Nicotinic Acid Metabolism

IV. FERREDOXIN-DEPENDENT REDUCTION OF 6-HYDROXYNICOTINIC ACID TO 6-OXO-1,4,5,6-TETRAHYDRONICOTINIC ACID

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SUMMARY

The characteristics and partial purification of a clostridial enzyme that catalyzes the reversible reduction of 6-hydroxy- nicotinic acid to 6-oxo-1,4,5,6-tetrahydronicotinic acid are described. This enzyme requires reduced ferredoxin or reduced methyl viologen dye as the electron donor. The enzyme is extremely sensitive to inactivation by air and iron-chelating reagents.

Previous reports from this laboratory have described the isolation of 6-hydroxynicotinic acid and 6-oxo-1,4,5,6-tetrahydronicotinic acid from the reaction media of a clostridial species grown on nicotinic acid (1). An enzyme that catalyzes the reversible oxidation of nicotinic acid to HNA has been purified from extracts of this microorganism (2). Since fortified crude extract of this Clostridium can metabolize HNA as well as nicotinic acid (1), this degradation was studied to define further the fermentation of nicotinic acid in this microorganism and the role of 6-oxo-1,4,5,6-tetrahydronicotinic acid.

This paper reports the partial purification of an enzyme which catalyzes the reversible reduction of HNA to the 4,5-dihydro-derivative, 6-oxo-1,4,5,6-tetrahydronicotinic acid. This enzyme requires reduced ferredoxin or reduced methyl viologen dye as the electron donor for the reduction of HNA.

METHODS

The nicotinic acid-fermenting Clostridium was grown and harvested and cell-free extracts were prepared by methods described previously (3). Clostridium pasteurianum starter cultures were a gift from Dr. W. Lovenberg. These cells were grown on a sucrose medium with nitrogen gas as the sole nitrogen source for two transfers. Then the medium was supplemented with ammonium sulfate (4). Ferredoxin was purified from these cells by a modification of the method of Mortenson (5, 6). The final preparations had an A280:A390 ratio of at least 0.74. Clostridium acetobutylicum ferredoxin was a gift of Dr. J. C. Rabinowitz. C. pasteurianum ferredoxin was also purchased from Worthington.

Ferredoxin was assayed with the hydrogenase system from Clostridium kluyveri (7).

Pyruvate was assayed by the method of Friedemann and Haugen (8). Nicotinic acid was assayed spectrophotometrically, following its reaction with cyanogen bromide (9).

Preparative and analytic disc gel electrophoreses were done on the standard Canalco system. Brinkman free flow electrophoresis was done in 20 mM potassium phosphate buffer, pH 7.4, at about 1500 volts, 200 mA, 5-10°, and with slow dosing and buffer flow. Enzymatic activity appeared in tubes 16 through 20. The electrophoresis could not be done in glyceral concentrations greater than 30% because of marked decreases in mobility.

Hydroxyapatite was purchased from Clarkson Chemical Company. DEAE-celluloses were purchased from Reeve Angel. They were all washed with acid and base as previously described (10), suspended in the buffer to be used, and, prior to use, washed extensively on the column with the eluting buffer until the pH and conductivity of the eluting buffer and eluate were the same.

Protein was determined by the method of Lowry et al. (11) after precipitation with 12% trichloracetic acid.

Cobinamide monocyanide was a gift from Dr. P. Renz. The 4,5-dihydro derivative of 6-hydroxynicotinic acid (i.e. 6-oxo-1,4,5,6-tetrahydronicotinic acid) was synthesized by a method developed by Dr. Tsai.* All other reagents and enzymes were commercial preparations: dithiothreitol, glutathione, sodium pyruvate, TPN, TPNH, 2,3,5-triphenyl tetrazolium chloride, pig heart isocitric dehydrogenase, and isocitric lactone from Calbiochem; 2-mercaptoethanol, α,α'-dipyridyl, and 1,10-ortho-
phenanthroline from Eastman; sodium dithionite from Fisher; streptomycin from Nutritional Biochemicals; ammonium sulfate (enzyme grade) from Mann; and 6-hydroxynicotinic acid from Aldrich. The 6-hydroxynicotinic acid was recrystallized from 50% aqueous acetic acid prior to determination of the ultraviolet extinction coefficients. All other chemicals were reagent grade or better.

RESULTS

Identification of 6-Oxo-1,4,5,6-tetrahydronicotinic Acid as Product of HNA Reduction

In preliminary experiments the disappearance of HNA, as catalyzed by cell-free extract, was accompanied by the appearance of a compound having a strong absorption band with a maximum at 275 nm. To isolate this product, reaction mixtures from many separate preliminary experiments were treated with 2% perchloric acid and, after centrifugation to remove the precipitated protein, the supernatant solutions were pooled; the resulting solution was adjusted to pH 3.5 and was extracted continuously for 24 hours with diethyl ether. The ether extract was dried with MgSO4 and evaporated to dryness. The residual was crystallized and sublimed as previously described (1). The sublimate consisted of colorless crystals. This product was identified as 6-oxo-1,4,5,6-tetrahydronicotinic acid on the basis of its melting point (219-225°, with decomposition), ultraviolet light absorption spectrum (Fig. 1), and infrared spectrum. All of these properties were identical with those obtained with an authentic synthetic sample of this compound; further, there was no depression of melting point on admixture of the enzymatic and synthetic product. Since this compound is the 4,5-dihydropyridine derivative of HNA for convenience we refer to it simply as the "dihydro" derivative.

Spectrophotometric Measurements of HNA Reduction

As shown in Fig. 1, HNA has appreciable absorbance at 310 nm (εM = 3.54 × 10³), whereas the dihydroderivative absorbs only slightly at this wavelength (εM = 0.38 × 10³). At 275 nm the molar absorbance of HNA is 3.66 × 10³ and that of the dihydroderivative is 11.2 × 10³ (repurification of the sample used in Fig. 1 gave this higher value). With crude extracts very little dihydroderivative accumulated so that the activity of the enzyme could be estimated by simply measuring the change in absorbance at 310 nm due to the disappearance of HNA. With partially purified enzyme preparations the dihydroderivative accumulated; however, the disappearance of HNA and the appearance of the dihydroderivative could be measured simultaneously from absorbance determinations at 275 nm and at 310 nm. Thus, the concentration (millimolar) of dihydroderivative equals:

\[ \frac{A_{275} - A_{310}}{0.35} \]

and the concentration (millimolar) of HNA equals:

\[ \frac{A_{310} - (0.93 \times \text{max dihydroderivate})}{3.5} \]

Since this rate of reaction in air was only 7% that under anaerobic conditions, all assays were carried out in atmosphere of argon or helium.

Cofactor Requirements

In studies with relatively crude enzyme fractions obtained by treating crude extracts with streptomycin and dithiothreitol, as described below, it was found that pyruvate, ferredoxin, CoA,
TABLE II
Incubation with C. kluyveri hydrogenase

The complete system contained the following components in 1 ml: Tris-HCl buffer, pH 7.8, 20 mM; HNA, 10 mM; C. kluyveri hydrogenase preparation, 9 mg. Experiment 1 also contained 91 mg of C. pasteurianum ferredoxin and 68 mg of protein from an ammonium sulfate fraction of the nicotinate grown cells. Experiment 2 contained 100 µg of C. acidivorans ferredoxin and 220 µg of crude enzyme protein which had been passed through Sephadex G-25. Reactions were run in 5-ml Warburg vessels gassed with hydrogen. After equilibration the reaction was started by addition of the enzyme from the side arm.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HNA consumed</th>
<th>Hydrogen uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles/90 min/mg protein</td>
<td></td>
</tr>
<tr>
<td>1. Complete</td>
<td>66</td>
<td>72</td>
</tr>
<tr>
<td>Minus ferredoxin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Minus ammonium sulfate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. Complete</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Minus HNA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Minus hydrogenase</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

and phosphate or arsenate were required for maximal activity (Table I). In other similar experiments it was found that diithiothreitol, glutathione, TPNH, and cobinamide had no effect on the reaction. Moreover, in none of these experiments was HNA reduced to nicotinic acid.

The need for ferredoxin suggested that this cofactor might be an electron carrier for the reduction of HNA. The roles of pyruvate, CoA, and orthophosphate might then be concerned simply with the generation of reduced ferredoxin as occurs in the phosphorolastic reaction (12). This interpretation was strengthened by the discovery that molecular hydrogen could replace pyruvate as the electron donor when HNA reduction is coupled to the ferredoxin-dependent hydrogenase system of C. kluyveri. This is illustrated by the data in Table II which show that in this coupled system the amount of hydrogen consumed is equal to the amount of HNA that disappears, when corrections are made for the small amount of endogenous hydrogen uptake in the absence of HNA. In the experiments described, the complete system contained the hydrogenase preparation from C. kluyveri which had been freed of ferredoxin by passage over DEAE-cellulose (7), an extract of the nicotinate-fermenting Clostridium, clostralid ferredoxin, and HNA. Little or no reaction occurred in the absence of any one of these components.

Assay of HNA Reductase Activity

Further evidence that pyruvate serves only as an electron donor is shown by the fact that reduced ferredoxin, produced nonenzymatically by reaction with dithionite, will support the enzymatic reduction of HNA. This observation led to the development of a simple assay system for measuring the HNA reductase activity by reaction with dithionite, will support the enzymatic reduction of HNA. This observation led to the development of a simple assay system for measuring the HNA reductase activity.

Fig. 2 presents some of the characteristics of this assay system. The reaction is linear with time at pH 6.4 and 7.4. At these pH values the reaction is also linear with enzyme concentration until over one-third of the substrate has been consumed. Fig. 2B presents the relation of HNA consumption to protein concentration at pH 7.4. The dependence of the reaction on substrate and dithionite is shown in Fig. 2C and D, respectively. Maximal activity is obtained with 15 to 20 mM dithionite. The enzyme appears to be saturated with 2 mM HNA. The dependence of was initiated by the further addition of 0.1 ml of a freshly prepared 0.2 M solution of sodium dithionite. The stock solution of sodium dithionite was made up in 0.1 M NaOH which had been gassed for 1 min in a 12-mm test tube. This solution had a pH of about 7.0 and was stable for at least 1 hour if kept stopped at 0°. The tubes were finally flushed with inert gas, stoppered, and incubated at 25° for 20 to 30 min. The reactions were stopped by the addition of 0.1 ml of 20% perchloric acid. After 5 min, when the decomposition of excess dithionite was complete, the samples were adjusted to pH 7.0 and centrifuged. From the ultraviolet absorbance of the supernatant solution at 310 and 275 µg, the amount of HNA which had disappeared and the amount of dihydro derivative formed were calculated as described above. One unit of HNA reductase activity is defined as the amount of enzyme that catalyzes the reduction of 1.0 µmole of HNA per min under the standard assay conditions.

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the results on a difference in absorbance at two wave lengths prevented use of concentrations of HNA much below 2 mm; with lower concentrations, the absorbance of control samples containing no substrate could not be ignored and reduced the sensitivity of the assay.

The effect of pH on the reaction is shown in Fig. 3. The maximum rate occurs at about pH 6.5. Tris-HCl and potassium phosphate buffers give comparable rates. The rapid decline in activity below pH 5.6 may be partly due to destruction of ferredoxin in acid (4).

**Purification of HNA Reductase**

The dithionite assay system with potassium phosphate buffer, pH 7.4, and *C. pasteurianum* ferredoxin, 91 pg per ml, was used to follow the purification.

**Crude Extract**—Cells were grown on nicotinic acid and frozen in liquid nitrogen. A suspension was formed by thawing 60 g of frozen cell paste in an equal volume of 0.05 M potassium phosphate buffer, pH 7.4, containing 0.03% Na₂S · 9H₂O. The suspension was passed through a French pressure cell, treated for 5 min with a few crystals of purified DNase, and centrifuged for 30 min at 30,000 × g. The supernatant solution was diluted 1:1 with water. This extract lost appreciable activity on standing several hours in the ice bath; therefore, the next step was carried out immediately.

**Streptomycin Treatment and Heat Step**—Streptomycin was added to the crude extract to give a final concentration of 1% (w/v). After 10 min a dithiothreitol solution adjusted to pH 7 was added to give a final concentration of 20 mM. After 15 min the large precipitate was removed by centrifugation. The activity in the supernatant solution was stable for at least several days in stopped tubes at 5°C. This precipitation resulted in only a 1.1- to 1.3-fold purification but removed large amounts of 260-nm absorbing material. The supernatant solution was placed in a stainless steel centrifuge bottle, HNA was added to a final concentration of 1 mM, and the extract was gassed with argon, stoppered, and heated for 30 min in a 62°C water bath. It was then cooled in ice, and the precipitate was removed by centrifugation.

**Ammonium Sulfate Fraction 1**—The straw-colored supernatant solution from Step 2 was fractionated by addition of solid ammonium sulfate at 5°C. The deep red-brown fraction precipitating between 55 and 75% ammonium sulfate was collected and dissolved in 0.05 M potassium phosphate buffer (pH 7.4) containing either 0.03% Na₂S · 9H₂O or 2 mM dithiothreitol.

**Heat and Ammonium Sulfate Fraction 2**—An aliquot of the preparation from Step 3 was gassed with argon, stoppered, and heated at 72°C for 30 min in the presence of 1 mM HNA. The brownish supernatant solution was refractionated by stepwise preparation with consecutive additions of ammonium sulfate. The precipitates were taken up in the same phosphate buffer as in Step 3. Table III summarizes the results of this purification scheme. The protein precipitating between 55 and 65% saturation in Step 4 represented a purification of about 20 fold with a yield of 43%.

Attempts at further purification were associated with large losses in enzymatic activity and decreases in specific activity. These steps included: column chromatography on DEAE-cellulose and hydroxylapatite, preparative disc gel electrophoresis at pH 7.5 and 9.5, Brinkman free flow electrophoresis, batch gel steps, and protamine, acid, and acetone fractionation.

**Electron Carrier Requirement**

With partially purified preparations the reaction has an absolute requirement for an electron carrier. Fig. 4 shows the dependence of HNA loss on ferredoxin concentration. This *C. pasteurianum* ferredoxin had a $A_{260}\text{nm}:A_{260}\text{nm}$ ratio of 0.74. Assuming that it is 90% pure, the apparent $K_m$ for this ferredoxin is $1.6 \times 10^{-5}$ M.

Since dithionite can reduce other electron carriers, the capacity of various carriers to serve as electron donors for the reduction of HNA was explored. Safranin O, neutral red, and TPN had essentially no ability to substitute for ferredoxin in this reaction. In addition, a TPNH-generating system with pig heart isocitric dehydrogenase was inactive. Benzyl viologen was inactive but the dithionite reduced it to a poorly soluble brown derivative which is probably the 2-electron reduction product (13).

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**TABLE III**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total</th>
<th>Total</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units</td>
<td>%</td>
</tr>
<tr>
<td>1. Crude supernatant</td>
<td>159</td>
<td>5400</td>
<td>2440</td>
<td>0.45</td>
</tr>
<tr>
<td>2. Heat at 62°C for 30 min</td>
<td>145</td>
<td>1150</td>
<td>2520</td>
<td>2.2</td>
</tr>
<tr>
<td>3. Ammonium sulfate saturation (55-75%)</td>
<td>40</td>
<td>340</td>
<td>2120</td>
<td>6.2</td>
</tr>
<tr>
<td>4. Aliquot (5 ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat at 72°C for 30 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-55%</td>
<td>1</td>
<td>3.7</td>
<td>34</td>
<td>9.2</td>
</tr>
<tr>
<td>55-60%</td>
<td>2</td>
<td>5.1</td>
<td>56</td>
<td>11.0</td>
</tr>
<tr>
<td>60-65%</td>
<td>2</td>
<td>4.6</td>
<td>42</td>
<td>9.1</td>
</tr>
<tr>
<td>65-70%</td>
<td>1</td>
<td>4.8</td>
<td>10</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Specific activity is micromoles of HNA consumed per mg of protein per min.
Enzymatic oxidation of the dihydro derivative to HNA occurs readily in the presence of appropriate electron acceptors. Thus, with chemically prepared dihydro derivative as substrate the enzyme catalyzes reduction of benzyl viologen, methyl viologen, 2,3,5-triphenyltetrazolium dyes, and ferredoxin. In addition, enzyme catalyzed reduction of benzyl viologen, methyl viologen, and 6-hydroxynicotinic acid.

In these studies the incubation mixtures contained potassium phosphate buffer, pH 7.4, 50 mM; dihydro derivative, 0.4 mM; benzyl viologen neutralized to pH 7, 0.8 mM; and variable amounts of enzyme from Step 4, in 2-ml reaction volumes. The reactions were run at room temperature. The mixtures in Klett tubes were gassed for 2 min with argon prior to the addition of enzyme. The appearance of reduced benzyl viologen was followed in a Klett colorimeter with a No. 540 filter. A viologen standard solution was prepared by incubation with 5% glucose in Na₂CO₃, 0.1 M (12). Equilibrium was considered attained when the Klett reading was unchanged for 5 min. The reaction was then stopped with perchloric acid and neutralized and an aliquot was used to determine the amounts of HNA and the dihydro derivative. There was no reduction of benzyl viologen or formation of HNA in the absence of enzyme. With 26, 51, and 128 μg of enzyme protein the HNA at equilibrium accounted for 50 to 56% of the dihydro derivative added and the reduced benzyl viologen was 70 to 81% of the total benzyl viologen added. From these data the calculated $E'_{\text{a}}$ at pH 7.4 is −0.39 volts for this back reaction. The error in this determination resulting from oxygen left in solution, incomplete reaction, high blank from the oxidized benzyl viologen, and competing reactions for the dihydroderivative is probably about 10%.

**Stoichiometry**

Enzyme fractions from the first three steps in the purification always showed less dihydro derivative formation than HNA loss. This difference increased with the amount of enzyme and the extent of the reaction. In the 55 to 60% saturated fraction from Step 4, the amount of dihydro derivative formed is greater than 85% of the amount of HNA loss. These enzyme fractions catalyze the hydrolysis of the dihydro derivative to ammonia and an aldehyde. The lack of perfect stoichiometry in the reduction of HNA is probably attributable to this hydrolysis.

**Stability and Effect of Iron Chelation**

The enzyme from Step 3 is stable to freezing and thawing and storage for several months in liquid nitrogen and up to 50 hours at 5°C when flushed with argon (Fig. 6A). However, the enzyme is quite unstable in air at 5°C, losing over 50% of the activity in 24 hours. The addition of 10⁻⁴ M cobamidine cyanide (Factor B) accelerates the loss of activity in air. This may be due to the ability of vitamin B₉ derivatives to catalyze sulfhydryl oxidation (14). The incubations in air resulted in comparable losses of activity with ferredoxin or methyl viologen as the electron carriers.

**Reverse Reaction**

Enzymatic reduction of the dihydro derivative to HNA occurs readily in the presence of appropriate electron acceptors. Thus, with chemically prepared dihydro derivative as substrate the enzyme catalyzed reduction of benzyl viologen, methyl viologen, 2,3,5-triphenyltetrazolium dyes, and ferredoxin. In addition, a slow enzymatic conversion of the dihydro derivative to HNA without added electron carriers occurred in the presence of air. In all of these experiments over 95% of the added dihydro derivative could be accounted for as either HNA or unchanged dihydro derivative.

In the presence of tetrazolium dye the enzyme catalyzes quantitative conversion of the dihydro derivative to HNA. On the other hand, only 5% of the dihydro derivative is oxidized to HNA in the presence of equimolar amounts of methyl viologen and the dihydro derivative.

The enzymatic reduction of benzyl viologen by the dihydro derivative was used to estimate the $E'_{\text{a}}$ for the reverse reaction. In these studies the incubation mixtures contained potassium phosphate buffer, pH 7.4, 50 mM; dihydro derivative, 0.4 mM; benzyl viologen neutralized to pH 7, 0.8 mM; and variable amounts of enzyme from Step 4, in 2-ml reaction volumes. The reactions were run at room temperature. The mixtures in Klett tubes were gassed for 2 min with argon prior to the addition of enzyme. The appearance of reduced benzyl viologen was followed in a Klett colorimeter with a No. 540 filter. A viologen standard solution was prepared by incubation with 5% glucose in Na₂CO₃, 0.1 M (12). Equilibrium was considered attained when the Klett reading was unchanged for 5 min. The reaction was then stopped with perchloric acid and neutralized and an aliquot was used to determine the amounts of HNA and the dihydro derivative. There was no reduction of benzyl viologen or formation of HNA in the absence of enzyme. With 26, 51, and 128 μg of enzyme protein the HNA at equilibrium accounted for 50 to 56% of the dihydro derivative added and the reduced benzyl viologen was 70 to 81% of the total benzyl viologen added. From these data the calculated $E'_{\text{a}}$ at pH 7.4 is −0.39 volts for this back reaction. The error in this determination resulting from oxygen left in solution, incomplete reaction, high blank from the oxidized benzyl viologen, and competing reactions for the dihydroderivative is probably about 10%.

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Attempts were made to find agents which could stabilize the enzyme in the presence of air. Enzyme from Step 3 (0.1 to 0.5 ml) was mixed with various test materials in a tube, 10 × 100 mm, and incubated at 5°C for between 20 and 30 hours. The following compounds had no effect on the stability when compared with a water blank (loss of 50 to 80% of the activity): FeSO₄, MgCl₂, Na₃MoO₄ at 1 and 10 mm; HNA and the dihydro derivative; 2-mercaptoethanol, dithiothreitol, glutathione at 5 to 50 mm; ammonium sulfate 4 to 25% saturation; NaCl, KCl, NH₄Cl at 0.1 m; urea at 1.25 m; potassium phosphate buffer, pH 7.4, 50 mm; ATP and AMP at 1 mm; bovine serum albumin, 1 mg per ml, pyruvate, 5 mm. In addition to the effect of Factor B noted above, the following compounds produced a marked increase of enzyme inactivation in air: EDTA at pH 7 and combinations of FeSO₄ 2 mm plus HNA 1 mm or EDTA 1 mm. Stability at pH 6.0 and 6.4 was not appreciably different than that at pH 7.0. Below pH 6 the enzyme appears to be even less stable.

Glycerol and ethylene glycol in high concentrations stabilized the enzyme. Fig. 6B shows that these effects require concentrations of 30% and over. Na₂S·9H₂O also stabilized slightly with the maximal effect at 0.03%. The Na₂S effect appears to be additive with that of glycerol.

One preparation of enzyme which had been purified by Brinkman free flow electrophoresis and hydroxyapatite chromatography (specific activity 3.4 μmoles of HNA loss per mg per min) showed much less stability even when gassed with argon. This instability was accelerated by MN and FAD at 10⁻⁴ m, cobinamide cyanide at 5 × 10⁻⁴ m, and 2 mm FeSO₄ plus 50 mm potassium pyrophosphate buffer, pH 7.3. On the other hand, 2 mm FeSO₄ with or without 2 to 5 mm citrate buffer stabilized the enzyme to this inactivation.

During various purification attempts on the enzyme from Step 3, it became apparent that some preparations had more activity with methyl viologen as the electron carrier than with ferredoxin. Preparations were obtained with ratio activity with 1 mm methyl viologen and 0.15 to 0.20 mg per ml of ferredoxin of from 2 to greater than 40. The greatest differential loss of activity with ferredoxin was seen after chromatography on microgranular DEAE-cellulose (DE 32). Chromatography on DEAE-cellulose powder and advanced fibrous ion exchange cellulose (DE 23) did not produce as much loss of this activity.

Attempts to restore the activity with ferredoxin by addition of various metals, FMN, FAD, folate acid, boiled cell extract, or recombination of fractions were unsuccessful. Addition of inactive fractions to preparations active with ferredoxin produced no additive or inhibitory effect.

A preparation having essentially no activity with ferredoxin exhibited a linear change in rate with varying enzyme concentration when methyl viologen was the electron carrier. Furthermore, the apparent Kₘ for methyl viologen was the same as the enzyme from Step 4. This suggests that one enzyme catalyzes the reaction with either ferredoxin or methyl viologen as the electron carrier and that an alteration in the enzyme can lead to differential loss in its capacity to accept electrons from ferredoxin.

The known ability of DEAE-cellulose to bind metals (10), the rapid loss of enzymatic activity on incubation with EDTA, and the stabilizing effect of FeSO₄ in one preparation suggested that metals may be necessary for enzymatic activity. To test this possibility enzyme from Step 3 was incubated for 3 days at 5°C in tubes gassed with argon and containing 10 mm dithiothreitol plus either water, 5 mm α,α'-dipryridyl, or 5 mm o-phenanthroline.

![Figure 6](http://www.jbc.org/)

**Figure 6.** Stability of the enzyme. A, enzyme from Step 3 of purification was incubated in stoppered tubes at 5°C in an atmosphere of argon (O), in air (●), or in air plus 10⁻⁴ m cobinamide cyanide (Factor B) (▲). At each time the activity was compared with a sample frozen in liquid nitrogen. This stable frozen sample was taken as 100% activity. B, enzyme from Step 3 of purification was incubated for 20 to 24 hours with either ethylene glycol (●), glycerol (▲), or glycerol plus Na₂S·9H₂O 0.03% (■). They were then assayed by the dithionite assay system described in the text with ferredoxin as the electron carrier. As in A, activity was compared with a frozen aliquot with no additives.

**Table IV**

<table>
<thead>
<tr>
<th>VOLUME %</th>
<th>Methyl viologen</th>
<th>Ferredoxin C. pasteurianum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.8</td>
<td>6.9</td>
</tr>
<tr>
<td>α,α'-Dipryridyl</td>
<td>6.7</td>
<td>0.36</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The incubation mixtures with the chelators formed the characteristic colors of the ferrous complexes. The preparations were passed through a small Sephadex G-25 column to separate the enzyme from dye and assayed by the dithionite assay system described in the text. This system was run at pH 7.4 for 30 min at 25°C with either 0.15 mg per ml of *C. pasteurianum* ferredoxin or 1 mm methyl viologen.
incubation with FeSO₄ failed to restore the activity with ferredoxin. Phenanthroline treatment caused complete loss of activity with either electron carrier or with air in the reverse reaction.

The enzymatic activity can be located on disc gel electrophoresis by the appearance of the insoluble red formazan color when the gel is incubated with potassium phosphate buffer, pH 7.4, 100 mM; 2,3,5-triphenyltetrazolium, 4.5 mM; and the dihydro derivative, 2 mM. In the standard Tris-glycine buffer system with 7.5% acrylamide separating gel, the band of enzymatic activity migrated about half the distance of the tracking dye. Only one active band is present when the enzyme is layered on the stacking gel in glycerol and the buffer contains 0.03% Na₂S·9H₂O. This band (prior to staining) has a dark brown color similar to that of ferredoxin.

Furthermore, consecutive treatment of the enzyme from Step 3 with Brinkman free flow electrophoresis, hydroxylapatite chromatography at pH 7.5, and ammonium sulfate fractionation resulted in a preparation which appeared to be at least 50% pure by disc gel electrophoresis. The activity of this preparation with 1 mM methyl viologen was 22 times that with 0.15 mg per ml of ferredoxin. This preparation was dark brown and gave the characteristic spectrum of the ferrous complex on reaction with o-phenanthroline in excess sodium mersalyl and dithionite. It also contained acid-labile hydrogen sulfide as judged by the formation of methylene blue on incubation of the enzyme with N,N-dimethyl phenylenediamine (15).

Thus there is strong presumptive evidence that this enzyme is an iron-containing protein which is very labile to air oxidation and is inactivated by the removal of iron from the enzyme. Apparently, removal of part of this iron results in the loss of activity only with ferredoxin. The inability to reactivation the enzyme with iron alone is similar to the experience with ferredoxin (16).

**Identification of Ferredoxin in Nicotinic Acid-grown Cells**

Cells grown on nicotinic acid were disrupted in a French pressure cell and then treated by the acetone fraction and DEAE-cellulose chromatography method of Mortenson for isolation of ferredoxin (5). The dark brown fraction obtained by elution with 1 M Tris-HCl buffer substituted for ferredoxin in the C. kluyveri hydrogenase system and in the conversion of HNA to the dihydro derivative with the enzyme from Step 4. In this preparation the activity remained as C. pasteurianum ferredoxin in the 7.5% acrylamide analytic disc gel run at pH 0.5.

**Energy Yield from Fermentation of Nicotinic Acid by Whole Cells**

It has been established that, for each millimole of ATP produced (net) during the anaerobic fermentation of various substrates by microorganisms, 10 mg (dry weight) of microbial mass is produced (17). Therefore, to obtain an estimate of the amount of ATP generated in the fermentation of nicotinic acid, the yield of bacterial cells per mole of substrate fermented was determined. The *Clostridium* was grown in the presence of 0 to 0.5 g of nicotinate per 100 ml of culture medium. After maximal growth had occurred the cells were harvested, dried over P₂O₅, and weighed. The nicotinic acid remaining in the supernatant was assayed by the CNBr method. The average milligrams of dry cell weight per mmole of nicotinic acid consumed are 9.5 ± 1.3 S.D. Therefore, the fermentation of nicotinic acid by this *Clostridium* probably yields only 1 eq of ATP per mole of nicotinic acid consumed.

**DISCUSSION**

The first step in the degradation of nicotinic acid by aerobic and anaerobic organisms appears to be the same, i.e. the formation of 6-hydroxynicotinic acid. From this point the paths diverge sharply. Aerobic metabolism involves the oxidation of the HNA to 2,5-dihydropyridine or 2,6-dihydroxynicotinic acid, followed by further oxidation to N-formyl maleamic acid (18, 19). Oxygen or methylene blue dye is needed to accept the electrons from these oxidations.

The metabolism of HNA by the *Clostridium* described in this paper proceeds by a reduction to the 4,5-dihydro derivative of HNA. This reaction is strongly endergonic with a potential at pH 7.4 of about +0.30 volt. Since this oxidation of nicotinic acid to HNA is strongly exergonic (2) it might be expected that the reduction of HNA would be coupled with the oxidation of nicotinic acid. However, this appears not to be the case since TPNH serves as the primary electron acceptor in the oxidation of nicotinic acid and electron donors of lower potential than TPNH (viz. ferredoxin) are required for this reduction of HNA to the dihydroderivative. A particulate cell-free preparation of *Azotobacter vinelandii* has been described, that catalyzes the reduction of benzyl and methylviologen dyes by TPNH (20).

Perhaps there is a similar system in the nicotinic acid-fermenting *Clostridium* that can utilize TPNH generated in the oxidation of nicotinic acid for the reduction of ferredoxin. Such an enzyme has not yet been shown.

Alternatively, the reduced ferredoxin needed for HNA reduction could be derived from the oxidation of pyruvate, which is probably a later intermediate in nicotinic acid metabolism (1). This possibility is supported by the fact that the reduction of HNA in crude cell-free extracts requires the presence of pyruvate, phosphate, coenzyme A, and ferredoxin. These supplements are required also for the phosphoroclastic decomposition of pyruvate by other clostridia (21). In this reaction ferredoxin serves as an electron carrier.

The over-all fermentation of nicotinic acid by the present *Clostridium* involves the formation of two 3-carbon intermediates with one of these at the oxidation level of pyruvate (1). The oxidation of this compound could yield the 1 mole of carbon dioxide and acetate that accumulate as end products. If this oxidation is similar to the phosphoroclastic reaction it could yield the 1 eq of ATP (in the form of acetyl CoA or acetyl phosphate) expected on the basis of cellular mass yield. In addition, this oxidation could yield the reduced ferredoxin required for the reduction of HNA to the dihydro derivative.

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