THE ROLE OF GLYCEROL IN THE BIOSYNTHESIS OF THE PYRIDINE RING OF NICOTINE

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James R. Fleeker

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This is to certify that the

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L. M. Bjerrum
Major professor

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ABSTRACT

THE ROLE OF GLYCEROL IN THE BIOSYNTHESIS OF THE PYRIDINE RING OF NICOTINE

by James R. Fleeker

The purpose of this study was to determine where the carbon atoms of glycerol were incorporated into the pyridine moiety of nicotine. Glycerol-1,3-C^{14} and glycerol-2-C^{14} were employed as precursors and were fed hydroponically to newly grown roots of intact Nicotiana rustica. The nicotine produced was isolated as the dipicrate. Nicotine was oxidized to nicotinic acid which was then converted to 2-hydroxynicotinic acid via several intermediates. The 2-hydroxynicotinic acid was decarboxylated and the resulting 2-hydroxypyridine reduced to valerolactam. The lactam was hydrolyzed, then methylated to the betaine which was heated in KOH at 350°C for ten minutes. The acetic and propionic acids which resulted from this cleavage were degraded carbon by carbon and the carbon dioxide collected and counted. When nicotinic acid-4,6-C^{14} was degraded in this manner, all the radioactivity was found in the propionic acid, thus the cleavage of the betaine is between the 3 and 4 carbons of the pyridine ring.

Glycerol-1,3-C^{14} labelled the pyridine ring at the 4 and 6 positions, with a significant amount of activity in the 2 and 3 carbon atoms. Glycerol-2-C^{14} labelled the ring almost exclusively in carbon atom 5.

The results of this study are discussed and compared to the results of other studies on compounds containing the pyridine moiety.

THE ROLE OF GLYCEROL IN THE BIOSYNTHESIS OF THE PYRIDINE RING OF NICOTINE

By

James R. Fleeker

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Finally, I would like to thank my wife and children for occasionally doing without my presence during these years of study.

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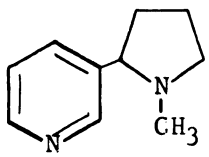
INTRODUCTION

The biogenesis of the pyridine ring of a number of natural products has been under study for some time by several groups of investigators. The compound perhaps receiving the most attention with regard to the biogenesis of its pyridine ring has been nicotinic acid, because of its nutritional role as a vitamin. The early investigations were carried out with animals and microorganisms, eventually tryptophan being found as the general precursor for this substance throughout the animal kingdom and in some microorganisms, such as the fungi (1,2).

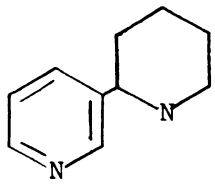
Early investigations by Yanofsky (55) established that tryptophan did not serve as precursor to nicotinic acid in bacteria. An exception to this is Xanthomonas pruni (3). Likewise in higher plants the indole moiety was not incorporated into the vitamin (4). A signal communication by Ortega and Brown (5), late in the last decade, indicated that a four-carbon and a three-carbon compound formed nicotinic acid in Escherichia coli. Shortly after this, these authors (6) reported that in the same organisms, glycerol-1,3-C^{14} as well as succinate-2,3-C^{14} were efficiently incorporated into the ring carbons of nicotinic acid. On the other hand, succinate-1,4-C^{14} provided labelled carbon almost exclusively to the carboxyl portion of nicotinic acid. Due to the lack of a degradation for the pyridine moiety of nicotinic acid, the labelling patterns within the ring were not determined.

Meanwhile a new role for nicotinic acid was found in higher plants-that of precursor for ricinine in Ricinus communis (7,8), and the pyridine rings of anabasine and nicotine in Nicotiana glauca (9) and

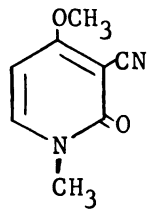
and Nicotiana rustica (10) respectively. Again, tryptophan, labelled with C^{14} in the indole nucleus did not serve as a precursor for nicotinic acid and thus did not bring about incorporation of C^{14} into these alkaloids (11,8).



nicotine



anabasine



ricinine

In an initial investigation by Waller and Henderson (12), succinate-1,4-C^{14} resulted in ricinine that had 75% of the activity in the cyano group, whereas the succinate labelled in the methylene carbons produced only ring labelled alkaloid. Glycerol was also incorporated into the molecule but labelling patterns were not determined.

The results of incorporation studies conducted by Dawson and Christman (13) on nicotine from Nicotiana tobacum led these investigators to conclude that the internal carbons of citric acid formed the 2, 3, and 4 atoms in the pyridine nucleus. However, their data were not supported by C^{14} distribution patterns due to the lack of a degradation for the ring. Since then, this group has developed such a degradation (14) but has not published any findings as to incorporation studies.

The investigations of Griffith and Byerrum (15) contradict the conclusions of Dawson and Christman. The former group found that acetate-2-C^{14} and succinate-2,3-C^{14} labelled the 2 and 3 carbons of the pyridine ring preferentially with almost 80% of the ring activity. The radioactivity residing in these positions was essentially equally divided, in

both cases, between these two positions. The nicotine labelled from succinate-2,3-C^{14} had twice the activity as that obtained from acetate-2-C^{14}.

Griffith et al. (45) reported that glycerol-C^{14} was efficiently incorporated into the pyridine ring of nicotine. But since a complete degradation had not been developed for this ring that would allow isolation of all the carbon atoms, no data were published as to what portion of the pyridine ring is derived from glycerol. The purpose of this study was to develop such a degradation and to determine what carbons of the pyridine ring of nicotine originate from glycerol.

EXPERIMENTAL AND RESULTS

Preparation of the Plants

The plants employed in this study were Nicotiana rustica. They were raised in a plant growth chamber with a constant light source of sixteen hours per day and a constant daylight and a constant night-time temperature. Wooden flats containing vermiculite (heat expanded mica) were used to grow the plants. When the plants were about 12-15 cm tall, the roots were cut off to within a centimeter of the root base and transferred to 125 ml conical flasks containing an inorganic nutrient solution (16). The nutrient solution was replaced every three or four days. The flasks were covered with black paper in order to prevent the growth of algae. At the end of about fifteen days the new root system had developed sufficient branching for the feeding of precursors.

Determination of Radioactivity

Counting of the C^{14} was done with both Geiger and Scintillation methods. Carbon dioxide-C^{14} 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 of the counter was determined and the samples corrected for self-absorption. All other compounds were counted in a Packard Scintillation Spectrometer Model 3314. The counting system was one ml of water containing the material to be counted and 10 ml of counting solution. For the counting solution, 100 g of napthalene, 10 g of 2,5-

diphenyloxazole, and 250 g of 5-phenyloxazoyl-benzene were dissolved in one liter of dioxane (all of these materials were spectrophotometric grade). The compounds that were counted by liquid scintillation were checked for quenching. All samples were counted to 10,000 events.

Administration of Radioactive Precursors and Isolation of Labelled Nicotine

Precursors labelled with carbon-14 were purchased from Volk Radiochemical Company. They were checked for radiopurity by exposure of X-ray film to chromatograms and chemical purity by paper chromatography against authentic non-labelled samples. The C^{14} activity was checked against the radioactivity claimed.

The plants, in batches of 36, were fed the precursors in the following manner: The plants were removed from the nutrient solution, the roots rinsed in water, and patted dry with tissue paper. They were then placed in 125 ml conical flasks containing the precursor. One ml of the precursor, in an aqueous solution containing 2.44×10^{-2} mmoles and 5 uc per ml, was fed per plant. This solution was taken up within two hours, so an additional ml of water was added to the flasks to insure absorption of any remaining material into the roots. After four hours of contact with the feeding solution, the plants roots were quickly rinsed with water, cut up in small pieces, and placed in a 3-liter round bottom flask for distillation. The rinse water was collected and the amount of unabsorbed precursor determined by radioassay.

The plant material was mixed with a liter of water and about 10 g of calcium oxide, then the mixture steam distilled until dilute silico-

tungstic acid showed no alkaloid to be present in the distillate. The distillate was collected in a few ml of 6 N hydrochloric acid. The solution was evaporated to dryness under diminished pressure, then the residue taken up in 0.5 N sodium hydroxide and steam distilled as before into dilute hydrochloric acid. The liquid was again evaporated to dryness and taken up in a minimum amount of methanol. To this was added an equal volume of a saturated solution of picric acid in methanol. The yellow precipitate that resulted was allowed to form over a 60 minute period, then collected on a small funnel. The crystals were washed twice with methanol. Finally the material was taken up in a minimum amount of boiling water and the solution allowed to cool overnight. The slender, yellow, needle-like crystals which resulted were collected on a filter funnel, washed once with water, and dried and stored in a desiccator. The crystals melted at 223-4°C.* The recorded melting point is 224-225°C (17).

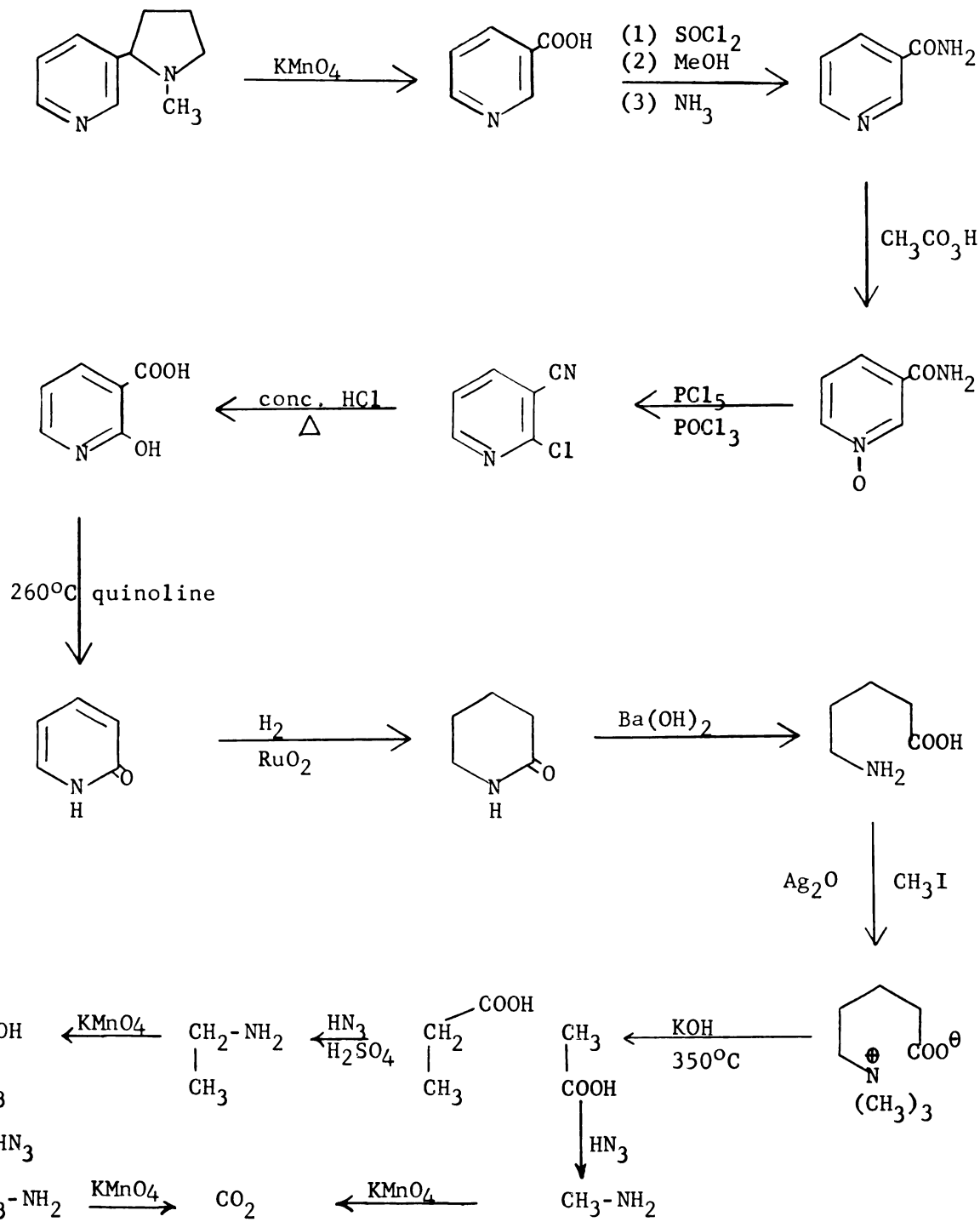
Degradation of Nicotine

In Figure 1 are summarized the degradation reactions employed in this study.

The weighed nicotine dipicrate was steam distilled from a solution of 6 N potassium hydroxide. Complete distillation of the nicotine was tested with silico-tungstic acid. The distillate was diluted to a known volume with water. This solution was then divided into four portions for oxidation, each portion being diluted with enough non-

*All melting points are uncorrected.

Figure 1


KMnO4
COOH
(1) SOCl2
(2) MeOH
(3) NH3
CONH2
CH3CO3H
CONH2
PCl5
POCl3
CN
Cl
conc. HCl
△
COOH
OH
260°C quinoline
H2
RuO2
Ba(OH)2
COOH
NH2
Ag2O
CH3I
KOH
350°C
COO⊖
(CH3)3
HN3
H2SO4
KMnO4
KMnO4
KMnO4
HN3
CO2

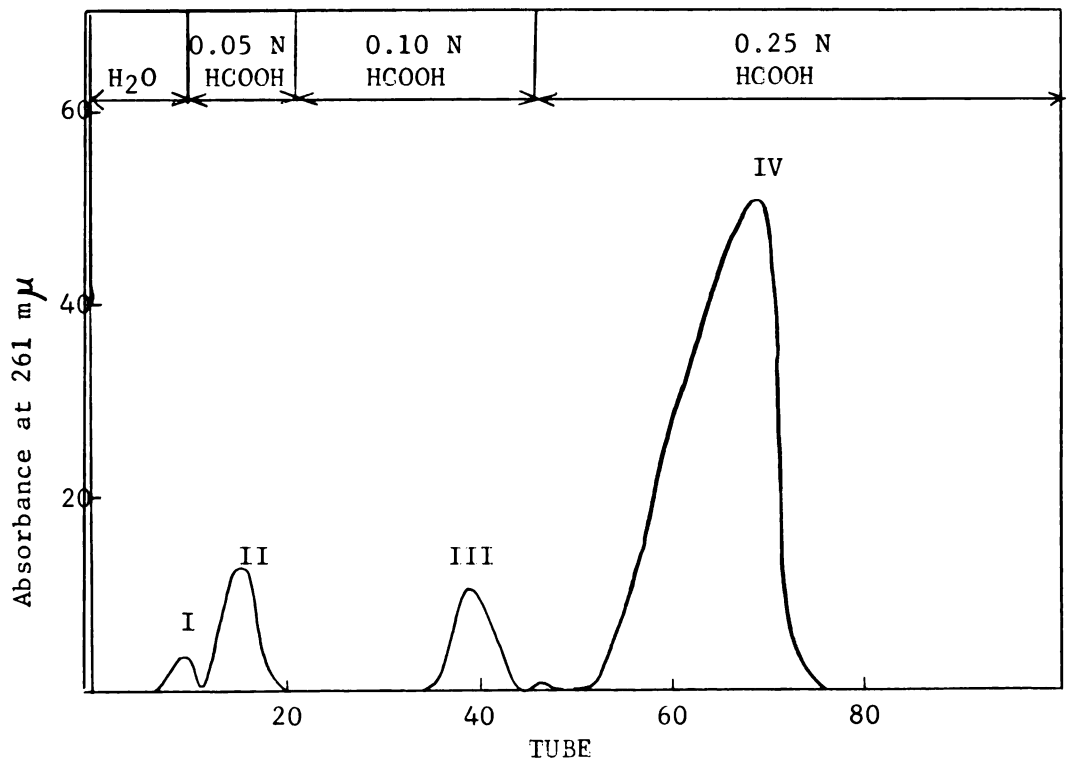
radioactive nicotine to bring the total amount of nicotine in each portion to 2.50 g. This still insured sufficient activity for counting purposes.

The oxidation of nicotine was carried out in a 4 liter conical flask. One of the four portions to be oxidized was placed in the flask and diluted to 1700 ml with water. A large stirring bar was added and the flask placed on a stirring plate. A solution of 19.2 g of potassium permanganate in 1700 ml of water was then added to the vigorously stirred solution over a period of 30 minutes; a molar ratio of 8:1 potassium permanganate to nicotine was employed (18). After the addition of permanganate was completed, the flask was placed on the steam bath for 16 hours. The excess permanganate was destroyed by adding 25 ml of ethanol, after which the solution was cooled somewhat and filtered through a medium-sintered glass funnel. The manganese dioxide which remained on the funnel was washed twice with 200 ml portions of boiling water and the wash water collected with the filtrate. The pale yellow filtrate, cooled by now to room temperature was filtered through a fine-sintered glass funnel; the resulting clear, colorless filtrate was acidified with 6 N hydrochloric acid in order to remove the carbonate from the solution and evaporated to dryness by warming under reduced pressure. The salts were then taken up in a minimum amount of water and titrated to a phenolphthalein end-point with 1 N potassium hydroxide.

The above solution, containing the potassium salt of nicotinic acid, was placed on a Dowex 1-X8 formate column, 15.2 cm^2 x 117 cm, prepared by the method of Preiss and Handler (19). The liquid was allowed to sink

into the resin bed, then the flask which contained the solution was washed with 5 ml of water and this wash solution also allowed to sink into the bed of the column. The Dowex column was then eluted with water, 0.05 N, 0.10 N, and 0.25 N formic acid in that order. Assay

Figure 2. Purification of Nicotinic Acid on Dowex 1-formate 15.2 cm^2 x 117 cm



was by absorbance at 261 mu and 100 ml fractions were collected. Figure 2 shows the elution diagram. There were four main peaks, the nicotinic acid present in peak IV was identified by its ultraviolet absorption spectra (20). Nicotinic acid, which was almost pure, was obtained by pooling the peak IV fractions and evaporating the liquid to dryness by warming under diminished pressure. After desiccation in vacuo the residue gave a melting point of 235-6°C. The recorded value is 232°C (21). The nicotinic acid obtained from this oxidation averaged 72% of the theoretical value or 5.5 g from 10 g of nicotine.

Nicotinamide

The dry nicotinic acid, about 5.5 g or 45 mmoles, was placed in a 300 ml round bottom flask and 9.1 ml (126 mmoles) of thionyl chloride carefully added to this. A condenser equipped with a calcium sulfate drying tube was quickly fitted onto the flask and the solution heated under reflux on the steam bath for two hours (22). At the end of this time the excess thionyl chloride was removed by warming under reduced pressure. The crude nicotinyl chloride hydrochloride was converted to the methyl ester hydrochloride by refluxing with 15 ml of dry methanol for 30 minutes; again the condenser was fitted with a calcium sulfate drying tube. The excess methanol was removed by evaporation under reduced pressure, leaving a clear, colorless oil (22). The flask was plugged with a glass stopper and cooled in an ice bath. After the ester had been cooled, 100 ml of concentrated, aqueous ammonia at 0°C was quickly added to the flask, then the flask stoppered and swirled in an ice bath until the ester was completely in solution. The solution was then kept for 18 to 20 hours at 0°C (23).

In order to obtain the nearly pure nicotinamide, the solution was evaporated to dryness under reduced pressure and the residue dried in a vacuum desiccator. The residue was then extracted four times by refluxing with 400 ml portions of dry benzene for 20 minutes, after which the benzene was decanted off into a 1 liter flask and the solution allowed to cool to room temperature. The resulting nicotinamide crystals were removed by filtration, combined, and dried in an oven at 80°C. The average yield was 4.9 g or 89% of the theoretical. The product melted at 126-7°C, while the reported value is 129°C (23).

Nicotinamide-1-oxide

The nicotinamide, about 40 mmoles, was placed in a 300 ml round bottom flask with 50 ml of glacial acetic acid, then 10 ml of 30% hydrogen peroxide (88 mmoles) cautiously added (24). The solution was refluxed for four hours on a steam bath.

After cooling, the liquid was diluted with 100 ml of water and evaporated to dryness under reduced pressure. The residue was dried in a vacuum desiccator overnight then dissolved in 20 ml of boiling water, 4 ml of ethanol added to this, and cooled to 4°C. The crystals which were obtained were collected on a filter and dried at 100°C for two hours. The pure compound decomposes over a wide temperature range when heated, preventing a melting point determination. The average yield was 4.2 g or 75% of the theoretical from nicotinamide. Elemental analysis gave the following results:

$C_6H_6N_2O_2$*	Found:	C, 52.08%	H, 4.29%	N, 20.35%
(138.12)	Calculated:	C, 52.10%	H, 4.38%	N, 20.30%

*Elemental analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

2-Chloronicotinonitrile

The thoroughly dried nicotinamide-1-oxide, about 30 mmoles, was placed in a 100 ml round bottom flask with 9.3 g of phosphorus pentachloride (45 mmoles) and 12.5 ml of phosphorus oxychloride (25). After the flask had been fitted with a condenser equipped with a drying tube, it was immersed in an oil bath and heated for one and one-half hours at 115-120°C.

The phosphorus oxychloride was removed by evaporation under reduced pressure, care being taken that the 2-chloronicotinonitrile did not sublime. The solid was mixed with 30 ml of an ice-water mixture and allowed to stand overnight at 0°C. The precipitate was collected on a medium-sintered glass filter, then stirred for five minutes with 30 ml of cold 0.5 N sodium hydroxide and the liquid removed by filtration. This was repeated once again and the solid washed with 20 ml portions of cold water until the filtrate was neutral to pH paper. The solid was then dried in a vacuum desiccator. The dried material was placed in a Soxhlet thimble, 33 x 94 mm, over 10 g of anhydrous sodium carbonate. The light brown solid was extracted in a Soxhlet extractor with 100 ml of dry ethyl ether. The ether solution was boiled for a minute with some decolorizing charcoal, filtered, and the resulting colorless filtrate evaporated to dryness, leaving the white crystals of 2-chloronicotinonitrile. After drying, the average yield was 1.6 g or 39% of the theoretical. The melting point was 106-107°C and the reported value is 107°C (24).

2-Hydroxynicotinic acid

The 2-chloronicotinonitrile, about 11.5 mmoles, was placed in a 25 ml flask with 12 ml of concentrated hydrochloric acid. A condenser was placed on the flask and the latter immersed in an oil bath at 125°C for nine hours (24). Upon cooling, long white crystals appeared. The excess liquid was evaporated and the residue taken up in 30 ml of 6 N formic acid. This solution was likewise evaporated to dryness under reduced pressure. The solid was then dried in a vacuum desiccator overnight and recrystallized from a minimum amount of boiling water. The slender, colorless, needles melted at 256°C and weighed between 1.35 and 1.40 g, or 83-85% of the theoretical. The recorded melting point is 256°C. Elemental analysis gave the following results:

$C_6H_5NO_3$	Found:	C, 51.88%	H, 3.68%	N, 10.08%
(139.11)	Calculated:	C, 51.80%	H, 3.62%	N, 10.06%

2-Hydroxypyridine

The apparatus employed for the decarboxylation of 2-hydroxynicotinic acid consisted of a 50 ml reaction flask equipped with a valve controlled inlet tube for nitrogen gas and a microcondenser to prevent the refluxing quinoline from being carried into the barium hydroxide and sulfuric acid traps. At the top of the condenser was a tube leading to the traps. The first trap consisted of 6 N sulfuric acid and two additional traps contained a solution of barium hydroxide (26).

The 2-hydroxynicotinic acid, about 10 mmoles, was placed in the reaction vessel with 20 ml of redistilled quinoline and 0.5 g of copper chromite catalyst, freshly prepared by the method of Lazier and Arnold (27). The reaction flask was immersed in a silicone oil bath and heated be-

tween 250-60°C. Each hour the system was swept with carbon dioxide-free nitrogen and the barium carbonate collected and weighed to determine how far the reaction had proceeded. When over 90% of the theoretical amount of barium carbonate had been collected, about five hours, the oil bath was removed, and the traps disconnected from the condenser. After the reaction solution had cooled somewhat, the liquid was diluted with 15 ml of 1.0 N sodium hydroxide and the quinoline removed by steam distillation. The aqueous solution which remained was acidified with 0.5 N hydrochloric acid and evaporated to dryness. The residue was then taken up in a minimum amount of water and placed on a Dowex 50-X12 sodium column.* The solution containing the 2-hydroxypyridine hydrochloride was allowed to sink into the resin bed and the flask which contained the solution rinsed with 5 ml of water and the wash solution also placed on the column. The column, 47 cm x 8.5 cm^2, was eluted with water and assay was by absorbance at 295 mu. Figure 3 shows the elution diagram. Usually only one ultraviolet absorbing peak was present but occasionally a very minor peak of 2-hydroxynicotinic acid was present. The compounds were identified by their ultraviolet spectra (28,29). The fractions containing the 2-hydroxypyridine were pooled and evaporated to dryness. The melting point was 105-6°C for the residue and the yield averaged 90% of the theoretical, about 840 to 850 mg of product. The recorded melting point is 106-7°C (29).

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*T. M. Jackanicz, unpublished data.

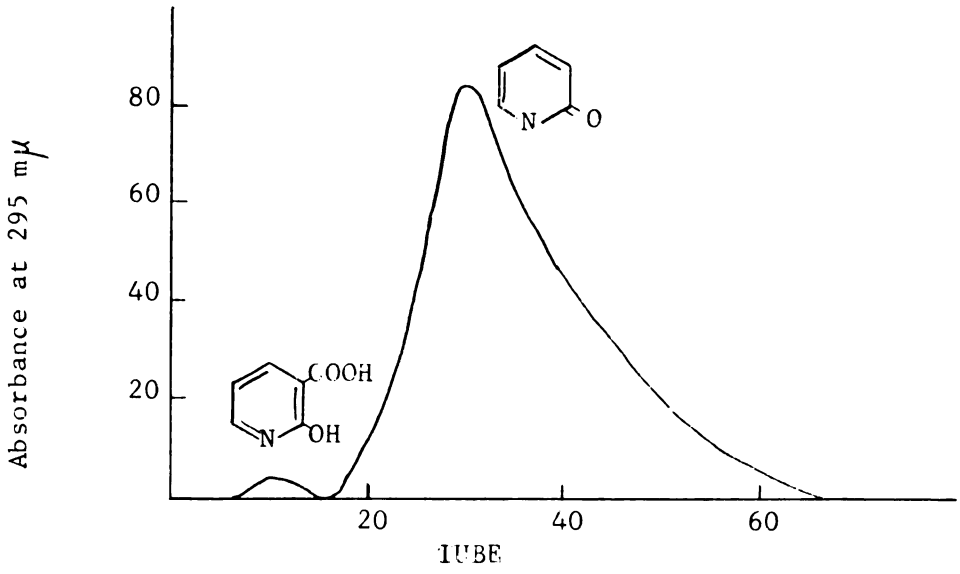


Figure 3. Purification of 2-Hydroxypyridine On Dowex 50-sodium.

δ-Valerolactam

The 2-hydroxypyridine from the preceding reaction was placed in a hydrogenation flask with 30 ml of ethanol and 75-100 mg of Englehardt brand ruthenium dioxide catalyst. The flask was placed in a Paar rocking-high pressure apparatus under 1000 p.s.i. of hydrogen for two hours. During the reaction time the temperature was raised slowly so as to be at 100°C at the end of the reaction period (30). When the apparatus had cooled and the flask removed, the catalyst was separated by filtration. The filtrate was assayed for any unreduced 2-hydroxypyridine by absorbance at 295 mu. The reaction was essentially quantitative in that only a few micrograms of 2-hydroxypyridine remained unreduced. The filtrate was evaporated by warming *in vacuo* to a viscous

colorless oil which crystallized slowly in a vacuum desiccator. The material was not characterized but used directly in the next step of the degradation.

5-Amino pentanoic acid

The crude lactam, about 9 mmoles, was placed in a 250 ml round bottom flask with a solution of 3.15 g of the octahydrate of barium hydroxide (20 meq.) in 30 ml water. The solution was gently refluxed six hours, allowed to cool to room temperature, and brought to pH 6-7 with carbon dioxide (31). After standing 30 minutes, the barium carbonate was removed from the mixture by filtration and the clear and colorless filtrate collected in a 250 ml round bottom flask. The barium carbonate was washed once with water and the filtrate and wash solution evaporated to a viscous oil. By placing the oil in a vacuum desiccator overnight, it crystallized to a white solid. The solid was used directly in the next reaction step, but a non-labelled sample, when recrystallized from ethanol-ether gave a melting point of 156-7°C. The reported value is 154-5°C (32).

5-Trimethylamino-pentanoic acid

The amino acid from the preceding reaction was placed in a 300 ml round bottom flask with 50 ml methanol, silver oxide freshly prepared from 12.0 g of silver nitrate (70 mmoles), and 3.2 ml (50 mmoles) of methyl iodide (33). A stirring bar was placed in the flask and the flask fitted with a Liebig condenser equipped with a calcium sulfate drying tube. The flask was immersed in an oil bath at 70°C and refluxed, with stirring, for 12 hours. During this period, the reaction vessel was removed from light to prevent decomposition of the silver salts.

At the end of the reaction time, the solution was cooled and the silver salts removed by filtration through an F-sintered glass funnel. The clear and colorless filtrate was collected in a round bottom flask and evaporated to a small volume; the solution was then transferred, by rinsing with methanol, to a 50 ml flask with a neck 16 cm long. The solution was then evaporated to a very viscous oil that crystallized on drying in a vacuum desiccator. When a portion of the betaine was re-evaporated with dilute hydrochloric acid, and recrystallized from ethanol-ether, the resulting crystals melted at 213-215°C. No melting point is recorded for this compound in the literature; however elemental analysis gave the following:

$C_8H_{18}NO_2Cl$	Found:	C, 48.83%	H, 9.13%	N, 7.07%
(195.69)	Calculated:	C, 49.09%	H, 9.26%	N, 7.15%

Propionic and Acetic acids

To the dried betaine from the previous reaction was added 10 g of potassium hydroxide. The flask was then placed in a metal bath at 300°C, rapidly heated to 350°C and held at that temperature for ten minutes (24). Early in the reaction a large volume of trimethylamine was released.

Upon cooling the potassium hydroxide solidified and was taken up in water. The solution was acidified with 6 N sulfuric acid and steam distilled, 300 ml of distillate being collected. The distillate was titrated potentiometrically to pH 8.0 with standard potassium hydroxide and evaporated to dryness. The yield of acids at this point, assuming equal amounts of acetic and propionic acid had been produced, was be-

tween 20 and 40%. The poorer yields were obtained when the betaine was not dry, thus it is essential to have the dry crystals for the cleavage reaction.

The acids were separated by column chromatography. The salts were dissolved in about 0.2 ml of water. To this solution a drop of phenol red solution was added and solid potassium bisulfate added until a pink color was obtained. The mixture, containing now the free acids, was extracted five times with 3 ml portions of 5% n-butanol in chloroform. The combined chloroform extracts were then placed on a column prepared from 70 g of Chromosorb*, 56 ml of 0.5 N sulfuric acid (24), and suspended in chloroform (33).

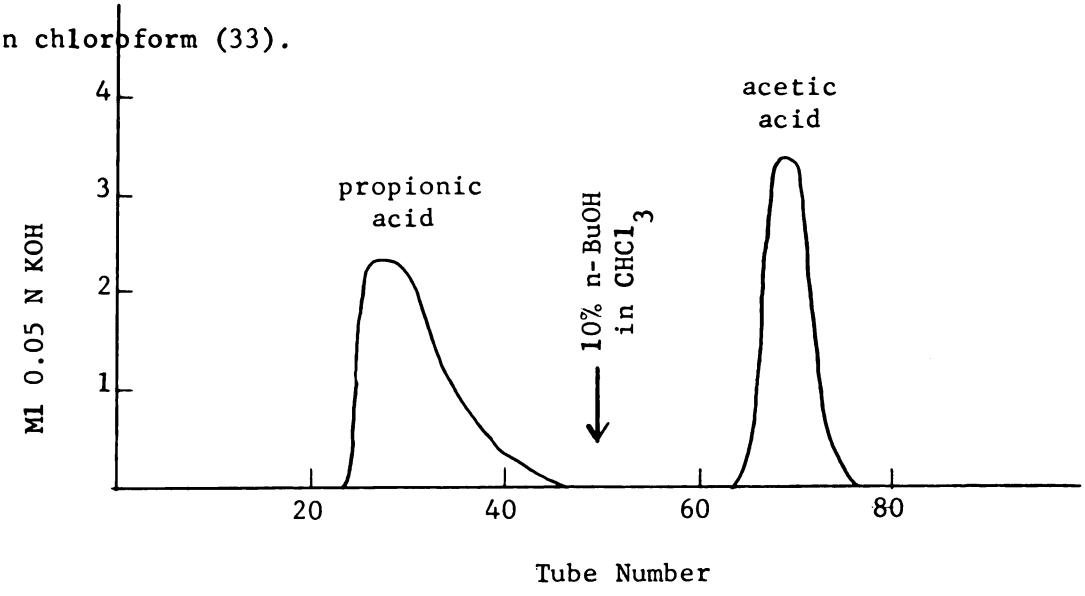


Figure 4. Separation of Propionic and Acetic Acids On A Celite-H_2SO_4-Chloroform Column

*A course type of distomacious earth from Fisher Scientific Co., Chicago, Illinois.

The column was first eluted with chloroform to remove the propionic acid, then with 10% n-butanol in chloroform to remove acetic acid. Figure 4 shows the elution diagram from a non-labelled sample of betaine. With labelled samples, titration with aqueous 0.05 N potassium hydroxide was used only to determine whether elution of a particular acid was complete. In Figure 4 assay of the non-labelled acids was done entirely by titration. Titrations were to a bromthymol blue end-point and an equal volume of ethanol added to the chloroform solution in order to prevent phase separation during titration. Scintillation counting was employed for labelled samples. Three ml of the eluant was mixed with 10 ml of counting solution for this assay.

The fractions from the individual peaks were collected and a few ml of concentrated ammonia added to form the salts of the acids. The solvents were then removed by evaporation.

The potassium salts of the acids were taken up in dilute sulfuric acid and steam distilled. The distillate was titrated as before potentiometrically, with standard potassium hydroxide, then evaporated to a known volume. Aliquots were removed for counting and the rest saved for further degradation.

Carbon by Carbon Degradation of Propionic and Acetic Acid

The degradation of propionic and acetic acids were carried out according to the procedure of Phares (34). The apparatus used for the decarboxylation step consisted of a 25 ml reaction flask, equipped with a sweep-tube, and traps which were connected to the flask. The first tube of the traps consisted of 5% potassium permanganate in 0.5 N sulfuric acid, the next two contained barium hydroxide.

For the degradation of propionic acid, 0.5 mmoles of potassium propionate in solution was placed in the reaction flask and the water evaporated. After cooling the flask, the salt was dissolved in 0.3 ml of 100% sulfuric acid, then recooled and 50 mg (0.77 mmoles) of sodium azide added. The azide was partially dissolved and the flask connected to the traps. The flask was placed in a water bath at 35°C and the temperature raised, over a period of 30 minutes, to 60-70°C. After about 30 minutes at this temperature, the system was swept for 10 minutes with carbon dioxide-free nitrogen. The resulting barium carbonate was plated, dried, weighed, and counted.

The ethylamine was recovered by first replacing the permanganate scrubber with 5.0 ml of 0.2 N sulfuric acid, then adding 3 ml of 5 N sodium hydroxide through the sweep tube and sweeping the system for fifteen minutes with nitrogen, maintaining the bath temperature at 90-100°C.

The ethylamine sulfate solution was transferred to a 25 ml flask and 5 ml of 5% potassium permanganate and 2.5 ml of 1.5 N sodium hydroxide added. The flask was tightly stoppered and placed in a 90-100°C bath for 15 minutes. After cooling, the solution was acidified with sulfuric acid and steam distilled. The distillate was titrated, concentrated, an aliquot removed for counting, and the remaining dried for further degradation.

About 0.3 mmoles of sodium acetate were employed for degradation by the same procedure that was used for propionate, proportionately smaller amounts of sulfuric acid and sodium azide were used. The initial

bath temperature was 45°C and raised over a period of 10 minutes to 70°C, then held at this temperature for 45 minutes. The methylamine was collected in the same manner as the ethylamine. The barium carbonate was also collected as before.

The methylamine sulfate was placed in a 25 ml flask with 3 ml of 5% potassium permanganate and 4.5 ml of 0.5 N sodium hydroxide. After the flask was stoppered, it was placed in a water bath at 90-100°C for 15 minutes. After this period the traps were connected and sulfuric acid added to the solution through the sweep tube. The flask was again placed in the water bath at 90-100°C and the system swept for 15 minutes. The barium carbonate was isolated and assayed as before.

Mechanism of Cleavage of 5-Trimethylamino pentanoic acid

This unusual reaction was first discovered by Varrentrap (35) when he fused oleic acid with solid potassium hydroxide and obtained acetic acid and palmitic acid in good yields. Apparently, the double bond migrated from the 9,10 position to the 2,3 position before fission of the molecule. Edmed (36) later confirmed this using octadec-2-enoic acid.

Cornforth and Hunter studied the mechanism of this type of cleavage using hexenoic acid-1-C^{14} (37). They found that cleavage of the 2-hexenoic acid-1-C^{14} with potassium hydroxide at 350°C gave only unlabelled butyric acid and acetic acid-1-C^{14}. Cornforth et al., have also employed this reaction in the cleavage of 6-trimethylaminoheptanoic acid, an intermediate in their degradation of cholesterol, obtaining, with the pure compound, only acetic and n-pentanoic acids. The pentanoic acid was further degraded to propionic and acetic acids via 2-pentanoic

acid (23). In the case of the betaine they postulated this mechanism: (1) elimination of trimethylamine; (2) migration of the resulting double bond to the 2,3 positions; and (3) cleavage at this position to the two acids (34).

We re-examined this mechanism for 5-trimethylaminopentanoic acid by preparing it from nicotinic acid-4,6-C^{14}. The latter compound was prepared via the reaction sequence in Figure 5.

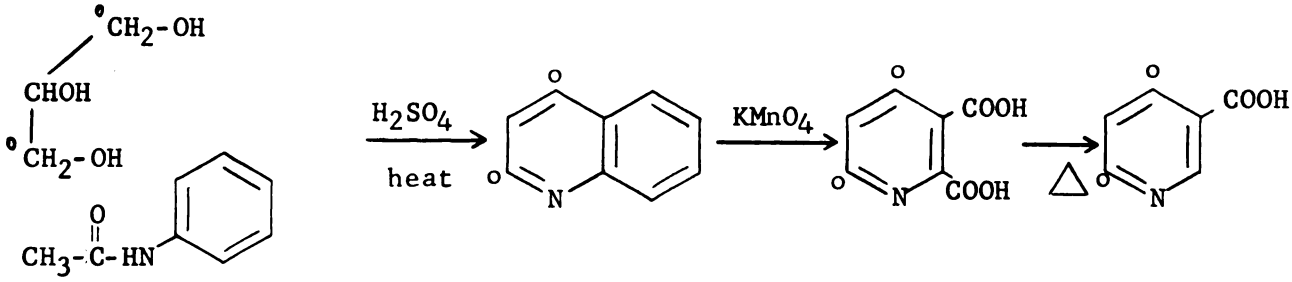


Figure 5. Procedure for the Synthesis of Nicotinic Acid-4,6-C^{14}.

Quinoline-2,4-C^{14}

To a 500 ml round bottom flask containing 100 mg of glycerol-1,3-C^{14} with 0.2 mcuries of radioactivity, the following reagents were added in this order: 21.5 g glycerol, 7.76 g acetanilide, 2.0 g ferrous sulfate, 3.55 g boric acid, 4.2 g nitrobenzene, and 18.2 g concentrated sulfuric acid (36). A reflux condenser with a calcium sulfate drying tube was fitted to the flask. The flask was heated cautiously with a micro-burner to a low simmer. After an half hour of simmering, the solution was heated at a moderate reflux for three hours.

After cooling somewhat, 30 ml of water was added to the solution and the solution steam distilled. A total of 150 ml of water and nitrobenzene were collected; then the solution was cooled once again. A solution of 34 g of sodium hydroxide in 100 ml of water was added carefully to the flask and the free quinoline steam distilled into a 3-liter, 3-necked, round bottom flask. The distillation was continued until no precipitate formed with silico-tungstic acid. The quinoline was not characterized but used directly in the next reaction.

Quinolinic acid-4,6-C^{14}

The solution prepared above was brought to a volume of one liter with water and 7.0 g of potassium hydroxide added. The necks of the flask were fitted with a dropping funnel, Liebig condenser, and a glass stopper. An oil bath was placed under the flask and the solution brought to a gentle reflux. Over a period of three hours, 74 g of potassium permanganate dissolved in 1.5 liters of water were added to the solution through the dropping funnel. After the addition was completed, the solution was refluxed an additional 15 hours (39).

The solution was allowed to cool, filtered through an M-sintered glass funnel, and the manganese dioxide washed twice with 100 ml portions of boiling water. The washings were added to the filtrate. The resulting filtrate was refiltered through an F-sintered glass funnel. The light yellow filtrate was acidified with 6 N hydrochloric acid and evaporated under reduced pressure. The residue was taken up in a minimum amount of water and titrated with 1 N potassium hydroxide to a phenolphthalein end-point. The liquid was placed on a Dowex 1-formate column and purified in the manner as described earlier for

nicotinic acid (19), the assay however was by absorbance at 268 mu. Figure 6 shows the elution diagram for the compound. The quinolinic acid was identified by its ultraviolet absorption spectra (40). After the fractions containing the labelled quinolinic acid were pooled and evaporated to dryness, the material was chromatographed with authentic quinolinic acid. The spots had identical R_f values in two different solvent systems. The labelled material was not weighed or further characterized, though a previous non-radioactive sample, after recrystallization from water, gave a melting point of 194-5°C. The recorded value is 195°C.

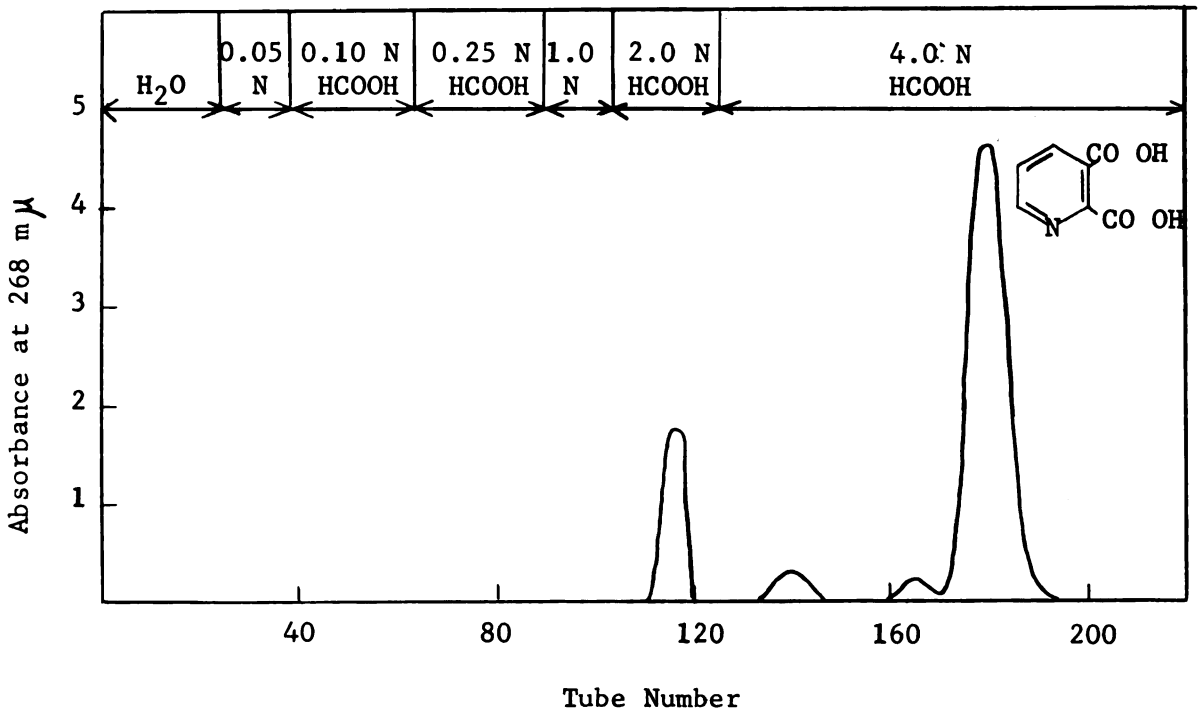


Figure 6. Purification of Quinolinic Acid-4,6-C^{14} On Dowex 1-formate

Nicotinic acid-4,6-C^{14}

The quinolinic acid-4,6-C^{14} prepared above was placed in a 300 ml round bottom flask with 50 ml of cyclohexanol. A Liebig condenser was fitted to the flask. Using a micro-burner, the solution was heated until all the acid was dissolved; this was accompanied by vigorous carbon dioxide evolution (41). When the carbon dioxide evolution had subsided the solution was refluxed an additional 15 minutes, then allowed to cool. The cyclohexanol was removed by steam distillation.

Some norite was added to the light brown solution, then the mixture heated to boiling and filtered hot. The filtrate was evaporated to dryness, and the residue taken up in a minimum amount of boiling water. Upon cooling, white crystals of nicotinic acid separated. These were collected on a funnel. The product weighed 1.33 g or 23% of the theoretical from acetanilide. The melting point was 232-4°C and the specific activity was 1.01 uc/mmole. Chromatography showed only one ultra-violet absorbing spot which was identical to authentic nicotinic acid; and a radioautograph also gave only one spot which was identical to authentic nicotinic acid.

Degradation and Cleavage of Nicotinic acid-4,6-C^{14}

The labelled nicotinic acid was degraded as described above, producing the betaine labelled in the 3 and 5 positions. The propionic acid and acetic acid which were obtained by potassium hydroxide fusion were separated chromatographically on a Celite-sulfuric acid-chloroform column. Every second tube was counted by liquid scintillation and the others titrated. Almost all the radioactivity was found in the

propionic acid; thus the cleavage was between the 2 and 3 positions of the carbon chain.

Results

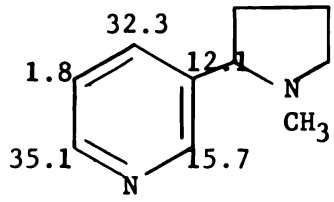
Nicotine from plants fed glycerol-1,3-C^{14} and glycerol-2-C^{14} was degraded by the procedure described above. Each carbon of the pyridine ring was isolated as barium carbonate, the C^{14} measured and the specific activity determined. The activity of the pyridine ring was determined from 2-hydroxypyridine. The data obtained are summarized in Table 1; most of the activity in the pyridine ring of nicotine obtained from plants fed glycerol-2-C^{14} was located in carbon 5. The 2 and 3 positions had little activity while the 4 and 6 positions were almost inactive. In contrast, almost all the carbons in the ring, obtained after feeding glycerol-1,3-C^{14}, had considerable C^{14} with the exception of the 5 position. Almost 70% of the C^{14}, in this case, was divided essentially equally between carbons 4 and 6; the 2 and 3 positions had 15.7 and 12.1% respectively. One may perhaps see the distribution pattern more clearly in Figure 7, where the carbon atoms of the pyridine ring are represented with their respective incorporations, expressed as percent. The ratio of the specific activity of the precursor fed to the specific activity of the nicotine dipicrate isolated is defined as the dilution. The dilutions for glycerol-1,3-C^{14} and glycerol-2-C^{14} are 288 and 316 respectively.

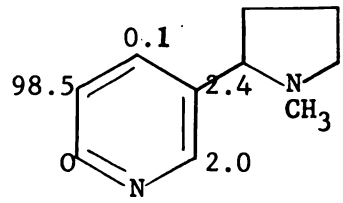
Table 1. Distribution of C^{14} in the Pyridine Ring of Nicotine From Plants Fed Glycerol-1,3-C^{14} and Glycerol-2-C^{14}

Compound	Carbon atom of ring	Glycerol-1,3-C^{14}		Glycerol-2-C^{14}	
		Sp. Act.	Percent*	Sp. Act.	Percent
		mμc/mM		mμc/mM	
Nicotine dihydrochloride**		26.90		335.0	
2-Hydroxypyridine	2,3,4,5,6	15.90	100.0	211.0	100.0
Barium carbonate from carbon 1 of acetic acid	2	2.50	15.7	4.1	2.0
Barium carbonate from carbon 2 of acetic acid	3	1.93	12.1	5.0	2.4
Barium carbonate from carbon 1 of propionic acid	4	5.13	32.3	0.2	0.1
Barium carbonate from carbon 2 of propionic acid	5	0.29	1.8	208.0	98.5
Barium carbonate from carbon 3 of propionic acid	6	5.59	35.1	0	0

*Percent C^{14} in pyridine moiety of nicotine

**Specific activity of nicotine after mixing non-radioactive nicotine with isolated radioactive nicotine.





Nicotine from glycerol-1,3-C^{14} Nicotine from glycerol-2-C^{14}

Figure 7. Percent incorporation of C^{14} for the pyridine ring of nicotine from the administration of glycerol-1,3-C^{14} and glycerol-2-C^{14}.

DISCUSSION

The results of this study provides convincing evidence that the glycerol carbon atoms are incorporated <u>in toto</u> into the 4, 5, and 6 carbon atoms of the pyridine ring of nicotine. This investigation, along with the results of Griffith and Byerrum (15), indicate that the pyridine nucleus is formed from a four-carbon and a three-carbon metabolite. This is illustrated in Figure 8. Griffith and Byerrum demonstrated that the methylene carbons of succinate were incorporated into the 2 and 3 carbons of the pyridine ring, and this finding, along with the results of the present study, strongly suggest a mechanism in which a four-carbon dicarboxylic acid condenses with a three-carbon compound that is closely related to glycerol. The precise nature of the compounds which condense to form the ring is a matter of conjecture at this point of our investigations, but some possibilities will be discussed later.

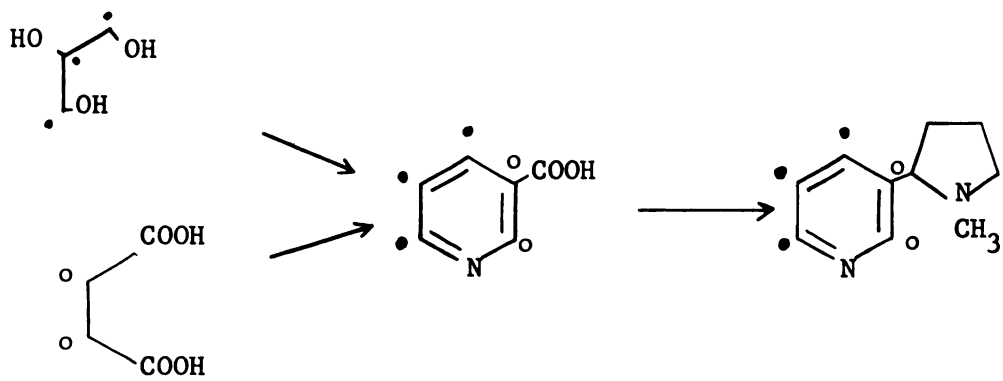


Figure 8.

Returning to the data presented earlier, we see that in the nicotine originating from glycerol-1,3-C^{14}, not all the C^{14} resided in the 4, 5, and 6 atoms of the ring. In fact, almost 30% of the activity was located in carbons 2 and 3. This fact can readily be explained if we consider the metabolism of glycerol. Glycerol can enter glycolysis by phosphorylation and oxidation to dihydroxyacetonephosphate, and can be subsequently metabolized to acetate. The acetate resulting from glycerol-1,3-C^{14} would have C^{14} mainly in the methyl carbon, whereas acetate-1-C^{14} would be formed from glycerol-2-C^{14}. Acetate-1-C^{14} is not incorporated to a significant extent into the pyridine ring (42), whereas acetate-2-C^{14}, and succinate-2,3-C^{14} formed from it via the tricarboxylic acid cycle (TCA cycle), are both efficiently incorporated into carbons 2 and 3 of the ring. Thus, we would expect activity in these two positions from glycerol-1,3-C^{14}. Conversely we would expect little or no activity in these positions of the ring from glycerol-2-C^{14}.

It would be informative here to mention some results from other laboratories on the biogenesis of the pyridine moiety in compounds of plant origin, especially those that have been published since the start of this investigation.

Waller and Henderson (43) working with ricinine from Ricinus communis found that the administration of succinate-2,3-C^{14} and acetate-2-C^{14} resulted in ricinine labelled to a high degree in the 2 and 3 carbon atoms of the ring. Scheidt and his group (44) found that aspartic acid-3-C^{14} labelled ricinine primarily in carbon atom 3.

Assuming identical pathways, these latter results contradicted those of Griffith, Hellman, and Byerrum (45) in that only 45% of the activity in the pyridine ring of nicotine resided in carbon 3 when aspartic acid-3-C^{14} was administered to tobacco plants. They postulated that this amino acid was converted to a symmetrical dicarboxylic acid such as fumarate or succinate before incorporation into the pyridine ring.

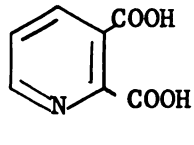
Essery et al. (46) demonstrated that glycerol-1,3-C^{14} was considerably incorporated into ricinine, with 45% of the C^{14} divided between the 4 and 6 carbon atoms, while only 2% was in the number 5 atom of the ring. Glycerol-2-C^{14} labelled the number 5 carbon preferentially with 39% of the C^{14} activity while the 4 and 6 positions were almost inactive. It appears then that a C-4 compound closely related to aspartic acid or succinate as well as a C-3 compound related to glycerol are the natural precursors to ricinine.

Leete and his group, utilizing a degradation developed in their laboratory for nicotinic acid, were able to obtain C^{14} distribution patterns for anabasine, an alkaloid, obtained from Nicotiana glauca, which is an isomer of nicotine (47). Administration of acetate-2-C^{14} led to the information of radioactive anabasine which had 37% of the total activity in the pyridine ring. By degrading this ring it was determined that all the activity was located at carbons 2 and 3 and was distributed equally between these positions. They suggested that the acetate-2-C^{14} entered the TCA cycle, leading to the formation of succinate-2,3-C^{14} which is a known precursor of anabasine (41). This same group of investigators later found that glycerol-2-C^{14} gave

anabasine with 32% of the pyridine ring radioactivity in carbon 5. The 4 and 6 positions had only 2% each (48). Apparently then, in anabasine, the pyridine moiety is derived from a C-4 and a C-3 unit as was the case with ricinine.

As mentioned earlier, the ring atoms of nicotinic acid are incorporated *in toto* into nicotine and ricinine. In a very significant study by Gross et al., using Mycobacterium tuberculosis, aspartic acid-1,4-C^{14}-N^{15} was shown to be incorporated directly, including its nitrogen atom, into nicotinic acid (49). The C^{14} was located in the carboxyl group. Glycerol also serves as a precursor of nicotinic acid in this same organism, but labelling patterns have not been studied for the ring.

Recently Leete (50) has postulated a biogenesis pathway for nicotinic acid in bacteria and plants, a summary of which is shown in Figure 9. This scheme appears to be compatible with our present knowledge, although controversy has arisen over what role quinolinic acid, I, plays in the biosynthesis of nicotinic acid. Lingens (51) found that in nicotinic acid deficient mutants of *E. coli*, there was no growth and cinchomeric acid, II, accumulated. On the other hand, a cell free system from *E. coli* was shown to incorporate quinolinic acid-C^{14} into nicotinic acid and



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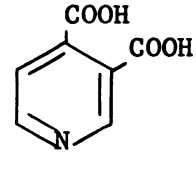

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nicotinamide adenine dinucleotide (52). In regards to multicellular organisms, Dawson and Christmas (53) reported that tritium-labelled quinolinic acid was not incorporated into the nicotine of Nicotiana tabacum, however, this group gave no experimental details. Using both in vivo and in vitro systems, Hadwiger et al. (54) found that Castor plants, Ricinus communis, converted quinolinic acid to nicotinic acid and nicotinic acid mononucleotide. At the same time they reported that corn seedlings also transformed radioactive quinolinic acid to radioactive nicotinic acid.

These investigations led us to conclude that quinolinic acid is probably an intermediate in the synthesis pathway of nicotinic acid. The study on the tobacco plant should be repeated.

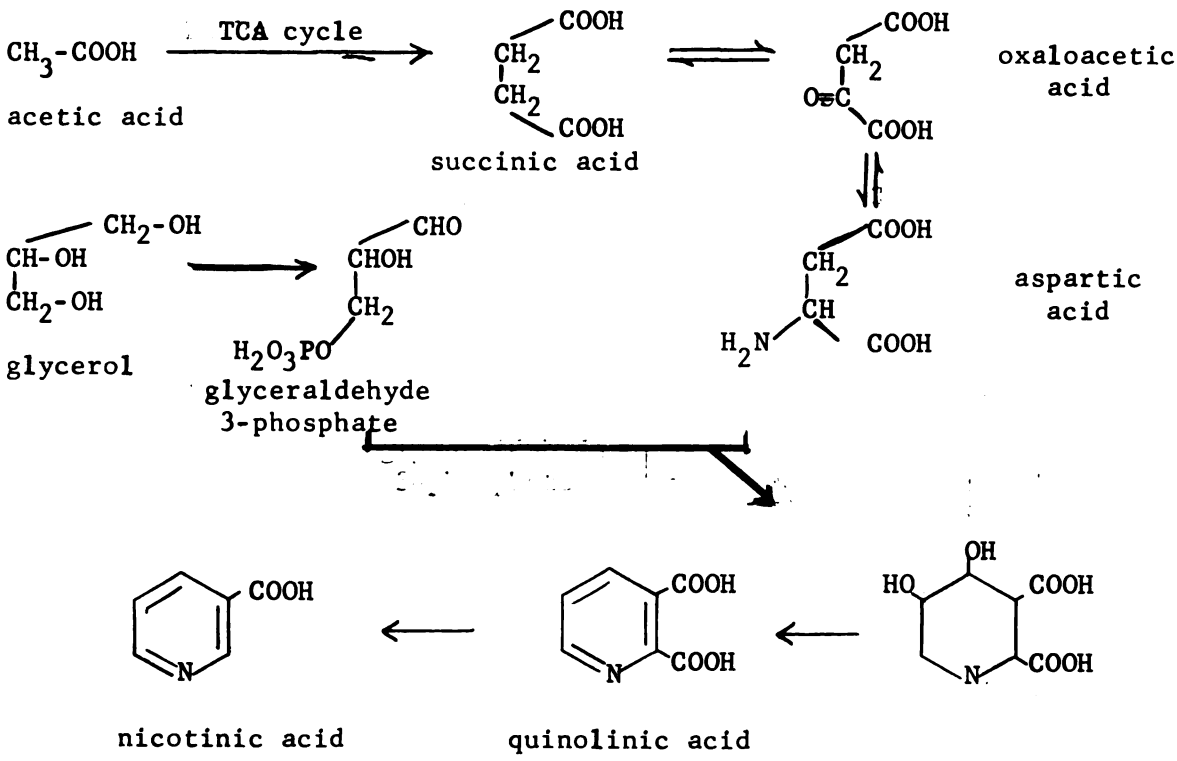

Figure 9.

Leete postulates that the compounds condensing to form the pyridine ring are glyceraldehyde-3-phosphate and aspartic acid. Glyceraldehyde-3-phosphate, which is closely related to glycerol in metabolism, could very likely be the compound that condenses to form the 4, 5, and 6 positions of nicotinic acid. Likewise aspartic acid could contribute the carboxyl group and the 2 and 3 ring positions; however, it is still possible for a compound such as oxaloacetate to condense with the glyceraldehyde and later incorporate the amine group. Aspartic acid is still more reasonable as a precursor if one accepts the results of Mothes using E. coli. The condensation of the methylene carbon of aspartic acid with the aldehyde group of glyceraldehyde-3-phosphate would be similar to the condensation of oxaloacetate and acetyl-coenzyme A by the ubiquitous condensing enzyme.

Since the metabolites contributing to the pyridine ring in plants and bacteria are active in metabolism, the best way of demonstrating the pathway would be through condensation of the proposed precursors in the presence of isolated enzymes responsible for the chemical conversions involved. If the biogenesis of nicotinic acid in bacteria and in higher plants includes the same intermediates, and indeed this appears to be so, then plants are probably not the best organisms with which to conduct initial enzyme studies. Bacteria are much easier and quicker to grow, and also offer the use of mutants. Of course, the final chemical conversion of nicotinic acid to the alkaloid under study must be carried out using plant extracts. In the meantime, synthesis of likely precursors with radio-labelling can still give useful information on possible intermediates involved in nicotine and nicotinic acid synthesis in plants.

SUMMARY

1. A degradation procedure was devised for nicotinic acid, permitting the isolation of all the carbons of the molecule.
2. Isotopically labelled glycerol was administered to Nicotiana rustica and found to be incorporated in toto into the 4, 5, and 6 carbons of the pyridine moiety of nicotine.
3. Glycerol-2-C^{14} labelled the pyridine ring with 98% of the activity in carbon 5. Glycerol-1,3-C^{14} produced radioactivity throughout the pyridine ring. Between carbons 4 and 6, almost 70% of the radioactivity was equally divided, whereas carbon 5 had less than 2%.
4. These results are discussed and compared to the results of other workers investigating the pyridine moiety of natural compounds of plant and bacterial origin.

BIBLIOGRAPHY

1. C. Heidelberger, E. P. Abraham, and S. Lepkovsky, J. Biol. Chem., 179, 151 (1949).

2. C. W. H. Partridge, D. M. Bonner, and C. Yanofsky, J. Biol. Chem., 194, 269 (1952).

3. D. Davis, L. M. Henderson, and D. Powell, J. Biol. Chem., 189, 543 (1951).

4. J. Grimshaw and L. Morrion, Nature, 181, 112 (1958).

5. M. V. Ortega and G. M. Brown, J. Am. Chem. Soc., 81, 4437 (1959).

6. M. V. Ortega and G. M. Brown, J. Biol. Chem., 235, 2939 (1960).

7. E. Leete and F. H. B. Leitz, Chem. and Ind., 1957, 1572.

8. G. R. Waller and L. M. Henderson, J. Biol. Chem., 236, 1186 (1961).

9. E. Leete, Chem. and Ind. London, 1957 1270.

10. R. F. Dawson, D. R. Christmas, R. C. Anderson, M. L. Salt, A. F. D'Adams, and U. Weiss, J. Am. Chem. Soc., 78, 2645 (1956).

11. L. M. Henderson, J. F. Someroski, D. R. Rao, P. H. Wu, T. Griffith, and R. U. Byerrum, J. Biol. Chem., 234, 93 (1959).

12. G. R. Waller and L. M. Henderson, Biochem. Biophys. Res. Commun., 5, 5 (1961).

13. R. F. Dawson and D. R. Christman, Chem. and Eng. News, 39, 40 (1961).

14. D. R. Christman, R. F. Dawson, K. I. C. Karlstrom, J. Org. Chem., 29, 2394 (1964).

15. T. Griffith and R. U. Byerrum, Biochem. Biophys. Res. Commun., 10, 293 (1963).

16. S. A. Brown and R. U. Byerrum, J. Am. Chem. Soc., 74, 1523 (1952).

17. T. A. Henry, The Plant Alkaloid, The Blakiston Co., Philadelphia, 1949, p. 39.

18. W. L. Alworth, R. C. De Selms, and H. Rapaport, J. Am. Chem. Soc., 86, 1611 (1964).

19. J. Preiss and P. Handler, J. Biol. Chem., 233, 488 (1958).

20. K. M. Jones, Biochem. J., 73, 719 (1959).

21. B. L. Lamberts and R. U. Byerrum, J. Biol. Chem., 233, 939 (1958).

22. S. M. McElvain and R. Adams, J. Am. Chem. Soc., 45, 2738 (1923).

23. E. B. La Forge, J. Am. Chem. Soc., 50, 2480 (1929).

24. E. C. Taylor and A. J. Crovetti, J. Org. Chem., 19, 1633 (1954).

25. E. C. Taylor and A. J. Crovetti, Org. Syn., Vol. 37, John Wiley & Sons, Inc., N. Y., 1957, p. 12.

26. C. Walling and K. B. Wolfstirn, J. Am. Chem. Soc., 69, 852 (1947).

27. W. H. Lazier and H. R. Arnold, "Organic Synthesis," Coll. Vol. II, John Wiley & Sons, Inc., New York, N. Y., 1943, p. 142.

28. D. E. Hughes, Biochem. J., 60, 303 (1955).

29. G. W. Erving and E. A. Steck, J. Am. Chem. Soc., 68, 2181 (1946).

30. M. Freifelder and G. R. Stone, J. Org. Chem., 26, 3805 (1961).

31. H. McKennis, L. B. Turnbull, H. N. Wingfield, and L. J. Dewey, J. Am. Chem. Soc., 80, 1634 (1958).

32. W. Treibs and H. Reinheckel, Chem. Ber., 89, 51 (1956).

33. J. W. Cornforth, G. D. Hunter, and G. Popjak, Biochem. J., 54, 597 (1953).

34. E. F. Phares, Arch. Biochem. Biophys., 33, 173 (1951).

35. F. Vorrentrap, Liebig Ann., 35, 196 (1840).

36. F. Edmed, J. Chem. Soc., 73, 362 (1898).

37. G. D. Hunter and G. Popjak, Biochem. J., 50, 163 (1951).

38. R. H. F. Manske and M. Kulka, "Organic Reactions," Vol. VII, John Wiley & Sons, Inc., New York, N. Y., 1953, p. 59.

39. S. Hoogewerff and W. A. Van Dorp, Rec. Trav. Chim., 1, 122 (1882).

40. S. W. Moline, H. C. Walker, and B. S. Schweigert, J. Biol. Chem., 234, 880 (1959).

41. M. Kulka, J. Am. Chem. Soc., J. Am. Chem. Soc., 68, 2472 (1946).

42. T. Griffith and R. U. Byerrum, Science, 129, 1485 (1959).

43. G. R. Waller and L. M. Henderson, Biochem. Biophys. Res. Commun., 5, 5 (1961).

44. U. Scheidt, G. Boeckh-Behrens, and A. M. Delluva, Zeit. Phys. Chem., 330, 63 (1962).

45. T. Griffith, K. P. Hellman, and R. U. Byerrum, Biochem., 1, 336 (1962).

46. J. Essery, P. F. Juby, L. Marion, and E. Trumbell, J. Am. Chem. Soc., 84, 4597 (1962).

47. A. R. Friedman and E. Leete, J. Am. Chem. Soc., 85, 2141 (1963).

48. E. Leete and A. R. Friedman, J. Am. Chem. Soc., 86, 1224 (1964).

49. D. Gross, H. R. Schuette, G. Hubner, and K. Mathes, Tetrahedron Letters, 1963, 541.

50. E. Leete, Science, 147, 1000 (1965).

51. F. Lingens, Angew. Chem., 72, 920 (1960).

52. A. J. Andrepli, M. Ikeda, Y. Nishizuka, and O. Hayaishi, Biochem. Biophys. Res. Commun., 12, 92 (1963).

53. D. R. Christman and R. F. Dawson, Biochem., 2, 182 (1963).

54. L. A. Hadwiger, S. E. Bodei, G. R. Waller, and R. K. Ghalson, Biochem. Biophys. Res. Commun., 13, 466 (1963).

55. C. Yanofsky, J. Bacterial., 68, 577 (1954).