Nicotinic Acid Metabolism

II. THE ISOLATION AND CHARACTERIZATION OF INTERMEDIATES IN THE FERMENTATION OF NICOTINIC ACID

(Received for publication, October 25, 1965)

L. TSAI, IRA PAGTANY,* AND E. R. STAETMAN

From the Laboratory of Biochemistry, Section on Enzymes, National Heart Institute, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

6-Hydroxynicotinic acid was demonstrated to be the first intermediate in the degradation of nicotinic acid by a clostridium. In the presence of pyruvate, other intermediates accumulated; two of these were identified, one as 1,4,5,6-tetrahydro-6-oxonicotinic acid, and the other as α-methylene-glutaric acid.

The pathways for nicotinic acid degradation may be considered as involving successive oxidative and reductive steps to 1,4,5,6-tetrahydro-6-oxonicotinic acid, then through some unidentified steps to α-methylene-glutaric acid, which is subsequently converted to propionic and acetic acids and carbon dioxide.

Pyruvate and lactate were shown not to be obligatory intermediates in the production of propionate.

This microorganism has a high content of $B_{12}$ coenzyme, but its function in nicotinic acid fermentation is not yet understood.

EXPERIMENTAL PROCEDURE

The organism was grown on the media described previously in 300-liter fermentation tanks under a nitrogen atmosphere (1).

Yields were about 1 g of cells (wet weight) per liter. The cells were harvested with a Sharples centrifuge, lyophilized, and stored in a desicator at $-15^\circ$. Cell-free extracts were prepared by suspending 6 g of dried cells in 60 ml of 0.03 M potassium phosphate buffer, pH 7.3, containing 0.03% Na$_2$S .7H$_2$O, and passing this through a French pressure cell. The particulate material was sedimented at 16,000 $\times$ g for 1 hour and discarded. The supernatant fraction could be stored in aliquots in a liquid nitrogen freezer for 2 months without appreciable loss of activity.

6-Hydroxynicotinic acid was measured by its absorption at 290 mp at pH 7.3. It could also be distinguished from nicotinic acid by its failure to react with cyanogen bromide (2). Pyruvate was isolated from reaction mixtures as the 2,4-dinitrophenyl-hydrazone, and its specific activity was determined on the chromatographically purified material (3). Propionate, acetate, and lactate were isolated by chromatography on Celite (4). Carbon dioxide was collected in NaOH and was precipitated and plated as barium carbonate. Protein was measured by sulfo-salicylic acid precipitation (5). The biuret reaction gave falsely high values which were probably due to a substance present in the crude bacterial extracts. Other details are described in a previous paper (1).

6-Hydroxynicotinic acid was purchased from Light Chemical Company, sodium pyruvate from Calbiochem, and α-methylene-glutaric acid from Aldrich. γ-Hydroxy-γ-methyl-α-ketoglutaric acid was a gift from Dr. A. Marcus, Beltsville, Maryland.

Elemental analyses were made by Micro-Tech Laboratories, Skokie, Illinois. All melting points were determined on a Kofler hot stage apparatus. Ultraviolet absorption spectra were measured with a Cary recording spectrophotometer, model 14M; infrared spectra were determined by Mrs. K. Warren on a Beckman IR-7 spectrometer; nuclear magnetic resonance spectra were measured with a Varian Associates model A-60 spectrometer. Vapor phase chromatography was performed on two different columns: (a) a 9-foot column of 0.75% SE-30 on Gas-Chrom P and (b) a 6-foot column of 1% QF-1 on Gas-Chrom P.

RESULTS AND DISCUSSION

Isolation of 6-Hydroxynicotinic Acid

A mixture of nicotinic acid, 20 μmoles; potassium phosphate (pH 7.3), 100 μmoles; and crude bacterial French press extract containing 28 μg of protein was incubated in a total volume of
2.0 ml for 30 min at 30° under helium. The reaction was stopped with 0.25 ml of 10% perchloric acid, and the precipitate was centrifuged and discarded. The supernatant was passed through a Dowex 50-H⁺ column (0.8 × 5 cm) which was washed with water, and 5.0-ml fractions were collected. Fractions 3 and 4 contained material which had an ultraviolet absorption spectrum identical with that of 6-hydroxynicotinic acid (Fig. 1). Approximately 10 to 20% of nicotinic acid could be accounted for as 6-hydroxynicotinic acid. This material was evaporated to dryness, dissolved in 0.2 ml of water, and chromatographed in two solvent systems: I, methyl ethyl ketone-tert-butyl alcohol-H₂O-NH₄OH (40:30:20:10), and II, methyl ethyl ketone-tert-butyl alcohol-H₂O-dimethylamine (40:40:20:1). Under ultraviolet light, "quenching" spots were observed on the chromatograms with RF values identical with those of authentic 6-hydroxynicotinic acid, i.e. 0.36 for Solvent I and 0.25 for Solvent II.

**Isolation of Intermediates A and B**

When nicotinic acid was decomposed in the presence of large amounts of pyruvate, intermediates other than 6-hydroxynicotinic acid were found to accumulate. To facilitate the isolation and characterization of these intermediates, a large scale experiment was performed as follows. A mixture containing 0.2 M 7-¹⁴C-nicotinic acid (0.21 mC per mmole), 0.2 M sodium pyruvate; 0.1 M potassium phosphate (pH 7.0), 0.015% Na₂S.7H₂O, and 96 g of dried cells in a total volume of 2,400 ml was incubated for 2+ hours at 30° under a helium atmosphere. The reaction was stopped by the addition of 60% perchloric acid with stirring to pH 2.0. The insoluble material was sedimented at 15,000 × g for 30 min, and the precipitate was discarded. The supernatant was brought to pH 7.0 with KOH. The precipitate of KClO₃ was removed by centrifugation. The liquid phase was passed through a Dowex 50-H⁺ column (5 × 45 cm) to remove nicotinic acid. The effluent solution was acidified with 6 N H₂SO₄ to pH less than 2.0 and was continuously extracted with ether for 24 hours. The ether-soluble material was concentrated to a small volume under a stream of helium, diluted to 10 liters with water, adjusted to pH 5.0, and applied to a Dowex 1-formate column (6 × 15 cm). The column was washed with water, followed by NH₄-formate buffer, pH 4.0, of increasing molarity (0.05 to 0.53 M) (Fig. 2). Fractions of 15 ml each were collected, and 0.0-ml aliquots were counted in a liquid scintillation spectrometer. The optical density at 270 mμ was also monitored. As can be seen from Fig. 2, this chromatographic procedure resolved the ether extract into major radioactive components, hereafter referred to as Intermediates A, B, and C. Fractions containing Intermediates A were pooled, as were those containing Fraction B, and lyophilized. The material was redissolved in water, adjusted to pH 2.0 or less with H₂SO₄, and extracted continuously into ether for 24 hours. The ether-soluble material was concentrated to a small volume, diluted with 100 ml of H₂O, and again lyophilized. This material was taken up in the appropriate organic solvent and crystallized as described below. The yields of recrystallized A and B were 76 and 17 mg, respectively.

**Identification of Intermediate A as 1,4,5,6-Tetrahydro-6-oxonicotinic Acid**

The material from tubes 60 to 76 was crystallized from acetone-chloroform, followed by sublimation at 110°/0.05 mm. Colorless crystals were obtained, m.p. 214–220° (with decomposition). Further recrystallizations did not improve the melting point.

\[
\text{C}_6\text{H}_7\text{NO}_3
\]

Calculated: C 51.06, H 5.00, N 9.93

Found: C 51.06, H 4.99, N 9.50

The ultraviolet absorption spectra were determined: \( \lambda_{\text{max}} \) \text{nm} (e = 1.47 × 10³) (Fig. 3); \( \lambda_{\text{max}} \) \text{nm} (e = 1.40 × 10³) (Fig. 3). The infrared spectrum (KBr pellet) showed absorption at 1705 cm⁻¹ (strong) (acid C=O); 1665 cm⁻¹ (broad, strong) (amide C=O), 3220 and 3130 cm⁻¹ (bonded NH), and 2720 to 2580 cm⁻¹ (acid OH, bonded) (Fig. 4).

The methyl ester of Intermediate A was obtained by treating a crude sample, 28 mg, in 2 ml of methanol with a large excess of

---

**Fig. 1. Ultraviolet absorption spectrum of 6-hydroxynicotinic acid.**

A, authentic sample; B, reaction product; in 0.01 M potassium phosphate buffer, pH 7.3.

**Fig. 2. Chromatogram of the intermediates on a Dowex 1-formate column.**

---

**Fig. 3.** Ultraviolet absorption spectrum of 6-hydroxynicotinic acid. A, authentic sample; B, reaction product; in 0.01 M potassium phosphate buffer, pH 7.3.
The ester was recrystallized from chloroform-cyclohexane; yield, 18.2 mg; m.p. 93-94°C; \( \lambda_{\text{max}} \) 277 m\( \mu \) \( (\epsilon = 1.49 \times 10^4) \); no change in alcoholic KOH.

\[ \text{C}_7\text{H}_8\text{NO}_3 \]

Calculated: C 54.20, H 5.85, N 9.03

Found: C 54.19, H 5.96, N 8.77

The infrared spectrum (chloroform) showed absorption at 1704 cm\(^{-1}\) (strong) (conjugate ester C\( =\)O), 1656 cm\(^{-1}\) (strong) (amide C\( =\)O), and 3408, 3250, and 3140 cm\(^{-1}\) (nonbonded and bonded N-H) (Fig. 3). The nuclear magnetic resonance spectrum was determined in CDCl\(_3\) (Fig. 7) with tetramethylsilane as internal reference.

A sample of the methyl ester, 14 mg, in 2 ml of acetic acid and 1 mg of PtO\(_2\) was shaken with hydrogen at atmospheric pressure and room temperature for 8 hours. The catalyst was filtered, and the filtrate was evaporated under reduced pressure to dryness. The residue was sublimed at 100°C/0.05 mm of Hg. Colorless sublimate was collected; yield, 10 mg; m.p. 107-109°C; no ultraviolet absorption.

\[ \text{C}_7\text{H}_8\text{NO}_3 \]

Calculated: C 53.49, H 7.05, N 8.91

Found: C 53.57, H 7.05, N 8.58

The infrared spectrum (chloroform) showed absorptions at 1740 cm\(^{-1}\) (strong) (ester C\( =\)O), 1667 cm\(^{-1}\) (amide C\( =\)O), and 3405 and 3240 cm\(^{-1}\) (nonbonded and bonded N-H) (Fig. 6).

This methyl ester was identical in melting point and infrared spectrum with an authentic sample of methyl 5-ketonipecotate prepared as follows. A mixture of 0.5 g of 6-hydroxynicotinic acid in 10 ml of 0.75 M NH\(_4\)OH and 0.2 g of Rh-Al\(_2\)O\(_3\) (5%) catalyst was shaken with hydrogen at 25 p.s.i. and room temperature for 4 hours. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to 2 ml; acidification gave a precipitate of 5-ketonipecotic acid. The dried acid was suspended in 10 ml of methanol and treated with an ethereal solution of diazomethane. After removal of solvent, the residue was sublimed in a vacuum to furnish methyl 5-ketonipecotate, m.p. 107-109°C.

The chromatographic behavior of Intermediate A, its infrared absorption between 1600 and 1800 cm\(^{-1}\), and its reactivity toward diazomethane are consistent with the properties of a carboxylic acid. Examination of the infrared spectrum of the methyl ester of A reveals (a) a secondary amide group \( (\lambda_{\text{max}} \) 1656 cm\(^{-1}\) and 3408 cm\(^{-1}\), Fig. 3) which is present in a cyclic structure since it lacks the absorption of an amide II band at about 1550 cm\(^{-1}\) (6), and (b) an \( \alpha,\beta \)-unsaturated ester, \( (\lambda_{\text{max}} \) 1704 cm\(^{-1}\), Fig. 4), shifting to 1740 cm\(^{-1}\) upon catalytic hydrogenation (Fig. 6). The elemental analysis of Intermediate A, \( \text{C}_7\text{H}_8\text{NO}_3 \), indicated that it contains 2 more hydrogen atoms than 6-hydroxynicotinic acid; the presence of an \( \alpha,\beta \)-unsaturated ester and the lactam function suggest the possible structure to be Ia or Ib.

\[ I_a \]

\[ I_b \]

FIG. 3. Ultraviolet absorption spectrum of Intermediate A in methanol.

FIG. 4. Infrared absorption spectrum of Intermediate A (KBr pellet).
Consideration of the ultraviolet absorption spectrum and analysis of the nuclear magnetic resonance spectrum of the methyl ester of A led conclusively to the assignment of Structure 1a for Intermediate A. In the nuclear magnetic resonance spectrum of the methyl ester of A in CDCl₃ (Fig. 7), the complex multiplet at δ = 2.63 ppm can be attributed to -CH₂CH₂- at positions 4 and 5, the singlet at δ = 3.78 ppm to the -CH₃ group of the ester, the broad peak at δ = 8.08 ppm to N—H, and the doublet at δ = 7.31 ppm to an olefinic proton adjacent to a N— or O— function. That this olefinic proton is definitely adjacent to the NH function was demonstrated by observing the spectrum in the presence of trace of D₂O upon which the N—H absorption at 8.08 ppm disappeared while the doublet at 7.31 ppm collapsed into a singlet. None of these spectral features...
can be accounted for by Structure Ib. In accordance with the International Union of Pure and Applied Chemistry rules of nomenclature, a compound of Structure Ia is designated as 1,4,5,6-tetrahydro-6-oxonicotinic acid.

Identification of Intermediate B as α-Methyleneglutaric Acid

The material from tubes 230 to 290 (Fig. 2) was purified by recrystallization from ethyl acetate-cyclohexane. The pure compound, m.p. 130-130.5⁰, had no ultraviolet absorption above 225 mµ, decolorized neutral permanganate instantly, and showed a broad absorption band in the infrared spectrum (KBr pellet) at 1710 to 1730 cm⁻¹ (acid C=O).

C₆H₁₀O₄

Calculated: C 50.12, H 5.60
Found: C 50.00, H 5.60

Quantitative hydrogenation was conducted in a microhydrogenator (7) with palladium on charcoal (5%) as catalyst; 1.646 mg of Intermediate B absorbed 0.25 ml of H₂ (an equimolar quantity to a compound with the formula C₆H₁₀O₄). The catalyst was removed by filtration, and the filtrate was evaporated to dryness under nitrogen. The residue, m.p. 66-69⁰, was taken up in 0.5 ml of methanol and treated with excess diazomethane in ether. After standing for 1 hour at room temperature, the solution was concentrated, and samples were analyzed by vapor phase chromatography in two different columns. In both cases a single peak was obtained; it had a retention time (12.6 min for Column a at 73⁰ and 2.4 min for Column b at 86⁰ under 10 p.s.i. of carrier gas) identical with that of an authentic sample of dimethyl α-methylglutarate.

The above characteristics indicated that Intermediate B is probably α-methylene glutaric acid. This identity was firmly established by comparison of Intermediate B with an authentic sample of α-methylene glutaric acid prepared according to Buchman, Reims, and Schlatter (8).

Utilization of Intermediates

Metabolism of 6-Hydroxynicotinic Acid—Incubation of 6-hydroxynicotinic acid with cell-free extracts that completely utilized nicotinic acid did not result in any disappearance of the 6-hydroxynicotinic acid (Table I). When pyruvate was added, however, 6-hydroxynicotinic acid was completely utilized and was not converted to nicotinic acid. In the conversion of nicotinic acid to 6-hydroxynicotinic acid, electrons are lost from the molecule. These ultimately appear in the fermentation products since the over-all fermentation can be represented by a balanced equation (9). It is likely that pyruvate supplies the electrons that are lacking in 6-hydroxynicotinic acid and allows its metabolism in this system, which ordinarily carries out the tightly coupled metabolism of nicotinic acid. Hydrogen gas, another possible source of electrons, was unable to act like pyruvate. It is not known if it is utilized by these extracts.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Experiment A</th>
<th>Experiment B</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-¹⁴C-Nicotinate</td>
<td>49</td>
<td>9.5</td>
</tr>
<tr>
<td>¹⁴C-Tetrahydro-6-oxonicotinate</td>
<td>49</td>
<td>9.2</td>
</tr>
<tr>
<td>α-Methylene glutarate, synthetic</td>
<td>40</td>
<td>9.2</td>
</tr>
</tbody>
</table>

* Results are given as percentage of label in ¹⁴CO₂.
* Results are given as micromoles of CO₂.

Pyruvate requirements for decomposition of 6-hydroxynicotinate

Each reaction mixture contained 50 µmoles of potassium phosphate buffer (pH 7.3) and 18 mg of enzyme in a total volume of 1.0 ml. Where indicated, there were 10 µmoles of nicotinate, 10 µmoles of 6-hydroxynicotinate, and 30 µmoles of sodium pyruvate. The gas phase was helium except for incubation with hydrogen gas. Incubation time was 30 min.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Addition</th>
<th>Amount consumed (µmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinate</td>
<td>None</td>
<td>10</td>
</tr>
<tr>
<td>6-Hydroxynicotinate</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>6-Hydroxynicotinate</td>
<td>Pyruvate</td>
<td>10</td>
</tr>
<tr>
<td>6-Hydroxynicotinate</td>
<td>H₂</td>
<td>0</td>
</tr>
</tbody>
</table>

Table I

Metabolism of 1,4,5,6-tetrahydro-6-oxonicotinate and α-methylene glutarate by dried bacterial cells

Incubations were conducted for 1 hour at 30⁰ in Warburg flasks with helium as the gas phase. Each flask in Experiment A contained 50 µmoles of potassium phosphate buffer (pH 7.3), 50 µg of dried bacterial cells, and, where indicated, 7-¹⁴C-nicotinate, 10 µmoles; ¹⁴C-tetrahydro-6-oxonicotinate, 1.3 µmoles; and ¹⁴Cα-methylene glutarate, 1.3 µmoles, in a total volume of 2.0 ml. Each flask in Experiment B contained (in micromoles) Tris-HCl, 100; reduced glutathione, 5; FeSO₄, 5; potassium phosphate (pH 7.3), 5; CaO, 0.02; dried bacterial cells containing 30 µg of protein; and, where indicated, 7-¹⁴C-nicotinate, 10 µmoles; and α-methylene glutarate, 10 µmoles in a total volume of 2.0 ml, pH 7.3.

Table II

Metabolism of 1,4,5,6-tetrahydro-6-oxonicotinate and α-methylene glutarate by dried bacterial cells

Each reaction mixture contained 50 µmoles of potassium phosphate buffer (pH 7.3), 1.646 mg of Intermediate B absorbed 0.25 ml of H₂ (an equimolar catalyst, was removed by filtration, and the filtrate was evaporated to dryness under nitrogen. The residue, m.p. 66-69⁰, had no ultraviolet absorption above 225 mµ, decolorized neutral permanganate instantly, and showed a broad absorption band in the infrared spectrum (KBr pellet) at 1710 to 1730 cm⁻¹ (acid C=O).

C₆H₁₀O₄

Calculated: C 50.12, H 5.60
Found: C 50.00, H 5.60

Quantitative hydrogenation was conducted in a microhydrogenator (7) with palladium on charcoal (5%) as catalyst; 1.646 mg of Intermediate B absorbed 0.25 ml of H₂ (an equimolar quantity to a compound with the formula C₆H₁₀O₄). The catalyst was removed by filtration, and the filtrate was evaporated to dryness under nitrogen. The residue, m.p. 66-69⁰, was taken up in 0.5 ml of methanol and treated with excess diazomethane in ether. After standing for 1 hour at room temperature, the solution was concentrated, and samples were analyzed by vapor phase chromatography in two different columns. In both cases a single peak was obtained; it had a retention time (12.6 min for Column a at 73⁰ and 2.4 min for Column b at 86⁰ under 10 p.s.i. of carrier gas) identical with that of an authentic sample of dimethyl α-methylglutarate.

The above characteristics indicated that Intermediate B is probably α-methylene glutaric acid. This identity was firmly established by comparison of Intermediate B with an authentic sample of α-methylene glutaric acid prepared according to Buchman, Reims, and Schlatter (8).

Utilization of Intermediates

Metabolism of 6-Hydroxynicotinic Acid—Incubation of 6-hydroxynicotinic acid with cell-free extracts that completely utilized nicotinic acid did not result in any disappearance of the 6-hydroxynicotinic acid (Table I). When pyruvate was added, however, 6-hydroxynicotinic acid was completely utilized and was not converted to nicotinic acid. In the conversion of nicotinic acid to 6-hydroxynicotinic acid, electrons are lost from the molecule. These ultimately appear in the fermentation products since the over-all fermentation can be represented by a balanced equation (9). It is likely that pyruvate supplies the electrons that are lacking in 6-hydroxynicotinic acid and allows its metabolism in this system, which ordinarily carries out the tightly coupled metabolism of nicotinic acid. Hydrogen gas, another possible source of electrons, was unable to act like pyruvate. It is not known if it is utilized by these extracts.

Metabolism of 1,4,5,6-tetrahydro-6-oxonicotinate and α-Methylene glutarate by Cell-free Extracts—Previous studies showed that the fermentation of nicotinate by washed cell suspensions of the clostridium involves a mechanism in which one half of the CO₂ and one half of the propionate carboxyl carbon are derived from carbon atom 7 (carboxyl group of nicotinate); the other half of each fermentation product is derived from carbon atom 6 of nicotinate (1). The data of Table II suggest that a similar fermentation is catalyzed by suspensions of dried cells. Thus, 1 mole of CO₂ is derived from the carboxyl group of nicotinate. To test the possibility that 1,4,5,6-tetrahydro-6-oxonicotinate and α-methylene glutarate are normal intermediates in the over-all fermentation of nicotinate, the ability of suspensions of dried cells to metabolize these compounds was examined. For these studies, the ¹⁴C-tetrahydro-6-oxonicotinate and ¹⁴Cα-methylene glutarate that had been isolated as products of 7-¹⁴C-nicotinate decomposition (see above) were used. The data of Table II show that, with 7-¹⁴C-nicotinate, half of the labeled carbon in each compound is converted to CO₂. Although the amounts of labeled substrates were not sufficient to permit an accurate measurement of the acetate and propionate formed, it was shown that most of the labeled carbon unaccounted for as CO₂ was incorporated into propionate, the other labeled product derived from carbon atom 6 of nicotinate (1). In a separate experiment, in which unlabeled, synthetic α-methylene glutarate was used as substrate, it was shown that 1 mole of CO₂ is produced for each mole of substrate fermented (last line, Table II). These data are therefore consistent with the tentative conclusion that tetrahydro-6-oxonicotinate and α-methylene glutarate are normal intermediates in the fermentation of nicotinate.
1812

Nicotinic Acid Metabolism. II

Vol. 241, No. 8

COOH

\[ \text{CH}_2\text{CH}_2\text{COOH} \]

0.02; FeSO\textsubscript{4}, 5; and 22.5 mg of protein.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Specific radioactivity</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-\textsuperscript{14}C-nicotinate</td>
<td>4620</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>Propionate</td>
<td>202</td>
<td>48.0</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>3.3</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>CO\textsubscript{2}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-\textsuperscript{14}C-nicotinate</td>
<td>7420</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Propionate</td>
<td>720</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO\textsubscript{2}</td>
<td>25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From considerations of the structures of the three intermediates that have been isolated, it is supposed that the decomposition of nicotinic acid involves successive oxidative and reductive steps to form 1,4,5,6-tetrahydro-6-oxonicotinate, followed by a series of unidentified steps to form a-methyleneglutarate; this is illustrated in Fig. 8. On the basis of previous studies with variously labeled nicotinate substrates (1), it seems likely that the further conversion of a-methyleneglutarate involves the formation of two identical C\textsubscript{3} derivatives or of two dissimilar C\textsubscript{3} compounds that are in equilibrium with each other.\textsuperscript{1} Ultimately the two C\textsubscript{3} compounds are converted to propionate, acetate, and CO\textsubscript{2}. This is illustrated by the lower part of Fig. 8.

In view of the fact that various C\textsubscript{3} compounds (lactate, pyruvate, and glycerol) are fermented by the organism, it seemed possible that lactate or pyruvate could be the hypothetical C\textsubscript{3} intermediate (1). To test this possibility, the decomposition of 7-\textsuperscript{14}C-nicotinate or 6-\textsuperscript{14}C-nicotinate by cell-free extracts was studied in the presence of a relatively large pool of unlabeled pyruvate. The results are summarized in Table III. It can be seen that with either labeled substrate the specific radioactivity of propionate formed was very much greater than the specific activities of the reisolated pyruvate. This result precludes an obligatory role of free pyruvate in the conversion of either C-6 or C-7 of nicotinate to propionate. The fact that the specific activity of the propionate is considerably less than that of the added nicotinate is attributed to the dilution of labeled propionate with unlabeled propionate present in the crude extract and possibly also to the formation of propionate from the pool of unlabeled pyruvate. Table III shows that lactate isolated from Experiment B had a relatively low specific isotopic content; therefore lactate cannot be an obligatory intermediate in the conversion of C-7 of nicotinate to propionate.

In considering possible mechanisms for the conversion of a-methyleneglutarate to propionate, a mechanism involving y-hydroxy-\gamma-methyl-\alpha-ketoglutarate appeared reasonable since the latter compound occurs in plants, where it has been shown to undergo enzymatic cleavage to 2 moles of pyruvate (10). However, neither y-hydroxy-\gamma-methyl-\alpha-ketoglutarate nor its lactone is metabolized by cell-free extracts. Whereas this appears to rule out these compounds as normal intermediates per se, the possibility that a derivative (viz. the CoA derivative) is involved cannot be excluded.

\[ \text{CH}_3\text{COOH} + \text{CO}_2 + \text{CH}_3\text{CH}_2\text{COOH} \rightarrow 2 \{ \text{C}_3\text{-Compds.} \} \]

Fig. 8. Pathways of nicotinic acid degradation

Fig. 9. Absorption spectra of B12 coenzyme. A, native coenzyme; B, after exposure to tungsten light; C, after addition of KCN.

\textsuperscript{1} An alternative, less likely possibility is that the synthesis or subsequent degradation of a-methyleneglutarate involves the formation of a symmetrical C\textsubscript{4} intermediate (1).
Isolation of Cobamide Coenzymes

During isolation of the intermediates of nicotinate metabolism, it was noted that the organism contains rather large amounts of a reddish compound that was partially purified and was identified as a vitamin B<sub>12</sub> derivative. To isolate the compound, 1 g of dried cells was extracted with 80% ethanol at 70° for 30 min. The B<sub>12</sub> coenzyme was purified by phenol extraction followed by ion exchange chromatography (11). The absorption spectrum is similar to that of a benzimidazole cobamide type of coenzyme (Fig. 9). On exposure to tungsten light, a characteristic peak appears at 350 μm, and on the addition of cyanide, a characteristic peak at 367 μm is seen. Based on the absorption spectrum, the content of coenzyme is 0.084 μmole per g of dried cells.

In view of the fact that this level of coenzyme is much above average for most microorganisms and is of the same order of magnitude as those found in bacteria in which the major energy metabolism is concerned with fermentation processes in which at least one step is catalyzed by a B<sub>12</sub> coenzyme-dependent enzyme (12, 13), it is tempting to believe that the high level of B<sub>12</sub> coenzyme in the nicotinic acid-fermenting clostridium reflects a role of this coenzyme in one of the steps of nicotinic acid decomposition. However, attempts to establish such a role have given ambiguous results. Thus, the over-all conversion of 7-<sup>14</sup>C-nicotinate to <sup>14</sup>CO<sub>2</sub> is partially inhibited by large amounts of intrinsic factor; however, this inhibition is not overcome by benzimidazole cobamide coenzyme, suggesting that the inhibition is unrelated to B<sub>12</sub> binding. Nevertheless, the inability to resolve the system for a B<sub>12</sub> coenzyme does not eliminate its participation, since it may be involved in a step that is not rate-limiting in our assay, or it may be tightly bound to an enzyme as in the methylmalonyl-CoA-succinyl CoA mutase studied by Mazumder, Sasakawa, and Ochoa (14).

REFERENCES
Nicotinic Acid Metabolism: II. THE ISOLATION AND CHARACTERIZATION OF INTERMEDIATES IN THE FERMENTATION OF NICOTINIC ACID
L. Tsai, Ira Pastan and E. R. Stadtman


Access the most updated version of this article at http://www.jbc.org/content/241/8/1807

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/241/8/1807.full.html#ref-list-1