Short Communications

The Incorporation of [$^{14}\text{C}_4$]Aspartic Acid into Nicotinic Acid *N*-Glucoside in *Nicotiana tabacum*

By T. A. SCOTT and A. L. DEVONSHIRE*

Department of Biochemistry, University of Leeds, Leeds LS2 9LS, U.K.

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Radioactivity from [$^{14}\text{C}_2$]- and [$^{14}\text{C}_3$]-aspartic acid is incorporated into C-2 and C-3 of the pyridine ring of nicotine in *Nicotiana tabacum* (Jackanicz & Byerrum, 1966), whereas in bacteria C-2, C-3 and C-4 of aspartic acid give rise to C-2, C-3 and C-7 of nicotinic acid (Scott, Bellion & Matthey, 1969). It therefore seems likely that C-7 of nicotinic acid is derived from C-4 of aspartic acid in *N. tabacum*, but this has not hitherto been demonstrated. The present work describes the incorporation of [$^{14}\text{C}_4$]-aspartic acid into the nicotinic acid moiety of nicotinic acid *N*-glucoside [*N*-(D-glucopyranosyl)- β -carboxypyridinium hydroxide] in *N. tabacum* var. White Gold. Nicotinic acid *N*-glucoside was first reported by Mizusaki, Tanabe, Kisake & Tamaki (1970) as a product of the glucosylation of nicotinic acid in *N. tabacum* var. Bright Yellow.

Seeds of *N. tabacum* var. White Gold were supplied by the Research Department of Carreras Tobacco, Co., Basildon, Essex, U.K. The seeds were germinated and the plants grown in hydroponic culture as described by Scott & Glynn (1967). [$^{14}\text{C}_7$]Nicotinic acid and [$^{14}\text{C}_4$]aspartic acid were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Excised roots were blotted on soft tissue and weighed. A solution of DL-[$^{14}\text{C}_4$]aspartic acid (1.62 mCi/mmol; 4.1 mg in 0.5 ml of water) was spread evenly over the surface of 100 g of roots. After 5 h at 25°C most of the solution was absorbed by the roots. Incubation was continued for 18 h at 25°C in hydroponic medium (Scott & Glynn, 1967). The roots were then homogenized with water (500 ml) and protein was precipitated by heating at 100°C for 10 min. Insoluble material was removed by centrifugation and the supernatant was dried by rotary evaporation. The residue was dissolved in water (25 ml) and placed on a column (24 cm $\times$ 2.6 cm diam.) of Dowex 1 X8 (formate form). After elution of the column with a linear 0–4 M gradient of formic acid (total volume 660 ml), the first radioactive fractions (effluent volume

10–80 ml) were dried by rotary evaporation. At this stage the product was still impure and contained large amounts of nicotine. Further purification was achieved on a column (24 cm $\times$ 2.6 cm diam.) of Dowex 50 (H^+ form) eluted with 1 M-hydrochloric acid (800 ml). Nicotinic acid *N*-glucoside appeared at peak effluent volume 400 ml in a total volume of 100 ml. Some minor impurities were well separated and nicotine was retained by this column. The product still contained ninhydrin-positive material, which was removed, together with traces of other u.v.-absorbing compounds, by t.l.c. on Kieselgel G (E. Merck A.-G., Darmstadt, Germany) in five different solvent systems: phenol-water-aq. ammonia (sp. gr. 0.88) (160:4:1, w/v/v), R_F 0.40–0.44; butan-1-ol-propan-1-ol-water-1 M-ammonia (25:50:15:1, by vol.), R_F 0.52–0.62; butan-1-ol-acetic acid-water (12:3:5, by vol.), R_F 0.1–0.2; ethanol-water-aq. ammonia (sp. gr. 0.88) (18:1:1, by vol.), R_F 0–0.10; propan-2-ol-water-acetic acid (2:2:1, by vol.), R_F 0.5–0.6. Nicotinic acid *N*-glucoside was located by radioautography, and the appropriate area was scraped from the plate and eluted with water. A final purification was achieved by chromatography on Dowex 50 (H^+ form) as described above. The yield was calculated from the extinction at 265 nm (ϵ_{265} 6840 l $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$). The yield of nicotinic acid *N*-glucoside was equivalent to 0.9 mg of nicotinic acid. Unlabelled nicotinic acid (5 mg) was added as carrier. The mixture was made 6 M with respect to hydrochloric acid and hydrolysed by heating at 120°C for 30 min. The nicotinic acid was purified and the distribution of radioactivity within the molecule was determined by the method of Scott (1971). After correction for dilution with the unlabelled carrier, C-7 of the nicotinic acid contained 7.0 $\mu\text{Ci}/\text{mmol}$ of C, which corresponds to a 233-fold dilution of the radioactivity in C-4 of the aspartic acid. The remainder of the molecule contained an average of 0.044 $\mu\text{Ci}/\text{mmol}$ of C, indicating that very little of the radioactivity in C-4 of aspartic acid had been incorporated into the pyridine-ring carbon atoms of the nicotinic acid.

* Present address: Rothamsted Experimental Station, Harpenden, Herts., U.K.

Naturally occurring nicotinic acid *N*-glucoside has been described only once before in the literature, after a different isolation procedure (Mizusaki *et al.* 1970). An authentic sample was therefore prepared by a modification of the method of these authors. 3-Carbamoyl-*N*-(2',3',4',6'-tetra-*O*-acetyl- β -glucopyranosidyl)pyridinium chloride was prepared by the method of Haynes & Todd (1950). A 1.0g portion of this compound was treated with nitrous anhydride to convert the amide group into a carboxyl group by the method of Atkinson & Morton (1960). The resulting acetylated derivative was hydrolysed by heating at 55°C for 2 h in 2% (w/v) hydrobromic acid, and the hydrolysate was placed on a column of Dowex 50 (H^+ form) and eluted with 1M-hydrochloric acid. Unchanged material was retained and the nicotinic acid *N*-glucoside was eluted in the early fractions, which were dried by rotary evaporation. The resulting yellow oil was dissolved in methanol and dried over anhydrous sodium sulphate. After removal of the drying agent, nicotinic acid *N*-glucoside hydrochloride was precipitated as a white powder by the addition of dry ethyl acetate. As the hydrochloride was very hygroscopic, it was converted into the hydroxide by treatment with excess of silver oxide. After treatment with hydrogen sulphide, silver sulphide and silver chloride were removed by filtration and the solution was freeze-dried. After recrystallization from acetone-water, the nicotinic acid *N*-glucoside hydroxide had m.p. 232–234°C (Found: C, 47.3; H, 5.4; N, 4.5. Calc. for $C_{12}H_{17}NO_8$: C, 47.5; H, 5.6; N, 4.6%). The yield was 200mg (30%). Mizusaki *et al.* (1970) reported m.p. 233–234°C for this compound; in their synthesis deacetylation with hydrobromic acid preceded the treatment with nitrous anhydride and column chromatography was not used for the purification of intermediates.

The synthetic and the natural compound could not be separated by t.l.c. in the five systems described above. The u.v.-absorption spectra of the two compounds were superimposable (λ_{max} . 265nm, ϵ_{265} 68401·mol⁻¹·cm⁻¹) and the same spectral shift was shown in the presence of

potassium cyanide (λ_{max} . 315nm). The low-resolution mass spectrum of the hydrochloride showed major peaks at *m/e* 123, 105, 78 and 51. No molecular ion was detected, but identical mass spectra were given by the synthesized and the natural nicotinic acid *N*-glucoside hydrochloride.

With the purification methods described above, 100g of fresh excised root of *N. tabacum* var. White Gold was found to contain 0.11 μ mol of free nicotinic acid and 22 μ mol of nicotinic acid *N*-glucoside.

Label from [¹⁴C₂]- and [¹⁴C₃]-aspartic acid is diluted 100–700-fold during its incorporation into the pyridine ring of nicotine (Jackanicz & Byerrum, 1966). This compares well with the 233-fold dilution reported in the present work for the incorporation of [¹⁴C₄]aspartic acid into nicotinic acid *N*-glucoside. The result confirms that C-4 of aspartic acid gives rise to C-7 of nicotinic acid in *Nicotiana* and supports the view that the total carbon skeleton of aspartic acid, with the exception of C-1, is utilized in the synthesis of nicotinic acid.

Nicotinic acid *N*-glucoside is a useful natural trapping agent for nicotinic acid in *Nicotiana*. In the root its pool size is about 20 times that of free nicotinic acid; for isotopic-tracer studies it has an advantage over nicotine in that C-7 of the nicotinic acid moiety is preserved.

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