Glutamete as a Precursor for the Pyrrolidine Ring of Nicotine*

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Recently it has been demonstrated that the amino acid, ornithine, is an efficient precursor of the pyrrolidine ring of nicotine in the tobacco plant (1, 2). Although there is considerable evidence that ornithine and glutamic acid may give rise to the pyrrolidine ring of proline through a common intermediate in animal systems (3) and in microorganisms (4), there has been little study of this metabolic relationship in higher plants. Morgan and Marion (5) isolated radioactive glutamic acid and proline from extracts of alfalfa plants that were fed C14-labeled ornithine. On the other hand, Naylor and Tolbert (6) reported no evidence of C14-labeled proline, ornithine, or arginine in leaves of barley seedlings after administration to the detached leaves of glutamic acid uniformly labeled with C14. Hence, it seemed of interest to test glutamic acid as a precursor of the pyrrolidine ring of nicotine in the tobacco plant. In the present work, glutamic acid-2-C14, fed to tobacco plants, was found to be converted to radioactive nicotine. Degradation of the nicotine revealed that positions 2 and 5 of the pyrrolidine ring were focal points of C14 labeling.

EXPERIMENTAL

The plants (Nicotiana rustica L.) were grown for 2 to 3 months to a height of 5 to 7 inches in a greenhouse before hydroponic administration of glutamic acid-2-C14.

Uptake of Glutamic Acid

Before administration of the radioactive glutamic acid, it was necessary to ascertain whether this amino acid was toxic in low concentrations or whether root microorganisms might alter or destroy the glutamic acid before absorption. Each of four tobacco plants was fed 2 mg. of D,L-glutamic acid in an Erlenmeyer flask containing about 50 ml. of an inorganic nutrient solution prepared as described by Byerrum and Wing (7). Each flask also contained 0.5 mg. of Aureomycin and a commercial fungicide1 to inhibit the growth of microorganisms. After 48 hours, the plants were removed from the flasks and each solution was analyzed for glutamic acid by the method of Gordon (8). In this procedure, the glutamic acid was oxidized under controlled conditions with sodium hypochlorite and the excess hypochlorite

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1 A mercury-containing fungicide, Ubavit Neu (Scheringes-Kahlbaum, A. G. Berlin). The fungicide was soluble only to the extent of about 1 part per million in the nutrient solution.

Radioactivity of Nicotine from Plants Fed Glutamic Acid-2-C14

D,L-Glutamic acid2 was fed to a group of tobacco plants under conditions previously described (1), except that the plant roots were shielded from light during the feeding period since this was shown to promote the synthesis of nicotine. Each plant received 0.35 mg. of D,L-glutamic acid-2-C14, having a radioactivity of 8 x 106 c.p.m. when measured with a Tracerlab proportional flow counter and a Nuclear-Chicago model 192 X scaler. After 5 days an additional 0.35 mg. of the radioactive glutamic acid was administered to each plant; 9 days later the plants were harvested, and the nicotine, isolated as the dipicrate (9), had a specific activity of 4.4 x 106 c.p.m. per mmole.

Since it was evident that glutamic acid was incorporated into the nicotine molecule, glutamic acid-2-C14 was administered to about 250 plants in amounts varying from 0.7 to 5.16 mg. per plant (ranging from 1.6 x 103 to 1.2 x 104 c.p.m. per plant). The nicotine was isolated and yielded about 5.0 gm. of radioactive nicotine dipicrate which was utilized for the degradation studies.

Degradation of Radioactive Nicotine and Isolation of Carbon from Position 2 of Pyrrolidine Ring

Radioactive nicotine was oxidized to potassium bicarbonate, nicotinic acid, and methylamine with neutral potassium permanganate according to the procedure of Laisbin (10). To accomplish this degradation, nicotine (0.39 gm.) was recovered from 1.5 gm. of nicotine dipicrate by an acetotropic distillation with water from an alkaline medium, as has been described by Smith (11). The solution of potassium permanganate (prepared by dissolving 2.34 gm. of KMnO4 in water and adjusting the volume to 80 ml.) was added in 5 ml. aliquots to the nicotine in about 250 ml. of water at 3 to 5 minute intervals. The

2 The D,L-glutamic acid-2-C14 was purchased from Tracerlab, Inc., Boston, Massachusetts.
mixture was heated on a steam bath for 10 hours to complete the oxidation. The manganese dioxide was removed by filtration and the filtrate evaporated to dryness at about 50° under reduced pressure. The residue was dissolved in water, the solution acidified with nitric acid, and the liberated carbon dioxide conveyed in a stream of nitrogen through a saturated solution of barium hydroxide. The barium carbonate was washed with water and dried at 110° for 1 hour. The acidified solution was neutralized with ammonium hydroxide and evaporated under reduced pressure. The residue was dissolved in water, the solution acidified with nitric acid, and the liberated carbon dioxide evaporated to dryness under diminished pressure. The residue was sublimed at 150° (at about 1 mm.) to yield nicotinic acid of m.p. 230° (recorded m.p., 232°). An infrared spectrum of the product was identical with the spectrum obtained with authentic nicotinic acid.

The nicotinic acid (100 mg.) was mixed with an excess of calcium oxide and decarboxylated by heating it (12) under an atmosphere of nitrogen. The resulting pyridine was distilled into a solution of methanol saturated with picric acid, and the picrate was recrystallized from water.

\[ \text{C}_{16}\text{H}_{17}\text{N}_{4}\text{O}_4 \] (308.2)

Calculated: C 42.68, H 2.62, N 16.18

Found: C 42.91, H 2.75, N 16.14

The residue, containing the carboxyl carbon of the nicotinic acid, was acidified and the liberated carbon dioxide was swept in a stream of nitrogen into a saturated solution of barium hydroxide. The resulting barium carbonate was separated as described previously.

**Degradation of Nicotine with Isolation of Carbon from Position 5 of Pyrrolidine Ring**

**Preparation of Cotinine (Compound I)—**A procedure was developed for isolating carbon 5 of the pyrrolidine ring of nicotine as shown in Fig. 1. To accomplish the degradation, a nonradioactive sample of the alkaloid was first converted to cotinine essentially by the method of Pinner (13). According to this procedure, 32 ml. of bromine in 80 ml. of 80 per cent acetic acid were added, with cooling, to 14 ml. of 95 per cent nicotine in 65 ml. of 80 per cent acetic acid. The resulting oily dibromocotinine perbromide crystallized as yellow-orange needles after hot water was added to the mixture.

The crystalline compound was separated by filtration and covered with 6 N hydrochloric acid. Zinc dust was added slowly, with cooling, to reduce the perbromide to cotinine. The addition of sodium hydroxide precipitated zinc hydroxide, which was removed by filtration. The filtrate was made strongly alkaline with sodium hydroxide and extracted with chloroform. Upon evaporation of the chloroform, an oily residue remained which was distilled between 170-175° under about 1 mm. pressure, yielding 6 to 7 gm. of cotinine as a colorless or pale yellow oil.

The neutralization equivalent was determined on another sample of this material dissolved in 85 per cent ethanol. The sample first had been dried for 10 hours over Drierite in a vacuum desiccator. This treatment was sufficient to remove the water of hydration (14). The theoretical neutralization equivalent was 194; found, 195. The crystalline compound (II) became red when exposed to vapors of cyanogen bromide, a characteristic of the substituted amino acid reported previously by McKennis et al. (15).

**Preparation of 4-(3'-Pyridyl)-4-Methylaminobutyric Acid (Compound III)—**Since 4-(3'-pyridyl)-4-methylaminobutyric acid (II) tends to cyclize at pH 7 or below (15), the amino group was benzoylated to prevent ring closure by the method suggested by Steiger (16) for amino acids. To this end, 600 mg. of the 4-(3'-pyridyl)-4-methylaminobutyric acid (II) were dissolved in 3 ml. of 1 N sodium hydroxide in an 8 inch test tube which was placed in an ice bath, 4 ml. of 1 N sodium hydroxide

![Fig. 1. Scheme for degradation of nicotine with separation of carbon 5 of the pyrrolidine ring.](http://www.jbc.org/)
and 0.4 ml. of benzoyl chloride were then introduced simultaneously into this solution. These reagents were added dropwise with vigorous stirring over a period of 15 to 20 minutes. The mixture at this stage developed a bright red color. The test tube was then shaken in an ice bath until the odor of benzoyl chloride had disappeared. During this period (about 2 to 3 hours) the temperature of the bath was permitted to rise to room temperature. The solution was then acidified with 1 N hydrochloric acid to pH 3.5 while the tube was cooled in an ice bath, and the precipitated benzoic acid was removed by filtration and washed with a few ml. of water; the washings were added to the filtrate. The filtrate was made alkaline to litmus with dilute ammonium hydroxide, concentrated to dryness under diminished pressure at 40–50° and extracted with cold n-butanol. The butanol extract was evaporated to dryness under reduced pressure at 50–60°, the residue was dissolved in methanol, and this solution was filtered into a small suction flask containing 5 to 10 ml. of water. Methanol was removed from the filtrate by gentle heating of the filtration flask, and colorless needles of 4-(3'-pyridyl)-4-N-methylbenzoylaminobutyric acid (III) were crystallized. The material was purified by recrystallization from water, and the crystals melted at 170°. The average yield of several trials was about 230 mg. (25 per cent).

C₁₇H₁₆N₄O₄ (396.3)
Calculated: C 68.44, H 6.08, N 9.40
Found: C 68.42, H 6.09, N 9.38

The neutralization equivalent, determined in 85 per cent ethanol, was 300. The crystalline material was not hygroscopic and gave no color when exposed to vapors of cyanogen bromide.

Preparation of 4-(3'-Pyridyl)-4-N-Methylbenzoylaminobutyramide (Compound IV)—An ethereal solution of diazomethane was prepared by adding 400 mg. of nitrosomethylurea to 20 ml. of 40 per cent potassium hydroxide under a layer of 25 ml. of ethyl ether. The yellow ethereal solution of diazomethane was decanted and dried over pellets of potassium hydroxide for 15 to 20 minutes. Then 150 mg. of dry 4-(3'-pyridyl)-4-N-methylbenzoylaminobutyric acid (III) were added in a small suction flask and the mixture was stirred with a glass-covered magnetic stirring bar until the esterification reaction had been completed, as was ascertained by solution of the crystalline material. This reaction was accompanied by evolution of nitrogen and usually required 6 to 10 hours. Occasionally, it was necessary to prepare and add more of the diazomethane solution to the reaction mixture. After the esterification had been completed, the solution was warmed and the diazomethane and ether were evaporated at room temperature in a stream of air.

The residue, a pale yellowish semisolid material, was dissolved in about 25 ml. of methanol which had been saturated with ammonia at 0°. The stoppered flask was permitted to stand, with occasional shaking, for about 100 hours at room temperature. The methanol was then removed under reduced pressure at 30–40°. The residue was dissolved in about 5 ml. of chloroform and treated with charcoal. The filtrate from this treatment was heated and dry ether was added until incipient precipitation had begun. The product crystallized as colorless plates which melted at 141–142°. The material gave a positive ferric hydroxamate test for primary amides. The average yield of several trials was about 130 mg. (approximately 85 per cent).

C₁₇H₁₆N₄O₄ (297.4)
Calculated: C 68.65, H 6.45, N 14.13
Found: C 68.72, H 6.47, N 14.23

C₁₇H₁₆N₄O₄ (298.3) washed with 2 to 3 ml. of water, and dried in a vacuum over Drierite. The average yield in several trials was about 50 mg. (50 per cent).

Decarboxylation of 4-(3'-Pyridyl)-4-N-Methylbenzoylaminobutyramide (IV)—150 mg. of the 4-(3'-pyridyl)-4-N-methylbenzoylaminobutyramide (IV) were introduced into a 100 ml., three-necked conical flask which was part of a glass assembly used for decarboxylation. The crystalline material was dissolved in a solution of sodium hypobromite which had been prepared by dissolving 0.02 to 0.03 ml. of bromine in 12 ml. of 0.25 N carbonate-free sodium hydroxide. A stream of nitrogen was passed through the assembly into a saturated solution of barium hydroxide. The reaction mixture was warmed to 70–80° and maintained in this temperature range for 15 to 20 minutes. After the apparatus had been permitted to cool, the solution was acidified by the addition of 3 ml. of 1 N hydrochloric acid. The liberated carbon dioxide was swept into the barium hydroxide solution and the barium carbonate was removed by filtration, washed with 2 to 3 ml. of water, and dried in a vacuum over Drierite. The average yield in several trials was about 50 mg. (50 per cent).

Preparation of Thioureia Derivative of 3-(3'-Pyridyl)-3-N-Methylbenzoylaminopropylamine (V)—The acidified reaction mixture, remaining after the decarboxylation of 4-(3'-pyridyl)-4-N-methylbenzoylaminobutyramide (IV), was made alkaline to litmus with 1 N sodium hydroxide and extracted with chloroform. The chloroform was removed under reduced pressure and the residue was dissolved in about 3 ml. of methanol. Phenylisothiocyanate (0.06 ml.) was added and the solution was refluxed for 10 minutes. Upon addition of water dropwise to the hot solution, shiny colorless plates began to appear. After the solution had cooled the precipitate was collected, recrystallized from methanol and water, and dried in a vacuum over Drierite (m.p., 196–198°). The yield was about 75 mg. (approximately 40 per cent).

A qualitative test indicated that sulfur was present in the crystalline material.

C₁₇H₁₆N₄O₄ (404.5)
Calculated: C 68.29, H 5.98, N 13.85
Found: C 68.42, H 6.08, N 13.97

Degradation of Nicotine Isolated from Plants Fed Glutamic Acid-2-C^{14}

For purposes of degradation, the radioactive nicotine diprole (3.8 gm.), obtained as described above, was diluted with 10.2 gm. of nonradioactive recrystallized nicotine diprole, and the radioactive disintegration of samples of the thoroughly ground and mixed material was counted. The nicotine was then recovered as the hydrochloride, as already described, and the hydrochloride was dissolved in 80 per cent acetic acid. 2 gm. of cotinine and 0.465 gm. of 4-(3'-pyridyl)-4-methylaminobutyric acid (II) were prepared as already described. Benzoacylation of 0.450 gm. of this substance and recrystallization of the
product yielded 0.163 gm. of 4-(3'-pyridyl)-4-N-methylbenzoylaminobutyric acid (III) which melted at 169-170°. Esterification of this compound with diazomethane and ammonolysis of the ester with ammonia in methanol yielded 0.142 gm. of 4-(3'-pyridyl)-4-N-methylbenzoylaminobutyramide (IV), which melted at 141-142°. Decarboxylation of this amide yielded 0.056 gm. of barium carbonate. The thiourea derivative of 3-(3'-pyridyl)-3-N-methylbenzoylaminopropylamine (V) was also prepared. The recrystallized product (0.078 gm.) melted at 197-198°.

Samples of each intermediate of the degradation were plated and counted.

### RESULTS AND DISCUSSION

The results of the degradation of nicotine, from plants fed glutamic acid-2-C¹⁴ with isolation of carbon 2 of the pyrrolidine ring, are shown in Table I. Through cleavage of the pyrrolidine ring, the carbon atom from position 2 of the ring was converted to the carboxyl carbon of potassium nicotine, and the carbon atoms from positions 3, 4, and 5 were oxidized to potassium bicarbonate. For counting purposes, the carbon atoms from positions 3, 4, and 5 were isolated as barium carbonate after the potassium bicarbonate was decomposed by acid. This barium carbonate (Line 2) possessed approximately half of the radioactivity found originally in the nicotine. Approximately the same radioactivity was found in the nicotine (Line 3). The decarboxylation indicated that about 90 per cent of the radioactivity of the nicotine was in the carboxyl carbon (Line 4), which was originally the carbon at position 2 of the pyrrolidine ring of nicotine. The pyridine ring (Line 5) possessed only a small fraction of the total radioactivity of the nicotine.

Previous studies of the degradation of nicotine obtained from plants fed ornithine-2-C¹⁴ (1, 2) have led to the postulation of a symmetrical intermediate along the route of biogenesis of the pyrrolidine ring. If the pyrrolidine ring were formed from glutamic acid by way of a symmetrical intermediate, without cleavage of the amino acid carbon chain, the radioactivity would probably be distributed equally between positions 2 and 5 of the ring. The second degradation was carried out in an attempt to isolate both the carbon atom from position 5 of the pyrrolidine ring and also the intact residual carbon skeleton of the nicotine molecule. The data obtained from examination of the intermediates and products of the degradation to isolate the carbon 5 of the pyrrolidine ring are presented in Table II.

The specific activities of the intermediates in the degradation are given in Table I. A symmetrical intermediate along the route of biogenesis of the nicotine molecule, possessed a specific activity approximately equal to that of the barium carbonate. From the data in Table I it is evident that the greater part of the radioactivity in the amine derivative is associated with the carbon atom which joins the pyridine ring and the sidechain (i.e. the carbon originally from position 2 of the pyrrolidine ring).

Several possible symmetrical intermediates have been suggested (17) to explain the pattern of C¹⁴-labeling resulting from the incorporation of ornithine-2-C¹⁴ into the pyrrolidine ring of nicotine. The results of the present study indicate that glutamic acid is also incorporated into the pyrrolidine ring by way of one or more biosynthetic pathways which involve symmetrical intermediates. The nature of these intermediates and their relative significance in the biosynthesis of nicotine are at the present time a matter of conjecture. Glutamic acid, proline, and ornithine appear to be interconvertible in animal systems (3) and in microorganisms (4) by way of glutamic-γ-semialdehyde and Δ¹-pyrroline-5-carboxylic acid. If Δ¹-pyrroline-5-carboxylic acid exists in the metabolism of the tobacco plant, the symmetrical compounds pyrrole or pyrrolidine may conceivably be formed from it. Succinic acid, arising from glutamic acid by way of the tricarboxylic acid cycle, has also been suggested (17). However, as will be discussed, ornithine was incorporated into nicotine to a greater extent than was glutamic acid when the two were fed under similar experimental conditions. Hence, it does not seem likely that succinic acid is an important intermediate in the biosynthesis of nicotine since the main route of succinic acid synthesis from ornithine would probably be by way of glutamic acid.

In the present work, glutamic acid-2 C¹⁴ was administered to tobacco plants under the same conditions employed in a previous study by Dewey et al. (1) with ornithine-2-C¹⁴. The incorporation of radioactivity into nicotine after administering glutamic
acid-2-C\textsuperscript{14} was about 1 to 2 per cent of that found after feeding ornithine-2-C\textsuperscript{14}. The comparatively low level of incorporation of the glutamic acid-2-C\textsuperscript{14} was probably due in part to the effects of dilution, since there is a relatively large reservoir of free glutamic acid in the tobacco plant compared to the reservoirs of free ornithine or proline. Commoner and Varda (18) and Roberts and Wood (19) have investigated free amino acids in tobacco leaves. Glutamic acid was detected in larger quantities than was proline, whereas no ornithine and only traces of arginine were reported. Furthermore, since glutamic acid is a very active metabolic intermediate in the synthesis of protein, glutamine, or carbohydrate, the rate of turnover of C\textsuperscript{14} in the free amino acid reservoir was probably rapid compared to the rate of nicotine synthesis.

SUMMARY

1. Nicotiana rustica plants that had been fed glutamic acid-2-C\textsuperscript{14} synthesized radioactive nicotine.

2. A degradation of the radioactive nicotine, which permitted isolation of carbon 2 of the pyrrolidine ring, demonstrated that this carbon atom contained about 45 per cent of the original radioactivity of the nicotine. The pyridine ring was shown to contain about 10 per cent and carbons 3, 4, or 5 of the pyrrolidine ring contained the remaining 45 per cent of the original radioactivity of the nicotine.

3. A procedure was developed for the degradation of the nicotine molecule, employing well established reactions, which permitted isolation of the carbon atom from position 5 of the pyrrolidine ring. The residual carbon skeleton of the molecule was recovered as a derivative of a substituted aliphatic primary amine. The intermediates produced at each step of this degradation were isolated.

4. Radioactive nicotine, obtained from plants fed glutamic acid-2-C\textsuperscript{14}, was degraded by this procedure. Half of the C\textsuperscript{14} of the nicotine was associated with carbon 5 of the pyrrolidine ring. The remaining radioactivity was shown to be present in the amine derivative and, from the previous degradation, was shown to be located primarily in the carbon which was originally at position 2 of the pyrrolidine ring of nicotine.

5. The pattern of labeling of nicotine from plants administered glutamic acid-2-C\textsuperscript{14} supports the postulation of a symmetrical intermediate along the pathway of incorporation of the amino acid into the pyrrolidine ring.

REFERENCES

Glutamate as a Precursor for the Pyrrolidine Ring of Nicotine
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