Microbial Metabolism of Pyridinium Compounds

METABOLISM OF 4-CARBOXY-1-METHYLPYRIDINIUM CHLORIDE, A PHOTOLYTIC PRODUCT OF PARAQUAT

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1. A bacterium, *Achromobacter* D, isolated from garden soil by elective culture, utilized *N*-methylisonicotinic acid (4-carboxy-1-methylpyridinium chloride) as sole carbon source. 2. Extracts of *N*-methylisonicotinate-grown cells oxidized this substrate only after supplementation with a source of nicotinamide nucleotides and then consumed 1 mol of O₂ and released 1 mol of CO₂/mol of *N*-methylisonicotinate supplied. 3. The *N*-methyl group of the substrate was released as methylamine whereas the five C atoms of the pyridine ring were accounted for as succinate and formate. The CO₂ evolved by extracts was believed to derive from the carboxyl group on C-4 of the heterocyclic ring. 4. The immediate precursor of the succinate end-product was succinic semialdehyde; the inducible nature of succinic semialdehyde dehydrogenase in *N*-methylisonicotinate-grown cells supported this finding. 5. There was no evidence for monohydroxylation of the ring, but the time sequence of the appearance of the end-products indicated that the oxygen-requiring, NADH-requiring and decarboxylation steps clearly preceded the formation of methylamine and succinate. 6. The results are consistent with the oxidative cleavage of a partially reduced heterocyclic ring followed by several hydrolytic and dehydrogenase steps resulting in the appearance of the end-products.

The bipyridylum herbicides, in particular diquat (I) and paraquat (II), presently find widespread use in agricultural and horticultural practice. Their unique advantage lies in the rapid loss of residual herbicidal activity in the soil where they become tightly bound by ionic bonding to soil colloids (Calderbank, 1968). Weber & Scott (1961) have reported that paraquat was readily degraded by micro-organisms when bound to kaolinite clays but when absorbed to montmorillonite clays the amount of paraquat degraded was very small. It has been shown by X-ray crystallography that in a clay mineral of the montmorillonite type, the planar paraquat molecule lies flat in a 0.3 nm (3 Å) spacing between the silicate sheets of the mineral, where its accessibility to microbial attack must be severely hindered. Weber & Coble (1968), who examined the ¹⁴CO₂ released from ring-labelled diquat by pure cultures of micro-organisms, confirmed that addition of montmorillonite stopped CO₂ evolution whereas kaolinite had no effect.

The slow decomposition of [¹⁴C]paraquat, even when firmly absorbed to soils, occurred with the formation of radioactive but non-phytotoxic products. Some 60% of the added label, however, was still recoverable after 5 months (Calderbank & Tomlin-

son, 1968). Bozarth *et al.* (1965) have observed the ability of many micro-organisms to tolerate up to 1000 p.p.m. of paraquat when supplemented with a carbohydrate source, but the majority of bacterial and fungal cultures could reduce paraquat only to the stable, coloured free-radical (III). *Corynebacterium fascians* and *Clostridium pasteurianum* decomposed some 20-40% of a 10 p.p.m. solution of paraquat but Baldwin *et al.* (1966) isolated a strain of *Lipomyces starkeyi* which utilized paraquat and several related pyridine compounds as a source of N and could decompose 20 p.p.m. of it completely. From either [Me-¹⁴C]- or [ring-¹⁴C]-paraquat, over 80% of the radioactivity was released as CO₂ in 4 weeks at 24°C, but no intermediates were found.

Both in the laboratory (Slade, 1965) and under field conditions on the plant surface (Slade, 1966) paraquat underwent photolysis by u.v. light. A likely early product in photodecomposition was the unsaturated aminoaldehyde (IV) and, among the other fragments arising from photolysis, CO₂ has been detected (Calderbank, 1968). Slade (1965) showed that the two principal products of paraquat photolysis were methylamine and the 4-carboxy-1-methylpyridinium ion (*N*-methylisonicotinic acid). The latter compound was also found as a product of paraquat degradation by a pseudomonad (Bozarth *et al.*, 1966).

The *N*-methylisonicotinate formed under field

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conditions on the plant surface by photolysis of paraquat was not translocated from the leaves, nor was it taken up by roots from the soil, because plants became rapidly desiccated by the herbicidal action of paraquat. *N*-Methylisonicotinate was, however, rapidly decomposed in soil (Calderbank, 1968). Micro-organisms with this ability were isolated from soil by Wright & Cain (1969) and by Orpin *et al.* (1971), but the metabolic pathways by which these organisms degraded *N*-methylisonicotinate appeared to differ considerably.

In the present paper we describe the metabolism of *N*-methylisonicotinate by an *Achromobacter*, washed suspensions of which released stoichiometric amounts of methylamine from the *N*-methyl group of the molecule (Wright & Cain, 1969). No other products accumulated with washed cells, but by using cell-free extracts, we have been able to show that the carbon skeleton of *N*-methylisonicotinate can be accounted for as CO₂, formate, succinate and methylamine (Wright & Cain, 1970). A synopsis of some of these results was presented at the International Colloquium on Pesticide and Herbicide Degradation at Ghent, Belgium (Cain *et al.*, 1970).

Materials and Methods

Organism

The isolation of the organism used and its cultivation in large volumes have been described by Wright & Cain (1969).

Preparation and use of extracts

Packed washed cells were crushed in a Hughes press (Hughes, 1951) and the crush was mixed with an equal volume of ice-cold potassium phosphate buffer (pH 7.6, 0.05 M). After standing for 30 min at 4°C, the suspension was centrifuged at 100000g for 1 h at 2°C in an MSE High-Speed 50 centrifuge. The clear supernatant, containing 12–35 mg of protein/ml, was pipetted off carefully. Ammonium sulphate fractionation of these extracts was done by

adding the solid salt over a period of 30 min at 0°C with constant (magnetic) stirring and constant adjustment to pH 7.6 with dil. KOH, and then centrifuging off the precipitated protein (5000g, 30 min). Fractions precipitated by adding 0.23 g of (NH₄)₂SO₄/ml of extract, (F₁); a further 0.15 g of (NH₄)₂SO₄/ml of extract (F₂); and a further 0.15 g of (NH₄)₂SO₄/ml of extract yet again (F₃) [i.e. a final (NH₄)₂SO₄ concentration 0.53 g/ml of extract] were usually collected, redissolved in ice-cold potassium phosphate buffer and dialysed against 0.05 M-phosphate buffer, pH 7.6 for 2 h to remove ammonium sulphate. The enzyme(s) catalysing the oxidation of NADH by *N*-methylisonicotinate without the rapid re-formation of NADH (see Fig. 1, curve 3) were localized in the F₃ fraction. Where NAD⁺-reducing activity was occasionally found in this fraction, it could be removed by repeating the fractionation with ammonium sulphate.

Extracts, particularly those used in preparative-scale procedures for chemical isolations, frequently needed supplementing with reduced nicotinamide nucleotide-generating systems rather than with substrate quantities of the reduced cofactors. NADH was generated by adding NAD⁺ (0.2–1 μmol), ethanol (50 or 100 μmol) and yeast alcohol dehydrogenase (EC 1.1.1.1) (11 or 22 EC units) made up in potassium phosphate buffer, pH 7.6 (100 μmol). NADPH was produced by NADP⁺ (0.2 μmol), glucose 6-phosphate (100 μmol), MgCl₂ (1 μmol) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (1–4 EC units) in the same buffer.

Determinations

Methylamine was detected by t.l.c. on silica gel or kieselguhr (0.25 mm-thick) plates with solvent H and was determined colorimetrically or enzymically after microdiffusion in Conway units for 18 h as described by Wright & Cain (1969).

Formate was detected in reaction mixtures by the chromotropic acid method of Grant (1948). It was determined in portions (0.5 ml) of the distillate

(25ml) from steam distillation of acidified, deproteinized (2ml of 1M-H₂SO₄) extracts, after it had been reduced with Mg ribbon and HCl, by the sensitive acetylacetone method of Nash (1953). Formate was sometimes measured with a crude formate dehydrogenase preparation from methylamine-grown cells of *Pseudomonas* AMI (Eady & Large, 1968). The test cuvette contained extract (0.1 ml, equivalent to 3 mg of protein), NAD⁺ (1 μmol), a formate sample containing 0–0.7 μmol and potassium phosphate buffer, pH 7.0 (50 μmol), in a total volume of 3 ml. The reference cuvette contained all components except NAD⁺. The reaction was started by the addition of the formate sample to the test cuvette and the increase in *E*₃₄₀ was followed to completion. This extinction ultimately began to fall owing to the presence of NADH oxidase activity in the extracts, but the initial formate concentration was obtained by extrapolating the linear extinction-decrease curve back to zero time. This spectrophotometric method was not used for accurate formate determinations but only as a confirmation of its presence.

Succinate was determined manometrically at pH 7.4 and 40°C by using a succinoxidase preparation from a homogenate of ox heart (Umbreit *et al.*, 1964). Deproteinized samples from *N*-methylisonicotinate incubations were continuously extracted at pH 2.0 with diethyl ether (75 ml) for 5 h and the ethereal solution was carefully evaporated to dryness. The residue was dissolved in the minimum volume of buffer and made up to a known volume. Samples were transferred to the side-arms of Warburg flasks for manometric determinations of gas exchanges. As a routine, controls containing known amounts of succinate were subjected to this extraction and assay procedure as a check on recovery values; these averaged about 85%.

Protein was measured by the biuret method of

Gornall *et al.* (1949), with bovine serum albumin [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] as a standard.

Manometric procedures

O₂ uptake and CO₂ evolution by washed-cell suspensions and by extracts were measured at 30°C by standard manometric procedures (Umbreit *et al.*, 1964). CO₂ was measured by the direct method. When further examination of the metabolic products of *N*-methylisonicotinate oxidation in Warburg flasks was necessary, cell suspensions were removed by centrifugation at 10000*g* for 30 min, or extract protein was precipitated by the addition of 0.2 vol. of fresh 10% (w/v) metaphosphoric acid or of 0.2 ml of 1M-H₂SO₄.

Spectrophotometry

U.v.-absorption spectra were measured with a Unicam SP.800 recording spectrophotometer. Enzyme-catalysed reactions involving nicotinamide nucleotides were followed at 340 nm in cuvettes of 10 mm light-path by using the SP.820 constant-wavelength attachment. Where necessary the scale was expanded tenfold to detect weak activity. The cuvette contents were maintained at 30±0.2°C by a water-jacketed cuvette holder. Enzyme units are expressed in μmol of product formed or substrate changed/min.

Chemical syntheses

Derivatives of *N*-methylpyridinium chloride (V–XVI) were synthesized and tested for activity with *N*-methylisonicotinate-grown cells and their extracts. For comparative purposes, in the structures shown

all tautomeric compounds are in the enol forms. The keto (pyridone) form is chemically dominant in the case of 2- and 4-substituted hydroxy compounds (Klingsberg, 1962). The 4-carboxyl-1-methylpyridinium chloride (*N*-methylisonicotinic acid, V) was synthesized by methylating isonicotinic acid with methyl iodide in dimethylformamide (Slade, 1966), followed by passage down a column (50 cm × 5 cm) of De-Acidite FF (Cl⁻ form), and the effluent was evaporated to dryness *in vacuo*. This material was used as prepared for bulk growth media, but small amounts were recrystallized twice from methanol-diethyl ether to give pale-yellow crystals, m.p. 264°C. 4-Carboxy-1-ethylpyridinium chloride (VI), m.p. 274–276°C; 4-carboxy-1-*n*-propylpyridinium chloride, m.p. 244–246°C; 4-phenyl-1-methylpyridinium chloride (VII), m.p. 44–45°C, and 3-carboxy-1-methylpyridinium chloride, m.p. 242–244°C, were prepared and recrystallized in a similar manner. *N*-Methylpyridinium chloride (VIII) was prepared by methylating pyridine by the method of Kosower (1955). The product was recrystallized from methanol-diethyl ether and was stored in a desiccator; it was too deliquescent to allow accurate determination of its melting point.

N-Methyl-4-carboxypyrid-2-one (IX) was obtained by the alkaline ferricyanide oxidation of the isopropyl ester of *N*-methylisonicotinic acid (Fronk & Mosher, 1959). It was recrystallized from methanol to give m.p. 252°C.

N-Methyl-3-hydroxyisonicotinic acid (X) was synthesized in three steps. 3-Hydroxyisonicotinic acid was prepared from cinchomeric (pyridine-3,4-dicarboxylic) acid via 3-aminoisonicotinic acid, and the carboxyl group was then esterified with methanol and SOCl₂ (Crum & Fuchsmann, 1966). 3-Hydroxyisonicotinic acid methyl ester (0.3 g) was then mixed with 2-aminopyridine (0.183 g) and heated to 150°C, then cooled rapidly before being left overnight. The product was washed with 3 ml of chloroform and then 2 ml of methanol; the washed material (20 mg) was recrystallized to give m.p. 254°C (J. D. Crum, personal communication). Dr. Crum also provided us with 100 mg of this compound.

N-Methylpyrid-4-one (XII) was synthesized by *N*-methylating 4-aminopyridine with methyl iodide and oxidizing the betaine derivative to the 4-imine with freshly prepared silver oxide. Refluxing for 10 h with alkali yielded a paste of the *N*-methylpyrid-4-one which was recovered by extraction with benzene in a Soxhlet extractor (Tschitschibabin & Ossetrowa, 1925). The picrate derivative melted at 187–190°C (literature value, 186–187°C).

N-Methyl-3-hydroxypyridinium chloride (XIII) was obtained by *N*-alkylation of 3-hydroxypyridine with methyl iodide in benzene (Albert & Phillips, 1956) to give a product which, after recrystallization, melted at 110°C.

N-Methyl-3-hydroxypyrid-4-one (XIV) was provided by Dr. H. de Koning, University of Amsterdam, The Netherlands, and *N*-methylpyrid-2,4-dione (XV) by Dr. H. C. van der Plas, Agricultural College, Wageningen, The Netherlands.

Attempts to reduce *N*-methylisonicotinic acid with sodium borohydride by the method of Lyle *et al.* (1955) resulted in the formation of a product tentatively identified by Dr. J. R. Blackborow, Department of Inorganic Chemistry, University of Newcastle upon Tyne, by n.m.r. as *N*-methyl-3,4,5,6-tetrahydroisonicotinic acid (XVI).

N-Methylsuccinamic acid was prepared from succinic anhydride and methylamine (Flitsch, 1964). After crystallization from acetone and then from methanol, the product melted at 107–109°C (literature value, 111°C).

N-Methylmaleamic acid was prepared similarly from maleic anhydride (Mehta *et al.*, 1960). After repeated recrystallization from methanol, the m.p. (156°C) was still lower than the literature value (167°C).

γ -(*N*-Methylamino)butyric acid hydrochloride was prepared by acid hydrolysis of *N*-methylpyrrolidone with conc. HCl (McElvain & Vozza, 1949). The m.p. was 119–120°C after recrystallization from ethanol.

Succinic semialdehyde was obtained by two methods. The original specimens used in this study were prepared by the Chloramine-T oxidation of monosodium glutamate (Dakin, 1917) and stored at –20°C. The 2,4-dinitrophenylhydrazone derivative of this product melted at 196–199°C. Stoolmiller & Abeles (1966) gave a value of 199°C. Later batches were obtained by the acid hydrolysis of diethyl-formylsuccinate (Jakoby, 1962).

Succinic dialdehyde was prepared from 2,5-dimethoxytetrahydrofuran by hydrolysis in 0.6 M-HCl until complete solution, then the aldehyde was salted out with CaCl₂ and extracted with diethyl ether (Fakstorp *et al.*, 1950). This product, because it was liquid, was characterized as its bis-2,4-dinitrophenylhydrazone, m.p. 271–272°C. Values in the literature range from 266–268°C (Cope & Keller, 1956) to 280°C (Jones *et al.*, 1961). Because of this variation and because the 2,5-dimethoxytetrahydrofuran itself gave a yellow crystalline derivative with the acid 2,4-dinitrophenylhydrazine (presumably after hydrolysis), the identity and purity of the succinic dialdehyde was checked by g.l.c. On a 2 m Apiezon L column at 80°C and an N₂ carrier gas flow-rate of 55 ml/min the furan showed two peaks with retention times of 423 and 522 s respectively. The redistilled succinic dialdehyde showed only a single peak at 261 s.

Maleic semialdehyde was prepared by Mr. G. K. Watson of this Department from furoic acid by bromination and alkaline hydrolysis of the resulting product by the method of Fecht (1905).

N-Methylphthalimide, m.p. 134°C, was synthesized from phthalic anhydride and methylamine (Wright & Cain, 1969).

Other chemicals

The following pyridine derivatives were obtained from R. Emanuel Ltd., Alpertton, Middx., U.K., and were either redistilled or recrystallized as appropriate: *N*-methylpyrid-2-one (XI); *N*-methylpiperid-2-one; *N*-methylpiperid-4-one; *N*-methylpiperidine; 3-hydroxypyridine, cinchomeric (pyridine-3,4-dicarboxylic) acid and *N*-methylpyrrolid-2-one. Other compounds from this source included 2,5-dimethoxytetrahydrofuran, vinylacetic acid and 4-phenylpyridine. NAD⁺, NADP⁺, NADH and NADPH were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and purified enzymes from Boehringer Corp. (London) Ltd., London W.5, U.K. All other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K.

Chromatography

Paper chromatography was done on Whatman no. 1 paper by the descending method in the following solvent systems (all proportions by vol.): A, ethanol–conc. NH₃ (sp.gr. 0.88)–water (20:1:4); B, butan-1-ol–acetic acid–water (4:1:2); C, butan-1-ol–ethanol–0.5 M-NH₃ (7:1:2); D, propan-2-ol–0.5 M-NH₃–water (20:1:2) and E, propan-1-ol–conc. NH₃ (sp.gr. 0.88) (3:2). Acidic substances were detected on paper chromatograms by spraying with 0.05% (w/v) Bromophenol Blue in aq. 0.1% citric acid. Acidic dinitrophenylhydrazones, although visible as yellow spots on untreated sheets, were also sprayed with 0.1 M-NaOH to intensify the colour of weak spots as well as to develop their characteristic colours in alkali.

T.l.c. was performed on plates coated with a 0.25 mm-thick layer of silica gel activated by heating at 120°C for 0.5 h. The plates were developed in the following solvent systems (all proportions by volume): F, light petroleum (b.p. 40–60°C)–diethyl ether–formic acid (28:12:1); H, 2-methylbutan-2-ol–propan-1-ol–conc. NH₃ (sp.gr. 0.88) (13:1:6); J, 2 M-KCl; K, ethyl formate–light petroleum (b.p. 40–60°C)–propionic acid (50:50:7.7).

Succinic acid and other dicarboxylic acids were characterized by the retention times of their dimethyl and bistrimethylsilyl esters during g.l.c. The dimethyl esters were prepared as described by Burchfield & Storrs (1962) but with *N*-methyl-*N*-nitrosourea as a diazomethane source. Trimethylsilyl esters were obtained by the method of Dalglish *et al.* (1966). The esters were analysed on glass columns (2.25 m × 5 mm) of Apiezon L or SE30 at various flow rates and column temperatures in a Pye Series 104 gas chromatography assembly. The carrier gas was N₂. Com-

ponents issuing from the columns were detected by a flame ionization detector with a detector oven temperature of 250°C.

Radioisotope experiments

The preparation of labelled *N*-methylisonicotinate, the measurement of radioactivity in this compound and other end-products and the instrumentation used are described by Wright & Cain (1972).

Results

Oxidation of N-methylisonicotinate by cell suspensions

Washed suspensions of *Achromobacter* D grown with *N*-methylisonicotinate rapidly oxidized this substrate, consuming 4 mol of O₂/mol, approx. 70% of that necessary for complete oxidation according to eqn. (1):

The methyl ester of *N*-methylisonicotinate was oxidized more slowly than *N*-methylisonicotinate itself. Such suspensions also oxidized *N*-ethylisonicotinate- (4-carboxy-1-ethylpyridinium chloride) at the same rate as *N*-methylisonicotinate but showed no oxidation of *N*-(*n*-propyl)isonicotinate- (4-carboxy-1-*n*-propylpyridinium chloride) or 4-phenyl-1-methylpyridinium chloride (Cain *et al.*, 1970). Cells grown with *N*-methylisonicotinate similarly failed to oxidize the isomeric 3-carboxy-1-methylpyridinium chloride (*N*-methylnicotinate) or isonicotinic acid. Only very slow rates of oxidation by *N*-methylisonicotinate-grown cells were recorded for the 2- and 3-hydroxy-substituted *N*-methylisonicotinic acids (IX and X) and for compound (XII); the compounds (XI), (XIII), (XIV), (XV), 1-methylpiperid-2- and -4-ones and 1-methylpiperidine stimulated no O₂ uptake above the endogenous rate. Metabolism via a monohydroxylated compound as found with many other pyridine compounds (Behrman & Stanier, 1957; Gherna *et al.*, 1965; Houghton *et al.*, 1968; Holcenberg & Stadtman, 1969; Orpin *et al.*, 1971) therefore seemed unlikely. Neither *N*-methylisonicotinate nor isonicotinate was hydroxylated by the model hydroxylating system of Udenfriend (Cain *et al.*, 1970), although this system hydroxylated several other pyridine compounds in ring positions identical with those found with microbial systems (Houghton *et al.*, 1968).

Oxidation of N-methylisonicotinate and analogues by cell-free extracts

Unsupplemented cell-free extracts did not oxidize *N*-methylisonicotinate, but the addition of NADH or an NADH-generating system, or NAD⁺ itself, resulted in the oxidation of *N*-methylisonicotinate with

Table 1. O_2 uptake and CO_2 evolution during the metabolism of *N*-methylisonicotinate by supplemented extracts

Warburg flasks (double side-arm) contained: crude extract equivalent to 16mg of protein; NAD^+ , $0.2\mu\text{mol}$; potassium phosphate buffer, pH 7.6, $100\mu\text{mol}$; *N*-methylisonicotinate as shown. KOH (20%, w/v) in the centre well for O_2 uptake flasks (0.2ml) was replaced by $2M-H_2SO_4$ (0.2ml) in the second side-arm in those flasks used for CO_2 measurement; CO_2 bound by the buffer was released by tipping the acid into the main compartment. The reaction was followed until O_2 uptake had ceased.

<i>N</i> -methylisonicotinate supplied (μmol)	O_2 consumed (μmol)	CO_2 evolved (μmol)
0	0.3	0.4
2	2.5	2.2
4	4.5	4.7
5	5.6	5.3

the uptake of 1 mol of O_2 and the evolution of 1 mol of CO_2 (Table 1). This CO_2 was derived exclusively from the 4-carboxyl group as was shown with *N*-methyl[carboxyl- ^{14}C]isonicotinate by Wright & Cain (1972). The requirement for NADH was specific; NADPH or an NADPH-generating system did not substitute for NADH. Supplemented extracts (like washed cells) also oxidized *N*-ethylisonicotinate but not *N*-(*n*-propyl)isonicotinate.

When this reaction was followed spectrophotometrically at 340nm, an increased rate of NADH oxidation by crude extracts was observed on the addition of *N*-methylisonicotinate. This reaction, however, did not go to completion but was followed by the reduction of the newly formed NAD^+ (Fig. 1a). These two stages could be separated by ammonium sulphate fractionation of the extracts. The F_3 fraction oxidized NADH to completion in the presence of *N*-methylisonicotinate without subsequent reduction of the resulting NAD^+ , but the addition of the F_2 fraction or of crude extract at this stage led to the reduction of the NAD^+ (Fig. 1a). Examination

Fig. 1. Involvement of nicotinamide nucleotides in the metabolism of *N*-methylisonicotinate by extracts of *Achromobacter D*

(a) The oxidation and reduction of NADH by extracts demonstrated spectrophotometrically at 340nm. Curve 1, endogenous rate of NADH ($1\mu\text{mol}$) oxidation by crude extract (1.5mg of protein); curve 2, oxidation of NADH ($1\mu\text{mol}$) and subsequent reduction of the NAD^+ by crude extract after the addition of *N*-methylisonicotinate ($1\mu\text{mol}$) at zero time; curve 3, oxidation of NADH by *N*-methylisonicotinate in the presence of the F_3 fraction (see the Materials and Methods section) (1.5mg of protein); curve 4, reduction of the NAD^+ formed in 3 by the addition at 22min of crude extract (1.5mg of protein). (b) *N*-Methylisonicotinate mediated reduction of NAD^+ by crude extracts. Curve 5, the delayed reduction of NAD^+ ($1\mu\text{mol}$) by *N*-methylisonicotinate catalysed by crude extract (1.5mg of protein); curve 6 shows the inactivity of the F_3 fraction with NAD^+ . Cuvettes contained, in addition to the components mentioned above, potassium phosphate buffer, pH 7.6, $250\mu\text{mol}$, in a total volume of 3ml .

Table 2. *Stoichiometry of N-methylisonicotinate oxidation by the NADH-supplemented F₃ fraction of extracts*

Cuvettes contained: potassium phosphate buffer, pH 7.6, 150 μ mol; substrate and NADH as indicated; extract (F_3 fraction) equivalent to 3.2 or 6.4 mg of protein and water to a total volume of 3 ml. Reactions were started by the addition of *N*-methylisonicotinate after establishing the endogenous rate of NADH oxidation. Results are corrected for endogenous NADH oxidation.

<i>N</i> -Methylisonicotinate supplied (μ mol)	NADH supplied (μ mol)	NADH oxidized by <i>N</i> -methylisonicotinate (μ mol)	<i>N</i> -Methylisonicotinate/NADH ratio
0.1	0.5	0.09	1.10:1
0.2	0.5	0.19	1.05:1
0.3	1.0	0.31	0.97:1
0.7*	1.0	0.65	1.08:1

* Experiment performed with 6.4 mg of protein.

of the stoichiometry of the NADH oxidation by the F_3 fraction showed that 1 mol of NADH was oxidized/mol of *N*-methylisonicotinate (Table 2). Crude extracts and the F_2 fraction also oxidized *N*-methylisonicotinate when catalytic amounts of NAD^+ itself were added to extracts, taking up 1 mol of O_2 and forming 1 mol of CO_2 /mol of substrate. These extracts, but not the F_3 fraction, caused an *N*-methylisonicotinate-dependant reduction of NAD^+ characterized by a consistent 3–4 min lag (Fig. 1b). To conserve the more expensive NADH, large-scale incubations for isolating metabolites were therefore usually supplemented with NAD^+ . The F_3 fraction metabolized *N*-methylisonicotinate with the same stoichiometry for O_2 , but only when NADH or an NADH-generating system was supplied (Fig. 2). In the presence of NAD^+ this fraction did not catalyse disappearance of *N*-methylisonicotinate (λ_{max} . 265 nm), O_2 uptake or CO_2 evolution (Wright & Cain, 1972).

In an attempt to trap an intermediate, the carboxyl group was blocked by preparing the methyl ester of *N*-methylisonicotinic acid. Washed suspensions of *N*-methylisonicotinate-grown cells, however, oxidized the methyl ester almost as rapidly as the free acid. Supplemented extracts again formed 1 mol of CO_2 (from the carboxyl group) and consumed 1 mol of O_2 /mol of the ester, so the extracts must have contained an active esterase hydrolysing the ester bond.

The enzyme system in the F_3 fraction showed a high degree of specificity for the *N*-methylisonicotinate molecule as well as for NADH. Of the several pyridinium compounds examined, only *N*-ethyl- and *N*-(*n*-propyl)-isonicotinates (*N*-alkyl group enlarged) and the methyl ester of *N*-methylisonicotinate were utilized when the reaction was followed either spectrophotometrically or manometrically. The enzyme required an alkylated N atom in the substrate and a carboxyl group on C-4 because isonicotinic acid and *N*-methylnicotinic acid were not oxidized by extracts. None of the compounds (VII–XVI) was attacked in these systems nor did any of them give rise to meta-

Fig. 2. *Oxidation of N-methylisonicotinate by cell-free extracts*

Warburg flasks contained: potassium phosphate buffer, pH 7.6, 100 μ mol; NAD^+ , 1 μ mol or NADH, 2 μ mol; *N*-methylisonicotinate, 2 μ mol; an NADH-generating system (see the Materials and Methods section); crude extract (equivalent to 13 mg of protein) or the F_3 fraction (equivalent to 15 mg of protein). O_2 uptake is shown for: Δ , crude extract with no NAD supplement, or the F_3 fraction + NAD^+ ; $\circ$, crude extract + NADH-generating system; $\bullet$, F_3 fraction + NADH.

bolic end-products identical with or analogous to those obtained from *N*-methylisonicotinate (Table 3).

End-products of the metabolism of N-methylisonicotinate by supplemented extracts

When the enzyme-catalysed oxidation of *N*-methylisonicotinate was followed by spectrophotometric scanning of the complete u.v. spectrum, no

Table 3. Oxidation of NADH by *N*-methylisonicotinate and its analogues

Cuvettes contained: F₃ fraction, 0.2ml (equivalent to 2.3mg of protein); potassium phosphate buffer, pH 7.6, 100 μ mol; NADH, 1 μ mol and substrate, 1 μ mol, in a total volume of 3ml. The rate of NADH oxidation was calculated over the first 3 min (during which it was linear) and was corrected for the endogenous rate. Compounds XI, XII, XIII, XIV and XV were inactive in this system.

Substrate	NADH oxidized (nmol/min)
1-Methylisonicotinate	225
1-Ethylisonicotinate	200
1-Methylisonicotinate methyl ester	190
1-(<i>n</i> -Propyl)isonicotinate	64
3-Hydroxy-1-methylisonicotinate	0
2-Hydroxy-1-methylisonicotinate	0
1-Methylnicotinate	0
1-Methylnicotinate methyl ester	0
1-Methylpyridinium ion	0
1-Methyl-3,4,5,6-tetrahydroisonicotinate	0

new u.v.-absorbing components were detected during the reaction nor was there ever any u.v.-absorbing material present in the deproteinized supernatant when oxidation had ceased. Such a result would be expected either by complete saturation of the pyridine ring of the substrate with loss of its aromatic character, or by ring cleavage and degradation to simple aliphatic end-products. Three such products were isolated in addition to the CO₂ arising from the 4-carboxyl group, i.e. methylamine, succinate and formate. These three compounds effectively account for the C₆-skeleton of the 1-methylpyridinium nucleus and were formed with the stoichiometry shown in eqn. (2) (X = H-acceptor molecule):

Methylamine. Washed suspensions of *Achromobacter* D formed methylamine from *N*-methylisonicotinate (Wright & Cain, 1969). Crude extracts similarly effected a cleavage of the heterocyclic ring when supplemented with NAD⁺ or NADH. This primary amine was identified as methylamine in deproteinized supernatants by t.l.c. on Kieselguhr plates in solvent J (*R_F* 0.79). Determinations by the dinitrofluorobenzene method of Dubin (1960) showed recoveries of 86% of the theoretical amounts (Table 4). The exclusive origin of this amine in the *N*-methyl group of *N*-methylisonicotinate and its absolute

identity with methylamine were shown by using *N*-[Me-¹⁴C]isonicotinate (Wright & Cain, 1969). The oxidation of *N*-ethylisonicotinate similarly gave rise to ethylamine but recoveries were usually lower. No propylamine was formed from *N*-(*n*-propyl)isonicotinate.

Succinate. Paper chromatography and t.l.c. of the acidic ether-soluble components of deproteinized reaction mixtures after oxidation of *N*-methylisonicotinate showed the presence of an acid which was absent if boiled extracts were used or if the incubation was done under N₂. This acid formed no dinitrophenylhydrazone, nor did it decolorize permanganate, suggesting the absence of a keto group or an unsaturated bond. Its *R_F* values in solvents A (0.2) and E (0.53) on paper, and solvents F (0.70) and J (0.56) on silica-gel t.l.c. were similar to those of succinate and unlike those of other saturated acids. This acid was also distinct in its chromatographic properties on t.l.c. from the anticipated unsaturated maleic acid or fumaric acid. The dimethyl and trimethylsilyl esters of this unknown acid were prepared after its extraction into ether from an incubation mixture. These esters had retention times on g.l.c. identical with those of the equivalent esters of authentic succinic acid (Table 5). The identity of this acid with succinic acid was further strengthened by the isolation of the crystalline material from a large-scale (800 μ mol of *N*-methylisonicotinate) experiment. The solid was recovered from the ether extract by evaporation to dryness and was recrystallized several times from ethyl acetate to yield 30mg of white crystals, m.p. 184–185°C. Succinic acid melted at 187–188°C and the mixed m.p. was 186–188°C. The mass spectrum of the natural product was prepared for us by Mr. P. Kelly of the Department of Chemistry, University of Newcastle upon Tyne, on an A.E.I. model MS12 mass spectrometer. No molecular ion (*m/e* 118) was obtained for the free acid, but the spectrum showed large peaks at *m/e* 100, 74, 73, 56, 55, 45, 44, 28 and 18. Small peaks were visible at *m/e*

101, 72, 31, 30 and 29. These mass-to-charge peaks can be attributed to fragments which are entirely consistent with the structure of the unknown being that of succinic acid. Further, authentic succinic acid gave an identical pattern.

The yields of succinic acid formed by NADH- or NAD⁺-supplemented extracts from *N*-methylisonicotinate were measured with a succinoxidase preparation. Amounts corresponding to some 75% of that theoretically possible were obtained (Cain *et al.*, 1970).

Formate. Many pyridine compounds give rise to

Table 4. *Formation of methylamine from the oxidation of N-methylisonicotinate-supplemented extracts*

Warburg flasks contained: extract, equivalent to 22mg of protein; NAD⁺, 0.2 μ mol; *N*-methylisonicotinate, 5 μ mol; potassium phosphate buffer, pH 7.6, 100 μ mol; 20% (w/v) KOH in centre well, 0.2ml; the total volume was 3ml. When O₂ uptake ceased the flask contents were treated as described by Wright & Cain (1969) and assayed for methylamine by the method of Dubin (1960). Controls containing no substrate or boiled extract, or methylamine (5 μ mol) to establish recovery values, were treated identically.

Extract	Substrate added	Amine present (μ mol)	Net methylamine formed (μ mol)	Recovery of metabolic methylamine (%)
Crude	None	2.00	—	—
Crude	Methylamine	4.93	2.93	—
Crude	<i>N</i> -Methylisonicotinate	4.50	2.50	86
Crude	<i>N</i> -Methylformamide	2.00	0	0
None	<i>N</i> -Methylisonicotinate	0	—	—
Crude (boiled)	<i>N</i> -Methylisonicotinate	1.90	0	—

Table 5. *Identification by g.l.c. of the saturated dicarboxylic acid produced by the metabolism of N-methylisonicotinate*

The dry ethereal solutions of the acids were converted into their dimethyl esters (Expt. 1) or to their bistrimethylsilyl esters (Expt. 2) as described in the Materials and Methods section. n.d., not determined.

	Ester examined	Column	...	Retention time (s)		
				Apiezon L	SE30	
					80°C	100°C
Expt. 1.	Oxalic acid	Column temp.	...	120°C		
	Malonic acid			90	n.d.	n.d.
	Succinic acid			138	n.d.	n.d.
	Glutaric acid			288	294	156
	Natural product from <i>N</i> -methylisonicotinate oxidation			516	n.d.	n.d.
				288	294	162
Expt. 2.	Natural product plus authentic succinic acid*			288	294	162
	Succinic acid					300
	Natural product from <i>N</i> -methylisonicotinate oxidation					300
	Natural product plus authentic succinic acid*					300

* Indicates only one symmetrical peak produced by these two components.

formate as a metabolic product (Behrman & Stanier, 1957; Houghton *et al.*, 1968) and the failure to detect more than 1 mol of CO₂ from *N*-methylisonicotinate when four of the remaining C atoms in the molecule could be accounted for as succinate suggested that

the remaining C atom might appear as formate or a compound at this oxidation level. Washed-cell suspensions of *Achromobacter* D, after growth on *N*-methylisonicotinate, oxidized formate immediately, whereas succinate-grown cells showed a lag of some

Table 6. *Production of formate from N-methylisonicotinate by supplemented extracts of Achromobacter D*

Warburg flasks contained: potassium phosphate buffer, pH 7.6, 100 μ mol; crude extract equivalent to 11 mg of protein; *N*-methylisonicotinate as shown; NAD⁺, 2 μ mol; 20% (w/v) KOH in the centre well, 0.2 ml; the total volume was 3 ml. At the end of O₂ uptake, the flask contents were acidified with 2M-H₂SO₄, and steam distilled to give approx. 25 ml of distillate. The distillate volume was made up to 25 ml and samples (0.5 ml) were assayed for formate by the method of Nash (1953) as described in the Materials and Methods section. The contents of control flasks containing no formate or boiled extract were similarly treated. Samples containing known amounts of formate were included in the steam distillation and subsequent analysis to maintain a check on recoveries; this averaged about 80%.

	Substrate supplied (μ mol)	Formate measured (μ mol)	Net formate produced (μ mol)	Formate/ <i>N</i> -methylisonicotinate molar ratio
Expt. 1.	None	0.1	—	
	Formate (0.5)	0.40	0.30	
	<i>N</i> -Methylisonicotinate (0.5)	0.38	0.28	0.93
Expt. 2.	None			
	<i>N</i> -Methylisonicotinate (4)		3.24*	0.81
	<i>N</i> -Methylisonicotinate (5)		4.29*	0.86
	<i>N</i> -Methylisonicotinate (10)		8.20*	0.82

* Corrected for 88% recovery of added formate in a control run.

30 min. *N*-Methylformamide was not oxidized by either suspension (Cain *et al.*, 1970). Formate was recovered by steam distillation from the deproteinized reaction mixtures of NAD⁺-supplemented extracts and *N*-methylisonicotinate when O₂ uptake had ceased. After reduction with Mg and HCl, the distillate gave the characteristic colour with chromotropic acid (Grant, 1948). No colour was produced if the reduction was omitted, indicating that the metabolic product was not formaldehyde. Determination of formate (Nash, 1953) in samples of the steam distillate gave recoveries of over 80% of the theoretical yield (Table 6). As a routine, samples of authentic formate were steam distilled at the same time to check recoveries. The identity of the isolated material with formate was confirmed by demonstrating its activity with the formate dehydrogenase of *Pseudomonas* AMI and its recrystallization to constant specific radioactivity as sodium formate in an isotope-trapping experiment (Wright & Cain, 1972).

Time-course of appearance of the end-products

To establish whether there was a specific order of appearance of the end-products which might indicate the way in which the heterocyclic substrate was being degraded, the time-course of their appearance was followed by taking samples at short intervals from a series of identical Warburg flasks, which enabled O₂,

CO₂, methylamine and formate to be measured for each sample. While each of the products was present before 4 min, CO₂ evolution was the most rapid and was complete after 10 min (Fig. 3). Within this period the rates of formate production and O₂ uptake were quite fast but slowed appreciably when CO₂ evolution was complete. Methylamine formation was the slowest. At 10 min, for instance, 4.8 μ mol of CO₂ but only 2.6 μ mol of methylamine had been formed, showing that the release of the *N*-methyl group was either slower or occurred much later in the metabolic sequence. This was confirmed on a much shorter time-scale by using ¹⁴C-labelled *N*-methylisonicotinate and measuring the percentage recovery of label in the CO₂ and methylamine. At intervals from 10 s to 2 min, approximately twice as much CO₂ as methylamine was produced (Fig. 4).

Formation of end-products by fractionated extracts

The four compounds CO₂, succinate, formate and methylamine were formed by crude extracts from *N*-methylisonicotinate when supplemented with nicotinamide nucleotides and at the completion of oxygen uptake they were present in the approximate proportion 1:1:1:1. When the F₃ fraction supplemented with NADH was substituted as the source of enzyme, this pattern was radically different. This fraction consumed O₂ and released stoichiometric amounts of

Fig. 3. Rates of formation of the products of *N*-methylisonicotinate metabolism by extracts

A series of Warburg flasks each contained: potassium phosphate buffer, pH 7.6, 100 μmol; crude extract equivalent to 18 mg of protein; *N*-methylisonicotinate 5 μmol; NAD⁺, 2 μmol; 20% (w/v) KOH (0.2 ml) or 2M-H₂SO₄ (0.2 ml) as appropriate, the total volume was 3 ml. The reaction was followed manometrically for O₂ and CO₂, and at intervals the reaction in one of the flasks was stopped by adding acid. After deprotonization, methylamine and formate were measured in the supernatants. ●, O₂ uptake; ○, CO₂ formation; △, methylamine produced; ▲, formate produced.

CO₂ from *N*-methylisonicotinate but at the completion of O₂ uptake, the amounts of CO₂, succinate, formate and methylamine were in the proportion 1:0.22:0.09:0.15. Subsequent addition of crude extract led to the appearance of further succinate, formate and methylamine. These results demonstrate that the NADH-requiring O₂-consuming decarboxylation step(s) catalysed by the F₃ fraction occurred early in the metabolic sequence and clearly preceded those conversions giving rise to the established end-products.

The product of the action of the F₃ fraction on *N*-methylisonicotinate has not been isolated or identified.

Precursors of methylamine, formate and succinate in N-methylisonicotinate metabolism

The absence of any new u.v.-absorbing products during oxidation of *N*-methylisonicotinate by washed cells or by nicotinamide nucleotide-supplemented extracts led us to examine the activity of some

Fig. 4. Rates of formation by extracts of CO₂ and methylamine as determined with isotopically labelled substrates

Two series (1 and 2) of double-side-arm flasks each contained: extract, 0.5 ml (equivalent to 16 mg of protein) and potassium phosphate buffer, pH 7.6, 100 μmol, in the main compartment. In series 1, one side-arm contained *N*-methyl[carboxyl-¹⁴C]isonicotinate, 2 μmol (5.9 × 10⁴ d.p.m.); the second side-arm, 2M-H₂SO₄, 0.2 ml, and the centre well 20% (w/v) KOH, 0.2 ml. The reaction in individual flasks of this series was stopped at intervals by tipping the acid, then the flask contents were shaken at 30°C for a further 10 min to allow complete absorption of evolved CO₂ by the alkali. In series 2, one side-arm contained *N*-[Me-¹⁴C]isonicotinate, 2 μmol (3.2 × 10⁴ d.p.m.), the second side arm, 2.5M-NaOH, 0.2 ml, and the centre well 2M-H₂SO₄, 0.2 ml. After the reaction had been stopped in individual flasks at intervals by tipping the alkali, the flasks were left shaking for 18 h at 30°C to allow complete absorption of methylamine by the H₂SO₄. Samples of the alkali from series 1 and the acid from series 2 were counted for radioactivity in the scintillation spectrometer. The values recorded in the figure are corrected for recoveries of ¹⁴CO₂ (100%) and ¹⁴CH₃NH₂ (54%) obtained in control flasks. ●, Recovery of ¹⁴C in CO₂ and ○, in methylamine.

putative intermediates with these enzyme sources, and to attempt the trapping of such precursors for isolation and characterization.

The decarboxylation of *N*-methylisonicotinate at

C-4 would give rise to either 1-methylpyridinium chloride (VIII) or, if the decarboxylation is oxidative, compound (XII). Hydroxylation of 1-methylpyridinium chloride (VIII) could conceivably yield compounds (XI) or (XII), but compound (XIII), the 3-hydroxy isomer, would be preferred. Although none of the hydroxy compounds was oxidized by washed suspensions or by supplemented extracts, 1-methylpyridinium chloride was oxidized by washed suspensions after a lag period of some 45 min (Cain *et al.*, 1970) and methylamine was formed in the supernatant. In most experiments, however, the yields of methylamine at the end of O₂ uptake were well below those produced from *N*-methylisonicotinate by the same cell suspensions: occasional batches of washed cells that rapidly oxidized *N*-methylisonicotinate showed no O₂ uptake at all with 1-methylpyridinium chloride. Further, NAD⁺- or NADH-supplemented extracts, which had an unimpaired ability to oxidize *N*-methylisonicotinate to CO₂, methylamine, succinate and formate, neither oxidized nor produced methylamine or succinate from 1-methylpyridinium chloride.

Similarly, washed suspensions took up O₂ when supplied with *N*-methylsuccinamate (XVII) (2 mol of O₂/mol) and formed methylamine from it (0.5 mol/mol). Supplemented extracts, however, again failed to form succinate or methylamine from *N*-methylsuccinamate although a control flask containing *N*-methylisonicotinate produced these two end-products in the expected 1:1 ratio.

N-Methylmaleamic acid (XVIII) and γ -(*N*-methylamino)butyric acid were not oxidized by washed suspensions grown with *N*-methylisonicotinate nor did they form methylamine, succinate or maleate when incubated with extracts.

Role of succinic semialdehyde and succinic dialdehyde

The characterization of the saturated succinate, rather than maleate or fumarate, as an end-product of metabolism of the aromatic pyridine ring, and the demonstration that *N*-methylsuccinamate was not the precursor of the methylamine and succinate formed by extracts prompted us to investigate the aldehyde derivatives of succinate as possible precursors of the succinate product, particularly as the semialdehyde had been previously found as a metabolite of a substituted pyridine compound, pyridoxine (Sparrow *et al.*, 1969).

Detection of succinic semialdehyde. Succinic semialdehyde was formed in incubation mixtures of washed cells and *N*-methylisonicotinate when semicarbazide was added as a trapping agent. Cells from a 1-litre overnight (exponential phase) culture were washed aseptically with buffer and redistributed in 100 ml of 0.1 M-potassium phosphate buffer, pH 7.0, between two flasks. Semicarbazide-HCl, adjusted to pH 7.0, was added to both flasks to give a final concentration of 50 mM and to one of the flasks sodium *N*-methylisonicotinate (100 mg) was also added. The flasks, containing a total solution volume of 50 ml, were incubated with shaking for 4 days at 30°C, at which time the cell suspension was centrifuged off. To the supernatants from each flask was added 0.4% (w/v) 2,4-dinitrophenylhydrazine in 2 M-HCl (20 ml) and the mixture warmed to 80°C for 10 min. After cooling, the acidic dinitrophenylhydrazones were extracted first into ethyl acetate (30 ml) and then into 10% (w/v) Na₂CO₃ (30 ml). The carbonate was adjusted to pH 1.0 with conc. HCl and re-extracted with ethyl acetate (20 ml). After concentration of the ester phase (containing the unknown 2,4-dinitrophenylhydrazones) to a small

Table 7. *Chromatographic and spectrophotometric identification of the product of N-methylisonicotinate metabolism as the acidic 2,4-dinitrophenylhydrazone derivatives*

For experimental details see the text. Two values for *R_F* indicate two spots with the pure compound.

	<i>R_F</i> value		$\lambda_{\max.}$ of 2,4-dinitrophenylhydrazones (nm)	
			In 10% (w/v) Na ₂ CO ₃	In 1 M-KOH
	Solvent C	Solvent D		
2,4-Dinitrophenylhydrazone				
Product from <i>N</i> -methylisonicotinate	0.58	0.59	380	475, 525
Succinic semialdehyde	0.60	0.61	380	476, 525
α -Oxoglutarate	0.14	0.16	379	410, 528
Pyruvate	0.56, 0.76	0.59, 0.74	371	442, (shoulder 525)
Maleic semialdehyde	0.53	0.56	470	474
Glyoxylate	0.46	0.52	448	452
Oxaloacetate	0.27	0.34	380	426, (shoulder 525)

volume, samples were chromatographed on paper, yielding two spots with R_F values, in solvents C and D of 0.58 and 0.59 respectively, very similar to those of the dinitrophenylhydrazone of succinic semialdehyde and well separated from those of the dinitrophenylhydrazones of pyruvate, α -oxoglutarate, oxaloacetate, glyoxylate and maleic semialdehyde. The areas of the unknown spots, corresponding to succinic semialdehyde 2,4-dinitrophenylhydrazone from paper chromatography in solvents C and D of a larger-scale experiment, were eluted with 2 ml of water; the eluates were concentrated and dissolved in 1 M-KOH or 10% (w/v) Na_2CO_3 and their spectra were measured both in the u.v. and the visible ranges. The wavelengths of the absorption maxima were identical with those of the corresponding derivative of synthetic succinic semialdehyde (Table 7).

Succinic semialdehyde was also detected as a product of *N*-methylisonicotinate metabolism in the absence of a trapping agent by using NADH-supplemented crude extracts. The reaction was followed by the decrease in E_{340} and was stopped by the addition of 2,4-dinitrophenylhydrazine in 2M-HCl when the E_{340} value began to rise again (see Fig. 1). The acidic 2,4-dinitrophenylhydrazones were separated by t.l.c. on Kieselguhr in solvent H, yielding a spot of R_F 0.43. The R_F value of the synthetic succinic semialdehyde derivative was also 0.43.

In experiments with both washed cells and extracts, control incubations without *N*-methylisonicotinate gave no acidic 2,4-dinitrophenylhydrazones.

Dehydrogenase activity for succinic semialdehyde and succinic dialdehyde. The rate of NAD^+ reduction at the expense of succinic semialdehyde was 13–15-fold higher in extracts prepared from *N*-methylisonicotinate-grown cells than in extracts prepared from cells grown with succinate or malate (Table 8). The succinic semialdehyde dehydrogenase activity was

localized in the F_2 fraction of crude extracts. The rate of formation of NADH by crude extracts in the presence of succinic dialdehyde was about one-sixth of the value for the semialdehyde and was the same in extracts from either *N*-methylisonicotinate- or succinate-grown cells.

The product of the succinic semialdehyde dehydrogenase reaction was confirmed by incubating crude extract with NAD^+ and *N*-methylisonicotinate. When NADH formation ceased, the acidified reaction mixture was extracted with equal volumes of diethyl ether. The ether layer was dried with anhydrous Na_2SO_4 , then it was concentrated to small volume and the concentrate was treated with diazomethane (Burchfield & Storrs, 1962). Samples of the methylated product were examined by g.l.c. and revealed material with the same retention time as dimethylsuccinate on both Apiezon L and SE30 columns. Succinic acid (as its dimethyl ester) could not be detected when succinic dialdehyde or maleic semialdehyde were substituted for succinic semialdehyde in the incubation system. Similarly, no succinic acid was formed from succinic semialdehyde by the F_3 fraction of extracts from *N*-methylisonicotinate-grown cells.

Other carbonyl intermediates. The use of semicarbazide to trap succinic semialdehyde in experiments performed with supplemented extracts was unsuccessful. In these experiments, however, the neutral dinitrophenylhydrazones (i.e. those left in the ethyl acetate layer after extraction with Na_2CO_3) were also examined. The ester layer from the carbonate extraction was concentrated to a small volume and streaked on to t.l.c. plates of silica gel which were developed in solvent K. In addition to unchanged 2,4-dinitrophenylhydrazine, which ran with the solvent front, two bands at R_F 0.69 and 0.77 were obtained. They were not present in control incubation

Table 8. NAD^+ -mediated dehydrogenase activities for succinic semialdehyde and succinic dialdehyde in extracts of *Achromobacter D*

Cuvettes contained: extract equivalent to 0.6mg of protein; potassium phosphate buffer, pH7.6, 150 μmol ; substrate, 1 μmol ; NAD^+ , 2 μmol . Reactions were started by the addition of substrate and the reduction of the NAD^+ was followed with a recording spectrophotometer at 340nm. The two values for succinic semialdehyde dehydrogenase activity were obtained with extracts prepared from separate batches of cells (a) and (b).

Enzyme		Specific activity (nmol/min per mg of protein) in extracts from cells grown with	
		<i>N</i> -Methylisonicotinate	Succinate
Succinic semialdehyde dehydrogenase	(a)	63	4
	(b)	78	6
Succinic dialdehyde dehydrogenase	(a)	12	10

mixtures containing no substrate but otherwise treated in the same way. These two carbonyl compounds have not been identified but neither of them was succinic dialdehyde, which formed an insoluble bisdinitrophenylhydrazone that remained at the origin in solvent K.

Discussion

The isolation of succinate and formate as end-products of the carbon skeleton of the heterocyclic nucleus suggested its cleavage into C₁ and C₄ compounds, a feature that has since been confirmed by radioisotopic experiments which also showed that the actual site of cleavage was the bond between C-2 and C-3 of the pyridinium ring (Wright & Cain, 1972). The requirement for O₂ and NADH shown by the F₃ fraction before *N*-methylisonicotinate was metabolized and the 1:1 ratio of these components observed, originally led us to suspect the involvement of a mono-oxygenase, hydroxylating the ring of *N*-methylisonicotinate. The inactivity of its 2- and 3-hydroxy derivatives (IX) and (X), and of all the other hydroxylated compounds, (XI) to (XV), tested both with washed cells and with supplemented extracts, and the inability of the Udenfriend model system to hydroxylate *N*-methylisonicotinate (Cain *et al.*, 1970) strongly imply an alternative mechanism.

The reaction catalysed by the F₃ fraction required NADH and O₂ and evolved CO₂, all at 1 mol/mol of *N*-methylisonicotinic acid. The rates of O₂ uptake and CO₂ production during the reaction do not parallel each other (Fig. 3) and the release of the 4-carboxyl group as CO₂ depends on the addition of NADH; NAD⁺ is, in this instance, only a poor substitute (see Table 1 and Fig. 2 in Wright & Cain, 1972). Further, a simple direct decarboxylation at C-4 can be ruled out because 1-methylpyridinium chloride (VIII) is not oxidized or converted into methylamine and succinate by supplemented extracts which had an unimpaired ability to oxidize *N*-methylisonicotinate to these end-products. We therefore believe that the NADH and O₂ requirements are probably distinct and could be accounted for by an initial reduction of the pyridinium ring at C-4 and C-5, or at N-1 and C-4, by NADH with concomitant loss of the carboxyl group at C-4. It is significant that although *N*-methylisonicotinate stimulated NADH oxidation by the F₃ fraction, the tetrahydro derivative (XVI), in which these atoms are already saturated, did not (Table 3). Reduction of the ring at C-4 either directly or after isomerization is, in any case, necessary to account for the appearance of succinate rather than maleate or fumarate among the end-products of metabolism, and the radioisotopic experiments (Wright & Cain, 1972) strongly suggest that C-4 and C-5 of the ring do indeed appear as the two methylene C atoms of succinate. This reduction

must take place early in metabolism because the F₃ fraction, although requiring the 1 mol of reduced nicotinamide nucleotide needed in the overall reaction, cannot oxidize succinic semialdehyde or form methylamine or succinate from *N*-methylisonicotinate.

The O₂-requiring step consuming 1 mol of O₂/mol of *N*-methylisonicotinate, although, we believe, not concurrent with the decarboxylation step (1 mol of CO₂/mol of this substrate) (Fig. 3), clearly follows it closely because both reactions were catalysed by the F₃ fraction, which cannot form succinate and formate from *N*-methylisonicotinate. The stoichiometry is consistent with a dioxygenase cleavage of the partially reduced pyridinium ring, which radioisotopic experiments indicate must occur between C-2 and C-3. The product of this reaction would be γ -(*N*-formyl-*N*-methylamino)vinylacetaldehyde (see Scheme 2). Hydrolysis of the *N*-formyl group would account for the appearance of the formate (Table 6), which has been shown by radioisotopic experiments to arise from C-2 (Wright & Cain, 1972), and would leave γ -(*N*-methylamino)vinylacetaldehyde; such neutral aldehydes have been found as products of *N*-methylisonicotinate metabolism by extracts. Hydrolysis of γ -(*N*-formyl-*N*-methylamino)vinylacetaldehyde to vinylacetaldehyde cannot precede the loss of the *N*-formyl group otherwise *N*-methylformamide rather than methylamine would be formed. *N*-Methylformamide, however, was not oxidized by washed cells (Cain *et al.*, 1970) nor was it hydrolysed to methylamine and formate by extracts which formed these products from *N*-methylisonicotinate (Table 4). Formate was produced from this substrate faster than was methylamine (Fig. 3), so they clearly could not arise from the hydrolysis of the same intermediate substrate.

Although such a direct oxygenase cleavage of the double bond between C atoms, neither of which is hydroxylated, is unprecedented, there is chemical support for such a reaction. The oxidation of phenol and catechol by peracetic acid or perbenzoic acid yields the same ring-cleavage product, *cis,cis*-muconate, as does the enzymic cleavage of the ring by catechol 1,2-dioxygenase. Cornforth *et al.* (1962, 1966) have shown that the action of peracetic acid on a dihydronicotinamide splits the ring between C-2 and C-3 to form an *N*-formylated carboxylic acid intermediate, which on further oxidation yields succinate (Scheme 1). Succinate in this reaction arises from the C-3–C-6 fragment of the ring just as it does in our putative metabolic pathway for *N*-methylisonicotinate degradation (Scheme 2).

Even without direct enzymic evidence for this dioxygenase attack at present, some of the alternatives can be eliminated as follows.

(i) Both 2-hydroxy- and 3-hydroxy-*N*-methylisonicotinates were not oxidized by washed suspensions

Scheme 1. Acid-catalysed nucleophilic addition to and peracetic acid oxidation of 1,4-dihydro-1-methylnicotinamide (Cornforth *et al.*, 1962)

Scheme 2. Putative pathway of *N*-methylisonicotinate degradation by *Achromobacter D*

A, *N*-methylisonicotinate; B, *N*-methyl-1,4-dihydroisonicotinate; C, γ -(*N*-formyl-*N*-methylamino)vinylacetaldehyde; D, γ -(*N*-methylamino)vinylacetaldehyde; E, γ -(*N*-methylamino)vinylacetate; F, succinic semi-aldehyde. The end-products, succinate, formate and methylamine are underlined.

or supplemented extracts. A 2,3-dioxygenase cleavage of the 2-hydroxy isomer would in any case form an *N*-carboxyl group which could only with difficulty give rise to the formate known to arise from C-2 (Wright & Cain, 1972). It is possible that a 3-hydroxy-dihydro-*N*-methylisonicotinate is the substrate for this ring-cleavage reaction, with the hydroxyl group originating from water and not O₂.

(ii) 2,3-Dihydroxy-*N*-methylisonicotinate has been suggested as a possible intermediate (Orpin *et al.*, 1971). Cleavage by a 2,3-dioxygenase between these hydroxyl groups as required by the radioisotopic

results would yield an unsaturated dicarboxylic acid, no neutral aldehydes (which we have found) and the same problem of formate production as in (i).

(iii) The same arguments would apply as for (i) and (ii) even with reduced 4,5- or 1,4-dihydropyridine nuclei.

(iv) The radioisotopic results and the nature of the end-products preclude ring cleavage elsewhere.

Two alternative pathways leading to the known end-products are available for the further metabolism of γ -(*N*-methylamino)vinylacetaldehyde. A direct

hydrolysis of this intermediate to succinic dialdehyde and methylamine followed by a dehydrogenase oxidation first to succinic semialdehyde and then to succinate may be ruled out by the very low succinic dialdehyde dehydrogenase activities in *N*-methylisonicotinate-grown cells (Table 8), the failure to find succinic dialdehyde among the neutral aldehydes produced by *N*-methylisonicotinate and the observation that succinic dialdehyde is not converted into succinate by NAD^+ -supplemented extracts. The alternative route involving first an oxidation of γ -(*N*-methylamino)-vinylacetaldehyde to the corresponding acid, γ -(*N*-methylamino)vinylacetate, followed by hydrolysis to succinic semialdehyde and methylamine is more likely, for two reasons. First because chemical hydrolysis of these tautomeric imines to a primary amine and an aldehyde is well established (Smith, 1965). Secondly, both of these products have been detected as intermediates in *N*-methylisonicotinate metabolism (Tables 4 and 7) and extracts catalysed the oxidation of succinic semialdehyde to succinate by an NAD^+ -mediated dehydrogenase, the amounts of which were elevated some 15-fold when the organism was grown on *N*-methylisonicotinate rather than on succinate (Table 8). It is unlikely that γ -(*N*-methylamino)vinylacetate is directly cleaved to methylamine and vinylacetate because the latter was neither oxidized by washed cells nor converted into succinate by extracts. Reduction of γ -(*N*-methylamino)vinylacetate to γ -(*N*-methylamino)butyrate or routes involving *N*-methylsuccinamate, *N*-methylmaleamate or maleate can similarly be ruled out because none of these compounds formed succinate or methylamine when incubated with supplemented extracts.

The metabolic pathway that we have proposed, in which the sequence of end-products agrees with their rates of appearance (Fig. 3) and based on the arguments above, is shown in Scheme 2. This pathway requires 1 mol of NADH to reduce *N*-methylisonicotinate and 1 mol of NAD^+ to oxidize succinic semialdehyde to succinate. These requirements account for (i) the sequence of partial NADH oxidation followed by the reduction of the NAD^+ , observed with crude extracts (Fig. 1a), (ii) the observation that aldehydes could be obtained if the reaction with crude extracts was stopped when NADH oxidation ceased and (iii) the inability of the F_3 fraction to convert *N*-methylisonicotinate into succinate because it could not reduce NAD^+ .

The F_2 fraction of crude extracts catalysed the NAD^+ -mediated oxidation of succinic semialdehyde to succinate to produce more NADH for ring reduction. The lag of about 3 min before *N*-methylisonicotinate metabolism begins, observed when crude extracts are supplemented with NAD^+ rather than NADH (Fig. 1b) can thus be attributed to the time needed by an endogenous hydrogen donor to reduce NAD^+ to the NADH necessary to initiate the

primary reduction step in the metabolism of *N*-methylisonicotinate.

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References

- Albert, A. & Phillips, J. N. (1956) *J. Chem. Soc. London* 1294
- Baldwin, B. C., Bray, M. F. & Geoghegan, M. J. (1966) *Biochem. J.* **101**, 15P
- Behrman, E. J. & Stanier, R. Y. (1957) *J. Biol. Chem.* **228**, 923
- Bozarth, G. A., Funderburk, H. H., Curl, E. A. & Davis, D. E. (1965) *Proc. S. Weed Conf.* **18**, 615
- Bozarth, G. A., Funderburk, H. H. & Curl, E. A. (1966) *Weed Soc. Amer. Abst.* 55
- Burchfield, H. P. & Storrs, E. E. (1962) *Biochemical Applications of Gas Chromatography*, p. 532 et seq., Academic Press, New York
- Cain, R. B., Wright, K. A. & Houghton, C. (1970) *Meded. Rijksfac. Landbouwwetensch. Gent* **35**, 785
- Calderbank, A. (1968) *Advan. Pest Contr. Res.* **8**, 199
- Calderbank, A. & Tomlinson, T. E. (1968) *Outlook Agr.* **5**, 252
- Cope, A. C. & Keller, R. T. (1956) *J. Org. Chem.* **21**, 141
- Cornforth, J. W., Ryback, G., Popjak, G., Donninger, C. & Schroepfer, G. L. (1962) *Biochem. Biophys. Res. Commun.* **9**, 371
- Cornforth, J. W., Cornforth, R. H., Donninger, C., Popjak, G., Ryback, G. & Schroepfer, G. L. (1966) *Proc. Roy. Soc. Ser. B* **163**, 436
- Crum, J. D. & Fuchsman, C. H. (1966) *J. Heterocycl. Chem.* **3**, 252
- Dakin, H. D. (1917) *Biochem. J.* **11**, 79
- Dalglish, C. E., Horning, E. C., Horning, M. G., Knox, K. L. & Yarger, K. (1966) *Biochem. J.* **101**, 792
- Dubin, D. T. (1960) *J. Biol. Chem.* **235**, 783
- Eady, R. R. & Large, P. J. (1968) *Biochem. J.* **106**, 245
- Fakstorp, J., Raleigh, D. & Schniepp, L. E. (1950) *J. Amer. Chem. Soc.* **72**, 869
- Fecht, H. (1905) *Ber. Deut. Chem. Ges.* **38**, 1272
- Flitsch, W. (1964) *Chem. Ber.* **97**, 1542
- Fronk, M. H. & Mosher, H. S. (1959) *J. Org. Chem.* **24**, 196
- Gherna, R. L., Richardson, S. H. & Rittenberg, S. C. (1965) *J. Biol. Chem.* **240**, 3669
- Gornall, A. G., Bardawill, C. H. & David, M. A. (1949) *J. Biol. Chem.* **177**, 751
- Grant, W. M. (1948) *Anal. Chem.* **20**, 267

- Holcenberg, J. S. & Stadtman, E. R. (1969) *J. Biol. Chem.* **244**, 1194
- Houghton, C. H., Wright, K. A. & Cain, R. B. (1968) *Biochem. J.* **106**, 5P
- Hughes, D. E. (1951) *Brit. J. Exp. Pathol.* **18**, 131
- Jakoby, W. B. (1962) *Methods Enzymol.* **5**, 774
- Jones, L. A., Hancock, C. K. & Seligman, R. B. (1961) *J. Org. Chem.* **26**, 228
- Klingsberg, E. (1962) *Heterocyclic Compounds: Pyridine and its Derivatives*, parts I and III, Interscience, New York
- Kosower, E. M. (1955) *J. Amer. Chem. Soc.* **77**, 3883
- Lyle, R. E., Perlowski, E. F., Troscianic, H. J. & Lyle, G. G. (1955) *J. Org. Chem.* **20**, 1761
- McElvain, S. M. & Voza, J. F. (1949) *J. Amer. Chem. Soc.* **71**, 896
- Mehta, N. B., Phillips, A. P., Lui, F. F. & Brooks, R. E. (1960) *J. Org. Chem.* **25**, 1102
- Nash, T. (1953) *Biochem. J.* **55**, 416
- Orpin, C. G., Knight, M. & Evans, W. C. (1971) *Biochem. J.* **122**, 58P
- Slade, P. (1965) *Nature (London)* **207**, 515
- Slade, P. (1966) *Weed Res.* **6**, 158
- Smith, P. A. S. (1965) *Open Chain Nitrogen Compounds*, part I, p. 297, Benjamin, New York
- Sparrow, L. G., Ito, P. P. K., Sundaram, T. K., Zach, D., Nyns, E. J. & Snell, E. E. (1969) *J. Biol. Chem.* **244**, 2590
- Stoolmiller, A. C. & Abeles, R. H. (1966) *J. Biol. Chem.* **241**, 2764
- Tschitschibabin, A. E. & Ossetrowa, E. D. (1925) *Ber. Deut. Chem. Ges.* **58**, 1708
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964) *Manometric Techniques*, 4th edn., p. 195, Burgess Publishing Co., Minneapolis
- Weber, J. B. & Coble, H. D. (1968) *J. Agr. Food Chem.* **16**, 475
- Weber, J. B. & Scott, D. C. (1961) *Science* **152**, 1401
- Wright, K. A. & Cain, R. B. (1969) *Soil Biol. Biochem.* **1**, 5
- Wright, K. A. & Cain, R. B. (1970) *Biochem. J.* **118**, 52P
- Wright, K. A. & Cain, R. B. (1972) *Biochem. J.* **128**, 561