SOME NOVEL CONCEPTS ON THE BIOSYNTHESIS AND BIOGENESIS OF TOBACCO ALKALOIDS

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I. Introduction

Alkaloids are basic substances of plant origin which contain a cyclic nitrogenous nucleus. In *Nicotiana* plants the alkaloids are 3-pyridyl derivatives. However, not all pyridine-containing compounds found in tobacco, such as nicotinic acid, are basic in nature although they usually are referred to as "alkaloidal materials."

Among the many alkaloidal materials found in *Nicotana,* nicotine is considered to be the principal alkaloid in commercial tobaccos, incluing *N. tabacum* and *N. rustica.* However, nornicotine appears to be the main alkaloid in more species of *Nicoticana* than does nicotine, and anabasine is the third most important (Smith and Smith 1942). In addition to nicotine, nornicotine, and anabasine, many other alkaloidal materials were found to be present in green tobacco including myosmine, nicotinic acid, anatabine and many other 3-pyridyl compounds of minute quantity (Jeffrey and Tso 1955; Tso and Jeffrey 1959). The chemical structure of major tobacco alkaloids and related compounds are shown in Figure 1.

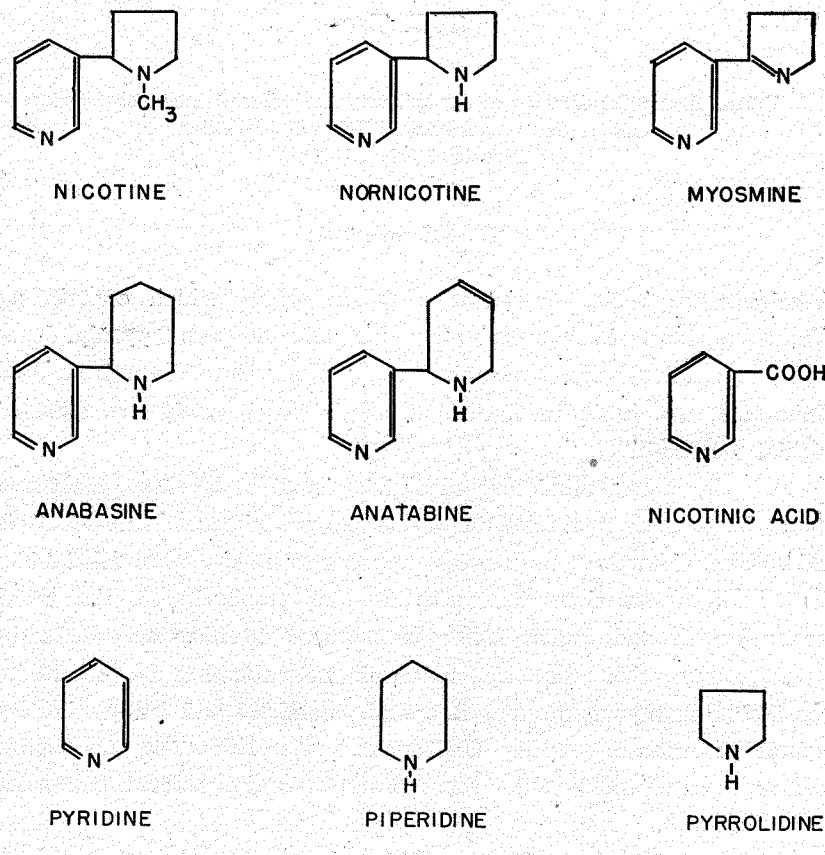
Numerous studies have been published on the biosynthesis and biogenesis of tobacco alkaloids. Recent advances in fundamental science and technology make possible further studies in this field. This paper will discuss some novel concepts on (1) the loci of alkaloid formation, (2) the pathways of alkaloid formation, (3) the interconversion of alkaloids, and (4) the metabolism of alkaloids in tobacco plants.

II. The Loci of Alkaloid Formation

Nath (1934–35) was the first one to make tobacco-tomato grafts in order to study alkaloid translocation. Since then this technique has been widely

FIGURE 1

MAJOR TOBACCO ALKALOIDS AND RELATED COMPOUNDS



used to study the location of alkaloid formation. Numerous studies (Dawson 1948, 1952, Mothes 1955) indicated that nicotine was formed in the tobacco root and translocated to the top through xylem, nornicotine is the demethylation product of nicotine in the shoot of a plant, and anabasine can be formed in both root and shoot. The evidence (Mothes 1955) that the root is the site of nicotine formation are: (a) that the discovery of nicotine in the bleeding sap of the alkaloid plant adduced the probability that translocation of nicotine was from the root to the scion, (b) that nicotine is not equally distributed within the whole scion of tomato graft on tobacco but is concentrated chiefly in one sector, (c) nicotine was found to be synthesized in excised roots from sterile

culture, and (d) that tobacco scions when grown on tomato stocks, accumulated no appreciable amount of nicotine, while large quantities of nicotine were found in tomato scions which were grown on tobacco stocks. However, there are evidence of other sites: (a) a slight nicotine synthesis existed within young, isolated leves and detached leaves (Cromwell 1943; Bose et al 1956; Mothes 1923) and (b) that young tobacco embryos grafted on tomato developed tobacco scions containing nicotine (Mashkortsev and Sirotenko 1951).

To study this problem, an experiment was performed in this laboratory (Tso and Jeffrey 1957) employing grafting technique involving *Nicotiana tabacum,* L. var. Robinson Medium Broadleaf, and tomato, *Lycopersicum esculentum* Mill. var. Rutgers and a stable isotope, N^{15}. The isotope was supplied in the form of $KN^{15}O_3$ in the nutrient solution in which the grafted plant grew. The results are shown in Table 1. In the grafted plant composed of a tobacco shoot on a tomato root alkaloids were not detected in the root, but were found in the shoot with 6.50 atom percent N^{15} excess in nicotine and 6.22 percent in nornicotine. These data indicate that nicotine as well as nornicotine can be formed in the shoot of the test plant without the presence of tobacco root. On the other hand, when a tomato shoot was grafted on a tobacco root, both root and shoot were found to contain alkaloids with considerable N^{15} excess and nornicotine had a much higher atom percent of N^{15} excess than that of nicotine, particularly in the root. These data indicate that nornicotine can be formed in the root and is not always formed by way of nicotine. This finding also suggests that nornicotine may possibly be a precursor of nicotine.

Table 1. Alkaloid Content (mg/plant part) and N^{15} Excess (atom %) in Grafted Plants

Sample	Root		Shoot	
	Nic.	Nornic.	Nic.	Nornic.
Tobacco Scion/Tomato Stock				
At time of transfer to N^{15} sol'n	0.02	0.00	11.50	2.10
After 4 wks. in N^{15} sol'n	0.00	0.00	40.90	8.90
N^{15} excess			6.50	6.22
Tomato Scion/Tobacco Stock				
At time of transfer to N^{15} sol'n	4.80	1.90	14.90	1.30
After 4 wks. in N^{15} sol'n	12.40	1.90	60.10	12.50
N^{15} excess	6.43	9.38	5.30	7.49

III. The Pathways of Alkaloid Formation

1. *The Incorporation of C^{14}*

In this investigation, three *Nicotiana* species, *N. glauca, N. glutinosa,* and

N. rustica were used in which anabasine, nornicotine, and nicotine, respectively, are the principal alkaloids. The source of C^{14} was $C^{14}O_2$ by photosynthesis or $NaHC^{14}O_3$ in water solution. The results (Tso et al 1961b, Tso 1961) showed that although carbon from $NaHC^{14}O_3$ solution can enter the plant through the roots and be incorporated into different compounds, from the viewpoint of alkaloid biosynthesis and of total C^{14} recovery, it is a very poor source in comparison with $C^{14}O_2$ introduced by photosynthesis.

Thirty minutes after one hour of exposure of these experimental plants in $C^{14}O_2$ atmosphere, C^{14} activity was found in sugars, organic acids, pigments, and amino acids in order of decreasing activity, but no activity was detected in alkaloids. After a period of eight more days, C^{14} had been gradually incorporated into the alkaloids. This was accompanied by a change of activity in organic compounds, particularly by a relative decrease in the activity of free sugars and amino acids. These findings indicate that alkaloid formation is a stepwise process probably involving sugars, organic acids, and amino acids. Evidence showing that compounds from these groups served as possible precursors of alkaloids will be discussed later in the sections concerning the ring formation. As shown in Table 2, nicotine appeared to have the highest specific C^{14} activity of any of the alkaloids separated from these three species. This is expected in *N. rustica,* and is explainable in *N. glutinosa,* in which nornicotine can be formed through nicotine demethylation. In *N. glauca,* the slightly higher specific activity in nicotine than in anabasine was probably contributed by the presence of an active methyl carbon in the nicotine molecule.

Table 2. The Incorporation of C^{14} and N^{15} into Alkaloids

Plants and Alkaloids	C^{14}	N^{15}
	Counts/Sec/m mole	Atom % Excess
N. rustica		
Nicotine	1.69×10^5	13.68
Nornicotine	3.19×10^4	9.72
Anabasine	1.50×10^4	1.92
N. glutinosa		
Nicotine	1.79×10^5	9.19
Nornicotine	1.80×10^4	7.51
Anabasine	4.21×10^4	7.05
N. glauca		
Nicotine	3.06×10^4	2.81
Nornicotine	1.78×10^4	0.67
Anabasine	1.39×10^4	7.80

2. *The Incorporation of N^{15}*

N^{15} was supplied to the plants through the nutrient solution in the form of $KN^{15}O_3$ with 30 atom percent excess. The incorporation of N^{15} into the alkaloids was selective (Tso 1961). The atom percent of N^{15} excess, as shown in Table 2, was found to be the highest in the anabasine of *N. glauca* and in the nicotine of *N. rustica.* In *N. glutinosa,* nicotine was found to have a higher N^{15} excess than nornicotine. In another test, when N^{15} was supplied to *N. glutinosa* throughout the whole growth period instead of for only 3 weeks before and after topping as used in Table 2, the atom percent of N^{15} excess in nicotine and nornicotine was 15.65 and 17.61, respectively. Thus, under this condition, nornicotine, the principal alkaloid of *N. glutinosa,* had a higher N^{15} excess than nicotine.

3. *The Incorporation of H^3*

H^3 was supplied to *N. rustica* var. *brasilia* in solution as tritiated water. Plants were grown in this solution for various lengths of time. Preliminary results concerning H^3 incorporation into alkaloid is shown in Table 3. One set of plants was analyzed immediately following the root absorption of H^3 water, the other set was allowed to grow without H^3 for a period of 13 to 16 days. A sharp increase of H^3 activity in the plant was observed from 30 to 150 minutes. After that time, only very slight change in total H^3 activity in the plant was found up to 70 hours. It appears that an equilibrium of H^3 between the plant and the solution was reached in 150 minutes. H^3 was incorporated into alkaloids, of which 95 percent was nicotine, 60 minutes after the initial exposure. The H^3 activity in alkaloid increased as time of exposure increased. However, H^3 activity gradually decreased from the alkaloids when exposed plants were grown in normal solution for 13 to 16 days. This may have resulted from (a) the dilution of newly formed alkaloid free or low in H^3, (b) loss of H^3 containing alkaloids through plant metabolism, or (c) the substitution of H^3 by regular hydrogen.

Table 3. H^3 Incorporation into Alkaloids

Time of Exposure to H^3 Water	Time between Exposure and Analysis	H^3 Activity in Alkaloids* Counts/Sec/m mole	Calculated Total H^3 Activity in Plant Counts/Sec.
30 min.	30 min.	1.44×10^2	6.69×10^5
30 min.	16 days	0	3.58×10^4
150 min.	30 min.	7.94×10^2	7.49×10^6
150 min.	16 days	0	3.27×10^4
70 hrs.	30 min.	1.72×10^3	9.65×10^6
70 hrs.	13 days	5.54×10^2	4.16×10^4

* 95% of the alkaloid was nicotine

4. *The pyridine ring*

Since nicotinic acid, or pyridine-3-carboxylic-acid, contains the pyridine ring, it and closely related compounds have received most attention in studies of the biogenesis of the tobacco alkaloid. Although the carboxyl carbon of nicotinic acid and its ethyl ester had been shown to be non-available for nicotine biogenesis (Dawson et al 1953), the pyridine ring of nicotinic acid is reported to be incorporated into nicotine of the tobacco root (Dawson et al 1956), as well as nicotine of intact tobacco plants (Tso and Jeffrey 1956). These results do not explain the mechanism of formation of the pyridine ring *per se.*

One idea of the origin of pyridine ring is centered in tryptophan 3-hydroxyanthranilic acid (3-hydroxy-o-aminobenzoic acid) and trigonelline in view of their metabolic relationship with nicotinic acid. However, Bowden (1953) stated that nicotine obtained from tobacco plants fed tryptophan labelled in the beta position with C^{14} exhibited no radioactivity. Aronoff (1956a, 1956b), feeding 3-hydroxyanthranilic acid carboxyl-C^{14} to excised pea shoots, did not find detectable radio trigonelline, although this system converts nicotinic acid rapidly to trigonelline. Another school of thought on the origin of the pyridine ring is centered in lysine. However, nicotine obtained from *N. tabacum* which was fed lysine-2-C^{14} was found to be inactive (Leete 1955, 1956). Radioactive nicotinic acid was obtained through oxidation of nicotine from plants fed with glutamate-2-C^{14} (Lamberts and Byerrum 1958) acetate-2-C^{14}, pyruvate-1-C^{14}, or pyruvate-3-C^{14} (Griffith and Byerrum 1959).

5. *The pyrrolidine ring*

Ornithine-2-C^{14} was reported to be incorporated into the pyrrolidine ring of nicotine in *N. rustica* (Dewey et al 1955, Dewey 1956). It was suggested (Leete 1955) that a systematic change takes place as follows: ornithine—putrescine—4 amino-butanal—pyrrolidine. Dewey (1956) indicated that ornithine and leucine are converted to the same symmetrical intermediate, succinic acid, before incorporation into nicotine. Because of the possible metabolic inter-relationship between ornithine and glutamic acids, glutamate 2-C^{14} was fed to tobacco plants and radioactivity was found in the pyrrolidine ring (Lamberts and Byerrum 1958). Acetate-1-C^{14} also was found to contribute C^{14} in the pyrrolidine ring of nicotine (Griffith and Byerrum 1959).

6. *The piperidine ring*

Much less work has been reported concerning the origin of the piperidine ring, as anabasine is less important than either nicotine or nornicotine. By supplying lysine-2-C^{14} or hydroxylysine—C^{14} to excised leaves, Aronoff (1956b) obtained only slight radioactivity in anabasine. Leete (1956) obtained radioactive anabasine by feeding lysine-2-C^{14} to *N. glauca.*

7. *The N-methyl group*

The origin of the N-methyl group of nicotine was studied extensively by Byerrum and his associates as summarized by Dewey (1956). C^{14} and deuterium labelled methyl group of methionine, was demonstrated to give rise to the methyl group of nicotine through transmethylation (Dewey et al 1954). Labelled carbon from formaldehyde, glycine, serine, betaine, choline, glycolate, formate, and bicarbonate were all reported to be incorporated into the methyl group of nicotine (Dewey 1956).

8. *General Studies*

As distinct from studies on the incorporation of a single element or the origin of a particular ring structure, investigations of the general distribution of organic constituents are in progress in this laboratory. Alkaloids, sugars, organic acids, and amino acids are determined in normal plants and in those abnormally developed due to mineral deficiencies, genetic tumorous, or those to which growth regulators are applied. These observations are being made in order to seek a possible product (alkaloid)—precursor relationship (Tso et al 1960, Tso and McMurtrey 1960). Available results indicated that (a) glucose, (b) glutamic acid, (c) asparagine, and (d) proline are related to alkaloid formation. Among them, glucose had been reported to be an immediate precursor for anabasine (Aronoff 1956a), and glutamate was demonstrated to be incorporated into nicotine (Lamberts and Byerrum 1958).

IV. The Interconversion of Alkaloids

It is known that nicotine demethylation is one of the main sources of nornicotine formation (Dawson 1950). It has been suggested that nornicotine may be an immediate precursor of nicotine (Tso and Jeffrey 1957). These two alkaloids are both composed of a pyridine ring and a pyrrolidine ring and such conversions are readily possible through methylation and demethylation. Anabasine, which contains a pyridine ring and a piperidine ring, might be derived from nicotine through expansion of a pyrrolidine to piperidine ring at the expense of the N-methyl carbon. To investigate these changes, three experiments were performed (Tso and Jeffrey 1959). The results are shown in Table 4.

The recoveries of supplied N^{15} from alkaloids in experimetal plants varied from 47.9, 20.4, and 11.3 percent in experiments 1, 2, and 3, respectively. The major portion of the recovered alkaloidal-N^{15} in *N. rustica* supplied with either anabasine or nornicotine was nicotine. However, when nicotine was supplied to *N. glauca* the major portion of it was recovered as its demethylation product, nornicotine. Through oxidization of the recovered alkaloids, it was found that in all cases the N^{15} excess in the pyridine ring and in the pyrrolidine or

piperidine ring was almost equal. Since more N^{15} was recovered in alkaloidal than other fractions, it appeared that there was a more direct conversion among alkaloids rather than a total breakdown of the supplied alkaloids and construction of new ones from the general metabolic pool.

Table 4. Alkaloid Conversion in *Nicotiana* Plants

Experiments	Total N^{15} supplied as Alkaloids			N^{15} recovered in Alkaloids		
	Nic	Nor	Anab	Nic	Nor	Anab
(1) Nornicotine supplied to *N. rustica* var. *brasilia*						
weight mg		200		1,054	49.4	40.1
N^{15} excess A%*		12.08		0.59	4.89	8.69
Re. Dist. %**				48.8	20.8	27.4
(2) Anabasine supplied to *N. rustica* var. *brasilia*						
weight mg			300	1,071	39.1	23.9
N^{15} excess A%			11.07	0.44	0.73	6.25
Rel. Dist. %				70.3	4.6	22.0
(3) Nicotine supplied to *N. gluca*						
weight mg	300			5.4	37.8	198
N^{15} excess A%	5.82			2.12	3.79	0.13
Rel. Dist. %				5.87	79.2	12.9

* Atom percent
** Relative distribution of N^{15} in alkaloids

In another experiment, N^{15} labelled nicotinic acid was supplied to intact *N. rustica* (Tso and Jeffrey 1956) and found to be incorporated into nicotine molecule. Experiments with excised root cultures showed similar results (Dawson et al 1956). This indicates that the pyridine ring may serve as a nucleus in alkaloid interconversion.

V. The Metabolism of Alkaloids

It is interesting to note that in tobacco the high concentration of alkaloids which would be very toxic to animals has no similar effect on plant cells. James (1953) summarized the current suggestions of reasons for the presence of alkaloids in plants, as follows: (a) alkaloids serve as protection against insects and herbivores, (b) alkaloids are detoxification products, (c) alkaloids may include regulatory substances such as nicotinic acid, (d) alkaloid may serve as reserves, (e) alkaloids are useful to the existence of the plant, and (f) alkaloids are waste products. Many of these suggestions have not received experimental support.

In the studies described in Table 4, a considerable portion of the supplied N^{15} excess was recovered in alkaloids other than those added or in organic compounds of the plant. This indicates that alkaloids are not inert or waste products. In order to observe the role of alkaloids in plant metabolism, C^{14}-N^{15} doubly-labelled nicotine was supplied to *N. rustica* var. brasilia (Tso and Jeffrey 1961). The results are shown in Table 5. Of the original C^{14}, 34.4 percent was recovered and the rest was presumably lost by respiration. The C^{14} activity was found in alkaloids, free amino acids, pigments, free organic acids, and free sugars. In hydrolyzate, C^{14} activity was found in furfural, amino acids, sugars, and organic acids. Of the original N^{15} excess, 91.6 percent was recovered, mostly in the insoluble residue; the rest were distributed between the proteins, alkaloids, free amino acids, and pigments.

Table 5. Recovery of C^{14} and N^{15} in *N. rustica* supplied with doubly-labelled Nicotine

	C^{14} Activity	N^{15} Excess	
	Counts/sec/plant	Atom%	mg/plant
Original C^{14} N^{15} nicotine	1.89×10^{6}	4.152	1.250
Recovery:			
Ethanol Extracts	2.30×10^{5}		
Pigment	1.20×10^{4}	0.029	0.011
Alkaloids	1.51×10^{5}	0.377	0.158
Amino acids	2.23×10^{4}	0.105	0.092
Organic acids	4.28×10^{3}		
Sugars	2.73×10^{3}		
Hydrolyzate	7.16×10^{4}		0.250*
Plant Tissue residue	2.52×10^{3}	0.413	0.408
Remainder in feeding solution and nutrient sol'n	3.46×10^{5}		0.221

* Summation of different fractions

It is clear from this experiment as well as from other demonstrations (Leete 1959, Griffith et al 1960) that alkaloids take an active part in plant metabolism.

VI. Summary

Through the application of new techniques and the employment of isotopic materials, recent studies have resulted in new concepts concerning biosynthesis and biogenesis of tobacco alkaloids.

The formation of tobacco alkaloids is a stepwise process. Available evidence indicates that the pathways of carbon for alkaloid formation probably involve sugars, organic acids, and amino acids. The incorporation of hydrogen seemed

to require a shorter time, but a constant turnover appeared to take place. The incorporation of nitrogen to alkaloids of different species is selective. The final step for the transformation of immediate precursor to nicotine and nornicotine can be completed either in the root or in the shoot of a tobacco plant.

Alkaloids are not inert or waste products. They are interconvertable and take an active part in plant metabolism.

菸草植物鹼之形成與演化

左 天 覺

菸草中植物鹼之形成與演化，素爲生物界所重視，各方試驗研究，因方法之不同，而結果亦各異。刻由於研究設備之進步及同位素之應用，所獲之結果，產生對此一問題之新觀念與認識。

(一) 菸草植物鹼係循一有系統之步驟而形成。其所含之三原素，各依特殊途徑，經一定過程，在不同種類之菸草植物中，最終組成不同之植物鹼。

(二) 形成植物鹼之最後階段，在菸草植物之任何部份均可完成，並不限於根部。

(三) 菸草植物鹼並非廢物或最終產物。各植物鹼不獨可以互相演變，且都積極參加所在植物之新陳代謝作用。

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