Biochem. J. (1972) **128**, 561–568
Printed in Great Britain

Microbial Metabolism of Pyridinium Compounds

RADIOISOTOPE STUDIES OF THE METABOLIC FATE OF 4-CARBOXY-1-METHYLPYRIDINIUM CHLORIDE

By K. A. WRIGHT and R. B. CAIN*
Microbiology Group, Department of Botany, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, U.K.

(*Received* 17 *December* 1971)

Extracts of *Achromobacter* D formed CO_2, methylamine, succinate and formate as metabolic end-products from *N*-methylisonicotinic acid (4-carboxy-1-methylpyridinium chloride). The origin of the CO_2 in the 4-carboxyl group and of the methylamine in the *N*-methyl group of *N*-methylisonicotinate was demonstrated with *carboxyl*-^{14}C- and *N-Me*-^{14}C-labelled substrates respectively. The carbon skeletons of formate and succinate were shown to arise from the C-2 and the C-3–C-6 atoms of the heterocyclic ring respectively by using *N*-methyl[2,3-$^{14}C_2$]isonicotinate. This result is consistent with ring cleavage by the organism between C-2 and C-3.

Suitably supplemented extracts of *Achromobacter* D degraded the heterocyclic ring of *N*-methylisonicotinic acid (4-carboxy-1-methylpyridinium chloride) to formate, succinate and methylamine; the 4-carboxyl group was released as CO_2. The only precursor of these end-products so far implicated either by chemical identification or by the enzyme pattern in *N*-methylisonicotinate-grown cells was succinic semialdehyde (Wright & Cain, 1972). The biodegradation of *N*-methylisonicotinate to succinic semialdehyde could be effected in several ways, but the characterization of a C_4 and a C_1 product from *N*-methylisonicotinate suggested a cleavage between C-2 and C-3 of the pyridine ring. To confirm the possible site of cleavage of the heterocyclic ring, as well as the origin of some of the other metabolic products in the *N*-methylisonicotinate molecule, we have examined the radioactive products, and in the case of succinate the distribution of radioactivity among the carbon atoms, arising from *N*-methylisonicotinate appropriately labelled with ^{14}C.

Materials and Methods

Organism

The origin of *Achromobacter* D, the method of growing it in large batches and the preparation and fractionation of extracts from it are described in the preceding paper (Wright & Cain, 1972).

Manometry

O_2 uptake and CO_2 output by washed cells and extracts were measured by conventional Warburg manometry in flasks of approx. 20ml capacity at 30°C. The centre well contained, where appropriate, 0.2ml of 20% (w/v) KOH for trapping CO_2, and the total fluid volume was adjusted to 3ml.

Chemicals

N-Methylisonicotinic acid was synthesized by methylating isonicotinic acid with methyl iodide in dimethylformamide and passing the product down a column of De-Acidite FF (Cl^- form) as described by Wright & Cain (1969). *N*-Methylisonicotinic acid labelled in the *N*-methyl group was prepared similarly with [^{14}C]methyl iodide (20Ci/mol) obtained from The Radiochemical Centre, Amersham, Bucks., U.K. This material, after purification and dilution with *N*-methylisonicotinate, had a specific radioactivity of 3.3mCi/mol. *N*-Methylisonicotinic acid labelled in the 4-carboxyl group was prepared identically, starting from [*carboxyl*-^{14}C]isonicotinic acid (The Radiochemical Centre) and unlabelled methyl iodide, to give a product of specific radioactivity 0.1Ci/mol. *N*-Methyl[2,3-$^{14}C_2$]isonicotinic acid was given by Dr. B. C. Baldwin of I.C.I. Jealott's Hill Research Station, Bracknell, Berks., U.K., who prepared it from *NN'*-dimethyl[2,3,2',3'-$^{14}C_4$]bipyridinium chloride (paraquat) (Dunn *et al.*, 1966) by u.v. photolysis (Slade, 1965). The photolytic products were chromatographed on paper in solvent B (described below) and the labelled *N*-methylisonicotinic acid was eluted. Rechromatography of a sample on a Whatman no. 4 paper strip followed by radioactivity counting in a Tracerlab strip scanner showed only one significant radioactive component (Fig. 1), which was not further purified, but which was diluted with *N*-methylisonicotinic acid to a final specific radioactivity of

*Present address: Biological Laboratories, University of Kent, Canterbury, Kent, U.K.

1.05×10^4 d.p.m./μmol. The labelled paraquat from which this compound was produced, was synthesized from [1,2-$^{14}C_2$]acetylene (i.e. uniformly labelled) and the synthetic route used (Dunn *et al.*, 1966) maintains this uniform labelling pattern. Because the ring of *N*-methylisonicotinic acid is symmetrical about the N-1–C-4 axis, the radioactive product is effectively *N*-methyl[2,3,5,6-$^{14}C_4$]isonicotinic acid. We have continued to assume that each of the carbon atoms in the ring-labelled material has the same specific radioactivity.

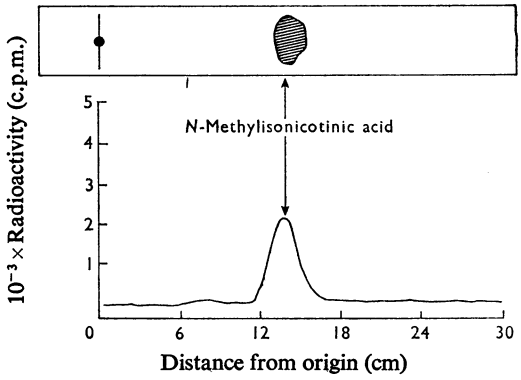


Fig. 1. *Radiochemical purity of N-methyl*[2,3-$^{14}C_2$]-*isonicotinic acid produced by ultraviolet photolysis of* [2,3,2′,3′-$^{14}C_4$]*paraquat*

For experiment details see the text.

Chromatography

The following solvent systems were used: A, ethanol–conc. NH_3 (sp.gr. 0.88)–water (20:1:4, by vol.) and B, butan-1-ol–acetic acid–water (4:1:2, by vol.).

Radioactivity measurements

Areas of radioactivity on paper chromatograms were accurately detected with a Tracerlab (Weybridge, Surrey, U.K.) 4π chromatogram strip scanner with a gas mixture of propane+argon (2:98) at a flow rate of 350ml/min.

Aqueous samples (0.1ml) were counted for radioactivity in a Packard Tri-Carb scintillation counter (model 3003 with model 526 automatic control) in screw-cap glass vials containing 2ml of 2-methoxyethanol and 3ml of scintillator [2,5-diphenyloxazole

Table 1. *Release of* $^{14}CO_2$ *from N-methyl*[*carboxyl-*^{14}*C*]*isonicotinate by extracts of Achromobacter D*

Double side-arm Warburg flasks contained: 2M-H_2SO_4 in the second side-arm, 0.2ml; 30% (w/v) KOH in the centre well (0.2ml); phosphate buffer, pH7.6, 100μmol; *N*-methyl[*carboxyl-*^{14}C]isonicotinate, 2μmol (50000d.p.m.); crude extract equivalent to 18mg of protein, or ammonium sulphate-fractionated extract equivalent to 30mg of protein (total volume 3ml). The reaction was followed by measuring O_2 uptake and, when the reaction was complete, bound CO_2 was released by tipping H_2SO_4 from the second side-arm. Flasks were shaken for a further 15min for KOH to absorb this bound CO_2 and samples were taken for counting of radioactivity.

Extract	Supplement	O_2 uptake (μmol)	$10^{-2} \times {}^{14}CO_2$ released (d.p.m.)	Recovery of added radioactivity in $^{14}CO_2$ (%)
Crude	None	0	10	2
Crude	NADH-generating system	2.4	331	66
Crude	NADH (2μmol)	2.5	440	88
Crude (boiled)	NADH-generating system	0	0	0
Crude	NAD^+ (1μmol)	2.5	375	75
30–50%-satd. fraction (F_2)*	NAD^+ (1μmol)	2.0	355	71
50–70%-satd. fraction (F_3)*	NAD^+ (1μmol)	0	43	9
50–70%-satd. fraction (F_3)*	NADH (1μmol)	1.9	360	72

* Fraction precipitated from crude extracts at 4°C by saturation within the indicated limits with ammonium sulphate (Wright & Cain, 1972).

(5g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.3g) per litre of toluene]. Corrections for counting efficiency were made for each sample by the channels-ratio method (Herberg, 1965). A minimum of 10000 counts was measured.

Radioactive CO_2 released by substrate metabolism was trapped in 20% (w/v) KOH in the centre well of conventional Warburg flasks, any residual CO_2 absorbed in the weakly alkaline (pH7.6) buffer solution of the main compartment being displaced by addition of 0.2ml of 2M-H_2SO_4. The filter-paper strip was transferred to a graduated tube and the centre well washed with 3×0.3ml vol. of water, the washings were added to the graduated tube and the contents (including the filter-paper strip) were made up to a known volume. Portions (0.1ml) of this solution were counted for radioactivity.

Degradation of [^{14}C]succinate

The carboxyl groups of labelled succinate were degraded to CO_2 by treating with NaN_3 and fuming H_2SO_4 essentially as described by Phares & Long (1955). Batches of succinic acid (25mg) were degraded in a pear-shaped flask (10ml capacity) attached to an acid–permanganate scrubber (10ml of 5M-H_2SO_4 and 5g of $KMnO_4$). The effluent CO_2 was bubbled into 30% (w/v) KOH (1ml) and the radioactivity in 0.1ml portions of this solution measured by scintillation counting.

Determinations

Protein, succinate, methylamine and formate were determined as described by Wright & Cain (1972).

Results

Origin of metabolic CO_2 in the carboxyl group of N-methylisonicotinate

Among the four end-products formed by the action of NAD^+- or NADH-supplemented extracts on *N*-methylisonicotinate, CO_2 was believed to arise exclusively from the 4-carboxyl group, although an additional contribution could have come from some further oxidation of the formate or succinate also produced by these extracts.

This problem was investigated by oxidizing *N*-methylisonicotinate, labelled either in the ring or in the substituent groups, with whole cells, with crude extracts, or alternatively with the 50–70%-saturated ammonium sulphate (F_3) fraction from these extracts. This fraction formed little formate and no succinate from *N*-methylisonicotinate (Wright & Cain, 1972), but consumed O_2 and released CO_2 when supplemented only with NADH. Washed cell suspensions released 80% of the radioactivity as CO_2 from *N*-methyl[*carboxyl*-^{14}C]isonicotinate but less than 3% of the radioactivity from the 2,3-$^{14}C_2$ and *N*-*Me*-^{14}C substrates, confirming the origin of the CO_2 in the 4-carboxyl group of this substrate. Recoveries of 65–90% of the available radioactivity as CO_2 were also made from *N*-methyl[*carboxyl*-^{14}C]isonicotinate when appropriate supplemented extracts were used (Table 1). The F_3 fraction of extracts (Wright & Cain, 1972) produced CO_2 rapidly only when the specific requirement for NADH was satisfied (Fig. 2). NAD^+ would not replace NADH for either O_2 uptake (Wright & Cain, 1972) or CO_2 production by this extract fraction.

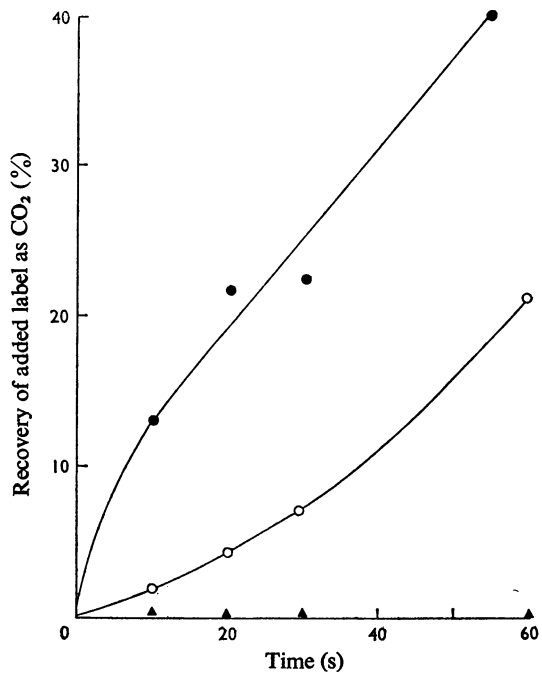


Fig. 2. *Formation of $^{14}CO_2$ from N-methyl[carboxyl-^{14}C]isonicotinate by extracts of Achromobacter D*

Duplicate double-side-arm Warburg flasks contained: crude extract equivalent to 15mg of soluble protein; *N*-methyl[*carboxyl*-^{14}C]isonicotinate, 2μmol (262000d.p.m./μmol); NAD^+ or NADH, 2μmol; phosphate buffer, pH7.6, 100μmol and 20% KOH in the centre well, 0.2ml. The total volume was 3ml. Portions (0.1ml) of the KOH solution from separate flasks were counted for radioactivity after the reaction had been stopped at suitable intervals by tipping 2M-H_2SO_4 from the second side-arm and shaking for a further 15min to allow the KOH to absorb any bound CO_2. Extract supplemented with ●, NADH; ○, NAD^+; ▲, no supplement.

Since both crude and fractionated extracts produced negligible $^{14}CO_2$ from the ring or methyl group, and gave comparably high recoveries of $^{14}CO_2$ only from *N*-methyl[*carboxyl*-^{14}C]isonicotinate, we conclude that the 4-carboxyl group of this substrate is the major source of the metabolic CO_2.

Origin of methylamine in the N-methyl group of N-methylisonicotinate

The oxidation of *N*-methylisonicotinate by washed cell suspensions yields methylamine (Wright & Cain, 1969). Several putative precursors of this primary amine, such as *N*-methylmaleamic acid or *N*-methylsuccinamic acid, were not oxidized by washed suspensions or by supplemented extracts, nor did they give rise to methylamine under conditions where its recovery from controls containing *N*-methylisonicotinate was complete (Wright & Cain, 1972). That this methylamine originated in the *N*-methyl group of *N*-methylisonicotinate after the enzymic action of extracts was confirmed by trapping the amine as *N*-methylphthalimide (Table 2), after which the derivative was crystallized to constant specific radioactivity. No radioactivity from the *N*-methyl group was trapped in the KOH of the Warburg flasks, confirming that the methyl group was not further oxidized by these extracts and did not contribute to the CO_2 found as a metabolic product.

Origin of succinate in the pyridine ring of N-methylisonicotinate

The identification of the acidic end-product of *N*-methylisonicotinate metabolism by supplemented extracts as succinate, rather than the unsaturated analogues maleate or fumarate expected from the aromatic pyridine ring (Wright & Cain, 1972), led us to re-confirm this identification with an isotopic-trapping experiment. Labelled succinate was formed in an incubation mixture by the oxidation of 20μmol of *N*-methyl[2,3-$^{14}C_2$]isonicotinate (2.1×10^5 d.p.m.) with NAD^+-supplemented extracts. When O_2 uptake ceased, the mixture was deproteinized and to half of the supernatant, succinic acid (520mg) was added and dissolved. The succinic acid was extracted from the acidified supernatant with diethyl ether, recovered by evaporating the solvent and recrystallized to constant specific radioactivity (Table 3). From the remainder of the deproteinized reaction mixture, the radioactive acidic components were extracted directly with diethyl ether. After concentration of the

Table 2. *Identification of* [^{14}C]*methylamine as a metabolic product of N-*[*Me*-^{14}C]*isonicotinate by isotope trapping as N-*[*Me*-^{14}C]*phthalimide*

Warburg flasks contained: extract equivalent to 28mg of soluble protein; NAD^+, 1μmol; *N*-[*Me*-^{14}C]isonicotinate, 10μmol (802000d.p.m.); phosphate buffer, pH7.6, 100μmol; 20% (w/v) KOH (0.2ml) in the centre well. The total volume was made up to 3ml with water. After the contents had been mixed, the flasks were incubated at 30°C and the course of the reaction was followed by observing the rate of O_2 uptake. When this had gone to completion (1mol of O_2/mol of substrate) (about 1h), the flask contents were deproteinized by adjusting to pH1 with conc. HCl. Samples (0.1ml) of the supernatant and of the KOH from the centre well were counted to determine recovery of added radioactivity. To the remainder of the supernatant from each experimental flask were added methylamine hydrochloride (0.5g), sodium acetate (1g) and excess of phthalic anhydride (1.3g) sufficient to react with all the available methylamine. The radioactive methylphthalimide was isolated and recrystallized to constant specific radioactivity (Wright & Cain, 1969).

Sample		Radioactivity (d.p.m.) In sample	Total supplied or recovered
N-[*Me*-^{14}C]Isonicotinate supplied		8020	802000
Total radioactivity recovered in supernatant at end of oxidation		7620	762000
Total radioactivity trapped in KOH of centre well			Nil
N-[*Me*-^{14}C]Phthalimide isolated (theoretical maximum was 668d.p.m./mg)			
1st recrystallization	(9.5mg)	5000	533d.p.m./mg
2nd recrystallization	(10.6mg)	5000	476d.p.m./mg
3rd recrystallization	(10.5mg)	5170	492d.p.m./mg
4th recrystallization	(10.1mg)	5210	516d.p.m./mg*

* This represents 77% recovery of added radioactivity in the methylamine.

Table 3. *Isolation of radioactive succinic acid as a metabolic product of N-methyl*[2,3-$^{14}C_2$]*isonicotinate and N-methyl*[*carboxyl*-^{14}C]*isonicotinate*

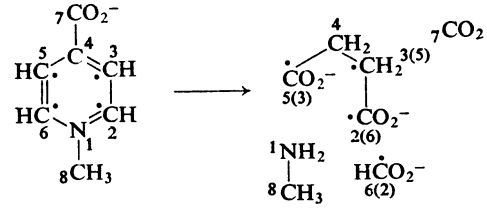
Large capacity (32ml) Warburg flasks contained: crude extract equivalent to 18mg protein; *N*-methyl[2,3-$^{14}C_2$]-isonicotinate, 20μmol (2.1×10^5 d.p.m.) or *N*-methyl[*carboxyl*-^{14}C]isonicotinate, 20μmol (5×10^5 d.p.m.); NAD^+, 2μmol; potassium phosphate buffer, pH7.6, 200μmol; 20% (w/v) KOH in the centre well, 0.5ml. The total volume was 6ml. After the oxidation of the substrate was complete, the contents of the main compartment were deproteinized and to half of the supernatant was added succinic acid (520mg in Expt. A; 500mg in Expt. B). This succinic acid was extracted with 5×30ml volumes of diethyl ether, then the combined ethereal solutions were evaporated to dryness and the residue was crystallized to constant specific radioactivity.

	Sample	Radioactivity
Expt. A.	Radioactivity supplied, 10μmol of *N*-methyl[2,3-$^{14}C_2$]isonicotinate	1.05×10^5 d.p.m.
	Radioactivity available for succinic acid formation from cleavage of the ring into a C_1 and a C_4 compound (75% of that supplied)	7.88×10^4 d.p.m.
	Theoretical radioactivity in reisolated succinic acid (520mg) = $(7.88\times10^4)/520$	150d.p.m./mg
	Radioactivity found in succinic acid:	
	(i) residue from ether extraction	100d.p.m./mg
	(ii) 1st recrystallization	98d.p.m./mg
	(iii) 2nd recrystallization	98d.p.m./mg
	(iv) 3rd recrystallization	105d.p.m./mg
	Hence recovery of radioactivity in succinate from ring-labelled substrate is 70%.	
Expt. B.	Radioactivity supplied in 10μmol of *N*-methyl[*carboxyl*-^{14}C]isonicotinate	2.5×10^5 d.p.m.
	Radioactivity obtained in succinic acid sample (12.2mg)	101d.p.m.
	But 500mg of succinic acid were added, therefore total radioactivity in succinate = $(500\times101)/12.2$	4.14×10^3 d.p.m.
	Hence recovery of radioactivity in succinate from carboxyl-labelled substrate is 1.7%	

ethereal solution, portions were chromatographed together with succinic acid on Whatman no. 1 paper in solvent A. The radioactivity co-chromatographed exactly with the authentic acid.

Similar experiments with *N*-methyl[*carboxyl*-^{14}C]-isonicotinate showed that the carboxyl group at C-4 contributed less than 2% to succinate formation (Table 3).

The likely origin of succinate in the pyridine ring of *N*-methylisonicotinate is therefore the four C atoms C-2(3)–C-5(6), with C-2(3) and C-5(6) forming the carboxyl groups of the product. Since C-4 of the ring-labelled *N*-methylisonicotinate is unlabelled, two-thirds of the radioactivity present in the isolated succinate should be in the carboxyl groups, with the remaining one-third equilibrated between the methylene carbon atoms because of the symmetrical nature of the succinate molecule (Scheme 1). Samples of the isolated succinate together with appropriate controls to establish the recovery rate were degraded by the method of Phares & Long (1955) in which the two



Scheme 1. *Distribution of radioactivity in the products of N-methyl*[2,3-$^{14}C_2$]*isonicotinate metabolism by extracts of Achromobacter D*

carboxyl groups are oxidized to CO_2. In a typical experiment (Table 4), the succinate isolated from *N*-methylisonicotinate in an isotope-trapping experiment gave a specific radioactivity of 436d.p.m./mg. If the distribution of label is that described above, two-thirds of the radioactivity (288d.p.m./mg) should occur in the carboxyl groups and be recoverable as

Table 4. *Degradation of [^{14}C]succinate to determine distribution of radioactivity*

Samples (25mg) of ^{14}C-labelled and unlabelled succinic acids as controls to determine recovery rates, and of succinic acid isolated from an *N*-methyl[2,3-$^{14}C_2$]isonicotinate oxidation (similar to that described in Table 3) were degraded to CO_2 by the method of Phares & Long (1955). Values in parentheses are the number of samples counted. S.D., standard deviation of the mean.

Sample	Radioactivity of sample (d.p.m./mg)	Radioactivity of CO_2 (d.p.m./mg±S.D.)
Succinic acid	3	0
[1,4-$^{14}C_2$]Succinic acid	35750	6616±425 (6)
[2,3-$^{14}C_2$]Succinic acid	18550	34
[^{14}C]Succinic acid from ring-labelled *N*-methylisonicotinate	436*	244±39 (2)†

* Equivalent to 288d.p.m./mg in the carboxyl groups assuming two-thirds of the radioactivity is located in these groups.

† Corrected for 18.5±2.0% recovery by this method as indicated with [1,4-$^{14}C_2$]succinic acid. Actual sample counts were 44 and 46d.p.m./mg.

Table 5. *Isolation of radioactive formate as a metabolic product of N-methyl[2,3-$^{14}C_2$]isonicotinate and N-methyl[carboxyl-^{14}C]isonicotinate*

The incubation mixtures were similar to those for Table 3 except that extract equivalent to 36mg of protein was used. After oxidation was complete and the flask contents had been deproteinized with 2M-H_2SO_4 (0.2ml), the formic acid was recovered by steam distillation and the distillate neutralized with NaOH. Sodium formate (1g or 500mg) was added, dissolved and the solution evaporated to dryness. The sodium formate crystals were washed with diethyl ether and recrystallized to constant specific radioactivity from aqueous methanol.

	Sample	10^{-2} × Radioactivity (d.p.m.)	Experimental recovery (%)
Expt. A.	*N*-methyl[2,3-$^{14}C_2$]isonicotinate supplied	780	
	Radioactivity from this molecule available for formate assuming ring cleavage into a C_1 and a C_4 compound (25%)	195	
	Radioactivity found in recovered sodium formate:		
	(i) After 1st ether wash	145	
	(ii) After 2nd ether wash	150	
	(iii) 1st recrystallization	132	
	(iv) 2nd recrystallization	135	70
Expt. B.	*N*-methyl[*carboxyl*-^{14}C]isonicotinate supplied	2500	
	Radioactivity reisolated in sodium formate sample (10.4mg)	1.12	
	But 500mg sodium formate was added therefore total radioactivity in sodium formate = (500×1.12)/10.4	52	2.1

CO_2 after oxidation. The experimental values were 244±39 (S.D.) and are clearly consistent with the origin of the succinate moiety in a cleavage of the pyridine ring between C-2(6) and C-3(5).

Origin of formate in the pyridine ring of N-methylisonicotinate

Of the seven C atoms available in *N*-methylisonicotinate, six are accounted for in three of the known end-products, CO_2, methylamine and succinate. The identification of formate as the remaining product (Wright & Cain, 1970) suggested that this might be the other product of ring cleavage into a C_4 and a C_1 compound postulated above. *N*-Methyl[2,3-$^{14}C_2$]-isonicotinate was oxidized by NAD^+-supplemented extracts as described above and the formic acid was recovered from the acidified reaction supernatants by steam distillation. The distillate was adjusted to pH7.0 with dil. NaOH and unlabelled sodium form-

ate was added to the neutralized solution. The sodium formate solution was then slowly evaporated to dryness, the crystals were washed twice with diethyl ether and the material was then recrystalled twice (to constant specific radioactivity) from aqueous methanol. Approx. 70% of the available radioactivity was recovered in this recrystallized product. Experiments with *N*-methyl[*carboxyl*-^{14}C]isonicotinate showed that the carboxyl group contributed only 2% to the formate moiety (Table 5).

Discussion

The use of appropriately labelled *N*-methylisonicotinate and the crystallization to specific radioactivity of the metabolic end-products provides convincing confirmation of their identities with the methylamine, formate and succinate suggested by their other chemical properties (Wright & Cain, 1972). Further, the origin from *N*-methyl[2,3-$^{14}C_2$]-isonicotinate of labelled formate and succinate with the radioisotopic distribution described can only arise from a cleavage of the heterocyclic nucleus into a C_4 and a C_1 compound. The presence of two-thirds of the available radioactivity in the carboxyl groups of succinate and the absence of any appreciable labelling of succinate from *N*-methyl[*carboxyl*-^{14}C]isonicotinate precludes a mechanism involving succinate formation from carboxylation of a possible C_3-precursor by the CO_2 released from *N*-methylisonicotinate during oxidation. Similarly the nearly quantitative recoveries of radioactivity from *N*-[*Me*-^{14}C]isonicotinate as [^{14}C]methylamine rule out the methyl group as an (albeit unlikely) contributor to the succinate molecule.

The cleavage of the heterocyclic nucleus into C_4 and C_1 compounds indicated by these results is consistent with most other reports of aerobic pyridine ring cleavage by micro-organisms. Behrman & Stanier (1957) and Gauthier & Rittenberg (1971*a*,*b*) with nicotinic acid, Sparrow *et al.* (1969) with pyridoxal, Houghton *et al.* (1968) with 2- and 3-hydroxypyridine, Houghton *et al.* (1969) with 4-hydroxypyridine and Watson & Cain (1972) with pyridine itself have all shown the formation of formate as a metabolic product of the heterocyclic nucleus together with a C_4 acid. Indeed the appearance of formate can almost be regarded as an indicator of an intermediate *N*-formyl compound, although Gauthier & Rittenberg (1971*b*), using crystalline 2,5-dihydroxypyridine oxygenase from *Pseudomonas putida*, were unable to detect *N*-formylmaleamic acid as the ring cleavage product of 2,5-dihydroxypyridine and concluded that the pure enzyme also catalysed the simultaneous hydrolysis of this putative intermediate to formate and maleamic acid.

It would appear from our studies that pyridinium compounds, in which the electron-density distribution around the heterocyclic nucleus may differ significantly from that of ring-substituted pyridines, are nevertheless degraded by an overall similar mechanism. Superficially, this is perhaps surprising, because the distribution of the positive charge of a pyridine salt over the atoms of the nucleus severely hinders electrophilic substitutions in the deactivated pyridine ring and probably prevents the attack by the electrophilic FeO^{2+}, a species which Ingraham (1966) suggested occurred in hydroxylations of the monooxygenase type. Nucleophilic substitutions, however, are promoted by the electron distribution in pyridine, and particularly pyridinium nuclei, and it may be for this reason that enzymic hydroxylation of pyridine compounds frequently occurs by the incorporation of the O atom from water.

There are clearly quite distinct enzymically catalysed routes by which *N*-methylisonicotinate degradation can be effected. The bacterium isolated by Orpin *et al.* (1971) hydroxylated this compound in the 2-position and then demethylated the ring N atom before further ring hydroxylation occurred, so that any influence of the *N*-alkyl substitution would disappear quite early in the course of degradation. In the *Achromobacter* D system, however, the *N*-methyl group persisted until its release, late in the metabolic sequence, as methylamine (Wright & Cain, 1972), and its influence would continue up to the stage of ring cleavage. We have been able to eliminate monohydroxylation of the *N*-methylisonicotinate molecule as an intermediate stage (Wright & Cain, 1970) and have no evidence to support the possible generation of the 2,3-diol of *N*-methylisonicotinate by *Achromobacter* D, as has been inferred in our system by Orpin *et al.* (1971).

The elucidation of the metabolic routes by which *Lipomyces starkeyi* can utilize paraquat (Baldwin *et al.*, 1966) and related pyridinium compounds such as monoquat, 4,4-bipyridyl, *N*-methylpyridinium, 1,2-dimethylpyridinium, 1-methyl-2,2′-bipyridyl, 1,1-di(carboxymethyl)-4,4′-bipyridinium, 1,1-di-(β-hydroxyethyl)-4,4′-bipyridinium and 2- and 4-hydroxypyridine only as a nitrogen source (J. R. Anderson, personal communication) would be of considerable interest in this respect.

This work was supported by grant no. B/SR/2440 to R. B. C. from the Science Research Council, which is most gratefully acknowledged. We are particularly indebted to Dr. B. C. Baldwin and Dr. J. R. Anderson of I.C.I., Jealott's Hill Research Station, for their kindness in supplying cultures, radioactive compounds and unpublished results.

References

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
Dunn, M. J., Martin, R. J., Roberts, N. L., Slade, P., Standen, H. & Walker, P. (1966) *J. Chem. Soc. C* 1468
Gauthier, J. J. & Rittenberg, S. C. (1971*a*) *J. Biol. Chem.* **246**, 3737
Gauthier, J. J. & Rittenberg, S. C. (1971*b*) *J. Biol. Chem.* **246**, 3743
Herberg, R. J. (1965) *Packard Technical Bulletin* no. 15, Packard Instrument Co., Downers Grove
Houghton, C., Wright, K. A. & Cain, R. B. (1968) *Biochem. J.* **106**, 51P
Houghton, C., Watson, G. K. & Cain, R. B. (1969) *Biochem. J.* **114**, 75P
Ingraham, L. L. (1966) *Compr. Biochem.* **14**, 24
Orpin, C. G., Knight, M. & Evans, W. C. (1971) *Biochem. J.* **122**, 58P
Phares, E. F. & Long, M. V. (1955) *J. Amer. Chem. Soc.* **77**, 2556
Slade, P. (1965) *Nature (London)* **207**, 515
Sparrow, L. G., Ito, P. P. K., Sundaram, T. K., Zach, D., Nyns, E. J. & Snell, E. E. (1969) *J. Biol. Chem.* **244**, 2590
Watson, G. K. & Cain, R. B. (1972) *Biochem. J.* **127**, 44P
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**, 543