Biochem. J. (1972) 130, 879–893
Printed in Great Britain

Microbial Metabolism of the Pyridine Ring

FORMATION OF PYRIDINEDIOLS (DIHYDROXYPYRIDINES) AS INTERMEDIATES IN THE DEGRADATION OF PYRIDINE COMPOUNDS BY MICRO-ORGANISMS

By C. HOUGHTON* and R. B. CAIN†
Microbiology Group, Department of Botany, University of Newcastle upon Tyne, Newcastle upon Tyne NE3 7RU, U.K.

(*Received* 22 *May* 1972)

1. Several species of micro-organisms that were capable of utilizing pyridine compounds as carbon and energy source were isolated from soil and sewage. Compounds degraded included pyridine and the three isomeric hydroxypyridines. 2. Suitable modifications of the cultural conditions led to the accumulation of pyridinediols (dihydroxypyridines), which were isolated and characterized. 3. Three species of *Achromobacter* produced pyridine-2,5-diol from 2- or 3-hydroxypyridine whereas an uncommon *Agrobacterium* sp. (N.C.I.B. 10413) produced pyridine-3,4-diol from 4-hydroxypyridine. 4. On the basis of chemical isolation, induction of the necessary enzymes in washed suspensions and the substrate specificity exhibited by the isolated bacteria, the initial transformations proposed are: 2-hydroxypyridine → pyridine-2,5-diol; 3-hydroxypyridine → pyridine-2,5-diol and 4-hydroxypyridine → pyridine-3,4-diol. 5. A selected pyridine-utilizer, *Nocardia* Z1, did not produce any detectable hydroxy derivative from pyridine, but carried out a slow oxidation of 3-hydroxypyridine to pyridine-2,3-diol and pyridine-3,4-diol. These diols were not further metabolized. 6. Addition of the isomeric hydroxypyridines to a model hydroxylating system resulted in the formation of those diols predicted by theory.

Although pyridine itself has only been detected in the rayless golden rod, *Aplopappus hartwegi* (Buehrer *et al.*, 1939), many other simple derivatives of pyridine (Renz, 1936; Manske & Marion, 1942; Powell & Strange, 1953) and its fully reduced analogue piperidine (Spath & Englander, 1935; Yurashevskii & Stepanov, 1939) are known in Nature. Most of these simple compounds are of restricted distribution, in contrast with the nicotinamide nucleotides which have essential metabolic roles in all living forms, and the pyridine- and piperidine-based alkaloids (Marion, 1950), which are widespread among plants. On the death and decay of the host species these pyridine compounds are returned to the soil.

Pyridine compounds are also released into the environment by the industrial and domestic use of coal, which gives rise to pyridine bases that are employed extensively as organic solvents and inevitably find their way into effluents. Further, the application of naturally occurring and chemically synthesized pesticides has become a widespread agricultural practice. Alkaloids such as nicotine, anabasine and cavadine have long had established usage in insecticide formulations, and more recent research (Bernstein *et al.*, 1966; Mohr *et al.*, 1968) has indicated the fungicidal activity of, for instance, 2-hydroxypyridine 1-oxide against *Microsporum* infections and of 2,6-dichloro-4-phenylpyridine-3,5-dicarbonitrile against *Alternaria*, *Venturia inaequalis*, *Cladosporium fulvum* and *Plasmopara*. Martin (1966) and Clapham *et al.* (1967) have reported the effective weed control given by 2,3,5-trichloro-4-pyridinol, 2,3,5,6-tetrachloro-4-pyridinol and the corresponding pyridoxyethanol; paraquat (1,1′-dimethyl-4,4′-bipyridilium dichloride), diquat (1,1′-diethylene-2,2′-bipyridilium dibromide) and picloram (4-amino-3,5,6-trichloropicolinic acid) currently have very wide agricultural use as herbicides (Calderbank, 1968).

Many of these pyridine compounds, both natural and synthetic, are ultimately degraded because their concentrations do not increase substantially in the soil environment. The carbon–nitrogen skeleton of pyridine is thus mineralized and these elements recycled. Such recycling is essential because, whereas a degree of biological recalcitrance is desirable in any pesticide or herbicide if it is to function economically, continued addition of compounds which are completely resistant to degradation can soon lead to the accumulation of concentrations that are potentially toxic or otherwise unacceptable. As part of an investigation of the microbial metabolism of pyridine-based

* Present address: Department of Biological Sciences, Napier College of Science and Technology, Colinton Road, Edinburgh EH10 5DT, U.K.
† Present address: Biological Laboratories, University of Kent, Canterbury, Kent, U.K.

herbicides (Wright & Cain, 1969, 1972), we studied the frequency and nature of the dihydroxypyridine compounds (called pyridinediols hereafter) arising from the microbial metabolism of simple pyridine compounds. Two of these diols were reported as microbial products for the first time by Houghton *et al.* (1968).

Materials

Chemicals and chemical syntheses

Melting points were measured with a Gallenkamp melting-point apparatus and are uncorrected. 2-Hydroxypyridine and 3-hydroxypyridine were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and were recrystallized twice from diethyl ether to give white crystalline products, m.p. 107°C and 124–125°C respectively. 4-Hydroxypyridine from the same source or from R. N. Emanuel Ltd., Alperton, Middx., U.K., was received in a very crude state and was purified by vacuum sublimation [120°C, 93 N/m² (0.7 mmHg)] followed by two recrystallizations from ethanol–diethyl ether (1:5, v/v) to give a white powder, m.p. 148–150°C.

Pyridine-2,5-diol was prepared by an Elbs oxidation of 2-hydroxypyridine with $K_2S_2O_8$ (Behrman & Pitt, 1958). The crude reddish-brown powder was recrystallized twice from ethanol and further purified by vacuum sublimation [130–180°C, 160 N/m² (1.2 mmHg)] to yield a white powder. This material darkened at 220°C and decomposed between 240° and 250°C (Behrman & Pitt, 1958; den Hertog *et al.*, 1950; Adams & Govindachari, 1947). The diacetate derivative was obtained by heating the diol (100 mg) at 40–50°C with an excess of acetic anhydride (0.5 ml) for 15 min. The reaction mixture was dried over NaOH pellets in a vacuum desiccator and the residue recrystallized from ethyl acetate to give crystals, m.p. 68–70°C.

Pyridine-2,3-diol was isolated from the mother liquors left from the crystallization of pyridine-2,5-diol prepared above. The first crop of crystals (predominantly the 2,5-diol) was discarded, but a second small crop was obtained at 4°C and recrystallized from ethanol giving white crystals, m.p. 246–247°C (den Hertog *et al.*, 1950). Pyridine-3,4-diol was prepared by three methods depending upon the availability of mimosine.

(*a*) Pyrolysis of mimosine (Adams *et al.*, 1945). Mimosine {β-[*N*-(3-hydroxypyrid-4-one)]-α-aminopropionic acid} (100 mg) was placed in a vacuum sublimination apparatus operating at 266.6 N/m² (2 mmHg) and rapidly heated to 220–250°C on a sand bath. An orange–yellow solid (41 mg, 74% yield) collected on the 'cold finger'. This material exhibited polymorphism, melting initially at about 80°C, resolidifying and finally remelting at 180°C. After three recrystallizations from ethanol, a pale-yellow compound was obtained, m.p. 236°C.

(*b*) Hydrolysis of mimosine (Hegarty *et al.*, 1964). Mimosine (100 mg) was refluxed with 0.1 M-HCl (2 ml) for 3 h and the solution evaporated to dryness *in vacuo*. The mixture of unchanged mimosine and pyridine-3,4-diol was separated by paper chromatography and the product eluted with water. The eluate contained 42 mg of pyridine-3,4-diol (76% yield) which was chromatographically pure.

These two methods had the advantage of simplicity and the direct formation of reasonably clean preparations; however, mimosine is very expensive, and we are very grateful to Dr. R. D. Court for his gift of the natural product. In the interim, a search for a route from a cheaper source showed that Elbs oxidation of 3-hydroxypyridine resulted in the formation of 2,3-, 2,5- and 3,4-diols in which the yield of the 3,4-diol was very low (Behrman & Pitt, 1958). We finally adopted an Elbs oxidation of 4-hydroxypyridine by the following method (*c*). 4-Hydroxypyridine (11 g), NaOH (20 g) and $K_2S_2O_8$ (33 g) were dissolved in water (375 ml). The mixture was stirred at 75°C for 3 h with a further addition of $K_2S_2O_8$ (26 g) after 2 h. The 4-hydroxypyridine 3-sulphate was then hydrolysed by heating the solution for 30 min after acidifying to pH 0.75 at 100°C. After cooling, the solution was adjusted to pH 6.5 and evaporated to dryness *in vacuo* (E. J. Behrman, personal communication). The solid residue was dried over P_2O_5 and continuously extracted with propan-2-ol in a Soxhlet apparatus. The propanolic extract was evaporated to dryness and the residue dissolved in the minimum volume of water. This solution was then passed down a column of Dowex 2 (X8; OH^- form; 200 mesh; 400 ml bed vol.) and eluted with water. The first few fractions contained a mixture of 4-hydroxypyridine and pyridine-3,4-diol, but subsequent fractions contained only the diol. Fractions containing the mixture were concentrated and again chromatographed. Those fractions containing only the diol were evaporated to dryness *in vacuo* to yield a pale-yellow powder (1 g), m.p. 236°C. Alternatively, the propanolic extract was concentrated and the unchanged 4-hydroxypyridine and pyridine-3,4-diol were separated by preparative-scale paper chromatography. The diacetate derivative of pyridine-3,4-diol was prepared in a manner identical with that used for the 2,5-diol isomer and was recrystallized twice from ethyl acetate to give a product, m.p. 138°C. den Hertog *et al.* (1950) recorded a value of 139–140°C.

Pyridine-2,4-diol was not formed in our hands by the fusion of 4-hydroxypyridine in a NaOH melt (van Schickh *et al.*, 1936). Synthesis was eventually accomplished via a cyclization procedure (Errera, 1898; den Hertog, 1946).

Pyridine-1,2-diol (*N*-hydroxypyrid-2-one) was syn-

thesized by the method of Newbold & Spring (1948), to give a white crystalline product, m.p. 150°C. The isomeric pyridine-1,3-diol (3-hydroxypyridine *N*-oxide), m.p. 191°C, was prepared by the perbenzoic acid oxidation of 3-hydroxypyridine (Shaw, 1949) and recrystallized from methanol. Pyridine-1,4-diol (*N*-hydroxypyrid-4-one) was obtained from pyridine *N*-oxide by the two-step procedure of Ochiai (1953). It was recrystallized from ethanol to give a pale-yellow powder, m.p. 243°C.

Inorganic chemicals were analytical-reagent-grade material from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Micro-organisms

Isolation of pure strains of micro-organisms. Micro-organisms with the ability to utilize pyridine as sole source of carbon, nitrogen and energy, and its hydroxy derivatives as principal carbon source, were obtained by elective culture either in shaken flasks or in soil percolation columns of the type described by Audus (1946). Sewage (1 ml) from an activated-sludge plant or a few crumbs of soil (0.5g) were added to 250ml-capacity conical flasks containing 100ml of a basal medium as follows: K_2HPO_4, 1g/l of water; KCl, 0.25g/l of water; $MgSO_4,7H_2O$, 0.25g/l of water (sterilized separately and added aseptically to the cooled medium); trace-element solution, 1ml; and organic heterocyclic substrates, 1g/l of water. The pH was adjusted to 7.5. The trace-element solution contained (per ml): $FeSO_4,7H_2O$, 40μg; $MnSO_4,4H_2O$, 40μg; $ZnSO_4,7H_2O$, 20μg; $CuSO_4,5H_2O$, 5μg; $CoCl_2,7H_2O$, 4μg; $Na_2MoO_4,2H_2O$, 5μg; $CaCl_2,6H_2O$, 0.5μg; NaCl, 1mg. The basal medium was supplemented with yeast extract (0.5g/l) where necessary and was sterilized by autoclaving. Organic compounds, whose heat-stability was suspect, were sterilized by filtration and added to the sterile basal medium. Pyridine was sterilized in this way for molar-growth-yield studies because 20–25% of the substrate was lost on autoclaving. After two sub-cultures the now enriched microflora was plated on the above basal medium solidified with 2% (w/v) agar.

Columns packed with coke breeze or gravel were inoculated with soil or sewage and percolated with the basal medium until heavy growth appeared on the coke or gravel support. Loopfuls of this growth were plated out directly. Stock cultures were kept at 4°C on slopes of nutrient agar and solidified basal medium.

Growth of the micro-organisms in large batches. Starter cultures of the organisms were inoculated from the appropriate solid medium into 100ml of basal medium supplemented with yeast extract where necessary and adjusted to the appropriate pH (Table 1). The flasks were then incubated at 30°C in a New Brunswick Gyrotory shaker until growth was in the exponential phase. The flask contents were then used as inocula for 1-litre batches of the same media in 2-litre conical flasks and incubation was continued to maintain exponential growth. The 1-litre cultures were then transferred to 10- or 20-litre batches of media. With this large inoculum, incubation at 30°C for 24h (48h for the *Agrobacterium*) gave good cell yields with high enzymic activity. The 10- or 20-litre cultures were aerated by forced aeration at approx. 5–7 litres of air/min dispersed through several coarse (porosity 0) sintered-glass discs. The air flow was measured by means of a calibrated flowmeter (Rotameter; Griffin and George Ltd., Wembley, Middx., U.K.).

The bacteria were harvested in an Alfa-Laval (Brentwood, Middx., U.K.) continuous-flow centrifuge operating at 8000rev./min, washed *in situ* with 2 litres of water and recentrifuged at 3600rev./min in a Super Medium centrifuge (MSE Ltd., Crawley, Surrey, U.K.).

The cells were then used immediately for respiration and metabolic studies or stored at −20°C.

Table 1. *Optimum conditions for growth and yield of cells from organisms degrading pyridine compounds*

The optimum substrate concentrations, the optimum pH for growth and the effects of added yeast extract were determined in growth experiments in 250ml flasks with side arms suitable for nephelometry and containing 75ml of the appropriate medium as described in the legend to Fig. 4. Cell-yield values were obtained from large batch cultures (see the Methods section).

Organism	Substrate added	Optimum substrate concentrations (%, w/v)	Yeast extract added (%, w/v)	Optimum pH	Average cell yield (g wet wt./litre of medium)
Nocardia Z1	Pyridine	0.1	None	8.0	2
Achromobacter G2	2-Hydroxypyridine	0.1	None	7.0	3
Achromobacter 7N	3-Hydroxypyridine	0.1	0.05	6.0	3
Achromobacter 2L	3-Hydroxypyridine	0.1	0.05	7.0	3
Agrobacterium 35S	4-Hydroxypyridine	0.025	0.015	8.0	0.75

Suspensions of the *Agrobacterium* with high metabolic activity towards 4-hydroxypyridine and pyridine-3,4-diol were ensured only by resuspending the washed cell paste in 0.1 M-Na_2HPO_4–KH_2PO_4 buffer, pH 8.2, containing 0.025% (w/v) of 4-hydroxypyridine and incubating for 1 h. This suspension was centrifuged, the cells were washed once with cold water and the resuspended cells used immediately or stored frozen.

Preparation of freeze-dried cells and cell-free extracts. Freeze-dried samples of bacteria were prepared by removing water from dense suspensions of cells frozen as thin shells at −70°C. For manometric studies, the dried preparation was suspended in ice-cold 0.1 M-potassium phosphate or tris–HCl buffers at pH 7.5 to a concentration of 20 mg/ml. Extracts were obtained by crushing the frozen cell pastes at −20°C with a Hughes press (Hughes, 1951) or by grinding the pastes with a pestle and mortar under liquid N_2 (Cain *et al.*, 1968). The thawed crush was taken up in twice its weight of buffer and centrifuged at 3000*g* to remove broken-cell debris. The particulate fraction was separated from the soluble material by centrifugation at 100000*g* for 1 h.

Growth experiments. Growth was followed nephelometrically after standardizing the nephelometer with a suspension of the appropriate organism of known count or dry wt./ml. Cultures were grown in 250 ml conical flasks to which a side-arm tube, neatly fitting the nephelometer, had been fused. When necessary, small samples of the medium were removed aseptically for measurement of the substrate concentration.

Quantitative determinations

Enzymic reactions and chemically catalysed changes in substrate concentrations were measured spectrophotometrically by a spectral scan (220–350 nm) at fast speed with a Pye Unicam SP. 800 recording spectrophotometer. Colorimetric determinations were made with the SP. 600 single-beam instrument.

Pyridine and its derivatives possess an extended conjugated double-bond structure and absorb u.v. light at certain specific wavelengths. The amounts of these compounds remaining in media were calculated from their known molar extinction coefficients (ϵ in litre·mol^{-1}·cm^{-1}) at specific wavelengths. Pyridine was determined spectrophotometrically at 257 nm (ϵ 2880), 2-hydroxypyridine at 293 nm (ϵ 5890), 3-hydroxypyridine at 313 nm (ϵ 3020) and 4-hydroxypyridine at 254 nm (ϵ 16510).

The respective concentrations of 3-hydroxypyridine, pyridine-2,3-diol and pyridine-3,4-diol in solutions containing all three compounds were determined by measuring the extinction at three wavelengths, chosen so that the extinction due to one of the components was much greater than the others. The solution of the three simultaneous equations (1)–(3) enabled the concentration to be determined as follows:

$$E_{313} = C_x X_5 + C_y Y_3 + C_z Z_3 \quad (1)$$

$$E_{297} = C_x X_4 + C_y Y_2 + C_z Z_2 \quad (2)$$

$$E_{273} = C_x X_3 + C_y Y_1 + C_z Z_1 \quad (3)$$

where C_x, C_y and C_z are the concentrations of 3-hydroxypyridine, pyridine-2,3-diol and pyridine-3,4-diol respectively. The molar extinction values X, Y and Z at each of these wavelengths and pH 7.5 are listed in Table 2.

By solving the two simultaneous equations (4) and (5) the quantities of 4-hydroxypyridine and pyridine-

Table 2. *Spectroscopic data used in calculating the concentrations of the hydroxypyridine*

Compound	pH	Wavelength (nm)	ϵ (litre·mol^{-1}·cm^{-1})	Symbol in equations (1)–(7)
3-Hydroxypyridine	6.0	276	2300	X_1
		320	2420	X_2
	7.5	273	1895	X_3
		297	1620	X_4
		313	3020	X_5
4-Hydroxypyridine	8.5	254	16510	S_1
		273	2640	S_2
Pyridine-2,3-diol	7.5	273	3000	Y_1
		297	8150	Y_2
		313	4500	Y_3
Pyridine-2,5-diol	6.0	276	1300	R_1
		320	5620	R_2
Pyridine-3,4-diol	7.5	273	9720	Z_1
		297	2100	Z_2
		313	360	Z_3
	8.5	254	3780	Z_4
		273	9000	Z_5

3,4-diol were determined in reaction mixtures containing both:

$$E_{254} = C_s S_1 + C_z Z_4 \quad (4)$$
$$E_{273} = C_s S_2 + C_z Z_5 \quad (5)$$

where C_s and C_z are the concentrations of 4-hydroxypyridine and pyridine-3,4-diol at pH 8.5 respectively.

The quantities of 3-hydroxypyridine and pyridine-2,5-diol in reaction mixtures containing both were determined by solving the two simultaneous equations (6) and (7):

$$E_{276} = C_r R_1 + C_x X_1 \quad (6)$$
$$E_{320} = C_r R_2 + C_x X_2 \quad (7)$$

where C_x and C_r are the concentration of 3-hydroxypyridine and pyridine-2,5-diol at pH 6.0 respectively. The molar extinction values S, R, X, and Z at the wavelengths in equations (4)–(7) are given in Table 2.

NH_3 was distilled from reaction mixtures or culture solutions in Conway (1962) micro-diffusion units and collected in 1 ml of 0.01 M-H_2SO_4 in the centre well. After 24 h at room temperature the acid solution was diluted to 6 ml and the NH_4^+ content determined by reading the E_{420} after the addition of Nessler's reagent (0.3 ml). Control reaction mixtures containing no NH_3 and standards containing known amounts of NH_3 were taken through the same procedure.

Protein was determined by the biuret method of Gornall *et al.* (1949) with bovine serum albumin as a standard.

The uptake of O_2 and the evolution of CO_2 by whole cells or extracts at 30°C were measured by standard manometric techniques (Umbreit *et al.*, 1957).

Chromatography

Separation of monohydroxypyridine and pyridinediols was achieved on Whatman no. 1 paper in the following solvent systems (all proportions by vol.): (1) butan-1-ol – conc. HCl – water (10:2:25); (2) butan-1-ol–acetic acid–water (4:1:5, upper layer); (3) ethanol–conc. NH_3 (sp.gr. 0.88)–water (20:1:4); (4) butan-1-ol – benzene – water (1:9:10, upper layer). Chromatography was usually performed in the descending direction but solvent (3) was used for ascending paper chromatography. Whatman 3MM paper and solvent (1) were used for preparative experiments.

The preparative separation of pyridine-3,4-diol from 4-hydroxypyridine was effected by thin-layer chromatography on glass plates (20 cm × 20 cm) coated with kieselguhr G (1 mm thick), dried horizontally for 10 min and then activated at 100°C for 30 min. The plates were developed with solvent (5), chloroform–methanol–water–formic acid (98% v/v) (100:20:19:0.8, by vol.; organic phase used).

Pyridine and its hydroxy derivatives were detected by their absorbance (pyridine) or blue fluorescence (pyridinediols) in u.v. light. All the hydroxy compounds gave very specific colours when sprayed with aq. 2% (w/v) $FeCl_3$ solution, which made their initial identification clear even when their R_F values were similar. Other spray reagents included Folin–Ciocalteau reagent (the BDH product diluted with an equal volume of water) followed by a saturated solution of Na_2CO_3, and 1% (w/v) solution of 2,6-dichloro-*p*-benzoquinone-4-chloroimine in toluene followed by dilute NH_3 solution (approx. 1 M).

Results

Isolation of bacteria capable of degrading the isomers of hydroxypyridine

Of several isolates obtained from enrichments of garden soil with the 2- and 3-isomers of hydroxypyridine, three were selected for detailed study. Two of these strains, 7N and 2L, grew well on 3-hydroxypyridine and resembled *Achromobacter cycloclastes* in morphology and most physiological and biochemical characters. Strain 7N was selected for its ability to accumulate large amounts of pyridine-2,5-diol in the medium and thus provide a convenient source of this compound. Strain G2, also tentatively identified as an *Achromobacter*, was obtained from similar enrichments with 2-hydroxypyridine.

Only one species of bacterium that could metabolize 4-hydroxypyridine completely was isolated during sampling of numerous soil sites, sewage and industrial-effluent outfalls in the Tyneside area over several months. This organism was obtained from a sample of aerated sewage by elective culture with 0.025% (w/v) 4-hydroxypyridine. Concentrations of 0.05% (w/v) or more proved inhibitory to growth. This strain, 35S, was identified by the National Collection of Industrial Bacteria as an *Agrobacterium* distinct from *Agrobacterium radiodurans* and *Agrobacterium radiobacter*. It has been deposited with the N.C.I.B. under accession number 10413. This aerobic, Gram-negative, peritrichously flagellated organism had a thick (1 μm) capsule as a consequence of which its growth in liquid medium produced characteristic feathery and stringy clumps in an otherwise faintly turbid medium; such clumps were readily dispersed by vigorous shaking. *Agrobacterium* 35S grew much more slowly than other isolates even under optimum conditions (Table 1). Addition of more yeast extract increased the cell yields but gave no increase in assimilation of 4-hydroxypyridine nor in the enzymic activity of washed-cell suspensions.

Those strains isolated on pyridine or 4-hydroxypyridine did not grow at the expense of 2- or 3-hydroxypyridine within a 14-day test period; similarly strains 2L, G2 and 7N isolated on the latter isomers were unable to utilize pyridine or 4-hydroxypyridine.

Table 3. *Oxidation of the hydroxypyridines by washed suspensions of the Achromobacter strains G2 and 2L and of Agrobacterium 35S*

Warburg flasks contained: Na_2HPO_4–KH_2PO_4 buffer, pH7.0, 100μmol; 20% (w/v) KOH in the centre well, 0.2ml; cell suspension, 0.5ml (8mg for strain G2, 12.1mg for strain 2L and 7.2mg for strain 35S, as cell dry wts.). The total volume was 2.5ml. O_2 uptakes were followed until completion and corrected for endogenous respiration. N.D., Not determined. The differences in O_2 uptake are between the value for the growth substrate and that for the pyridinediol used.

		O_2 uptake when supplied with substrate shown			
Organism	Growth substrate	Growth substrate (mol of O_2/mol)	Pyridine-2,5-diol (mol of O_2/mol)	Pyridine-3,4-diol (mol of O_2/mol)	Difference (mol of O_2/mol)
Achromobacter G2	2-Hydroxypyridine	4.23	3.82	N.D.	0.41
Achromobacter 2L	3-Hydroxypyridine	3.25	2.78	N.D.	0.47
Agrobacterium 35S	4-Hydroxypyridine	3.10	N.D.	2.10	1.0

After a lag period of 3–4 days, however, strain 2L transferred from 2-hydroxypyridine began to grow well on 3-hydroxypyridine; similarly strain G2 grew on the 2-isomer after transfer from 3-hydroxypyridine medium. *Agrobacterium* 35S failed to grow on some 20 other pyridine or piperidine compounds tested, whether or not they carried a 4-hydroxy substituent. This may well be a reflection of the extreme specificity of the 4-hydroxypyridine 3-hydroxylase in this organism (G. K. Watson, C. Houghton & R. B. Cain, unpublished work).

Metabolism of 2- and 3-hydroxypyridine by the Achromobacter strains

Washed suspensions of *Achromobacter* G2 grown on 2-hydroxypyridine oxidized this substrate and pyridine-2,5-diol rapidly and without a lag period. Complete oxidation of these two substrates [equations (8) and (9)] required 5 and 4.5mol of O_2/mol of substrate supplied (Table 3), but the recorded uptakes with washed suspensions were usually about 70–80% of theory. Nevertheless, the difference in O_2 uptake between 2-hydroxypyridine and pyridine-2,5-diol obtained with this bacterium (0.41mol of O_2/mol of substrate) is close to that (0.5mol of O_2/mol of substrate) required by theory:

$$C_5H_5NO + 5O_2 \rightarrow 5CO_2 + H_2O + NH_3 \quad (8)$$

2-Hydroxypyridine

$$C_5H_5NO_2 + 4\tfrac{1}{2}O_2 \rightarrow 5CO_2 + H_2O + NH_3 \quad (9)$$

Pyridine-2,5-diol

Strain G2 also carried out a slow oxidation of pyridine-2,3-diol and pyridine-2,4-diol, consuming 1.5 and 3.5mol of O_2/mol of substrate respectively. The two 3-hydroxypyridine utilizers *Achromobacter* 2L and 7N, in contrast, oxidized only 3-hydroxypyridine and pyridine-2,5-diol, but again with a difference in O_2 consumption of about 0.5mol/mol of substrate (Table 3). None of the other seven pyridinediols elicited any O_2 uptake above the endogenous rate with washed suspension harvested in the exponential, early- or late-stationary phases. Crude extracts of all three bacteria prepared from cells grown on their appropriate hydroxypyridine isomer oxidized only pyridine-2,5-diol of the eight isomeric diols.

After growth on fumarate+NH_3, washed suspensions and extracts of these strains failed to oxidize the heterocyclic substrate and showed no inducible response within 2h.

Accumulation of pyridine-2,5-diol in cultures of Achromobacter 7N. When strain 7N grew in cultures containing 3-hydroxypyridine, pronounced changes in the u.v. spectrum of the liquid medium at the late exponential phase of growth indicated that a product was accumulating in considerable yield. The absorption peaks of 3-hydroxypyridine at 246 and 277nm disappeared and were replaced by a new peak at 230nm and the colour of the medium became dark green. Addition of $FeCl_3$ reagent to samples of the medium taken at this time produced a pink–red coloration suggesting the presence of pyridine-2,5-diol (den Hertog *et al.*, 1950). Paper chromatography of samples with the appropriate markers in solvent (1) confirmed the presence of residual 3-hydroxypyridine (R_F 0.66) and pyridine-2,5-diol (R_F 0.90). This diol was isolated and characterized as follows. A 10-litre vessel containing 8 litres of 3-hydroxypyridine medium was given a 2-litre inoculum of an exponential-phase culture of strain 7N grown on this medium. The 10-litre culture was vigorously aerated (5 litres of air/min) for 5–7h at 30°C and the aeration rate then decreased to 200ml/min. Accumulation of the diol began almost immediately and was followed spectrophotometrically in samples of the medium, the extinction data being interpreted quantitatively as described in the Methods section. When the maximum amount of diol had accumulated (approx. 43% of that theoretically possible) (Fig. 1) the culture was centrifuged and the supernatant concentrated, first to 1 litre under N_2 by using a circulatory cyclone evaporator operating at 22–28°C. This concentrated solution was then ad-

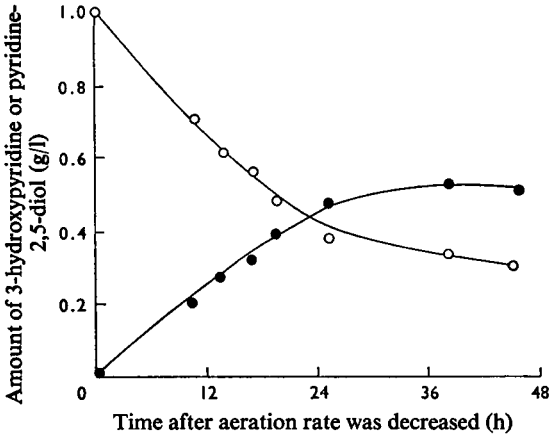

Fig. 1. *Conversion of 3-hydroxypyridine into pyridine-2,5-diol in cultures of Achromobacter 7N*

After 7h of vigorous aeration (5 litres of air/min), the aeration rate was decreased to 200ml of air/min. Spectrophotometric measurements were begun at this time (zero) and continued for 48h. Extinction values were converted into concentrations of o, 3-hydroxypyridine, and •, pyridine-2,5-diol, as described in the Methods section.

justed to pH7 with 5M-NaOH and dried *in vacuo*. The reddish-coloured solid was thoroughly dried over P_2O_5 and then exhaustively extracted with propan-2-ol under N_2 in a Soxhlet apparatus. The propanolic extract was left overnight at −20°C and the precipitate (3.3g) was filtered off. Concentration of the filtrate *in vacuo* under N_2 followed by cooling again to −20°C yielded a further 0.6g of diol. The combined precipitates were recrystallized twice from ethanol and then vacuum-sublimed [130–180°C at 200N/m² (1.5mm-Hg)]. A pure white powder was obtained which had no distinct melting point but darkened at 220°C and decomposed between 245–255°C. These values closely resemble those of chemically synthesized pyridine-2,5-diol (Behrman & Pitt, 1958; den Hertog *et al.*, 1950). The diacetate derivative, after recrystallization from ethyl acetate, had m.p. 68–70°C which was not depressed by admixture with authentic material (m.p. 68–70°C). The u.v. spectra of the isolated and authentic pyridine-2,5-diol were identical, with absorption maxima at 230 and 320nm. At 320nm, ϵ for the natural product was 5650litre·cm^{-1}·mol^{-1}; Behrman & Pitt (1958) gave a value of 5620.

Formation of pyridine-2,5-diol by other bacteria. Under normal growth conditions, with shaken cultures or forced aeration, pyridine-2,5-diol did not accumulate in cultures of strains G2 and 2L grown with 2- and 3-hydroxypyridine respectively, and appeared in smaller amounts in cultures of 7N. If cultures of G2 or 2L showing visible growth were then kept stationary or the forced aeration was decreased or stopped altogether, pyridine-2,5-diol was formed by these bacteria from their respective hydroxypyridine. Concentrations of the diol, however, never approached those accumulated by strain 7N. Restoration of adequate aeration to cultures of G2 and 2L which were showing the presence of pyridine-2,5-diol led to its rapid disappearance.

Metabolism of 4-hydroxypyridine by Agrobacterium N.C.I.B. 10413

Washed suspensions of *Agrobacterium* 35S grown on 4-hydroxypyridine oxidized only 4-hydroxypyridine of the three isomeric hydroxypyridines and only pyridine-3,4-diol of the three dihydroxypyridines (1,4-, 2,4- and 3,4-diols) carrying a hydroxyl group at the C-4 position. Of the eight pyridine diols tested, only pyridine-3,4-diol was oxidized without a lag period (Fig. 2). The oxidation of pyridine-2,5-diol showed a sigmoid curve for O_2 uptake with this organism, suggesting a rapid adaptive response, but this very brief lag period could be considerably extended by growing the organism on carefully purified 4-hydroxypyridine instead of the usual commercial product. Total O_2 uptakes, obtained with appropriately grown washed suspensions, were approximately 3mol of O_2/mol of pyridine-3,4-diol (Table 3). The complete oxidation of each substrate by washed suspensions also resulted in the accumulation of 1mol of NH_3 (Table 4).

Pyridine-3,4-diol never accumulated to detectable amounts in shaken or stationary cultures of *Agrobacterium* 35S grown under the usual culture conditions, so these conditions were varied to facilitate isolation of the diol. Accumulation of pyridine-3,4-diol was little affected by the concentration of 4-hydroxypyridine used as substrate in growth experiments, but it increased considerably with increase in pH of the medium, maximum amounts persisting at pH8.5. A larger-scale experiment was then carried out with thick suspensions of washed cells in a poorly aerated medium to minimize further metabolism of the accumulating diol. Under these conditions nearly quantitative conversion of 4-hydroxypyridine into pyridine-3,4-diol was achieved (Fig. 3). At the end of the experiment, the cells were removed by centrifugation, the supernatant solution was freeze-dried and the pyridine-3,4-diol extracted from the dry residue with two 5ml portions of hot ethanol. The ethanolic solution was concentrated to 1ml *in vacuo* at 35°C and applied as a band to t.l.c. plates of kieselguhr G, which were developed in solvent (5). This solvent clearly separated the diol (R_F 0) from traces of residual 4-hydroxypyridine (R_F 0.89). The diol band was scraped off the plates into water (20ml) and the kieselguhr filtered off. The u.v. spectrum of this filtrate

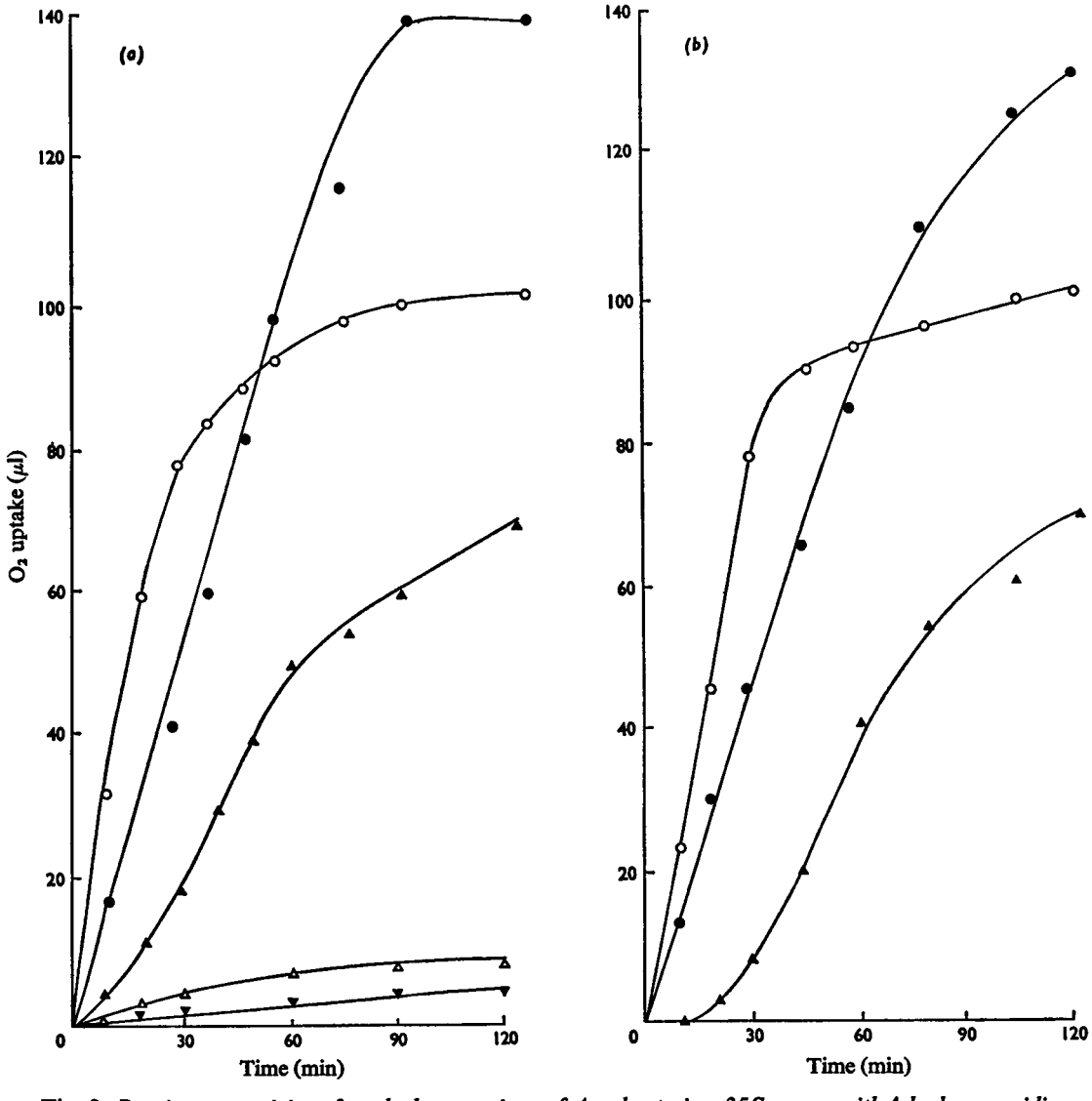

Fig. 2. *Respiratory activity of washed suspensions of Agrobacterium 35S grown with 4-hydroxypyridine*

Warburg flasks contained in a total volume of 2.5ml: Na_2HPO_4–KH_2PO_4 buffer, pH8.0, 100μmol; substrate, 2μmol; cell suspension, 0.5ml, equivalent to 7.2mg dry wt. in (*a*) and 10.6mg dry wt. in (*b*). ●, 4-Hydroxypyridine; ○, pyridine-3,4-diol; ▲, pyridine-2,5-diol; ▼, pyridine-2,4-diol; △, pyridine-1,4-diol. (*a*) Cells grown with commercial 4-hydroxypyridine. (*b*) Cells grown with purified 4-hydroxypyridine. The results were corrected for endogenous respiration.

and that of a solution of pyridine-3,4-diol were identical. The filtrate was freeze-dried, the residue dissolved in a little ethanol and subsequently used for chromatographic comparison with authentic pyridine-3,4-diol. Chromatography in one or two dimensions in four solvent systems indicated its identity with the authentic material (Table 5).

Isolation of pyridine-degrading bacteria

Contrary to general experience, we did not find it difficult to isolate pyridine-degrading micro-organisms. Several strains of bacteria isolated by elective culture were capable of degrading this heterocyclic substrate, though most isolates have been actinomycetes. Percolating columns packed with coke

Table 4. *Formation of NH_3 from 4-hydroxypyridine and pyridine-3,4-diol by washed suspensions of Agrobacterium 35S*

Oxidation was performed in Warburg flasks containing: Na_2HPO_4–KH_2PO_4 buffer, pH7.5, 100μmol; substrate as shown; cell suspension, 0.5ml (equivalent to 13.3mg dry wt.); 20% (w/v) KOH in the centre well, 0.02ml; water to a final volume of 2.5ml. When O_2 uptake returned to the endogenous rate, the cells were removed by centrifugation and the supernatants carefully removed. The NH_3 was distilled from 0.5ml samples in Conway units and then assayed quantitatively with Nessler's reagent. A control mixture containing no substrate was treated identically and used as the blank.

Substrate supplied (μmol)		NH_3 formed (μmol)	Recovery of ring nitrogen (%)
4-Hydroxypyridine	(2)	1.9	96
	(4)	3.9	
Pyridine-3,4-diol	(2)	1.6	85
	(4)	3.6	

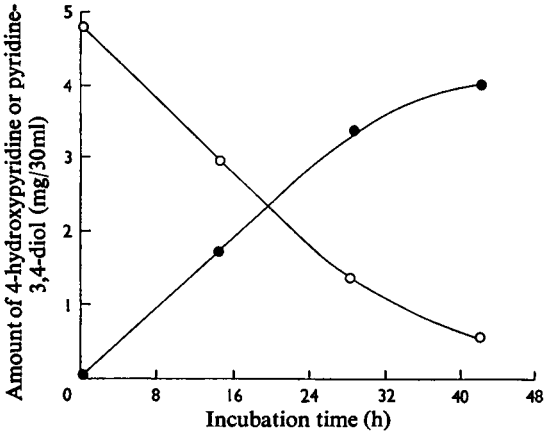

Fig. 3. *Conversion of 4-hydroxypyridine into pyridine-3,4-diol by Agrobacterium 35S under conditions of inadequate aeration*

The reaction mixture consisted of: 4-hydroxypyridine, 4.8mg; tris–HCl buffer, pH8.5, 2nmol; cell suspension equivalent to 143mg dry wt. The total volume was 30ml in a 100ml flask. This flask and a control flask containing no 4-hydroxypyridine were incubated at 30°C without shaking in a water bath. At intervals, 0.05ml of the reaction mixture from test and control flasks were transferred to silica cuvettes and diluted to 3ml with water. By using the control sample as a reference, a complete spectral scan in the u.v. range was made and the concentrations of substrate and product were determined from the absorbance values as described in the Methods section. o, 4-Hydroxypyridine; ●, pyridine-3,4-diol.

breeze, inoculated with a few crumbs of soil and continuously percolated at 30°C with basal medium containing pyridine as sole carbon and nitrogen source, quickly formed a profuse pink growth of bacteria on the coke-breeze surface. After 10–14 days of incubation, this growth consisted of an almost pure culture of pyridine-degrading organisms (R. B. Cain, unpublished work; F. W. Moore, personal communication), which could easily be isolated by plating out on solidified pyridine basal medium. The ability of such isolates to utilize alkyl-substituted pyridines has varied widely; an occasional isolate utilized all the isomeric picolines (methylpyridines), whereas rather more utilized only 4-methylpyridines. No strain isolated from a pyridine enrichment has grown with any lutidine (dimethylpyridine) or collidine (trimethylpyridine) isomer. A *Nocardia* species, Z1, which metabolized pyridine as sole carbon, nitrogen and energy source was selected for further study. It grew best at pH8.0 and pyridine concentrations in the range 0.1–0.2% (v/v); higher concentrations of pyridine greatly extended the lag period. The addition of yeast extract to the growth medium had two effects. At low concentrations (0.005–0.02%, w/v) the initial lag phase of growth was shorter, but the final cell yield and the rate of assimilation of pyridine were both increased (Fig. 4). The addition of yeast extract at 0.05% (w/v) decreased the initial uptake of pyridine; during the subsequent exponential phase of growth, however, this uptake was very rapid, resulting in a higher cell yield produced in a shorter time with an overall acceleration of pyridine assimilation. *Nocardia* Z1 had a very limited heterocyclic nutritional spectrum. Although pyridine supported good growth, the following compounds were unable to support growth of the organism over a concentration range of 0.01–0.2% (v/v or w/v): 2-, 3- and 4-methylpyridines; 2-, 3- and 4-ethylpyridines; 2,3-, 2,4-, 2,6- and 3,5-dimethylpyridines; picolinic acid, nicotinic acid and isonicotinic acid; 2-, 3- and 4-hydroxypyridines; pyridine *N*-oxide. Under the incubation conditions used, the pyridinediols autooxidized to quinones and were not tested. No heterocyclic or aliphatic compound was accumulated in

Table 5. *Comparison of the properties of synthetic pyridinediols with the natural compounds isolated from culture filtrates and incubation mixtures*

For details see the text. N.D., not determined.

Compound	m.p. (°C)	$\lambda_{max.}$ (nm)	ϵ at $\lambda_{max.}$ (litre·mol^{-1}·cm^{-1})	Colour of $FeCl_3$ complex	ϵ of $FeCl_3$ complex at $\lambda_{max.}$ (litre·mol^{-1}·cm^{-1})	Chemical analysis (%)			R_F in solvent			
						C	H	N	(1)	(2)	(3)	(5)
Pyridine-2,3-diol	246–247	297	8150	Blue	1755	54.1	4.5	12.6	0.81	N.D.	N.D.	N.D.
Material isolated from *Nocardia* Z1 incubation	246–247	297	8100	Blue	1750	53.7	4.6	12.3	0.81	N.D.	N.D.	N.D.
Pyridine-3,4-diol	139*	273	10100	Purple	1220	54.1	4.5	12.6	0.69	0.55	0.46	0
Material isolated from *Nocardia* Z1 incubation	136*	273	10150	Purple	1200	53.8	4.3	N.D.	0.68	0.54	0.46	0
Material isolated from *Agrobacterium* incubation	N.D.	273	N.D.	Purple	N.D.	N.D.	N.D.	N.D.	0.69	0.54	0.45	0

* m.p. of the diacetate.

Fig. 4. *Effect of yeast extract concentration on the utilization of pyridine by Nocardia Z1*

Erlenmeyer flasks (250ml) with side arms contained 75ml of basal medium with 0.1% (v/v) pyridine as the only source of carbon and nitrogen (○), or the same medium supplemented with 0.01% (w/v) of yeast extract (●). Flasks were shaken at 30°C and growth was followed nephelometrically. Pyridine concentration was determined spectrophotometrically on 1ml samples removed aseptically and centrifuged to remove cells. ——, Removal of pyridine; – – – –, growth of *Nocardia* Z1.

pyridine-containing media supporting the growth of *Nocardia* Z1.

Metabolism of pyridine by Nocardia Z1

Freshly harvested washed suspensions of *Nocardia* Z1 oxidized pyridine in the Warburg respirometer, consuming 3–3.5mol of O_2 and forming 0.9mol of NH_3/mol of pyridine consumed. These suspensions did not oxidize the picolines, pyridine *N*-oxide or any of the mono- or di-hydroxypyridines with the exception of 3-hydroxypyridine. This substrate was oxidized at a variable but always very low rate, approximately one-thirtieth of that with pyridine itself (Fig. 5*a*). Neither the cell-free extract nor the particulate material from *Nocardia* cells, disrupted by the Hughes press, ultrasonic disintegration or grinding under liquid N_2, metabolized pyridine even after supplementation with cysteine (0.01–10mM), tetrahydrofolate (0.01–1mM) or substrate amounts of NADH, NADPH, FAD or FMN either alone or in any combination.

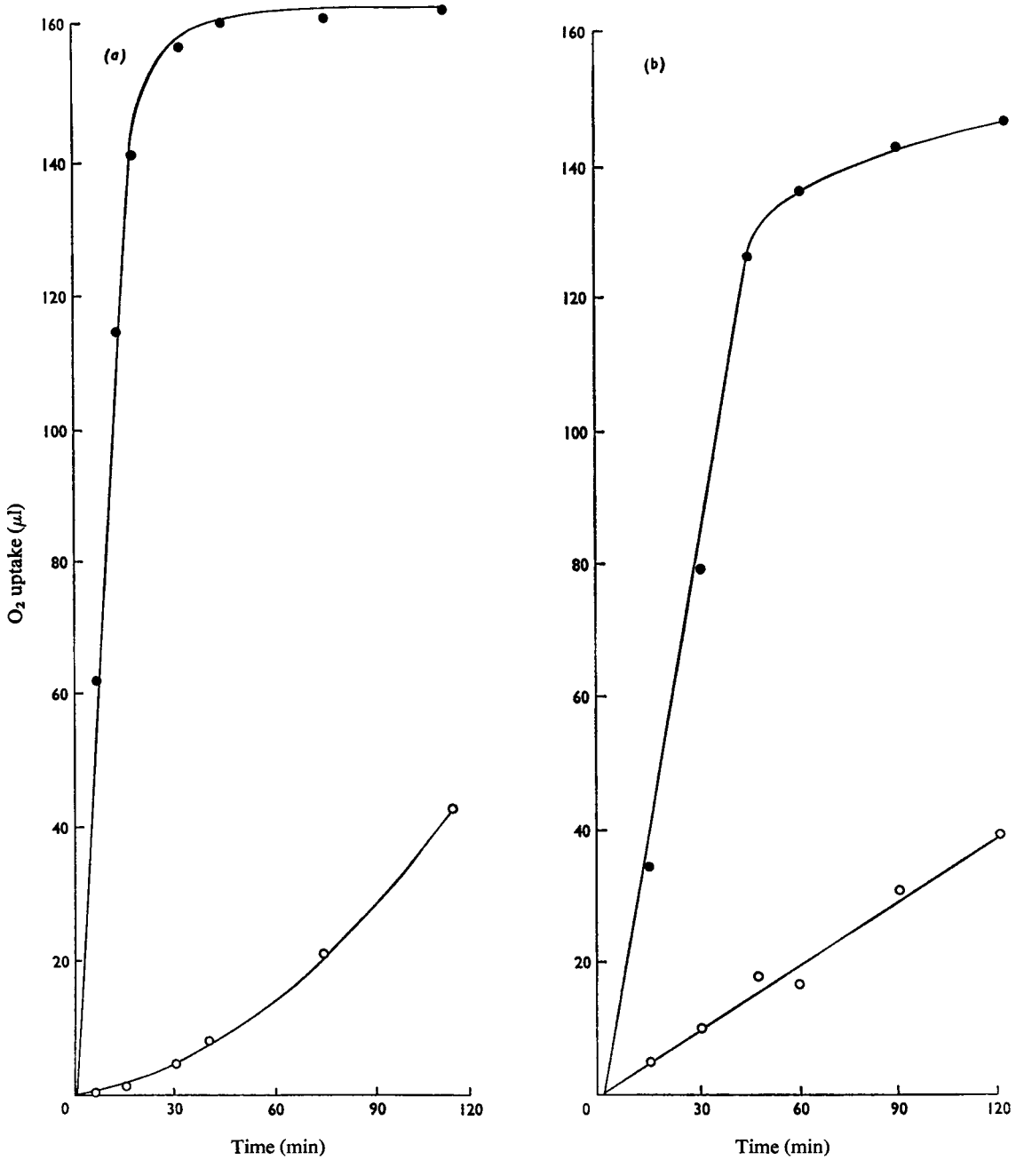

Fig. 5. *Oxidation of pyridine and 3-hydroxypyridine by Nocardia Z1*

Suspensions of (*a*) fresh washed cells (25.6mg dry wt./ml) and (*b*) freeze-dried cells resuspended in water (20mg dry wt./ml) were incubated with: substrate, 2μmol; Na_2HPO_4–KH_2PO_4 buffer, pH8.0, 100μmol; KOH (0.2ml) in the centre well of Warburg flasks containing a total solution volume of 2.5ml. Results are corrected for endogenous respiration. ●, Pyridine; ○, 3-hydroxypyridine.

A suspension of freeze-dried cells oxidized 3-hydroxypyridine at 12% of the rate with pyridine itself and without the sigmoid response (Fig. 5*b*), but other hydroxylated pyridine derivatives were not oxidized at rates in excess of endogenous respiration.

Oxidation of 3-hydroxypyridine by Nocardia Z1. Although the rate of oxidation of 3-hydroxypyridine by pyridine-grown cells was too low to suspect it as an intermediate in pyridine metabolism, it was clear that this organism could metabolize 3-hydroxypyridine further. Suspensions of *Nocardia* Z1 in buffer containing 3-hydroxypyridine produced a reddish-brown colour on further incubation and addition of $FeCl_3$ reagent to a sample of this solution, after removing the cells gave a blue coloration suggesting the presence of a pyridinediol. Boiled cells of *Nocardia* Z1 were unable to oxidize 3-hydroxypyridine, to produce a coloration in the incubation medium or excrete a compound that reacted with $FeCl_3$.

The contents of experimental flasks giving the $FeCl_3$ reaction were dried *in vacuo* at 40°C and the unknown compounds extracted from the solid residue with 2×5ml portions of hot ethanol. After concentrating the ethanolic solution to 0.2ml, the compounds were separated by paper chromatography in solvent (1). Two pyridinediols were present in the ethanolic extracts; R_F values (0.81 and 0.69) and the colour changes with $FeCl_3$ spray (blue and reddish-violet) were characteristic of pyridine-2,3-diol and pyridine-3,4-diol respectively.

When the reaction was done on a larger scale and its course followed spectrophotometrically, the conversion of 3-hydroxypyridine into the two pyridinediols was almost quantitative (Fig. 6). Prolonged incubation of the reaction mixture led to a slow disappearance of the diols which continued if the cells were removed, but did not occur even when cells were present if further incubation was done anaerobically. In one series of experiments, aerobic incubation was continued until spectrophotometric analysis showed that the pyridinediol content had fallen to one-half of its maximum, at which stage the cells were removed by centrifugation, washed and resuspended in separate reaction mixtures containing one or other of the two pyridinediol isomers. No decrease in the pyridinediol content occurred over a period of 1h, suggesting that these organisms had not developed the enzymic capacity to metabolize the pyridinediols in the first incubation system. The slow disappearance of these compounds was therefore probably an auto-oxidation effect.

From several litre-scale incubations, totalling in all about 10 litres, isolation of the two diols was achieved. The 10-litre volume was concentrated to approximately 200ml in a circulatory cyclone evaporator operating under N_2 at 30°C; the remaining 200ml was freeze-dried. The resulting dry solid was continuously extracted with propan-2-ol under N_2 in a Soxhlet apparatus until the hot propanol dripping from the extraction thimble no longer gave a phenol reaction with the Folin–Ciocalteau reagent. The propanolic extract was then evaporated to dryness *in vacuo* and the residue dissolved in the minimum volume of hot ethanol. The diols were separated by preparative-scale paper chromatography, the two isolated bands located, cut out, and the diols eluted from the paper strips with water before being freeze-dried.

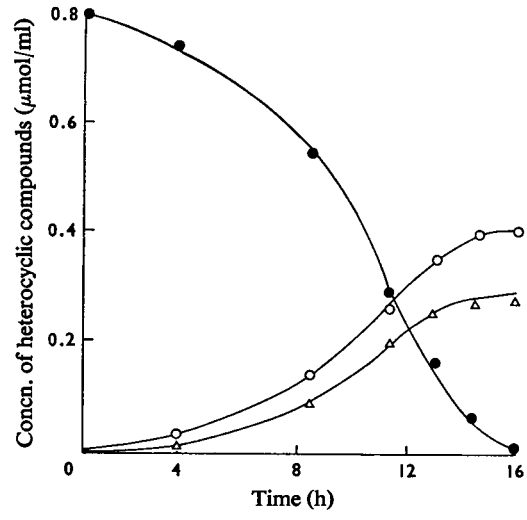

Fig. 6. *Conversion of 3-hydroxypyridine into pyridine-2,3-diol and pyridine-3,4-diol by washed suspensions of pyridine-grown Nocardia Z1*

The following components were incubated at 30°C (in 250ml conical flasks) in a metabolic shaker bath operating at 100 oscillations/min: 20ml of a pyridine-grown *Nocardia* Z1 suspension (equivalent to 366mg dry wt.); 3-hydroxypyridine, 80μmol; Na_2HPO_4–KH_2PO_4 buffer, pH7.5, 2mmol; water to a total volume of 100ml. The control flask contained no substrate. Samples (3ml) removed during the incubation were centrifuged to remove the cells and the u.v. spectra of the diluted supernatants recorded. The spectrophotometric data were interpreted quantitatively by solving the simultaneous equations (1)–(3) (see the Methods section). ●, 3-Hydroxypyridine; ○, pyridine-2,3-diol; △, pyridine-3,4-diol.

Pyridine-2,3-diol was obtained as a reddish-coloured powder (60mg). After two recrystallizations from ethanol an off-white material, m.p. 246°C, was obtained with the properties summarized in Table 5. The i.r. spectra of the natural product and synthetic pyridine-2,3-diol in KBr discs were identical.

The crude preparation of pyridine-3,4-diol was

Table 6. *Non-enzymic hydroxylation of pyridine compounds by the model system*

The incubation medium contained: pyridine compound, 120μmol; potassium ascorbate, 284μmol; $FeSO_4$, 30μmol; EDTA, 160μmol; Na_2HPO_4–KH_2PO_4 buffer, pH5.5, 600μmol in a total volume of 6ml. The solutions were maintained at 35°C for 2h and aerated with a continuous stream of air through a sintered-glass disc (porosity 1). Solutions containing the hydroxypyridines usually darkened in colour during this period. After freeze-drying the reaction mixture, the diols were extracted from the residue with 4×25ml portions of hot ethanol. The ethanolic extract was concentrated and chromatographed on paper in solvent (1).

Substrate	Products		
	R_F in solvent (1)	Colour with $FeCl_3$	Identity
Pyridine	None	None	—
2-Hydroxypyridine	0.80	Blue	Pyridine-2,3-diol
	0.85	Pink	Pyridine-2,5-diol
3-Hydroxypyridine	0.71	Purple	Pyridine-3,4-diol
	0.80	Blue	Pyridine-2,3-diol
	0.86	Pink	Pyridine-2,5-diol
4-Hydroxypyridine	0.69	Purple	Pyridine-3,4-diol
Isonicotinic acid	None	None	—

purified by vacuum sublimation [160°C, 267N/m^2 (2mmHg)], yielding a pale-yellow material (16mg) which exhibited polymorphism but finally melted at 185°C. The m.p. of the diacetate derivatives of the natural product (136°C) and pyridine-3,4-diol (139°C) were not depressed on admixture. A comparison of the other properties of the natural and synthetic pyridine-3,4-diols (Table 5) confirmed their identity.

Non-enzymic hydroxylation of heterocyclic compounds

Hydroxylation of various aromatic compounds at electron-rich sites occurred when they were added to a model hydroxylating system containing ascorbate, Fe^{2+} ions, EDTA and O_2 (Udenfriend *et al.*, 1954). Pyridine and its hydroxyl derivatives were examined in this system as a means of confirming the presence of electronegative sites likely to undergo hydroxylation by mono-oxygenases.

Pyridine itself underwent no hydroxylation when added to an aerated (Fe^{2+}+ascorbate+EDTA) system. The presence of a hydroxyl group in the heterocyclic nucleus markedly increased its reactivity because both 2- and 3-hydroxypyridines were hydroxylated in the model system, each giving a mixture of pyridinediols (Table 6). 2-Hydroxypyridine formed the isomeric pyridine-2,3- and -2,5-diols whereas 3-hydroxypyridine gave pyridine-2,3-, -2,5- and -3,4-diols. 4-Hydroxypyridine was hydroxylated only at the 3-position to form pyridine-3,4-diol. No diols with an *N*-substituted hydroxyl (e.g. pyridine-1,2-, -1,3- or -1,4-diols) were synthesized in the model system from 2-, 3- or 4-hydroxypyridine.

Discussion

The N atom of pyridine is electronegative in relation to the C atom and renders the molecule asymmetric, giving it a certain degree of polarity not possessed by the benzene ring. The pyridine ring is thus relatively electron-deficient and resists electrophilic substitutions especially at C-2, C-4 and C-6. Further, the N atom possesses a lone pair of electrons by which pyridine can form pyridinium salts, resulting in the distribution of a full positive charge over the ring carbons; pyridinium compounds are thus even more resistant to electrophilic attack. The N atom exerts least influence on the C-3 position; the chemical reactions of 3-hydroxypyridine, for example, closely resemble those of phenol. The 2-hydroxy isomer, in contrast, shows only weak phenolic properties and in aqueous solution exists predominantly (340:1) in the tautomeric pyridone form. 4-Hydroxypyridine is even less phenolic, the pyridone form predominating in the ratio 2200:1 (Meislich, 1962).

The mechanism by which the Udenfriend model system (Udenfriend *et al.*, 1954) hydroxylates aromatic compounds was originally attributed to the hydroxyl radical (HO·) as the attacking species from studies of the interaction of Fe^{2+} and H_2O_2 (Fenton's reagent) (Breslow & Lukens, 1960). The Udenfriend system, however, does not hydroxylate in the manner of a Fenton reagent nor does it appear to involve H_2O_2, HO_2· or HO· (Norman & Radda, 1962; Ullrich & Staudinger, 1966). Addition to the incubation mixture of compounds able to scavenge free radicals from solutions (e.g. quinol) prevented hydroxylation when H_2O_2 was substituted for O_2 in the Udenfriend system, but hydroxylation proceeded

normally when O_2 was employed (Bade & Gould, 1968), suggesting that hydroxylation was not caused by a free-radical attack. The hydroxylation mechanism may therefore be ionic and there is evidence supporting the role of electrophilic substitutions occurring in other model systems (Ullrich *et al.*, 1971).

No hydroxylation of pyridine took place in the Udenfriend system (Table 6), indicating that pyridine is resistant to electrophilic substitution. Because substituents withdrawing electrons, such as 4-carboxyl, further emphasize the electron deficiency of the heterocyclic ring, rendering electrophilic hydroxylation even more difficult, it was anticipated that isonicotinic (pyridine-4-carboxylic) acid would also fail to undergo hydroxylation in the Udenfriend model system. No derivative was in fact produced.

On the other hand, the presence of a hydroxyl group in the aromatic ring system renders it much more susceptible to electrophilic attack because the O atom of the substituent hydroxyl group shares its lone pair of electrons with the aromatic ring. This increases the electron density of the ring in general but more specifically in regions *ortho* and *para* to the hydroxyl substituent. 2-Hydroxypyridine would therefore be expected to yield the 2,3-diol as a product of *ortho*-hydroxylation and the 2,5-diol by *para*-hydroxylation since C-2 and C-5 are activated most by the original hydroxyl group. Similarly, 3-hydroxypyridine would be expected to form the 2,3- and 3,4-diols by *ortho*-hydroxylation and the 2,5-diol by a *para*-attack. Because of the combined but opposite effects of the N atom and 4-hydroxyl group, further hydroxylation of 4-hydroxypyridine would probably yield only the 3,4-diol. These predicted products were precisely those obtained when the monohydroxypyridine isomers were hydroxylated with the Udenfriend model system (Table 6). It should be recalled that the predominant tautomers in the case of 2- and 4-hydroxypyridine possess not the electron-donating hydroxyl (enol) group but the electron-withdrawing pyridone (oxo) configuration. Presumably the enol tautomers are hydroxylated in the model system although they are present in much lower concentrations than the oxo tautomers.

Hydroxylation of pyridine derivatives such as nicotine (Hochstein & Dalton, 1965) and nicotinic acid (Hunt *et al.*, 1958) by enzymes obtained from micro-organisms occurs from water rather than from molecular O_2 as found with the model system and microbial hydroxylations of substituted homocyclic compounds. Hayaishi (1969) suggested that this type of hydroxylation, which was also known to occur in the hydroxylation of purines and pyrimidines, took place preferably with hydrophilic substrates. Alternatively the electron deficiency of the heterocyclic ring may prevent an attack by a species such as the electrophilic FeO^{2+}, which Ingraham (1966) has suggested may occur in reactions catalysed by mono-oxygenases that incorporate molecular O_2 into aromatic molecules. The values of approximately 0.5 mol of O_2 consumed/mol of substrate utilized as the difference in O_2 uptake between 2- or 3-hydroxypyridine and pyridine-2,5-diol observed when these compounds were oxidized by washed suspensions of the *Achromobacter* species (Table 3), suggests that the additional O_2 in the diol was, in this case, incorporated from water:

$$\begin{aligned} XH + H_2O + NAD^+ &\rightarrow XOH + NADH + H^+ \\ NADH + H^+ + \tfrac{1}{2}O_2 &\rightarrow NAD^+ + H_2O \end{aligned} \qquad (10)$$

Thus the presence of an electron-donating group on the ring does not overcome the effect of the ring nitrogen and cause a mono-oxygenase-mediated reaction by the *Achromobacter* carrying out this transformation.

The *Agrobacterium*, on the other hand, required 1 mol of O_2/mol of 4-hydroxypyridine oxidized to pyridine-3,4-diol; this is the value expected for a mono-oxygenase reaction incorporating an atom of molecular O_2 into the hydroxyl group (Hayaishi, 1969):

$$XH + O_2 + NADH + H^+ \rightarrow XOH + NAD^+ + H_2O \qquad (11)$$

Such a reaction has subsequently been confirmed by the discovery and characterization in *Agrobacterium* of a 4-hydroxypyridine 3-hydroxylase, probably an FAD-mediated mono-oxygenase (G. K. Watson, C. Houghton & R. B. Cain, unpublished work).

Thus two distinct diols were synthesized: a *para*-diol, 2,5-dihydroxypyridine, and an *ortho*-diol, 3,4-dihydroxypyridine, both possessing diol configurations typical of those found in the catabolic pathways for the degradation of homocyclic compounds. An *ortho*-diol has not, we believe, been detected in any previously elucidated catabolic pathway involving pyridine-based compounds, and the enzyme causing its formation, 4-hydroxypyridine 3-hydroxylase, may be the first described FAD-mediated hydroxylase able to incorporate atmospheric O_2 into a pyridine. Our difficulty in isolating this micro-organism suggests that this metabolic pathway is not widespread.

The formation of the 2,3- and 3,4-diols from 3-hydroxypyridine by pyridine-grown *Nocardia* Z1 was clearly an unusual case of non-specific hydroxylation because neither diol appeared to be metabolized further. The *ortho*-diols formed are nevertheless precisely those predicted by the Udenfriend model system (Table 6). The common intermediate pyridine-2,5-diol and the common pathway in the *Achromobacters* for its subsequent metabolism (R. B. Cain,

C. Houghton & K. A. Wright, unpublished work) may explain the result of growth experiments in which those bacteria isolated with either 2- or 3-hydroxypyridine could grow on the isomeric substrate after a short lag period, whereas the organism utilizing 4-hydroxypyridine and employing a quite different metabolic route involving the 3,4-diol was unable to utilize the other isomers, at least within 14 days.

The work was supported by a grant to R. B. C. by the Science Research Council which is gratefully acknowledged. We thank Dr. E. J. Behrman for his advice and comments on the Elbs oxidation of 4-hydroxypyridine, Dr. B. Straughan for the i.r. spectra and Dr. K. A. Wright for synthesizing some of the pyridine derivatives. We are particularly indebted to Dr. R. D. Court of the Commonwealth Scientific and Industrial Research Organization, St. Lucia, Queensland, Australia, for the gift of several grams of mimosine.

References

Adams, R. & Govindachari, T. R. (1947) *J. Amer. Chem. Soc.* **69**, 1806
Adams, R., Cristol, S. J., Anderson, A. A. & Albert, A. A. (1945) *J. Amer. Chem. Soc.* **67**, 89
Audus, L. J. (1946) *Nature (London)* **158**, 419
Bade, M. G. & Gould, B. S. (1968) *Biochim. Biophys. Acta* **156**, 425
Behrman, E. J. & Pitt, B. M. (1958) *J. Amer. Chem. Soc.* **80**, 3717
Bernstein, J., Chen, J. L. & Cyr, G. N. (1966) *U.S. Patent* 3 904 (cl. 167–58)
Breslow, R. & Lukens, L. N. (1960) *J. Biol. Chem.* **235**, 292
Buehrer, T. F., Mason, C. M. & Crowder, J. A. (1939) *Amer. J. Pharm.* **111**, 105
Cain, R. B., Bilton, R. F. & Darrah, J. A. (1968) *Biochem. J.* **108**, 797
Calderbank, A. (1968) *Advan. Pest Contr. Res.* **8**, 127
Clapham, A. J., Kenyon, S. & Bell, R. S. (1967) *Proc. Northwest. Weed Contr. Conf.* **21**, 395
Conway, E. J. (1962) *Microdiffusion Analysis and Volumetric Error*, 2nd. edn., Crosby, Lockwood and Sons Ltd., London
den Hertog, H. J. (1946) *Rec. Trav. Chim. Pays-Bas* **65**, 129
den Hertog, H. J., Wilbaut, J. P., Schepman, F. R. & van der Wal, A. A. (1950) *Rec. Trav. Chim. Pays-Bas Belg.* **69**, 700
Errera, G. (1898) *Ber. Deut. Chem. Ges.* **31**, 1682
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751
Hayaishi, O. (1969) *Annu. Rev. Biochem.* **38**, 21
Hegarty, M. P., Court, R. D. & Thorne, P. M. (1964) *Aust. J. Agr. Res.* **15**, 168
Hochstein, I. I. & Dalton, B. P. (1965) *Biochem. Biophys. Res. Commun.* **21**, 644
Houghton, C., Wright, K. A. & Cain, R. B. (1968) *Biochem. J.* **106**, 51P
Hughes, D. E. (1951) *Brit. J. Exp. Pathol.* **32**, 97
Hunt, A. L., Hughes, D. E. & Lowenstein, J. M. (1958) *Biochem. J.* **69**, 170
Ingraham, L. L. (1966) *Compr. Biochem.* **14**, 424
Manske, R. H. F. & Marion, L. (1942) *Can. J. Res. Sect. B* **20**, 87
Marion, L. (1950) *Alkaloids* **1**, 167
Martin, R. J. (1966) *U.S. Patent* 3 249 419
Meislich, H. (1962) *Pyridine Its Deriv.*, part 3, p. 619
Mohr, G., Erdman, D., Lust, S. & Schneider, G. (1968) *Int. Symp. Crop Protection 20th.* 1293
Newbold, G. F. & Spring, F. S. (1948) *J. Chem. Soc. London* 1846
Norman, R. O. C. & Radda, G. K. (1962) *Proc. Chem. Soc. London* 138
Ochiai, E. (1953) *J. Org. Chem.* **18**, 534
Powell, J. F. & Strange, R. E. (1953) *Biochem. J.* **54**, 205
Renz, J. (1936) *Hoppe-Seyler's Z. Physiol. Chem.* **244**, 153
Shaw, E. (1949) *J. Amer. Chem. Soc.* **71**, 67
Spath, E. & Englander, G. (1935) *Ber. Deut. Chem. Ges. B* **68**, 2218
Udenfriend, S., Clark, C. T., Axelrod, J. & Brodie, B. B. (1954) *J. Biol. Chem.* **208**, 731
Ullrich, V. & Staudinger, H.-J. (1966) in *Biological and Chemical Aspects of Oxygenases* (Bloch, K. & Hayaishi, O., eds.), p. 235, Maruzen Co. Ltd., Tokyo
Ullrich, V., Ruf, H.-H. & Mimoun, H. (1971) *Biochem. J.* **125**, 9P
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957) *Manometric Techniques*, 3rd. edn., pp. 1–76, Burgess, Minneapolis
van Schickh, O., Binz, A. & Schulz, A. (1936) *Ber. Deut. Chem. Ges. B* **69**, 2593
Wright, K. A. & Cain, R. B. (1969) *Soil Biol. & Biochem.* **1**, 5
Wright, K. A. & Cain, R. B. (1972) *Biochem. J.* **128**, 543
Yurashevskii, N. K. & Stepanov, S. I. (1939) *J. Gen. Chem. USSR* **9**, 2203