The Pyridine Nucleotide Cycle and Its Role in the Biosynthesis of Ricinine by *Ricinus communis* L.*

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SUMMARY

The pyridine moieties of the pyridine nucleotides, nicotinic acid mononucleotide, nicotinic acid adenine dinucleotide, and nicotinamide adenine dinucleotide, were shown to be incorporated into ricinine by *Ricinus communis* L. with an efficiency comparable to that of quinolinic acid, nicotinic acid, and nicotinamide, which had been established previously as being efficient precursors of this alkaloid. All of these pyridine compounds except quinolinic acid were postulated to be closely related in a cyclic form, and they could function as a source of metabolic intermediates for the biosynthesis of ricinine. It was shown that nicotinamide was readily converted to nicotinic acid and *N*-methyl nicotinamide *in vivo*; nicotinic acid was converted to nicotinamide adenine dinucleotide and *N*-methyl nicotinonic acid (trigonelline) *in vitro*; nicotinamide adenine dinucleotide was readily converted to nicotinamide and *N*-methyl nicotinamide *in vivo*.

The biosynthesis of pyridine compounds in higher plants has been studied extensively by determining labeling patterns which characterize the incorporation of various isotopically labeled precursors into the pyridine alkaloids, ricinine (1-7), nicotine (8-11), and anabasine (12, 13). The present evidence indicates that the pyridine ring of these alkaloids and of nicotinic acid (14) produced by microorganisms is formed from aspartic acid and a glycerol derivative. Recent experiments on the biosynthesis *in vitro* of quinolinic acid in a system prepared from *Escherichia coli* (15) also support the theory of the formation of the pyridine ring from glycerol and aspartate. However, the reports that aspartate-2-14C gives rise to nicotinic acid-7-14C in *Serratia marcescens* (16) and that formate-14C and acetate-14C but not glycerol-14C are incorporated into the ring carbons of nicotinic acid in a partially purified enzyme system from *Clostridium butyricum* (17) suggest that alternate pathways for pyridine ring biosynthesis may be operating in some organisms. Pertinent review articles by Ramstad and Agurell (18) and by Leete (19) have appeared recently. In previous studies from this laboratory (7, 20), it was shown that quinolinic acid was an efficient precursor of ricinine in intact *Ricinus communis* L. plants. The evidence indicated that quinolinic acid may serve as a key intermediate in the formation of pyridine compounds in higher plants. The conversion of quinolinic acid into nicotinic acid mononucleotide was also shown *in vitro* in plants (20), microorganisms (21, 22), and animals (23, 24).

It was suggested by Joshi and Handler (25) that a portion of the nicotinamide produced *in vitro* by the action of nicotinamide adenine dinucleotide glycohydrolase may be reutilized by deamidation of nicotinamide and conversion to NAD via the Priess-Handler pathway (26). Cyclic schemes for the degradation and resynthesis of NAD have been proposed by Joshi and Handler (25) and Sarma, Rajalakshmi, and Sarma (27). These schemes were postulated when the available evidence indicated that the nicotinyl moiety enters the pathway of NAD biosynthesis de novo at the level of free nicotinic acid. The recent discovery that the nicotinyl moiety enters the pathway de novo as nicotinic acid mononucleotide which is formed from quinolinic acid and 5-phosphoribosylpyrophosphate (20-24) provides the basis for the formulation of a pyridine nucleotide cycle as shown in Fig. 1. Gholson (28) has recently summarized the evidence for the formation and degradation of the pyridine nucleotides in biological systems and has suggested that the cycle exists in plants, animals, and microorganisms.

The recent discovery of hepatic nicotinamide mononucleotide pyrophosphorylase (29) offers a potential "short circuit" of the cycle between nicotinamide and NAD. However, the
quantitative contribution of this reaction to NAD biosynthesis has yet to be evaluated.

The objective of this investigation was to determine whether the pyridine ring moiety of the pyridine nucleotides was incorporated into ricinine and to measure the efficiency of these compounds as alkaloid precursors. This study was also designed to investigate the occurrence of the pyridine nucleotide cycle in the castor plant and its relationship to the biosynthesis of ricinine.

**EXPERIMENTAL**

**Materials**

*Labeled Compounds Used*—$^{14}$C-Labeled nicotinic acid and nicotinamide and aniline-$^{14}$C were purchased from New England Nuclear. Quinolinic acid-2,3,7,8-$^{14}$C was synthesized (10) by the condensing glycerol and uniformly labeled aniline-$^{14}$C. N-methylnicotinic acid-$^{14}$C and N-methylnicotinamide-$^{14}$C were synthesized as follows. About 10 μmoles of nicotinic acid-
7-14C or nicotinamide-7-14C solution were brought to dryness in a 5-ml round bottom flask. To the residue, 0.5 ml of methyliodide was added and the mixture was refluxed under an air condenser for 6 hours in an oil bath at about 50°. The product was taken to dryness and the residue was dissolved in a minimum amount of water. The labeled N-methyl nicotinic acid or N-methyl nicotinamide was further purified by paper chromatography with Whatman No. 3MM filter paper. The radiochemical yield of each compound was 80%.

The pyridine nucleotides, nicotinic acid adenine dinucleotide-7-14C and nicotinic acid mononucleotide-7-14C, were prepared from incubation of nicotinic acid-7-14C with extracts of Bacillus subtilis (30) and isolated on a Dowex 1 formate column (26). NAD-7-14C was prepared by incubation of nicotinic acid-7-14C with extracts of Saccharomyces fragilis (28) and isolated on a Dowex 1 formate column (26). NAD-7-14C was prepared by incubation of nicotinic acid-7-14C in a desiccator over P2O5 under vacuum. Dried residues obtained from plant extracts were extracted with 7 ml, 1.5 ml, 1.0 ml, 1.0 ml volumes of water; after each treatment the supernatant fraction was recovered by centrifugation. All supernatant fractions were combined and an aliquot was used for counting the total radioactivity recovered (Packard model 314A Tri-Carb liquid scintillation spectrometer). Samples of the plant extracts (0.1 ml) were spotted on 1-inch strips of Whatman No. 1 paper and chromatographed in four different solvent systems: 85% isopropyl alcohol, n-butyl alcohol-H2O-acetic acid (4:2:1), n-butyl alcohol saturated with 3% NH4OH, and acetic acid-H2O (7:3). Radioactive peaks on the chromatograms were detected with a Nuclear-Chicago 4π Autoradiograph II paper strip counter and were identified by comparing them to standard chromatograms of authentic compounds obtained under the same conditions.

The NAD spot was eluted and further identified by conversion to NADH with alcohol dehydrogenase as follows. To 0.35 ml of NAD-14C eluent were added 2.7 ml of Tris buffer (pH 10.0, 1.0 mm), 0.1 ml of alcohol dehydrogenase (3.0 mg per ml, Sigma), and 0.1 ml of ethanol (95%) to start the reaction in a 1-cm spectrophotometric cell. The absorption at 340 μm rose significantly with a small comcomitant fall at 260 μm. The reaction was terminated by exposure of the contents of the cell to boiling water for 1 min. NADH and unchanged NAD were separated on a DEAE-cellulose column (0.7 X 12 cm) by batchwise elution with ammonium bicarbonate (32).

The standard reaction mixture consisted of phosphate buffer (pH 7.0), 0.67 mM; MgCl2, 10.0 μM; EDTA, 8 μM; ATP, 20.0 μM; L-methionine, 5.0 μM; S-adenosyl-L-methionine, 0.1 μM; nicotinic acid-7-14C, 0.1 μM, 2.22 cpm × 10^-4; and 1 ml of the homogenate to a total volume of 2 ml. The mixture was incubated at 30° for 12 hours. An addition of 200 μg of streptomycin and penicillin was made in some experiments to eliminate the possibility of microbial action during the long incubation period. The antibiotics did not affect the results. The reaction was terminated with 30% perchloric acid and centrifuged. The supernatant solution was neutralized with 1 N KOH. The clear supernatant solution was cochromatographed with authentic compounds in five solvent systems: 85% isopropyl alcohol, 60% n-propyl alcohol, n-butyl alcohol-H2O-acetic acid (4:2:1), n-butyl alcohol saturated with 15% NH4OH and 95% ethanol-1 M ammonium acetate (7:3), pH 5.0. The products were detected on the basis of fluorescence in ultraviolet light (254 μm). Radioactive peaks were located with a Nuclear-Chicago 4π Autoradiograph II paper strip counter. NAD-14C eluted from the paper chromatogram was further identified by conversion into NADH-14C with alcohol dehydrogenase as described previously. The product was placed on a DEAE-cellulose column in the presence of carrier NADH. The NADH recovered from the column was found to contain all the radioactivity. N-Methylnicotinic acid-14C was diluted with 50.0 mg of carrier N-methylnicotinic acid and purified by recrystallization to constant specific activity. Other radioactive peaks found on the paper chromatograms were not identified.

Cloning in Vivo of NAD-7-14C.—To each of two uniform 1-week-old castor seedlings, 10 μl (specific activity, 13.5 mCi per mmole, 6.55 μCi per μl) of NAD-7-14C were introduced by means of stem absorption. One plant was harvested 1 hour later and...
Incorporation of quinolinic acid-2,3,7,8-14C, nicotinic acid-7-14C, nicotinamide-7-14C, nicotinic acid adenine dinucleotide-7-14C, N-methylnicotinic acid-8-14C, and N-methylnicotinamide-8-14C into ricinine by intact Ricinus communis L. plants.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor</th>
<th>Ricinine</th>
<th>Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Amount administered</td>
<td>Specific activity</td>
</tr>
<tr>
<td>Quinolinic acid-2,3,7,8-14C</td>
<td>0.26 mcC/mole</td>
<td>1,660.0</td>
<td>1.35 mcC/mole</td>
</tr>
<tr>
<td>Nicotinic acid-7-14C</td>
<td>10.00 mcC/mole</td>
<td>92.4</td>
<td>3.91 mcC/mole</td>
</tr>
<tr>
<td>Nicotinamide-7-14C</td>
<td>11.00 mcC/mole</td>
<td>57.7</td>
<td>3.31 mcC/mole</td>
</tr>
<tr>
<td>Nicotinic acid mononucleotide-7-14C</td>
<td>12.40 mcC/mole</td>
<td>19.5</td>
<td>11.85 mcC/mole</td>
</tr>
<tr>
<td>Nicotinic acid adenine dinucleotide-7-14C</td>
<td>12.40 mcC/mole</td>
<td>43.6</td>
<td>3.30 mcC/mole</td>
</tr>
<tr>
<td>NAD-7-14C</td>
<td>13.50 mcC/mole</td>
<td>29.7</td>
<td>1.57 mcC/mole</td>
</tr>
<tr>
<td>N-methylnicotinicotinic acid-8-14C</td>
<td>10.00 mcC/mole</td>
<td>61.2</td>
<td>1.89 mcC/mole</td>
</tr>
<tr>
<td>N-methylnicotinamide-8-14C</td>
<td>7.50 mcC/mole</td>
<td>79.4</td>
<td>0.79 mcC/mole</td>
</tr>
</tbody>
</table>

* Ten-day-old plants were used. The duration of the experiment was 96 hours. Percentage incorporation was calculated by dividing the total radioactivity recovered by the total radioactivity administered.

RESULTS

The results of 96-hour ricinine biosynthesis studies from plants administered nicotinic acid mononucleotide-7-14C, nicotinic acid adenine dinucleotide-7-14C, NAD-7-14C, quinolinic acid-2,3,7,8-14C, nicotinamide-7-14C, N-methyl nicotinic acid-8-14C, and N-methylnicotinamide-8-14C are presented in Table I. The amounts of precursors administered were nearly equal except for quinolinic acid-2,3,7,8-14C. Considerably more of this compound was administered since its specific activity was low. In Fig. 2A, data are shown comparing the efficiencies of the same precursors listed in Table I, but in the former instances information from experiments conducted for shorter times are included. N-methylnicotinic acid and N-methyl-nicotinamide were less efficient precursors of ricinine than were the compounds in the pyridine nucleotide cycle shown in Fig. 1. It is proposed, therefore, that N-methylnicotinic acid and N-methylnicotinamide be placed outside the cycle. N-methyl-nicotinic acid is known to occur in corn and its occurrence in castor plants is reported herein (Table III). N-methylnicotinic acid can be converted to nicotinic acid, nicotinamide, and NAD (30). N-methylnicotinamide has not been previously reported in plants, and its occurrence in castor plants is shown in Table II. The roles of nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide in the biosynthesis of NAD in animal systems (26, 34, 35) and bacteria (36) have been well established, and it is likely that the same pathway occurs in higher plants.

In an attempt to understand better the role of NAD, nicotinic acid, nicotinamide, and quinolinic acid in the biosynthesis of ricinine, an experiment was performed in which the same dosage of each precursor was used, and the results from this experiment are presented in Fig. 2B. The percentage incorporation of NAD, nicotinic acid, and nicotinamide at the same dosage level (900 mpmoles per plant) was nearly the same. Quinolinic acid was the most efficient precursor of ricinine; however, previous studies have shown that the efficiency of quinolinic acid and nicotinic acid varies inversely with the amount administered (7). Thus the significance of the higher efficiency of quinolinic acid shown in Fig. 2B is difficult to assess at this time. The high concentration of quinolinic acid (1650 mpmoles per plant) administered to castor plants used in the experiment reported in Fig. 2A is the reason for the slightly lower incorporation as compared with NAD and nicotinamide in that series of experiments. The relative percentage incorporation of these precursors may depend upon the level and activity of the enzymes involved in their metabolism, i.e. deamidase, transmethylase, phosphatase, and NAD glycohydrolase; however, all precursors from the cycle in Fig. 1 and quinolinic acid are incorporated rapidly into ricinine as shown in Fig. 2A and B. It has been proposed that quinolinic acid is one of the first pyridine compounds formed in the biosynthesis of pyridine compounds in plants (7, 10, 20) and microorganisms (21). From experiments in vitro with castor plant homogenates, it was found that quinolinic acid was converted to nicotinic acid mononucleotide and subsequently to nicotinic acid (30). One possible explanation for the higher efficiency of quinolinic acid as a precursor can be that free nicotinic acid is not in the direct route of ricinine biosynthesis and that it is utilized only by competition with quinolinic acid for the formation of nicotinic acid mononucleotide (Fig. 1). Also, free nicotinic acid can be utilized for the production of N-methyl-
nicotinic acid a widely occurring alkaloid in higher plants (37), or it can be stored as niacinogen (38). Results of precursor competition studies\(^1\) between quinolinic acid and nicotinic acid showed that the percentage incorporation of quinolinic acid-2,3,7,8\(^{14}\)C into ricinine was not markedly affected when diluted 10-μmoles of quinolinic acid-2,3,7,8\(^{14}\)C (specific activity, 90.7 μC per mmole) with and without 36 μmoles of nicotinic acid were administered to castor plants, and the percentage incorporation

\(^1\) Experiments were performed under the same conditions used for those in Table I. In the labeled quinolinic acid experiment, 4
TABLE II

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Total radioactivity administered</th>
<th>Total radioactivity recovered</th>
<th>Product</th>
<th>Distribution of radioactivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinamide-7-14C</td>
<td>μM</td>
<td>16.0</td>
<td>10.4</td>
<td>Nicotinic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N-methyl nicotinamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NAD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ricinine and others</td>
</tr>
</tbody>
</table>

* Percentages of distribution of radioactivity reported here are averaged values taken from paper chromatograms of each solvent system described under "Experimental."

TABLE III

Conversion in vitro of nicotinic acid-7-14C into NAD and N-methyl nicotinamide

The standard reaction mixture consisted of phosphate buffer (pH 7.0), 0.67 mM; MgCl2, 10.0 mM; EDTA, 8 μM; ATP, 20.0 μM; L-methionine, 5.0 μM; S-adenosyl-L-methionine, 0.1 μM; nicotinic acid-7-14C, 0.1 μM, 2.22 cpm X 10^-4; and 1 ml of the homogenate to a total volume of 2 ml. The mixture was incubated at 30°C for 12 hours.

<table>
<thead>
<tr>
<th>Components of reaction mixture</th>
<th>Radioactive components recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid</td>
<td>NAD</td>
</tr>
<tr>
<td>Complete</td>
<td>1871</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>1803</td>
</tr>
<tr>
<td>Minus EDTA</td>
<td>1966</td>
</tr>
</tbody>
</table>

TABLE IV

Cleavage in vitro of NAD-7-14C by young castor plants

<table>
<thead>
<tr>
<th>Duration of experiment</th>
<th>Total radioactivity administered</th>
<th>Distribution of radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td>mgC</td>
<td>Product*</td>
</tr>
<tr>
<td>1</td>
<td>65.5</td>
<td>Ricinine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-methyl nicotinamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unchanged NAD and others</td>
</tr>
<tr>
<td>96</td>
<td>65.5</td>
<td>Ricinine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-methyl nicotinamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unchanged NAD and others</td>
</tr>
</tbody>
</table>

* Products were first recovered from DEAE cellulose column chromatography and further identified by paper chromatography in the solvent systems described in the text.

of 14C was 4.3 and 5.4, respectively. In the labeled nicotinic acid experiment, 60.6 mmoles of nicotinic acid 7-14C (specific activity, 12.4 mC per millimole) with and without 720 mmoles of quinolinic acid were administered to castor plants, and the percentage incorporation of 14C was 11.9 and 20.7, respectively.

1 Castor cotyledons were homogenized in a Potter homogenizer with 4 volumes of 0.1 M potassium phosphate buffer, pH 7.0. After centrifugation at 10,000 x g for 20 min at 4°C, supernatants were assayed for protein, quinolinic acid decarboxylase (21), and nicotinic acid mononucleotide pyrophosphorylase (35). Nicotinic acid mononucleotide was determined by the formation of nicotinic acid-7-14C from nicotinic acid mononucleotide-7-14C as detected by paper chromatography. The values obtained in units of nitrogen moles of nicotinic acid mononucleotide per hour per mg of protein were: quinolinic acid decarboxylase, 0.06 to 0.08; nicotinic acid mononucleotide pyrophosphorylase, 0.15 to 0.24; and nicotinic acid mononucleotide, 2.6.
43–45). In rabbit tissue, NAD is cleaved into fragments by two distinct pathways, i.e. to nicotinamide by NAD nucleotidase and to nicotinamide mononucleotide by NAD pyrophosphatase (43). Nicotinamide mononucleotide was not isolated in the experiment in Table IV. It would be included under “others” in that table.

**DISCUSSION**

In the present studies, it was shown that the pyridine ring moieties of nicotinic acid mononucleotide and NAD can be readily incorporated into ricinidine with efficiencies of the same order of magnitude as those of nicotinic acid and nicotinamide which have been previously reported (46). These efficient precursors of ricinidine could therefore be functioning in a cyclic manner (Fig. 1). In this study, the following cyclic conversions were shown:

\[
\text{In vivo: nicotinamide} \rightarrow \text{nicotinic acid} \quad (1)
\]

\[
\text{In vitro and in vivo: nicotinic acid} \rightarrow \text{NAD} \quad (2)
\]

\[
\text{In vivo: NAD} \rightarrow \text{nicotinamide} \quad (3)
\]

Reactions 2 and 3 may have involved the mononucleotides, nicotinic acid mononucleotide and nicotinamide mononucleotide, as intermediates which remained undetected by the methods used.

Leete and Leitz (2) first suggested that the pyridine nucleotides might be involved in the biosynthesis of ricinidine; however, the experimental proof was not attempted until recently when the C-labeled compounds became available. They suggested a biogenetic scheme which employed nucleophilic attack by hydroxide ion at carbon atoms 2 and 4 of NAD. Recently, Robinson (47) reported the occurrence of an enzyme in young castor seedlings that catalyzed the oxidation of L-methylnicotinonitrile since this enzyme is not highly specific for the 1-alkyl group it might be active in the formation of nicotinonitrile nucleotide analogue which may be a key intermediate in the biosynthesis of ricinidine. The nicotinonitrile nucleotide analogue is not readily available; however, preliminary experiments with N-methyl nicotinonitrile-S-14C as a precursor of ricinidine indicated that it was about 10% as effective as N-methyl nicotinamide. Work is in progress to determine the effectiveness of several pyridine nitriles as precursors of ricinidine.

The metabolic relationships of quinolinic acid, nicotinic acid, N-methyl nicotinic acid, nicotinamide, N-methyl nicotinamide, nicotinic acid mononucleotide, nicotinic acid adenine dinucleotide, and NAD have been examined and these compounds have been implicated in the biosynthesis of ricinidine by Ricinus communis L. The pathway for the biosynthesis of ricinidine is still not completely understood. Although it appears that quinolinic acid and the compounds in the pyridine nucleotide cycle are nearly equivalent as precursors of ricinidine, the detailed steps in the conversion of the particular pyridine compound to ricinidine remain to be elucidated.

**REFERENCES**


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