Phytochemical Studies on the Tobacco Alkaloids

Part IX. Question for Nornicotine as a Precursor of Nicotine

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dl-Ornithine-2-¹⁴C, *dl*-methionine-¹⁴CH₃ and partially racemized *l*-nornicotine-2,5-¹⁴C were administered to aseptically grown excised roots (*N. rustica* var. Brasilia). Incorporation of their radioactivity to nicotine was compared. The extent of their radioactive incorporation to nicotine was high in the order of ornithine, methionine and nornicotine; incorporation of radioactivity of nornicotine to nicotine was extraordinarily low. ¹⁵N-Labeled nornicotine was also fed to the same materials and ¹⁵N distribution was examined. Most of ¹⁵N still remained in the nornicotine reisolated. Marked amounts of ¹⁵N were located in the ethanol-insoluble fraction, the amino acid fraction and the substances having chromatographic *R_F* value close to that of nicotine. Only small amount of ¹⁵N was incorporated to the isolated nicotine.

Nornicotine is generally accepted to be a direct precursor of nicotine in tobacco plants. From these findings, however, it can be said that the biosynthesis of nicotine can occur through other routes without going through nornicotine.

INTRODUCTION

After administration of nornicotine-¹⁵N to tobacco plants, Tso and Jeffrey¹⁾ have isolated nicotine labeled with ¹⁵N from the plants, and proposed nornicotine as a precursor of nicotine. Later, they have studied the incorporation rate of ³H in the tobacco alkaloids and found that the relative change of the ³H specific activities of the alkaloids can best be explained on the supposition of nornicotine as a direct precursor of nicotine.²⁾ Leete³⁾ has also postulated nornicotine as a direct precursor of nicotine in his biosynthetic scheme of nicotine from ornithine.

However, our results described in the preceding paper⁴⁾ indicate that the nornicotine

produced in the tobacco root is biosynthesized through at least two different pathways: exclusive incorporation of C-2 of ornithine to C-2 and equal incorporation to C-2 and C-5 of the pyrrolidine ring. The proposed explanation for the nornicotine having equal activities at C-2 and C-5 from ornithine-2-¹⁴C is that the nornicotine may be derived from nicotine by demethylation.⁵⁾ Accordingly, neither of the two biosynthetic routes can supply nornicotine which can serve as a precursor of nicotine having equal activities at C-2 and C-5 from ornithine-2-¹⁴C. This results seem to be consistent with our previous results^{6,7)} of a series of studies of the optical properties of the tobacco alkaloids. However, the results described in the previous paper

1) T. C. Tso and R. N. Jeffrey, *Arch. Biochem. Biophys.*, **80**, 46 (1959).

2) T. C. Tso and R. N. Jeffrey, *Arch. Biochem. Biophys.*, **97**, 4 (1962).

3) E. Leete, "Biogenesis of Natural Compounds", Pergamon Press, 1963, p. 739.

4) S. Mizusaki, T. Kisaki and E. Tamaki, *This Journal*, **29**, 714 (1965).

5) S. Mizusaki, T. Kisaki and E. Tamaki, *This Journal*, **29**, 78 (1965).

6) T. Kisaki and E. Tamaki, *Arch. Biochem. Biophys.*, **94**, 252 (1961).

7) T. Kisaki and E. Tamaki, *Naturwissenschaften*, **47**, 541 (1960).

cannot exclude a possibility by which some of the nornicotine can be synthesized from ornithine-2-¹⁴C and can be methylated to give nicotine labeled equally at C-2 and C-5. This is the biosynthetic mode which is presumed by many authors as those cited above. Hence, it is desirable to make another study to persuade whether or not nornicotine can be a direct precursor of nicotine in tobacco plants. To this end, we have examined on incorporation of the label to nicotine by feeding nornicotine-¹⁵N, nornicotine-2,5-¹⁴C, ornithine-2-¹⁴C and methionine-¹⁴CH₃ to aseptic cultures of excised roots to avoid any microbial action. It has been well known that ornithine is a good precursor of the pyrrolidine ring of nicotine, and methionine is a common methyl donor in plants and is incorporated in the methyl moiety of nicotine in tobacco plants.

EXPERIMENTAL

Excised root cultures

An aseptic culture of tobacco root (*Nicotiana rustica* var. Brasilia) was performed in a 100 ml of flask containing 30 ml of medium according to Solt's method.⁸⁾ The third successive cultures of the root were used for the feeding experiment.

Tracers

dl-Ornithine-2-¹⁴C (26.0 mc/mM) was purchased from California Corporation for Biochemical Research. *dl*-Methionine-¹⁴CH₃ (2.3 mc/mM) was purchased from Daiichi Chemical Co. Nornicotine-2,5-¹⁴C was prepared in the following way. Nicotine-2,5-¹⁴C, which was biosynthesized from ornithine-2-¹⁴C by excised tobacco roots, was converted to nornicotine by feeding to excised *Nicotiana tabacum* "Cherry Red" grown on tomato stock. The radioactive nornicotine⁹⁾ was purified in a same method¹⁰⁾ as described previously; specific activity, 1.10×10^7 c.p.m./mM. Nornicotine-¹⁵N was prepared in the following way. *Nicotiana glutinosa* plants were grown in sand by supplying (¹⁵NH₄)₂SO₄ (30% ¹⁵N atom excess) as nitrogen source. A crude alkaloid fraction extracted from the plants was fractionally distilled to give a nornicotine fraction boiling at 123°C/6.5 mm and after several recrystallizations of the dipicrate, it melted at 187~9°C, $[\alpha]_D^{24} = -71.70$. Oxidation of small portion of the nornicotine with neutral KMnO₄ solution gave nicotinic acid. Upon ¹⁵N analyses, it was shown that ¹⁵N atom excess % was 5.49 at the pyridine nitrogen and 5.67 at the pyrrolidine nitrogen.

Each culture solution of 30 flasks containing 4 weeks old tobacco roots was aseptically renewed with 20 ml of culture solution containing either 6.51 mg of nornicotine-¹⁵N having 5.58 ¹⁵N atom excess % (adjusted at pH=6.0 with acetic acid), *dl*-ornithine-2-¹⁴C¹¹⁾, *dl*-methionine-¹⁴CH₃, or nornicotine-2,5-¹⁴C, respectively. After 2-week additional culture period the media of each flask were combined and used for the analyses.

Administration of tracers

Each culture solution of 30 flasks containing 4 weeks old tobacco roots was aseptically renewed with 20 ml of culture solution containing either 6.51 mg of nornicotine-¹⁵N having 5.58 ¹⁵N atom excess % (adjusted at pH=6.0 with acetic acid), *dl*-ornithine-2-¹⁴C¹¹⁾, *dl*-methionine-¹⁴CH₃, or nornicotine-2,5-¹⁴C, respectively. After 2-week additional culture period the media of each flask were combined and used for the analyses.

Analytical procedure

Aseptic cultures fed with radioactive compounds were filtered through a fritted glass filter and separated to roots (average fresh weight, approximately 12g) and culture fluids. After acidification with acetic acid, the culture fluids were concentrated in vacuo. The concentrate and the roots were again combined and were subjected to steam distillation on alkalization. The steam distillate was caught in hydrochloric acid solution. According to the Brown and Barthel,¹²⁾ an aliquot of the distillate was treated with sodium nitrite to nitrosify secondary amine tobacco alkaloids and again steam distilled to separate nicotine from nornicotine. The nicotine content was determined spectrophotometrically by the U. V. method¹³⁾ and the radioactivity was determined after drying an aliquot of the distillate on a planchet. The cultures fed with nornicotine-¹⁵N were filtered through a fritted glass filter and the roots remaining on the filter were washed several times with water (fresh weight, 11.939 g). The roots were then extracted repeatedly with 100 ml of 70% ethanol containing 0.1% acetic acid. The culture media, washings and ethanol extracts were combined and concentrated to dryness in vacuo. To the concentrate, 51 mg of nicotine and 55 mg of nornicotine were added as carriers, respectively and, after alkalization with ammonia, alkaloids were extracted continuously with chloroform. The chloroform extract was liquid-chromatographed on

11) *d*-Form of ornithine appears not to be assimilated by the roots.

12) C. V. Brown and W. F. Barthel, *Ind. Eng. Chem., Anal. Ed.*, **15**, 740 (1943).

13) C. O. Willits, M. L. Swain, J. A. Conelley and B. A. Brice, *Anal. Chem.*, **22**, 430 (1950).

8) M. L. Solt, *Plant Physiol.*, **32**, 480 (1957).

9) The nornicotine obtained here is *l*-predominant nornicotine.

10) T. Kasaki and B. Tamaki, *This Journal*, **28**, 492 (1964).

FIG. 1. Paperchromatograms of the Fractionated Alkaloids from the Excised Tobacco Root Culture.

Condition in paperchromatography is similar to that described in the preceding paper.⁴⁾

- I Chloroform extract from ammoniacal aqueous solution
- II Ether eluate from alumina column
- III 2% methanol-ether eluate from alumina column
- IV Chloroform extract from pH=5 aqueous solution
- a HR fraction b Nicotine c Nornicotine

alumina column (3×20 cm). As shown in Fig. 1, nicotine was obtained in the ether eluate, and nornicotine and two other Koenig-positive spots (HR) having high R_F values appeared in the 2% methanol-ether eluate. The nicotine was further purified by repetition of recrystallization of its picrate. The 2% methanol-ether eluate was shaken with N hydrochloric acid and the hydrochloric acid solution was adjusted to pH 5.4~5.6 and the substances corresponding to HR were continuously extracted with chloroform. The chloroform extract was again shaken with N hydrochloric acid and reextracted with chloroform at the same pH. On three repetition of this procedure, HR was separated from nornicotine. The mother liquors were combined and, after alkalization with NaOH, nornicotine was extracted with chloroform. The amino acid fraction was concentrated in vacuo and treated with Dowex 50×4 (H⁺) ion exchange resin. Elution of the column with 2N ammonia released amino acid which had been adsorbed. Auto-

FIG. 2. Autoradiogram (a) and Parerchromatogram (b) of Alkaloid of the Sample Fed with Nornicotine-2,5-¹⁴C.

Conditions in paperchromatography and radioautography are similar to those described in the preceding paper.⁴⁾

- A. Nicotine
- E. Nornicotine

radiography was made in the similar manner to the preceding paper.⁴⁾ ¹⁵N analysis was performed by mass spectrometer at Institute of Physical and Chemical Research, Tokyo, Japan. Radioactive assay was made by Hitachi RDF-4 windowless gasflow GM counter.

RESULTS AND DISCUSSION

The results of radioactive incorporation to nicotine of either ornithine, methionine or nornicotine labeled with ¹⁴C fed to the excised root culture are shown in Table I. *dl*-Ornithine-2-¹⁴C and *dl*-methionine-¹⁴CH₃ were in-

TABLE I. COMPARISON OF INCORPORATION ON VARIOUS PRECURSORS TO NICOTINE IN EXCISED ROOT CULTURE

Tracer	Amount fed (c.p.m.) (μ M)		Nicotine yield (mg)	Total activity in Nicotine (c.p.m.)	Radioactive incorporation (%)
<i>dl</i> -Ornithine-2- ¹⁴ C*	7.94×10^5	40	9.6	4.05×10^4	5.1
<i>dl</i> -Methionine- ¹⁴ CH ₃ *	8.03×10^5	40	10.2	2.81×10^4	3.5
Nornicotine-2,5- ¹⁴ C	4.02×10^5	46	8.8	7.50×10^2	0.19

* Original tracers were diluted with either cold *l*-ornithine or *l*-methionine, respectively.

TABLE II. DISTRIBUTION IN VARIOUS FRACTIONS OF ¹⁵N OF NORNICOTINE FED TO EXCISED ROOT CULTURE

Fraction	N(mg)	¹⁵ N atom excess (%) ^{c)}	¹⁵ N excess (mg)	Incorporation (%) ^{d)}
Nornicotine administered	36.97	5.58	2.06	—
Ethanol-insoluble fraction	19.03	0.07	0.013	0.63
Amino acid fraction	9.08	0.39	0.035	1.7
Nicotine ^{a)}	12.76	0.04	0.005	0.24
Nornicotine ^{a)}	9.76	1.92	0.19	9.2
HR fraction ^{b)}	2.29	1.51	0.035	1.7

a) The sample was diluted with corresponding carrier. b) See the text c) Atom excess (%) value can be obtained to three places of decimals by the mass spectrometer employed but, since the last numerical value is not always reliable, it was cut off. d) Ratio of ¹⁵N excess(mg) of individual fraction to that of the originally added nornicotine.

corporated into nicotine to a large extent. However, only a small extent of radioactivity of nornicotine appeared in the nicotine. Same result was obtained in feeding experiment of either *l*- or *d*-nornicotine-U-³H to the excised root culture.¹⁴⁾

As proposed by Leete, it is properly considered that the biosynthetic route to nicotine from ornithine would involve several steps. On the other hand, nornicotine and methionine can be considered as a direct precursor of nicotine as a methyl acceptor and a methyl donor, respectively. Since it has been accepted that methionine is a common biological methyl donor¹⁵⁾ in tobacco plants, it is properly thought that its utilization to nicotine cannot be low. And, if nornicotine is a precursor of nicotine, it is expected that largest incorporation of radioactivity to nicotine must be found with nornicotine feeding. However, the result in Table I is inconsistent with this expectation,

casting doubt about nornicotine as a direct precursor of nicotine. Fig. 2 illustrates radioautogram of alkaloid of the sample fed with nornicotine-2,5-¹⁴C. A considerable amount of radioactivity was found in the substance(s) (HI) having close *R_F* value to nicotine, but no radioactivity was found in nicotine(A).

In a similar experimental condition to the feeding experiment of radioactive material, the nornicotine labeled with ¹⁵N was fed to excised root culture. Table II shows a comparison of distribution of ¹⁵N of nornicotine to nicotine and to other various fractions. Only small amount of ¹⁵N was detected in the ethanol-insoluble fraction. Most of ¹⁵N was still remained in the HR fraction which has close *R_F* values to that of nicotine on paperchromatogram shown in Fig. 1 and which seems to correspond to the substance having close *R_F* values to nicotine in Fig. 2. The results indicate that nornicotine can be metabolically transformed to various compound, mainly to the HR fraction, but can

14) Unpublished data.

15) B. Ladesic, Z. Devide, N. Pravdic and D. Keglevic, *Arch. Biochem. Biophys.*, **97**, 556 (1962).

scarcely be utilized to nicotine.

According to the results of the comparative study by these tracer feeding experiments described in this paper, it seems to be inconceivable that nornicotine is a direct precursor of nicotine in tobacco roots.

At least, it can be said that in *Nicotiana* roots the biosynthesis of nicotine can occur

through other route(s) without going through nornicotine. Therefore, at which step in the biosynthesis of nicotine methylation takes place still remains to be answered.

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