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Studies of the Catabolic Pathway of Degradation of Nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45: Removal of the Amino Group from 2-Aminomuconic Semialdehyde

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***Pseudomonas pseudoalcaligenes* JS45 utilizes nitrobenzene as the sole source of nitrogen, carbon, and energy. Previous studies have shown that degradation of nitrobenzene involves the reduction of nitrobenzene to nitrosobenzene and hydroxylaminobenzene, followed by rearrangement to 2-aminophenol, which then undergoes *meta* ring cleavage to 2-aminomuconic semialdehyde. In the present paper, we report the enzymatic reactions responsible for the release of ammonia after ring cleavage. 2-Aminomuconic semialdehyde was oxidized to 2-aminomuconate in the presence of NAD by enzymes in crude extracts. 2-Aminomuconate was subsequently deaminated stoichiometrically to 4-oxalocrotonic acid. No cofactors are required for the deamination. Two enzymes, 2-aminomuconic semialdehyde dehydrogenase and a novel 2-aminomuconate deaminase, distinguished by partial purification of the crude extracts, catalyzed the two reactions. 4-Oxalocrotonic acid was further degraded to pyruvate and acetaldehyde. The key enzyme, 2-aminomuconate deaminase, catalyzed the hydrolytic deamination that released ammonia, which served as the nitrogen source for growth of the organism.**

Nitrobenzene is one of the top 50 industrial chemicals produced in the United States; over 1.6 billion pounds was produced in 1995 (26). Because of its toxicity, nitrobenzene is listed as a priority pollutant by the U.S. Environmental Protection Agency (9). Degradation of nitrobenzene has been detected in various waste streams and sludges (3, 18, 19) and in soil enrichment cultures (4, 13). Three different pathways for the catabolism of nitrobenzene have recently been identified (2, 14, 15). One pathway, described for mixed cultures, involved the anaerobic reduction of nitrobenzene to aniline (2). Subsequent transfer of the culture fluid to an aerobic reactor allows the aerobic degradation of aniline, probably by a pathway involving the dioxygenase-catalyzed removal of the amino group (24). The second pathway, found in a *Comamonas* sp., proceeds via an initial dioxygenation to a nitrohydrodiol, which spontaneously decomposes to catechol accompanied by the release of nitrite (15). The third pathway (14), which seems to be more common among isolates from contaminated groundwater, is initiated with the partial reduction of nitrobenzene, via nitrosobenzene, to hydroxylaminobenzene, which is enzymatically rearranged to 2-aminophenol. Ring cleavage of 2-aminophenol yields 2-aminomuconic semialdehyde. The reduction pathway has been studied in *Pseudomonas pseudoalcaligenes* JS45 (14), and the nitroreductase (23) and 2-aminophenol 1,6-dioxygenase (12) have been purified and characterized. However, the mechanism of the subsequent metabolism of 2-aminomuconic semialdehyde and the release of ammonia is unknown. The process is known to require NAD, but it is not clear whether NAD is required for oxidation of the aldehyde before the removal of the amino group (14). We report here the details of the reactions that convert 2-aminomuconic semialdehyde to intermediates of the catechol *meta* cleavage pathway.

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MATERIALS AND METHODS

Bacteria and growth conditions. *P. pseudoalcaligenes* JS45 was maintained on an agar-solidified, nitrogen-free medium (BLKN) (1) with nitrobenzene provided in the vapor phase via a tube (5 by 60 mm) plugged with cotton and placed in the lid of the petri plate (14). For growth in liquid medium, 1 liter of BLKN containing nitrobenzene (2.5 mM) was inoculated with strain JS45 and then incubated with shaking (250 rpm) at 30°C until the A_{600} reached about 0.24. Cells were harvested by centrifugation.

Preparation of crude extracts. Cells were suspended in 4 ml of phosphate buffer (50 mM, pH 8.0) in the presence of ascorbate (5 mM) and $FeSO_4$ (0.05 mM) and were broken by two passages through a French pressure cell at 20,000 lb/in^2. The resulting suspension was centrifuged at 100,000 × *g* for 60 min, and the pellet was discarded. The supernatant fluid was stored at −70°C until use.

Enzyme assays. Because 2-aminomuconic semialdehyde is unstable and spontaneously transforms to picolinic acid (7, 14, 16), coupled assays for 2-aminomuconic semialdehyde dehydrogenase in the presence of NAD were started with 2-aminophenol, which is converted to the semialdehyde by 2-aminophenol 1,6-dioxygenase present in crude extracts. 2-Aminophenol 1,6-dioxygenase activity was estimated by monitoring the initial increase in A_{380} (12, 14). 2-Aminomuconic semialdehyde dehydrogenase activity was monitored by the subsequent decrease in A_{380} and the increase in A_{326}. In some assays, the activity of 2-aminomuconate semialdehyde dehydrogenase was measured by using 2-hydroxymuconic semialdehyde (E_{375} = 44 $mM^{-1} \cdot cm^{-1}$) as the substrate because of the instability of 2-aminomuconic semialdehyde (7, 16). 2-Aminomuconate deaminase activity was measured by the decrease in A_{326} (E_{326} = 16.5 $mM^{-1} \cdot cm^{-1}$) (7).

Isolation of 2-aminomuconate. A reaction mixture (16 ml) containing crude extract (0.038 mg of protein/ml) and NAD (0.1 mM) was incubated for 6 min with 2-aminophenol (0.1 mM). The reaction was stopped by the addition of KOH (final concentration of 0.1 M). The pH was adjusted to 9.5 by addition of 55 ml of phosphate buffer (50 mM, pH 7.0), and the solution was applied to a Q-Sepharose (Pharmacia, Uppsala, Sweden) column (1.5 by 6 cm) preequilibrated with phosphate buffer (25 mM, pH 8.0). 2-Aminomuconate was eluted with a stepwise gradient of 0 to 0.2 M NaCl in phosphate buffer (25 mM, pH 8.0) (NaCl concentrations and volumes were as follows: 0.0 M, 10 ml; 0.05 M, 10 ml; 0.10 M, 20 ml; 0.15 M, 30 ml; 0.18 M, 50 ml; and 0.2 M, 20 ml).

Isolation of 4-oxalocrotonic acid. A reaction mixture containing 2-aminophenol (0.13 mM), NAD (0.13 mM), crude extract (35 μg of protein/ml), and EDTA (10 mM) in 1.5 liters of phosphate buffer (50 mM, pH 8.0) was incubated at room temperature for 60 min. The reaction was terminated by addition of 40 ml of HCl (4 M). A white crystalline precipitate that formed on standing was removed by centrifugation, and the acidified supernatant was extracted with ethyl acetate. The ethyl acetate was removed by flash evaporation, and the residue was dried overnight in a desiccator with anhydrous calcium sulfate. The residue was dissolved in 10 ml of anhydrous ethanol, and the insoluble material was removed by centrifugation. Concentration of the solution yielded 11 mg of a light yellow precipitate subsequently identified as 4-oxalocrotonic acid.

Separation of the activities of 2-aminomuconic semialdehyde dehydrogenase and 2-aminomuconate deaminase. A Hitrap Q 5-ml column (Pharmacia) was equilibrated with phosphate buffer (50 mM, pH 7.0), loaded with 15 ml of crude extracts (2.7 g of protein/ml), and washed with 20 ml of phosphate buffer. Proteins were eluted with a 60-ml linear gradient of 0 to 0.5 M NaCl in 50 mM phosphate buffer. Fractions (2.0 ml) were collected and assayed for enzyme activities and protein concentration.

Analytical methods. Spectrophotometric analyses were performed on a Cary 3E UV-visible spectrophotometer (Varian Associates, Sunnyvale, Calif.). Infrared spectra of solid 4-oxalocrotonic acid in KBr pellets were obtained with a Magna IR spectrometer 750 (Nicolet Instrument, Madison, Wis.). Ammonia release was measured by a standard method (17). Acetaldehyde was determined by measuring the decrease in A_{340} concomitant with NADH oxidation in the presence of alcohol dehydrogenase. Pyruvate was estimated by a similar method with excess lactate dehydrogenase (22). Thin-layer chromatography of 2,4-dinitrophenylhydrazones was carried out on precoated cellulose plates (LK2F linear cellulose plates; Whatman, Clifton, N.J.) with two solvent systems: solvent A, *n*-butanol–ethanol–0.5 M NH_4OH (7:1:2), and solvent B, isopropanol–H_2O–0.5 M NH_4OH (20:2:1). No detection reagent was needed for the bright yellow derivatives of pyruvate and 4-oxalocrotonic acid with 2,4-dinitrophenylhydrazine (21).

Chemicals. 4-Oxalocrotonic acid was prepared by the method of Lapworth (11) from the potassium salt of diethyl 2,4-hexadiene-5-hydroxy-1,6-dioate, which was obtained from condensation of diethyl oxalate and ethyl crotonate in the presence of potassium metal in toluene, as described by Wiley and Hart (30). NADH was from Boehringer Mannheim (Indianapolis, Ind.). All other chemicals were from Sigma (St. Louis, Mo.) or Aldrich (Milwaukee, Wis.).

RESULTS

Spectral changes during metabolism of 2-aminophenol by crude extracts. When crude extracts were mixed with a solution of 2-aminophenol in phosphate buffer (50 mM, pH 8.0), a maximal absorbance corresponding to 2-aminomuconic semialdehyde appeared rapidly at 380 nm. The subsequent slow disappearance of the A_{380} was accompanied by the appearance of a maximum absorbance at 264 nm. The absorbance at 264 nm was due to picolinic acid, which forms spontaneously from 2-aminomuconic semialdehyde, as described previously (7, 12, 14, 20). When NAD was included in the reaction mixture, picolinic acid was not formed. Instead, a maximum absorbance at 330 to 335 nm appeared and then disappeared slowly. When the experiment was repeated in the presence of lactate dehydrogenase and pyruvate, the maximum absorbance was at 326 rather than 335 nm (Fig. 1). The highest A_{326} was 60% of the A_{335} obtained in the absence of lactate dehydrogenase and pyruvate. These observations suggested that the absorbance at 335 nm was due to the formation of NADH and an unknown compound with a maximum absorbance at 326 nm.

Isolation and identification of 2-aminomuconate. The results described above were confirmed by the isolation of the unknown compound with a Q-Sepharose column. NADH (absorbance peaks at 264 nm and 340 nm) was eluted with 0.15 M NaCl, and a compound with only one maximum absorbance peak at 326 nm was eluted with 0.18 M NaCl. The absorbance at 326 nm of the 0.18 M NaCl fraction did not decrease when lactate dehydrogenase and pyruvate were added. When the latter preparation was incubated with crude extracts of JS45, the release of ammonia was detected, indicating that the amino group was still associated with the compound. The UV-visible spectrum of the compound was identical to that reported for 2-aminomuconic acid (7). At pH 13 the maximal absorbance of the compound shifted to 333 nm, and the process was reversible, as previously described (7). However, at pH 1 the maximal absorbance shifted slowly to 305 nm. After acidification, the absorption maximum did not shift back to 326 or 333 nm when the pH was adjusted to 7 or higher. Instead, the maximum appeared at 295 nm under neutral conditions and at 350 nm at an alkaline pH (0.1 M KOH). The spectral changes after acidification were exactly the same as those of synthetic 4-oxalocrotonic acid (2-hydroxymuconic acid). The observation is consistent with the spontaneous conversion of 2-aminomuconic acid to 2-hydroxymuconic acid by acidification (7). The observations of release of ammonia and formation of 2-hydroxymuconic acid confirmed that the unknown compound was 2-aminomuconic acid (hereafter referred to 2-aminomuconate due to its instability under acidic conditions).

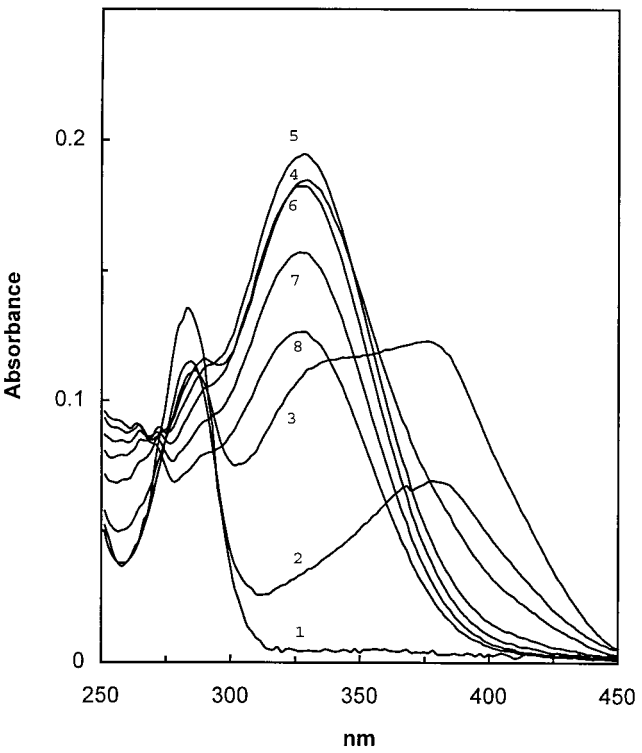

FIG. 1. Spectral changes associated with the metabolism of 2-aminophenol. 2-Aminophenol (0.05 mM) was incubated with crude extracts of JS45 (0.038 mg of protein/ml) in the presence of NAD (0.05 mM), lactate dehydrogenase (0.4 U/ml), and pyruvate (0.4 mM) in phosphate buffer (50 mM, pH 8.0). Recordings were taken at time zero (curve 1), 10 s (curve 2), 1 min (curve 3), 3 min (curve 4), 5 min (curve 5), 7 min (curve 6), 10 min (curve 7), and 14 min (curve 8).

Enzymatic deamination of 2-aminomuconate. When the 2-aminomuconate preparation was incubated with crude extracts of JS45, 2-aminomuconate disappeared with concomitant release of ammonia (Fig. 2). No cofactors were required. Heat-treated (60°C for 5 min) crude extracts did not catalyze the conversion. These observations indicated that an enzymatic reaction was responsible for the removal of the amino group from 2-aminomuconate.

Isolation and identification of 4-oxalocrotonic acid, the product of enzymatic deamination. Crude extracts were used to accumulate large quantities of the product of the deamination reaction. The freshly prepared solution of the product in phosphate buffer (25 mM, pH 7.3) initially absorbed strongly at 295 nm, but the peak subsequently decreased and a new peak appeared at 237 nm. The spectral changes are identical to those of authentic 4-oxalocrotonate. The enol form (maximal absorbance at 295 nm) isomerizes to the keto form (maximal absorbance at 237 nm) in aqueous solution (6, 22, 29). The maximal absorbance of the enzymatic product shifted reversibly to 305 nm at pH 1 and to 350 nm at pH 13; the behavior was identical to that of authentic 4-oxalocrotonic acid. The identity of the isolated compound was further confirmed by comparison of its infrared spectrum with that of authentic 4-oxalocrotonic acid.

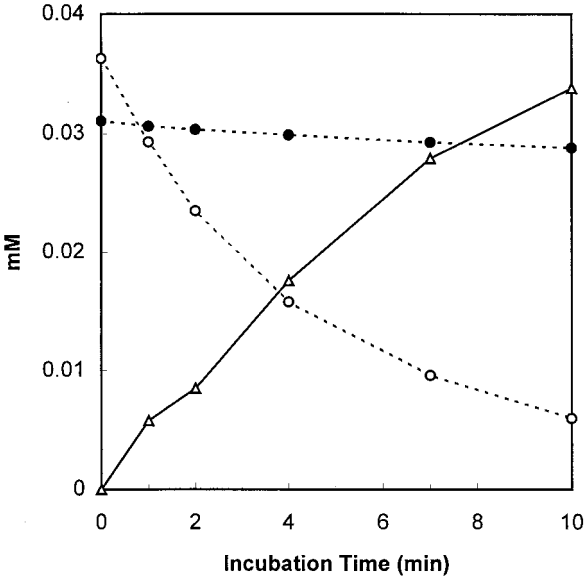

FIG. 2. Deamination of 2-aminomuconate. 2-Aminomuconate (0.036 mM) was incubated with crude extracts (0.026 mg of protein/ml) in phosphate buffer (50 mM, pH 8.0, 0.15 M NaCl). ○, 2-aminomuconate; △, ammonia; ●, 2-aminomuconate in the control without crude extract.

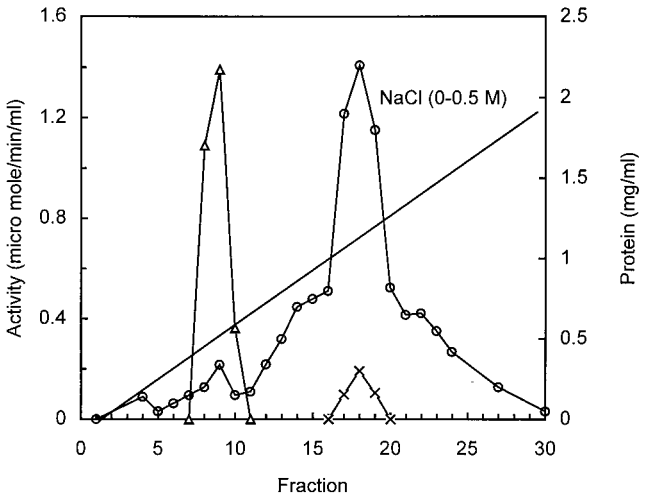

FIG. 3. Hitrap Q separation of 2-aminomuconic semialdehyde dehydrogenase and 2-aminomuconate deaminase activities in crude extracts. ×, 2-aminomuconic semialdehyde dehydrogenase; △, 2-aminomuconate deaminase (measured with 2-hydroxymuconase semialdehyde as the substrate); ○, protein.

Degradation of 4-oxalocrotonic acid to pyruvate and acetaldehyde. Enzymes in crude extracts of *P. pseudoalcaligenes* JS45 catalyzed the conversion of 4-oxalocrotonic acid to products which did not absorb in the UV region. When NADH was subsequently added to the reaction mixture, the A_{340} did not decrease significantly. However, the absorbance of NADH at 340 nm decreased rapidly upon the addition of alcohol dehydrogenase and again upon the addition of lactate dehydrogenase. These observations suggested that acetaldehyde and pyruvate were formed from 4-oxalocrotonic acid (22). The identity of pyruvate was further confirmed by thin-layer chromatography. The 2,4-dinitrophenylhydrazone of the product of the reactions gave bright yellow spots with R_f values of 0.53 in solvent A and 0.50 in solvent B. The chromatographic behavior was identical to that of a pyruvate 2,4-dinitrophenylhydrazone standard. When 4-oxalocrotonic acid (0.26 mM) was incubated with crude extracts (0.15 mg of protein/ml) in the presence of 1 mM $MgCl_2$ for 3 h, pyruvate (0.1 mM) and acetaldehyde (0.14 mM) were recovered.

Separation of the activities of 2-aminomuconic semialdehyde dehydrogenase and 2-aminomuconate deaminase. The two enzyme activities were well separated with a Hitrap Q column (Fig. 3). In the presence of NAD (0.1 mM), enzymes (0.05 mg of protein/ml) in fraction 18 catalyzed the stoichiometric transformation of 2-aminophenol (0.028 mM) to 2-aminomuconate (0.025 mM) with the production of 0.026 mM NADH, which indicated that 2-aminomuconic semialdehyde dehydrogenase coeluted with 2-aminophenol 1,6-dioxygenase. The activity of 2-aminomuconic semialdehyde dehydrogenase was much higher than that of 2-aminophenol 1,6-dioxygenase either in crude extracts or in fraction 18, because the amount of 2-aminomuconic semialdehyde reached a maximum in 1 min and no accumulation of picolinic acid, the spontaneous product of 2-aminomuconate semialdehyde, was observed under the assay conditions used. The fast enzymatic conversion of 2-aminomuconic semialdehyde is a good strategy for the cells of *P. pseudoalcaligenes* JS45 to prevent the formation of picolinic acid. However, it was not possible to determine the specific activity of 2-aminomuconic semialdehyde dehydrogenase in the coupled assay.

When fraction 9 was incubated with 2-aminomuconate, the decrease in absorbance at 326 nm was concomitant with a shift of the absorbance to 295 nm and with the appearance of a peak at 237 nm (Fig. 4). The spectral changes clearly indicated that 2-aminomuconate was transformed to 4-oxalocrotonic acid by

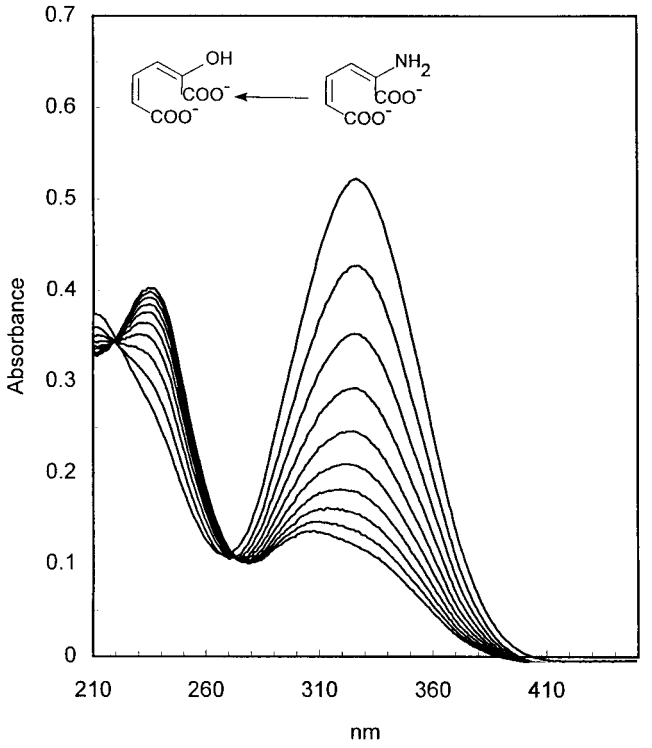

FIG. 4. Spectral change during deamination of 2-aminomuconate. Reactions were catalyzed by partially purified 2-aminomuconate deaminase (0.001 mg of protein/ml). Recordings were taken at 1-min intervals from top to bottom at 326 nm.

2-aminomuconate deaminase in fraction 9. Stoichiometric conversion of 2-aminomuconate (0.033 mM) to 4-oxalocrotonic acid (0.032 mM) and ammonia (0.030 mM) was observed when 2-aminomuconate was incubated with fraction 9. The specific activity of 2-aminomuconate deaminase was 5.4 μmol · min^{-1} · mg of protein^{-1} in fraction 9, versus 0.2 μmol · min^{-1} · mg of protein^{-1} in crude extracts.

DISCUSSION

Previous experiments (14) indicated that 2-aminomuconic semialdehyde is degraded by crude extracts of *P. pseudoalcaligenes* JS45 in the presence of NAD. However, the intermediate(s) was not identified. In the present work, isolation of the intermediate 2-aminomuconate indicated that NAD was used to oxidize the aldehyde moiety of 2-aminomuconic semialdehyde prior to deamination. The enzymatic transformation of 2-aminomuconic semialdehyde to 2-aminomuconate in the presence of NAD has been previously observed only in cat and rat liver, but 2-aminomuconate was only tentatively identified in the reaction mixture (7). Because 2-aminomuconic semialdehyde was extremely unstable and cyclized to picolinic acid, Ichiyama et al. (7) used 2-hydroxymuconic semialdehyde as a substitute substrate for investigating the properties of 2-aminomuconate semialdehyde dehydrogenase. 2-Hydroxymuconate semialdehyde dehydrogenase is an enzyme in the catechol *meta* cleavage pathway in several microorganisms (5, 6, 8, 10, 22). We are currently investigating whether and how the 2-aminomuconic semialdehyde dehydrogenase from *P. pseudoalcaligenes* JS45 is related to the 2-hydroxymuconic semialdehyde dehydrogenase of the catechol *meta* cleavage pathway.

There are four known general mechanisms for the elimination of amino groups: (i) transamination, (ii) hydrolysis, (iii) deamination without the participation of H_2O by ammonia-lyases, and (iv) oxidative deamination (28). The last mechanism, catalyzed by amino acid dehydrogenases or oxidases, requires the participation of NAD(P), FAD, or other acceptors. It was previously proposed that 2-aminomuconate is reductively deaminated to 2-ketoadipate in cat liver cells in the presence of NADH or NADPH by 2-aminomuconate reductase during the metabolism of tryptophan (7, 16). The amino group appeared to have been removed by hydrolysis, and a double bond of 2-aminomuconate was reduced. The details of the mechanism were not recorded. In a recent textbook of biochemistry, however, two enzymes, a hydratase and a dehydrogenase, were proposed to be involved in the transformation of 2-aminomuconate to 2-ketoadipate in the pathway of tryptophan degradation (27). In contrast, 2-aminomuconate was deaminated to 2-hydroxymuconic acid (4-oxalocrotonic acid) by crude extracts of JS45 in the absence of added cofactors, which clearly indicated that the reaction is hydrolytic. We designated the enzyme 2-aminomuconate deaminase (systematic name, 2-aminomuconate aminohydrolase). The structure of 2-aminomuconate is not similar to those of the substrates for any of the five sub-subclasses of hydrolases acting on carbon-nitrogen bonds listed in *Enzyme Nomenclature* (28) (EC 3.5.x.). To our knowledge, *trans*-4-amino-6-carboxy-2-oxo-hexa-3,5-dienoate, an intermediate in bacterial metabolism of 5-aminosalicylic acid, is the only similar metabolic intermediate previously described (25). That compound was deaminated to fumarylpyruvate by hydrolysis. These two hydrolytic enzymes appear to represent a new sub-subclass acting on carbon-nitrogen bonds in unsaturated linear amino acids. The characterization of 2-aminomuconate deaminase is under way.

Based on our results, we propose three final steps in the pathway for degradation of nitrobenzene by *P. pseudoalcaligenes* JS45 (Fig. 5). 2-Aminomuconic semialdehyde is oxidized in an NAD-dependent dehydrogenation to 2-aminomuconate, which is hydrolytically deaminated to 4-oxalocrotonic acid. 4-Oxalocrotonic acid slowly isomerizes spontaneously to three forms (6, 22, 29). Preliminary experiments indicate that enzymes in crude extracts seem to enhance the process. Then, 4-oxalocrotonic acid is further degraded to pyruvate and acetaldehyde as in the catechol *meta* cleavage pathway (5, 6, 10, 22). The participation of the two unusual dehydrogenase and deaminase enzymes in the reductive pathway, taken together with the participation of the unusual nitroreductase (23), mutase (14), and dioxygenase (12), raises intriguing questions about the origin and evolution of the pathway. Cloning and sequencing of the genes, currently in progress, will allow a more complete understanding of the source of the genes. Under aerobic conditions, the reductive pathway and the oxidative pathway of biodegradation of nitrobenzene produce a common intermediate, 4-oxalocrotonic acid. The oxidative pathway is simple and involves an initial dioxygenase reaction and subsequent metabolism of catechol by a common *meta* cleavage (15). However, the pathway requires 2 mol of molecular oxygen to produce 4-oxalocrotonic acid. If nitrobenzene serves as the nitrogen source for growth, the reduction of nitrite to ammonia would require 3 mol of NAD(P)H. In contrast, the more complex and unusual reductive pathway requires only 1

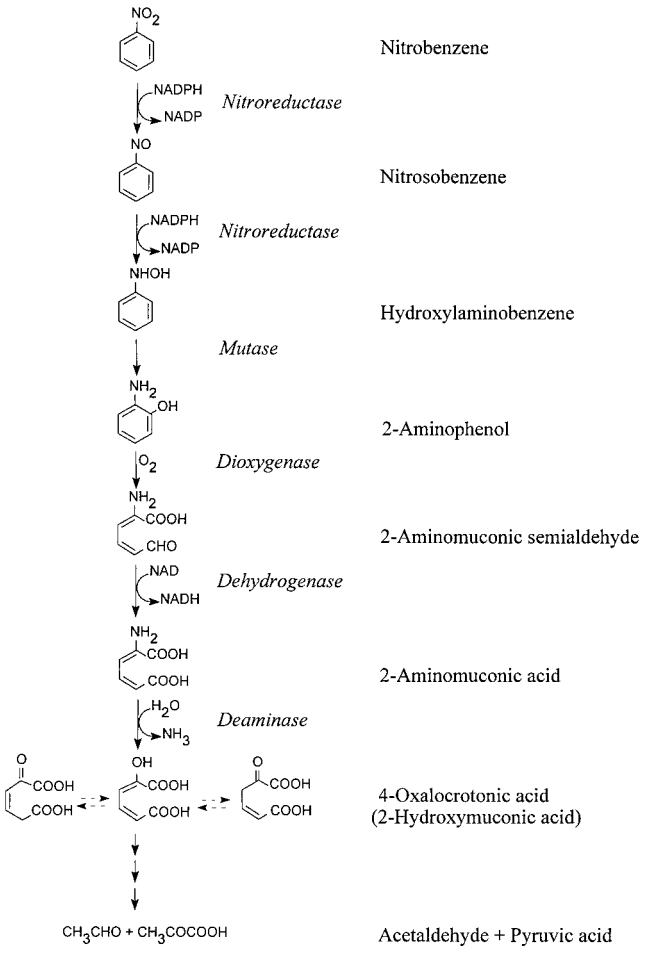

FIG. 5. Proposed pathway of biodegradation of nitrobenzene by *P. pseudoalcaligenes* JS45.

mol of oxygen and 1 mol of reducing equivalents to convert nitrobenzene to 4-oxalocrotonic acid and ammonia (Fig. 5). The selective advantages of the reductive pathway in an ecosystem where nitrobenzene is the sole source of carbon, nitrogen, and energy and where oxygen is limiting, as it is in contaminated aquifers, are obvious. The advantages of the strategy would explain why strains isolated from a variety of contaminated subsurface sites use the reductive pathway (15).

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