Biochem. J. (1972) **127**, 819–831
Printed in Great Britain

The Bacterial Oxidation of Picolinamide, a Photolytic Product of Diquat

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(*Received* 3 *November* 1971)

The pathway of oxidation of picolinamide (pyridine-2-carboxamide) by a Gram-negative rod has been elucidated. Under conditions of high pH, restricted aeration and high substrate concentration, whole cells released 2,5-dihydroxypyridine into culture supernatants. Sodium arsenite at 5mM caused whole cells to accumulate 6-hydroxypicolinate, and, at 1mM, pyruvate, in culture media. Whole cells oxidized picolinamide, picolinate, 6-hydroxypicolinate, maleamate and maleate without lag. Cell-free extracts converted picolinamide into picolinate, and hydroxylated picolinate to 6-hydroxypicolinate. The hydroxylase was particulate, but could be solubilized by ultrasonic treatment; it required NAD^+ for activity, and did not require molecular oxygen. 2,5-Dihydroxypyridine was converted into maleamate and formate by an oxygenase requiring GSH and Fe^{2+}. Maleamate was deamidated to maleate, and maleate isomerized to fumarate, by unsupplemented extracts.

The bipyridylium herbicide Diquat (6,7-dihydrodipyrido[1,2-*a*:2′,1′-*c*]pyrazinediium ion) is now used extensively for desiccation or total foliage destruction. Its value lies in the fact that it is active on any photosynthetic tissue, can be applied at low rates in aqueous solution and is immediately inactivated on reaching the soil by absorption on clay minerals and organic matter. In this state it is protected from leaching and can accumulate in the soil system. It is, however, known that Diquat does disappear slowly, although it seems relatively resistant to biological degradation. Nothing is known of its metabolism, if any, in higher plants.

Diquat is degraded photochemically, a process accelerated by surface adsorption, to the 1,2,3,4-tetrahydro-1-oxopyrido[1,2-*a*]-5-pyrazinium ion (Slade & Smith, 1967; Smith & Grove, 1969), which further decomposes to picolinamide (pyridine-2-carboxamide) and picolinate (pyridine-2-carboxylate) (Calderbank, 1968; Smith & Grove, 1969). A bacterium capable of growing on picolinamide has been isolated; the present paper describes the pathway by which picolinamide is oxidized by this bacterium. A preliminary report of this work has been published (Orpin *et al.*, 1971).

Materials and Methods

Microbiological methods

Organism and culture conditions. A short, Gram-negative rod-shaped bacterium capable of utilizing picolinamide as a sole source of carbon and nitrogen was isolated from garden soil perfused with medium containing (g/litre): KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 0.5; $MgSO_4,7H_2O$, 0.2; $CaCl_2,6H_2O$, 0.02; $FeSO_4,7H_2O$, 0.005; $ZnSO_4,7H_2O$, 0.002; $CuSO_4,5H_2O$, 0.002; $CoSO_4$, 0.002; picolinamide, 0.1. The medium was adjusted to pH7.0 with 10M-NaOH, and the organism was grown in the perfusion medium with the picolinamide concentration increased to 0.3g/litre. It was maintained on this medium solidified with 20g of agar/litre. All the media were sterilized by autoclaving at 120°C for 30min. Starter cultures consisting of 35ml of growth medium supplemented with 0.2g of Oxoid yeast extract/litre were incubated at 25°C with aeration (Gallenkamp Orbital shaker, 130 strokes/min). When fully grown, these cultures were used to inoculate 300ml of growth medium contained in 1-litre baffled flasks, which were incubated at 25°C under forced aeration. These were harvested after 2–3 days, while growth was still logarithmic, by centrifugation in a bench-model Sharples Supercentrifuge. The cells were washed twice with five times the pellet volume of 67mM-sodium phosphate buffer (pH7.0) (Umbreit *et al.*, 1957) by centrifugation at 3000*g* for 15min, and either stored as a pellet at −20°C or used immediately. Before use, stored cells were incubated with 0.2g of picolinamide/litre, in 67mM-sodium phosphate buffer, pH7.0, for 1h at 25°C. The cells were then centrifuged at 3000*g* for 15min; the pellet was washed twice with five times the pellet volume of, and resuspended in, the same buffer.

Preparation of cell-free extracts. Thick suspensions of cells (30–50mg dry wt./ml in 67mM-sodium phosphate buffer, pH6.8 or 7.0) were disrupted by the use of either a Biox X-press (A. B. Biox, Nacka,

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Sweden) or an MSE–Mullard 60W ultrasonic disintegrator. The disrupted cell suspensions were centrifuged at 3000*g* for 15min at 0°C in an MSE 18 centrifuge, to remove the unbroken cells and large debris. The crude extract so obtained was used either directly or centrifuged further at 18000*g* for 20min and the particulate fraction (*P*1 or *Pu*1, *P*1 referring to extracts prepared with the X-press, *Pu*1 to those prepared with the ultrasonic disintegrator) removed. In some experiments, the supernatant (*S*1 or *Su*1) was then centrifuged at 100000*g* for 30min at 0°C, in an MSE 65 centrifuge, yielding a brick-red particulate fraction (*P*2 or *Pu*2) and the supernatant (*S*2 or *Su*2).

Analytical methods

Spectrophotometry. Spectrophotometric determinations were carried out in Beckman DB or Unicam SP.800 recording spectrophotometers. Infrared spectra were determined with a Perkin–Elmer Infracord model 137E with KCl or KBr discs.

Manometry. Gaseous exchange was measured by the direct method of Dixon (1952) with conventional Warburg constant-volume respirometers.

Determinations. Protein concentrations were determined by the method of Gornall *et al.* (1949). Interference by the amide group in picolinamide and maleamate was negligible at the concentration of these compounds present in reaction mixtures.

Picolinamide was determined either spectrophotometrically at 263nm ($\epsilon_{263} = 4050$) or, when interfering compounds were present, by the measurement of ammonia liberated on acid hydrolysis. After being deproteinized at pH7.0, the supernatant was acidified to pH4 with 2M-HCl and made up to a known volume with water. Two 1ml samples were then taken, placed in capped tubes and evaporated to near dryness on a boiling-water bath under a stream of air; one sample was then removed, to serve as the unhydrolysed control, and the other subjected to acid hydrolysis. Conc. HCl (1 drop) was added, and the sample covered and left in the boiling-water bath for a further 30min, after which time the ammonia present in both samples was determined by a modification of the ninhydrin method of Brown *et al.* (1969). The reagent was prepared by the following method: ninhydrin (1.45g) and hydrindantin (0.22g) were dissolved in 2-methoxyethanol (160ml) in a black reagent bottle. Nitrogen was bubbled through the solution for 30min; 25ml of 4M-sodium acetate buffer, pH5.5, and 155ml of de-ionized water were added, and N_2 was bubbled through for a further 30min. The reagent was stored under N_2. Then 1ml of this reagent was added to each sample in which it was desired to determine ammonia, and the mixture heated on a boiling-water bath for 10min, after which time maximum colour production had been reached. The volume in each tube was adjusted to 10ml with water, and the E_{570} read against a sample prepared under identical conditions from a control experiment containing no picolinamide. It was assumed that the ammonia formed, corrected for endogenous ammonia in the unhydrolysed control, was all formed by the hydrolysis of the picolinamide.

Picolinate was measured spectrophotometrically at 264nm at pH1 ($\epsilon_{264} = 7800$). In experiments with cell-free extracts, it was first purified; the reaction mixture was deproteinized at pH7.0, evaporated to dryness under N_2, and the picolinate extracted into methanol (4 × 2ml portions). This extract was evaporated to 1ml and chromatographed quantitatively in solvent (*a*) (see below). The material running at the same R_F as picolinate was eluted into methanol, the solution was evaporated under N_2, the residue dissolved in a known volume of water, acidified to pH1 and the E_{264} was measured. A calibration curve was prepared, all the samples going through this procedure, to minimize errors from losses incurred during the isolation procedure.

6-Hydroxypicolinate was determined spectrophotometrically at 310nm at pH1 ($\epsilon_{310} = 4450$). In experiments with cell-free extracts it was first purified by chromatography and estimated in the same manner as picolinate.

2,5-Dihydroxypyridine was determined spectrophotometrically at 320nm at pH7.0, or, when NAD(P)H was present, at 310nm, and quantified by reference to a calibration curve. In experiments with cell-free extracts it was first purified by chromatography and estimated in the same manner as picolinate.

Fumaramate and maleamate were determined by the measurement of ammonia liberated on acid hydrolysis, as described for the determination of picolinamide.

Fumarate and maleate were determined by the permanganate method of Scott & Powell (1948) after quantitative t.l.c. of deproteinized reaction mixtures in solvent (*f*).

Formate was reduced to formaldehyde with magnesium turnings (Eegriwe, 1937) and the formaldehyde determined by the chromotropic acid method of MacFayden *et al.* (1945).

Ammonia, when liberated in enzymic reaction mixtures, was measured by using double-side-arm Warburg flasks. The experiments were initiated by the addition of substrate from one side arm to the reaction mixture in the main chamber of the flask, and terminated by the addition of alkali from the second side arm. The ammonia liberated from the reaction mixture was absorbed by acid in the centre well of the flask. The flasks were kept shaking for a further 2h to allow the acid to absorb all the ammonia liberated. The filter paper in, and other

contents of, the centre well were removed and placed in a centrifuge tube, eluted with 5ml of water and centrifuged to sediment small particles of cellulose. The supernatant was decanted and the ammonia present measured with the ninhydrin reagent, prepared and used as described above for the determination of picolinamide.

Chromatography. Chromatography was carried out on thin layers of silica gel [Kieselgel G; E. Merck A.G., Darmstadt, Germany, supplied by Camlab (Glass) Ltd., Cambridge, U.K.] on 20cm × 20cm or 20cm × 10cm glass plates. For analytical purposes 0.25mm layers were used; for preparative purposes 1.0mm layers were used. The plates were allowed to dry at room temperature and finally dried at 120°C for 30min. The following solvents were used: (*a*) the upper phase of butan-1-ol–acetic acid–water (5:1:4, by vol.); (*b*) 1M-ammonium acetate–ethanol (1:8, v/v); (*c*) ethanol–aq. NH_3 soln. (sp.gr. 0.88)–water (20:1:4, by vol.); (*d*) pyridine–light petroleum (b.p. 60–80°C) (1:2, v/v); (*e*) light petroleum (b.p. 60–80°C)–ethyl formate–propionic acid (26:14:3, by vol.); (*f*) the upper layer of diethyl ether–benzene–formic acid (14:6:5, by vol.), saturated with water; (*g*) butan-1-ol–ethanol–water (7:1:2, by vol.). Standard compounds were run with unknown samples wherever possible. The R_F values for some metabolites of picolinamide are given in Table 1.

Compounds that fluoresced or absorbed light on chromatograms were detected under u.v. light at 254nm (Chromatolite; Hanovia Lamps Ltd., Slough, Bucks., U.K.). Weakly absorbing materials were detected in the same manner after spraying with ethanolic 0.05% sodium fluorescein. 2-Carboxypyridines were detected by spraying with freshly prepared aq. 3% (w/v) ferrous sulphate, which produced brown spots. Phenolic compounds were detected by spraying with Folin–Ciocalteu reagent, followed by 4% (w/v) Na_2CO_3, giving blue spots on a white background. Carbonyl compounds were detected by spraying with 0.1% 2,4-dinitrophenylhydrazine in 2M-HCl, giving yellow 2,4-dinitrophenylhydrazones, which absorb u.v. light. The chromatograms were then exposed to ammonia fumes, when the derivatives of some carbonyl compounds became brown. Unsaturated compounds were detected by spraying with 0.05M-$KMnO_4$ in 3% (w/v) metaphosphoric acid, giving white spots on a pink background.

Deproteinization. Deproteinization was effected by the addition of 25% (w/v) trichloroacetic acid to the protein solution, to give a 5% final concentration of trichloroacetic acid, where acid precipitation was acceptable. Deproteinizing at neutral pH was accomplished by treatment with 0.2vol. of satd. $Ba(OH)_2$-20% (w/v) $ZnSO_4$ (2:1, v/v), followed by removal of the precipitate by centrifugation. Deproteinization at neutral pH was desirable to avoid adsorption of pyridinium ions formed at low pH values on the precipitated protein.

Chemicals

Buffers. Tris–HCl, tris–maleate, citrate–sodium phosphate and boric acid–sodium tetraborate buffers were prepared as described by Gomori (1955). Sodium phosphate buffer was prepared by the method of Umbreit *et al.* (1957).

Reagents. Picolinate, 2-picoline, nicotinamide and isonicotinate were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.; 2-hydroxypyridine, 3-hydroxypyridine, 4-hydroxypyridine and maleamate from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; 2,6-dihydroxypyridine hydrochloride and 2,3-dihydroxypyridine from Ralph N. Emanuel

Table 1. *R_F values of picolinamide and metabolites*

For experimental details see the text.

Compound	Solvent ...	R_F of compound (*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)
Picolinamide		0.67	0.73	0.54	—	—	0.46	—
Picolinate		0.28	0.43	0.45	—	—	0.07	—
6-Hydroxypicolinate		0.37	0.50	0.52	—	—	0.33	—
2,5-Dihydroxypyridine		0.68	0.71	0.68	—	—	0.22	—
Maleamate		0.61	—	0.28	—	—	0.42	—
Maleate		0.54	—	0.27	—	—	0.47	—
Fumarate		0.75	—	0.61	—	—	0.90	—
Pyruvate		—	—	0.43	0.47	—	—	—
Pyruvate 2,4-dinitrophenylhydrazone		—	—	—	—	0.32	—	0.53

Ltd., London S.E.1, U.K.; picolinate-*N*-oxide and 2-hydroxymethyl-3-hydroxypyridine hydrochloride from Kodak Ltd., West Kirby, Liverpool, U.K. All were purified by recrystallization. All other chemicals were of reagent grade from commercial sources; all solvents were redistilled before use.

Syntheses. The following materials were synthesized by the methods of the respective authors; picolinamide and isonicotinamide (pyridine-4-carboxamide) (Vogel, 1956); 3-hydroxypicolinate (Sheehan, 1966); 5-hydroxypicolinate (Duesel & Scudi, 1949); 6-hydroxypicolinate (Newbold & Spring, 1949; Boekelheide & Lehn, 1961); 2,4-dihydroxypyridine (von Schickh *et al.*, 1936); 2,5-dihydroxypyridine (Behrman & Pitt, 1958); fumaramate (Greiss, 1879).

3,4-Dihydroxypyridine was synthesized by an adaptation of the method of Wibaut & Kleipool (1947) for the synthesis of *N*-methyl-3,4-dihydroxypyridine: meconic acid (1g), synthesized by the method of Wibaut & Kleipool (1947), was heated in a sealed tube with aq. NH_3 soln. (sp.gr. 0.88; 1g) in 20ml of water, at 100°C for 8h. The resulting 2,6-dicarboxylate was decarboxylated by heating in a sealed tube for 4h at 230°C, and the 3,4-dihydroxypyridine produced purified by treatment of the reaction mixture with charcoal, followed by evaporation to dryness under reduced pressure until crystals formed. The mixture was kept at 4°C overnight and filtered. The yield of 3,4-dihydroxypyridine was 0.6g, m.p. 236–239°C (lit. 240–241°C).

Results

Experiments with whole cells

Investigations of cultures. The supernatant of cultures was examined spectrophotometrically during growth on picolinamide. It was found that under conditions of restricted aeration, high substrate concentration and alkaline pH, E_{320} increased. If the pH was allowed to rise to 8 or higher, the supernatant became green and then brown. Tests on the supernatant before the appearance of the green colour showed that there was an accumulation of a compound giving a blue colour with Folin–Ciocalteu reagent. The optimum conditions for the production, at 25°C, of this compound were determined as pH7.5 and 1g of picolinamide/litre, with slight aeration. The nature of this compound was investigated. Washed cells (0.7g dry wt.), suspended in 350ml of 67mM-phosphate buffer, pH7.5, containing 1g of picolinamide/litre, were incubated at 25°C and shaken gently by hand every 30min. At the end of 3h, when E_{320} was at a maximum, the cells were centrifuged down, and the supernatant was evaporated to dryness *in vacuo* at a temperature not exceeding 35°C in a rotary evaporator previously flushed with nitrogen. The residue was extracted into propan-2-ol (5×20ml), and this extract concentrated under nitrogen to approx. 5ml. This was then chromatographed by using solvents (*c*) and (*f*). Solvent (*c*) resolved six components, three of which had the same R_F values and characteristics when viewed under u.v. light as picolinamide, picolinate and 2,5-dihydroxypyridine. Authentic 2,5-dihydroxypyridine was partially oxidized in this solvent and chromatographed as four components, one of which was unchanged 2,5-dihydroxypyridine. The three other components from the incubation mixture ran at the same R_F values as the oxidation products of the authentic 2,5-dihydroxypyridine. The spots corresponding to picolinate and 2,5-dihydroxypyridine were scraped off, eluted with propan-2-ol and their u.v. spectra, i.r. spectra and melting points found to be practically identical with those of authentic materials (m.p. of authentic picolinate = 135–137°C, isolated material = 135–136°C; m.p. of authentic 2,5-dihydroxypyridine = 248°C, isolated material = 245–247°C). The diacetyl derivative of 2,5-dihydroxypyridine and the *p*-toluidide derivative of picolinate were prepared and found to have similar melting points (68°C and 104°C respectively) to the corresponding derivatives of the isolates (68–69°C; 103°C). The mixed melting points were unchanged. Solvent (*f*) resolved three components in addition to picolinamide, two of which were identified, by their R_F, appearance in u.v. light, spectral characteristics and melting point, as picolinate and 2,5-dihydroxypyridine. Each compound was inseparable from authentic material when chromatographed in solvents (*a*), (*b*) and (*c*).

No conversion of picolinamide into picolinate or 2,5-dihydroxypyridine occurred in experiments conducted with boiled cells.

Uptake of oxygen. Manometric experiments showed that picolinamide-grown cells oxidized picolinamide, picolinate, 6-hydroxypicolinate, maleamate and maleate without lag. Oxygen uptake was 4.5, 4.0, 3.6, 1.9 and 1.8mol/mol respectively (Fig. 1). Oxygen uptake was stimulated by 2-hydroxypyridine, but spectrophotometric examination of the supernatant showed that it was not oxidized. None of the monohydroxypyridines, 2,3-dihydroxypyridine, 2,4-dihydroxypyridine, 2,5-dihydroxypyridine, 2,6-dihydroxypyridine, 3-hydroxypicolinate, 5-hydroxypicolinate or fumaramate was oxidized. Glucose-grown cells oxidized none of these compounds immediately, although once more 2-hydroxypyridine stimulated oxygen uptake; picolinamide and picolinate were oxidized after a lag period of 1h.

Inhibitor studies. The following inhibitors were used in attempts to cause an accumulation of intermediates of picolinamide and picolinate metabolism by whole-cell suspensions: cyanide, fluoride, arsenite, *p*-chloromercuribenzoate, iodoacetamide, 8-hydroxyquinoline, EDTA and sodium diethyldithiocarbamate.

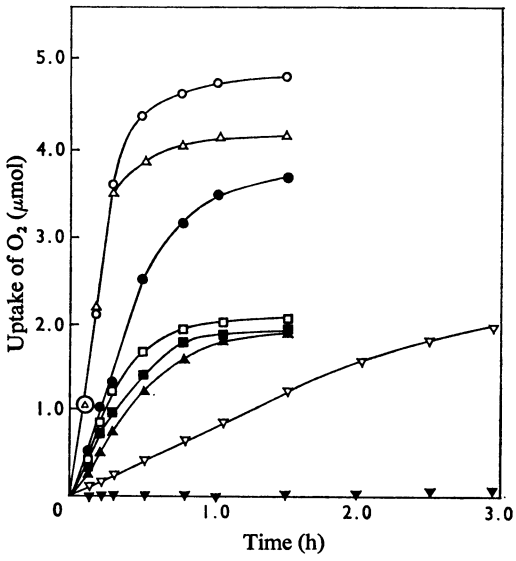


Fig. 1. *Uptake of oxygen by washed cell suspensions of picolinamide-grown cells*

Warburg flasks contained, in a working volume of 2.8ml: phosphate buffer, pH6.8, 190μmol; cell suspension, 8mg dry wt.; substrate, 1μmol. The reaction was started by the addition of substrate from the side arm. The centre well contained 0.2ml of 20% (w/v) KOH. Temperature, 30°C; atmosphere, air. The results are corrected for endogenous uptake. The substrates were: ○, picolinamide; △, picolinate; ●, 6-hydroxypicolinate; □, maleamate; ■, fumarate; ▲, maleate; ▽, 2-hydroxypyridine; ▼, fumaramate, 3- and 4-hydroxypyridine, 2,3-, 2,4-, 2,5- and 2,6-dihydroxypyridine and 3- and 5-hydroxypicolinate.

Oxygen uptakes were measured manometrically; the supernatants were examined spectrophotometrically and tested for the presence of keto acids by the Rothera (1908) test. In the presence of 1mM-sodium arsenite, oxygen uptake was decreased to 2.8mol/mol and a compound giving a blue colour with the Rothera test accumulated. This compound was isolated from a scaled-up reaction mixture. Whole cells (200mg dry wt.) were suspended in 250ml of 50mM-phosphate buffer, pH7.0, containing 1mM-sodium arsenite and 0.5mM-picolinamide, and incubated at 30°C in a 1-litre baffled flask in an orbital shaker; the reaction was monitored spectrophotometrically. When E_{263} indicated a complete oxidation of picolinamide, the incubation mixture was evaporated under reduced pressure to about 25ml, made acid to Congo Red with 1M-HCl, and extracted five times with 50ml portions of diethyl ether. The extract was dried over anhydrous $MgSO_4$ for 24h, the drying agent was filtered off, and the filtrate evaporated to approx. 5ml under reduced pressure. Samples were then chromatographed with solvents (*c*) and (*d*); both showed the presence of a compound with the same R_F as pyruvate. An excess of 2,4-dinitrophenylhydrazine in 2M-HCl was then added to the extract, and the mixture left for 2h, after which time a light precipitate was visible. This was filtered off, recrystallized from ethyl acetate and examined chromatographically in solvents (*c*), (*e*) and (*g*). The only component present corresponded to the 2,4-dinitrophenylhydrazone of authentic pyruvate. The melting point of the derivative (216°C) and that of pyruvate 2,4-dinitrophenylhydrazone (218°C) were similar, and the mixed melting point was not depressed (216–218°C).

In the presence of 5mM-sodium arsenite, the oxygen uptake decreased to 0.6mol/mol, and a spectral examination revealed the appearance of a peak at 310nm. This compound was isolated from a scaled-up reaction mixture: washed whole cells (1.0g dry wt.) were suspended in 500ml of 50mM-phosphate buffer, pH7.0, containing 5mM-sodium arsenite and 1mM-picolinamide, in a 1-litre baffled flask. The suspension was incubated at 30°C in an orbital shaker and the reaction was monitored spectrophotometrically at 310nm. When E_{310} had reached a constant maximal value, the cells were removed by centrifugation and the supernatant was concentrated *in vacuo* to approx. 25ml. It was then passed down a column of Zeo-Karb 225 SRC 14 resin (H^+ form) and eluted with water. The fractions containing the material absorbing at 310nm were combined and evaporated to dryness under reduced pressure, to yield a pale-brown solid. This was sublimed *in vacuo* to yield 81mg of pale-pink material, which was identical with 6-hydroxypicolinate in i.r. and u.v. spectra and on chromatography. The melting point of the isolated material was 270–273°C; that of authentic 6-hydroxypicolinate was 273–275°C, and the mixed melting point was 270–273°C.

Pyruvate and 6-hydroxypicolinate were also formed from picolinate under the same conditions, with oxygen uptakes of 2.6 and 0.55mol/mol respectively.

Experiments with cell-free extracts

Crude cell-free extracts prepared by using the X-press were tested manometrically for their ability to oxidize potential intermediates at pH7.0, with the spectrophotometric examination of the reaction mixture supernatants at the end of the experiment. Oxygen uptake was observed without lag when unsupplemented extract was incubated with picolinamide or picolinate. Oxygen uptake terminated at 0.54 and 0.51mol/mol respectively (Table 2); the rate of oxidation of picolinamide was only 40% of that of picolinate. Spectrophotometric examination

Table 2. *Oxygen uptake of cell-free extracts and fractionated cell-free extracts incubated with picolinamide and picolinate*

Double-side-arm Warburg flasks contained, in a working volume of 2.6ml: sodium phosphate buffer, pH7.0, 175μmol; extract as described below; substrate, 4μmol. The centre well contained 0.2ml of 20% (w/v) KOH. The second side arm contained 0.2ml of 3M-HCl to absorb any NH_3 liberated. Temperature, 30°C; atmosphere, air. Oxygen-uptake values are corrected for endogenous respiration. The whole extract or fractions *S*1, *Su*1, *S*2 and *Su*2 each contained 15mg of protein/ml (1ml of each was used where described); the particulate fractions used (*P*1, *P*2, *Pu*1, *Pu*2) were each centrifuged from 1.0ml of whole extract. PA, picolinate; PN, picolinamide; 6-HPA, 6-hydroxypicolinate. (d), Dialysed.

X-press extracts					Ultrasonic extracts				
Extract	Substrate	Final uptake of O_2 (μl)	Uptake of O_2 (mol/mol)	Product	Extract	Substrate	Final uptake of O_2 (μl)	Uptake of O_2 (mol/mol)	Product
Whole	PN	48	0.54	6-HPA	Whole	PN	48	0.54	6-HPA
Whole	PA	46	0.51	6-HPA	Whole	PA	46	0.52	6-HPA
*P*1	PN	2	—	—	*Pu*1	PA	4	—	—
*S*1	PN	0	—	PA	*Su*1	PA	43	0.48	6-HPA
*P*2	PN	3	—	—	*Pu*2	PA	0	—	—
*S*2	PN	0	—	PA	*Su*2	PA	44	0.49	6-HPA
*P*1+*S*1	PN	47	0.53	6-HPA	*Pu*1+*Su*1	PA	47	0.52	6-HPA
*P*1+*S*2	PN	45	0.52	6-HPA	*Pu*1+*Su*2	PA	45	0.50	6-HPA
*P*1+*P*2	PN	2	—	—	*Pu*1+*Pu*2	PA	2	—	—
*P*2+*S*2	PN	4	—	—	*Pu*2+*Su*2	PA	44	0.49	6-HPA
*P*1	PA	47	0.52	6-HPA	*Su*2 (d)	PA	0	—	—
*S*1	PA	4	—	—	*Su*2 (d)+*Pu*2	PA	0	—	—
*P*2	PA	7	—	—	*Su*2 (d)+*Pu*2+ NAD^+ (1μmol)	PA	43	0.48	6-HPA
*S*2	PA	0	—	—	*Su*2 (d)+NAD^+ (4μmol)	PA	45	—	6-HPA

of the flask contents, after deproteinization at pH7 and centrifugation, showed that in the flasks containing picolinamide or picolinate, the spectrum of the substrate had been replaced by one with $\lambda_{max.}$ at 310nm. Boiled cell-free extract did not give any oxygen uptake or spectral change when incubated with either substrate. 3-Hydroxypicolinate, 5-hydroxypicolinate, 6-hydroxypicolinate, 2-hydroxypyridine, 2,3-dihydroxypyridine, 2,4-dihydroxypyridine, 2,5-dihydroxypyridine and 2,6-dihydroxypyridine were not oxidized. The dihydroxypyridines were incubated at pH6.8 to minimize autoxidation.

Hydrolysis of picolinamide. The crude cell-free extract was fractionated by centrifugation and fractions *P*1, *P*2, *S*1 and *S*2 were tested for their ability to oxidize picolinamide (Table 2). None of the fractions when tested separately showed oxygen uptake in the presence of picolinamide, though spectral investigation of the supernatants showed a decrease in E_{263} in the flasks containing the *S*1 and *S*2 fractions. Fraction *P*1 combined with either fraction *S*1 or *S*2 resulted in an oxygen uptake of 0.52–0.54mol/mol. From a scaled-up reaction mixture, fraction *S*1 was found to convert picolinamide into picolinate with the stoicheiometric release of ammonia. Picolinate was isolated by this procedure: fraction *S*1 (280mg of protein) was incubated with 100μmol of picolinamide in 50ml of 67mM-phosphate buffer, pH7.0, at 30°C with shaking. The reaction was followed spectrophotometrically at 263nm, and when E_{263} was constant, after 1h, the whole reaction mixture was deproteinized at pH7 and centrifuged at 3000*g* for 10min. The supernatant was evaporated to dryness under reduced pressure in an atmosphere of nitrogen, at a temperature not exceeding 35°C. The resulting solid was extracted with ethanol (5×25ml) and this extract was concentrated to 5ml under nitrogen. The concentrate was chromatographed with solvent (*a*), which resolved one major component with R_F 0.28, and which absorbed u.v. light and became brown on spraying with $FeSO_4$. The concentrate was evaporated to dryness under a stream of nitrogen and dissolved in 20ml of water. Then 20ml of 10% (w/v) $CuSO_4$ was added and the precipitated copper salt filtered off. The solid was suspended in water and decomposed with a stream of H_2S. The CuS was removed by centrifugation, and the clear supernatant was evaporated *in vacuo* until crystals formed, when it was chilled to −20°C overnight. The crystals were filtered off and dried in air at 60°C to constant weight (7.5mg). The purified compound exhibited chromatographic properties identical with those of picolinate in five solvent systems, and had identical u.v. and i.r. spectra. The melting points of the isolate (135°C) and authentic material (136–137°C) were similar and the mixed melting point was not depressed (134–137°C).

In crude cell-free extracts, picolinamide deamidase activity had a pH optimum of 7.0; no activity occurred in 50mM-citrate–phosphate buffer below pH4.2; at pH9.5 in 50mM-boric acid–sodium tetraborate buffer, activity was still 68% of that in 67mM-phosphate buffer at pH7.0 (Fig. 2). Dialysis of fraction *S*1 against 200vol. of 10mM-phosphate buffer, pH7.0, at 4°C for 18h resulted in no loss of activity over undialysed extracts. The activity was unaffected by incubation under nitrogen. Isonicotinamide and nicotinamide were not deamidated by crude cell-free extracts.

Hydroxylation of picolinate. Fractions *P*1 and *S*1 combined converted picolinamide into a compound with $\lambda_{max.}$ 310nm, as did the *P*1 fraction with picolinate. The product was isolated from a scaled-up reaction mixture: fraction *P*1 (0.25g dry wt.) was suspended in 50ml of 20mM-phosphate buffer, pH7.0, containing 100μmol of picolinate, and was incubated at 30°C with shaking. When E_{310} of the supernatant was constant the whole reaction mixture was centrifuged at 38000*g* for 20min at 0°C and the supernatant was passed down a column of Zeo-Karb (SRC14) resin (H^+ form). The column was eluted with water, and the fractions containing material absorbing at 310nm were combined, evaporated to dryness *in vacuo* and sublimed *in vacuo* to yield 24mg of pale-pink crystals. The isolated compound was chromatographed in solvents (*a*), (*b*), (*c*) and (*f*) and found to be inseparable from authentic 6-hydroxypicolinate. It had a similar melting point (272°C) to authentic material (273–275°C), the mixed melting point (272–275°C) was not depressed, and it had identical u.v.-spectral characteristics both with the 6-hydroxypicolinate isolated from cultures inhibited with 5mM-sodium arsenite and with authentic material.

During the course of these experiments, a cell-free extract was prepared by ultrasonic disintegration, fractionated, and the fractions were assayed for activity (Table 2). The *Su*1 fraction converted both picolinamide and picolinate into 6-hydroxypicolinate; the *Pu*1 and *Pu*2 fractions were ineffective in hydroxylating picolinate. A considerable amount of picolinate hydroxylase activity was lost from the *Su*1 fraction after centrifuging at 100000*g* for 30min, since the rate of hydroxylation of picolinate by fraction *Su*2 was only 25% of that with fraction *Su*1. Fractions *Su*2 and *Pu*2 combined resulted in activity comparable with that of the original *Su*1 fraction. This showed that a particulate fraction was necessary for fraction *Su*2 to be completely active. Fraction *Su*2, after dialysis for 16h against 100vol. of 20mM-phosphate buffer, pH7.0, would not hydroxylate picolinate, even when combined with fraction *Pu*2. Dialysed fraction *Su*2 hydroxylated picolinate completely in the presence of stoicheiometric quantities of NAD^+, either aerobically or under

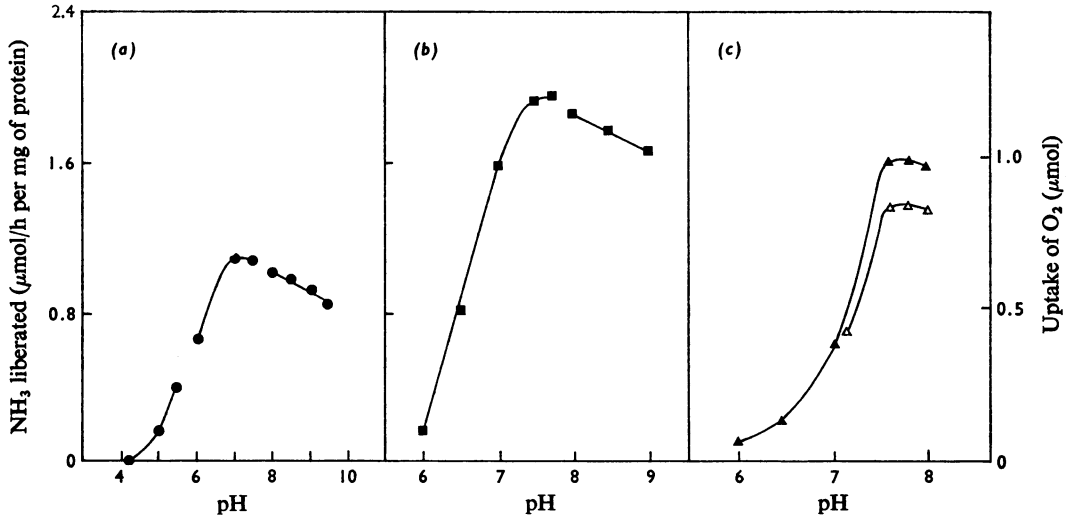


Fig. 2. *pH–activity curves for (a) picolinamide deamidase, (b) maleamate deamidase and (c) 2,5-dihydroxypyridine oxygenase*

(*a*) Picolinamide deamidase was assayed in double-side-arm Warburg flasks containing, in a working volume of 2.2ml: buffer, 90μmol; extract (*S1*), 15mg of protein; picolinamide, 12μmol. The reaction was started by the addition of substrate from one side arm, and stopped by the addition of 0.5ml of 20% (w/v) KOH from the other. The ammonia liberated was absorbed in 0.3ml of 3M-HCl in the centre well and after absorption was measured by the ninhydrin method. The buffers used were: pH4.2–5.6, 50mM-citrate–phosphate; pH6.0–7.7, 50mM-phosphate; pH8.0–9.5, 50mM-borate. Temperature, 30°C; atmosphere, air. (*b*) Maleamate deamidase was assayed in the same manner and under the same conditions as picolinamide deamidase. The buffers used were: pH6.0–7.5, 67mM-phosphate; pH8.0–9.0, 50mM-borate. (*c*) 2,5-Dihydroxypyridine oxygenase was assayed in single-side-arm Warburg flasks containing, in a working volume of 2.8ml: buffer, 120μmol; enzyme (*S1*), 30mg of protein; 2,5-dihydroxypyridine, 5μmol; GSH, 30μmol; $FeSO_4$, 30μmol. The reaction was started by the addition of substrate from the side arm. The centre well contained 0.2ml of 20% (w/v) KOH. The buffers used were: pH6.0–8.0, 50mM-phosphate (▲); pH7.1–8.0, 50mM-tris–HCl (△). Temperature, 30°C; atmosphere, air.

nitrogen. FAD, FMN and NAD(P)H had no effect. Fraction *Pu2* contained a high NAD(P)H oxidase activity and, on the addition of catalytic quantities of NAD^+ to dialysed fractions *Su2+Pu2*, the stoicheiometric hydroxylation of picolinate took place.

6-Hydroxypicolinate was not oxidized by cell-free extracts, even though they were prepared under a variety of conditions.

Oxidation of 2,5-dihydroxypyridine. Crude cell-free extracts, when prepared with the Biox X-press, were unable to oxidize 2,5-dihydroxypyridine. If, however, cells were broken ultrasonically in 50mM-phosphate buffer containing 1mM-2-mercaptoethanol, then the 38000*g* supernatant fraction was capable of degrading 2,5-dihydroxypyridine slowly. All activity was lost on the dialysis of this fraction for 15h at 4°C against 200vol. of 20mM-phosphate buffer, pH7.0, containing 1mM-2-mercaptoethanol. Activity could be restored partly by the addition of GSH to the extracts and almost completely by the addition of GSH and Fe^{2+} (Table 3). Oxygen was found to be obligatory; no oxidation of 2,5-dihydroxypyridine occurred in experiments conducted anaerobically in the presence of GSH and Fe^{2+}, even in the presence of Methylene Blue. L-Cysteine and Fe^{2+} separately stimulated aerobic activity by 10%. NAD(P), oxidized or reduced, flavin nucleotides and metals other than Fe^{2+} did not stimulate activity; αα′-bipyridyl gave 90% inhibition at 0.5mM. Oxygen uptake was 1mol/mol of substrate oxidized. The pH optimum of the reaction was measured manometrically, by using 50mM-phosphate and 50mM-tris–HCl buffers. The optimum was near pH7.5 (Fig. 2); no incubations were run above pH8.0 owing to excessive oxidation of the substrate. Most of the activity was confined to the fractions precipitated between 30 and 50% saturation with ammonium sulphate.

Table 3. *Oxidation of 2,5-dihydroxypyridine by the 38000g supernatant fraction of cell-free extracts*

Single-side-arm Warburg flasks contained, in a working volume of 2.8ml: sodium phosphate buffer, pH6.8, 155μmol; 2,5-dihydroxypyridine, 5μmol; supernatant fraction, 30mg of protein, and, where applicable, L-cysteine, 23μmol; GSH, 23μmol; ascorbate, 23μmol; $FeSO_4,7H_2O$, 2.3μmol. The reaction was started by tipping in the substrate. Temperature, 30°C; atmosphere, air.

	Uptake of O_2			
	Extract (undialysed)		Extract (dialysed)	
Additions	(μl/15min)	(% of maximum)	(μl/15min)	(% of maximum)
None, basal conditions	3.2	15	0	0
L-Cysteine	3.5	17	0	0
GSH	14.2	70	2.6	12
L-Cysteine+Fe^{2+}	3.6	18	2.0	10
GSH+Fe^{2+}	20.7	100	19.6	95
Ascorbate	3.2	15	1.8	9
Ascorbate+Fe^{2+}	3.0	13	2.6	12

This fraction was used to isolate the reaction products: 500mg of protein (30–50%-satd. ammonium sulphate fractions) was incubated at 25°C in 100ml of 50mM-phosphate buffer, pH7.5, containing 5mmol of GSH, 1mmol of 2,5-dihydroxypyridine and 0.5mmol of $FeSO_4$. The reaction was monitored spectrophotometrically in deproteinized samples. When 2,5-dihydroxypyridine had disappeared, the reaction mixture was deproteinized at pH7.0, the precipitate removed by centrifugation and the supernatant made alkaline with 1ml of aq. NH_3 soln. (sp.gr. 0.880). The supernatant was evaporated to dryness under reduced pressure, acidified to Congo Red with 2M-HCl and extracted with 5×25ml o boiling ethanol. The extract was made alkaline with aq. 4M-NH_3 soln. and evaporated to dryness under reduced pressure. The resulting solid was dissolved in water and chromatographed in solvent (*f*). Only one compound could be detected by spraying with the permanganate reagent or sodium fluorescein; this was not found in the control preparation. The compound was separated chromatographically in solvent (*f*), bulked and eluted with water. The eluate was concentrated to dryness under reduced pressure and extracted into hot ethanol; this extract was then evaporated to dryness, yielding a white solid. This material chromatographed as one component in solvents (*a*), (*c*) and (*f*), and was inseparable from maleamate in these solvents. When the chromatograms were sprayed with 10% (v/v) HCl and heated at 120°C for 30min, and then sprayed with the ninhydrin–hydrindantin reagent, both the isolated material and maleamate gave a purple spot. The melting point of the isolate (170–171°C) was similar to that of authentic maleamate (172–173°C) and the mixed melting point (170–172°C) was not depressed.

A sample (5mg) was refluxed with 3ml of ethanolic 10% (w/v) KOH for 30min, neutralized with 2M-HCl and evaporated to dryness. The residue was chromatographed in solvents (*a*), (*c*) and (*f*). The compound corresponding to maleamate had disappeared and a second compound corresponding to fumarate was present. After chromatography in solvent (*f*), the product was eluted with water and the eluate evaporated to dryness under nitrogen to yield a white solid. The melting point of this product was measured (sublimed at 202°C) and found to be similar to fumarate (sublimed at 200°C). A second sample (5mg) was hydrolysed by refluxing in 3ml of 2M-HCl for 30min. Neutralization and evaporation to dryness followed by chromatography in solvents (*a*), (*c*) and (*f*) showed its conversion into maleate. The melting point of the acid-hydrolysis product was identical with that of maleate (130°C); their mixed melting point was unchanged. The acid hydrolysis was repeated and the ammonia liberated measured. For each mol of maleate generated, 1mol of ammonia was released. The behaviour of the oxidation product of 2,5-dihydroxypyridine during alkaline and acid hydrolysis was identical with that of maleamate.

Maleamate contains four of the original five carbon atoms of 2,5-dihydroxypyridine. To discover the fate of the remaining carbon atom (probably C-6) a search for the accumulation of a C-1 compound was instigated, and formaldehyde and formate were sought in reaction mixtures, formate being first reduced to formaldehyde. The formaldehyde was recovered by steam-distillation; the distillate was frozen at −70°C in solid CO_2. Formaldehyde was detected only in those preparations that had been reduced with magnesium turnings, in quantities

suggesting the production of 1mol of formate for each mol of 2,5-dihydroxypyridine oxidized. The stoicheiometry of 2,5-dihydroxypyridine cleavage was studied in the following experiment. Single-side-arm Warburg flasks contained, in a final volume of 2.8ml: phosphate buffer, pH7.0, 180μmol; 2,5-dihydroxypyridine, 5μmol; $FeSO_4$, 2.7μmol; GSH, 27μmol; enzyme, 25mg of protein. The enzyme was the partially purified preparation used above, with excess of ammonium sulphate removed by dialysis against 10mM-phosphate buffer, pH7.0, containing 1mM-2-mercaptoethanol, for 16h. A control flask was used to correct for any ammonia liberated by acid hydrolysis of GSH. 2,5-Dihydroxypyridine was converted quantitatively into maleamate and formate.

Deamidation and oxidation of maleamate. Maleamate was deamidated by the 38000*g* supernatant fraction, but activity was lost during precipitation with either ammonium sulphate or ethanol. Cell-free extracts prepared ultrasonically in 50mM-phosphate buffer, pH7.0, had only 60% of the activity of those prepared in the same buffer containing 1mM-2-mercaptoethanol. Activity was measured by the liberation of ammonia, in the same manner as picolinamide deamidase. Optimum deamidation occurred at pH7.5, and 84% of this activity was present at pH9.0. There was little activity below pH6 (Fig. 2).

The incubation mixture at pH7.5 was deproteinized with 1ml of 3M-H_2SO_4 and the supernatant fluid extracted with 5×10ml of diethyl ether; the ether was evaporated to dryness and the residue dissolved in 0.5ml of aq. 0.1M-NH_3 soln. When this was chromatographed in solvent (*f*), two compounds, maleamate and maleate, were found, although the recovery was not quantitative. It was thought, by analogy with the observation of Behrman & Stanier (1957), that maleate was being isomerized to fumarate, so fumarase activity was blocked by using *meso*-tartrate. In the presence of 10mM-*meso*-tartrate, the stoicheiometric production of fumarate from maleamate and maleate was demonstrated. No deamidation of fumaramate occurred under the same conditions. The 38000*g* fraction, in the presence of 10mM-*meso*-tartrate, converted maleate quantitatively into fumarate (Table 4).

These results indicate the oxidation of picolinamide by this organism to be via the pathway depicted in Scheme 1. The only step not demonstrated enzymically is the conversion of 6-hydroxypicolinate into 2,5-dihydroxypyridine. That fumarate is eventually produced has been conclusively demonstrated, and it is at this point that the C_4 skeleton enters the central pathways of intermediary metabolism.

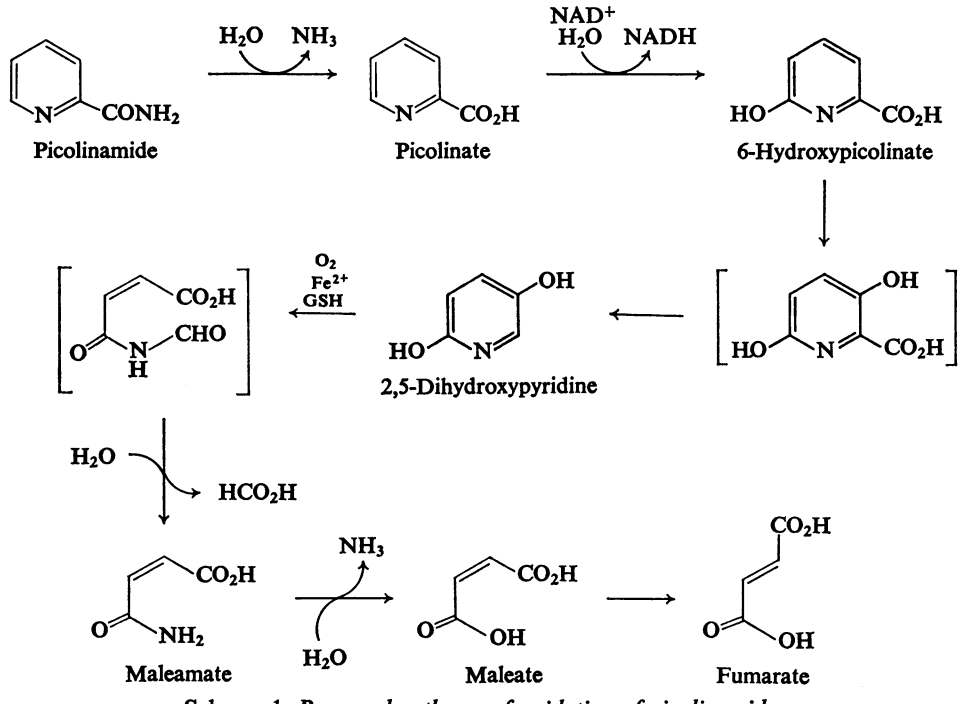
Discussion

The pathway of oxidation of picolinamide has been shown to be mediated by an inducible enzyme system. This pathway has intermediates common

Table 4. *Stoicheiometry of 2,5-dihydroxypyridine, maleamate and maleate oxidation*

(1) Conditions for 2,5-dihydroxypyridine oxidation. Single-side-arm Warburg flasks contained, in a working volume of 2.8ml: sodium phosphate buffer, pH6.8, 175μmol; $FeSO_4,7H_2O$, 2.3μmol; GSH, 23μmol; 2,5-dihydroxypyridine, 5μmol or 10μmol; 30–50%-satd. ammonium sulphate fraction of cell-free extract, 25mg of protein. The experiment was started by the addition of substrate from the side arm. (2) Conditions for maleamate oxidation. Double-side-arm Warburg flasks contained in the main compartment, in a working volume of 2.1ml: sodium phosphate buffer, pH7.5, 140μmol; 38000*g* supernatant protein, 10mg; *meso*-tartrate, 17μmol; substrate, 5 or 10μmol; the centre well contained 0.5ml of 3M-HCl. One side arm contained 0.4ml of 20% KOH; the experiment was started by the addition of substrate from the other side arm, and terminated after 1h by the addition of the KOH. (3) Conditions for maleate oxidation were as for maleamate oxidation, but single-side-arm Warburg flasks were employed, and the KOH and HCl were omitted. Fumarate was measured after 1h of incubation. All flasks were incubated at 30°C; atmosphere, air. Oxygen-uptake values are corrected for endogenous respiration.

Enzyme fraction	Substrate supplied	Products (μmol)				Uptake of O_2 (μmol)
		Maleamate	Formate	Maleate	Ammonia	
30–50%-satd. ammonium sulphate	2,5-Dihydroxypyridine, 5.0μmol	4.9	4.9	—	—	4.8
	2,5-Dihydroxypyridine, 10.0μmol	10.2	9.6	—	—	10.0
38000*g* supernatant	Maleamate, 5.0μmol	—	—	4.8	4.7	—
	Maleamate, 10.0μmol	—	—	9.8	9.8	—
	Maleate, 5.0μmol	—	—	4.7	—	—
	Maleate, 10.0μmol	—	—	9.6	—	—



Scheme 1. *Proposed pathway of oxidation of picolinamide*

with oxidation pathways of certain other substituted pyridines in that 2,5-dihydroxypyridine is an intermediate and that ring-fission eventually yields maleamate. This has been demonstrated for nicotinate (Behrman & Stanier, 1957), nicotine (Gherna, 1964), 2-hydroxypyridine and 3-hydroxypyridine (Houghton *et al.*, 1968). Similarly, 6-hydroxylation of the pyridine nucleus has been demonstrated in those compounds that have an unsubstituted carbon atom in this position: nicotinate (Ensign & Rittenberg, 1964), isonicotinate (Ensign & Rittenberg, 1965), nicotine (Gherna, 1964) and picolinate (Dagley & Johnson, 1963). The production of a 6-hydroxylated intermediate does not, however, mean that 2,5-dihydroxypyridine is necessarily on the pathway of oxidation. 6-Hydroxy intermediates may be subsequently hydroxylated in the 2-position, as in the alternative pathways of nicotine (Gherna, 1964) and nicotinate (Ensign & Rittenberg, 1964) oxidation, to generate a 2,6-dihydroxy compound. The evidence presented here, however, shows that in this case 2,5-dihydroxypyridine is an intermediate even though it did not accumulate in any cell-free system and was not oxidized by whole cells. It is possible that 2,5-dihydroxypyridine released into the supernatant of cultures is only a by-product of the oxidative pathway and not a main component of that pathway, but the fact that it is oxidized in a cell-free system precludes this possibility.

Let us now consider the reactions leading to the production of 2,5-dihydroxypyridine. The picolinamide deamidase, though not purified, exhibited similar characteristics to other deamidases; no cofactors were shown to be necessary, maximum activity occurred at a slightly alkaline pH and activity was maintained at a high rate even at pH9.0. Essentially similar properties have been shown by Hughes & Williamson (1953) in a nicotinamide deamidase, by Halpern & Grossowicz (1957) for asparaginase and by Archibald (1944) and Klingman & Handler (1958) for glutaminase. The reaction appears to be one of a simple hydrolytic cleavage of the carbon–nitrogen bond of the amide group.

The picolinate hydroxylase in this system is a particulate enzyme that can be solubilized by ultrasonic treatment. Oxygen is not obligatory, since the reaction can take place anaerobically with the solubilized enzyme in the presence of NAD^+, which is reduced during the reaction. This indicates a hydration of a double bond and subsequent dehydrogenation. NAD^+ was required in stoicheiometric quantities, though it could be continuously regenerated by the particulate NADH oxidase system of the fractionated cell-free extract, which contains the cytochrome system. Hunt *et al.* (1958) showed that the oxygen incorporated into the hydroxyl group during the hydroxylation of nicotinate was derived from water. The oxidizing agent was not

identified, but it was thought that the dehydrogenation was linked with molecular oxygen via the cytochrome system. Until now NAD^+ has not been positively implicated as a cofactor in the 6-hydroxylation of any pyridine nucleus. The system of Dagley & Johnson (1963), hydroxylating picolinate, is probably a similar reaction involving a different cofactor for the dehydrogenation step: the addition of NAD^+ or NADH to their reaction mixtures did not affect the rate of hydroxylation. The system would operate anaerobically in the presence of Methylene Blue and the enzyme was soluble after centrifugation at 10500*g* for 45min, so it is probable that some of the cytochrome system was present to oxidize the primary electron acceptor, which was not identified.

Hydroxylation by successive hydration and dehydrogenation steps is not uncommon in the oxidation of the pyridine nucleus. All the 6-hydroxylating systems described differ from systems hydroxylating the aromatic ring in requiring neither molecular oxygen, nor thiols, nor metal ions. The picolinate hydroxylase studied here is no exception.

The steps during the conversion of 6-hydroxypicolinate into 2,5-dihydroxypyridine are not clear. Assuming that no dehydroxylation occurs, the ways in which this conversion could occur are: (*a*) by the hydroxylation of 6-hydroxypicolinate to 3,6-dihydroxypicolinate, followed by decarboxylation; (*b*) the decarboxylation of 6-hydroxypicolinate to 2-hydroxypyridine, followed by hydroxylation at C-5. System (*a*) could not be tested, since 3,6-dihydroxypicolinate was not available and could not be synthesized. Nevertheless it seems more likely, since 2-hydroxypyridine was not oxidized either by whole cells or cell-free extracts under any conditions. It has not been possible to determine the fate of the carboxyl carbon atom; it is presumably lost in a non-oxidative decarboxylation reaction similar to the conversion of 4,5-dihydroxyphthalate into protocatechuate (Ribbons & Evans, 1960).

The oxygen uptake of 1mol/mol and requirement of Fe^{2+} during the cleavage of 2,5-dihydroxypyridine is in agreement with the work of Behrman & Stanier (1957). They, however, did not implicate a thiol or reducing agent in the reaction. GSH was required during 2,5-dihydroxypyridine cleavage by cell-free extracts of the picolinamide-oxidizing organisms, and this system is therefore essentially similar to that of Houghton *et al.* (1968), who showed that 2,5-dihydroxypyridine oxygenase from an organism oxidizing 3-hydroxypyridine required L-cysteine in addition to Fe^{2+} for maximum activity. The pH optimum of their system was 7.0, whereas that from the picolinamide-oxidizing organism was rather higher, at pH7.5.

The work on the 2,5-dihydroxypyridine oxygenase in the present paper does not suggest any mechanism for ring-fission. Behrman & Stanier (1957) suggest a cleavage between C-5 and C-6 of the pyridine ring, to yield *N*-formylmaleamate, which is in turn hydrolysed to formate and maleamate. Such a cleavage, too, seems probable in the system studied here, as 1mol of 2,5-dihydroxypyridine yields 1mol each of formate and maleamate, with an oxygen consumption of 1mol/mol. This oxygen uptake precludes the occurrence of 2,3,6-trihydroxypyridine, which has been suggested as the ring-fission substrate during the oxidation of nicotine (Gherna, 1964) and nicotinate (Ensign & Rittenberg, 1964).

The deamidation of maleamate appears to be a hydrolytic cleavage of the carbon–nitrogen bond of the amide group. No cofactor or metal ions were shown to be necessary. Treatment with ammonium sulphate inactivated the enzyme in this system, though both the maleamate deamidases of Houghton *et al.* (1968) could be purified by this precipitation method. Behrman & Stanier (1957) did not attempt to purify their maleamate deamidase.

C. G. O. thanks the Ministry of Agriculture, Fisheries and Food for a post-graduate studentship.

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