Degradation of *o*-Nitrophenol and *m*-Nitrophenol by a *Pseudomonas putida*

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A *Pseudomonas putida* strain that grew on *o*-nitrophenol (ONP) and *m*-nitrophenol (MNP) as sole sources of carbon and nitrogen was isolated from soil. The microorganism was unable to metabolize *p*-nitrophenol. Growing and resting cells of *P. putida* degraded ONP releasing nitrite and MNP releasing ammonium. Enzymes involved in the metabolism of ONP or MNP were inducible by ONP or MNP, respectively. ONP was degradable in a cell-free system. The initial enzyme converted ONP to catechol and nitrite. The enzyme required NADPH as a coenzyme and its specific activity was 15 μmol min^{-1} (g of protein)$^{-1}$. Catechol was further degraded through the ortho-cleavage pathway to β-ketoadipic acid. Attempts to demonstrate the degradation of MNP in a cell-free system failed and the pathway of the MNP metabolism remains unknown.

Many pesticides, drugs, dyes, and explosives are nitroaromatic compounds. Numerous publications deal with the microbial metabolism of nitroaromatic compounds. A complete microbial mineralization was found for nitrobenzoic acid (Cartwright and Cain, 1959), nitroaniline (Zeyer and Kearney, 1983), and nitrophenol derivatives (Germanier and Wuhrmann, 1963; Gundersen and Jensen, 1956; Jensen and Lautrup-Larsen, 1967; Raymond and Alexander, 1971; Simpson and Evans, 1953; Spain, 1979; Spain et al., 1979; Sundhakar-Barik et al., 1976), whereas only a partial degradation of nitrotoluene derivatives (McCormick et al., 1976) has been reported.

Two enzymatic systems exist for the removal of nitro substituents from nitroaromatic compounds. One involves an initial reduction of the nitro substituent by a nitroreductase, followed by a release of ammonium. Reduction of various nitroaromatic compounds through this pathway was demonstrated in cell-free systems under aerobic (Tewfik and Evans, 1966; Villanueva, 1964) and anaerobic (McCormick et al., 1976; Yamashina, 1954; Yamashina et al., 1954) conditions. In the other system, the nitro substituent is directly removed in the form of nitrite from the aromatic core. This direct removal was demonstrated by Spain (1979), who isolated an enzyme that converted *p*-nitrophenol (PNP) to hydroquinone and nitrite. The reaction required oxygen and NADPH and the enzymatic activity was stimulated by FAD. The enzyme was specific for PNP and did not attack any isomers of PNP.

In this report we present data on the aerobic microbial degradation of *o*-nitrophenol (ONP) and *m*-nitrophenol (MNP). Degradation of ONP and release of nitrite by growing and resting cells of *Pseudomonas* spp. has been described (Germanier and Wuhrmann, 1963; Simpson and Evans, 1953). A metabolism through catechol as an intermediate was assumed but no direct proof for a particular pathway has so far been given. Germanier and Wuhrmann (1963) reported the degradation of MNP coupled to a release of ammonium by a *Pseudomonas* sp., whereas Raymond and Alexander (1971) described a partial metabolism of MNP to nitrohydroquinone by an unidentified bacterium. We are not aware of any reports describing a total degradation of ONP and MNP by either the same microorganism or a cell-free system.

MATERIALS AND METHODS

Chemicals. ONP, MNP, PNP, and catechol were purchased from Aldrich Chemical Company, Milwaukee, WI. [^{14}C]ONP (sp act. 6.8 mCi/mM) was from Pathfinder Laboratories, Inc., St. Louis, MO, and [^{14}C]PNP (sp act. 1.8 mCi/mM) from Mallinckrodt, St. Louis, MO. The radiochemical purity of [^{14}C]ONP and [^{14}C]PNP was determined by HPLC and found to be 98%. *cis,cis*-Muconic acid was a gift from L. N. Ornston, Yale University, New Haven, CT; β-ketoadipic acid was obtained from Sigma Chemical Co., St. Louis, MO; yeast extract was from Difco Laboratories, Detroit, MI; chloramphenicol, bovine albumin, NADPH, and FAD were from Calbiochem-Behring Corp., La Jolla, CA.

Medium and Culture Conditions. The composition of the basal medium has been previously reported (Zeyer and Kearney, 1982). The basal medium was supplemented with substrates as indicated under Results and sterilized by filtration through a 0.45-μm GA-6 membrane filter (Gelman Sciences, Inc., Ann Arbor, MI). All cultures were incubated under aerobic conditions at 26 °C on a rotary shaker.

Isolation of *Pseudomonas putida*. Basal medium (10 mL) supplemented with 1 mM ONP or MNP as a sole source of carbon and nitrogen was inoculated with soil samples from various sources and incubated. The disappearance of the yellow color in the medium was taken as an indicator of a degradation of ONP or MNP. When a culture turned colorless, a sample (2 mL) was transferred into 10 mL of fresh medium. After about 5–10 transfers, samples of the culture were streaked out on plates containing basal medium, 2% agar, and 1 mM ONP or MNP. After an incubation time of a few days, several microorganisms were isolated that grew on ONP or MNP as a sole source of carbon and nitrogen. One organism grew on both ONP and MNP. This organism was identified as a *P. putida* according to Bergey's manual (Buchanan and Gibbons, 1974). The major characteristics of the organisms were white to buff, smooth colonies, Gram-negative, fluorescent, growth on glucose but not on xylose or maltose, no hydrolysis of gelatin and soluble starch, no denitrification, and positive oxidase and arginine dihydrolase reaction. All experiments described in this report were performed with this strain.

Preparation of Cell Suspensions and Enzyme Extracts. *P. putida* was cultured on basal medium supplemented with 1 mM ONP and 0.02% yeast extract (pH 7.5). Yeast extract was added to increase the cell density. A decrease in concentration of ONP during incubation was determined by HPLC. As soon as ONP was depleted, the cells were harvested by centrifugation (4000*g*, 30 min, Sorvall RC-2B) and washed 3 times with phosphate buffer (0.05 M, pH 7.5). For the preparation of a cell suspension, the sediment was suspended in phosphate buffer (0.05 M, pH 7.5, about 100 mL of buffer/1 g wet weight of cells) and 1 mM chloramphenicol was added to stop further

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enzyme synthesis. For the preparation of an enzyme extract, the sediment was suspended in phosphate buffer (0.02 M, pH 7.5, about 12 mL of buffer/1 g wet weight of cells) and the suspension was subjected to intermittent (7 × 15 s) sonic vibration with a Brownwill Biosonic IV ultrasonic system (300-W acoustical power). The resulting sonic extract was centrifuged (30000*g*, 15 min) and the supernatant was used as the enzyme extract. All procedures were carried out at <5 °C.

Cell suspensions and enzyme extracts of *P. putida* precultured on basal medium supplemented with 1 mM MNP and 0.02% yeast extract (pH 6.5) were prepared by using the same methods. However, the pH of the phosphate buffer was 6.5.

Cell suspensions and enzyme extracts were always used within 2 h after preparation and incubated with selected substrates under aerobic conditions at 26 °C.

Determination of Protein, Ammonium, Nitrite, Radioactivity, and Absorption Spectra. The protein content of cultures, cell suspensions, and enzyme extracts was measured by the modified method of Lowry for whole cells (Herbert et al., 1971) with bovine albumin as a standard. Ammonium was determined by the method of Weatherburn (1967). Nitrite was measured by using the Griess-Jlosvay reaction as modified by Montgomery and Dymock (1961). The distribution of radioactivity while incubating [^{14}C]PNP in a *P. putida* culture was determined by pulling CO_2 free air through the culture and subsequently through 10 mL of 0.2 N NaOH to absorb the liberated CO_2. The amount of radioactivity in the culture and in the NaOH trap at the end of the incubation time was measured by taking 1-mL samples, adding 10 mL of Ready-Solv HP scintillation fluid (Beckman), and counting the samples in a Searle Mark II liquid scintillation counter (Searle Analytic, Inc.). Absorption spectra were recorded on a Beckman Model 25 spectrophotometer.

Analysis of ONP, MNP, and PNP in Culture and Cell Suspensions. ONP, MNP, and PNP in cultures or cell suspensions were analyzed by taking a 1-mL sample, adding 0.1 mL of a concentrated pH 2 buffer solution, and extracting the sample with 1 mL of ethyl acetate. ONP, MNP, and PNP were extractable with an efficiency of >98% by this procedure. A 25-μL sample of the ethyl acetate extract was injected into a HPLC system, consisting of a U6K injector, a Model 6000A and a Model M 45 pump, a Model 660 solvent programmer, a Model 440 absorbance detector operating at a wavelength of 254 nm, and a Model 730 data module (all instruments from Waters Associates, Milford, MA). The separation was performed using an 8-mm 5-μm Radial-PAK C18 column (Waters) and water–acetonitrile (65:35) plus 0.1% acetic acid as a solvent. At a flow rate of 2 mL/min, the following retention times were observed: for ONP, 7.6; for MNP, 4.8; for PNP, 4.3 min. A quantitative determination was achieved by comparing the peak areas of samples with the peak areas of standards of known concentrations.

Analysis of ONP Degradation in a Cell-Free System. Enzyme assays were carried out in phosphate buffer (0.02 M, pH 7.5) containing ONP, NADPH, and enzyme extract. Some assays (see Results) were supplemented with FAD. The volume of the assay and the concentrations of substrate, cofactors, and protein are indicated under Results. The reaction in the assay was started by adding the enzyme extract. Kinetic data on the degradation of ONP in an enzyme assay was obtained spectrophotometrically. The enzyme assay was prepared in a cuvette (1-cm pathway) and the decrease of the absorbance at 410 nm was monitored on a Beckman Model 25 spectrophotometer connected to a recorder. A decrease in the absorbance of 0.1 min^{-1} corresponded to a degradation rate of 30 μmol L^{-1} min^{-1}. The specific activity was expressed on a protein basis and indicated as μmol min^{-1} (g of protein)$^{-1}$.

[^{14}C]ONP was used as a substrate to study the distribution of products in a cell-free system. Samples (1 mL) of the cell-free system incubated with [^{14}C]ONP (about 70 000 dpm/mL) were treated with 1 mL of methanol to stop the reaction and centrifuged (7000*g*, 30 min) at 4 °C to precipitate the denatured proteins. After the nitrite content was measured, the supernatant was analyzed by TLC and HPLC.

For the TLC analysis, silica gel 60F-254 plates (20 × 20 cm, Merck) were used. All samples were separated in two-dimensional chromatography using two different solvent systems: (A) benzene–methanol–acetic acid (45:8:4); (B) ethyl acetate–acetic acid–water (30:20:10). Selected standards showed the following R_f values: for ONP, 0.70 (A) and 0.92 (B); for catechol, 0.45 (A) and 0.88 (B); for *cis,cis*-muconic acid, 0.39 (A) and 0.84 (B); for β-ketoadipic acid, 0.20 (A) and 0.69 (B). After separation, nonlabeled standards were localized under UV light and ^{14}C-labeled compounds were localized by autoradiography (X-ray film NS-5T, Kodak). The UV absorption of β-ketoadipic acid is poor (Stanier and Ingraham, 1954) and therefore high concentrations had to be applied on TLC plates. HPLC analysis (instrument and column as mentioned above) of the supernatant was performed by injecting a 25-μL sample and running a linear gradient from water–acetonitrile (94:6) to 100% acetonitrile within 7 min at a constant flow rate of 2 mL/min. Water and acetonitrile contained 0.1% acetic acid. Selected standards showed the following retention times and molar extinction coefficients (ε, in water, pH 7) at 254 nm: for ONP, retention time = 7.7 min, and ε = 3800; for catechol, 5.5 and 500; for *cis,cis*-muconic acid, 4.7 and 17 300; for β-ketoadipic acid, 2.7 and <50. For quantitative determinations, the HPLC effluent at a particular retention time was collected and the amount of radioactivity was measured in a liquid scintillation counter and compared to the total radioactivity in the sample.

RESULTS

Degradation of ONP, MNP, and PNP by Growing Cells of *P. putida*. The degradation of ONP or MNP as a sole source of carbon and nitrogen by *P. putida* was highly dependent on the pH of the medium (Table I). ONP was rapidly metabolized at pH 7.5 and the culture turned colorless. No apparent degradation was found at pH 7 and only a slow degradation was observed at pH 8. In contrast, MNP was rapidly metabolized at pH 6.5, but no degradation was found at pH 6. The culture growing on MNP at pH 6.5 turned distinctly orange-red after 1–2 days and was slightly brownish after 3 days. At pH 7–8, cultures on MNP grew poorly and turned increasingly dark brown after a few days. The pK_a of ONP and MNP is 7.2 and 8.3, respectively. At the pHs where metabolism occurred, ONP was mainly dissociated whereas MNP was not charged. Hence, metabolism does not seem to be a function of the charge. Gundersen and Jensen (1956) observed a similar pronounced pH dependence for the degradation of PNP. Products obtained by the degradation of ONP and MNP are listed in Table II. The nitro substituent of ONP was released as nitrite, whereas an accumulation of ammonium was found in cultures growing on MNP. The protein content of both cultures was around 0.046 mg/mL, which corresponds to a nitrogen content of about 0.5 mM [if one assumes a nitrogen content of 16% of the proteins (Herbert et al., 1971)]. Additional studies

Table I. Degradation of ONP and MNP by *P. putida* at Different pH Values

pH of medium[a]	substrate recovered in culture, mM[b]	
	ONP	MNP
5	1.35	1.50
6	1.43	1.49
6.5	1.47	<0.02
7	1.50	0.87
7.5	<0.02	0.90
8	0.45	1.17
9	1.26	1.40

[a] 10 mL of basal medium plus 1.5 mM ONP or MNP. 0.5 mL of a culture grown on basal medium plus 1.5 mM ONP (pH 7.5) was used as an inoculum. [b] Incubation time for cultures on ONP was 2 days and for cultures on MNP 3 days. Data shown are the mean values of four independent experiments. The standard deviation of each value is below 0.16.

Table II. Products of the Degradation of ONP and MNP by *P. putida*

substrate and pH of medium[a]	substrate and products recovered in culture after an incubation time of 4 days			
	substrate, mM	NH_4^+, mM	NO_2^-, mM	protein, mg/mL
ONP (pH 7.5)	<0.02	<0.02	0.51	0.048
MNP (pH 6.5)	<0.02	0.74	<0.01	0.044

[a] 10 mL of basal medium plus 1 mM ONP or MNP.

Table III. Degradation of PNP by *P. putida*

substrates and pH of medium[a]	analysis of products after an incubation time of 7 days			
	$^{14}CO_2$ evolved, %	^{14}C in culture, %	substrates in culture, mM	
one substrate				
[^{14}C]PNP (pH 7)	<0.1	95	PNP:	0.43
two substrates				
[^{14}C]PNP + ONP (pH 7.5)	<0.1	96	PNP:	0.43
			ONP:	<0.02
[^{14}C]PNP + MNP (pH 6.5)	<0.1	94	PNP:	0.48
			MNP:	<0.02

[a] 10 mL of basal medium plus substrate (0.5 mM of each). The amount of ^{14}C initially added (about 20 000 dpm/mL) was considered to be 100%.

revealed that growth of *P. putida* was increasingly inhibited at concentrations above 1.5–2 mM and stopped completely at 2.5 mM ONP or MNP. *P. putida* was unable to degrade PNP as a sole source of carbon and nitrogen (Table III). To increase the sensitivity of the degradation test, [^{14}C]PNP (0.5 mM) was used as a substrate and the evolved CO_2 was entrapped. After an incubation period of 7 days, 86–95% of the PNP and the radioactivity were recovered in the culture and less than 0.1% $^{14}CO_2$ was liberated. The degradation of [^{14}C]PNP was also determined in presence of ONP or MNP as additional substrates. ONP and MNP were completely metabolized, indicating that 0.5 mM PNP had no toxic effect on *P. putida*. The degradation of PNP, however, was not significant.

Degradation of ONP and MNP by Resting Cells of *P. putida*. Cells of *P. putida* were precultured in the presence of ONP or MNP, harvested, and suspended in buffer. The degradation of ONP and MNP by these cell suspensions is outlined in Table IV. Cells precultured in presence of ONP rapidly degraded ONP and released nitrite. These cells did not metabolize MNP. In contrast, MNP precultured cells rapidly metabolized MNP, releasing ammonium, but did not degrade ONP. Even in suspensions that did not metabolize any ONP or MNP, some ammonium was detectable at the end of the incubation time. This may be due to a release of previously accumulated ammonium.

Suspensions of cells precultured in the presence of ONP or MNP showed no activity on PNP, which is in agreement with the data presented in Table III.

Degradation of ONP and MNP by an Enzyme Extract of *P. putida*. Growing and resting cells of *P. putida* degraded MNP and thereby produced ammonium. By analogy to previous reports (Tewfik and Evans, 1966; Villanueva, 1964) it was assumed that the strain had nitroreductase activity. All attempts, however, to demonstrate the reduction of the nitro substituent of MNP in a cell-free system failed. Application of neither the enzyme assay described for the degradation of 3,5-dinitro-*o*-cresol to 3-amino-5-nitro-*o*-cresol by Tewfik and Evans (1966) nor the assay used by Villanueva (1964) to demonstrate the metabolism of *p*-dinitrobenzene to *p*-nitroaniline allowed us to detect a conversion of MNP.

In contrast, a modification of the enzyme assay used by Spain (1979) to demonstrate the metabolism of PNP led to the degradation of ONP (Table V). ONP was completely metabolized in the presence of NADPH and nitrite was released in stoichiometric amounts. No significant degradation was observed in the absence of NADPH or in the presence of a heat-inactivated enzyme extract. In contrast to the data reported by Spain (1979), only a partial degradation of ONP took place within 20 min in the presence of NADPH/FAD. Analysis of samples withdrawn at 5-min intervals from the enzyme assays revealed that ONP was initially degraded in the presence of NADPH and NADPH/FAD at the same rate. However,

Table IV. Degradation of ONP and MNP by Resting Cells of *P. putida*

preculture medium	substrate (1 mM) added to cell suspension[a]	substrate and products in cell suspension after an incubation time of 2.5 h, mM[b]		
		substrate	NH_4^+	NO_2^-
basal medium + 1 mM ONP + 0.02% yeast extract (pH 7.5)	ONP (pH 7.5)	<0.02[c]	0.20	0.95
	MNP (pH 6.5)	1.02	0.12	<0.01
basal medium + 1 mM MNP + 0.02% yeast extract (pH 6.5)	ONP (pH 7.5)	0.94	0.12	<0.01
	MNP (pH 6.5)	<0.02[c]	0.85	<0.01

[a] The cell suspensions prepared from the precultures were divided into two volumes, to which the indicated substrates were added. The pH was adjusted to the value indicated in parentheses. The protein content of all suspensions was 0.8 mg/mL. [b] Data shown are the mean values of two experiments. [c] The concentration of ONP and MNP, respectively, during incubation was determined every 5 min by HPLC. The concentration dropped linearly. ONP was completely degraded after 2.4 h and MNP was degraded after 1.7 h. The specific activity was estimated to be 9–12 μmol min^{-1} (g of protein)$^{-1}$.

Table V. Degradation of ONP by an Enzyme Extract of *P. putida*

substrate, enzyme, and coenzymes in assay[a]				substrate and product in assay after an incubation time of 20 min	
ONP, mM	enzyme extract,[b] mg of protein/mL	NADPH, mM	FAD, mM	ONP, mM[c]	NO_2^-, mM[c]
0.2	1.1	0.8	0.2	0.07	0.14
0.2	1.1	0.8		<0.02	0.19
0.2	1.1		0.2	0.19	<0.01
0.2	1.1			0.20	<0.01
0.2	1.1[d]	0.8		0.20	<0.01

[a] Assay was carried out in 5 mL of buffer. [b] *P. putida* was precultured on basal medium plus 1 mM ONP plus 0.02% yeast extract (pH 7.5). [c] Samples (1 mL) were taken every 5 min, treated with 1 mL of methanol, and analyzed by HPLC as indicated under Materials and Methods. However, nonlabeled ONP was used in the assay and for a quantitative determination the peak area was compared with a standard. Data shown are the mean values of three experiments. [d] Enzyme extract inactivated in boiling water for 10 min.

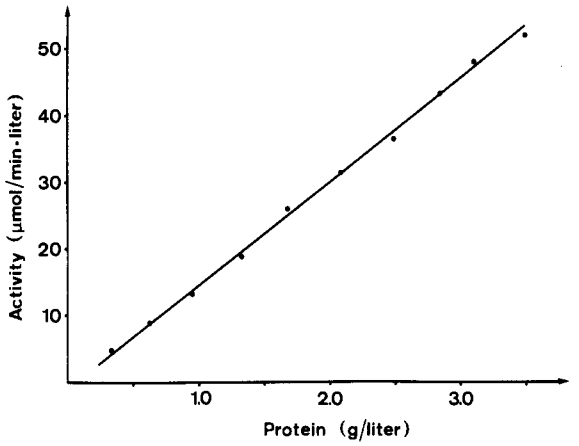

Figure 1. Rate of the ONP degradation as a function of the amount of protein in the enzyme assay. *P. putida* was precultured on basal medium plus 1 mM ONP plus 0.02% yeast extract (pH 7.5). The enzyme assay was carried out in 2 mL of buffer containing 0.1 mM ONP plus 0.4 mM NADPH plus enzyme extract. The ONP degradation was recorded spectrophotometrically. The data shown are the mean values of three experiments. The standard deviation of the activity of 3.5 g of protein/L is 8.0.

the degradation rate decreased rapidly in the assay containing NADPH/FAD; the concentration of ONP reached a plateau at 0.07 mM and was not further metabolized.

The degradation of ONP in the enzyme assays reported in Table V was determined by HPLC because FAD interfered with the spectrophotometric assay. An aqueous solution of ONP (pH 7.5) had an absorption maximum at 410 nm (ϵ = 3300), a wavelength where FAD also absorbed strongly (ϵ = 6600, pH 7.5). However, the finding that FAD was not required for the metabolism of ONP allowed a spectrophotometric measurement of the degradation at 410 nm. The degradation rate of ONP as a function of the amount of protein present in the enzyme assays is shown in Figure 1. The rate increased linearly with increasing amounts of protein. The specific activity was constant and calculated to be 15 μmol min^{-1} (g of protein)$^{-1}$, which is about 50% higher than the activity found in resting cells (Table IV).

Products of ONP Degradation by an Enzyme Extract. [^{14}C]ONP was used as a substrate to facilitate the identification of the products of the enzymatic degradation.

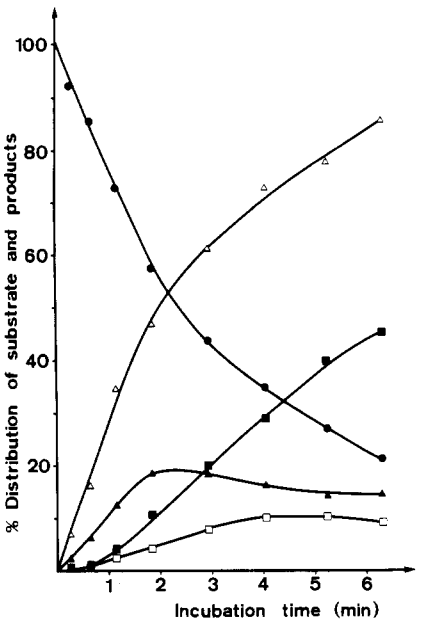

Figure 2. Products of the ONP degradation by an enzyme extract of *P. putida*. *P. putida* was precultured on basal medium plus 1 mM ONP plus 0.02% yeast extract (pH 7.5). The enzyme assay was carried out in 15 mL of buffer containing 0.2 mM [^{14}C]ONP plus 0.8 mM NADPH plus enzyme extract (2.7 mg/mL). Samples were taken periodically and analyzed as indicated under Materials and Methods. 0.2 mM nitrite was considered to be 100%. (●) ONP; (□) catechol; (▲) *cis,cis*-muconic acid; (■) β-ketoadipic acid; (△) nitrite.

An assay containing [^{14}C]ONP, NADPH, and enzyme extract was incubated and samples were taken periodically and analyzed (Figure 2). A qualitative analysis of the samples by TLC followed by autoradiography showed a rapid decrease of the ONP concentration and the appearance of two minor products and one major product. The products were tentatively identified by two-dimensional cochromatography with standards. The spots on the autoradiography film matched with standards of catechol, *cis,cis*-muconic acid (minor products), and β-ketoadipic acid (major product). Traces of two to three other intermediates were also observed on the film. These compounds were not identified. On HPLC, the samples showed distinct peaks for ONP (7.7 min) and *cis,cis*-muconic acid (4.7 min); however, no peaks were detected for catechol and β-ketoadipic acid. A determination of the molar extinction coefficients (see Materials and Methods) at 254 nm (operational wavelength of the HPLC detector) indicated that in contrast to ONP and *cis,cis*-muconic acid, good responses cannot be expected for catechol and β-ketoadipic acid. Only concentrated (>1 mM) standard solutions of catechol and β-ketoadipic acid exhibited significant peaks on the HPLC chromatogram.

A quantitative analysis of the samples was performed by HPLC (Figure 2). Some 90–95% of the total radioactivity of each sample injected into the HPLC could be collected at the retention times for ONP, catechol, *cis,cis*-muconic acid, and β-ketoadipic acid, respectively. The total amount of radioactivity in each sample remained constant during incubation, which indicated that no $^{14}CO_2$ was evolved.

DISCUSSION

Growing cells of *P. putida* degraded ONP releasing nitrite and MNP producing ammonium. Resting cells pregrown in presence of ONP degraded ONP releasing nitrite but were inactive on MNP. In contrast, MNP pregrown cells metabolized MNP producing ammonium

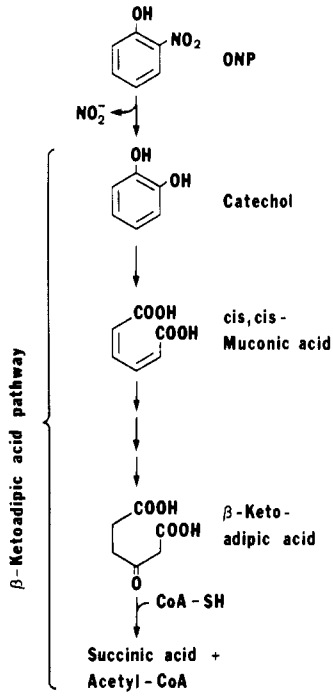

Figure 3. Proposed pathway of ONP degradation.

but did not degrade ONP. This suggested that *P. putida* has two separate, inducible, enzyme systems to metabolize nitrophenols: an oxidative system for ONP and a reductive system for MNP. The total degradation of ONP and MNP by pure strains of microorganisms has been reported, but each organism was specific for ONP or MNP, respectively (Germanier and Wuhrmann, 1963; Simpson and Evans, 1953). We are not aware of published research that indicates the presence of an oxidative and a reductive enzymatic system for the degradation of nitroaromatic compounds in the same microorganism.

Attempts to study the pathway of MNP degradation failed. We were unable to determine any intermediates such as *m*-aminophenol or resorcinol in growing cultures or in cell suspensions. Using similar enzyme assays as reported for the reduction of *p*-dinitrobenzene to *p*-nitroaniline (Villanueva, 1964) and the conversion of 3,5-dinitro-*o*-cresol to 3-amino-5-nitro-*o*-cresol (Tewfik and Evans, 1966), we were unable to demonstrate conversion of MNP to *m*-aminophenol by a nitroreductase. MNP is probably not metabolized through *m*-aminophenol as an intermediate. Germanier and Wuhrmann (1963) also found no evidence for MNP degradation through *m*-aminophenol and resorcinol. Resting cells of *Pseudomonas* sp. precultured on MNP were simultaneously adapted to MNP and 1,2,4-benzenetriol but not to *m*-aminophenol and resorcinol. It cannot be excluded that *P. putida* initially oxidizes MNP to nitrohydroquinone or *p*-nitrocatechol, which may be primary substrates for a nitroreductase. This assumption is supported by a previous publication (Raymond and Alexander, 1971), which described the microbial conversion of MNP to nitrohydroquinone.

The pathway of ONP degradation was investigated in a cell-free system. The data suggested metabolism of ONP through catechol and *cis,cis*-muconic acid to β-ketoadipic acid (Figure 3). The initial enzyme catalyzed the conversion of ONP to catechol and nitrite. The enzyme required NADPH as a coenzyme and the reaction was incomplete in presence of NADPH/FAD. An NADPH-dependent enzyme that converted PNP to hydroquinone and nitrite was reported (Spain, 1979). This enzyme was inactive on ONP and its activity on PNP was stimulated by FAD. Our enzymatic data were obtained by using a crude enzyme extract. Purification of the enzyme will be required to allow the accurate determination of its properties. Numerous papers deal with the metabolism of catechol to β-ketoadipic acid and the reaction sequence is known as the β-ketoadipic acid pathway (Stanier and Ornston, 1973). None of the enzymes involved in this pathway require any coenzymes, but the activity of one enzyme (*cis,cis*-muconate lactonizing enzyme) is stimulated in presence of manganese ions (Sistrom and Stanier, 1954). Crude enzyme extracts, however, were reported to have a sufficient concentration of manganese ions to allow a fast conversion of catechol to β-ketoadipic acid (Stanier et al., 1950; Hayaishi and Stanier, 1951), which is in accordance with our data.

Registry No. ONP, 88-75-5; MNP, 554-84-7; NADPH, 53-57-6; catechol, 120-80-9; β-ketoadipic acid, 689-31-6; ammonium, 14798-03-9.

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