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BIOCHEMICAL STUDIES ON TOBACCO ALKALOIDS—VI.
BIOSYNTHESIS OF NICOTINE THROUGH NORNICOTINE

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Abstract—^{15}N-nornicotine and L-methionine-methyl-^{14}C were supplied to tobacco plants (*Nicotiana tabacum* L. cv. Connecticut Broadleaf) through the root. ^{15}N excess and ^{14}C activity were found in nicotine isolated from these experimental plants thus indicating that nornicotine can serve as an immediate precursor of nicotine. Methyl-^{14}C, known to be very active biologically, was incorporated at a higher rate than ^{15}N-nornicotine except in an early stage in the root where there was a high relative incorporation of ^{15}N in nicotine. The relative incorporation of ^{15}N-nornicotine and methyl-^{14}C from methionine to nicotine, however, is very similar in the root or the shoot. The main site of methylation, starting from 4 days after feeding, was in the tobacco shoot.

INTRODUCTION

THE ORIGIN of the methyl carbon of nicotine has been the subject of many investigations.[1–4] The methyl group of methionine has been demonstrated to give rise to the methyl group of nicotine,[3,4] and such transmethylation could take place either as an intermediate step during the biosynthesis of nicotine (either following or during the formation of the pyrrolidine ring) or as the final step (the transmethylation of nornicotine to nicotine). Nornicotine itself can be formed through demethylation of nicotine,[5,6] but this transformation occurs only at a later stage of tobacco plant development. It has been suggested therefore that since these two alkaloids are readily interconvertible[7] through transmethylation or oxidative demethylation[8] nornicotine may be an immediate precursor of nicotine.[6]

The formation of nornicotine prior to that of nicotine has been demonstrated in two independent experiments employing isotopes: (*a*) A higher ^{15}N atom per cent excess was found in nornicotine than in nicotine when ^{15}N was supplied in the form of $K^{15}NO_3$ to the nutrient solution where tobacco–tomato grafted plants were grown[6] and (*b*) a higher H^3 activity was found in nornicotine than in nicotine if the tobacco plants were grown in a nutrient solution containing tritiated water.[9]

The object of the present experiment was to determine whether nornicotine was capable of acting as an immediate precursor of nicotine.

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RESULTS AND DISCUSSION

A. Apparent Absorption of Supplied Material

The residue of the feeding solution, including repeated washings, was examined for remaining methionine and nornicotine. During the 6 hr of root feeding, the plant absorbed from 84 to 97·4% of the supplied methionine ($2{\cdot}20$–$2{\cdot}55 \times 10^7$ cpm) and 96·7 to 98·9% (1·538–1·574 mg) of the supplied nornicotine, despite the fact that these labeled materials were supplied at the same concentrations of $1{\cdot}5 \times 10^{-1}$ mmole. Previous reports[1,7] indicated the absence of microbiological activities within this short feeding period and the labeled materials were absorbed by the plant in their original form.

B. Incorporation of ^{14}C into Nicotine

Nicotine isolated as dipicrate from each plant was purified through repeated crystallization and purity was proven by melting point and paper chromatography. The ^{14}C activity in nicotine is shown in Table 1. The specific activity of nicotine isolated from root of test plants

TABLE 1. ^{14}C ACTIVITY AND ^{15}N EXCESS IN NICOTINE FROM EXPERIMENTAL PLANTS

Plant	Time between feeding and plant analysis (days)	Nicotine from root			Nicotine from shoot		
		Weight (mg)	Specific activity (cpm/mm)	^{15}N excess (at. %)	Weight (mg)	Specific activity (cpm/mm)	^{15}N excess (at. %)
I	2	25·0	$1{\cdot}68 \times 10^6$	0·238	87·0	$1{\cdot}56 \times 10^6$	0·008
II	4	21·6	$1{\cdot}32 \times 10^6$	0·009	44·0	$1{\cdot}65 \times 10^6$	0·022
III	6	26·2	$4{\cdot}68 \times 10^5$	0·012	85·0	$9{\cdot}07 \times 10^5$	0·022
IV	8	14·6	$3{\cdot}67 \times 10^5$	0·008	81·0	$7{\cdot}06 \times 10^5$	0·016

varied from $3{\cdot}67 \times 10^5$ to $1{\cdot}68 \times 10^6$ cpm/mm, and that isolated from shoot varied from $7{\cdot}06 \times 10^5$ to $1{\cdot}65 \times 10^6$ cpm/mm.

Earlier publications have shown that the radioactivity in the nicotine isolated from tobacco plants which had been fed methionine-methyl-^{14}C was located in the methyl group.[1,4] This has been assumed here since no uncatalyzed exchange of methyl groups between methionine and nicotine was found to take place during the feeding period.[3]

C. Incorporation of ^{15}N into Nicotine

The nicotine content in the test plants, Connecticut Broadleaf tobacco, is relatively low under regular conditions. This fact was taken into consideration during feeding of labeled materials. Therefore, only a small amount of nornicotine was supplied to the test plants in order to maintain a condition for nicotine formation as close to normal as possible. Consequently, the total ^{15}N excess supplied from nornicotine was also limited, which resulted in the relatively low atom per cent of ^{15}N excess found in isolated nicotine.

The experimental error in ^{15}N determinations, however, is within 0·002%. The data presented in Table 1 are averages of two sets of independent determinations, and in most cases the two sets of data were identical and it is evident that there is definite incorporation of ^{15}N into the nicotine molecule.

To examine whether nornicotine was incorporated into nicotine as such or as a degradation compound, a separate experiment[7] was carried out. *N. rustica* var. *brasilia* which is known to tolerate high nicotine content, was supplied with 200 mg of uniformly ^{15}N labeled nornicotine via the roots. After 7 days, 52% of the supplied ^{15}N was recovered in alkaloids from the test plant, and 23% of the supplied ^{15}N appeared in nicotine which was found to have its pyridine and pyrrolidine rings equally labeled with ^{15}N. Based on this evidence, it is clear that ^{15}N-nornicotine was incorporated as such into the nicotine molecule.

D. Relative Incorporation of ^{14}C *and* ^{15}N

The changes with time of the relative incorporation of ^{14}C in nicotine expressed as the ratio of specific activity of recovered nicotine to that of supplied methionine, and similarly, the

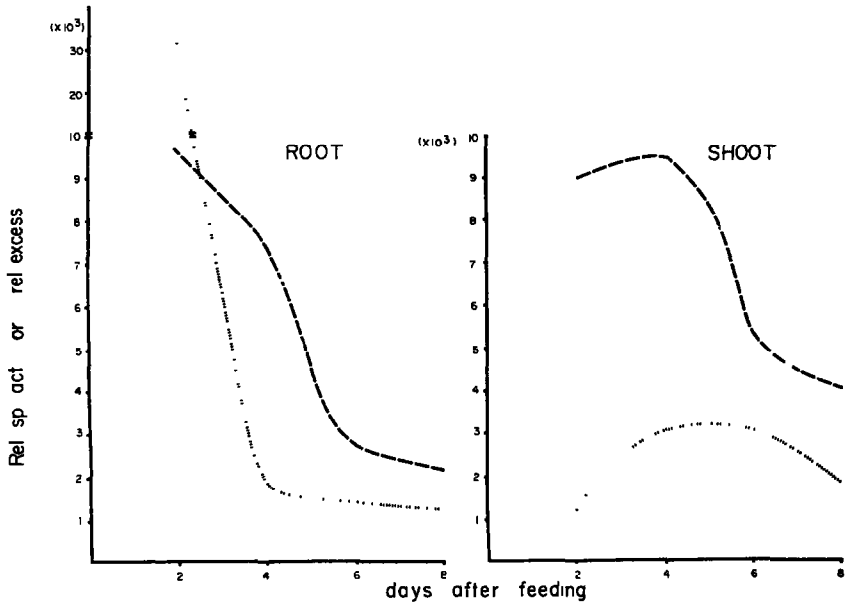

FIG. 1. RELATIVE SPECIFIC ACTIVITY OF ^{14}C AND RELATIVE EXCESS OF ^{15}N IN ISOLATED NICOTINE.

..... ^{15}N

--------- ^{14}C

relative incorporation of ^{15}N expressed as the ratio of atom per cent ^{15}N excess of recovered nicotine to that of supplied nornicotine are shown in Fig. 1.

A high ^{15}N excess was found in nicotine obtained from the root 2 days after feeding. It may be explained that a high proportion of newly absorbed nornicotine was immediately methylated in the root area within this early period. This newly transformed nicotine has yet to be translocated or diluted. Aside from this case, a higher rate of ^{14}C than of ^{15}N incorporation, ranging from 1·60 to 7·98 times, was observed in nicotine from the root and shoot of all other experimental plants shown in Fig. 1. This higher rate of ^{14}C incorporation is expected since the methyl group is known to be very active in the biological system.

In the nicotine of the root, the highest incorporation of both ^{14}C and ^{15}N was found in the plant 2 days after feeding and gradually decreased with the increase of time. On the nicotine of

the shoot, the highest incorporation of both ^{14}C and ^{15}N was found in the plant 4 days after feeding, and then gradually decreased. Beginning 4 days after feeding, a constantly higher incorporation of ^{14}C and ^{15}N was found in the nicotine obtained in shoots than in roots. It appears to indicate that the absorbed labeled methionine and nornicotine were gradually translocated to the shoot, and in this period the main site of transmethylation is in the shoot. This is even more evident in view of the fact that a greater amount of nicotine was present in the shoot than in the root and therefore a higher dilution occurred in the shoot area.

The ratio of relative incorporation of ^{14}C and relative incorporation of ^{15}N at different stages after feeding is shown in Fig. 2. Except in the case of nicotine in the root after 2 days'

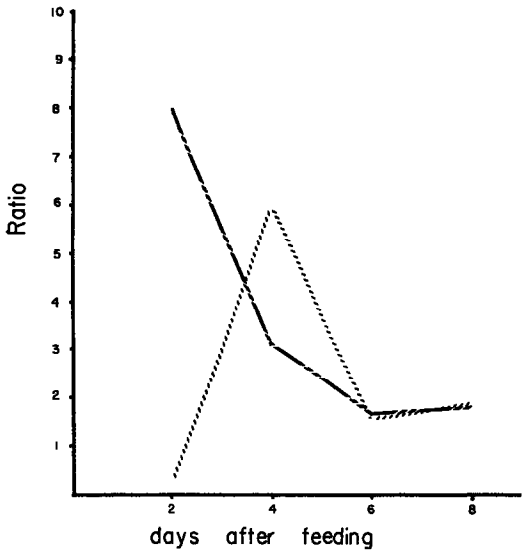

FIG. 2. RATIO OF RELATIVE INCORPORATION OF ^{14}C TO ^{15}N IN ISOLATED NICOTINE.

,,,,,,,,,,, Root

—·—·—·— Shoot

feeding when there was a high ^{15}N incorporation and, therefore, a low ^{14}C–^{15}N ratio, all the other ratios followed a general pattern of gradually decreasing value as the time increased. This fact indicates that ^{14}C in labeled nicotine takes an active part in the metabolic system.

E. Recovery of ^{14}C and ^{15}N

No attempt was made to recover the supplied isotopes completely. Available data, however, would indicate the general trend of incorporation and metabolism of these materials.

Considering the experimental plant as a whole, nicotine only maintained a relatively small portion of ^{14}C supplied as ^{14}C-methyl methionine, ranging from 1·5 to 5·1% (expressed as percentage of apparent absorption). Other ethanol extractable materials, including free amino acids, organic acids, and sugars contained 11·8 to 14·3%. Acid hydrolyzates of the residue had 5·8 to 6·2%, and the non-hydrolyzed residue, of which a great proportion is probably lignin, had incorporated 3·6 to 12·3%.

Recovery of ^{15}N was not studied as extensively as ^{14}C because the quantity of ^{15}N excess supplied to the plants was small. The recovery of ^{15}N in nicotine is from 0·8 to 4·2% of

absorbed amount of ^{15}N as nornicotine. The presence of higher than normal quantity of nornicotine in the experimental plants, 1–2% in root and 3–5% of total in shoot, indicates that some supplied ^{15}N nornicotine may yet have to be methylated or metabolized.

EXPERIMENTAL

^{15}N-nornicotine, obtained through biosynthesis, has an ^{15}N excess of 7·154 at. %. L-Methionine-methyl-^{14}C, obtained from commercial supply, has a specific activity of 10 mc/mm mole. It was diluted with inactive L-methionine for feeding. The purity of these compounds was examined through paper chromatography and scannography.

Experimental plants of *Nicotiana tabacum* L. cv. Connecticut Broadleaf were grown in nutrient solution until they flowered and were then decapitated. Individual decapitated plants, about 3 ft tall, were taken to the isotope hood and their roots were immersed in a beaker containing 22·38 mg of L-methionine-methyl-^{14}C and 22·23 mg of ^{15}N-nornicotine in 25 ml of water. Most of this feeding solution was absorbed by the plant root in about 3 hr. A small amount of fresh water was then added for continuous feeding for another 3 hr. The plant root was then washed and the plant was returned to the conventional nutrient solution. Four plants so fed were allowed to grow for different periods before harvest (2, 4, 6, and 8 days). During this period, the plants were grown in an isotope hood with continuous light of 260 ft-candles at the top of the plant and 45 ft-candles at the lowest leaf.

The root and the shoot from each plant was harvested separately. The plant material was extracted in a blendor with 80% ethanol, taking into consideration the water content of the material. This tissue-ethanol mass was allowed to remain at room temperature for 2 days before filtration. This extraction was repeated three times.

The ethanol extract was concentrated under reduced pressure, and pigments were removed through ether extraction. Alkaloids were obtained by extracting the residue with ether under alkaline condition. Nicotine was isolated by azeotropic distillation and further purified through repeated crystallization as dipicrate.

A portion of plant tissue after the ethanol extraction was hydrolyzed for 24 hr with 6 N HCl. Total ^{14}C activity in plant tissue residue was obtained through combustion. The ^{14}C activity was measured with a Tri-carb liquid scintillation spectrometer using a toluene–absolute ethanol (450 : 50) solvent system with POPOP (100 mg) and PPO (15 g) as scintillators. A few samples containing pigments were measured with a Nuclear Measurements Corp. PC-3 Internal Proportional Counter.* All activity data reported are corrected. ^{15}N was measured by a mass spectrometer as previously described.[6]

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* Mention of specific trade names is made for identification only and does not imply any endorsement by the U.S. Government.