Biochem. J. (1992) 282, 675–680 (Printed in Great Britain)

Bacterial metabolism of 5-aminosalicylic acid

Initial ring cleavage

Andreas STOLZ,* Bernd NÖRTEMANN† and Hans-Joachim KNACKMUSS*‡

*Institut für Mikrobiologie der Universität Stuttgart, Azenbergstrasse 18, 7 Stuttgart 1, Federal Republic of Germany, and †Fachgebiet Chemie und Chemische Verfahrenstechnik, Universität Paderborn, Postfach 1621, 4790 Paderborn, Federal Republic of Germany

The metabolism of 5-aminosalicylate (5AS) by a bacterial strain, *Pseudomonas* sp. BN9, was studied. Intact cells of *Pseudomonas* sp. BN9 grown with 5AS oxidized 5AS and 2,5-dihydroxybenzoate (gentisate), whereas cells grown with gentisate oxidized only the growth substrate of all substituted salicylates tested. Cell extracts from *Pseudomonas* sp. BN9 catalysed the stoichiometric reaction of 1 mol of oxygen with 1 mol of 5AS to a metabolite with an intense u.v.-absorption maximum at 352 nm (pH 8.0). This metabolite was accumulated under neutral conditions, but was rapidly destroyed at acid pH. It was identified by m.s. and acid-catalysed deamination to fumarylpyruvate (*trans*-2,4-dioxohept-5-enedioic acid) as *cis*-4-amino-6-carboxy-2-oxohexa-3,5-dienoate, thus demonstrating direct cleavage of the monohydroxylated substrate 5AS to a non-aromatic ring-fission product. The enzyme responsible for conversion of 5AS was shown to be Fe(II)-dependent and to be distinct from gentisate 1,2-dioxygenase in strain BN9.

INTRODUCTION

Amino- and hydroxy-naphthalenesulphonic acids (ANS and HNS) are building blocks of azo dyes and thus constitute a major contaminant of aqueous ecosystems. The isolation of a mixed bacterial community growing with 6-aminonaphthalene-2-sulphonic acid (6A2NS) as sole source of carbon, nitrogen, sulphur and energy has been reported from this laboratory (Nörtemann *et al.*, 1986). Basically two bacterial strains were involved in the total degradation of 6A2NS. Strain BN6 converted 6A2NS into equimolar amounts of 5-aminosalicylic acid (5AS) and utilized only the pyruvate released from the breakdown of the sulphonated aromatic ring. The excreted 5AS was taken up and degraded by a second strain of the mixed culture, *Pseudomonas* sp. BN9. In contrast with the generally known degradative pathways for aromatic compounds, 5AS was directly subject to ring cleavage without being converted into an arenediol. The present publication describes this unusual ring-fission reaction catalysed by a 5AS 1,2-dioxygenase.

EXPERIMENTAL

Materials

Bacteria. Strain BN9 has been isolated and described by Nörtemann *et al.* (1986). *Moraxella* sp. OA3 (Crawford *et al.*, 1975) was kindly provided by R. C. Bayly, Monash University, Prahran, Australia.

Chemicals. 5AS and vitamins were obtained from Sigma, Deisenhofen, Germany. 3-Amino- and 3-hydroxy-salicylate were purchased from EGA-Chemie, Steinheim, Germany. 4-Aminosalicylate was supplied by Serva, Heidelberg, Germany, and gentisate by Fluka, Buchs, Switzerland. Biochemicals were supplied from Boehringer, Mannheim, Germany. All other chemicals used for mineral salts media and buffer solutions were obtained from Merck, Darmstadt, Germany.

Methods

Culture conditions. For cultivation a mineral medium was used which contained per litre: 6 g of $Na_2HPO_4,2H_2O$, 1 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 50 mg of $Ca(NO_3)_2,4H_2O$, 200 mg of $MgSO_4,7H_2O$, 10 mg of Fe(III)/NH_4/citrate and 0.1 ml of a trace-element solution described by Pfennig & Lippert (1966), without iron salt and EDTA. This medium was supplemented by a vitamin solution described by Sharak-Genthner *et al.* (1981).

5AS and the vitamin solution were sterilized by membrane filtration (pore size 0.2 μm; Sartorius, Göttingen, Germany); all other solutions were autoclaved. 5AS was added to batch cultures at concentrations of 0.5–2 mM.

To prevent autoxidation of 5AS in the growth medium, the lag phase of strain BN9 with this substrate must be kept as short as possible. Therefore strain BN9 was usually pregrown with nutrient broth (Difco, Detroit, MI, U.S.A.), transferred to a mineral medium containing 0.5 mM-5AS + 0.1 g of nutrient broth per litre and then finally to the mineral medium with 5AS (2 mM) containing the vitamin solution.

For the production of larger amounts of cells, strain BN9 was grown in a 10-litre fermenter (Bio-Mag 1; BCC, Göttingen, Germany).

Flasks were incubated at 30 °C on a rotary shaker at 100 rev./min. Solid media were prepared by addition of 1.5% Agar no. 1 (Oxoid, Basingstoke, Hants, U.K.) to the medium which contained the appropriate carbon source.

Oxygen-uptake experiments. Oxygen uptake was measured at 25 °C with an oxygen electrode (YSI 5350, Yellow Springs Instrument Co., Yellow Springs, CO, U.S.A.). For experiments with resting cells, the washed biomass was resuspended in 80 mM-Na_2HPO_4/KH_2PO_4 buffer, pH 7.4. The cell suspension (2.9 ml) was transferred to the reaction chamber of the oxygen electrode and the endogenous respiration was measured for 3 min. Thereafter 100 μl of a 50 mM solution of the respective test substrate was added. The oxygen uptake was determined and corrected for endogenous respiration.

Protein estimation and enzyme assays. Cell extracts were prepared by disrupting the cells in a French Press (SLM Aminco; SLM Instruments Inc., Urbana, IL, U.S.A.) with an internal pressure of 1.1×10^8 Pa. Cells and cell debris were removed by

Abbreviations used: ANS, aminonaphthalenesulphonic acid; 5AS, 5-aminosalicylate; 6A2NS, 6-aminonaphthalene-2-sulphonic acid; HNS, hydroxynaphthalenesulphonic acid; NEM, *N*-ethylmaleimide; R_t, retention time.

‡ To whom correspondence should be addressed.

centrifugation at 100000 *g* for 1 h (Centrikon T-1055; Kontron, Zürich, Switzerland).

One unit of enzyme activity is defined as the conversion of 1 μmol of substrate/min.

Protein content of cell-free extracts was determined by the method of Bradford (1976). BSA was used as a standard.

The following enzyme activities were determined by the use of spectrophotometric methods in silica cuvettes of 10 mm pathlength by use of a Kontron Uvikon 820 spectrophotometer. Gentisate 1,2-dioxygenase was measured by the method of Wheelis *et al.* (1967). For 5AS 1,2-dioxygenase the cuvettes contained, in 1 ml, 50 μmol of Tris/HCl, pH 8.0, and 0.3 μmol of 5AS. The reaction was started by addition of cell-free extract. The increase in absorbance was recorded at 350 nm. Fumarylpyruvate hydrolase was assayed by the method of Hagedorn *et al.* (1985).

Partial purification of the fumarylpyruvate hydrolase of strain BN9. Protein purification was performed with a f.p.l.c. system as described elsewhere (Kuhm *et al.*, 1990). Strain BN9 was grown with gentisate, and a crude extract was prepared and applied to a Mono Q column (HR 5/5; Pharmacia, Uppsala, Sweden). Protein was eluted with 30 ml of a linear gradient of Tris/HCl (20 mM, pH 8.0) to Tris/HCl (20 mM, pH 8.0) plus 500 mM-NaCl at a flow rate of 1 ml/min; 0.5 ml fractions were collected. The maximum activity of fumarylpyruvate hydrolase was eluted at a concentration of 0.16 M-NaCl.

Analytical methods. 5AS and its metabolites were analysed by reverse-phase h.p.l.c. (h.p.l.c. data and chromatography control station model 840, equipped with a programmable multi-wavelength detector model 490; Waters Associates, Milford, MA, U.S.A.). A reverse-phase column [125 mm × 4.6 mm (internal diameter); Bischoff, Leonberg, Germany], packed with 5 μm particles of Lichrosorb RP8 (Merck) was used. The separated compounds were detected photometrically by simultaneous measurements at 210 nm and 350 nm. The following solvent system was used: 90% (v/v) water, 10% (v/v) methanol and an ion-pair reagent according to the instructions of the manufacturer (low-u.v. PIC A; Waters). The usual flow rate was 0.8 ml/min. Ammonia was determined with glutamate dehydrogenase by the method of Da Fonseca-Wollheim *et al.* (1974).

To determine the u.v. spectra and the stability of *cis*-4-amino-6-carboxy-2-oxohexa-3,5-dienoate, 5AS was quantitatively oxidized to this metabolite by a cell-free extract of strain BN9 and protein removed by ultrafiltration (PM 10; Amicon, Beverly, MA, U.S.A.).

U.v. spectra were detemined with a Kontron 810P spectrophotometer (Kontron, Eching, Germany). Mass spectra were recorded on an MAT 311A spectrometer (Varian, Palo Alto, CA, U.S.A.).

Conversion of the ring-fission product of 5AS into fumarylpyruvate. For preparation of a fumarylpyruvate standard, a cell-free extract from gentisate-grown cells of *Moraxella* sp. OA3 was incubated with gentisate (0.3 mM in 100 mM-Tris/HCl buffer, pH 8) and the reaction was simultaneously monitored spectrophotometrically and by h.p.l.c. analysis. The increase in absorbance at 330 nm was correlated with the formation of a product [h.p.l.c. analysis: R_t = 3.9 min, R_t of gentisate as reference = 7.0 min; solvent system 50% (v/v) water, 50% (v/v) methanol, 0.1% (v/v) H_3PO_4, flow rate 0.8 ml/min; reverse-phase column as above but 25 cm long]. After complete conversion of gentisate, the reaction mixture was acidified with HCl to pH 2. The spectrophotometrically observed isomerization of maleylpyruvate to fumarylpyruvate (Lack, 1959) correlated with the results of h.p.l.c. analysis: a decrease in signal intensity at R_t = 3.9 min corresponded to an increase in a broad peak (R_t = 5.2 min). With the acid h.p.l.c. solvent system, the compound with R_t = 5.2 min showed an absorption maximum at 336 nm (measured *in situ*) and was thus identified as fumarylpyruvate.

5AS was oxidized by a cell-free extract from strain BN9. In order to identify the product, the reaction mixture was acidified with HCl to pH 2 and precipitated protein was removed by centrifugation. The reaction product in the supernatant was analysed and identified by h.p.l.c. as fumarylpyruvate (R_t = 5.2 min; *in situ* $\lambda_{max.}$ = 336 nm).

RESULTS

Oxygen uptake by whole cells

In general, ring cleavage of aromatic compounds during aerobic metabolism is mediated by a dioxygenolytic mechanism. Thus gentisate is broken down by 1,2-dioxygenation (Tanaka *et al.*, 1957; Lack, 1959; Crawford *et al.*, 1975). Because of the structural analogy of 5AS and gentisate, it was suggested that 5AS might also be subject to an analogous 1,2-dioxygenation (Nörtemann *et al.*, 1986). Therefore the oxygen uptake of resting cells after cultivation with different carbon sources was determined.

After growth with 5AS, cells of strain BN9 showed an increased oxygen uptake with 5AS (0.28 μmol of O_2/min per mg of protein) and gentisate (0.33 μmol of O_2/min per mg of protein). Gentisate-grown cells oxidized gentisate (0.45 μmol of O_2/min per mg of protein), but were completely inactive with 5AS. These observations did not distinguish between an unspecific gentisate dioxygenase in 5AS-grown cells with activity for 5AS and two different enzymes, one exclusively for gentisate and another one for 5AS.

Neither 5AS- nor gentisate-grown cells could oxidize 4-amino-, 4-hydroxy-, 5-fluoro-, 5-chloro-, 5-bromo-, 5-iodo-, 3- or 4-chloro-salicylate.

5AS metabolism in cell-free extracts

Turnover of 5AS by cell extracts of 5AS-grown cells of strain BN9 caused a bathochromic shift of the absorption maximum from 330 nm to 352 nm together with a considerable increase in intensity of the maximum. The same enzyme preparation catalysed the oxidation of gentisate to maleylpyruvate, which shifted the absorption maximum from 318 nm to 330 nm (Nörtemann *et al.*, 1986). Oxygen uptake by cell-free extracts converting 5AS was correlated with the bathochromic shift observed photometrically (Fig. 1). The total amount of oxygen consumed at the expense of 5AS was strictly proportional to the initial concentration of the organic substrate: 1 mol of oxygen was consumed per mol of 5AS added (Fig. 1*a*). When the same reaction mixtures were analysed in a parallel set of experiments spectrophotometrically, it was shown that the increase in absorbance at 350 nm was strictly proportional to the amount of oxygen consumed. The increase in absorbance at 350 nm was linearly correlated with the amount of 5AS initially present in the cuvette (Fig. 1*b*). When the reaction was analysed by h.p.l.c., the peak for 5AS (R_t = 17.3 min) decreased and the formation of a single product was observed (R_t = 12.3 min). The absorption spectrum of the product measured *in situ* during h.p.l.c. showed an absorbance maximum at 352 nm. It was therefore concluded that the product observed by h.p.l.c. was identical with the reaction product observed spectrophotometrically. Ammonia was not released in the course of the reaction. From the results described above it was concluded that 5AS catabolism was initiated by a dioxygenase analogous to gentisate 1,2-dioxygenase.

The yellow ring-cleavage product of 5AS was not further metabolized by the cell-free extract at a significant rate. The amount of product formed increased in the presence of L-

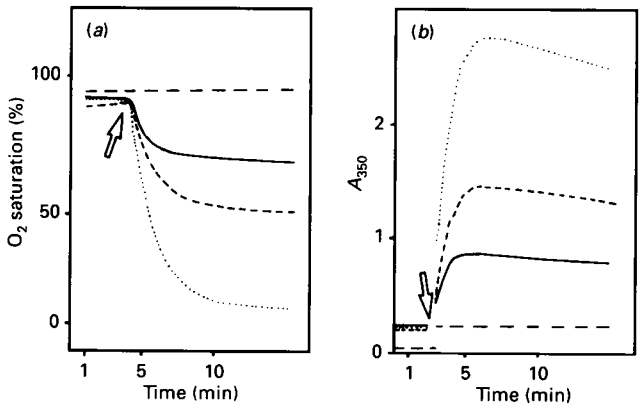


Fig. 1. Comparison of the polarographic (*a*) and spectral changes (*b*, at 350 nm) during 5AS oxidation by cell-free extracts of 5AS-grown cells of strain BN9

The reaction mixtures contained: 100 mM-Tris/HCl, pH 8.0, and 0.95 mg of protein/ml. 5AS was added at the time indicated by the arrow so that the initial concentration (— —) was 50 μM (——), 100 μM (– – – –) and 200 μM (· · · ·). The controls contained 100 μM-5AS and instead of the cell-free extract the corresponding volume of water.

ascorbic acid (5 mM), whereas the reaction rate remained unaffected under these conditions. Stabilization of enzymes by ascorbic acid has already been described for Fe(II)-containing dioxygenases (Suda *et al.*, 1951; Flamm & Crandall, 1963; Mitchell *et al.*, 1963). The amount of product formed clearly corresponded to the initial amount of 5AS added. Thus the absorption coefficient of the product could be determined by converting different concentrations of 5AS and recording the increases in absorbance at 350 nm (ϵ_{350} 12100 litre·mol^{-1}·cm^{-1}, pH 8.0, reaction coefficient 10200 litre·mol^{-1}·cm^{-1} based on $\epsilon_{5AS,350}$ 1900 litre·mol^{-1}·cm^{-1}). The absorption spectrum did not change significantly between pH 8.0 and 12.8. When the pH value of the solution was shifted to pH 1 the absorption maximum at 352 nm remained constant, but its intensity decreased significantly ($\epsilon_{352nm,pH1}$ 9100 litre·mol^{-1}·cm^{-1}).

To determine the stability of the compound, it was incubated at different pH values and the amount recovered after different time intervals was determined by h.p.l.c. At pH 6.4–12 the compound was stable at room temperature. The ring-fission product, however, was labile under acidic conditions, and its half-life at pH 2 was about 30 min.

The apparent K_m value for 5AS, calculated from Lineweaver–Burk plots, was 1.4×10^{-4} M. The specific activities of cell-free extracts after growth of strain BN9 with 5AS were about 0.8–1.2 units/mg of protein. After growth with gentisate, nutrient broth or acetate, the specific activity of 5AS 1,2-dioxygenase in cell-free extracts was less than 0.05 unit/mg of protein.

If cell-free extract (1.7 mg of protein/ml) was incubated with various Fe^{2+}-chelating agents such as 8-hydroxyquinoline, *o*-phenanthroline or 2,2′-dipyridyl (each 0.1 mM; 15 min), the enzyme was completely inactivated. In contrast, the enzyme showed 67% residual activity after incubation with 1 mM-Tiron (4,5-dihydroxy-1,3-benzene-3,5-disulphonic acid disodium salt). Cell-free extract, which had lost its 5AS 1,2-dioxygenase activity after 14 h storage at 4 °C, was re-activated to 37% of its original activity by incubation with 2 mM-$(NH_4)_2Fe(SO_4)_2$ over a period of 6 h. From these results it was concluded that the 5AS-converting enzyme was an Fe^{2+}-containing dioxygenase and thus resembles gentisate 1,2-dioxygenase (Crawford *et al.*, 1975).

M.s. of the reaction product

The product formed from 5AS turnover by cell-free extracts was rapidly destroyed under acidic conditions. We therefore tested the possibility of producing the metabolite by whole cells and thus of circumventing tedious deproteinization procedures. Metabolism of maleylpyruvate, the ring-cleavage product of gentisate, is inhibited in certain bacteria by the addition of *N*-ethylmaleimide (NEM) (Crawford & Frick, 1977). In the presence of NEM (or maleimide), oxidation of 5AS by a cell suspension of *Pseudomonas* sp. BN9 gave rise to a pronounced increase in absorbance at 350 nm. This was not found in control experiments with 5AS or NEM alone. A decrease in absorbance was observed in the presence of 5AS and bacteria (Fig. 2*a*). This was due to metabolism of 5AS by the cells (molar absorption coefficient of 5AS: ϵ_{350} = 1900 litre·mol^{-1}·cm^{-1}). Analysis of the reaction mixtures by h.p.l.c. (Figs. 2*b* and 2*c*) showed rapid conversion of 5AS by the cells in the absence of NEM and excretion of small

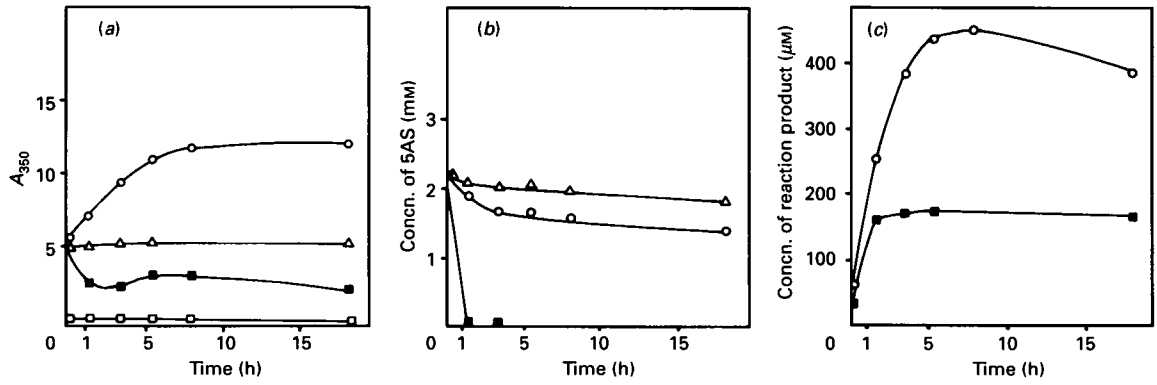


Fig. 2. Incubation of resting cells from strain BN9 with 5AS and NEM

Strain BN9 was grown with 5AS. At the end of the exponential growth phase cells were centrifuged and resuspended to an absorbance (A_{546}) of 7 in sodium/potassium phosphate buffer (50 mM, pH 7.4). Cell suspension (10 ml) was incubated in 100 ml Erlenmeyer flasks at 30 °C on a rotary shaker (100 rev./min) and the following additions were made: 22 μmol of 5AS (■), or 22 μmol of 5AS and 10 μmol of NEM (○), or 10 μmol of NEM (□). To detect possible spontaneous reactions of 5AS and/or NEM, the cell-free control contained in 10 ml of sodium/potassium phosphate buffer, 22 μmol of 5AS and 10 μmol of NEM (△). After different time intervals, samples were taken, the cells precipitated by centrifugation (12000 rev./min, 5 min; Biofuge A; Heraeus-Christ, Osterode, Germany), and the supernatants analysed. (*a*) Absorbance of the samples was determined spectrophotometrically after appropriate dilution. The initial absorbance is due to 5AS (ϵ_{350} = 1900 litre·mol^{-1}·cm^{-1}). The concentrations of (*b*) 5AS and (*c*) the reaction product were determined by h.p.l.c.

Table 1. Mass spectrum of 4-amino-6-carboxy-2-oxohexa-3,5-dienoate

The ring-fission product of 5AS was isolated as described in the Results section and analysed by m.s. The intensities are referred to the base peak *m/z* 44.

m/z	Intensity (% of base peak)	Assignment
185	1.3	M^+
167	0.5	$M^+ - H_2O$
140	2.0	$M^+ - CO_2H$
127	10.7	$M^+ - CHCO_2H$
122	7.8	$(M^+ - H_2O) - CO_2H$
112	2.2	$M^+ - COCO_2H$
99	18.0	$M^+ - CHCOCO_2H$
86	5.6	$M^+ - CNH_2[CH]_2CO_2H$
71	4.0	$M^+ - CNH_2CHCOCO_2H$

amounts of the ring-fission product. In the presence of NEM, turnover of 5AS was significantly inhibited, but larger amounts of the ring-cleavage product were excreted (Fig. 2*b* and 2*c*). Obviously NEM inhibited the subsequent reaction in the pathway even more than 5AS 1,2-dioxygenase.

In aerobic aqueous solutions, 5AS underwent autoxidation (Nörtemann *et al.*, 1986). The amount of 5AS lost by spontaneous autoxidation was determined in a cell-free control (Fig. 2*b*) and subtracted from the amount of 5AS converted by the cells in the presence of NEM. Thus an almost quantitative conversion of 5AS into the ring-fission product by NEM-inhibited cells was found (Figs. 2*b* and 2*c*).

In order to isolate the product, the experiment was repeated using a more concentrated cell suspension (100 ml; $A_{546} = 15$). Thus from 2 mM-5AS initially present 1.5 mM was converted. The culture supernatant was carefully adjusted to pH 3.5 and rapidly extracted with ethyl acetate. Under these conditions the residual 5AS was not extracted into the organic layer. The organic solvent was removed by flash evaporation and the dry residue immediately analysed by m.s. According to the data given in Table 1, the structure of the ring-cleavage product of 5AS was determined by m.s. to be 4-amino-6-carboxy-2-oxohexa-3,5-dienoate. With high-resolution techniques, the composition of the molecular ion at *m/z* 185 corresponded to the molecular formula $C_7H_7NO_5$.

Conversion of the ring-cleavage product of 5AS into fumarylpyruvate (*trans*-2,4-dioxohept-5-enedioic acid)

At pH 2 the absorption maximum of the ring-cleavage product of 5AS ($\lambda_{max.} = 352$ nm) was replaced by a new absorption maximum at $\lambda_{max.} = 332$ nm. No isosbestic points were observed during this reaction. The end product of the acid-catalysed reaction showed a typical pH-dependence of the absorption spectrum ($\lambda_{max.,pH\,7.3} = 342$ nm; $\lambda_{max.,pH\,13} = 350$ nm), which was very similar to that described for fumarylpyruvate. Fumarylpyruvate has been described as a metabolite of gentisate and as a product of acid-catalysed isomerization of maleylpyruvate (Tanaka *et al.*, 1957; Lack, 1959). The identity of the product formed from the ring-cleavage product of 5AS and fumarylpyruvate was further confirmed by h.p.l.c. (see the Experimental

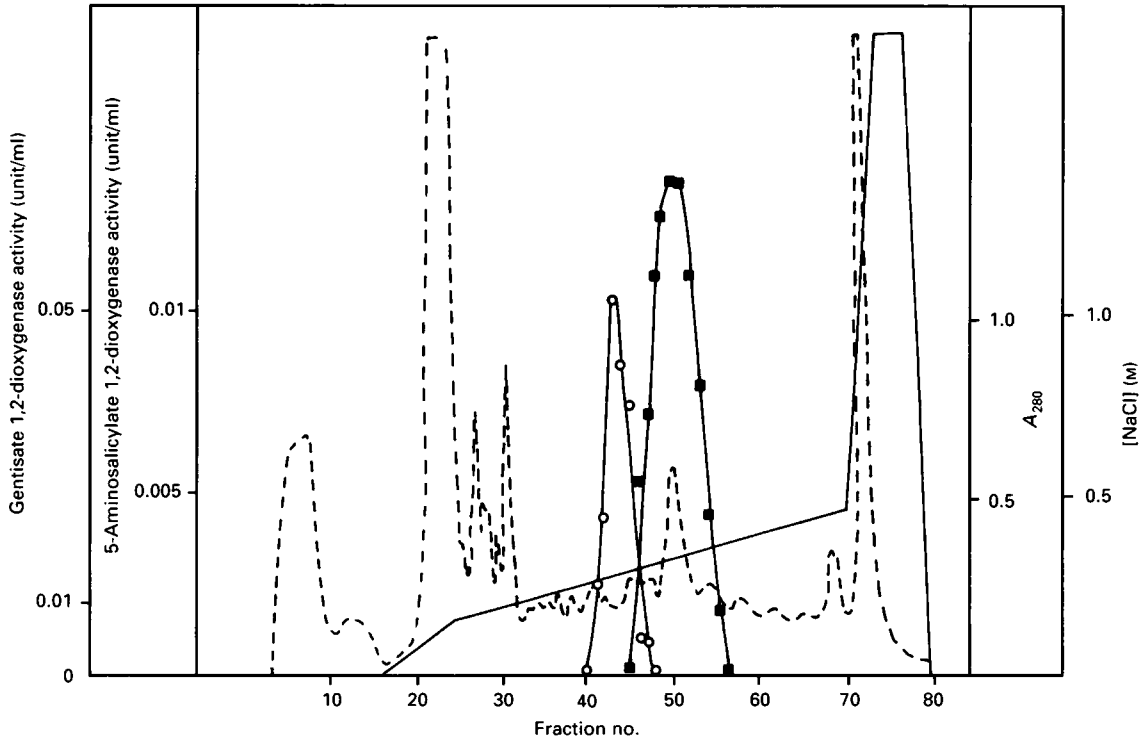


Fig. 3. Elution profile from a Mono Q column of a crude extract prepared from 5AS-grown cells of strain BN9

The anion-exchange column (1 ml bed volume, Mono Q HR5/5; Pharmacia) was equilibrated with 10 ml of Tris/HCl (pH 8.0, 20 mM). A crude extract from 5AS-grown cells of strain BN9 (5.9 mg of protein) was layered on the column. The column was washed with 8 ml of Tris/HCl (pH 8.0, 20 mM) and the protein was eluted with 27 ml of a gradient of NaCl (0–0.45 M-NaCl). The flow rate was 0.4 ml/min. Fractions of volume 0.6 ml were collected and assayed. Further chromatography conditions are described in the Experimental section. Enzyme activity profiles were 5-aminosalicylate 1,2-dioxygenase (○) and gentisate 1,2-dioxygenase (■). The protein content was determined spectrophotometrically at 280 nm (----).

section). In addition, the h.p.l.c. analysis showed that in the course of the reaction at pH 2 a transient intermediate was formed [solvent system 90% water, 10% methanol, ion-pair reagent; R_t (product from 5AS) = 8.9 min, R_t (intermediate) = 6.8 min]. A u.v. spectrum of this intermediate measured *in situ* showed an absorption maximum at 355 nm (pH 7.2).

After growth with gentisate, strain BN9 synthesized a fumarylpyruvate hydrolase which was partially purified by f.p.l.c. (see the Experimental section). The protein fraction containing the fumarylpyruvate hydrolase activity did not react with the ring-cleavage product of 5AS. If preincubated at pH 2 for 16 h and neutralized, an immediate decrease in the A_{340} was measured on addition of fumarylpyruvate hydrolase. During acid treatment, stoichiometric amounts of ammonia were released from the ring-cleavage product of 5AS.

The results presented above indicate that the monohydroxylated aromatic compound 5AS is directly cleaved by a dioxygenase type of enzyme yielding 4-amino-6-carboxy-2-oxohexa-3,5-dienoic acid.

Separation of gentisate 1,2-dioxygenase from 5AS 1,2-dioxygenase

Identification of the product formed from 5AS by cell-free extracts of strain BN9 showed that both 5AS and gentisate are subjected to 1,2-ring cleavage. It was of interest to see whether 5AS and gentisate were converted by the same enzyme. That two separate activities, a gentisate 1,2- and a 5AS 1,2-dioxygenase, must exist in cell-free extracts of 5AS-grown cells became evident by comparison of relative activities towards the two substrates during storage of the extracts. After 20 h at room temperature in 100 mM-Tris/HCl, pH 8.0, the activity with 5AS was completely abolished, whereas 45% of the initial gentisate 1,2-dioxygenase was still present. Under all conditions tested (different pH values, different solvents, inhibition by iron-chelating agents), the 5AS 1,2-dioxygenase was significantly less stable than the gentisate 1,2-dioxygenase. In addition, the two enzyme activities were clearly separated by anion-exchange chromatography (Fig. 3). Although recovery of the total activity for 5AS and gentisate was rather low (0.5% and 5% respectively), it could clearly be demonstrated that the 5AS 1,2-dioxgenase exhibited no activity with gentisate and that the gentisate 1,2-dioxygenase did not oxidize 5AS.

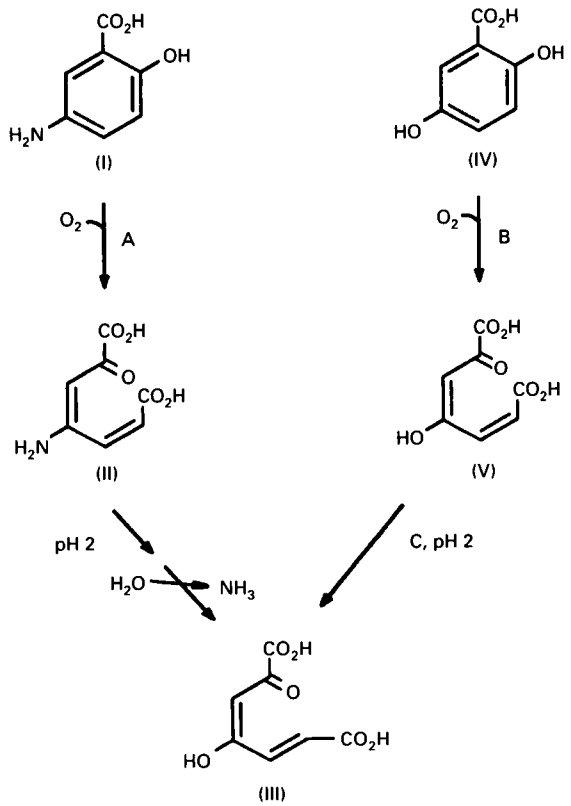
DISCUSSION

Considerable knowledge exists about the fate of 5AS in mammalian metabolism. 5AS is a structural element of the azo compound sulphasalazine (salicylazosulphapyridine), which has been used for more than 40 years in the treatment of human ulcerative colitis. 5AS is released from sulphasalazine by reductive cleavage of the azo bond under anaerobic conditions by the intestinal flora and has been identified as the active agent of the drug (Peppercorn & Goldman, 1972; Khan *et al.*, 1977). Mammals excrete 5AS either unchanged in the faeces or as conjugates such as *N*-acetyl- or *N*-glucopyranosyl-5-aminosalicylate (Tjørnelund *et al.*, 1989). Activated leucocytes convert 5AS into gentisate, salicylate and various unidentified products (Dull *et al.*, 1987). In contrast, nothing is known about bacterial metabolism of 5AS.

Substrates for ring cleavage of aromatic compounds by aerobic bacteria usually carry at least two hydroxy groups either *ortho* or *para* to each other (Dagley, 1975). Until now direct cleavage of a monohydroxylated aromatic has been proposed only for the assimilation of 2-carboxy-1-hydroxynaphthalene and 5-chlorosalicylate (Kiyohara & Nagao, 1977; Crawford *et al.*, 1979). It is generally believed that the existence and the arrangement of two hydroxy groups in the *ortho* or *para* position is required to permit the shift of electrons involved in the ring-fission reaction (Dagley, 1975). The degree of activation of the aromatic ring exerted by a hydroxy group can be compared with that produced by an amino group by use of the Hammett equation. The σ coefficients for $-NH_2$ are $\sigma_{meta} = -0.16$ and $\sigma_{para} = -0.66$, and for $-OH$ $\sigma_{meta} = +0.12$ and $\sigma_{para} = -0.37$ (Beyer & Walter, 1981). Thus the σ values show that not only the OH but also an NH_2 group activates the aromatic nucleus in the *ortho* and *para* position for an electrophilic attack. This effect is even more pronounced than that of the hydroxy group.

Until now we were unable to produce enough ring-cleavage product of 5AS of sufficient purity to prove its structure by n.m.r. techniques. However, m.s. data support the proposed structure of *cis*-4-amino-6-carboxy-2-oxohexa-3,5-dienoate. In addition the stoicheiometry of the reaction of 5AS with oxygen and the acid-catalysed transformation of the ring-cleavage product into fumarylpyruvate are compatible with the proposed ring-cleavage mechanism of 5AS (Scheme 1).

The *cis* configuration of the metabolite could be deduced from the complex spectral changes observed during the acid-catalysed conversion into fumarylpyruvate. The reaction from *cis*-4-amino-6-carboxy-2-oxohexa-3,5-dienoate to fumarylpyruvate (*trans*-6-carboxy-2,4-dioxo-5-hexenoic acid) and ammonia involves two steps, a *cis–trans* isomerization and hydrolysis of the C–N bond. These reactions could occur by the hydrolysis of the ring-fission product yielding ammonia and maleylpyruvate, followed by isomerization to fumarylpyruvate. Alternatively *cis–trans*



Scheme 1. Proposed reactions of 5AS and its ring-fission product compared with the pathway for the degradation of gentisate

Key to enzymes: A, 5-aminosalicylate 1,2-dioxygenase; B, gentisate 1,2-dioxygenase; C, maleylpyruvate isomerase. Key to compounds: (I) 5AS; (II) *cis*-4-amino-6-carboxy-2-oxohexa-3,5-dienoic acid; (III) fumarylpyruvate; (IV) gentisate; (V) maleylpyruvate. The degradative pathway for gentisate was taken from Tanaka *et al.* (1957).

isomerization could precede hydrolysis. In the second case *trans*-4-amino-6-carboxy-2-oxohexa-3,5-dienoic acid would be the intermediate, which could be hydrolysed to fumarylpyruvate. The h.p.l.c. analysis of the reaction mixture clearly showed an intermediate with an absorption maximum at 355 nm (pH 7.2). Since maleylpyruvate shows an absorption maximum between pH 7 and 8 at 332 nm (Hagedorn *et al.*, 1985), it could be excluded as intermediate. Therefore we propose that isomerization precedes deamination. The ring-fission product of gentisate is maleylpruvate (*cis*-6-carboxy-2,4-dioxo-5-hexenoic acid), which in neutral aqueous solutions exists as the stable *cis*-isomer (Lack, 1959). The ring-fission product of 5AS should have the same stereochemistry as maleylpyruvate and the *cis* configuration at C-5–C-6 should be equally stable in both compounds.

The ring-fission product of 5AS can exist in two tautomeric forms as imine or enamine. Imines have been found as transient intermediates in the reactions of D- and L-amino acid oxidases and glutamate dehydrogenase (Porter & Bright, 1972; Hochreiter *et al.*, 1972). Generally imines are easily hydrolysed in aqueous solutions to the corresponding oxo compounds. Surprisingly 4-amino-6-carboxy-2-oxohexa-3,5-dienoate was stable in neutral aqueous solutions. The rate of hydrolysis of different ketimines in aqueous solutions is determined by tautomerism and/or resonance and steric hindrance (Culbertson, 1951). A stabilization against hydrolysis of imines as tautomeric enamines by conjugation and tautomerism can also be deduced from the published half-lives (t_H) of various aliphatic imines. For glutamate dehydrogenase the existence of the imine formed has only indirectly been proved; 2-aminopenta-2,4-dienoic acid has a $t_H = 9.5$ min and 2-amino-4-oxopent-2-enoic acid a $t_H > 20$ h (Hochreiter *et al.*, 1972; Marcotte & Walsh, 1978). Obviously the conjugation system of N–C=C–C=O [the enaminone structure (Greenhill, 1977)] stabilizes these compounds against hydrolysis. In the case of 4-amino-6-carboxy-2-oxohexa-3,5-dienoate its enamine structure is stabilized by conjugation with both the 2-oxo function and the C-5–C-6 double bond, and therefore the compound is stable in aqueous solutions at pH 7.

The cleavage of a monohydroxylated aminated benzene ring has already been demonstrated for 3-hydroxyanthranilate 3,4-dioxygenase, which is involved in mammals but not in most bacteria in the metabolism of tryptophan and the synthesis of NAD (Stanier & Hayaishi, 1951; Decker *et al.*, 1961; Vescia & Di Prisco, 1962; Koontz & Shiman, 1976; Foster & Moat, 1980). In contrast, certain bacteria metabolize anthranilate (2-aminobenzoate) via 5-hydroxyanthranilate to gentisate (Ladd, 1962; Cain, 1968). Thus, as shown for 5AS, direct oxidative cleavage of a monohydroxylated amino aromatic ring seems to be a rather unusual reaction among bacterial catabolic systems.

Received 10 May 1991/1 August 1991; accepted 15 August 1991

REFERENCES

Beyer, H. & Walter, W. (1981) Lehrbuch der Organischen Chemie, S. Hirzel Verlag, Stuttgart

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Cain, R. B. (1968) Antonie van Leeuwenhoek **34**, 417–432

Crawford, R. L. & Frick, T. D. (1977) Appl. Environ. Microbiol. **34**, 170–174

Crawford, R. L., Hutton, S. W. & Chapman, P. J. (1975) J. Bacteriol. **121**, 794–799

Crawford, R. L., Olson, P. E. & Frick, T. D. (1979) Appl. Environ. Microbiol. **38**, 379–384

Culbertson, J. B. (1951) J. Am. Chem. Soc. **73**, 4818–4823

Da Fonseca-Wollheim, F., Bergmeyer, H. U. & Gutmann, I. (1974) in Methoden der Enzymatischen Analyse (Bergmeyer, H. U., ed.), pp. 1850–1853, Verlag Chemie, Weinheim

Dagley, S. (1975) Essays Biochem. **11**, 81–138

Decker, R. H., Kang, H. H., Leach, F. R. & Henderson, L. M. (1961) J. Biol. Chem. **236**, 3076–3082

Dull, B. J., Salata, K., van Langenhove, A. & Goldman, P. (1987) Biochem. Pharmacol. **36**, 2467–2472

Flamm, W. G. & Crandall, D. I. (1963) J. Biol. Chem. **238**, 389–396

Foster, J. W. & Moat, A. G. (1980) Microbiol. Rev. **44**, 83–105

Greenhill, J. V. (1977) Chem. Soc. Rev. **6**, 277–294

Hagedorn, S. R., Bradley, G. & Chapman, P. J. (1985) J. Bacteriol. **163**, 640–647

Hochreiter, M. C., Patek, D. R. & Schellenberg, K. A. (1972) J. Biol. Chem. **247**, 6271–6276

Khan, A. K. A., Piris, J. & Truelove, S. C. (1977) Lancet **ii**, 892–895

Kiyohara, H. & Nagao, K. (1977) Agric. Biol. Chem. **41**, 705–707

Koontz, W. A. & Shiman, R. (1976) J. Biol. Chem. **251**, 368–377

Kuhm, A. E., Schlömann, M., Knackmuss, H.-J. & Pieper, D. H. (1990) Biochem. J. **266**, 877–883

Lack, L. (1959) Biochim. Biophys. Acta **34**, 117–123

Ladd, J. N. (1962) Nature (London) **194**, 1099–1100

Marcotte, P. & Walsh, C. (1978) Biochemistry **17**, 5613–5619

Mitchell, R. A., Kang, H. H. & Henderson, L. M. (1963) J. Biol. Chem. **238**, 1151–1155

Nörtemann, B., Baumgarten, J., Rast, H. G. & Knackmuss, H.-J. (1986) Appl. Environ. Microbiol. **52**, 1195–1202

Peppercorn, M. A. & Goldman, P. (1972) J. Pharmacol. Exp. Ther. **181**, 555–562

Pfennig, N. & Lippert, K. D. (1966) Arch. Mikrobiol. **55**, 245–256

Porter, D. J. T. & Bright, H. J. (1972) Biochem. Biophys. Res. Commun. **46**, 571–576

Sharak-Genthner, B. R., Davis, C. L. & Bryant, M. P. (1981) Appl. Environ. Microbiol. **42**, 12–19

Stanier, R. Y. & Hayaishi, O. (1951) Science **114**, 326–330

Suda, M., Takeda, Y., Sujishi, K. & Tanaka, T. (1951) J. Biochem. (Tokyo) **38**, 297–302

Tanaka, H., Sugiyama, S., Yano, K. & Arima, K. (1957) Bull. Agric. Chem. Soc. Jpn. **21**, 67–68

Tjørnelund, J., Hansen, S. H. & Cornett, C. (1989) Xenobiotica **19**, 891–899

Vescia, A. & Di Prisco, G. (1962) J. Biol. Chem. **237**, 2318–2324

Wheelis, M. L., Palleroni, N. J. & Stanier, R. Y. (1967) Arch. Mikrobiol. **59**, 302–314