ABSTRACT

THE INÇORPORATION OF ASPARTATE AND MALATE INTO THE PYRIDINE RING OF NICOTINE

by Theodore M. Jackanicz

Nicotinic acid is a precursor of the pyridine ring of nicotine in tobacco metabolism. In animals and certain molds such as Neurospora crassa, tryptophan is metabolized to nicotinic acid. But in higher plants and microörganisms such as Escherichia coli, this relationship does not exist. In previous investigations glycerol and a four carbon dicarboxylic acid, intimately related to the tricarboxylic acid cycle, have been shown to be precursors. But the exact nature of the dicarboxylic acid directly incorporated into the pyridine ring, has not yet been elucidated.

In an attempt to study the incorporation of dicarboxylic acids which might be used for pyridine ring biosynthesis, aspartic and malic acids were employed as possible precursors. Although procedures have been developed previously for the degradation of the pyridine ring, in the present study a method for the isolation of each carbon of the ring was devised which required fewer steps and resulted in a greater over-all yield than was the previous case. Therefore much less nicotine was required in each degradative study.

Aspartate-3-C^{14} and malate-3-C^{14} were hydroponically fed to Nicotiana rustica plants. The nicotine was isolated as the dipicrate, converted to the hydrochloride, and oxidized to nicotinic acid. The nicotinic acid was methylated to trigonelline acid sulfate which was oxidized to N-methyl-2-pyridone-5-carboxylic acid. Decarboxylation of the pyridone-carboxylic acid and catalytic reduction of the ensuing product produced N-methyl-2-piperidone. Hydrolysis of the piperidone, subsequent methylation to the betaine, followed by potassium hydroxide fusion, yielded acetic acid from carbons 5 and 6 of the original pyridine ring, and propionic acid from carbons 2, 3, and 4. The over-all yield of the fatty acids from nicotine was about 15%. Therefore, about 2 g of nicotine was ample starting material for each degradation.

When aspartic acid-3-C^{14} was studied, 57% of the label resided in carbon 3 of the pyridine ring, whereas 38% was in carbon 2. When malic acid-3-C^{14} was employed, 61% of the tracer appeared in carbon 3, whereas 38% was in carbon 2. The remaining small percentage of radioactive carbon was scattered among the other carbon atoms.

These results are discussed and compared to the experiments of other workers in the field of nicotinic acid biosynthesis.

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By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1965

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to Dr. Richard U. Byerrum for his guidance, patience, and encouragement during this investigation.

In addition, I wish to thank the National Institutes of Health for providing funds to aid in this study.

Finally, I wish to thank my wife, Sandra, for uncomplainingly enduring numerous lonely evenings over the past three years.

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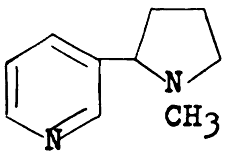
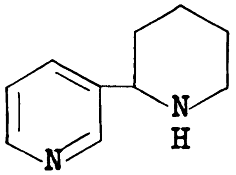
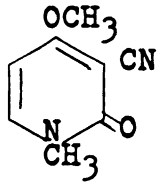
INTRODUCTION

The pyridine ring occurs in several alkaloids as well as the vitamin niacin, and several coenzymes. Previous research has shown that nicotinic acid is a precursor of the pyridine rings of nicotine and anabasine as well as the pyridone moiety of ricinine (1,2,3). Thus the problem of pyridine ring synthesis in nicotine can be viewed as a study of nicotinic acid biosynthesis.


Nicotine


Anabasine


Ricinine

In 1949, Heidelberger et al. found that rats could convert tryptophan to nicotinic acid (4). It was also demonstrated that molds such as *Neurospora crassa* could synthesize nicotinic acid from tryptophan (5). But three separate groups found that tryptophan was not converted to nicotinic acid in higher plants (2,6,7). Yanofsky also reported the same phenomenon in *Escherichia coli* (8). Therefore a different pathway must exist in these latter systems.

Ortega and Brown found that in resting *E. coli* cells, glycerol-1,3-C^{14} or succinate-2,3-C^{14} labeled mainly the pyridine ring of nicotinic acid (9,10). But when succinate-1,4-C^{14} was fed to this organism, most of the label was found in the carboxyl group of nicotinic acid. From their limited degradation data, they concluded that glycerol or a closely related metabolite was condensed with a four carbon dicarboxylic acid in such a way that one of the carboxyl groups of the acid became the carboxyl group of nicotinic acid.

As previously mentioned, nicotinic acid was also found to be a precursor of ricinine. Waller and Henderson fed several C^{14} labeled precursors to castor bean seedlings. They decided, as did Ortega and Brown, that glycerol or a related three-carbon compound condensed with a four-carbon dicarboxylic acid so that one of the carboxyl groups became the carboxyl group of nicotinic acid (11).

Three groups have studied the Nicotiana alkaloids, nicotine and anabasine. Leete found that acetate-2-C^{14} was incorporated into the pyridine ring of anabasine (12). Griffith and Byerrum demonstrated that acetate-2-C^{14} was efficiently incorporated into the pyridine ring of nicotine, and that it was a vastly superior source compared to acetate-1-C^{14} (13). Subsequently they demonstrated that both glycerol-1,3-C^{14} and glycerol-2-C^{14} were extensively incorporated into the pyridine ring. From their incorporation data, they concluded that glycerol was incorporated as a three carbon piece without fission of a carbon-carbon bond (14).

In contrast, Dawson and Christman have studied nicotine biosynthesis in Nicotiana tobaccum and believe that glycerol is converted to a two carbon compound which then condenses with citric acid to yield an intermediate which eventually forms nicotinic acid (15). They based their conclusion on the fact that in their studies, glycerol-2-C^{14} was incorporated twice as efficiently as glycerol-1,3-C^{14} into the pyridine ring. This indicated that one of the labeled carbons in the 1,3-C^{14} labeled species was cleaved.

Up until this point, the biosynthetic studies mainly concerned incorporation studies into the pyridine moiety, with no specific degradation to ascertain the actual location of radiocarbon. Then in 1963, Griffith and Byerrum developed a partial degradation sequence whereby carbons 2, 3, and 6 were isolated, and their specific activities determined (16). After feeding acetate-2-C^{14} and succinate-2,3-C^{14}, they found that about 75% of the label was equally distributed between carbons 2 and 3 with only a few percent located in carbon 6. The radioactivity of the nicotine after feeding succinate was twice as great as nicotine from the acetate.

More recently, Fleeker fed both glycerol-1,3-C^{14} and glycerol-2-C^{14} and determined the specific activity of each carbon atom in the pyridine ring of the biosynthesized nicotine (17). He found that 98% of the glycerol-2-C^{14} was incorporated into carbon 5. When glycerol-1,3-C^{14} was fed, almost 70% of the label was equally divided between carbons 4 and 6, indicating that glycerol can provide carbons 4, 5, and 6 of the ring. Almost 30% of the label resided in carbons 2 and 3. The conversion of glycerol to acetate, via glycolysis, and the entrance of the acetate into the tricarboxylic acid cycle can explain this additional incorporation.

As previously mentioned, Griffith and Byerrum found that succinate-2,3-C^{14} was incorporated into carbons 2 and 3 of the ring. Preliminary experiments in this laboratory with aspartate-3-C^{14} had indicated that about half of the incorporated label resided at carbon 3 of the pyridine ring, and it was postulated that an equal percentage was located at carbon 2. But since no other ring carbons could be isolated at that time, it was not possible to draw any definite conclusions concerning the biosynthesis of the pyridine ring from aspartate. Since both succinate and aspartate were incorporated into the pyridine ring, it would be of interest to see whether aspartate, or another closely related asymetric metabolite, were incorporated with randomization of the label between carbons 2 and 3, or whether the tracer might be specifically distributed, favoring one carbon atom over the other. If the specific activity of carbons 2 and 3 of the pyridine ring were appreciably different after feeding aspartate-3-C^{14}, such results would indicate that aspartate was a more direct intermediate than succinate or some other symmetrical metabolite of the tricarboxylic acid cycle. These results would also disclose perhaps which of the two carboyxl groups becomes the carboxyl group of nicotinic acid.

Several other degradations of the pyridine ring have been published; all of them begin with nicotinic acid as the product of the oxidation of nicotine or anabasine.

Fleeker converted nicotinic acid to 5-trimethylaminopentanoic acid by eight separate reactions (17). The betaine was then cleaved to acetate and propionate which were degraded, via the Schmidt Procedure, to carbon dioxide, and the specific activity of each carbon of the original pyridine ring was determined. Leete has developed a degradation whereby nicotinic acid is converted to 1,3-dimethyl-2-phenylpiperidine (42,47). This product was divided into two samples. One portion yielded carbons 2 and 3 of the original pyridine ring after two additional reactions; the remaining sample was used to obtain carbon 6 after four reactions and finally carbon 5 after three more reactions. Carbon 4 was then obtained by difference.

Finally, Christman and Dawson have published a procedure for degradation of the pyridine ring of nicotine (48). Nicotinic acid was converted to nicotinamide which was methylated to trigonelline amide and then oxidized with potassium ferricyanide. The products of the oxidation were a mixture of the corresponding 2- and 6-pyridones of trigonelline amide, which were separated by column chromatography. The two samples were then separately treated as follows. The amide function was hydrolyzed to a carboxyl group which was then decarboxylated to N-methyl-2-pyridone. One of the pyridone samples would more accurately be termed N-methyl-6-pyridone, since it represented the carbon atoms of the original pyridine ring in a different manner than the other pyridone sample. The pyridone, through six additional reactions, yielded pentanoic acid which was degraded by the Schmidt Procedure to obtain each separate carbon atom of the pyridine ring.

Therefore, the purpose of this study was to investigate the incorporation of aspartate-3-C^{14} and malate-3-C^{14} into nicotine and to develop improved methods for degrading the pyridine ring. Utilizing the degradative procedure developed in this investigation revealed that about 60% of the tracer resided in carbon 3 while about 40% appeared in carbon 2 after feeding both aspartate-3-C^{14} and malate-3-C^{14}. Carbons 4, 5, and 6 contained essentially no radioactivity. Dilution data revealed that aspartate might be a more direct precursor of the pyridine ring than malate.

EXPERIMENTAL AND RESULTS

Preparation of Plants

Due to their relatively high nicotine content, Nicotiana rustica, var. humilus plants were used for the present experiments. The plants were grown in a commercial plant growth chamber at a day temperature of 27°C and a night temperature of 25°C, exposed to 425 foot candles of light for 16 hours per day. The seeds were planted in a flat of vermiculite (commercial name for heat expanded mica) and usually had germinated in ten to fourteen days. Between one and two weeks after germination, each seedling was transplanted into flats, at a distance of 5 cm from its neighbor. The plants were watered as necessary (usually every other day) and once a week were treated with a nutrient solution of 0.1 g $MgSO_4 \cdot 7H_2O$, 0.6 g $Ca(NO_3)_2 \cdot 4H_2O$, and 0.1 g K_2HPO_4 per liter.

At a height of 12-15 cm the plants had reached the optimum state for feeding experiments. Since the rate of de novo nicotine synthesis is proportional to the rate of new root growth (18), the roots were cut to a length of about one cm, and the residual vermiculite rinsed with tap water. A cotton plug was wrapped around the stem of each plant for support and each was placed in a 125 ml Erlenmeyer flask containing sufficient nutrient solution to cover the roots (19). To promote new root formation and decrease the growth of photosynthetic microörganisms, the flasks were wrapped with black construction paper (20). Every four or five days the nutrient solution was discarded, the roots washed with tap water, and new nutrient solution added.

Feeding of Plants

After two to three weeks of hydroponic growth, new root systems had developed and the plants were ready for nicotine synthesis studies. The plants were removed from the Erlenmeyer flasks, the roots rinsed with distilled water, then blotted dry with tissue paper, and inserted into 15 cm x 5 cm beakers containing the desired precursor. Each plant received 0.5 ml of aqueous solution containing 10 uc of C^{14} present as 2.44×10^{-2} mmoles of the compound fed. During the metabolic period, a few drops of water were added to each beaker to keep the roots moist and also to rinse the containers to insure maximum uptake of precursor.

Aspartate-3-C^{14} and malate-3-C^{14}, which were used as precursors in the present study, were purchased from Volk Radiochemical Company. Radioautography indicated that the compounds were radiochemically pure. The specific activity of each sample was checked.

Isolation of Nicotine

After a four hour feeding period, the roots and beakers were rinsed with distilled water, and the washings saved for the later determination of unabsorbed precursor. The plants were cut into small pieces and ground in a Model CB-5 Waring Blendor for one minute at high speed using a ratio of one liter of water per fifteen plants. The slurry was transferred to a three liter, ground glass, round bottom flask and was made alkaline with calcium oxide. The mixture was heated with a burner, the nicotine distilled azeotropically with water, and the mixture collected in a two liter ground glass round bottom flask, containing a few ml of 6N HCl. To avoid charring the plant material, water was added at intervals to the distillation flask so that the volume was never less than one liter.

The distillation was continued until the distillate failed to give a precipitate with silicotungstic acid, a general reagent for detecting alkaloids (21). The distillate was evaporated to dryness _in vacuo_ leaving crude nicotine dihydrochloride. The white precipitate was dissolved in a few ml of water, transferred to a steam distillation flask, made alkaline, and steam distilled. Again the distillate was collected in 6N HCl, evaporated to dryness _in vacuo_, and the crude product dissolved in a small volume of methanol. Addition of an equal volume of picric acid saturated methanol to this solution precipitated nicotine dipicrate as a yellow amorphous mass. The nicotine dipicrate was filtered on a medium porosity fritted funnel, and was recrystallized from water yielding yellow needles melting at 224-226°C*. The reported value is 224-225°C (22).

*All melting points were corrected.

Degradation of Nicotine

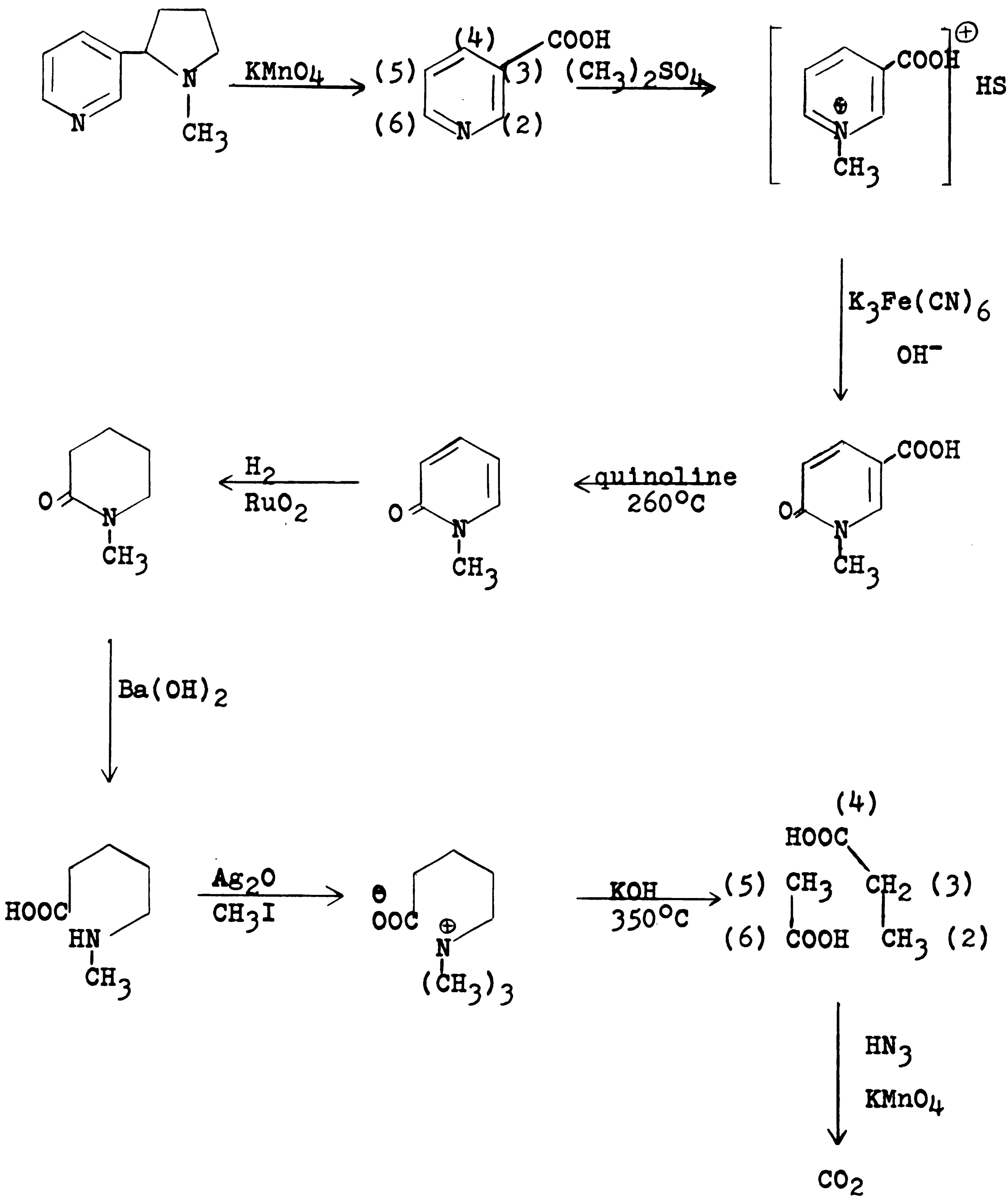
After aspartate-3-C^{14} and malate-3-C^{14} were found to be efficient nicotine precursors, the next step was to degrade the pyridine ring to determine the specific activity of each carbon atom. The degradation sequence is shown in Figure 1.

Nicotinic acid

The weighed nicotine dipicrate was transferred to a steam distillation flask, dissolved in water, and made alkaline with sodium hydroxide pellets. The nicotine was steam distilled into 6N HCl until no precipitate with silicotungstic acid was observed in the distillate. The nicotine dihydrochloride was transferred to a four liter Erlenmeyer flask and about one liter of water was added. The pH was adjusted to 9 with dilute potassium hydroxide. Then two liters of potassium permanganate solution (eight moles per mole of nicotine) was added dropwise over a one hour period while the nicotine solution was stirred vigorously. The flask was heated on a steam bath to complete the reaction and to settle the manganese dioxide which had precipitated (23).

After 16 hours of heating the solution, ethanol was added until the purple permanganate color disappeared. The suspension was filtered first through a medium and then a fine porosity fritted funnel. The manganese dioxide was washed three times with 50 ml portions of hot water and the washings were added to the filtrate. The water was removed

Figure 1.


N
N
CH$_3$
KMnO$_4$
(4)
COOH
(5)
(3)
(6)
N
(2)
(CH$_3$)$_2$SO$_4$
COOH
N
CH$_3$
HSO$_4$
K$_3$Fe(CN)$_6$
OH$^-$
COOH
O
N
CH$_3$
quinoline
260°C
O
N
CH$_3$
H$_2$
RuO$_2$
O
N
CH$_3$
Ba(OH)$_2$
HOOC
HN
CH$_3$
Ag$_2$O
CH$_3$I
OOC
N
(CH$_3$)$_3$
KOH
350°C
(4)
HOOC
(5)
CH$_3$
CH$_2$
(3)
(6)
COOH
CH$_3$
(2)
HN$_3$
KMnO$_4$
CO$_2$

in vacuo, and the white residue of impure nicotinic acid was dissolved in about 50 ml water. Formic acid, 6N, was added to give a solution of pH 1 to release the carbon dioxide resulting from carbons 3, 4, and 5 of the pyrrolidine ring plus the N-methyl carbon. The solution was then titrated to a phenolphthalein endpoint with 2N potassium hydroxide. The solution was concentrated to about 10 to 20 ml, placed on a column of Dowex-1-formate, and eluted by the method of Preiss and Handler (24). For a sample of 750 mg nicotine, a column 60 cm x 3.5 cm sufficed.

Elution of the column with water and three different concentrations of formic acid, released three ultraviolet absorbing species plus nicotinic acid. A typical elution pattern is shown in Figure 2. The peaks were measured by determining the absorption at 261 mu in a Beckman DB Spectrophotometer. The nicotinic acid fractions were combined and the total quantity of compound present determined by measuring the ultraviolet absorption, and comparing it to a standard curve prepared from nicotinic acid samples of known concentration. The identity of nicotinic acid was confirmed by ultraviolet spectroscopy (31), paper chromatography, thin-layer chromatography, and its melting point of 233-235°C. The published melting point is 232°C (20).

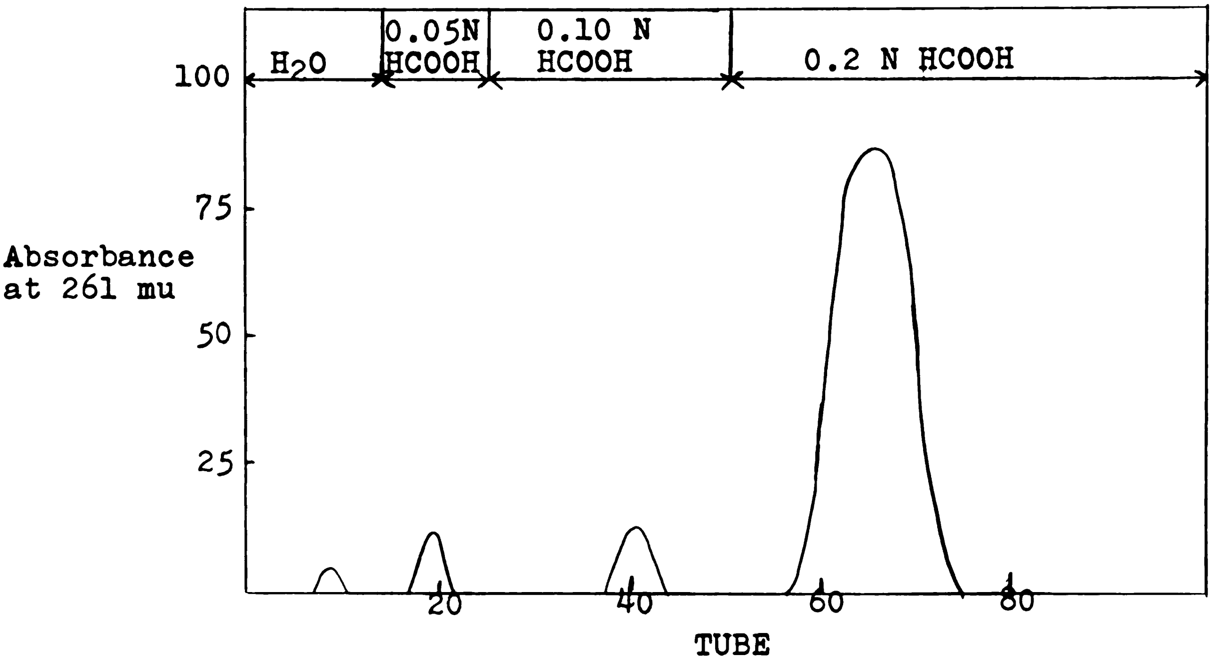


Figure 2. Purification of Nicotinic Acid on Dowex-1-Formate.

<u>Trigonelline acid sulfate</u>

The nicotinic acid obtained in the previous reaction was diluted with cold compound to give 4.3 g of nicotinic acid (35 mmoles) which with 5 ml of dimethyl sulfate were transferred to a 100 ml round bottom flask, equipped with a Liebig condenser and a calcium chloride drying tube. The sample was heated at 130°C for four hours and every 15 to 30 minutes the solution was stirred by means of a magnetic bar (25). The flask was then cooled in ice, and the contents were diluted with 50 ml of water plus 3.5 ml of 0.5 N H_2SO_4.

A little Norite was added, the flask heated on the steam bath for a few minutes, and the mixture was filtered through a medium porosity fritted funnel. The solution was concentrated to about 10 ml by heating on a hot plate, and was then diluted with absolute ethanol to provide a solution 90% in ethanol. Crystals started to form after a few minutes. After about 30 minutes at room temperature, the beaker was placed in the refrigerator for 30 minutes to facilitate further crystallization. The crystals were filtered on Whatman No. 1 filter paper in a Büchner funnel. Concentration of the mother liquid, again followed by addition of alcohol to 90% yielded a second crop of crystals to give a total quantity of 6.4 g of trigonelline acid sulfate which corresponded to 77% yield. Both samples melted at 198-200°C. The recorded value is 199-201°C (25). If one saves the final mother liquid and separately uses it in the next reaction, an additional 300 mg of somewhat less pure N-methyl-2-pyridone-5-carboxylic acid can be obtained.

N-methyl-2-pyridone-5-carboxylic acid

Over a 90 minute period, 71 ml of a 32% solution of potassium ferricyanide was added dropwise to a stirred solution of 6.4 g of trigonelline acid sulfate dissolved in 93 ml 2.5 N sodium hydroxide (26). After 45 minutes of additional stirring at room temperature, the pH was adjusted to 3.7 with 6 N H_2SO_4. Slowly needle-like crystals started to form and after 1 hour at room temperature, the flask was cooled in the refrigerator for an additional hour. The crystals were filtered through Whatman No. 1 filter paper in a Büchner funnel and were washed with ice cold water until they were white. The washings and mother liquid were combined, the pH of the solution was lowered to 3.5, and the above purification steps were repeated to yield a second crop of crystals. A total of 2.7 g of product was obtained which corresponded to 64% yield. An additional 150 mg of product can be obtained by continuous ether extraction of the final mother liquid. The crystals melted at 240-242°C; the published value for N-methyl-2-pyridone-5-carboxylic acid is 240-241°C (26). The compound was further characterized by its ultraviolet absorption peaks at 254 mu and 298 mu (27) and by an elemental analysis which gave the following results:

$C_7H_7NO_3$* Found: C, 54.90%; H, 4.62%; N, 9.13%

(153.18) Calculated: C, 54.90%; H, 4.61%; N, 9.21%

Since N-methyl-2-pyridone-3-carboxylic acid has a melting point of 183-184°C and an ultraviolet absorption maximum at 320 mu (27), there is no question as to the possibility of a mixture of the two acids being present.

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*Microanalysis performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

N-methyl-2-pyridone

All of the pyridone carboxylic acid from the previous reaction (2.70 g), 25 ml of redistilled quinoline, and 900 mg of freshly prepared copper chromite catalyst (28) were placed in a two neck 50 ml round bottom flask. The flask was fitted with a reflux condenser, immersed in a silicone oil bath, and heated at 250-260°C until carbon dioxide was no longer collected in a barium hydroxide trap which was connected to the top of the condenser (29). The reaction was usually completed in about five hours, whereupon the mixture was cooled, and filtered through a medium porosity fritted funnel into a long neck distillation flask. The quinoline was removed by steam distillation. When the distillate no longer gave a precipitate with silicotungstic acid, all of the quinoline had been removed. The residue was extracted five times with 70 ml portions of chloroform. Removal of the solvent left a yellow oil with ultraviolet absorption peaks at 225 and 298 mu. The published values for N-methyl-2-pyridone are 226 and 300 mu (30).

N-methyl-2-piperidone

All of the N-methyl-2-pyridone from the previous reaction, 30 ml of 95% ethanol, and 200 mg of Englehard brand ruthenium dioxide catalyst were transferred to a 100 ml flask designed especially for reductions. The flask was placed in a Paar high pressure rocking bomb under 1000 pounds of pressure for two hours and was slowly heated to

100°C over this period (32). The mixture was filtered through a fine porosity fritted funnel to remove the catalyst. The lack of ultraviolet absorption at 298 mu indicated essentially complete reduction. The solvent was removed *in vacuo* leaving a very pale yellow oil.

N-methyl-5-aminopentanoic acid

All of the product from the previous reaction was refluxed in 75 ml of water for six hours with 6 g of the octahydrate of barium hydroxide (33). The flask was cooled, chips of dry ice were added to precipitate the barium ions, and the barium carbonate precipitate was removed by filtration through Whatman No. 42 filter paper in a Büchner funnel. Removal of the water under reduced pressure left an oil which crystallized under vacuum desiccation and melted at 125-126°C. The reported value for N-methyl-5-aminopentanoic acid is 126-127°C (34).

5-trimethylaminopentanoic acid

All of the acid from the previous experiment was dissolved in 75 ml of methanol and transferred to a 250 ml round bottom flask. Twenty g of freshly prepared silver oxide and 5.0 ml of methyl iodide were added (35). A stirring bar was placed in the flask and a Liebig condenser, fitted with a calcium chloride drying tube, was inserted. The flask was placed in a 70°C oil bath and stirred for 12 hours. During the reaction period, the flask was placed in a dark room to lessen silver salt decomposition.

The silver salts were removed by filtering through a fine porosity fritted funnel, and the solvent was collected in a 50 ml long neck round bottom flask. Evaporation of the methanol yielded an oil which crystallized under vacuum desiccation. The betaine melted at 225-228°C (dec.) which compares favorably with Christman's value of 225°C (dec.) (48).

Propionic and acetic acids

Ten g of potassium hydroxide was added to the betaine and the long neck flask was immersed in a metal bath which had been preheated to 300°C (35). The temperature was quickly raised to 350°C and held there ± 5°C for 10 minutes. The flask was cooled, the contents were dissolved in water, and then transferred to a 125 ml steam distillation flask. All residual volatile alkaline and neutral compounds were removed by steam distillation of about 250 ml of water. Addition of excess 6 N H_2SO_4 to the remaining solution generated propionic and acetic acids which were steam distilled and titrated to pH 8 with standard sodium hydroxide, as determined with a pH meter.

Assuming that equal quantities of acetic and propionic acids were present, the yield of acids from N-methyl-2-pyridone-5-carboxylic acid was about 40%. The water was removed under reduced pressure leaving the sodium salts of the acids, which were dissolved in about 0.2 ml water. Then one drop of phenol red was added, followed by the addition of solid potassium bisulfate until the indicator turned yellow. At this point the distinctive odor of the fatty acids was noticeable. The mixture was extracted with five 3 ml portions of 5% n-butyl alcohol in chloroform and placed on a Chromosorb* column 51 cm x 2.7 cm. The column had been prepared by mixing 70 g of Chromosorb with 56 ml of 0.5 N H_2SO_4 and then suspending the mixture in chloroform previously equilibrated with 0.5 N H_2SO_4 (36).

Elution of the column with chloroform removed the propionic acid; 10% n-butyl alcohol in chloroform eluted the acetic acid. The contents of each tube from the column was mixed with an equal volume of methanol and titrated to a bromothymol blue endpoint with standard sodium hydroxide. See Figure 3 for a typical elution pattern. The tubes containing the two acids, as determined by titration, were treated separately as follows.

The chloroform and methanol were removed <u>in vacuo</u> leaving the sodium salt in a few ml of water. The solution was transferred to a steam distillation flask, acidified with 6 N H_2SO_4, and the fatty acid was steam distilled. A small aliquot was removed for paper chromatography to check for cross contaimination between the two acids or the

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*A coarse type of diatomaceous earth from Fisher Scientific.

presence of other acids (37). The remaining acid was titrated with standard sodium hydroxide to pH 8 as measured with a pH meter, and was concentrated to a known volume. Aliquots were removed for counting and the rest used for further degradations.

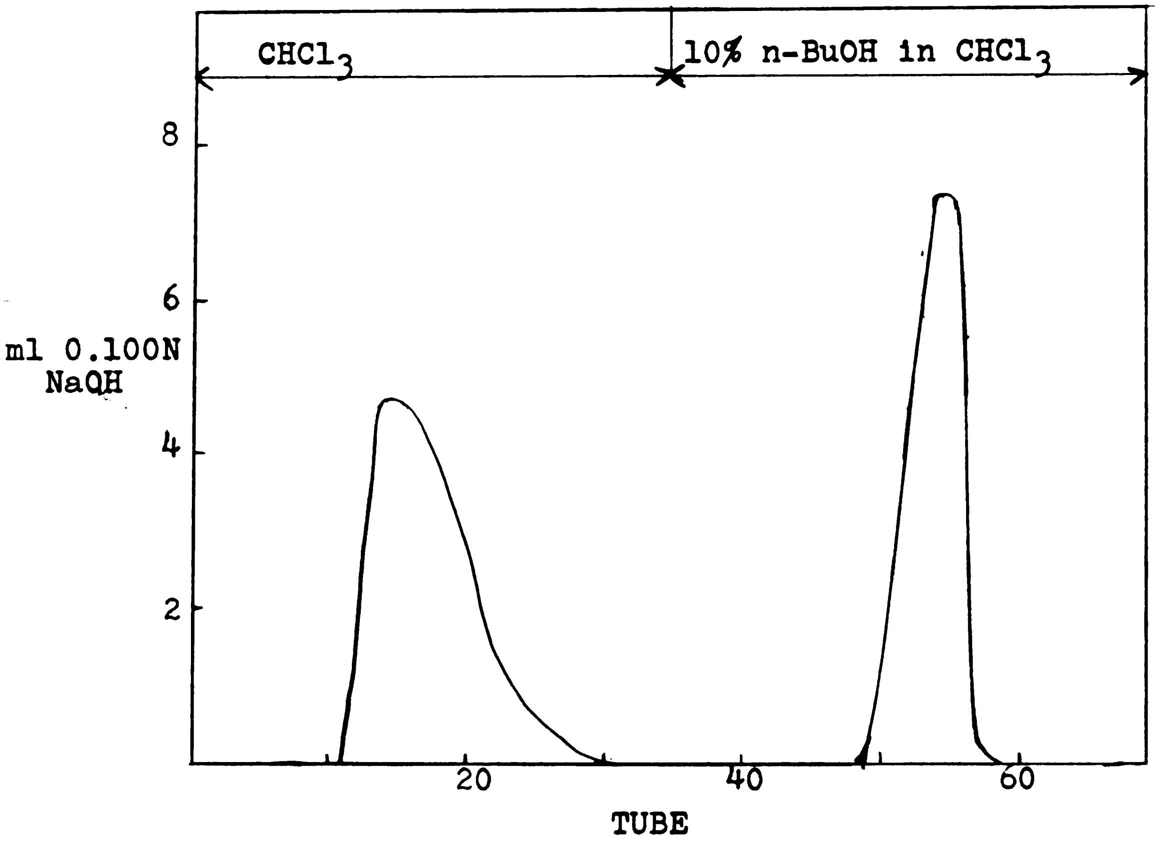


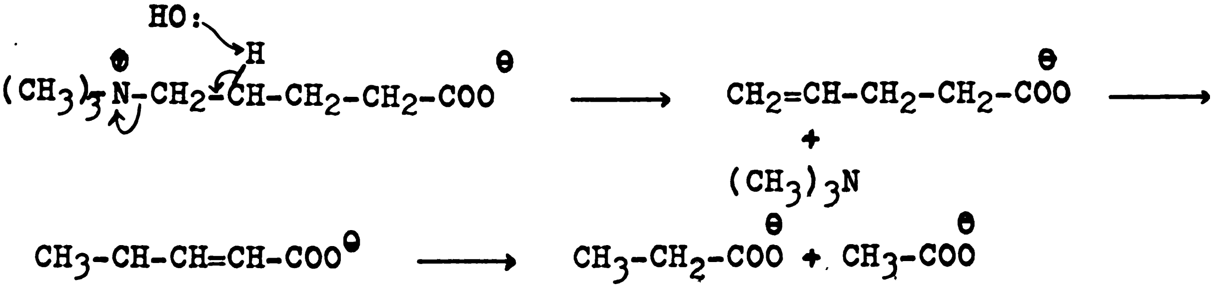
Figure 3. Separation of Propionic and Acetic Acids on Chromosorb.

Mechanism of Cleavage of 5-trimethylaminopentanoic acid

In 1840, Varrentrap found that fusion of potassium hydroxide pellets with oleic acid yielded acetic and palmitic acids (38). Edmed observed a similar phenomenon by converting octadec-2-enoic acid to hexanoic and acetic acids (39). In addition Cornforth and Hunter cleaved 2-hexenoic acid-1-C^{14} to butyric acid plus acetic acid-1-C^{14} (40). Also, Cornforth et al. found that when 6-trimethylaminoheptanoic acid was fused with potassium hydroxide, pentanoic and acetic acids were the only products. All of these reactions bear striking similarities to the cleavage of the pentanoic acid betaine.

As final proof of the mechanism of the cleavage, Fleeker chemically synthesized nicotinic acid-4,6-C^{14} and via a somewhat different degradative scheme than described in the present study, converted it to 5-trimethylaminopentanoic acid (17). Potassium hydroxide cleavage, followed by separation and subsequent radioassay of the acids, indicated that cleavage occurred between carbons two and three of the betaine.

Cornforth postulates a cleavage mechanism as follows:



$$(CH_3)_3\overset{\oplus}{N}\text{-}CH_2\text{-}\overset{\overset{HO:}{H}}{C}H\text{-}CH_2\text{-}CH_2\text{-}CO\overset{\ominus}{O} \longrightarrow CH_2{=}CH\text{-}CH_2\text{-}CH_2\text{-}CO\overset{\ominus}{O} + (CH_3)_3N \longrightarrow$$

$$CH_3\text{-}CH\text{-}CH{=}CH\text{-}COO^{\ominus} \longrightarrow CH_3\text{-}CH_2\text{-}CO\overset{\ominus}{O} + CH_3\text{-}CO\overset{\ominus}{O}$$

Degradation of Propionic and Acetic Acids

For the complete degradation of propionic acid, about 0.5 mmole of the sodium salt was dried under vacuum in a 10 ml pear shaped ground glass flask (41). The flask was cooled in ice, then 0.3 ml of 100% H_2SO_4 was added, and the flask was gently shaken to dissolve the sodium propionate. Again the flask was cooled and 50 mg (0.77 mmole) of sodium azide was added. The mixture was warmed and gently shaken to partially dissolve the azide. The flask was connected to a train consisting of a tube of 5% potassium permanganate in 0.5 N H_2SO_4 and a tube of saturated barium hydroxide to trap the carbon dioxide. Nitrogen was then bubbled through the system and the reaction temperature was raised to 35°C with a water bath. Over a 30 minute period the temperature was raised to 60-70°C, and held there for an additional 30 minutes at which time the reaction was essentially completed. The barium carbonate was filtered, dried and prepared for radioactivity assay.

In order to recover the resulting ethylamine, the permanganate tube was replaced with a tube containing 5 ml of 0.2 N H_2SO_4; 3.5 ml of 5 N NaOH was added to the reaction flask to liberate the ethylamine. The system was swept for 15 minutes with the bath temperature at 90-100°C.

The ethylamine sulfate solution was transferred to a 50 ml round bottom flask containing 5 ml of 5% potassium permanganate, the flask was tightly stoppered, and the mixture was heated in a 90-100°C water bath for 15 minutes. The solution was transferred to a steam distillation apparatus, acidified with 6N H_2SO_4, and the acetic acid steam distilled. The distillate was titrated, concentrated to a small volume and transferred to a 10 ml pear shaped flask where it was dried under vacuum.

The dry sodium acetate (about 0.3 mmoles) was treated in the same manner as the sodium propionate, using proportionally smaller quantities of sulfuric acid and sodium azide. However, the initial bath temperature was 45°C and was raised in 10 minutes to a final temperature of 70°C which was maintained for 45 minutes.

The methylamine was recovered in the same manner as the ethylamine. The methylamine sulfate was transferred to a 25 ml pear shaped flask containing 3 ml of 5% potassium permanganate; 0.6 ml of 5N NaOH was added, the flask was tightly stoppered, and the contents were heated for 15 minutes at 90-100°C. The flask was cooled, connected to the usual train, and 6N H_2SO_4 was added to release the carbon dioxide. The system was swept for 15 minutes with heating, and the barium carbonate was treated in the usual manner.

Determination of Radioactivity

Carbon dioxide was counted as barium carbonate in a Nuclear-Chicago proportional gas flow counter, Model D-47, with an automatic sample changer and Nuclear-Chicago Model

192A ultrascaler. The efficiency was determined and samples corrected for self absorption. All other counting was performed in a Packard Tricarb Scintillation Spectrometer, Model 3314. A one ml aqueous aliquot of the radioactive sample was mixed with ten ml of a solution consisting of 100 g of reagent grade naphthalene, 10 g of 2,5-diphenyl-oxazole, and 250 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene, dissolved in 1 liter of dioxane. All reagents were spectroquality. The efficiency was determined and all compounds checked for quenching. Since the absolute counting efficiencies were calculated, radioactive determinations in both systems could be compared.

RESULTS

As mentioned above, the propionic and acetic acids obtained from the potassium hydroxide cleavage were degraded so that each separate carbon atom of the original pyridine ring could be isolated and its specific activity determined. The results of this investigation are shown in Table 1. When aspartate-3-C^{14} was used as a precursor, 57% of the label resided at carbon 3 of the pyridine ring and 38% at carbon 2. Essentially the same results were observed with malate-3-C^{14} experiments. Sixty-one percent of the radioactivity appeared at carbon 3 of the pyridine ring and 38% at carbon 2. In both experiments, an insignificant percentage of the tracer appeared at carbons 4, 5, and 6. A visual presentation of the data appears in Figure 4.

Dilutions* for aspartate ranged from 360 to 800 with an average value of 600. For malate the values ranged from 1260 to 3270 with an average value of 2200.

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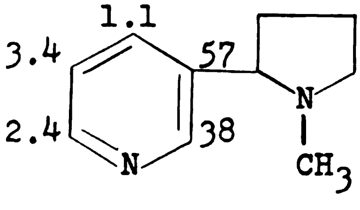
*Specific activity of the precursor divided by the specific activity of the nicotine isolated.

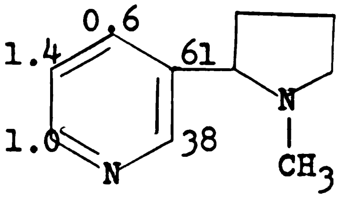
Table 1. Distribution of C^{14} in the Pyridine Ring of Nicotine From Plants Fed Aspartate-3-C^{14} and Malate-3-C^{14}.

	Aspartate-3-C^{14}		Malate-3-C^{14}	
Compound	Sp. Act. cpm/MM	Percent*	Sp. Act.	Percent
N-methyl-5-aminopentanoic acid	8480	100	2350	100
Barium Carbonate (carbon 2)	3220	38	894	38
Barium Carbonate (carbon 3)	4840	57	1430	61
Barium Carbonate (carbon 4)	93.4	1.1	19	0.6
Barium Carbonate (carbon 5)	288	3.4	33	1.4
Barium Carbonate (carbon 6)	204	2.4	24	1.0

*Percentage of radioactivity in a carbon atom as compared to N-methyl-5-aminopentanoic acid.



Nicotine from aspartate-3-C^{14}



Nicotine from malate-3-C^{14}

Figure 4. Percent incorporation of C^{14} for the pyridine ring of nicotine from feeding experiments with aspartate-3-C^{14} and malate-3-C^{14}.

DISCUSSION

The results of the present experiments indicate that aspartic and malic acids are efficient precursors of the pyridine ring of nicotine. Furthermore the two acids seem to be incorporated so that their 2 and 3 carbons respectively, become the 2 and 3 carbons of the pyridine moiety. The presence of label in carbon 2 of the pyridine ring after feeding the 3-labeled precursors can be explained by assuming that some of the malate is dehydrated to fumarate and that some of the aspartate is deaminated to fumarate. Some of the aspartate can also be deaminated to oxalacetate which can then be metabolized to furmarate via malate. Since fumarate is a symmetrical compound, the label is randomized between carbons 2 and 3. The fumarate is then converted back to malate and aspartate. In effect, these malate and aspartate molecules now have their isotope equally distributed between carbons 2 and 3. These randomized species can now enter the pool of the carbon 3-labeled precursors and proceed into the pyridine ring. Of course one must assume that the inter-conversion of these four carbon acids is such as to allow this randomization process to occur to the extent that was observed.

One method of reducing randomization would be to feed the precursor to the tobacco plants for a shorter time period, perhaps for one hour instead of the usual four hours. Although the actual incorporation of carbon-14 into nicotine would decrease during a shorter metabolic period, the 3-labeled precursor would be less susceptible to randomization by interconversion among other four carbon dicarboxylic acids.

Since the label in carbons 2 and 3 of the pyridine ring is not equally distributed after feeding aspartate-3-C^{14} and malate-3-C^{14}, succinate, fumarate, and any other symmetrical four carbon dicarboxylic acid may be eliminated as direct precursors of the pyridine ring. This leaves malate, aspartate, and oxalacetate as possible choices. But perhaps some other unknown four carbon acid could be the actual precursor.

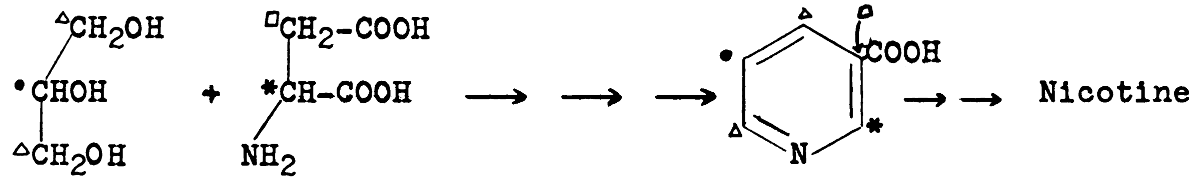
As mentioned earlier, aspartate has a lower dilution factor than malate, and therefore seems to be a more direct pyridine ring precursor. Dilution data, however, possess limited value due to effects such as metabolic pool sizes and turnover rates of these pools. If aspartate is a more direct precursor of carbons 2 and 3 of the pyridine ring than malate, it must have a dilution equal to or lower than malate, since the malate would be diluted by non-radioactive intermediates between malate and aspartate in the pathway. However, if oxalacetate were in effect the actual precursor, conceivably the aspartate might be converted to oxalacetate faster than malate is, and thereby seem to be a more immediate precursor of the ring. On the other hand, if the malate pool were considerably larger than the aspartate pool, the malate-C^{14} would be diluted to a greater extent with non-radioactive compound than aspartate was, and therefore appear to be a more remote ring precursor than aspartate.

In order to determine the isotope distribution in the pyridine ring, it was necessary to devise a degradative procedure which permitted the isolation of each individual carbon atom. The degradation developed in this study proved to be shorter, faster to perform, and required less starting material than previous procedures. Nicotine was oxidized to nicotinic acid which was methylated to trigonelline. Ferricyanide oxidation of this product yielded only N-methyl-2-pyridone-5-carboxylic acid. The corresponding isomer is not observed when trigonelline is oxidized instead of trigonelline amide. After four additional reactions 5-trimethylaminopentanoic acid is obtained. This product, when fused with potassium hydroxide, yielded acetic acids from carbons 5 and 6 of the original pyridine ring, and propionate from carbons 2, 3, and 4. The over-all yield of fatty acids from nicotine was about 15%, and as a result about 2 g of nicotine sufficed for starting material.

As previously mentioned, nicotinic acid is a precursor of the pyridine rings of nicotine and anabasine, as well as the pyridone ring of ricinine. Therefore, subsequent discussions will in effect concern nicotinic acid biosynthesis. Several other research groups studying alkaloid biosynthesis have also been able to isolate carbons 2 and 3 of the pyridine ring after feeding carbon-14 labeled tracers. Leete fed acetate-2-C^{14} to *Nicotiana glauca* plants and isolated radioactive anabasine (42). Essentially all of the pyridine ring activity was equally distributed between carbons 2 and 3. Juby fed succinate-2,3-C^{14} to castor bean seedlings (43). Isolation of ricinine synthesized by these plants and subsequent degradation of it, disclosed that carbons 2 and 3 each contained about 38% of the label. The remaining activity was associated with the cyanogen carbon which originally was the carboxyl carbon of nicotinic acid.

In the two previous experiments, carbons 2 and 3 were equally labeled, and no information could be obtained as to the exact manner in which these precursors were incorporated into the ring. But Gross and co-workers chose to use aspartate-1,4-C^{14},N^{15} in a nicotinic acid biosynthesis study in Mycobacterium tuberculosis (44). They found that 48% of the radioactivity fed could be accounted for in the carboxyl group of the isolated nicotinic acid. Since during biosynthesis of nicotinic acid one of the carboxyl groups of aspartate was cleaved, the maximum expected incorporation of carbon-14 would be 50% of the original precursor. The 48% value is in excellent agreement. Furthermore, their mass spectrometric N^{15}/C^{14} ratio was 0.91, indicating that the nitrogen of the aspartate was incorporated with the carbon skeleton. These observations support the role of aspartate as the four carbon dicarboxylic acid precursor of nicotinic acid.

Since Fleeker found that glycerol-1,3-C^{14} was incorporated into carbons 4 and 6 and that glycerol-2-C^{14} was incorporated into carbon 5 (17), a plausible view of nicotine synthesis is:



Two other research groups have attacked nicotinic acid biosynthesis, but at a point subsequent to the condensation of the two units which form the ring system. Since nicotinic acid is a six carbon compound and the two suspected precursors contain seven carbons, somewhere along the pathway, one carbon atom must be cleaved from one of the intermediates. Of course, there is the possibility that this carbon is lost during the condensation reaction by some type of concerted mechanism. But Andreoli et al. investigated quinolinic acid (2,3-pyridine-dicarboxylic acid) as a possible precursor of nicotinic acid (45). They prepared a cell-free enzyme system from E. coli which was incubated with quinolinic acid and 5-phosphoribosyl-1-pyrophosphate for three hours. They then identified nicotinic acid, nicotinic acid ribonucleotide, deamido-NAD, and NAD as reaction products in a ratio of about 42:15:1:4. These researchers believed that they had prepared a crude enzyme system which catalyzed the condensation of quinolinic acid and 5-phophoribosyl-1-pyrophosphate to the corresponding nucleotide, which was then decarboxylated to nicotinic acid ribonucleotide. Furthermore they postulated that the nicotinic acid obtained was a product of the cleavage of the nucleotide, since when the 5-phosphoribosyl-1-pyrophosphate was omitted from the reaction medium, no nicotinic acid was detected.

In separate experiments, Hadwiger et al. studied quinolinic acid metabolism in two different systems (46). First, quinolinic acid-2,3,7,8-C^{14} was fed to corn seedlings. Radioactive nicotinic acid was isolated from the plants, thus confirming the fact that quinolinic acid can be converted to nicotinic acid. In a second experiment quinolinic acid-2,3,7,8-C^{14} was fed to castor bean seedlings, and radioactive ricinine was isolated. This finding again illustrated that quinolinic acid can be metabolized to nicotinic acid. In addition, quinolinic acid was found to be about 20 times more efficient than nicotinic acid as a ricinine precursor. This phenomenon can be explained by postulating that "free" nicotinic acid is actually not a ricinine precursor. Instead, as in the previously mentioned situation with E. coli, nicotinic acid ribonucleotide appeared to be an intermediate, produced by the decarboxylation of quinolinic acid ribonucleotide. In this case, then, nicotinic acid when fed, would first be converted to the ribonucleotide before being metabolized to ricinine. Perhaps no such enzyme exists which catalyzes the condensation of nicotinic acid with 5-phosphoribosyl-1-pyrophosphate to the ribonucleotide. Then the conversion would be non-specifically mediated by the quinolinic acid enzyme, and therefore proceed at a much slower rate. Or if such an enzyme does exist, the equilibrium might favor cleavage over condensation.

Hadwiger et al. also prepared a cell-free enzyme system from castor bean seedlings which catalyzed the conversion of quinolinic acid to nicotinic acid, nicotinic acid ribonucleotide, and an unknown nicotinic acid derivative (46). These compounds were the products of a four hour incubation period. But when the reaction time was lowered to one hour, nicotinic acid ribonucleotide was the only product observed. Again this result points toward a ribosyl derivative of quinolinic acid as the actual species which is decarboxylated to nicotinic acid ribonucleotide. This product can then be hydrolyzed to nicotinic acid, or proceed via several biosynthetic reactions to various pyridine compounds.

Thus, assuming that aspartate is the four carbon dicarboxylic acid precursor for the 2 and 3 carbons and the carboxyl group, and that quinolinic acid is an intermediate in the pathway, Figure 5 illustrates a possible metabolic sequence for the biosynthesis of nicotinic acid.

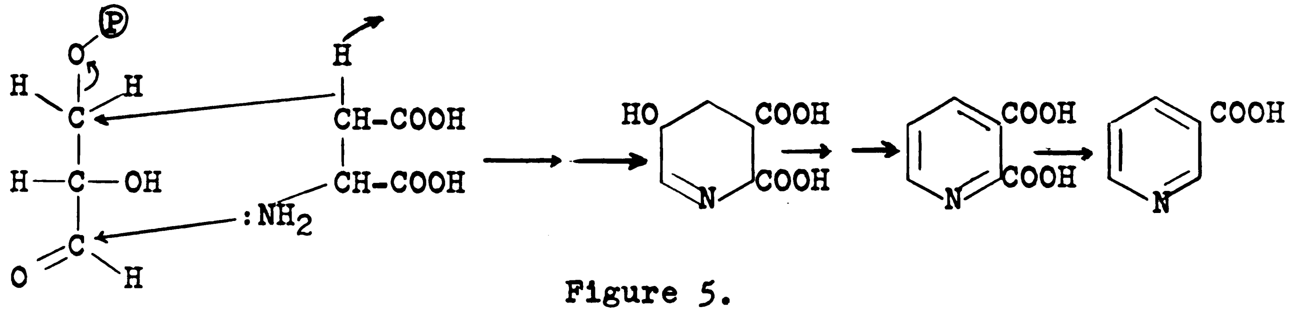


Figure 5.

Leete has recently proposed a biosynthetic scheme similar to the one in Figure 5 (49). He suggests that carbon 3 of glyceraldehyde-3-phosphate is the source of carbon 6 of the pyridine ring, and that carbon 1 of the aldehyde becomes carbon 4 in the ring. Additional information could be obtained concerning the three carbon precursor for the 4, 5, and 6 carbons of the pyridine ring of nicotine by feeding a compound such as glyceraldehyde-3-phosphate, labeled in either carbon 1 or carbon 3. With such an experiment, one should be able to determine the orientation of this three carbon moiety in its incorporation into the pyridine ring and therefore, determine which carbon becomes carbon 6 of the ring and which becomes carbon 4.

The actual determination of the intermediates before quinolinic acid in the biosynthesis of the pyridine ring is indeed a formidable task. One possible method of attack on the problem would be to feed aspartate-C^{14} to tobacco plants for a very short metabolic period, perhaps 15 to 30 minutes. Two dimensional paper chromatography of an extract of the roots of these plants should separate the various carbon-14 labeled compounds present. Using radioautography and standard chromatographic solvent systems, one should then be able to identify aspartate metabolites which are not related to pyridine ring synthesis. Hopefully, one or more unique spots would appear on the chromatograms, corresponding to the possible intermediates shown in Figure 5. After identifying these possible intermediates, they probably could be synthesized and labeled with carbon-14. Feeding these labeled compounds to tobacco plants and isolation of the nicotine, would determine whether genuine precursors had been employed. Of course, the final proof that these are actual precursors, would be the ability of a cell-free tobacco root preparation to convert the suspected intermediates to quinolinic acid or some other pyridine compound. Unfortunately the outlined approach is a very formidable one and requires much additional laboratory investigation. But it is not an unrealistic suggestion and could well help to unravel the pyridine ring enigma.

SUMMARY

1. A unique nicotinic acid degradation was devised which permitted isolation of all of the carbons of the pyridine ring individually.
2. Aspartate-3-C^{14} and malate-3-C^{14} were fed to <u>Nicotiana rustica</u> tobacco plants and found to be incorporated almost exclusively into carbons 2 and 3 of the pyridine ring of nicotine.
3. When aspartate-3-C^{14} was fed, 57% of the tracer resided in carbon 3 and 38% in carbon 2. Malate-3-C^{14} labeled carbon 3 with 61% of the isotope and carbon 2 with 38%.
4. These results are discussed and compared to data of other researchers in the general area of nicotinic acid biosynthesis.

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