Bacterial metabolism of 5-aminosalicylic acid: enzymic conversion to L-malate, pyruvate and ammonia

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5-Aminosalicylate (5AS) was converted to L-malate, pyruvate and ammonia by cell-free extracts from *Pseudomonas* sp. BN9 in the presence of glutathione. In the absence of glutathione, 5AS was oxidized to the ring-fission product *cis*-4-amino-6-carboxy-2-oxo-hexa-3,5-dienoate (*cis*-ACOHDA). Glutathione catalysed the spontaneous isomerization of *cis*-ACOHDA to its *trans*-isomer. The same reaction was catalysed by light and by acidic conditions. *trans*-ACOHDA was enzymically deaminated to fumarylpyruvate (*trans*-2,4-dioxo-5-hexenoate). The *trans*-ACOHDA hydrolase was induced after growth of *Pseudomonas* sp. BN9 with 5AS, but not after growth with acetate or nutrient broth. At the fumarylpyruvate stage, the metabolism of 5AS converged with a pathway described for the degradation of gentisate. Fumarylpyruvate was cleaved by *Pseudomonas* sp. BN9 to fumarate and pyruvate.

Introduction

5-Aminosalicylate (5AS) was identified as an intermediate in the degradation of 6-aminonaphthalene-2-sulphonic acid (6A2NS) and related azo dyes by a mixed bacterial community (Nörtemann *et al.*, 1986; Haug *et al.*, 1991). Two bacterial strains participated in the total degradation of 6A2NS. *Pseudomonas* sp. BN6 converted 6A2NS into an equimolar amount of 5AS and utilized only the pyruvate released from the breakdown of the sulphonated aromatic ring. 5AS was degraded after interspecies metabolite transfer by *Pseudomonas* sp. BN9. In contrast with the generally known degradative pathways for aromatic compounds, 5AS was subject to direct ring fission without the intermediate formation of a dihydroxylated aromatic compound, being cleaved by a dioxygenase in *Pseudomonas* sp. BN9 to *cis*-4-amino-6-carboxy-2-oxo-hexa-3,5-dienoate (Stolz *et al.*, 1992). The further metabolism of *cis*-4-amino-6-carboxy-2-oxo-hexa-3,5-dienoate is described here.

Methods

Bacterial strains and media. *Pseudomonas* sp. BN9 has been isolated and described by Nörtemann *et al.* (1986). *Pseudomonas acidovorans*

DSM 50053 was kindly provided by Dr M. Schlömann, Stuttgart. Bacteria were cultivated in a mineral medium which contained, per litre: 6 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1 g KH_2PO_4 , 1 g $(\text{NH}_4)_2\text{SO}_4$, 50 mg $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 200 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg $\text{Fe}(\text{III})/\text{NH}_4/\text{citrate}$ and 0.1 ml of a trace element solution described by Pfennig & Lippert (1966), without iron salt and EDTA. The medium was supplemented by a vitamin solution described by Sharak-Genthner *et al.* (1981) and 5AS (4 mM) was added as the sole carbon and energy source. *Pseudomonas acidovorans* was grown in the same mineral medium as used for strain BN9, but with gentisate (2 mM) as the carbon source. For some experiments bacteria were grown in nutrient broth (Difco).

Analytical methods. 5-Aminosalicylate (5AS) and its metabolites were analysed by ion-pair reverse-phase HPLC (HPLC data and chromatography control station model 840, equipped with a programmable multi-wavelength detector model 490; Waters Associates). A reverse-phase column (125 mm $\times$ 4.6 mm i.d.; Bischoff), packed with 5 μm particles of LiChrospher 100 RP-8 (Merck), was used. The solvent system was 90% water/10% methanol (v/v) and tetra-*n*-butylammonium phosphate as the ion-pair reagent (low-UV PIC A; Waters). The usual flow-rate was 0.8 ml min⁻¹. The separated compounds were detected photometrically by simultaneous measurement at 210 and 350 nm.

Ammonia was determined using glutamate dehydrogenase according to Da Fonseca-Wollheim *et al.* (1974). Cell-free extracts were dialysed for 3 h against a 1000-fold volume of sodium/potassium phosphate buffer (25 mM, pH 7.4, 4 °C) before the release of ammonia from 4-amino-6-carboxy-2-oxo-hexa-3,5-dienoate (ACOHDA) was assayed. This treatment removed ammonia from the cell-free extracts without affecting the relevant enzyme activities.

Molar absorption coefficient of *trans*-ACOHDA. Different amounts of *trans*-ACOHDA were dissolved in 1 ml Tris/HCl buffer, pH 8.0, and 20 μl cell-free extract was added (2.8 mg protein ml⁻¹). The decrease in A_{350} was determined spectrophotometrically. After the reaction had stopped, 30 μl 6 mM-NADH was added. Further spectrophotometric measurements were at 340 nm. Lactate dehydrogenase (10 μl of an

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Abbreviations: ACOHDA, 4-amino-6-carboxy-2-oxo-hexa-3,5-dienoate; 6A2NS, 6-aminonaphthalene-2-sulphonate; 5AS, 5-aminosalicylate.

aqueous solution 10 mg ml⁻¹; Boehringer) was added and lactate-dehydrogenase-dependent NADH oxidation determined. The amount of pyruvate formed from *trans*-ACOHDA was calculated from the difference in pyruvate concentration before and after the conversion of *trans*-ACOHDA by cell-free extracts. No pyruvate formation was detected in a control experiment containing only cell-free extract without the addition of *trans*-ACOHDA.

Preparation of cell-free extracts. Cells suspended in 50 mM-Tris/HCl buffer, pH 8.0, were disrupted with a French press (Aminco, SLM Instruments) at 1.1×10^8 Pa. Cells and cell debris were removed by centrifugation at 100 000 g for 1 h at 4 °C.

Protein estimation and enzyme assays. The protein content of cell-free extracts was determined by the method of Bradford (1976), using bovine serum albumin as a standard. One unit of enzyme activity was defined as the conversion of 1 µmol substrate min⁻¹. For fumarylpyruvate hydrolase, the cuvettes contained 0.1 µmol fumarylpyruvate in 1 ml 100 mM-Tris/HCl, pH 8.0. The enzyme assay was started by the addition of protein. The molar absorption coefficient (ϵ_{340}) of fumarylpyruvate was 9400 l mol⁻¹ cm⁻¹ (Tanaka *et al.*, 1957).

For detecting *trans*-ACOHDA hydrolase, the reaction mixture contained, in 610 µl, 60 µM-Tris/HCl buffer, pH 7.5, 30 nmol *trans*-ACOHDA and 20 µl of cell extract from gentisate-grown cells of *P. acidovorans* [10 mg protein ml⁻¹, 0.13 U fumarylpyruvate hydrolase (mg protein)⁻¹]. The reaction was started by the addition of 50 µl of the fraction tested, and the decrease in A_{350} was measured.

Preparation of *cis*-ACOHDA. 5AS (15 mg) was dissolved in 100 ml Na/K phosphate buffer (20 mM, pH 7.5). The pH of the solution was determined using an electrode, and was periodically adjusted to 7.5–8.0 by addition of NaOH. Cell-free extract (1.5 ml, 7.2 mg protein) was added and substrate turnover was analysed by HPLC. After the complete conversion of the substrate, 15 mg solid 5AS and 1.5 ml cell-free extract were added a second time. In this way 120 mg of 5AS was oxidized to *cis*-ACOHDA. Finally, 500 ml cold ethanol was added. The mixture was centrifuged, and the pellet was discarded. Diethyl ether (1800 ml) was added to the supernatant. This mixture was incubated at 4 °C for 12 h without shaking, and a yellow precipitate containing *cis*-ACOHDA was obtained. The supernatant was then decanted carefully, the yellow precipitate was redissolved in water, and the solvent was evaporated. The ring-fission product was purified by TLC (20 cm × 20 cm plates, 0.1 cm thickness, Silica Gel 60 PF_{254nm}; Merck). The solvent system was chloroform/methanol/25% (v/v) ammonia solution (40:40:20, by vol.). The yellow ring-fission product had an R_F of 0.55; it was eluted with methanol and the solvent was evaporated.

Preparation of *trans*-ACOHDA. Strain BN9 was grown with 5AS (4 mM) as sole source of carbon and energy. After complete consumption of 5AS, the culture supernatant was concentrated by evaporation. *trans*-ACOHDA was isolated from this solution by preparative HPLC (HPLC Pump 64, Knauer) with a Schoeffel GM 770 detector (Kratos) and an attached recorder (BD 8 multi range, Kipp & Zonen). The signals were integrated with an Autolab System 1 (Spectra Physics). A reverse-phase column (250 mm × 16 mm, Bischoff, Leonberg, Germany) packed with 5 µm particles (LiChrosorb PR8; Merck) was used. The solvent consisted of 80% water/20% methanol, by vol., and tetra-*n*-butylammonium phosphate as ion-pair reagent (low-UV PIC A; Waters). Fractions were collected directly behind the detector.

Protein chromatography. A fast protein liquid chromatography system (Pharmacia) was used; it consisted of two pumps (P-500), a liquid chromatography controller (LCC-500), a motor valve (MV-7), an anion-exchange column (Mono Q HR 5/5), a UV monitor (UV-1), a fraction collector (FRAC-100), and a recorder (REC-482). The anion-exchange column (1 ml bed volume; Mono Q HR5/5; Phar-

macia) was equilibrated with 15 ml 20 mM-Tris/HCl, pH 8.0. A cell-free extract from 5AS-grown cells of strain BN9 (5.1 mg protein) was layered on the column. The column was washed with 5 ml 20 mM-Tris/HCl buffer, pH 8.0 and protein was eluted with 30 ml of a linear gradient of NaCl (0–0.5 M-NaCl). The flow rate was 0.8 ml min⁻¹. Fractions of 0.5 ml were collected and assayed.

Gas chromatography. The following protocol was used to esterify pyruvic acid and fumaric acid (Bulletin 748G, Supelco). A sample (6 ml) was acidified to approximately pH 2 using 0.1 ml aqueous H₂SO₄ (50%, v/v). To 1 ml of this acidified sample were added 2 ml methanol and 0.4 ml 50% (v/v) H₂SO₄; the tube was stoppered and incubated for 30 min at 55 °C. Then 1 ml H₂O and 0.5 ml chloroform were added, the sample was gently inverted 20 times and centrifuged for 3 min at 3000 g. A sample (5 µl) of the chloroform phase was injected into a gas chromatograph (model 437A, United Technologies Packard; column: GP 15% SP-1220/1% H₃PO₄ on 100/120 Chromosorb WAW, 6 ft × $\frac{1}{4}$ in SS, Supelco). The injector temperature was 180 °C, the oven temperature 120 °C and the flame ionization detector temperature was 250 °C. The flow rate was 13 ml min⁻¹ with nitrogen as carrier gas.

trans-ACOHDA (0.1 mM in 4 ml 50 mM-Tris/HCl, pH 8.0) was converted by a cell-free extract from strain BN9. The sample was then treated as described above. After the conversion of *trans*-ACOHDA, two signals with the same retention times as the methyl esters of pyruvate (R_t = 4.4 min) and fumarate (R_t = 9.5 min) were detected. In contrast, these signals were not found in the original samples of *trans*-ACOHDA or the cell-free extract.

Chemicals. Fumaric acid was prepared as described by Talley *et al.* (1959), using fumaric acid monoethyl ester as the starting material for the synthesis. Maleylpyruvate and fumarylpyruvate were enzymically prepared from gentisate as described by Stolz *et al.* (1992). 5-Aminosalicylate was obtained from Sigma. Fumaric acid monoethyl ester was purchased from EGA-Chemie. Gentisate was from Fluka. Other biochemicals were from Boehringer. The chemicals used for mineral salts media and buffer solutions were from Merck.

Results

Degradation of 5-aminosalicylate by cell-free extracts to L-malate, pyruvate and ammonia

Cell-free extracts from *Pseudomonas* sp. BN9 oxidized 5-aminosalicylate (5AS) to a metabolite with an intense UV absorption maximum at 352 nm, previously identified as *cis*-4-amino-6-carboxy-2-oxo-hexa-3,5-dienoate (*cis*-ACOHDA; Stolz *et al.*, (1992); Fig. 4). Further metabolism of this ring-fission product was observed only when reduced glutathione (GSH) or dithiothreitol (DTT) was added (Nörtemann *et al.*, 1986).

A cell-free extract from strain BN9 (0.38 mg protein) was incubated simultaneously with 5AS (200 nmol) and GSH (200 nmol, in 1 ml 100 mM-Tris/HCl, pH 8.0). An immediate increase in absorbance ($\lambda_{\max}$ = 325 nm, $\Delta A_{352} \text{ min}^{-1}$ = 0.06) was followed by a slow turnover of *cis*-ACOHDA ($\Delta A_{352} \text{ min}^{-1}$ = 0.01). When GSH was replaced by the same concentration of DTT the turnover rate of *cis*-ACOHDA was only 48% of the rate found with GSH.

When 5AS (0.2 mM) was oxidized by cell-free extracts in the presence of GSH, 72% of the initial 5AS

concentration was recovered as ammonia. In a control experiment, 5AS was converted by cell-free extract in the absence of GSH. As expected, the formation of the ring-fission product was not accompanied by the release of ammonia.

The formation of pyruvate and L-malate from 5AS was demonstrated using specific enzyme tests (Vassault, 1985; Burlina, 1985). From the initial concentration of 5AS, 86% was recovered as pyruvate and 79% as L-malate.

Spontaneous reactions of cis-ACOHDA in the presence of glutathione, light and acids

GSH and other thiols catalyse spontaneous *cis-trans* isomerizations and form addition products at carbon-carbon double bonds (Morgan & Friedman, 1938; Lack, 1961; Seltzer & Lin, 1979). Therefore, a purified preparation of *cis*-ACOHDA was incubated with GSH (1 mM) without cell-free extract. Two spontaneous reactions were observed. A rapid conversion of the substrate ($R_t = 8.9$ min) to a new compound ($R_t = 6.8$ min) was detected by HPLC. This product was not detected in the absence of GSH (Fig. 1*b*). The *in situ* spectrum of the reaction product showed an absorption maximum at 355 nm. When the same reaction was assayed spectrophotometrically, the absorption maximum shifted slowly to 320 nm (Fig. 1*a*). It was shown by HPLC that this slow reaction was connected with a turnover of the product formed in the quick reaction. DTT (1 mM) could replace GSH in both reactions. The reaction rate with DTT was only half that observed with GSH. Oxidized glutathione (GSSG) did not show any effect. The product with an absorption maximum at 320 nm was most probably the addition product of glutathione with ACOHDA. Similar products have been described for the reaction of glutathione with fumarylpyruvate and fumarylacetone (Lack, 1961; Seltzer & Lin, 1979). The conversion of the ring-fission product to the new compound with an absorption maximum at 355 nm was also catalysed by light in the absence of GSH. *cis*-ACOHDA (70 μ M in 100 mM-Tris/HCl, pH 8.0) was incubated in a cuvette for 4 h in the sunlight, and was completely converted to the new compound. No reaction was detected in a control experiment protected from the light.

During the acid-catalysed transformation of *cis*-ACOHDA to fumarylpyruvate, an intermediate was formed which was tentatively identified as *trans*-ACOHDA (Stolz *et al.*, 1992). The product formed from *cis*-ACOHDA in the presence of light and GSH (or DTT) was shown by HPLC retention times and *in situ* spectra to be identical with the intermediate formed in the course of the acid-catalysed reaction.

Fig. 1. Spontaneous reaction of *cis*-ACOHDA with GSH (or DTT). (a) Overlay spectra, recorded every 20 min. Arrows indicate absorption maxima. The cuvette contained, in a total volume of 1 ml, 100 μ M Tris/HCl, pH 8.0, 0.1 μ M *cis*-ACOHDA and 1 μ M GSH. (b) HPLC analysis. The reaction mixtures contained, in a total volume of 200 μ l, 20 μ M Tris/HCl, pH 8.0, 20 nmol *cis*-ACOHDA and 200 nmol GSH (circles) or 200 nmol DTT (triangles). As a control, *cis*-ACOHDA (■) was incubated under the same conditions, but without the addition of thiols. Concentrations of *cis*- (filled symbols) and *trans*-ACOHDA (open symbols) were determined by HPLC.

Isolation of trans-ACOHDA from culture supernatants

Two metabolites absorbing at 350 nm were found in supernatants from 5AS-grown cultures by HPLC. They were identified by their retention times, *in situ* spectra and enzymic conversion as *cis*- and *trans*-ACOHDA. After growth of strain BN9 with 5AS (4 mM) as sole source of carbon and energy, about 40 μ M-*cis*-ACOHDA and 20 μ M-*trans*-ACOHDA were found in the culture fluid after complete turnover of 5AS. *trans*-ACOHDA was purified from the culture supernatant by preparative HPLC (see Methods). At pH 8, *trans*-ACOHDA had an absorption maximum at 355 nm. At pH 1, the absorption

Fig. 2. Absorption spectra of *cis* (a) and *trans*- (b) ACOHDA at pH 1 (---), pH 8 (—) and pH 13 (····). *cis*-ACOHDA was purified by preparative TLC and *trans*-ACOHDA by preparative HPLC (see Methods). The compounds were mixed with the required amounts of acid (HCl) or base (NaOH) and a spectrum was recorded immediately.

maximum shifted to 380 nm. This bathochromic shift was not found with the *cis* isomer (Fig. 2). At pH 2, the half-lives for *cis*- and *trans*-ACOHDA were 29 min and 95 min, respectively. Fumarylpyruvate was identified as the reaction product by its UV spectrum and its HPLC retention time. The deamination of *trans*-ACOHDA to fumarylpyruvate proceeded by the formation of three isosbestic points, at 346 nm, 278 nm and 231 nm.

Enzymic conversion of *trans*-ACOHDA to fumarate and pyruvate

When *trans*-ACOHDA was incubated with cell-free extract from strain BN9 grown with 5AS A_{350} decreased almost to zero. The disappearance of *trans*-ACOHDA was accompanied by the formation of two new HPLC signals. The retention times of these two compounds (3.4 and 4.9 min) were identical with those of pyruvate and fumarate. Maleate could be clearly distinguished by its retention time (4.2 min) from fumarate. When maleate (100 μ M) was incubated with the same crude extract for 30 min, no isomerization to fumarate was found. The formation of fumarate and pyruvate from *trans*-ACOHDA was confirmed by GC of the methyl esters (see Methods). The amount of pyruvate formed always correlated with the initial concentration of *trans*-ACOHDA. The ϵ_{350} of *trans*-ACOHDA was determined to be 13900 l mol⁻¹ cm⁻¹.

Formation of fumarylpyruvate from *trans*-ACOHDA

Theoretically, there are two different pathways which result in the formation of pyruvate, fumarate and ammonia from *trans*-ACOHDA. One possibility is the hydrolytic cleavage of *trans*-ACOHDA to pyruvate and fumaramide, followed by the hydrolysis of fumaramide to fumarate and ammonia. The other possibility is the hydrolysis of the imino-function of *trans*-ACOHDA to fumarylpyruvate and hydrolysis of the latter to fumarate

Fig. 3. elution profile from a Mono Q column of a crude extract prepared from 5AS-grown cells of strain BN9. *trans*-ACOHDA hydrolase (○) and fumarylpyruvate hydrolase (●) activities were measured. The protein content was determined as A_{280} (—). The chromatography conditions are described in Methods.

and pyruvate. To test the first hypothesis, fumaramide (50 μM) was incubated together with *trans*-ACOHDA (100 μM ; in 50 mM potassium/sodium phosphate buffer pH 7.4) with cell-free extract from strain BN9 and the turnover of the substrate(s) was analysed by HPLC. *trans*-ACOHDA was readily converted to pyruvate and fumarate. In contrast, fumaramide was not attacked at all and was thus excluded as an intermediate. Cell-free extracts of strain BN9 contained fumarylpyruvate hydrolase activity [0.34 U (mg protein)⁻¹]. The enzymic activities with *trans*-ACOHDA and fumarylpyruvate were separated by column chromatography. It was hard to detect protein fractions which converted *trans*-ACOHDA to fumarylpyruvate by spectrophotometric assay, because the substrate and the product showed absorption maxima at 354 and 345 nm, respectively. Therefore, as a source of the fumarylpyruvate hydrolase, a cell-free extract from *P. acidovorans* DSM 50053 grown with gentisate was added to each fraction obtained by anion-exchange chromatography (Fig. 3). The fraction with the highest activity with *trans*-ACOHDA (fraction 23, Fig. 3) was incubated with *trans*-ACOHDA in the absence of crude extract from *P. acidovorans*. As a result, the absorption maximum shifted from 354 nm to 345 nm. When a fraction containing the partially purified fumarylpyruvate hydrolase (fraction 32, Fig. 3) was added to this reaction mixture, the absorbance maximum at 345 nm disappeared completely. No activity was observed when the fumarylpyruvate hydrolase activity alone was incubated with *trans*-ACOHDA in the absence of other components of the crude extract. The conversion of *trans*-ACOHDA to fumarylpyruvate by fraction 32 was also shown by HPLC. The second product from the hydrolysis of *trans*-ACOHDA was ammonia. Different concentrations of *trans*-ACOHDA (40–140 μM) were incubated with the most active fraction from the column chromatography. The amounts of fumarylpyruvate and ammonia formed were strictly dependent on the initial concentration of the substrate. For all concentrations, we recovered equimolar amounts of fumarylpyruvate and 80–100% of the calculated amount of ammonium ion. After growth of strain BN9 with 5AS, the specific activities of cell-free extracts with *trans*-ACOHDA were 0.12–0.15 U (mg protein)⁻¹. After growth with acetate or nutrient broth, the specific activity of *trans*-ACOHDA hydrolase was less than 0.005 U (mg protein)⁻¹.

Discussion

Aromatic amines are produced on a large scale by the chemical industry. They are used as intermediates in the production of drugs, dyes, pesticides and various other chemicals. Furthermore, they have been found as intermediates in the metabolism of azo dyes, aminonaph-

thalenesulphonic acids, and nitro-substituted aromatic compounds under both aerobic and anaerobic conditions (Durham, 1958; Cartwright & Cain, 1959; Wheeler *et al.*, 1975; Hallas & Alexander, 1983; Nörtemann *et al.*, 1986; Rafii *et al.*, 1990; Haug *et al.*, 1991; Schackmann & Müller, 1991; Chung *et al.*, 1992). Certain aromatic amines, as (substituted) benzidine(s) or naphthylamine(s), are carcinogenic (Weisburger & Weisburger, 1966). There is therefore considerable interest in the microbial degradation of this class of aromatic compounds. 5-Aminosalicylate was identified as an intermediate in the bacterial degradation of aminonaphthalenesulphonates and related azo dyes (Nörtemann *et al.*, 1986; Haug *et al.*, 1991). Furthermore, it is the active agent in many drugs for the treatment of human ulcerative colitis (Peppercorn & Goldman, 1972; Khan *et al.*, 1977; Dull *et al.*, 1987; Tjørnelund *et al.*, 1989).

Fig. 4 shows the proposed degradative pathway for the aerobic bacterial metabolism of 5AS. It involves two enzymes which were specifically induced after growth of strain BN9 with 5AS, 5AS 1,2-dioxygenase and *trans*-ACOHDA hydrolase. In *Pseudomonas* sp. BN9, 5AS 1,2-dioxygenase was distinct from the gentisate 1,2-dioxygenase (Stolz *et al.*, 1992). Nevertheless, it was shown that the gentisate 1,2-dioxygenases from *P. acidovorans* ATCC 17438 and *P. testosteroni* ATCC 49249 also oxidized 5AS, but with significantly reduced V_{max} (1–7%) compared to gentisate as substrate and a high K_m (3.0–4.5 mM) (Harper & Lipscomb, 1990*a, b*). 5AS 1,2-dioxygenase and gentisate 1,2-dioxygenases obviously oxidize 5AS to the same product (Harper & Lipscomb, 1990*b*; Stolz *et al.*, 1992). 5AS 1,2-dioxygenase resembles gentisate 1,2-dioxygenase in various aspects (Crawford *et al.*, 1975; Stolz, 1989; Stolz *et al.*, 1992). This suggests that 5AS 1,2-dioxygenase may be evolutionarily related to gentisate 1,2-dioxygenase. This could explain that it is easy to isolate pure cultures from the environment that degrade 5AS. In contrast we were unable to isolate bacteria that grow with 3- or 4-aminosalicylate (unpublished results).

Unfortunately, we could not detect any enzyme activity in crude extracts that converted *cis*-ACOHDA to the *trans*-isomer. This could be due to the instability of this activity in a cell-free system, wrong assay conditions or a lack of such an enzyme in the strain. The spontaneous isomerization of the ring-fission product in the presence of thiols demonstrated that the last of these was possible. The non-enzymic isomerization of maleylpyruvate to fumarylpyruvate and maleylacetone to fumarylacetone has been reported (Lack, 1961; Seltzer & Lin, 1979). Nevertheless, the excretion of *trans*-ACOHDA by strain BN9 growing with 5AS and the induction of a *trans*-ACOHDA hydrolase after growth of strain BN9 with 5AS suggested that *trans*-ACOHDA

Fig. 4. Proposed pathway for the degradation of 5-aminosalicylate by *Pseudomonas* sp. BN9.

was a natural metabolite in the degradation of 5AS by strain BN9. *trans*-ACOHA hydrolase was the second enzyme which was newly found during this study. Only in the eukaryotic metabolism of tryptophan has a comparable enamine intermediate been found as a product of 3-hydroxyanthranilate. This compound, α -aminomuconate, is reductively deaminated to α -ketoadipate (Ichiyama *et al.*, 1965; Nishizuka *et al.*, 1970).

The degradative pathway of 5AS converges at the stage of fumarylpyruvate with the degradative pathway for gentisate, which is used by most Gram-negative bacteria (Crawford & Frick, 1977; Hagedorn *et al.*, 1985). This is the first observation of the involvement of fumarylpyruvate hydrolase in a degradative pathway distinct from the gentisate pathway. Therefore, the question arises whether fumarylpyruvate hydrolase

belongs to the same operon as gentisate 1,2-dioxygenase or is regulated separately.

The degradative pathway of 5AS differs from the bacterial metabolism of all other aminoaromatic compounds studied before. Aniline, methylanilines, and 2- and 4-aminobenzoate are usually deaminated initially by dioxygenases, which form (substituted) catechol(s) (Durham, 1956; Taniuchi *et al.*, 1964; Bachofer, 1976; Helm & Reber, 1979; Aoki *et al.*, 1983; Appel *et al.*, 1984; Latorre *et al.*, 1984; Raabe *et al.*, 1984). Certain bacteria metabolize 2-aminobenzoate via 5-hydroxyanthranilate to gentisate (Ladd, 1962; Cain, 1968). Only for the metabolism of 2-aminobenzoate by the denitrifying *Pseudomonas* strain KB740 has a pathway via an aminated non-aromatic intermediate (2-amino-5-oxo-cyclohex-1-ene-carboxyl-CoA) been found (Altenschmidt *et al.*, 1990; Altenschmidt & Fuchs, 1992). The present demonstration of novel degradative pathways for 2-aminobenzoate by *Pseudomonas* strain KB740 and 5AS by *Pseudomonas* sp. BN9 suggests that other aminobenzenes could also be metabolized by bacteria without the intermediate formation of *o*- or *p*-diphenols.

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