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Homolytic carbon to phosphorus bond scission of some phosphonates catalyzed by bacterial carbon-phosphorus lyase

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Abstract

The production of volatile degradation products of phosphonates was monitored to investigate the mechanism involved in the biodegradation of propylphosphonic acid and phenylphosphonic acid by *Rhizobium* sp MMM101a. The biodegradation of propylphosphonic acid gave rise to the production, in decreasing order, of propane, methane, ethane, 1-butene, propene, isobutene, butane and ethene. The formation of these degradation products was strongly reduced by adding catalase to the growing cultures indicating the involvement of peroxides in the biodegradation mechanism. OH° radical scavengers did not reduce the rate of biodegradation, and therefore these radicals appear not to be involved. Addition of ascorbate, a known hydroxylating agent in biological systems, increased the amount of biodegradation products. The involvement of iron in the degradation was indicated and was optimal at a concentration of 950 μM. This suggests the involvement of a metalloenzyme involving iron and peroxide. The decomposition of phenylphosphonic acid yielded benzene and biphenyl. No phenol could be detected, again suggesting that OH° radicals were not involved in the biodegradation. The presence of deuterated benzene did not result in the occurrence of biphenyl consisting of one nondeuterated and a deuterated ring, which is chemically more likely. It therefore appears that the degradation of the phosphonates occurs on a multicentered enzyme. The diversity of the products generated by this bacterium from phosphonates, many of them due to rearrangement of the carbon moiety of the substrate molecule, suggests an overall involvement of superoxide radicals in the homolytic carbon to phosphorus bond scission.

Introduction

Phosphonates are a class of organophosphorus molecules characterized by one or more carbon to phosphorus (C-P) bonds. They find a very wide application in industrial processes, agriculture and consumer goods. Phosphonate molecules as hydroxyethylenediphosphonic acid (D2010), aminotrimethylenephosphonic acid (D2000), ethylenediaminetetramethylenephosphonic acid (D2041), hexamethylenediaminetetramethylenephosphonic acid (D2051) and diethylenetriaminepentamethylenephosphonic acid (D2060) are used as sequestrants in laundry activities, boilers and cooling systems. They are disposed in water (via domestic and industrial effluents) (Gledhill, 1991). Phosphonate pesticides as glyphosate (Roundup^{TM}) and glufosinate (Basta^{TM}) are present in soils via agricultural activities (Pipke et al. 1987). In addition, accidental releases of these phosphonate compounds can be expected. Since the C-P bond is highly resistant to chemical and thermal decomposition, the study of the biodegradation of these compounds is of major importance to prevent accumulation of these compounds and their derivatives in the environment (Schowanek and Verstraete, 1990a).

Two different biological cleavage mechanisms for the C-P bond have been described which are tightly Pi regulated. A third mechanism that would not be controlled by the PHO regulon, and which as a consequence is not Pi regulated, might also be possible (Wanner and Metcalf, 1992). The strain used in these experiments also shows Pi regulated phosphonate degradation; however this regulation is not as tight as the regulation reported observed with other strains (Schowanek and Verstraete, 1990a).

The first degradative pathway involves phosphonoacetaldehyde hydrolase, also called phosphonatase. This enzyme was originally described in *Bacillus cereus* where it catalyzed the hydrolytical cleavage of phosphonoacetaldehyde to acetaldehyde and Pi (La Nauze and Rosenberg, 1968). The second pathway found in a variety of strains has been given the trivial name 'C-P lyase'. This degradation mechanism is especially encountered in the degradation of alkyl and arylphosphonates. The mechanism of this reaction and part of the genetic regulation require further elucidation (Metcalf and Wanner, 1993). These authors suggest two possible modes of action. In the type A mode, there could be a redox cleavage of the C-P bond by a mechanism involving redox chemistry at the P center, e.g. a reduction of the phosphonate at the P center revealing a carbon chain and phosphoric acid. Oxidation in a second step would then release Pi.

$$RCH_2PO_3H2 \xrightarrow[Reduction]{2H^+} RCH_3 + [P(OH)_3]$$
$$\xrightarrow{Tautomerisation} HPO(OH)_2 + R \xrightarrow[Oxidation]{O_2^{-\cdot}} H_3PO_4 + R \tag{1}$$

A second proposed mode (type B) could consist of a direct oxidation of the phosphonate.

$$RPO_3H_2 \xrightarrow[Oxidation]{O_2^{-\cdot}} H_3PO_4 + R \tag{2}$$

Several authors presume the involvement of radical-mediated degradation (Cordeiro et al., 1986; Shames et al., 1987; Wanner and Metcalf, 1992). In the type B mode, it is assumed that superoxide radicals are involved (Wanner and Metcalf, 1992). The degradation of alkylphosphonates normally produces Pi and the respective alkylchain, as reported by Cordeiro et al. (1986); Shames et al. (1987); Wacket et al. (1987). No reports are known to us about the modification of the carbon chain after the action of a C-P lyase. Only Cordeiro et al. (1986) mentioned a minor modification of the carbon skeleton to an additional unsaturation. In this paper, rearrangements in the carbon moiety of organophosphonates with formation of polycondensates were monitored.

Materials and methods

Reagents and glassware

Propylphosphonic (PrPn) acid and deuterated biphenyl(D_{10}) were purchased from Sigma Chemical Co., phenylphosphonic acid (PhPn) from Janssen Chemicals, Beerse Belgium. Deuterated benzene(D_6), toluene(D_8) were from Aldrich Chemical Company Inc. All other reagents were of the highest purity commercially available. All media and stock solutions were prepared with Milli-Q water (Milli-Q System; Waters Associates, Inc.) with a resistance (R) > 18 $M\Omega\ cm^{-1}$. Catalase from beef liver with an activity of 260000 U/ml was purchased from Boehringer Mannheim GmbH as a solution in ethanol and glycerol. All standard gasses were purchased from L'Air Liquide (Belgium). Each piece of glassware was cleaned meticulously by washing it with P-free detergent (Ecover, Malle Belgium), followed by rinsing with distilled water and was soaked for at least 12 h in 10% HNO_3 to avoid any contamination. Thereafter the glassware was finally rinsed 4 times with Milli-Q water. Background levels of P never exceeded 2 μM of P. Accordingly, growth was never observed in P-free media ($OD_{600} <$ 0.030).

Medium

The growth medium (BMM) was modified from Schowanek and Verstraete (1990b). It consisted of the following per liter of medium: 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid], 200 mg KCl, 200 mg $MgSO_4 \cdot 7H_2O$, 25 mg $CaCl_2.2H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$, 1325 mg NH_4Cl, 3000 mg glucose, 3380 mg sodium succinate, 3070 mg glycerol. The pH was adjusted to 7.2 with NaOH 1 M. For solid plates, noble agar (Difco Laboratories) was supplemented (15 g/l) prior to autoclaving. Yeast extract (Difco laboratories) was made phosphate free according to Weimberg and Orton (1963) and was filtersterile (0.2 μm) added to the autoclaved medium to a final concentration of 0.05 g/l.

Phosphonate and KH_2PO_4 (Pi) stock solutions were prepared with Milli-Q water and were of a concentration of 100 mM. Phosphate (Pi) or phosphonates were filtersterilised and aseptically supplemented to the autoclaved medium in appropriate concentrations.

Microorganisms

The bacterium used in this study, *Rhizobium* sp. MMM101a, was isolated from an enrichment culture from an activated sludge plant (Schowanek and Verstraete, 1990a). The strain was able to use phosphonic acid in the presence of phosphate (Schowanek and Verstraete, 1990a). It was grown on BMM agar plates for 3 days at a temperature of 28 °C with propylphosphonate as the sole P source. The strain was maintained at a temperature of 4 °C, and was routinely subcultured every month.

Degradation experiments

In spite of meticulous control of the cultivation of the inoculum, experimental results differed for each inoculum used. Therefore when different degradation conditions were compared, the same inoculum culture was used for all samples of a test series. For a given series thus prepared, results were reproducible.

Degradation experiments of propylphosphonic acid (Pn) were typically executed in sealed tubes (Bellco glass inc.) of 26 ml containing 6 ml of medium supplemented with Pi or Pn to a final concentration of 300 μM. In experiments evaluating the effect of catalase, different amounts of catalase were added prior to inoculation. In control experiments, catalase was inactivated by heating it to 100 °C. Activity of the catalase, both before and after the experiment was monitored by O_2 release from H_2O_2.

The initial atmosphere in the tubes consisted of 95.5% (v/v) O_2 and 4.5% (v/v) CO_2. Tubes where incubated upside down to avoid any gas leakage through the stopper. The tubes were incubated in the dark for 5 days at 28 °C with shaking at 78 rpm.

Degradation of phenylphosphonic acid was executed in washbottles with 100 ml of BMM medium. The bottles were inoculated with a fresh culture of MMM101a (10^9 cells/ml) at a concentration of 6% (v/v). The inlet (the immersed tube) of the washbottle was closed with a stopper. The outlet of the bottle was connected to a large ORBO^{32} tube (Supelco inc., USA). Uninoculated bottles served as controls. In each bottle 10 ppm of deuterated benzene(D_6), toluene(D_8) and biphenyl(D_{10}) was added. The addition of the deuterated compounds before incubation served to permit the calculation of the extraction efficiency of the formed compounds in the medium during degradation. Moreover, they served to determine the origin of the degradation products. The use of different containers were compared to the gasmeasurements. When using larger containers with more volume, more reaction products were trapped allowing a detection of traces degradation products. This was necessary because the used method for biphenyl determination was less sensitive compared to the method of the gasmeasurement. Controls done with washbottles and propylphosphonate revealed the same results as in the small containers. The bottles were incubated at a temperature of 28 °C on a linear shaker, at a speed of 80 reciprocations per minute for a period of 5 days. Bottles were covered from light to avoid photodegradation. After incubation, the stopper at the inlet of the bottle was removed to allow flushing with N_2 during 15 minutes at a temperature of 80 °C. This last step served to trap volatile degradation products on the ORBO^{32} tube. The ORBO^{32} trap was opened and the granulated activated carbon content was desorbed by 2 ml CS_2 according to the manufacturer. Samples were stored in 2 ml closed vials with septum (Chrompac). The vials were stored at −70 °C till further analysis.

Phosphonate stocksolutions were monitored for interfering impurities by H- NMR (Jeol Instruments), D_2O served as solvent.

Analytical facilities

Optical densities were measured with a Kontron UV spectrophotometer at a wavelength of 600 nm.

Analysis of the enriched gaseous degradation products was done according to Vandepitte et al. (1994). The CS_2 extracts from the ORBO^{32} tubes were analyzed by a Varian Gas Chromatograph coupled to a Finnigan Magnum 500 ion trap mass spectrometer. The Chromatograph was equipped with a DB 5.625 capillary column (J&W Scientific). The start temperature was 35 °C and was held for 3.33 minutes. Then the temperature was raised gradually to 200 °C at a rate of 25.1 °C/minute and was finally kept at this temperature during 5 minutes. Injector temperature was 300 °C. Manifold temperature was set at 220 °C. The injected sample volume consisted of 2 μl and was injected in splitless mode.

The mass detector was operated in chemical ionization (CI) mode and in electronical ionization (EI) mode. The ionization gas that served in CI mode, was methanol. The manifold temperature of the mass detector was kept at 220 °C and the scan rate was 1 second.

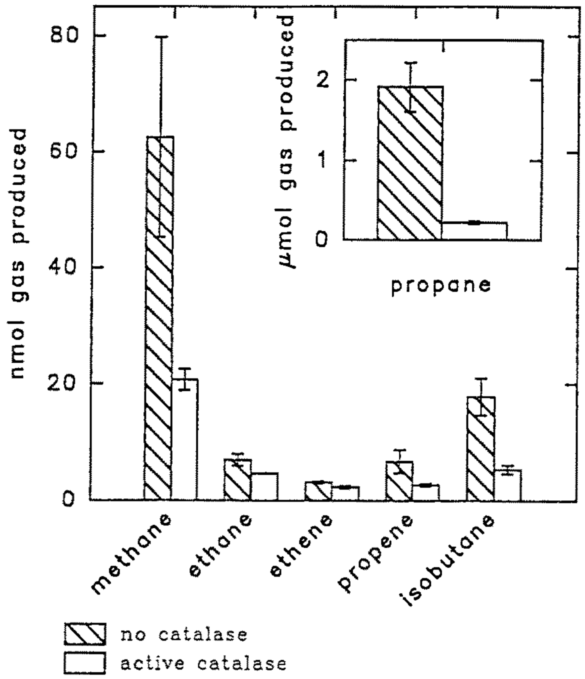

Fig. 1. Volatile degradation products from propylphosphonate (300 μM, i.e. 18 μmol/tube) by biological action of MMM101a. The reference test was made in the absence of active catalase. A series of tests were also performed in the presence of 4.3 U/ml active catalase. Results are means of triplicates, errorbars represent the standard deviation.

Results

Degradation of propylphosphonic acid

Propylphosphonate served as a model compound to evaluate the degradation of alkylphosphonates, and to obtain insights in the degradation mechanism of this class of xenobiotics. In our experiments, propylphosphonate served as the sole P-source. Two types of controls were used: uninoculated tubes and inoculated tubes with Pi as P-source. The former were to monitor abiotic release of degradation products, whereas the latter observed possible occurrence of gaseous degradation products during growth on BMM medium phosphate in absence of propylphosphonate. Oxygen was necessary for growth. Increasing the O_2 concentration to 95.5% strongly increased the degradation velocity (data not shown).

The final bacterial concentration, after 5 days of incubation, was the same for growth on Pi and propylphosphonate as indicated by the OD and by direct bacterial counting. Bacterial concentrations up to 10^9 organisms per ml were observed corresponding to an OD of 3.2.

When propylphosphonate was incubated with *Rhizobium* sp. MMM101a as inoculum, a spectrum of volatile degradation products was formed (Fig. 1). The major degradation product was propane, followed by methane, isobutane, propene, ethane and ethene. The molar ratios to the initial amount of substrate supplied were 1/1.1, 1/30, 1/90, 1/250, 1/360 and 1/600 respectively.

As peroxide production is a common phenomenon in the oxidative respiration (Brock and Madigan, 1991), the involvement of biologically produced H_2O_2 on the degradation of propylphosphonate was investigated by adding catalase to growing bacterial cultures at a final concentration of 4.3 U/ml. Catalase was verified to be active throughout the 5 days of incubation. Figure 1 illustrates the results obtained with addition of 4.3 U/ml catalase. As a reference, cultures where no catalase and where inactivated catalase was added (data not shown) were also incubated. A significant decrease in the amount of propane, methane and ethene production as degradation products of propylphosphonate was observed in the presence of catalase (Fig. 1). The control with inactivated catalase (data not shown) was similar to the control without added catalase. Results of an experiment with 0.8 U/ml instead of 4.3 U/ml catalase did not differ from the results obtained with 4.3 U/ml catalase (data not shown).

Phosphonic acids are very susceptible to free radicals (Schowanek and Verstraete, 1990a). H_2O_2 can generate radicals. Hence, these radicals might be directly involved in the degradation of phosphonates. In order to trap any reactive OH° formed during the biodegradation of the propylphosphonic acid, the medium was either supplemented with propylgallol (300 μM) or saturated with $CaCO_3$ in another experiment. These two compounds typically trap reactive OH° at the applied concentrations (Vick and Zimmerman, 1987; Duget and Anselme, 1989; Schowanek and Verstraete, 1990c). Propylgallol and $CaCO_3$ did not show any toxicity in the applied concentrations as was shown by normal growth of the strain in the presence of either of these compounds. For this test series, the degradation products were the same as in the reference cultures (data not shown). Apparently both radical scavengers were unable to alter the metabolic pattern i.e. the range and the amount of degradation products. Alternatively, OH° radicals are not directly involved.

Table 1. Methane, ethene, ethane and propane release from propylphosphonate (100 mM. i.e. 0.6 mmol/tube) by biological action of MMM101a in the absence and the presence of ascorbate (150 mM) and by abiological action of H_2O_2 (80 μM) and ascorbate.

Inoculum	nmol of gaseous degradation product detected in the headspace			
	methane	ethene	ethane	propane
MMM 101a	57	nd.	nd.	3
MMM 101a + ascorbate	61	10	230	842
H_2O_2 + ascorbate	5	10	27	392

nd. not detected

Table 2. Biodegradation products (mmol) from propylphosphonate (300 μM. i.e. 18 μmol/tube) by the biological action of MMM101a in the presence of different Fe^{2+} concentrations.

Degradation product	130 μM	400 μM	680 μM	950 μM	1230 μM	1510μM
			Fe^{2+}			
propane	300 ± 200	1080 ± 40	1180 ± 10	1250 ± 50	1100 ± 140	1120 ± 20
methane	45 ± 11	38 ± 0.6	37 ± 0.6	35 ± 3.4	38 ± 4.8	34 ± 0.7
ethane	11 ± 1.8	9.5 ± 0.9	10.5 ± 1.9	7.9 ± 3.5	9.8 ± 2.2	9.0 ± 0.3
ethene	3.4 ± 0.4	3.3 ± 0.1	3.52 ± 0.02	3.7 ± 0.1	3.4 ± 0.4	3.4 ± 0.4
isobutene	5.5 ± 4.3	2.1 ± 4.1	3.2 ± 6.4	5.2 ± 4.5	nd.	3.9 ± 5.0
1-butene	6.5 ± 1.2	6.6 ± 0.9	6.7 ± 0.1	6.6 ± 1.5	6.7 ± 1.4	5.8 ± 1.1
propene	5.6 ± 0.8	7.1 ± 0.9	7.7 ± 0.7	11.9 ± 0.5	12.8 ± 0.9	12.5 ± 0.0
butane	4.9 ± 0.5	5.1 ± 0.24	6.7 ± 0.1	7.7 ± 0.8	5.2 ± 0.6	5.4 ± 0.1

nd. not detected

In another experiment ascorbate (150 mM) was added as a reductant to a culture growing on propylphosphonate as a P-source. In order to match the high concentration of ascorbate, biodegradation experiments were executed with 100 mM propylphosphonic acid. Under the given conditions, biodegradation of propylphosphonate (100mM) gave relatively low yields of methane and propane only (Table 1). However by adding ascorbate to the growing cultures, production of ethene and ethane was also observed. The amount of propane increased by a factor 273, whereas the amount of methane remained constant.

The influence of iron, an important factor in radical generation (Roberfroid, 1987), was investigated by adding different concentrations of iron (added as $FeSO_4$) to the BMM medium with propylphosphonate, at a concentration of 300 μM, as the sole P-source. The lowest concentration of iron tested, i.e. 130 μM, was sufficient for optimal growth (Schowanek and Verstraete, 1990b). After 5 days of incubation with MMM101a, the released gases were quantified as a measure for the degradative activity of the strain. Production of propane, methane, ethane, ethene, isobutene, 1-butene, propene and butane was observed (Table 2). Propane was present in the highest concentration, with a significant maximum at 950 μM $FeSO_4$. The same maximum was observed for propene and butane, however much lower amounts were formed. The concentrations of the other gaseous compounds i.e. methane, ethane, ethene, isobutene and butene were independent of the applied iron concentrations (Table 2) and not significantly different (α = 0.05) for a given gaseous degradation product.

Mimicking the biological degradation reaction by adding different H_2O_2 concentrations to uninoculated test tubes containing BMM with 300 μM propylphosphonate revealed methane, ethene and propene production by the abiotic reaction (Fig. 2). Remarkably, no other gases could be observed. A similar experiment but using 50.000, 100.000 and 1000.000 μM propylphosphonate and 80 μM H_2O_2 revealed a different range of degradation products, similar to those

Table 3. Abiological methane, ethene, ethane, propene and isobutene production from different propylphosphonate concentrations by the action of hydrogen peroxide (80 μM).

Applied propylphosphonate	nmol of gaseous degradation product detected in the headspace				
	methane	ethene	ethane	propane	isobutene
50000 μM	30	10	5	nd.	40
100000 μM	44	13	6	nd.	198
1000000 μM	160	168	413	2	107474

nd. not detected

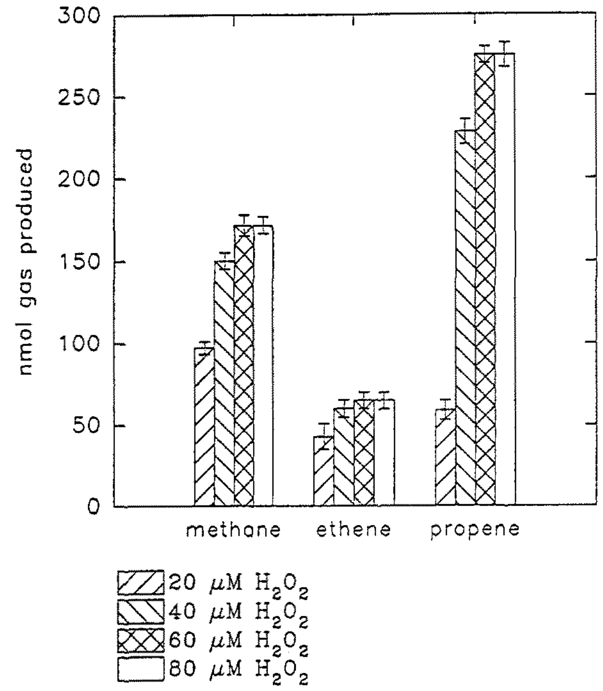

Fig. 2. Abiotic methane, ethene and propene production from propylphosphonate (300 μM, i.e. 18 μmol/tube) by action of hydrogen peroxide. Results are means of triplicates, errorbars represent the standard deviation.

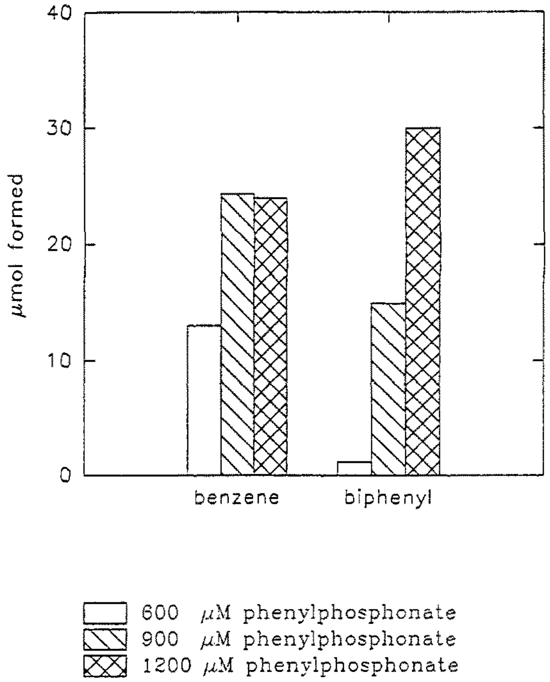

Fig. 3. Biological benzene and biphenyl production from phenylphosphonate by biological action of MMM101a.

observed in the biodegradation reactions (i.e. methane, ethene, ethane, propane, and isobutene (Table 3)). It should be noted that the concentration of propane in all these chemical degradation experiments was very low.

Mimicking the biodegradation reaction in the presence of ascorbate was carried out by adding hydrogen peroxide (80 μM) and ascorbate (150 mM) to a test tube with propylphosphonate (10.000 μM) (Table 1). The following degradation products and respective molar ratio's were found: methane 1/11000, ethene 1/5600, ethane 1/2000 and propane 1/152. These results were in accordance with the biological degradation in the presence of ascorbate. However, ethane and propane were observed at higher concentrations in the bioreaction, i.e. 8× and 2×, respectively.

Biodegradation of phenylphosphonic acid

Experiments with 600, 900 and 1200 μM phenylphosphonate as the sole P-source were executed with 100 ml cultures in washbottles. In all tests, benzene and

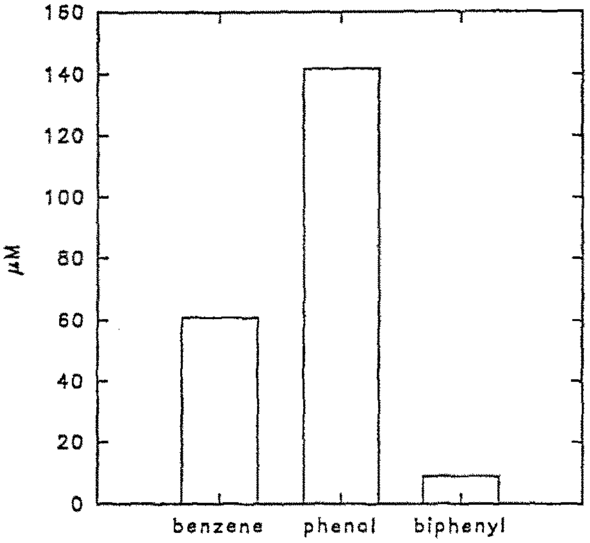

Fig. 4. Abiotic benzene, phenol and biphenyl production from phenylphosphonate (1200 μM) by the action of hydrogen peroxide.

biphenyl were observed (Fig. 3). Concurrent with the degradation of propylphosphonic acid, a condensation product of the arylchain, biphenyl, was also observed. To examine whether the biphenyl came from two benzyl radicals or from a condensation between an activated benzyl group (from the degradation of phenylphosphonic acid) and a previously formed benzene molecule, deuterated benzene (15 mM) was added to the medium. Analysis of the medium yielded the added deuterated compound, undeuterated benzene and biphenyl. Despite several efforts, no biphenyl nor another condensate of a deuterated ring structure and a nondeuterated ring, could be observed. These findings indicate that the biphenyl originated from the combination of two phenylradicals from the degradation of phenylphosphonate and not from a recombination of a phenylradical and another arylstructure present in the medium i.e the deuterated benzene. The latter reaction is however much more evident, if it is considered pure chemically (Pryor, 1966). It should be noted that phenol is a normal byproduct of the chemical Fenton oxidation of benzene (Walling, 1975). Simulation of the degradation by adding the Fenton reagent to uninoculated tubes with phenylphosphonate (1200 μM) indeed resulted in the occurrence of phenol, benzene and very small amounts of biphenyl (Fig. 4). In the biodegradation experiments, phenol was never detected.

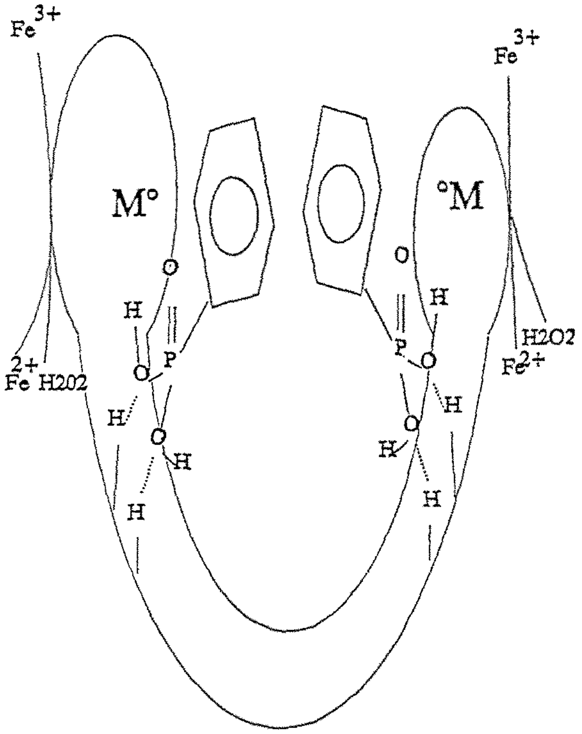

Fig. 5. Hypothetical reaction mechanism of phenylphosphonate at the C-P Lyase enzyme.

Discussion

The degradation of phosphonates is most probably mediated by a radical mechanism involving peroxide produced during respiration. This is supported by the formation of a wide range of degradation products. The occurrence of degradation products of propylphosphonate that are recombinations of the carbon chain of the phosphonate as butane and butene (Fig. 1) is also indicative of the radical reaction mechanism. The possibility mimic the bioreaction by the Fenton reaction (Table 3; Fig. 2) is supporting the concept that radicals of some type are involved in the breakdown. The effect of peroxide on the degradation of organic molecules in the presence of metals as iron is known as the Fenton reaction and has previously been documented (Plimmer et al., 1967; Wood, 1988; for a review Walling et al., 1975). The inhibition of the degradation reaction by catalase can been considered as evidence that peroxides participate in the reaction (Berneis, 1963).

The increase in the levels of gases released by iron addition (Table 2) is most probably caused by a more efficient functioning of the C-P cleavage mechanism.

The oxidation or reduction of metals is certainly a possible source of free radicals in vivo. This is supported by the occurrence of such species in cellular metabolism (Leach, 1954; Isenberg, 1964). Endogeneously produced free radical intermediates are sometimes important in anabolic and catabolic pathways. Hydroxylation by hydroxyl free radicals is common in biological systems, a classical example being the conversion of tyramine into hydroxytyramine, which is mediated by L-ascorbic acid (Udenfriend et al., 1954). Ascorbic acid in abundance, together with metal ions in the living organism is an ideal system for the production of free radicals. In the presence of copper ions, it is likely that the initial reaction is as follows (Yamazaki and Piette, 1961):

$$AH_2 + Cu^{2+} - > Cu^{+} + AH^{\circ} + H^{+} \tag{3}$$

(Where AH_2 is the reduced form of ascorbic acid, and AH° is the organic free radical derived from the substrate). However, other metals such as iron may take the role of copper. It has been claimed that such a free radical mechanism is the main pathway in the ascorbic acid oxidase and in peroxidase reactions (Yamazaki and Piette, 1961). In our experiments, the effect of added iron was pronounced (Table 2). This suggests the action of a metallo oxido-reductase enzyme in the degradation of the phosphonates. It seems likely that, compared to the biological reaction, a more vigorous reaction occurred in the pure chemical Fenton reagent reaction, converting propane to other compounds. Increasing the propylphosphonate concentration gave rise to other gaseous degradation products, due to another substrate oxidant ratio, resulting in another radical reaction scheme.

The absence of phenol in the biodegradation of phenylphosphonate and the observation that neither propylgallate nor CO_3^{2-} inhibited the degradation reaction strongly indicates that OH° radicals are not directly involved in the degradation mechanism. However they could play a role as an intermediate in the phosphonate degradation process. Instead of a OH° radical, another very reactive oxygen species e.g. $O_2^{-\circ}$ can be involved in the degradation mechanism by the C-P lyase. According to Walling (1975) the fastest OH° reactions are diffusion controlled, so it is conceivable that a system might generate a hydroxyl radical in juxtaposition to an absorbed substrate, and immediately convert the resulting radical to a stable product by oxidation by a suitable positioned metal ion (possibly just oxidized in the OH° forming process). The observation of biphenyl containing two undeuterated ring structures instead of a deuterated and an undeuterated structure (as is most likely if considered chemically (Pryor, 1966)) is indicative of a condensation which occurs between two phenylradicals formed very close one to another, for example on a multicenter enzyme. A hypothesis for the mechanism is given in Figure 5. Iron is oxidized with H_2O_2 from the respiratory chain. This generates a radical, trapped on the enzyme (stabilized by a molecule M). This radical, possibly a reactive oxygen species such as $O_2^{-\circ}$ could be transferred to the phosphonate. In this hypothesis, several reactions occur on the same enzyme and at the same time, resulting in recombinations of the radicals.

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