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THE INCORPORATION OF Δ^1-PYRROLINE-5-CARBOXYLIC ACID INTO NICOTINE

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Abstract—DL-Δ^1-Pyrroline-5-carboxylic acid-5-^{14}C, a possible precursor of nicotine, was fed to *Nicotiana rustica* for 6 hr. The following percentage distribution of ^{14}C was found after partial degradation of the isolated nicotine: pyridine ring, 5·9 per cent; carbon 2′, 47·8 per cent; and carbon 3′, 1·1 per cent. More than 40% of the remaining radioactivity was located in carbon 5′. Although DL-Δ^1-pyrroline-5-carboxylic acid-5-^{14}C was incorporated symmetrically as are other precursors of the pyrrolidine ring of nicotine, its low incorporation into nicotine (0·04 per cent) indicates that it probably is not on the main pathway of pyrrolidine ring biosynthesis.

INTRODUCTION

CHEMICAL degradation of nicotine isolated from intact tobacco plants after metabolism of glutamate-2-^{14}C,[1,2] ornithine-2-^{14}C,[3,4] acetate-2-^{14}C,[5,6] and other ^{14}C-labeled metabolites[7] has demonstrated that carbons 2′ and 5′, as well as carbons 3′ and 4′, of the pyrrolidine ring of nicotine are equally labeled. These results indicate that precursors are incorporated into the pyrrolidine ring of nicotine via a symmetrical intermediate. From initial ^{14}C studies Leete[8] postulated that Δ^1-pyrroline was the probable symmetrical intermediate. However, subsequent studies with ^{15}N-labeled ornithine-2-^{14}C indicated that the δ-amino nitrogen of ornithine, and not the α-amino nitrogen, participated in the formation of the pyrrolidine ring of nicotine.[9] Since the δ-amino group of ornithine is lost in the formation of glutamic semialdehyde, the pathway of ornithine incorporation into nicotine via glutamic semialdehyde, DL-Δ^1-pyrroline-5-carboxylic acid (abbreviated as PC) and Δ^1-pyrroline is open to question.

Recently Leete[10] and Mizusaki *et al.*[11] have proposed that ornithine is incorporated into the pyrrolidine ring of nicotine via the symmetrical compound putrescine and 4-(*N*-methyl)-aminobutyraldehyde. Mizusaki *et al.* observed the formation of 4-(*N*-methyl)-aminobutyraldehyde after metabolism of ornithine-2-^{14}C by tobacco roots. Radioactive 4-(*N*-methyl)-aminobutyraldehyde, as well as putrescine, were efficient precursors of labeled

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nicotine.[11] The present work was undertaken to clarify the pathway for the formation of the pyrrolidine ring of nicotine. PC, a proposed intermediate between ornithine and the hypothetical symmetrical intermediate Δ^1-pyrroline, was labeled with ^{14}C at carbon 5 and administered to *Nicotiana rustica*. The resulting labeling pattern of the pyrrolidine ring of the isolated nicotine is interpreted in relation to the proposed pathways via Δ^1-pyrroline or putrescine.

RESULTS AND DISCUSSION

The ^{14}C distribution in nicotine after 6 hr of PC-5-^{14}C metabolism by *Nicotiana rustica* is shown in Table 1. Carbon 2′ of nicotine contained 47·8 per cent of the total radioactivity

TABLE 1. DISTRIBUTION OF ^{14}C IN NICOTINE AFTER 6 hr OF PC-5-^{14}C METABOLISM

Degradation product	Carbons of nicotine	Radioactivity (dpm/mmole)	Radioactivity (%)
N,Benzoyl*meta*nicotine	Pyridine, 2′,3′,4′,5′, N-CH_3	5290 ± 60*	100·0
Nicotinic acid	Pyridine, 2′	2680 ± 50	50·7
$BaCO_3$	2′	2530 ± 40	47·8
Pyridine perchlorate	Pyridine	310 ± 10	5·9
N,*N*-Dimethyl-β-alanine·HCl	3′,4′,5′, N-CH_3	2710 ± 100	51·2
Benzoic acid	3′	60 ± 10	1·1

* Standard deviation from the mean as determined from the mean of a series of replicate samples.

in nicotine whereas approximately 6 per cent of the label was located in the pyridine ring. The sum of the radioactivity in the remaining carbons (3′, 4′, 5′, and *N*-CH_3) was about 51 per cent of the total radioactivity. Carbon 3′ contained 1·1 per cent. Individual values for carbons 4′, 5′, and *N*-CH_3 could not be accurately determined due to low yields. However, the counting data for these three carbons indicate that more than 40 per cent of the radioactivity of nicotine was located in carbon 5′. Since previous studies have shown that carbons 2′ and 5′, as well as carbons 3′ and 4′, are labeled equally after feeding radioactive precursors,[1–7] it is reasonable to assume that carbon 4′ contained approximately 1 per cent, carbon 5′ contained 48 per cent and the remaining 1 per cent was located in the *N*-methyl group. The data are summarized in Fig. 1.

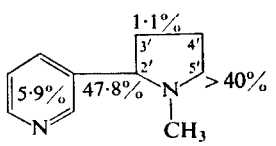

FIG. 1. PERCENT DISTRIBUTION OF RADIOACTIVITY IN NICOTINE AFTER 6 hr OF Δ^1-PYRROLINE-5-CARBOXYLIC ACID-5-^{14}C INCORPORATION BY *N. rustica*.

The results in Table 1 clearly indicate that PC-5-^{14}C was incorporated into the pyrrolidine ring via a symmetrical intermediate. However, it is not clear by what pathway PC-5-^{14}C was

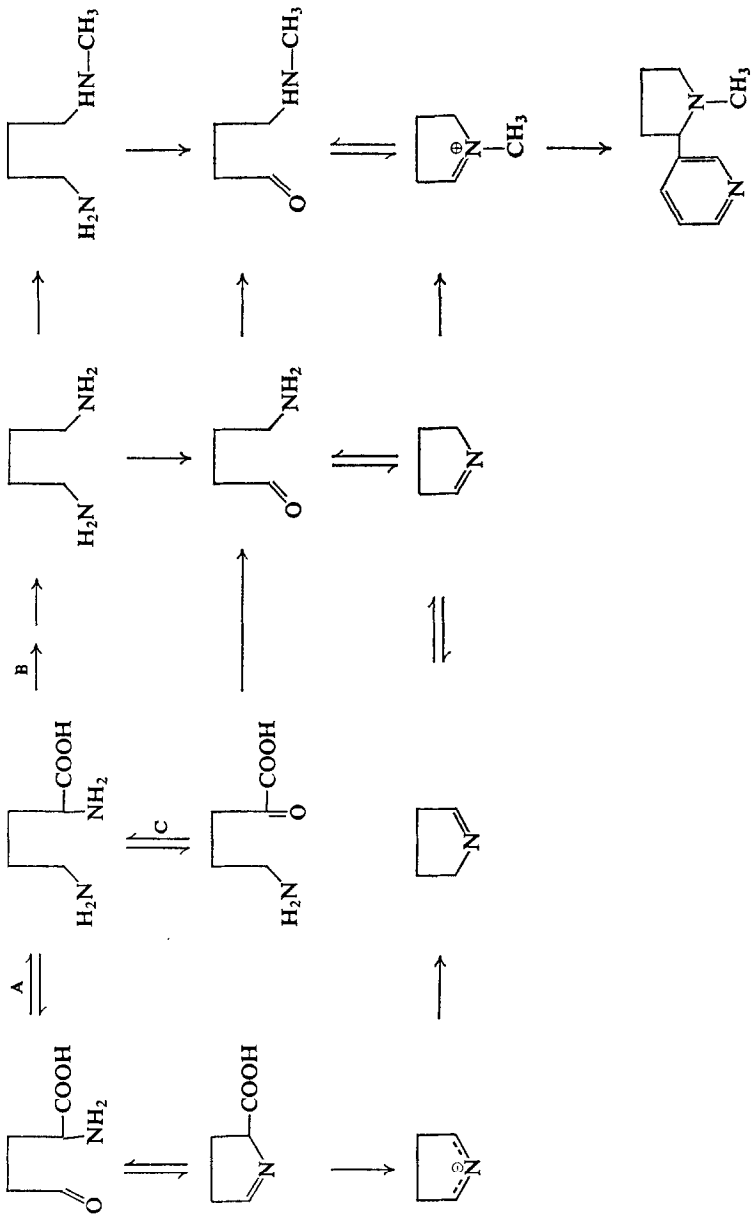

FIG. 2. HYPOTHETICAL PATHWAYS FOR THE INCORPORATION OF ORNITHINE INTO NICOTINE.

incorporated into the pyrrolidine ring of nicotine. Figure 2 shows three hypothetical pathways for the biosynthesis of the pyrrolidine ring of nicotine starting with ornithine. Pathway A proceeds via PC and Δ^1-pyrroline before incorporation into nicotine. Pathway C proceeds via α-keto-δ-aminovaleric acid. If pathway A was the sole or predominant physiological pathway, it is anticipated that PC-5-^{14}C would be extensively incorporated into nicotine. However, after 6 hr only about 0·04 per cent of the radioactivity originally present in the feeding solution was recovered in the isolated nicotine. Mizusaki *et al.*,[11] using excised tobacco roots, observed after 5 hr the following percentage incorporation of glutamate-U-^{14}C, ornithine-2-^{14}C, and putrescine-1,4-^{14}C into nicotine: 3·2, 14·5 and 24·3 per cent, respectively. Therefore, the present evidence indicates that PC is not directly on the main pathway of nicotine biosynthesis. It is probable that PC was converted to ornithine prior to incorporation into nicotine. The many reactions required to convert PC to ornithine, and eventually to nicotine, would explain its low incorporation.

Although this study makes the involvement of PC in the pathway of nicotine biosynthesis dubious, it does not completely eliminate Δ^1-pyrroline as an intermediate. If ornithine was incorporated via pathway B, oxidation of putrescine would yield 4-aminobutyraldehyde, which is in equilibrium with its cyclic form, Δ^1-pyrroline. Subsequent methylation would yield 4-(*N*-methyl)-aminobutyraldehyde, the compound observed by Mizusaki *et al.*[11] after feeding ornithine-2-^{14}C to excised tobacco roots. If oxidation and methylation occurred in the above order, two symmetrical intermediates (putrescine and Δ^1-pyrroline) would be involved in the biosynthesis of the pyrrolidine ring of nicotine. However, if methylation occurs prior to oxidation, Δ^1-pyrroline would not be an intermediate. Schütte, Maier, and Mothes[12] have shown that *N*-methylputrescine is incorporated *in toto* into the pyrrolidine ring of nicotine. However, the physiological significance of this observation is not clear since the presence of *N*-methylputrescine has not been demonstrated in tobacco plants.

No direct evidence exists to exclude the possibility that ornithine is metabolized by pathway C to α-keto-δ-aminovaleric acid, 4-aminobutyraldehyde (Δ^1-pyrroline), 4-(*N*-methyl)-aminobutyraldehyde before incorporation into nicotine. However, since Mizusaki *et al.*[11] observed that putrescine was more readily incorporated into nicotine than ornithine, it is probable that pathway C is not the primary pathway of pyrrolidine ring biosynthesis.

Although extensive evidence is now available to indicate that pathway B is the preferential pathway for pyrrolidine ring biosynthesis, several uncertainties remain. The sequence of reactions between putrescine and 4-(*N*-methyl)-aminobutyraldehyde has not yet been determined. In addition, it is necessary to assume a fast equilibrium between ornithine and α-keto-δ-aminovaleric acid which results in exchange of the α-amino nitrogen before the formation of the symmetrical intermediate, putrescine, since it has been shown that the α-amino nitrogen does not participate in the pyrrolidine ring formation.[9]

EXPERIMENTAL

The starting material for the synthesis of DL-Δ^1-pyrroline-5-carboxylic acid-4-^{14}C was diethyl acetamidomalonate-2-^{14}C purchased from ICN. The procedure was essentially that employed by Strecker.[13] An elemental analysis* gave the following values: Found: C, 39·39; H, 5·49; N, 8·86; Cl, 22·58 per cent. Calc. for $C_5H_8NO_2Cl$: C, 40·10; H, 5·35; N, 9·35; Cl, 23·70 per cent.

Fifty mg of PC-5-^{14}C with a specific activity of $6{\cdot}36 \times 10^9$ dpm/mmole was dissolved in 25 ml of water and

* Performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

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carefully adjusted to pH 6–7 with 0·2 N NaOH. The radioactive material was administered to twenty-five plants for 6 hr as previously described.[14] Ninety-eight per cent of the radioactivity in the feeding solution was absorbed by the plants. The isolated nicotine was degraded by the method of Liebman *et al.*[15] as modified by Zielke *et al.*[6]

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