APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1991, p. 812–819
0099-2240/91/030812-08$02.00/0

Vol. 57, No. 3

Pathway for Biodegradation of *p*-Nitrophenol in a *Moraxella* sp.

JIM C. SPAIN[1]* AND DAVID T. GIBSON[2]

Air Force Engineering and Services Center, Tyndall Air Force Base, Florida 32403-6001,[1] and Department of Microbiology and Biocatalysis Research Group, University of Iowa, Iowa City, Iowa 52242[2]

Received 9 October 1990/Accepted 2 January 1991

A *Moraxella* strain grew on *p*-nitrophenol with stoichiometric release of nitrite. During induction of the enzymes for growth on *p*-nitrophenol, traces of hydroquinone accumulated in the medium. In the presence of 2,2′-dipyridyl, *p*-nitrophenol was converted stoichiometrically to hydroquinone. Particulate enzymes catalyzed the conversion of *p*-nitrophenol to hydroquinone in the presence of NADPH and oxygen. Soluble enzymes catalyzed the conversion of hydroquinone to γ-hydroxymuconic semialdehyde, which was identified by high-performance liquid chromatography (HPLC)-mass spectroscopy. Upon addition of catalytic amounts of NAD^+, γ-hydroxymuconic semialdehyde was converted to β-ketoadipic acid. In the presence of pyruvate and lactic dehydrogenase, substrate amounts of NAD were required and γ-hydroxymuconic semialdehyde was converted to maleylacetic acid, which was identified by HPLC-mass spectroscopy. Similar results were obtained when the reaction was carried out in the presence of potassium ferricyanide. Extracts prepared from *p*-nitrophenol-grown cells also contained an enzyme that catalyzed the oxidation of 1,2,4-benzenetriol to maleylacetic acid. The enzyme responsible for the oxidation of 1,2,4-benzenetriol was separated from the enzyme responsible for hydroquinone oxidation by DEAE-cellulose chromatography. The results indicate that the pathway for biodegradation of *p*-nitrophenol involves the initial removal of the nitro group as nitrite and formation of hydroquinone. 1,4-Benzoquinone, a likely intermediate in the initial reaction, was not detected. Hydroquinone is converted to β-ketoadipic acid via γ-hydroxymuconic semialdehyde and maleylacetic acid.

Nitroaromatic compounds are released into the environment through their wide use as dyes, pesticides, plasticizers, explosives, and solvents. The presence of the nitro group causes such compounds to be more resistant to biodegradation than the unsubstituted analogs. There have been many reports (7, 14–16, 18, 20–22, 24) on the biodegradation of nitrophenols, but little is known about the catabolic pathways involved.

Degradation of *o*-nitrophenol by *Pseudomonas putida* involves replacement of the nitro group with a hydroxyl group and subsequent conversion of the catechol to β-ketoadipate via *cis,cis*-muconate (24). Simpson and Evans (18) suggested that the initial step in the bacterial degradation of *p*-nitrophenol (PNP) involved a similar oxidative removal of the nitro group. Munnecke and Hsieh (14) detected hydroquinone as an early metabolite in PNP degradation by a pseudomonad and proposed that it was hydroxylated to form 1,2,4-benzenetriol prior to *ortho* ring fission. In contrast, Raymond and Alexander (16) suggested that a *Flavobacterium* sp. converted PNP to 4-nitrocatechol as the first step in complete degradation.

In a preliminary communication (21), we described an enzyme in a *Moraxella* strain that replaced the nitro group with a hydroxyl group as the initial reaction in PNP degradation. In the present report, we describe additional characterization of the enzyme and the complete pathway for degradation of PNP by the *Moraxella* sp.

MATERIALS AND METHODS

Bacteria and culture conditions. A *Moraxella* species was isolated from activated sludge by selective enrichment with PNP (21). Cultures were grown in a minimal salts medium (21) at pH 7.0 with forced aeration at 30°C. The basal medium was supplemented with PNP (150 mg/liter) and yeast extract (0.1%).

Preparation of cell extracts. Cell extracts were prepared by treatment with a French pressure cell (21). Crude extracts were separated into soluble and particulate fractions by centrifugation (177,000 × *g*) for 1 h. The pellet, which contained nitrophenol oxygenase activity, was suspended in 0.02 M phosphate buffer and stored at −70°C. The supernatant fluid was subjected to ammonium sulfate precipitation. Proteins that precipitated between 35 and 55% ammonium sulfate saturation were dialyzed overnight against 0.02 M phosphate buffer (pH 7.0) and stored at −70°C.

Enzyme assays. PNP oxidation was assayed spectrophotometrically as described previously (21). The extinction coefficient of PNP at 420 nm and pH 7.0 was 7,000 M^{-1} cm^{-1}. Hydroquinone oxygenase and benzenetriol oxygenase were measured polarographically in a Clark-type oxygen electrode. Reaction mixtures contained (in a final volume of 1.8 ml) 0.05 mM hydroquinone or 1,2,4-benzenetriol, 20 mM phosphate buffer (pH 7.0), and cell extract (0.1 to 0.5 mg of protein).

Ion-exchange chromatography. A column (2.0 by 6.0 cm) was packed to a height of 4.0 cm with DEAE-cellulose (Whatman DE 52). The column was equilibrated with 0.02 M sodium-potassium phosphate buffer (pH 7.0), loaded with 50 mg of protein (35 to 55% ammonium sulfate fraction), and washed with 50 ml of phosphate buffer. Proteins were eluted with a 300-ml linear gradient of 0 to 0.4 M KCl in 0.02 M phosphate buffer. Fractions (5.0 ml) were collected and assayed for enzyme activity and protein concentration.

Analytical methods. Thin-layer chromatography was carried out on precoated cellulose sheets (Eastman, Rochester, N.Y.) developed with butanol-acetic acid-water (12:3:5). Aldehydes and ketones were visualized by spraying with 2,4-dinitrophenylhydrazine (0.1% in 3 N HCl). Acids were visualized by spraying with bromocresol green (0.1% in methanol).

* Corresponding author.

Spectrophotometric measurements were obtained with a Beckman model 25 or Cary model 219 spectrophotometer. Protein content was estimated by the biuret method (6). Nitrite was measured by the Griess-Ilosvay reaction (13). The Rothera test for β-keto acids was performed as described by Kilby (10). High-performance liquid chromatography (HPLC) was carried out by a modification of the method of Schlömann et al. (17). The stationary phase was a Spherisorb C_8 column (10 μm), 250 mm by 4.6-mm internal diameter (Alltech Associates, Deerfield, Ill.). The mobile phase (solvent system A) was acetonitrile-water-trifluoroacetic acid (50:950:1), and the flow rate was 1.5 ml/min. The A_{210} was monitored with a diode array detector (Hewlett-Packard Co., Palo Alto, Calif.).

HPLC-mass spectroscopy (MS) was carried out with a Hewlett-Packard model 5987A mass spectrometer equipped with a Vestec thermospray interface. The stationary phase was the same as for HPLC (above). The mobile phase (solvent system B) was the same as solvent system A with the addition of 0.01 M ammonium acetate. The flow rate was 1.5 ml/min. β-Ketoadipic acid and levulinic acid were quantitated by monitoring positive single ions at 117 and 134 atomic mass units (amu). Maleylacetic acid, *cis*-acetylacrylic acid, and *trans*-acetylacrylic acid were quantitated by monitoring negative single ions at 113 amu. No other ions were detected at significant levels. The mobile phase used for these three compounds (solvent system C) was methanol–0.1 M ammonium acetate–acetic acid (100:900:4). The hydroquinone ring fission product prepared biologically was quantitated by positive single-ion monitoring at 141 amu and negative single-ion monitoring at 143 amu. No other ions were detected at significant levels. The mobile phase was solvent system B. Capillary column gas chromatography-mass spectral analyses were performed with a 5987 GC-MS (Hewlett-Packard Co.) with a fused silica column and an electron impact ionization detector.

Chemicals. 1,2,4-Benzenetriol and 2,4-dinitrophenylhydrazine were from Aldrich Chemical Co. (Milwaukee, Wis.). Hydroquinone, 2,2′-dipyridyl, 2,6-dichlorophenol-indophenol, *o*-phenanthroline, iodoacetamide, 5,5′-dithiobis-(2-nitrobenzoic acid), *p*-hydroxymercuribenzoate, rotenone, amytal, dicumerol, cytochrome *c*, NAD^+, NADH, $NADP^+$, NADPH, flavin adenine dinucleotide, levulinic acid, and β-ketoadipic acid were from Sigma Chemical Co. (St. Louis, Mo.). Acriflavin neutral was from Nutritional Biochemicals (Cleveland, Ohio). *trans*-Acetylacrylic acid was from PCR Inc. (Gainesville, Fla.). *cis*-Acetylacrylic acid was prepared as described by Schlömann et al. (17). Metapyrone and SKF 525-A were gifts from Carl Cerniglia at the National Center for Toxicological Research, Jefferson, Ark. Maleylacetate was prepared from 1,2,4-benzenetriol by incubation with extracts from resorcinol-grown cells of *Pseudomonas* strain ORC as described by Chapman and Ribbons (3). *Pseudomonas* strain ORC was kindly provided by Peter Chapman (U.S. Environmental Protection Agency, Gulf Breeze, Fla.). In some experiments maleylacetate standards were prepared by incubation of 3-chlorocatechol with crude extracts from chlorobenzene-grown cells of *Pseudomonas* sp. strain JS6 (19). The method was similar to that used by Tiedje et al. (23). Reaction mixtures contained (in 1.0 ml) 3-chlorocatechol (10^{-4} M), cell extract (0.25 mg of protein), and Tris buffer (pH 7.0) (10^{-2} M). Production of maleylacetate was monitored by measuring the increase in A_{242}.

TABLE 1. Substrates oxidized by PNP-grown cells of a *Moraxella* sp.[a]

Assay substrate	Oxygen uptake (μmol/min/mg of protein) after growth on:	
	Glucose	PNP
PNP	<0.01	0.41
Hydroquinone	<0.01	0.75
1,2,4-Benzenetriol	0.07	0.36
4-Nitrocatechol	<0.01	0.10
Catechol	<0.01	<0.01

[a] Oxygen consumption was measured polarographically as described in Materials and Methods for hydroquinone oxygenase. All substrate concentrations were 0.05 mM.

RESULTS

Growth on PNP. The *Moraxella* sp. grew on PNP as the sole source of carbon and released nitrite in stoichiometric amounts. It could use nitrate or ammonium salts but not nitrite or PNP as the nitrogen source. Concentrations of PNP above 150 mg/liter inhibited growth.

Accumulation of metabolites. Cultures grown on succinate underwent an induction period prior to growth on PNP. During the induction period, traces of hydroquinone accumulated in the culture fluid and then disappeared with the onset of PNP degradation. 2,2′-Dipyridyl, an iron chelator, inhibits certain aromatic ring cleavage enzymes that require ferrous ions for their activities (2). When PNP-grown cultures were incubated with radiolabeled PNP in the presence of 2,2′-dipyridyl, the PNP was converted stoichiometrically to hydroquinone. The results suggested that hydroquinone was an early intermediate in the catabolic pathway and that the enzyme responsible for further degradation of hydroquinone required ferrous iron for activity.

Oxidation of aromatic compounds by cell suspensions. Washed-cell suspensions of the PNP-grown *Moraxella* sp. oxidized PNP, hydroquinone, and 1,2,4-benzenetriol rapidly and without a lag. 4-Nitrocatechol was oxidized more slowly, and catechol did not stimulate oxygen uptake (Table 1). Glucose-grown cells oxidized 1,2,4-benzenetriol slowly and did not oxidize any of the other aromatic substrates tested.

Conversion of PNP to hydroquinone. In a preliminary communication (21), we reported that enzymes associated with particulate fractions of cell extracts prepared from the *Moraxella* isolate catalyzed the conversion of PNP to hydroquinone. NADPH served as the electron donor, and flavin adenine dinucleotide stimulated the activity. More detailed characterization of the enzyme activity follows.

The initial rate of the reactions was highest at pH 7.5 to 8.0, but the reaction rate was not linear at pH values above 7.0. Therefore, all assays were conducted at pH 7.0. Several metal ions were tested for the ability to stimulate PNP oxidation. Reaction mixtures were preincubated with the metal ions (0.1 M) for 5 min before the reaction was initiated by the addition of NADPH. All of the metal ions tested reduced the rate of the reaction. Preincubation with ferrous ions reduced the activity by 60%; magnesium, manganese, and zinc ions were less inhibitory.

Kinetic studies. The affinity of the enzyme in the particulate fraction of cell extracts for PNP and NADPH was determined spectrophotometrically. The apparent K_m for PNP was 6.0×10^{-6} M; substrate inhibition occurred at PNP

TABLE 2. Effects of inhibitors on PNP oxidation[a] by a washed particulate fraction of cell extract

Inhibitor	Concn (M)	% of initial activity
Metapyrone	5×10^{-4}	101
Carbon monoxide	—[b]	100
SKF 525-A	5×10^{-4}	100
2,6-Dichlorophenol-indophenol	1×10^{-5}	64
Cytochrome *c*	1×10^{-4}	31
2,2'-Dipyridyl	5×10^{-3}	98
o-Phenanthroline	5×10^{-4}	100
Iodoacetamide	5×10^{-4}	100
5,5'-Dithiobis-(2-nitrobenzoic acid)	5×10^{-4}	80
p-Hydroxymercuribenzoate	5×10^{-5}	2
Rotenone	1×10^{-4}	110
Amytal	5×10^{-4}	105
Dicumarol	1×10^{-4}	53
Acriflavin	2×10^{-4}	3
Picric acid	5×10^{-7}	12
2,4-Dinitrophenol	5×10^{-6}	45
4-Nitrocatechol	2×10^{-5}	42

[a] PNP oxidation was assayed spectrophotometrically as described in Materials and Methods.

[b] —, Reaction mixture was allowed to equilibrate with a 1:1 mixture of carbon monoxide and air.

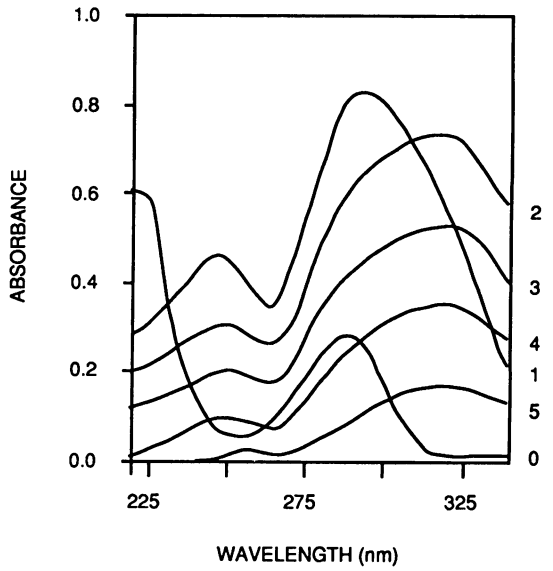

FIG. 1. Spectral changes during enzymatic oxidation of hydroquinone by cell extracts of the *Moraxella* sp. Reaction mixtures contained 0.53 mg of protein (crude cell extract), 8.5×10^{-5} M hydroquinone, and 20 mM phosphate buffer (pH 7.0) in a final volume of 1.0 ml. The reaction was initiated by the addition of cell extract. UV spectra were determined before extract was added (spectrum 0), after 1.0 min (spectrum 1), 5.0 min (spectrum 2), 8.5 min (spectrum 3), 12.0 min (spectrum 4), and 15 min (spectrum 5).

concentrations above 5×10^{-5} M. The apparent K_m for NADPH was 3.2×10^{-5} M.

The affinity of the enzyme for oxygen was determined polarographically. Double reciprocal plots of $1/V_0$ versus $1/[O_2]$, where V_0 is the initial velocity, were hyperbolic. Plots of $1/V_0$ versus $1/[O_2]^2$ were linear in the range of 1.8×10^{-5} to 4.8×10^{-5} M, and the apparent K_m was 3.6×10^{-5} M.

Inhibition studies. The cytochrome P-450 inhibitors SKF 525-A, metapyrone, and carbon monoxide did not inhibit PNP oxidation (Table 2). Inhibition by 2,6-dichlorophenol-indophenol and cytochrome *c* suggested that they were able to accept electrons from some component of the PNP-oxidizing system.

The iron chelators 2,2'-dipyridyl and *o*-phenanthroline did not inhibit the oxidation of PNP. Iodoacetamide was not an inhibitor, but another sulfhydryl reagent, 5,5'-dithiobis-(2-nitrobenzoic acid), was slightly inhibitory, and *p*-hydroxymercuribenzoate was a powerful inhibitor. Inhibition by acriflavin suggests the participation of a flavin in the reaction.

Substrate specificity. The particulate enzyme that oxidized PNP was specific. It oxidized 4-nitrocatechol at 11% of the rate observed with PNP. 4-Nitrocatechol also inhibited PNP oxidation (Table 2). Nitrobenzene, *o*-nitrophenol, *m*-nitrophenol, 5-nitrosalicylate, 4-nitrobenzoate, 2,4-dinitrophenol, *p*-nitrophenyl sulfate, *p*-nitrophenyl phosphate, *p*-nitrophenyl acetate, and potassium nitrate did not stimulate oxygen uptake or nitrite release by the PNP oxygenase.

Hydroquinone metabolism. Soluble fractions of crude cell extracts catalyzed the oxidation of hydroquinone with concomitant uptake of 1 mol of oxygen per mol of substrate. When crude extracts were fractionated with ammonium sulfate, maximum hydroquinone-oxidizing activity precipitated between 35 and 55% ammonium sulfate saturation. The specific activity of a typical preparation was 0.48 μmol/min/mg of protein.

Identification of the product of hydroquinone oxidation. When reactions were carried out in the spectrophotometer with crude cell extracts, the low A_{288} due to hydroquinone was replaced by a broad, transient peak absorbing at 290 to 320 nm with a shoulder at 240 to 245 nm (Fig. 1). After 20 min, the reaction mixture showed no significant absorbance in the UV region of the spectrum. The results suggested that hydroquinone was converted to an intermediate which was further metabolized by enzymes in the crude extracts.

When cell extracts were subjected to ammonium sulfate precipitation and dialysis prior to the spectrophotometric assays, the absorbance at 290 to 320 nm appeared as before but remained stable. Identical results were observed with dialyzed crude cell extracts. The UV absorbance of the hydroquinone oxidation product was abolished upon acidification to pH 1.5. The spectrum was shifted by changes in pH near neutrality. At pH 6.0, the absorbance maximum appeared at 320 nm; at pH 8.0, the absorbance maximum shifted to 290 nm. The spectrum at pH 7.0 gave a broad absorbance maximum at 290 to 320 nm. The extinction coefficient at 320 nm, calculated by assuming complete conversion, was 11,000 M^{-1} cm^{-1} at pH 7.0. HPLC analysis of the reaction mixture revealed a single UV-absorbing compound with a retention time of 2.49 min in solvent A, and the peak area was proportional to the concentration of hydroquinone initially added. The negative-ion spectrum of the hydroquinone oxidation product gave a single ion at 141 amu, and the positive-ion spectrum gave a single ion at 143 amu (Table 3) when the reaction mixture was analyzed by HPLC-MS. The reaction was carried out on a larger scale, and the ring fission product was extracted with ethyl acetate after acidification. Thin-layer chromatographic analysis revealed the presence of a major yellow band with an R_f of 0.76. The compound turned deep orange upon treatment with 2,4-dinitrophenylhydrazine and gave an acid reaction with bromocresol green. Attempts to isolate and crystallize

TABLE 3. HPLC and HPLC-MS analysis of potential intermediates in the degradation of PNP by a *Moraxella* sp.[a]

Compound	Solvent system	Retention time (min)	UV absorbance maximum (nm)	Major ion (amu)
β-Ketoadipic acid	A	3.62	<200	
	B	3.40		117,[b] 134[b]
Levulinic acid	A	4.51	<200	
	B	4.22		117,[b] 134[b]
Maleylacetic acid	A	3.16	<200	
	C	1.87		113[c]
cis-Acetylacrylic acid	A	4.22	<200	
	C	2.18		113[c]
trans-Acetylacrylic acid	A	6.41	225	
	C	2.49		113[c]
Hydroquinone ring fission product	A	2.48		
	B	2.65		141,[c] 143[b]

[a] Reaction mixtures or standards prepared as described in the text were centrifuged and injected directly into the HPLC.
[b] Positive-ion monitoring.
[c] Negative-ion monitoring.

the orange compound were unsuccessful; the product was unstable and decomposed during extraction and subsequent workup. Gas chromatography-MS analysis of the crude product revealed a compound that gave the mass spectrum shown in Fig. 2. The spectrum shows a molecular ion at *m/z* 142, a base peak at *m/z* 113 (M^+—COOH), and major fragments at *m/z* 113 (M^+—CHO) and *m/z* 71 (M^+—CH=CH—COOH). The fragmentation pattern, together with the properties listed above, suggests that the metabolite formed from hydroquinone is γ-hydroxymuconic semialdehyde. No attempt was made to determine whether the double bond was *cis* or *trans*. The characteristics of the compound agree well with the preliminary descriptions by Larway and Evans (12) and Darby et al. (5) for the compound formed from hydroquinone by other pseudomonads.

Properties of the hydroquinone-oxidizing enzyme. After ammonium sulfate precipitation and dialysis, the enzyme preparation required added ferrous ions for maximum rates of hydroquinone oxidation, and no activity was detected in the presence of 10 mM 2,2′-dipyridyl. Other metal ions did not substitute for ferrous ions. The rate of the enzymatic oxidation was not markedly affected by variations in pH between 6.2 and 8.0. In an air-saturated buffer, the apparent K_m for hydroquinone was 1.5×10^{-5} M and substrate inhibition occurred above 2×10^{-4} M. When cell extracts were allowed to catalyze the complete oxidation of 7×10^{-5} M hydroquinone, subsequent additions of hydroquinone were oxidized at progressively lower rates until activity disappeared altogether. Activity was not restored by the addition of ferrous sulfate. The loss of activity during catalysis was not due to a buildup of a toxic product, because fresh extract added to the spent reaction mixture oxidized hydroquinone at the normal rate.

Metabolism of γ-hydroxymuconic semialdehyde. Fractions of cell extracts that precipitated between 35 and 55% ammonium sulfate saturation catalyzed the disappearance of γ-hydroxymuconic semialdehyde in the presence of NAD^+ (Fig. 3). When NAD^+ was added to reaction mixtures containing cell extract and γ-hydroxymuconic semialdehyde, the A_{320} decreased rapidly. The rate of the NAD^+-dependent decrease in absorbance was proportional to the amount of protein in the reaction mixture. Two minutes after the addition of NAD^+, a spectrum of the reaction mixture showed no absorbance in the UV region. $NADP^+$, NADH, and NADPH could not substitute for NAD^+, and NADH inhibited the NAD^+-dependent conversion. Subsequent experiments indicated that NAD^+ was required in catalytic amounts and that NADH did not accumulate during the reaction. The results suggested the operation of a recycling mechanism for regeneration of the NAD^+.

The reaction mixture gave a strong positive reaction in the Rothera test for β-keto acids. Liquid chromatography-MS analysis of the reaction mixture showed stoichiometric conversion of the ring fission product to β-ketoadipic acid. The β-ketoadipic acid was identified and quantitated by comparison with an authentic standard (Table 3).

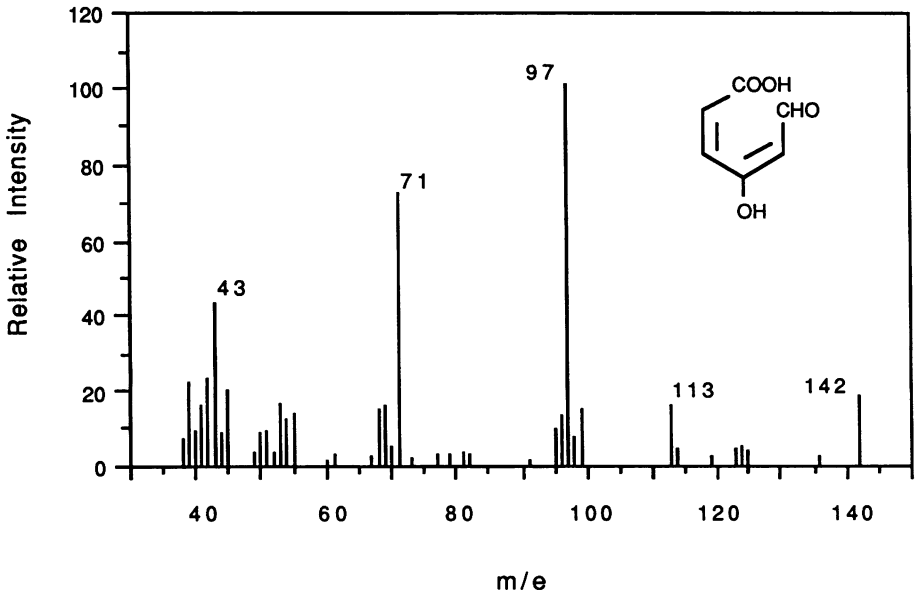

FIG. 2. Mass spectrum of the metabolite isolated after the enzymatic oxidation of hydroquinone.

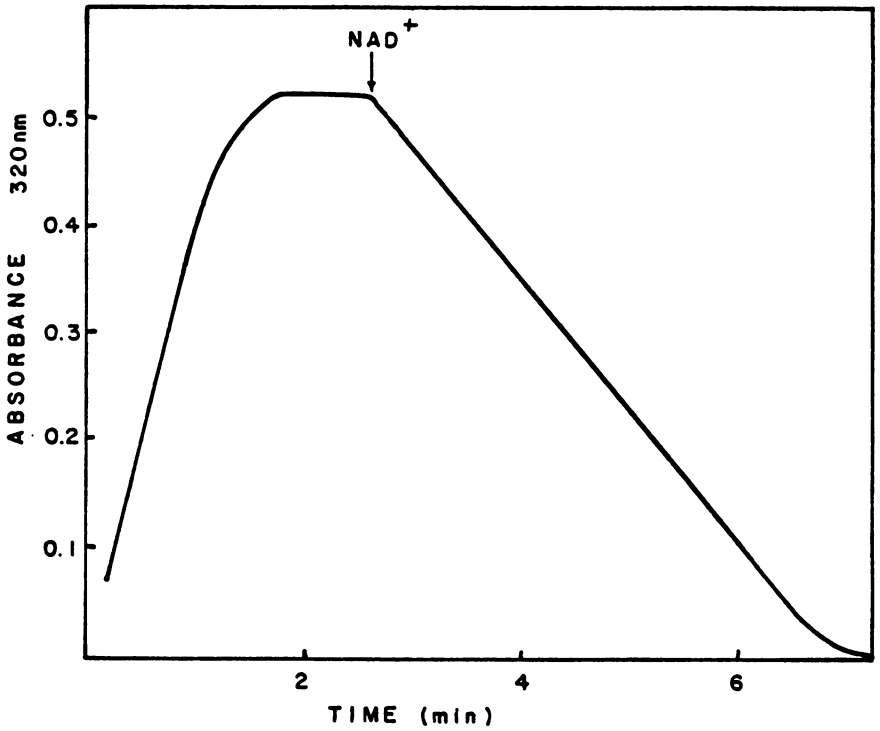

FIG. 3. NAD^+-dependent disappearance of γ-hydroxymuconic semialdehyde. Hydroquinone oxidation was monitored spectrophotometrically by measuring the increase in the A_{320} of a reaction mixture containing 0.05 mM hydroquinone, 0.65 mg of protein (35 to 55% ammonium sulfate fraction), and 20 mM phosphate buffer (pH 7.2) in a final volume of 1.0 ml. The reaction was initiated by the addition of hydroquinone. NAD^+ was added at the indicated time to a final concentration of 0.05 mM.

The operation of a recycling mechanism for NAD^+ suggested that the ring fission product is oxidized and then reduced prior to the formation of β-ketoadipic acid. When the oxidation of hydroquinone was carried out in the presence of an excess of lactic dehydrogenase and pyruvate, the addition of NAD^+ resulted in the disappearance of the UV absorbance maximum at 290 to 320 nm and the appearance of a new absorbance maximum at 240 to 245 nm. HPLC and liquid chromatography-MS analysis of the reaction mixture revealed a single major product that cochromatographed with authentic maleylacetic acid. In a separate experiment, hydroquinone (5×10^{-5} M) was converted to the ring fission product by the partially purified cell extract. The addition of potassium ferricyanide (10^{-4} M) followed by NAD^+ (10^{-4} M) to the reaction mixture resulted in the stoichiometric formation of maleylacetic acid. The reaction was carried out on a larger scale, and the maleylacetate was extracted with ethyl acetate. Liquid chromatography-MS analysis gave a major negative ion at 113 amu. The product slowly decomposed during storage to a mixture of *cis*- and *trans*-acetylacrylic acids which were identified by HPLC and liquid chromatography-MS by comparison with authentic standards. Attempts at derivatization with 2,4-dinitrophenylhydrazine or esterification with BF_3 methanol led to formation of the corresponding derivatives of acetylacrylic acid. These properties of the compound formed by oxidation of γ-hydroxymuconic semialdehyde were thus identical to those reported for maleylacetic acid by Chapman and Ribbons (3).

Oxidation of 1,2,4-benzenetriol. PNP-grown cells oxidized 1,2,4-benzenetriol (Table 1). Extracts prepared from PNP-grown cells also retained the ability to catalyze 1,2,4-benzenetriol oxidation. The specific activity of a typical 35 to 55% ammonium sulfate fraction with 1,2,4-benzenetriol was 0.188 μmol of O_2 per min per mg of protein, the reaction required 1.03 mol of O_2 per mol of 1,2,4-benzenetriol, and ferrous ions were required for enzyme activity. Specific activity with 1,2,4-benzenetriol as a substrate was only 25 to 50% of the activity observed with hydroquinone.

The enzyme responsible for 1,2,4-benzenetriol oxidation was more stable during storage on ice than the enzyme that catalyzed hydroquinone oxidation, and it did not lose activity during catalysis. In addition, extracts that had completely lost the ability to oxidize hydroquinone still catalyzed the oxidation of 1,2,4-benzenetriol.

DEAE-cellulose chromatography. A 35 to 55% ammonium sulfate fraction of a *Moraxella* cell extract was fractionated by DEAE-cellulose column chromatography (Fig. 4). The results show the separation of two protein fractions that oxidize hydroquinone and 1,2,4-benzenetriol. Although there was some overlap in activity, fraction 36 oxidized hydroquinone (specific activity, 0.62) and showed no activity with 1,2,4-benzenetriol. Fraction 47 oxidized 1,2,4-benzenetriol (specific activity, 0.74) and did not oxidize hydroquinone. These observations, together with the differences observed in stability and catalysis, clearly demonstrate that different enzymes are responsible for the oxidation of hydroquinone and 1,2,4-benzenetriol.

Characterization of the 1,2,4-benzenetriol oxidation product. When cell extracts were incubated with 1,2,4-benzenetriol, the initial absorbance maximum at 286 nm disappeared and was replaced by a new maximum at 242 to 245 nm. The final spectrum was identical to that reported for maleylacetic acid by Chapman and Ribbons (3). The single compound

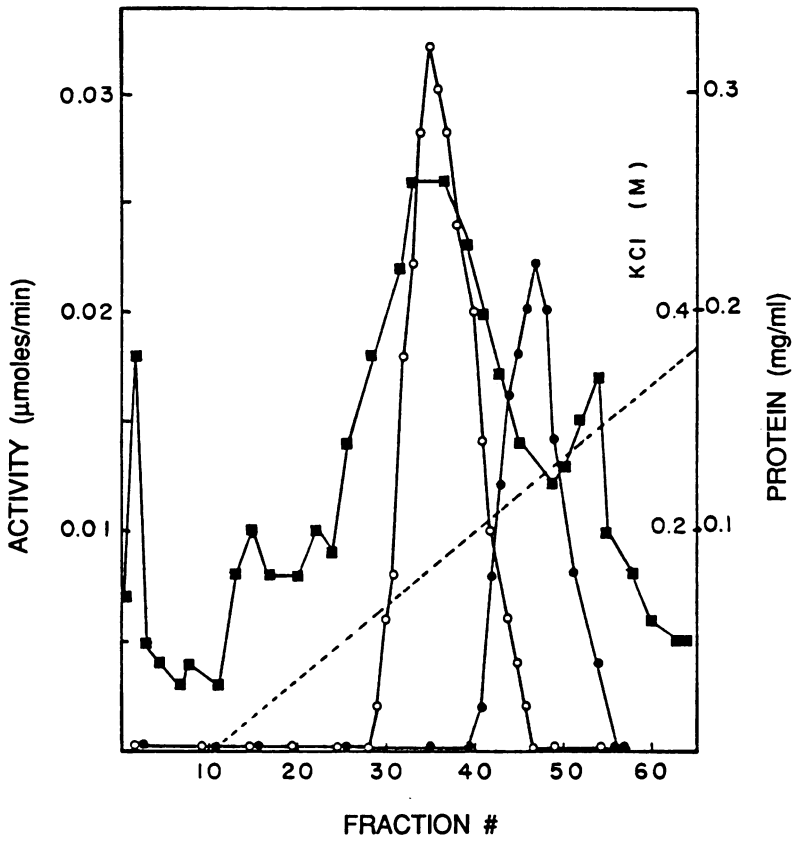

FIG. 4. DEAE-cellulose separation of hydroquinone-oxidizing and 1,2,4-benzenetriol-oxidizing activities in cell extracts. *Moraxella* cell extract (35 to 55% ammonium sulfate fraction) was subjected to chromatography on DEAE-cellulose as described in Materials and Methods. Symbols: ○, hydroquinone oxidation; ●, 1,2,4-benzenetriol oxidation; ■, protein.

detected by HPLC and HPLC-MS cochromatographed with authentic maleylacetic acid.

The results indicate that the enzyme in *Moraxella* extracts is a ring fission dioxygenase which oxidizes 1,2,4-benzenetriol to maleylacetic acid and is similar to an enzyme reported by Chapman and Ribbons (3) for a *Pseudomonas* sp. grown on resorcinol.

When cell extracts were incubated with 1,2,4-benzenetriol (10^{-4} M) and NADH (2×10^{-4} M), maleylacetate did not accumulate. Instead, the trihydroxy compound was completely converted to β-ketoadipate, which was identified and quantitated by HPLC-MS. The results indicate the presence of maleylacetate reductase, but attempts to assay the enzyme directly were not successful.

DISCUSSION

Previous work has shown conclusively that PNP is converted to hydroquinone by enzymes in *Moraxella* extracts (21) (Fig. 5). At that time, it was suggested that the initial reaction product was *p*-benzoquinone, which was subsequently reduced to hydroquinone by an NADH-dependent quinone reductase. The quinone was not isolated, however, because the PNP-oxidizing enzyme and the quinone reductase were membrane bound and could not be separated. Zeyer and Kocher (25) have recently reported the purification of an enzyme from *P. putida* that converts *o*-nitrophenol to catechol and nitrite. They suggested that the reaction was analogous to that of the PNP oxygenase in the *Moraxella* sp., primarily because of the stoichiometry of the reaction. In both instances 2 mol of NADH was required for conversion of the nitrophenol to the corresponding dihydroxy compound. Although the two nitrophenol oxygenases seem to catalyze similar reactions, the properties of the enzymes are different. The enzyme from *P. putida* was soluble and nonspecific and required magnesium or manganese ions. It was not stimulated by flavin adenine dinucleotide or flavin mononucleotide. In contrast, the enzyme from the *Moraxella* sp. was particulate and specific and required flavin adenine dinucleotide for maximum activity. Both enzymes exhibited substrate inhibition by the nitrophenols, and their affinities for their respective substrates were similar.

The initial replacement of the nitro group by a hydroxyl group in the *Moraxella* sp. was also suggested by Simpson and Evans (18) for a *Pseudomonas* sp. grown on PNP. This contrasts with the initial conversion of PNP to 4-nitrocatechol suggested by Raymond and Alexander (16) for a *Flavobacterium* sp. The stimulation of oxygen uptake in PNP-grown cells of the *Moraxella* sp. by 4-nitrocatechol suggests that it is a substrate analog of PNP. Nitrite was released from 4-nitrocatechol by washed cells but not by particulate-enzyme preparations. We found no evidence of enzymes in the *Moraxella* sp. that could hydroxylate PNP to form 4-nitrocatechol. In addition, PNP is converted to hydroquinone at rates sufficient to account for the growth of the organism.

The mass spectral analyses in this report provide the first clear identification of γ-hydroxymuconic semialdehyde. The

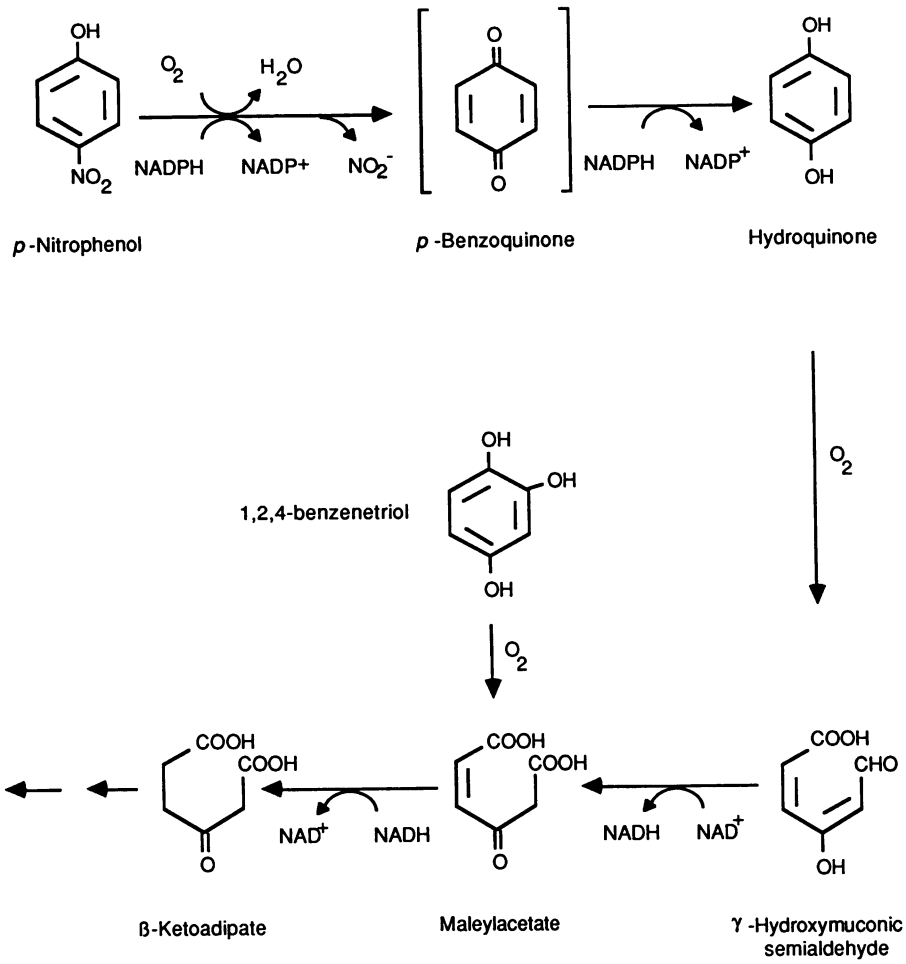

FIG. 5. Proposed pathway for degradation of PNP and 1,2,4-benzenetriol.

dioxygenase-catalyzed conversion of hydroquinone to γ-hydroxymuconic semialdehyde was initially proposed by Larway and Evans (12) for a *Pseudomonas* sp. grown on hydroquinone. The properties of the enzyme they characterized were similar to those of the enzyme in the *Moraxella* sp. described in this report. Both enzymes required ferrous ions and were inhibited by dipyridyl; however, the enzyme from the *Pseudomonas* sp. was stimulated by cysteine or ascorbate, whereas the *Moraxella* enzyme was not. A similar ring fission mechanism for hydroquinone in a strain of *P. putida* grown on 4-ethylphenol was suggested by Darby et al. (5). In each instance, the reaction seems to involve ring fission by a dioxygenase analogous to the reaction described by Lack (11) for conversion of gentisic acid to maleylpyruvate and by Chapman and Dagley (1) for conversion of homogentisic acid to maleylacetoacetate. The ring fission product in both of these instances contained a *cis* double bond which was isomerized to the *trans* configuration prior to further metabolism. It was later shown that in other bacteria, maleylacetoacetate (8) or maleylpyruvate (4) could be hydrolyzed without prior isomerization.

Previous workers (5, 12) have demonstrated the conversion of hydroquinone to β-ketoadipic acid by bacterial cell extracts. When we carried out the reaction with extracts from the *Moraxella* sp., inclusion of reagents that oxidize NADH blocked the recycling of the electron carrier and led to the stoichiometric accumulation of maleylacetate. To our knowledge, this is the first direct evidence that maleylacetic acid is an intermediate in the pathway. In preliminary experiments, we were not able to detect maleylacetate reductase in cell extracts. However, the conversion of both hydroquinone and 1,2,4-benzenetriol to maleylacetate in the absence of NADH and to β-ketoadipate in the presence of NADH argues for the operation of a maleylacetate reductase. Larway and Evans (12) were also unable to detect maleylacetate reductase in cell extracts prepared from their resorcinol-grown pseudomonad, even though maleylacetate was rapidly degraded by intact cells. It is possible that maleylacetate reductase is closely associated with the enzymes that produce maleylacetate or that maleylacetate undergoes a *cis*-to-*trans* isomerization prior to reduction.

Our results indicate that 1,2,4-benzenetriol is not an intermediate in the pathway for degradation of PNP. We found no evidence for conversion of hydroquinone to 1,2,4-benzenetriol prior to ring fission as suggested by Munnecke and Hsieh (14) for a *Pseudomonas* sp. grown on PNP and by Karasevich and Ivoilov (9) for a yeast grown on *p*-hydroxybenzoate. Therefore, the presence of a 1,2,4-benzenetriol ring fission enzyme in PNP-grown cells remains to be explained.

On the basis of our results and on analogy with other systems, we propose the pathway shown in Fig. 5 for the

metabolism of PNP by the *Moraxella* sp. The evidence for participation of 1,4-benzoquinone in the pathway is circumstantial; purification of the enzymes involved will be required before the reaction mechanism can be rigorously demonstrated.

ACKNOWLEDGMENTS

We thank Shirley Nishino for HPLC analyses, Mike Henley for mass spectral analyses, Peter Chapman for helpful discussions, the late W. C. Evans for providing us with unpublished information relating to PNP and hydroquinone degradation, and Peggy Lee for typing the manuscript.

REFERENCES

1. **Chapman, P. J., and S. Dagley.** 1962. Oxidation of homogentisic acid by cell-free extracts of a *Vibrio*. J. Gen. Microbiol. **28**:251–256.
2. **Chapman, P. J., and D. J. Hopper.** 1968. The bacterial metabolism of 2,4-xylenol. Biochem. J. **110**:491–498.
3. **Chapman, P. J., and D. W. Ribbons.** 1976. Metabolism of resorcinylic compounds by bacteria: alternative pathways for resorcinol catabolism in *Pseudomonas putida*. J. Bacteriol. **125**:985–998.
4. **Crawford, R. L.** 1976. Degradation of homogentisate by strains of *Bacillus* and *Moraxella*. Can. J. Microbiol. **22**:276–280.
5. **Darby, J. M., D. G. Taylor, and D. J. Hopper.** 1987. Hydroquinones as the ring-fission substrates in the catabolism of 4-ethylphenol and 4-hydroxyacetophenone by *Pseudomonas putida* JD1. J. Gen. Microbiol. **133**:2137–2146.
6. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum protein by means of the biuret reaction. J. Biol. Chem. **177**:751–766.
7. **Gunderson, K., and H. L. Jensen.** 1956. A soil bacterium decomposing organic nitro compounds. Acta Agric. Scand. **6**:100–114.
8. **Hopper, D. J., P. J. Chapman, and S. Dagley.** 1968. Enzymic formation of D-malate. Biochem. J. **110**:798–800.
9. **Karasevich, Y. N., and V. S. Ivoilov.** 1978. Preparatory metabolism of *para*-hydroxybenzoic acid in the yeast *Candida tropicalis*. Mikrobiologiya **46**:846–856.
10. **Kilby, B. A.** 1951. The formation of β-ketoadipic acid by bacterial fission of aromatic rings. Biochem. J. **49**:671–674.
11. **Lack, L.** 1959. The enzymic oxidation of gentisic acid. Biochim. Biophys. Acta **34**:117–123.
12. **Larway, O., and W. C. Evans.** 1965. Metabolism of quinol and resorcinol by soil pseudomonads. Biochem. J. **95**:52.
13. **Montgomery, H. A. C., and J. F. Dymock.** 1961. The determination of nitrite in water. Analyst **86**:414–416.
14. **Munnecke, D. M., and D. P. H. Hsieh.** 1974. Microbial decontamination of parathion and *p*-nitrophenol in aqueous media. Appl. Microbiol. **28**:212–217.
15. **Nyholm, N., P. Lindgaard-Jorgensen, and N. Hansen.** 1984. Biodegradation of 4-nitrophenol in standardized aquatic degradation tests. Ecotoxicol. Environ. Safety **8**:451–470.
16. **Raymond, D. G. M., and M. Alexander.** 1971. Microbial metabolism and cometabolism of nitrophenols. Pest. Biochem. Physiol. **1**:123–130.
17. **Schlömann, M., P. Fischer, E. Schmidt, and H.-J. Knackmuss.** 1990. Enzymatic formation, stability, and spontaneous reactions of 4-fluoromuconolactone, a metabolite of the bacterial degradation of 4-fluorobenzoate. J. Bacteriol. **172**:5119–5129.
18. **Simpson, J. R., and W. C. Evans.** 1953. The metabolism of nitrophenols by certain bacteria. Biochem. J. **55**:XXIV.
19. **Spain, J. C., and S. F. Nishino.** 1987. Biodegradation of 1,4-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **53**:1010–1019.
20. **Spain, J. C., P. A. Van Veld, C. A. Monti, P. H. Pritchard, and C. R. Cripe.** 1984. Comparison of *p*-nitrophenol biodegradation in field and laboratory test systems. Appl. Environ. Microbiol. **48**:944–950.
21. **Spain, J. C., O. Wyss, and D. T. Gibson.** 1979. Enzymatic oxidation of *p*-nitrophenol. Biochem. Biophys. Res. Commun. **88**:634–641.
22. **Sudhakar-Barik, R. Siddaramappa, and N. Sethunathan.** 1976. Metabolism of nitrophenols by bacteria isolated from parathion-amended flooded soil. Antonie van Leeuwenhoek **42**:461–470.
23. **Tiedje, J. M., J. M. Duxbury, M. Alexander, and J. E. Dawson.** 1969. 2-4 D metabolism: pathway of degradation of chlorocatechols by *Arthrobacter* sp. J. Agric. Food Chem. **17**:1021–1026.
24. **Zeyer, J., and P. C. Kearney.** 1984. Degradation of *o*-nitrophenol and *m*-nitrophenol by a *Pseudomonas putida*. J. Agric. Food Chem. **32**:238–242.
25. **Zeyer, J., and H. P. Kocher.** 1988. Purification and characterization of a bacterial nitrophenol oxygenase which converts *ortho*-nitrophenol to catechol and nitrite. J. Bacteriol. **170**:1789–1794.