Radical-Based Dephosphorylation and Organophosphonate Biodegradation

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Abstract: Products resulting from the degradation of organophosphonates by *Escherichia coli* are identified and used as a basis for evaluating mechanisms which may be the chemical basis of the biodegradation. One mechanistic hypothesis which is consistent with the biodegradation products involves radical-based dephosphorylation. Chemical modeling of this process is achieved by the reaction of alkylphosphonic acids with lead(IV) tetraacetate and electrochemical oxidation at a platinum anode.

Organophosphonates are so readily degraded by *Escherichia coli*[1] that this class of organic functionality can serve as the sole source of phosphorus during microbial growth. This implies that the carbon to phosphorus bond of organophosphonates[2] can be cleaved by certain biological systems. The importance of the chemistry exploited during the biodegradation is difficult to understate given its direct relevance to detoxification of herbicides, insecticides, antibiotics, and agents of chemical warfare[3] that contain the phosphonic acid moiety. The challenge of providing a satisfying chemical explanation for the biodegradation tests our understanding of organophosphorus chemistry. Thus beyond an obvious environmental rationale for the study of organophosphonate biodegradation, such an endeavor can provide unique insights into fundamental organic chemistry.

One mechanism which has been proposed[1f] as the chemical basis for the biodegradation involves radical-based dephosphorylation (Scheme I). Gauging the relevance of this chemical mechanism to *E. coli* degradation of organophosphonates is complicated by the failure of the degrading activity to survive lysis of *E. coli* cells. In this account, hypothetical chemical mechanisms including radical-based dephosphorylation are evaluated according to their ability to account for the products of the biodegradation of organophosphonates. Simultaneously, chemical models of the biodegradation are used to ascertain whether radical-based dephosphorylation is chemically reasonable and if the conditions necessary for such a process are attainable in a biological system.

Results

Alkylphosphonate Biodegradation. Earlier studies of *E. coli* degradation of methylphosphonate have been extended to isotopically labeled material.[1f] Growth of *E. coli* on $^{13}CH_3PO_3H_2$ and $CD_3PO_3H_2$ yields $^{13}CH_4$ and CD_3H, respectively. No deuterium-substituted methane species other than CD_3H were detected during degradation of $CD_3PO_3H_2$. In order to establish whether the hydrocarbon production could account for all of the inorganic phosphate formed, *E. coli* cells grown on methylphosphonate were harvested and exhaustively digested with acid. Inorganic phosphate thus formed was quantitated and compared to the amount of methane formed during the *E. coli* growth. The ratio of inorganic phosphate:hydrocarbon which was obtained indicated that the moles of inorganic phosphate generated during the degradation of methylphosphonate coincided with the moles of methane which were formed.

The remainder of the organophosphates of Table I can be divided into groups distinguished by the nature of the alkyl side chain attached directly to phosphorus. Either these side chains were branched alkanes (**3**, **4**, **5**, **6**) or they contained sp^2 hybridization (**7**, **8**, **9**, **10**). Although neither *tert*-butyl- (**6**) or isopropylphosphonate (**5**) were degraded, isobutyl- (**3**) and cyclopropylmethylphosphonate (**4**) were readily broken down. Phenyl- (**7**), vinyl- (**8**), and allylphosphonate (**9**) could function as a sole phosphorus source while benzylphosphonate (**10**) proved to be the exception. *E. coli* were unable to degrade esterified alkyl-

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Scheme I

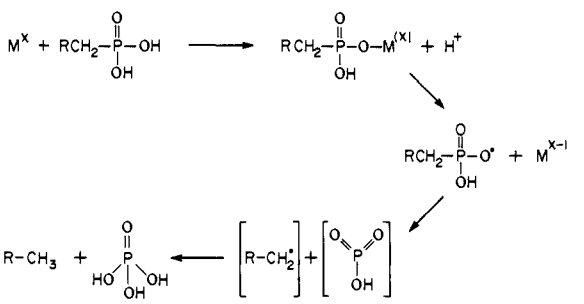


Table I

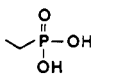
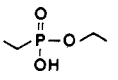
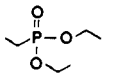
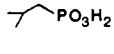
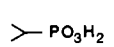
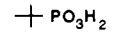
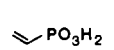
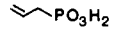
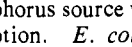
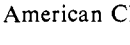
	Organophosphonate	G - growth NG - no growth	Products
1a	$CH_3PO_3H_2$	G	CH_4
1b	$^{13}CH_3PO_3H_2$	G	$^{13}CH_4$
1c	$CD_3PO_3H_2$	G	CD_3H
2a		G	(0.5%)
2b		NG	
2c		NG	
3		G	 (1%)
4a		G	 (2%)
5		NG	
6		NG	
7		G	
8		G	
9		G	
10		NG	

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Table II

$$R\text{-}PO_3H_2 \xrightarrow[DMF,\ 110°]{Pb(OAc)_4}$$

R	Products (% Yield)		Mole ratio (alkane : alkene)
CH_3CH_2- 2a	CO_2 (60%)	—, = (2.2%)	6 : 1
$CH_3CH_2CH_2-$ 11	CO_2 (52%)	, (3.8%)	3 : 1
$CH_3CH_2CH_2CH_2-$ 12	CO_2 (50%)	, (1%)	4 : 1
$CH_3CH(CH_3)H$ 5	CO_2 (45%)	, (11%)	3 : 1
$CH_3C(CH_3)_2H$ 6	CO_2 (41%)	, (6%)	1 : 1

Table III

$$R\text{-}PO_3H_2 \xrightarrow[DMF,\ 110°]{Pb(OAc)_4,\ Cu(OAc)_2}$$

R	Products (% Yield)		Mole ratio (alkane : alkene)
CH_3CH_2- 2a	CO_2 (63%)	—, = (4%)	1 : 11
$CH_3CH_2CH_2-$ 11	CO_2 (53%)	, (8.6%)	1 : 15
$CH_3CH_2CH_2CH_2-$ 12	CO_2 (51%)	, (3.5%)	1 : 11
$CH_3CH(CH_3)H$ 5	CO_2 (47%)	, (29%)	1 : 480
$CH_3C(CH_3)_2H$ 6	CO_2 (40%)	, (26%)	1 : 41

phosphonates. Neither the monoethyl or diethyl ester of ethylphosphonic acid (**2b**, **2c**) supported *E. coli* growth. For all of the alkylphosphonates which were able to function as the sole source of phosphorus during *E. coli* growth, alkanes were the predominant product although small levels of alkene were also formed.

An *E. coli* K-12 strain was used for all of the degradation experiments. Each colony initially grown on medium containing inorganic phosphate was able to grow when transferred to medium where methylphosphonate was the only source of phosphorus. Upon initial transfer to the alkylphosphonate medium, there was a pronounced lag phase during which little growth was observed. This lag time prior to onset of vigorous growth disappeared during subsequent transfers to fresh medium where alkylphosphonates were the only source of phosphorus.

The purity of the alkylphosphonates used in the study was a preeminent consideration particularly in relation to the presence of inorganic phosphate and alkenylphosphonate contaminants. Aside from its presence causing spurious growth observations, contaminating inorganic phosphate led to a drastic reduction of hydrocarbon generation even with those organophosphonates readily degraded by *E. coli*. Virtually all of the organophosphonates, whether synthesized or purchased from commercial sources, possessed some level of inorganic phosphate contamination. Therefore, all organophosphonates examined in the growth/degradation experiments were purified by anion exchange chromatography or by reverse phase (C-18) high pressure liquid chromatography (HPLC). No inorganic phosphate could be detected in the purified organophosphonates.

It was also imperative to establish that the small levels of alkene products formed during biodegradation of alkylphosphonates were unique to the biodegradation and not due to alkenylphosphonate contamination of the alkylphosphonates. Both cyclopropylmethylphosphonate and ethylphosphonate were synthesized by condensation of a trialkyl phosphite with an appropriate alkyl halide. Capillary gas chromatographic separation[4] with flame ionization detection did not reveal any diethyl vinylphosphonate in the product resulting from triethyl phosphite reaction with ethyl iodide. Condensation of triisopropyl phosphite with cyclopropylmethyl bromide did result in a 0.25 mol % diisopropyl 3-butenylphosphonate contaminant in unpurified diisopropyl cyclopropylmethylphosphonate. This alkenylphosphonate was completely removed by high pressure liquid chromatography with a reverse-phase column. After removal of the alkyl esters,[5] high field (400 mHz) NMR analysis[6] failed to reveal any olefinic resonances in the NMR of ethyl- or cyclopropylmethylphosphonate. These various lines of evidence indicate that the alkene products formed during the biodegradation of alkylphosphonates were not due to spurious contamination of the phosphorus sources.

Chemical Models. Reaction of alkylphosphonic acids with lead(IV) tetraacetate to form alkane and alkene products (Table II) has proven to be a general, albeit low yielding, reaction. A fivefold molar excess of alkylphosphonic acid is dissolved with the lead(IV) tetraacetate in dry dimethylformamide. This homogeneous solution is degassed under nitrogen atmosphere. No detectable reaction occurs at room temperature or if the degassing under nitrogen procedure is omitted. Upon heating to 110 °C product formation is observed simultaneous with formation of a large quantity of a white, solid precipitate. This precipitate is insoluble in water or organic solvents. Digestion with hot, concentrated nitric acid leads to formation of inorganic phosphate as indicated by ^{31}P NMR.

During optimization of the reaction, a wide range of solvents were examined. These included tetrahydrofuran, dioxane, acetonitrile, benzene, chloroform, *tert*-butyl alcohol, and acetic acid. Dimethylformamide afforded the highest product yield. As an alternative to heating to 110 °C, the reaction can proceed at 55 °C when irradiated with 3500-Å light. Yields were not significantly higher for the photolysis reaction. Unlike reaction of lead(IV) tetraacetate with alkylcarboxylic acids, the presence of lithium chloride in the reaction with alkylphosphonates does not

(1) (a) Zeleznick, L. D.; Meyers, T. C.; Titchener, E. B. *Biochim. Biophys. Acta* **1963**, *78*, 546. (b) James, E. A., Jr.; Meyers, T. C.; Titchener, E. B. *Fed. Proc. (Abstr.)* **1965**, *24*, 440. (c) Harkness, D. R. *J. Bacteriol.* **1966**, *92*, 623. (d) Alam, A. U.; Bishop, S. H. *Can. J. Microbiol.* **1969**, *15*, 1043. (e) Tamari, M. *J. Gen. Appl. Microbiol.* **1977**, *23*, 49. (f) Cordeiro, M. L.; Pompliano, D. L.; Frost, J. W. *J. Am. Chem. Soc.* **1986**, *108*, 332. (g) Wackett, L. P.; Shames, S. L.; Venditti, C. P.; Walsh, C. T. *J. Bacteriol.*, in press.

(2) The bond energy for a C–P bond is estimated to be 60–65 kcal/mol. (a) Hudson, R. F. *Pure Appl. Chem.* **1964**, *9*, 371. (b) Roberts, J. D.; Caserio, M. C. *Basic Principles of Organic Chemistry*; W. A. Benjamin, Inc.: New York, 1964; p 1201.

(3) For a useful overview see: (a) Hilderbrand, R. L. *The Role of Phosphonates in Living Systems*; CRC Press, Inc.: Boca Raton, 1983. (b) Hori, T.; Horiguchi, M.; Hayashi, A. *Biochemistry of Natural C–P Compounds*; Japanese Association for Research on the Biochemistry of C–P Compounds: Japan, 1984.

(4) GC analysis could readily detect 0.2 mol % alkenylphosphonate contaminant in alkylphosphonate.

(5) McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M. C. *Tetrahedron Lett.* **1977**, 155.

(6) ^1H NMR analysis was capable of detection of 0.5 mol % alkenylphosphonate contaminant in alkylphosphonate.

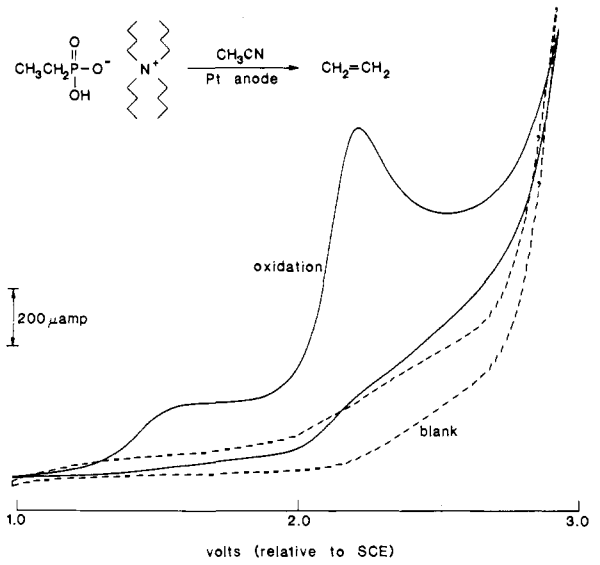


Figure 1.

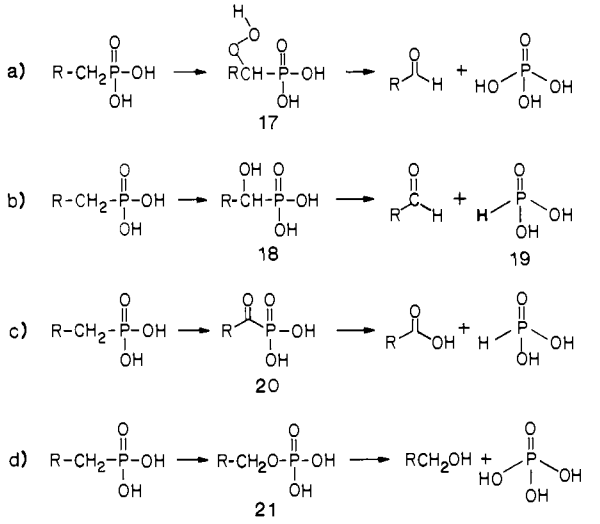
lead to any detectable alkyl halide formation.[7] No hydrocarbon products were observed when lead(IV) tetraacetate was reacted with ethyl ethylphosphonate (**2b**) and diethyl ethylphosphonate (**2c**).

When lead(IV) tetraacetate is used alone in the oxidations, an excess of alkane relative to alkene is generally formed (Table II). Addition of copper(II) acetate leads to a reversal in the product ratio (Table III). The excess of alkene to alkane is particularly pronounced in the oxidation of isopropyl- and *tert*-butylphosphonate. The overall yields of alkane and alkene are also significantly higher upon inclusion of copper(II) acetate in the reaction. Reaction of alkylphosphonate with copper(II) acetate in the absence of lead(IV) tetraacetate does not afford any detectable alkane or alkene products.

Electrochemical oxidation of alkylphosphonates was expected to provided an accurate determination of the oxidation potential of alkylphosphonates. Acetonitrile as solvent with tetra-*n*-butylammonium tetrafluoroborate as supporting electrolyte was chosen due to the stability of the combination at higher oxidation potentials.[8] Both the counter and working electrodes were platinum. Most experiments examined the oxidation of the monotetra-*n*-butylammonium salt of ethylphosphonic acid. The monotetra-*n*-butylammonium salt was used to control the ionization state of the organophosphonate being oxidized while enhancing the solubility of the organophosphonate in acetonitrile. All manipulations associated with weighing electrolyte, transferring solutions, and assembling the electrochemical cell were done under inert nitrogen atmosphere so as to avoid introduction of water into the cell.

During controlled-potential oxidations in excess of +2.0 V (SCE), gas evolution was observed at the counter electrode. Analysis of this gas indicated the presence of ethene and butene. These products apparently arise from the well-precedented reductive cleavage of quarternary ammonium salts.[9] Given our interest in the gaseous products formed during electrochemical oxidation of organophosphonates, a divided cell was used where the counter electrode was completely separated from the working

Scheme II



electrode headspace. Control experiments were run with the assembled cell containing solvent and supporting electrolyte. Even when the oxidation potential was maintained at +2.20 V for 2 h, no hydrocarbon products were observed in the working electrode headspace.

Figure 1 shows a typical cyclic voltammogram of ethylphosphonate electrochemical oxidation. A major current peak occurs at 2.20 V with a smaller peak at 1.68 V. Controlled-potential oxidation of a stirred solution of ethylphosphonate at both 2.20 and 1.68 V (SCE) produced ethene with low current efficiencies. Similar oxidation of propylphosphonate generated propene. Neutralization of the alkylphosphonate in excess of pH 7.0 resulted in voltammograms which looked almost identical with the electrochemical oxidation of tetra-*n*-butylammonium hydroxide in acetonitrile.[10]

Discussion

Chemical precedent suggests that alkylphosphonate degradation might involve initial insertion of atomic or molecular oxygen at the carbon α to the phosphorus (paths a, b, c, and d of Scheme II). The resulting α-hydroperoxy **17**,[11] α-hydroxy **18**,[12] α-keto **20**,[13] or phosphate monoester **21**[14] could then decompose or be easily degraded by the biological system.[15,16] However, the products of *Escherichia coli* degradation of methylphosphonate clearly indicate that this is not the case. Formation of ^{13}C-labeled methane during *E. coli* degradation of $^{13}CH_3PO_3H_2$ rigorously establishes that the methane being produced during the biodegradation is directly derived from methylphosphonate. The lack of deuterium label loss in the CD_3H generated during the biodegradation of $CD_3PO_3H_2$ indicates that the α-carbon of methylphosphonate is not first oxidized, the carbon to phosphorus

(7) (a) Kochi, J. K. *J. Am. Chem. Soc.* **1965**, *87*, 2500. (b) Kochi, J. K. *J. Org. Chem.* **1965**, *30*, 3265.

(8) (a) Eberson, L.; Nyberg, K. *Adv. Phys. Org. Chem.* **1976**, *12*, 1. (b) Fleischmann, M.; Pletcher, D. *Tetrahedron Lett.* **1968**, 6255.

(9) (a) Mann, C. K.; Barnes, K. K. *Electrochemical Reactions in Nonaqueous Systems*; Marcel Dekker, Inc.: New York, 1970; Chapter 9. (b) Finkelstein, M.; Petersen, R. C.; Ross, S. D. *J. Am. Chem. Soc.* **1959**, *81*, 2361. (c) Ross, S. D.; Finkelstein, M.; Petersen, R. C. *J. Am. Chem. Soc.* **1960**, *82*, 1582. (d) Mayell, J. S.; Bard, A. J. *J. Am. Chem. Soc.* **1963**, *85*, 421. (e) Finkelstein, M.; Petersen, R. C.; Ross, S. D. *Electrochim. Acta* **1965**, *45*, 187. (f) Littlehaites, J. D.; Woodhall, B. J. *Discuss. Faraday Soc.* **1968**, *45*, 187. (g) Dubois, J. E.; Monvernay, A.; Lacaze, P. C. *Electrochim. Acta* **1970**, *15*, 315.

(10) Simonson, L. A.; Murray, R. W. *Anal. Chem.* **1975**, *47*, 290.

(11) (a) Mikolojczyk, M.; Midura, W.; Grzejszczak, S. *Tetrahedron Lett.* **1984**, 2489. (b) Mikolajczyk, M.; Brzejszczak, S.; Midura, W.; Popielarczyk, M.; Omelanczuk, J. In *Phosphorus Chemistry. Proceedings of the 1981 International Conference*; Comstock, M. J., Ed.; American Chemical Society: Washington D.C., 1981; Chapter 11.

(12) Horner, L.; Roder, H. *Chem. Ber.* **1970**, *103*, 2984.

(13) Berlin, K. D.; Taylor, H. A. *J. Am. Chem. Soc.* **1964**, *86*, 3862.

(14) Quin, L. D.; Kisalus, J. C.; Mesch, K. A. *J. Org. Chem.* **1983**, *48*, 4466.

(15) Degradation by pathway a and pathway d would provide inorganic phosphate directly. Pathways b and c would lead to generation of phosphorous acid **19** which could be oxidized to inorganic phosphate. Such oxidation has been observed in a microbial system: Malacinski, G. M.; Konetzka, W. A. *J. Bacteriol.* **1967**, *93*, 1906.

(16) One also could conceive of reductive cleavage of the carbon to phosphorus bond or addition of the elements of water across the carbon to phosphorus bond. The nearest chemical precedent for these processes involves hydrogenolysis and hydrolysis of phosphonium salts and ylides. Similar chemistry has not been observed for alkylphosphonic acids. See: (a) Johnson, W. A. *Ylid Chemistry*; Academic: New York, 1966. (b) Wadsworth, W. S., Jr.; Emmons, W. D. *J. Am. Chem. Soc.* **1961**, *83*, 1733.

bond cleaved, and the resulting formate, formaldehyde, or methanol reduced to methane. There is the possibility that the process responsible for methane generation from methylphosphonate is operating concurrent with one of the degradative mechanisms outlined in Scheme II. However, the mole ratio of methane to inorganic phosphate produced indicates that if nonhydrocarbon-generating biodegradaton is occurring, such a pathway is responsible for only a minor portion of the methylphosphonate degraded.

One chemical mechanism which can account for the products of *E. coli* degradation of methylphosphonate is radical-based dephosphorylation. As depicted in Scheme I such a process would be initiated by generation of a phosphonyl radical. Subsequent fragmentation of this reactive intermediate would lead to monomeric metaphosphate and a methyl radical. Abstraction of a hydrogen atom by the methyl radical would yield methane gas. Radical-based dephosphorylation is thus consistent with the formation of CD_3H during *E. coli* degradation of $CD_3PO_3H_2$. Presumably, the monomeric metaphosphate would react rapidly with water to form the inorganic phosphate necessary for the survival of the microbe.

One centerpiece of radical-based dephosphorylation is the alkyl radical. A number of the organophosphonate biodegradation products identified in Table I support the notion of an alkyl radical intermediate. For instance, radical-based dephosphorylation of cyclopropylmethylphosphonate would proceed through a cyclopropylmethylcarbinyl radical. Such radicals undergo ring-opening reactions to afford 1-butene. Formation of this alkene during the biodegradation of cyclopropylmethylphosphonate is compelling evidence in favor of radical intermediates.

The ratio of cyclopropylmethane to 1-butene can provide an indication of the rate constant for hydrogen atom abstraction at the enzyme active site. If the product ratio reflects only the partitioning of the cyclopropylcarbinyl radical between ring opening (1.3×10^8 s^{-1})[17] to butenyl radical and hydrogen atom abstraction leading to cyclopropylmethane, the 50:1 ratio of cyclopropylmethane to 1-butene suggests a rate constant of 6.5×10^9 s^{-1} for hydrogen atom abstraction at the enzyme active site. This assumes that the rate constant of hydrogen atom abstraction by the butenyl radical is substantially greater than the rate constant[18] for cyclization of the butenyl radical back to cyclopropylcarbinyl radical. There is no way currently to determine if such an assumption is correct. Thus although the ratio of products indicates that the rate of hydrogen atom abstraction by cyclopropylcarbinyl radical is rapid, 6.5×10^9 s^{-1} must be considered an upper limit.

It is conceivable that the system which oxidizes the alkylphosphonate to the phosphonyl radical is also capable of oxidizing the alkyl radical to a carbonium ion. Subsequent loss of a proton would afford the ethene or isobutene observed during the biodegradation of ethylphosphonate and isobutylphosphonate. The ratio of alkane:alkene would then be a function of the rate of the second one-electron oxidation vs. the rate of hydrogen atom abstraction by the alkyl radical. Given the rapid rate of hydrogen atom abstraction suggested in the biodegradation of cyclopropylmethylphosphonate, the frequency of oxidative elimination of alkyl radical at the enzyme active site may be rather low. This is consistent with the amounts of ethene and isobutene generated during *E. coli* degradation of ethyl- and isobutylphosphonate.

Table I also emphasizes that the biodegradation is not limited to alkylphosphonic acids. Phenyl-, vinyl-, and allylphosphonates are all degraded to products consistent with radical-based dephosphorylation. *E. coli* inability to exploit isopropyl-, *tert*-butyl-, and benzylphosphonate as phosphorus sources (Table I) is rather surprising given the pronounced stability of the alkyl radical which would result from radical-based dephosphorylation of these organophosphonates. Thus while radical-based dephosphorylation can reliably account for the products formed during biodegradation of a given organophosphonate, this mechanistic interpretation is a far less useful tool relative to predicting which organophosphonates are susceptible to *E. coli* degradation.

The formation of alkane and alkene products during *E. coli* and lead(IV) tetraacetate degradation of alkylphosphonates is an important link between the biodegradation and chemical model system. However, the chemical model has been rather fragile precedent for radical-based dephosphorylation[19] given the conversions (1–2% based on Pb(IV) consumed) of alkylphosphonate to hydrocarbon. Closer scrutiny of the reaction of lead(IV) tetraacetate with alkylphosphonates affords an explanation for the low conversion and suggests the parameters to be followed for optimization of the reaction.

For all of the alkylphosphonates, the moles of carbon dioxide formed during the chemical reaction (Table II) vastly outstrip the hydrocarbon production. Carbon dioxide undoubtedly arises from the oxidative decarboxylation of the acetate ligands of the lead(IV), and this points to the inefficiency of the initial exchange of the alkylphosphonate for the acetate ligands. The ligation of the alkylphosphonates with the lead is likely bidentate given the lack of reaction of the monoethyl and diethyl ester of ethylphosphonate. Product formation in the chemical reaction can be viewed in terms of an intermediate alkyl radical. Loss of an electron followed by a proton leads to alkenes while hydrogen atom abstraction by the alkyl radical results in alkane product. The involvement of a radical chain reaction is implicated by the complete cessation of hydrocarbon formation upon exposure of the reaction to oxygen.

Summing the moles of carbon dioxide, alkene, and alkane formed (Table II) accounted for only one-half of the lead(IV) consumed during the reaction. This observation and the large quantities of intractable solid formed during the reaction suggested that the alkyl radical intermediates may be undergoing some reaction other than oxidative elimination or hydrogen atom abstraction. An efficient trap of the alkyl radical was therefore sought and subsequently found with copper(II) acetate. Intermediate alkyl free radical oxidation by copper(II) is precedented to be quite rapid.[20] As expected, there was a pronounced change in the ratio of alkene to alkane product subsequent to addition of copper(II) acetate to the lead(IV) tetraacetate oxidation with alkene formation becoming predominant (Table III). The presence of copper(II) acetate in the reaction of lead(IV) tetraacetate with alkylphosphonic acids also increased the overall yield of hydrocarbon products without altering the amount of carbon dioxide generated (Table III). For the reactions of isopropyl- and *tert*-butylphosphonic acids, 70–80% of the lead(IV) loss could be accounted for subsequent to addition of copper(II) acetate.

Another entry route into the type of radical-based dephosphorylation outlined in Scheme I might involve homolysis of phosphonyl peroxides. Unfortunately, phosphonyl peroxides which have been synthesized do not undergo homolytic scission of the peroxide bond.[21] We therefore focused on electrochemical oxidation of organophosphonates, which has an advantage of providing an oxidation potential for removal of electrons from the phosphonate moiety. Formation of ethene from ethylphosphonate and propene from propylphosphonate indicates that electrochemical oxidation of alkylphosphonates is leading to cleavage of the carbon to phosphorus bond. The main current peak at 2.20 V (SCE) for the monoanion of ethylphosphonic acid is at the limit of the oxidative stability of the electrolyte/solvent combination. This current peak and the production of olefinic products are quite

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similar to electrochemical oxidations of carboxylic acids[22] (the Kolbe oxidation). Unfortunately, both current peaks at 1.68 and 2.20 V (SCE) are far in excess of oxidation potentials possessed by enzymes in *E. coli*. Certainly the oxidation potential of the phosphonate moiety could be perturbed to more accessible levels by the biological system, but to what extent this can be accomplished is not yet clear.

Conclusion

Given the structural diversity of the organophosphonates degraded by *E. coli*, the ability of radical-based dephosphorylation to correctly predict the products of the biodegradation is noteworthy. The necessary chemical precedent for radical-based dephosphorylation is provided via the alkylphosphonate reaction with lead(IV) tetraacetate and electrochemical oxidation. However, chemical degradation of alkylphosphonates along the lines of the radical-based dephosphorylation of Scheme I requires forcing conditions which are difficult to translate to the microbial system. Therefore, although it has opened an entirely new area of organophosphorus chemistry, radical-based dephosphorylation of the type formulated in Scheme I does not fulfill all of the criteria necessary for a definitive explanation of the chemistry exploited during *E. coli* cleavage of the carbon to phosphorus bond of organophosphonates.

Experimental Section

General. Chemicals were purchased from Aldrich, Sigma, or Alpha. Unless otherwise specified, all organophosphonates used in *E. coli* growths were purified by chromatography on AG 1×8 (Bio-Rad) with a linear triethylammonium bicarbonate gradient (0.1–0.5 N) at pH 7.0. Fractions which eluted prior to inorganic phosphate were concentrated, and the triethylammonium bicarbonate was removed with a 2-propyl alcohol azeotrope followed by passage through a Dowex 50 (H^+ form) column. Concentration under high vacuum afforded the free acid. Calibration gases used as the internal standards were purchased from Alltech Associates, Inc.

^{1}H NMR spectra were recorded on a Varian XL-400 spectrometer and chemical shifts reported in parts per million relative to internal tetramethylsilane (CH_4Si, $\delta = 0.0$) with $CDCl_3$ as solvent and to sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP, $\delta = 0.0$) when D_2O was the solvent. ^{13}C NMR were recorded on a Varian XL-400 spectrometer and chemical shifts reported in parts per million relative to internal acetonitrile (CH_3CN, $\delta = 3.69$). A Nicolet 300 spectrometer was used for all ^{31}P NMR and shifts recorded relative to an external phosphoric acid standard. A Cary 14 double-beam spectrophotometer was used for optical density measurements. High pressure liquid chromatography (HPLC) was run on a Waters Associates ALC/510/R401/6000 system. Cyclic voltammetry was performed on an EG&G Princeton Applied Research Corporation Model 173 Potentiostat/Galvanostat driven by a Model 175 Universal Programmer.

Alkane and alkene analyses were carried out on a Hewlett Packard HP5890A gas chromatograph fitted with a Hewlett Packard HP3392A integrator. Volatile product mixtures were separated on gas chromatographic columns which included 0.19% picric acid on Graphpac GC, 80% Porapak N + 20% Porapak Q, Carbosphere, or Alumina F1 purchased from Alltech Associates, Inc. Flame ionization detection was used for products formed during *E. coli* degradation of alkylphosphonates as well as electrochemical oxidations. Thermal conductivity detection was used during quantitation of volatile products formed during reaction of lead(IV) tetraacetate with alkylphosphonates. Products were characterized by coinjection with authentic samples as well as GC–MS analysis on a Hewlett Packard 5970 mass spectrometer and comparison of fragmentation patterns with those of authentic samples. Alkene contaminants in diethyl vinylphosphonate and diisopropyl cyclopropylmethylphosphonate were analyzed by gas chromatography on a 0.25 μm fused silica SE-54 capillary column (J&W Scientific, Inc.) and flame ionization detection.

Synthesis of Cyclopropylmethylphosphonate. Cyclopropylmethyl bromide (5.0 g, 0.037 mol) and triisopropyl phosphite (7.2 g, 0.037 mol) were refluxed together at 140 °C overnight. The diester thus formed was vacuum distilled to yield 5.1 g (0.023 mol, 62%) of diisopropyl cyclopropylmethylphosphonate. Analysis of the diisopropyl cyclopropylmethylphosphonate on a fused silica capillary column found small contaminating levels of diisopropyl 3-butenylphosphonate (0.25 mol %). The identity of the contaminant was established by coinjection with diisopropyl 3-butenylphosphonate prepared by condensation of triisopropyl phosphite with 4-bromo-1-butene. This trace contaminant was eliminated by purification of the diisopropyl cyclopropylmethylphosphonate by HPLC on a semipreparative μ-Bondapak C-18 column (60:40, water:acetonitrile elution). After HPLC purification, no diisopropyl 3-butenylphosphonate could be detected by the gas chromatographic analysis.

Transesterification proceeded by reaction for 5 h with neat bromotrimethylsilane (13.9 g, 0.09 mol) at room temperature under a nitrogen atmosphere. Excess bromotrimethylsilane was subsequently removed under vacuum. Diethyl ether (30 mL) was added to the resulting oil and the solution extracted three times with water (20 mL) to obtain 2.8 g of the free acid (0.02 mol, 90%) that was further purified by standard anion exchange chromatography (see General). ^{1}H NMR ($CDCl_3$) 0.25–0.3 (m, 2 H), 0.55–0.60 (m, 2 H), 0.9–1.0 (m, 1 H), 1.65–1.75 (dd, 2 H), 7.5–8.0 (s, 2 H); ^{13}C NMR (D_2O) 2.32 (J_{PCCC} = 4.6 Hz), 2.84 (J_{PCC} = 11.2 Hz) 29.57 (J_{PC} = 134.4 Hz).

Reaction of Alkylphosphonic Acids with Lead(IV) Tetraacetate. Alkylphosphonic acid (1 mmol) and lead(IV) tetraacetate (0.2 mmol) were transferred to a 50-mL, two-necked, round-bottomed flask and a balloon attached to modify any pressure increase during the course of the reaction. The reaction apparatus was attached to a gas manifold, anhydrous DMF added to dissolve the reagents, and the resulting solution degassed by alternate evacuation and introduction of nitrogen. After disconnecting the reaction vessel from the gas manifold, the reaction was allowed to proceed for 1 h in an oil bath at 110 °C. At the end of the reaction the vessel was immersed in liquid nitrogen for a few minutes to completely collapse the attached balloon. The balloon was then removed and the flask allowed to warm to room temperature (**BEHIND A SAFETY SHIELD**!) and product gases analyzed by GC. The yields and ratios reported were the average of three trials.

Electrochemistry. For all of the electrochemical measurements, both working and counter electrodes were platinum (0.25 cm^{-2}). Prior to each run, both electrodes were immersed in concentrated nitric acid for 15 min, rinsed with distilled, deionized water, and dried under a nitrogen stream. A three-compartment electrochemical cell was used where the counter electrode headspace was completely segregated from the working electrode headspace. Contact between the reference and counter electrode solutions and the working electrode solution was through a Vycor frit. The electrochemical cell was airtight. All manipulations of solvent, electrolyte, organophosphonate, as well as assembly of the electrochemical cell were carried out in a Vacuum/Atmospheres inert atmosphere container. Once assembled and during subsequent cyclic voltammetry, the cell was sealed under nitrogen atmosphere.

Tetra-*n*-butylammonium tetrafluoroborate was recrystallized from hexane/ethyl acetate and dried under vacuum at 60 °C for 24 h. Acetonitrile was distilled under nitrogen from phosphorus pentoxide. Counter electrode, working electrode, and reference electrode compartments all contained solutions of 0.5 M tetra-*n*-butylammonium tetrafluoroborate in dried acetonitrile. The reference electrode consisted of a ferrocene/ferrocinium hexafluorophosphate couple calibrated at the end of each oxidation relative to SCE.

The alkylphosphonates were dissolved in water and tetra-*n*-butylammonium hydroxide (40% solution) was added to pH 3.5. After concentration under vacuum, the oil was azeotroped three times with dry acetonitrile and dried under vacuum for 24 h. The evacuated flask containing the organophosphonate was transferred to the inert atmosphere container and a solution of the organophosphonate in acetonitrile prepared. This solution was transferred via syringe to the electrochemical cell to make the counter and working electrode solutions 0.01 M in alkylphosphonate. Two oxidation waves were observed with a peak potentials at 1.68 and 2.20 V. Controlled-potential oxidation at both 1.68 and 2.20 V provided ethene with current efficiencies of 0.1% and 0.2%, respectively.

***E. coli* Growths.** All glassware used for *E. coli* growths was immersed in 8 M nitric acid for a 12-h period followed by multiple rinsings with distilled, deionized water. Agarose (Type II, medium EEO purchased from Sigma) was electrophoretically eluted as a 6 mm thick slab gel with distilled, deionized water containing trizma·HCl (64 mM, pH 7.0).

E. coli RB 791 (W3110 lacL8I^q), an *E. coli* K-12 variant, was maintained at −20 °C on 50% aqueous glycerol. The aqueous solution contained glucose (26 mM), magnesium sulfate (1.0 mM), trizma·HCl (64 mM), sodium chloride (8.0 mM), ammonium chloride (19 mM), thiamine (0.014 mM), and inorganic phosphate (0.2 mM) at pH 7.0.

Growth on solid medium consisted of streaking *E. coli* from the glycerol freezes onto plates containing glucose (26 mM), magnesium sulfate (1 mM), trizma·HCl (64 mM), sodium chloride (8 mM), ammonium chloride (19 mM), thiamine (0.014 mM), phosphonate (0.2 mM), and purified agar (1.5% wt/v). Simultaneous to application to solid media

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containing organophosphonate, *E. coli* from the glycerol freeze were spread on control plates containing all of the growth medium components minus the organophosphonate.

Growth in liquid suspension entailed transfer of a single colony into a sterile test tube containing 5 mL of growth medium. The growth medium was identical with that used for growths on solid media with the exception that the agarose was omitted. Test tubes were shaken at 37 °C for 12–18 h or until a cloudy suspension developed. An inoculant (1.5 mL) was subsequently transferred from the test tube growth to a flask (145 mL total volume) containing 50 mL of growth solution. All containers, transfers, and solutions were sterile. The final growth flask was then sealed. After the flask was shaken for 12–18 h, a sample of the headspace of the sealed flask was withdrawn with a gas-tight syringe and analyzed by gas chromatography.

Quantitation of Inorganic Phosphate and Hydrocarbon Formed during *E. coli* Degradation of Methylphosphonates.[23] Methane produced during a growth was quantitated via the appropriate response factor relative to an ethane internal standard. Cells from growths in the sealed flasks were harvested by centrifugation (10000 g for 10 min) at 0 °C. After removal of the supernatant, the cells were twice washed with salt solution (trizma·HCl (64 mM), ammonium chloride (19 mM), and sodium chloride (8 mM)) and harvested by centrifugation (10000 g for 10 min) at 0 °C. The pellet was resuspended in 5 mL of salt solution and a portion (0.1 mL) transferred to each of three borosilicate test tubes. A range of concentrations of inorganic phosphate standards were added to separate test tubes. To each of the tubes containing *E. coli* cells and inorganic phosphate standards was added 5.0 M sulfuric acid followed by heating at 150 °C for 3 h. After the tubes were cooled, hydrogen peroxide (30% aqueous solution) was introduced to each tube and heating once again begun at 150 °C. The tubes containing cell debris were removed as soon as the clear solution began to yellow while the inorganic phosphate standards continued to be heated for 1.5 h at 150 °C. Subsequently 1.8 mL of a solution of ammonium molybdate (1.8 mM) was added to all of the tubes followed by 0.12 mL of Fiske and Subbarow reducer (obtained from Sigma). All of the tubes were then heated at 100 °C and cooled to room temperature, and the optical density of each tube measured at 812 nm. The amount of inorganic phosphate in the digested cell solutions was quantitated by comparison of optical density at 812 nm to the calibration curve derived from the inorganic phosphate standards.

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The Separation of the Intramolecular Isotope Effect for the Cytochrome P-450 Catalyzed Hydroxylation of *n*-Octane into Its Primary and Secondary Components

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Abstract: The intramolecular isotope effect for the cytochrome P-450b ω-hydroxylation of [1,1,1-$^2H_3$]-*n*-octane was separated into its primary and secondary components by the method of Hanzlik (Hanzlik et al. *J. Am. Chem. Soc.* **1985**, *107*, 7164). The primary isotope effect was found to lie between 7.3 and 7.9 while the secondary isotope effect was found to lie between 1.09 and 1.14. These data are consistent with a highly symmetrical transition state with 15% of the observed isotope effect being due to secondary isotope effects. Although the system was found to depart from the rule of the geometric mean, the phenomenon could not be attributed to tunneling.

Recently, we reported that the intrinsic isotope effect associated with the cytochrome P-450 catalyzed hydroxylation of the methyl group in *n*-octane was 9.8.[1] The large magnitude of this isotope effect and the even larger values reported by others[2,3] suggest that tunneling may contribute significantly to the reaction rate. For example, on the basis of Bell's analysis, the maximum allowable semiclassical isotope effect for cleavage of a carbon hydrogen vs. carbon deuterium bond would be 9 at 37°.[4] Unfortunately, the isotope effects associated with cytochrome P-450 aliphatic hydroxylation have invariably been reported as a combination of primary and secondary effects thereby precluding any assessment of the role tunneling might play in these reactions. We now report the separation of primary and secondary isotope effects, by the method of Hanzlik et al.,[5] for the aliphatic hydroxylation of *n*-octane. These data are consistent with a highly symmetrical transition state and indicate that up to 15% of the observed isotope effect is due to secondary isotope effects.

Experimental Section

Materials. *N,O*-Bis(trimethylsilyl)trifluoroacetamide and acetonitrile were obtained from Pierce Chemical Co., diethyl ether was obtained from J. T. Baker, and pentane was obtained from Burdick and Jackson. All biochemicals were obtained from Sigma Chemical Co.

Instrumentation. Gas chromatography was performed with an HP 5840A gas chromatograph modified for use with a J&W DB-5 or DB-1 capillary column. Difference spectra were recorded on an HP 8451A UV spectrophotometer. GC/MS analysis of the product alcohols was performed on a VG 7070H mass spectrometer in the selected ion recording mode, interfaced to a HP-5710A GC fitted with a J&W DB-5 fused silica capillary column. The derivatized metabolities were cold trapped at 40 °C, and then the temperature was ramped at 20 deg/min to 90 °C followed by isothermal elution. Mass spectral parameters were as follows: dwell, 5 ms; ionizing voltage, ca. 70 eV; source temperature 200–205 °C. The deuterium incorporation in each substrate was determined by bleeding each compound into the source of the mass spectrometer at a steady rate through the reference inlet and monitoring the ion current of the various isotopically substituted species with selected ion recording of the molecular ion. The mass spectrometric parameters for the substrates were the same as those for the analysis of the product alcohols except the dwell time was increased to 50 ms. The measured intensity of each ion monitored was corrected for the natural isotopic abundance of ^2H, ^{13}C, ^{14}C, ^{18}O, ^{29}Si.

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