Studies on the Biogenesis of the Ring Systems of Nicotine*

THOMAS GRIFFITH, KENNETH P. HELLMAN AND RICHARD U. BYERRUM

From the Kedzie Chemical Laboratory, Michigan State University, East Lansing, Michigan

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Dawson et al. (1) have shown that the pyridine ring of nicotinic acid is utilized to form the pyridine ring of nicotine in sterile cultures of tobacco root. Since studies with animals and some microorganisms have demonstrated that tryptophan is converted to nicotinic acid (2), tryptophan might reasonably be expected to yield the pyridine ring of nicotine. However, experiments have shown conclusively that in tobacco and some other higher plants there was essentially no conversion of C¹¹ or tritium-labeled tryptophan to compounds containing the pyridine ring (3, 4, 5). It was, therefore, necessary to examine other compounds as possible intermediates in the biosynthesis of the ring systems of nicotine. The methyl carbon of acetate, but not the carboxyl carbon, recently was shown to yield nicotine labeled significantly in the pyridine ring (6). This finding led to the postulate that in some higher plants the pyridine ring may be synthesized from simple metabolic intermediates related to acetate.

The present study is an investigation of the incorporation of several C¹¹-labeled compounds, which might be interconvertible with acetate in metabolism, into nicotine in intact tobacco plants. In addition, a partial degradation of nicotine which allows for isolation of carbon from the 3 position of the pyridine ring is described.

EXPERIMENTAL

Nicotiana rustica plants were grown for 2 to 3 months in a greenhouse to a height of 12 to 15 cm. They were taken from the greenhouse, the roots were removed and discarded, and the plants were placed in an inorganic nutrient solution (7) to allow new roots to develop. A period of 2 weeks was usually sufficient. The radioactive compounds were fed to the plants by the hydroponic technique described previously which insures absorption of the isotope in 3 to 4 hours (3). Each plant was supplied with 2.44 × 10⁻⁵ moles of a given compound with a specific activity of 4.10 × 10⁶ c.p.m. per nmole.

The plants were harvested 7 days after administration of the labeled precursor, and nicotine was isolated and purified as the dipicrate (7).

Oxidation of Pyrrolidine Ring of Nicotine

The pyridine ring was oxidized by addition of nitric acid and was collected as barium carbonate. Nicotinic acid was isolated as the silver salt. This was decomposed with hydrogen sulfide, and the nicotinic acid was purified by three to five sublimations (140° at about 1 mm Hg). The melting point of nicotinic acid prepared in this way was 232 to 233° (recorded m.p. 232°).

Decarboxylation of Nicotinic Acid—Nicotinic acid was decarboxylated by dry distillation of its calcium salt (10). Pyridine was recovered and purified as the picrate. The residue from the distillation was acidified yielding calcium carbonate which was collected as barium carbonate. This carbonate represented carbon from the 2 position of the pyrrolidine ring.

Oxidation of Pyridine Ring of Nicotine

Spray-oxidation of nicotine isomethiodide hydriodide (1-methyl-3-(2'-[1-methylpyrrolidyl]) pyridinium iodide hydriodide) I—Nicotine was degraded to isolate carbon from the 3 position of the pyridine ring by the series of reactions shown in Fig. 1. As the first step in the degradation, nicotine was methylated by the procedure reported by Pictet and Généquand (11). In a typical experiment, nicotine, 1.00 g, was dissolved in 50 ml of water, and 2 ml of 47% hydrogen iodide were added. The final pH of the solution was 5.2. Water was removed under reduced pressure and residual nicotine hydriodide dissolved in 50 ml of methanol. The solution was cooled in an ice bath and 2.0 ml of methyl iodide were added. The reaction mixture was refluxed for 12 hours. Upon cooling the mixture, nicotine isomethiodide hydriodide crystallized as yellow needles and was collected on a sintered glass filter. After two additional recrystallizations, the melting point of the compound was 220 to 222° (reported m.p., 209° (11)). The yield was 3.85 g (76%).

C₁₁H₁₈N₂I₂ (432.2)

Calculated: C 30.57, H 4.21, N 6.49, I 58.74

Found: C 30.58, H 4.28, N 6.68, I 58.68

Unchanged nicotine was recovered from the methanolic filtrates as the dipicrate. About 10% of the original nicotine could be recovered in this way.

To confirm the identification of nicotine isomethiodide hydriodide, the hydriodide was converted to nicotine isomethiodide by treatment of 2 g of the preparation with 20 ml of a saturated solution of sodium carbonate. The solution was heated gently and the water removed under reduced pressure. The dry residue was extracted with chloroform and the chloroform removed by evaporation. This residue was extracted with about 10 ml of methanol. Nicotine isomethiodide precipitated from the meth-

* This work was supported by a grant from the Tobacco Industries Research Committee.

1 All melting points were uncorrected.
the melting point of the anhydrous material was 115 to 116°. A reported value for the monohydrate is 116° (12).

Preparation of L-Hygrinic Acid (1-Methylpyrrolidine-2-carboxylic Acid) III—The method used for the preparation of hygrinic acid was essentially that of Karrer and Widmer (12). In a usual experiment, nicotine isomethiodide hydridioxide (3.80 g) was dissolved in 34 ml of water, and 29 ml of a solution of potassium ferricyanide (30 g per 100 ml) were added. The solution was cooled in an ice bath, layered with 50 ml of benzene, and 29 ml of 12 N potassium hydroxide were added in small increments with stirring. The reaction mixture was warmed to room temperature, saturated with potassium carbonate and extracted with 300 ml of benzene. The benzene was removed under reduced pressure, and the residual oil, impure N-methylnicosetine (II), was oxidized with chromic acid without further purification. To accomplish the oxidation, the N-methylnicosetine was dissolved in 8 ml of water, 29 ml of chromic acid solution (15 g chromium trioxide and 23 g sulfuric acid per 100 ml) were added, and the mixture was refluxed for 5 hours. The excess of chromate was then reduced by the addition of sulfur dioxide, and the excess of sulfur dioxide was expelled by gentle boiling. A hot, saturated barium hydroxide solution was added, carefully avoiding an excess, to precipitate hydrous chromium oxide and barium sulfate. The bulky precipitate was collected on a sintered glass filter and washed with 200 ml of hot water. The combined filtrates were concentrated to a volume of 25 ml under reduced pressure, and an excess of copper carbonate was added. This mixture was boiled for 2 minutes and the excess copper carbonate filtered. The filtrate was evaporated to dryness and the cupric ion was precipitated by hydrogen sulfide. The resulting 1-methylpyrrolidine was not collected. The residue, containing the carboxyl carbon of the acid, was acidified and the liberated carbon dioxide was swept in a stream of nitrogen into a solution of barium hydroxide. The barium carbonate was separated by filtration and dried in a vacuum desiccator.

The yield was 28 mg (78%).

Degradation of Radioactive Nicotine and Determination of C14—In most cases insufficient radioactive nicotine for the degradation was isolated from tobacco plants fed the C14-labeled precursors. The radioactive nicotine was therefore diluted with an appropriate quantity of nonradioactive nicotine when necessary.

Radiochemicals were purchased from Volk Radio Chemical Company, 5412 North Clark Street, Chicago 40, Illinois. The determinations of C14 were made with a Tracerlab proportional flow counter and a Nuclear-Chicago model 192X scaler. All counts were corrected for self absorption. Elementary analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

RESULTS AND DISCUSSION

Incorporation of C14 into Nicotine—The extent of incorporation of the radioactivity of several C14-labeled compounds into nicotine is shown in Table I. When these compounds were fed under essentially identical conditions, nicotine with the highest specific activity was formed in plants fed glyceral-1,3-C14 followed in decreasing order by propionate-2-C14, acetate-2-C14, acetate-1-C14, and propionate-1-C14 in their ability to provide radioactive carbon for nicotine synthesis. The nicotine from the plants fed glyceral was over 50 times as radioactive as nicotine from plants fed propionate-1-C14.

Incorporation of C14 into Pyrrolidine Ring of Nicotine—Previous studies have demonstrated the incorporation of glutamate-2-C14 (8) and ornithine-2-C14 (14, 15) into nicotine by intact tobacco plants. The radioactivity from both compounds was located almost exclusively in the pyrrolidine ring of nicotine. Isolation of the 2 and 5 carbons of the pyrrolidine ring showed approximately equal distribution of radioactivity between these two positions after either of the two labeled amino acids had been fed (8, 14). It may be inferred that during the conversion of...
The nicotinic acid accounted for 50% of the C14. The nicotine contained 42% of the C14 of the original nicotine, whereas the acid was decarboxylated and the carboxyl carbon recovered as at least two major pathways by which propionate can be metabolized. Flavin et al. (16, 17) have shown that propionate can be carboxylated to methyhnalonate which subsequently rearranges to succinate. On the other hand, Giovanelli and Stumpf (18), with the use of peanut mitochondria, showed that propionate can carboxylated to methyhnalonate which subsequently rearranges to succinate. On the other hand, Giovanelli and Stumpf (18), with the use of peanut mitochondria, showed that propionate can

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nicotine dipicrate</th>
<th>Dilution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate-1-C14</td>
<td>0.44 c.p.m./mole X 10^-4</td>
<td>946</td>
</tr>
<tr>
<td>Acetate-2-C14</td>
<td>1.01</td>
<td>436</td>
</tr>
<tr>
<td>Propionate-1-C14</td>
<td>0.04</td>
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<td>Propionate-1-C14</td>
<td>0.05</td>
<td>8,225</td>
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<tr>
<td>Propionate-2-C14</td>
<td>1.76</td>
<td>230</td>
</tr>
<tr>
<td>Propionate-2-C14</td>
<td>2.30</td>
<td>177</td>
</tr>
<tr>
<td>Glycerol-1,3-C14</td>
<td>3.05</td>
<td>134</td>
</tr>
<tr>
<td>Glycine-1,3-C14</td>
<td>2.88</td>
<td>142</td>
</tr>
</tbody>
</table>

* Dilution was calculated by dividing the specific activity of the precursor fed by the specific activity of the nicotinic dipicrate.

TABLE II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maximal specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Propionate-2-C14</td>
</tr>
<tr>
<td></td>
<td>c.p.m./mole X 10^-4</td>
</tr>
<tr>
<td>1. Nicotine dipicrate</td>
<td>2.6</td>
</tr>
<tr>
<td>2. Barium carbonate</td>
<td>1.1</td>
</tr>
<tr>
<td>3. Nicotinic acid</td>
<td>1.3</td>
</tr>
<tr>
<td>4. Pyridine picrate</td>
<td>1.0</td>
</tr>
<tr>
<td>5. Barium carbonate†</td>
<td>0.3</td>
</tr>
</tbody>
</table>

† Barium carbonate from carbons 2, 3 and 4 of the pyrrolidine ring. The specific activity was multiplied by 3.

Degradation of the pyrrole ring of nicotine isolated from plants fed glycerol-1,3-C14 (Column 3 of Table II) showed that 6% of the C14 of nicotine was located in carbon 2 of the pyrrole ring. If the assumption again is made that equal quantities of C14 are in carbons 2 and 5, about 20% of the C14 in the nicotine molecule would be equally distributed between carbons 2 and 4. Thus, the pattern of labeling of the pyrrole ring after feeding glycerol-1,3-C14 was similar to the labeling subsequent to the administration of acetate-2-C14 (6) and propionate-2-C14. However, with labeled glycerol, a considerably larger part of the total C14 of the nicotine was located in the pyridine ring. It is well established that glycerol may be phosphorylated and converted to acetate through glycolysis. Glycerol-1,3-C14 would yield acetate-2-C14, and thus the same distribution of C14 in the pyrrole ring would be expected after administering either glycerol-1,3-C14 or acetate-2-C14. Therefore both propionate and glycerol, along with acetate (6), appear to enter the pyrrole ring of nicotine by way of the tricarboxylic acid cycle and glutamate.

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cept the 3 carbon. The loss of these carbons resulted in a loss of radioactivity amounting to 13% of the radioactivity of the original nicotine in the acetate experiment and 21% in the propionate experiment. Barium carbonate from the carboxyl carbon of the hygronic acid (originally the 3 position of the pyridine ring of nicotine) contained 13% of the original radioactivity of the nicotine in both the acetate and propionate experiment. Therefore, about 50% of the radioactivity in the pyridine ring was in the 3-position after feeding acetate-2-C\(^14\), whereas about 40% was in the 3-position after feeding propionate-2-C\(^14\).

Although the exact position of the remaining radioactivity of the pyridine ring was not ascertained, it could reasonably be argued that it might reside predominately in the 2-position after feeding either acetate-2-C\(^14\) or propionate-2-C\(^14\).

The two pathways for propionate metabolism, which were discussed in an earlier section, indicate that if propionate-2-C\(^14\) were oxidized to acetate, which in turn would enter the tricarboxylic acid cycle, succinate labeled in carbons 2 and 3 would result. Propionate formed in turn from the labeled succinate, by way of methylmalonate, would be labeled in carbons 2 and 3 tending to randomize radioactivity between these positions in the propionate pool. Acetate-2-C\(^14\), of course, would also form propionate-2,3-C\(^14\). A similar randomization of radioactivity would occur if propionate were in equilibrium with methylmalonate without previous oxidation to acetate. Therefore, if propionate-2-C\(^14\) were the precursor of carbons 2 and 3 of the pyridine ring, carbon 2 would contain a quantity of C\(^14\) similar to carbon 3 following these reactions which randomize the C\(^14\); and carbons from these two positions would account for most of the C\(^14\) of the pyridine ring.

It has been demonstrated that the pyridine ring of nicotinic acid is converted into nicotine (1). It is reasonable to assume in the present study that nicotinic acid or a related metabolite serves as an intermediate for the synthesis of nicotine. During the conversion to nicotine, nicotinic acid would be decarboxylated (1). Thus, if the 2 and 3 carbons of propionate were incorporated into the pyridine ring, it is possible that the carboxyl carbon of propionate would become the carboxyl carbon of nicotinic acid. If such an incorporation of propionate into nicotinic acid occurred, no radioactivity from propionate-1-C\(^14\) or acetate-1-C\(^14\) would be found in the pyridine ring of nicotine. Such a hypothesis is borne out by the results of this and a previous study (6).

Propionate-2-C\(^14\) was converted to nicotine to a greater extent than acetate-2-C\(^14\). The fact that when propionate and acetate are fed under similar conditions, propionate yields nicotine of greater specific activity is an indication that propionate is a more immediate precursor of nicotine than acetate.

Several intermediate compounds would undoubtedly exist between propionate and the pyridine ring. It has been shown in several microorganisms and animals that propionate can be metabolized to \(\beta\)-alanine (21). \(\beta\)-Alanine, in part because of its 3 amino group, would appear to be a logical intermediate in the conversion of propionate to the pyridine ring.

These considerations, along with the data presented in Table III, provide strong evidence for the hypothesis that propionate contributes its 2 and 3 carbons to the 3 and 2 position of the pyridine ring and that acetate is incorporated into the pyridine ring by way of propionate.

The distribution of C\(^14\) in the pyrrolidine ring of nicotine from plants fed glycerol-1,3-C\(^14\) was similar to the distribution when acetate-2-C\(^14\) was the precursor (Table II and (6)). Thus it is likely that at least part of the glycerol which entered nicotine was metabolized by way of glycolysis to acetate. However, as can be seen in Table II, the pyridine ring contained about 60% of the C\(^14\) of the original nicotine after feeding glycerol-1,3-C\(^14\). If all the glycerol, during its incorporation into nicotine, were metabolized to acetate, only 40% of the original C\(^14\) would be expected to reside in the pyridine ring. Therefore, glycerol must have been utilized for pyridine ring synthesis by a metabolic pathway which excludes acetate.

Table III

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maximal specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate-2-C(^14)</td>
</tr>
<tr>
<td></td>
<td>c.p.m./mole X (10^3)</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>1.6</td>
</tr>
<tr>
<td>Nicotinic acid isomethiodide</td>
<td>1.6</td>
</tr>
<tr>
<td>Hygric acid</td>
<td>1.4</td>
</tr>
<tr>
<td>Barium carbonate†</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* The reported values are the averages of two determinations.
† Barium carbonate resulting from the decarboxylation of hygric acid.

SUMMARY

**Nicotiana rustica** plants fed acetate-1-C\(^14\), acetate-2-C\(^14\), propionate-1-C\(^14\), propionate-2-C\(^14\), and glycerol-1,3-C\(^14\) synthesized radioactive nicotine. The labeled glycerol yielded nicotine with the greatest specific radioactivity when all these precursors were administered under similar conditions.

Radioactive nicotine was degraded to obtain carbon 2 of the pyrrolidine ring as barium carbonate. When tobacco plants were fed propionate-2-C\(^14\), carbon 2 of the pyrrolidine ring of nicotine contained 12% of the C\(^14\) and 42% of the C\(^14\) was associated with carbons 3, 4 and 5. When glycerol-1,3-C\(^14\) was the precursor fed, 6% of the C\(^14\) was associated with carbon 2, and 27% with carbons 3, 4, and 5.

If the assumption is made that a symmetrical intermediate is formed during the incorporation of these precursors into the pyrrolidine ring, an equal quantity of C\(^14\) would be associated with carbons 2 and 5, and this pattern of labeling would be consistent with the postulate that both propionate and glycerol entered the tricarboxylic acid cycle and were converted to glutamate during the synthesis of the pyrrolidine ring of nicotine.

The pyridine ring of nicotine from plants fed propionate-2-C\(^14\) contained 39% of the C\(^14\) whereas 57% of the C\(^14\) was located in the pyridine ring when glycerol-1,3-C\(^14\) was the precursor.

A degradation procedure employing established reactions was described which permitted isolation of carbon 3 of the pyridine ring. Nicotine synthesized in plants fed propionate-2-C\(^14\) and acetate-2-C\(^14\) was degraded by this procedure. In both experiments, approximately one-half of the C\(^14\) of the pyridine ring.
was associated with carbon 3. The implications of these results with regard to pyridine ring biosynthesis are discussed.

REFERENCES
Studies on the Biogenesis of the Ring Systems of Nicotine
Thomas Griffith, Kenneth P. Hellman and Richard U. Byerrum


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