STUDIES ON TOBACCO ALKALOIDS. II. THE FORMATION OF NICOTINE AND NORNICOTINE IN TOBACCO SUPPLIED WITH N^{15} [1,2]

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The preceding paper (11) reports the changes in nicotine and nornicotine content as related to growth conditions of two selections of Nicotiana plants which contain principally nornicotine. Certain results obtained proved difficult to explain on the basis of current theories which state that nicotine synthesis is conducted predominantly in the root (4) and subsequent conversion to nornicotine takes place only in the leaf (5). It appeared that some of the uncertainties might be cleared up by means of experiments employing N^{15}.

MATERIALS AND METHODS

A common variety of Maryland tobacco, *Nicotiana Tabacum* L. var. Robinson Medium Broadleaf, and of tomato, *Lycopersicum esculentum* Mill. var. Rutgers, were used in these experiments. Plants of these varieties were used as intact individuals and also after tongued inarch grafting of each scion on the other stock. In some instances grafts of tobacco on tobacco and tomato on tomato were also made in an effort to distinguish between the anatomical or mechanical effects of grafting and the physiological effects due to presence of unlike tissue.

The plants were grown in solution culture using McMurtrey's C_1 solution (7) modified as necessary to use N^{15}. Of the 225 ppm of N in this solution, 150 ppm were derived from KNO_3 and HNO_3, each of which contained 30 atom percent excess N^{15}. Thus, the nutrient solution contained about 45 ppm of N^{15} as nitrate, or about 20 atom percent excess N^{15}. All the nitrogen in this solution was applied as nitrate. Detached leaf culture following the techniques of Dawson (3) was also employed.

Tobacco samples were treated as indicated in table I. Nicotine was obtained from the fractional steam distillate using MgO. The nicotine was purified by picrate precipitation but nornicotine was not purified satisfactorily from the distillation residue by picrate precipitation, due to the considerable amounts of other alkaloids present. A partial purification was conducted by distillation with steam from 5 % NaOH and extraction of the distillate with ethyl ether. Since this solution was still impure, a new method of purification of nornicotine was developed in which the Craig countercurrent apparatus (2) was used. In the course of this development the separation of a mixture prepared from nicotine, nornicotine, and anabasine was investigated by means of this apparatus,

TABLE I

SEPARATION OF FRACTIONS FROM TOBACCO SAMPLES

A. Green sample, 10 gm green tissue
  1. Extract with 50 % (final conc) acetone
  2. Filter
  3. From one portion of filtrate, determine total alkaloid by distilling with NaOH and NaCl and make spectrophotometric analysis on distillate (10)
  4. From another portion, determine nicotine and nornicotine by paper chromatography, elute spots and make spectrophotometric analysis of each (10)

B. Remainder of green sample—extract with 0.1 *N* HCl
  Residue—keep in 80 % alcohol for amino acid study
  Extract—concentrate, neutralize, fractionally distill from MgO
    Distillate
      1. Concentrate
      2. Make nicotine picrate
      3. Recrystallize
      4. Dissolve in 5 % NaOH
      5. Steam distill
      6. Check spectrum for purity
      7. Kjeldahl
      8. Oxidize NH_3 to N_2
      9. Analyze with mass spectrograph for N^{15}
    Residue
      1. Make to 5 % NaOH
      2. Steam distill
      3. Make distillate to 5 % NaOH
      4. Extract into ethyl ether
      5. Separate nornicotine by countercurrent distribution
      6. Check fractions by paper chromatography
      7. Combine nornicotine fractions
      8. Make to 5 % NaOH
      9. Steam distill
      10. Check purity spectrophotometrically
      11. Kjeldahl
      12. Oxidize NH_3 to N_2
      13. Analyze with mass spectrograph for N^{15} derived from nornicotine

using various organic solvents and buffers of various pH values saturated with each other. The results of certain of these experiments are shown in figure 1. The best separation of nornicotine from nicotine and anabasine, and also from other constituents of tobacco extracts, was found with chloroform as a stationary phase and 0.2 M KH_2PO_4-NaOH buffer of pH 7.0 as an aqueous moving phase. After 30 transfers under these conditions nearly all of the nornicotine had passed tube 20, whereas none of the impurities determinable by paper chromatography (10) had done so. The picrate of nornicotine which had been separated in this manner was found to have no depression of mixed melting point with authentic nornicotine picrate.

Nicotine and nornicotine which had been purified

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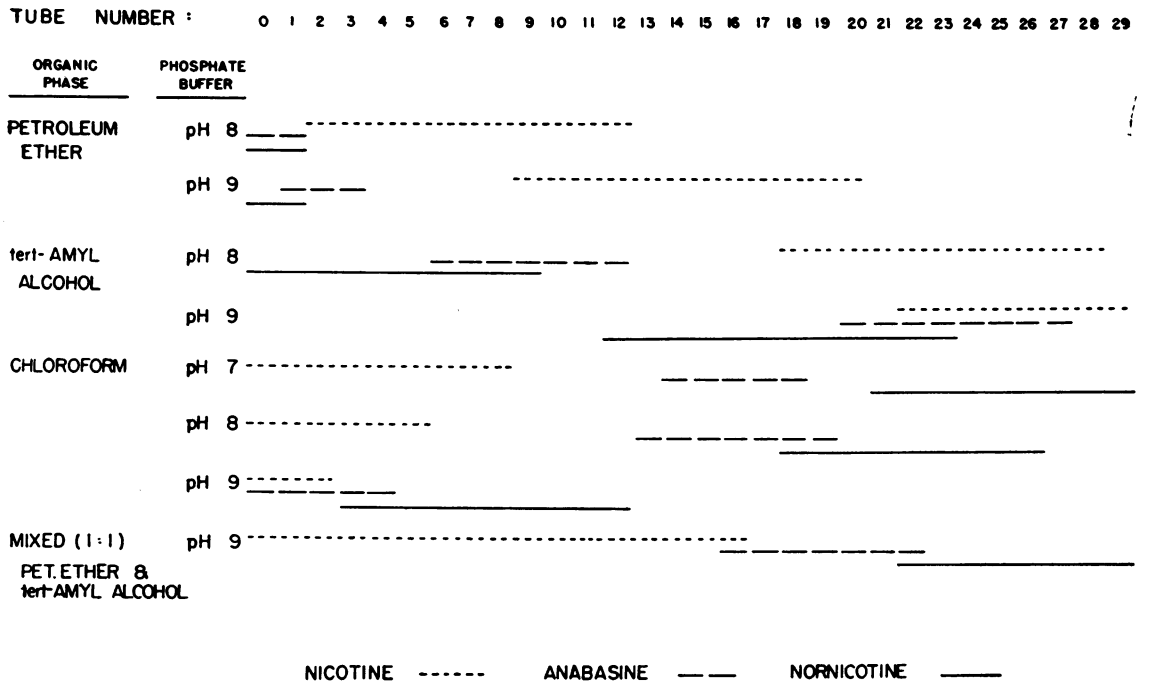

FIG. 1. Countercurrent distribution of nicotine, nornicotine, and anabasine. The organic phase moves and the aqueous buffer is stationary except when chloroform is used. The best separation was obtained with chloroform and pH 7 buffer.

from tobacco extracts, as above, were each converted to ammonia by the Kjeldahl method and the ammonia was oxidized to nitrogen with hypobromite and measured with the mass spectrometer as described by Rittenberg (8).

EXPERIMENTS AND DISCUSSION

PRELIMINARY N^{15} EXPERIMENT: The use of N^{15} can provide proof of the course of chemical reactions only in cases where it can be proven that exchange of nitrogen within the compound in question does not occur (1). Since exchange of nitrogen in other compounds has been reported (13), a preliminary experiment was necessary to prove that this did not occur in the pyridine alkaloids.

Nicotine was supplied to tomato plants in regular solution culture by adding one gram of nicotine per liter of the nutrient solution and readjusting the pH. Analysis showed that nicotine went into the tomato plants, as such, and built up an alkaloid concentration similar to that in tobacco plants at the corresponding growth stage. These N^{14} nicotine-containing tomato plants were transferred into a nicotine-free nutrient solution, which contained 20 % N^{15} excess in the form of nitrate, where they grew for two more weeks. They were then harvested and analyzed as shown in table II. It will be noted that no pyridine alkaloids were found in the tomato plant before nicotine was supplied, which may be considered to be at variance with the findings of Wahl (12). Actually, Wahl claimed a nicotine content of 1 to 2 mg per 100 gm dry substance, or about 1 to 2 ppm of fresh tissue. Since the average fresh weight of the tomato plants at this stage was 33 gm, this would correspond to 0.03 to 0.07 mg per plant which would be determinable without the rigorous concentration procedures used by Wahl. In 4 years' work we have never found nicotine in the strain of tomato plants here used, but 1 ppm would be near the limit of detection by our procedure.

The difference in nicotine content before and after transfer of the tomato plants from the nicotine-containing nutrient solution (table II, (c) vs (d) and (e)) is large, probably due to low values for (c), but the small quantity of N^{15} found in the nicotine of the plants grown in 20 % excess N^{15}-containing nutrient solution for two weeks makes it seem improbable that any significant synthesis occurred. In two independent samples only 0.006 % and 0.007 % excess of N^{15} over the average natural abundance of 0.367 atom percent were found in nicotine isolated from the tomato plants of this experiment. An ammonium sulfate blank analyzed 0.004 % excess N^{15} and nicotine isolated from a tobacco plant grown in N^{14} nutrient culture analyzed 0.004 % excess. The excess N^{15} found in the nicotine from tomato plants was not much greater than that found in the blank samples. Thus evidence of substitution of N^{15} for N^{14} in nicotine was not obtained. Since such substitution is presumably a property of the compound rather than of the plant, one would expect the same results in the tobacco plant if a similar experiment could be performed with it.

PRELIMINARY GRAFTING EXPERIMENT: In order to use grafting techniques in connection with the investigation of the synthesis of alkaloids in the plant, it is

TABLE II

EXPERIMENT ON THE POSSIBLE SUBSTITUTION OF NITROGEN IN TOBACCO ALKALOIDS
(MG ALKALOID/PLANT)

SAMPLE (WHOLE TOMATO PLANT)	TOTAL ALKALOID *	NICOTINE	NORNICOTINE
a. Before nicotine feeding	Not found	Not found	Not found
b. Grown 3 days in nutrient solution containing N^{14} nicotine (1 gm/l)	19	19	Not found
c. Grown 2 wks in nutrient solution containing N^{14} nicotine (1 gm/l)	40	40	Not found
d. Plant from c transferred to nicotine-free, $N^{15}O_3^-$-containing nutrient solution and grown 2 additional wks	79	66	9.5
N^{15} excess	..	*0.007 %* **	..
	..	*0.006 %* ***	..
e. Plants from c transferred to N^{14} nutrient solution and grown 2 additional wks	71	62	7.4

* Including other alkaloids besides nicotine and nornicotine.
** Reference sample 1—Regular nicotine from tobacco (N^{14} culture), 0.004 % excess N^{15}.
*** Reference sample 2—$(NH_4)_2SO_4$, 0.004 % excess N^{15}.

necessary to know the extent to which the presence of the graft union influences the physiological processes of the plant. Few experiments of this kind are described in the literature and none under the experimental conditions used here. Consequently, preliminary experiments were conducted in which plants of tobacco grafted on tobacco and of tomato grafted on tomato were compared with intact plants of the same strains. The tobacco plants were grown in nutrient solution (7) and the tomato in this plus 0.5 gm of nicotine per liter. The growth of the plants was interrupted by the injury resulting from grafting, so the grafted plants were not as large and did not contain as much nicotine as the intact plants of the same age. However, each part of these plants contained about the same amount of alkaloids as the corresponding part of younger intact plants of the same size. Thus it appears that grafting within a given strain does not have sufficient effect on the ability of these strains to synthesize, translocate, or degrade these alkaloids to be detected in the presence of the general inhibitory effect of the graft injury.

The above-described experiment concerns the mechanical effects of grafting but does not concern the phenomenon of graft incompatibility between different plant species. In an effort to measure this effect between the strains involved, plants were grown consisting of roots and tops of tobacco with tomato stems in between, and similar ones containing roots and tops of tomato and intervening tobacco stems. Both types were grown in nutrient solution without added nicotine. In these "sandwich" plants the alkaloid content of the various parts was quite variable from plant to plant. The difference in alkaloids between the twice-grafted plants, part for part, and ungrafted plants of the same size, and bearing the same kind of root, is not great in most cases. Thus the evidence of significant graft incompatibility influencing the translocation of nicotine or possible precursors across the union was not obtained.

GRAFTING EXPERIMENTS USING N^{15}: Plants composed of tobacco shoots grafted on tomato roots were used principally. Samples were taken 1) at the time of grafting, 2) six weeks later, just before transplanting the grafted plants into N^{15} solution, and 3) after four weeks in N^{15} solution, at normal harvesting maturity. Corresponding samples of intact tobacco and tomato plants grown under similar conditions were harvested at the same times for reference. Table III shows the results expressed as mgs of alkaloid found per plant part. Analyses were made on combined samples from four to six plants, so as to have enough material for N^{15} determinations.

At the time of grafting 3.7 mg of total alkaloid were found in the tobacco roots and 13.4 mg in the tobacco shoots. Since the tomato plants contained nothing reported as any pyridine alkaloid by the methods used, the initial alkaloid content of the grafted plants composed of tobacco scions on tomato roots was also approximately 13.4 mg. Six weeks later, when some of the grafted plants were transferred from the nutrient solution containing N^{14} to that containing approximately 20 atom percent excess N^{15} as nitrate, the total alkaloid content of the tomato stock was negligible, and that of the tobacco scion of these same plants was 16.2 mg. Since the grafted plants were not cut loose from the tobacco roots until a few days after the grafts were made, the increase during this period is not considered to be significant. However, in the four weeks following the transfer to N^{15} the total alkaloid content rose to about four times the level at grafting and the nornicotine level was proportionately more. The alkaloid increase in the plants transferred to new N^{14} nutrient solution was not so great, but was still two to three times. Since great care was taken to see that the tobacco scion was not permitted to develop roots, and

none were found inside the graft union, the conclusion appears to be inescapable that nicotine alkaloids were synthesized in these plants composed of tobacco scion and tomato stock. Further proof of this is provided by the mass spectrographic analysis of the N^{15} excess in nicotine and nornicotine purified from these plants at harvest. Each of two nicotine samples contained nearly 6.5 atom percent excess N^{15} and a nornicotine sample contained about 6.2 %.

Admittedly, the N^{15} content of the alkaloids indicates that not more than one-half of the excess was found which would be expected if N^{15} had been incorporated in the proportion of 20 % excess in which it was present in the nutrient solution into all of the increased amount of nicotine found subsequent to transfer into N^{15} enriched solution. Thus it appears that more than one-half of this increased nicotine found after transfer to the N^{15} enriched solution may have been formed from nitrogen compounds without excess N^{15}, and so presumably was present in the plants at the time of transfer; but the remainder appears to have been formed from nitrogen which was present as 20 % excess $N^{15}O_3^-$ in the solution into which the plants composed of tomato root and tobacco top were transferred.

The increase, during ten weeks, of the alkaloid content in the two lots of grafted plants with tomato roots was only about 43 and 25 mg, whereas the increase in the two grafted lots with tobacco roots was 92 and 166 mg, and in the intact tobacco plants 344 mg. So it is clear that the majority of the alkaloid synthesis normally takes place in tobacco roots, but it also appears that synthesis through all the steps from nitrate in the nutrient solution to nicotine or nornicotine can take place in plants composed of tobacco scions on tomato stock. Since it seems likely that amino acids or closely related compounds form a part of the chain of precursors of nicotine, and they are probably formed by the tomato plant as well as the tobacco plant, we cannot say on the basis of present information which steps can take place in which part of the plant. Evidently some critical step ordinarily takes place principally in the tobacco root and much of the synthesis of alkaloid is probably completed there.

Since the N^{15} excess in the nutrient solution of certain lots was 20 atom percent during the last four weeks of the experiment and no excess N^{15} existed in the grafted plants before that period, one would expect that 1) a higher percentage of N^{15} excess would exist in the alkaloid being formed most extensively during this period as compared to the earlier period, and 2) in the case of a given alkaloid a greater N^{15} excess would exist in the plant part in which it was synthesized than in the plant part to which it was being translocated. The results here presented show a higher percentage excess of N^{15} in the nicotine from the tobacco root of the grafted plant than in the tomato shoot, confirming the previous workers' findings as to the direction of translocation of the majority of the nicotine. However, they also show a higher percentage excess of N^{15} in the nornicotine from the root of these plants (9.4 atom %) than in the nicotine from the root (6.4 %), or the nicotine (5.3 %), or nornicotine (7.5 atom %) from the shoot. This cannot be explained on the basis of demethylation of

TABLE III

ALKALOID CONTENT (MG/PLANT PART) AND PERCENTAGE EXCESS N^{15} IN THE ALKALOIDS OF GRAFTED AND UNGRAFTED PLANTS

SAMPLE	ROOT			SHOOT		
	TOTAL ALKALOIDS *	NICOTINE	NORNICOTINE	TOTAL ALKALOIDS *	NICOTINE	NORNICOTINE
Intact plants						
Tobacco						
At grafting time	3.7	3.0	0.4	13.4	10.5	0.9
At harvest	90.5	69.5	17.2	271.0	215.0	48.8
Tomato						
At grafting time	0	0	0	0	0	0
At harvest	0	0	0	0	0	0
Grafted plants						
Tobacco scion on tomato stock						
At time of transfer to new solution	0.3	0.2	0	16.2	11.5	2.1
After 4 wks in N^{15} solution	0.5	0	0	55.6	40.9	8.9
N^{15} excess in the alkaloids	...	....	....	....	*6.49 %*	*6.22 %*
	...	....	....	....	*6.50 %*	....
After 4 wks in new N^{14} solution	0.4	0	0	38.2	28.7	7.3
Tomato scion on tobacco stock						
At time of transfer to new solution	6.8	4.8	1.9	16.7	14.9	1.3
After 4 wks in N^{15} solution	14.8	12.4	1.9	80.6	60.1	12.5
N^{15} excess in the alkaloids	...	*6.43 %*	*9.38 %*	....	*5.30 %*	*7.49 %*
After 4 wks in new N^{14} solution	28.8	22.2	5.4	141.3	106.0	25.8

* Including alkaloids besides nicotine and nornicotine.

nicotine to nornicotine in the shoot and translocation back to the root. Also, it cannot be explained as resulting demethylation of root nicotine. It would appear from the results that the root nicotine might have been derived from root nornicotine and that in this experiment most of the leaf nornicotine arose by translocation of root nornicotine rather than by demethylation of shoot nicotine. These data do not prove that nicotine was formed from nornicotine in the root, but they show that the majority of the nornicotine could not have come from nicotine under the conditions of the experiment. The quantity of root nornicotine did not increase appreciably during the last four weeks of the experiment, which is not surprising if it was being used up both by methylation to nicotine in the root and by translocation as nornicotine to the shoot.

ALKALOID CHANGES DURING AIR-DRYING: In order to study this problem further, two intact Robinson plants were grown in the same manner as the checks of the previous experiment, except that they were grown in about 20 % excess N^{15} solution in the form of nitrate for the last four weeks. Sampling was done as in part 3a of the preceding paper (11). That is, samples for immediate analysis consisted of 1) alternate leaves from each of two plants, 2) one longitudinal half of the stem of each, and 3) one longitudinal half of the roots of each. The other sample of each plant part was composed of the remaining halves of both plants after drying for one week in the greenhouse. The results are shown in table IV.

The amount of both nicotine and nornicotine found in the root and stem samples, which had been dried for a week, was much less than in the initial amount but the proportion of each to the total alkaloid content was not changed significantly. However, in the leaf the total alkaloid content did not decrease significantly, but the majority of the nicotine was converted to nornicotine. Thus the present experiment confirms previous workers in showing that the conversion of nicotine to nornicotine occurs in the leaves but does not occur to an appreciable extent under these conditions in the stalk or root.

In addition to the above-mentioned data, the amount of excess N^{15} in the purified alkaloids of the leaf is shown in table IV. Two samples of the purified initial leaf nicotine were separately Kjeldahlized and analyzed by mass spectrography to give some indication of the reproducibility of results. There was not sufficient N^{15} alkaloid available to permit replication of all samples. The percentage excess N^{15} does not appear to be significantly different between the initial nicotine and nornicotine samples and the nicotine sample after a week's drying. However, the nornicotine sample at the later date appears to be appreciably higher in N^{15}. The only explanation of this increase, if real, which has been proposed so far is that additional nornicotine was synthesized from precursors which were not steam distillable or had no ultraviolet absorption maximum near 259 mμ, but which were present in the leaves when detached. Though this explanation cannot be verified at this time, it may be noted that a decrease of 10 to 20 % in alkaloid on a per plant basis usually occurs during curing of tobacco and decreases of 39.5 and 28 % were observed in the root and stem in this material, whereas a decrease of only 1.2 % was observed in the case of this leaf sample. The increase in N^{15} excess from about 6 % in the other samples to about 9 % in the leaf nornicotine during air-drying could be explained if we could assume the synthesis from precursors containing 20 % excess N^{15} of an amount of nornicotine equal to 18 % of the amount of alkaloid present. This would also explain the failure to observe significant loss of total alkaloid in the leaf. It would also be in line with results, presented below, on detached leaf culture.

DETACHED LEAF CULTURE: Table V shows the results of an experiment designed to investigate further the ability of detached leaves to synthesize alkaloids. The leaves of two tobacco plants which had been grown in regular N^{14} culture solution were used. Alternate leaf samples were taken in the same way as in the previous experiments, One group, 276 gm, was used for green-sample analysis, the other group, 272 gm, for detached leaf culture. The petiole of each leaf in the latter group was inserted into a nutrient solution containing 20 % excess N^{15} in the form of nitrate. The growth of micro-organisms in the nutrient solution was minimized by immersing the container in cold running water as suggested by Dawson

TABLE IV

THE TRANSFORMATION OF NICOTINE TO NORNICOTINE DURING AIR-DRYING
(MG ALKALOID/PLANT PART)

PLANT PART	INITIAL CONTENT			CONTENT AFTER 1 WK AIR-DRYING		
	TOTAL ALKALOID	NICOTINE	NORNICOTINE	TOTAL ALKALOID	NICOTINE	NORNICOTINE
Root	49.2	38.3	8.8	29.8	24.5	4.1
Stem	37.9	32.5	4.0	27.4	22.6	3.6
Leaf	110.3	83.8	20.0	109.0	17.4	79.3
			Excess N^{15} in leaf alkaloids			
%	....	*7.05*	*6.25*	....	*6.26*	*9.24*
Mg	....	1.02	0.24	....	0.19	1.39
%	....	*6.80*	....	....	....	....
Mg	....	0.99	....	....	....	....

TABLE V

CHANGES IN ALKALOID CONTENT RESULTING FROM ONE WEEK OF DETACHED LEAF CULTURE

	TOTAL ALKALOID CONTENT *	NICOTINE	NORNICOTINE
	mg	*mg*	*mg*
Original alkaloid content	614	489	90
After 1 wk, detached leaf culture in N^{15} solution	688	534	128
Excess N^{15}	...	*0.010 %* **	*0.010 %* **
	...	*0.009 %* ***	....

* Including alkaloids other than nicotine and nornicotine.

** Reference sample 1—Regular nicotine from tobacco in N^{14} culture, 0.004 % excess N^{15}.

*** Reference sample 2—$(NH_4)_2SO_4$, 0.004 % excess N^{15}.

(3). Compressed air was also supplied into the solution, as was done with rooted plants.

Leaf samples were analyzed after one week of detached leaf culture. The results are expressed as mg alkaloid per sample corresponding in size to the average number of leaves per plant. The total alkaloid content in the leaves increased from 614 mg to 688 mg, or 12 %. The nicotine content increased from 489 mg to 534 mg, a 9 % increase. The nornicotine content increased from 90 mg to 128 mg, an increase of 42 %. However, only 0.010 and 0.009 % of N^{15} excess was found in nornicotine. These observed excess N^{15} figures are higher than the percent N^{15} excess found in the N-atom substitution experiment, but cannot be considered to be significant since the reference natural-abundance samples averaged an "excess" of 0.004 % N^{15}. Thus they do not constitute proof of the formation of alkaloids by complete synthesis from N^{15} nitrate in detached leaf culture. Contamination of the "purified" alkaloids with 30 ppm of a nitrogen-containing compound of about the same molecular weight but containing 20 % excess N^{15} would result in the average observed excess found in these three alkaloid samples over that found in the two reference samples. On the other hand, the increase in amount of total and individual alkaloids can hardly be explained as due to sampling error. The leaves in each sample, with almost identical initial weight, came from the same plants and a marked increase in nicotine, nornicotine, and independently determined total alkaloid was observed following detached leaf culture. These results agree with the indications obtained in the air-drying experiment, that tobacco alkaloids are formed through intermediate nitrogen-containing compounds or precursors.

In view of what is now known of intermediary metabolism, we would suspect that the synthesis of any molecule as complex as nicotine or nornicotine would occur in a number of steps. These reactions may occur principally in different parts of the plant. The experimental techniques formerly available did not permit one to distinguish between these steps. Dawson has utilized the evidence that small quantities of nicotine are present in the root tip together with the evidence from his grafting experiments to assert that nicotine is synthesized in the roots. The results presented here could be explained on the assumption that some N^{14} nitrogen had been taken up by the roots of the plant and had undergone the critical transformation into potential alkaloid-forming, nitrogen-containing compounds which were present at least in the leaves; but, in this case, the final conversion to alkaloids was not completed until after the leaves were removed from the stem, during the period of detached leaf culture.

CONCLUSION

Results presented here, which were obtained by employing N^{15}, tend to confirm and extend those reported in Part I of this paper (11). These results indicate that the synthesis of nicotine alkaloids follows a stepwise course involving a number of reactions and possibly alternate pathways. They appear to proceed through nitrogen-containing precursors which do not react as alkaloids to the analytical methods used in these experiments. In the case of plants growing under normal conditions it is evident that the rate-limiting step of this synthesis is carried out in the root more extensively than elsewhere. It is also evident that the synthesis of some of the nicotine is completed there. This is in line with the previous findings of Dawson (4) and many others. However, evidence is also presented that nicotine may be synthesized in the detached leaf, but if this occurs it is not a complete synthesis from inorganic nitrogen but is a conversion of nitrogen-containing compounds from a form in which they do not react to the analytical methods used. These methods are sensitive to most pyridyl compounds.

Nornicotine is generally known to be a minor constituent of the alkaloid fraction of many strains of *N. Tabacum* and a major constituent of certain strains of this species (6) and of most of the other species of Nicotiana which have been studied (9). It has been stated (5) that it is produced only in the leaf at the expense of nicotine. The present experiments confirm previous observations that nicotine can be converted to nornicotine in the leaf. However, since nornicotine was detected in the root at an early stage of seedling growth, as described in the previous paper (11), and was found in the work reported here to contain a higher percentage excess N^{15} than the nicotine in plants shifted into N^{15}-containing nutrient culture during their later growth, it appears that nornicotine can be formed independently either in root or in leaf of tobacco plants.

If the synthesis of nicotine passes through nornicotine some of the results can be explained. In that case the nornicotine of most strains of tobacco appears to remain, during the early stages of growth, at a level below that which is detectable by the methods which have been used in the past. In the later stages of growth both of the strains used in this work devel-

oped a high nornicotine content. Much of this nornicotine derives from the nicotine present in the leaves, but as shown in the previous paper the nornicotine content may continue to rise after nicotine is no longer detectable. This evidence, combined with the higher N^{15} content of the isolated nornicotine, requires the consideration of the possibility that some of the nitrogen may be synthesized into organic precursors of the alkaloids in the roots and then into nornicotine without having been converted to nicotine. The present results do not indicate whether translocation from the roots to the leaves of these predominantly nornicotine-containing strains occurred principally as nornicotine or as a precursor of it.

The differences in results obtained in these experiments from those of other workers may be explained as follows:

a) The use of an ultraviolet spectrophotometric method for total alkaloid determination and of paper chromatographic and countercurrent distribution methods for alkaloid separation and identification permits the estimation of much smaller quantities, especially of nornicotine in the presence of nicotine.

b) The use of N^{15} permits one to distinguish nitrogen taken in after a change in culture solution from that taken in before.

c) Different strains of plants were selected to make the changes involving nornicotine more pronounced so that they could be more readily detected.

d) Different techniques were used in the growth of the plants.

The results of these experiments provide evidence of a much more complicated set of reactions involved in the synthesis and transformations of the tobacco alkaloids than have been recognized previously, but much more data will be required before the course of all the reactions can be defined. Further studies on the alkaloid precursors and on the possibility of methylation of nornicotine to nicotine in tobacco plants will be helpful in understanding these reactions.

Summary

1. A study of the formation of nicotine and nornicotine has been conducted employing tongued inarch grafting, detached leaf culture, and isotopic nitrogen (N^{15}) as nitrate in nutrient solution culture. *Lycopersicum esculentum* Mill. var. Rutgers and *Nicotiana Tabacum* L. var. Robinson Medium Broadleaf, a tobacco variety known to contain relatively large proportions of nornicotine, were used.

2. Nicotine synthesis in plants composed of tobacco scions on tomato stock was observed in far larger quantities than would be expected on the basis of previous studies. Grafted plants were transferred to a nutrient solution containing 20 % excess N^{15} as nitrate. After four weeks' growth in this solution the alkaloids isolated from these plants contained about 6 % excess N^{15}.

3. In plants composed of tomato scions on tobacco stocks, grown in N^{15} nutrient solution after grafting, the nornicotine isolated from the roots contained a higher N^{15} excess than did the nicotine, indicating root synthesis of nornicotine independent of nicotine. The leaf nornicotine also contained a greater excess of N^{15} than did leaf nicotine, indicating translocation of nornicotine to the leaf.

4. The results here presented may be explained by an hypothesis that alkaloid synthesis is a stepwise process and the distinctive property of the root in this process is the synthesis of some alkaloid precursor(s).

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