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Phytochemical Studies on the Tobacco Alkaloids. XII. Identification of γ-Methylaminobutyraldehyde and its Precursor Role in Nicotine Biosynthesis

Shigenobu Mizusaki, Takuro Kisaki[1], and Einosuke Tamaki

Division of Plant Biochemistry, Central Research Institute, Japan Monopoly Corporation, 1-28-3, Nishishinagawa, Shinagawa-ku, Tokyo, Japan

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Abstract. **γ-Methylaminobutyraldehyde (N-methylpyrroline) labeled with ^{14}C was isolated from tobacco roots which had metabolized ornithine-2-^{14}C. It was labeled most strongly 4 hours after adding ornithine-2-^{14}C to the root, also labeled by putrescine-1,4-^{14}C and methionine-$^{14}CH_3$, and observed in the root but not in the aerial portions of tobacco plants. γ-Methylaminobutyraldehyde when added back to the root was an efficient precursor of nicotine. Identity of γ-methylaminobutyraldehyde from tobacco roots was confirmed by comparison with the authentic compound.**

The existence of γ-methylaminobutyraldehyde (N-methylpyrroline) in the biosynthetic pathway of nicotine indicates that the methyl group must be introduced before condensation of the pyridine moiety with the pyrrolidine moiety.

It has been well established that the pyridine ring of nicotine is derived from nicotinic acid (4), and the pyrrolidine ring from ornithine (7, 16), putrescine (17), glutamic acid, and proline (14). The mechanism of the condensation of the 2 rings was discussed by Byerrum and his group (15), Leete *et al.* (18), Dawson (5), and Mothes and Schutte (21). Byerrum and his co-workers (2, 6) demonstrated that the methyl group is introduced from methionine and labile C_1-fragments. However, intermediates between these precursors and nicotine and the mechanism of the reaction have remained largely unexplored.

Ladesic and Tso (13) and Schroter (22) have reported that nornicotine can serve as a precursor of nicotine. However, our previous paper (11) demonstrated that while nicotine always occurs in the pure *l*-form in tobacco plants, nornicotine isolated from tobacco roots is predominantly *d*-form. We also reported that radioactive nicotine and nornicotine isolated from sterile root cultures of *Nicotiana rustica* fed DL-ornithine-2-^{14}C differed in the distribution of radioactivity between C-2 and C-5 in the pyrrolidine ring, and that only small amounts of ^{15}N were incorporated into nicotine by administration of ^{15}N-labeled nornicotine (20). These findings led to the inference that the biosynthesis of nicotine occurs through a route(s) that does not involve nornicotine. Alworth and Rapoport (1) have also excluded nornicotine as a precursor of nicotine from experiments with $^{14}CO_2$ in *Nicotiana glutinosa*.

One of the problems in nicotine biosynthesis is the step at which the methyl group is introduced. One aim of this work was to examine the possibility that methylation takes place before condensation of the pyridine moiety with the pyrrolidine moiety. In our previous paper (12) γ-methylaminobutyraldehyde (N-methylpyrroline) was shown to be involved in nicotine biosynthesis by tobacco plants, and in this paper we describe a more detailed account of this investigation.

Materials and Methods

Plant Materials. An aseptic root culture of *Nicotiana rustica* var. brasilia was maintained in a 100 ml Erlenmyer flask containing 30 ml of medium prepared according to the method of Solt (24). The cultures were grown for 3 weeks prior to addition of labeled compound. Plants of *Nicotiana tabacum* variety Bright Yellow, *Lycopersicum esculentum* (Fukuju, No. 2), *Datura stramonium*, and *Atropa belladonna* were grown hydroponically for about 4 weeks. Detached root segments and leaf disks from each plant were used for the feeding experiments with DL-ornithine-2-^{14}C.

Labeled Compounds. DL-Ornithine-2-^{14}C monohydrochloride (26 mc/m mole) was purchased from California Corporation for Biochemical Research. Glutamic acid-U-^{14}C (3.84 mc/m mole) was purchased from the Radiochemical Centre, Amersham, Buchs. Putrescine-1,4-^{14}C hydrochloride (3.5 mc/m mole), nicotinic acid-7-^{14}C (2 mc/m mole), DL-

[1] Present address: Department of Biochemistry, Michigan State University, East Lansing, Michigan, until fall of 1968, thereafter the Central Research Institute, Japan Monopoly Corporation, Tokyo, Japan.

methionine-$^{14}CH_3$ (2.3 mc/m mole), and γ-aminobutyric acid-1-^{14}C (1.3 mc/m mole) were purchased from Daiichi Chemical Company. Nicotinic acid-^3H was prepared by the Wilzbach procedure in Daiichi Chemical Company. The purity of these chemicals was re-examined by column chromatography before use, and they proved to be entirely free of contaminants as shown amino acid analysis with an amino acid analyzer and by ^{14}C flow monitor of the effluent.

Preparation of γ-Methylaminobutyraldehyde. γ-Aminobutyraldehyde diethylacetal was prepared as described by Hasse *et al.* (9); b. p., 80 to 83°/6 mm. Formyl-γ-aminobutyraldehyde diethylacetal was prepared by letting a mixture of γ-aminobutyraldehyde diethylacetal and ethylformate stand for 2 days at room temperature; b. p., 154 to 155°/2 mm. γ-Methylaminobutyraldehyde diethylacetal was prepared according to the procedure for preparing a secondary amine from anilide (8) as follow. To a solution of 0.4 g of lithium aluminum hydride in 15 ml ether was slowly added a solution of 2.0 g of formyl-γ-aminobutyraldehyde diethylacetal in 12 ml ether. Afterwards sufficient water added dropwise to decompose the excess lithium aluminum hydride and then 10 ml of 20% sodium carbonate solution. The mixture was extracted with ether. After drying over anhydrous potassium carbonate and filtering, the ether solution was evaporated to a yellow oil, which on distillation gave a colorless oil; b. p., 85 to 86°/4mm. (yield, 90%). γ-Methylaminobutyraldehyde was made by treating its acetal with same volume of 2 N HCl for 30 minutes at 60°.

Administration of Labeled Compounds. For experiments with excised root culture, most of the culture solution from 3 flasks was aseptically withdrawn by pipette, and 1 μc of the tracer in 1 ml of sterile water was added back to each flask. After incubation at 30° for designated periods, the root mass and the media of each flask were analyzed separately. Studies on ornithine metabolism in various plants were also run with excised root segments and leaf disks. Two g of root tissue from each hydroponically grown plant were transferred into Petri dishes and then 3 μc of DL-ornithine-2-^{14}C in 0.3 ml of sterile water was added. In experiments with leaf disks, on the flat bottom of a Petri dish were pipetted 10 spots of 50 μl aliquots of the tracer solution each containing 0.1 μc of DL-ornithine-2-^{14}C. Leaf disks of 1 cm diameter were carefully placed on each spot in contact with the solution without intervention of air bubbles so that the solution was infiltrated into the disks under vacuum. Tracer solutions were almost completely absorbed into the disks within 60 minutes. After 5 hours incubation at 30°, the excised roots and leaf disks from each plant were analyzed.

Preparation of Sample for Analyses. The roots and leaf disks, after incubation, were immediately ground in a mortar with 10 ml of 75% aqueous alcohol containing 0.2% acetic acid. The mixture was filtered through a fritted glass filter. This extraction was repeated 4 times with 10 ml of 75% aqueous alcohol containing 0.2% acetic acid. The alcohol extracts were combined and the volume was reduced *in vacuo* below 40°. Insoluble matter, which formed during concentration, was removed by centrifugation. The supernatant was reduced *in vacuo* to dryness below 40°. The residue was taken up a small volume of 0.2 M citrate buffer (pH, 2.2) and the volume was adjusted to 5 ml. Samples were stored in a deep-freezer until analyzed.

Isolation of Nicotine. Nicotine was obtained by steam distillation with 10 mg of nicotine added to the steam distillate as carrier (20). The steam distillate was caught in an excess of hydrochloric acid solution and the hydrochloric acid solution was concentrated to dryness. The residue was dissolved in a small volume of methanol. This solution was subjected to gas chromatography as described by Yasumatsu and Akaike (25).

Isolation of γ-Methylaminobutyraldehyde. Five μc of DL-ornithine-2-^{14}C in a small amount of water was administered to either 5 flasks of sterile cultures of *N. rustica* or 5 g of the roots excised from hydroponically grown *N. tabacum*. After 5 hours incubation at 30°, the ethanol soluble fraction was obtained as described above. Metabolites of ornithine were isolated by a Amberlite IR-120 column equilibrated with citrate buffer and detected by ^{14}C flow monitor system. The fraction Y-2 corresponding to Y-2 peak shown in figure 1 was separated on a 50 cm long column with 0.38 M citrate buffer at pH 4.26 or a 15 cm column with 0.38 M citrate buffer at pH 5.28. The fractions were pooled and passed through a column of Dowex 50 × 4 (H^+ form, 2.0 × 13 cm). The column was washed with water and eluted with N HCl. The effluent was collected in 60 fractions of 5 ml each. Material in fraction 38 to 45 was detected by radioactivity and by Dragendorff's reagent. These fractions were combined and concentrated to dryness in a partial vacuum below 40°. The residue was dissolved in a small volume of water and an aliquot used in subsequent tests: Preparation of 2,4-dinitrophenylhydrazone, paper chromatography, and color test with various reagents such as Dragendorff's reagent, Koenig reagent, ninhydrin, *o*-aminobenzaldehyde, ammoniacal $AgNO_3$ and $KMnO_4$ solution.

Preparation of 2,4-Dinitrophenylhydrazone. An aliquot of the radioactive Y-2 sample was added to authentic γ-methylaminobutyraldehyde which had prepared by hydrolyzing 100 mg of its acetal with N HCl. The 2,4-dinitrophenylhydrazone was obtained by the usual methods. Recrystallization was repeated from 90% ethanol until constant melting point and constant specific activity were obtained.

Analytical Procedures. Individual metabolites were separated by aid of a Beckman/Spinco Model

120 Amino Acid Analyzer and radioactivity determined by Packard Tri-Carb flow monitor system. Two ml of the solution prepared from the extracts was introduced on a column either of 150- or 50 cm length for measurement of neutral and acidic amino acids or basic amino acids, respectively. Gas chromatography was performed with a Shimazu Model GC-1B.

Results

Metabolism of Ornithine. In order to examine the possible precursor of intermediates in nicotine biosynthesis among the metabolites of ornithine, metabolism of DL-ornithine-2-^{14}C in tobacco root was studied first.

A typical flowgram of metabolite of DL-ornithine-2-^{14}C is shown in figure 1. After 5 hours incubation radioactivity was found in compounds metabolically related to ornithine such as glutamine, proline, glutamic acid, citrulline, γ-aminobutyric acid, and arginine. Besides these compounds several unknown radioactive substances were observed and designated Y-1, A-1, A-2, A-3, and Y-2. Y-1, A-1, A-2, and A-3 were eluted early from the ion-exchange column of 150 cm length, and Y-2 was eluted between histidine and arginine with the column of 50 cm length. Y-1 and Y-2 gave a yellow color with ninhydrin. Y-2 and arginine were

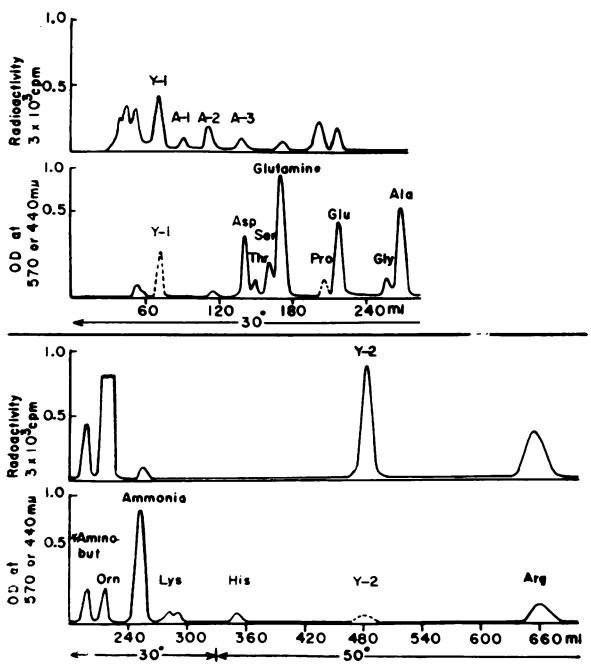

FIG. 1. Flowgram of the metabolites of DL-ornithine-2-^{14}C which had been fed to excised root culture of *N. rustica*. Upper part of this figure is the elution pattern for a 150 cm long column with 0.2 M citrate buffer at pH 3.25 and the lower part for a 50 cm long column eluted with 0.38 M citrate buffer at pH 4.26.

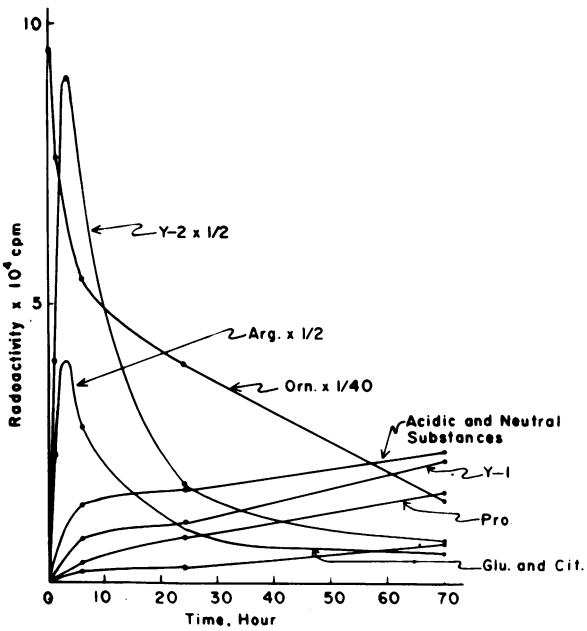

FIG. 2. Time course of radioactive incorporation of DL-ornithine-2-^{14}C into individual metabolite in excised root culture of *N. rustica*.

labeled most strongly among these compounds in this time period.

Figure 2 shows the result from time course experiment with DL-ornithine-2-^{14}C. As the feeding time increased, ornithine was rapidly metabolized and Y-1, proline, glutamic acid, citrulline, and the acidic and neutral fractions gained radioactivity slowly. On the other hand, Y-2 and arginine acquired a marked amount of radioactivity in a few hours. Radioactivity of both substances reached a maximum at 4 hours, at which time radioactivity of Y-2 was approximately 2.5 times as high as that of arginine. Thereafter ^{14}C in this unknown decreased very rapidly with time. Such changes indicated that Y-2 was a very active metabolite and had a high turnover rate. Upon the administration of the isolated Y-2 fraction to excised root cultures, incorporation of ^{14}C of Y-2 into nicotine was examined. The result shown in table I indicated

Table I. *Incorporation of Y-2 into Nicotine*

Isolated Y-2 fraction was added back to excised root culture of *N. rustica* and incubated for 24 hours at 30°. Percentage of incorporation was determined by dividing total activity of the nicotine by the activity of Y-2 fraction fed. Radioactivity of the Y-2 fraction fed was 6 × 10^5 cpm.

Experiment	Radioactivity incorporation
	%
1	7.03
2	30.1
3	33.0

that the substance was a very effective precursor of nicotine.

Identification of γ-Methylaminobutyraldehyde. In order to obtain some information about the structure of Y-2, other various labeled compounds were fed to excised root culture of *N. rustica*. Y-2 was labeled not only by ornithine-2-^{14}C but also putrescine-1,4-^{14}C or methionine-$^{14}CH_3$, but not by glutamic acid-U-^{14}C or γ-aminobutyric acid-1-^{14}C (table II). Also, neither nicotinic acid-U-^{3}H nor carboxyl-^{14}C, the pyridine ring of which serves as a precursor of the pyridine ring of nicotine, gave radioactive Y-2. These observations seem to indicate that Y-2 should possess a butane skeleton derived from ornithine or putrescine and methyl group derived from methionine, but neither a pyridine ring nor carboxyl group from nicotinic acid.

Table II. *Incorporation of Various Labeled Compounds into Y-2, Arginine and Nicotine*

Duration of the experiment was 5 hours.

Tracer	Y-2	Arginine	Nicotine
	%	%	%
Putrescine-1,4-^{14}C	1.1	0	24.3
Glutamic acid-U-^{14}C	0	0	3.2
Nicotinic acid-U-^{3}H	0	0	9.5
Nicotinic acid-7-^{14}C	0	0	0
DL-Methionine-$^{14}CH_3$	0.13	0	5.5
γ-Aminobutyric acid-1-^{14}C	0	0	0
DL-Ornithine-2-^{14}C	0.90	0.36	14.5

The latter fact was further confirmed by its negative reaction with Koenig reagent and by the absence of ultraviolet absorption corresponding to a pyridine moiety. Y-2 also had the following properties: a positive reaction with Dragendorff's reagent; instantly decolorized $KMnO_4$ solution; reduced ammoniacal $AgNO_3$ in cold with mirror formation; reacted with 2,4-dinitrophenylhydrazine in 2 N HCl; did not react with *o*-aminobenzaldehyde; gave a yellow color with ninhydrin. These properties of Y-2 indicated that this compound had carbonyl and amino groups. Since purification of Y-2 appeared to be difficult due to its unstable character, the isolated fraction Y-2 was compared with authentic material for chemical behavior. Y-2 was chromatographically indistinguishable from authentic γ-methylaminobutyraldehyde in 4 different solvent systems: *n*-butyl alcohol-acetic acid-water (4:1:5, R_F = 0.28), ter-butyl alcohol-formic acid-water (5:1:1:, R_F = 0.59), *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl (1:1:1, R_F = 0.48), *n*-butyl alcohol saturated with water (R_F = 0.13). The radioactivity of Y-2 cochromatographed with γ-methylaminobutyraldehyde. Constant specific activity persisted through several recrystallizations of the 2,4-dinitrophenylhydrazone of a mixture of Y-2 and the authentic aldehyde.

Anl., Found: C, 41.44 H, 5.05 N, 21.03

Calcd: for $C_{11}H_{16}O_4N_5Cl_1$; C, 41.53 H, 5.08 N, 21.13

The elution positions of Y-2 and the authentic compound on the amino acid analyzer were identical. On the basis of these observations, it was concluded that Y-2 was γ-methylaminobutyraldehyde or its cyclized form, N-methylpyrroline.

Occurence of γ-Methylaminobutyraldehyde. In order to determine a site of γ-methylaminobutyraldehyde biosynthesis in the tobacco plant and to examine whether other plants besides tobacco could synthesize it from ornithine, DL-ornithine-2-^{14}C was fed to the detached root and leaf disk of tobacco plant and some other solanaceous plants. Soluble fraction from each material was analyzed with an amino acid analyzer and flow monitor system. In all cases, there was a considerable incorporation of ^{14}C into arginine, indicating that the added ornithine penetrated into an active site of metabolism (table III). Only the root but not the leaf of tobacco plants had the ability to synthesize γ-methylaminobutyraldehyde from ornithine. Since the site of nicotine biosynthesis is located mainly in the root, it is consistent that this compound is detected in the root but not in the aerial portions of tobacco plant. For the roots of *Datura* and *Atropa* plants, which produced tropane alkaloid, a radioactive peak was observed in the same elution position as γ-methylaminobutyraldehyde. But no radioactivity corresponding to γ-methylaminobutyraldehyde was observed in both the root and leaf of the tomato plant and the leaf of *Datura* and *Atropa*.

Table III. *Incorporation of DL-Ornithine-2-^{14}C into γ-Methylaminobutyraldehyde (γ-MABA) and Arginine in Some Solanaceous Plants*

Radioactivity of the DL-ornithine-2-^{14}C fed was 5.79 × 10^6 cpm. The duration of the experiment was 5 hours.

Plant material	Radioactivity incorporated γ-MABA	Arginine
	cpm × 10^{-4}	*cpm* × 10^{-4}
Sterile root culture of *N. rustica*	3.35	0.49
Root of hydroponically grown *N. tabacum*	6.44	6.30
Leaf of *N. tabacum*	0	1.21
Root of hydroponically grown *Lycoperisicum esculentum*	0	3.16
Root of hydroponically grown *Datura stramonium*	0.29	3.90
Leaf of *Datura stramonium*	0	7.85
Root of hydroponically grown *Atropa belladonna*	7.76	0.92
Leaf of *Atropa belladonna*	0	5.00

Discussion

Ornithine is converted to glutamic acid and proline via glutamic-γ-semialdehyde, and citrulline and arginine by the ornithine cycle in higher plants (3, 10). Moreover, in tobacco plant ornithine enters the biosynthetic route of nicotine which involves γ-methylaminobutyraldehyde as an intermediate. γ-Methylaminobutyraldehyde, which is considered to exist in equilibrium with its cyclic form, N-methylpyrroline, has now for the first time been identified as an effective precursor of nicotine.

It has been established that the C-2 of ornithine is incorporated equally into C-2 and C-5 of nicotine (7, 16). Putrescine and mesomeric anion of Δ^1-pyrroline as possible symmetric intermediates have been proposed to explain the pattern of ^{14}C-labeling resulting from incorporation of ornithine-2-^{14}C into the pyrrolidine ring of nicotine (15, 17). From the data on the administration of ^{15}N-labeled ornithine-2-^{14}C to excised root of *N. tabacum*, it was suggested by Leete *et al.* (18) that as the first step in nicotine biosynthesis from ornithine, α-deamination takes place yielding α-keto-δ-aminovaleric acid (Δ^1-pyrroline-2-carboxylic acid). However, it does not seem likely that α-keto-δ-aminovaleric acid is an important intermediate in the biosynthesis of nicotine since in the present experiments putrescine was more efficient precursor of γ-methylaminobutyraldehyde and nicotine than ornithine (table III). The main route of γ-methylaminobutyraldehyde and nicotine synthesis from ornithine would probably be by way of putrescine. Recently, Leete (19) proposed that putrescine is the symmetrical intermediate between ornithine and the pyrrolidine ring.

Schroter and Neuman (23) reported that α-methylornithine can serve as a precursor of nicotine; this compound may give γ-methylaminobutyraldehyde by deamination and decarboxylation. However, it would be impossible for C-2 in α-methylornithine to distribute equally in C-2 and C-5 of the pyrrolidine ring of nicotine by such a reaction. Accordingly, for the metabolic sequence from ornithine to γ-methylaminobutyraldehyde at least 3 routes are possible as pictured in figure 3. As mentioned before, it would be more probable that ornithine is converted to γ-methylaminobutyraldehyde via putrescine and methylation of γ-aminobutyraldehyde (Δ^1-pyrroline) or putrescine takes place.

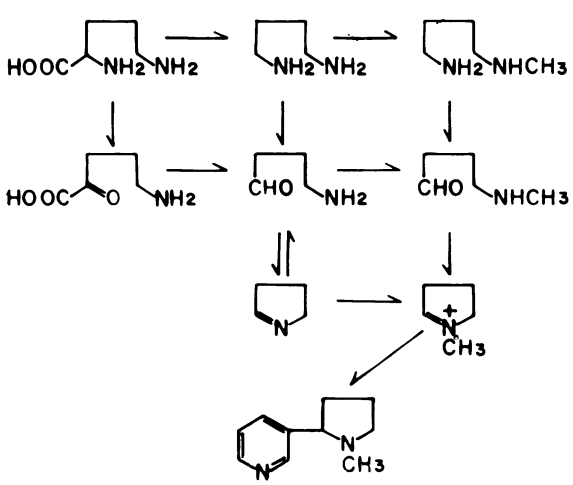

FIG. 3. Hypothetical biosynthetic pathway for nicotine.

The occurrence of γ-methylaminobutyraldehyde as an effective precursor of the nicotine biosynthesis supports our previous conclusion (12, 20) that nicotine is synthesized through a route(s) which does not involve nornicotine. Dawson and coworkers (4) have shown that the tritium labeled on C-6 of nicotinic acid was less efficiently incorporated into nicotine than the hydrogens labeled on the other carbon of nicotinic acid and the active intermediate may be a 1,6-dihydronicotinic acid derivative. It is now proposed that N-methyl-Δ^1-pyrroline would condense with 1,6-dihydronicotinic acid derivative to yield an intermediate, which undergoes decarboxylation and hydride abstraction to give nicotine.

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