Microbial Metabolism of the Pyridine Ring

METABOLIC PATHWAYS OF PYRIDINE BIODEGRADATION BY SOIL BACTERIA

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1. Two bacteria, a *Bacillus* sp. and a *Nocardia* sp. (strain Z1) were isolated from soil by enrichment with 0.1% (v/v) pyridine and grew rapidly on this compound as sole C, N and energy source. The monohydroxypyridines, tetrahydropyridine, piperidine and some other analogues were not utilized for growth or oxidized by washed suspensions of either bacterium. 2. Cell-free extracts were unable to metabolize pyridine even after supplementation with a variety of cofactors or protecting agents. Treatment of cells with toluene led to rapid loss of the ability to oxidize pyridine. 3. In the presence of 10 mM-semicarbazide at pH 6.0, *Nocardia* Z1 accumulated a semialdehyde identified as its 2,4-dinitrophenylhydrazone by chromatography, mixed melting point, mass spectrometry and isotope trapping from [2,6-¹⁴C]pyridine as glutarate semialdehyde. 4. Extracts of this bacterium prepared from cells grown with pyridine or exposed to the gratuitous inducer 2-picoline, contained high activities of a specific glutarate semialdehyde dehydrogenase. 5. Cells grown with pyridine or glutarate also contained a glutaric dialdehyde dehydrogenase, an acyl-CoA synthetase and elevated amounts of isocitrate lyase but no glutaryl-CoA dehydrogenase. 6. *Bacillus* 4 accumulated in the presence of 10 mM-semicarbazide several acidic carbonyl compounds from pyridine among which was succinate semialdehyde. Extracts of this bacillus after growth of the cells with pyridine contained an inducible succinate semialdehyde dehydrogenase in amounts at least 50-fold over those found in succinate-grown cells. 7. Two mutants of this bacillus, selected for their inability to grow on pyridine were deficient in succinate semialdehyde dehydrogenase. 8. In the presence of 0.2 mM-KCN, washed suspensions of *Bacillus* 4 accumulated formate and possibly formamide from pyridine. The use of [¹⁴C]pyridine showed that formate was derived from C-2 of the pyridine ring. 9. The organism had a specific formamide amidohydrolase cleaving formamide quantitatively to formate and NH₃. 10. Formate was further oxidized by the particle fraction. There was no soluble formate dehydrogenase in extracts.

Pyridine and its alkyl derivatives, obtained from coal or by synthesis, are widely used as industrial solvents which inevitably find their way into effluents. Pyridine was tried as a soil antiseptic some 60 years ago, but Buddin (1914) found that instead of a decrease in the soil microflora, large numbers of a few species developed that were able to decompose the pyridine. The disappearance of pyridine and quinoline in soil by biological agencies was also claimed by Robbins (1917) and confirmed by Funchess (1917) who found that pyridine and quinoline were almost as good a source of nitrogen for corn crops as NaNO₃. The explana-

tion for this remarkable observation presumably involved the liberation of the heterocyclic-N as NH₃ or some compound which could equally well be nitrified.

The availability of pyridine as a substrate for microbial growth in these early studies was confined to comparatively few bacteria (Koser & Rettger, 1919; den Dooren de Jong, 1926; Ostroff & Henry, 1939), but actinomycetes frequently predominated if pyridine formed the sole source of C, N and energy for growth (Horvath, 1943; Moore, 1949; Ensign & Rittenberg, 1963). Pyridine in sewage was rapidly removed by activated sludge (Grunwald, 1970) from which Stafford & Cally (1970) later isolated a bacterium capable of growing at the expense of pyridine or 4-picoline (4-methylpyridine). In our hands the isolation of pyridine-degrading bacteria by elective culture has invariably been successful,

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although isolates have, with few exceptions, been actinomycetes (Houghton & Cain, 1972).

The problem of analysing the metabolic pathways used to degrade pyridine has so far proved intractable. The appearance of the pyridine N as NH_3 was implied by Funchess' (1917) studies and confirmed by Moore (1949), Stafford & Calley (1970) and by Houghton & Cain (1972) who obtained nearly quantitative recovery of the ring N in this form, but the fate of the carbon skeleton has not previously been reported, due in part to the continued failure of investigators to obtain cell-free extracts that could degrade pyridine irrespective of the precautions adopted or supplementary cofactors added. We have met the same problem but, by using a combination of appropriate trapping agents and inhibitors, mutant strains of a pyridine-degrading bacterium and $[^{14}\text{C}]$ pyridine, we now have evidence for two distinct metabolic routes of biodegradation of this noxious industrial solvent.

Materials

Chemicals

Pyridine was redistilled before use to b.p. 114–115°C.

$[2-^{14}\text{C}]$ Pyridine was prepared from $[6-^{14}\text{C}]$ nicotinic acid (The Radiochemical Centre, Amersham, Bucks., U.K.) by decarboxylation with a copper chromite catalyst at 240°C essentially by the method of Scott (1967). The liberated radioactive pyridine was collected on a cooled 'finger' and separated from traces of nicotinic acid (some of which was also vaporized at this temperature) by distillation from an aqueous solution into a test tube, cooled in liquid N_2 . Decarboxylation of 20 mg of nicotinic acid (31 mCi/mol) resulted in the production of 1.6 mg of pyridine (29.2 mCi/mol).

$[3-^{14}\text{C}]$ Pyridine was prepared in low yield by a Skraup synthesis of quinoline from $[2-^{14}\text{C}]$ glycerol followed by oxidation to quinolinic acid with H_2O_2 and successive decarboxylations of this product, first to nicotinic acid (Scott & Glynn, 1967) and then to pyridine with the copper chromite catalyst described above. Nitrobenzene, 0.07 ml; $\text{FeSO}_4 \cdot 5\text{H}_2\text{O}$, 40 mg; boric acid, 70 mg; aniline sulphate, 163 mg; conc. H_2SO_4 , 0.2 ml and $[2-^{14}\text{C}]$ glycerol, 101 mg (50 μCi , The Radiochemical Centre) were heated in a 5 ml round-bottomed flask fitted with a condenser at 140°C for 4 h. Water (0.3 ml) was added to the cooled mixture and excess of nitrobenzene was removed by steam distillation. Then 8 M-NaOH (1 ml) was added and the mixture steam-distilled again. The first 10 ml of distillate containing the quinoline was made up to 25 ml with water. Acetic acid (0.5 ml) and cupric acetate (500 mg) were added. With the mixture at 70°C, 30% (v/v) H_2O_2 (7 ml) was added dropwise over a 10 min period. A transient muddy-

brown suspension was replaced by a blue precipitate of copper quinolate, which was removed by filtration and washed with water. The copper quinolate was suspended in water and H_2S passed for 1 h at 70°C. The resulting CuS was removed by filtration, and the solution of quinolinic acid remaining was then freeze-dried. The residue was dissolved in acetic acid (10 ml) and refluxed for 6 h to produce nicotinic acid. This solution was evaporated to dryness and the nicotinic acid mixed with copper chromite catalyst (200 mg). Decarboxylation at 240°C resulted in the production of 0.51 mg of $[3-^{14}\text{C}]$ pyridine (0.23 μCi). The radiochemical purity of the labelled pyridine samples was confirmed by paper chromatography in solvent A when only one radioactive spot (R_F 0.28) was observed in each case.

The symmetry of the pyridine ring about the N atom means that the $[2-^{14}\text{C}]$ - and $[3-^{14}\text{C}]$ -pyridine samples synthesized above react metabolically as $[2,6-^{14}\text{C}]$ pyridine and $[3,5-^{14}\text{C}]$ pyridine respectively; they are so referred to in the Results and Discussion sections.

Glutarate semialdehyde. 4,4'-Dicarboethoxybutyraldehyde (20 ml), prepared by the method of Warner & Moe (1948), was added to 0.2 M- H_2SO_4 (200 ml) and autoclaved at 172 kPa (25 lb/in 2) (130°C) for 8 h. After cooling, the solution was saturated with NaCl and extracted with diethyl ether (4 $\times$ 50 ml). The combined ether extracts were dried with anhydrous MgSO_4 and the ether was removed under reduced pressure. Distillation at 120°C and 133.3 Pa (1 mm of Hg) gave 6 g of a colourless oil, which was stored at -20°C. After a few days the oil solidified due to formation of the tripolymer (Piacenti & Pino, 1961). The 2,4-dinitrophenylhydrazone had m.p. 140–141°C after sintering at 128°C. Hall & Howe (1951) reported a melting point of 139–140°C after sintering at 125°C. Glutarate semialdehyde was further purified by chromatography. Some 50 mg was dissolved in water (0.3 ml), heated at 100°C for 10 min to hydrolyse the polymer, and chromatographed on Whatman no. 3 paper in solvent A. The glutarate semialdehyde (R_F 0.80) was eluted with water. Solutions were stored at -20°C and depolymerized just before use.

Succinate semialdehyde was prepared by the method described by Wright & Cain (1972) and also by hydrolysis of diethylformylsuccinate (Jakoby, 1962). The 2,4-dinitrophenylhydrazone had m.p. 196–199°C. Reported values range from 194–196°C (Motoki, 1961) to 204°C (Conia & Gore, 1963).

Succinic dialdehyde was prepared by the method of Wright & Cain (1972) and maleic semialdehyde by the bromination and alkaline hydrolysis of 2-furoic acid (Fecht, 1905).

Glutaconic dialdehyde was obtained by the reaction of ethyl chlorosulphonate with pyridine in NaOH (Baumgarten, 1924). Glutaric dialdehyde

was obtained as a commercial 25% aqueous solution (BDH Chemicals Ltd., Poole, Dorset, U.K.) and its concentration determined by the method of Frigerio & Shaw (1969). Glutaryl-CoA was prepared from glutaric anhydride and determined by the methods of Simon & Shemin (1953).

Organisms

The isolation of *Nocardia* Z1 from soil was described by Houghton & Cain (1972). *Bacillus* 4, also isolated by enrichment from soil, was a small Gram-positive bacillus forming small, oval central spores which were difficult to stain. The red colonies which formed on nutrient agar or pyridine mineral agar were catalase-positive. Nitrate was reduced to nitrite and starch was hydrolysed by the bacterium which has been tentatively identified as a pigmented strain of *B. subtilis*. Cultures of both organisms were maintained on pyridine mineral agar.

Growth of bacteria in large batches

The media and other methods used for the large-scale growth of *Nocardia* Z1 have already been described (Houghton & Cain, 1972) and *Bacillus* 4 was grown in the same way. In the culture of mutant strains unable to utilize pyridine, mineral media were supplemented with succinate (0.5 g/l) and $(\text{NH}_4)_2\text{SO}_4$ (0.25 g/l) as well as pyridine (0.5 g/l).

Methods

Production of *Bacillus* 4 mutants

Strains blocked in their ability to utilize pyridine and its metabolites were obtained without a selection procedure from a suspension of *Bacillus* 4 in minimal growth medium, treated with *N*-methyl-*N'*-nitronitrosoguanidine (300 µg/ml) for 5.5 h (90% kill). The survivors were plated on succinate mineral agar. Plates containing about 100 colonies were replica plated on to pyridine and succinate agar. Colonies which would grow on succinate but not on pyridine were purified by restreaking and tested for growth in liquid pyridine mineral medium. Isolates which would grow under these conditions were discarded.

The mutants were grown on pyridine+succinate mineral medium containing $(\text{NH}_4)_2\text{SO}_4$ as N source. Four mutants, strains B-1, B-2, B-4 and B-5 that would grow on pyridine+succinate but not degrade the pyridine were obtained. Washed cell suspensions of these mutants grown in this manner would not oxidize pyridine. A fifth mutant, strain B-3, utilized the pyridine in the growth medium and cell suspensions also oxidized pyridine as rapidly and with the same O_2 uptake as the wild type, but this organism, unlike the others, accumulated small amounts of succinic semialdehyde and an unidentified derivative from pyridine.

Preparation of cell-free extracts

Extracts were prepared by rupture of frozen cell pastes in the Hughes (1951) press without abrasive at -20°C , followed by extraction of the crushed cells with 2–3 vol. of 50mM-Tris-HCl buffer, pH 8.0. The suspension was then subjected to several 1 min periods of ultrasonic disintegration in the MSE-Mullard 150W instrument with alternate cooling in an ice bath. Small batches of cells could be conveniently ruptured by ultrasonic disintegration alone. Unbroken cells were removed by centrifugation at 15000 rev./min (35000g) in a cooled MSE High Speed 18 centrifuge and the lighter particulate material was then removed by centrifugation for 1–2 h at 50000 rev./min (120000g) in the $10\times 10\text{ml}$ angle rotor of an MSE Super Speed 50 centrifuge.

Investigations of metabolism of pyridine by extracts

Extracts of *Bacillus* 4 were tested for their ability to degrade pyridine under a wide range of conditions. Extracts were prepared in 50mM-Tris-HCl buffer, pH 8.0 or 100mM-potassium phosphate buffer, pH 7.0. Both crude extract and high-speed supernatants were tested. The particle fraction was resuspended in the buffers and also tested. A range of additions to the buffers, tested in an attempt to stabilize the pyridine degrading enzymes, included: 30% (v/v) glycerol, 10% (v/v) acetone, 10% (v/v) ethanol, 0.1% (w/v) mercaptoethanol and 1mM-dithiothreitol.

Pyridine degradation was tested by three types of assay: (i) pyridine disappearance was followed by ΔE_{257} (ϵ for pyridine = $32401\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$) or manometrically by O_2 uptake; (ii) NAD^+ or NADP^+ was included and increase in ΔE_{340} or O_2 uptake determined; or (iii) NADH or NADPH were included and $-\Delta E_{340}$ determined in the presence and absence of pyridine. Only high-speed supernatant could be tested by this spectrophotometric method because of the high NAD(P)H oxidase activity of crude extracts. Pyridine oxidation could be measured manometrically when the flasks contained a reduced nicotinamide nucleotide-regenerating system (Watson *et al.*, 1974) and in the presence of a regenerating system it was also possible to measure metabolism of pyridine at $-\Delta E_{257}$.

A range of coenzymes were also included alone and in combination in some incubations; among those used were: ATP and Mg^{2+} (both at 0.33mM), FAD, FMN or riboflavin (10 µM), biotin (10 µM), CoA (0.33mM), tetrahydrofolic acid (0.33mM) and thiamine pyrophosphate (33 µM). Among metal ions tested were Fe^{2+} , Fe^{3+} , K^+ , Na^+ , Mn^{2+} , Cu^{2+} and Zn^{2+} (0.1mM).

Enzyme assays

Except where otherwise stated, enzyme specific activities are recorded in units (µmol of substrate

disappeared or product formed/min) per mg of protein.

Glutarate semialdehyde dehydrogenase (glutarate semialdehyde-NAD⁺ oxidoreductase) (EC 1.2.1.20). This was determined in assay mixtures containing: cell-free extract (0.5–2.5 mg of protein); Tris-HCl buffer, pH 8.0, 100 μ mol; NAD(P)⁺, 1 μ mol; and glutaric semialdehyde, 1 μ mol in a total volume of 3.0 ml. The increase in E_{340} was measured for the first 1–2 min.

Glutaric dialdehyde dehydrogenase (EC 1.2.1.-). This was measured in similar assay mixtures containing: cell-free extract (0.5–2.5 mg of protein); potassium phosphate buffer, pH 7.0, 100 μ mol; NAD(P)⁺, 1 μ mol; and glutaric dialdehyde, 1 μ mol in a total volume of 3.0 ml. The increase of E_{340} was measured.

Acyl-CoA synthetase (EC 6.2.1.3). The assay mixtures contained, in a total volume of 2.5 ml: dicarboxylic acid, 100 μ mol; ATP, 10 μ mol; MgSO₄, 10 μ mol; CoA, 0.25 μ mol; mercaptoethanol, 10 μ mol; hydroxylamine, pH 7.0, 1 mmol; Tris-HCl buffer, pH 7.5, 100 μ mol and extract (0.5–2 mg of protein). A control contained no dicarboxylic acid. The mixtures were incubated at 30°C for 2 h after which 1 M-HCl (1 ml), 10% (w/v) trichloroacetic acid (1 ml) and 5% (w/v) FeCl₃ in 0.1 M-HCl (1 ml) were added. Protein was removed by centrifugation and the E_{500} recorded. The determination was calibrated with standards of glutaric anhydride in 1 M-hydroxylamine.

Glutaryl-CoA dehydrogenase [glutaryl-CoA-(acceptor) oxidoreductase (decarboxylating)] (EC 1.3.99.7). This was determined by the methods of Numa *et al.* (1964).

Isocitrate lyase (EC 4.1.3.1). This was determined by the method of Olson (1959). A linear rate of increase of E_{252} due to the formation of glyoxylate semicarbazone ($\epsilon = 124001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) was found after an initial 2–5 min lag period.

Succinate semialdehyde dehydrogenase (succinate semialdehyde-NAD⁺ oxidoreductase) (EC 1.2.1.24). This was determined in assay mixtures containing: extract (0.25–1 mg of protein); Tris-HCl buffer, pH 8.0, 100 μ mol; NAD(P)⁺, 1 μ mol; and succinate semialdehyde, 2 μ mol. The increase of E_{340} was measured for the first 1–2 min.

Amidase (acylamide amidohydrolase) (EC 3.5.1.4). This was measured in incubation mixtures (total volume 40 ml) containing: extract (5–10 mg of protein); Tris-HCl buffer, pH 8.0, 1 mmol; formamide, 160 μ mol. The remaining formamide was measured in 1 ml samples by the amide assay described under 'Determinations', and the NH₃ produced by the enzyme reaction in 0.2 ml samples with Nessler's reagent after microdistillation in Conway units (Houghton & Cain, 1972). Because this enzyme would not hydrolyse higher homologues

of formamide, it was strictly a formamide amido-hydrolase and is so referred to in the Results section.

Manometric methods

O₂ uptake by washed cell suspensions was determined at 30°C by using the Gilson constant-pressure respirometer. Flasks contained cell suspension; Tris-HCl or potassium phosphate buffer, 100 μ mol and substrate, 3 mol, in a total volume of 2.8 ml with 0.2 ml of 20% (w/v) KOH in the centre well. The dry wt. of a cell suspension was determined by drying samples (1 ml) at 100°C on weighed planchets.

Chromatography

Paper chromatography was performed on Whatman no. 1 paper in a descending direction. The solvents used were (all proportions by volume): A, butan-1-ol-acetic acid-water (12:3:5); B, butan-1-ol-0.5 M aq. NH₃-ethanol (7:2:1); C, propan-2-ol-conc. NH₃ (sp.gr. 0.88)-water (20:1:2); D, butan-1-ol-water-ethanol (7:2:1); E, ethanol-conc. NH₃ (sp.gr. 0.88)-water (18:1:1); F, phenol-water (4:1); G, propan-1-ol-water (7:3); H, propan-1-ol-water (4:1); J, butan-1-ol saturated with 0.5 M aq. NH₃ (organic layer used). T.l.c. was performed on Eastman silica-gel Chromagram Sheets (Eastman Kodak Co., Rochester, N.Y., U.S.A.). The solvents used were: K, hexane-ethyl formate-acetic acid (5:3:1); L, light petroleum (b.p. 80–100°C)-ethyl acetate-propionic acid (65:35:7.7); M, hexane-acetic acid-xylene (5:1:3); N, butan-1-ol-chloroform-acetic acid (4:15:1); P, ethanol-conc. NH₃ (sp.gr. 0.88)-water (25:4:3).

Pyridine was detected under u.v. light as a brown spot on a fluorescent background. 2,4-Dinitrophenylhydrazones were visible by their yellow colour, which was converted into a stronger reddish-brown coloration by spraying with 1 M-NaOH. Amino acids were detected by dipping the chromatograph in 0.25% (w/v) ninhydrin in acetone and heating at 100°C for 5 min. Organic acids were detected with 0.05% (w/v) Bromophenol Blue in 50% (v/v) aq. ethanol, adjusted as necessary with citric acid or aq. NH₃ solution. Unsaturated acids were detected by their bleaching of 0.05 M-KMnO₄ in 0.1 M-H₂SO₄. Glutarate semialdehyde was located with 0.2% (w/v) 2,4-dinitrophenylhydrazine in 2 M-HCl, forming a deep-yellow spot on a paler background.

Isolation of 2,4-dinitrophenylhydrazones

Glutarate semialdehyde produced by the metabolism of pyridine by *Nocardia* Z1 cells in the presence of semicarbazide was identified as its 2,4-dinitrophenylhydrazone. A typical incubation mixture contained, in 400 ml: *Nocardia* Z1 cells, 4 g dry wt.; potassium phosphate buffer, 1 mmol,

Table 1. *Chromatographic characterization of the 2,4-dinitrophenylhydrazones derivatives of some carbonyl compounds found in cultures of pyridine-degrading bacteria*

Pyruvate exhibited two spots in solvents B, C, D and L. n.d., Not determined. For details, see the text.

	R_F values					
	Paper chromatography in solvent			T.l.c. in solvent		
	B	C	D	K	L	M
2,4-Dinitrophenylhydrazone of						
Glutarate semialdehyde	0.59	0.54	0.92	0.81	0.71	0.29
Succinate semialdehyde	0.52	n.d.	0.60	0.73	0.38	0.16
Pyruvate	0.52, 0.70	0.45, 0.70	0.51, 0.73	0.65	0.14, 0.21	0.13
2-Oxoglutarate	0.10	n.d.	n.d.	0.02	0	0
Derivative X	0.71	0.70	0.78	0.39	0.14	0.02

containing semicarbazide-HCl, 4mmol adjusted to pH6.0 and pyridine, 400 μ mol. Pyridine degradation was determined by measuring $-ΔE_{257}$ and semicarbazone formation by $ΔE_{235}$. After 2h the cells were removed by centrifugation and 10ml of 0.2% (w/v) 2,4-dinitrophenylhydrazine in 2M-HCl was added. The mixture was left overnight and then extracted twice with ethyl acetate.

The combined extracts were shaken with 10% (w/v) Na_2CO_3 solution (100ml) for 1min and the layers separated. T.l.c. of the yellow ethyl acetate layer in solvent L showed that no neutral aldehyde dinitrophenylhydrazone but only excess 2,4-dinitrophenylhydrazine, which ran at the solvent front, was present. The red-brown Na_2CO_3 layer was acidified with conc. HCl and the acidic 2,4-dinitrophenylhydrazones extracted back into ethyl acetate (30ml). The ethyl acetate phase was dried with anhydrous $MgSO_4$, evaporated to dryness and the residue recrystallized from water.

Similar incubations containing, for example, cells of *Bacillus* mutant B-3, 180mg; potassium phosphate buffer, pH6.0, 1mmol (containing semicarbazide-HCl, 1mmol) and pyridine, 100 μ mol were used for isolation of 2,4-dinitrophenylhydrazones after pyridine degradation by this organism.

2,4-Dinitrophenylhydrazones were characterized by both paper chromatography and t.l.c. (Table 1), by their light-absorption maxima in 10% (w/v) Na_2CO_3 and 1M-NaOH (Wright & Cain, 1972) and by reduction to the corresponding amino acid by catalytic hydrogenation over PtO_2 (Chamberlain & Dagley, 1968).

Isolation of glutaric acid

The product of glutarate semialdehyde metabolism was isolated from an incubation mixture containing, in a total volume of 30ml: extract of pyridine-grown *Nocardia* Z1 cells, 20.6mg of protein; Tris-HCl buffer, pH8.0, 1mmol; NAD^+ , 50 μ mol

and glutarate semialdehyde, 40 μ mol. The formation of NADH was measured at 340nm and, at the end of the reaction, 1M-HCl was added and denatured protein removed by centrifugation. The supernatant was continuously extracted with diethyl ether for 12h, the extract dried with anhydrous $MgSO_4$ and evaporated to dryness. The product was identical by paper chromatography and t.l.c. with glutaric acid.

Determinations

Pyridine was measured by E_{257} ($\epsilon = 32401 \cdot mol^{-1} \cdot cm^{-1}$ at pH7.0).

Protein was determined by the Folin method (Lowry *et al.*, 1951) with crystalline bovine serum albumin [Sigma (London) Chemical Co., Kingston-upon-Thames KT2 7BH, Surrey, U.K.] as standard.

Formate was obtained from incubation mixtures (3–5ml) acidified with 1M- H_2SO_4 after steam distillation in a Markham still. Distillate (20vol.) was collected in 1M-NaOH (0.5ml) and the solution evaporated to dryness. The sodium formate was reduced with Mg and HCl to formaldehyde and determined with acetylacetone or chromotropic acid (Wood & Gest, 1957).

Succinate semialdehyde and glutarate semialdehyde were measured by the extinction of their 2,4-dinitrophenylhydrazones in 10% (w/v) Na_2CO_3 solution ($\epsilon = 10450$ and 15600 at 374nm respectively).

Formamide and other amides were determined by conversion into their hydroxamates and reaction with $FeCl_3$. Mixtures containing an amide, 0–5 μ mol; 2.5M-NaOH, 0.2ml and 2M-hydroxylamine hydrochloride, 0.2ml, were incubated at 40°C for 40min. Water, 1ml; 3M- HNO_3 , 0.2ml and iron alum solution [2g of $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4 \cdot 24H_2O$ and 50ml of 3M-HCl in 1 litre of water], 0.4ml were added and E_{490} was measured after 10min. The amount of colour formed was rather variable and standards were always determined at the same time as unknowns.

Radioactivity measurements

Samples were counted for radioactivity in a Packard Tri-Carb scintillation counter (model 3390) in glass vials containing 10 ml of Insta-Gel scintillation cocktail (Packard Instruments Ltd., Caversham, Berks., U.K.). Corrections for counting efficiency were made by the external-standard ratio method. A minimum of 10 000 counts was measured.

Areas of radioactivity on paper chromatograms were detected with a Tracerlab (Weybridge, Surrey, U.K.) 4π chromatogram strip scanner at various scan speeds.

Mass spectrometry

Mass spectra were measured and analysed by Dr. P. Kelly, Department of Chemistry, University of Newcastle upon Tyne.

Results

Growth Experiments

Both *Nocardia* Z1 and *Bacillus* 4 would utilize pyridine as sole carbon and nitrogen source for growth. Examination of culture filtrates showed that no u.v.-absorbing products accumulated during pyridine degradation. The ring N was released as NH_3 ; in a typical experiment with *Bacillus* 4 an 80% recovery of NH_3 was obtained from the available pyridine N in a 20 litre culture.

Houghton & Cain (1972) reported that *Nocardia* Z1 would not grow on a wide range of other pyridine compounds including the methyl-, ethyl-, dimethyl- and hydroxy-pyridines and pyridine carboxylic acids. The growth characteristics of *Bacillus* 4 were very similar. No growth occurred on any of the isomeric methyl-, ethyl-, amino- or hydroxy-pyridines; on picolinic acid, nicotinic acid or isonicotinic acid, or on piperidine each provided at 0.1% (w/v or v/v) as sole C and N sources. Benzene and nitrobenzene (0.1%, v/v) would not act as growth substrates when $(\text{NH}_4)_2\text{SO}_4$ (0.5%, w/v) was included as N source.

Metabolism of pyridine by washed cell suspensions

Pyridine was rapidly oxidized by washed cell suspensions of *Nocardia* Z1 (15–20 μl of O_2 /h per mg dry wt. of cells) and *Bacillus* 4 (35–55 μl of O_2 /h per mg dry wt. of cells) in manometric experiments with a total O_2 uptake of 2.5–3.0 mol/mol of pyridine. The oxidation of pyridine was inducible. Suspensions of *Nocardia* Z1 grown on succinate, glutarate or glucose oxidized pyridine only very slowly, whereas suspensions of *Bacillus* 4 grown on succinate did not oxidize pyridine at a detectable rate.

After pyridine degradation by suitably induced cell suspensions was complete, the cells were removed by centrifugation and the u.v. spectrum of the supernatant was recorded. Complete disappearance of the

characteristic absorption peaks of pyridine was found and no new peaks were produced. The supernatants were examined for the presence of carbonyl compounds by addition of 2,4-dinitrophenylhydrazine and isolation of derivatives as described in the Methods section and for acidic derivatives by paper chromatography and t.l.c. of ether extracts of the acidified supernatants in solvents A and E. No end-products of pyridine degradation were found with either organism. Washed cells of *Bacillus* 4, grown with pyridine, did not oxidize any of the isomeric monohydroxypyridines.

Metabolism of pyridine by cell-free extracts

Houghton & Cain (1972) were unable to demonstrate degradation of pyridine by extracts of *Nocardia* Z1 and identical results were obtained in the present study with extracts of *Bacillus* 4. Extracts were prepared as rapidly as possible and, when a low-speed centrifugation was used for removal of unbroken cells, could be tested as early as 20 min after rupture of cells. No evidence for metabolism of pyridine was obtained under any of the conditions described in the Methods section. In contrast, the cells of both organisms would still rapidly oxidize pyridine after storage at -20°C for up to 1 year. Treatment of washed suspensions for 1 h at 30°C in 50 mM-Tris-HCl, pH 8.0, containing 2% (v/v) toluene or 0.2% (w/v) sodium dodecyl sulphate led to some 90% fall in pyridine-oxidation rate. Dutton & Evans (1969) have reported a fatty acid inhibitor, present in particles of *Rhodopseudomonas palustris* that inhibited metabolism of benzoate by whole cells. Neither particles nor soluble extracts of *Nocardia* Z1, however, inhibited oxidation of pyridine by cell suspensions.

Despite our failure to demonstrate degradation of pyridine by extracts, evidence has been obtained for intermediates of pyridine metabolism. The pathways used by *Nocardia* Z1 and *Bacillus* 4 appear to be different so the results obtained with each organism are described separately.

Metabolism of pyridine by *Nocardia* Z1

Formation of glutarate semialdehyde. Glutarate semialdehyde formed during metabolism of pyridine by *Nocardia* Z1 was identified as its 2,4-dinitrophenylhydrazone.

The rates of O_2 uptake and total O_2 consumption during pyridine metabolism by washed cell suspensions were decreased in the presence of semicarbazide at pH 6.0. The effect increased with increasing semicarbazide concentration and at 10 mM the rate of O_2 uptake was decreased by some 80%, whereas the total O_2 uptake was decreased from 3 to 2 mol/mol of pyridine. Spectrophotometric examination of the flask contents, after removal of cells by centrifugation, showed that a new absorption peak at 235 nm was

present, suggesting the formation of a semicarbazone. No semicarbazone was formed in the absence of pyridine and no change in the spectrum of pyridine occurred when shaken with semicarbazide in the absence of cells.

To obtain sufficient material for a positive identification the semicarbazone was converted into the 2,4-dinitrophenylhydrazone in the large-scale incubation mixture described in the Methods section. Yellow-brown needles were isolated, m.p. 140°C after sintering at about 125°C. The derivative had a melting point identical with that of glutarate semialdehyde 2,4-dinitrophenylhydrazone and a mixed melting point showed no depression. Paper chromatography and t.l.c. showed that the isolated derivative had an R_F identical with that of the derivative of glutarate semialdehyde in solvents B, C, D, K, L and M (Table 1). The amino acid produced by catalytic hydrogenation of the 2,4-dinitrophenylhydrazone was identified as 5-aminovalerate by comparison with the authentic amino acid in solvents A (R_F 0.53), E (R_F 0.22) and F (R_F 0.86). The absorption maxima of several oxo acid 2,4-dinitrophenylhydrazones are characteristic (Wright & Cain, 1972) and both the isolated derivative and glutarate semialdehyde 2,4-dinitrophenylhydrazone had identical absorption spectra in 10% (w/v) Na_2CO_3 solution and 1 M-NaOH with maxima at 374 and 425 nm respectively. The identity of the isolated derivative was confirmed when its mass spectrum showed a parent ion of mass 296 corresponding to $\text{C}_{11}\text{H}_{12}\text{N}_4\text{O}_6$. This formula is

equivalent to $\text{C}_5\text{H}_8\text{O}_3$ for the free carbonyl compound, consistent with its identity as glutarate semialdehyde.

Labelled glutarate semialdehyde 2,4-dinitrophenylhydrazone was isolated in a similar way from the products of metabolism of [2,6- ^{14}C]pyridine (Table 2). Radioactivity was incorporated into glutarate semialdehyde with no decrease in specific radioactivity showing that the C skeleton of glutarate semialdehyde was probably derived directly from the pyridine ring.

These results suggested that glutarate semialdehyde was a metabolite of pyridine by *Nocardia* Z1. The amount of glutaric semialdehyde 2,4-dinitrophenylhydrazone formed in replicate experiments was calculated from its molar extinction in 10% (w/v) Na_2CO_3 solution and showed that 10–15% of the stoichiometric amount accumulated from the available pyridine.

Metabolism of glutarate semialdehyde. Glutarate semialdehyde was metabolized by an NAD(P) $^{+}$ -dependent dehydrogenase that was induced by growth of *Nocardia* Z1 on pyridine. About a 50-fold increase of activity was found in extracts of cells grown on pyridine compared with that present in extracts of cells grown on succinate. The activity of cells grown on glutarate itself was also increased, but was still 20-fold lower than that in fully-induced extracts (Table 3). When resting-cell suspensions of succinate-grown cells were shaken in the presence of pyridine (Fig. 1), the induction of pyridine oxidation (measured manometrically in whole cells) was

Table 2. Isolation of radioactive glutarate semialdehyde 2,4-dinitrophenylhydrazone after metabolism of [2,6- ^{14}C]pyridine by *Nocardia* Z1

The incubation mixture contained, in a total volume of 10 ml: pyridine-grown *Nocardia* Z1 cells (equivalent to 35.3 mg dry wt.); potassium phosphate buffer, pH 6.0, 500 μmol ; semicarbazide-HCl adjusted to pH 6.0, 150 μmol ; [2,6- ^{14}C]pyridine, 20 μmol (135 000 d.p.m.). A control flask contained no pyridine. After shaking for 2 h at 30°C the cells were removed by centrifugation and 0.5 ml of 0.2% (w/v) 2,4-dinitrophenylhydrazone in 2 M-HCl was added to the supernatants. After 12 h at room temperature the mixtures were extracted with ethyl acetate and the acidic 2,4-dinitrophenylhydrazones, then re-extracted back into 50 ml of 10% (w/v) Na_2CO_3 solution. The E_{375} of this solution was 0.69 corresponding to the formation of 2.2 μmol of [^{14}C]glutarate semialdehyde 2,4-dinitrophenylhydrazone, a yield of 11% from the available pyridine. Glutarate semialdehyde 2,4-dinitrophenylhydrazone (30 mg) was added to the carbonate solutions which were acidified with conc. HCl and the derivatives extracted into ethyl acetate. The ethyl acetate was removed *in vacuo* and the residues recrystallized three times from water. After each recrystallization 0.5–1.0 mg of the derivative was dissolved in toluene (2 ml) and 10 ml of Insta-Gel scintillator was added; the counting efficiency of the coloured solutions was 70–80%. The counts were corrected for background (28 d.p.m./mg) from the material reisolated from the control incubation without pyridine.

Sample	Radioactivity (d.p.m./mg)
Reisolated glutarate semialdehyde 2,4-dinitrophenylhydrazone	
(i) 1st recrystallization	567
(ii) 2nd recrystallization	612
(iii) 3rd recrystallization	604
	} mean value = 608

Specific radioactivity of the available [2,6- ^{14}C]pyridine was 6750 d.p.m./ μmol .

Total label in the reisolated derivative (608×30) was 18 240 d.p.m.

Therefore specific radioactivity in the original 2.2 μmol of glutarate semialdehyde 2,4-dinitrophenylhydrazone was 8290 d.p.m./ μmol .

Table 3. *Glutarate semialdehyde dehydrogenase and glutaric dialdehyde dehydrogenase in extracts of Nocardia Z1*

Nocardia Z1 was grown on the substrates indicated, harvested during exponential growth and activity of glutarate semialdehyde dehydrogenase and glutaric dialdehyde dehydrogenase determined as described in the Methods section. n.d., Not determined.

Growth substrate	Nicotinamide nucleotide used	Glutarate semialdehyde dehydrogenase (μmol of NAD(P)H/min per mg of protein)	Glutaric dialdehyde dehydrogenase (μmol of NAD(P)H/min per mg of protein)
Pyridine	NADP ⁺	0.34	0.046
	NAD ⁺	0.275	0.185
Glutarate	NADP ⁺	0.017	n.d.
	NAD ⁺	0.015	n.d.
Succinate	NADP ⁺	0.006	0.009
	NAD ⁺	0.008	0.034

Fig. 1. *Simultaneous induction of pyridine oxidation and glutarate semialdehyde dehydrogenase activity in Nocardia Z1*

A 20-litre batch of *Nocardia* Z1, grown on 1 g of succinate/litre for 24 h was harvested, washed with water and resuspended in mineral-salts medium at 30°C containing 0.1% (v/v) pyridine to a final cell density of 17 mg dry wt./ml. The suspension was incubated with shaking at 30°C and the pyridine concentration maintained at 0.1% (v/v) throughout the experiment by spectrophotometric determination and addition of the appropriate amount of pyridine. At the times indicated, a 30 ml and a 5 ml sample were removed, centrifuged and the cells washed with water. The smaller sample was used immediately to measure the initial rate of oxidation of pyridine in the Gilson respirometer (○). The 30 ml sample was centrifuged and from the pellet, extracts were prepared by sonication. Glutarate semialdehyde dehydrogenase (●) and glutaric dialdehyde dehydrogenase (△) were assayed as described in the Methods section.

closely paralleled by an increase in the specific activity of glutarate semialdehyde dehydrogenase in extracts from the cells.

The pyridine analogues 2-fluoropyridine and 2-, 3- and 4-picoline were also tested for their ability to induce pyridine oxidation in incubation mixtures

similar to those described in Fig. 1. 2-Picoline (0.1%, v/v) acted as an inducer for pyridine oxidation ($4 \mu\text{l}$ of O_2/h per mg dry wt. of cells), but it was not itself oxidized, or otherwise metabolized, by the induced cells nor would it support growth of *Nocardia* Z1. Concomitant with this gratuitous induction of pyridine oxidation was the induction of glutarate semialdehyde dehydrogenase (specific activity 0.11 units/mg of protein) by the 2-picoline analogue. The other analogues failed to induce pyridine oxidation or the dehydrogenase.

Glutarate semialdehyde dehydrogenase required thiols for maintenance of full activity and was very sensitive to heavy metals. It showed strict specificity for its glutarate semialdehyde substrate, but crude extracts and a 13-fold purified preparation both contained NAD⁺- and NADP⁺-dependent dehydrogenase activity with the NAD⁺ rates some 85% of those obtained with NADP⁺ (Table 3). Although the enzyme had a pH optimum of 9.3 for the NADP⁺-dependent activity and 9.8 for the NAD⁺-dependent activity (measured in 0.3 M-glycine-KOH buffer), these activities were probably catalysed by the same protein because the elution profile of the two activities from a column (40 cm $\times$ 2.5 cm diam.) of Sephadex G-200 in Tris-HCl buffer (pH 8.0) was identical and the ratio of NAD⁺/NADP⁺ activities remained constant at 1.38:1 through a 3-step (13-fold) purification procedure (Watson, 1972). Further, the activities with NAD⁺ and NADP⁺ were lost at an identical rate ($t_{1/2} = 70$ min at 50°C) when the enzyme was heat-denatured. At the appropriate pH optimum the NADP⁺-dependent enzyme showed K_m 0.1 mM for NADP⁺ and 70 μM for glutaric semialdehyde; the corresponding values for the NAD⁺-dependent activity were 0.42 mM and 0.14 mM. Although not a substrate, succinate semialdehyde was a good competitive inhibitor with K_i 0.6 mM. Maleate semialdehyde, glutaconic dialdehyde, crotonaldehyde, butyraldehyde, glyoxalate and 2-oxoadipate would not substitute for glutarate semialdehyde.

The metabolism of 1 mol of glutarate semialdehyde

Fig. 2. Oxidation of pyridine and some putative intermediary metabolites by washed cells of *Nocardia Z1*

In (a) Gilsen flasks contained in a total volume of 2.8 ml: cell suspension (equivalent to 42 mg dry wt. of cells from a culture grown with pyridine as C, N and energy source); potassium phosphate buffer, pH 7.5, 100 μmol; and pyridine (Δ), glutaric dialdehyde (○), glutarate semialdehyde (■), or glutarate (▲), 3 μmol. Results are corrected for the endogenous respiration of 56 μl/h. There was 0.2 ml of 20% (w/v) KOH in the centre well. In (b), flasks contained in 2.8 ml of solution: cell suspension (equivalent to 50 mg dry wt.); Tris-HCl buffer, pH 8.0, 100 μmol; and substrate, 2 μmol. There was 0.2 ml of 20% (w/v) KOH in the centre well. The oxidation of pyridine (Δ); 1,2,3,6-tetrahydropyridine (●); piperidine (□); 2- and 3-hydroxypiperidine (○) is corrected for the endogenous respiration of 90 μl/h.

required 1 mol of NAD(P)⁺ and the reaction product was identified as glutarate after isolation from the incubation mixture as described in the Methods section. The isolated material had an R_f identical with that of glutaric acid in solvents A (R_f 0.82), G (R_f 0.28), H (R_f 0.09), N (R_f 0.84) and M (R_f 0.52). The product was detected with Bromophenol Blue sprays, but was not visible under u.v. light and did not decolorize a permanganate spray showing that it was not an unsaturated acid.

Metabolism of glutaric dialdehyde. Glutaric dialdehyde was rapidly oxidized by cell suspensions of pyridine-grown *Nocardia Z1* (Fig. 2) and extracts of these cells contained a glutaric dialdehyde dehydrogenase, which had a pH optimum of 8.0 and utilized NAD⁺ at some 5 times the rate of NADP⁺ (Table 3).

Activity of this enzyme increased only sixfold during the induction of pyridine oxidation, whereas the activity of glutarate semialdehyde dehydrogenase increased nearly 60-fold (Fig. 1 and Table 3).

No evidence was found for the formation of glut-

aric dialdehyde during pyridine degradation. Neutral 2,4-dinitrophenylhydrazones, for instance, were not obtained from the oxidation of pyridine by resting cell suspensions in the presence of semicarbazide, nor was radioactive glutaric dialdehyde recovered in isotope-trapping experiments in which [¹⁴C]pyridine was metabolized by cell suspensions in the presence of an excess of glutaric dialdehyde.

Metabolism of glutarate. Because glutarate was identified as the product of glutarate semialdehyde metabolism by the specific dehydrogenase, the metabolism of the dicarboxylic acid by *Nocardia Z1* was examined. No direct evidence for glutarate formation was found; for example no radioactivity was incorporated into glutarate when [¹⁴C]pyridine was degraded in the presence of a large excess of glutarate though in this case, it is likely that the uninduced intact cell may be impermeable to the dicarboxylate anion. Suspensions of cells grown with pyridine would oxidize glutarate only after a lag phase of 20–30 min, whereas glutarate semialdehyde

and glutaric dialdehyde were oxidized immediately (Fig. 2). *Nocardia* Z1 when grown on glutarate as sole C source gave extracts containing low activities of an acyl-CoA synthetase; a similar activity with glutarate as the CoA-acceptor was present in extracts obtained from cells grown with pyridine or succinate (Table 4). No glutaryl-CoA dehydrogenase was found in extracts of pyridine- or glutarate-grown cells.

Some suggestion that the products of pyridine oxidation were yielding acetyl-CoA came from the induction of isocitrate lyase in pyridine- or glutarate-grown cells to activities some 15- to 20-fold higher than those found in bacteria grown with pyruvate or glucose (Table 5).

Metabolism of pyridine by *Bacillus* 4

Formation of succinate semialdehyde. The wild-type strain of *Bacillus* 4 did not produce any neutral or acidic 2,4-dinitrophenylhydrazones from the metabolism of pyridine in incubation mixtures containing semicarbazide, subsequently treated with acidic 2,4-dinitrophenylhydrazine.

Succinate semialdehyde was identified, however, after the metabolism of pyridine by cell suspensions of *Bacillus* mutant strain B-3 in potassium phosphate buffer, pH 6.0, containing 10 mM-semicarbazide. In experiments with *Nocardia* Z1 (see the Methods section) only a single product was formed, but incubations with mutant B-3 yielded a mixture of 2,4-dinitrophenylhydrazones. Succinate semialdehyde 2,4-dinitrophenylhydrazone (R_F 0.52, 0.60,

Table 4. *Acyl-CoA synthetase activity in extracts of Nocardia* Z1

Nocardia Z1 was grown on the substrates indicated and cells were harvested during exponential growth. Acyl-CoA synthetase was assayed as described in the Methods section. In Expt. 2, crude extracts were used in each case and the activities corrected for hydroxamate formation in the absence of dicarboxylic acid.

Expt. 1	Growth substrate	CoA acceptor	Reaction mixture	Hydroxamate formed (μ mol)
Pyridine	Glutarate		Complete	1.3
			Minus	0.7
			glutarate	
			Minus CoA	0.4
			Minus ATP and Mg^{2+}	0.1
Expt. 2	Growth substrate	CoA acceptor	Specific activity of acyl-CoA synthetase (μ mol/h per mg of protein)	
Pyridine	Succinate	Glutarate	4.40	
			0.16	
			0.15	
			0.08	
Glutarate	Glutarate		0.16	
Succinate	Glutarate		0.20	

Table 5. *Isocitrate lyase activity in extracts of Nocardia* Z1

Nocardia Z1 was grown on the substrates indicated and the cells harvested during exponential growth. Extracts were prepared by sonication and isocitrate lyase determined as described in the Methods section.

Growth substrate	Isocitrate lyase activity (μ mol/min per mg of protein)
Glutarate	0.32
Pyridine	0.27
Butyrate	0.19
Succinate	0.053
Glucose	0.018
Pyruvate	0.014

0.38 and 0.16 in solvents B, D, L and M respectively) was formed, but usually together with the corresponding derivatives of pyruvate and occasionally 2-oxoglutarate. The identity of the succinate semialdehyde derivative was confirmed by its reduction to, and co-chromatography with, authentic 4-amino-butyrate in solvents A (R_F 0.50), E (R_F 0.38) and F (R_F 0.77). Small amounts of an unidentified compound (derivative X) were also formed during metabolism of pyridine by strain B-3 (Table 1).

The 2,4-dinitrophenylhydrazone of this compound showed an unusual and characteristic reaction with NH_3 vapour in which the original pale-yellow spots on paper or silica-gel t.l.c. plates formed an intense brown colour. The derivatives of succinate semialdehyde, pyruvate and 2-oxoglutarate were not affected by NH_3 vapour.

No glutarate semialdehyde 2,4-dinitrophenylhydrazone was formed by the wild-type or mutant *Bacillus*.

Metabolism of succinate semialdehyde. Extracts of *Bacillus* 4 cells, grown on pyridine, contained high activities of succinate semialdehyde dehydrogenase. The activity of cells grown on succinate was always at least 50-fold lower (Table 6). Identical results were obtained with succinate semialdehyde prepared by either of the two methods described in the Materials section.

Very small amounts of succinate semialdehyde dehydrogenase activity were found in extracts of the mutant strains B-1 and B-2, which were unable to oxidize or otherwise degrade pyridine after growth in pyridine+succinate medium (Table 6), but the mutant B-3 contained very nearly the fully-induced wild-type enzyme activity. Since this strain oxidized pyridine, produced succinate semialdehyde from it and contained the dehydrogenase converting succinate semialdehyde into succinate, its inability to grow at the expense of pyridine alone must be due to a metabolic lesion remote from these early steps.

Several other analogues of succinate semialdehyde were also tested for activity with extracts of the

Table 6. Succinate semialdehyde dehydrogenase activity in wild-type and mutant strains of *Bacillus* 4

Bacteria were grown on the appropriate medium to exponential phase when they were harvested and extracts prepared. Enzyme activities were determined as described in the Methods section.

Organism	Growth substrate	Nicotinamide nucleotide used	Specific activity (μmol of NAD(P)H/min per mg of protein)
Wild-type	Pyridine	NAD ⁺	0.52
		NADP ⁺	0.095
	Succinate	NAD ⁺	0.006
		NADP ⁺	0.001
Mutant B-1	Pyridine+succinate	NAD ⁺	0.66
		NAD ⁺	0.018
Mutant B-2	Pyridine+succinate	NAD ⁺	0.023
Mutant B-3	Pyridine+succinate	NAD ⁺	0.48

Fig. 3. Production of formate and NH_3 during pyridine metabolism by cyanide-treated suspensions of *Bacillus* 4

The incubation mixture, shaken at 30°C in a tightly stoppered flask to avoid loss of KCN, contained in total volume of 50 ml: a washed suspension of pyridine-grown *Bacillus* 4 [equivalent to 225 mg dry wt. in experiment (a) (main graph) and 320 mg dry wt. in experiment (b) (inset)]; Tris-HCl buffer, pH 8.0, 1 mmol; KCN 10 μmol ; and pyridine, 100 μmol . At the times indicated, a 5 ml sample was taken, the cells were removed by centrifugation and pyridine (○) and NH_3 (▲) determined in the supernatants. Formate (●) was measured after steam-distillation as described in the Methods section.

wild-type *Bacillus* grown on pyridine. No significant activity (less than 2% of the rate with succinate semialdehyde) was found with glutarate semialdehyde, maleate semialdehyde, succinic dialdehyde, crotonaldehyde, glyoxalate, butyraldehyde, propionaldehyde, acetaldehyde or formaldehyde.

Production of formate and formamide. Formate was recognized as a product of pyridine metabolism

when it was isolated from resting-cell suspensions of *Bacillus* 4 degrading pyridine in the presence of KCN to inhibit further oxidation of formate by the electron-transport system. KCN inhibited the rate of pyridine oxidation by some 90% at 0.2 mM, but at this concentration, the accumulation of 0.65–0.80 mol of formate/mol of pyridine occurred. A typical experimental result is shown in Fig. 3. No formate was produced in the absence of KCN or pyridine. No colour was obtained with acetylacetone or chromotropic acid without the $\text{Mg} + \text{HCl}$ reduction step in the test for formate (Wood & Gest, 1957) indicating that the metabolic product was not formaldehyde.

In an earlier experiment, evidence was found for the occurrence of formamide as a likely precursor of the formate. Under conditions similar to those in Fig. 3, a consistent lag in the production of NH_3 from pyridine occurred (Fig. 3, inset) and when similar reaction mixtures were tested for amides, some 40 μmol of a product reacting with hydroxylamine under the conditions of the formamide assay (see the Methods section) accumulated from the pyridine during the first 30 min. This intermediate subsequently disappeared within the next 60 min with the production of formate and NH_3 . We have been unable to accumulate this intermediate in more recent experiments where recovery of 85% of the stoichiometric amount of formate was not unusual (Table 7).

Experiments with [^{14}C]pyridine confirmed the origin of the formate. The identity of formate with a radioactive product of [2,6- ^{14}C]pyridine metabolism was suggested by paper chromatography of the metabolic product as its ammonium salt in solvents E (R_F 0.58) and J (R_F 0.17) where the radioactivity co-chromatographed with ammonium formate; it was confirmed by the isolation of radioactive sodium formate in an isotope-trapping experiment and recrystallization of this salt to constant specific radioactivity (Table 7). Recovery of radioactivity in sodium formate from [2,6- ^{14}C]pyridine was approx.

Table 7. Identification of [^{14}C]formate as a product of [^{14}C]pyridine metabolism by *Bacillus* 4

Incubation mixtures contained, in a total volume of 20 ml: suspension of *Bacillus* 4 (equivalent to 103 mg/dry wt.); Tris-HCl buffer, pH 7.5, 0.75 mmol; KCN, 4 μmol ; and pyridine, 20 μmol (74200 d.p.m. of [2,6- ^{14}C]pyridine or 56400 d.p.m. of [3,5- ^{14}C]pyridine). The mixtures were shaken at 30°C for 2.5 h in stoppered flasks to avoid loss of HCN arising from hydrolysis of the KCN and formate production was measured in an identical pilot vessel containing unlabelled pyridine. After 2.5 h, when 17 μmol of formate had accumulated, the cells were removed from all the radioisotope incubations by centrifugation, 0.5 ml of 1 M- H_2SO_4 was added to the supernatants and the mixtures were steam-distilled into 1 ml of 1 M-NaOH. Sodium formate (500 mg) was added and the distillate (approx. 200 ml) was evaporated to dryness *in vacuo*. The sodium formate was washed with diethyl ether and recrystallized twice from aq. methanol. Samples of the recrystallized material were dissolved in 1 ml of water, added to 10 ml of Insta-Gel scintillator and counted for radioactivity.

Sample	Radioactivity in sample (d.p.m.)	$10^{-3} \times$ Total radioactivity (d.p.m.)	Recovery of radio- activity available in pyridine (%)*
1. [2,6- ^{14}C]Pyridine supplied		74.2	—
Isolated sodium formate			
1st recrystallization (14.6 mg)	923	31.6	50.1
2nd recrystallization (10.3 mg)	686	33.3	52.8
2. [3,5- ^{14}C]Pyridine supplied		56.4	—
Isolated sodium formate			
1st recrystallization (9.2 mg)	24	1.3	2.7
2nd recrystallization (19.1 mg)	43	1.1	2.4

* Corrected for only 17 μmol of formate produced from 20 μmol of available pyridine at time of measurement.

50%, consistent with the origin of the formate in C-2 of the pyridine ring. In contrast, little radioactivity was incorporated into sodium formate from [3,5- ^{14}C]pyridine (Table 7).

Metabolism of formate and formamide. Washed cell suspensions of *Bacillus* 4, grown on pyridine, oxidized formate (1.7 μl of O_2/h per mg dry wt. of cells) and formamide (6.0 μl of O_2/h per mg dry wt. of cells) without a lag. Cells grown on succinate would also oxidize formate and formamide, but at a rate only some 20–40% of that found with pyridine-grown cells. Extracts of *Bacillus* 4 were examined for the presence of a soluble formate dehydrogenase (Johnson & Quayle, 1964), but no activity was detected. This result, together with the inhibition of formate metabolism by KCN, suggested that formate was oxidized by a cytochrome-linked particulate system as found in other bacteria degrading heterocyclic compounds (Wright & Cain, 1972; Watson *et al.*, 1974).

Formamide was metabolized by high-speed supernatants with the formation of stoichiometric amounts of NH_3 . The specific activity of the formamide amidohydrolase in extracts of cells grown on pyridine (0.21–0.40 unit/mg of protein) and succinate (0.38–0.46 unit/mg of protein) was similar, suggesting that the enzyme was constitutive. The amidase was inactive, however, with methylformamide, acetamide, propionamide, urea or nicotinamide.

Discussion

The main problem found in all investigations of

pyridine degradation is the continued failure of extracts to metabolize pyridine under a wide range of conditions. The reasons for this failure remain obscure, for the effects of many cofactor and stabilizer additions have been examined alone and in combination. It would seem unlikely that the activity of even a very labile enzyme would remain undetected when extracts, which could be tested within 20–30 min of cell rupture, were derived from whole cells in which the ability to oxidize pyridine was maximal and which could be stored for up to 1 year at -20°C without appreciable loss of this activity. The possibility remains that some intact structural organization in the cell essential for pyridine degradation is lost during extract preparation or destroyed in the broken cells by exposure to the solvent effect of pyridine, or the enzyme(s) involved are extremely oxygen-sensitive like the nitrogenase of *Klebsiella pneumoniae* ($t_{1/2} = 45\text{ s}$) (Eady *et al.*, 1972).

Fate of the pyridine ring

Because of the failure to obtain extracts degrading pyridine, the evidence for metabolic intermediates is indirect and has depended on the isolation of degradation products and the demonstration where possible of the appropriate enzymes catalysing their further metabolism. In both organisms, the first unequivocally identified products were semialdehydes. The position of these semialdehydes as

Scheme 1. Proposed metabolic pathways for the biodegradation of pyridine by soil bacteria

In both organisms an initial reduction of pyridine produces 1,4-dihydropyridine (I) which, in the case of *Nocardia* Z1 undergoes a hydrolytic N-C-2 ring cleavage and subsequent deamination to glutaric dialdehyde (VII) followed by successive oxidations to glutarate semialdehyde (VIII), glutarate (IX) and glutaryl-CoA (X). In *Bacillus* 4, on the other hand, hydrolysis or a dioxygenase attack at the C-2-C-3 double bond forms *N*-formylaminovinylaldehyde [4-(*N*-formyl)aminobut-3-en-1-ol] (II), which undergoes immediate oxidation to the corresponding semialdehyde, *N*-formylaminovinylacetate [4-(*N*-formyl)aminobut-3-enoic acid] (III). Hydrolysis of the C-6-N bond liberates formamide and succinate semialdehyde (IV), the former of which is attacked by a specific formamide aminohydrolase to produce formate and NH₃, whereas IV is further oxidized to succinate (V) by an equally specific dehydrogenase. Compounds shown in brackets have been detected or shown to be involved in the reactions concerned. Compounds in brackets are hypothetical.

intermediary metabolites was further emphasized by the induction of the specific dehydrogenases after grown on pyridine to activities some 50-fold higher than those found after growth on other unrelated substrates.

Glutarate semialdehyde dehydrogenase and pyridine oxidation were induced in *Nocardia* Z1 by the gratuitous inducer 2-picoline strongly implicating the enzyme as physiologically important in pyridine metabolism in this actinomycete. Succinate semialdehyde dehydrogenase activity was substantially decreased in mutants of *Bacillus* 4 blocked in their ability to grow on or oxidize pyridine, an indication that in this bacillus too, succinate semialdehyde metabolism was linked with that of pyridine oxidation.

The remaining C-atom of the pyridine ring, the other four of which are accounted for in the *Bacillus* 4 pathway by succinate semialdehyde, was found in formate. Radioisotope experiments (Table 7) showed that formate was produced by cells of *Bacillus* 4 from C-2 of the pyridine ring, suggesting that a ring fission between C-2 and C-3 probably occurred. In other known instances, e.g. the metabolism of 2,5-dihydroxypyridine (Behrman & Stanier, 1957; Gauthier & Rittenberg, 1971), 3,4-dihydroxypyridine (Watson *et al.*, 1974) and 1-methyl-4-carboxypyridinium (Orpin *et al.*, 1972; Wright & Cain, 1972), formate derived from the pyridine nucleus invariably originated from an *N*-formyl intermediate produced by a C-2-C-3 ring cleavage. The fate of the *N*-formyl group may differ, however, as a result of subsequent hydrolyses. Usually the N-C-2 bond is hydrolysed (as in the above examples) yielding the amino compound and formate; alternatively (Scheme 1) hydrolysis at C-6-N could result in the production of formamide. Although formamide was not consistently found during the metabolism of pyridine by KCN-poisoned washed cells, its precursor-product relationship with formate and NH_3 (Fig. 3), its rapid oxidation by cell suspensions and the presence in extracts of high amidohydrolase activity specific for formamide strongly suggested the involvement of formamide in the metabolism of *Bacillus* 4.

At present, the metabolic steps between pyridine and the semialdehydes produced from it by *Nocardia* Z1 and *Bacillus* 4 remain speculative. The formation of these fully reduced intermediates was itself surprising because the corresponding unsaturated analogues would be anticipated from a ring cleavage of the aromatic pyridine nucleus. Their appearance suggested that pyridine metabolism involved a reduction step which must occur early on because maleic semialdehyde, the unsaturated analogue of succinate semialdehyde, was neither formed from pyridine nor metabolized by extracts of *Bacillus* 4. Similarly glutaconic dialdehyde, a known product of the chemical ring fission of pyridine (Baumgarten,

1924) was not further metabolized by extracts of either *Nocardia* Z1 or *Bacillus* 4. The unsaturated analogues of glutaric semialdehyde, 4-formylvinylacetate and 4-formylcrotonate are too unstable to test. A reductive step has already been noticed in the metabolism of the analogous nicotinic acid by a *Clostridium*, but this was not unexpected in an anaerobic environment (Tsai *et al.*, 1966).

The principal product of hydrolysis of a dihydropyridine (Shaw, 1937; Barnes, 1960) is glutaric dialdehyde. This compound was oxidized by cell suspensions of *Nocardia* Z1 at rates comparable with that of pyridine itself (Fig. 1*a*) and extracts contained a labile glutaric dialdehyde dehydrogenase, which was inhibited in Tris-HCl buffers and utilized NAD^+ some fivefold faster than NADP^+ (Table 3). The enzyme differed in all these properties from glutaric semialdehyde dehydrogenase as well as in undergoing very little increase in activity during induction of *Nocardia* Z1 in pyridine medium (Fig. 2) so there was little doubt that the two dehydrogenases were distinct.

The production of glutarate from glutarate semialdehyde by the inducible glutarate semialdehyde dehydrogenase suggested that glutarate may be a metabolite of pyridine in *Nocardia* Z1, but after growth on pyridine, glutarate was oxidized rather slowly and only after a lag, which probably reflected the need to induce a transport system for glutarate into the cells for similar permeability problems have been recorded with other carboxylic acids such as acetate (Karlsson & Barker, 1948), tartrate (Shilo & Stanier, 1957), *cis,cis*-muconate (Ornston & Stanier, 1966) and β -carboxymuconate (Meagher *et al.*, 1972) in species of *Pseudomonas*.

Extracts of *Nocardia* Z1 cells were able to convert glutarate into glutaryl-CoA, but the specific activity was rather low (0.16–0.20 $\mu\text{mol/h}$ per mg of protein) and we think it unlikely that glutaryl-CoA synthetase is involved in pyridine metabolism even though similar low activities (0.22 and 0.40 $\mu\text{mol/h}$ per mg of protein) have been recorded in extracts of two pseudomonads grown on glutarate (Nishizuka *et al.*, 1960; Ichihara & Miyaki, 1961; Hoet & Stanier, 1970). The formation of glutaryl-CoA by a thiol transferase, such as occurs in rat liver mitochondria (Besrat *et al.*, 1969) and in other *Nocardia* species (Rann, 1973), may provide the alternative mechanism of glutarate activation. No evidence for further metabolism of glutaryl-CoA was sought, but in *Pseudomonas fluorescens* this proceeds by decarboxylation to crotonyl-CoA and β -oxidation to acetyl-CoA (Numa *et al.*, 1964). An indication that acetyl-CoA units were formed during pyridine metabolism was provided by the much higher isocitrate lyase activities found in extracts of *Nocardia* Z1 grown on pyridine or glutarate as compared with pyruvate or glucose.

Putative early steps in pyridine metabolism; reduction, oxidation or hydrolysis?

Although the similarity in chemical properties of benzene and pyridine is mentioned by many authors (e.g., Mosher, 1950; Barnes, 1960; Palmer, 1967) the N atom of pyridine permits the formation of pyridinium salts. Both pyridine and pyridinium compounds resist electrophilic substitutions especially at C-2, C-4 and C-6 so metabolism of these compounds by hydroxylation would not be expected. Cain *et al.* (1970) found that pyridine and *N*-methylisonicotinate were not hydroxylated by a ferrous-ascorbate-EDTA model hydroxylating system and the biodegradation of *N*-methylisonicotinate by an *Achromobacter* has subsequently been shown not to involve any of the monohydroxy derivatives of the pyridinium compound (Wright & Cain, 1972; Preston & Cain, 1974). Pyridine-grown cells of *Bacillus* 4 did not oxidize any of the three isomeric monohydroxypyridines and *Nocardia* Z1, even though carrying out a non-specific hydroxylation of 3-hydroxypyridine (Houghton & Cain, 1972), oxidized it at a rate only about 10% of that of pyridine. Benzene, the homocyclic analogue of pyridine, is metabolized by a benzene dioxygenase to a peroxide and thence via the dihydrodiol (Axccl & Geary, 1973) to catechol (Gibson *et al.*, 1968). Similar transformations of pyridine would result in the formation of 2,3- or 3,4-dihydroxypyridine, but these diols as well as 2,5-dihydroxypyridine were not metabolized by cell-free extracts or oxidized by cell suspensions of *Nocardia* Z1 (Houghton & Cain, 1972) or *Bacillus* 4. Hydroxylation mechanisms are therefore unlikely.

The complete oxidation of both pyridine and glutarate semialdehyde consumes $5\frac{1}{2}$ mol of O_2 /mol of substrate (eqns. 1 and 2);

Consequently, in purely chemical terms, the conversion of pyridine into glutarate semialdehyde requires no oxidative step and it is possible to write mechanisms for reductive and hydrolytic cleavage of pyridine to glutaric dialdehyde and glutarate semialdehyde. Fig. 1 nevertheless shows that oxidation of glutarate semialdehyde (but not the dialdehyde) consumed approx. 1 mol of O_2 less than that required for the oxidation of the same amount of pyridine, so that this semialdehyde must originate from pyridine by a mechanism involving, or linked to, an oxidative step. In the case of *N*-methylisonicotinate metabolism, where both NADH-utilizing and O_2 -consuming reactions were observed, Wright & Cain (1972) suggested first a reduction of the pyridinium ring with the resulting dihydropyridine compound undergoing an oxygenase cleavage to form successively a

dialdehyde and a semidialdehyde. An analogous reaction can be envisaged in the case of pyridine (Scheme 1). First, there is a chemical precedent for the formation of glutaric dialdehyde from a dihydropyridine (I) (Barnes, 1960); secondly, we must account in both pathways for the formation of the two fully-reduced semialdehydes, which do not arise from their immediate unsaturated precursors; thirdly, the more reduced derivatives 1,2,3,6-tetrahydropyridine and piperidine were not oxidized by pyridine-grown cells (Fig. 1b) so reduction clearly does not proceed beyond a dihydropyridine; and finally the C-2-C-3 ring cleavage indicated by the radioisotope study in the *Bacillus* pathway (Table 7) would involve a double bond of 1,4-dihydropyridine and resemble the oxygenase-like peracetic acid+ozone oxidation of *N*-methyl dihydronicotinamide (Cornforth *et al.*, 1966) or the hydrolytic cleavage of the cyclohexane ring in the reductive metabolism of benzoic acid by *Rhodopseudomonas palustris* (Dutton & Evans, 1969) in both of which a saturated dicarboxylic acid was formed. As C-2 ultimately becomes a carboxyl in both pathways it must be unsubstituted. The necessity for a free C-2 during pyridine metabolism by *Nocardia* Z1 was confirmed by the organism's inability to oxidize 2-picoline or 2-fluoropyridine.

Whether a dialdehyde is produced by a hydrolytic or an oxygenative mechanism from the suspected dihydropyridine (I), the *N*-distal carbonyl group (C-3 of the pyridine ring) must in the case of *Bacillus* 4 undergo immediate oxidation because succinic dialdehyde was not a precursor of succinate semialdehyde in this organism. Dialdehydes containing N such as II (Scheme 1) could have only an extremely short existence as free compounds and they would be unlikely to leave the enzyme surface before undergoing further modification, otherwise they would immediately cyclize to substituted pyrroles (Harries, 1901).

Scheme 1 summarizes the possible steps in the metabolic routes for pyridine oxidation.

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