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THE INCORPORATION OF [2,3,7-^{14}C] NICOTINIC ACID INTO NICOTINE BY *NICOTIANA TABACUM*

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Abstract—[2,3,7-^{14}C] Nicotinic acid, prepared from [U-^{14}C] aniline hydrogen sulphate, was administered to tobacco plants via the roots in hydroponic culture. Radioactive nicotine was isolated from the plant and purified as the dipicrate. The dipicrate was converted into nicotine, which was oxidized to nicotinic acid. The nicotinic acid was degraded stepwise to give the radioactivity of each carbon atom. A feature of this degradation is the rapid hydrogenation of nicotinic acid with rhodium on charcoal as the catalyst; there is a 50 per cent simultaneous decarboxylation to give hexahydronicotinic acid, piperidine and CO_2. Radioactivity was detected and measured in positions 2 and 3 of the nicotinic acid, with negligible activity in positions 4, 5 and 6. This shows that the pyridine ring of nicotinic acid does not become symmetrical during its conversion into nicotine; the attachment of the pyrrolidine moiety occurs only at the site from which the carboxylic acid group is lost, i.e. at position 3.

INTRODUCTION

IN THE biosynthesis of nicotine from nicotinic acid by *Nicotiana tabacum*, the carboxyl group (C-7) of the nicotinic acid is lost.[1] It is therefore reasonable to assume that the carboxyl group is replaced by the pyrrolidine ring. Indeed, in an electronic mechanism for the synthesis of nicotine, proposed by Dawson[2] and now widely accepted, a 1,6-dihydropyridine intermediate is attacked by an N-methyl-Δ'-pyrrolidinium cation at position 3, with simultaneous decarboxylation. This mechanism also explains the labilization of hydrogen at C-6 of the nicotinic acid, which occurs during the conversion into nicotine.[3] Nevertheless, it has not hitherto been shown that the attachment of the pyrrolidine moiety occurs exclusively at position 3 of nicotinic acid. In the present work, [2,3,7-^{14}C] nicotinic acid was administered to plants of *Nicotiana tabacum*. The resulting nicotine was oxidized to nicotinic acid, which was then degraded to determine the distribution of radioactivity within the molecule.

RESULTS AND DISCUSSION

The results of the degradation of the radioactive nicotinic acid are given in Tables 1 and 2. As expected, the chemically synthesized, substrate nicotinic acid is labelled equally in positions 2, 3 and 7, whereas the nicotinic acid derived from nicotine is labelled equally in positions 2 and 3; C-7 of the substrate nicotinic acid is lost by decarboxylation. Taking into account the loss of C-7, the dilution of specific activity during the metabolism of nicotinic acid to nicotine was 35·6 times.

Taking the results for the nicotine-derived nicotinic acid, the radioactivity of each carbon atom was calculated as follows. The value for C-2 243 counts/min is obtained from the

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[1] R. F. DAWSON, *J. Am. Chem. Soc.* **75**, 5114 (1953).

[2] R. F. DAWSON, *Science in Progress* (Edited by W. R. BRODE), p. 117. Yale University Press (1962).

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

values for C-3,4,5,6 and C-2,3,4,5. Taking this value for C-2 and 124 counts/min for C-2,6, the activity is −5, corrected to zero. From C-3,6 the value for C-3 is therefore 260 counts/min. By similar calculations a value of 23 counts/min is obtained for C-4, and −28 counts/min for C-5. Alternative calculations give low or negative values for C-4,5 and 6. If it is assumed that C-4,5 and 6 have no radioactivity, the maximum error of the counts in this experiment is 11·5 per cent.

TABLE 1. THE DEGRADATION OF RADIOACTIVE NICOTINIC ACID

The method of degradation is shown in Fig. 2 and is described in the text. A sample of authentic [2,3,7-^{14}C] nicotinic acid was diluted 350 times for the degradation. In the preparation of nicotinic acid from the nicotine extracted from the plant, this sample underwent a 30-fold dilution.

	Radioactivity (above background) (counts/min at infinite thickness of $BaCO_3$)	
Origin of CO_2	Authentic [2,3,7-^{14}C] nicotinic acid (measured)	Nicotinic acid from nicotine (measured)
2,6	380	124
3,6	392	130
2,3,5,6	385	118
3,4,5,6	190	63
2,3,4,5,6	310	99
2,3,4,5,6,7	393	82

TABLE 2. RADIOACTIVITY OF THE INDIVIDUAL CARBON ATOMS OF NICOTINIC ACID, CALCULATED FROM THE RESULTS IN TABLE 1

	Radioactivity (above background) (counts/min at infinite thickness of $BaCO_3$)			
	Authentic [2,3,7-^{14}C] nicotinic acid		Nicotinic acid from nicotine	
Carbon atom (measured directly)	measured	corrected for dilution	measured	corrected for dilution
2	790	276500	243	6290
3	780	273000	260	7800
4	10	—	23	—
5	30	—	−28	—
6	30	—	−5	—
7	794	277900	0·0	—

Similar calculations were used for the calculation of values for the authentic [2,3,7-^{14}C] nicotinic acid. The error of the degradation method, due chiefly to the dependence on difference calculations, is still apparent. It can therefore be concluded that the samples of nicotinic acid degraded in this experiment were labelled equally in positions 2 and 3 and at no other position in the ring. Thus in the synthesis of nicotine from nicotinic acid, the pyrrolidine moiety becomes bound exclusively at position C-3 and there can be no stage in the conversion in which the 6-membered ring becomes symmetrical.

Clearly, the degradation method is only suitable for the determination of a simple labelling pattern in a sample of nicotinic acid of fairly high radioactivity.

EXPERIMENTAL

Preparation of [2,3,7-^{14}C] *Nicotinic Acid*

This was a modification of the method of Pastan, Tsai and Stadtman.[4] Nitrobenzene (42 mg), glycerol (216 mg), ferrous sulphate (20 mg), boric acid (35·5 mg) and [U-^{14}C] aniline sulphate (80 mg specific activity 1·25 μc/mg) were mixed in a 5-ml capacity round-bottomed flask fitted with a condenser. Conc. H_2SO_4 (182 mg) was added with stirring and the mixture was heated at 140° for 4 hr on an oil bath.

Water (0·3 ml) was added to the cooled mixture and excess nitrobenzene was removed by steam distillation. After making the mixture strongly alkaline by the addition of 8 N NaOH (1 ml), the mixture was steam distilled again; the first 10 ml of steam distillate (which contained the quinoline) was made up to 25 ml with water, and glacial acetic acid (0·5 ml) and cupric acetate (500 mg) were added. With the solution at 65–70°, 30% H_2O_2 (7 ml) was added over a period of 10 min. The transient brown suspension was replaced by a blue precipitate of copper quinolinate, which was removed by filtration and washed with warm distilled water (10 ml). The precipitate was dried and resuspended in water (15 ml). H_2S was passed for 1 hr at 70°. The black precipitate of copper sulphide was removed by filtration and the resulting solution of quinolinic acid was taken to dryness under a stream of nitrogen at 80°. The residue was dissolved in glacial acetic acid (25 ml) and boiled under reflux for 6 hr. The acetic acid solution was evaporated to dryness under reduced pressure, and the residual nicotinic acid was sublimed at 140° and 1 mm pressure. 16·0 mg of nicotinic acid were obtained (29 per cent of theoretical). Radio-autograms showed that the nicotinic acid was radiochemically pure in three different solvent systems: *n*-butanol: propyl alcohol: water: ammonia (1:2:1:trace); *n*-butanol:formic acid:water (15:3:2); sec-butanol:formic acid: water (15:3:2).

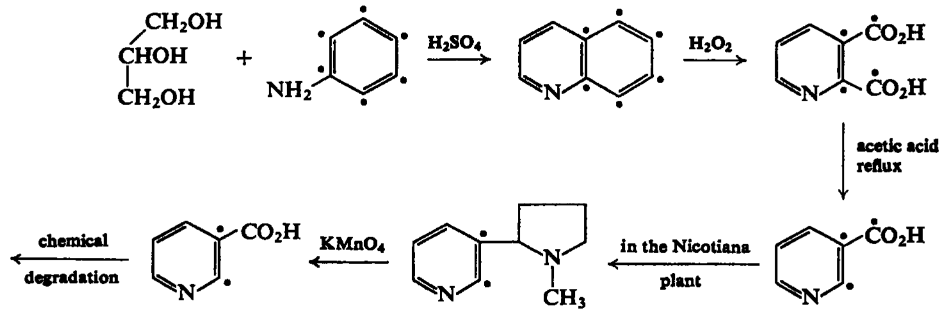

FIG. 1. SCHEMATIC SUMMARY OF THE SYNTHESIS OF [2,3,7-^{14}C] NICOTINIC ACID, ITS ADMINISTRATION TO NICOTIANA, AND THE OXIDATION OF THE RESULTING NICOTINE TO NICOTINIC ACID.

Administration of Nicotinic Acid to Tobacco Plants

Tobacco plant seeds (*Nicotiana tabacum* var. *virginia*) were germinated on moist blotting paper at 25° and grown to a height of 20 cm in soil. They were then transferred to hydroponic

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[4] I. PASTAN, L. TSAI and E. R. STADTMAN, *J. Biol. Chem.* **239**, 902 (1964).

inorganic nutrient medium (INM) of the following composition (per litre): 83 mg KH_2PO_4; 83 mg $(NH_4)_2SO_4$; 83 mg KCl; 330 mg $Ca(NO_3)_2$; 0·7 mg $FeCl_3$. The plants were grown in a greenhouse at 30° in natural light.

A plant 40 cm in height was selected and all lateral roots cut off. New roots were allowed to grow over a period of 10 days in INM. The plant was removed from the medium and immersed in a solution (10 ml) of [2,3,7-^{14}C] nicotinic acid (10 mg, total activity 6 μc), pH 6·6. 24 hr later the roots were immersed in INM (10 ml). After this had been absorbed, the plant was allowed to metabolize the nicotinic acid in continuous light for 7 days, with its roots immersed in 200 ml of INM.

Extraction of Nicotine

The leaves and roots were partly homogenized in an M.S.E. "Atomix" blender with 0·1 N H_2SO_4 (5 ml of acid per gm fresh wt of tissue). After boiling under reflux for 4 hr, the suspension was cooled and filtered. The filtrate was extracted with ether (3 × 200 ml), to remove acidic and neutral ether-soluble material. 5 N NaOH was then added until the solution was alkaline to phenolphthalein, and the solution was again extracted with ether (4 × 200 ml). This ether extract which contained the nicotine, was dried over anhydrous sodium sulphate. The sodium sulphate was removed by filtration, the ether was evaporated under a stream of nitrogen, and the residue was dissolved in 10 ml absolute alcohol. To this was added saturated ethanolic solution of picric acid (10 ml). The nicotine dipicrate was collected by filtration and washed with cold absolute alcohol. The yield of nicotine dipicrate was 18 mg. This was diluted to 50 mg with unlabelled nicotine dipicrate.

Oxidation of Nicotine to Nicotinic Acid

Nicotine was obtained by steam distilling a solution of its dipicrate (50 mg) in 2 N sodium hydroxide (15 ml). 5 ml of distillate were collected, and made up to 10 ml with an equal volume of 2 N H_2SO_4. Potassium permanganate (100 mg) was added, and the solution heated under reflux in a water bath for 45 min. After cooling, the reaction mixture was made alkaline with solid NaOH and left overnight. The precipitate was centrifuged down (1200 rev/min; 1 hr), washed with 20 ml of hot distilled water, and the washings added to the supernatant. The pooled washings and supernatant were adjusted to pH 3 and passed through charcoal Norite A (20 g) in a Buchner funnel. The Norite A was washed with distilled water (200 ml), and the nicotinic acid was eluted with ethanol: 0·3% NH_4OH (1:1 v/v) (200 ml; 20 vol./g of Norite A). The alcohol eluate was evaporated to dryness and the residue was redissolved in water (5 ml). 5 N NaOH (1 drop) was added and the solution was boiled to remove ammonia. The solution was made strongly acidic by the addition of conc. HCl and evaporated to dryness under reduced pressure. Sublimation of the residue at 140° and 1 mm gave 10·3 mg of nicotinic acid hydrochloride (yield, 85 per cent of theory). This was diluted with unlabelled nicotinic acid, to give sufficient material for the radiochemical degradation.

The Radiochemical Degradation of Nicotinic Acid

Since rhodium was reported to catalyse the rapid and specific hydrogenation of conjugated systems,[5] it was tried as a catalyst for the hydrogenation of nicotinic acid. Nicotinic acid (20 mg) (I) in 2 N H_2SO_4 (5 ml) in the presence of 5% rhodium on charcoal (10 mg) becomes rapidly hydrogenated at room temperature and atmospheric pressure. Hydrogenation was

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[5] D. CHRISTMAN and R. F. DAWSON, *Biochemistry* **2**, 182 (1963).

complete after 1 hr, but it was accompanied by about 50 per cent decarboxylation. The resulting CO_2 represents C-7 of the nicotinic acid.

The hydrogenation mixture contained approximately equimolar proportions of hexahydronicotinic acid (nipecotic acid) (II) and piperidine (III). Without removing the catalyst, the solution was made strongly alkaline by the addition of 5 N NaOH. Benzoyl chloride (30 mg) was then added and the mixture stirred rapidly for one hour, while cooling in ice. During this time, the solution was kept alkaline to phenolphthalein by the occasional addition of 5 N NaOH. The benzoyl piperidine (IV) was extracted from the solution by shaking with diethyl ether (3 × 10 ml). This extract was blown to dryness in a stream of nitrogen. A solution (5 ml) containing $KMnO_4$ (10 mg) was added to the residue and the mixture was heated for 30 min on a boiling-water bath. The MnO_2 was removed by filtration and washed with hot water. The filtrate and washings were evaporated to dryness under reduced pressure and conc. HCl (5 ml) was added to the residue. This solution was boiled under reflux for 12 hr to hydrolyse the N-benzoyl-5-amino-valeric acid (V). The resulting 5-amino-valeric acid (VI) was purified and degraded as shown in Fig. 2 according to the methods of Strassman and Weinhouse.[6] Values were thus obtained for the radioactivity of C-2,6, C-2,3,5,6, and C-2,3,4,5,6.

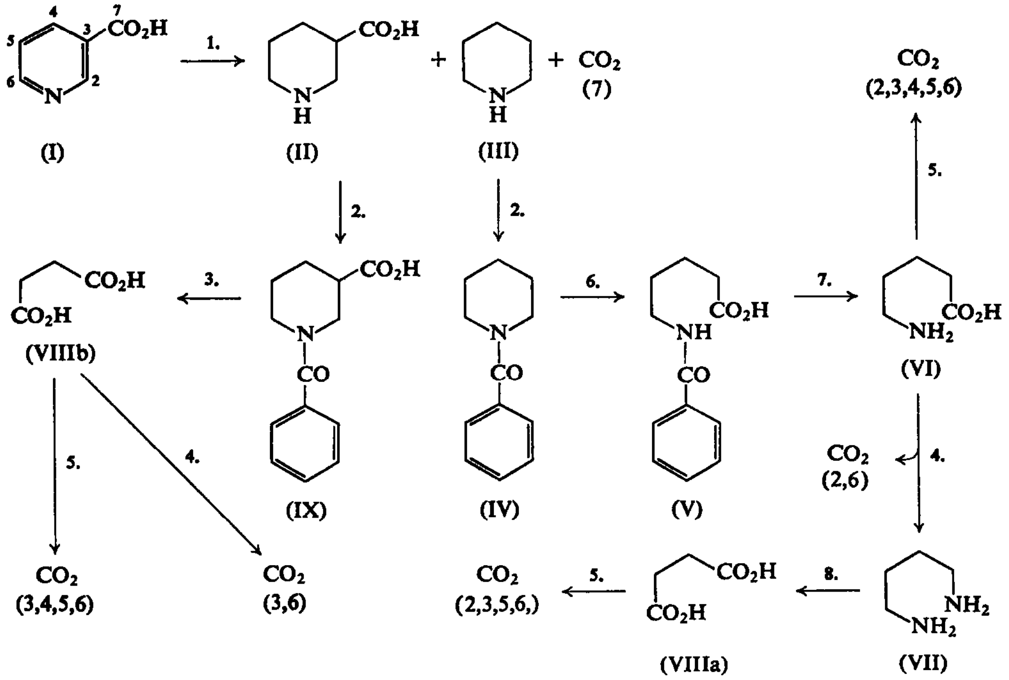

FIG. 2. REACTIONS USED IN THE DEGRADATION OF RADIOACTIVE NICOTINIC ACID.

I, nicotinic acid; II, nipecotic acid; III, piperidine; IV, benzoylpiperidine; V, benzoyl-5-amino-valeric acid; VI, 5-amino-valeric acid; VII, 1,4-diamino-butane; VIII, succinic acid; IX, benzoyl-nipecotic acid.

1. catalytic hydrogenation over rhodium-charcoal catalyst; 2. benzoyl chloride; 3. prolonged oxidation with acidic $KMnO_4$; 4. NaN_3 conc. H_2SO_4; 5. total combustion; 6. controlled oxidation with $KMnO_4$ at pH 7; 7. reflux with 6 N HCl; 8. oxidation with alkaline $KMnO_4$.

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[6] M. STRASSMAN and S. WEINHOUSE, *J. Am. Chem. Soc.* **75**, 1680 (1953).

The alkaline, aqueous solution of N-benzoyl nipecotic acid (IX) was acidified with 2 N H_2SO_4 and maintained at 80°, while titrating with 1% $KMnO_4$. Titration was stopped when the red colour persisted for 1 min. The resulting succinic acid was purified and degraded as shown in Fig. 2 according to Strassman and Weinhouse.[6] Values were thus obtained for the radioactivity of C-3,6 and C-3,4,5,6.

Measurement of Radioactivity

Samples were totally burnt by heating with the combustion fluid of Van Slyke and Folch,[7] or submitted to specific decarboxylation reactions. The apparatus was flushed with CO_2-free N_2, and the effluent gases were passed through 5 ml of 5% (w/v) $KMnO_4$ in N H_2SO_4 and then 5 ml of carbonate-free 2 N NaOH. $BaCO_3$ was precipitated from the NaOH solutions by the addition of an equal volume of carbonate-free 5% (w/v) $BaCl_2$. Triplicate $BaCO_3$ disks were plated on filter paper with a standard stainless-steel filter assembly and washed with water and acetone, and the radioactivity was determined in a windowless gas-flow counter (Tracerlab SC-16). All counts were corrected to infinite thickness with the aid of a self-absorption curve.

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[7] D. D. VAN SLYKE and J. FOLCH, *J. Biol. Chem.* **136,** 509 (1940).