# Studies on the Biosynthesis of the Pyrrolidine Ring of Nicotine*

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ABSTRACT: A degradation of nicotine which resulted in fission of the pyrrolidine ring to yield oxalic acid, methylamine, and 3-pyridyl methyl ketone was developed, which, along with previously utilized degradations, permitted assessment of $^{14}$C in each carbon of the ring. After a biosynthetic period of 2 days with [2-$^{14}$C]acetate, radioactivity was found to be evenly distributed between positions 2 and 5, and also between positions 3 and 4, but the quantity of $^{14}$C in positions 3 and 4 was almost twice as great as that in positions 2 and 5. The labeling pattern was consistent with the hypothesis that acetate was converted by way of the tricarboxylic acid cycle to glutamate which proceeded through a symmetrical intermediate to form the pyrrolidine ring.

When [1-$^{14}$C]acetate (Wu *et al.*, 1962) or [2-$^{14}$C]glutamate (Lamberts and Byerrum, 1958) was administered to tobacco plants, the nicotine synthesized was labeled equally in the 2 and 5 positions of the pyrrolidine ring, and little or no $^{14}$C was incorporated into the 3 and 4 positions. This labeling pattern was consistent with the hypothesis that acetate was converted by way of the tricarboxylic acid cycle to glutamate which then yielded the pyrrolidine ring through a symmetrical intermediate (Lamberts and Byerrum, 1958; Leete, 1958). When additional $^{14}$C-labeled precursors, such as [2-$^{14}$C]acetate, [2-$^{14}$C]propionate, or [1,3-$^{14}$C]glycerol (Wu *et al.*, 1962), were fed to tobacco plants, the nicotine synthesized was labeled in all of the carbons of the pyrrolidine ring. In these experiments, therefore, it was not possible to test the proposed biosynthetic pathway involving the tricarboxylic acid cycle until a degradation could be developed which would permit an estimation of $^{14}$C in each ring carbon. Such a degradation procedure is described in the present report. This degradation has been used to analyze the pyrrolidine ring for $^{14}$C in nicotine isolated from plants metabolizing [2-$^{14}$C]acetate during a 2-day period.

## Experimental

*Growth of Plants and Uptake of $^{14}$C-Labeled Compounds. Nicotiana rustica* plants used in this study were grown from seeds in a constant-temperature growth chamber to a height of 6–7 in. The roots were cut from the plants and new roots were allowed to develop for about 2 weeks in a nutrient solution (Brown and Byerrum, 1952), at which time the root system became a dense mat. The plants were then judged ready to be fed the desired $^{14}$C-labeled compounds. The $^{14}$C-labeled compounds were fed hydroponically by a procedure which permitted their absorption by the plants in a 2- to 3-hour period. (Henderson *et al.*, 1959).

Each plant was fed 1 ml of a solution containing $2.44 \times 10^{-5}$ mole of sodium [2-$^{14}$C]acetate containing 5–10 μc of $^{14}$C. The plants were then allowed to grow for approximately 48 hours under alternating conditions of 12 hours of light and dark. The light intensity at the top of the plants was 500–600 foot-candles. After the 2-day metabolism period, the plants were harvested and nicotine was isolated and purified as the dipicrate (Brown and Byerrum, 1952).

*Degradation of Nicotine.* To obtain sufficient nicotine for degradation, calculated amounts of nonradioactive nicotine were added to the radioactive samples. Three procedures were employed to degrade nicotine to ascertain $^{14}$C in the various positions of the pyrrolidine ring.

The first degradation, described in detail previously (Lamberts and Byerrum, 1958), led to the isolation of carbon 2 of the pyrrolidine ring as well as carbons 3, 4, and 5 of the ring and the *N*-methyl carbon as a group. Nicotine was treated with neutral potassium permanganate, resulting in the formation of potassium nicotinate and 4 moles of potassium bicarbonate per mole of nicotine. The potassium bicarbonate was obtained for counting as $BaCO_3$. Nicotinic acid was isolated and decarboxylated. The carboxyl group, which was recovered as $BaCO_3$, represented carbon 2 of the pyrrolidine ring.

The second degradation of nicotine provided for the isolation of carbon 5 of the pyrrolidine ring. Except for a modification in the preparation of 4-(3′-pyridyl)-4-*N*-methylbenzoylaminobutyric acid, all other steps were followed exactly as described previously (Lamberts and Byerrum, 1958). Nicotine was first treated with bromine to give dibromocotinine perbromide, which was then converted to cotinine by treatment with Zn in 0.6 N HCl. The cotinine was subjected to alkaline hydrolysis which resulted in opening of the lactam ring to yield the unstable compound, 4-(3′-pyridyl)-4-methylaminobutyric acid. This compound was stabilized against cyclization by benzoylation in alkaline medium to yield

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4-(3′-pyridyl)-4-*N*-methylbenzoylaminobutyric acid,[1] which in turn was converted to 4-(3′-pyridyl)-4-*N*-methylbenzoylaminobutyramide by treatment with diazomethane and ammonia. The resulting amide was decarboxylated by the Hoffmann procedure, and the amide carbon, originally carbon 5 of the pyrrolidine ring, was recovered as $BaCO_3$. The amine, 3-(3′-pyridyl)-3-*N*-methylbenzoylaminopropylamine resulting from the decarboxylation, was isolated as a solid thiourea derivative.

The third degradation resulted in a fission of the pyrrolidine ring to yield oxalic acid, methylamine, and 3-pyridyl methyl ketone as shown in Figure 1.

*Preparation of Dibromocotinine.* The preparation of dibromocotinine was initiated from the perbromide of dibromocotinine which had been made by bromination of nicotine according to the method described by Lamberts and Byerrum (1958). Four g of this orange crystalline compound, dibromocotinine perbromide, was suspended in 15–20 ml of water. Sulfur dioxide was bubbled slowly into the suspension with cooling until all of the crystals had gone into solution. Usually, filtration was needed at this point to remove impurities. Solid anhydrous $K_2CO_3$ (*ca.* 3.2 g) was added to the colorless filtrate in small portions until the mixture became definitely alkaline, at which time a copious amorphous precipitate appeared and gas was no longer evolved. When more water (15–20 ml) was added, the amorphous precipitate changed to fine white needles upon cooling. These crude crystals were collected by filtration and rinsed well with cold water. For recrystallization, 8–10 ml of methanol was added slowly to the crude crystals, during which procedure the mixture was heated on a water bath, not above 50°. As soon as the crystals had gone into solution, heat was removed; water was added dropwise until cloudiness persisted, and the mixture was placed in the cold to permit the collection of colorless needles of dibromocotinine. The recrystallized colorless needles were dried in a vacuum desiccator. The compound had the same melting point (125°) as reported by Pinner (1893). The average yield in several experiments was about 2.0 g (yield 87%).

*Hydrolysis of Dibromocotinine.* Dibromocotinine (1.4 g) was suspended in 20 ml of $H_2O$ to which 5 g of $Ba(OH)_2 \cdot 8\ H_2O$ (2.5 mM) was added in a 100-ml micro-Kjeldahl flask. The mixture was refluxed for 15–20 minutes on a steam bath with shaking. The resulting dark-brown liquid was steam distilled until the distillate was no longer alkaline to litmus paper. About 650 ml of distillate (a) was collected, which was made acidic with dilute HCl, and was treated further for isolation of the oxime of 3-pyridyl methyl ketone. The portion (b) that remained in the reaction flask was cooled and saved for the isolation of oxalic acid.

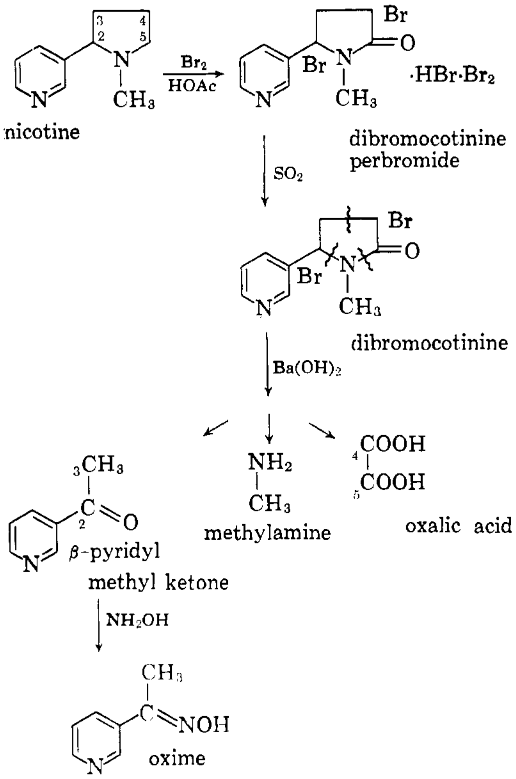

FIGURE 1: Degradation of the pyrrolidine ring of nicotine to isolate carbons 2 and 3 as 3-pyridyl methyl ketone and carbons 4 and 5 as oxalic acid.

*Preparation of Oxime of 3-Pyridyl Methyl Ketone.* The acidified distillate (a) from the steam distillation described was evaporated to dryness on a steam bath with the aid of a stream of air swept over the surface of the liquid. The dry residue (*ca.* 0.2 g), which was a mixture of hydrochlorides of 3-pyridyl methyl ketone and methylamine, was dissolved in 2.0 ml of water and was made alkaline to litmus paper with a dilute NaOH solution. Methylamine was evolved at this point, but was not collected. To the mixture was added a freshly prepared alkaline hydroxylamine reagent for preparation of the oxime (LeForge, 1928).

The mixture was then covered with a watch glass and was heated gently on a steam bath for 10–15 minutes. Fan-shaped crystals appeared when the solution stood at room temperature for at least 12 hours. The colorless crystals of 3-pyridyl methyl ketone oxime were recrystallized several times from hot benzene. The yield was 32 mg, and the melting point was 114.5–115° (cor) as compared with the reported mp, 112° (Engler and Kiby, 1889).

*Anal.* Calcd for $C_7H_8N_2O$ (136): C, 61.76; H, 5.88;

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[1] In order to facilitate the isolation of 4-(3′-pyridyl)-4-*N*-methylbenzoylaminobutyric acid and to improve the yield (to 35%), a modification (Krampl, 1961) of the previously described procedure was employed. Following the removal of the bulk of benzoic acid at *p*H 3.5, the filtrate was extracted several times with ether to remove the residual benzoic acid; the solution was then brought to *p*H 6.5 with dilute $NH_4OH$. The mixture was taken to dryness under reduced pressure. Water (5–10 ml) was added to dissolve the salts, followed by dropwise addition of dilute acetic acid until maximum precipitation of 4-(3′-pyridyl)-4-*N*-methylbenzoylaminobutyric acid took place. The crude precipitate was collected and was recrystallized from methanol and water.

N, 20.58. Found[2] authentic: C, 61.86; H, 5.89; N, 20.42. Found experimental: C, 61.80; H, 5.91; N, 20.62.

*Isolation of Oxalic Acid.* The mixture (b) left in the reaction flask after the steam distillation described earlier was treated as follows in order to isolate oxalic acid which represented carbons 4 and 5 of the pyrrolidine ring. The liquid was stoppered and was allowed to stand for at least 2 days at room temperature. The solid sediment which had settled was collected by filtration, dissolved in 1.0 N HCl, and filtered, if needed, to remove impurities. The acidic solution was taken to dryness under reduced pressure at or below 30°. Excess HCl was removed by sweeping a current of air over the residue. About 450 mg of dry material which contained the oxalic acid was collected. Pure oxalic acid was isolated by vacuum sublimation of the crude residue (*ca.* 1 mm pressure at 50–60°) (Clarke and Davis, 1921). The yield was 55.0 mg; mp 189.5° (cor), mixed mp 189.5–190° (cor).

*Anal.* Calcd for $C_2H_2O_4$ (90): C, 26.66; H, 2.22. Found: C, 26.57; H, 2.36.

For uniform comparison of $^{14}C$ content, all compounds isolated in the degradation were converted to barium carbonate before determining their radioactivities (Van Slyke *et al.*, 1951). Barium carbonate samples were counted in a flow counter (Nuclear Chicago Corp., Model 192). All counts were corrected for self-absorption.

## Results

The results of the three partial degradations of nicotine formed during a 2-day metabolism period in plants fed [2-$^{14}C$]acetate are shown in Tables I through III. All analyses were run in triplicate. The figures presented represent averages of these analyses.

Table I presents the results of the degradation designed principally to ascertain the $^{14}C$ in carbon 2 of the pyrrolidine ring, and it can be seen that carbon 2 possessed 11.1% of the total radioactivity of the nicotine molecule based on the $^{14}C$ analysis of nicotine dipicrate. In addition, 51.1% of the $^{14}C$ was associated with carbons 3, 4, 5, and *N*-methyl of the pyrrolidine ring.

TABLE I: Isolation of Carbon 2 of the Pyrrolidine Ring of Nicotine after Administration of [2-$^{14}C$]Acetate to Tobacco Plants.

| | Specific Activity (cpm/mmole × $10^{-3}$) | Per Cent $^{14}C$ Distribution in Nicotine |
|---|---|---|
| Nicotine dipicrate | 4.5 | 100 |
| Barium carbonate (from carbons 3, 4, 5, and *N*-methyl of pyrrolidine ring) | 2.3 | 51.1 |
| Nicotinic acid | 2.2 | 48.9 |
| Barium carbonate (carbon 2 of pyrrolidine ring) | 0.5 | 11.1 |

In Table II is a summary of results of the second degradation with isolation of carbon 5 of the pyrrolidine ring. Carbon 5 possesses 11% of the $^{14}C$ of the nicotine molecule. It will be noted that the $^{14}C$ in position 5 is approximately equal to the amount located in position 2.

TABLE II: Isolation of Carbon 5 of the Pyrrolidine Ring of Nicotine after Administration of [2-$^{14}C$]Acetate.

| Compound | Specific Activity (cpm/mmole × $10^{-3}$) | Per Cent $^{14}C$ Distribution of Radioactivity |
|---|---|---|
| Nicotine dipicrate | 4.5 | 100 |
| 4-(3′-Pyridyl)-4-methylbenzoylaminobutyric acid | 4.7 | |
| 4-(3′-Pyridyl)-4-methylbenzoylaminobutyramide | 4.8 | |
| Barium carbonate (carbon 5 of pyrrolidine ring) | 0.5 | 11.0 |
| Thiourea derivative of 3-(3′-pyridyl)-3-*N*-methylbenzoylaminopropylamine | 4.1 | 90.4 |

The radioactivity of various compounds obtained in the third degradation, in which oxalic acid and the oxime of 3-pyridyl methyl ketone were isolated, is shown in Table III. The oxalic acid, representing carbon atoms

TABLE III: Degradation of the Pyrrolidine Ring of Nicotine to Isolate Carbons 2 and 3 as 3-Pyridyl Methyl Ketone and Carbons 4 and 5 as Oxalic Acid.

| Compounds | Specific Activity (cpm/mmole × $10^{-3}$) | Per Cent $^{14}C$ Distribution in Nicotine |
|---|---|---|
| Nicotine dipicrate | 4.5 | 100 |
| Dibromocotinine | 4.5 | 100 |
| Oxime of 3-pyridyl methyl ketone | 3.0 | 66.7 |
| Oxalic acid (carbons 4 and 5 of pyrrolidine ring) | 1.3 | 28.9 |

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[2] The microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

4 and 5 of the pyrrolidine ring, possessed 28.9% of the radioactivity of nicotine, whereas the oxime, representing the pyridine ring plus carbons 2 and 3 of the pyrrolidine ring, possessed about 66.7% of the nicotine $^{14}$C activity. If the $^{14}$C content of oxalic acid and of the oxime are subtracted from the total activity of nicotine, the radioactivity in the *N*-methyl group should be obtained. Using this procedure about 4.4% of the $^{14}$C activity of nicotine is calculated to reside in the *N*-methyl carbon.

It was calculated that 17.8 and 17.9% of the radioactivity of the nicotine molecule reside in positions 3 and 4, respectively, of the pyrrolidine ring. The specific activity of carbon 4 was obtained by subtracting the specific activity of carbon 5 (Table II) from the specific activity of oxalic acid (Table III). The specific activity of carbon 3 was obtained by subtracting the specific activity of nicotinic acid (Table I) from the specific activity of 3-pyridyl methyl ketone oxime (Table III).

The results indicate that about 37.7% of the $^{14}$C in the nicotine molecule resides in the pyridine ring after feeding the plants [2-$^{14}$C]acetate for 48 hours. Figure 2 summarizes the results of $^{14}$C analyses performed on the pyrrolidine ring, giving the per cent of $^{14}$C located in each carbon of the ring as well as the calculated $^{14}$C content of the pyridine ring and *N*-methyl group.

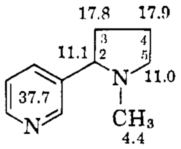

FIGURE 2: Per cent $^{14}$C content in the pyrrolidine ring carbons and other portions of nicotine following administration of [2-$^{14}$C]acetate.

## Discussion

Previous studies from our laboratory (Griffith and Byerrum, 1959; Wu *et al.*, 1962) were concerned with the labeling of the pyrrolidine ring of nicotine following administration of $^{14}$C-labeled acetate, propionate, glycerol, and aspartate. When [1-$^{14}$C]acetate was administered, essentially all of the $^{14}$C within the pyrrolidine ring was equally distributed between positions two and five. The degradation methods then available did not permit localization of all of the radioactive carbon in the pyrrolidine ring when other labeled precursors were fed, but nonetheless suggested formation of the ring by way of the tricarboxylic acid cycle. $\alpha$-Ketoglutarate in the tricarboxylic acid cycle appeared to yield glutamate, which was converted to the pyrrolidine ring with a symmetrical compound as an intermediate. In the present study in which a degradation scheme was described for the pyrrolidine ring which permitted assessment of $^{14}$C in each carbon, the results are again consistent, during a 2-day feeding period, with incorporation of acetate into the pyrrolidine ring by way of the tricarboxylic acid cycle, glutamate, and a symmetrical intermediate.

Evidence is available, however, which suggests that the proposed pathway is not the only mechanism for synthesis of the pyrrolidine ring. For instance, [2-$^{14}$C]-acetate in the 2-day feeding period contributed about 11% of the $^{14}$C of the pyrrolidine ring to the 2 position. If, however, short feeding periods were used such that the carbon of acetate would circulate through the tricarboxylic acid only a limited number of times, less and less $^{14}$C should be found in the 2 position, and more and more should be located in carbons 3 and 4. It was shown, however (Wu *et al.*, 1962), that in feeding periods of 1, 2, and 3 hours, 18%, 20%, and 22%, respectively, of the $^{14}$C in the pyrrolidine ring was located in the 2 carbon. Recently Alworth *et al.* (1964) administered $^{14}CO_2$ to tobacco plants to study the biosynthesis of nicotine, and estimated more $^{14}$C to be in the 3 and 4 positions of the pyrrolidine ring than would be expected if $CO_2$ were converted to glutamate through glycolysis and the tricarboxylic acid cycle. Therefore, indications are either that there are two pathways for glutamate synthesis, as reflected in labeling of the pyrrolidine ring, or that unknown factors such as long-term equilibration of relatively inert metabolic pools affect the observed labeling of the ring.

Since feeding of labeled acetate during short periods has been done exclusively in the light, whereas longer periods of metabolism have involved alternating periods of light and dark, it is possible that one or another pathway might predominate depending on light conditions employed during the experiment. Recent experiments by Hiller (1964), however, suggest that glutamate is synthesized by *Chlorella* via the tricarboxylic acid cycle in both light and dark, and thus speculation that glutamate is synthesized by two different pathways must be explored with further experiments.

It should be noted that experiments concerned with the biosynthesis of the pyrrolidine ring of nicotine can also provide information about the biosynthesis of glutamate since the mechanism of conversion of the carbon chain of glutamate to the pyrrolidine ring seems clear. Thus, it is possible to study the biosynthesis of metabolically active intermediates by studying the biosynthesis of a relatively stable major metabolic product.

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# Immunochemical Studies on Blood Groups. XXXIII. The Effects of Alkaline Borohydride and of Alkali on Blood Group A, B, and H Substances*

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ABSTRACT: Degradation of blood group A, B, and H substances by alkali with or without sodium borohydride causes destruction of almost all of the serine and threonine and of the internal *N*-acetylgalactosamine of the blood group substances. Some destruction of galactose also occurs. Most of the other amino acid constituents remained unchanged but some destruction of arginine and histidine was also found. In the presence of reducing agent, α-aminobutyric acid and additional alanine were formed from threonine and serine and an additional peak was found on the chromatograms after valine. The findings suggest that oligosaccharide side chains bearing the A, B, and H determinants are linked to the serine and threonine probably by an *N*-acetylgalactosamine residue, but the possibility of linkage by a galactose cannot be excluded.

Earlier studies from this laboratory (Schiffman *et al.*, 1964a,b; Lloyd and Kabat, 1964) have shown that treatment of blood group A, B, and H substances with alkali in the presence of sodium borohydride degrades these substances with the appearance of low molecular weight dialyzable reduced oligosaccharides, some of which showed highly potent specific blood group activity as measured by inhibition of precipitation or hemagglutination with their corresponding specific antibodies. In their study of the effects of alkali on mucopolysaccharides, Anderson *et al.* (1964a,b, cf. 1963) reported that a sample of hog mucin A + H blood group substance upon treatment with alkali showed destruction of 8% of its threonine and 28% of its serine, showing that the sugars were released by a β-carbonyl elimination (cf. Ballou, 1954) of *O*-substituted serine and threonine. The reduced oligosaccharide fragments identified (Lloyd and Kabat, 1964) indicated that the oligosaccharide chains liberated by this reaction were further degraded by a peeling reaction (Whistler and BeMiller, 1958; Ballou, 1954) from the reducing end. In connection with our studies on the structure of the blood group substances an effort was made to study the effects of the alkaline elimination reaction with and without borohydride (or borodeuteride) on the amino acid and sugar compositions of the dialyzable and nondialyzable portions of blood group A, B, and H substances. The data confirm and extend the findings of Anderson *et al.* (1964a,b) that selective destruction of serine and threonine occur, and also establish that the *N*-acetylgalactosamine in the interior of the oligosaccharide chain is selectively destroyed in the A, B, and H substances. More recently in studies of a polysaccharide from a human colloid breast carcinoma, alkaline liberation of *N*-acetylgalactosamine was accompanied by destruction of threonine (Adams, 1965). Glycosidic linkages of sugars or amino sugars to serine and threonine have been shown to exist in other polysaccharides (Lindahl and

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