Microbial Metabolism of the Pyridine Ring

METABOLISM OF 2- AND 3-HYDROXYPYRIDINES BY THE MALEAMATE PATHWAY IN *ACHROMOBACTER* SP.

By RONALD B. CAIN,* CHARLES HOUGHTON† and KEITH A. WRIGHT

Microbiology Group, Department of Botany, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K., and Biological Laboratories, University of Kent, Canterbury CT2 7NJ, Kent, U.K.

(Received 19 October 1973)

1. Washed suspensions of two *Achromobacter* species (G2 and 2L), capable of growth upon 2- and 3-hydroxypyridine respectively as sources of C and N, rapidly oxidized their growth substrate pyridine-2,5-diol (2,5-dihydroxypyridine) and the putative ring-cleavage product maleamate without a lag. Suspensions derived from fumarate plus $(\text{NH}_4)_2\text{SO}_4$ cultures were unable to do so. 2. Extracts of both bacteria oxidized pyridine-2,5-diol with the stoichiometry of an oxygenase forming 1 mol of NH_3 /mol of substrate. 3. Heat-treated extracts, however, formed maleamate and formate with little free NH_3 . 4. The conversion of maleamate into maleate plus NH_3 by extracts of strain 2L, fractionated with $(\text{NH}_4)_2\text{SO}_4$, and the metabolism of maleamate and maleate to fumarate by extracts of both strains demonstrated the existence of the enzymes catalysing each reaction of the maleamate pathway in these bacteria. 5. The pyridine-2,5-diol dioxygenase (mol.wt. approx. 340000) in extracts of these *Achromobacter* species required Fe^{2+} ($1.7\ \mu\text{M}$) to restore full activity after dialysis or treatment with chelating agents; the enzyme from strain 2L also had a specific requirement for L-cysteine (6.7 mM), which could not be replaced by GSH or dithiothreitol. 6. The oxygenase was strongly inhibited in a competitive manner by the isomeric pyridine-2,3- and -3,4-diols.

Houghton & Cain (1972) described the isolation of certain Gram-negative bacteria that utilized the three isomers of hydroxypyridine. The metabolic pathway used by *Agrobacterium* to degrade 4-hydroxypyridine and its metabolite pyridine-3,4-diol had several novel features and has been described in the two preceding papers (Watson *et al.*, 1974a,b). Three strains of *Achromobacter*, isolated at the same time, produced pyridine-2,5-diol from both 2- and 3-hydroxypyridine (Houghton & Cain, 1972). The present paper gives evidence that this intermediate is degraded by the maleamate pathway in the two most metabolically active strains.

Materials

Microbiological

The isolation of the *Achromobacter* strains G2, 2L and 7N, their growth characteristics and the production of washed suspensions and cell-free extracts have been described (Houghton & Cain, 1972).

* Present address: Biological Laboratories, University of Kent, Canterbury CT2 7NJ, Kent, U.K.

† Present address: Department of Biological Sciences, Napier College of Science and Technology, Edinburgh EH10 5DT, U.K.

Crude extracts, although consuming O_2 at the expense of pyridine-2,5-diol, did not accumulate any detectable organic products from this substrate so were treated in a variety of ways to effect the accumulation of formate, maleamate, maleate and fumarate.

Extracts of G2 (2-hydroxypyridine utilizer). Crude extracts in 0.05 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 7.0, containing about 14 mg of protein/ml were heated at 55°C for 10 min with constant stirring. The precipitated protein was removed by centrifugation at 15000g for 15 min. This preparation converted pyridine-2,5-diol into maleamate.

After fractionation with solid $(\text{NH}_4)_2\text{SO}_4$, the fraction of these same crude extracts that was precipitated between 20 and 30% (w/v) saturation (termed the G_1 fraction) was able to convert maleamate or maleate into fumarate but would not further metabolize fumarate.

Extracts of 2L (3-hydroxypyridine utilizer). Crude extracts, in the same buffer as above, were heated at 58°C for 10 min but were otherwise treated identically to the G2 extracts. Heat-treated 2L extracts converted pyridine-2,5-diol into a mixture of maleamate and fumarate with the former predominating (see the Results section).

When crude extracts of the 2L organism were fractionated with solid $(\text{NH}_4)_2\text{SO}_4$, that fraction which precipitated between 20 and 30% saturation (termed

Table 1. *Partial purification of the pyridine-2,5-diol dioxygenase in extracts of Achromobacter 2L*

Details of the purification procedures are described in the text. One unit of activity is defined as the disappearance of 1 μ mol of pyridine-2,5-diol/min and is equivalent to a $-\Delta E_{320}$ /min of 1.87 under the conditions of the assay.

Material	Volume (ml)	Total activity (units)	Recovery of activity (%)	Protein (mg)	Specific activity (units/mg of protein)	Purification factor
1. Crude extract	45	185	100	290	0.64	—
2. Heat-treated extract (55°C, 10 min)	44	166	90	105	1.6	2.5
3. 20–30%-satd.-(NH ₄) ₂ SO ₄ fraction	3	120	65	32	3.4	5.3
4. Sephadex G-200 eluate (fractions 15–19, pooled)	20	77	42	7	11.0	17.0
5. 0–50%-satd.-(NH ₄) ₂ SO ₄ fraction of pooled eluate	2.5	46	25	3	15.3	24.0

the L₁ fraction) was able to hydrolyse maleamate to maleate plus NH₃ but could not isomerize the maleate so formed.

When crude heat-treated extracts of either bacterium were centrifuged at 150000g for 1–2 h in the 10×10 ml angle-head of an MSE SuperSpeed 50 ultracentrifuge, the small particles were completely removed. Such extracts were able to accumulate formate from pyridine-2,5-diol. Concentrated crude extracts of both bacteria that had not been subjected to this treatment often showed O₂ uptakes in excess of that required for the dioxygenase-catalysed oxidation of pyridine-2,5-diol, attributable to the oxidation of the formate so produced to CO₂.

Partial purification of pyridine-2,5-diol dioxygenase. Packed cells (20 g wet wt.) were ruptured in the Hughes press (Hughes, 1951) and the crush was suspended at 10°C in 2 vol. of 0.05 M-Na₂HPO₄-KH₂PO₄ buffer, pH 7.0. The unbroken cells and cell debris were removed by centrifugation at 50000g for 30 min to leave the crude extract (step 1). The crude extract was then subjected with constant stirring to a temperature of 55°C for 10 min in a jacketed water bath, the temperature of which was controlled within $\pm 0.1^\circ\text{C}$ by a 'Circotherm' unit (Shandon Scientific Co., London N.W.10, U.K.). The mixture was rapidly cooled in an ice bath and the coagulated protein removed by centrifugation at 15000g for 30 min (step 2). The supernatant (44 ml) was stirred continuously in a cold-room at 4°C during the slow addition of solid powdered (NH₄)₂SO₄ (4.7 g; 20% saturation). The solution was left for 30 min and the small precipitate removed by centrifugation and discarded. Further (NH₄)₂SO₄ (3 g) was added to bring the concentration to 30% saturation. The precipitate formed after being left for 30 min was collected by centrifugation, the pellet washed carefully with a saturated neutral solution of (NH₄)₂SO₄, drained and dissolved in 3 ml of 0.05 M-Na₂HPO₄-KH₂PO₄ buffer, pH 7.0 (step 3). The material from step 3 was placed on the

surface of a column (45 cm×2.5 cm) of Sephadex G-200 equilibrated with the same buffer containing 6.7 mM-L-cysteine. The column was eluted with the cysteine-phosphate buffer, pH 7.0, by downward flow at 30 ml/h at 4°C; 4 ml fractions were collected. Protein was monitored by E₂₈₀. The oxygenase activity was well separated from most of the protein and eluted at about 70 ml, coincident with a small subsidiary protein peak. Fractions 15–19 were pooled (20 ml) (step 4). The protein in this solution was precipitated by the addition of solid (NH₄)₂SO₄ (6.3 g; 50% saturation), collected by centrifugation at 50000g for 15 min and redissolved in 2.5 ml of 0.05 M-Na₂HPO₄-KH₂PO₄ buffer, pH 7.0, containing 6.7 mM-L-cysteine. This solution was dialysed against 500 ml of the same buffer for 2 h and then used immediately (step 5). The results of a purification of the *Achromobacter* 2L enzyme are summarized in Table 1.

Chemical

The isomeric pyridinediols were prepared and purified as described by Houghton & Cain (1972). After its unequivocal identification as pyridine-2,5-diol, the material accumulating in large amounts in cultures of *Achromobacter* 7N growing with 3-hydroxypyridine was a far more convenient source of this compound than was the Elbs oxidation of 2-hydroxypyridine (Behrman & Pitt, 1958). The natural product was isolated and recrystallized from the culture filtrates as described by Houghton & Cain (1972). Maleic acid (m.p. 130°C) and maleamic acid (m.p. 171–172°C) were obtained as white crystals by recrystallization of the commercially available products from diethyl ether and ethanol-ether (2:1, v/v) respectively. Sauers & Cotter (1961) recorded a melting point of 165–168°C for maleamic acid.

Fumaramic acid was obtained by deamination of L-asparagine as follows. L-Asparagine (14 g) was dissolved in 0.04 M-Na₂HPO₄-KH₂PO₄ buffer, pH 6.7

(1170ml), and the solution heated at 100°C in a steam bath for 24h. After being cooled, 580ml of the solution was passed through a column of Dowex 50 ion-exchange resin (X8; H⁺ form; bed vol. 330ml), which adsorbed residual aspartic acid and asparagine. The effluent was collected and the Dowex column regenerated before the second batch of reaction mixture was applied. The combined effluents were then passed down a column of Dowex 2 (X8; OH⁻ form; bed vol. 280ml), which adsorbed fumaramic acid and phosphate ions. The former was eluted with 1.6 litres of 1M-formic acid and the eluted material evaporated to dryness *in vacuo* at 40°C. The residue was dissolved in the minimum volume of distilled water at 50–60°C, filtered hot and the filtrate left at 4°C for 24h. A white crystalline solid (0.62g), m.p. 226°C but subliming at 215°C by the capillary-tube method, was obtained after several recrystallizations from water. Talley *et al.* (1959) recorded a melting point of 216.5–217.5°C for this compound. Other chemicals used were AnalaR grade materials from BDH Chemicals Ltd., Poole BH12 4NN, Dorset, U.K.

The nomenclature of the hydroxypyridines and pyridinediols is that of Watson *et al.* (1974a).

Methods

Quantitative determinations

Enzyme reactions and changes in substrate concentrations were measured spectrophotometrically with a Pye Unicam SP.800 recording spectrophotometer fitted with the SP.820 constant-wavelength attachment, scale-expansion facilities and a constant-temperature cell housing maintained at 30°C by circulating water. Colorimetric determinations were made with the Pye Unicam SP.600 single-beam instrument.

Pyridine-2,5-diol has a light-absorption maximum at 320nm (ϵ 5620 litre·mol⁻¹·cm⁻¹) and its disappearance in cultures or incubation mixtures was followed spectrophotometrically at this wavelength. Maleamate and maleate have significantly lower molar extinction coefficients at 240–290nm than has fumarate so that the formation of fumarate from the two precursors led to increases in extinction over this wavelength range. Addition of crystalline fumarate hydratase (EC 4.2.1.2) to a reaction mixture accumulating fumarate produced an immediate fall in extinction of the solution owing to the formation of malate which has negligible u.v. light absorption at these wavelengths. For convenience, experiments were carried out at 290nm where extinction coefficients for maleamate, maleate and fumarate are 34.1, 16.4 and 170 litre·mol⁻¹·cm⁻¹ respectively.

Maleamate, maleate and fumarate in reaction mixtures were determined quantitatively by titration with 10mM-NaOH after chromatographic separation

as the acids on a column (30cm×1cm diam.) of silicic acid prepared and used essentially by the methods described by Swim & Utter (1957). Preliminary experiments with known amounts of material showed recoveries in excess of 90% for all three acids.

Formate and NH₃ were determined and O₂ uptakes measured manometrically by the methods described by Houghton & Cain (1972).

Protein concentrations were measured by the biuret method of Gornall *et al.* (1949) and spectrophotometrically by the method of Warburg & Christian (1941). These techniques were calibrated with recrystallized bovine serum albumin [Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K.]. Measurements on the same protein samples by these two methods showed that biuret determinations were higher by 3.6-fold. Results from spectrophotometric measurements were therefore increased by this amount.

Enzyme assays

Pyridine-2,5-diol 5,6-dioxygenase (EC 1.13.11.9) was measured in two ways: (i) by O₂ consumption, either in the Warburg respirometer (Umbreit *et al.*, 1957), or where the addition to reaction mixtures of further components would interfere with spectrophotometric assay by using an oxygen electrode with a 3ml capacity cell (Rank Bros., Bottisham, Cambs., U.K.) linked to a Unicam AR-45 slave recorder (Watson *et al.* 1974b); (ii) spectrophotometrically at 320nm and 30°C in silica cuvettes (10mm light-path) containing Na₂HPO₄–K₂HPO₄ buffer, pH7.0 (100μmol), freshly prepared FeSO₄ (30nmol), substrate and extract in a total volume of 3ml.

Certain crystalline enzyme preparations were used as markers to determine the molecular weight of the dioxygenase (see below). Enzyme activities in the eluted fractions were measured as follows: L-glutamate dehydrogenase by the method of Cooper *et al.* (1958); L-lactate dehydrogenase by the method of Kubowitz & Ott (1943); L-malate dehydrogenase by the method of Mehler *et al.* (1948); fumarate hydratase by the method of Racker (1950).

Chromatography

Paper chromatography of the organic acids was done by the ascending method on Whatman no. 1 paper in the following solvent systems: 1, ethanol–conc. NH₃ (sp.gr. 0.88)–water (20:1:4, by vol.); 2, diethyl ether–benzene (7:3, v/v, and containing sufficient formic acid to make the solution 3M; the mixture was then saturated with water and the organic phase used). To locate organic acids, the chromatograms were dried at 95°C for 30min to remove formic acid from the solvent and then sprayed with an aqueous solution of Bromophenol Blue (50mg/100ml) made slightly alkaline with aq. 2M-NH₃ giving yellow spots on a blue background.

Alternatively, the chromatograms were dried for about 5 min at 95°C and sprayed with the same solution but also containing 200 mg of citric acid to produce a yellow background with blue spots. Acids with unsaturated double bonds also showed up well after spraying with a solution of pyridine-acetic anhydride (9:1, v/v) or 0.05% (w/v) KMnO_4 in aq. 50% (v/v) ethanol.

Determination of enzyme molecular weight

The technique of Whitaker (1963) with marker proteins of known molecular weight was used to calibrate a column (90 cm $\times$ 0.9 cm diam.) of Sephadex G-200. Preparations of pyridine-2,5-diol dioxygenase purified to step 4 (approx. 1.5 mg of protein) were concentrated to 4 ml by dialysis against a 15% (w/v) solution of CarboWax 4000 (BDH Chemicals Ltd.) for 15 h at 4°C. To this solution was added 250 μg of crystalline L-glutamate dehydrogenase (EC 1.4.1.3; mol.wt. 1×10^6 ; Koch-Light Laboratories Ltd., Colnbrook SL3 0BZ, Bucks., U.K.), 250 μg of crystalline beef heart L-lactate dehydrogenase [EC 1.1.1.27; mol.wt. 1.35×10^5 ; Sigma (London) Chemical Co. Ltd.], 100 μg of crystalline L-malate dehydrogenase [EC 1.1.1.37; mol.wt. 4×10^4 ; Sigma (London) Chemical Co. Ltd.], 200 μg of crystalline fumarate hydratase [EC 4.2.1.2; mol.wt. 2.05×10^5 ; Sigma (London) Chemical Co. Ltd.] and 1 mg of Blue Dextran 2000 [mol.wt. 2×10^6 ; Pharmacia (G.B.) Ltd., London W.13, U.K.], and the mixture applied to the top of the gel column. The proteins were eluted with 0.05 M Na_2HPO_4 - KH_2PO_4 buffer, pH 7.0, containing 6.7 mM-L-cysteine and 1 ml fractions collected. The pyridine-2,5-diol dioxygenase activity from extracts of both bacteria was eluted around fractions 56-63.

Results and Discussion

Achromobacter 7N, which quantitatively converted 3-hydroxypyridine into pyridine-2,5-diol under conditions of decreased aeration and even accumulated this diol in large amounts in normally aerated cultures (Houghton & Cain, 1972), had only a restricted ability to further metabolize the diol, so for subsequent metabolic studies the two strains G2 and 2L, capable of growth at the expense of 2- and 3-hydroxypyridine respectively, were used.

Washed suspensions of strain G2, grown with 2-hydroxypyridine, oxidized this substrate and pyridine-2,5-diol without a lag as described by Houghton & Cain (1972), but also oxidized the putative metabolic products maleamate and maleate consuming 2.5 mol of O_2 /mol of these acids. Similar suspensions of strain 2L obtained from 3-hydroxypyridine cultures would oxidize only maleamate (1.5 mol of O_2 /mol of maleamate). The defective strain 7N would

oxidize neither acid. After growth with fumarate plus $(\text{NH}_4)_2\text{SO}_4$, none of these bacteria could oxidize its respective heterocyclic substrate, pyridine-2,5-diol or the two organic acids.

Oxidation of pyridine-2,5-diol by crude cell-free extracts

Crude extracts of both G2 and 2L strains, prepared from cells grown with their appropriate hydroxypyridine substrate, oxidized only pyridine-2,5-diol of the eight isomeric pyridinediols examined; 1 mol of O_2 /mol of the 2,5-diol was consumed provided that the protein concentration of the extracts exceeded 4 mg/ml. Extracts more dilute than this showed little or no O_2 uptake. The uptake of O_2 was paralleled by a concomitant loss of the E_{320} owing to the disappearance of pyridine-2,5-diol. The end products of metabolism by crude extracts had no u.v. absorption. No such loss of E_{320} occurred when the reaction was carried out under a N_2 atmosphere.

After complete oxidation of pyridine-2,5-diol by extracts of *Achromobacter* G2 or 2L, NH_3 could be detected among the end products. Quantitative determinations showed that extracts of both micro-organisms produced 0.87-0.93 mol of NH_3 /mol of substrate oxidized.

Metabolism by heat-treated extracts

Extracts of strains G2 and 2L, heated for 10 min at 55° and 58°C respectively, oxidized pyridine-2,5-diol with the same O_2 stoichiometry as crude extracts but accumulated acidic products. Paper chromatography of the products of pyridine-2,5-diol metabolism by heat-treated strain G2 extracts showed the presence of maleamic acid; when heat-treated extracts of strain 2L were used, both maleamic and smaller amounts of fumaric acid were observed (Table 2).

When the experiments were repeated, the acidic products separated on a silicic acid column and measured quantitatively, heat-treated strain G2 extracts converted 10 μmol of pyridine-2,5-diol into 8.7 μmol of maleamic acid. Similar extracts of *Achromobacter* 2L gave 5.2 μmol of maleamic acid and 2.1 μmol of fumaric acid. Fumaramate was never detected as a product of either pyridine-2,5-diol or maleamate metabolism. NH_3 production by these heat-treated extracts was approx. 5-10% of that found when fresh crude extracts were used.

The other major product of pyridine-2,5-diol metabolism by extracts was formate, which accumulated under conditions where further oxidation of formate was prevented; e.g. by removing the particle fraction from heat-treated (55°C, 10 min) extracts by ultracentrifugation. When such clarified extracts (5 ml, containing 80 mg of extract protein) were incubated at 30°C with Na_2HPO_4 - KH_2PO_4 buffer (pH 7.0,

Table 2. Identification of the metabolic products of pyridine-2,5-diol produced by extracts of *Achromobacter*

For experiments with extracts of strain G2 the reaction mixtures contained: $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 7.0, 250 μmol ; pyridine-2,5-diol, 20 μmol ; heat-treated extract (55°C, 10 min) (3 ml; equiv. to 42 mg of extract protein); distilled water to a total volume of 7 ml. These were placed in the main compartment of large (30 ml capacity) Warburg flasks and incubated at 30°C. After O_2 uptake had ceased, the solutions were deproteinized by acidification to pH 2 with 10% (w/v) metaphosphoric acid and the organic acids extracted with 5×20 ml portions of diethyl ether. The ethereal extracts were evaporated to dryness *in vacuo*, the residue was dissolved in ethanol and the organic acids were separated by paper chromatography. For experiments with extracts of strain 2L, reaction mixtures contained: $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 7.0, 1 mmol; pyridine-2,5-diol, 16 μmol ; heat-treated extract (58°C, 10 min) (5 ml; equiv. to 75.5 mg of protein); distilled water to a total volume of 150 ml. These were placed in 250 ml capacity Erlenmeyer flasks, incubated at 30°C with slow shaking and the reaction was followed spectrophotometrically at 320 nm on samples removed from the incubation mixture. When all the substrate had disappeared, the solutions were treated as above. Organic acids on the chromatograms were located with the Bromophenol Blue–citric acid spray, and further identified by their u.v. absorbance and their reaction with the pyridine–acetic anhydride reagent. No compounds were found in control incubations without pyridine-2,5-diol. n.t., Not tested.

Compound	R_F in solvents:		Reaction with pyridine–acetic anhydride	Absorbance in u.v. light
	1	2		
Compound from incubations with strain G2 extracts	0.37	0.53	—	—
Compounds from incubations with strain 2L extracts	0.37	0.53	—	+
	0.20	0.91	+	+
Maleamic acid	0.37	0.53	—	+
Maleic acid	0.16	0.57	+	+
Fumaramic acid	0.34	n.t.	+	+
Fumaric acid	0.20	0.91	—	+

Fig. 1. Conversion of maleamate and maleate into fumarate by extracts of *Achromobacter* G2

The reaction was followed spectrophotometrically at 290 nm and 30°C in cuvettes containing: $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 7.0, 60 μmol ; the G_1 fraction of an extract of organism G2, 0.2 ml (equiv. to 3.2 mg of protein); distilled water to make the final volume to 3 ml; in trace (1) sodium maleamate, 28 μmol ; in trace (2) sodium maleate, 28 μmol ; in trace (3) sodium maleamate, 28 μmol plus potassium *meso*-tartrate, 30 μmol . The reference cuvette contained all components except the substrates. Crystalline fumarate hydratase (about 40 EC units) was added at the time indicated by the arrows.

250 μmol) and pyridine-2,5-diol (5 μmol), total volume 7 ml, in 30 ml capacity Warburg flasks and the reaction was followed till O_2 uptake ceased, analysis of the flask contents by the method of Nash (1953) showed that 4.9 μmol of formate was produced from 5 μmol of pyridine-2,5-diol.

Conversion of maleamate and maleate into fumarate

Extracts of *Achromobacter* G2 grown with 2-hydroxypyridine were treated with $(\text{NH}_4)_2\text{SO}_4$ as described in the Methods section and the fraction (G_1) precipitated between 20–30% saturation was used to demonstrate spectrophotometrically the production of fumarate from maleamate and maleate (Fig. 1). Addition of 10 mM-*meso*-tartrate, a known inhibitor of fumarate hydratase (Massey, 1953), enhanced the production of fumarate in these experiments presumably by inhibiting the residual endogenous fumarate hydratase in the extracts, but accumulation of fumarate never approached the theoretical amount, presumably owing to the formation of an equilibrium mixture of maleate and fumarate. Addition of crystalline fumarate hydratase (about 40 EC units) after 30 min led to an immediate loss of the E_{290} as the accumulated fumarate was used and the maleate–fumarate equilibrium displaced in the direction of fumarate. Fumarate was confirmed in the original reaction mixtures by paper chromatography of the diethyl ether extracts after acidification of the cuvette contents with 10% (w/v) metaphosphoric acid.

Fig. 2. Conversion of maleamate into maleate by extracts of *Achromobacter* 2L

Cuvettes contained: $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 7.0, $300\text{ }\mu\text{mol}$; the L_1 fraction of an extract of organism 2L, 0.1 ml (equiv. to 2.4 mg of protein); distilled water to make the final volume to 3 ml . The reaction was started by the addition of sodium maleamate, $28\text{ }\mu\text{mol}$. When the decrease in E_{290} ceased, 0.05 ml of the G_1 fraction of an extract of organism G2 was added (at arrow A). This fraction can convert the accumulated maleate into fumarate and resulted in an increase in E_{290} though this was not followed to completion. Crystalline fumarate hydratase (about 40 EC units) was added at arrow B. The reference cuvette received all additions except the original sodium maleamate.

Similar reaction mixtures containing extracts of the 3-hydroxypyridine-utilizer *Achromobacter* 2L were unable to produce an increase in E_{290} even when tartrate was added. The accumulation of fumarate from maleamate and maleate was eventually observed in reaction mixtures containing heat-treated (58°C , 10 min) cell-free extracts of this organism. This treatment destroyed endogenous fumarate hydratase in these extracts since added fumarate was not degraded.

Conversion of maleamate into maleate and NH_3

Whereas crude extracts oxidizing pyridine-2,5-diol to completion produced approx. 1 mol of NH_3/mol of substrate, heat-treated extracts that accumulated maleamate and formate from the diol showed only very low NH_3 production, suggesting that the fate of the pyridine-N was first the amide group of maleamate and thence NH_3 formed by deamidation of the amide to maleate. The fraction (L_1) of *Achromobacter* 2L extracts which was precipitated by $(\text{NH}_4)_2\text{SO}_4$ between $20\text{--}30\%$ saturation catalysed the formation of maleate from maleamate ($28\text{ }\mu\text{mol}$) (Fig. 2). The E_{290} of the cuvette contents decreased 0.155 over a period of 65 min , equivalent to the formation of $28\text{ }\mu\text{mol}$ of maleate from maleamate. A sample removed from a second cuvette with identical contents was analysed

for NH_3 and showed the production of $25.5\text{ }\mu\text{mol}$ of NH_3 from $28\text{ }\mu\text{mol}$ of maleamate. The presence of maleate was confirmed by extracting the acidified cuvette contents with $5\times 20\text{ ml}$ portions of diethyl ether, evaporating the ethereal extracts to dryness and dissolving the residue in the minimum volume of ethanol. Paper chromatography of the ethanolic extract in solvents 1 and 2 showed the presence of maleic acid (R_F 0.15 and 0.56 respectively in these solvents). When fumaramate was substituted for maleamate, only $1.7\text{ }\mu\text{mol}$ of NH_3 was produced in the same incubation period and no trace of fumaric acid was detected by paper chromatography.

Maleate was not degraded further in the above system, but if to a reaction mixture in which maleate had accumulated was added (Fig. 2, arrow A) the G_1 fraction of an extract of organism G2 (capable of converting maleate into fumarate), there was an immediate increase in E_{290} as maleate was isomerized to fumarate. This increase in E_{290} was rapidly and completely reversed by the subsequent addition of crystalline fumarate hydratase (Fig. 2, arrow B). If the cuvette contents were analysed by paper chromatography at this stage, only fumaric acid (R_F 0.20 and 0.90 in solvents 1 and 2 respectively) could be detected.

Since fumaramate was produced neither from pyridine-2,5-diol or maleamate, nor converted into fumarate plus NH_3 by any of these extracts at a significant rate, the metabolic pathway in these bacteria is therefore:

Pyridine-2,5-diol dioxygenase in extracts of *Achromobacter* G2 and 2L

The stoichiometry for pyridine-2,5-diol and O_2 in the reaction with crude extracts suggested the presence of a dioxygenase similar to that described by Behrman & Stanier (1957) in *Pseudomonas putida*, growing at the expense of nicotinic acid, and to that implicated in other pseudomonads, with nicotine or picolinamide as their C and N sources (Tabuchi, 1955; Gherna & Rittenberg, 1962; Orpin *et al.*, 1972).

The oxygenase activity in crude extracts was stable for several days at 4°C and for at least several weeks at -20°C . In this crude form, the enzyme withstood exposure for 10 min at 60°C without loss of activity but after the same period at 65°C only 25% of the activity remained. The enzyme purified to step 4 (Table 1) was much less stable and lost activity rapidly in air ($t_{1/2} \approx 12\text{ h}$) even in the presence of cysteine.

The enzyme was partially purified from both *Achromobacter* strains, but that from strain 2L, which was eluted from Sephadex G-200 coincident with a

Table 3. *Specific requirement for L-cysteine for protection and reactivation of pyridine-2,5-diol dioxygenase in extracts of Achromobacter 2L*

In Expt. 1, the protecting agents were included in the following reaction mixtures: $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 7.0, 100 μmol ; extract protein (purified to step 4 of Table 1), 0.4 ml (equiv. to 80 μg of protein); distilled water to a final volume of 2.97 ml. These mixtures, except for one control, were preincubated at 4°C for 6 h, after which they were transferred to cuvettes together with pyridine-2,5-diol (0.2 μmol) and FeSO_4 (30 nmol). The enzyme activity was then measured spectrophotometrically. In Expt. 2, L-cysteine at the concentration shown was included in the standard assay mixture and assays were carried out immediately. The enzyme used was the same as in Expt. 1 above.

Protecting agent added	$-\Delta E_{320}/\text{min}$	Enzyme activity (% of control value)
Expt. 1. None (assayed immediately)	0.016	100
None	0.008	50
Acetone (10%, v/v)	0.008	50
Ethanol (10%, v/v)	0.011	66
GSH (2 mM)	0.008	50
Mercaptoethanol (2 mM)	0.008	50
Dimercaptopropanol (2 mM)	0.008	50
Dithiothreitol (2 mM)	0.008	50
L-cysteine (2 mM)	0.058	358
Expt. 2. None	0.018	100
L-Cysteine (0.1 mM)	0.050	270
L-Cysteine (3.3 mM)	0.128	710
L-Cysteine (6.7 mM)	0.234	1310
L-Cysteine (10 mM)	0.176	970

Table 4. *Inhibition of pyridine-2,5-diol dioxygenase from Achromobacter 2L by the isomeric pyridinediols*

Cuvettes contained: $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 7.0, 100 μmol ; extract, 1 ml (equiv. to 0.5 mg of protein); the inhibiting pyridinediol, 0.5 μmol ; distilled water to 2.9 ml. The cuvette contents were preincubated at 30°C for 5 min and the reaction was started by addition of pyridine-2,5-diol (0.5 μmol ; 0.1 ml). The reference cuvette contained all components except substrate. The $-\Delta E_{320}/\text{min}$ of the non-inhibited control was 0.195.

Pyridinediol isomer added as inhibitor	Enzyme activity (% of control value)
None	100
Pyridine-1,2-diol	18
Pyridine-1,3-diol	85
Pyridine-1,4-diol	49
Pyridine-2,3-diol	18
Pyridine-2,4-diol	23
Pyridine-2,6-diol	70
Pyridine-3,4-diol	11

et al., 1972). The oxygenase present in extracts of *Achromobacter* G2 was not protected by cysteine nor was activity stimulated by cysteine after storage for 5 days at 4°C.

The enzymic activity of extracts prepared from either organism was completely lost on dialysis against phosphate buffer, by incubation with or dialysis against 0.1 μM -8-hydroxyquinoline and by $(\text{NH}_4)_2\text{SO}_4$ fractionation. Addition of 6.7 mM-L-cysteine and fresh Fe^{2+} (1.7 μM) to these preparations completely restored oxygenase activity; Mn^{2+} , Co^{2+} , Cu^{2+} , Ni^{2+} , Zn^{2+} , Mo^{5+} and Fe^{3+} (0.1 μM –0.1 mM) in the presence or the absence of cysteine were without effect.

Determinations of the molecular weight of pyridine-2,5-diol dioxygenase (purified as far as step 4, Table 1) by the method of Whitaker (1963) on Sephadex G-200 gave values of 340000 for the strain G2 enzyme and 330000 for the strain 2L preparation. These values are very similar to that of pyridine-3,4-diol dioxygenase from *Agrobacterium* sp. (Watson *et al.*, 1974b) and of the same order of magnitude as that of the crystalline pyridine-2,5-diol dioxygenase (242000) obtained from *P. putida* by Gauthier & Rittenberg (1971a).

Extracts of either strain G2 or 2L consumed O_2 only with pyridine-2,5-diol of the eight isomeric diols. These isomers, however, displayed various degrees of inhibition when they were preincubated for 5 min with the partially purified (step 5) enzyme from strain 2L. The *ortho*-substituted compounds (pyridine-1,2-,

small subsidiary protein peak, required 6.7 mM-L-cysteine for maximum activity. Mercaptoethanol, dimercaptopropanol, dithiothreitol or GSH did not stimulate activity (Table 3) in *Achromobacter* 2L extracts, whereas preincubation with dithiothreitol plus Fe^{2+} would restore activity of the enzyme from *P. putida* (Gauthier & Rittenberg, 1971a) and GSH plus Fe^{2+} would do so with the enzyme from a Gram-negative bacterium utilizing picolinamide (Orpin

-2,3- and -3,4-diols) produced the greatest degrees of inhibition (in the range 80–90%), whereas those isomers with *meta*- and *para*-substitution patterns were generally much less effective (Table 4). The inhibition produced by the 2,3- and 3,4-diol isomers was competitive. It is noteworthy that those isomers which are either the substrate or the most effective competitive inhibitors of the pyridine-2,5-diol dioxygenase are also those which are produced by treating 2- and 3-hydroxypyridines with the ascorbate-Fe²⁺-EDTA model hydroxylating system (Udenfriend *et al.*, 1954; Houghton & Cain, 1972). The hydroxylases of the *Achromobacter* strains G2 and 2L are clearly much more specific because they produced only the *para*-substituted pyridine-2,5-diol from both hydroxypyridine isomers.

Our purest preparation of the oxygenase still produced maleamate and formate from pyridine-2,5-diol, not *N*-formylmaleamate which would be expected as the product of a C-5–C-6 dioxygenase cleavage of this heterocyclic substrate (Behrman & Stanier, 1957). In view, however, of the careful ¹⁸O studies done with the crystalline oxygenase from *P. putida* by Gauthier & Rittenberg (1971*b*), in which they showed that the products were maleamate and formate and that *N*-formylmaleamate was neither formed by nor a substrate for the enzyme, we have considered it unnecessary to pursue this investigation further.

This work was supported by research grants from the Science Research Council and The Royal Society to R. B. C. which are gratefully acknowledged. C. H. was in receipt of a S.R.C. studentship.

References

- Behrman, E. J. & Pitt, B. M. (1958) *J. Amer. Chem. Soc.* **80**, 3717–3718
- Behrman, E. J. & Stanier, R. Y. (1957) *J. Biol. Chem.* **228**, 923–945
- Cooper, J., Srere, P. A., Tabachnick, M. & Racker, E. (1958) *Arch. Biochem. Biophys.* **74**, 306–314
- Gauthier, J. J. & Rittenberg, S. C. (1971*a*) *J. Biol. Chem.* **246**, 3737–3742
- Gauthier, J. J. & Rittenberg, S. C. (1971*b*) *J. Biol. Chem.* **246**, 3743–3748
- Gherna, R. L. & Rittenberg, S. C. (1962) *Bacteriol. Proc.* **27**
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Houghton, C. & Cain, R. B. (1972) *Biochem. J.* **130**, 879–893
- Hughes, D. E. (1951) *Brit. J. Exp. Pathol.* **32**, 97–109
- Kubowitz, F. & Ott, P. (1943) *Biochem. Z.* **314**, 94–117
- Massey, V. (1953) *Biochem. J.* **55**, 172–177
- Mehler, A. H., Kornberg, A., Grisolia, S. & Ochoa, S. (1948) *J. Biol. Chem.* **174**, 961–977
- Nash, T. (1953) *Biochem. J.* **55**, 416–421
- Orpin, C. G., Knight, M. & Evans, W. C. (1972) *Biochem. J.* **127**, 819–831
- Racker, E. (1950) *Biochim. Biophys. Acta* **4**, 211–214
- Sauers, C. K. & Cotter, R. J. (1961) *J. Org. Chem.* **26**, 6–10
- Swim, H. E. & Utter, M. F. (1957) *Methods Enzymol.* **4**, 584–595
- Tabuchi, T. (1955) *J. Agr. Chem. Soc. Japan* **29**, 219–226
- Talley, A. E., Fitzpatrick, T. J. & Porter, W. L. (1959) *J. Amer. Chem. Soc.* **81**, 174–175
- Udenfriend, S., Clark, C. T., Axelrod, J. & Brodie, B. B. (1954) *J. Biol. Chem.* **208**, 731–739
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957) *Manometric Techniques*, 3rd edn., pp. 1–76, Burgess, Minneapolis
- Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384–396
- Watson, G. K., Houghton, C. & Cain, R. B. (1974*a*) *Biochem. J.* **140**, 265–276
- Watson, G. K., Houghton, C. & Cain, R. B. (1974*b*) *Biochem. J.* **140**, 277–292
- Whitaker, J. R. (1963) *Anal. Chem.* **35**, 1950–1953