

BIOCHEMICAL STUDIES ON TOBACCO ALKALOIDS—VII. BIOSYNTHESIS OF ALKALOIDS TRIPLY LABELED WITH ^{14}C , ^3H , AND ^{15}N

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Abstract—*Nicotiana rustica* var. *brasilia* plants were grown inside a specially constructed chamber and supplied with $^{14}\text{CO}_2$, $^3\text{H}_2\text{O}$, and ^{15}N as nitrate. Despite the fact that the $^{14}\text{CO}_2$ activity was only half that of HTO, the incorporation of ^{14}C into alkaloids was higher than ^3H . Generally, the highest specific activity in nicotine of ^{14}C and ^3H , as well as the highest excess ^{15}N , was found in the leaves of the middle part of a plant. In plants of this variety sampled at an actively growing stage, a considerably high percentage of activity was found in alkaloids other than the principal alkaloid (nicotine), including nornicotine, anatabine, and anabasine. From the incorporation of ^{14}C and ^3H , a special role of anatabine in the nornicotine–nicotine conversion system is indicated. Generally, the ratios of $^{14}\text{C}/^{15}\text{N}$, $^3\text{H}/^{15}\text{N}$, and $^{14}\text{C}/^3\text{H}$ in nicotine was found to increase from root to top leaves; this is parallel to the distribution of the amount of nicotine found in these plants.

INTRODUCTION

OF THE three elements C, H and N which compose the molecules of principal tobacco alkaloids, ^{14}C labeling has been most widely studied;^{1,2} tritium and ^{15}N labeling have been investigated less extensively. We previously reported³ that ^{14}C from $^{14}\text{CO}_2$ is incorporated in a stepwise manner through sugars, organic acids, and amino acids into the alkaloid, and that this process requires more than 90 min. Tritium from $^3\text{H}_2\text{O}$ was detected in the alkaloids 30 min after a 1-min exposure; this indicated that the incorporation of ^3H was through an exchange rather than via a stepwise synthesis.⁴ We reported the incorporation of ^{15}N , supplied as nitrate, to be selective to the molecule of the major alkaloid characteristically present in a particular plant.³

The present paper reports the employment of all three isotopes, ^{14}C , ^3H and ^{15}N , and the results obtained in a study of their relative incorporation into tobacco alkaloids through biosynthesis. Alkaloid and other labeled materials thus produced will be used for further metabolic investigations.

RESULTS AND DISCUSSION

1. ^{14}C and ^3H Activity in Total Alkaloids from Various Plant Parts

Samples from four plants of the first crop were separated into respective plant parts, including roots, stems, leaves, suckers, and flowers and the total alkaloids obtained from the ethanol extract by steam distillation. Activity of ^{14}C and ^3H in total alkaloids was measured

¹ G. S. ILYIN, *Biokhimiya* **21**, 108 (1956).

² E. LETTE. In *Biogenesis of Natural Substances* (Edited by M. GATES), Chapter VIII. University of Minnesota, Minneapolis, (1959).

³ T. C. Tso, *Arch. Biochem. Biophys.* **92**, 248 (1961).

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

and the results are shown in Table 1. Comparing various plant parts from this crop, alkaloids in the roots had the highest ^{14}C incorporation, followed by leaf, and then flower and stem. The lowest incorporation of ^{14}C in alkaloids was found in suckers.

TABLE 1. ^{14}C AND ^3H ACTIVITY IN THE TOTAL ALKALOIDS FROM COMPOSITE SAMPLES OF THE FOUR PLANTS OF THE FIRST CROP

Plant part	Disintegrations/min/m-mole $\times 10^7$		$^{14}\text{C}/^3\text{H}$ Ratio
	^{14}C	^3H	
Leaves			
1 and 2 (bottom)	4.86	4.93	0.98
3 and 4	4.83	3.99	1.21
5 and 6	5.07	3.99	1.27
7 and 8	5.90	4.44	1.33
9 and 10	5.58	3.94	1.42
11 and 12	5.74	3.40	1.69
Average	5.33	4.11	1.30
Root	6.09	4.00	1.52
Stem	4.93	3.76	1.31
Sucker	4.34	3.03	1.43
Flower	4.94	2.90	1.70

^3H incorporation in alkaloids from various plant parts did not follow a pattern similar to that of ^{14}C . Alkaloids in leaves were found to have the highest ^3H incorporation, followed by alkaloids from roots, stems, suckers, and flowers, in decreasing order. Since most ^3H is incorporated into alkaloid molecules through exchange rather than through a stepwise synthesis, as is ^{14}C ,^{3,4} the plant part which has the largest surface area (such as the leaf) would have the highest rate of ^3H incorporation.

Despite the fact that the ^{14}C activity supplied was only half that of ^3H , the incorporation of ^{14}C into alkaloids was generally higher than ^3H , except in the bottom leaves where the incorporation of ^{14}C and ^3H was almost equal. The $^{14}\text{C}/^3\text{H}$ activity ratio in alkaloids increased systematically from the bottom toward the top leaves. The highest specific activity of either ^{14}C or ^3H , however, was found in the center leaves (7th and 8th) which were at the most developed stage physiologically (Table 1).

2. ^{14}C and ^3H Activity in Nicotine from Various Plant Parts

Plant materials used for this study were obtained from the second crop which was supplied with 50 mc of ^{14}C and 100 mc of ^3H , which is about half the dosage used for the first crop. The four plants were harvested and separated into roots and leaves, and the latter subdivided into three groups according to their location on the stem. ^{14}C and ^3H activity found in purified nicotine is shown in Table 2. Due to an extra supply of nitrate nitrogen as a source of ^{15}N 4 weeks before decapitation to two of the four plants (A and B), these were still actively growing when harvested. Plant D, however, showed signs of overmaturity. These facts may contribute to the inconsistency of the results among four plants (Table 2).

Since 95 per cent of the total alkaloid in this variety is nicotine, the incorporation of ^{14}C and ^3H into nicotine would be expected to be similar to the previously discussed incorporation of these elements into total alkaloids. Generally, the highest specific activity of ^3H in nicotine was found in the middle leaves, and that of ^{14}C in top leaves. The $^{14}\text{C}/^3\text{H}$ ratio was lowest in

bottom leaves, and increased stepwise toward top leaves. A much lower than average $^{14}\text{C}/^3\text{H}$ ratio was found in leaves of Plant D. Since Plant D was more mature than others, it is possible that a comparatively higher turnover and therefore less ^{14}C remained in nicotine. Furthermore, a higher exchange of ^3H in the nicotine molecule would occur.

TABLE 2. ^{14}C AND ^3H ACTIVITY (DISINTEGRATIONS/MIN PER M-MOLE $\times 10^6$) IN NICOTINE FROM VARIOUS PLANT PARTS OF THE SECOND CROP

Plant	Root			Bottom leaves (1-4)			Middle leaves (5-8)			Top leaves (9-12)		
	^{14}C	^3H	$^{14}\text{C}/^3\text{H}$ Ratio	^{14}C	^3H	$^{14}\text{C}/^3\text{H}$ Ratio	^{14}C	^3H	$^{14}\text{C}/^3\text{H}$ Ratio	^{14}C	^3H	$^{14}\text{C}/^3\text{H}$ Ratio
A	19.3	4.13	4.67	20.6	3.18	6.47	35.1	6.33	5.55	34.2	6.21	5.50
B	22.1	6.56	3.37	26.8	4.84	5.53	34.0	5.35	6.36	32.9	4.22	7.79
C	16.9	5.45	3.10	31.9	5.59	5.70	24.1	3.73	6.46	24.7	3.98	6.20
D	12.9	2.58	5.01	24.0	6.73	3.56	22.3	7.20	3.09	27.0	7.55	3.57
Average	17.8	4.68	3.81	25.8	5.09	5.06	28.9	5.65	5.12	29.7	5.49	5.40

3. Relative Activity in Individual Alkaloids

The individual alkaloids nornicotine, anabasine, anatabine, and nicotine were separated by paper chromatography, and the combined activity ($^{14}\text{C} + ^3\text{H}$) of each was measured. The results are expressed in relative percentage of total alkaloid activity, as shown in Table 3.

TABLE 3. RELATIVE AMOUNT (%) OF COMBINED ACTIVITY (^{14}C AND ^3H) FOUND IN INDIVIDUAL ALKALOIDS* FROM VARIOUS PLANT PARTS OF THE SECOND CROP

Plant	Root				Bottom leaves (1-4)				Middle leaves (5-8)				Top leaves (9-12)			
	Nor.	Anab.	Anat.	Nic.	Nor.	Anab.	Anat.	Nic.	Nor.	Anab.	Anat.	Nic.	Nor.	Anab.	Anat.	Nic.
A	8.7	8.7	10.5	72.1	4.1	2.5	5.1	88.3	1.3	1.3	4.0	93.4	1.5	1.5	4.6	92.3
B	24.8	14.1	23.0	38.4	2.2	1.2	3.1	94.2	2.2	3.5	5.3	89.0	3.1	2.7	3.7	90.5
C	4.3	3.0	4.7	88.0	3.0	2.0	5.6	89.4	0.9	0.7	2.1	96.3	1.0	0.9	3.8	94.4
D	4.5	3.0	4.8	87.7	2.6	1.9	3.6	91.9	3.2	1.7	5.8	89.3	2.2	1.6	4.4	91.8
Average	10.6	7.2	10.8	71.6	3.0	1.9	4.4	91.0	1.9	1.8	4.3	92.0	1.9	1.7	4.1	92.3

* Abbreviations: Nor. = Nornicotine; Anab. = Anabasine; Anat. = Anatabine; and Nic. = Nicotine.

As the principal alkaloid of *N. rustica* plants, nicotine has the highest amount of total activity. Anatabine ranks next to nicotine in total activity, except in one case (plant B roots) where nornicotine was slightly higher. Nornicotine generally ranks third and anabasine lowest in total activity. Although the amount of each alkaloid was too small to be quantitatively measured from each plant part, the average proportion of nicotine, anatabine, nornicotine, and anabasine, by visual comparison of paper chromatograms from leaf sample, is 94:2:2:2, and from root sample it is 95:2:2:1. From calculations of the amount of each alkaloid and the relative activity, there appears to be a relatively higher specific activity in anatabine than in anabasine; this agrees with our earlier report.³ Although the pathway

of alkaloid interconversion is not yet clear, this result may suggest a closer relationship between nicotine and anatabine than between nicotine and anabasine.

Plants A and B, which received additional nitrogen supply in the form of ^{15}N nitrate, showed an extended growth as compared to Plants C and D. In the roots of Plants A and B, the relative activity of individual alkaloids was quite different from those of Plants C and D, especially the ratio between nornicotine and nicotine. Since nornicotine is considered a precursor of nicotine in *Nicotiana* plants,⁵ the present data show that in Plant A and B, where alkaloid synthesis is still very active, some of the newly formed nornicotine remains to be converted to nicotine. The higher relative activity of anatabine further indicates the possible role of anatabine in the nornicotine-nicotine formation system.

4. ^{15}N Excess in Nicotine

Nicotine was the only alkaloid with sufficient yield for a precise ^{15}N determination. The results of ^{15}N excess determination in nicotine as atom percent, are shown in Table 4. Nicotine in the roots and bottom leaves of Plant B had a higher ^{15}N excess than in Plant A, which again indicates a more active alkaloid synthesis was taking place in B compared with A. Of the three groups of leaves, nicotine in the middle leaves had a higher ^{15}N excess as compared to either the bottom or top leaves. These results agree with those of ^{14}C and ^3H specific activities found in the total tobacco alkaloids (Table 1). The bottom leaves of Plant A had the lowest ^{15}N excess. This may have been due to the fact that Plant A was relatively more mature than Plant B; and the bottom leaves were among the most senescent.

TABLE 4. ^{15}N EXCESS (ATOM %) IN NICOTINE FROM VARIOUS PLANT PARTS OF THE SECOND GROUP

Plant	Root	Bottom leaves (1-4)	Middle leaves (5-8)	Top leaves (9-12)
A	5.00	4.01	5.29	4.74
B	5.40	4.94	5.15	4.40
Average	5.20	4.48	5.22	4.57

5. Triple Labeling of Nicotine

Plants A and B, in which nicotine was labeled with ^{14}C , ^3H and ^{15}N , were used to examine the relative labeling of these three elements. Ratios between ^{14}C , ^3H and ^{15}N were calculated to indicate the relative incorporation of these elements in nicotine at various positions on the plants. For easier comparison, the ^{15}N excess (atom %) were multiplied by 10^6 . The results are shown in Table 5.

From the root to top leaves, the incorporation of ^{14}C in nicotine, relative to ^{15}N , increased consistently, although the slope leveled gradually between the middle and top leaf area. The incorporation of ^3H relative to ^{15}N in nicotine was lower in root and in bottom leaves, and slightly higher in middle leaves and top leaves but the overall change was small. The $^{14}\text{C}/^3\text{H}$ ratio is low in roots due to a low ^{14}C incorporation and high ^3H exchange, and this ratio also increased toward the top leaves.

⁵ B. LADESIC and T. C. Tso, *Phytochem.* 3, 541 (1964).

TABLE 5. ACTIVITY RATIOS IN NICOTINE TRIPLY LABELED WITH ^{15}N , ^{14}C , AND ^3H FROM DIFFERENT PARTS OF THE PLANT

Plant	Root			Bottom leaves			Middle leaves			Top leaves		
	$^{14}\text{C}/^{15}\text{N}$	$^3\text{H}/^{15}\text{N}$	$^{14}\text{C}/^3\text{H}$	$^{14}\text{C}/^{15}\text{N}$	$^3\text{H}/^{15}\text{N}$	$^{14}\text{C}/^3\text{H}$	$^{14}\text{C}/^{15}\text{N}$	$^3\text{H}/^{15}\text{N}$	$^{14}\text{C}/^3\text{H}$	$^{14}\text{C}/^{15}\text{N}$	$^3\text{H}/^{15}\text{N}$	$^{14}\text{C}/^3\text{H}$
A	3.86	0.83	4.67	5.13	0.79	6.47	6.63	1.20	5.55	7.21	1.31	5.50
B	4.09	1.21	3.37	5.42	0.98	5.53	6.60	1.04	6.36	7.47	0.96	7.79
Average	3.98	1.02	4.02	5.28	0.89	6.00	6.62	1.12	5.96	7.34	1.14	6.65

The total nicotine content in roots, bottom leaves, middle leaves, and top leaves was 5.75, 11.50, 28.25, and 38.0 mg in Plant A, and 7.25, 14.25, 28.0, and 44.5 mg in Plant B, respectively. The average of two plants gives a relative distribution of 7.3, 14.5, 31.7, and 46.5 per cent in those plant parts, respectively. This indicates a high accumulation of nicotine in upper leaves at this stage of growth. The ratio of $^{14}\text{C}/^{15}\text{N}$, $^3\text{H}/^{15}\text{N}$, and $^{14}\text{C}/^3\text{H}$, also shows a similar tendency.

EXPERIMENTAL

Nicotiana rustica var. *brasilia* plants were used in this study. The experiments were carried out in an enclosed growth chamber especially constructed for isotope labeling work.⁶ Two crops were produced, and four plants of each crop were used. Each plant, about 6 in. in height and with five leaves, was transplanted into 10 l. of nutrient solution.

The temperature was maintained at $78 \pm 2^\circ\text{F}$, r.h. 78–80%. Light intensity was 1500 foot-candles at the top of the plants for 15 hr a day before topping, and for 17 hr a day after topping.

In the first crop no ^{15}N was used; in the second crop ^{15}N was supplied to two of four test plants in the form of nitrate, 1 g of ^{15}N per plant. ^{14}C was supplied in the form of $^{14}\text{CO}_2$ in the atmosphere, and tritium was supplied as tritiated water in the nutrient solution supplied to each plant. In the first crop 99.6 mc of ^{14}C and 200 mc of ^3H were used. In the second crop, 50 mc of ^{14}C , and 100 mc of ^3H were used. $^{14}\text{CO}_2$ concentration was adjusted to 1000 ppm or nearly three times as much as the average amount of CO_2 in the air. The maximum total activity of ^{14}C inside the chamber was 0.75 mc. Assuming that there were 220 g of water in the air at 80% relative humidity, and that the total capacity of the chamber was 11,000 l. the maximum ^3H activity in the air would be about 1.1 mc. In the second crop, the maximum ^3H activity in the air would be only 0.55 mc.

Plants were decapitated after about 4 weeks of growth (early flower stage) and harvested approximately 3 weeks later. The first crop was grown in the chamber 44 days, while the second crop was grown 40 days. The root, stem, suckers, flowers, and leaves were harvested separately. The method of extraction was similar to that described previously.⁴ Briefly, we extracted the plant material twice with 70% ethanol, in a Waring Blendor* taking into consideration the amount of water in the tissue and allowing 7 min for each extraction. About 40 ml of ethanol solution was used for each 10 g of fresh material at each extraction, and an additional 20 ml was used for washing.

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

⁶ J. H. SMITH, F. E. ALLISON and J. F. MULLINS, *Misc. Publ. U.S. Dept. Agr.* No. 911, (1962).

Total alkaloids were obtained through steam distillation of the extract after evaporation of ethanol under reduced pressure at 40°. The quantity of total alkaloids, determined by means of u.v. spectrophotometry, was evaluated as nicotine. The mixed alkaloid distillate was transferred into ether and the individual alkaloids separated chromatographically. Purification of each alkaloid was achieved by countercurrent distribution⁷ and by picrate crystallization.

¹⁴C and ³H activity were measured with a Tri-Carb* and also a Nuclear-Chicago (Model 723) Scintillation Spectrometer,* using toluene–absolute ethanol (450:50) system with POPOP (100 mg) and PPO (1.5 g) as scintillators. All data were corrected.

¹⁵N determination was made by Rittenberg's method.⁸ Alkaloids were converted to ammonia which was oxidized to nitrogen with hypobromite. The ¹⁵N excess was measured with a mass spectrometer.

Relative amount of combined activity (¹⁴C and ³H) in individual alkaloid from various plant parts was measured with an Actigraph* radio-chromatogram scanning system.

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* See footnote on p. 291.

⁷ T. C. Tso and R. N. JEFFREY, *Plant Physiol.* 32, 86 (1957).

⁸ D. RITTENBERG, In *Preparation and Measurement of Isotope Tracers* (Edited by D. W. WILSON), p. 31. Edward Brothers, Ann Arbor, Michigan (1948).