The Pathway of Nicotinic Acid Oxidation by a Bacillus Species*

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Bacteria able to grow with nicotinic acid as a sole source of carbon and nitrogen have been isolated by a number of investigators (1-5). Hughes et al. (6-10) found that Pseudomonas fluorescens initiates degradation of nicotinic acid by a hydroxylation to form 6-hydroxy nicotinic acid and established that the responsible enzyme is located in a cell wall membrane fraction. An initial hydroxylation in the 6-position was also reported by Harary (11, 12) for a Clostridium species which ferments nicotinic acid, and by Behrman and Stanier (13) for a strain of P. fluorescens. The latter authors (13) showed that subsequent reactions, mediated by enzymes contained in the soluble fraction of disrupted cells, catalyze the oxidative decarboxylation of 6-hydroxy nicotinic acid to 2,5-dihydroxypyridine and its cleavage to maleamic and formic acids. The former compound undergoes hydrolytic deamination to maleic acid, which is then isomerized to fumaric acid.

This paper describes a different pathway of nicotinic acid oxidation utilized by a Bacillus species isolated from soil.

EXPERIMENTAL PROCEDURE

The experimental organism was isolated from soil by conventional elective culture techniques. The medium employed had the following composition: K2HPO4 13.3 g; KH2PO4 4.0 g; (NH4)2SO4 1.0 g; MgSO4·7H2O 0.1 g; carbon source (nicotinic acid or glucose) 2.0 g; trace salts solution, 10 ml; water to 1 liter. The trace salts solution was sterilized separately and added aseptically to the base medium. It contained 2.0 g of CaCl2·2H2O; 1.0 g of MnSO4·4H2O, and 0.5 g of FeSO4·7H2O per liter of 0.1 N HCl. Yeast extract, 0.05%, was sometimes added to the complete medium to increase the growth rate. Stock cultures were maintained on the above medium, which was solidified with 1.5% agar.

Large batches of cells were grown in 12-liter, round bottomed flasks containing 8 liters of medium inoculated with 800 ml of a shake culture in the log growth phase. Cultures were incubated at 30° with forced aeration. After approximately 24 hours of incubation, at which time the cultures had reached the maximum stationary phase and were deep blue in color, the cells were harvested in a Sharples centrifuge and stored unwashed as a thick cell paste at -18°. Cell yields of from 3 to 4 g, wet weight, per liter of medium were obtained.

For the most part, cell-free extracts were prepared with a Hughes press. Frozen cell paste, 10 g, was forced through the orifice of a previously chilled (-18°) Hughes press block by means of a hand-operated Carver press, transferred to 30 ml of 0.02 M potassium phosphate buffer, pH 6.8, and extracted for 30 minutes at 4° with intermittent stirring. A few milligrams of DNase were added to decrease the extreme viscosity of the mixture. Occasionally extracts were obtained by subjecting suspension of 10 g of cells in 35 ml of buffer to sonic oscillation for 6 minutes at full power in a Raytheon 10-kc magnetostriuctive sonic oscillator. After either treatment, unbroken cells and debris were removed by centrifugation at 18,000 x g for 30 minutes. The supernatants contained approximately 25 to 30 mg of protein per ml, assayed by the spectrophotometric technique of Warburg and Christian (14).

Spectrophotometric data were obtained with a Bausch and Lomb 505 recording spectrophotometer, or in some cases, with a Beckman model DU spectrophotometer. Oxygen consumption was measured at 30° in an air atmosphere by conventional manometric techniques (15).

Nicotinic acid, 6-hydroxy nicotinic acid, and nicotinic acid-carboxyl-14C were purchased from the California Corporation for Biochemical Research. Glutaconic acid, 2-, 3-, and 4-hydroxy-pyridine, and 2,6-dihydroxy pyridine hydrochloride, were obtained from the Aldrich Chemical Company. The procedure of Behrman and Pitt (16) was followed for the synthesis of 2,3- and 2,5-dihydroxy pyridine. We gratefully acknowledge the gift of maleamic acid from the American Cyanamid Company and of a sample of 2,3-dihydroxy pyridine from Dr. E. J. Behrman.

RESULTS

A nicotinic acid enrichment culture which had been inoculated with soil and incubated on a reciprocal shaker for 1 week at 30° developed a deep blue color. Stract plate from this culture on nicotinic acid agar showed a variety of colonies after several days of incubation, including some surrounded by a bright blue zone as well as the pseudomonad type usually observed from such enrichment cultures. Repeated streaking of these pigment-forming colonies resulted in the isolation of a pure culture of a large, somewhat pleomorphic, gram-positive, spore-forming, rod-shaped bacterium which grows well with nicotinic acid as the sole source of carbon and nitrogen, and produces from this substrate a bright blue soluble pigment. Its aerobic metabolism and spor production identify this isolate as a member of the genus Bacillus; a more complete description of the bacterium has been given elsewhere (17).

Respiration studies showed that glucose-grown resting cells oxidized nicotinic acid only after a pronounced lag, whereas nicotinic acid-grown cells oxidized the homologous substrate im-
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The oxidation of nicotinic acid by glucose-grown resting cells (O--O), and of nicotinic acid (●●●), 6-hydroxynicotinic acid (O--O), 2,6-dihydroxypyridine (●●●●●), and 2,5-dihydroxypyridine and maleamic acid (X---X) by nicotinic acid-grown resting cells. Reaction mixtures contained 1.0 ml of washed cells (500 Klett units, No. 60 filter) suspended in 0.03 M potassium phosphate buffer, pH 7.0, 3 μmoles of substrate, and water to a total volume of 2.1 ml. Endogenous respiration has been subtracted (glucose-grown cells, 27 μl per hour; nicotinic acid-grown cells, 23 μl per hour).

mediated (Fig. 1), thus indicating that the oxidation system is an inducible one. Cells grown on nicotinic acid also oxidized 6-hydroxynicotinic acid and 2,6-dihydroxypyridine without a lag, although the rate of metabolism of the latter compound was significantly lower than that of nicotinic acid (Fig. 1). Maleamic acid and 2,5-dihydroxypyridine, two intermediates in the pathway of nicotinic acid oxidation by pseudomonads (13), were not oxidized.

Cell-free extracts of nicotinic acid-grown cells catalyzed the oxidation of nicotinic acid and 6-hydroxynicotinic acid with the uptake of 1.0 and 0.5 μmole of oxygen per μmole of substrate, respectively (Fig. 2). The oxidation of 2,6-dihydroxypyridine could not be demonstrated. Numerous variations were introduced in the preparation and testing of the extracts in an effort to demonstrate activity with 2,6-dihydroxypyridine, but without success. Included were variations in the pH of the extraction or assay systems over the range of 6.0 to 9.5, additions of cell debris to the extracts, and supplementation of the extracts with various coenzymes and metal salts.¹ Several artificial electron acceptors, including methylene blue, brilliant cresyl blue, and ferricyanide, markedly stimulated the rate but not the extent of nicotinic acid oxidation by most extracts. Dialysis of an extract against cold phosphate buffer for 18 hours resulted in a complete loss of activity.

To localize the enzymes catalyzing the two oxidative steps, a cell paste was divided into two portions, one of which was disrupted by sonic oscillation and the other by the Hughes press. The extracts obtained were then centrifuged at 18,000 × g for 30 minutes, and portions of the resulting supernatants were re-centrifuged for 1 hour at 105,000 × g. Both the 18,000 × g supernatants and the 105,000 × g supernatants and particulates, were resuspended in buffer and tested for activity with and without added methylene blue. The 18,000 × g supernatants catalyzed oxidation of both nicotinic acid and 6-hydroxynicotinic acid without dye, although the rates were markedly increased in its presence (Table 1). On the other hand, the 105,000 × g supernatants were inactive without methylene blue, but oxidized both substrates rapidly in its presence.

The active fractions were precipitated from the 18,000 and 105,000 × g supernatants by 0.2 to 0.4 saturated ammonium sulfate. These fractions were yellow-green in color and required the presence of methylene blue for activity. No attempts at further purification were made.

Crude extracts were also tested for activity with other substrates; the pertinent data are given in Fig. 3. The extracts were similar to the resting cells in that they did not oxidize 2,5-dihydroxypyridine, but differed by showing considerable activity maleamic and maleic acids. The failure of resting cells to oxidize the latter substrates may thus be ascribed to a lack of permeability, as was also found for P. fluorescens (13).

After the enzymatic oxidation of nicotinic acid or 6-hydroxynicotinic acid reached completion, reaction mixtures showed a new ultraviolet absorption spectrum with maxima at 264 and 322 nm.
When such a reaction mixture was deproteinized with alcohol, and acid added to a pH of approximately 2, a new spectrum with maxima at 224 and 300 μm was found. Neutralization of the acidified sample did not restore the original spectrum but yielded a new one with peaks at 234 and 322 μm (Fig. 4).

The spectra in acid and after neutralization are characteristic of

![Graph](http://www.jbc.org/)

**Fig. 4.** The spectra of reaction mixtures after enzymatic oxidation of nicotinic acid or 6-hydroxynicotinic acid. ——O, before acid treatment; ——O, after acid treatment; O—O, reneutralized.

2,6-dihydroxypyridine both in positions of the maxima and in ratios of absorbances of the peaks (see Table II).

Since the two-step enzymatic oxidation of nicotinic acid and the one-step oxidation of 6-hydroxynicotinic acid led to the same product, as judged from the absorption spectra, and since this product was converted (by acid treatment) to what appeared to be 2,6-dihydroxy pyridine, it was assumed that the initial oxidations resulted in hydroxylations at the 2- and 6-positions, forming 2,6-dihydroxynicotinic acid. To test this possibility, enzymatic oxidation of nicotinic acid-carboxyl-14C, was followed until it was completely converted to the material absorbing at 264 and 322 μm. The reaction mixture was deproteinized with alcohol and an aliquot was dried on a planchet and counted. A drop of dilute hydrochloric acid was then added to the planchet, which was again dried and recounted. The residue, taken up in water, gave the characteristic 2,6-dihydroxy pyridine spectrum. Before acid treatment the sample gave 115 c.p.m. over background; after acidification and redrying the count was indistinguishable from background. These data show that the acid treatment resulted in the loss of the carboxyl group of the starting nicotinic acid and provides strong evidence that the material absorbing at 264 and 322 μm is 2,6-dihydroxynicotinic acid.

Substrate quantities of 2,6-dihydroxynicotinic acid were prepared enzymatically. Three 500-ml flasks, each containing about 450 mg of extract protein, 100 mg of nicotinic acid, and 1 μm methylene blue in 100 ml of 0.02 M phosphate buffer, pH 7.5, were shaken at 30°C. The reaction was followed spectrophotometrically until no further increase in absorbance at 322 μm occurred. The flask contents were combined and dried in a vacuum. The residue was extracted with boiling absolute methanol in a nitrogen atmosphere, the desired compound going into solution under these conditions.

### Table I

**Location of nicotinic acid- and 6-hydroxynicotinic acid-oxidizing activity in extracts of disrupted cells after centrifugation**

The flask contents were supernatant or sediment protein or both as indicated, 3.0 μmoles of substrate, 0.1 pmole of methylene blue (MB) where noted, and 0.02 M phosphate buffer, pH 7.2, to 2.1 ml. The data were corrected for oxygen uptake in the absence of substrate.

<table>
<thead>
<tr>
<th>Centrifugal fraction</th>
<th>Protein (mg)</th>
<th>Specific activity*</th>
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<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Nicotinic acid</td>
<td>6-Hydroxynicotinic acid</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>-MB +MB</td>
<td>-MB +MB</td>
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<tr>
<td>Hughes press</td>
<td></td>
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<td></td>
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<tr>
<td>A. 18,000 X g supernatant</td>
<td>14.6</td>
<td>2.7</td>
<td>4.1</td>
<td>2.3</td>
</tr>
<tr>
<td>B. 105,000 X g supernatant</td>
<td>6.3</td>
<td>0</td>
<td>5.2</td>
<td>0</td>
</tr>
<tr>
<td>C. 105,000 X g sediment</td>
<td>4.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D. B + C.</td>
<td>8.7</td>
<td>2.4</td>
<td>2.5</td>
<td></td>
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<tr>
<td>Sonic oscillator</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>E. 18,000 X g supernatant</td>
<td>19.0</td>
<td>3.2</td>
<td>4.4</td>
<td>2.8</td>
</tr>
<tr>
<td>F. 105,000 X g supernatant</td>
<td>18.3</td>
<td>0</td>
<td>3.6</td>
<td>0</td>
</tr>
<tr>
<td>G. 105,000 X g sediment</td>
<td>5.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H. F + G.</td>
<td>18.2</td>
<td>3.4</td>
<td>2.7</td>
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</table>

* Oxygen uptake at 20 minutes.
Attempts to crystallize the product directly from methanol by concentrating the solution in a vacuum and holding at −18° were unsuccessful. A major difficulty was the instability of the compound and its conversion to a blue and then a brown product. Decomposition was relatively slow in alkaline solution, and a partial purification was obtained by precipitating the product from ammoniacal methanol by the addition of an equal volume of ether. The amorphous precipitate that formed was collected on a sintered glass filter and washed with ether, and then dried. A portion of the product was dissolved in water and placed on a DEAE-cellulose column, 2.5 × 17 cm, which previously had been washed with ammonium hydroxide and then water. Gradient elution with ammonium hydroxide removed the compound in a narrow band. The fractions showing maxima at 264 and 322 nm were pooled and dried in a vacuum. A light amber material, 11 mg, which became pale blue on prolonged standing, was obtained.

The partially purified product, assumed to be an unhydrated ammonium salt, had molar extinction coefficients of 7,900 and 15,900 at 264 and 322 nm, respectively. A comparison of these values with the averages of those calculated from the absorption spectra of a number of enzymatic reaction mixtures (9,000 at 264 nm, 18,500 at 322 nm) indicated that the isolated product contained about 10% impurities.

Another portion of the crude product was absorbed on a column of Dowex 1 (Cl−) and removed by gradient elution with HCl. The only eluate fractions showing ultraviolet absorption had maxima at 225 and 300 nm, which were the same as those of the acidified reaction mixture shown in Fig. 4. These fractions were pooled and dried in a vacuum. A comparison of the properties of the product with those of an authentic sample of 2,6-dihydroxypridine (Table II) established its identity. The conclusive identification of 2,6-dihydroxypridine as the product formed by acid treatment, plus the data from the labeling experiment, confirms that the unaltered enzymatic product is 2,6-dihydroxynicotinic acid which is readily decarboxylated by acid.

The course of oxidation of 2,6-dihydroxynicotinic acid by nicotinic acid-grown resting cells is shown in Fig. 5; data for nicotinic acid and 2,6-dihydroxypyridine oxidation by the same cell suspension are included for comparison. The rate of oxidation of nicotinic acid and its dihydroxy derivative were essentially the same while total oxygen uptake differed by 1 μmole per μmole of substrate, as would be expected if the dihydroxy derivative is an intermediate in the metabolic pathway. As was previously found, the rate of 2,6-dihydroxypridine oxidation was relatively slow.

The utilization of 2,6-dihydroxypridine by nicotinic acid-grown cells, albeit at a slower rate, suggested that this compound could also be in the metabolic sequence. If so, it would have to be formed by a nonoxidative decarboxylation of 2,6-dihydroxynicotinic acid. Numerous attempts to demonstrate a nonoxidative decarboxylation of the dipyridol were unsuccessful with either resting cells or cell-free extracts. Instead, the data indicated that the removal of the carboxyl group is an oxidative process. In a typical experiment, a dilute suspension of nicotinic acid-grown cells was placed in a quartz Thunberg tube and 2,6-dihydroxynicotinic acid was added to the side arm. The substrate was tipped in and the changes in absorbance at 264 and 322 nm were observed spectrophotometrically, with a similar cuvette containing cells but no substrate used as a blank. Essentially no change
occurred during a 60-minute anaerobic period; the subsequent introduction of air induced a rapid disappearance of the substrate (Fig. 6).

During growth on nicotinic acid, variable amounts of 2,6-dihydroxynicotinic acid accumulated in the medium. The quantity depended on the initial substrate concentration; accumulation did not occur at low nicotinic acid levels (Table III). If the accumulated acid spontaneously decarboxylated at the pH of the growth medium, then the bacteria would be provided with a constant source of the dipyridol and could be induced to utilize it. This could explain the oxidation of the dipyridol by nicotinic acid-grown resting cells. If so, such adaptation would not be expected under conditions in which accumulation of 2,6-dihydroxynicotinic acid did not occur. To test this possibility, cells grown in media of different nicotinic acid concentrations were assayed for their ability to oxidize 2,6-dihydroxypyridine. The data show (Table III) that the rate of oxidation was essentially the same for all suspensions and independent of either the starting substrate concentration or the amount of 2,6-dihydroxynicotinic acid that had accumulated in the growth medium. The suggested explanation for the metabolism of the dipyridol does not appear tenable and an alternate one is presented below.

**DISCUSSION**

Nicotinic acid is oxidized by the isolated *Bacillus* species via a series of induced enzyme reactions. The oxidation of 6-hydroxynicotinic acid by nicotinic acid-grown resting cells and extracts thereof indicates that this compound is the first intermediate in the metabolic sequence. An initial hydroxylation at the 6-position has been demonstrated in all of several studies of nicotinic acid oxidation by species of *Pseudomonas* (6-10, 13), *Clostridium* (11, 12), and now *Bacillus*. It is interesting that with the *Pseudomonas* species the initial hydroxylating enzyme is a component of a particular cell wall membrane complex (6-10, 13), whereas the enzyme appears to be soluble in the *Bacillus* species.

Resting cells oxidized nicotinic acid and 6-hydroxynicotinic acid essentially to completion, whereas extracts obtained from these cells were limited to a two- and one-step oxidation of the respective compounds. In the enzymatic oxidations, 2,6-dihydroxynicotinic acid was produced stoichiometrically from both substrates. The identification of this compound was based upon its acid decarboxylation to a product identified as 2,6-dihydroxypyridine. The rapid oxidation of 2,6-dihydroxynicotinic acid by nicotinic acid-grown resting cells is a further indication of its role as a metabolic intermediate.

A search of the literature revealed only two references to 2,6-dihydroxynicotinic acid. Guthzeit and Laska (19) reported its synthesis by means of an acid hydrolysis of the ethyl ester prepared by heating isoacetic acid ethyl ester with ammonia. The synthesis was repeated by Hughes (6), who presented a spectrum for his product which is identical with that of 2,6-dihydroxypyridine. In view of the facile acid decarboxylation of 2,6-dihydroxynicotinic acid demonstrated in this paper, the acid step in the above procedure indicates that it was indeed 2,6-dihydroxypyridine which was synthesized. In fact, this di- pyridol has been prepared by a similar decarboxylation of 2,6-dihydroxynicotinic acid with dilute HCl (20) and by the action of concentrated HCl on ethylidene-2,6-dihydroxynicotinate (21). The evidence thus suggests that 2,6-dihydroxynicotinic acid has not been previously synthesized or described.

The failure of enzymes to oxidize beyond the level of 2,6-dihydroxynicotinic acid remains unexplained. The possibility that a cofactor or metal loss was responsible is not excluded although the addition of a large variety of such compounds failed to induce more complete oxidations. Since 2,6-dihydroxynicotinic acid is accumulated by intact cells during growth on moderate concentrations of nicotinic acid, the enzyme or enzymes responsible for its further metabolism is rate-limiting and perhaps may be labile and easily inhibited or inactivated.

Although the enzymatic cleavage of the pyridine ring could not be demonstrated, the oxidation of maleamic acid by extracts of nicotinic acid-grown cells implicates this compound as a product of ring cleavage as it also is in the *Pseudomonas* pathway (13).

**Fig. 6.** The utilization of 2,6-dihydroxynicotinic acid by nicotinic acid-grown resting cells in the absence and presence of oxygen. Cuvettes contained 0.7 μmole of 2,6-dihydroxynicotinic acid, 0.1 ml of cells (350 Klett units, No. 60 filter), and phosphate buffer, pH 7.2, to 3.0 ml. Measured spectrophotometrically at indicated wave lengths.
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The merging of the two pathways can be envisioned as resulting from a common intermediate, 2,3,6-tripyridol, being formed by the oxidative decarboxylation of 2,6-dihydroxynicotinic acid by the *Bacillus* species and by the oxidation of 2,5-dipyridol by the pseudomonads (Fig. 7). Further oxidation of the tripyridol would yield N-formylmaleamic acid, postulated in the Behrmann-Stanier scheme (13), and then maleamic acid.

The oxygen requirement for decarboxylation of 2,6-dihydroxynicotinic acid by resting cells supports the postulate of the tripyridol. The production of the blue pigment both from nicotinic and 2,6-dihydroxynicotinic acid also bears on this point. A blue pigment, first described by Bucherer (22) and Wenusch (23), and recently studied by Gries et al. (24), is also formed during nicotine metabolism by *Arthrobacter* species. It has recently been shown (17) that this pigment and one formed by the spontaneous oxidation of 2,6-dipyridol (25) have the characteristics of azaquinones which arise from the chemical oxidation of tripyridols (26-28). Recently, direct spectrophotometric evidence has been obtained for the formation of 2,3,6-tripyridol from 2,6-dipyridol by the *Arthrobacter* species mentioned above.3

Assuming that the tripyridol is both the precursor of the pigment and the intermediate just previous to ring cleavage, then the oxidation of 2,6-dipyridol by nicotinic acid-adapted cells may be explained without its being in the pathway. As the first step in the spontaneous oxidation of the dipyridol to pigment, the tripyridol would be formed (Fig. 7), which in the presence of active cells would be further oxidized via maleamic acid. The initial abiological reaction is slow and thus would control the over-all rate of oxidation observed.

**SUMMARY**

A *Bacillus* species was isolated (from soil) which uses nicotinic acid as its sole carbon source and produces a soluble blue pigment from this substrate. The organism oxidized nicotinic acid via 6-hydroxynicotinic acid and 2,6-dihydroxynicotinic acid; the enzymes involved in the hydroxylations were soluble. The latter compound was produced enzymatically and obtained in purified form. It is suggested that its further metabolism involves an oxidative decarboxylation to 2,3,6-tripyridol followed by ring cleavage and the formation of maleamic acid. Although 2,6-dihydroxypyrindine is slowly oxidized by resting cells, the evidence indicates that it is not an intermediate in nicotinic acid oxidation but enters the metabolic pathway by its spontaneous oxidation to the tripyridol.

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The Pathway of Nicotinic Acid Oxidation by a *Bacillus* Species
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