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CONVERSION OF NICOTINE TO NORNICOTINE IN HARVESTED TOBACCO: FATE OF THE METHYL GROUP[1]

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Studies on the origin of nornicotine in tobacco, recently reviewed by Mothes (14), have been made by several investigators but the mechanism of its formation still remains obscure. Indeed there may be more than one mechanism. Dawson (5) and Il'in (11) have shown that in some varieties nornicotine is formed in the leaves from nicotine translocated from the roots. In other varieties demethylation alone cannot explain the origin of the entire nornicotine pool. Thus, Tso and Jeffrey (25) found that *Nicotiana tabacum* L. var. Robinson Medium Broadleaf grown in nutrient solution containing N^{15} produced nornicotine which had a higher percentage excess of N^{15} than the nicotine from the same plant. Clearly, some of this nornicotine must have had an independent origin.

The post-harvest increase of nornicotine observed in some varieties appears to be associated with a simultaneous decrease of nicotine (26, 8) and may thus be distinct from de novo synthesis. This inverse relationship has lead to the widely-held view that here the nornicotine does arise from nicotine by demethylation.

Invoking known metabolic reactions, two general mechanisms by which nicotine may be converted to nornicotine can be formulated (6): A, through transmethylation, or B, through oxidative demethylation. The first possibility has little experimental support although Bose et al. (2) have reported a slight increase in nornicotine when nicotine was incubated in the presence of tobacco tissue homogenates and ethanolamine. In the absence of ethanolamine no conversion was detected.

The oxidative pathway has received more support than has transmethylation. Wenkert (27) has treated it theoretically and suggests nicotine 1′-oxide as an intermediate. On an experimental basis, Egri (7) and Il'in (12) both consider the oxidative mechanism to be of prime importance. Egri reports that nicotine 1′-oxide infiltrated into tobacco leaves gives rise to an increase in nornicotine. Il'in has shown that the demethylation of nicotine is coupled with an enhanced oxygen uptake.

We undertook the present investigation to see if a study of the fate of the methyl group might provide a more adequate experimental basis for a choice between the alternative mechanisms.

MATERIALS & METHODS

LABELED NICOTINES: Randomly labeled L-nicotine-C^{14} (specific activity 9 μc/mmole) was isolated from plants of an inbred line of *Nicotiana tabacum* L. var. Oxford 1–181. The plants were grown hydro-

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ponically at the Radiological Nutriculture Laboratory from the seedling stage to maturity in an atmosphere enriched with $C^{14}O_2$ within a hermetically sealed chamber especially designed for the purpose (17, 19). During the period of C^{14} assimilation the fresh weight of the plants increased from an average of 4 g to an average of 2.2 kg per plant.

The volatile bases, evolved during flue-curing of the leaves from the labeled plants, were isolated as the crude silicotungstates. The nicotine was obtained from an alkaline solution of these by azeotropic distillation (20) through a Widmer column into a solution of hydrochloric acid. Excess acid was removed by repeated concentration at reduced pressure and then the nicotine was regenerated as the free base by adding excess Dowex-21-OH resin to a solution of the hydrochloride. The concentration of L-nicotine-C^{14} in the final solution was determined spectrophotometrically using constants reported in the literature (28). The purity of the final product was checked by exposing two-dimensional chromatograms to x-ray film. No radioactive contaminants were detected after an exposure of 3 weeks.

Nicotine-N-methyl-C^{14} was prepared by methylating L-nornicotine with formaldehyde-C^{14} according to the method described by Markwood (13), but using a closed system to prevent loss of radioactivity and to avoid the use of excess formaldehyde. The nicotine-N-methyl-C^{14} was isolated by azeotropic distillation. It was assayed and tested for purity by the methods described above for nicotine-C^{14}. The specific activity of the N-methyl-C^{14}, calculated from the nominal value of the commercial sample of formaldehyde-C^{14}, was one millicurie per millimole.

PLANT MATERIAL & INFILTRATION TECHNIQUES: Tobacco plants were grown in the greenhouse in aerated Hoagland's solution supplemented with A_5 micronutrients (10, Method B, soln 1) and with ammonium nitrate to a final concentration of 0.002 M. Two varieties were grown: one, a flue-cured type known as Oxford 1–181, normally contains nicotine as the major alkaloid with only minor amounts of nornicotine; the other, a dark cigar type (Univ. of Kentucky LN 36–13–57, No. 82), contains nicotine as the major alkaloid during its vegetative period but is capable of transforming the nicotine almost completely to nornicotine when air cured (8). Consequently, the cured leaf of the latter variety contains nornicotine as the major alkaloid and only small amounts of nicotine. We refer to this variety as "converter".

A leaf on a mature plant was selected and prepared for transpirational infiltration with labeled nicotine. For this, an area on the adaxial surface in the basal region of the midrib was wiped free of epidermal hairs and ringed with silicone grease to confine the solution being fed. The leaf was then cut off under water about two centimeters below the prepared area and transferred, with the cut end still submerged, onto an elevated operating screen.

Infiltration was initiated immediately. A measured volume—usually 100 µl—of the radioactive solution was deposited within the silicone dam. An incision severing the vascular bundles was then made through the droplet (4, 16) with a pointed scalpel. The tip of the blade was worked through an arc to expose the entire cross-section of the conducting tissue to the administered fluid. The leaf began absorbing the droplet immediately at a rapid rate, and the supply of water to the basal end was then removed. During the uptake, care was taken to prevent the development of an air lock by keeping the incision flooded with repeated applications of the labeled solution. When the desired amount of material had been taken up, the solution was washed in by similar applications of water. The rate of uptake could be controlled within limits by manipulating the factors which influence transpiration. Under our conditions an average leaf absorbed 2 to 3 ml of fluid in less than 1 hour.

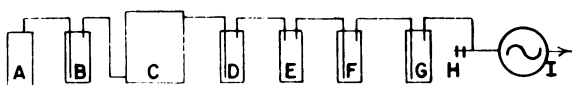

FIG. 1. Schematic diagram of curing apparatus. A: CO_2 absorber; B: rehumidifier (H_2O); C: curing chamber; D: scrubber (2 N H_2SO_4); E: trap; F and G: scrubbers (1 N NaOH); H: flow-control valve; I: pump.

CURING & COLLECTING METABOLIC CO_2: The infiltrated leaf was weighed and then transferred into a curing chamber fitted with inlet and outlet cocks. Pickle jars (1-gal) served the purpose in the earlier experiments; subsequently, flat chambers, 39.5 × 30 × 3.8 cm (internal dimensions) were assembled from Lucite panels ⅜ inch thick. The sealed container was connected to a train of accessories (fig 1). It was covered with a double thickness of heavy black cloth to exclude light.

Air was drawn through the system by a pump, I. Carbon dioxide in the incoming air was adsorbed by Caroxite in tube A. The air was rehumidified by bubbling through water in B. It then passed over the leaf supported on a screen shelf in C and through 2 N H_2SO_4 in D and 1 N NaOH in F and G. The flow rate was adjusted to about 100 ml per minute by short circuting the flow through valve H. Tube A was renewed as required and the contents of tubes F and G were collected at 24 hour intervals. The curing period was determined by the appearance of the leaf. In individual experiments it lasted from 188 to 291 hours. The temperature was maintained at 23 ± 2 C.

The CO_2 absorbed in traps F and G during each interval was precipitated by cautious addition of $BaCl_2$ solution in slight excess. The precipitated carbonate was collected by centrifugation and was wash-

ed three times with water, or until indicator strips showed the pH to be near 7. Trap G rarely yielded a precipitate. If found, it was combined with that in F at the first washing.

One experiment was designed to test the effect of anaerobic conditions on the metabolism of nicotine. For this, the leaf was split lengthwise after infiltration and half was cured aerobically as described; the other, anaerobically in a stream of nitrogen scrubbed through Oxorbent. After insertion of the leaf, the anaerobic chamber was flushed with a rapid stream of nitrogen before it was connected to the absorption train.

EXTRACTION: The cured leaf was cut up into boiling 95 % ethanol. After standing at room temperature (usually overnight) the ethanol was decanted and the strips were disintegrated in a blendor in 30 % ethanol. The homogenate was filtered with the aid of suction through Whatman No. 41H paper on a small Buchner-type funnel. The residue was re-extracted three more times in the blendor with 30 % ethanol; once by resuspending in hot 30 % ethanol; and finally, with hot water. No more radioactivity could be extracted from the residue after this sequence of treatments.

The filtrates were combined, their volume was measured, and then a small portion was removed for radioassay. The remaining extract was concentrated at reduced pressure at temperatures below 40 C. Usually 1 ml of the final concentrate represented the solubles from about two grams of fresh tissue.

CHROMATOGRAPHIC ANALYSIS OF EXTRACTS: Samples of the extract were spotted on Whatman No. 1 paper for two directional chromatography. The volumes applied represented known amounts of tissue —routinely 200 mg fr wt, but ranged from 150 to 300 mg. Three solvent systems were used. Phenol-water [prepared by adding 39 ml of H_2O per 100 g of redistilled phenol (*23*)] and sec-butanol-formic acid-water were used alternatively for the first direction. The latter solvent was prepared by mixing sec-butanol with a stock solution of formic acid (formic acid 88–90 %, 1,120 ml; H_2O, 880 ml) in a volume ratio of three to one (*9*). For the second direction *n*-butanol-glacial acetic acid-water (100–22–50 v/v/v) was used (*23*). The acidic solvents were prepared just before use.

Multiple chromatograms were prepared to permit spraying with a variety of reagents, and also to provide sufficient material for further diagnostic tests on eluates. The chromatograms were inspected under both long- and short-wave ultraviolet light. Any fluorescent or quenching areas thus revealed were outlined.

Radioautograms of the chromatograms were prepared by exposure to x-ray film, routinely for 3 weeks, but adjusted in individual cases to suit the circumstances.

Radioactive spots on the chromatograms were identified by chromatographic position; by their behavior toward spray reagents; by elution and cochromatography with authentic specimens, and by inference from their response to chemical treatment. To illustrate, one radioactive spot suspected to be serine from its chromatographic position and reaction with ninhydrin, cochromatographed with authentic serine in the three solvent systems. It also yielded a volatile radioactive fragment precipitable with dimedon when oxidized with periodate (*15*) along with carrier serine. The original spot was therefore identified as serine.

Classes of compounds were characterized by employing spray reagents described in the literature (*1, 21, 23*): thus, ninhydrin for amino acids; diazotized aromatic amines for phenols; Koenig's reaction and Dragendorff's reagent for pyridine derivatives, etc.

Differentiating reagents were used where available. For nornicotine, the isatin reagent of Stephens and Weybrew (*22*), and an *o*-benzoquinone reagent adapted from the observations of Trautner and Roberts (*24*), when used in conjunction with chromatographic position, may be classified as such. We prepare the *o*-benzoquinone reagent by mixing one volume of 0.01 M catechol with 2.5 volumes of 1 M phosphate buffer, pH 7.4, and adding one volume of 0.04 M $K_3Fe(CN)_6$ solution. With this, nornicotine and some other secondary amines react immediately to produce burgundy-red spots.

Choline was identified by the distinctive color it forms with Dragendorff's reagent (*1*, p 361); by its reaction with 2,2′,4,4′,6,6′-hexanitrodiphenylamine (*1* p 352), and by cochromatography with an authentic specimen.

RADIOASSAY: For radioassay, C^{14}-containing materials were plated onto aluminum planchets at densities below 0.5 mg/cm^2. Consequently corrections for self absorption were not required. A planchet spinner, mounted under a hair dryer which supplied warm air, was used in plating solutions or thin suspensions. Solids, such as $BaCO_3$ and insoluble residues, were first suspended in ethanol by grinding in a Ten Broeck homogenizer.

Nicotine solutions were injected into a drop of dilute oxalic acid solution on the planchet before spreading. Such plates retain their original levels of radioactivity over extended periods.

For quantitative purposes the radioactivity of all plates was counted in triplicate with a thin mica end-window beta counter. The customary corrections were applied. The average of the three counts was used in computing the data reported here.

RESULTS

METABOLISM OF RANDOMLY LABELED NICOTINE: Randomly labeled nicotine was chosen for the early stages of the investigation because it provided an easy means of detecting its conversion to nornicotine. Futhermore, it was expected that fragments from the randomly labeled compound would also provide an

TABLE I

DISTRIBUTION OF RADIOACTIVITY FROM RANDOMLY LABELED NICOTINE-C^{14} IN 2 DIFFERENT STRAINS OF TOBACCO

VARIETY		FED	SOLUBLE	CO_2	RESIDUE	RECOVERED
Converter*	cpm $\times 10^{-3}$	412.6	349.2	18.3	6.5	374.0
	%	(100)	(84.6)	(4.5)	(1.6)	(90.7)
Oxford-1–181**	cpm $\times 10^{-3}$	49.5	46.1	None	None	46.1
	%	(100)	(93.0)	(0)	(0)	(93.0)

* Fresh weight: 10.4 g; curing period: 188 hr; Σ CO_2 collected: 6.6 mmoles.
** Fresh weight: 14.3 g; curing period: 247 hr; Σ CO_2 collected: 18.1 mmoles.

opportunity for the recognition of concomitant processes.

In table I are compared the results from a typical experiment with the converter with those obtained from a single experiment with the Oxford 1–181 variety. It is established from the data that leaves of the former are capable of oxidizing nicotine carbon to CO_2, whereas leaves of the latter are not.

From radioautograms from chromatograms of the soluble fractions it is further established that nicotine is metabolically active in the converter strain, but not in the Oxford 1–181 variety where unchanged nicotine represented all of the detectable radioactivity. The converter showed nornicotine as the major radioactive component in this fraction. Hence, in agreement with the results obtained by Schröter (18) with *N. glauca* Grah., we may conclude that here too nicotine is converted to nornicotine. Moderate amounts of radioactivity were also present in residual nicotine, serine, and choline. A few unidentified components containing minor amounts of radioactivity were also present.

In table I it is shown that 4.5 % of the total radioactivity was recovered in the CO_2 fraction. This suggests that the CO_2 may have been derived from a single carbon atom of the nicotine molecule. On this assumption, the expected figure would be 10 % if conversion were complete. In this experiment conversion was estimated to be only about 60 % complete from counts made directly on the chromatograms. Allowing for this, the observed recovery in the CO_2 fraction would be about three-fourths of that expected on the single carbon hypothesis. The carbon atom most likely to be involved would be the N-methyl carbon. Had the $C^{14}O_2$ originated in the ring carbons, the chromatograms should have revealed the appropriate fragments. Our chromatograms did not provide any evidence for this possibility.

METABOLISM OF SPECIFICALLY LABELED NICOTINE: To obtain more obvious evidence concerning the origin of the CO_2 carbon, nicotine–N–methyl–C^{14} was infiltrated into converter leaves. In Table II are summarized the results from one such experiment. The respired CO_2 accounted for 66.7% of the total radioactivity fed proving that the N-methyl group is metabolized to CO_2.

The data in table II show again that conversion was incomplete. Chromatograms of the soluble fraction confirmed this since residual nicotine produced the most prominent spot on the radioautograms. It accounted for 51 % of the soluble activity, serine for 11 % and choline for 1 %. About 11 % of the soluble activity remained at the origin. The remainder was present in unidentified compounds.

EFFECT OF ANAEROBIC CONDITIONS: The results of the preceding experiments have shown that the conversion of nicotine to nornicotine is associated with the oxidation of the methyl carbon to CO_2. The process, therefore, must be oxygen dependent, and manipulation of the oxygen supply might be expected to reveal the locus of the oxygen requirement. Following this reasoning, a converter leaf was infiltrated with nicotine–methyl–C^{14}. It was then split along the midrib and one portion was cured aerobically; the other anaerobically (table III). Absence of oxygen abolished the conversion of the methyl carbon to CO_2 although it did not suppress CO_2 evolution from endogenous substrates to the same degree. No radioactivity could be detected in the anaerobic CO_2, whereas 27 % of the radioactivity available to the aerobic section was recovered as CO_2.

Chromatograms of the soluble fraction revealed further that anaerobic conditions prevented the spread of radioactivity from the methyl group to the metabolites normally present in the aerobic experiments. More than 99 % of the soluble activity remained in the nicotine. Trace amounts of radioactivity were

TABLE II

DISTRIBUTION OF RADIOACTIVITY FROM NICOTINE-N-$C^{14}H_3$ IN CONVERTER LEAF*

FRACTION	CPM $\times 10^{-3}$	%
Fed	1,188.2	100.0
Soluble	207.2	17.4
Residue	56.0	4.7
CO_2	792.8	66.7
Recovered	1,056.0	88.8

* Fresh weight: 31.8 g; curing period: 291 hr; Σ CO_2 collected: 19.4 mmoles.

TABLE III

EFFECT OF AEROBIC & ANAEROBIC CONDITIONS ON DISTRIBUTION OF RADIOACTIVITY FROM NICOTINE-N-$C^{14}H_3$ IN CONVERTER LEAF

	AEROBIC HALF*		ANAEROBIC HALF**	
	CPM $\times 10^{-3}$	% of RECOVERED	CPM $\times 10^{-3}$	% of RECOVERED
Fed		2,650 $\times 10^3$ CPM***		
Soluble	571	65.6	1,562†	99.3†
Residue	63.9	7.3	11.4	0.7
CO_2	236.0	27.0	None	0
Total	871	99.9	1,573	100

* Fresh weight: 19.5 g; curing period: 192 hr; Σ CO_2 collected: 6.6 mmoles.
** Fresh weight: 18.5 g; curing period: 192 hr; Σ CO_2 collected: 5.3 mmoles.
*** Overall recovery: $\frac{(871 + 1573) \times 10^3 \times 100}{2650 \times 10^3} = 92.3\ \%$.

† Nicotine accounts for more than 99 % of this radioactivity.

detected in only two other spots, both unidentified. Neither serine nor choline was radioactive.

A parallel analysis of the aerobic portion revealed the normal distribution of radioactivity. The highest level appeared in unchanged nicotine. Serine, choline, and the unidentified spots all contained radioactivity.

INSOLUBLE RESIDUE: The data presented in the tables and also from unreported experiments show that the insoluble residue retains radioactivity which appears to be related to the oxidation of the methyl group of nicotine. Either C^{14} was not incorporated into this fraction (table I, Oxford 1—181), or its incorporation was greatly reduced (table III, anaerobic column) when the methyl carbon was prevented from entering the CO_2 pool.

The radioactive component(s) could not be extracted by continuous treatment with chloroform, ether, or acetone; nor hydrolyzed with 6 N HCl or 5 N NaOH at 100 C for 12 hours. This behavior suggested that radioactivity might be associated with lignin. Accordingly, the residue described in table II was used to isolate lignin by the method of Byerrum, et al. (3). The yields were 9.7 and 9.5 % in two parallel isolations. Both lignin samples contained radioactivity, but only about ten per cent of the total was recovered from the demethylation (3) of each. Most of the radioactivity remained in the reaction mixture; its nature remains in doubt.

The insoluble residues were not investigated further. Nevertheless these limited observations allow the conclusion that, under some conditions, the methyl group of nicotine may contribute its carbon atom to the lignin pool.

DISCUSSION

The main objective of these experiments was to provide a basis for a choice between the two possible pathways of the nicotine to nornicotine conversion. We succeeded in establishing the ultimate fate of the methyl group by tracing it to CO_2, but the initial and intermediary steps still remain obscure. The full picture is likely to emerge only after one or more of the unknown intermediates has been identified. Meanwhile the evidence for the oxidative nature of the overall reaction has been considerably augmented.

The anaerobic experiment failed to identify the locus of the oxygen requirement. The results can be interpreted in two ways: A, that the effect of oxygen is direct, involving the nicotine molecule itself; or, B, indirect, by regulating the supply of some energy-rich compound such as ATP which would be required for activation if the methyl group of nicotine were removed through a transmethylation reaction prior to oxidation. Although unlikely, the latter possibility cannot be ruled out on the basis of present evidence.

A direct effect of oxygen could mean that the nicotine must first be activated by an oxidative step. Such a concept has been proposed by Wenkert (27) who suggested nicotine 1′-oxide as an intermediate. It is supported by the work of Egri (7) who has reported that feeding of the oxide to tobacco leaves results in an increase of nornicotine. In our experiments we could not detect radioactivity in the nicotine 1′-oxide region on our chromatograms.

If we follow our observation and admit the possibility that nicotine 1′-oxide may not be a participant in the conversion, the oxygen requirement could be explained in still another way. The methyl group itself may first be oxidized (perhaps to the level of formaldehyde) and then transferred to a carrier molecule from which it could be further oxidized to CO_2. Mothes (14) would explain the oxygen-linked demethylation of nicotine reported by Il'in (12) and Egri (7) in a similar manner and would equate the carrier molecule with the folic acid system. Although tempting, the identification with folic acid in

this particular demethylation still lacks experimental verification and discounts the possibility that unknown carriers may exist. It is clear that additional information will be needed before the problem is solved.

Summary

The post-harvest conversion of nicotine to nornicotine by leaves from a strain of cigar tobacco has been studied with the aid of randomly and specifically C^{14}-labeled nicotines. The experiments represent an attempt to trace the fate of the methyl group and to provide data for a choice between possible mechanisms of the reaction.

The results reported have confirmed the conversion of nicotine to nornicotine and have established that the conversion is coupled with the oxidation of the methyl carbon to CO_2. The existing evidence for the oxidative nature of the overall reaction has been considerably augmented although the initial steps of the reaction remain obscure.

Incidental observations on the spread of carbon from the methyl group of nicotine to other metabolic pools such as serine, choline, and some unidentified compounds have been made. These observations also allowed the conclusion that the methyl group of nicotine can contribute carbon to lignin.

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