Biosynthesis of the *Nicotiana* Alkaloids. XI. Investigation of Tautomerism in N-Methyl-Δ^1-pyrrolinium Chloride and Its Incorporation into Nicotine[1]

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Abstract: Reaction of N-methyl-Δ^1-pyrrolinium-2-^{14}C chloride with acetonedicarboxylic acid yielded hygrine which was labeled solely at C-2. This result indicates that the 1,2 double bond in the pyrrolinium salt is not capable of a tautomeric shift to the 1,5 position. The administration of N-methyl-Δ^1-pyrrolinium-2-^{14}C chloride to *Nicotiana tabacum* plants yielded radioactive nicotine (18% incorporation) which had all its activity located at C-2′ of the pyrrolidine ring. Therefore the randomization of activity which occurs at C-2′ and C-5′ of the pyrrolidine ring of nicotine when ornithine-2-^{14}C is the precursor must occur prior to the formation of the N-methyl-Δ^1-pyrrolinium salts. The following metabolic sequence is suggested: ornithine → putrescine → N-methylputrescine → 4-methylaminobutanal → N-methyl-Δ^1-pyrrolinium salt.

More than 10 years have elapsed since it was first discovered that the administration of ornithine-2-^{14}C to *Nicotiana* plants yielded nicotine which was labeled equally at C-2′ and C-5′ of the pyrrolidine ring.[2,3] Recently it has been observed that the δ- but not the α-amino group of ornithine is utilized for the formation of the pyrrolidine ring of nicotine.[4] This result is apparently in conflict with the later work of Schröter and Neuman[5] who found that α-N-methylornithine-N-$^{14}CH_3$ served as a precursor of the N-methyl group of the pyrrolidine ring. Finally the whole status of ornithine as a true biosynthetic precursor of the pyrrolidine ring of nicotine has been questioned by Rapoport[6] who found that a short-term carbon dioxide-^{14}C feeding to *N. glutinosa* plants afforded labeled nicotine which had unsymmetrical labeling in the pyrrolidine ring.

One unsettled problem concerning the incorporation of ornithine-2-^{14}C into nicotine is the stage at which a symmetrical intermediate is formed so that the pyrrolidine ring becomes labeled equally at C-2′ and C-5′. We suggested in a recent review[7] that 4-methylaminobutanal (I) is an intermediate, and that this compound cyclizes to the N-methyl-Δ^1 pyrrolinium salt (II) (Figure 1). Equal labeling at C-2′ and C-5′ of the pyrrolidine ring is achieved by postulating a tautomeric equilibrium between II and III. It is assumed that the pyridine moiety of nicotine becomes attached at C-2 of this pyrroline derivative. In support of this hypothesis, Kisaki, Mizusaki, and Tamaki[8] have isolated radioactive 4-methylaminobutanal from tobacco plants which had been fed ornithine-2-^{14}C. Furthermore the administration of this biosynthetic radioactive 4-methylaminobutanal to tobacco plants yielded radioactive nicotine.

In view of these results it was desirable to check whether the N-methyl-Δ^1-pyrrolinium salt II is capable of undergoing the postulated tautomeric change to III. The Δ^1-pyrrolinium salt labeled with ^{14}C at C-2 was prepared by the following route. 4-Chlorobutyronitrile-1-^{14}C (VI) was obtained by refluxing an ethanolic solution of 1-bromo-3-chloropropane with aqueous potassium cyanide-^{14}C.[9] On refluxing the nitrile with a solution of hydrogen chloride in ethanol, containing the required amount of water for hydrolysis, ethyl 4-chlorobutyrate-1-^{14}C (V) was obtained. N-Methyl-2-pyrrolidone-2-^{14}C (IV) was obtained by heating the chloro ester with methylamine in benzene in a sealed tube.[10] Partial reduction of IV with lithium aluminum hydride afforded 4-methylaminobutanal-1-^{14}C which cyclized to N-methyl-Δ^1-pyrrolinium chloride in the presence of hydrochloric acid.[11] This salt had an absorption maximum in the ultraviolet at 267 mμ (ε 2240) which is characteristic for a protonated azomethine group.[12] Reaction of the pyrrolinium salt with acetonedicarboxylic acid yielded radioactive hygrine (VII).[11,13] If the double bond in II is mobile between the 1,2 and 1,5 positions, activity in the hygrine would be equally divided between C-2 and C-5. The hygrine was degraded by the scheme illustrated in Figure 2. Wolff–Kischner reduction of hygrine yielded 1-methyl-2-propylpyrrolidine (VIII), which was converted to its methiodide and subjected to a Hofmann degradation. The expected product was IX, since the only carbon–hydrogen bond, on a carbon β to the quaternary nitrogen, which can be oriented parallel to the carbon–nitrogen bond being cleaved, is at C-1′ of the propyl side chain. Hydrogenation and methylation of the Hofmann product did in fact yield 1-dimethylaminoheptane methiodide (XII),

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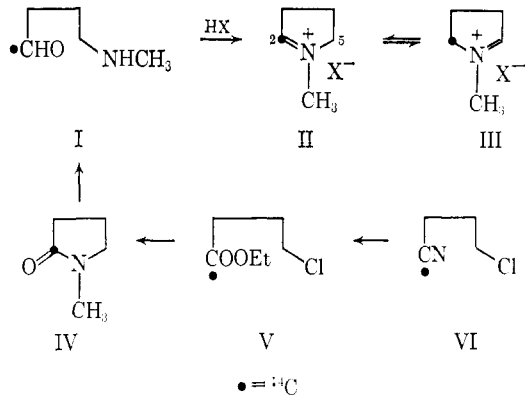

Figure 1.

Table I

	Spec act., dpm/mM × 10^{-5} Expt 1	Expt 2
Hygrine and Its Degradation Products		
Hygrine oxime	2.26	1.43
1-Dimethylaminoheptane methiodide	2.24	1.41
Formaldehyde dimethone (C-5)	0	0
Hygric acid	2.20	1.40
N-Methyl-2-phenyl-Δ^2-pyrroline oxalate	2.21	1.35
Benzoic acid (C-2)	2.20	1.38
Nicotine and Its Degradation Products		
Nicotine diperchlorate	3.67	
Nicotinic acid	3.65	
3-Nitro-5-(3′-pyridyl)pyrazole	3.70	
Pyridine oxalate	0	

no 4-dimethylaminoheptane methiodide being detected. A second Hofmann degradation on XII yielded 1-heptene (XI), which was oxidized to heptane-1,2-diol with osmium tetroxide and cleaved to hexanal and formaldehyde (derived from C-5 of hygrine) with butanal-1-^{14}C was added to acetonedicarboxylic acid using Galinovsky's conditions.[11] In the second experiment the isolated pyrrolinium chloride was used. The hygrine obtained from both experiments was found to have all its activity located at C-2, indicating

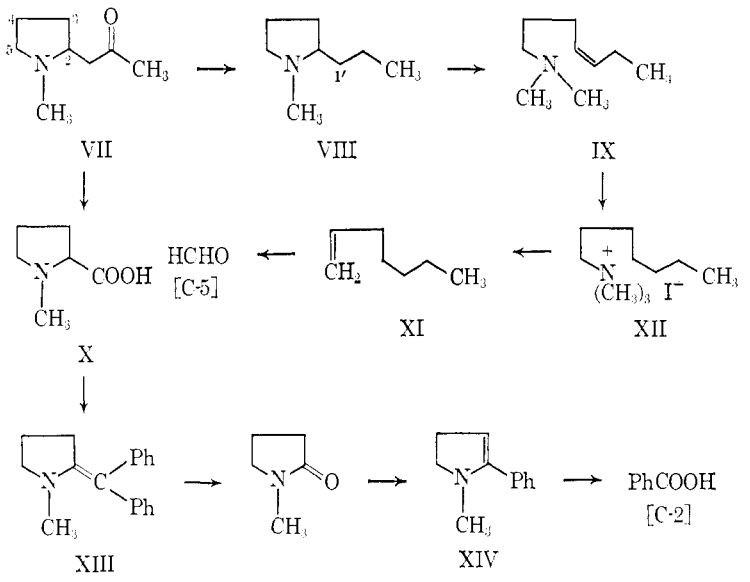

Figure 2. Degradation of the radioactive hygrine.

sodium metaperiodate. In a second series of reactions hygrine was oxidized with chromic acid affording hygric acid (X),[14] which was converted to its ethyl ester and treated with excess phenylmagnesium bromide. The resultant tertiary alcohol was dehydrated by refluxing with *p*-toluenesulfonic acid in acetic acid yielding XIII.[15] Ozonolysis yielded benzophenone and N-methyl-2-pyrrolidone which was treated with phenylmagnesium bromide affording 2-phenyl-N-methyl-Δ^2-pyrroline (XIV).[16] Oxidation of this compound with permanganate yielded benzoic acid (derived from C-2 of hygrine). Activities of these degradation products are recorded in Table I. In experiment 1 no attempt was made to isolate the N-methyl-Δ^1-pyrrolinium salt, and an aqueous acetic acid solution of 4-methylamino-

that the suggested tautomerism of II to III does not occur under the conditions of our experiments. In order to discover whether this tautomerism is possible *in vivo* the N-methyl-Δ^1-pyrrolinium-2-^{14}C chloride was administered to *Nicotiana tabacum* plants which were grown hydroponically. Uptake of the tracer from the inorganic nutrient solution was rapid and the plants were harvested after 3 days. Nicotine was isolated as its diperchlorate and its activity indicated a specific incorporation of 3.3% and an absolute incorporation of 18.2%.[17] Degradation by previously described methods[18] indicated that all the activity of the nicotine was located at C-2′ of the pyrrolidine ring. In Figure 3 we depict a new scheme for the biosynthesis of the pyrrolidine ring of nicotine which is consistent with this result and also enables us to

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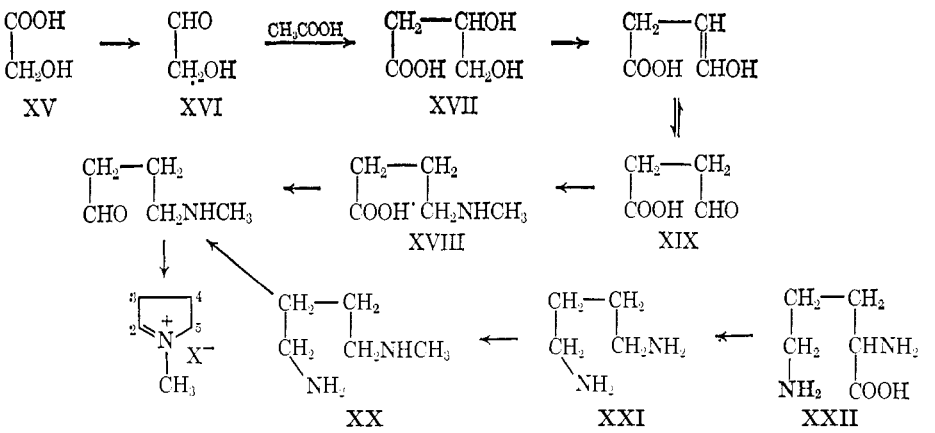

Figure 3. New hypothesis for the biosynthesis of the pyrrolidine ring.

rationalize the unsymmetrical labeling which occurs after a short-term $^{14}CO_2$ feed. We consider that ornithine (XXII) is decarboxylated to putrescine (XXI) which is then methylated yielding N-methylputrescine (XX). This compound has recently been shown[19] to be incorporated intact, without degradation, into the N-methylpyrrolidine ring of nicotine. Oxidation of the primary amino group of N-methylputrescine affords 4-methylaminobutanal,[20] the precursor of the N-methyl-Δ^1-pyrrolinium salt. It has been pointed out by Rapoport[21] that glycolic acid (XV) is one of the initial products of photosynthesis in tobacco leaves, administration of $^{14}CO_2$ yielding glycolic acid with equal labeling on both carbons. This labeled glycolic acid could be reduced to glycolic aldehyde (XVI) which on condensation with acetate would yield 3,4-dihydroxybutyric acid (XVII). Dehydration would afford succinsemialdehyde (XIX), which on transamination and N-methylation yields 4-methylaminobutyric acid (XVIII). Reduction provides the required 4-methylaminobutanal. The participation of active glycolic acid in this tentative scheme would lead to a higher level of activity at C-4 and C-5 of the pyrroline, and a greater amount of activity at C-4′ and C-5′ in the pyrrolidine ring of nicotine, as found by Rapoport.[6] At this time the relative importance of these two pathways cannot be determined; however we are willing to concede that the route from ornithine may represent a minor or aberrant pathway for the biosynthesis of the pyrrolidine ring of nicotine.

Experimental Section

Melting points are corrected. Radioactivity measurements were carried out in a Nuclear Chicago liquid scintillation spectrometer, Model 724, using as solvents either toluene or dioxane–water with the usual scintillators.[22]

4-Chlorobutyronitrile-1-^{14}C. 1-Bromo-3-chloropropane (50 g) dissolved in ethanol (55 ml) was added to potassium cyanide-^{14}C (10 g, 0.5 mcurie)[23] dissolved in water (15 ml) and the mixture refluxed for 3 hr. After cooling, water (60 ml) and chloroform (20 ml) were added, and the heavier chloroform layer separated. The aqueous layer was extracted with more chloroform (six 20-ml portions). The combined chloroform extract was washed with 20% calcium chloride solution (three 30-ml portions) and dried over calcium chloride. After removal of the chloroform the residue was distilled yielding 4-chlorobutyronitrile-1-^{14}C (9.2 g, bp 180–200° (760 mm)).

Ethyl 4-Chlorobutyrate-1-^{14}C. 4-Chlorobutyronitrile-1-^{14}C (9.2 g) was added to absolute ethanol (100 ml) which had been saturated with hydrogen chloride. Water (2.0 ml) was added, and the mixture was refluxed for 16 hr. The residue obtained on evaporation of the reaction mixture was dissolved in water (50 ml) and extracted with benzene (three 50-ml portions). The benzene extract was washed with 10% sodium carbonate solution and then dried over sodium sulfate. Distillation yielded ethyl 4-chlorobutyrate-1-^{14}C (5.2 g, bp 184–186° (760 mm).

N-Methyl-2-pyrrolidone-2-^{14}C. Ethyl 4-chlorobutyrate-1-^{14}C (2.43 g), methylamine (3 ml), and benzene (5 ml) were heated in a sealed glass tube at 110° for 16 hr. Crystals of methylamine hydrochloride (0.9 g) were filtered off and washed with benzene. The combined benzene filtrates were distilled yielding N-methyl-2-pyrrolidone-2-^{14}C (2.3 g, bp 198–202° (760 mm)) having an activity of 5.55×10^4 dpm/mg.

Reduction of N-Methyl-2-pyrrolidone-2-^{14}C and Reaction with Acetonedicarboxylic Acid. Experiment 1. N-Methyl-2-pyrrolidone-2-^{14}C (9.9 g, 2.25×10^5 dpm/mM[24]) was dissolved in ether (200 ml) and lithium aluminum hydride (55 ml of a 2.1% solution) in ether added. The mixture was refluxed for 1 hr, and then added to a mixture of ice (50 g), water (100 ml), and acetic acid (12 ml). The ether was removed from this mixture by evaporation on a rotary evaporator *in vacuo*. The resultant aqueous solution having a pH of 4.5 was allowed to stand at room temperature for 3 hr. Sodium hydroxide (1 *N*, 200 ml) was added to this solution and added to a mixture containing acetonedicarboxylic acid (16 g), 0.1 *M* potassium dihydrogen phosphate (500 ml), 1 *N* sodium hydroxide (250 ml), and water (500 ml). The pH of the resultant solution was 10.2. After standing at room temperature for 5 days the solution was acidified with concentrated hydrochloric acid and evaporated on a steam bath in a current of air until the volume was reduced to 300 ml. This solution was extracted with ether and the residual aqueous solution made strongly alkaline with 25% sodium hydroxide. Continuous ether extraction of this solution for 12 hr yielded crude hygrine which was distilled (6.8 g, bp 75–80° (20 mm). This hygrine was dissolved in ethanol (30 ml) and picric acid (10 g) in hot ethanol (20 ml) added. On standing a short time crystals of *dl*-hygrine picrate (7.1 g), mp 153–154°, separated. This picrate was suspended in a mixture of concentrated hydrochloric acid (10 ml) and water (10 ml) and the picric acid filtered off. The pale yellow filtrate was extracted with ether until colorless and then evaporated. The residual syrup was basified with 25% sodium hydroxide and extracted with ether. Evaporation of the dried (sodium sulfate) ether extract yielded hygrine (2.62 g) which was used in subsequent degradations without dilution.

Experiment 2. The reduction of N-methyl-2-pyrrolidone-2-^{14}C with lithium aluminum hydride was carried out as in experiment 1. However at the end of the reduction period the reaction

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(23) Purchased from Nuclear Chicago Corp.

(24) Material of this activity was obtained by dilution of the previously obtained N-methyl-2-pyrrolidone-2-^{14}C.

mixture was made strongly alkaline by the addition of 25% sodium hydroxide. The ether layer was then extracted with 2 *N* hydrochloric acid (three 50-ml portions). The aqueous solution was evaporated to dryness *in vacuo* at 20°, then finally dried over solid potassium hydroxide in a vacuum desiccator. The semicrystalline residue was completely soluble in chloroform (ethanol free) and had an absorption in the infrared in this solvent at 1665 cm^{-1} due to the quaternary azomethine group. Addition of benzene to this chloroform solution caused the separation of N-methyl-Δ^1-pyrrolinium-2-^{14}C chloride as a pale yellow syrup which could not be obtained crystalline. The salt was hygroscopic and became dark brown after several weeks. The ultraviolet spectrum in 95% ethanol had λ_{max} 267 mμ (ε 2240). This absorption maximum disappeared on the addition of a drop of ammonia to the ethanol solution. Acidification with hydrochloric acid regenerated the maximum at 267 mμ. The N-methyl-Δ^1-pyrrolinium chloride was dissolved in water, made basic by the addition of 1 equiv of sodium hydroxide, and then allowed to react with acetonedicarboxylic acid as described in experiment 1. The resultant hygrine was purified as before *via* its picrate.

dl-**Hygrine Oxime.** Hygrine (0.1 g) was added to a solution of hydroxylamine hydrochloride (0.5 g) in 10% sodium carbonate solution (20 ml), and the mixture was allowed to stand at room temperature for 24 hr. The ether extract of this solution was dried over sodium sulfate and then evaporated leaving a residue which was crystallized from petroleum ether (bp 60–70°) affording colorless needles of *dl*-hygrine oxime, mp 121–122° (lit.[25] 125°).

Degradation of the *dl*-Hygrine-^{14}C. a. To Obtain Activity at C-5. Hygrine (0.934 g) was refluxed with 95% hydrazine (4 ml) for 16 hr. Water and solid potassium hydroxide were then added and the hygrine hydrazone which separated as an oil was extracted with ether. The ether was evaporated without drying and the residual oil dissolved in ethanol (10 ml) in which sodium (1 g) had previously been dissolved. The solution was heated in a sealed tube at 150° for 12 hr. The contents of the tube were acidified with hydrochloric acid and evaporated to dryness. The residue was dissolved in a little water and made strongly alkaline with potassium hydroxide. The N-methyl-2-propylpyrrolidine which separated as an oil was extracted with ether and dried over solid potassium hydroxide. The residue obtained on evaporation of the ether was refluxed with a mixture of methyl iodide (5 ml) and methanol (20 ml) for 4 hr. Attempts to obtain the methiodide crystalline were not successful. The residue obtained on evaporation of the above solution was dissolved in water (20 ml), shaken with silver hydroxide (from 2 g of silver nitrate and sodium hydroxide), filtered, and evaporated to dryness. The residue was heated at 180° (0.01 mm), and the distillate was hydrogenated in ethanol (30 ml) in the presence of platinum oxide (0.4 g) at 2 atm for 2 hr. Methyl iodide (3 ml) was added to the filtered solution. After standing overnight the solution was evaporated and the semicrystalline residue (1.58 g) dissolved in chloroform and chromatographed on Woelm alumina (60 g, activity III). The column was developed with chloroform, then with 3–5% ethanol in chloroform when 1-dimethylaminoheptane methiodide (0.525 g), mp 141–142°, was eluted. This was identical (infrared, mixture melting point) with authentic material.[26] This methiodide was subjected to a Hofmann degradation followed by cleavage of the resultant 1-heptene with osmium tetroxide and sodium metaperiodate using previously described procedures.[27]

b. To Obtain Activity at C-2. Hygrine (0.55 g) dissolved in water (10 ml) containing concentrated sulfuric acid (1.5 ml) was oxidized with chromium trioxide (1.2 g). The resultant hygric acid (0.26 g), mp 160–170°, was isolated by the procedure of Lukeš, *et al.*[14] This hygric acid was dissolved in a mixture of ethanol (15 ml) and benzene (20 ml) containing five drops of concentrated sulfuric acid. After refluxing overnight, sodium carbonate solution was added and the benzene layer separated. The aqueous layer was extracted with more benzene and the combined benzene extracts were washed with 20% calcium chloride solution. Evaporation of the dried (sodium sulfate) benzene solution afforded ethyl hygrate (0.27 g) as a mobile oil, having an infrared spectrum identical with an authentic specimen. Further degradation of the ethyl hygrate was carried out as previously described.[15] Activities of hygrine and its degradation products are recorded in Table I.

Administration of N-Methyl-Δ^1-pyrrolinium-2-^{14}C Chloride to *N. tabacum* and Isolation of the Nicotine. Four *N. tabacum* plants (3 months old) were grown hydroponically, where the roots of the plants were suspended in an aerated inorganic nutrient solution.[28] N-Methyl-Δ^1-pyrrolinium-2-^{14}C chloride (15.5 mg, 1.45×10^6 dpm) was added to the nutrient solutions. Essentially all the activity was absorbed by the roots within 48 hr. After 3 days the plants (wet weight 528 g) were harvested and nicotine was isolated as previously described,[18] as the diperchlorate (179 mg from 117 mg of distilled nicotine). The nicotine was oxidized with concentrated nitric acid[11] yielding nicotinic acid and 3-nitro-5-(3′-pyridyl)-pyrazole. The nicotinic acid was decarboxylated by heating with calcium oxide, the evolved pyridine being collected as its oxalate. The activities of the radioactive nicotine and its degradation products are recorded in Table I.

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