Biochem. J. (1974) **140**, 277–292
Printed in Great Britain

Microbial Metabolism of the Pyridine Ring

THE METABOLISM OF PYRIDINE-3,4-DIOL (3,4-DIHYDROXYPYRIDINE) BY *AGROBACTERIUM* SP.

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(*Received* 19 *October* 1973)

1. Pyridine-3,4-diol (3,4-dihydroxypyridine, 3-hydroxypyrid-4-one), an intermediate in 4-hydroxypyridine metabolism by an *Agrobacterium* sp (N.C.I.B. 10413), was converted by extracts into 1 mol of pyruvate, 2 mol of formate and 1 mol of NH_3 at pH 7.0. 2. Formate, but not the alternative likely product formamide, was further oxidized fivefold faster by 4-hydroxypyridine-grown washed cells than by similar organisms grown on succinate. 3. The oxidation of pyridine-3,4-diol by crude extracts at pH 8.5 required 1 mol of O_2/mol of substrate, produced 1 mol of acid and led to the formation of formate and a new compound with an extinction maximum of 285 nm (Compound I). This step was believed to be mediated by a new labile dioxygenase ($t_{\frac{1}{2}}$ = 4h at pH 7.0, 4°C) cleaving the pyridine ring between C-2 and C-3. 4. Many of the properties of this pyridine-3,4-diol dioxygenase paralleled those of the extradiol ('meta') oxygenases of aromatic-ring cleavage. The extreme lability of the enzyme has so far precluded extensive purification. 5. Compound I showed changes in the u.v.-absorption spectrum with pH but after acidification it was converted into a new product, 3-formylpyruvate, with an extinction maximum now at 279 nm. 6. Both Compound I and 3-formylpyruvate were metabolized by extracts but at very different rates. The slower rate of metabolism of Compound I was nevertheless consistent with that of pyridine-3,4-diol metabolism. 7. On acidification Compound I released about 0.65 mol of NH_3 and has been identified as 3-formiminopyruvate. 8. 3-Formylpyruvate was hydrolysed to formate and pyruvate (K_m 2 μM) by an acylpyruvate hydrolase active against several other dioxo homologues. The activity of this enzyme was much lower in extracts of succinate-grown cells.

Agrobacterium 35S, isolated from a sample of aerated sewage, was the only organism from many attempted enrichments with 4-hydroxypyridine over a period of 2 years which grew on 4-hydroxypyridine as major C and N source. Pyridine-3,4-diol was identified as a metabolite of 4-hydroxypyridine when it accumulated in cultures under conditions of high pH and restricted aeration (Houghton & Cain, 1972) and was subsequently isolated as the product of the partially purified 4-hydroxypyridine-3-hydroxylase, an FAD-dependent mono-oxygenase (Watson *et al.*, 1974). The present paper reports the further metabolism of pyridine-3,4-diol by this organism. A new pathway for the metabolism of the pyridine ring is described involving a probable oxygenase-catalysed extradiol ('meta') ring fission of pyridine-3,4-diol, followed by hydrolysis and deamination with the eventual formation of NH_3, pyruvate and formate as the end products. Preliminary results have been reported (Houghton *et al.*, 1968).

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Materials and Methods

Organism

The isolation, identification and growth in batch culture of *Agrobacterium* 35S (N.C.I.B. 10413) were described by Houghton & Cain (1972).

Preparation of cell-free extracts

Cell-free extracts and the high-speed supernatant fraction from them were prepared as described by Watson *et al.* (1974), but the crush was extracted with an equal volume of 50mM-potassium phosphate buffer, pH 7.0, containing 30% (v/v) glycerol. Excessive dilution of extracts was avoided because pyridine-3,4-diol dioxygenase activity was rapidly lost in dilute extracts.

Chemicals

4-Hydroxypyridine and the pyridinediols were prepared or purified as described by Houghton & Cain (1972). *N*-Methyl-3-hydroxypyrid-4-one was provided by Dr. H. de Koning, Laboratory for Organic Chemistry, University of Amsterdam, The Netherlands. A very generous gift of mimosine, β-[*N*-(3-hydroxypyrid-4-one)]-α-aminopropionic acid, was donated by Dr. R. D. Court, Commonwealth Scientific and Industrial Research Organisation, St. Lucia, Queensland, Australia. The nomenclature for the monohydroxy- and dihydroxypyridines is that of Houghton & Cain (1972) and Watson *et al.* (1974).

Phosphate and Tris–HCl buffers were prepared as described by Watson *et al.* (1974). Reduced glutathione and dithiothreitol were products of the Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BHT, U.K. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Chemical syntheses

3-*Formylpyruvate.* Ethyl ethoxymethylenepyruvate (1.72g), prepared by the method of Kuhn & Lutz (1963), was dissolved in 100ml of dry diethyl ether and 0.36g of water and 10ml of 2M-methanolic sodium methoxide were added. The mixture was stirred for 2h and the red–brown precipitate extracted into 50ml of water and freeze-dried. The red–brown solid was stored desiccated at −20°C. For the preparation of 3-formylpyruvate, 20mg of this crude solid was dissolved in the minimum volume of water, streaked at the origin of a sheet of Whatman no. 3 paper (57cm×46cm) and run in a solvent system of ethanol–conc. NH_3 (sp.gr. 0.88)–water (20:1:4, by vol.). 3-Formylpyruvate (R_F 0.20), detected under u.v. light was separated from an unidentified compound (R_F 0.60) and large amounts of red–brown material which remained at or near the origin. The 3-formylpyruvate band was eluted into the minimum volume of water and either used immediately or stored at −20°C for no longer than 1 week, after which it was repurified by the above procedure.

A yellow precipitate was formed on the addition of an acidic solution of 2,4-dinitrophenylhydrazine to solutions of 3-formylpyruvate prepared as above but the derivative had a low melting point. (melting began at about 125°C). Srinivasan *et al.* (1956), reported a melting point of 200–201°C for 3-formylpyruvate bis-2,4-dinitrophenylhydrazone. The determination of 3-formylpyruvate in these solutions is described below.

2,4-*Dioxovalerate*, 2,4-*dioxohexanoate and* 2,4-*dioxoheptanoate.* The methyl esters of these 2,4-dioxo acids were prepared from diethyloxalate and either acetone, butan-2-one or pentan-2-one respectively, by the method of Royals (1945). The sodio-derivative of the methyl ester of 2,4-dioxovalerate was precipitated during the reaction and was collected by centrifugation. The corresponding 2,4-dioxohexanoate and 2,4-dioxoheptanoate esters were extracted with benzene and purified by distillation under reduced pressure. The free acids of all three esters were prepared by saponification with 1 or 2 equivalents of NaOH, followed by acidification and extraction into diethyl ether (Lehninger & Witzemann, 1942). The solid residues left after evaporation of the ether were purified by recrystallization from carbon tetrachloride and vacuum sublimation [60–80°C; 1.33kN/m² (10mmHg)]. Some of the properties of these dioxo acids are presented in Table 1.

Enzyme assays

The enzymic activity of extracts and u.v.-absorption spectra were measured with a Unicam SP. 800 spectrophotometer as described by Watson *et al.* (1974). All activities were measured at 30°C.

Pyridine-3,4-diol dioxygenase (*EC* 1.13.11.–). This was normally assayed spectrophotometrically by the decrease in E_{273} in assay mixtures containing in a total volume of 3.0ml: high-speed supernatant extract, 0.1–0.3ml; potassium phosphate buffer, pH7.0, 100μmol; pyridine-3,4-diol, 0.3μmol.

Table 1. *Properties of the* 2,4-*dioxo acids*

n.d., Not determined.

Compound	M.p. (°C)	Reported m.p.* (°C)	M.p. of 2,4-dinitrophenylhydrazone (°C)	$\lambda_{max.}$ (nm)	ε at $\lambda_{max.}$ (litre·mol^{-1}·cm^{-1})	Reaction in thiobarbituric acid test
3-Formylpyruvic acid	n.d.	—	200–201†	279	21500	ε_{549} 82500
2,4-Dioxovaleric acid	98	98	280	295	12200	No reaction
2,4-Dioxohexanoic acid	82–83	83	198–200	296	9200	No reaction
2,4-Dioxoheptanoic acid	56	56	162–163	296	8900	No reaction

* Values reported by Breusch & Keskin (1945).

† Value reported by Srinivasan *et al.* (1956).

O_2 uptake by pyridine-3,4-diol dioxygenase was followed with a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) attached to a Unicam AR.45 recorder. The recorder was calibrated by the addition of excess of solid sodium dithionite to 3ml of air-saturated buffer under which condition the full deflexion of the pen was equivalent to 0.69μmol of O_2.

Catechol 2,3-dioxygenase (metapyrocatechase) (EC 1.13.11.2). This was measured in assay mixtures containing in a total volume of 3.0ml: high-speed supernatant, 0.2–0.5ml; potassium phosphate buffer, pH7.0, 100μmol; catechol, 1μmol. The increase in E_{375} owing to the formation of 2-hydroxymuconic semialdehyde ($\varepsilon = 27000$ litre·mol^{-1}·cm^{-1}) was determined (Kojima *et al.*, 1961).

Acylpyruvate hydrolase (*EC* 3.7.1.–). This was assayed in mixtures containing in a total volume of 3.0ml: high-speed supernatant 0.025–0.10ml; Tris–HCl buffer, pH8.0, 100μmol; the acylpyruvate, 0.2μmol. The rate of decrease of E_{279} (for 3-formylpyruvate) or E_{275} (for the other dioxo acids) was determined.

Determinations

Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin [Sigma (London) Chemical Co. Ltd.] as a standard.

Pyridine-3,4-diol could be measured by its absorption at 273nm, although the theoretical absorbance ($\varepsilon = 10100$ litre·mol^{-1}·cm^{-1}) was decreased by up to 30% in incubation mixtures containing protein. It was therefore determined by a fresh calibration curve made under the precise conditions of the particular assay involved. NH_3 was determined after distillation into 1M-H_2SO_4 in Conway (1957) units by the phenol–hypochlorite method of Russell (1944) or with Nessler's reagent [6.8g of 'Sigma Ammonia Colour Concentrate' in 100ml of 10% (w/v) NaOH]. The E_{420} was measured after 10min against a blank of 1M-H_2SO_4 and the reagent.

Pyruvate was determined enzymically by the oxidation of NADH at 340nm in the presence of lactate dehydrogenase (10μg; Sigma type III, from ox heart) and excess of NADH (Kubowitz & Ott, 1943).

Formate concentrations were measured either by colorimetric methods after steam distillation of the free acid from acidified metabolite solutions or by means of a formate dehydrogenase preparation where acid conditions were to be avoided. Formic acid was steam distilled from acidified pyridine-3,4-diol or 3-formylpyruvate incubation mixtures in a Markham still. Distillate equivalent to 20vol. of the sample was collected, neutralized and concentrated to 2ml. Formate was determined, after reduction to formaldehyde with Mg and HCl, by its colour reaction with chromotropic acid (Grant, 1948) or acetylacetone (Nash, 1953). The exact procedures used were those of Wood & Gest (1957). Tests with known amounts of formate indicated that the recovery of formate by this method was 85–90%.

The need also arose, however, to determine formate in the presence of Compound 1, an acid-labile intermediate of pyridine-3,4-diol metabolism. It was necessary therefore to use a means of formate determination that avoided acid isolation conditions but was nevertheless suitably specific for formate. Johnson *et al.* (1964) described a method with the soluble formate dehydrogenase from *Pseudomonas oxalaticus* grown on oxalate. The original strain of this organism has been lost and in the present investigation formate dehydrogenase was partially purified from methanol-grown *Pseudomonas* AM1 (N.C.I.B. 9133) by the method of Johnson & Quayle (1964) except that their heat treatment was omitted. To determine the amount of formate present in samples of incubation mixtures, 0.5 EC unit of formate dehydrogenase and 0.5μmol of NAD^+ were added to a buffered solution (pH7–8) containing up to 0.2μmol of formate in a final volume of 1.0 or 1.5ml. The reaction was followed to completion by the increase in E_{340} and the low linear NADH oxidase activity evident at the end of this stage was extrapolated back to zero time to obtain the total theoretical increase in extinction.

3-Formylpyruvate was determined by the thiobarbituric acid test of Weissbach & Hurwitz (1959) except that the periodate oxidation was not required. To 0.5ml of test solutions were added 0.5ml of 0.5M-HCl and 2ml of 2-thiobarbituric acid solution (300mg dissolved in the minimum volume of 1M-NaOH, acidified to pH2 with 1M-HCl and made up to 100ml). The mixture was heated at 100°C for 10min and the E_{549} determined.

3-Formylpyruvic acid was not isolated as a pure solid compound, so to determine the concentration of 3-formylpyruvate in its solutions for calibrating the thiobarbiturate assay it was first hydrolysed to formate and pyruvate and the pyruvate estimated enzymically with NADH and lactate dehydrogenase (Kubowitz & Ott, 1943). 3-Formylpyruvate was not a substrate for lactate dehydrogenase. Hydrolysis mixtures containing 5ml of 3-formylpyruvate solution (2–3mM) and 5ml of 1M-NaOH were heated at 100°C. Before and at intervals during hydrolysis, samples were removed and the thiobarbituric acid test was performed. After 2h, 98% hydrolysis to pyruvate had occurred and the mixture was adjusted to pH7.0 with 1M-HCl. A control mixture containing 2mM-sodium pyruvate in 0.5M-NaOH gave a quantitative recovery under these conditions. After three duplicate determinations of the pyruvate formed from the 3-formylpyruvate solutions on test, an average value of ε 82500 litre·mol^{-1}·cm^{-1} (±764 S.E.M.) was

determined for the red pigment formed in the thiobarbituric acid test. A value of ε 81 500 litre·mol^{-1}·cm^{-1} was reported by Kuhn & Lutz (1963) for the crystalline pigment.

Solutions of 3-formylpyruvate showed changes with pH in their u.v.-absorption spectra that were very similar to those reported for other dioxo acids and their esters (Fig. 1). An ε value at 279nm and pH8.0 of 21500 litre·mol^{-1}·cm^{-1} (±603 S.E.M.) was calculated for 3-formylpyruvate from three determinations of the pyruvate formed on alkaline hydrolysis. When the extinction changes were plotted as a function of pH, points were obtained that closely fitted titration curves based on pK_a values of 2.0 and 5.0 for ionization of the carboxyl and enol hydroxyl groups respectively of 3-formylpyruvate.

Determination of protein molecular weights by gel chromatography

The molecular weights of pyridine-3,4-diol dioxygenase and 3-formylpyruvate hydrolase were determined by gel chromatography on Sephadex G-200 as described by Andrews (1965). To assist stabilization when the molecular weight of the labile pyridine-3,4-diol oxygenase was determined, 10% (v/v) glycerol was added to the equilibrating buffer (50mM-Tris–HCl, pH8.0); the usual 30% (v/v) concentration decreased the flow to an unworkably slow rate.

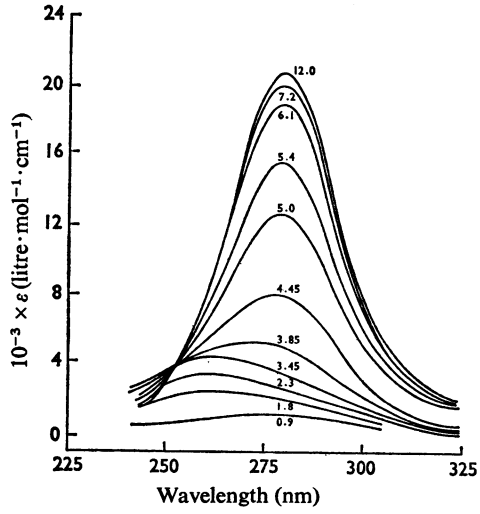


Fig. 1. *U.v.-absorption spectra of* 3*-formylpyruvate*

Solutions of 3-formylpyruvate (0.08mM) were adjusted to the pH values indicated on the curves and the light-absorption spectra recorded.

Formation and identification of pyruvate 2,4*-dinitrophenylhydrazone*

Pyruvate was formed from pyridine-3,4-diol in incubation mixtures containing in a total volume of 60ml: high-speed supernatant (16.1mg of protein); potassium phosphate buffer, pH7.0, 5mmol; pyridine-3,4-diol, 20μmol. The mixture was magnetically stirred at 30°C to obtain good aeration and after 1h a further 15μmol of pyridine-3,4-diol was added. After a further 1h, 5ml of 2,4-dinitrophenylhydrazine (0.4%, w/v, in 2M-HCl) was added, the mixture left overnight and the precipitated protein removed by centrifugation. The mixture was extracted with ethyl acetate (20ml) and the acidic 2,4-dinitrophenylhydrazones re-extracted from the ethyl acetate layer into 10% (w/v) Na_2CO_3 solution. The red–brown carbonate layer was acidified with conc. HCl and the 2,4-dinitrophenylhydrazones were extracted into ethyl acetate (10ml), which was dried with anhydrous $MgSO_4$ and decreased to a small volume. The solution was allowed to evaporate slowly to dryness when sufficient yellow–brown crystals were formed for a melting-point determination. Samples were also chromatographed on Whatman no. 1 paper with authentic pyruvate 2,4-dinitrophenylhydrazone in the following solvents (all proportions by volume): A, butan-1-ol – aq. 0.5M-NH_3 – ethanol (7:2:1); B, propan-2-ol – conc. NH_3 (sp.gr. 0.880) – water (20:1:2). T.l.c. of the dinitrophenylhydrazones was performed on plastic-backed silica-gel sheets (Eastman Kodak Co., Rochester, N.Y., U.S.A.). The solvents used were: hexane–ethyl formate–propionic acid (5:3:1, by vol.); D, light petroleum (b.p. 80–100°C)–ethyl formate–propionic acid (65:35:7.7, by vol.); E, hexane–xylene–acetic acid (5:3:1, by vol.). 2,4-Dinitrophenylhydrazones were detected on chromatographs by their yellow colour or by their dark-brown absorbance under u.v. light.

The identity of pyruvate 2,4-dinitrophenylhydrazone was confirmed by its reduction to alanine with H_2 over platinum black as described by Chamberlain & Dagley (1968), followed by paper chromatography in solvents F, ethanol–conc. NH_3–water (18:1:1, by vol.); G, butan-1-ol–acetic acid–water (12:3:5, by vol.) and H, phenol–water (4:1, v/v). Amino acids were detected with a spray of 0.25% (w/v) ninhydrin in acetone followed by heating at 100°C for 5min.

Manometric methods

O_2 uptake by cell suspensions and cell-free extracts was determined by standard manometric procedures at 30°C (Umbreit *et al.*, 1964). Reaction mixtures contained cell suspension or extract, buffer and substrate in a total volume of 2.8ml with 20% (w/v) KOH (0.2ml) in the centre well. In some experiments extracts were supplemented with an NADH-regenerating system as described by Watson *et al.* (1974).

The dry weight of such cell suspensions was determined by drying to constant weight at 100°C on weighed planchets. CO_2 evolution during the metabolism of 3-formylpyruvate was determined in a N_2 atmosphere obtained by purging the flasks and manometers for 15min with O_2-free N_2.

Results

Experiments with whole cells

Washed suspensions of 4-hydroxypyridine-grown cells rapidly oxidized pyridine-3,4-diol, 3-formylpyruvate and pyruvate with an O_2 uptake of about 3.5, 2.0 and 1.5mol of O_2/mol of substrate (Fig. 2) compared with the theoretical O_2 uptake for their complete oxidation to CO_2 and water of 4.5, 3.0 and 2.5mol respectively. Since the formation of only 85% of the stoicheiometric amount of NH_3 from pyridine-3,4-diol also occurred (Houghton & Cain, 1972) there was evidently some incorporation of metabolites into cell material. Pyridine-3,4-diol was not oxidized at a significant rate by washed suspensions of cells grown on succinate.

When the oxidation of pyridine-3,4-diol was followed in the presence of 5mM-potassium arsenite the rate of O_2 uptake was decreased and the total O_2 uptake decreased to about 2.5mol/mol of pyridine-3,4-diol. The cells were removed by centrifugation, the flask contents examined by formation of 2,4-dinitrophenylhydrazones and their separation by paper chromatography as described in the Materials and Methods section showed that pyruvate was present. The isolated 2,4-dinitrophenylhydrazone formed two spots, R_F 0.50 and 0.70 in solvent A, as did authentic pyruvate 2,4-dinitrophenylhydrazone. No significant amounts of oxo acids were formed in the control flask containing no pyridine-3,4-diol.

Formate was oxidized (4μl of O_2/h per mg dry wt. of cells) by cell suspensions grown on 4-hydroxypyridine with a total O_2 uptake of 0.5mol/mol of formate. Succinate-grown cell suspensions would also oxidize formate but at a decreased rate (0.9μl/h per mg dry wt. of cells). Formamide was not oxidized by either of these cell suspensions.

Experiments with cell-free extracts

End products of pyridine-3,4-diol metabolism by high-speed supernatant extracts. At pH7.0–7.5, the metabolism of pyridine-3,4-diol by cell-free extracts occurred with the loss of the characteristic u.v. spectrum, leaving no u.v.-absorbing products. The metabolic products were identified as pyruvate, formate and NH_3.

Pyruvate was identified by formation of the 2,4-dinitrophenylhydrazone in the large-scale incubation mixture described in the Materials and Methods section. The isolated derivative melted at 213–216°C, whereas authentic pyruvate 2,4-dinitrophenylhydrazone had a melting point of 216°C. The identity of the isolated material was confirmed by paper chromatography and t.l.c. in solvents A–E. The R_F values of the isolated material and pyruvate 2,4-dinitrophenylhydrazone were identical in solvents A (two spots, R_F 0.52, 0.73), B (two spots, R_F 0.45, 0.70), C (R_F 0.73), D (R_F 0.64) and E (R_F 0.20). The isolated material was reduced by catalytic hydrogenation to the corresponding amino acid which was identified as alanine by paper chromatography with authentic alanine in solvents F (R_F 0.28), G (R_F 0.32) and H (R_F 0.61). The absorption spectra of the isolated derivative and pyruvate 2,4-dinitrophenylhydrazone in 10% (w/v) Na_2CO_3 solution ($\lambda_{max.}$ 373nm) and 1M-NaOH ($\lambda_{max.}$ 445nm) were identical. Pyruvate

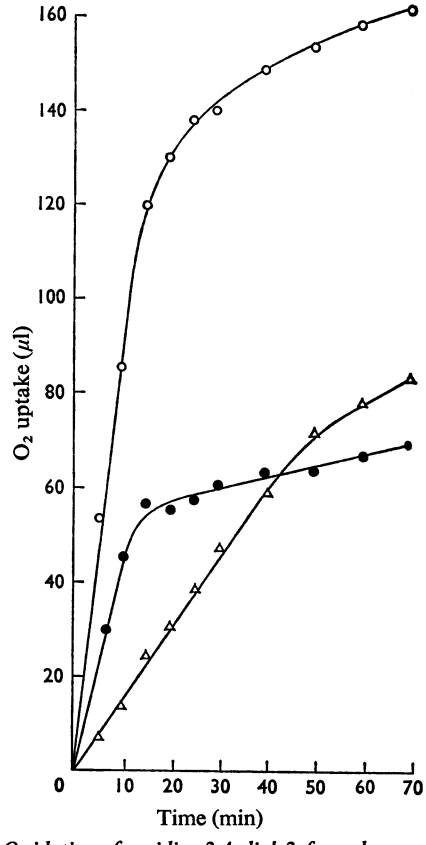


Fig. 2. *Oxidation of pyridine-3,4-diol, 3-formylpyruvate and pyruvate by washed cell suspensions*

Warburg flasks contained: Tris–HCl buffer, pH8.0, 150μmol; washed cells (equiv. to 49mg dry wt.); pyridine-3,4-diol (○), 3-formylpyruvate (△) or pyruvate (●), 2μmol. The results are corrected for the endogenous O_2 uptake of 1.3μl/min.

was estimated in the presence of NADH and lactate dehydrogenase and the results indicated that up to 0.87mol of pyruvate/mol of pyridine-3,4-diol was formed by the high-speed supernatant.

Formate was identified as a metabolic product by two methods. Formic acid could be steam-distilled from reaction mixtures acidified after the metabolism of pyridine-3,4-diol by high-speed supernatant. In a series of determinations with either the chromotropic acid or acetylacetone method for the colorimetric determination of formate, the results indicated that between 1.3 and 1.7mol of formate were formed from 1mol of pyridine-3,4-diol. The results of two such determinations are detailed in Table 2. No colour was produced if the Mg–HCl reduction of formate to formaldehyde was omitted, indicating that the metabolic product was not formaldehyde. When formate production was confirmed by the coupled NADH assay with formate dehydrogenase and NAD^+ (see the Materials and Methods section), the results of several independent determinations indicated that 1.3–1.4mol of formate were formed during the metabolism of 1mol of pyridine-3,4-diol. No NADH formation occurred in the absence of formate dehydrogenase or of pyridine-3,4-diol in this assay procedure.

NH_3 was detected either by Nessler's reagent or by the phenol–hypochlorite method. The results of several determinations showed that about 0.8mol of NH_3/mol of pyridine-3,4-diol was released by the high-speed supernatant but the yield of NH_3 was decreased to 0.15mol/mol of substrate in the presence of crude extracts containing particulate material. No NH_3 was formed by crude extracts in the presence of an NADH-regenerating system.

Fig. 3 shows the production of pyruvate, NH_3 and formate arising from the metabolism of pyridine-3,4-diol by high-speed supernatant extract at pH7.0. Formate was formed at about twice the initial rate and in almost double the amounts of pyruvate and NH_3 which suggested that 2mol of formate and 1mol each of pyruvate and NH_3 arose from the pyridine ring.

Pyridine-3,4-diol dioxygenase. Pyridine-3,4-diol was oxidized by high-speed supernatant extracts in manometric experiments with an O_2 uptake of 1.0mol/mol of substrate (Watson *et al.*, 1974). The O_2 uptake was increased to 1.2–1.3mol/mol in the presence of resuspended particulate material or crude extract and to 1.6mol/mol with crude extract in the presence of an NADH-regenerating system. Examination of the flask contents after the metabolism of pyridine-3,4-diol by extracts showed that the metabolic products had no visible or u.v. absorption.

Rather large amounts of extract (5–10mg of protein/Warburg flask) were required for complete oxidation of pyridine-3,4-diol because the enzyme responsible was inactivated during its action; with small amounts of extract, only part of the added substrate was oxidized. When such extracts were stored at 4°C for more than 12h the ability to oxidize pyridine-3,4-diol was nearly always completely lost. Metabolism of pyridine-3,4-diol did not occur when heat-denatured (100°C for 5min) fresh extracts were tested or when O_2 was excluded by performing the assay under N_2. This result, the stoicheiometry for O_2 and the lack of any nicotinamide nucleotide or other cofactor requirement suggested that pyridine-3,4-diol was probably metabolized by a dioxygenase. Extracts from cells grown on 4-hydroxypyridine had rather variable pyridine-3,4-diol dioxygenase activities of 10–50nmol/min per mg of protein, but no activity was detected in extracts prepared from cells grown on succinate.

Pyridine-3,4-diol dioxygenase activity (10mg of extract protein/ml) was very labile in air with a

Table 2. *Production of formate from pyridine-3,4-diol by extracts of Agrobacterium*

Warburg flasks contained in a total volume of 2.8ml: high-speed supernatant (6.8 or 8.2mg of protein in Expts. 1 and 2 respectively); potassium phosphate buffer, pH7.0, 200μmol; either no substrate, pyridine-3,4-diol (3μmol) or formate (2.5 or 5.0μmol). When O_2 uptake from the metabolism of pyridine-3,4-diol was complete, 0.5M-H_2SO_4 (0.2ml) was added and the precipitated protein removed by centrifugation. The acid reaction mixtures were steam-distilled and formate was estimated by the acetylacetone or chromotropic acid method as described in the Materials and Methods section.

Substrate supplied (μmol)	Formate measured (μmol)	Net formate produced* (μmol)	Formate/pyridine-3,4-diol molar ratio
Expt. 1. (acetylacetone method)			
None	0	—	—
Pyridine-3,4-diol	4.32	4.70	1.60
Expt. 2. (chromotropic acid method)			
None	0.10	—	—
Pyridine-3,4-diol	3.62	4.05	1.35

* Values obtained are corrected for the 92 and 87% recovery obtained with the known amounts of formate in Expts. 1 and 2 respectively.

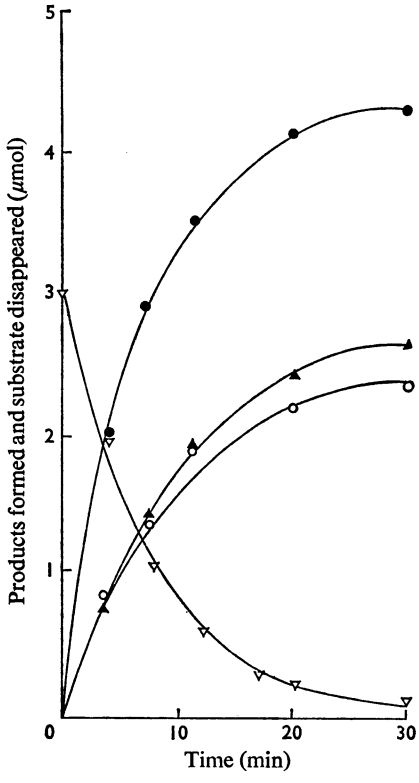


Fig. 3. *Metabolism of pyridine-3,4-diol by high-speed supernatant extracts at pH 7.0*

The incubation mixture contained in a total volume of 30ml: high-speed supernatant (7.0mg of protein); potassium phosphate buffer, pH7.0, 3mmol; pyridine-3,4-diol, 3μmol. At intervals samples (5ml) were removed, added to 0.5M-H_2SO_4 (0.5ml) to stop the reaction and pyridine-3,4-diol (▽), formate (●), pyruvate (▲) and NH_3 (○) were determined. Formate was measured by the formate dehydrogenase method in this experiment.

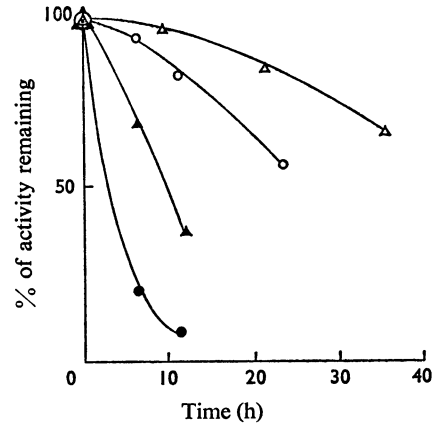


Fig. 4. *Stabilization of pyridine-3,4-diol oxygenase*

High-speed supernatant was prepared in the absence of glycerol and stored at 4°C under the following conditions: (a) no additions (●); (b) with the addition of 30% (v/v) glycerol (▲); (c) saturated with and stored under N_2 (○); (d) with the addition of 30% (v/v) glycerol and also saturated with and stored under N_2 (△). At the times indicated pyridine-3,4-diol oxygenase activity was determined as described in the Materials and Methods section.

half-life ($t_{\frac{1}{2}}$) of 3–4h at pH7.0 and 4°C. Loss of activity occurred even more rapidly in diluted extracts. Oxidizing agents such as potassium ferricyanide (0.5mM) or ammonium persulphate (1mM) rapidly denatured the enzyme which had $t_{\frac{1}{2}}$ of only 16 and 6min respectively with these reagents. Activity was stabilized to some extent by the addition of glycerol and, for this reason, 30% (v/v) glycerol was usually included in the buffer used for preparation of cell-free extracts. Storage of extracts under N_2 resulted in substantial stabilization that was further increased by the inclusion of glycerol (Fig. 4). Other stabilizing agents were either inhibitory or had no effect. Acetone at 10% (v/v), used for the stabilization of other dioxygenases (Nozaki *et al.*, 1963), strongly inhibited pyridine-3,4-diol dioxygenase. Mercaptoethanol (10mM), GSH, dithiothreitol, EDTA, ascorbate or cysteine (all at 1mM) did not stabilize activity. Some stabilization was obtained in the presence of pyridine-3,4-diol (0.1mM) itself but the substrate analogues, mimosine, *N*-methyl-3-hydroxypyrid-4-one and 4-hydroxypyridine caused more rapid inactivation.

Pyridine-3,4-diol dioxygenase activity was completely lost after dialysis for 4h. Fe^{2+} is an essential cofactor for many dioxygenases (Hayaishi, 1966) but no evidence for an Fe^{2+} requirement for pyridine-3,4-diol dioxygenase was obtained. The lost activity of dialysed extracts could not be restored by anaerobic incubation with mercaptoethanol (10mM), ascorbate or dithiothreitol (1mM) or by reduction with $NaBH_4$ (1mg) (Nozaki *et al.*, 1968) in the presence or the absence of Fe^{2+} (10μM). No activity was restored by the addition of any of Na^+, Mg^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+} or Al^{3+} (all at 10μM) to the dialysed preparation. Pyridine-3,4-diol dioxygenase was, however, strongly inhibited by metal-chelating agents, such as 2,2′-dipyridyl and *o*-phenanthroline, and by thiol reagents such as *p*-chloromercuribenzoate.

A wide range of substrate analogues was strongly inhibitory, especially those containing a 3- or a 4-hydroxy or a 3,4-dihydroxy substituent (Table 3). The inhibition by mimosine and *N*-methyl-3-hydroxypyrid-4-one for instance was competitive.

Table 3. *Effect of enzyme inhibitors and substrate analogues on the activity of pyridine-3,4-diol dioxygenase*

Reaction mixtures, which contained in a total volume of 3.0ml: 0.2ml of high-speed supernatant prepared in buffer containing 30% (v/v) glycerol; 100μmol of potassium phosphate buffer, pH7.0; inhibitor as shown, were incubated in an ice bath for 15min. The mixture was then warmed to 30°C, 0.3μmol of pyridine-3,4-diol added and the decrease in E_{273} followed. The reference cuvette contained all components except substrate. Control samples without inhibitor had an activity of 20nmol/min.

Inhibitor added	Concentration (mM)	Activity remaining (% of control value)
(a) Chelating and thiol reagents		
2,2'-Bipyridyl	0.1	20
o-Phenanthroline	0.1	41
EDTA	1	102
8-Hydroxyquinoline	0.1	92
Sodium azide	0.1	73
Iodoacetate	1	70
Iodoacetamide	1	56
p-Chloromercuribenzoate	0.1	22
(b) Substrate analogues		
Mimosine {β-[*N*-(3-hydroxypyrid-4-one)]-α-aminopropionate}	0.1	13
Pyridine-1,4-diol	0.1	22
N-Methyl-3-hydroxypyrid-4-one	0.1	32
4-Hydroxypyridine	0.1	57
3-Hydroxypyridine	0.1	59
Pyridine-2,6-diol	0.1	73
Pyridine-2,4-diol	0.1	78
Pyridine-1,3-diol	0.1	80
2-Hydroxypyridine	0.1	84
Pyridine-2,3-diol	0.1	93
Pyridine-2,5-diol	0.1	96

Only pyridine-3,4-diol of the eight isomeric pyridinediols was oxidized by extracts but mimosine and *N*-methyl-3-hydroxypyrid-4-one were slowly metabolized at 3 and 3.5% respectively of the rate with pyridine-3,4-diol. It is possible, but unlikely, that these rates may be due to slow chemical hydrolysis or to enzymic demethylation by the extracts. The high-speed supernatant extract also contained low catechol 2,3-oxygenase activity (2–3nmol/min per mg of protein) so that the formation of 2-hydroxymuconic semialdehyde could be demonstrated at 375nm (Kojima *et al.*, 1961). The catechol 2,3-oxygenase activity was also very labile but could be stabilized by the incorporation of 10% (v/v) acetone into the extract. It is not yet certain whether a separate enzyme is present or whether pyridine-3,4-diol dioxygenase can also attack catechol slowly; the difference in acetone stabilization suggests that these two dioxygenases are distinct.

A pH optimum of 7.5 was determined for pyridine-3,4-diol dioxygenase activity assayed with the oxygen electrode. The enzyme had an approximate average molecular weight by gel chromatography on Sephadex G-200 of 330000 (mean of three separate experiments with different extract preparations).

Formation of Compound I

When the metabolism of pyridine-3,4-diol by extracts containing 0.2–0.4mg of protein/ml, was followed spectrophotometrically at pH8.5 in Tris–HCl buffer, a new compound with an absorption maximum at 285–286nm was formed. This compound will be referred to as Compound I. At pH8.0 a transient increase in extinction at 285nm occurred and was followed by a decrease; below pH8.0 no 285nm-absorbing material accumulated. That the formation of Compound I was enzymically catalysed and not an artifact caused by the effect of Tris–HCl buffer or alkaline pH was shown by the inability of heat-denatured extracts to cause any change in the spectrum of pyridine-3,4-diol.

To determine whether Compound I was a ring-fission product of pyridine-3,4-diol, the stoicheiometry of O_2 uptake during Compound I formation was examined with the oxygen electrode. The results indicated that 1mol of O_2 was required for the metabolism of 1mol of pyridine-3,4-diol at pH8.5, suggesting that Compound I was a product of the oxygenation of pyridine-3,4-diol. No change occurred when the reaction was run in N_2-flushed buffer under N_2.

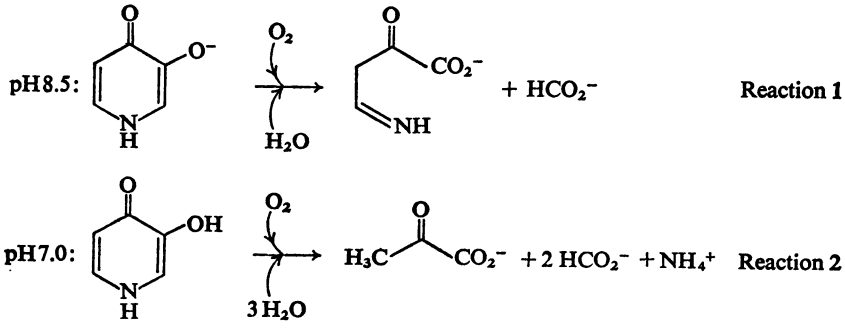
When this reaction was carried out in an automatic titrating unit with unbuffered reaction mixtures containing 32.1mg of extract protein in a total volume of 100ml, pre-adjusted to either pH7.0 or 8.5, the addition of 10μmol of pyridine-3,4-diol (at the appropriate pH) led to the consumption of 20.9 and 10.6μmol of NaOH respectively to maintain these pH conditions during the oxidation. This result showed that only one net acid group was created per mol of pyridine-3,4-diol oxidized under conditions in which Compound I was formed (Reaction 1, Scheme 1). The production of two acid groups/mol of the diol at pH7.0 was consistent with its further oxidation (Reaction 2, Scheme 1) to the recognized end products: 2mol of formate, 1mol of pyruvate and 1mol of NH_3 (i.e. net formation of two acid groups) (cf. Fig. 3).

If the pH of incubation mixtures, in which Compound I had formed at pH8.5, was now readjusted to pH7.0 with 0.1M-HCl, a decrease of the extinction at 285nm occurred as Compound I was metabolized: the final products had no u.v. absorption. These final products were formate, pyruvate and NH_3 just as in experiments performed at pH7.0 throughout. To implicate Compound I as a direct metabolite of pyridine-3,4-diol, however, it was necessary to show that Compound I was metabolized to these end products as rapidly as pyridine-3,4-diol under the appropriate conditions. After the readjustment of reaction mixtures from pH8.5 to pH7.0, the rate of Compound I metabolism was often insufficient to account for the overall rate of pyridine-3,4-diol metabolism to its end products, but it was possible that during accumulation of Compound I from pyridine-3,4-diol at pH8.5 and 30°C, some inactivation of the enzymes responsible for the further metabolism of Compound I may have occurred at this pH. Compound I was therefore allowed to accumulate in incubation mixtures at pH8.5 and the enzyme responsible for its formation destroyed by heat-denaturation at 100°C for 5 min. The incubation mixtures were then adjusted to one of a range of pH values and further amounts of freshly prepared high-speed supernatant extract added. Under these conditions a pH optimum of 7.2 was determined for the subsequent disappearance of Compound I (measured spectrophotometrically at 285nm) (Fig. 5) and at this pH value the rate of disappearance of this compound was commensurate with the overall rate of pyridine-3,4-diol oxidation. Compound I thus fulfilled the criterion of an obligatory intermediate in pyridine-3,4-diol metabolism.

The enzymes responsible for the formation and metabolism of Compound I could also be partially distinguished by heat-treatment of high-speed supernatant extracts at 50°C for 10min. After this treatment a slow formation of Compound I from pyridine-3,4-diol was found even at pH7 but considerable inactivation of the pyridine-3,4-diol dioxygenase activity also occurred and it was not possible to use this technique either for purification of pyridine-3,4-diol oxygenase or for studies on the accumulation of Compound I. Attempts to separate the dioxygenase from other proteins by $(NH_4)_2SO_4$ fractionation, adsorption on calcium phosphate gel or molecular sieving on Sephadex G-200 have so far been unsuccessful in the absence of a convenient stabilization procedure. The glycerol-containing buffers used in protecting the enzyme preclude these standard methods.

Properties of Compound I

When solutions of Compound I ($\lambda_{max.}$ 285nm at pH8.5) were acidified, a new light-absorption maximum was found. At pH3–4 the spectrum showed a broad absorption maximum at 250–260nm; a further decrease to pH1 gave a further decrease in the extinction at 250–260nm. Gradual addition of 1M-NaOH to this acidified solution resulted in a shift of the absorption maximum from 260 to 279nm between pH4 and 5. The extinction at this wavelength



Scheme 1. *Metabolism of pyridine-3,4-diol by extracts at pH 8.5 and 7.0 yielding 1 and 2 net equivalents of acid respectively*

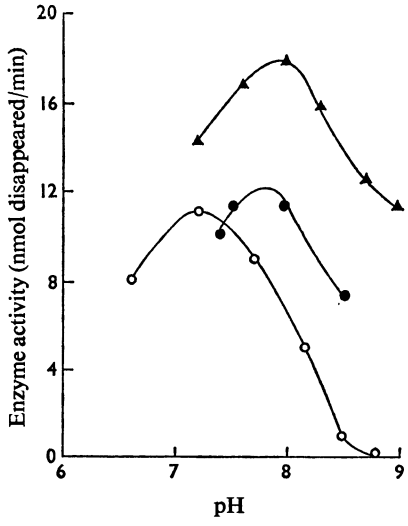


Fig. 5. *Effect of pH on the metabolism of Compound I and its acid-breakdown product*

(*a*) Compound I was allowed to form in an incubation mixture containing in a total volume of 30ml: high-speed supernatant (7.2mg of protein); Tris–HCl buffer, pH8.5, 1.5mmol; pyridine-3,4-diol, 3μmol. The incubation mixture was heated at 100°C for 5min, cooled and samples (5ml) were adjusted to various pH values with 0.1M-HCl. The subsequent metabolism of Compound I (○) was then followed in cuvettes containing in a total volume of 3.0ml: solution of Compound I from above, 2.0ml (containing approx. 0.15μmol of Compound I); Tris–HCl buffer of the required pH, 50μmol; high-speed supernatant (0.36mg of protein). (*b*) Compound I was allowed to form in a similar incubation mixture containing high-speed supernatant extract (6.4mg of protein), but the incubation mixture was then acidified to pH2 with 1M-HCl and, after 30min, samples (5ml) were readjusted to various pH values with 1M-NaOH. The metabolism of the acid-breakdown product (Compound II; 3-formylpyruvate) (●) was followed in cuvettes containing in a total volume of 3.0ml: acid-treated Compound I solution, 2.0ml; Tris–HCl buffer of the required pH, 50μmol; high-speed supernatant (0.08mg of protein). In both experiments samples of a control mixture (containing no pyridine-3,4-diol) were taken through all the procedures and used in the reference cuvettes. (*c*) The pH versus activity profile for authentic 3-formylpyruvate (▲) is shown for comparison. This was assayed as described in the Materials and Methods section with samples of high-speed supernatant extract containing 0.4mg of protein.

continued to increase up to pH8, above which both $\lambda_{max.}$ and E were constant to pH13 (cf. Fig. 1). The addition of extract protein to solutions of acid-treated and reneutralized Compound I did not alter the new absorption maximum at 279nm back to the original 285nm. The spectrum of incubation mixtures containing Compound I at pH8.5 was not altered on heat treatment (100°C for 5min) nor by addition of 1M-NaOH to pH13 followed by readjustment to pH8.5.

These results indicated that acidification of Compound I formed a new product, Compound II, with a distinct light-absorption spectrum. This material was also metabolized by fresh high-speed supernatant extracts with a complete loss of the E_{279}. The pH optimum for this reaction was 7.8 but the rate at pH8.5 was still about 50% of the maximum value, whereas the metabolism of Compound I itself was virtually nil at this pH (Fig. 5). At its pH optimum, 7.2, Compound I was degraded at 30nmol/min per mg of extract protein. Its acid-breakdown product Compound II not only showed a different pH optimum (7.8) for its subsequent metabolism, but at that pH was degraded at 140nmol/min per mg of extract protein. These differences are further confirmation that Compounds I and II were distinct entities.

Identity of Compound II with 3-formylpyruvate

The u.v. absorption spectra of Compound II over a wide range of pH conditions were identical with those of 3-formylpyruvate (Fig. 1). Addition of acidic $FeCl_3$ solution (which contained sufficient HCl to convert any Compound I into Compound II) to reaction mixtures containing Compound I resulted in an orange–brown colour whereas pyridine-3,4-diol produced a violet colour before accumulation of Compound I had occurred. Under these conditions 3-formylpyruvate also formed an orange–brown colour. The formation of 3-formylpyruvate on acidification of Compound I was further suggested by the identity of the bis-2,4-dinitrophenylhydrazone with that of 3-formylpyruvate on paper chromatography in solvent A and was confirmed by the positive results of the thiobarbituric acid test on reaction mixtures in which the oxidation of pyridine-3,4-diol at pH8.5 had occurred (see Fig. 7). Under the acid conditions of the test, conversion of Compound I into Compound II would occur so the formation of Compound I at pH8.5 in these experiments resulted in a simultaneous appearance of a positive reaction in the thiobarbituric acid test. The light-absorption spectrum of the red pigment produced in the test by both authentic 3-formylpyruvate and acid-treated Compound I was identical, with an absorption maximum at 549nm. The thiobarbituric acid test is specific for compounds containing the R–CO–CH_2–CHO group but an absorption maximum at 549nm is characteristic of 3-formylpyruvate (R = CO_2H) (Weissbach & Hurwitz, 1959). Malonic dialdehyde (R = H) also forms a pink colour but with an absorption maximum at 532nm (Waravdekar & Saslaw, 1957). No colour was formed in control

incubations containing no substrate or with heat-denatured extracts.

After readjustment of incubation mixtures containing Compound I from pH8.5 back to pH7.0, the further metabolism of Compound I was paralleled by an equivalent decrease in the amount of material reacting in the thiobarbituric acid test (Fig. 6). Metabolism of pyridine-3,4-diol at pH7.5 (where no increase in E_{285} could be detected) resulted in a transient accumulation of material reacting in the thiobarbituric acid test, so some formation of Compound I (or 3-formylpyruvate) therefore occurred although it was later further metabolized (Fig. 6).

If stoicheiometric conversion of Compound I into 3-formylpyruvate by the acid treatment was assumed, the extinction obtained in the thiobarbituric acid test could be used to measure the amount of Compound I formed from pyridine-3,4-diol. The results of several determinations indicated that up to 0.75mol of Compound I was formed from 1mol of pyridine-3,4-diol at pH8.5. From this result and the extinction values obtained when pyridine-3,4-diol was converted into Compound I at pH8.5, an approximate value of $\varepsilon = 18000$ litre·mol^{-1}·cm^{-1} at 285nm may be calculated for Compound I.

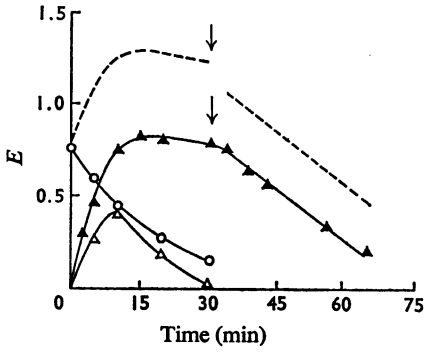


Fig. 6. *Formation of a compound reacting in the thiobarbituric acid test during the metabolism of pyridine-3,4-diol at pH* 8.5 and 7.5

The two incubation mixtures contained in a total volume of 30ml: high-speed supernatant (10.2mg of protein); Tris–HCl buffer, pH8.5 or 7.5, 1.5mmol; pyridine-3,4-diol, 3μmol. The increase of E_{285} (due to Compound I) at pH8.5 (----) and the decrease of E_{275} (due to pyridine-3,4-diol) (○) at pH7.5 were recorded against a control mixture containing no pyridine-3,4-diol. At regular intervals samples (0.5ml) were removed from each incubation mixture, added to 0.5M-HCl and heated with thiobarbituric acid solution as described in the Materials and Methods section. The E_{549} of each sample from the incubations at pH8.5 (▲) and at pH7.5 (△) was determined. At the time indicated by the arrow the incubation mixture at pH8.5 was carefully adjusted to pH7.0 with 1M-HCl and measurements were continued.

Structure of Compound I

After identification of 3-formylpyruvate as the compound formed after acidification of Compound I, it was possible to make a tentative identification of the structure of Compound I itself. The structure was deduced from analyses of the amounts of formate, NH_3 and pyruvate formed during the production of Compound I from pyridine-3,4-diol at pH8.5 and the products released after acidification or during further metabolism of Compound I.

During the formation of Compound I at pH8.5 only small amounts of pyruvate and NH_3 were formed, indicating that Compound I retained the pyridine-N. In a typical experiment 4.4μmol of Compound I was formed from 6μmol of pyridine-3,4-diol (Fig. 7); the small quantities of pyruvate (0.75μmol) and NH_3 (0.75μmol) formed in this experiment represented the complete metabolism of the remaining pyridine-3,4-diol and together all these products accounted for utilization of 87% of the substrate. During the metabolism of pyridine-3,4-diol at pH8.5, amounts of formate approaching 1mol/mol of substrate were formed, whereas at pH7.0 up to 2mol of formate/mol of substrate appeared. The initial rate of formate production, although rather less than that of Compound I, was very much higher than the rate of pyruvate or NH_3 production. This formation of Compound I and formate in the ratio of 1:1 from pyridine-3,4-diol (Fig. 7) confirmed that Compound I contained four C atoms.

The acidification of 4.4μmol of Compound I resulted in its conversion into the C_4 compound 3-formylpyruvate (see the previous section) and in the release of 2.8μmol of NH_3, a yield of 65% from the available Compound I; no further formate was released on acidification (Fig. 7). Additional formate was formed, however, during the subsequent metabolism of Compound I after the incubation mixture had been readjusted to pH7.0. Metabolism of Compound I (3μmol) under these circumstances resulted in the production of 2.8μmol of NH_3, 2.4μmol of pyruvate and 1.7μmol of formate. The low yields of formate in some of these experiments were possibly due to further oxidation of formate by contaminating particulate material in the high-speed supernatant or to some solubilization of the particulate formate dehydrogenase in this organism. The results suggested a likely true proportion of 1:1:1 for pyruvate, formate and NH_3 production during the metabolism of Compound I as found for pyridine-3,4-diol itself and were consistent with the proposed structure of Compound I as 3-formiminopyruvate.

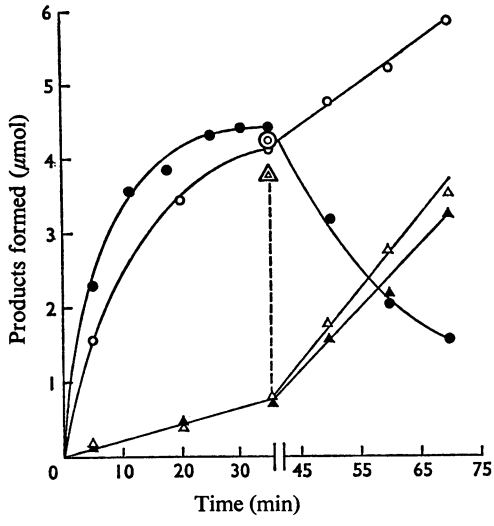


Fig. 7. *Time-course of pyruvate, formate and NH_3 production during the formation, acidification and further metabolism of Compound I*

The incubation mixture contained in a total volume of 60ml: high-speed supernatant (9.6mg of protein); Tris–HCl buffer, pH8.5, 3mmol; pyridine-3,4-diol, 6μmol. At the times indicated samples (6ml) were added to 0.5M-NaOH to stop the reaction. After 35min 1M-HCl was carefully added to the incubation mixture to decrease the pH to 7.0 and at this time an additional sample (6ml) was acidified to pH2 with 1M-HCl (0.5ml). As the reaction proceeded at pH7.0 further samples (6ml) were added to 0.5M-NaOH. Compound I (●) was determined by the thiobarbituric acid test; pyruvate (▲) with NADH and lactate dehydrogenase; NH_3 (△) by the phenol–hypochlorite method after microdistillation; formate (○) with NAD^+ and formate dehydrogenase. The amounts of formate (◎) and NH_3 (◬) formed after acidification are also shown. Control estimations, especially important for NH_3, were carried out on samples from a control mixture containing no pyridine-3,4-diol. After adjustment to pH7.0 at 35min, the appearance of pyruvate, NH_3 and formate were not, in this experiment, followed to completion, but only sufficiently to demonstrate their subsequent production from Compound I. Experiments in which the final yields of these products from Compound I were established are described in the text.

Metabolism of 3-formylpyruvate

The compound produced from 3-formiminopyruvate by acid hydrolysis has been identified as 3-formylpyruvate. This compound was rapidly metabolized by high-speed supernatant extracts at pH7.0 (Fig. 5), as indicated by a progressive and complete loss of the E_{279} of freshly prepared 3-formylpyruvate solutions and the simultaneous loss of the reaction of 3-formylpyruvate in the thiobarbituric acid test. An acylpyruvate hydrolase with specific activities of 40–100nmol/min per mg of protein was found in extracts produced from cells grown on 4-hydroxypyridine, but extracts of cells grown on succinate contained very low activities of about 2nmol/min per mg of protein. A slow non-enzymic hydrolysis of 3-formylpyruvate occurred in assay mixtures containing heat-denatured extract and the results of enzymic determinations were corrected for this activity.

No O_2 uptake occurred during the metabolism of 3-formylpyruvate by high-speed supernatant although the thiobarbituric acid test indicated that the 3-formylpyruvate had been metabolized. An O_2 uptake approaching 0.5mol/mol of 3-formylpyruvate did occur, however, with crude extracts containing particulate material, indicating that a product was further metabolized by particulate material: this product was formate. In manometric experiments under an atmosphere of N_2, no CO_2 evolution occurred during the action of high-speed supernatants on 3-formylpyruvate even though its complete utilization was again effected under these conditions. Oxaloacetate, a possible metabolite of 3-formylpyruvate, was not decarboxylated to pyruvate (a known end product of pyridine-3,4-diol metabolism) by active extracts under N_2 at rates in excess of its non-enzymic decomposition.

The products of 3-formylpyruvate metabolism were identified as pyruvate and formate. Pyruvate was identified by the formation, extraction and chromatography of its 2,4-dinitrophenylhydrazone derivative as described in the Materials and Methods section. A derivative was formed after metabolism of 3-formylpyruvate that formed two spots in solvent A (R_F 0.52 and 0.70) as did authentic pyruvate 2,4-dinitrophenylhydrazone (R_F 0.50 and 0.70). No oxaloacetate 2,4-dinitrophenylhydrazone (R_F 0.27 in solvent A) was detected during or after metabolism of 3-formylpyruvate. 3-Formylpyruvate itself formed a bis-2,4-dinitrophenylhydrazone that had the distinct R_F values of 0.38 and 0.56 in solvent A. Considerable amounts of the 3-formylpyruvate derivative also remained at the origin in this solvent, indicating the likely presence of polymeric material that would account for the low melting point (see the Materials and Methods section). Nearly stoicheiometric amounts of pyruvate were formed from 3-formylpyruvate by high-speed supernatant extracts (Table 4).

The colorimetric estimation of formate resulting from the metabolism of 3-formylpyruvate by high-speed supernatant extracts showed that about 0.8mol of formate/mol of 3-formylpyruvate was formed. The production of formate was confirmed by the addition of NAD^+ and formate dehydrogenase to assay mixtures containing 3-formylpyruvate and high-speed supernatant, when the formation of about

Table 4. *Formation of pyruvate from 2,4-dioxo acids by extracts of 4-hydroxypyridine-grown Agrobacterium*

The metabolism of 3-formylpyruvate was followed by the change in E_{279} in mixtures containing in a total volume of 3.0ml: high-speed supernatant (1.2mg of protein); Tris–HCl buffer, pH 8.0, 100μmol; 3-formylpyruvate, 0–0.37μmol. At the end of the reaction the mixture was heated at 100°C for 5min, cooled and lactate dehydrogenase (10μg) and NADH (0.6μmol) were added. Pyruvate was determined from the total decrease in E_{340}. The formation of pyruvate from the other dioxo acids was determined in similar assay mixtures but containing high-speed supernatant with 0.45mg of protein.

Substrate supplied	(μmol)	Pyruvate formed (μmol)	Yield (% of theoretical value)
Formylpyruvate	0	0	—
	0.93	0.087	94
	0.185	0.165	89
	0.280	0.270	97
	0.370	0.330	90
2,4-Dioxovalerate	0.25	0.246	98
	0.50	0.510	102
2,4-Dioxohexanoate	0.125	0.125	100
2,4-Dioxoheptanoate	0.125	0.121	97

70% of the stoicheiometric amount of NADH occurred.

Properties of the acylpyruvate hydrolase

When measured in high-speed supernatant extracts without further purification, the acylpyruvate hydrolase activity was dependent on protein concentration; the pH optimum in Tris–HCl buffer with 3-formylpyruvate as substrate was pH8.0. The pH–activity profile was similar to that found for the metabolism of the product formed by acidification of Compound I (Fig. 5), a further indication that this product was 3-formylpyruvate. The acylpyruvate hydrolase was fairly heat-labile ($t_{\frac{1}{2}}$ 3min at 55°C in Tris–HCl buffer, pH8.0).

The activity in the extracts was not specific for 3-formylpyruvate but would hydrolyse other acylpyruvates with the formation of pyruvate and the corresponding fatty acid (Table 4). The enzyme was most active with 2,4-dioxovalerate though the apparent K_m value was lowest with the presumed natural substrate in this organism, 3-formylpyruvate (Table 5).

The hydrolysis of 3-formylpyruvate was irreversible; no increase in E_{279} occurred in reaction mixtures containing formate, pyruvate and high-speed supernatant extracts in a variety of concentrations and pH values. Enzyme activity was increased 62% by CN^- (0.1mM) and 32% by EDTA (1mM) probably by the removal of some metal contaminant in the extract or buffer because Zn^{2+}, Ni^{2+} and particularly Cu^{2+} inhibited the enzyme (22, 39 and 68% respectively) at 0.1mM. The average molecular weight of the acylpyruvate hydrolase, assayed with 3-formylpyruvate, was determined by gel chromatography on Sephadex G-200. The mean value obtained from three separate extract preparations was 72000 (±5000).

Table 5. *Kinetic properties of the acylpyruvate hydrolase*

Assay mixtures contained in a total volume of 3.0ml: high-speed supernatant (0.45mg of protein); Tris–HCl buffer, pH8.0, 100μmol; substrate, 0.1μmol. The reaction was followed by decrease in E_{279} with 3-formylpyruvate or E_{295} with the other dioxo acids. $V_{max.}$ was corrected for a slow decrease of absorbance in the absence of extract.

Substrate	$V_{max.}$ (nmol/min)	K_m (μM)
3-Formylpyruvate	39	2
2,4-Dioxovalerate	60	11
2,4-Dioxohexanoate	18	7
2,4-Dioxoheptanoate	18	7

Metabolism of formate by cell-free extracts

Formate was oxidized (3μl/h per mg of protein) by crude extracts or by the particulate material derived from them, but not by high-speed supernatant extracts. Formamide was not oxidized by crude extracts, by the particle fraction or by the high-speed supernatant. No $NAD(P)^+$-dependent soluble formate dehydrogenase could be detected in extracts.

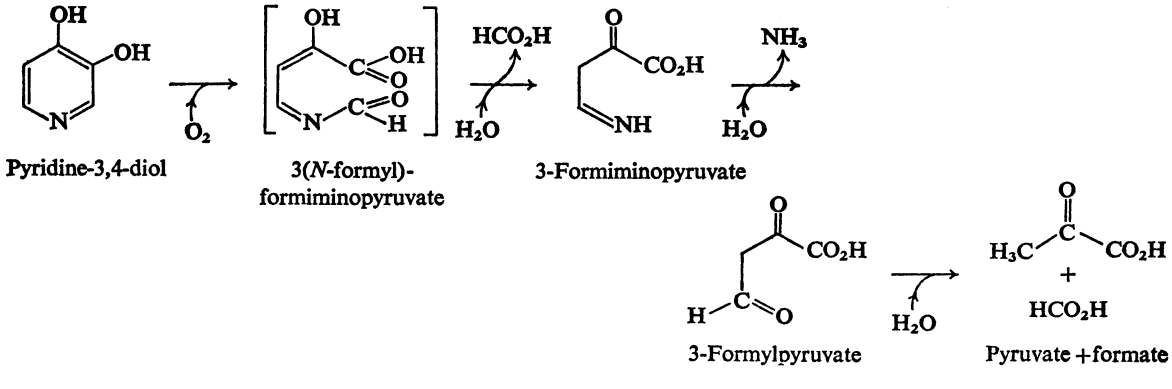
Discussion

The metabolic pathway proposed for the metabolism of 4-hydroxypyridine by *Agrobacterium* 35S as the result of these studies is shown in Scheme 2. The position of pyridine-3,4-diol as an intermediary metabolite was first established by Houghton & Cain (1972) with intact cells and confirmed by isolating

pyridine-3,4-diol as the product of 4-hydroxypyridine-3-hydroxylase (Watson *et al.*, 1974). The lack of a requirement for a nicotinamide nucleotide or other cofactor and the absolute O_2 requirement of the enzyme responsible for the further metabolism of pyridine-3,4-diol suggested that it was a dioxygenase. This dioxygenase was very labile but could be stabilized to some extent by glycerol or by storage under anaerobic conditions. In this respect the enzyme is rather similar to catechol 2,3-dioxygenase (metapyrocatechase) (Kojima *et al.*, 1961), which could be stabilized in the presence of 10% (v/v) acetone in the working buffers (Nozaki *et al.*, 1963). In the presence of oxygen, Fe^{2+} at the active site is oxidized to Fe^{3+} and lost from the enzyme (Nozaki *et al.*, 1968). The mechanism of stabilization by organic solvents is unknown but Nozaki *et al.* (1968) discovered that acetone was a competitive inhibitor of catechol 2,3-dioxygenase as well as a stabilizer, indicating that it probably had a specific effect at the active site. Acetone was also an inhibitor of pyridine-3,4-diol dioxygenase although it did not in this case act as a stabilizer. It is noteworthy that extracts also contained low catechol 2,3-dioxygenase activity that was stabilized by acetone. It is not yet known whether this activity is due to pyridine-3,4-diol dioxygenase or to a separate enzyme, possibly induced by impurities in the rather crude 4-hydroxypyridine used as growth substrate. The best known heterocyclic cleaving oxygenase is pyridine-2,5-diol dioxygenase, which has been studied in *Pseudomonas putida* grown on nicotinic acid (Behrman & Stanier, 1957; Gauthier & Rittenberg, 1971*a*,*b*) in two *Achromobacter* strains grown on 2- and 3-hydroxypyridine respectively (Cain *et al.*, 1974) and in a Gram-negative organism (Orpin *et al.*, 1972) grown on picolinamide. This dioxygenase in each of these bacteria, requires Fe^{2+} and a thiol donor such as dithiothreitol, cysteine or GSH for activity. No evidence of a metal requirement for pyridine-3,4-diol dioxygenase has so far been discovered and anaerobic incubation in the presence of Fe^{2+} and a wide variety of thiol reagents did not restore lost activity. The lability of the enzyme has made its purification difficult and it is likely that a metal requirement may not become apparent until some purification has been effected. Although significant inhibition of pyridine-3,4-diol dioxygenase was obtained with the iron-chelating agents *o*-phenanthroline and 2,2′-dipyridyl, Nozaki *et al.* (1968) have shown with catechol 2,3-dioxygenase that other nitrogen bases such as *m*-phenanthroline and quinoline, which are very poor metal chelators, were even more inhibitory, so that *o*-phenanthroline and 2,2′-dipyridyl may inhibit other than as metal-chelating agents.

The pyridine-3,4-diol dioxygenase activity described here and the pyridine-2,5-diol dioxygenase reported by Cain *et al.* (1974) were very specific and would not oxygenate any of the other isomeric pyridinediols tested. A wide range of hydroxylated pyridines, especially those with a 3,4-dihydroxy substituted ring, were, however, inhibitory to the pyridine-3,4-diol dioxygenase. The molecular weight of this dioxygenase was 330000, similar to the reported values of 300000 (Houghton *et al.*, 1968) and 242000 (Gauthier & Rittenberg, 1971*a*) for pyridine-2,5-diol dioxygenase.

The position of ring cleavage in an *o*-dihydroxy-aromatic compound is affected by one of two types of oxygenase. The rapid aerobic inactivation, but stabilization with glycerol or under anaerobic conditions, is characteristic of extradiol-type ('meta') dioxygenases. Further, pyruvate and formate, typical end products of extradiol cleavage of aromatic compounds (Dagley *et al.*, 1964), were formed after metabolism of pyridine-3,4-diol by high-speed supernatant extracts. These results, together with the establishment of the site of ring cleavage (see below),



Scheme 2. *Metabolic pathway for the biodegradation of pyridine-3,4-diol*

clearly establish pyridine-3,4-diol dioxygenase as an extradiol ('meta') dioxygenase.

The identity of Compound I with 3-formiminopyruvate was suggested by the metabolism of pyridine 3,4-diol at pH8.5 to formate and Compound I in virtually equivalent amounts whereas acidification of Compound I resulted in the release of NH_3 and the formation of 3-formylpyruvate. Imines, like Compound I, which are not further substituted with alkyl or aryl groups on the N atom have not been described in the literature except as postulated transient intermediates during chemical syntheses. Their acid hydrolysis to NH_3 and the corresponding aldehyde is well known (Smith, 1965). Indeed, the stability of Compound I under neutral and alkaline conditions and to heat is rather surprising and may be due to the stabilizing effect of the conjugated enol group in the ionized tautomer. The identification of such a C_4 intermediate immediately restricts the possible sites of ring fission of pyridine-3,4-diol to the bond between C-2 and C-3 or that between C-5 and C-6. The fission of an aromatic ring between unsubstituted atoms is extremely rare and it is difficult to predict the formation of an intermediate that could form 3-formylpyruvate by acid-treatment after a ring fission between C-5 and C-6 of the pyridine nucleus. After ring fission between C-2 and C-3, however, the expected product of a dioxygenase attack would be 3-(*N*-formyl)-formiminopyruvate from which hydrolysis would release the *N*-formyl group as formate and result in the formation of Compound I (3-formiminopyruvate); these were the two products observed (Fig. 7). We have not attempted to demonstrate the formation of 3-(*N*-formyl)formiminopyruvate, the postulated product of pyridine-3,4-diol dioxygenase, which might be expected as the product only of the purified enzyme. A similar *N*-formyl compound, *N*-formylmaleamic acid, was initially thought to be the product of pyridine-2,5-diol dioxygenase (Behrman & Stanier, 1957), but more recent studies with the crystalline dioxygenase have shown that the products were in fact maleamate and formate and that synthetic *N*-formylmaleamate was not a substrate of pyridine-2,5-diol dioxygenase and could not be detected during the reaction (Gauthier & Rittenberg, 1971*b*). Hydrolysis must therefore occur at the oxygenase active site. The possibility of an enzymic hydrolysis of 3-(*N*-formyl)formiminopyruvate to formamide and 3-formylpyruvate during the further metabolism of pyridine-3,4-diol was excluded by the identification of Compound I as an *N*-containing intermediate (Fig. 7) and also by the demonstration that both extracts and whole cells were unable to oxidize formamide whereas they did oxidize formate.

The tautomeric forms of the intermediates in Scheme 2 require some discussion. Pyridine-3,4-diol probably exists mainly as the 3-hydroxypyrid-4-one form in neutral solution; its u.v. spectrum, for instance, is very similar to that of *N*-methyl-3-hydroxypyrid-4-one which must exist in this form. The pK_a of 8.6 for ionization of the hydroxyl group also corresponds closely to the pK_a for 3-hydroxypyridine (8.7) but not 4-hydroxypyridine (11.1) (Albert & Phillips, 1956), an indication that ionization of the -OH group at C-3 occurs. 3-Formylpyruvate and 3-formiminopyruvate are shown in Scheme 2 in the oxo form; the ionized enol form, however, has been directly demonstrated in the case of 3-formylpyruvate by determination of the pK_a for ionization of the hydroxyl group; the very similar absorption spectra of Compound I and 3-formylpyruvate suggests that the former compound also exists normally in the ionized enol form. The conjugate system of the hypothetical 3-(*N*-formyl)-formiminopyruvate extends over six atoms and is very similar to the electron distribution of 2-hydroxymuconic semialdehyde, the product of catechol 2,3-oxygenase; it might be expected therefore that 3-(*N*-formyl)formiminopyruvate would have an absorption maximum similar to 2-hydroxymuconic semialdehyde around 375nm.

The one gap in our postulated pathway is the demonstration of the enzymically catalysed conversion of pyridine-3,4-diol or Compound I into 3-formylpyruvate. 3-Formylpyruvate was, however, rapidly metabolized by extracts of *Agrobacterium* 35S when grown on 4-hydroxypyridine, whereas extracts of cells grown on succinate would hydrolyse 3-formylpyruvate to formate and pyruvate only at a very decreased rate. An alternative route for 3-formylpyruvate metabolism might involve its oxidation to oxaloacetate and decarboxylation of this oxo acid to pyruvate, but this mechanism was excluded when it was found that the unsupplemented high-speed supernatant would metabolize 3-formylpyruvate anaerobically with no CO_2 evolution, but was unable to decarboxylate oxaloacetate to pyruvate or produce formate under the same conditions.

The metabolism of similar 2,4-dioxo acids to pyruvate and the corresponding fatty acid by extracts of liver and kidney from a wide range of animals was reported by Meister & Greenstein (1948). The K_m value of these extracts for 2,4-dioxovalerate was extremely high (about 10mM) but the acylpyruvate hydrolase activity of *Agrobacterium* 35S had an affinity for 3-formylpyruvate and the other dioxo acids some three orders of magnitude lower, a feature which would explain our failure to detect 3-formylpyruvate in incubation mixtures and substantiate the specific function of this enzyme in pyridine-3,4-diol degradation.

This pathway of 4-hydroxypyridine metabolism is probably of somewhat restricted distribution in Nature. *Agrobacterium* 35S was isolated only after a very extensive search of many soil, water and sewage samples from both urban and rural sources (Houghton

& Cain, 1972). 4-Hydroxypyridine itself has not been reported to occur naturally but its halogenated derivatives such as pyrichlor (2,3,5-trichloro-4-hydroxypyridine) are added to the environment as herbicides (Clapham *et al.*, 1967) and the pyridine-3,4-diol moiety is found naturally in the mimosine molecule (Marion, 1950). It is not surprising therefore that a mechanism for its biodegradation has evolved.

This work was supported by grants from the Science Research Council (B/SR/2440) and the Royal Society to R. B. C. which are gratefully acknowledged.

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