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THE METABOLISM OF NICOTINE-1′-OXIDE IN EXCISED *NICOTIANA GLUTINOSA* LEAVES

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Abstract—Aqueous solutions of nicotine-1′-oxide-^{14}C and nicotine-^{14}C were absorbed through the stems of excised *Nicotiana glutinosa* leaves. After varying metabolic periods, the alkaloids were reisolated from the excised leaves and separated by TLC. The ^{14}C content of the regions of the chromatographs corresponding to nicotine and nornicotine were determined by liquid scintillation counting. It was found that nicotine-1′-oxide was converted into both nicotine and nornicotine by the mature *N. glutinosa* leaf. The extent of the conversion of nicotine-1′-oxide to nornicotine approximated the extent of the conversion of nicotine to nornicotine. However, it was found that nicotine was converted faster than nicotine-1′-oxide to nornicotine, and that nicotine-1′-oxide was converted to nicotine faster than it was converted to nornicotine. In young *N. glutinosa* leaves it was found that little nicotine-1′-oxide was converted to nornicotine, while the conversion of nicotine-1′-oxide to nicotine was still observed. The increased conversion of nicotine-1′-oxide to nornicotine that was observed as the plants matured could be directly correlated with the increased ability of the maturing *N. glutinosa* leaves to convert nicotine to nornicotine. The experimental results are readily explained by proposing that the *N. glutinosa* leaves catalyze a reduction of nicotine-1′-oxide to nicotine and that the observed conversion of nicotine-1′-oxide to nornicotine occurs only via the nicotine so produced. It is concluded, therefore, that nicotine-1′-oxide is not an intermediate in the conversion of nicotine to nornicotine in alkaloid containing *N. glutinosa* leaves.

INTRODUCTION

THE CONVERSION of nicotine to nornicotine in *Nicotiana* plants is one of the most extensively investigated examples of alkaloid interconversions. Since Dawson's pioneering work with grafted plants indicated that nornicotine was largely formed in the aerial portion of the *Nicotiana* plant by demethylation of nicotine translocated from the roots,[1] the conversion of nicotine to nornicotine has also been investigated in curing *Nicotiana* leaves[2,3] and as a function of the age of the plant.[4] The substrate specificity of the demethylation process has been investigated,[5] including a series of studies regarding the chirality of the substrate nicotine and nornicotine involved.[6] Nevertheless the details of this alkaloid interconversion process remain unknown.

In 1954 Wenkert proposed that amine N-oxides might serve as active intermediates in several *in vivo* alkaloid transformations, including the demethylation of nicotine to nornicotine in *Nicotiana*.[7] Nicotine-1′-oxide has also been proposed as an intermediate in the degradation of nicotine by bacteria.[8] Stepka and Dewey[9] tested the proposal that nicotine-1′-oxide

[1] R. F. DAWSON, *Am. J. Botany* **32**, 416 (1945).
[2] T. C. TSO and R. N. JEFFREY, *Plant Physiol.* **32**, 86 (1957).
[3] W. STEPKA and L. J. DEWEY, *Plant Physiol.* **36**, 592 (1961).
[4] T. C. TSO and R. N. JEFFREY, *Plant Physiol.* **31**, 433 (1956).
[5] R. F. DAWSON, *J. Am. Chem. Soc.* **73**, 4218 (1951).
[6] T. KISAKI and E. TAMAKI, *Archs Biochem. Biophys.* **92**, 351 (1961).
[7] E. WENKERT, *Experientia* **10**, 346 (1954).
[8] E. WADA and K. YAMASAKI, *Science* **117**, 152 (1953).
[9] W. STEPKA and L. J. DEWEY, *Plant Physiol.* **39**, 283 (1964).

could be an intermediate in the nicotine to nornicotine conversion by examining this process in curing tobacco leaves. They observed that under conditions where nicotine itself was demethylated to nornicotine, nicotine-1′-oxide was not converted to nornicotine, and concluded that nicotine-1′-oxide was not an intermediate in the conversion. As part of an extensive study on the specificity of the nicotine demethylation process, Kisaki and Tamaki[10] tested for nicotine-1′-oxide conversions in alkaloid-free *N. tabacum* leaves obtained from grafted plants. Although these workers reported a slight conversion of nicotine-1′-oxide to nornicotine (3·7 per cent), they attributed this conversion to secondary formation from the nicotine that was observed to be produced from the added nicotine-1′-oxide. They also concluded that nicotine-1′-oxide was not an intermediate in the demethylation process.

Schröter[11] has investigated the nicotine to nornicotine conversion in *Nicotiana* leaf extracts and concluded that the nicotine demethylation process is not a transmethylation reaction, but is an oxidative process. Nicotine-1′-oxide would be a likely intermediate in such an oxidative demethylation. Since the enzymatic reactions occurring in curing tobacco leaves or in alkaloid-free leaves might differ from the reactions occurring in nongrafted *Nicotiana* leaves containing the normal quantities and distribution of nicotine alkaloids, Schröter's observations prompted us to reinvestigate the possibility that nicotine-1′-oxide functions as an intermediate in the nicotine demethylation process.

RESULTS AND DISCUSSION

Ring-labeled nicotine-^{14}C, prepared by $^{14}CO_2$ biosynthesis, was converted to nicotine-1′-oxide-^{14}C. This labeled substrate was supplied to excised leaves from mature *Nicotiana glutinosa* plants, a tissue that is known to be converting nicotine to nornicotine under normal growth conditions. The alkaloid fraction was isolated from the excised leaves after a given

TABLE 1. CONVERSION OF NICOTINE-1′-OXIDE TO NICOTINE AND NORNICOTINE BY EXCISED LEAVES FROM MATURE *N. glutinosa*

Compound supplied*	Recovered in alkaloids† (net dpm)		Recovered ratio $\left(\frac{\text{nornicotine-}^{14}C}{\text{nicotine-}^{14}C}\right)$
	Nornicotine	Nicotine	
Nicotine-1′-oxide^{14}C	770 (4·5%)	770 (4·5%)	1·0
Nicotine-^{14}C	1790 (10%)	6100 (36%)	0·3

* An aqueous solution of the labelled compound ($1{\cdot}7 \times 10^4$ dpm of each compound (2 μc/mmole)) was absorbed through the stems of excised leaves and metabolized for a 24-hr period; 12 hr of illumination followed by 12 hr of darkness.

† Of the total ^{14}C supplied to the excised leaves, 86 per cent was recovered in the alkaloidal fraction in the nicotine-^{14}C experiment and 16 per cent in the nicotine-1′-oxide-^{14}C experiment. The percentages recovered from the chromatographs as nicotine and nornicotine are indicated in parentheses.

[10] T. KISAKI and E. TAMAKI, *Nippon Nogei Kagaku Kaishi* **38**, 549 (1964).
[11] H. B. SCHRÖTER, *Deutsh. Acad. Naturwissen., Halle* **3**, 157 (1966).

period, fractionated by means of TLC and the various pyridine alkaloids detected with the König reagent.[12] The extent of the nicotine alkaloid interconversion was established by determining the amount of ^{14}C present in each detected alkaloid region. The efficiency of the conversion of nicotine-1′-oxide-^{14}C to nornicotine-^{14}C was compared to that of nicotine-^{14}C, supplied to the leaf under the same conditions. The results of such an experiment are summarized in Table 1.

The data summarized in Table 1 show that when supplied to excised *N. glutinosa* leaves under identical conditions, the conversions of nicotine-1′-oxide and nicotine to nornicotine occur to comparable extents. These results are contrary to what one would have predicted from the previously reported experiments concerning the metabolism of nicotine-1′-oxide by *Nicotiana* plant material.[9,10] Two different interpretations of the data in Table 1 seemed reasonable. Either the added nicotine-1′-oxide was efficiently reduced to nicotine in the leaf and the nicotine then demethylated via unknown intermediates, or nicotine-1′-oxide, formed reversibly from nicotine, was an active intermediate on the pathway from nicotine to nornicotine.

TABLE 2. RELATIVE RATES OF NICOTINE AND NICOTINE-1′-OXIDE METABOLISM IN EXCISED *N. glutinosa* LEAVES

Compound supplied*	Metabolic period† (hr)	Recovered activity in alkaloids (net dpm)		Recovered ratio $\left(\frac{\text{nornicotine-}^{14}C}{\text{nicotine-}^{14}C}\right)$
		Nornicotine	Nicotine	
Nicotine-1′-oxide-^{14}C	12	100	1190	0·08
	48	3960	5250	0·8
Nicotine-^{14}C	12	2700	9870	0·3
	48	6020	2300	2·6

* $1{\cdot}7 \times 10^4$ dpm of each compound (2 μc/mmole) was used for each experiment.
† The metabolic period consisted of alternating 12-hr periods of illumination and darkness.

A series of additional experiments were carried out with excised *N. glutinosa* leaves in order to distinguish between these two possibilities. All experiments with mature *N. glutinosa* leaves confirmed the observation that nicotine-1′-oxide is converted into both nornicotine and nicotine by this tissue. Although the total amount of added radioactivity recovered in the alkaloidal fraction varied from experiment to experiment, the ratio of nornicotine-^{14}C to nicotine-^{14}C determined in the recovered alkaloids permits a direct analysis and comparison of the metabolism of nicotine-1′-oxide under different conditions. As shown in Table 2, experiments designed to establish the relative rates of these alkaloid interconversions showed that the conversion of nicotine to nornicotine was faster than the conversion of nicotine-1′-oxide to nornicotine, and that the conversion of the nicotine-1′-oxide to nicotine was faster than the conversion of the oxide to nornicotine. These observations could be partially due to slower penetration of the more polar N-oxide into the cell. However, the observation that nicotine-1′-oxide is converted into nicotine more rapidly than it is converted into nornicotine suggests that the conversion of the nicotine-1′-oxide to nornicotine is taking place via nicotine.

[12] E. HODGSON, E. SMITH and F. E. GUTHRIE, *J. Chromatog.* **20**, 176 (1965).

Additional evidence for this view was obtained by carrying out the same experiments with excised leaves from young *N. glutinosa* plants (about 6, 9 and 12 weeks from seeding).

As shown in Table 3 (part A) the conversion of nicotine-1′-oxide to nornicotine in the young *N. glutinosa* leaves is less than the conversion observed with older leaves (Tables 1 and 2). The young *N. glutinosa* leaves, however, are still capable of converting nicotine-1′-oxide to nicotine. The relative efficiencies of these conversions in young leaves leads to the low nornicotine-^{14}C/nicotine^{14}-C ratios in the recovered alkaloids. The *N. glutinosa* plants used in part A of Table 3 were allowed to grow for additional 3-week periods and the experiments repeated (parts B and C). The ratios of nornicotine-^{14}C/nicotine-^{14}C in the recovered alkaloids in Table 3 shows that increasing plant age results in an increasing ability to convert nicotine-1′-oxide to nornicotine.

TABLE 3. EFFECT OF PLANT AGE UPON CONVERSION OF NICOTINE-1′-OXIDE TO NICOTINE AND NORNICOTINE

	Metabolic† period (hr)	Recovered activity in alkaloids (net dpm)		Recovered ratio $\left(\frac{\text{nornicotine-}^{14}\text{C}}{\text{nicotine-}^{14}\text{C}}\right)$
		Nornicotine	Nicotine	
A. Nicotine-1′-oxide* supplied to leaves from 6-week-old *N. glutinosa* plants	12	50	630	0·08
	24	60	1860	0·03
	48	260	3200	0·08
B. Nicotine-1′-oxide* supplied to leaves from 9-week-old plants	12	50	1160	0·04
	24	430	1080	0·4
	48	290	610	0·5
C. Nicotine-1′-oxide* supplied to leaves from 12-week-old plants	12	440	1400	0·3

* A total of 10^4 dpm of nicotine-1′-oxide^{14}C (6 μc/mmole) was used for each experiment.
† The metabolic period consisted of alternating 12-hr periods of illumination and darkness.

Although nornicotine is often stated to be the major alkaloid of *N. glutinosa*, young *N. glutinosa* plants contain nicotine as the major alkaloid and the conversion of nicotine to nornicotine occurs as the plant matures.[4] The increase in the amount of nicotine-1′-oxide converted to nornicotine relative to that converted to nicotine with increasing plant age can be directly correlated with the increasing ability of the *N. glutinosa* leaf to convert nicotine to nornicotine. When supplied with nicotine-^{14}C, the leaves of younger *N. glutinosa* plants used in part A established a nornicotine-^{14}C/nicotine-^{14}C ratio of 0·25 after 24 hr. After six additional weeks of growth (part C), addition of the same amount of nicotine-^{14}C resulted in a nornicotine-^{14}C/nicotine-^{14}C ratio of 1·2 in the alkaloids recovered after 24 hr.

The data presented in Tables 1–3 show conclusively that nicotine-1′-oxide can be effectively converted into nicotine and nornicotine by intact *N. glutinosa* leaves. As noted in the Experimental section the nicotine-1′-oxide-^{14}C was stable in aqueous solution at 5° for several months. When incubated at 30° for 24 hr with boiled *N. glutinosa* leaf extracts there was a detectable amount (365 net dpm) of conversion of the nicotine-1′-oxide-^{14}C to nicotine-^{14}C. However, since the amount of this nonenzymatic reduction to nicotine is significantly

less than the amount of reduction observed in experiments with the intact *N. glutinosa* leaves, we feel the nicotine-1′-oxide to nicotine conversion reported in this paper represents an enzymatic activity of the *N. glutinosa* leaf. No nonenzymatic formation of nornicotine was observed.

The data reported here do not completely eliminate the possibility that nicotine-1′-oxide is an obligatory intermediate in the conversion of nicotine to nornicotine in *Nicotiana* leaves. It is possible, for example, that the metabolically active form of nicotine-1′-oxide exists only as an enzyme-bound intermediate and that the data obtained reflect barriers to membrane transport of the added nicotine-1′-oxide and the potential for reversible conversions of nicotine and nicotine-1′-oxide. However, the data are readily explained by proposing that the *N. glutinosa* leaf catalyzes a reduction of nicotine-1′-oxide to nicotine, and that the observed conversion of nicotine-1′-oxide-^{14}C to nornicotine-^{14}C occurs only via the nicotine-^{14}C so produced. While studies with cell-free *Nicotiana* extracts will be necessary in order to obtain definitive results, our present results lead us to conclude that nicotine-1′-oxide is not an intermediate in the conversion of nicotine to nornicotine in intact *Nicotiana* leaves where this reaction is occurring as a portion of the normal metabolism of the plant.

EXPERIMENTAL

Radioactive nicotine-^{14}C was prepared biosynthetically by exposing *Nicotiana glutinosa* plants to an atmosphere of $^{14}CO_2$. The crude radioactive nicotine alkaloid fractions were obtained from the plants according to published procedures.[13] After the addition of carrier nicotine (Eastman Organic Chemicals) the nicotine-^{14}C was isolated and purified by chromatography on a 1-μ thick layer of silica gel GF-254 (E. Merck) using the NH_4OH, $CHCl_3$, CH_3OH solvent system of Hodgson *et al.*[12] The nicotine region was located by u.v. light and eluted from the silica gel with $CHCl_3$. A portion of the nicotine-^{14}C was converted to nicotine-1′-oxide-^{14}C.[14] The nicotine-1′-oxide-^{14}C was also purified by the thick layer silica gel chromatography method described above and its identity established by comparison of the TLC properties and i.r. spectrum with those of authentic nicotine-1′-oxide (dipicrate, m.p. 164–165° dec. Anal. (Calc. for $C_{10}H_{14}NO_2.2C_6H_3N_3O_7$: C, 41·5; H, 3·2; N, 17·6. Found: C, 41·8; H, 3·2; N, 17·3 per cent). The location of the oxygen at the 1′-position was confirmed by NMR. The oxide in $CDCl_3$ showed a normal 3-substituted pyridine spectrum from 7·2 to 8·5 ppm but the methyl peak found at 2·18 ppm in nicotine now appeared as two lower field peaks at 2·8 and 3·0 ppm. The chemical and radiochemical purity of the nicotine-^{14}C and nicotine-1′-oxide-^{14}C solutions were established by TLC followed by ^{14}C counting of the developed chromatograph. More than 99 per cent of the total ^{14}C activity was located in the detected pyridine alkaloid spot.

The *N. glutinosa* plants were grown in a greenhouse in soil. For the experiments described in Tables 1 and 2, two large leaves (6–8 g total tissue) were excised from plants that were beginning to bud. For the experiments reported in Table 3, two leaves from younger *N. glutinosa* plants were chosen. The cut stems of the leaves were placed in a small test-tube and the radioactive compound absorbed in about 1 ml of water. After the leaves had absorbed several additional 1-ml portions of H_2O, the stems were transferred to a beaker of water for the remainder of the metabolic period. During the indicated periods of illumination, laboratory fluorescent lighting was supplemented with direct illumination from a 300-W, low temperature, narrow-beam spot lamp. The experiments were terminated by grinding the leaves in 50% aqueous acetone and the crude alkaloidal fraction isolated by extraction procedures.[13]

The total crude alkaloidal fraction isolated from the two leaves was spotted on a 500-μ silica gel TLC plate, the separation of nicotine and nornicotine effected by a $CHCl_3$, CH_3OH, NH_4OH solvent system,[12] and the pyridine alkaloids detected by the König reagent. The colors produced by the König reagent were allowed to fade, the regions of the chromatograph corresponding to nicotine and nornicotine were scraped into scintillation vials and 1 ml of CH_3OH, followed by 10 ml of a toluene solution of PPO, was added. Random, non-alkaloidal areas of the chromatographs collected for background determinations generally counted 40–50 cpm.

Although the nicotine-1′-oxide was stable for several months when stored in aqueous solution at 5°, incubation of 10^4 dpm of nicotine-1′-oxide-^{14}C with *N. glutinosa* leaf homogenates for 24 hr at 30° resulted in the detection of 365 net dpm as nicotine. Since prior boiling of the plant leaf homogenate increased the

[13] W. L. ALWORTH, R. C. DESELMS and H. RAPOPORT, *J. Am. Chem. Soc.* **86**, 1608 (1964).
[14] E. C. TAYLOR and N. E. BOYER, *J. Org. Chem.* **24**, 275 (1959).

activity found in the nicotine region slightly, this background conversion is ascribed to nonenzymatic reduction. No nornicotine-^{14}C was detected after these incubations.

NMR spectra were taken on a Varian A-60 spectrometer, i.r. spectra were taken on a Beckman IR-8 spectrometer, ^{14}C analyses were made with a Beckman LS-200 series liquid scintillation counter using external standardizations, m.ps. were determined on a calibrated Fisher-Johns apparatus and chemical analyses were performed by G. I. Robertson, Jr., Florham Park, New Jersey.

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