Metabolism of Aniline by *Rhodococcus erythropolis* AN-13[†]

Kenji AOKI, Ryu SHINKE and Hiroshi NISHIRA

Department of Agricultural Chemistry, Faculty of Agriculture,
Kobe University, Rokko, Kobe 657, Japan

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The metabolic pathway of aniline was examined in *Rhodococcus erythropolis* AN-13 that was isolated from soil when aniline was provided as a sole source of carbon and nitrogen. *cis, cis*-Muconic acid and β -ketoadipic acid were detected by thin-layer chromatography in an incubation mixture containing aniline and resting cells of this strain. These two carboxylic acids were also formed from catechol, when the substrate was incubated with cell-free extract of aniline-grown cells, and characterized spectrally as crystalline samples. Ammonia was released from aniline by resting cells. The cell-free extract of aniline-grown cells had a strong catechol 1,2-dioxygenase activity. Catechol, once formed from aniline, was apparently converted so rapidly to *cis, cis*-muconic acid that it could not be isolated. These results suggest that *R. erythropolis* AN-13 converted aniline to catechol with the release of ammonia and then mineralized catechol ultimately to inorganic end products, H₂O and CO₂, through the β -ketoadipic acid pathway.

Microbial degradation of aromatic amine-based herbicides leads to the accumulation of the corresponding aniline derivatives in soil.¹⁾ Many studies on microbial and chemical actions on the accumulated halogenated anilines have been reported: acylation of *p*-bromoaniline,²⁾ oxidation of the amino group of 4-chloroaniline to a nitro group,³⁾ hydroxylation of 3- and 4-chloroaniline,^{4,5)} and conversion of chloroanilines to azobenzenes⁶⁾ and triazenes.⁷⁾ But few papers have described a metabolic pathway for the conversion of the halogenated anilines to inorganic end products in microorganisms.⁸⁾

Unhalogenated aniline, an intermediate in the microbial degradation of isopropyl *N*-phenylcarbamate, is metabolized by some microorganisms,^{9,10)} and the physiology of aniline degradation by the organisms was investigated.^{11,12)} Walker and Harris¹³⁾ reported that washed cells of an aniline-grown microorganism¹⁴⁾ release ammonia from the amino group of aniline. Bachofer *et al.*¹⁵⁾ showed that a *Nocardia* mutant forms catechol from aniline with the incorporation of two oxygen

atoms. Russel¹⁶⁾ stated that the cell-free extract of an aniline-grown *N. calcearea* strain metabolizes catechol to *cis, cis*-muconic acid. But a total biodegradation pathway for mineralization of aniline is unknown.

We¹⁷⁾ reported that seven strains of *Rhodococcus erythropolis* isolated from soil degrade aniline rapidly and maintain the ability to grow on aniline as a sole source of carbon and nitrogen. *R. erythropolis* AN-13 was selected for metabolic studies of aniline, because of all strains tested it grew best on aniline. We found several metabolites in the degradation of aniline by this strain. In this report we report isolation and identification of these aniline metabolites and propose a pathway for the biodegradation of aniline to inorganic end products by *R. erythropolis* AN-13.

MATERIALS AND METHODS

Growth of culture. *R. erythropolis* AN-13 isolated from soil was used throughout this study.¹⁷⁾ For the growth medium, three solutions were prepared separately. Solution A contained 3 g of KH₂PO₄, 6.5 g of Na₂HPO₄·12H₂O, 0.5 g of NaCl and 0.2 g of yeast extract

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(Daigo Eiyo, Osaka, Japan) in 700 ml of water (pH 6.8). Solution B contained 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.5 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and ZnCl_2 in 150 ml of water. Solution C contained 2.69 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 3 g of aniline hydrochloride (extra pure grade, Wako Pure Chem., Osaka) in 125 ml of water; 5 N NaOH was added to pH 6.8 and the volume was brought to 150 ml. Solutions A and B were autoclaved, and solution C was sterilized by filtration. The three solutions were mixed at room temperature. The agar medium for storage of the strain was prepared as follows: Solution A containing 15 g of agar was autoclaved and mixed with sterile solutions B and C at 50 to 60°C, and the mixture was put into a test tube and solidified.

R. erythropolis AN-13 was subcultured in 20 ml of growth medium for 3 days at 30°C with shaking, transferred to 400 ml of the medium in a 3-liter flask and cultured for 3 days at 30°C on a reciprocating shaker. Cells were collected by centrifugation at $9000 \times g$ for 20 min, washed twice with 0.1 M K-Na phosphate buffer (pH 7.1) and stored at 5°C. About 1.5 g of wet cells was obtained per 400 ml of medium.

Formation of metabolites from aniline by resting cells. Aniline-grown cells (optical density of 4 at 660 nm, 1.6 mg·dry cell/ml) were incubated with 30 mM aniline in 10 ml of 75 mM K-Na phosphate buffer (pH 7.1) at 30°C for 0.5 to 48 hr with shaking. The incubation mixture was harvested at various times, and cells were removed by centrifugation at $19,000 \times g$ for 20 min. The supernatant was adjusted to pH 2 with 4 N H_2SO_4 and extracted twice with ethyl ether. The combined extract was evaporated to dryness. The residue dissolved in acetone was applied to a TLC (thin-layer chromatography) aluminium sheet (Silica Gel 60F₂₅₄, Merck, Darmstadt), and chromatographed in solvent system A of 20% $\text{KCl}^{18)}$ and solvent system B of isopropyl ether-formic acid (99%)–water (90: 7: 3)¹⁹⁾ for 20 and 40 min, respectively, at room temperature. Metabolite I (Met-I, *cis,cis*-muconic acid), Met-I' (*cis,trans*-muconic acid) and catechol were detected with ultraviolet (UV) light at 254 nm. For Met-II (β -keto adipic acid), 3% SeO_2 was sprayed on, and the spots were detected by heating at 120°C for 20 min.²⁰⁾

Release of ammonia from aniline. Aniline-grown cells (optical density of 4 at 660 nm) were incubated with 7 mM aniline in 5 ml of 75 mM K-Na phosphate buffer (pH 7.1) at 30°C for 1 to 24 hr with shaking. An incubation mixture without aniline was used as a control. These mixtures were harvested at various times, and cells were removed by centrifugation at $19,000 \times g$ for 20 min. We determined ammonia in the supernatant fluid by a modification²¹⁾ of the Conway method and remaining aniline by the method of Thompson²²⁾ using a diazo coupling reaction.

Isolation of Met-I. One gram of aniline-grown wet cells was suspended in 10 ml of 0.1 M K-Na phosphate buffer (pH 7.1) and disrupted with a Kubota 200 M ultrasonic oscillator (Kubota Shoji, Tokyo, Japan) at 180 W for 20 min. The cell-free extract was centrifuged at $19,000 \times g$ for 20 min to remove cell debris and intact cells; the supernatant was put into a 200-ml brown flask containing 100 mg of catechol in 100 ml of 0.1 M K-Na phosphate buffer (pH 7.1). The mixture was incubated at 30°C for 3 hr with slow shaking and then adjusted to pH 2.3 with 4 N H_2SO_4 . Precipitated protein was removed by centrifugation at $9000 \times g$ for 20 min; the supernatant fluid was evaporated below 35°C to 25 ml. This concentrate was saturated with $(\text{NH}_4)_2\text{SO}_4$, readjusted to pH 2.3 and centrifuged again at $9000 \times g$ for 20 min to remove the precipitate. The supernatant was extracted three times with ethyl ether and the extract was then evaporated to dryness. The product was dissolved in ethanol and crystallized by the addition of water and storage at 5°C for 2 days. The crude crystals were recrystallized in ethanol–water, and prismatic, colorless crystals appeared; their dry weight was 8 mg.

Isolation of Met-I'. The metabolites of aniline were prepared by the procedure described under "Formation of metabolites from aniline by resting cells." After they were chromatographed (TLC in solvent system B), Met-I' was visualized with UV light and extracted with acetone from the silica gel. A mass of Met-I' was obtained from Met-I as follows: Crude crystals of Met-I were boiled in water for 10 min; the solution was concentrated with an evaporator until it became turbid; it was kept at 5°C for 2 days; and colorless crystals (6 mg) were obtained.

Isolation of Met-II.²³⁾ To the incubation mixture used for the formation of Met-I was added 0.1 ml of 10 mM MnCl_2 . Incubation was continued at 30°C with slow shaking until the Rothera reaction²⁴⁾ of the mixture was dense pink-violet. After incubation for about 20 hr, the mixture was adjusted to pH 2.3 with 4 N H_2SO_4 and centrifuged at $9000 \times g$ for 20 min to remove precipitated protein; the supernatant fluid was concentrated to 20 ml below 40°C with an evaporator. The concentrate was saturated with $(\text{NH}_4)_2\text{SO}_4$, readjusted to pH 2.3 and centrifuged again at $9000 \times g$ for 20 min. The supernatant was extracted three times with ethyl ether; the extract was evaporated to dryness. The product was dissolved in ethyl acetate and crystallized by the addition of *n*-hexane and storage at 5°C overnight. The crude crystals were recrystallized in ethyl acetate–*n*-hexane, and colorless needles (35 mg) were obtained.

Analytical methods. UV absorption spectra of Met-I and Met-I' were measured with a Hitachi 124 spectrophotometer (Hitachi, Tokyo) in 0.1 N NaOH. Mass spectra of crystalline Met-I, Met-I' and Met-II were analyzed with a Hitachi RMU-6MG mass spectrometer

at an ionization potential of 20 eV and a chamber temperature of 200°C. Samples were injected with a direct inlet system operating from 100 to 200°C. Gas chromatographic and mass spectrometric analyses of modified Met-I, Met-I' and Met-II were carried out with this spectrometer coupled to a gas chromatograph. A glass column packed with 2% OV-1 was used. Met-I and Met-I' were methylated with ethereal diazomethane generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide. Met-II was trimethylsilylated with *N,O*-bis(trimethylsilyl)acetamide in acetonitrile. Infrared (IR) absorption spectra of Met-I, Met-I' and Met-II were measured with a Hitachi EPI-S2 IR spectrophotometer as tablets in KBr.

Enzyme assay. Catechol 1,2-dioxygenase (EC 1.13.11.1) was determined spectrophotometrically by measuring the increase in absorbance at 260 nm.²⁵⁾ One unit of enzyme activity was defined as the amount of enzyme that formed 1 μmol of *cis,cis*-muconic acid per min at 24°C. Specific activity was defined as units per mg of protein. Protein concentrations were determined by the method of Lowry *et al.*²⁶⁾

RESULTS

Oxidation of aniline by resting cells

Aniline-grown resting cells of *R. erythropolis* AN-13 were incubated with aniline for 0.5 to 48 hr, and metabolic intermediates were examined by TLC. Met-I appeared after incubation for 0.5 hr, the densest spot being seen after 14-hr incubation after which it decreased. Met-II, on the other hand, first appeared after 4-hr incubation, showed the maximal density after 24-hr incubation and then decreased. Met-II was also formed from Met-I following incubation with cell-free extract of aniline-grown cells. Met-I, however, was not formed in an incubation mixture containing Met-II and this cell-free extract. These results indicate that Met-I was formed at the early stage of aniline degradation and was converted to Met-II, which was further metabolized. Met-I and Met-II were also detected in growing cultures. A small amount of Met-I' sometimes appeared on the plate with an increase of Met-I. The reason for its appearance will be given below.

Release of ammonia from aniline

Figure 1 shows that aniline was readily

FIG. 1. Release of Ammonia from Aniline by Resting Cells of *R. erythropolis* AN-13.

Aniline was incubated with resting cells at 30°C with shaking. After the cells were removed by centrifugation, ammonia (○—○) released and aniline (□—□) remaining in the supernatant fluid were determined.

degraded by resting cells and disappeared from the mixture after incubation for 10 hr. As aniline decreased, ammonia in the mixture increased. The maximal ammonia accumulation (5.8 $\mu\text{mol/ml}$) was seen after 10-hr incubation, when aniline had disappeared from the mixture. This value corresponded to 83% of the amino group of aniline originally in the incubation mixture. The amount of ammonia in the mixture was constant during incubation from 10 to 24 hr. Most ammonia released from aniline was therefore not incorporated into cells.

Identification of Met-I and Met-I'

Met-I was positive as to the UV spectrum and showed *R_f* values of 0.66 and 0.85 in solvent systems A and B, respectively, on TLC. The maximal UV absorption was seen at 257 nm in 0.1 N NaOH. Its melting point was 194°C. The mass spectrum of Met-I shows that there was a molecular ion at *m/z* 142 (M^+), which agreed with the empirical formula of $\text{C}_6\text{H}_6\text{O}_4$ (Fig. 2). Major fragment ions were at *m/z* 124 ($\text{M}-\text{H}_2\text{O}$) and 97 ($\text{M}-\text{COOH}$). The methyl derivative of Met-I showed one peak on gas chromatography and yielded major ion peaks in the mass spectrum at *m/z* 170 (M^+), 155 ($\text{M}-\text{CH}_3$), 139 ($\text{M}-\text{OCH}_3$) and 111 ($\text{M}-\text{COOCH}_3$).

cis,cis-Muconic acid has been reported to be readily isomerized to *cis,trans*-isomer by

FIG. 2. Mass Spectrum of Met-I.

FIG. 3. TLC Analyses of the Conversion of Met-I to Met-I' by Heating in Solvent Systems A (a) and B (b).

Met-I' was prepared by extraction from silica gel, after separation from other aniline metabolites by TLC. See the text for details. 1, Met-I; 2, heated Met-I; 3, Met-I'; 4, heated Met-I'; 5, *trans, trans*-muonic acid (authentic); and 6, heated *trans, trans*-muonic acid.

heating.²⁷⁾ The conversion from Met-I (*cis, cis*-form) to Met-I' (*cis, trans*-form) by heating was examined by TLC in this study. Figure 3 shows that Met-I and Met-I' had distinct *R_f* values from each other on TLC in solvent systems A and B. Although the *R_f* values of Met-I' were not changed by heating, the values of heated Met-I were low and agreed with those of Met-I'. The *R_f* values of authentic *trans, trans*-muonic acid (Nakarai Chem., Kyoto, Japan) were unchanged by heating, and differed from that of Met-I' in solvent system A and that of Met-I in solvent system B, as shown in Fig. 3. These results suggest that the small amount of Met-I' found in the incubation mixture was not a metabolic product of aniline, but was derived nonbiologically from Met-I during the preparation for metabolites.²⁸⁾

Met-I showed a characteristic IR absorption spectrum different from that of Met-I': Met-I had an absorption band at 1590 cm^{-1} for $-\text{HC}=\text{CH}-\text{HC}=\text{CH}-$, which is a symmetrical and conjugated diene derived from the *cis, cis*-form, and Met-I' ones at 1630 and 1600 cm^{-1} on the basis of an asymmetrical diene of the *cis, trans*-form. An absorption band was also seen at 1690 cm^{-1} for $-\text{COOH}$ in Met-I and Met-I'. Further, Met-I showed absorption at 1250 and 830 cm^{-1} , and Met-I' at 890 and 750 cm^{-1} , as described by Elvidge *et al.*²⁷⁾

The melting point of Met-I' was $189\text{--}190^\circ\text{C}$. Its maximal UV absorption was at 260 nm in 0.1 N NaOH. The mass spectra of Met-I' and its methyl derivative showed the same mass patterns as those of Met-I and its derivative, respectively. We thus identified Met-I and Met-I' as *cis, cis*- and *cis, trans*-muonic acid, respectively.

Identification of Met-II

Met-II had *R_f* values of 0.44 and 0.42 in solvent systems A and B, respectively, on TLC. Its melting point was $120\text{--}121^\circ\text{C}$ which is identical to that of authentic β -keto adipic acid (Sigma, Saint Louis). In the mass spectrum of Met-II, a molecular ion was not detected (Fig. 4). Major fragment ions were at m/z 142 ($\text{M}-\text{H}_2\text{O}$), 116 ($\text{M}-\text{COO}$), 101 ($\text{M}-\text{CH}_2\text{COOH}$), 73 ($\text{M}-\text{COCH}_2\text{COOH}$) and 56 ($\text{M}-\text{CH}_2\text{COOH}-\text{COOH}$). The trimethylsilylated derivative of Met-II showed one peak on gas chromatography. A molecular ion appeared at m/z 376, which shows that three trimethylsilyl residues were introduced into the Met-II molecule, and that this compound was reversibly interconverted from the keto form to the enol. The IR absorption spectrum of

FIG. 4. Mass Spectrum of Met-II.

Met-II was identical with that of the authentic β -keto adipic acid. These results show that Met-II was β -keto adipic acid.

Catechol 1,2-dioxygenase

The cell-free extract of aniline-grown cells of *R. erythropolis* AN-13 had a catechol 1,2-dioxygenase activity (0.14 units/mg $\cdot$ protein). On the other hand, cells grown in nutrient broth without aniline did not exhibit this enzymatic activity. When cells grown in nutrient broth were transferred to aniline medium, they formed the dioxygenase. These results suggest that catechol is one of the metabolic intermediates in the aniline degradation by *R. erythropolis* AN-13.

DISCUSSION

In this report we first propose a metabolic pathway for mineralization of aniline by a microbial culture (Fig. 5). *R. erythropolis* AN-13 converts aniline to catechol with the release of ammonia; catechol is attacked with two atoms of oxygen and converted to *cis,cis*-muconic acid; and this is mineralized through the β -keto adipic acid pathway to inorganic end products. The proposed pathway for aniline metabolism by *R. erythropolis* AN-13 is similar to that for 3,4-dichloroaniline metabolism by a *Pseudomonas putida* strain which is supposed to degrade it through 4,5-dichlorocatechol, 3,4-dichloromuconate, 3-chlorobutenolide and 3-chloro-4-keto adipate to succinate and acetate.⁸⁾

cis,cis-Muconic acid, β -keto adipic acid and ammonia were isolated and confirmed as metabolites in aniline degradation. Catechol, however, was not isolated from growing cul-

tures of *R. erythropolis* AN-13 or incubation mixtures containing aniline and resting cells. You and Bartha⁸⁾ could not isolate 4,5-dichlorocatechol in the 3,4-dichloroaniline degradation by the *P. putida* strain, although they isolated some other metabolites. In *R. erythropolis* AN-13, the cell-free extract of aniline-grown cells had a strong catechol 1,2-dioxygenase activity that catalyzed the formation of *cis,cis*-muconic acid from catechol. Catechol also seems to be one of the metabolites in aniline degradation. Bachofer *et al.*¹⁵⁾ observed the accumulation of catechol in aniline metabolism by a *Nocardia* mutant. In our strain, however, catechol, once formed from aniline, was presumably metabolized rapidly to *cis,cis*-muconic acid without accumulation.

In the liver of rabbits²⁰⁾ and in human erythrocytes,^{30,31)} aniline was reported to be oxidized to *p*-aminophenol. Sloane *et al.*³²⁾ reported that a *Mycobacterium smegmatis* strain oxidizes aniline to *p*-aminophenol. Citrate and manganese ions increase the ability of washed cells to hydroxylate aniline.³³⁾ But *R. erythropolis* AN-13 did not produce *p*-aminophenol from aniline in growing cultures nor did its resting cells. This strain did not form *o*-aminophenol from aniline. Further, resting cells of the aniline-grown strain did not take up a significant amount of oxygen in the presence of these aminophenols (Aoki *et al.*, unpublished data). These results show that *R. erythropolis* AN-13 converted aniline to catechol without the formation of *p*- or *o*-aminophenol as an intermediate.

Reiner and Hegeman³⁴⁾ isolated (–)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid as an intermediate in the formation of catechol from benzoic acid by a mutant of *Alcaligenes eutrophus*. Reiner³⁵⁾ showed that oxygen atoms of the two hydroxyl groups in the compound are derived from molecular oxygen. A metabolic intermediate may likewise be present in the formation of catechol from aniline by *R. erythropolis* AN-13. Additional studies are necessary to determine the intermediate by using a mutant of this strain.

R. erythropolis AN-13 used in this experi-

FIG. 5. Proposed Pathway for the Biodegradation of Aniline by *R. erythropolis* AN-13.

I, aniline; II, catechol; III, *cis,cis*-muconic acid; IV, (+)-muconolactone; and V, β -keto adipic acid.

ment grew well on aniline as a sole source of carbon and nitrogen, although aniline is a nonbiological, synthetic aromatic amine. This strain degraded aniline to catechol (presumably) and ammonia at an early stage of biodegradation, as already described. Because catechol is a common intermediate in the metabolism of tryptophan and many aromatic compounds, the organism could readily use it as a carbon source with released ammonia as the nitrogen source. *R. erythropolis* AN-13, thus, is able to grow on aniline as the sole and nitrogen source carbon.

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