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Benzene from Bacterial Cleavage of the Carbon–Phosphorus Bond of Phenylphosphonates

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An organism, identified as a strain of *Klebsiella pneumoniae*, was proved by direct assay to utilize ionic methyl phenylphosphonate as sole phosphorus source. One product from C–P-bond cleavage was identified as benzene by combined g.l.c.–mass spectrometry. The molar yield of benzene from the phosphonate was 89%.

Alkylphosphonates have great chemical stability as a result of their C–P bond. Synthetic alkyl- and aryl-phosphonates [e.g. $C_nH_{(2n+1)}P(O)(OR)OR'$ and $C_6H_5P(O)(OR)OR'$] are widely used in industry, in agriculture and in commercial products. Although they have been proved to be biodegradable (Cook *et al.*, 1978a), little is known about their metabolism.

The natural product dihydrogen (2-aminoethyl)-phosphonate is resistant to chemical attack (e.g. 8M-HCl at 150°C for 48h) but readily degraded through a defined pathway in *Bacillus cereus*. Hydrolysis of the intermediate (2-oxoethyl)phosphonate by phosphonoacetaldehyde hydrolase (EC 3.11.1.1) yields acetaldehyde and P_i (La Nauze *et al.*, 1970, 1977). Alkylphosphonates are not known in Nature, with the exception of 2-amino-4-methylphosphinobutyrate (Bayer *et al.*, 1972). Dihydrogen methylphosphonate is degraded by a strain of *Pseudomonas testosteroni*, which quantitatively releases the methyl group as methane (Daughton *et al.*, 1979). We now report an analogous reaction in which the phenyl group of phenylphosphonates is released as benzene on cleavage of the C–P bond.

Materials and Methods

O-Methyl hydrogen phenylphosphonate and dihydrogen phenylphosphonate were gifts from the Velsicol Chemical Corp., Chicago, IL, U.S.A. Mass spectra of these two compounds were obtained with a Finnigan 3300 Quadrupole mass spectrometer and Systems Industries 150 Data System by electron-impact ionization (70eV) and direct probe insertion. For the following mass spectra, the two most intense ions (relative to the base peak) are presented for every 14 mass units above m/e 34 (relative intensity > 1): O-methyl hydrogen phenylphosphonate, 39 (14.0),

43 (9.3), 50 (23.0), 51 (51.9), 65 (21.6), 74 (8.0), 77 (56.0), 78 (100.0), 91 (73.2), 94 (24.9), 108 (1.2), 112 (1.7), 125 (1.5), 128 (1.4), 141 (8.1), 142 (26.7), 153 (3.5), 158 (5.2), 171 (26.0) and 172 (M^+ , 27.5); dihydrogen phenylphosphonate, 50 (8.6), 51 (13.6), 66 (9.8), 74 (2.8), 76 (1.2), 77 (4.3), 94 (100.0), 158 (M^+ , 38.1) and 159 (3.0). The base peak (m/e 78) for O-methyl hydrogen phenylphosphonate confirmed the presence of the phenyl group, and the other peaks were consistent with the structure. Although the spectrum of dihydrogen phenylphosphonate had a weak m/e 78 peak and a base peak (m/e 94), which did not correspond to the expected fragmentation, the anticipated M^+ peak was present. All other chemicals were of the highest purity available commercially.

Glassware was cleaned by steeping in 5M-HNO₃ to minimize the amount of contaminative phosphorus compounds (Cook *et al.*, 1978b).

The bacterial isolate was maintained by monthly subculturing in a buffered-salts liquid medium containing limiting (0.1mM) ionic methyl phenylphosphonate and 5mM-*p*-hydroxybenzoate as carbon source (Cook *et al.*, 1978a). All experiments were done under aseptic conditions.

To study the disappearance of the phosphorus source, growth medium (30ml) with *p*-hydroxybenzoate and 0.1mM- P_i , ionic methyl phenylphosphonate or phenylphosphonate was prepared in 250ml Erlenmeyer flasks. Inoculation was with cells fully grown in an identical medium. The flasks were shaken at 30°C until the turbidity stabilized (about 3 days). Samples taken before and after growth were centrifuged (20000g for 20min at 4°C), and the supernatant fluid was stored at –20°C until assayed. Experiments were done at 30°C to monitor the headspace of 30ml cultures or standards in shaken 250ml screw-cap bottles sealed with Mininert Teflon valves (Pierce Chemical Co., Rockford, IL, U.S.A.). A single phosphorus source (0.2mM) was present. Most experiments were done with succinate (20mM) plus acetate (10mM) as carbon sources to repress synthesis of enzymes degrading aromatic compounds

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(Stevenson & Mandelstam, 1965), but some experiments were done with *p*-hydroxybenzoate (15 mM) as carbon source.

Phenylphosphonates were determined quantitatively as their benzyl esters after reaction with 3-benzyl-1-*p*-tolyltriazene (Aldrich Chemical Co., Metuchen, NJ, U.S.A.) (C. G. Daughton, A. M. Cook & M. Alexander, unpublished work). (Caution: 3-benzyl-1-*p*-tolyltriazene is a suspect carcinogen.) Samples (4 ml, adjusted to pH 7) were passed through glass columns (3 cm × 4 mm) of Bio-Rad AG 50W-X8 (H⁺ form; 50–100 mesh) resin. The first 10 drops of effluent were discarded, and 200 µl of effluent was dried at 125°C in a 9 ml screw-cap glass vial. 3-Benzyl-1-*p*-tolyltriazene (200 µl, 0.25 M in acetone) was added to the cooled residue, the vial was sealed with a Teflon-lined cap and the solution was refluxed for 20 min at 70°C. The acetone was evaporated under a stream of dry N₂, and distilled water (1 ml) and 6.25 M-HCl (4 drops) were added to destroy excess of benzylating reagent. The mixture was extracted with cyclohexane (2 ml; spectrographic grade), and the Na₂SO₄-dried organic phase was analysed by g.l.c. The benzyl esters were determined by using a flame-photometric detector (phosphorus mode), after separation on a 1.8 m × 2 mm glass column that had been silanized with 5% (w/v) dichlorodimethylsilane in toluene, packed with 5% (w/w) OV-210 on 80–100-mesh Gas-Chrom Q, and vapour-phase-deposited with Carbowax 20M (Ives & Giuffrida, 1970). Helium (30 ml/min) was the carrier gas. The column was temperature-programmed from 170°C to 225°C at 16°C/min for *O*-benzyl *O*-methyl phenylphosphonate (retention time 5.45 min) or was run isothermally at 225°C for *OO*-dibenzyl phenylphosphonate (retention time 9.8 min).

Benzene was determined quantitatively by g.l.c. with a flame-ionization detector. Samples of culture headspace (500 µl) were taken with a 1 ml gas-tight syringe and injected on to a 1.8 m × 2 mm stainless-steel Teflon-lined column packed with 80–100-mesh Porapak Q/S. Helium was the carrier gas, and the oven temperature was 200°C. Standards were prepared by adding benzene with a high-pressure syringe to water and diluting to 1.00 litre to give a 1.00 mM solution. Portions of this solution were immediately added to 250 ml screw-cap glass bottles and made to 30 ml with distilled water (for standards), with growth medium or with cultures of *Klebsiella pneumoniae* (for controls), and the bottles were sealed with Mininert valves. The amount of benzene (µmol) in the headspace was calculated by interpolation from the linear regression equations (coefficient of correlation 0.999) generated from the peak-heights obtained from the standards (0–6 µmol per bottle). Benzene was tentatively identified in the culture headspace by co-elution with authentic benzene on the Porapak column (retention time 5.50 min). The

identification of benzene was confirmed by interfacing the Porapak column with a Finnigan 3300 Quadrupole mass spectrometer equipped with a Systems Industries 150 Data System (column, 200°C; helium, 20 ml/min; ionizing voltage, 70 eV).

Negative-stained preparations of the organism were examined for cell-wall type and for inclusions by using a Phillips 300 electron microscope. Equal volumes of a suspension of cells in distilled water, 1% phosphotungstic acid (adjusted to pH 7.0 with KOH) and 0.05% bovine serum albumin were mixed, 1 drop of the mixture was applied to a Formvar-coated grid, and the blotted air-dried grid was examined.

Results and Discussion

A bacterium was isolated in media containing ionic methyl phenylphosphonate as sole phosphorus source (Cook *et al.*, 1978a). The bacterium was a non-motile oxidase-negative coccobacillus that had no cellular inclusions and usually occurred in pairs. Electron micrographs of the bacterium showed a wrinkled cell surface, clearly indicating a Gram-negative organism. The bacterium grew anaerobically on glucose with gas production, and it grew aerobically with glucose, lactose, xylose or citrate as carbon source. Lysine decarboxylase and urease reactions were positive. The organism had no arginine dihydrolase, ornithine decarboxylase or capacity for denitrification or phenylalanine deamination, and it did not produce H₂S or indole. We conclude that the organism is a strain of *Klebsiella pneumoniae*. The isolate has been deposited in the German Collection of Microorganisms (D.S.M., Grisebachstrasse 8, D-34 Göttingen, Federal Republic of Germany) and its accession number is pending.

K. pneumoniae grew in a medium containing 0.1 mM ionic methyl phenylphosphate or phenylphosphonate as sole phosphorus source and *p*-hydroxybenzoate as carbon source. There was no measurable growth in the absence of an added phosphorus source. Growth terminated with 58% and 27% utilization of the ionic methyl phenylphosphonate and ionic phenylphosphonate respectively. No loss of either phosphonate was observed in uninoculated controls, and there was no interference in the assay from controls with P_i as the phosphorus source. These findings confirm directly that phenylphosphonates are used as phosphorus sources by bacteria, complementing evidence obtained by a non-specific phosphorus assay (Cook *et al.*, 1978a,c).

Benzene was tentatively identified in the headspace above cultures grown with phenylphosphonates by co-chromatography. This finding was confirmed by mass spectrometry (Table 1). No benzene was released from cultures growing in media with P_i as phosphorus source or from sterile growth medium containing either phosphonate.

Table 1. *Mass-spectral data for benzene from bacterial cleavage of phenylphosphonates*

For experimental details see the text.

<i>m/e</i>	Relative intensity		
	Authentic benzene	Product from methyl phenylphosphonate	Product from phenylphosphonate
39	9.6	9.6	10.5
50	17.8	17.5	16.7
51	18.0	18.8	18.2
52	18.6	18.0	17.4
76	5.1	4.7	5.6
77	22.9	23.3	25.2
78	100.0	100.0	100.0
79	6.4	6.5	7.0

The doubling time of *K. pneumoniae*, assayed by release of benzene, was about 2.5 h. The benzene released from 6 μ mol of ionic methyl phenylphosphonate in cultures with succinate plus acetate as carbon sources was 89% of the theoretical maximum. The benzene concentration over cultures with P_i as phosphorus source remained unchanged for at least a week. When *p*-hydroxybenzoate was the carbon source, growth was slower on ionic methyl phenylphosphonate (but not on P_i), and the yield of benzene was only 19%. We do not know if *p*-hydroxybenzoate was a competitive inhibitor of phenylphosphonate cleavage or if some benzene was degraded when *p*-hydroxybenzoate was used as a carbon source.

The yield of benzene in media with 6 μ mol of ionic phenylphosphonate and succinate plus acetate as carbon sources was 20%. The reason for this low yield is not known, but the base peak (*m/e* 94) in the mass spectrum of dihydrogen phenylphosphonate may indicate the presence of phenol as a fragmentation product, which would result if the sample contained *O*-phenyl phosphate.

The biological production of benzene is unusual. To our knowledge, the only other reports of such an activity are a preliminary communication on the metabolism of triphenyl-lead acetate (Williams *et al.*,

1972) and the breakdown of phenylmercuric chloride (Tezuka & Tonomura, 1978, and references cited therein), though the benzene in the latter system was apparently not identified conclusively. In contrast, a proposed pathway for the degradation of benzenesulphonates, analogues of phenylphosphonates, shows catechol and sulphite as the initial products from cleavage of the C-S bond (Cain & Farr, 1968); the high molar growth yield with benzenesulphonate as a carbon source (Ripin *et al.*, 1971) is further evidence against the presence of a volatile intermediate in the C-S-bond cleavage.

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