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The Bacterial Oxidation of *N*-Methylisonicotinate, a Photolytic Product of Paraquat

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Two bacteria have been isolated that are capable of oxidizing *N*-methylisonicotinate, a photodegradation product of Paraquat (1.1′-dimethyl-4,4′-bipyridylium ion). *N*-Methylisonicotinate-grown cells of strain 4C1, a Gram-positive rod, oxidized 2-hydroxy-*N*-methylisonicotinate without lag. Cell-free extracts of these cells converted 2-hydroxyisonicotinate into 2,6-dihydroxyisonicotinate; the reaction did not require molecular oxygen. Maleamate was deamidated and maleate isomerized to fumarate by soluble enzyme systems. [^{14}C]Formaldehyde was isolated as the dimedone derivative from the supernatant of a cell suspension oxidizing *N*-[^{14}C]methylisonicotinate, and no [^{14}C]-methylamine was detected. Whole cells incubated with *N*-methyl[*carboxy*-^{14}C]isonicotinate released 95% of the radioactivity as $^{14}CO_2$. The second bacterium, strain 4C2, a Gram-negative rod, did not oxidize any of the mono- or di-hydroxypyridines or their *N*-methyl derivatives that were available or could be synthesized; nor did cell-free extracts oxidize any of these compounds. Methylamine was oxidized by whole cells without lag; cell-free extracts converted methylamine into formaldehyde when a soluble enzyme system requiring an electron acceptor was used; formaldehyde was oxidized to formate and formate to CO_2 by enzyme systems requiring NAD^+.

Paraquat (1,1′-dimethyl-4,4′-bipyridylium ion) is one of a series of herbicidally active bipyridyls, and is now used extensively for total foliage destruction. The Paraquat ion, being cationic, is readily adsorbed on anionic soil colloids and plant-leaf surfaces. In this state it is susceptible to photolytic degradation (Slade, 1966), and is converted eventually into *N*-methylisonicotinate and methylamine, both of which compounds enter the soil.

Wright & Cain (1969, 1970) have published reports about the degradation of *N*-methylisonicotinate by *Achromobacter* D. The pathway is thought not to involve any hydroxylated heterocyclic compounds, but rather a partial reduction of the pyridinium ring followed by a direct oxidative cleavage between C-2 and C-3. The resulting dialdehyde is considered to be hydrolysed to yield formate, methylamine and succinate. We describe here a new pathway for the oxidation of *N*-methylisonicotinate by a Gram-positive organism, a preliminary report of which has already been published (Orpin *et al.*, 1971). We also describe experiments with a Gram-negative organism, the results of which are consistent with the view that it oxidizes *N*-methylisonicotinate by a pathway similar to that used by *Achromobacter* D.

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Materials and Methods

Microbiological methods

Isolation of N-methylisonicotinate-oxidizing bacteria. Two bacteria able to grow with *N*-methylisonicotinate as sole source of carbon and nitrogen have been isolated. Strain 4C1, a Gram-positive rod, was isolated from soil perfused with the following medium (g/l): KH_2PO_4, 0.5; $(NH_4)_2SO_4$, 0.5; $MgSO,7H_2O$, 0.2; $CaCl_2,6H_2O$, 0.02; *N*-methylisonicotinate, 0.2. The pH was adjusted to 7.0 with NaOH.

Strain 4C2, a Gram-negative rod, was isolated from sewage sludge by using the same medium, but with a *N*-methylisonicotinate concentration of 0.5 g/l.

Growth and harvesting of cultures. The medium employed contained (g/l): KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 0.5; $MgSO_4,7H_2O$, 0.2; $CaCl_2,6H_2O$, 0.02; $FeSO_4,7H_2O$, 0.05; $ZnSO_4,7H_2O$, 0.02; $CuSO_4,5H_2O$, 0.02; $CoSO_4$, 0.02; either *N*-methylisonicotinate, 0.2 (strain 4C1) or 0.5 (strain 4C2) or glucose, 0.5. The pH was adjusted to 7.0 with NaOH. Starter cultures were supplemented with 0.1 g of Oxoid yeast extract/litre. The bacteria were maintained by monthly subculture on slopes of *N*-methylisonicotinate medium solidified by the addition of 2% (w/v) Oxoid no. 3 agar. All media were sterilized by autoclaving at 121°C for

20min. Cultures were grown at 25°C. Starter cultures (35ml) in 100ml conical flasks were used, when fully grown, to inoculate 300ml quantities of medium in 1-litre baffled conical flasks. Up to this scale, cultures were aerated by shaking at 130 strokes/min on a Gallenkamp Orbital Shaker. After 48h, 300ml cultures were used to inoculate 9-litre cultures grown under forced aeration. These cultures were harvested after 2–3 days, while growth was still exponential, by centrifugation in a bench-model Sharples Supercentrifuge. The cells were washed twice with five times the pellet volume of 67mM-sodium phosphate buffer, pH7.0, by centrifugation at 3000*g* for 15min, and either stored as a pellet at −20°C or used immediately. Before use, stored cells were incubated for 1h at 25°C in 67mM-sodium phosphate buffer, pH7.0, containing 0.2g of *N*-methylisonicotinate/litre. The cells were then centrifuged at 3000*g* for 15min, and the pellet was washed twice with five times the pellet volume of, and resuspended in, the same buffer.

Cell-free extracts. Cell-free extracts were prepared by several methods, all operations being performed at 0–4°C. The cells were suspended in buffer (50mg dry wt./ml) and disrupted by the use of an MSE–Mullard 60W ultrasonic disintegrator, or a Biox X-press (A. B. Biox, Nacka, Sweden) or a Hughes' (1951) press. The buffers used were sodium phosphate (20 and 67mM; pH6.0, 7.0 and 7.8), tris–HCl (20 and 40mM; pH7.4 and 8.0), tris–maleate (20 and 40mM; pH5.2, 6.0 and 7.0). The unbroken cells were removed by centrifugation (3000*g* for 15min, MSE 18 centrifuge, angle head). The crude extract was either used as it was or fractionated by centrifugation at 38000*g* for 30min to separate a particulate fraction.

Analytical methods

Thin-layer chromatography. Chromatography was performed on thin layers of silica gel (Kieselgel G; E. Merck, Darmstadt, Germany) on 20cm×20cm, 10cm×20cm or 5cm×20cm glass plates. For analytical purposes, 0.25mm layers were used, and for preparative purposes, 1.0mm layers. The plates were allowed to dry at room temperature and finally dried at 120°C for 30min. The following solvents were used: (*a*) the organic phase of butan-1-ol–acetic acid–water (5:1:4, by vol.); (*b*) aq. 1M-ammonium acetate–ethanol (1:8, v/v); (*c*) ethanol–aq. ammonia soln. (sp.gr. 0.88)–water (20:1:4, by vol.); (*d*) the organic phase of diethyl ether–benzene–90% formic acid (14:6:5, by vol.) saturated with water; (*e*) 0.5M-HCl, saturated with 3-methylbutan-1-ol. Standard compounds were run with unknown samples whenever possible.

Compounds that fluoresced or absorbed strongly were detected under u.v. light at 254nm (Hanovia Lamps Ltd., Slough, Bucks.). Weakly absorbing materials were detected in the same manner after spraying with ethanolic 0.05% sodium fluorescein.

Phenolic compounds were sprayed with Folin–Ciocalteu reagent, followed by 4% (w/v) Na_2CO_3, giving blue spots on a white background.

Unsaturated compounds were visualized by spraying with 0.05M-$KMnO_4$ in 3% (w/v) metaphosphoric acid, giving white spots on a pink background.

Thin-layer electrophoresis. Thin-layer electrophoresis was performed in a Shandon–Kuhn electrophoresis apparatus on 0.25mm layers of silica gel G supported on 20cm×20cm glass plates, at a constant current of 30mA. The layers were previously saturated with electrolyte. Two layers of Whatman 3MM chromatography paper were used as wicks. Compounds were detected in the same manner as on thin-layer chromatograms.

Measurements of oxygen uptake. Manometric experiments were conducted in conventional Warburg constant-volume respirometers. Gaseous exchange was measured by the direct method of Dixon (1952). The temperature was 30°C except where otherwise stated.

Spectrophotometry. Spectrophotometry was carried out in a Beckman DB or Unicam SP.800 recording spectrophotometer. Infrared spectra were determined by using a Perkin–Elmer Infracord model 137 with KCl or KBr discs.

Determinations. N-Methylisonicotinate was determined by measurement of its extinction at 265nm ($\epsilon_{265} = 4100$); 2-hydroxy-*N*-methylisonicotinate at 320nm at pH2 ($\epsilon_{320} = 7500$); 2-hydroxyisonicotinate at 279nm at pH2 ($\epsilon_{320} = 5020$); each was quantified against a calibration curve. Maleamate, maleate and ammonia were determined by the methods described by Orpin *et al.* (1972), methylamine by that of Dubin (1960), formate by that of Eegriwe (1937) and formaldehyde by that of MacFayden *et al.* (1945). Protein was determined by the biuret method of Gornall *et al.* (1949). No interference by the amide group of maleamate occurred at the concentrations of maleamate employed in reaction mixtures.

Detection and measurement of radioactivity. Samples for radioactivity assay were plated on aluminium planchets and counted by using a Geiger–Muller tube connected to an I.D.L. type 1700 scaler. Radioactive areas on certain chromatograms were detected by the methods of Dutton & Evans (1969). The radioactivity of certain chromatograms supported on 20cm×5cm glass plates was assayed by using a Packard model 7200 chromatogram scanner coupled to a model 385 recording rate-meter. Radioactive peaks were assayed quantitatively by cutting them out of the trace, weighing them and comparing these weights with those given by samples of known radioactivity counted under identical conditions.

Chemicals

Reagents. 2-Picoline, 4-picoline, isonicotinate, chromotropic acid and 2,6-dihydroxyisonicotinate were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K.; 2-, 3- and 4-hydroxypyridine and maleamate were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; 2,3-dihydroxypyridine was from Ralph N. Emanuel Ltd., London S.E.1, U.K.; 2,6-dihydroxypyridine hydrochloride was from Fluka, Buchs, Switzerland. All were purified by recrystallization. All other chemicals were of reagent grade from commercial sources. All solvents were redistilled before use.

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

Syntheses. The following materials were synthesized by published methods: 2,4-dihydroxypyridine (von Schickh *et al.*, 1936); 2,5-dihydroxypyridine (Behrman & Pitt, 1958); 3,4-dihydroxypyridine (Orpin *et al.*, 1972); *N*-methylisonicotinate (Slade, 1965); 2-hydroxy-*N*-methylisonicotinate (Fronk & Mosher, 1959); 3,4-dihydroxy-*N*-methylpyridine (Wibaut & Kleipool, 1947); 2,4-dihydroxy-*N*-methylpyridine (den Hertog & Buurman, 1956); 3-hydroxyisonicotinate (Duesel & Scudi, 1949); 3-hydroxy-*N*-methylpyridine (Albert & Phillips, 1956); fumaramate (Greiss, 1879). Other compounds were synthesized by the following methods.

(a) 2-Hydroxy-*N*-methylpyridine. Dry 2-hydroxypyridine (5g) was refluxed with methyl iodide (8g) in *NN*-dimethylformamide (25ml) for 1h under anhydrous conditions. The reaction mixture was concentrated under reduced pressure to yield a thick red syrup, which was distilled under reduced pressure to yield 3.5g of pale-yellow oil, b.p. 249°C (lit. 250°C).

(b) 4-Hydroxy-*N*-methylpyridine. Dry 4-hydroxypyridine (5g) was refluxed with methyl iodide (8g) in *NN*-dimethylformamide (25ml) for 2h under anhydrous conditions. The excess of methyl iodide and the solvent were removed under reduced pressure; the residue was triturated with water (3×30ml), filtered and the excess of iodine removed from the filtrate by treatment with 10% (w/v) silver nitrate until no more iodide was precipitated. The supernatant was treated with H_2S until no more silver sulphide was precipitated; the silver sulphide was removed by centrifugation in a bench centrifuge and the supernatant was evaporated to small volume under reduced pressure. On cooling, 4-hydroxy-*N*-methylpyridine separated as a white solid, which was dried *in vacuo* over P_2O_5. This material was recrystallized from ethanol, to yield 4.2g of white deliquescent crystals, m.p. 85°C (lit. 86°C).

(c) *N*-Methylpyridine. Dry pyridine (5g) was refluxed with methyl iodide (8g) in *NN*-dimethylformamide (25ml) for 1h under anhydrous conditions. The solvent was removed under reduced pressure and the residue crystallized twice from ethanol. The yield of *N*-methylpyridinium iodide was 10.8g (78%), m.p. 117–120°C (lit. 118–120°C). This was converted into the chloride by dissolving 1g portions in 10ml of water and passing the solution down a column of De-Acidite FF anion-exchange resin (Cl^- form). The chloride was evaporated to dryness under reduced pressure and recrystallized from ethanol; yield 0.5g (77%).

(d) 2-Hydroxyisonicotinate. The methods of Buchi *et al.* (1947) and Baumler *et al.* (1951) were attempted but were not successful. A synthesis modified from that of Boekelheide & Lehn (1961) did, however, yield the required material. Isonicotinate (2g) was refluxed with methanol (10ml) and conc. H_2SO_4 (5ml) on a water bath for 3h. The reaction mixture was neutralized with $KHCO_3$ and extracted with chloroform (10×10ml). The extract was dried over anhydrous Na_2SO_4 overnight, filtered and the solid washed with dry chloroform (2×20ml). The combined washings and filtrate were evaporated *in vacuo* to yield 1.9g of 4-methoxycarbonylpyridine, as a pale-yellow oil, which solidified on cooling to 0°C for 1h. 4-Methoxycarbonylpyridine (2g) was converted into the *N*-oxide by heating on a boiling-water bath with 30% (v/v) hydrogen peroxide (2ml) in acetic acid (5ml). After 2h a further 2ml of 30% H_2O_2 was added, and the mixture was refluxed for a further 2h. The mixture was evaporated to approx. 2ml; water (10ml) was then added and the mixture evaporated to dryness. This second distillation removed excess of hydrogen peroxide and any acetic acid not removed previously. The 4-methoxycarbonylpyridine *N*-oxide prepared thus was a pale-yellow chromatographically pure powder. The yield was 1.9g. 4-Methoxycarbonylpyridine *N*-oxide (0.9g) was refluxed with acetic anhydride (10ml) for 6h. The excess of acetic anhydride was removed *in vacuo* and the residue extracted with boiling methanol (4×10ml). The extract was treated with charcoal and evaporated to dryness to yield 0.56g of a pale-brown solid. A portion was subjected to a second treatment with charcoal and evaporated *in vacuo* until crystallization started. The mixture was then cooled to 0°C. After 3h the white crystals of 2-hydroxy-4-methoxycarbonylpyridine were filtered off and found to melt at 212°C (lit. 211–212°C). The yield was 47%. The 2-hydroxy-4-methoxycarbonylpyridine was hydrolysed with alkali; 0.25g was refluxed with 10% (w/v) NaOH (10ml) for 3h. The reaction mixture was neutralized with 5M-HCl, the precipitated salt was centrifuged off, and the supernatant evaporated to dryness. The solid was extracted with methanol (3×10ml) and the solvent removed under reduced

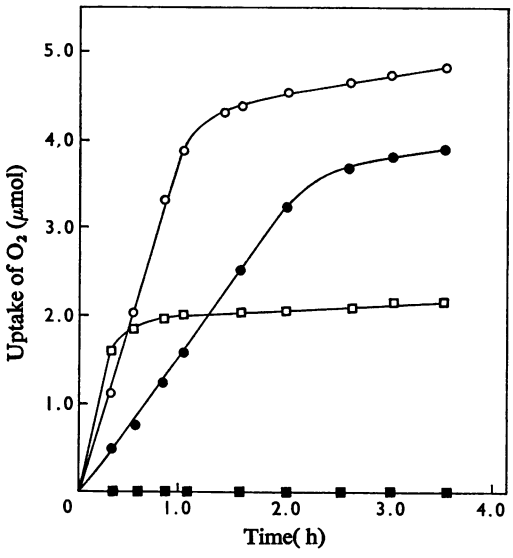


Fig. 1. *Oxidation of N-methylisonicotinate and potential metabolites by washed suspensions of N-methylisonicotinate-grown strain 4C1*

Single-side-arm Warburg flasks contained, in a working volume of 2.8ml: phosphate buffer, pH6.8, 200μmol; cells, 8mg dry wt.; substrate, 1μmol. The centre well contained 0.2ml of 20% (w/v) KOH. The reaction was started by the addition of the substrate from the side arm. Temperature, 30°C; atmosphere, air. Corrected for endogenous respiration. The substrates were: ○, *N*-methylisonicotinate; ●, 2-hydroxy-*N*-methylisonicotinate; □, fumarate; ■, 2-, 3- and 4-hydroxypyridine, 2,4-, 2,6- and 3,4-dihydroxypyridine and their *N*-methyl derivatives (isonicotinate, 2-hydroxyisonicotinate, 2,3- and 2,5-dihydroxypyridine, maleamate, fumaramate, maleate, methylamine and methanol).

pressure to yield 0.2g of a white solid, m.p. 315–325°C (decomp.).

Radioactive chemicals. *N*-[^{14}C]Methylisonicotinate was generously supplied by Dr. A. Calderbank, I.C.I. Jealotts Hill Research Station, Bracknell, Berks., U.K. [*carboxy*-^{14}C]Isonicotinate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Purification of N-[^{14}C]methylisonicotinate. Chromatography of *N*-[^{14}C]methylisonicotinate with solvents (*b*) and (*c*) resolved two radioactive components with each solvent. In solvent (*b*), *N*-methylisonicotinate ran at R_F 0.20 and the other component at R_F 0.09. In solvent (*c*), *N*-methylisonicotinate ran at R_F 0.28; the other component did not move from the origin. Solvent (*c*) was therefore used to purify the material. Crude *N*-[^{14}C]methylisonicotinate (0.5mg) was applied in strips on 20cm×20cm glass plates coated with 1.0mm layers of silica gel G. After development, the area corresponding to R_F 0.15–0.35 was scraped off, eluted with 10ml of water, neutralized with 1M-HCl, evaporated to dryness under reduced pressure, dissolved in boiling methanol (3×5ml), and evaporated to 1ml. The *N*-methylisonicotinate was precipitated in 20ml of sodium-dried diethyl ether. The ether was decanted off and the material allowed to dry in air, followed by drying overnight at 60°C. Chromatography in solvents (*a*), (*b*), (*c*) and (*d*) did not resolve any other radioactive component, nor any other component detectable by inspection in u.v. light (254nm).

Synthesis of N-methyl[carboxy-^{14}C]isonicotinate. *N*-Methyl[*carboxy*-^{14}C]isonicotinate (0.12Ci/mol) was *N*-methylated by the procedure of Slade (1965). The radioactive isonicotinate (30mg), diluted with carrier isonicotinate (60mg), was refluxed with methyl iodide (0.3g) in *NN*-dimethylformamide (5ml). Excess of methyl iodide and solvent were removed under reduced pressure and the iodide was converted into the chloride by passage through De-Acidite-FF (Cl^- form). The material was concentrated under reduced pressure at 30°C and chromatographed on silica gel G in solvents (*a*), (*b*) and (*c*). The only impurity detected by radioautography, after 2 weeks' exposure, was a trace of the original isonicotinate. This was removed by preparative t.l.c. in solvent (*a*). The area corresponding to *N*-methylisonicotinate was eluted with hot methanol and evaporated to dryness. The residue was redissolved in boiling methanol (3×5ml), evaporated to about 1ml under reduced pressure and the *N*-methylisonicotinate precipitated in sodium-dried diethyl ether (10ml). The resulting *N*-methyl[*carboxy*-^{14}C]isonicotinate had a specific radioactivity of 35mCi/mol.

Results

Oxidation of N-methylisonicotinate by strain 4C1

Strain 4C1 was found to metabolize *N*-methylisonicotinate most rapidly at 30°C at pH7–7.2, with a substrate concentration of 0.2g/litre. It would not grow at a pH below 4.5 or above 8.5. The optimum concentration of *N*-methylisonicotinate was found to be 0.2–0.25g/litre. Although *N*-methylisonicotinate alone was capable of providing sufficient carbon and nitrogen for growth, the addition of 1g of ammonium sulphate/litre resulted in a fivefold increase in the rate of growth on *N*-methylisonicotinate.

Experiments with whole cells. Cells grown on *N*-methylisonicotinate immediately oxidized this substrate, 2-hydroxy-*N*-methylisonicotinate and fumarate (Fig. 1). Isonicotinate, 2-hydroxyisonicotinate, 2,6-dihydroxyisonicotinate, 2,6-dihydroxy-*N*-methyl-

isonicotinate, 2-, 3- and 4-hydroxypyridine, 2-, 3- and 4-hydroxy-*N*-methylpyridine, 2,3-, 2,4-, 2,5-, 2,6- and 3,4-dihydroxypyridine, 2,4-, 2,6- and 3,4-dihydroxy-*N*-methylpyridine, 3-hydroxyisonicotinate, *N*-methylpyridine, pyridine, maleamate, fumaramate, maleate, methylamine and methanol were not oxidized within 4h. Glucose was oxidized after a lag period of 2h. Cells grown on glucose would only oxidize fumarate and glucose, the oxidation of fumarate proceeding at only 60% of the rate of oxidation of fumarate by *N*-methylisonicotinate-grown cells. The oxygen uptake of *N*-methylisonicotinate-grown cells with *N*-methylisonicotinate was 4–5 mol/mol, with 2-hydroxy-*N*-methylisonicotinate 3.5–5 mol/mol and with fumarate 2 mol/mol. Cells that had been stored as a pellet at −20°C lost their ability to oxidize 2-hydroxy-*N*-methylisonicotinate, unless they were preincubated with *N*-methylisonicotinate. Spectrophotometric examination over the range 210–340 nm, of the supernatant fluid of suspensions oxidizing either compound, did not show the presence of any other absorbing peaks.

Similarly, spectrophotometric examination and t.l.c. of the supernatant fluids of suspensions of cells in the logarithmic phase of growth and at the stationary phase, incubated under a variety of conditions of pH and buffer composition and in the presence of known enzyme inhibitors, did not reveal the presence of any compounds not present in control experiments. Of the inhibitors tested, inhibition of oxygen uptake was shown by cyanide, *p*-chloromercuribenzoate, iodoacetamide and 8-hydroxyquinoline, all at 1 mM (Table 1). No inhibition was shown with sodium arsenite, aminopterin or amethopterin at the same concentration.

Experiments with N-[^{14}C]methylisonicotinate. To study the fate of the methyl group of *N*-methylisonicotinate, *N*-[^{14}C]methylisonicotinate was incubated with a washed cell suspension in a double-side-arm Warburg apparatus. When oxidation was complete bound CO_2 was released by acidifying the reaction mixture with 0.4 ml of 3 M-HCl; the flasks were then shaken for a further 30 min to allow the CO_2 to be absorbed in the centre well. The cells were collected by centrifugation (bench centrifuge), washed

Table 1. *Effect of inhibitors on the oxidation of N-methylisonicotinate by washed cell suspensions of strain 4C1*

Single-side-arm Warburg vessels contained, in a working volume of 2.8 ml: phosphate buffer, pH 7.0, 200 μmol; cells, 7 mg dry wt.; inhibitor, 2.8 μmol; *N*-methylisonicotinate, 10 μmol. The centre well contained 0.2 ml of 20% (w/v) KOH. The reaction was started by the addition of the substrate from the side arm. Temperature, 30°C; atmosphere, air.

Inhibitor	Uptake of O_2 (μl after 15 min)
None	42
Sodium arsenite	43
Aminopterin	40
Amethopterin	43
Cyanide	3
Iodoacetamide	14
p-Chloromercuribenzoate	2
8-Hydroxyquinoline	23

Table 2. *Recovery of radioactivity from cultures oxidizing N-[^{14}C]methylisonicotinate*

Double-side-arm Warburg flasks contained, in a working volume of 2.3 ml: cells, 7 mg dry wt.; phosphate buffer, pH 7.0, 150 μmol; either *N*-[^{14}C]isonicotinate, 3 μmol (1 μCi), or *N*-methyl[*carboxy*-^{14}C]isonicotinate, 3 μmol (0.1 μCi). The centre well contained 0.2 ml of 20% (w/v) KOH. The reaction was started by the addition of the substrate from the side arm. Temperature, 30°C; atmosphere, air. When oxygen uptake was complete, the reaction mixture was acidified by the addition of 0.5 ml of 3 M-HCl from the second side arm. Radioactivity was determined on fractions prepared as described in the text.

		Percentage of initial radioactivity recovered						
					Supernatant			
Strain	*N*-Methylisonicotinate label	Total	Cells	CO_2	No treatment	Formaldehyde trapped	Methylamine trapped	Formic acid trapped
4C1	*methyl*-^{14}C	25	8	16	1	—	—	—
4C1	*methyl*-^{14}C	95	6	14	0	75	0	2
4C1	*carboxy*-^{14}C	98	3	95	0	—	—	—
4C2	*methyl*-^{14}C	95	5	89	1	—	—	—
4C2	*carboxy*-^{14}C	97	3	93	1	—	—	—

twice with 2ml of water and resuspended in 1ml of water. The washings were added to the supernatant. The filter paper from the centre well was eluted with water, and this was combined with the washings from the centre well. The three fractions, cells, supernatant and CO_2, were then plated directly and counted for radioactivity. At the end of the experiment only 25% of the radioactivity was recovered (Table 2), suggesting the loss of some volatile compound during handling as the physical loss was negligible. It was therefore decided to employ agents capable of retaining volatile C-1 compounds in solution, to prevent such loss.

The experiment was repeated with eight flasks, of which four were terminated at 30min and the rest when oxygen uptake had ceased. One flask from each series was then treated in each of the following ways. (*a*) To trap methylamine the supernatant was left acid. (*b*) Formate was trapped as the sodium salt by making the supernatant alkaline. (*c*) Formaldehyde was trapped as the dimedone derivative. The supernatant (1.0ml) was mixed with 2.0ml of 20% (w/v) dimedone in 50% (w/v) ethanol. The mixture was warmed gently for 10min and allowed to cool. (*d*) Methanol was first oxidized to formaldehyde, which was trapped as the dimedone derivative. A sample (1ml) of supernatant was added to an ice-cold mixture of conc. H_2SO_4 (1ml) and aq. satd. $K_2Cr_2O_7$ (5ml). The mixture was agitated for 5min and then diluted to 10ml and steam-distilled. The distillate was collected in a tube in an ice bath. The first 4ml of the distillate was collected and treated with 10ml of 20% (w/v) dimedone in aq. 50% (w/v) ethanol. The mixture was evaporated to dryness under reduced pressure at 40°C and the residue suspended in 1ml of water. The acid and alkaline supernatants did not retain radioactivity. Those treated with dimedone retained radioactivity even before oxidation, indicating that the radioactivity occurred in a volatile aldehyde, probably formaldehyde (Table 2).

[^{14}C]Formaldehyde was identified as a product of the reaction in a similar experiment in which 10μCi of *N*-[^{14}C]methylisonicotinate was used. Carrier formaldehyde (1mmol) was then added to the supernatant, followed by 10ml of 20% (w/v) dimedone in aq. 20% (w/v) ethanol. The mixture was warmed gently for 5min and kept at 4°C overnight. The precipitated material was filtered off and recrystallized from aq. 20% (v/v) ethanol to constant specific radioactivity (136c.p.m./mg). The m.p. of the derivative was 188°C, corresponding to that of the formaldehyde derivative (lit. 189°C); the mixed melting point was not depressed.

The distribution of radioactivity in a whole-cell system oxidizing *N*-methyl[*carboxy*-^{14}C]isonicotinate was studied, in the same manner as for *N*-[^{14}C]-methylisonicotinate. The results for incorporation of radioactivity into the cellular fraction, CO_2 and supernatant fractions are presented in Table 2. It is evident from these figures that approx. 95% of the radioactivity was liberated from the substrate as CO_2 by the time that oxygen uptake had ceased. The remaining activity was present in the cell fraction.

An attempt was made to isolate intermediates with the methyl group attached, by experiments involving short exposure to *N*-[^{14}C]methylnicotinate, followed by fractionation by the procedure of Dutton & Evans (1969). The only fraction containing appreciable radioactivity was the water-soluble fraction; the radioactivity in the neutral ether, acid ether and 24h ether extracts was extremely low. On radioautography, only two components were found in the water-soluble fraction. One of them was the substrate; the second component did not correspond to any of the substituted pyridines available for comparison and was not present in large enough quantity to isolate and characterize.

Experiments with cell-free extracts. Crude cell-free extracts were tested for their ability to oxidize the substrate and certain possible intermediate com-

Table 3. *Oxidation of potential intermediates by cell-free extracts of N-methylisonicotinate-grown strain* 4C1

Single-side-arm Warburg vessels contained, in a working volume of 2.8ml: phosphate buffer, pH7.0, 200μmol; Biox X-press cell-free extract, 17mg of protein; substrate, 1μmol. The centre well contained 0.2ml of 20% (w/v) KOH. The reaction was started by the addition of substrate from the side arm. Temperature, 30°C; atmosphere, air. Results are corrected for endogenous respiration.

	Uptake of O_2		
Substrate	After 15min (μl)	Final (μl)	mol/mol
N-Methylisonicotinate	0	0	0
2-Hydroxy-*N*-methylisonicotinate	4.5	12.4	0.55
Maleate	26	31	1.4
Maleamate	26	31	1.4
Fumarate	22	29	1.3

pounds by Warburg manometry. When the oxidation, if any, was complete, the reaction mixtures were deproteinized at neutral pH values (Orpin *et al.*, 1972) and examined by u.v. spectrophotometry. Crude cell-free extracts prepared in 67mM-phosphate buffer, pH7.0, and in 50mM-tris–HCl buffer, pH7.2, by using the Biox X-press, oxidized 2-hydroxyisonicotinate, maleamate, maleate and fumarate without lag (Table 3); *N*-methylisonicotinate, isonicotinate, 3-hydroxyisonicotinate, 2-hydroxy-*N*-methylisonicotinate, *N*-methylpyridine, 4-hydroxypyridine, 2,4-, 2,5-, 2,6- and 3,4-dihydroxypyridine, 2,6-dihydroxy-*N*-methylpyridine, 2,6-dihydroxyisonicotinate, 2,6-dihydroxy-*N*-methylisonicotinate, fumaramate or methylamine were not oxidized within 4h. The addition of NAD(P)H, FAD, FMN, GSH, L-cysteine, Cu^{2+} or Fe^{2+} to the reaction mixtures singly or in combination did not result in the oxidation of *N*-methylisonicotinate, isonicotinate, 2-hydroxy-*N*-methylisonicotinate, 2- and 4-hydroxy-*N*-methylpyridine, 2,4- and 2,6-dihydroxypyridine, 2,6-dihydroxyisonicotinate, 2,6-dihydroxy-*N*-methylisonicotinate or *N*-methylpyridine. Oxygen uptake during the oxidation of 2-hydroxyisonicotinate was 0.55mol/mol, maleamate 1.4mol/mol, fumarate 1.3mol/mol and maleate 1.4mol/mol.

Crude cell-free extracts when incubated with ATP and homocysteine in 67mM-phosphate buffer, pH7.0, did not oxidize or demethylate *N*-methylisonicotinate, 2-hydroxy-*N*-methylisonicotinate, *N*-methylpyridine, 2- and 4-hydroxy-*N*-methylpyridine, 2,4-, 2,6- and 3,4-dihydroxy-*N*-methylpyridine or 2,6-dihydroxy-*N*-methylisonicotinate. Oxidative activity was looked for by spectrophotometric examination of the deproteinized (pH7.0) supernatant, and t.l.c. in solvents (*a*), (*b*), (*c*) and (*d*) of the methanol extract of the supernatant after evaporation to dryness.

Crude cell-free extracts prepared ultrasonically in 67mM-phosphate buffer, pH7.0, could also oxidize 2-hydroxyisonicotinate; oxygen uptake was limited to 0.55mol/mol. When oxygen uptake was complete, the reaction mixture was deproteinized at neutral pH and examined spectrophotometrically. The spectrum of 2-hydroxyisonicotinate was replaced with one having extinction maxima at 234 and 343nm. On acidification to pH2 with 3M-HCl, the spectrum changed to one with $\lambda_{max.}$ 338nm; on being made alkaline (pH13) with NaOH, the solution quickly became purple. The deproteinized reaction mixture was evaporated to dryness on a water bath (40°C) under a stream of nitrogen. The residue was extracted with hot methanol (5×5ml); the combined extracts were evaporated to about 2ml, under nitrogen, on a water bath at 40°C. The concentrate was then chromatographed in solvents (*a*), (*b*) and (*d*). In all solvents only one major component was present, with R_F values similar to those of 2,6-dihydroxyisonicotinate. To obtain larger quantities of the material, the methanolic extract was chromatographed on 1mm silica-gel layers. When solvent (*a*) was used, however, the material became grey on the chromatography plates when they were dried in an air current. To avoid this, the area corresponding to R_F 0.55–0.72 was scraped off and eluted with 1M-acetic acid before the solvent evaporated from the plate. The acetic acid was then removed by bubbling nitrogen through the eluate in a test tube until 5min after no more acetic acid could be smelt. The liquid was then concentrated under nitrogen at 40°C, and the resulting pale-brown solid dried over P_2O_5. Infrared and u.v. spectra were identical with those of authentic 2,6-dihydroxyisonicotinate. The u.v.-spectral characteristics of authentic 2,6-dihydroxyisonicotinate were found to be: (*a*) at pH1, $\lambda_{max.}$ 339nm, ϵ_{339} 2900; (*b*) at pH7, $\lambda_{max.}$ 234nm and 343nm, ϵ_{234} 6150 and ϵ_{343} 5050. The characteristics of the isolated material were: (a) at pH1, $\lambda_{max.}$ 338nm, ϵ_{338} 2800; (b) at pH7, $\lambda_{max.}$ 234nm and 343nm, ϵ_{234} 6100 and ϵ_{343} 4950. The melting points of the isolated material (295°C, decomp.) and 2,6-dihydroxyisonicotinate (300°C, decomp.) showed close agreement, and their mixed melting point was not depressed (295°C, decomp.). 2,6-Dihydroxyisonicotinate was also generated anaerobically if Methylene Blue was added to the system; no loss of activity occurred if the crude cell-free extract was dialysed for 14h at 4°C against 10mM-phosphate buffer, pH7.0. Cell-free extracts centrifuged at 38000*g* for 30min did not hydroxylate 2-hydroxyisonicotinate, either aerobically or anaerobically.

Cell-free extracts prepared ultrasonically in either 67mM-phosphate buffer, pH7.0, or 50mM-tris–HCl buffer, pH7.5, and centrifuged at 38000*g* for 30min were found manometrically to oxidize maleamate (1.8mol/mol), maleate (1.7mol/mol) and fumarate (1.6mol/mol) but not fumaramate. To determine the reaction products with maleamate, a cell-free extract was prepared in phosphate buffer, incubated in double-side-arm Warburg flasks and the ammonia measured by the method described by Orpin *et al.* (1972). After the liberation of ammonia and equilibration, the reaction mixture was neutralized with 2M-HCl, deproteinized at neutral pH and the supernatant assayed for maleamate. It was found that for each mol of maleamate consumed, 1mol of ammonia was liberated. No deamidation of fumaramate occurred under similar conditions. This observation, combined with the observation that neither whole cells nor extracts oxidized fumaramate, suggests that deamidation to maleate, rather than isomerization to fumaramate, is the first step. To isolate the reaction products, maleamate (50mmol) was incubated with 10ml of cell-free extract (38000*g* supernatant containing 250mg of protein) and 10mM-*meso*-tartrate (an inhibitor of fumarase) in a total volume of 15ml of 67mM-phosphate buffer, pH7.0. After 2h the whole

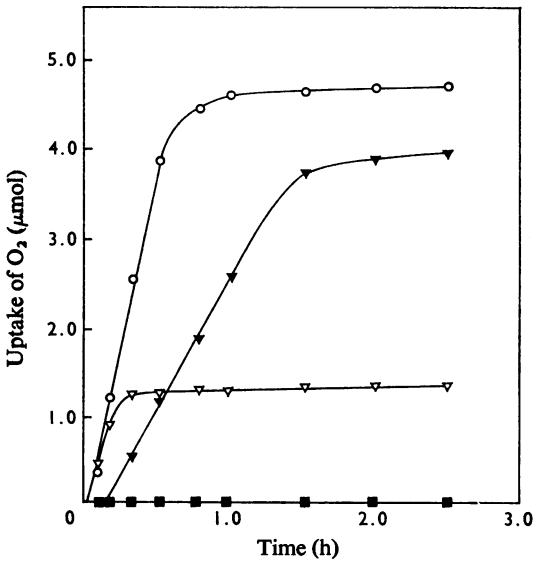


Fig. 2. *Oxidation of N-methylisonicotinate and potential metabolites by washed suspensions of N-methylisonicotinate-grown strain 4C2*

Single-side-arm Warburg flasks contained, in a working volume of 2.8ml: phosphate buffer, pH6.8, 200μmol; cells, 7mg dry wt.; substrate, 1μmol. The centre well contained 0.2ml of 20% (w/v) KOH. The reaction was started by the addition of the substrate from the side arm. Temperature, 30°C; atmosphere, air. Corrected for endogenous respiration. The substrates were: ○, *N*-methylisonicotinate; ▼, *N*-methylpyridine; ▽, methylamine; ■, 2,6-dihydroxyisonicotinate, 2,4-, 2,6- and 3,4-dihydroxypyridine, 2- and 4-hydroxypyridine and their *N*-methyl derivatives, isonicotinate, pyridine, 3-hydroxyisonicotinate, 2,3- and 2,5-dihydroxypyridine, fumaramate, maleate and maleamate.

reaction mixture was deproteinized and the supernatant fluid extracted with diethyl ether (5×10ml). The extract was evaporated to dryness and the residue dissolved in 2ml of aq. 0.1M-NH_3 soln. A control experiment, containing no maleamate, was treated similarly. The extracts were chromatographed in solvent (*d*). Two compounds not present in the control were detected by spraying with the potassium permanganate reagent. The two compounds ran at the same R_F values as fumarate and maleate respectively. The extract was then chromatographed as a band in solvent (*d*), and the areas corresponding to the two components were scraped off separately and eluted with water. The eluates were concentrated under nitrogen and chromatographed in solvents (*a*), (*c*) and (*d*). The material corresponding to fumarate was inseparable from authentic fumarate in all the solvent systems; the material corresponding to maleate co-chromatographed with authentic maleate in all the solvent systems.

The pH optimum of the enzyme was then determined, the liberation of ammonia from maleamate being used as the criterion of activity. The enzyme was found to have a broad pH optimum, with maximum activity between pH8.0 and 9.0.

Oxidation of N-methylisonicotinate by strain 4C2

The ability of washed cell suspensions of strain 4C2 to oxidize various possible intermediate compounds was studied manometrically, followed by the spectrophotometric investigation of the supernatant after the removal of the cells by centrifugation. *N*-Methylisonicotinate, fumarate and methylamine were oxidized without lag, with oxygen uptakes of 4.5, 2.3 and 1.2mol/mol respectively (Fig. 2). *N*-Methylpyridine was oxidized after a very short lag period of about 5min. Isonicotinate, pyridine, 2-hydroxy-*N*-methylisonicotinate, 2-, 3- and 4-hydroxypyridine, 2-, 3- and 4-hydroxy-*N*-methylpyridine 2,3-, 2,5- and 2,6-dihydroxypyridine, 2,6- and 3,4-dihydroxy-*N*-methylpyridine, 3,4-dihydroxy-*N*-methylisonicotinate, fumaramate, maleamate and maleate were not oxidized within 4h. Glucose-grown cells would oxidize only fumarate initially; no other compounds were oxidized except *N*-methylpyridine after a lag of 2h.

Since none of the heterocyclic compounds, with the exception of *N*-methylisonicotinate, was oxidized immediately by washed suspensions of *N*-methylisonicotinate-grown cells, it was decided to test their ability to oxidize certain aliphatic compounds that might be derived from the substrate after ring-fission. The rates of oxidation of succinate and malate were threefold and twofold respectively the rates of oxidation by succinate-grown cells. There was no difference in the rates of oxidation of fumarate, citrate, itaconate, oxaloacetate, glyoxylate, glycollate, pyruvate, acetate, propionate or butyrate. These differences might indicate succinate or some related compound to be the end product of *N*-methylisonicotinate oxidation.

The supernatant fluids of cultures actively metabolizing *N*-methylisonicotinate were examined for the presence of compounds derived from the substrate. Cultures were harvested either during the exponential phase of growth or during the resting stage, and the cells were removed by using the Sharples centrifuge. The supernatant fluid (5 litres) was evaporated to dryness at 30–40°C in a rotary evaporator. The solid was extracted with hot methanol (5×25ml), the extracts were combined, and the volume was decreased under nitrogen to about 10ml. This was chromatographed in solvents (*a*), (*b*), (*c*) and (*d*).

The chromatograms were examined in u.v. light before and after spraying with fluorescein; they were sprayed with Folin–Ciocalteu reagent or with ferric chloride. No phenolic compounds were detected and the only compound visible in u.v. light was identified as *N*-methylisonicotinate.

Experiments with N-[^{14}C]methylisonicotinate. Whole-cell experiments conducted as described above for strain 4C1 to determine the fate of ^{14}C from labelled *N*-methylisonicotinate showed that both the *N-methyl*-^{14}C and the *carboxy*-^{14}C were converted into carbon dioxide (Table 2). Not more than 5% of the radioactivity of either methyl-labelled material or carboxyl-labelled material was incorporated into the cellular material. The addition of *N*-methylpyridine, isonicotinate or 2-hydroxy-*N*-methylisonicotinate to the supernatant of cells oxidizing *N*-[^{14}C]methylisonicotinate did not trap any labelled material, as might have occurred if any labelled *N*-methylpyridine, isonicotinate or 2-hydroxy-*N*-methylisonicotinate had been released from the cells in quantities too small to be detected by chromatography of the untreated supernatant. Similarly, no additional radioactivity was retained in the supernatant of cell suspensions oxidizing *N*-methyl[*carboxy*-^{14}C]isonicotinate in the presence of isonicotinate or 2-hydroxyisonicotinate.

Experiments with cell-free extracts. Crude cell-free extracts oxidized succinate, succinic semialdehyde, methylamine, formaldehyde and formate without lag. Oxygen uptake terminated at 1.1 mol/mol with succinate, 1.4 mol/mol with succinic semialdehyde, 1.3 mol/mol with methylamine, 0.8 mol/mol with formaldehyde and 0.5 mol/mol with formate. *N*-Methylisonicotinate, isonicotinate, 2-hydroxyisonicotinate, 2-hydroxy-*N*-methylisonicotinate, 3-hydroxyisonicotinate, 2-, 3- and 4-hydroxypyridine, 2-, 3- and 4-hydroxy-*N*-methylpyridine, 2,3-, 2,4-, 2,5-, 2,6- and 3,4-dihydroxypyridine, 2,6-dihydroxyisonicotinate, 2,4-, 2,6- and 3,4-dihydroxy-*N*-methylpyridine, 2,6-dihydroxy-*N*-methylisonicotinate, pyridine, maleamate, fumaramate, maleate and methanol were not oxidized.

The addition of NAD(P)$^+$, GSH, L-cysteine, Cu^{2+} or Fe^{2+} to reaction mixtures did not result in the oxidation of any of the heterocyclic compounds by extracts prepared with the Biox X-press, Hughes (1951) press or ultrasonic disintegrator and sodium phosphate, tris–HCl or tris–maleate buffers. The rates of methylamine and succinic semialdehyde oxidation could, however, be augmented by the addition of NAD$^+$.

To identify the reaction products of methylamine oxidation a flask containing the crude cell-free extract prepared from 0.8 g dry wt. of cells (22 ml of suspension) was incubated with shaking in air at 30°C with methylamine hydrochloride (100 μmol) and 20 μmol of NAD$^+$. The methylamine concentration was determined at intervals on samples taken from the reaction mixture. When the methylamine had disappeared, the reaction mixture was deproteinized with trichloroacetic acid, centrifuged at 18000*g* for 30 min, and the supernatant assayed for formaldehyde, formate and methanol. None of them was detected, and it therefore appeared that the oxidation of methylamine had gone to completion. Manometric experiments showed that for each mol of methylamine oxidized, 1 mol of carbon dioxide and 1 mol of ammonia were liberated.

After dialysis of the crude extract against 200 vol of 10 mM-phosphate buffer, pH 7.0, for 16 h at 4°C, no oxidation of methylamine occurred, even if the extract was supplemented with NAD$^+$. If, however, ferricyanide was added, a small amount of oxidation occurred, and if NAD$^+$ and ferricyanide were added together, then full activity could be restored. The dialysed extract would not oxidize formaldehyde or formate unless supplemented with NAD$^+$; addition of ferricyanide had no effect on the rate of oxidation of formaldehyde and formate. It therefore appeared that the ferricyanide was essential for the oxidation of methylamine, even though only a little methylamine oxidation occurred when only ferricyanide was added. After the crude extract had been centrifuged at 100000*g* for 30 min, it oxidized only formaldehyde and formate. In the presence of ferricyanide only 10% of the added methylamine was oxidized, but if NAD$^+$ was added in excess, then methylamine was oxidized completely. Formaldehyde oxidation required 2 mol of NAD$^+$/mol, and that of formate, 1 mol/mol.

In view of the fact the NAD$^+$ was essential for the complete oxidation of methylamine but not for its partial oxidation, and that NAD$^+$ was required for the removal of formaldehyde from the system, it was thought that formaldehyde might be inhibiting the methylamine-oxidizing system. When formaldehyde and methylamine were added together to unsupplemented extracts, it was found that the formaldehyde was oxidized immediately but the methylamine was oxidized only when the formaldehyde had disappeared. The addition of NAD$^+$ only affected the rate of oxidation of both compounds, and not the order in which they were oxidized. If methylamine and formaldehyde were added to dialysed crude cell-free extract supplemented with ferricyanide, no oxidation of either methylamine or formaldehyde occurred. Further, if formaldehyde was added in excess to a system oxidizing methylamine, the oxidation of methylamine stopped immediately.

Succinic semialdehyde-oxidizing activity was lost after the cell-free extract was dialysed for 14 h against 10 mM-phosphate buffer, pH 7.0, but was regained by the addition of NAD$^+$ to the reaction mixture. The enzyme was precipitated between 50 and 60% saturation with ammonium sulphate. To identify the

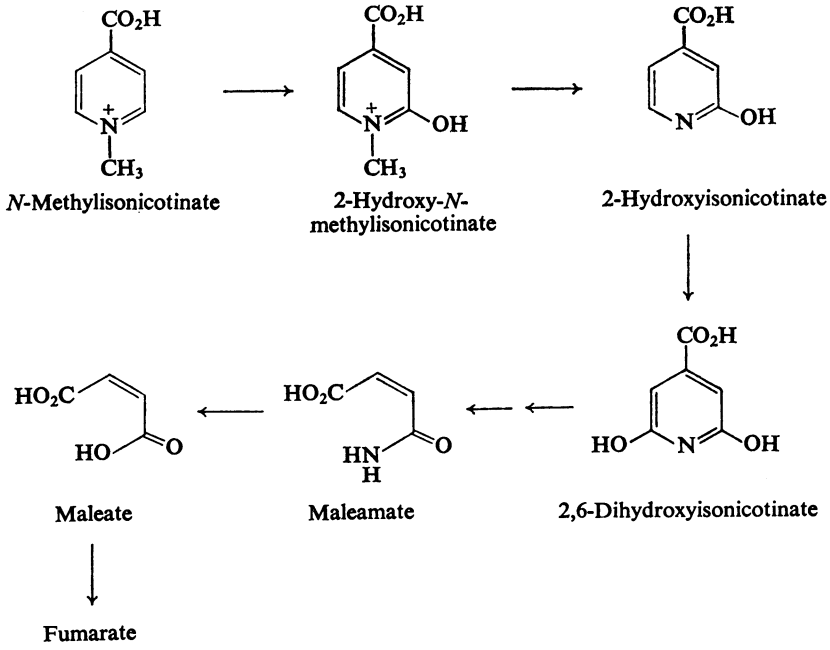
reaction products, the partially purified enzyme (10mg of protein) was incubated in a volume of 15ml with 0.75mmol of phosphate buffer, pH7.0, 50μmol of succinic semialdehyde and 50μmol of NAD^+. The reaction was monitored spectrophotometrically, and when E_{340} had reached a maximum the reaction mixture was deproteinized. The supernatant was evaporated to dryness under reduced pressure and the residue extracted with diethyl ether (5×10ml). The extract was concentrated and chromatographed in solvents (*a*) and (*d*). The only compound present that did not occur in the control co-chromatographed with succinate.

An experiment was also performed in a volume of 3.0ml containing 100μmol of phosphate buffer, pH7.0, 3mg of enzyme protein, 1μmol of succinic semialdehyde and 1μmol of NAD^+. The change in E_{340} was measured; purified extracts from *N*-methylisonicotinate-grown cells oxidized succinic semialdehyde at a rate of 1.36μmol/min per mg of protein and those from succinate-grown cells at 0.08μmol/min per mg of protein.

Discussion

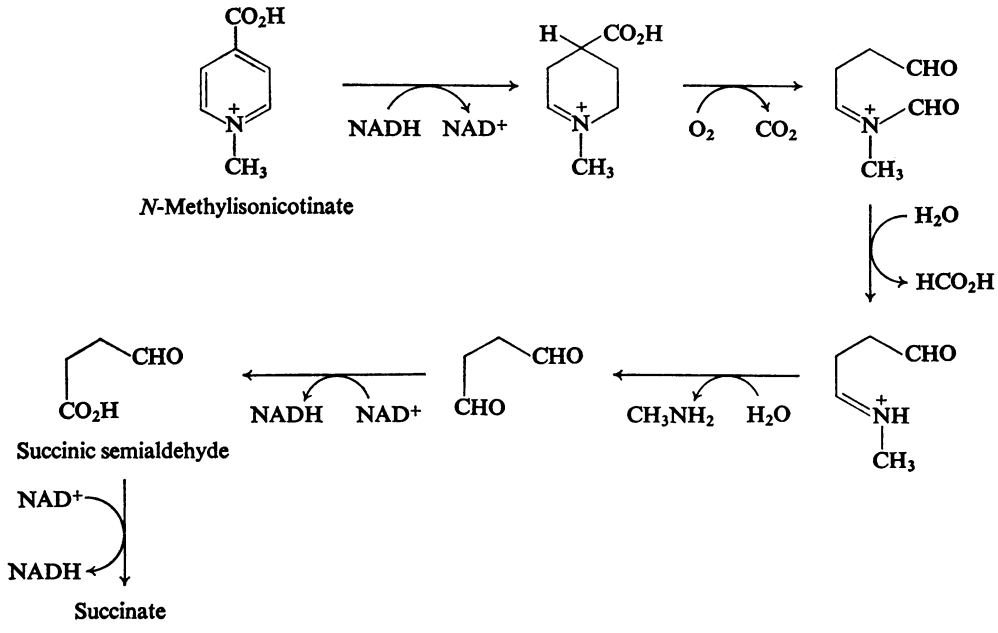
There appear to be two pathways by which *N*-methylisonicotinate may be oxidized: one as in strain 4C1 involving removal of the methyl group and oxidation to formaldehyde before ring-fission, and the other, as in strain 4C2, where ring-fission probably occurs before the liberation of methylamine from the *N*-methyl group of the aliphatic ring-fission product. With strain 4C2 no hydroxylation of the pyridinium ring has been demonstrated, and none of the monohydroxylated *N*-methylpyridines available was oxidized either by whole cells or by cell-free extracts alone or supplemented with a range of cofactors.

The first step in the catabolism of *N*-methylisonicotinate by strain 4C1 appears to be a hydroxylation at C-2, since 2-hydroxy-*N*-methylisonicotinate is oxidized without lag by whole cells; this step was not demonstrated enzymically. Since 2-hydroxyisonicotinate was oxidized in cell-free extracts, it seems likely that 2-hydroxy-*N*-methylisonicotinate is the substrate for demethylation. This, again, could not be demonstrated in cell-free extracts, so no results can be presented on the mechanism of demethylation. Ribbons (1971) has reported that the activity of many *O*-demethylases is very rapidly lost on exposure to air; *N*-demethylases may be similarly sensitive. That the methyl group is converted into formaldehyde has been shown by the isolation of [^{14}C]formaldehyde from cells incubated with *N*-[^{14}C]methylisonicotinate. 2-Hydroxyisonicotinate is hydroxylated by the crude cell-free extract; no requirement for any diffusible cofactor was demonstrated. The hydroxylation seems to be a hydration followed by dehydrogenation; the reaction occurs either anaerobically,



N-Methylisonicotinate — 2-Hydroxy-*N*-methylisonicotinate — 2-Hydroxyisonicotinate

Scheme 1. *Proposed pathway of oxidation of N-methylisonicotinate by strain 4C1*



Scheme 2. *Pathway proposed for N-methylisonicotinate oxidation by Achromobacter D (Cain et al., 1970)*

in the presence of an electron acceptor, or aerobically in the presence of a particulate (38000*g*) fraction. The nicotinate hydroxylase and 6-hydroxynicotinate hydroxylase of Ensign & Rittenberg (1964) are operative under the same conditions, but the particulate fraction was sedimented at 105000*g*. Since maleamate is oxidized in cell-free extracts, ring-fission is probably between C-2 and C-3 of the pyridine ring. That fumaramate is not oxidized suggests that the carboxyl atom at C-4 is removed either before or immediately after ring-fission, and that maleamate is not isomerized to fumaramate before deamidation. Neither the ring-fission substrate nor product could be identified, so no evidence on whether the carboxyl carbon atom at C-4 is removed before fission or immediately after fission has been obtained. That this carbon atom is not incorporated into the fumarate is demonstrated by the very low incorporation of radioactivity from *N*-methyl[*carboxy*-^{14}C]isonicotinate into cellular constituents. The proposed pathway for *N*-methylisonicotinate by strain 4C1 is depicted in Scheme 1.

Wright & Cain (1970) and Cain *et al.* (1970) have studied the oxidation of *N*-methylisonicotinate in *Achromobacter* D. No hydroxylating activity could be demonstrated in their cell-free extracts that were capable of releasing methylamine from the *N*-methylisonicotinate when supplemented with NAD^+ or NADH, even though concomitant oxygen uptake of 1 mol/mol was recorded. Further examination of the system showed NADH to be the active nucleotide; it was oxidized to NAD^+ and then regenerated. The carboxyl group on C-4 of the pyridinium ring was shown to be liberated as CO_2 and *N*-[2,3,5,6-^{14}C]-methylisonicotinate gave rise to [^{14}C]formate and [^{14}C]succinate in cell-free extracts supplemented with NAD^+. The formate arose from C-2 and the succinate from C-3 and C-6. It was suggested that the pyridinium ring is first partly reduced and then cleaved by an oxygenase to form a dialdehyde, which is hydrolysed to liberate formate, methylamine and succinic semialdehyde (Scheme 2). Cells grown on *N*-methylisonicotinate had succinic semialdehyde-oxidizing activities some 15-fold those prepared from succinate-grown cells.

The lack of any hydroxylating capacity in cell-free extracts of strain 4C2, the lack of oxidation by whole cells of any of the mono- or di-hydroxylated derivatives that were available, and the ability to convert succinic semialdehyde into succinate by cell-free extracts, is in agreement with the results obtained with *Achromobacter* D.

It is difficult to explain why no activity could be demonstrated towards *N*-methylisonicotinate in cell-free extracts supplemented with NAD^+ or NADH. If the pathway is the same, the enzyme responsible for the partial reduction of the pyridinium ring and the oxygenase responsible for ring-fission may be less stable than those induced in *Achromobacter* D.

In both systems, succinic semialdehyde is converted

into succinate; purified cell-free extracts of strain 4C2 grown on *N*-methylisonicotinate oxidized succinic semialdehyde 17 times more rapidly than purified cell-free extracts of succinate-grown cells. The generation of succinate explains the increase in succinate oxidation by strain 4C2 cells grown on *N*-methylisonicotinate over those grown on glucose, although the threefold increase in succinate oxidation is not as high as obtained with *Achromobacter* D. Both *Achromobacter* D and strain 4C2 release the ring nitrogen atom as methylamine; *Achromobacte* D, however, does not oxidize methylamine whereas strain 4C2 does, the pathway progressing via formaldehyde and formate to CO_2. Methanol is apparently not an intermediate. Although each enzyme was not purified, the results suggest that methylamine is oxidized to formaldehyde by a soluble enzyme, which can utilize ferricyanide but not oxygen as an oxidizing agent. This reaction is similar to the methylamine dehydrogenase of Eady & Large (1968), though their enzyme was not subject to product-inhibition as is the enzyme present in strain 4C2. The formaldehyde generated from methylamine is oxidized to formate. This reaction required NAD^+ for activity; formate, too, was oxidized by an NAD^+-dependent system, and both systems required NAD^+ in stoicheiometric amounts.

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References

Albert, A. & Phillips, J. N. (1956) *J. Chem. Soc. London* 1294

Baumler, J., Sorkin, E. & Erlenmeyer, H. (1951) *Helv. Chim. Acta* **34**, 496

Behrman, E. J. & Pitt, B. M. (1958) *J. Amer. Chem. Soc.* **80**, 3717

Boekelheide, V. & Lehn, W. L. (1961) *J. Org. Chem.* **26**, 428

Buchi, J., Labhart, P. & Ragaz, L. (1947) *Helv. Chim. Acta* **30**, 507

Cain, R. B., Wright, K. A. & Houghton, C. (1970) *Meded. Rijksfac. Landbouwwetensch., Gent* **35**, 785

den Hertog, H. J. & Buurman, D. J. (1956) *Rec. Trav. Chim. Pays-Bas* **75**, 257

Dixon, M. (1952) *Manometric Methods*, p. 51, Cambridge University Press, London

Dubin, D. T. (1960) *J. Biol. Chem.* **235**, 783

Duesel, B. F. & Scudi, J. V. (1949) *J. Amer. Chem. Soc.* **71**, 1866

Dutton, P. L. & Evans, W. C. (1969) *Biochem. J.* **113**, 525

Eady, R. R. & Large, P. J. (1968) *Biochem. J.* **106**, 245

Eegriwe, E. (1937) *Z. Anal. Chem.* **110**, 22

Ensign, J. C. & Rittenberg, S. C. (1964) *J. Biol. Chem.* **239**, 2285

Fronk, M. H. & Mosher, H. S. (1959) *J. Org. Chem.* **24**, 196

Gomori, G. (1955) *Methods Enzymol.* **1**, 138

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751

Greiss, P. (1879) *Chem. Ber.* **12**, 2117

Hughes, D. E. (1951) *Brit. J. Exp. Pathol.* **32**, 97

MacFayden, D. A., Watkins, H. D. & Anderson, P. R. (1945) *J. Biol. Chem.* **168**, 107

Orpin, C. G., Knight, M. & Evans, W. C. (1971) *Biochem. J.* **122**, 58P

Orpin, C. G., Knight, M. & Evans, W. C. (1972) *Biochem. J.* **127**, 819

Ribbons, D. W. (1971) *FEBS Lett.* **12**, 161

Slade, P. (1965) *Nature (London)* **207**, 515

Slade, P. (1966) *Weed Res.* **6**, 158

Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957) *Manometric Techniques*, 3rd edn., p. 168, Burgess Publishing Co., Minneapolis

von Schickh, O., Binz, A. & Schulz, A. (1936) *Chem. Ber.* **69**, 2593

Wibaut, J. P. & Kleipool, R. J. C. (1947) *Rec. Trav. Chim. Pays-Bas* **66**, 24

Wright, K. A. & Cain, R. B. (1969) *Soil Biol. Biochem.* **1**, 5

Wright, K. A. & Cain, R. B. (1970) *Biochem. J.* **118**, 52P