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METABOLISM OF NICOTINIC ACID IN TOBACCO PLANTS*

SHIGENOBU MIZUSAKI, YOKO TANABE, TAKURO KISAKI and EINOSUKE TAMAKI

Division of Plant Biochemistry, Central Research Institute, Japan Monopoly Corporation, 1-28-3, Nishishinagawa, Shinagawa-ku, Tokyo, Japan

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Abstract—^{14}C from nicotinic acid-6-^{14}C which was administered to tobacco plants was incorporated into nine pyridine compounds during 3 hr incubation; two of these were identified as nicotinic acid-*N*-glucoside and 6-hydroxynicotinic acid. Nicotinic acid was only incorporated into intermediates of the pyridine nucleotide cycle to a limited extent the main product being the glucoside. Nicotinic acid glucoside was incorporated into nicotine with the same efficiency as nicotinic acid, but the rate of the incorporation was markedly reduced when unlabelled nicotinic acid was also fed, suggesting that the glucoside is not involved in the direct route of nicotine biosynthesis.

INTRODUCTION

IT IS well known that the pyridine ring of nicotine is derived from nicotinic acid.[1] Frost *et al.*[2] reported that nicotinamide adenine dinucleotide could also function as precursor of the pyridine moiety and suggested that the pyridine nucleotide cycle operates in tobacco plants. In tobacco, however, the metabolism of nicotinic acid and the biosynthetic pathway from the acid to nicotine is almost entirely unknown.

We decided, therefore, to investigate the metabolism of nicotinic acid in tobacco and its role in the biosynthesis of tobacco alkaloids possessing a 3-substituted pyridine nucleus. During the course of this work, nicotinic acid-*N*-glucoside and 6-hydroxynicotinic acid were identified among the metabolites of nicotinic acid.

RESULTS AND DISCUSSION

Nicotinic Acid Metabolism

A typical elution diagram when the metabolites of nicotinic acid-6-^{14}C in tobacco are separated on Amberlite IR-120 is shown in Fig. 1. Table 1 shows a comparison of the distribution of radioactivity among the metabolites from roots and leaves. After a few hours incubation radioactivity was found in nicotinic acid glucoside (peak *N*-5), 6-hydroxynicotinic acid (peak *N*-7) and several other peaks from the chromatogram (Fig. 1), one of which was isolated from tobacco leaves and characterized as nicotianine.[3] Conversion of nicotinic acid-6-^{14}C to labelled 6-hydroxynicotinic acid was observed in both sterile root culture[4] and hydroponically grown root segments but not in the leaf. No nicotinic acid disappeared from the nutrient solution in which the tobacco plants were grown during the incubation period, indicating the absence of micro-organisms which are known to be able to convert

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[1] R. F. DAWSON, D. R. CHRISTMAN, A. D'ADAMO, M. L. SOLT and A. P. WOLF, *J. Am. Chem. Soc.* **82**, 2628 (1960).

[2] G. M. FROST, K. S. YANG and G. R. WALLER, *J. Biol. Chem.* **242**, 887 (1967).

[3] M. NOGUCHI, H. SAKUMA and E. TAMAKI, *Phytochem.* **7**, 1861 (1968).

[4] Unpublished data.

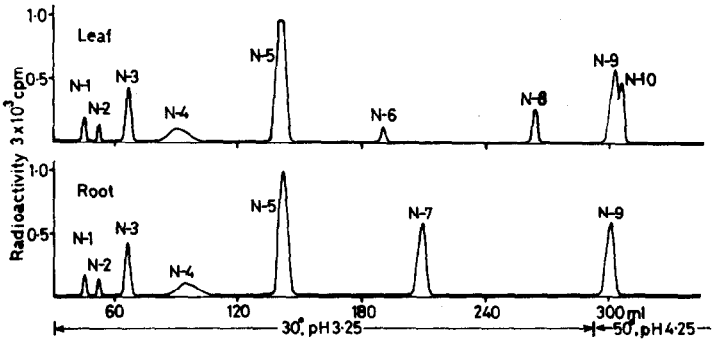

FIG. 1. ELUTION DIAGRAM OF THE METABOLITES OF NICOTINIC ACID-6-^{14}C PRODUCED IN TOBACCO PLANTS AND SEPARATED ON ION-EXCHANGE RESIN.

2·0 ml of the sample was introduced on a 150-cm long column of Amberlite I.R. 120 equilibrated with 0·2 M citrate buffer, pH 3·25. Chromatography was carried out at 30–50° as described by Spackman *et al.*[5a] *N*-5, nicotinic acid glucoside; *N*-7, 6-hydroxynicotinic acid; *N*-9, nicotinic acid; *N*-10, nicotianine.

nicotinic acid to 6-hydroxynicotinic acid.[5b, 6] In the leaf the majority of the label was found in nicotinic acid glucoside whereas in the roots most of the radioactivity was divided between nicotinic acid glucoside and 6-hydroxynicotinic acid.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN INDIVIDUAL METABOLITES OF NICOTINIC ACID-6-^{14}C FED TO TOBACCO PLANTS

Metabolite	Root (cpm × 10^{-4})		Leaf (cpm × 10^{-4})	
N-1	1·29	(0·65)	2·76	(1·38)
N-2	0·30	(0·15)	0·26	(0·13)
N-3	5·72	(2·86)	5·06	(2·53)
N-4	5·53	(2·77)	5·93	(2·97)
N-5*	46·8	(23·4)	92·0	(46·0)
N-6	0		0·93	(0·47)
N-7†	17·8	(8·90)	0	
N-8	0		3·12	(1·56)
Nicotinic acid	15·1	(7·55)	22·2‡	(11·10)

Nicotinic acid-6-^{14}C was administered to root segments and detached leaf and the tissues were incubated at 30° for 3 hr. Individual metabolites were separated by aid of ion-exchange column chromatography using an amino acid analyzer and radioactivity was determined by a flow monitor system. The figures in parentheses express the results as a percentage of the total radioactivity administered to tobacco plants.

* Nicotinic acid–*N*-glucoside.
† 6-Hydroxynicotinic acid.
‡ Nicotinic acid + nicotianine.

[5a] D. H. SPACKMAN, W. H. STEIN and S. MOORE, *Anal. Chem.* **30**, 1190 (1958).
[5b] I. HARARY, *J. Biol. Chem.* **227**, 823 (1957).
[6] L. TSAO, I. PASTAN and E. R. STADTMAN, *J. Biol. Chem.* **241**, 1807 (1966).

The results of a time-course experiment in which nicotinic acid-6-^{14}C was administered to roots are shown in Fig. 2. Nicotinic acid glucoside was rapidly labelled and declined rapidly after 2 hr, indicating that the compound must be metabolically active. A considerable amount of radioactivity was found in the eluate from the ion-exchange resin column with 0·2 N NaOH, most of which was present in nicotine.

Frost *et al.*[2] showed that upon the administration of labelled quinolinic acid, nicotinic acid or nicotinamide adenine dinucleotide to tobacco roots gave rise to label in the pyridine ring of nicotine and suggested that the pyridine nucleotide cycle operates in tobacco plants.

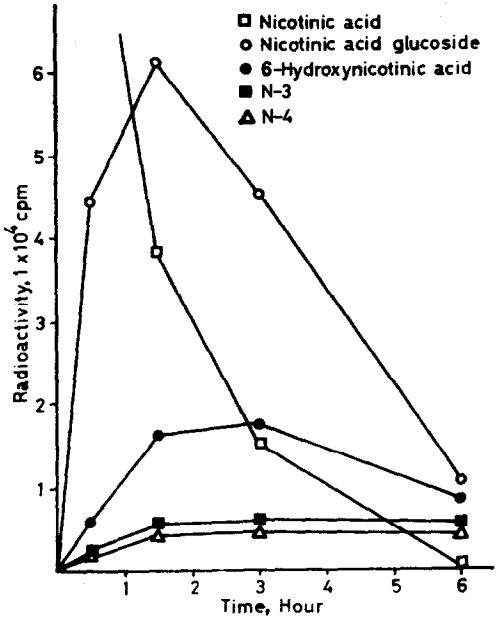

FIG. 2. TIME COURSES OF RADIOACTIVE INCORPORATION OF NICOTINIC ACID-6-^{14}C INTO INDIVIDUAL METABOLITES IN ROOT SEGMENTS OF HYDROPONICALLY GROWN *N. tabacum*.

Individual metabolites were separated with an amino acid analyzer and the radioactivity determined by a flow monitor system.

The present results show, however, that in tobacco leaves nicotinic acid-6-^{14}C is not readily incorporated into the intermediates of the cycle, but is mainly converted to nicotinic acid glucoside.

In order to examine the possible precursor role of the metabolites of nicotinic acid in the biosynthesis of nicotine, the isolated radioactive metabolites were administered separately to tobacco root segments and some isotopic competition experiments were undertaken. The results are shown in Tables 2 and 3. Nicotinic acid glucoside and unknown substance N-3 were incorporated into nicotine with the same efficiency as nicotinic acid. 6-Hydroxynicotinic acid was not incorporated into nicotine as observed by Dawson *et al.*[1] In isotopic competition studies, it was found that the incorporation of nicotinic acid into nicotine was not affected by the presence of unlabelled nicotinic acid glucoside during the incubation period (Table 3). On the other hand, in the presence of unlabelled nicotinic acid a great decrease was found in the percentage nicotinic acid glucoside incorporated into nicotine. These results indicate that nicotinic acid glucoside is not involved in the direct route of the

nicotine biosynthesis and is only utilized in the absence of exogeneous nicotinic acid. It is considered that the glucoside functions as storage substance like niacinogen.[7]

TABLE 2. INCORPORATION OF NICOTINIC ACID AND ITS METABOLITES INTO NICOTINE

Compound	Radioactive incorporation (%)
Nicotinic acid	9·20
Nicotinic acid glucoside	9·00
N-3	9·05
6-Hydroxynicotinic acid	0

Nicotinic acid-6-^{14}C and the isolated metabolites were added back to 2 g of root segments and the tissues were incubated at 30° for 6 hr. The percentage of the incorporation was determined by dividing the total radioactivity of the nicotine by the total radioactivity of the compound administered.

TABLE 3. EFFECT OF UNLABELLED NICOTINIC ACID GLUCOSIDE OR NICOTINIC ACID ON THE INCORPORATION OF NICOTINIC ACID-6-^{14}C OR NICOTINIC ACID-6-^{14}C GLUCOSIDE INTO NICOTINE

	Precursor		Incorporation (%) (Amount of unlabelled nicotinic acid or nicotinic acid glucoside)		
	Specific activity (cpm/mM)	Amount administered (cpm)	0 nm	1 nm	10 nm
Nicotinic acid	$8{\cdot}18 \times 10^9$	$5{\cdot}08 \times 10^5$	9·31	9·12	8·12
Nicotinic acid glucoside	$8{\cdot}87 \times 10^9$	$5{\cdot}11 \times 10^5$	9·00	1·96	0·17

0·062 nm of nicotinic acid-6-^{14}C with and without 1 nm and 10 nm of unlabelled nicotinic acid glucoside were administered to 2 g of root segments. 0·058 nm of nicotinic acid-6-^{14}C glucoside with and without 1 nm and 10 nm of unlabelled nicotinic acid were administered to 2 g of root segments. Duration of experiment was 6 hr. Percentage incorporation was determined by dividing the total radioactivity of the nicotine by the total radioactivity administered.

Identification of Nicotinic Acid-N-*Glucoside*

A marked amount of radioactivity was found in the substances which were not absorbed by the Dowex 1 × 8 formate column. Two radioactive substances were revealed in this fraction by paper chromatography using various solvent systems. One of these was the same as *N*-5 (Fig. 1) and the other was identified as nicotine. Upon the administration of various labelled compounds, such as nicotinic acid-7-^{14}C and D-glucose-U-^{14}C, tobacco root segments gave labelled *N*-5. Upon hydrolysis in 1 N NH_4OH at 100° for 30 min in a sealed tube, *N*-5 yield two spots which had identical R_f values as nicotinic acid and glucose respectively on paper chromatography. Crystalline *N*-5 was obtained from 1 kg of the roots treated with nicotinic acid as described in the Experimental section and gave only one quenching spot on paper chromatograms developed with various solvent systems after treatment by the method of Kodicek and Reddi.[8] The u.v. spectrum of the substance exhibited a sharp maximum at 263 nm which was displaced by a broad maximum at 350 nm upon reduction

[7] H. P. GHOSH, P. K. SARKAR and B. C. GUHA, *Nature* **198**, 4847 (1963).
[8] E. KODICEK and K. K. REDDI, *Nature* **168**, 475 (1951).

with sodium borohydride. These results indicate that the nitrogen atom of the pyridine ring is substituted by glucose, resulting in a pyridinium compound, so that the probable structure of compound *N*-5 is the *N*-glucoside of nicotinic acid. Final confirmation of the structure of *N*-5 was obtained by comparison with an authentic sample of nicotinic acid-*N*-glucoside prepared according to the method of Viscontini *et al.*[9] No depression of the melting point was observed, and the i.r. and u.v. spectra of both compounds were perfectly identical. These properties clearly show that the compound *N*-5 is *N*(D-glucopyranosyl)-β-carboxypyridinium hydroxide.

Identification of 6-Hydroxynicotinic Acid

The elution volumes of compound *N*-7 from an ion-exchange column of Dowex 1 formate and from the amino acid analyser was in good agreement with those of 6-hydroxynicotinic acid. *N*-7 was shown to be chromatographically identical to authentic 6-hydroxynicotinic acid in each of five solvent systems: *n*-butanol, acetic acid, water (4:1:5, R_f 0·68); tert-butanol, formic acid, water (5:1:1, R_f 0·80); *n*-butanol saturated with water (R_f 0·10); phenol saturated with water (R_f 0·63); 95% ethanol, 1 M ammonium acetate (pH 5·0) (7:3, R_f 0·58), and its specific activity was unchanged through several recrystallizations of a mixture with 6-hydroxynicotinic acid.

EXPERIMENTAL

Plant Materials and Labelled Compounds

Nicotiana tabacum "Bright Yellow" was grown hydroponically as described previously.[10] Detached root segments and detached leaf were used for the feeding experiments of labelled compounds. Nicotinic acid-7-^{14}C (10 mc/mM) and D-glucose-U-^{14}C (5 mc/mM) were purchased from Daiichi Chemical Company and nicotinic acid-6-^{14}C (26·2 mc/mM) from Radiochemical Centre, Amersham, Bucks.

Administration of Labelled Compounds

For the time-course experiment, 2 g of root tissue, about 5 cm long, from the tip were taken in Petri dishes and 1 μc of tracers in 0·5 ml of sterile water added as described previously.[10] Feeding to leaves was carried out by placing the petiole end down in the solution containing 1 μc of nicotinic acid-6-^{14}C. After incubation at 30° for designated periods, the roots and leaf were ground in a mortar with 10 ml of 75% EtOH. The mixture was filtered through a fritted glass filter. The alcohol extracts were evaporated to dryness at 40° under reduced pressure. The residue was taken up with a small volume of 0·2 M citrate buffer, pH 2·2, and the volume was adjusted to 5 ml. Samples were stored in a deep-freeze until analysed.

Isolation of Metabolites of Nicotinic Acid

5 μc of nicotinic acid-6-^{14}C in a small volume of sterile water was administered to 10 g of root segments. After incubation at 30° for 3 hr, roots were extracted with 75% EtOH as described above. The extract was evaporated to dryness under reduced pressure. The residue was dissolved in 20 ml H_2O and 5 g of activated charcoal (Darco G-60) added. After stirring for 5 hr the charcoal was filtered and washed several times with water. The materials absorbed on the charcoal were eluted in 200 ml of 60% EtOH with stirring. After concentration of the ethanol solution to 5 ml under reduced pressure, the concentrate was applied to a Dowex 1 × 8 formate column (2 × 20 cm). The column was eluted with increasing concentrations of formic acid (0·05–2 N). Fractions of 5 ml each were collected. Aliquots of each fraction were assayed for absorbance at 260 nm and for radioactivity. The fractions containing radioactive materials were then pooled separately and evaporated to dryness under reduced pressure.

The fraction containing nicotinic acid glucoside was evaporated to dryness and the residue dissolved in a small volume of H_2O. The solution was separated on Whatman No. 1 filter paper in *n*-butanol:acetic acid:water (4:1:5) and the band of nicotinic acid glucoside on the chromatogram cut out and extracted with distilled water.

[9] M. VISCONTINI, R. HOCHREUTER and P. KARRER, *Helv. Chim. Acta* **39**, 1620 (1956).
[10] S. MIZUSAKI, T. KISAKI and E. TAMAKI, *Plant Physiol.* **43**, 93 (1968).

To obtain sufficient quantities of metabolites to permit identification, 1 kg of root segments were used. Unlabelled nicotinic acid was added by dipping the roots in 1 l. of solution containing 1 g of the acid and the tissues were incubated at 30° for 3 hr. EtOH extraction and charcoal treatment were carried out as described above. The fraction containing nicotinic acid glucoside was obtained by chromatography on a Dowex 1 × 8 formate column (3 × 30 cm) and evaporated to dryness under reduced pressure. The residue was dissolved in a small volume of H_2O and EtOH was added to the solution until the solution became slightly turbid. After standing at 0° for a week, crystals of nicotinic acid glucoside were obtained and recrystallized 3 times more to yield 14 mg of colourless crystals, m.p. 233–234° (decomp.). (Found: C, 47·3; H, 5·5; N, 4·5. $C_{12}H_{17}O_8N_1$ required: C, 47·5; H, 5·7; N, 4·6%.)

Isolation of Nicotine and Analytical Methods

Nicotine was obtained by steam distillation and purified by gas chromatography previously described.[10] Radioactivity in individual metabolites was determined after separation by a Beckman/Spinco amino acid analyzer using a Packard Tri-Carb flow monitor system as previously described[10] or by a Beckman liquid scintillation counter.

Preparation of N(D-*glucopyranosyl*)-β-*Carbamoylpyridinium Bromide*

N(D-Glucopyranosyl)-β-carbamoylpyridinium bromide, m.p. 150–152°, was prepared according to the method of Viscontini *et al.*[9]

Preparation of N(D-*Glucopyranosyl*)-β-*Carboxypyridinium Hydroxide*

N(D-Glucopyranosyl)-β-carbamoylpyridinium bromide (1 g) in dry acetic acid (27 ml) was kept at −10° while an equal volume of N_2O was condensed in the reaction vessel.[11] The solution was kept at 10–15° for 1 hr and evaporated to dryness. The residue was dissolved in a small volume of H_2O and separated on a cellulose column (3 × 35 cm) with BuOH, acetic acid, H_2O (6:3:1). The fractions containing *N*(D-glucopyranosyl)-β-carboxypyridinium bromide were combined, concentrated to dryness, and crystallized from aqueous ethanol, giving colourless needles of *N*(D-glucopyranosyl)-β-carboxypyridinium bromide, m.p. 205–206°, yield, 0·7 g. The compound was then treated with Ag_2O and H_2S and recrystallization from aqueous EtOH afforded colourless needles of *N*(D-glucopyranosyl)-β-carboxypyridinium hydroxide, m.p. 233–234°.

[11] M. R. ATKINSON and R. K. MORTON, *Nature* **188**, 58 (1960).