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Microbial Metabolism of the Pyridine Ring

THE HYDROXYLATION OF 4-HYDROXYPYRIDINE TO PYRIDINE-3,4-DIOL (3,4-DIHYDROXYPYRIDINE) BY 4-HYDROXYPYRIDINE-3-HYDROXYLASE

By G. KEITH WATSON,* CHARLES HOUGHTON† and RONALD B. CAIN‡
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.

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1. The first metabolic step in the biodegradation of 4-hydroxypyridine by an *Agrobacterium* sp. was hydroxylation to form pyridine-3,4-diol. 2. Extracts required 1 mol of O_2 and 1 mol of NADH or NADPH for the conversion of 4-hydroxypyridine into pyridine-3,4-diol, suggesting that the enzyme responsible, 4-hydroxypyridine-3-hydroxylase, was a mixed function mono-oxygenase. 3. After treatment with acidic $(NH_4)_2SO_4$ the enzyme required FAD for activity; FMN and riboflavin would not substitute for FAD. 4. The rate of anaerobic reduction of FAD by NAD(P)H was increased more than tenfold in the presence of 4-hydroxypyridine, suggesting that the mechanism of hydroxylation was similar to that of other aromatic hydroxylases which are of the mono-oxygenase type. 5. The partially purified enzyme was extremely specific for its heterocyclic substrate but would utilize either NADH or NADPH. 6. 4-Hydroxypyridine-3-hydroxylase was strongly inhibited by high substrate concentration (above 0.5 mM) especially below pH 7.5. 8. The inflexion at pH 8.4 in a pK_m versus pH plot, together with strong inhibition by *p*-chloromercuribenzoate, suggested a role for thiol groups in substrate binding.

The pyridine ring occurs in Nature in the form of pyridine coenzymes (nicotinamide and pyridoxal derivatives), plant alkaloids and also in a range of other, less widely distributed, natural products (e.g. dipicolinic acid in *Bacillus* spores). Other pyridine compounds are added to the environment in the form of industrial effluents and herbicides. These pyridine derivatives do not accumulate but are degraded by microbial action in the environment. During a study of the microbial metabolism of the widely used bipyridinium herbicides, paraquat (1,1′-dimethyl-4,4′-bipyridilium dichloride) and diquat (1,1′-diethylene-2,2′-bipyridilium dibromide), micro-organisms were isolated that would degrade the monohydroxylated pyridines. 2-Hydroxypyridine and 3-hydroxypyridine were hydroxylated by *Achromobacter* strains (Houghton & Cain, 1972) to pyridine-2,5-diol which was then degraded by the maleamate pathway (Cain *et al.*, 1974), a route also used by *Pseudomonas putida* when grown on nicotinic acid (Behrman & Stanier, 1957) and by a pseudomonad growing at the expense of picolinamide (Orpin *et al.*, 1972). 4-Hydroxypyridine, however, was degraded by an *Agrobacterium* sp. and experiments with washed cell suspensions utilizing 4-hydroxypyridine under conditions of restricted aeration and high pH led to the isolation of pyridine-3,4-diol (Houghton & Cain, 1972).

Aromatic hydroxylation involves two principal mechanisms. Mono-oxygenases, such as salicylate hydroxylase (Yamamoto *et al.*, 1965; Katagiri *et al.*, 1965; Takemori *et al.*, 1969; White-Stevens & Kamin, 1972; White-Stevens *et al.*, 1972) and *p*-hydroxybenzoate hydroxylase (Hosakawa & Stanier, 1966; Yano *et al.*, 1969; Herp *et al.*, 1969; Howell *et al.*, 1972), are characterized by their incorporation of ^{18}O into the hydroxylated product from $^{18}O_2$ and by their requirement for a reduced nicotinamide nucleotide:

$$R\text{–}H + {}^{18}O_2 + NAD(P)H + H^+ \rightarrow R\text{–}{}^{18}OH + NAD(P)^+ + H_2{}^{18}O \quad (1)$$

An alternative mechanism of hydroxylation is involved in the hydroxylation of many heterocyclic compounds, such as uracil (Hayaishi & Kornberg, 1952), nicotinic acid (Hughes, 1955; Holcenberg & Stadtman, 1969; Hirschberg & Ensign, 1971*a*,*b*), nicotine (Hochstein & Dalton, 1967) or *N*-methylisonicotinic acid (Orpin *et al.*, 1972). Studies with $H_2{}^{18}O$ have shown that the O_2 incorporated into these hydroxylated products is derived from water with $NADP^+$, an artificial dye such as Methylene Blue,

* Present address: Unilever Research, Port Sunlight Laboratory, Port Sunlight, Wirral, Cheshire L62 4XN, U.K.

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

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

or a particulate electron-transport system linked to O_2 being required as electron acceptor:

$$R\text{–}H + H_2{}^{18}O \rightarrow R\text{–}{}^{18}OH + 2H^+ + 2e^-$$
$$2H^+ + 2e^- + X \rightarrow XH_2 \quad (2)$$
(X = electron acceptor)

Houghton & Cain (1972) predicted, from evidence based on O_2 uptakes during oxidation of 4-hydroxypyridine and pyridine-3,4-diol by washed cell suspensions of *Agrobacterium*, that 4-hydroxypyridine might be hydroxylated by an enzyme of the mono-oxygenase type, although there was at that time no precedent for the hydroxylation of a pyridine derivative by this type of enzyme. The results with cell-free extracts in the present paper provide evidence that a new mono-oxygenase, 4-hydroxypyridine-3-hydroxylase, may be involved. A short preliminary report has been published (Houghton *et al.*, 1969).

Materials and Methods

Nomenclature of hydroxylated pyridines

Hydroxylated pyridines can exist as alternative tauteromeric forms. The 2- and 4-isomers of hydroxypyridine, both as the solid and in solution, exist predominantly in the pyridone rather than the pyridinol form. The ratio of pyridone/pyridinol for 4-hydroxypyridine in solution, for instance, is 2200:1 (Meislich, 1962). 3-Hydroxypyridine, in contrast, exists mainly in the pyridinol form. The 'hydroxypyridine' nomenclature is thus used for all the monohydroxy isomers for convenience or where this trivial usage is unlikely to be misleading. Where we have proposed a likely EC name for the enzyme, the correct chemical nomenclature has been used.

The dihydroxylated pyridines also have alternative tautomeric forms. The 'pyridinediol' nomenclature has been used in reference works (e.g. Meislich, 1962), in our first paper in this series (Houghton & Cain, 1972) and on grounds of brevity is maintained here but, once again, compounds such as pyridine-3,4-diol undoubtedly exist in the 3-hydroxypyrid-4-one form in solution.

Chemicals

The 2-, 3- and 4-isomers of hydroxypyridine, pyridine-3,4-diol and the other isomeric pyridinediols were prepared or purified as described by Houghton & Cain (1972). *N*-Methylpyrid-4-one was prepared as described by Wright & Cain (1972). 2,6-Dicarboxypyrid-4-one was obtained from Pfaltz and Bauer Ltd., Northern Boulevard, Flushing, N.Y., U.S.A. 4-Quinolinol and 4-hydroxypteridine were obtained from Ralph Emanuel Ltd., Alperton, Middx., U.K. and other pyridine compounds from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K. NAD^+, $NADP^+$, NADH, NADPH, FAD, FMN, riboflavin, alcohol dehydrogenase and calcium phosphate gel were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K. The dry weight of suspensions of the gel was determined by drying samples to constant weight on weighed planchets at 100°C.

Buffers

Tris–HCl buffer (50mM) was prepared by the addition of 1M-HCl to a solution of Tris until the required pH was obtained, the solution was then diluted to a Tris concentration of 50mM and the pH rechecked. Potassium phosphate buffer was prepared by mixing equimolar solutions of K_2HPO_4 and KH_2PO_4 to obtain the required pH.

Organism

The isolation, identification, growth pattern and large-scale cultivation of *Agrobacterium* 35S have been described (Houghton & Cain, 1972).

Methods

Preparation of cell-free extracts. Extracts were usually prepared by rupture of packed frozen cells in the Hughes (1951) press without abrasive at −20°C followed by extraction of the crushed cells with 2vol. of ice-cold 50mM-Tris–HCl buffer, pH8.0. The suspension was then subjected to ultrasonic disintegration with an MSE–Mullard 60W cell disintegrator (MSE Ltd., Manor Royal, Crawley, Sussex, U.K.) for several periods of 1min with alternate cooling for 1min in an ice bath. This treatment resulted in considerable loss of pyridine-3,4-diol dioxygenase activity (Watson *et al.*, 1974) so when extracts able to oxidize pyridine-3,4-diol were required, the Hughes press crush was taken up in an equal volume of water and the sonication omitted. The extract was left in an ice bath for 15min and whole cells and heavy particulate material were removed by centrifugation at 25000*g* for 10min in the 10×10ml angle rotor of an MSE SuperSpeed 50 ultracentrifuge. This crude extract, containing suspended light particulate material, was clarified by further centrifugation at 120000*g* for 1–2h to produce a high-speed supernatant extract, which was carefully removed from the red-brown particulate layer. The remaining pellet was washed carefully with buffer and resuspended to the original volume in fresh buffer to give a particle fraction.

Assay of 4-hydroxypyridine hydroxylase. The oxidation of NADH or NADPH has been used as the standard assay for salicylate hydroxylase (Yamamoto *et al.*, 1965) and *p*-hydroxybenzoate hydroxylase (Hosakawa & Stanier, 1966) and was chosen as the

most convenient method for the assay of 4-hydroxypyridine hydroxylase.

The assay mixture contained, in a total volume of 2.9ml: 0.05–0.20ml of high-speed supernatant; 0.5μmol of NADH or NADPH; 100μmol of Tris–HCl buffer. Measurements of activity with NADH were performed at pH8.0 and with NADPH at pH7.5. The rate of NAD(P)H oxidation was followed by the decrease in E_{340} at 30°C for 3–5min before the addition of 0.1ml of 10mM-4-hydroxypyridine, after which the rate was followed for a further 2–4min. The hydroxylase activity was determined as the difference between the rates of NAD(P)H oxidation in the presence and the absence of 4-hydroxypyridine. One unit of enzyme was defined as the amount that caused the oxidation of 1μmol of NAD(P)H/min at 30°C and the specific activity as the number of units/mg of protein. FAD had no effect on the activity of high-speed supernatant extracts but 10μM-FAD was added to the standard assay mixture when the activity of purified extracts was determined. Under these standard conditions activity was linear with time for the first 2–3min and the rate was proportional to the amount of protein present up to at least 0.5mg of protein.

Purification of 4-hydroxypyridine hydroxylase. High-speed supernatant was carefully adjusted to pH7.0 with 0.1M-HCl and a solution of protamine sulphate added to give a final concentration of 1mg/10mg of protein. The supernatant was left for 10min, a small precipitate removed by centrifugation at 30000*g* for 10min and the supernatant adjusted to pH8.0 with 0.1M-NaOH. An aqueous calcium phosphate gel suspension was added to obtain a final concentration of 2mg dry wt. of suspension/mg of protein. The supernatant was stirred for 15min and the gel removed by centrifugation. This procedure resulted in a selective removal of much of the NADPH-dependent activity and was omitted when purified preparations containing high activity with NADPH were required.

Sufficient solid $(NH_4)_2SO_4$ was added to obtain 60% (w/v) saturation, the mixture stirred for 15min and the precipitate discarded. Further $(NH_4)_2SO_4$ was added to obtain 80% (w/v) saturation, the mixture stirred for 15min, centrifuged and the precipitate dissolved in the minimum volume of 50mM-Tris–HCl buffer, pH8.0. The dissolved $(NH_4)_2SO_4$ fractions were applied to a column (40cm×2.5cm diam.) of Sephadex G-200 and eluted in 3ml fractions with 50mM-Tris–HCl buffer, pH8.0, containing 10μM-FAD. The most active fractions (15–21) were combined and centrifuged at 120000*g* for 1–2h to remove residual particulate material. To obtain purified preparations that required FAD for activity, the $(NH_4)_2SO_4$ fractionation was performed at pH5.0 and the precipitate produced by 60–80% saturation was washed with neutral saturated $(NH_4)_2SO_4$ solution and stirred with saturated $(NH_4)_2SO_4$ solution at pH5.0 for 2–3h. The suspension was collected by centrifugation, again washed with neutral saturated $(NH_4)_2SO_4$ and dissolved in the minimum volume of 50mM-Tris–HCl buffer, pH8.0. The FAD-free enzyme was then applied to a Sephadex G-200 column as described above but the eluting buffer used did not contain FAD.

The purification procedure was completed at 4°C within 18h and the partially purified preparation used immediately.

Isolation of the product of 4-hydroxypyridine hydroxylase. A mixture of 15ml high-speed supernatant extract [in which the enzyme(s) responsible for further metabolism of pyridine-3,4-diol had been inactivated by the extract being left to stand at 0°C for 24h], 30mg of alcohol dehydrogenase, 5μmol of NAD^+ and 1.5mmol of Tris–HCl buffer, pH8.5, in a total volume of 50ml was rapidly stirred at 30°C, to obtain good aeration. At intervals over 12h a total of 0.3mmol of 4-hydroxypyridine and 1.5mmol of ethanol were added as the reaction was followed by spectrophotometric analysis of the incubation mixture.

The mixture was acidified to pH2.0 with 1M-HCl, precipitated protein removed by centrifugation, the solution readjusted to pH7 with 1M-NaOH and the mixture evaporated to dryness. The residue was extracted with hot propan-2-ol and the propanolic extract was concentrated, streaked at the origin of a sheet of Whatman no. 3 chromatography paper and run in solvent A. Unchanged 4-hydroxypyridine (R_F 0.64) was separated from a new compound (R_F 0.46) that formed a violet colour with $FeCl_3$ spray. The areas of paper containing this new compound were eluted with water and the eluate was freeze-dried to yield a yellow–brown solid (10mg).

Substrate specificity of 4-hydroxypyridine hydroxylase. The lack of activity by substrate analogues in the spectrophotometric assay for 4-hydroxypyridine hydroxylase was checked by chromatographic examination of the products of larger-scale incubations. These incubation mixtures contained, in a total volume of 30ml: 1.5ml of high-speed supernatant; 1.25mg of alcohol dehydrogenase; 1μmol of NAD^+; 100μmol of ethanol; 500μmol of Tris–HCl buffer, pH8.5. A control mixture containing denatured (100°C for 5min) extract was used for each substrate. The substrate analogue of 4-hydroxypyridine (5μmol) was added and the incubation mixture shaken at 25°C for 2h when a second 5μmol of substrate was added. After a further 2h, the mixtures were freeze-dried, extracted with hot propan-2-ol and filtered. The u.v.-absorption spectrum of a portion of the filtrate was recorded and the remainder concentrated and chromatographed in solvents A and B with authentic substrates. The R_F values of the substrates are detailed in Table 1.

Table 1. *Chromatographic characteristics and u.v.-absorption maxima of compounds tested as substrates for and products of 4-hydroxypyridine hydroxylase*

n.t., Not tested.

Substrate	R_F in solvents: 1	2	Colour produced by spraying with: $FeCl_3$	Folin–Ciocalteau reagent	U.v.-absorption maximum (nm)
4-Hydroxypyridine	0.64	0.65	Yellow	No reaction	256
2-Hydroxypyridine	0.71	0.73	Yellow	No reaction	295
3-Hydroxypyridine	0.63	0.65	Orange	Blue	243, 277, 308
4-Hydroxypteridine	0.45	0.33	Orange	No reaction	240, 315
4-Hydroxyquinoline	0.77	0.96	Orange	No reaction	230, 316, 323, 329
N-Methylpyrid-4-one	0.62	0.63	Yellow	No reaction	261
4-Aminopyridine	0.58	0.28	No reaction	No reaction	261
Pyridine,2,5-diol	n.t.	0.88	Red	Blue	320
Pyridine-2,3-diol	n.t.	0.83	Blue	Blue	297
Pyridine-3,4-diol	0.39	0.67	Violet	Blue	273
N-Methyl-3-hydroxypyrid-4-one	0.29	0.64	Violet	Blue	280

Determinations. Protein was determined by the Folin method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as standard.

O_2 uptake during the metabolism of 4-hydroxypyridine and pyridine-3,4-diol was determined by using the Warburg respirometer (Umbreit *et al.*, 1957). The flasks contained, in a total volume of 2.8ml: crude extract containing particulate material, or high-speed supernatant, 1–2ml; potassium phosphate buffer, pH7.5, 100μmol; 4-hydroxypyridine or pyridine-3,4-diol, 2 or 3μmol. When required either an NADH-regenerating system containing: NAD^+, 0.1μmol; ethanol, 10μmol; alcohol dehydrogenase, 0.2mg; or an NADPH-regenerating system containing: $NADP^+$, 0.1μmol; glucose 6-phosphate (K^+ salt) 10μmol; glucose 6-phosphate dehydrogenase, 0.1mg, was also added. The centre well of the flasks contained 0.2ml of 10% (w/v) KOH.

Chromatographic techniques. Paper chromatography of pyridine derivatives was done on Whatman no. 1 paper in the descending direction in the following solvents (all proportions by volume): A, ethanol–conc. NH_3 (sp.gr. 0.88)–water (20:1:4); B, butan-1-ol–conc. HCl–water (20:4:5); C, butan-1-ol–acetic acid–water (4:1:5, upper layer); D, butan-1-ol–methanol–water (20:13:7). Many of the pyridine derivatives could be detected under u.v. light. The position of hydroxylated compounds was confirmed by spraying with (*a*) 0.5% (w/v) $FeCl_3$ in 1M-HCl or (*b*) Folin and Ciocalteau's reagent (the BDH product) followed by 10% (w/v) Na_2CO_3.

Attempts were made to determine the molecular weight of 4-hydroxypyridine hydroxylase by gel chromatography on columns (90cm×0.9cm) of Sephadex G-200 as described by Andrews (1965). A purified preparation was also passed through a column (90cm×0.9cm) of Sepharose 4B gel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) packed by passing several bed volumes of 50mM-Tris–HCl buffer, pH8.0, through the column. Blue Dextran and ferritin (mol.wt. 500000) were used as markers.

Results

Houghton & Cain (1972) showed that washed suspensions of 4-hydroxypyridine-grown *Agrobacterium* oxidized, without a lag period, only pyridine-3,4-diol of the eight isomeric diols tested. They further noted that the 1mol/mol of substrate difference in total O_2 consumption between suspensions oxidizing 4-hydroxypyridine and pyridine-3,4-diol would be the result expected of a mono-oxygenase-catalysed hydroxylation of 4-hydroxypyridine. This feature was therefore further examined with extracts.

Oxidation of 4-hydroxypyridine and pyridine-3,4-diol by extracts

The results of experiments to investigate the oxidation of 4-hydroxypyridine and pyridine-3,4-diol by high-speed supernatant extracts and by crude extracts containing particulate material are shown in Table 2. The effect of a reduced nicotinamide nucleotide-regenerating system and the effects of 'aging' extracts by storage in air at 4°C for 2 days was also determined.

Only an extremely slow and incomplete oxidation of 4-hydroxypyridine occurred in the absence of substrate amounts of NADH, NADPH or the corresponding regenerating systems, but in the presence of a reduced nicotinamide nucleotide a rapid uptake of 2.1 and 2.6mol of O_2/mol of substrate occurred with freshly prepared high-speed supernatant extract and a crude extract containing the

Table 2. *Oxidation of 4-hydroxypyridine and pyridine-3,4-diol by extracts of Agrobacterium*

O_2 uptakes were measured manometrically at 30°C in reaction mixtures containing, in a total volume of 2.8ml: 1–2ml of extract derived from 4-hydroxypyridine-grown cells (approx. 8mg of protein); 100μmol of potassium phosphate buffer, pH 7.5; and 2 or 3μmol of substrate. The centre well contained 0.2ml of 20% (w/v) KOH. Either crude extracts containing the particulate fraction or high-speed supernatant extracts prepared as described in the Materials and Methods section were used. The extracts were either used immediately after preparation (fresh) or after storage at 4°C for 2–4 days in air ('aged'). The NADH-regenerating system, which was either present (+) or absent (–) comprised: 0.1μmol of NAD^+; 10μmol of ethanol; 0.2mg of alcohol dehydrogenase. The results are corrected for O_2 uptake in the absence of substrate.

Substrate	Type of extract used	NADH-regenerating system	O_2 uptake (mol/mol of substrate)
4-Hydroxypyridine	Crude, fresh	+	2.6
4-Hydroxypyridine	Crude, 'aged'	+	1.0
4-Hydroxypyridine	High-speed supernatant, fresh	+	2.1
4-Hydroxypyridine	High-speed supernatant, 'aged'	+	1.0
4-Hydroxypyridine	Crude or high-speed supernatant, fresh or 'aged'	–	0.1
Pyridine-3,4-diol	Crude, fresh	+	1.6
Pyridine-3,4-diol	Crude, fresh	–	1.2
Pyridine-3,4-diol	High-speed supernatant, fresh	+	1.0
Pyridine-3,4-diol	High-speed supernatant, fresh	–	1.0
Pyridine-3,4-diol	Crude or high-speed supernatant, 'aged'	+ or –	0

particulate fraction respectively. The rate of O_2 consumption and the total O_2 uptake were identical with either the NADH- or the NADPH-regenerating system described in the Materials and Methods section. When the regenerating systems were replaced by substrate amounts of NADH or NADPH, lower total O_2 uptakes were generally found owing to endogenous oxidation of some of the reduced nucleotide.

When 'aged' extracts were used, 4-hydroxypyridine was oxidized with an uptake of 1mol of O_2/mol of substrate only in the presence of an NADH-regenerating system. Pyridine-3,4-diol was not oxidized by such 'aged' extracts, later found to be due to inactivation of the very labile pyridine-3,4-diol oxygenase (Watson *et al.*, 1974). With fresh extracts the O_2 uptake for the diol was 1.0 and 1.2mol/mol of substrate with high-speed supernatant or crude extracts containing particulate material respectively. In the presence of an NADH-regenerating system the O_2 uptake by crude extracts containing the particle fraction was increased to 1.6mol/mol of pyridine-3,4-diol. This difference of 1mol of O_2 between the oxidation of 1mol of 4-hydroxypyridine and 1mol of pyridine-3,4-diol, by suitably supplemented extracts, was identical with that found with washed cell suspensions.

The O_2 uptake of 1mol during the metabolism of 4-hydroxypyridine by 'aged' extracts suggested that an oxidation of 4-hydroxypyridine only as far as pyridine-3,4-diol occurred under these conditions. The low u.v. absorption of the components of the regenerating system enabled the reaction to be followed spectrophotometrically (Fig. 1). The absorption peak of 4-hydroxypyridine at 256nm was altered to a new maximum at 280nm. Comparison of the new spectrum with that of a stoicheiometric amount of pyridine-3,4-diol showed that virtually complete conversion of 4-hydroxypyridine into pyridine-3,4-diol had occurred. The requirement for a reduced nicotinamide nucleotide was investigated spectrophotometrically at 340nm by the addition of 4-hydroxypyridine to cuvette reaction mixtures containing either NADH or NADPH and high-speed supernatant. A marked increase in the initially slow NAD(P)H oxidase rate was found and the amount of NAD(P)H subsequently oxidized was virtually identical with the amount of 4-hydroxypyridine supplied (Table 3).

Identification of pyridine-3,4-diol

The product of the aerobic metabolism of 4-hydroxypyridine by 'aged' extracts was isolated as described in the Materials and Methods section from an incubation mixture essentially similar to that used in Fig. 1. The isolated material had a melting point of 230°C with darkening and decomposition; den Hertog *et al.* (1950) reported darkening at 230°C with melting at 239–240°C. The diacetate had a melting point of 136–139°C. Houghton & Cain (1972) reported a melting point of 138°C and den Hertog *et al.* (1950) a melting point of 134–140°C for this derivative. The u.v.-absorption spectra of the isolated material in water, 0.1M-HCl and 0.1M-NaOH were identical with those of authentic pyridine-3,4-diol under the same conditions. The isolated material co-chromatographed with pyridine-3,4-diol in solvents A (R_F 0.39), B (R_F 0.72), C (R_F 0.64) and D (R_F 0.63). A blue colour was formed with Folin and Ciocalteau's reagent and a red–violet colour with $FeCl_3$. The light-absorption maximum of the $FeCl_3$

complex at 535–540nm was identical with that given when authentic pyridine-3,4-diol and $FeCl_3$ were mixed (Houghton & Cain, 1972). These results show convincingly that the product of 4-hydroxypyridine metabolism by 'aged' extracts was pyridine-3,4-diol and that the enzyme responsible, 4-hydroxypyridine hydroxylase, requires NAD(P)H and O_2 for activity. No pyridine-3,4-diol formation took place under a N_2 atmosphere with or without cofactor supplementation, nor could the O_2 requirement be replaced by ferricyanide.

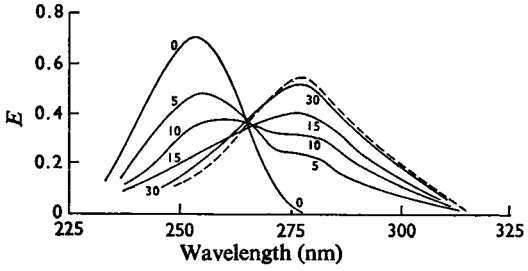


Fig. 1. *Conversion of 4-hydroxypyridine into pyridine-3,4-diol by 'aged' extracts of Agrobacterium followed spectrophotometrically*

A solution containing 1.5ml of high-speed supernatant extract ('aged' for 1 day at 4°C) (12mg of protein), 200μmol of Tris–HCl buffer, pH8.5, 2.5mg of alcohol dehydrogenase, 0.2μmol of NAD^+, 20μmol of ethanol and 2μmol of 4-hydroxypyridine, in a total volume of 6ml, was incubated at 30°C for 1h. At the times indicated on the curves (min), 0.6ml of the incubation mixture was added to 2.4ml of water and the spectrum rapidly scanned (——) from 220–320nm, with, as reference cell, 0.6ml of a similar incubation mixture containing no 4-hydroxypyridine that had also been diluted to 3ml with water. ---- is the spectrum of 0.2μmol of authentic pyridine-3,4-diol added to 0.6ml of the control incubation mixture and diluted to 3ml with water.

Purification of 4-hydroxypyridine hydroxylase

Suitable conditions for a complete purification of 4-hydroxypyridine hydroxylase were not discovered. The results of a partial purification are shown in Table 4. Only a fivefold increase in specific activity of the NADH-dependent enzyme was obtained in spite of very considerable removal of inert protein. The final preparations were used immediately since activity was rapidly lost on storage or on attempted further purification by DEAE-cellulose chromatography.

Properties of 4-hydroxypyridine hydroxylase

FAD requirement. Preparations of 4-hydroxypyridine hydroxylase that were treated with acidic $(NH_4)_2SO_4$ as described in the Materials and Methods section had very low activity in the absence of added FAD. The activity with both NADH and NADPH was restored by the addition of FAD (Fig. 2) but not by FMN or riboflavin (up to 10μM). The concentration of FAD required for half-maximal activity was about 0.2–0.3μM.

Anaerobic reduction of FAD. When NAD(P)H was incubated anaerobically with FAD, 4-hydroxypyridine and a partially purified preparation of 4-hydroxypyridine hydroxylase the E_{450} decreased as the FAD was reduced. The reaction was complete when an amount of $FADH_2$, equivalent to the added NADH or NADPH, was formed (Fig. 3). Reintroduction of air resulted in the instantaneous reversal of the E_{450} to its original value. In the absence of 4-hydroxypyridine, the anaerobic reduction of FAD occurred at a very much decreased rate. This slow rate was probably due to contaminating NAD(P)H oxidase activity, because purified extracts that had been held at 4°C for 1 week and had lost all 4-hydroxypyridine hydroxylase activity still showed the slow anaerobic FAD reduction in the presence or the

Table 3. *Oxidation of reduced nicotinamide nucleotides during the 3-hydroxylation of 4-hydroxypyridine by extracts of Agrobacterium*

The decrease in E_{340} was followed at 30°C in quartz cuvettes containing, in a total volume of 3ml: 100μmol of Tris–HCl buffer, pH8.0; 0.1–0.4μmol of 4-hydroxypyridine; either 0.7μmol of NADH and 0.15ml of high-speed supernatant extract (0.75mg of protein), or 0.7μmol of NADPH and 0.5ml of the same extract (2.5mg of protein). The reaction was followed until the rate of NAD(P)H oxidation was decreased to the rate of a control mixture containing no substrate and the total $-\Delta E_{340}$ corrected for this endogenous oxidation.

4-Hydroxypyridine supplied (μmol)	Reduced nucleotide used in test	$-\Delta E_{340}$	NAD(P)H oxidized (μmol)	Ratio of NAD(P)H oxidized/substrate used
0.1	NADH	0.19	0.09	0.90
0.2	NADH	0.42	0.20	1.00
0.3	NADH	0.58	0.28	0.93
0.4	NADH	0.79	0.38	0.95
0.2	NADPH	0.39	0.19	0.95
0.4	NADPH	0.75	0.36	0.90

Table 4. *Partial purification of 4-hydroxypyridine hydroxylase from extracts of Agrobacterium*

Fraction	Volume (ml)	Total protein (mg)	Reduced nucleotide used in assay	Total activity (units)	Specific activity (units/mg of protein)	Purification factor
1. High-speed supernatant	15	62	NADH	3.7	0.060	—
			NADPH	1.1	0.018	
2. Salmine sulphate (Sigma) supernatant	15	57	NADH	3.45	0.061	1
			NADPH	1.0	0.018	
3. Calcium phosphate gel supernatant	16	34	NADH	2.8	0.083	1.4
			NADPH	0.45	0.013	
4. Fraction precipitated between 60–80% satn. with $(NH_4)_2SO_4$ and redissolved	2	16	NADH	2.4	0.150	2.5
			NADPH	0.38	0.024	
5. Fractions 15–21 eluted from a Sephadex G-200 column and combined	18	6	NADH	1.8	0.300	5
			NADPH	0.27	0.045	

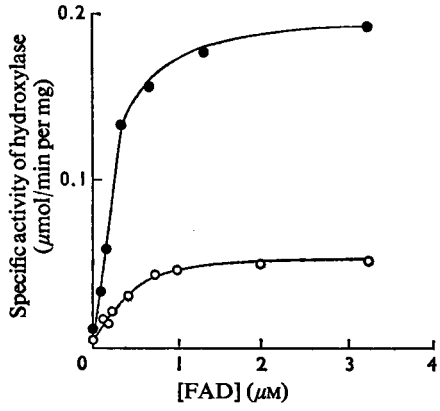


Fig. 2. *Requirement of partially purified 4-hydroxypyridine hydroxylase for FAD*

The specific activities of a sample of partially purified extract (0.22mg of protein) that had been treated with acidic $(NH_4)_2SO_4$ solution to remove flavin were measured in the standard assay at 30°C with the further addition to the cuvettes of FAD (0–10nmol) as shown. Activity was measured with ●, NADH and ○, NADPH.

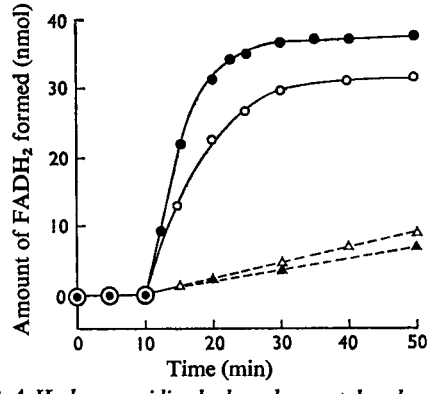


Fig. 3. *4-Hydroxypyridine hydroxylase-catalysed reduction of FAD by reduced nicotinamide nucleotides under anaerobic conditions*

The main vessel of a Thunberg-type cuvette, evacuated and flushed with O_2-free N_2 several times to render the contents anaerobic and maintained at 30°C, contained in a total volume of 2.9ml:0.031 unit of 4-hydroxypyridine hydroxylase (purified to step 5 in Table 4); 60nmol of FAD; 1μmol of 4-hydroxypyridine; 100μmol of Tris-HCl buffer, pH8.0. The reaction was started by the addition of ●, ▲, 38nmol of NADH or ○, △, 33nmol of NADPH from the side-arm. The amount of FAD reduced was calculated from an ε_{450} of 11300 litre·mol^{-1}·cm^{-1} for FAD and 980 litre·mol^{-1}·cm^{-1} for $FADH_2$. —— represents $FADH_2$ formed in the presence, and ---- $FADH_2$ formed in the absence, of 4-hydroxypyridine.

absence of 4-hydroxypyridine. The 2- or 3-isomers of hydroxypyridine, *N*-methylpyrid-4-one or phenol, which would not act as substrates in the aerobic reaction (see below) would also not substitute for 4-hydroxypyridine in the anaerobic reduction of FAD.

Substrate specificity. Both high-speed supernatant extracts and partially purified extracts used either NADH or NADPH although activity was greater with NADH. A decrease in the ratio of NADPH/NADH-dependent activity was found during the calcium phosphate gel treatment of the purification procedure, suggesting that more than one isofunctional enzyme might, perhaps, have been present. A complete separation of NADH- and NADPH-dependent activity, however, was not obtained in the absence of a suitable stabilizing procedure for the enzyme. The following compounds would not substitute for 4-hydroxypyridine (pyrid-4-one) with NADH in the standard assay: *N*-methylpyrid-4-one, 2- or

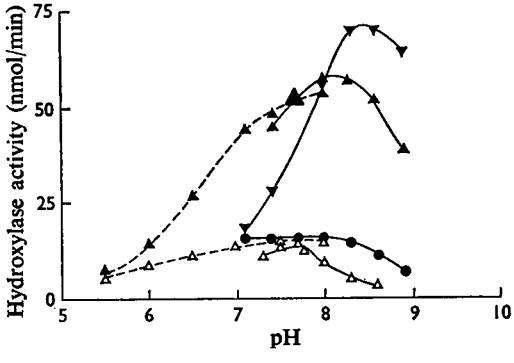


Fig. 4. *Effect of pH on the activity of 4-hydroxypyridine hydroxylase*

The assay mixtures contained, in a total volume of 3ml: enzyme (0.45mg of protein, purified to step 4 in Table 4); Tris–HCl or potassium phosphate buffer at the pH values indicated and containing 10μM-FAD, 100μmol; NADH or NADPH, 0.5μmol; 4-hydroxypyridine, 0–5μmol. The reaction rate was measured with NADH (solid symbols) and 4-hydroxypyridine at ●, 33μM; ▲, 0.33mM; ▼, 1.67mM; and with NADPH (open symbol) and 4-hydroxypyridine at △, 0.33mM. Values from pH5.5–8.0 were measured in potassium phosphate buffer (----) and from pH7.4–9.0 in Tris–HCl buffer (——).

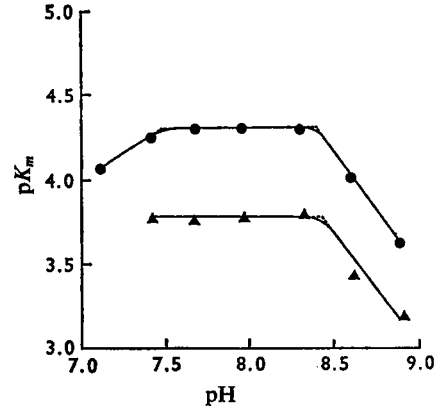


Fig. 5. *Effect of pH on the K_m values for NADH and 4-hydroxypyridine of 4-hydroxypyridine hydroxylase*

The K_m values were determined from double-reciprocal plots of results from experiments conducted at 30°C in which the concentrations of either NADH or 4-hydroxypyridine were varied in the presence of a constant concentration of the other substrate. All assay mixtures contained, in a total volume of 3ml: 0.3ml of enzyme (0.45mg of protein) (purified to step 4 in Table 4), 100μmol of Tris–HCl buffer at various pH values containing 10μM-FAD; and the substrates. The K_m value for NADH was determined with 0.05–0.5μmol of NADH in the presence of 1μmol of 4-hydroxypyridine and the K_m value for 4-hydroxypyridine with 0.05–5μmol of 4-hydroxypyridine in the presence of 0.5μmol of NADH. Variation with pH of the pK_m value (= $-\log_{10}K_m$) is shown for ●, NADH and ▲, 4-hydroxypyridine.

3-hydroxypyridine, any of the isomeric pyridinediols, 4-hydroxyquinoline, 4-hydroxypteridine, 2,6-dicarboxypyrid-4-one, 4-aminopyridine, 4-ethylpyridine, 2-, 3- or 4-picoline, isonicotinic acid, *N*-methylisonicotinic acid, pyridine, phenol or *p*-hydroxybenzoic acid. Some of these very similar analogues were also tested in the larger-scale incubation mixtures described in the Materials and Methods section. The results confirmed that only 4-hydroxypyridine was hydroxylated. The absorption spectrum of each substrate in the propan-2-ol extracts was identical with that of the boiled controls except for the 4-hydroxypyridine sample which had formed pyridine-3,4-diol (absorption at 273nm). Paper chromatography confirmed that no trace of hydroxylated products were formed from 2- or 3-hydroxypyridine, *N*-methylpyrid-4-one, 4-hydroxypteridine, 4-hydroxyquinoline or 4-aminopyridine.

pH optimum. In the standard assay mixture containing 0.33mM-4-hydroxypyridine with NADH as the cofactor the pH optimum was 8.0–8.3 in Tris–HCl buffer, and about pH8.0 in phosphate buffer. The position of maximum activity was, however, dependent on the concentration of 4-hydroxypyridine. At low 4-hydroxypyridine concentrations (10–100μM) specific activity was unchanged between pH7.0 and 8.3, but increasing the 4-hydroxypyridine concentration above 0.33mM led to substantial decreases in specific activity at pH7.0–7.5 because of substrate inhibition (Fig. 4). At higher concentrations of 4-hydroxypyridine (1.66mM) the pH optimum was 8.4 and remained unchanged at this value up to 5mM substrate concentration. When NADPH was used the pH optimum was 7.5.

Effect of pH on K_m values and substrate inhibition. In view of the complex effect of substrate concentration on the pH optimum, the effect of pH on K_m values and substrate inhibition was investigated. The results of a pK_m versus pH plot are given in Fig. 5. The apparent K_m of 0.18mM for 4-hydroxypyridine and 0.05mM for NADH remained constant between pH7.3 and 8.3. Above pH8.3 the pK_m values decreased, corresponding to an increase of K_m for both 4-hydroxypyridine and NADH. With NADH a decrease of pK_m value also occurred below pH7.3 but the K_m for 4-hydroxypyridine could not be accurately determined below pH7.1 owing to the extensive substrate inhibition. The apparent K_i for this substrate inhibition was determined by the method of Cleland (1970) from the equation:

$$K_i = [S_{max.}]^2/K_m \quad (3)$$

Table 5. *Effect of pH on the inhibitor constant (K_i) for substrate inhibition of 4-hydroxypyridine hydroxylase*

K_m values and substrate concentrations giving the maximum observable reaction rates [$S_{max.}$] at several pH values were determined from Lineweaver–Burk double-reciprocal plots as described in Fig. 5. The K_i values for 4-hydroxypyridine inhibition were calculated from eqn. (3) (see the text).

pH	[$S_{max.}$] (mM)	[$S_{max.}$]^2	K_m (mM)	Calculated K_i (mM)
7.10	0.20	0.04	0.18	0.22
7.35	0.40	0.16	0.18	0.89
7.95	0.83	0.70	0.18	3.9
8.60	2.0	4.0	0.37	11.0

where [$S_{max.}$] is the substrate concentration causing the maximum initial reaction rate. The variation of the K_i values for substrate inhibition of the hydroxylase with pH is shown in Table 5. With NADPH as electron donor the K_m value at the pH optimum of 7.5 was 0.37mM for 4-hydroxypyridine and 0.15mM for NADPH.

The theoretical basis for pK_m versus pH plots has been discussed by Dixon & Webb (1964). The inflexion at pH8.4 probably represents the ionization of a group in the free enzyme or substrate. The pK_a values of 4-hydroxypyridine are 11.1 and 3.3 for ionization of the hydroxyl and ring-nitrogen (Albert & Phillips, 1956) suggesting that the group ionizing at pH8.4 is situated on the enzyme. This pK_a value is very similar to that of 8.53, calculated by Benesch & Benesch (1955) for ionization of the thiol group of cysteine in proteins, a possible indication that thiol groups are involved in the binding of 4-hydroxypyridine and NADH to 4-hydroxypyridine hydroxylase. The inhibition at the lower pH values was not due to the increased anion content of the Tris–HCl buffer as was reported by Teng *et al.* (1972) for *p*-hydroxybenzoate hydroxylase. Decreasing the normal Tris–HCl buffer concentration from 33 to 10mM at pH7.5 by simple dilution with a corresponding decrease in the Cl^- content, did not alter the pattern of substrate inhibition at this pH.

Effect of inhibitors and metals. The results of inhibition studies are shown in Table 6. Activity was strongly inhibited by heavy metal ions, such as Cu^{2+} and Zn^{2+}, but inhibition could be reversed, to some extent, by the subsequent addition of EDTA or GSH. Fe^{2+} or Fe^{3+} had no effect on 4-hydroxypyridine hydroxylase activity. Metal chelators such as *o*-phenanthroline, 2,2′-dipyridyl and 8-hydroxyquinoline were usually only slightly inhibitory. Strong inhibition (80%) was obtained with 0.1mM-*p*-chloromercuribenzoate but not by iodoacetate.

No inhibition of the hydroxylase activity was obtained when 2- or 3-hydroxypyridine, pyridine-1,4-diol, 4-aminopyridine, isonicotinic acid, *N*-methylpyrid-4-one or phenol (all at 5μmol) were included with substrate in the standard assay mixture.

Table 6. *Effect of some inhibitors and metal ions on the activity of 4-hydroxypyridine hydroxylase*

Enzyme activities were measured in the standard assay system with NADH to which the compounds shown below were added. The reaction was started either immediately or after 15min preincubation at 30°C by addition of the substrate. Values obtained after a 15min preincubation are shown in parentheses.

Compound added	Concentration (mM)	Enzyme activity (% of control value)
None	—	100
$CuSO_4$	1	51
$ZnSO_4$	0.1	25
$ZnSO_4$+EDTA	0.1+1 respectively	76
$ZnSO_4$ + GSH	0.1+1 respectively	68
$FeSO_4$	0.1	101
$FeCl_3$	0.1	98
1,10-Phenanthroline	1	102 (85)
2,2′-Dipyridyl	1	100 (95)
8-Hydroxyquinoline	0.1	100 (90)
8-Hydroxyquinoline	1	103 (75)
KCN	1	104 (117)
Sodium iodoacetate	1	100 (120)
Sodium *p*-chloromercuribenzoate	0.1	20 (0)
Potassium arsenite	1	99 (89)
Sodium arsenate	1	101 (92)

Molecular size. Analysis of eluates from Sephadex G-200 columns during the purification procedure suggested that 4-hydroxypyridine hydroxylase was eluted with high-molecular-weight protein and nucleic acids and this result was confirmed when suitable marker proteins were included. Activity was eluted very near to the gel exclusion volume of the column.

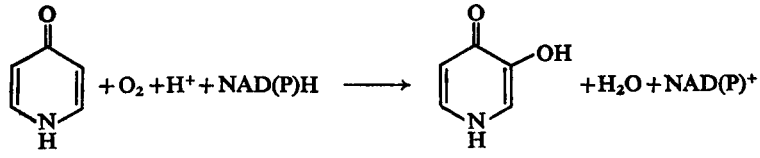
A similar result was obtained when gel chromatography of the partially purified enzyme was repeated on a Sepharose 4B column when the hydroxylase was eluted in the same fractions as the Blue Dextran marker indicating that the molecular weight of 4-hydroxypyridine hydroxylase was very high. An accurate value has not therefore been assigned to the enzyme which may form part of a larger aggregate.

Discussion

Agrobacterium 35S was isolated by Houghton (1969) by elective culture from sewage, in the presence of low concentrations (up to 0.025%, w/v) of 4-hydroxypyridine. Higher substrate concentrations

strongly inhibited growth. The organism grew slowly, even in the presence of yeast extract, with the production of copious capsular material that resulted in a characteristic growth form of slimy filaments and clumps in an otherwise almost clear medium. The slow growth and the heavy capsule production was reflected in low enzymic activity of extracts and low protein yields from harvested cells. Partly for these reasons, 4-hydroxypyridine hydroxylase has been difficult to purify but some of the properties of this new enzyme have been examined.

The hydroxylation of 4-hydroxypyridine could be studied in extracts in which the further metabolism of pyridine-3,4-diol was prevented by allowing the labile pyridine-3,4-diol oxygenase activity to inactivate over 1–2 days at 4°C (Watson *et al.*, 1974). Oxidation of 4-hydroxypyridine by these extracts only occurred in the presence of NADH or NADPH with an O_2 uptake of 1mol/mol of substrate during which spectrophotometric analysis showed that virtually stoicheiometric formation of pyridine-3,4-diol had occurred, a product confirmed by its isolation from a large-scale incubation mixture. The rate of NAD(P)H oxidation by high-speed supernatant extracts was increased by the addition of 4-hydroxypyridine whereupon 1mol of reduced nicotinamide nucleotide was oxidized by 1mol of 4-hydroxypyridine. These results suggest that the hydroxylation of 4-hydroxypyridine (pyrid-4-one) can be represented by:



and that 4-hydroxypyridine hydroxylase is thus a mixed-function mono-oxygenase. Experiments with $^{18}O_2$ and $H_2{}^{18}O$ that would confirm the proposed mechanism absolutely have to date been precluded by the cost of the isotope.

This hydroxylation of a pyridine compound by a probable mono-oxygenase is, so far, rare and has previously been described only for pyridine-2,6-diol oxidase, a mono-oxygenase with a flavin prosthetic group hydroxylating pyridine-2,6-diol to pyridine-2,3,6-triol (Holmes & Rittenberg, 1972*a*,*b*). All other pyridine-ring hydroxylases use an alternative mechanism involving nucleophilic attack by OH^- derived from water at the C-2 or C-6 position of an electron-deficient carboxylated pyridine ring; the resulting pseudobase is then dehydrogenated to form a pyridone derivative. The identification of the product of 4-hydroxypyridine hydroxylase, however, initially suggested that a different mechanism might be involved since nucleophilic attack of 4-hydroxypyridine by OH^- would result in the formation of a 2,4-substituted pyridine as in, for example, the alkali fusion of 4-hydroxypyridine to pyridine-2,4-diol (van Schickh *et al.*, 1936). The formation of pyridine-3,4-diol implied that the electron-donating OH^- group of the pyridinol tautomer of 4-hydroxypyridine was permitting electrophilic attack of the ring at the *ortho*-position. Electrophilic attack always results in the formation of 3- and 5-substituted derivatives; pyridine-3,4-diol is formed from 4-hydroxypyridine, for example, by the Elbs peroxidation and in the Udenfriend *et al.* (1954) model hydroxylating system (Houghton & Cain, 1972) where electrophilic or free-radicle attack is involved.

The mechanism of aromatic hydroxylation has been investigated in great detail with salicylate and *p*-hydroxybenzoate hydroxylase purified from pseudomonads. Both these enzymes contain FAD. 4-Hydroxypyridine hydroxylase was not sufficiently purified to permit reliable estimations of flavin content but, after treatment with acidic $(NH_4)_2SO_4$ and gel chromatography on Sephadex G-200, extracts were virtually inactive in the absence of FAD. Activity could be restored by the addition of FAD but not FMN or riboflavin; the apparent K_m value for FAD was about 0.2–0.3μM. A similar concentration restored half-maximum activity of imidazolylacetate mono-oxygenase (Maki *et al.*, 1969), but the K_m value for FAD of salicylate hydroxylase was rather lower (0.07μM), restoration of activity at this concentration requiring preincubation at 0°C for 10min (Yamamoto *et al.*, 1965). Our results suggest that 4-hydroxypyridine hydroxylase contains an FAD prosthetic group but confirmation awaits a complete purification.

A role for FAD in the hydroxylation of 4-hydroxypyridine was suggested by the anaerobic reduction of FAD by NAD(P)H, the rate of which was up to tenfold higher in the presence of 4-hydroxypyridine than when no 4-hydroxypyridine was present. A similar anaerobic reduction of FAD by reduced nicotinamide nucleotide was found with both salicylate and *p*-hydroxybenzoate hydroxylases. Katagiri *et al.* (1965) showed that the K_m value for NADH of salicylate hydroxylase was increased 400-fold in the absence of salicylate. A 10^4-fold decrease in the rate of reduction of *p*-hydroxybenzoate hydroxylase-bound FAD by NADPH in the absence of *p*-hydroxy-

benzoate was reported by Higashi *et al.* (1970*a*). These results have been attributed to the reduction of an enzyme–substrate–FAD complex followed by reaction with O_2 to form an oxygenated flavin intermediate (Spector & Massey, 1972), which breaks down to release the products and the free re-oxidized enzyme–FAD complex (Takemori *et al.*, 1969).

The increased rate of anaerobic FAD reduction by NADH catalysed by the enzyme in the presence of 4-hydroxypyridine could not be duplicated with several substrate analogues that similarly failed to act as substrates in the aerobic reaction. These results further emphasize that the anaerobic reduction of FAD was closely related to the hydroxylation of 4-hydroxypyridine and that the mechanism of 4-hydroxypyridine hydroxylase may be similar to that of *p*-hydroxybenzoate and salicylate hydroxylases. This very pronounced substrate specificity of 4-hydroxypyridine hydroxylase would account for the very limited nutritional spectrum of *Agrobacterium* 35S, the growth of which was supported only by 4-hydroxypyridine from a wide variety of other pyridine or piperidine compounds tested (Houghton & Cain, 1972). Another very characteristic property of aromatic hydroxylases is their inhibition by high substrate concentrations. The strong inhibition of 4-hydroxypyridine hydroxylase above 0.5mM-4-hydroxypyridine would also account for the growth inhibition of *Agrobacterium* 35S observed by these authors at 4-hydroxypyridine concentrations in liquid media above 0.025% (2.6mM).

Kinetic studies on *p*-hydroxybenzoate hydroxylase have shown that binding of substrate occurs by a two-step process (Nakamura *et al.*, 1970; Higashi *et al.*, 1970*a*,*b*; Teng *et al.*, 1972) and substrate inhibition may be due to binding of more than 1 molecule of substrate/molecule of enzyme to form an inactive enzyme–substrate complex. A possible role of thiol groups during the binding of substrate to imidazoleacetate mono-oxygenase was discovered by Okamoto *et al.* (1968). The inhibition of 4-hydroxypyridine hydroxylase by *p*-chloromercuribenzoate and the inflexion at pH8.4 in the pK_m versus pH plot (near to the pK_a for a cysteine residue in proteins) suggest a possible role for thiol groups in substrate binding by this enzyme. Many hydroxylases require metal ions. Dopamine-β-hydroxylase requires Cu^{2+} and uses ascorbic acid as electron donor (Friedman & Kaufman, 1965), and iron, as a component of non-haem iron protein, participates in the electron-transport system involved in microsomal and mitochondrial hydroxylases (reviewed by Hayaishi, 1969). Nicotinate hydroxylase, purified from a *Clostridium* sp. (Holcenberg & Stadtman, 1969) and *Bacillus* sp. (Hirschberg & Ensign, 1971*a*) also contained non-haem iron.

The conflicting evidence for metal requirements for aromatic hydroxylation was examined by Yamamoto *et al.* (1969), who examined three crystalline mono-oxygenases and found that no metals were present and that hydroxylation could occur without the participation of metal ions. No evidence for a metal requirement of 4-hydroxypyridine hydroxylase was obtained; no stimulation of activity was ever observed by metal ions nor were metal chelators inhibitory. This typical mono-oxygenase character further substantiates our belief that 4-hydroxypyridine hydroxylase is a (presently) rare example of a mono-oxygenase catalysing the hydroxylation of a heterocyclic nucleus.

We therefore propose for this enzyme the formal name: pyrid-4-one, reduced NAD(P)–oxygen oxidoreductase (3-hydroxylating) (EC 1.14.13.–). An acceptable trivial name is 4-hydroxypyridine-3-hydroxylase.

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References

Albert, A. & Phillips, J. N. (1956) *J. Chem. Soc. London* 1294–1304

Andrews, P. (1965) *Biochem. J.* **96**, 595–606

Behrman, E. J. & Stanier, R. Y. (1957) *J. Biol. Chem.* **228**, 923–945

Benesch, R. E. & Benesch, R. (1955) *J. Amer. Chem. Soc.* **77**, 5877–5881

Cain, R. B., Houghton, C. & Wright, K. A. (1974) *Biochem. J.* **140**, 293–300

Cleland, W. W. (1970) *Enzymes*, 3rd edn., **2**, 42

den Hertog, H. J., Wilbaut, J. P., Schepman, F. R. & van der Wal, A. A. (1950) *Rec. Trav. Chim. Pays-Bas Belg.* **69**, 700–710

Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., pp. 135–145, Longmans Green, London

Friedman, S. & Kaufman, S. (1965) *J. Biol. Chem.* **240**, 4763–4773

Hayaishi, O. (1969) *Annu. Rev. Biochem.* **38**, 21–44

Hayaishi, O. & Kornberg, A. (1952) *J. Biol. Chem.* **197**, 717–732

Herp, B., Calvin, M. & Hosakawa, K. (1969) *J. Biol. Chem.* **244**, 5644–5655

Higashi, N., Nakamura, S., Yano, K. & Arima, K. (1970*a*) *Agr. Biol. Chem. (Tokyo)* **34**, 964–965

Higashi, N., Shoun, H., Hiromi, K., Yano, K. & Arima, K. (1970*b*) *J. Biochem. (Tokyo)* **67**, 749–752

Hirschberg, R. & Ensign, J. C. (1971*a*) *J. Bacteriol.* **108**, 751–756

Hirschberg, R. & Ensign, J. C. (1971*b*) *J. Bacteriol.* **108**, 757–759

Hochstein, L. I. & Dalton, B. P. (1967) *Biochim. Biophys. Acta* **139**, 56–68

Holcenberg, J. S. & Stadtman, E. R. (1969) *J. Biol. Chem.* **244**, 1194–1203

Holmes, P. A. & Rittenberg, S. C. (1972*a*) *J. Biol. Chem.* **247**, 7622–7627

Holmes, P. A. & Rittenberg, S. C. (1972*b*) *J. Biol. Chem.* **247**, 7628–7633

Hosakawa, K. & Stanier, R. Y. (1966) *J. Biol. Chem.* **241**, 2453–2460
Houghton, C. (1969) Ph.D. Thesis, University of Newcastle upon Tyne
Houghton, C. & Cain, R. B. (1972) *Biochem. J.* **130**, 879–893
Houghton, C., Watson, G. K. & Cain, R. B. (1969) *Biochem. J.* **114**, 75P
Howell, L. G., Spector, T. & Massey, V. (1972) *J. Biol. Chem.* **247**, 4340–4350
Hughes, D. E. (1951) *Brit. J. Exp. Pathol.* **32**, 97–109
Hughes, D. E. (1955) *Biochem. J.* **60**, 303–310
Katagiri, M., Maeno, H., Yamamoto, S. & Hayaishi, O. (1965) *J. Biol. Chem.* **240**, 3414–3417
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Maki, Y., Yamamoto, S., Nozaki, M. & Hayaishi, O. (1969) *J. Biol. Chem.* **244**, 2942–2950
Meislich, H. (1962) *Pyridine and its Derivatives*, part 3, p. 619, Interscience, New York
Nakamura, S., Ogura, Y., Yano, K., Higashi, N. & Arima, K. (1970) *Biochemistry* **9**, 3235–3242
Okamoto, H., Nozaki, M. & Hayaishi, O. (1968) *Biochem. Biophys. Res. Commun.* **32**, 30–36
Orpin, C. G., Knight, M. & Evans, W. C. (1972) *Biochem. J.* **127**, 833–844
Spector, T. & Massey, V. (1972) *J. Biol. Chem.* **247**, 5632–5636
Takemori, S., Yasuda, H., Mihara, K., Suzuki, K. & Katagiri, M. (1969) *Biochim. Biophys. Acta* **191**, 58–68
Teng, N., Kotowycz, G., Calvin, M. & Hosakawa, K. (1972) *J. Biol. Chem.* **246**, 5448–5453
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 Publishing Co., Minneapolis
van Schickh, O., Binz, A. & Schultz, A. (1936) *Chem. Ber.* **69**, 2593–2605
Watson, G. K., Houghton, C. & Cain, R. B. (1974) *Biochem. J.* **140**, 277–292
White-Stevens, R. H. & Kamin, H. (1972) *J. Biol. Chem.* **247**, 2358–2370
White-Stevens, R. H., Kamin, H. & Gibson, Q. H. (1972) *J. Biol. Chem.* **247**, 2371–2381
Wright, K. A. & Cain, R. B. (1972) *Biochem. J.* **128**, 543–559
Yamamoto, S., Katagiri, M., Maeno, H. & Hayaishi, O. (1965) *J. Biol. Chem.* **240**, 2408–3413
Yamamoto, S., Takeda, H., Maki, Y. & Hayaishi, O. (1969) *J. Biol. Chem.* **244**, 2951–2955
Yano, K., Higashi, N. & Arima, K. (1969) *Biochem. Biophys. Res. Commun.* **34**, 1–7