The Role of Glycerol in the Biosynthesis of the Pyridine Moiety of Nicotine*

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EXPERIMENTAL PROCEDURE

Nicotiana rustica plants were grown in a growth chamber to a height of 12 to 15 cm. Before administration of radioactive compounds, the roots were cut from the plants and new root systems were allowed to develop for 2 weeks in a nutrient solution (8). The radioactive compounds were administered hydroponically, and each plant received 2.44 × 10−5 mole of the 14C-labeled substrate. Almost all the substrate was absorbed by the plants during a 4-hour period. At the end of this time, the plants were harvested and the nicotine was isolated as the dipicrate (8).

Degradation of Nicotine

The procedure for degradation of the pyridine ring is outlined in Fig. 1.

Oxidation of Nicotine—Radioactive nicotine was recovered from the dipicrate, and enough nonradioactive nicotine was added to the labeled material to give a total of 10 g of nicotine. The oxidation to nicotinic acid was carried out according to the procedure of Alworth, DeSelms, and Rapaport (9), in which potassium permanganate is used as the oxidizing agent in a molar ratio to nicotine of 8:1. A solution of 10.2 g of KMnO4 in 1700 ml of water was added over a period of 30 min to 2.5 g of nicotine in 1700 ml of water. The solution was heated on a steam bath for 16 hours, the MnO2 was removed, and the potassium salt of nicotinic acid, in a small volume, was placed on a Dowex 1-X8 (formate) column, 15.2 cm² × 110 cm. The column was prepared and eluted, and the fractions were assayed according to the procedure of Preiss and Handler (10). The tubes containing the nicotinic acid were collected and the solution was evaporated to dryness. The crystalline residue melted at 234–235°. The reported melting point is 232° (11). The yield was about 5.5 g from 10 g of nicotine, 72% of the theoretical.

Nicotinamide—The procedure of McElvain and Adams (12) and LaForge (13) was used to prepare nicotinamide. In the usual experiment, 5.5 g of nicotinic acid were mixed with 10 ml of thionyl chloride and the mixture was refluxed for 2 hours on a steam bath. The excess thionyl chloride was then removed by evaporation under reduced pressure. The crude nicotinyl chloride hydrochloride was converted to the methyl ester hydrochloride by refluxing it with 15 ml of dry methanol for 30 min. Again the excess liquid was removed by evaporation under diminished pressure, leaving a clear, colorless oil. The flask was cooled somewhat, and 100 ml of cold, concentrated aqueous ammonia were added; the flask was stoppered and allowed to stand for 18 hours at 0°.

In order to obtain the nearly pure nicotinamide, the solution was evaporated to dryness under reduced pressure, and the residue was placed overnight in a vacuum desiccator. The residue was extracted four times by refluxing it with 400-ml portions of dry benzene for 20 min, after which the solution was decanted into a 1-liter flask and allowed to cool to room temperature. The resulting nicotinamide crystals were dried in an oven at 80°. The average yield was 4.9 g, or 89% of the theoretical. The product melted at 126–127°; the reported melting point is 129° (13).

Nicotinamide 1-Oxide—The nicotinamide was mixed with 50 ml of glacial acetic acid and 10 ml of 30% H2O2. The solution...
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FIG. 1. Degradation procedure for the pyridine moiety of nicotine

was then refluxed for 4 hours on a steam bath (14), diluted with 100 ml of water, and evaporated to dryness under reduced pressure. After being dried overnight in a vacuum desiccator, the white residue was dissolved in 20 ml of boiling water, 4 ml of ethanol were added, and the solution was allowed to cool to room temperature. The crystals were filtered and dried at 100° for 2 hours. Additional crystals were obtained when the filtrate was cooled to 0°. The average yield was 4.2 g, 75% of the theoretical from nicotinamide. The compound did not give satisfactory melting point determinations, but elemental analysis gave the following result.

C₈H₈N₂O₂ (188.12)
Calculated: C 52.10, H 4.38, N 20.30
Found: C 52.08, H 4.29, N 20.35

2-Chloronicotinonitrile—Nicotinamide 1-oxide (4.2 g) was placed in a flask with 9.3 g of phosphorus pentachloride and 12.5 ml of phosphorus oxychloride (15). The flask was immersed in an oil bath and heated under reflux for 1½ hours at 115-120°. Phosphorus oxychloride was removed by evaporation at about 30° with the use of a water pump to reduce pressure. Under these conditions the 2-chloronicotinonitrile did not sublime. However, if the temperature was allowed to rise to 50°, some loss of 2-chloronicotinonitrile by sublimation was experienced. The solid was mixed with 30 ml of an ice-water mixture and allowed to stand overnight at 0°. The precipitate was collected, stirred for 5 min with 30 ml of ice-cold 0.5 N NaOH, and again filtered. This procedure was repeated once, and the solid was washed with 20-ml portions of cold water until the filtrate was neutral to pH paper. The solid was dried in a vacuum desiccator. The dried material was placed in a Soxhlet thimble, 33 x 94 mm, over 10 g of anhydrous sodium carbonate and was extracted with 100 ml of dry ethyl ether. The ether solution was boiled for 1 min with a small quantity of decolorizing charcoal and filtered, and the resulting colorless filtrate was evaporated to dryness, leaving white crystals of 2-chloronicotinonitrile. The average yield was 1.6 g, 39% of the theoretical. The melting point was 106-107°; the reported value is 107° (14).

2-Hydroxynicotinic Acid—The 2-chloronicotinonitrile (1.6 g) was heated under reflux for 9 hours with 12 ml of concentrated HCl (14). The liquid was removed by evaporation, and 30 ml of 6 N formic acid were added; this solution was also evaporated to dryness. The residue, 2-hydroxynicotinic acid, was recrystallized from boiling water. The crystals melted at 256°, which is the reported value for 2-hydroxynicotinic acid, and weighed between 1.35 and 1.40 g, or 83 to 85% of the theoretical. Elemental analysis gave the following result.

C₈H₇NO₂ (139.11)
Calculated: C 51.80, H 3.62, N 10.06
Found: C 51.88, H 3.68, N 10.08

2-Pyridone—The method of Walling and Wolfstirn (16) was
followed for the decarboxylation of 2-hydroxynicotinic acid. The acid obtained from the preceding reaction was refluxed in 25 ml of redistilled quinoline in the presence of 300 mg of copper chromite catalyst. The decarboxylation was usually over 90% complete at the end of 5 hours. The quinoline was removed by steam distillation; the resulting aqueous solution was acidified with dilute HCl, and the solution was evaporated to dryness. The residue was taken up in a minimal amount of water and the solution was placed on a Dowex 50-Na⁺ column. The column was eluted with water, and the eluent was assayed for absorption at 295 nm. The fractions containing the pyridone, identified by their ultraviolet spectrum, were collected and evaporated to dryness. The material melted at 105-106°C, whereas the reported value for 2-pyridone is 107°C (17). The average yield was 90%.

5-Aminopentanoic Acid—The pyridone from the preceding reaction was dissolved in 50 ml of ethanol containing 75 to 100 mg of finely divided RuO₂. The flask was then placed in a Parr rocking high pressure apparatus, under 1000 psi of H₂, for 2 hours (18). The temperature was raised over the 2-hour period to 100°C. After the solution was cooled, it was filtered to remove the RuO₂ and evaporated to a viscous oil. The reaction was essentially quantitative. The oil was taken up in 30 ml of water containing 3.15 g of Ba(OH)₂·8H₂O. The solution was refluxed gently for 6 hours, then cooled to room temperature (19). The barium ion was precipitated with CO₂ and the BaCO₃ was removed by filtration. The colorless filtrate was evaporated to a viscous oil which crystallized upon standing in a vacuum desiccator.

When the material was recrystallized from ether-ethanol, the melting point was 156-157°C (20). The yield from the 2-pyridone averaged about 95%.

5-Trimethylaminopentanoic Acid—The crystalline material from the preceding reaction was placed in a flask which contained 50 ml of methanol, AgO freshly prepared from 12 g of AgNO₃, and 3.2 ml of methyl iodide (21). This mixture was refluxed for 12 hours in the absence of light. The silver salts were then removed by filtration, and the resulting colorless filtrate was evaporated to a viscous oil which crystallized when placed in a vacuum desiccator. When this material was recrystallized from ether-ethanol, the crystals melted at 213-215°C and elemental analysis gave the following:

C₈H₁₈NO₂Cl (195.09)

Calculated: C 49.09, H 9.26, N 7.15
Found: C 48.89, H 9.13, N 7.07

Fusion of Betaine with KOH—The thoroughly dried betaine from the previous reaction was mixed with 10 g of KOH and fused at 350°C for 10 min (21). After the reaction mixture had cooled, the solid material was taken up in water, acidified with sulfuric acid, and steam-distilled. The distillate, containing acids produced in the fusion process, was neutralized with NaOH and the solution was evaporated to dryness. The free acids were taken up in 5% 1-butanol in chloroform according to the procedure of Elsden (22), and the propionic and acetic acids were separated on a Celite column as described by Cornforth, Hunter, and Popjak (21). Each acid obtained by chromatography was then chromatographed on paper with authentic samples of propionic acid and acetic acid for comparison. Two solvent systems, NH₄OH-95% ethanol (1:100) and NH₄OH-1-butanol-H₂O (2:72:16), were employed for chromatography. With both solvents the Rₚ values for the isolated materials correspond to the Rₚ values for the authentic acids. Radioautographs of these chromatograms revealed two radioactive spots corresponding in Rₚ value to propionic and acetic acids.

Propionic and acetic acid were degraded carbon by carbon by the Schmidt reaction according to the procedure described by Phares (23). After each decarboxylation, the resulting amine was oxidized to the corresponding aliphatic acid with KMnO₄. The CO₂ released during the Schmidt reaction was collected as barium carbonate, which was plated, dried, and weighed for future counting.

Counting of ¹⁴C—The radioactivity of barium carbonate samples was measured in a Nuclear-Chicago proportional flow counter equipped with an automatic sample changer. The counts were corrected for self-absorption. All other compounds were counted by liquid scintillation in a Packard Tri-Carb liquid scintillation spectrometer, model 3314. The efficiencies of the counters were determined, and all samples were counted to 10,000 events. Radioactive precursors were purchased from Volk Radiochemical Company.

Nicotinic Acid-4, 6-¹⁴C—Quinoline-2, 4-¹⁴C was prepared from acetonilide and glycerol-1,3-¹⁴C according to the procedure of Manske and Kulka (24). The radioactive quinoline was oxidized to quinolinic acid-4, 6-¹⁴C with KMnO₄ as described by Hoogerwerf and VanDorp (25). Nicotinic acid-4, 6-¹⁴C was obtained by refluxing the labeled quinolinic acid in cyclohexanol (20).

Mechanism of Cleavage of 5-Trimethylaminopentanoic Acid—Although Hunter and Popjak (27) have examined the cleavage mechanism when 2-hexanoic acid-1-¹⁴C is fused with KOH at 360°C, they did not examine the cleavage mechanism when betaines are treated in a similar manner. Such a study was carried out; the starting material for synthesis of the betaine to be studied was the nicotinic acid-4, 6-¹⁴C prepared previously. The betaine, 5-trimethylaminopentanoic acid, prepared by degrading nicotinic acid-4, 6-¹⁴C by the procedure described earlier, was fused with KOH and the resulting acids were separated as before. Essentially all of the radioactivity was observed in the propionic acid. This result showed that the carbon chain of the betaine was broken at positions 2 and 3 so that propionic acid was derived from carbon atoms 4, 5, and 6 of the pyridine ring of nicotine, and acetic acid from carbon atoms 2 and 3.

RESULTS

The results of the degradations of nicotine from plants fed glycerol-2-¹⁴C and glycerol-1, 3-¹⁴C are presented in Table I. Nearly all of the radioactivity in the pyridine ring of nicotine originating from plants fed glycerol-2-¹⁴C was in carbon 5. The ¹⁴C was distributed throughout the pyridine ring of nicotine after feeding glycerol-1, 3-¹⁴C, with carbon atoms 2, 3, 4, and 6 having significant labeling. Radioactivity was divided essentially equally between carbon atoms 4 and 6 with 85% of the ¹⁴C in the ring located in these two positions. In addition, about 28% of the ¹⁴C in the ring was nearly equally divided between carbon atoms 2 and 3. It will be noted that glycerol-1, 3-¹⁴C contributes a much larger quantity of ¹⁴C to positions 2 and 3 than does glycerol-2-¹⁴C.
Distribution of $^{14}C$ in pyridine ring of nicotine from plants fed glycerol-1,3-$^{14}C$ and glycerol-2-$^{14}C$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific activity</th>
<th>In pyridine portion</th>
<th>Specific activity</th>
<th>In pyridine portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine dihydrochloride</td>
<td>26.90</td>
<td>%</td>
<td>335.0</td>
<td>%</td>
</tr>
<tr>
<td>2-Pyridone</td>
<td>15.90</td>
<td>100.0</td>
<td>211.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Barium carbonate from C-1 of acetic acid</td>
<td>2.50</td>
<td>15.7</td>
<td>4.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Barium carbonate from C-2 of acetic acid</td>
<td>3.00</td>
<td>12.1</td>
<td>5.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Barium carbonate from C-1 of propionic acid</td>
<td>5.13</td>
<td>32.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Barium carbonate from C-2 of propionic acid</td>
<td>0.20</td>
<td>1.8</td>
<td>208.0</td>
<td>98.5</td>
</tr>
<tr>
<td>Barium carbonate from C-3 of propionic acid</td>
<td>5.50</td>
<td>35.1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Percentage of the $^{14}C$ in the pyridine portion of nicotine.
† Specific activity of nicotine after nonradioactive nicotine (1964).

DISCUSSION

The results presented here indicate that the carbon atoms of glycerol are incorporated in toto into positions 4, 5, and 6 of the pyridine ring. This finding is in agreement with experiments concerned with the biosynthesis of anabasine and ricinine mentioned earlier (4, 5). The considerable radioactivity in positions 2 and 3 of the ring derived from glycerol-1,3-$^{14}C$ can be explained if it is assumed that conversion of glycerol to acetate via the glycolytic pathway occurs, and that conversion of acetate to succinate by way of the tricarboxylic acid cycle then results. In addition, it is assumed that the incorporation of glycerol into the pyridine ring through the reactions of glycolysis and the tricarboxylic acid cycle proceed at a rate more or less equivalent to the rate of introduction of glycerol into positions 4, 5, and 6 of the ring. Glycerol-1,3-$^{14}C$ would cause acetate-2-$^{14}C$ through the reactions of glycolysis, and the succinate formed from acetate-2-$^{14}C$ through reactions of the tricarboxylic acid cycle would be labeled principally in the methylcy carbons. These two precursors, acetate-2-$^{14}C$ and succinate-2,3-$^{14}C$, have been shown to be incorporated into carbon atoms 2 and 3 of the pyridine ring, and a significant incorporation of $^{14}C$ from these compounds occurred in a short metabolism period of from 2 to 4 hours (1). On the other hand, glycerol-2-$^{14}C$ would produce acetate-1-$^{14}C$ and the succinate produced from it would therefore be labeled in the carboxyl groups. Neither carboxyl-labeled acetate nor carboxyl-labeled succinate is incorporated into the pyridine ring (28, 29).

The small percentage of $^{14}C$ in carbon atoms 2 and 3 of the pyridine ring originating from plants fed glycerol-2-$^{14}C$ is in contrast to Leete’s results with anabasine, which indicate considerable radioactivity in these positions of the pyridine ring after glycerol labeled at position 2 is fed. The metabolism time in our experiments, however, was a fraction of the time in those of Leete and Friedman (4) and, therefore, in our studies less randomization of $^{14}C$ would be expected.

SUMMARY

A new degradation of the pyridine ring of nicotine is described which permits isolation of each ring carbon. The location of the $^{14}C$ in the ring, with the use of this degradation, provides evidence that the carbon atoms of glycerol were incorporated in toto into positions 4, 5, and 6 of the pyridine ring. Over 98% of the $^{14}C$ in the pyridine ring derived from glycerol-2-$^{14}C$ was in carbon atom 5 after a 4-hour metabolism period.

The pyridine ring of nicotine formed from glycerol-1,3-$^{14}C$ had 68% of the radioactivity divided between carbon atoms 4 and 6, whereas carbon 5 had about 2% of the radioactivity. Some $^{14}C$ from glycerol-1,3-$^{14}C$ was found in positions 2 and 3 of the pyridine ring. It is proposed that this incorporation results from conversion of labeled glycerol, by way of glycolysis and the tricarboxylic acid cycle, to methylene-labeled succinate which has previously been found to yield the pyridine ring of nicotine labeled in positions 2 and 3.

REFERENCES

The Role of Glycerol in the Biosynthesis of the Pyridine Moiety of Nicotine
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