McBain, 1974). To our knowledge, the only reported exception to the inability of higher organisms to cleave this bond is the finding that rice plants cleave ionic *O*-ethyl phenylphosphonothioate, a hydrolytic product of Inezin, yielding ionic *O*-ethyl phosphorothioate (Endo et al., 1970); however, the authors did not eliminate the possibility of microbial attack.

Cook et al. (1978a) reported and reviewed the microbial utilization of representative classes of phosphorus-containing breakdown products of organophosphorus pesticides. They concluded that only one previous report gave definitive evidence of extensive utilization of a chemically stable organophosphorus compound (i.e., dimethyl hydrogen phosphate). The first direct evidence for the biological cleavage of the C–P bond of alkylphosphonates has recently been obtained (Daughton et al., 1979b), and the present study characterizes the metabolic pathway and its control. This is the first account of the complete metabolism to natural products of an organophosphorus xenobi-

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Table I. Ionic Organophosphonates

abbreviation	chemical name	chemical formula
MPn	dihydrogen methylphosphonate[a]	$CH_3P(O)(OH)_2$
IMPn	*O*-isopropyl hydrogen methylphosphonate[a]	$[(CH_3)_2CHO]CH_3P(O)OH$
PMPn	*O*-pinacolyl hydrogen methylphosphonate[a,e]	$[(CH_3)_3CCH(CH_3)O]CH_3P(O)OH$
EPn	dihydrogen ethylphosphonate[b]	$CH_3CH_2P(O)(OH)_2$
EEPn	*O*-ethyl hydrogen ethylphosphonate[b]	$(CH_3CH_2O)CH_3CH_2P(O)OH$
EETPn	*O*-ethyl hydrogen ethylphosphonothioate[b]	$(CH_3CH_2O)CH_3CH_2P(S)OH$
PrPn	dihydrogen propylphosphonate[c]	$CH_3CH_2CH_2P(O)(OH)_2$
EPrPn	*O*-ethyl hydrogen propylphosphonate[c]	$(CH_3CH_2O)CH_3CH_2CH_2P(O)OH$
AEPn	dihydrogen 2-aminoethylphosphonate[d]	$NH_2CH_2CH_2P(O)(OH)_2$

[a] Chemical Systems Laboratory, Aberdeen Proving Ground, MD. [b] Stauffer Chemical Co., Richmond, CA. [c] FMC Corporation, Middleport, NY (NIA 10656 and NIA 10637, respectively). [d] Sigma Chemical Co., St. Louis, MO. [e] 3,3-Dimethyl-2-yl hydrogen methylphosphonate.

otic, in this instance, one generated by facile hydrolysis of the chemical warfare agents sarin or soman.

EXPERIMENTAL SECTION

Substrates, Metabolites, and Reagents. The organophosphorus compounds used and their abbreviations, formulas, and sources are given in Table I. The free acid of PMPn was isolated from a crude salts mixture using the method of Harris et al. (1964). The resultant organic extract was purified by evaporative bulb-to-bulb distillation (116 °C, 67 mPa); a nearly colorless, viscous oil was collected at 25 °C. The IR spectra were obtained for IMPn and PMPn as neat liquid films and, for MPn (mp 94–102 °C), as a solid film between NaCl plates. The IR spectra matched those published for IMPn and MPn (Sadtler Research Laboratories, 1966) and the IR spectrum for PMPn agreed with published data (Thomas, 1974).

Anal. Calcd for MPn (CH_5O_3P): C, 12.5; H, 5.2; P, 32.3. Found: C, 12.3; H, 5.5; P, 32.5. Calcd for IMPn ($C_4H_{11}O_3P$): C, 34.8; H, 8.0; P, 22.4. Found: C, 35.0; H, 7.9; P, 22.4. Calcd for PMPn ($C_7H_{17}O_3P$): C, 46.7; H, 9.5; P, 17.2. Found: C, 46.7; H, 9.7; P, 17.4.

Mass spectra for MPn, IMPn, PMPn, EPn, and EEPn were obtained with a Finnigan 3300 Quadrupole mass spectrometer and Systems Industries 150 Data System by electron impact ionization (70 eV) and direct probe insertion. For the following mass spectra, at least the two most intense ions are presented for every 14 mass units above m/z 34 (rel intensity > 1.0). MPn, 45 (3.8), 47 (9.9), 48 (5.9), 55 (3.4), 65 (6.2), 69 (2.8), 78 (33.4), 79 (16.0), 80 (4.1), 81 (100.0), 96 (M^+, 38.6), 97 (7.3). IMPn, 44 (7.3), 45 (6.8), 47 (9.9), 48 (5.3), 59 (1.9), 65 (2.6), 78 (2.9), 79 (32.4), 80 (6.1), 96 (2.8), 97 (100.0), 123 (20.4), 138 (M^+, 1.1), 139 (20.4). PMPn, 39 (3.4), 41 (8.2), 45 (2.0), 55 (3.1), 57 (2.4), 65 (5.6), 69 (16.2), 79 (14.8), 80 (42.0), 96 (3.4), 97 (100.0), 123 (4.1), 124 (20.6), 175 (5.2), 180 (M^+, --), 181 (2.9). EPn, 43 (2.7), 47 (8.2), 63 (3.8), 65 (18.0), 81 (17.5), 82 (100.0), 91 (9.2), 93 (2.4), 109 (9.5), 110 (M^+, 6.6), 111 (16.0). EEPn, 43 (9.4), 45 (7.6), 57 (2.8), 63 (1.7), 65 (38.4), 81 (20.7), 82 (39.0), 93 (42.8), 94 (18.5), 110 (13.3), 111 (100.0), 123 (5.1), 127 (6.8), 138 (M^+, 6.3), 139 (36.7). The base peaks (m/z 97) for IMPn and PMPn verified that they were methylphosphonates. The data for MPn agreed with the tabular data of Griffiths and Tebby (1975), and those for IMPn were consistent with the mass spectrum of diisopropyl methylphosphonate (Occolowitz and White, 1963). The mass spectrum of EEPn (base peak of 111) proved that it was an ethylphosphonate. The mass spectra for all of these phosphonates were consistent with their structures, all yielding M + 1 peaks.

Methane, ethane, ethene, ethyne, propane, and propene at greater than 99.97, 99.0, 99.5, 99.6, 99.0, and 99.5% purity, respectively, were obtained from Linde, Keasbey, NJ. Pinacolyl alcohol (3,3-dimethyl-2-butanol, 99% purity), chloramphenicol, 2-bromoethylsulfonic acid (Na salt), and CS_2 (spectrographic grade) were obtained from Aldrich Chemical Co., Metuchen, NJ. Isopropyl alcohol (electronic grade) was purchased from Fisher Scientific Co., Fair Lawn, NJ. Dihydrogen methyl phosphate (di–monocycloammonium salt), sodium dimethylarsinate, and DL-methionine were obtained from Sigma Chemical Co., St. Louis, MO. Sodium methylarsonate was from Alfa Inorganics, Beverly, MA. *O*-Ethyl methylsulfonate was obtained from Eastman Kodak Co., Rochester, NY. Methylmercury chloride and ethylmercury chloride (standard references no. 4560 and 3400, respectively) were supplied by Environmental Protection Agency, Research Triangle Park, NC.

Analysis and Identification of Metabolites. Spectrophotometric assays were done using a Beckman DBG or a Bausch and Lomb Spectronic 88 spectrophotometer equipped with a micro flow-through cell of 1-cm pathlength. Gas chromatography (GC) was done with a Perkin-Elmer 3920 B chromatograph equipped with a flame ionization detector (FID) or with a flame photometric detector (FPD) (phosphorus mode). The identity of a compound, tentatively assigned by cochromatography with GC, was confirmed by obtaining mass spectra with a Finnigan 3300 Quadrupole mass spectrometer equipped with a Systems Industries 150 Data System.

The determination of isopropyl alcohol was done using GC–FID by direct aqueous injection and by formation of the nitrous acid ester, isopropyl nitrite. A 1.8 m × 2 mm i.d. stainless steel Teflon-lined column packed with 100/120 mesh Chromosorb 101 and vapor-phase deposited with Carbowax 20M was used for direct aqueous injection. The oven was programmed to 170 °C at 32 °C/min after 1 min at 80 °C and the helium flow rate was 35–60 mL/min. The limit of detection was 1 nmol. Methanol, ethanol, and isopropyl alcohol had nonadjusted retention times (t_R) of 3.35, 4.35, and 5.0 min, respectively. The nitrous acid ester of isopropyl alcohol was determined using a 10.8 m × 2 mm i.d. Teflon (FEP) column packed with 12% OS-124 (polyphenyl ether, 5 ring), 0.5% phosphoric acid, on 40/60 mesh Chromosorb T, run at 150 °C with nitrogen at 40 mL/min. Portions of bacterial cultures were passed through filters (0.2-μm pore size membranes), and 3.0 mL of the filtrate, 5 mL of 4.0 M $NaNO_2$, and 1 mL of 1.0 M H_2SO_4 were added to 12-mL glass centrifuge tubes; the tubes were sealed with polyethylene caps, and the contents were mixed. After 5 min, 1 mL of CS_2 was added, and the tube was stoppered and mixed for 30 s. The organic phase was transferred to a 2-mL volumetric flask, the extraction was repeated, and the organic phases were pooled and brought to 2.0 mL with CS_2. The organic phase contained the isopropyl nitrite (t_R of 1.8 min and limit of detection of 0.2 nmol). The CS_2 gave a broad peak from 3.2–14.0 min. For mass spectrographic identification of isopropyl alcohol,

direct aqueous injections were made on the Chromosorb 101 column, which was programmed to 170 °C at 20 °C/min after 1 min at 100 °C, and charge-exchange (chemical ionization) with helium was done at a source pressure of 133 Pa.

For the analysis of pinacolyl alcohol, 5-mL samples from supernatant fluids of cultures were extracted twice in 12-mL glass centrifuge tubes with 2.5 mL of CS_2, the emulsions being broken by centrifugation at 39000*g* for 15 min. The organic phases were pooled and brought to 5.0 mL. Pinacolyl alcohol was determined by GC–FID with a 3.6 m × 2 mm i.d. stainless steel column packed with 5% DEGS (stabilized) on 80/100 mesh Chromosorb W-HP that was programmed to 95 °C at 32 °C/min after 2 min at 80 °C with nitrogen or helium at a flow rate of 30–40 mL/min. The limit of detection was less than 0.1 nmol. The t_R values for CS_2 and pinacolyl alcohol were 1.0 and 2.7 min, respectively. The recovery of pinacolyl alcohol from growth medium fortified to 0.25 mM was 75%. For mass spectrographic identification, CS_2 extracts were concentrated 14-fold under a stream of dry nitrogen; the oven was operated at 80 °C with a helium flow rate of 15 mL/min, and the ionizing voltage was 70 eV.

Inorganic orthophosphate (Pi) was determined by the reduced heterophosphomolybdate method of Dick and Tabatabai (1977) with the procedural stipulation made by Cook et al. (1978b). This assay does not respond to labile phosphorus compounds. Total labile nonphosphonate phosphorus (e.g., esters, phosphite, etc.) was determined by a modified Bartlett procedure using the high molybdate concentration (Cook et al., 1978b). This involved developing the heterophosphomolybdate complex for 1 h instead of 10 min; the modified procedure gives complete conversion of phosphite to Pi. Cellular polyphosphate was extracted as Pi as described by Kaltwasser (1962), and the Pi was determined by the modified Bartlett procedure. Total phosphorus in cell pellets was determined using the procedure of Cook et al. (1978b).

The headspace of bacterial cultures was assayed for alkanes by GC–FID. Quantitation was by injection of 200-μL samples with a 1-mL gas-tight syringe onto a 1 m × 2 mm i.d. stainless steel column packed with 80/100 mesh Durapak (phenylisocyanate on Porasil C) (Applied Science Laboratories, Inc., State College, PA) at 23 °C, and interpolation from the linear regression equations generated from peak heights obtained from alkane standards (1.9–38.5 Pa). Standards were prepared by serial dilution at 101.3 kPa in 250-mL screw-cap glass bottles fitted with Mininert Teflon sampling valves. The moles (n) of alkanes in the culture headspaces were calculated from the ideal gas equation, $n = PVR^{-1}T^{-1}$, where P was pressure (Pa) determined by interpolation from the standard curves, V the headspace volume (L) (the water solubilities of methane and ethane are insignificant), T was the temperature in K of the experimental culture, and R the calculated gas constant (8295.5, 8273.9, and 8290.4 $L \cdot Pa \cdot K^{-1} \cdot mol^{-1}$ for methane, ethane, and propane, respectively). The t_R values for methane, ethane, ethene, propane, ethyne, and propene were 0.58, 0.77, 0.95, 1.23, 1.73, and 2.52 min, respectively, with base line resolution. The limits of detection for methane and ethane were less than 0.04 nmol.

For tentative confirmation of methane and ethane in the headspace, the samples were cochromatographed with authentic standards on a 1.8 m × 2 mm i.d. stainless steel column packed with 60/80 mesh 5 Å molecular sieve at 20 °C for methane or 150 °C for ethane. Cochromatography was also determined on the Durapak column at 2 °C and −90 °C (using a subambient accessory) for methane or at 23 °C for ethane and propane. Mass spectrometry of methane and ethane was done with the 5 Å molecular sieve column at temperatures of 30 and 150 °C, the helium carrier gas at flow rates of 20 and 15 mL/min, and electron impact ionization voltages of 18 and 19 eV, respectively.

A derivatization procedure was developed for the determination of MPn (Daughton et al., 1979c). The dibenzyl ester of MPn was quantitatively formed by benzylation with 3-benzyl-1-*p*-tolyltriazene (Aldrich Chemical Co.). (**Caution**: 3-benzyl-1-*p*-tolyltriazene is a suspect carcinogen.) The mass spectrographic identification of the benzyl derivatives was done on a 1.5 m × 2 mm i.d. glass column packed with 3% QF-1 on 80/100 mesh Gas-Chrom Q that was programmed from 140 to 225 °C at 15 °C/min with helium as the carrier gas and charge exchange with methane at a source pressure of 133 Pa.

IMPn and PMPn were determined by the combined derivatization/extraction procedure described by Daughton et al. (1976) using 1:1 ethyl acetate/methyl ethyl ketone as the extraction solvent. The methyl esters were analyzed by GC–FPD using a 1.8 m × 2.0 mm i.d. glass column that was silanized with 5% DMCS (dimethyldichlorosilane) in toluene and packed with 5% OV-210 on 80/100 mesh Gas-Chrom Q. The recoveries for 2–10 μM PMPn and 2–12 μM IMPn were 91–99% and 84–89%, respectively, with the limit of detection about 0.2 pmol.

Samples (5.0 mL) of culture headspace were checked for the presence of molecular oxygen by injection into 65-mL bottles sealed with Mininert valves and containing 5 mL each of 6 mM NaOH, 0.015% methylene blue in water, and 6% glucose in water. The solution had been made anoxic by boiling under a stream of N_2 until colorless (Conn, 1957). Immediate appearance of a blue coloration on addition of sample confirmed that the culture was not anoxic. The addition of N_2 caused no color change.

Growth assayed as turbidity or protein was measured by published procedures (Cook et al., 1978a,c).

Glassware. Glassware was cleaned by steeping in 5 M HNO_3 to reduce contaminative phosphorus to levels that would not support bacterial growth as detected by change in turbidity in media not fortified with phosphorus (Cook et al., 1978c).

Organism. The strain of *Pseudomonas testosteroni* used (Daughton et al., 1979a) could obtain phosphorus but not carbon for growth from several alkylphosphonates. It was grown in a salts solution buffered with Tris (tris(hydroxymethyl)methylamine) (Cook et al., 1978a) and supplemented with carbon and phosphorus sources. Inocula for experiments were prepared by growing cells from stock cultures in 30 mL of nutrient broth contained in a 250-mL Erlenmeyer flask shaken for 24 h at 30 °C. Portions (0.1 mL) of these cultures were inoculated into 30 mL of buffered salts medium (3% v/v) containing *p*-hydroxybenzoate as carbon source and one growth-limiting (0.1 mM) phosphorus source, and this culture was grown on the shaker at 30 °C. To obtain culture headspace for analysis, the bacteria were grown on a shaker at 30 °C in 30 mL of medium in 250-mL screwcap bottles closed with Mininert Teflon sampling valves. The medium contained limiting amounts of added phosphorus (0–0.11 mM) and 120 mg-atom/L of the carbon source. When the effect of chloramphenicol (10 μM) was studied, the culture used as inoculum was harvested by centrifugation, the cells were suspended in buffered salts solution (0.1 volume), and a 30% inoculum was used.

Growth experiments to study substrate utilization and the formation of metabolic products were done at 30 °C with 300 mL of medium in 1-L side-arm flasks using the

apparatus of Harvey et al. (1968). Samples were taken at intervals for the determination of turbidity. The bacteria were removed by centrifugation at 20000*g* for 10 min at 4 °C, and the supernatant fluid was used for analyses. When large amounts of cell material were required, 800 mL of culture was grown at 30 °C in the side-arm flask. The medium (Cook et al., 1978a) gave yields of about 1 mg of bacterial protein/mL with 1 mM phosphorus source and 50 mM *p*-hydroxybenzoate as carbon source. Cultures in the late logarithmic phase of growth were harvested by centrifugation at 20000*g* for 10 min at 4 °C. The cells were washed in buffered salts solution and stored at −20 °C until required for studies of cell extracts and cell suspensions.

To screen for organophosphorus compounds serving as sources of carbon, phosphorus, or nitrogen, *P. testosteroni* was grown with the required nutrient limitation, and these induced cells were the inocula for 30 mL of medium containing the test substance. Phosphorus was supplied at 0.1 mM, nitrogen at 2.5 mM, and carbon at 20 mg-atom/L.

Cell Suspensions and Cell-Free Extracts. Cells stored at −20 °C were thawed, suspended in extraction buffer (4 °C) that contained 0.1 mM dithiothreitol and 50 mM Tris (pH 7.5) or 50 mM histidine (pH 6.0), washed three times, and then resuspended to 250 mg wet weight/mL of the buffer. These cells were disrupted by two passages through an ice-cold French pressure cell (Aminco, Silver Spring, MD) at about 130 MPa, a treatment that reduced viable counts by about 99.8%. The suspension of disrupted cells was centrifuged at 40000*g* for 30 min at 4 °C, and the clear supernatant fluid was used for assays of enzyme activity. On occasion, the cell debris was resuspended in extraction buffer and assayed for enzyme activity.

Cell suspensions or cell-free extracts were used to study the cleavage of the isopropoxy group from IMPn. The complete reaction mixture contained 200 μmol of Tris buffer (pH 8.5), 10 μmol of $MgCl_2$, 1 μmol of EDTA, 1 μmol of dithiothreitol, 5 μmol of IMPn, and either cell extract (ca. 20 mg of protein) or cell suspension (250 mg wet weight of cells) in a final volume of 2.0 mL. The reaction was started by the addition of protein (or cell suspension), and assays were done at 30 °C. Reaction mixtures were sampled for isopropanol and MPn. Isopropyl alcohol (by direct aqueous injection) was determined directly from the mixtures. For the other determinations, the biological material was removed. Cell suspensions were centrifuged at 20000*g* for 10 min at 4 °C, and the supernatant fluid was used for the determinations. Protein in the reaction mixtures was precipitated at 4 °C by the addition of 0.16 mL of 70% (w/w) perchloric acid (4 °C). After 10 min on ice, the precipitate was removed by centrifugation at 40000*g* for 20 min at 4 °C. The supernatant fluid was neutralized with cold KOH, the resultant $KClO_4$ was removed by centrifugation at 10000*g* for 10 min at 4 °C, and the neutral supernatant fluid was used for the determinations.

Phosphonatase was assayed by the release of methane from MPn in 3-mL assay mixtures in 13-mL tubes sealed with serum stoppers. The assay of Cook et al. (1978c) was used with AEPn replaced by MPn and with 20 mg of protein per assay.

RESULTS AND DISCUSSION

Utilization of *O*-Alkyl Alkylphosphonates as Phosphorus Sources. At the end of growth of *P. testosteroni* in media containing about 0.1 mM IMPn or 0.1 mM PMPn as sole phosphorus source, no measurable IMPn remained, whereas about 40% of the PMPn was not utilized. Similar data were obtained by determining extracellular phosphorus before and after growth (Cook et al., 1978a). *P. testosteroni* did not grow in buffered salt solution containing 10 mM gluconate as a carbon source but without an added source of phosphorus. These data confirm that IMPn and PMPn are used as phosphorus sources by *P. testosteroni*. MPn, IMPn, and PMPn did not serve as carbon sources for the organism.

Table II. Mass Spectral Data for Pinacolyl Alcohol from PMPn

	relative intensities		
	pinacolyl alcohol		
m/z	published[a]	authentic std	unknown[b]
41	40.0	66.5	71.2
45	79.0	49.6	60.4
56	63.0	44.1	55.2
57	100.0	100.0	100.0
69	20.0	70.3	74.3
84	<1.0	8.9	12.8
87	25.0	32.8	34.6
101	<1.0	1.2	0.3
102 (M)	3.0	2.7	2.4

[a] Registry of Mass Spectral Data, Vol. I, p 97. [b] From culture grown on PMPn.

Cleavage of the Alkoxy Bond of *O*-Alkyl Alkylphosphonates. To determine if pinacolyl alcohol was formed from *O*-pinacolyl methylphosphonate, *P. testosteroni* was grown in buffered salt solution containing 60 mM adipate and 0, 0.25, or 0.5 mM PMPn or 0.5 mM Pi as phosphorus source. When cultures reached the stationary phase, the supernatant fluid was analyzed for pinacolyl alcohol. None was found in cultures lacking PMPn. Extracts from PMPn-grown cultures assayed by GC gave one peak, which cochromatographed with authentic pinacolyl alcohol. The mass spectral data are summarized in Table II. The results confirm that pinacolyl alcohol (M_r = 102) is released by *P. testosteroni* during growth, presumably from the pinacolyl moiety of PMPn. The results demonstrate that the cleavage product is not 2,3-dimethyl-2-butanol, which would have yielded a base peak at m/z 59 instead of m/z 57 as a result of α-carbon cleavage. The latter alcohol along with 2,3-dimethyl-1-butene have been reported as the only dealkylation products released from the aging of soman-inhibited eel cholinesterase (Michel et al., 1967). The concentration of pinacolyl alcohol released in cultures supplied with 0.25 and 0.50 mM PMPn was about 0.1 and 0.2 mM, respectively. The incomplete yield of pinacolyl alcohol from PMPn correlated with the incomplete utilization (40% remaining) of PMPn as a limiting phosphorus source.

P. testosteroni grew well in the salts solution containing 1 mM IMPn and 60 mM adipate, but only ca. 0.06 mM isopropyl alcohol was detected at the end of growth. Therefore, esterase activity was assayed in cell suspensions and cell extracts. Reaction mixtures from cells grown with Pi as phosphorus source formed no isopropyl alcohol. No isopropyl alcohol was observed in mixtures lacking IMPn but containing cells or extracts of cells grown with IMPn; isopropyl alcohol was recovered from isopropyl alcohol fortified mixtures. Reaction mixtures of cells or extracts of cells, which had been induced to grow on IMPn as phosphorus source, produced a compound from IMPn that gave a single peak; this peak cochromatographed with authentic isopropyl alcohol. The mass spectral data for isopropyl alcohol are summarized in Table III. The results confirm that the isopropyl moiety of IMPn was released as isopropyl alcohol (M_r = 60) by a soluble enzyme (system) of *P. testosteroni*. Yields of isopropyl alcohol of about 2 and 5 mM were obtained from 2.5 and 5.0 mM IMPn,

Table III. Mass Spectral Data for Isopropyl Alcohol from IMPn

	relative intensities		
m/z	authentic isopropyl alcohol	cell extract[a]	whole cells[a]
39	67.0	69.5	65.9
40	14.4	10.8	11.6
41	35.1	33.0	32.5
42	30.0	44.6	42.3
43	100.0	100.0	100.0
44	15.0	7.5	8.1
45	87.8	52.8	54.1
59	4.1	3.4	3.1
60 (M)	1.6	0.1	0.2

[a] From culture grown on IMPn.

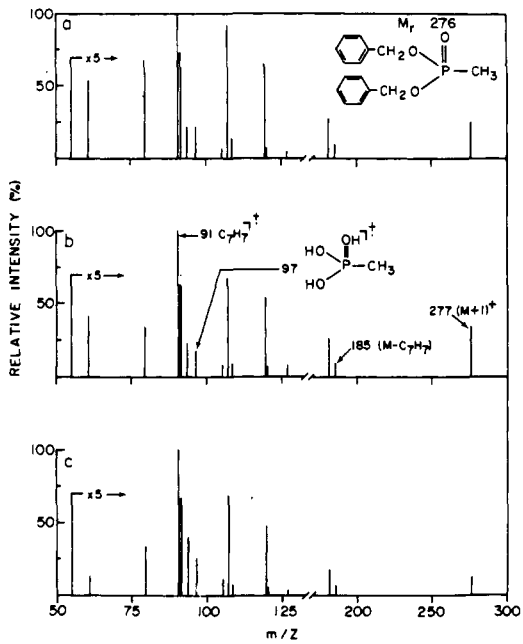

Figure 1. Mass spectra (chemical ionization) of benzylated derivatives of (a) authentic MPn, (b) product formed from IMPn by *P. testosteroni*, and (c) product formed from IMPn by cell extracts of *P. testosteroni*.

respectively, after incubation with cells or enzyme preparations from *P. testosteroni*. The quantitative yield of isopropyl alcohol from IMPn correlated with the complete utilization of IMPn as a limiting phosphorus source.

The reaction mixtures assayed for isopropyl alcohol were also assayed for MPn formation from IMPn. Controls without biological material gave no peaks, apart from benzylated IMPn in samples fortified with IMPn or benzylated MPn in samples fortified with MPn. IMPn was incubated with cells or extracts of cells grown with IMPn as phosphorus source, and a compound was detected whose benzyl derivative gave a peak by GC–FPD that cochromatographed with authentic samples of MPn treated in parallel. The mass spectra for these samples are shown in Figure 1. Charge exchange gave quasiparent peaks (M + 1 = 277) for the benzyl derivative of MPn, *O,O*-dibenzyl methylphosphonate. The mass spectra are dominated by that of the benzyl group, the base peak being $m/z = 91$ (tropylium ion); extensive rearrangement occurred, with combination of two benzyl fragments and ejection of the phosphinyl radical ($m/z = 181$). The spectra conclusively demonstrate that MPn was a phosphorus-containing product released upon cleavage of the isopropoxy group from IMPn.

Table IV. Production of Alkanes from Alkylphosphonates by *Pseudomonas testosteroni*

phosphorus source	P supplied, μmol	alkane formed, μmol		
		methane	ethane	propane
Pi	3.0	0.0	0.0	0.0
AEPn	3.0	0.0	0.0	0.0
methyl phosphate	3.0	0.0	0.0	0.0
MPn	0.30	0.29	0.0	0.0
	1.0	1.0	0.0	0.0
	3.0	3.2	0.0	0.0
IMPn	0.30	0.30	0.0	0.0
	1.0	0.97	0.0	0.0
	3.0	3.0	0.0	0.0
PMPn	2.0	1.6	0.0	0.0
	3.0	2.3	0.0	0.0
EPn	0.25	0.0	0.25	0.0
	1.0	0.0	1.1	0.0
	3.0	0.0	3.3	0.0
EEPn	0.28	0.0	0.27	0.0
	1.1	0.0	1.3	0.0
	3.4	0.0	3.8	0.0
PrPn	0.25	0.0	0.0	0.20
	1.0	0.0	0.0	0.67
	3.0	0.0	0.0	2.16
EPrPn	0.25	0.0	0.0	0.16
	1.0	0.0	0.0	0.49
	3.0	0.0	0.0	1.28

Table V. Mass Spectral Data for Methane Produced by *P. testosteroni* from MPn, IMPn, and PMPn

	relative intensities			
		alkane from		
m/z	authentic methane	MPn	IMPn	PMPn
12	0	0.1	0.5	0.0
13	0	0.4	0.4	0.3
14	0	1.3	1.5	1.4
15	50.6	48.4	47.7	48.2
16 (M)	100.0	100.0	100.0	100.0
17	0.0	1.1	1.7	0.0

Quantitation of MPn release from 2.5 μmol of IMPn by cell extracts showed formation of about 2.5 μmol of MPn. MPn is therefore the only phosphorus-containing cleavage product of IMPn. Thus, IMPn was enzymatically hydrolyzed to isopropyl alcohol and MPn.

Cleavage of the Carbon–Phosphorus Bond. Analysis of headspace samples showed that alkylphosphonates in sterile media did not spontaneously decompose to alkanes, and that alkanes above ambient levels were not detected when *P. testosteroni* was grown in media with 0.1 mM Pi, 0.1 mM methyl phosphate, or 0.1 mM AEPn as phosphorus source (Table IV). Samples of the headspace of cultures at the end of growth in media with limiting MPn, IMPn, or PMPn gave only one peak, and it cochromatographed with authentic methane. The identification of methane was confirmed by mass spectrometry (Table V). The yield of methane from MPn and IMPn was stoichiometric, and no traces of other alkanes were detected. The yield of methane was independent of carbon source (gluconate, β-hydroxybutyrate, or *p*-hydroxybenzoate). Methane was thus the sole carbon-containing cleavage product from the C–P bond of methylphosphonates. The yield of methane from PMPn was about 75% (Table IV), roughly consistent with the estimates (see above) of the incomplete metabolism of PMPn. The incomplete degradation of PMPn may indicate an inability of *P. testosteroni* to utilize one or more of the four optical isomers of this compound.

The headspace from cultures fully grown on EPn or EEPn gave only one peak, and it cochromatographed with

Table VI. Mass Spectral Data for Ethane Produced by *P. testosteroni* from EPn and EEPn

	relative intensities		
		alkane from	
m/z	authentic ethane	EPn	EEPn
26	0.2	0.2	0.4
27	1.6	1.6	1.3
28	100.0	100.0	100.0
29	14.8	15.3	15.2
30 (M)	37.3	37.8	37.5
31	0.8	0.6	0.4

authentic ethane. The identification of ethane was confirmed by mass spectrometry (Table VI); the base peak (*m/z* 28) was probably because of air (e.g., CO and N_2). Ethane was released stoichiometrically from EPn and from EEPn (Table IV). Therefore, ethane was the sole organic product from the cleavage of the C–P bond of ethylphosphonates. EETPn was not utilized by *P. testosteroni* as a phosphorus source, and no ethane was released from this thiophosphonate.

When PrPn or EPrPn was the phosphorus source, the headspace gave one major peak, and it cochromatographed with authentic propane; a minor peak (3 × base line noise) was also present, and it cochromatographed with propene. The yield of propane from the propylphosphonates was constant and seemingly not stoichiometric (Table IV), but utilization of EPrPn as sole limiting phosphorus source was complete (as assayed by benzylation and GC) so presumably these compounds were not pure.

Michel et al. (1967) hypothesized that propene may be a catabolic product from IMPn, but we never observed propene to be formed from IMPn, the data above demonstrating that the sole cleavage product of the ester bond in IMPn was isopropyl alcohol.

The release of alkanes proceeded under aerobic conditions inasmuch as the headspace of the cultures always contained oxygen. This is in contrast to the strict anaerobiosis required for methane production from CO_2 (Zeikus, 1977). Another difference between methane production from MPn and classical methanogenesis was the insensitivity of methane production by *P. testosteroni* to 2-bromoethylsulfonate (0.1 mM), a potent inhibitor of methanogenesis from CO_2.

The system of C–P bond cleavage in *P. testosteroni* is similar to the cleavage of the natural product (2-oxyethyl)phosphonate by the aerobe *Bacillus cereus*, a reaction in which acetaldehyde was demonstrated as the carbon-containing product (La Nauze et al., 1970). However, the mechanism of C–P bond cleavage in *P. testosteroni* must differ from the aldolase-like imine formation proposed by La Nauze et al. (1977) because the alkylphosphonates have no aldehydic group to react with the phosphonatase. We were unable to obtain cell-free extracts to cleave the C–P bond of MPn. The supernatant fluid of cell-free extracts had no activity and the particulate fraction did not give reproducible activity when tested under a range of conditions, including eleven alternative buffers (pH range 4.5–9.5), twelve other vitamins, coenzymes, and ion regimes and simplifications of the assay system.

Phosphate from the Phosphorus Moiety. When provided with limiting amounts of MPn as a phosphorus source, whole cells assimilated all the phosphorus while releasing methane. Neither whole cells under nongrowing conditions nor cell-free extracts generated methane, and thus no inorganic phosphorus product was released. Consequently, an alternative method had to be used to determine the identity of the phosphorus compound formed on cleavage of the C–P bond. For this purpose, cells induced to utilize Pi, MPn (or EPn), or AEPn were incubated in complete medium supplemented with 10 μM chloramphenicol to inhibit protein synthesis and, therefore, inhibit phosphorus assimilation.

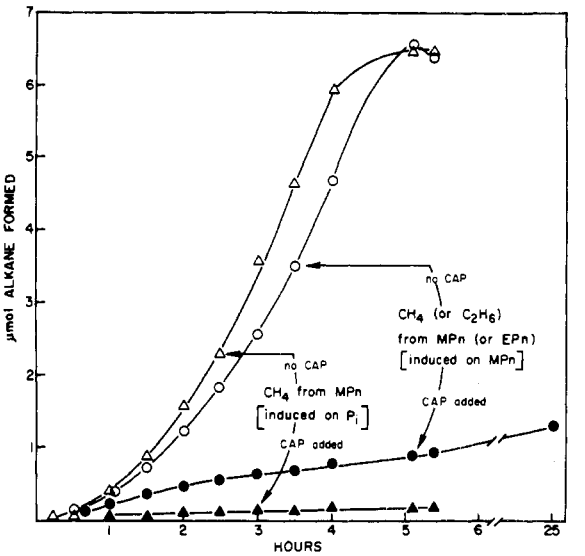

Figure 2. Effect of chloramphenicol (CAP) on alkane formation from MPn or EPn by *P. testosteroni*. The inoculum had been induced to grow on Pi, MPn, or AEPn (data points same as △).

In the absence of chloramphenicol, the utilization of 6 μmol of MPn by *P. testosteroni* (as indicated by methane formation) was exponential and complete, whether the inoculum had been induced to grow with Pi or MPn (Figure 2) or EPn (not shown). If cells induced to grow with Pi or AEPn were used as inoculum in the presence of chloramphenicol, little or no methane was released from MPn (Figure 2). Cells induced to grow with MPn (or EPn) and incubated for 25 h with MPn (or EPn) in the presence of chloramphenicol showed arithmetic production of methane (or ethane); the yield of alkane (1.3 μmol) represented about 22% of the substrate added. At 25 h, the supernatant fluids were analyzed for Pi and nonphosphonate phosphorus (as Pi), and the cell pellet was assayed for polyphosphate (as Pi) and total phosphorus (as Pi). Inorganic orthophosphate was detected as a major component of the labile phosphorus compounds in the supernatant fluid, and polyphosphate, a storage polymer of Pi in bacteria, was a major component of the phosphorus incorporated into the cells (Table VII). The assay for polyphosphate underestimates the true level of polyphosphate (Harold, 1966). The identities of the other phosphorus compounds in the system are unknown.

The data demonstrate that Pi was an end product of cleavage of the C–P bond of methyl- and ethylphosphonates by *P. testosteroni*. It is not clear whether Pi was the initial product, since phosphite (phosphonic acid) could have been liberated by reductive cleavage. However, the one documented phosphonatase is a hydrolase liberating Pi on cleavage of the C–P bond (La Nauze et al., 1970), so Pi may indeed be the initial product of cleavage.

Regulation of Phosphonatase Synthesis. *P. testosteroni* not previously exposed to MPn was suspended in the growth medium with chloramphenicol and MPn. These cells showed little or no capacity to degrade MPn (assayed by methane formation), whereas cells previously exposed to MPn did degrade MPn in the presence of chloramphenicol (Figure 2). In contrast, cells induced on

Table VII. Phosphorus Released from Phosphonates by Chloramphenicol-Treated *P. testosteroni*

compd added (6 μmol)	supernatant fluid[a] total labile P,[c] μmol	supernatant fluid[a] Pi,[d] μmol	cell pellet[b] total P (net),[e] μmol	cell pellet[b] polyphosphate,[f] μmol	alkane released, μmol	total P released,[g] μmol
none	0.00	0.01	0.0	0.0	0.0	0.01
MPn	0.57	0.40	0.63	0.32	1.35	1.20
EPn	0.48	0.33	0.54	0.22	1.19	1.02

[a] Twenty-five-hour sample (20000*g* for 10 min at 4 °C) of supernatant fluid. [b] Twenty-five-hour sample (20000*g* for 10 min at 4 °C) of washed cell pellet. [c] Nonphosphonate phosphorus by modified Bartlett (see Methods section). [d] Method of Dick and Tabatabai (1977). [e] Method of Cook et al. (1978b); inoculum had 0.63 μmol of P. [f] Phosphorus as polyphosphate by method of Kaltwasser (1962). [g] Sum of total nonphosphonate phosphorus in supernatant fluid and cell pellet.

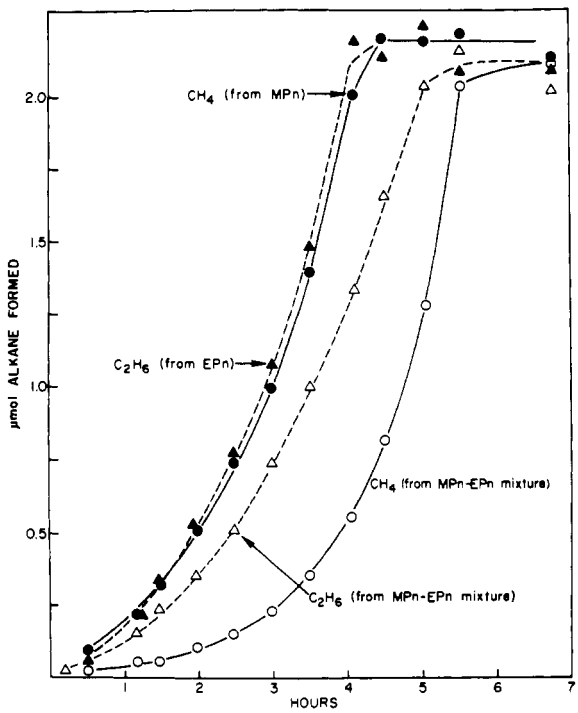

Figure 3. Production of alkane from MPn, EPn, or an MPn-EPn mixture by *P. testosteroni*. The inoculum had been induced to grow on EPn.

Pi or MPn grew equally well (as indicated by methane formation) when inoculated into MPn-containing medium (Figure 2). This behavior is characteristic of an inducible enzyme (system) (Schlegel, 1976). Synthesis of this inducible enzyme system is repressed by Pi (Daughton et al., 1979a).

Specificity of *P. testosteroni* for Alkylphosphonates. The exponential rate of release of methane from MPn was identical with the rate of ethane release from EPn in cultures induced for growth with EPn (Figure 3), MPn, or Pi (neither shown) as phosphorus source. When MPn and EPn were present simultaneously in cultures induced for growth with EPn (Figure 3), MPn, or Pi (neither shown), the rate of product formation from each phosphonate was depressed, methane release being more strongly inhibited than ethane release. We interpret this as competition for one enzyme (system), with EPn being the preferred substrate.

Cells induced to utilize AEPn released little methane from MPn in the presence of chloramphenicol (Figure 2), and AEPn, which is used as a phosphorus but not as a carbon or nitrogen source (Cook et al., 1978c), only slightly inhibited methane production from MPn during growth (not shown). Inability to split the C-N bond of amines is a characteristic of *P. testosteroni* (Stanier et al., 1966), but the hypothesized product of AEPn, ethylamine, has not been detected by GC/FID after growth on 1.0 mM AEPn. We suspect that there is a separate phosphonatase specific for AEPn in this pseudomonad. *P. testosteroni* also grew well with 0.1 mM phosphite as a phosphorus source. Phosphite (0.1 mM) suppressed the rate of methane release from MPn during growth, but the mechanism is unknown.

Table VIII. Activity of *P. testosteroni* on CH_3-Heteroatom Bonds

compd tested,[a] 2 μmol	products, μmol CH_4	products, μmol C_2H_6
none	0	2
$CH_3P(O)(OH)_2$	2	2
$CH_3OP(O)(OH)_2$	0	2
$CH_3As(O)(OH)_2$	0	2
$(CH_3)_2As(O)(ONa)$	0	2
$CH_3SO_2(OCH_2CH_3)$	0	2
$CH_3S(CH_2)_2CH(NH_2)COOH$	0	2
CH_3HgCl	0	0

[a] EPn (2 μmol) was supplied to cultures (30 mL) of *P. testosteroni* as phosphorus source to induce the enzyme(s) necessary to cleave the C-P bond, and ethane was produced concomitantly with growth. The test compounds were potential sources of methane.

The specificity of the phosphonatase for alkylphosphonates was determined by testing activity of the bacterium on other heteroatom–carbon bonds. Cells induced to utilize EPn were grown in the presence of EPn plus one of the test substrates, each of which was a potential methane source. The release of methane and ethane was monitored. No compound tested (except MPn) was converted to methane, and therefore none was a substrate for the phosphonatase during or after utilization of EPn (Table VIII). Methylmercury chloride (and ethylmercury chloride) totally inhibited growth. Thus, the enzyme (system) which cleaves the C–P bond of alkylphosphonates appears to be specific for the C–P bond. *P. testosteroni* does not cleave the C–P bond of phenylphosphonates (Cook et al., 1978a).

The only biological system reportedly releasing an alkane or arene by cleavage of a heteroatom–carbon bond is the cleavage of alkyl- and arylmercury compounds by a pseudomonad, but the identification of the products in that instance was only tentative (Tezuka and Tonomura, 1976). Microorganisms also act on the carbon–arsenic bond of methylarsonate, but the product is CO_2 (von Endt et al., 1968). We conclude that our phosphonatase (system) differs from those published.

Pathway. We have established the pathway of *O*-alkyl alkylphosphonate catabolism by *P. testosteroni* shown in Figure 4. The alkoxy group is removed first to yield the alcohol and the divalent alkylphosphonate. The latter is then cleaved to yield the alkane. Inorganic orthophosphate is the final phosphorus-containing product, but it is not established as the initial product. The utilization of the

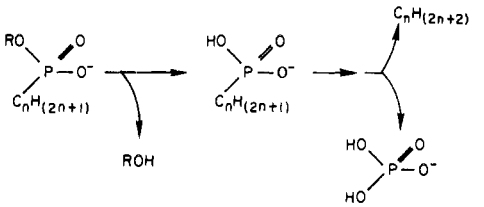

Figure 4. Pathway for the metabolism of *O*-alkyl alkylphosphonates by *P. testosteroni*.

alkylphosphonates occurred aerobically and only when they were present as sole phosphorus sources. The enzyme (system) that cleaves the C–P bond is specific for alkylphosphonates. This investigation is the first to establish (i) degradation of simple alkylphosphonates, (ii) the complete conversion of an organophosphorus toxicant to natural products, and (iii) the catabolic pathway for an alkylphosphonate.

Subsequent to completion of this research, Verweij et al. (1979) reported the presence of small quantities of presumably ionic methylphosphonates in certain surface waters. This is perhaps the first report of this class of compounds in natural ecosystems.

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