A new pathway for isonicotinate degradation by *Mycobacterium* sp. INA1

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An organism able to utilize isonicotinate aerobically as the sole source of carbon, nitrogen and energy was isolated from soil samples and identified as a *Mycobacterium* sp. No growth occurred on any other heteroaromatic substrate tested, except 2-hydroxyisonicotinate, which was shown to be an intermediate in isonicotinate metabolism. Degradation of isonicotinate involved two consecutive hydroxylations leading to 2,6-dihydroxyisonicotinate (citrazinate). The reactions were catalysed by two different dehydrogenases, which were apparently molybdenum-dependent. Citrazinate was activated by CoA via a synthetase reaction, and the resulting citrazyl-CoA ester was reduced by a NADPH-dependent reductase. In the presence of 1 mM-arsenite, cells accumulated 2-oxoglutarate during isonicotinate metabolism.

Introduction

Bacterial metabolism of isonicotinate was first described for a *Pseudomonas* sp. to proceed via 2-hydroxyisonicotinate and citrazinate (2,6-dihydroxyisonicotinate) (Ensign & Rittenberg, 1965). Gupta & Shukla (1979a) confirmed these first steps in isonicotinate breakdown for a *Micrococcus* sp. (formerly a *Sarcina* sp.) and proposed a degradation pathway involving hydrolysis of citrazinate to *cis*-aconitamide and *cis*-aconitate. A completely different pathway of isonicotinate metabolism occurs in *Bacillus brevis*, starting with a reduction of the aromatic ring and ring fission by an oxygenase reaction (Singh & Shukla, 1986). No mycobacterial strain has been reported so far to degrade isonicotinate, though some mycobacteria form isonicotinate from isonicotinic acid hydrazide, a well-known antitubercular drug (Toida, 1962; Youatt, 1962). In this paper we present a new pathway for isonicotinate degradation by *Mycobacterium* sp. INA1 that shares the first two hydroxylation steps with known routes but differs in the way that citrazinate is further metabolized.

Methods

Enrichment of organisms, media and culture conditions. Using conventional enrichment procedures, aerobic, isonicotinate-degrading bacteria were isolated from soil samples that had been preincubated at 30 °C for 3 months in the presence of 10 mM-isonicotinate. The medium contained the following components (mM): CaCl₂, 0.09; MnSO₄, 0.06; MgSO₄, 2.03; NaCl, 0.86; potassium phosphate, 10; isonicotinate, 8;

the final pH was 7.5. Vitamin solution (5 ml l⁻¹) (Genthner *et al.*, 1981) and trace element solution SL10 (1 ml l⁻¹) (Widdel *et al.*, 1983) were also added to the medium. For routine cultivation of strain INA1 the substrate concentration of the medium was raised to 16 mM, and the buffer was changed to 50 mM-potassium phosphate, pH 7.0; additionally, vitamins were omitted as their presence had no effect on growth. To determine molybdenum dependence, a molybdenum-free trace element solution was used and cells were transferred eight times to molybdenum-free medium. Cells were grown at 30 °C in baffled conical flasks on a rotary shaker (130 r.p.m.). Growth was followed as the OD₆₀₀ (1 cm light path) using an Uvikon 810 spectrophotometer.

Characterization of strain INA1

Electron microscopy. Washed cells were enclosed in small cubes of 0.5% (w/v) agar for easier handling. After successive fixation in 3% (w/v) glutaraldehyde and 1% (w/v) OsO₄, cells were dehydrated with acetone and embedded in resin B as described by Spurr (1969). To give contrast, 2% (w/v) uranyl acetate in 70% (v/v) acetone was included in the dehydration process. Ultrathin sections (90–100 nm) cut with glass knives were mounted on copper grids (300 mesh) covered with Formvar foils and poststained with 10% (w/v) lead citrate in 0.1 M-NaOH (modified from Venable & Coggeshall, 1965). Electron microscopy was done with a Philips EM 301 electron microscope at an acceleration voltage of 80 kV.

Detection of mycolic acids as cell wall components. Analysis of mycolic acids was done as described by Minnikin *et al.* (1975).

Identification and measurement of metabolites

High performance liquid chromatography. For reversed phase chromatography, a Kontron HPLC system was used that included a pump model 420, a mixer M 800/491, an RP18 column (220 × 4.6 mm, 5 µm particle size) and a detector model 430. The chromatography was controlled by a Kontron data system MT450. For separation of isonicotinate, 2-hydroxyisonicotinate and citrazinate, the solvent was 5% (v/v) methanol in 50 mM-potassium phosphate buffer (final pH 6.0) at a flow rate of 0.4 ml min⁻¹. The detection system was set at 260 or 345 nm for measurement and quantification of isonicotinate or

Abbreviation: NBT, Nitro Blue tetrazolium chloride.

citrazinate, respectively. For analysis of the citrazyl-CoA synthetase reaction as well as the citrazyl-CoA reductase reaction, the same solvent system was used except that the methanol concentration was raised to 15% (v/v) and detection was at 200 nm.

Spectrophotometry. UV spectra were recorded with a Uvikon 810 spectrophotometer (Kontron). For the quantitative determination of 2-hydroxyisonicotinate an ϵ_{310} of $4357 \text{ M}^{-1} \text{ cm}^{-1}$ was used (Gupta & Shukla, 1979a). Fluorescence spectra were determined using a Kontron spectrofluorometer SFM 23B.

Thin-layer chromatography of dinitrophenylhydrazones. Keto acids were identified by treatment with 0.1% dinitrophenylhydrazine in 2 M-HCl for 30 min at room temperature. The dinitrophenylhydrazones were extracted with ethyl acetate and analysed by TLC on silica gel using the solvent system light petroleum (b.p. 60–80 °C)/ethyl acetate/acetic acid (26:14:3, by vol.) (Shukla, 1974).

Oxygen consumption. This was measured with a Clark oxygen electrode (Rank Brothers).

Experiments with cell extracts

Preparation of cell extracts. Cells were harvested shortly before they reached the stationary growth-phase by centrifugation at 7000 g and washed once in 0.9% NaCl. The cells were resuspended to give a suspension of 35 g wet wt cells per 100 ml 50 mM-potassium phosphate buffer, pH 7.0, and preincubated with 0.3 mg lysozyme ml^{-1} for 20 min at 37 °C. Phenylmethylsulphonyl fluoride (1 μl of a 100 mM-stock solution in propan-2-ol ml^{-1}) and DNase I (0.3 mg ml^{-1}) were added, and cells were ruptured either by ultrasonic disruption or by passage through a French pressure cell. In the first case, 7 to 18 ml of cell suspension was sonicated in a Schoeller KLK 250 disintegrator for $5 \times 1 \text{ min}$ at 80 to 100% of maximal output with cooling intervals of 1 min. Prolonged sonication (5 to 6 min) was found to be optimal for isonicotinate dehydrogenase activity but lowered the 2-hydroxyisonicotinate dehydrogenase activity. Therefore cells were also ruptured using a SLM Aminco French pressure cell. A single passage at 140 to 200 MPa yielded reproducibly high activities for both enzymes. Cell debris was removed by centrifugation at 30000 g, and the clear supernatant was stored at -20°C . Protein was measured by the method of Bradford (1976).

Enzyme assays. All enzyme assays were done at 30 °C. One unit (U) is defined as the activity that converts 1 μmol of substrate min^{-1} . Isonicotinate dehydrogenase and 2-hydroxyisonicotinate dehydrogenase activities were measured at 535 nm as the reduction of the electron acceptor NBT (Nitro Blue tetrazolium chloride). In order to prevent precipitation of reduced NBT, Triton X-100 was added to the reaction mixtures. The molar absorption coefficient, ϵ , of NBT was determined to be $21.3 \text{ mM}^{-1} \text{ cm}^{-1}$ under the reaction conditions used. The citrazyl-CoA synthetase reaction was followed as product formation at 410 nm; the molar absorption coefficient, ϵ , of citrazyl-CoA was determined to be $3.6 \text{ mM}^{-1} \text{ cm}^{-1}$ at 410 nm and $1.9 \text{ mM}^{-1} \text{ cm}^{-1}$ at 340 nm by converting defined amounts of citrazinate to its CoA-ester in the presence of the synthetase and a 3- to 6-fold molar excess of CoA and ATP. The citrazyl-CoA reductase reaction could be measured as NADPH consumption as well as by citrazyl-CoA consumption at 340 nm, the molar absorption coefficient, ϵ , of both substrates together being $8.2 \text{ mM}^{-1} \text{ cm}^{-1}$. The reaction mixtures contained the following components: isonicotinate dehydrogenase assay, 40 mM-Tris/HCl, 2% (w/v) Triton X-100, 0.25 mM-NBT, 7.5 mM-isonicotinate, final pH 8.2; 2-hydroxyisonicotinate dehydrogenase assay, 40 mM-Tris/HCl, 3% (w/v) Triton X-100, 0.4 mM-NBT, 11.5 mM-2-hydroxyisonicotinate, final pH 8.7; citrazyl-CoA synthetase assay: 50 mM-Tris/HCl, 100 mM-KCl, 20 mM-MgCl₂, 0.5 mM-CoA, 2.5 mM-ATP, 5 mM-citrazinate, final pH 8.5; generally, 100 μg protein were added per ml of assay volume. In order to assay citrazyl-CoA reductase the following procedure was used. Firstly, citrazyl-CoA was synthe-

sized in a reaction mixture containing 40 mM-potassium phosphate, pH 7.0, 10 mM-MgCl₂, 0.05 mM-citrazinate, 0.05 mM-CoA, 0.05 mM-ATP and protein (approximately 20 $\mu\text{g ml}^{-1}$) from a fraction containing citrazyl-CoA synthetase activity but free of reductase activity after gel-filtration chromatography; the reaction was allowed to proceed until the A_{410} was constant. Subsequently, 0.05 mM-NADPH was added, and the complete reaction mixture served as a substrate solution. The reductase reaction was started by addition of approximately 50 μg protein per ml of assay volume. In order to account for citrazyl-CoA-independent NADPH oxidase activity, controls were done in which the substrate solution was replaced by 0.05 mM-NADPH in 40 mM-potassium phosphate, pH 7.0.

Gel electrophoresis. Gradient PAGE was done under non-denaturing conditions as described by Blaschke *et al.* (1991). Using the reaction mixtures described for enzyme assays, bands of dehydrogenase activities were visualized after the reduction of NBT. General staining of proteins was done with Coomassie blue (Weber & Osborn, 1969).

Gel-filtration chromatography. This was done on Sephacryl S-HR 300 (Pharmacia) using a Pharmacia XK 16 column (bed volume 190 ml) to which cell extract was applied. The protein was eluted with 50 mM-potassium phosphate, pH 7.0, at a flow rate of 0.3 ml min^{-1} . Chymotrypsinogen A (M_r 25000), egg albumin (45000), aldolase (158000), and ferritin (450000) (Boehringer Mannheim) were used as M_r markers under the same elution conditions.

Chemicals. 2-Hydroxyisonicotinate was produced by strain INA1, especially under conditions of oxygen restriction, and could be separated from the medium once cells had reached the stationary phase by precipitation with 1 M-HCl and subsequent filtration. The 2-hydroxyisonicotinate contained no isonicotinate but 1 to 5% citrazinate. Citrazinate did not react under the conditions used for 2-hydroxyisonicotinate dehydrogenase assay. *N*-Methylisonicotinate was a gift from Lonza (Visp, Switzerland). All the other heterocyclic compounds were obtained from Aldrich, Merck or Sigma.

Results

Enrichment of bacteria; growth, and characterization of strain INA1

From soil samples, three aerobic, rod-shaped bacteria (strains INA1 to INA3) were isolated that were able to degrade isonicotinate. Strains INA2 and INA3 were motile, Gram-negative organisms that were not further characterized. Strain INA1 was not motile and slightly pleomorphic. It stained Gram-negative and showed a positive aminopeptidase reaction. Although the latter is considered to be quite typical for Gram-negative bacteria, some Gram-positive bacteria give a positive reaction as well (Cerny, 1978). Electron microscopy of ultrathin sections of strain INA1 revealed no outer membrane structure (Fig. 1), and the organism was, therefore, considered to be Gram-positive. The cell wall contained mycolic acids, the methyl esters of the mycolic acids producing a triple band pattern when chromatographed on silica gel thin layers. Such multiple forms of mycolic acids are found only in mycobacteria and have different oxygen-containing functional groups (Minnikin *et al.*, 1975). Strain INA1 was thus classified as a *Mycobacterium* sp. The further characterization followed

Fig. 1. Ultrathin sections of strain INA1. w1, outer layer of cell wall (electron transparent); w2, inner layer of the cell wall (electron dense); cm, cell membrane; the bar represents 0.1 μ m.

the determinative key of Wayne & Kubica (1986) in *Bergey's Manual of Systematic Bacteriology*, which indicated that strain INA1 belonged to the species *Mycobacterium aurum*. However, strain INA1 showed several differences compared to the type strain of *M. aurum* (ATCC 23366): young cultures were coloured less intensely, the pattern of mycolic acids differed in one band, and the pattern of soluble cell proteins after gel electrophoresis showed several significant differences. Also, the type strain does not grow on isonicotinate. Thus, some uncertainty remains about the classification of strain INA1 at the species level. Growth of *Mycobacterium* sp. INA1 was slow, the doubling time on different substrates ranging from 5 to more than 20 h. On isonicotinate it was 9 h, and the cultures reached a final OD₆₀₀ of 1.4 to 1.5 (OD₆₀₀ = 1.0 corresponds to about 470 μ g dry wt cells per ml of medium). Concentrations up

to 40 mM-isonicotinate hardly inhibited growth. Besides isonicotinate, 2-hydroxyisonicotinate also supported growth but a shift to this substrate caused long lag phases (about 10 d). When cells were streaked on solidified medium containing 2-hydroxyisonicotinate, some larger colonies protruded from basal smear growth. Thus, the retarded growth of *Mycobacterium* sp. INA1 may have been due to the emergence of gain mutants. Isoniazid, *N*-methylisonicotinate, pyridine-4-aldehyde, citrazinate, 2,6-dihydropyridine, pyridine, nicotinate, picolinate, dipicolinate, pyridine-2,4-dicarboxylate and pyrrole-2-carboxylate did not serve as growth substrates. Pyridine-4-aldehyde, however, was transformed by cells of strain INA1 in some way, as could be seen from changes in the UV spectrum of the medium. Youatt (1962) described the dismutation of pyridine-4-aldehyde to the corresponding alcohol and acid by *Mycobacterium tuberculosis*.

Identification of intermediates

When strain INA1 was grown on isonicotinate, two aromatic metabolites could be detected in the medium and separated from each other by reversed phase HPLC. They were tentatively identified as 2-hydroxyisonicotinate and citrazinate on the basis of their absorption and fluorescence spectra (Table 1). Furthermore, the second metabolite co-eluted with authentic citrazinate in the reversed phase chromatography described above as well as in the paper chromatography system of Ensign & Rittenberg (1965). Authentic citrazinate also served as substrate for a CoA esterification reaction detected in cell extracts of strain INA1. The fact that strain INA1 did not grow on citrazinate might be due to the lack of an uptake system. When resting cells were incubated

Table 1. Identification of the aromatic metabolites detected in the medium as 2-hydroxyisonicotinate and citrazinate on the basis of their spectral properties

All spectra were recorded in 5% (v/v) methanol in 50 mM-potassium phosphate, pH 6.0. (The same solvent was used for their separation by HPLC.)

Metabolite		Authentic substance		Literature value*		Reference
<i>Absorption maxima (nm) of UV spectra†</i>						
		Isonicotinate	265	Isonicotinate	265	Singh & Shukla (1986)
Metabolite 1	312	2-Hydroxyisonicotinate	NA	2-Hydroxyisonicotinate	310	Gupta & Shukla (1979a)
Metabolite 2	343	Citrazinate	344	Citrazinate	345	Gupta & Shukla (1979a)
<i>Excitation/emission maxima (nm) of fluorescence spectra‡</i>						
Metabolite 1	317/396	2-Hydroxyisonicotinate	NA	2-Hydroxyisonicotinate	322/400	Gupta & Shukla (1978)
Metabolite 2	345/436	Citrazinate	346/435	Citrazinate	355/445	Gupta & Shukla (1979a)

NA, Not available.

* Singh & Shukla (1986) recorded spectra of the fermentation broth; Gupta & Shukla (1978, 1979a) did not mention the solvent.

† UV spectra were recorded from 240 to 400 nm.

‡ The reliability of fluorescence maxima was ± 2 nm.

Fig. 2. (a) Bands of isonicotinate dehydrogenase (lane 1) and 2-hydroxyisonicotinate dehydrogenase (lane 2) after gel electrophoresis using a 4 to 18% (w/v) polyacrylamide gradient and activity staining. (b) Calibration for M_r determination using the following markers: thyroglobulin (669000), ferritin from horse spleen (440000), catalase (232000), lactate dehydrogenase (140000) and bovine serum albumin (67000); ○, marker proteins; ■, isonicotinate dehydrogenase; ▲, 2-hydroxyisonicotinate dehydrogenase.

aerobically with 30 mM-isonicotinate in the presence of 1 mM-arsenite, (conditions which allow accumulation of oxo-acids by the cells) 2-oxoglutarate was the only oxoacid that accumulated in the supernatant. In a control experiment pyruvate was the main oxoacid produced when cells were incubated with 30 mM-fumarate and 1 mM-arsenite.

Isonicotinate dehydrogenase and 2-hydroxyisonicotinate dehydrogenase

Isonicotinate- and 2-hydroxyisonicotinate-oxidizing enzymes were detected in extracts of cells grown on isonicotinate, when NBT was used as electron acceptor. Neither activity was present in cells grown on glucose, but both were induced by the addition of 5 mM-isonicotinate to the growth medium 4 h before harvest. The pH optima of the reactions were at 8.2 and 8.7, respectively. Neither NAD^+ nor NADP^+ could replace NBT in the assay system. Oxygen served as an electron acceptor for 2-hydroxyisonicotinate dehydrogenase but not for isonicotinate dehydrogenase. Gradient non-denaturing PAGE followed by staining for enzyme activity produced different patterns when isonicotinate or 2-hydroxyisonicotinate were used as reaction substrates. By comparison with marker proteins, the M_r values were estimated to be 90000 and 390000 for isonicotinate and 2-hydroxyisonicotinate dehydrogenases, respectively (Fig. 2). These results supported the idea of two distinct enzymes. The M_r 90000 protein,

however, was not considered to be the physiologically active form of isonicotinate dehydrogenase for two reasons. The isonicotinate dehydrogenase yielded a much weaker activity band than did 2-hydroxyisonicotinate dehydrogenase, although the first enzyme was more active in cell extracts than the second one. Also, after gel filtration, isonicotinate dehydrogenase eluted at an M_r of approximately 300000, slightly after the 2-hydroxyisonicotinate dehydrogenase which eluted at an M_r of approximately 350000. When cells were grown on a molybdenum-free isonicotinate medium, the cell yield was only 75% of that in a control with 0.1 μM -molybdate in the growth medium (this control value was 2.4 g of wet cells l^{-1}), and the activities of isonicotinate and 2-hydroxyisonicotinate dehydrogenases were only 16 and 38% [the activities of the controls, i.e. 100% activity, were 0.23 and 0.09 U (mg protein) $^{-1}$, respectively, for the two enzymes]. The activity of citrazyl-CoA synthetase was not affected by the availability of molybdenum during growth, pointing to a specific molybdenum requirement of isonicotinate and 2-hydroxyisonicotinate dehydrogenases. An involvement of molybdenum in isonicotinate metabolism was further indicated by growth experiments. Growth in the absence of molybdate was specifically inhibited by its antagonist tungstate (10^{-7} M) when isonicotinate served as substrate.

Citrazyl-CoA synthetase

An enzyme activity [up to 0.50 U (mg protein) $^{-1}$] that metabolized citrazinate in the presence of ATP and CoA and gave rise to an intermediate with an absorption maximum at 385 nm (Fig. 3) was found in cell extracts of strain INA1. Reversed phase chromatography of the components revealed that CoA was consumed in the reaction, while ATP was hydrolysed to AMP and putatively PP_i (Fig. 4a). Citrazinate could be recovered from the citrazyl-CoA ester by alkaline hydrolysis as deduced from UV spectra fluorescence spectra and analysis by HPLC. The citrazyl-CoA synthetase required metal ions such as K^+ , and was inactivated by dialysis against Tris/HCl buffer but not against potassium phosphate buffer or Tris/HCl buffer + KCl (all buffers pH 7.0–7.5). Mg^{2+} was required only in the synthetase assay. The enzyme had a pH optimum at 8.5 in Tris/HCl buffer and an apparent M_r of 135000 as estimated by gel filtration.

Citrazyl-CoA reductase

Further catabolism of citrazyl-CoA by cell extracts of *Mycobacterium* sp. INA1 was dependent on NADPH and did not consume oxygen. Thus, neither a mono- or dioxygenase reaction nor a monooxygenase/reductase

Fig. 3

Fig. 3. Spectrophotometric observation of citrazinate metabolism. In the presence of a 5-fold excess of both CoA and ATP, 0.1 mM citrazinate (—) was transformed to its CoA ester (---) by cell extract. Then 0.15 mM-NADPH was added and further incubation led to a complete reduction of citrazyl-CoA (.....).

Fig. 4

Fig. 4. HPLC analysis of substrate consumption and product formation during the citrazyl-CoA synthetase and citrazyl-CoA reductase reactions. Citrazinate (0.1 mM) was incubated with equimolar amounts of CoA and ATP in the presence of citrazyl-CoA synthetase. Then 0.1 mM-NADPH and citrazyl-CoA reductase were added, and the whole mixture was incubated again. Only enzyme preparations that did not contain interfering activities were used. (a) Citrazyl-CoA synthetase reaction mixture assay before (—) and after (---) reaction. (b) Citrazyl-CoA reductase reaction mixture before (—) and after (---) incubation in the presence of citrazyl-CoA reductase and NADPH. (1) Citrazinate; (2) ATP; (3) AMP; (4) CoA; (5) citrazyl-CoA; (6) unidentified component; (7) NADP⁺ and the remaining NADPH and ATP, all three co-eluting; (8) product of citrazyl-CoA reduction.

reaction described recently by Langkau *et al.* (1990) was considered to occur in these mycobacteria. NADH could not replace NADPH in the reductase assay. Reduction of citrazyl-CoA led to the loss of absorption at 385 nm (Fig. 3) and would seem to occur at the aromatic ring, which is the only reasonable position for reduction. The stoichiometry of NADPH to citrazyl-CoA consumption was 0.85:1, when the decrease of the two substrates was measured at 340 nm [$\epsilon(\text{NADPH})_{340} = 6.3 \text{ mM}^{-1} \text{ cm}^{-1}$] and 410 nm [$\epsilon(\text{citrazyl-CoA})_{410} = 3.6 \text{ mM}^{-1} \text{ cm}^{-1}$], respectively. [The ΔA_{340} was corrected for the change due

to citrazyl-CoA consumption [$\epsilon(\text{citrazyl-CoA})_{340} = 1.9 \text{ mM}^{-1} \text{ cm}^{-1}$]. HPLC analysis of the reductase reaction mixture showed that a new product was formed that eluted slightly after citrazyl-CoA and that CoA was not liberated during the reaction (Fig. 4b). Therefore, we propose the product shown in Fig. 5 as a further metabolite in isonicotinate catabolism by *Mycobacterium* sp. INA1. Under the assay conditions used, the citrazyl-CoA reductase had an activity about 0.20 U (mg cell protein)⁻¹. The M_r of the enzyme was about 50000 as estimated by gel filtration.

Fig. 5. Proposed pathway for the degradation of isonicotinate by *Mycobacterium* sp. INA1.

Discussion

Degradation of isonicotinate by a mycobacterium was shown for the first time, to our knowledge, in this work. The degradative pathway involved two hydroxylations of the aromatic ring, an activation by CoA and a reductive step. (Fig. 5). This combination of known elements represents a new strategy for the microbial breakdown of heteroaromatics.

As was found for a *Pseudomonas* sp. by Ensign & Rittenberg and a *Micrococcus* sp. by Gupta & Shukla (1979a), isonicotinate metabolism proceeded via 2-hydroxyisonicotinate and citrazinate. Evidence for this was based mainly on absorption and fluorescence data as well as on the presence of an enzyme that metabolized citrazinate at considerable rates. The hydroxylating enzymes reduced an artificial electron acceptor and, therefore, we propose to classify them as dehydrogenases. In contrast to the analogous activities found in a *Micrococcus* sp. (Gupta & Shukla, 1979b), the dehydrogenase activities in *Mycobacterium* sp. INA1 seemed to be due to two distinct enzymes. Hydroxylation of the carbon adjacent to the heteroatom is the commonest mode of initial attack on the aromatic ring during the aerobic, microbial breakdown of heterocyclic substrates. It occurs, for example, in the degradation of nicotine

(Hughes, 1955), picolinate (Dagley & Johnson, 1963), nicotine (Hochstein & Rittenberg, 1959), quinoline (Grant & Al-Najjar, 1976), furan-2-carboxylate (Trudgill, 1969), and thiophene-2-carboxylate (Cripps, 1973). Further common features of these reactions seem to be that the incorporated hydroxyl group is derived from water but not from oxygen (Hunt *et al.*, 1958; Hirschberg & Ensign, 1959; Pereira *et al.*, 1988) and that they are affected by the availability of molybdate (Nagel & Andreesen, 1989; Koenig & Andreesen, 1989; Siegmund *et al.*, 1990). Several of the dehydrogenases involved have been shown to contain a molybdenum cofactor (Krüger *et al.*, 1987; Freudenberg *et al.*, 1988; Nagel *et al.*, 1989). Preliminary results obtained with the mycobacterial isonicotinate and 2-hydroxyisonicotinate dehydrogenases suggest that they, too, might be molybdoenzymes.

Activation of citrazinate or any other intermediate to the corresponding CoA ester has not been previously reported to occur in isonicotinate metabolism. Similar steps, however, are found in the aerobic metabolism of furan-2-carboxylate (Trudgill, 1969; Koenig & Andreesen, 1989) and thiophene-2-carboxylate (Cripps, 1973). In the case of these two substrates, however, activation by CoA is thought to be followed by hydrolytic ring fission, but not by reduction. Reductive steps are not

very common in the aerobic metabolism of heteroaromatics, and the compounds involved are generally not activated. This was shown for the degradation of isonicotinate by *Bacillus brevis* (Singh & Shukla, 1986), of *N*-methylisonicotinate by *Achromobacter D* (Wright & Cain, 1972), and of pyridine by a *Corynebacterium* sp. (Shukla & Kaul, 1974). Reduction of the ring is more widespread in the anaerobic catabolism of aromatic compounds (Evans & Fuchs, 1988). The successive combination of activation by CoA and reduction is known from the anaerobic degradation of benzoate by *Rhodospseudomonas palustris* (Hutber & Ribbons, 1983) that is thought to lead into a β -oxidation-like sequence for final breakdown. The final steps in isonicotinate metabolism by *Mycobacterium* sp. INA1 remain to be elucidated.

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