APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1981, p. 1159-1165
0099-2240/81/051159-07$02.00/0

Vol. 41, No. 5

Inhibition of Catechol 2,3-Dioxygenase from *Pseudomonas putida* by 3-Chlorocatechol

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Received 22 December 1980/Accepted 13 February 1981

Partially purified preparations of catechol 2,3-dioxygenase from toluene-grown cells of *Pseudomonas putida* catalyzed the stoichiometric oxidation of 3-methylcatechol to 2-hydroxy-6-oxohepta-2,4-dienoate. Other substrates oxidized by the enzyme preparation were catechol, 4-methylcatechol, and 4-fluorocatechol. The apparent Michaelis constants for 3-methylcatechol and catechol were 10.6 and 22.0 μM, respectively. Substitution at the 4-position decreases the affinity and activity of the enzyme for the substrate. Catechol 2,3-dioxygenase preparations did not oxidize 3-chlorocatechol. In addition, incubation of the enzyme with 3-chlorocatechol led to inactivation of the enzyme. Kinetic analyses revealed that both 3-chlorocatechol and 4-chlorocatechol were noncompetitive or mixed-type inhibitors of the enzyme. 3-Chlorocatechol (K_i = 0.14 μM) was a more potent inhibitor than 4-chlorocatechol (K_i = 50 μM). The effect of the iron-chelating agents Tiron and *o*-phenanthrolene were compared with that of 3-chlorocatechol on the inactivation of the enzyme. Each inhibitor appeared to remove iron from the enzyme, since inactive enzyme preparations could be fully reactivated by treatment with ferrous iron and a reducing agent.

The presence of halogen substituents appears to reduce or prevent the complete biodegradation of many aromatic compounds (1, 19). Several compounds are known to be oxidized to catechols, which are resistant to further degradation. The halogenated catechols accumulate presumably because they are unable to serve as substrates for ring-fission dioxygenases present in the organism. The accumulation of chlorocatechols during the microbial oxidation of chlorinated aromatic molecules has been reported by several authors (15, 17, 18, 25, 29).

Gibson et al. (15) reported the accumulation of halogenated catechols during the co-oxidation of chloro-, bromo-, and iodobenzene by *Pseudomonas putida*. They reported that the halogenated catechols appeared to inhibit the ring-fission dioxygenase present in the organism and proposed that the halocatechols might chelate the iron cofactor of the enzyme which is necessary for catalysis. These authors reported that the presence of 3-chlorocatechol would cause the accumulation of catechols from benzene and toluene (12, 15). This observation is unusual since catechols that are catabolic intermediates rarely accumulate in the culture medium. The mechanism of inhibition by 3-chlorocatechol has not been elucidated and has been investigated in this study.

In contrast, Dorn et al. (6) have reported the isolation of a strain of *Pseudomonas fluorescens* that is capable of utilizing 3-chlorobenzoate as a carbon source. The organism was isolated from sewage with a chemostat by enrichment initially on benzoate and was gradually replaced by 3-chlorobenzoate. When *P. fluorescens* was grown on benzoate, it was found to contain enzymes that would convert 3-chlorobenzoate to 3-chloro- and 4-chlorocatechols. The catechol 1,2-dioxygenase present in benzoate-grown cells exhibited little activity with the halogenated catechols. However, when the organism was cultured on 3-chlorobenzoate, a second catechol 1,2-dioxygenase was induced that would rapidly oxidize both 3-chloro- and 4-chlorocatechols. Further studies indicated that specific enzymes were also induced for subsequent degradative reactions (7, 20). In addition, organisms that are able to grow with chlorinated phenoxyacetic herbicides, such as 2,4-dichlorophenoxyacetic acid and 2-methyl-4-chlorophenoxyacetic acid, can be readily isolated from soil. The metabolism of these compounds has been extensively studied by the research groups of Evans (8-10) and Alexander (3, 27, 28). Thus, it appears that certain strains of bacteria may have evolved enzyme systems specifically for the degradation of

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chlorinated aromatic molecules. Alternatively, many organisms appear to oxidize chlorinated molecules using enzyme systems that normally function in the metabolism of non-halogenated compounds. These latter transformations often result in the accumulation of chlorinated catechols, which are resistant to further degradation.

This paper describes the inhibition by 3-chlorocatechol of the catechol dioxygenase from *P. putida*. Evidence is presented that indicates the inactivation of the ring-fission enzyme is due to the ability of 3-chlorocatechol to chelate the iron cofactor required for catalytic activity.

MATERIALS AND METHODS

Organism and growth conditions. The isolation and characterization of *P. putida* has been reported (14). The organism was isolated from soil by elective culture, with ethylbenzene as the sole carbon source. Subsequent observations indicated that the organism grew equally well with either ethylbenzene or toluene as the carbon source. The latter compound was used routinely as the growth substrate.

P. putida was maintained at 4°C on agar slants of mineral salts medium (26) containing 0.2% succinate. Cells were transferred from a slant to 100 ml of mineral salts broth (pH 7.2) containing 0.2% succinate in a 500-ml Erlenmeyer flask. Cultures were incubated at 27°C on a rotary shaker at 150 rpm for 12 h. A 5% (vol/vol) inoculum of the culture described above was used for further experiments.

Biotransformation experiments were conducted in 2-liter Erlenmeyer flasks containing 500 ml of mineral salts medium as described above. Carbon sources were added to the cultures according to the experimental design. Toluene was introduced to the culture medium as a vapor as previously described (4). Growth of the cultures was monitored turbidimetrically at 600 nm.

Large-scale (10-liter) cultures of *P. putida* were grown as previously described (24) under forced aeration at 30°C in a New Brunswick model M14 Microferm fermentor. The mineral salts basal medium was supplemented with peptone (5.0 g/liter; Difco Laboratories) to increase cell yields. Toluene was supplied as an additional carbon source for growth as an air-toluene mixture. Air was also supplied to the culture at a rate of 10 liters/min, and the culture was stirred at 500 rpm. Cells were harvested with an air-driven Sharples continuous-flow centrifuge. The cells were washed twice with 0.05 M KH_2PO_4 buffer, pH 7.2, and used in further studies. Unused cells were stored at −15°C until required.

Detection of 3-chlorocatechol. Quantitative measurement of 3-chlorocatechol accumulation in the culture medium was conducted at various times. A 5.0-ml portion of the culture was extracted with 15.0 ml of ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo at 30°C. The residue was dissolved in 0.5 ml of acetone and applied to a preparative thin-layer chromatography plate, precoated with Silica Gel 60. The solvent system used for chromatography was chloroform-acetone (80:20). 3-Chlorocatechol was located with the use of ultraviolet light. The region of the plate containing the metabolites was removed and extracted with 5.0 ml of methanol. A sample (0.1 ml) of the methanol extract was diluted to 1.0 ml with the same solvent, and the ultraviolet spectrum was recorded. The reference cuvette contained methanol.

Alternatively, samples of duplicate culture extracts were examined by analytical thin-layer chromatography. After development of the chromatograms as described above, compounds were located with the use of ultraviolet light and also by spraying with a 2% (wt/vol) solution of 2,6-dichloroquinone-4-chloroimide in methanol (Gibbs reagent; 11).

Isolation of 3-chlorocatechol. When 3-chlorocatechol accumulation in a culture had reached a maximum, the culture medium was extracted with 2 volumes of ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo at 30°C. The residue was dissolved in 1.0 ml of acetone, applied to several preparative thin-layer chromatography plates, and chromatographed as described above. 3-Chlorocatechol isolated from the chromatograms was further purified by recrystallization from petroleum ether.

Whole-cell studies. Determination of the rate of whole-cell oxidation of various substrates was conducted polarographically with a Clark-type oxygen electrode at 30°C, using 0.05 M KH_2PO_4 buffer, pH 7.0, which was saturated with air before use. The reaction mixture, in a total volume of 1.4 ml, contained buffer and a portion of cell suspension. The reaction was initiated with the addition of 200 nmol of the substrate in 10 μl of *N,N*-dimethylformamide. All rates were corrected for endogenous respiration of the cell suspension. Whole cells were unable to oxidize *N,N*-dimethylformamide, nor did this solvent inhibit enzymatic activity.

Partial purification of cell extracts. Preliminary studies indicated that catechol dioxygenase was most stable in extracts prepared in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5, containing 10% (vol/vol) acetone and 10% (vol/vol) glycerol (TAG buffer). Frozen cells were thawed and suspended in TAG buffer, pH 7.5 (3 g of cells per 10 ml of buffer). The suspension was chilled in ice and subjected to sonic oscillation at 40 kHz with a Bronwill Biosonik III ultrasonic disintegrator for 1.5 min (three 30-s exposures). Cell debris was removed after centrifugation at 30,000 × *g* for 1 h at 5°C. The clear supernatant solution was used as a source of crude cell extract.

The catechol dioxygenase from *P. putida* was partially purified by a procedure utilizing heat treatment, ammonium sulfate fractionation, and gel filtration chromatography. Crude cell extract was incubated at 50°C for 15 min. The solution was chilled in ice and centrifuged at 15,000 × *g* for 30 min. Finely powdered ammonium sulfate was added to the supernatant solution to 30% of saturation. The suspension was stirred for an additional 15 min and centrifuged at 15,000 × *g* for 30 min. The concentration of ammonium sulfate in the supernatant solution was increased to 50% of saturation. Centrifugation yielded a precipitate which was slowly dissolved in a minimal volume of 0.05 M TAG buffer, pH 7.5. Preliminary studies indicated that

enzyme activity in the ammonium sulfate fraction was stimulated when the enzyme was dialyzed for 12 h against TAG buffer, pH 6.8, containing 1 mM $FeSO_4$ and 1 mM dithiothreitol. The dialyzed fraction was applied to a column of Sephadex G-200 (2.7 by 31.0 cm). Protein was eluted from the column with TAG buffer, pH 7.5. Fractions from the column with the highest specific activities still oxidized catechol and 3-methylcatechol but did not degrade the products of ring-fission. The partially purified preparation was stable for 2 weeks when stored at 4°C under a nitrogen atmosphere.

Enzyme analyses. Catechol dioxygenase activity was determined polarographically at 30°C with a Clark-type oxygen electrode, using 0.05 M KH_2PO_4 buffer, pH 7.5, which was saturated with air before use. The reaction mixture, in a total volume of 1.4 ml, contained buffer and an amount of cell extract found to give a linear rate of O_2 consumption with respect to protein concentration. Reactions were initiated by the addition of 1.0 μmol of catechol or 1.0 μmol of 3-methylcatechol in 10.0 μl of distilled water. All rates were corrected for endogenous respiration. No autoxidation of the catechol substrates was observed under the assay conditions described.

Cell extracts were analyzed spectrophotometrically for the presence of catechol 1,2-dioxygenase (EC 1.13.11.1) by the procedure described by Hegeman (16). Catechol 1,2-dioxygenase activity was not detected in cell extracts of toluene-grown *P. putida*. Catechol 2,3-dioxygenase (EC 1.13.11.2) activity present in cell extracts of *P. putida* was monitored spectrophotometrically by the procedure of Nozaki et al. (22). When catechol was utilized as the substrate, the formation of α-hydroxymuconic semialdehyde was monitored at 375 nm (2; $\epsilon = 33{,}400\ mol^{-1}\ cm^{-1}$). When 3-methylcatechol was utilized as the substrate, the product of the reaction, 2-hydroxy-6-oxohepta-2,4-dienoate, was monitored at 388 nm (2; $\epsilon = 13{,}800\ mol^{-1}\ cm^{-1}$). The reaction mixture, in a total volume of 1.0 ml, contained 0.05 M KH_2PO_4 buffer, pH 7.5, and a suitable amount of protein. Reactions were initiated by the addition of 1.0 μmol of the catechol substrate. The reference cuvette contained all of the components of the reaction except the substrate.

Analytical methods. Ultraviolet and visible spectra were determined on a Beckman model 25 recording spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer model 137 spectrophotometer and were referenced to the absorptions of polystyrene. Crystalline samples were mulled in Nujol and placed between NaCl disks. Low-resolution mass spectra were determined on a DuPont-Consolidated Electrodynamics Corp. model 21-491 spectrometer and were referenced to assigned perfluoroalkane peak fragments. Melting points were obtained with a Büchi melting point apparatus and were uncorrected.

Whole-cell protein determinations were conducted after digestion of a sample of the cell suspension in 0.1 N NaOH at 100°C for 1 h. Protein concentration was determined by the method of Lowry et al. (21), with crystalline bovine serum albumin as the standard.

Chemicals. 3-Chlorocatechol was synthesized by a method analogous to that described by Dakin (5) for the synthesis of catechol from salicylaldehyde. 3-Chlorosalicylaldehyde was a generous gift from Lou Kapecek, Union Carbide Corp., South Charleston, W.Va. Chlorobenzene, *o*-phenanthroline, 1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt (Tiron), catechol, 3-methylcatechol, and 4-methylcatechol were purchased from the Aldrich Chemical Co., Milwaukee, Wis. Ammonium sulfate (enzyme grade) was from Schwarz/Mann Research Co., Orangebury, N.Y. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J., and was prepared as described by the manufacturer. Analytical and preparative thin-layer chromatography plates, precoated with Silica Gel 60 F-254, were from Brinkmann Instruments Inc., Westbury, N.Y. Organic solvents were purified by vacuum distillation before use. When necessary, catechol substrates were purified by vacuum sublimation. All other materials were of the highest purity commercially available and were used without further purification.

RESULTS

Preliminary observations. *P. putida* was unable to utilize chlorobenzene as a source of carbon and energy for growth. The ability of whole cells of *P. putida*, previously grown with either succinate or toluene, to oxidize various aromatic compounds was determined polarographically (Table 1). Cells grown with succinate exhibited low activities with the various substrates. Alternatively, toluene, chlorobenzene, and 3-methylcatechol were readily oxidized by whole cells grown on toluene. 3-Chlorocatechol was not oxidized by the organism and prevented the further oxidation of 3-methylcatechol.

Oxidation of chlorobenzene by *P. putida*. When *P. putida* was grown in 500-ml cultures of mineral salts broth containing 0.2% succinate and 0.05% chlorobenzene, a neutral product (compound A) was detected in the culture me-

TABLE 1. *Oxidation of various aromatic substrates by washed, whole cells of P. putida*

Substrate[a]	Sp act (nmol of O_2 consumed min^{-1} mg^{-1})[b]	
	Toluene grown	Succinate grown
Toluene	168.0	10.4
3-Methylcatechol	572.1	10.4
Chlorobenzene	94.6	10.4
3-Chlorocatechol	0.0	0.0
3-Chlorocatechol[c] + 3-methylcatechol	0.0	0.0

[a] Substrates were added in *N,N*-dimethylformamide (200 nmol/10 μl).

[b] Enzymatic activity and protein concentrations were determined as described in the text.

[c] 3-Chlorocatechol was incubated with whole cells for 1 min before the addition of 3-methylcatechol.

dium. Compound A (R_f 0.3) absorbed ultraviolet light and gave an intense brown color with Gibbs reagent. After 20 h, duplicate cultures were extracted to leave an oily residue. Purification of the residue led to the isolation of a crystalline product that melted at 43 to 45°C (compound A). The metabolite was identified as 3-chlorocatechol, as it had mass, ultraviolet, and infrared spectra identical to those of synthetic 3-chlorocatechol.

Oxidation of 3-methylcatechol by partially purified extracts. Partially purified extracts of *P. putida* catalyzed the oxidation of 3-methylcatechol to a product with a visible absorption spectrum (Fig. 1) and properties consistent with those reported for 2-hydroxy-6-oxohepta-2,4-dienoate (2). Thus, catechol 2,3-dioxygenase catalyzes the proximal extradiol cleavage of 3-methylcatechol. Partially purified extracts did not oxidize the ring-fission product.

Partially purified extracts oxidized catechol, 3-methylcatechol, 4-methylcatechol, and 4-fluorocatechol. Polarographic studies were conducted to determine kinetic parameters of compounds which serve as substrates for the catechol 2,3-dioxygenase. Enzyme activity was monitored at various concentrations of each of the substrates. Results of the investigation are summarized in Table 2. The enzyme exhibited greater affinity and activity for 3-methylcatechol than for catechol. Substitution at the 4-position of the catechol molecule decreases the affinity of the enzyme for the substrates.

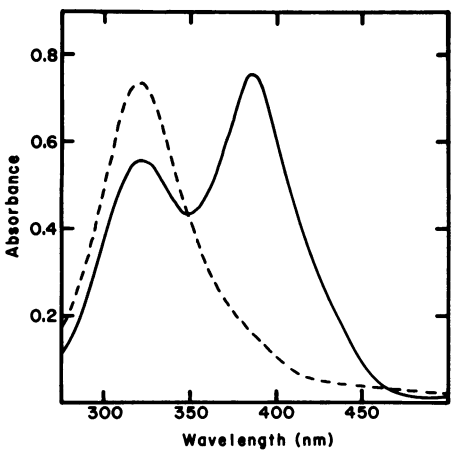
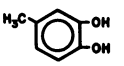
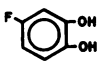


FIG. 1. *Absorption spectrum of the product of the oxidation of 3-methylcatechol by partially purified extracts. The reaction mixture (final volume, 1.0 ml) contained 50.0 nmol of 3-methylcatechol in 0.05 M KH_2PO_4 buffer, pH 7.5. Partially purified enzyme (10.0 μg) was added to initiate the reaction. The reference cuvette contained all of the components of the assay system except 3-methylcatechol. The absorption spectrum of the final product at pH 7.5 (——) was recorded on a Beckman model 25 recording spectrophotometer. The absorption spectrum of the product at pH 5.5 (- - -) was recorded after the addition of 10.0 μl of 2 N HCl to both cuvettes.*

TABLE 2. *Summary of the kinetic parameters of catechol 2,3-dioxygenase for various substrates*[a]

Compound	Structure	Km(μM)	Vmax
3-Methylcatechol		10.6	125.0
Catechol		22.0	43.0
4-Methylcatechol		1100.0	33.0
4-Fluorocatechol		62.5	9.0

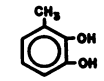
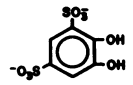
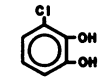
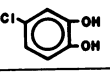
[a] Activity of the enzyme (8.4 μg of protein) was monitored polarographically as described in the text. Maximum activity (V_{max}) is expressed as nanomoles of O_2 consumed per minute.

Interaction of partially purified extracts and 3-chlorocatechol. Partially purified catechol 2,3-dioxygenase did not oxidize 3-chlorocatechol, 4-chlorocatechol, and Tiron. Kinetic analyses were conducted to determine the type of inhibition exhibited by the substrate analogs for the partially purified enzyme. In the analyses, various concentrations of 3-methylcatechol and the inhibitor were mixed in the cuvette, and the reaction was initiated by the addition of enzyme. Activity of the enzyme in each case was determined with 0.7 μg of protein. The Michaelis constant determined for 3-methylcatechol was 1.7 μM. The effect of various concentrations of 3-methylcatechol was determined in the presence of 5.0 mM, 0.2 μM, and 20 μM Tiron, 3-chlorocatechol, and 4-chlorocatechol, respectively. Results were used to prepare the Lineweaver-Burke plots.

A summary of the results is given in Table 3. The results indicated that Tiron is a competitive inhibitor of the enzyme, whereas 3-chlorocatechol and 4-chlorocatechol are noncompetitive or mixed-type inhibitors.

The effects of the iron-chelating agents Tiron and *o*-phenanthroline were compared to that of 3-chlorocatechol in the inactivation of catechol 2,3-dioxygenase. Samples of partially purified enzyme were treated with 1.0, 5.0, and 25.0 μmol of 3-chlorocatechol, Tiron, and *o*-phenanthroline, respectively. Activity of the enzyme was monitored spectrophotometrically with time

TABLE 3. *Summary of the inhibition of the catechol 2,3-dioxygenase by substrate analogs*

Compound	Structure	[I]	K_I
3-Methylcatechol			Km = 1.7 μM
Tiron		5×10^{-3} M	7.4 mM
3-Chlorocatechol		2×10^{-7} M	0.14 μM
4-Chlorocatechol		2×10^{-5} M	50.0 μM

(Fig. 2). The samples were incubated at 27°C throughout the analysis. The samples were then dialyzed for 12 h against 2 liters of TAG buffer, pH 7.5. After dialysis, the activity of the enzyme in each sample was determined (Table 4). The samples were further dialyzed against 1 liter of TAG buffer, pH 6.8, containing 1 mM dithiothreitol and 1 mM $FeSO_4$. Activity of the enzyme in each sample was again determined. The results of the investigation indicate that each of the compounds tested inactivate the enzyme by interaction with the iron cofactor. Complete activity of the enzyme was restored by dialysis of the enzyme against TAG buffer, pH 6.8, containing ferrous iron and a reducing agent.

DISCUSSION

P. putida oxidizes toluene via (+)-*cis*-1(*S*),2(*R*)-dihydroxy-3-methylcyclohexa-3,5-diene to 3-methylcatechol (13, 31). The latter metabolite serves as a substrate for a ring-fission dioxygenase present in the organism, and further metabolism ultimately leads to complete degradation. In contrast, chlorobenzene was unable to support the growth of *P. putida* when supplied as the sole carbon source. However, the organism has the ability to co-oxidize chlorobenzene through a *cis*-dihydrodiol to 3-chlorocatechol (Fig. 3). Further metabolism of 3-chlorocatechol does not occur since the metabolite inhibits the ring-fission dioxygenase present in the organism.

This study was conducted to investigate both the properties of the ring-fission dioxygenase and the mechanism of enzyme inhibition by 3-chlorocatechol. Cell extracts prepared from *P. putida* grown with toluene were found to contain a catechol 2,3-dioxygenase that was able to oxidize catechol and 3-methylcatechol. The presence of a catechol 1,2-dioxygenase was not detected in cell extracts. Thus, the ring cleavage of catechol substrates by *P. putida* appears to occur solely by the extradiol fission of the aromatic nucleus.

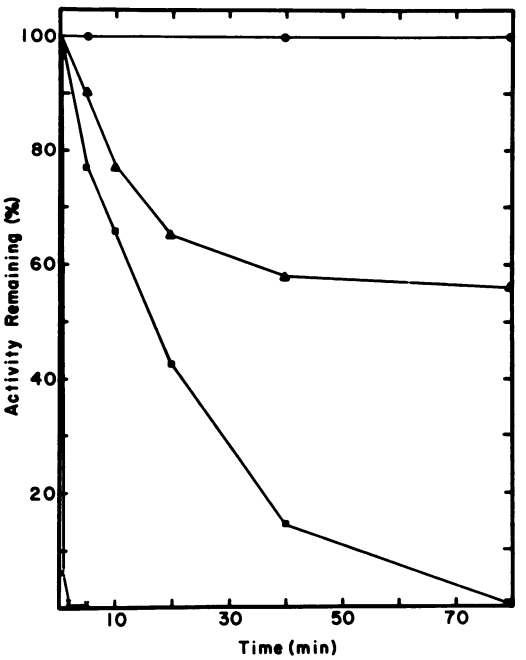


FIG. 2. *Inactivation of catechol 2,3-dioxygenase by iron-chelating agents. Samples of the enzyme (3.4 mg) were incubated at 27°C in the presence of various iron-chelating agents as follows: control, no additions (●); 5.0 μmol of Tiron (▲); 25.0 μmol of o-phenanthroline (■); 1.0 μmol of 3-chlorocatechol (×). Activity of the enzyme in each sample was monitored spectrophotometrically with time with 3-methylcatechol (1.0 μmol).*

TABLE 4. *Interaction of catechol 2,3-dioxygenase with iron-chelating agents*

Sample (treatment)[a]	Sp act (μmol of product formed min^{-1} mg^{-1})		
	Initial activity[b]	After inactivation[c]	After reactivation[d]
Control	4.16	2.47	4.49
3-Chlorocatechol	4.16	0.00	4.17
Tiron	4.16	3.57	4.25
o-Phenanthroline	4.16	0.00	4.21

[a] Samples of the enzyme were treated with the various inhibitors as described in the text.

[b] Initial activity of the sample before the addition of inhibitor.

[c] Activity present in the sample after treatment with the inhibitor and dialysis against 0.05 M TAG buffer, pH 7.5.

[d] Activity present in the sample after further dialysis against 0.05 M TAG buffer, pH 6.8, containing 1 mM $FeSO_4$ and 1 mM dithiothreitol.

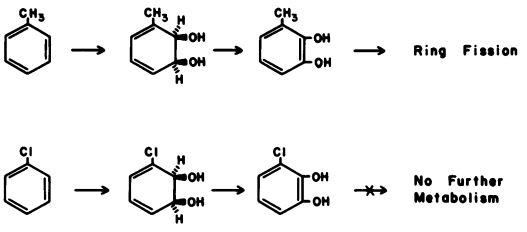


FIG. 3. *Initial reactions of the oxidation of toluene and chlorobenzene by P. putida (13, 15).*

Partial purification of catechol 2,3-dioxygenase from *P. putida* was achieved by a procedure utilizing heat treatment, ammonium sulfate fractionation, and gel filtration chromatography. The purification procedure was found to completely remove the enzyme that catalyzes subsequent degradation of the ring-fission product. Partially purified catechol 2,3-dioxygenase catalyzed the stoichiometric conversion of 3-methylcatechol to a product with properties identical to those reported for 2-hydroxy-6-oxohepta-2,4-dienoate (2). Thus, the enzyme catalyzes the proximal extradiol cleavage of 3-methylcatechol, which is consistent with a previous report of the activity of a catechol 2,3-dioxygenase with substituted catechols (23).

A wide variety of substituted catechols that were examined as possible substrates and the partially purified catechol 2,3-dioxygenase were shown to catalyze the oxidation of catechol, 3-methylcatechol, 4-methylcatechol, and 4-fluorocatechol. The enzyme exhibited greater affinity and activity for 3-methylcatechol (K_m = 10.6 μM) than for catechol (K_m = 22.0 μM). Substitution at the 4-position resulted in a decrease in the affinity and activity of the enzyme for the substrate. These observations suggest that substitution at the 3-position is essential for the greatest affinity of the catechol substrate for the active site of the enzyme.

3-Chlorocatechol was not oxidized by partially purified catechol 2,3-dioxygenase. Furthermore, incubation with 3-chlorocatechol resulted in inactivation of the enzyme. A kinetic analysis revealed that both 3-chlorocatechol and 4-chlorocatechol were noncompetitive or mixed-type inhibitors of the enzyme. 3-Chlorocatechol (K_i = 0.15 μM) was a more potent inhibitor than 4-chlorocatechol (K_i = 50 μM) and thus may be another indication of the specificity of the enzyme for 3-substituted catechols. Tiron appeared to be a competitive inhibitor of the enzyme.

The effects of the iron-chelating agents *o*-phenanthroline and Tiron were compared with that of 3-chlorocatechol on the inactivation of the catechol 2,3-dioxygenase. The enzyme was rapidly inactivated when incubated with 3-chlorocatechol and was slowly inactivated when treated with *o*-phenanthroline. Tiron also exhibited a slight inhibition or inactivation of the enzyme. Inactivation of the enzyme by each reagent appears to be due to the removal of the iron cofactor, because the samples of inactivated enzyme could be fully reactivated by treatment with ferrous iron and a reducing agent.

Wigmore and Ribbons (30) have previously stated that the co-oxidation of halogenated aromatic compounds may not proceed, even if a potential exists, due to the formation of inhibitory products. The accumulation of 3-chlorocatechol during the co-oxidation of chlorobenzene by *P. putida* is consistent with this statement. 3-Chlorocatechol was found to be a potent inhibitor of the catechol 2,3-dioxygenase in this organism. This investigation has provided substantial indirect evidence that the inactivation of the catechol 2,3-dioxygenase is due to the ability of 3-chlorocatechol to chelate the iron cofactor of the enzyme. Further studies with a purified enzyme are necessary to determine the exact mechanism of inactivation.

ACKNOWLEDGMENTS

This investigation was supported in part by grants ES-00537 from The National Institute of Environmental Health Sciences, and F-440 from The Robert A. Welch Foundation.

We thank Lou Kapecek for the generous gift of 3-chlorosalicylaldehyde and Roberta DeAngelis for assistance in preparing the manuscript.

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