

Studies on the Biosynthesis of the Pyridine Ring of Nicotine*

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Tobacco plants were fed three C^{14} -labeled substrates to study their role in the biosynthesis of the pyridine ring of nicotine. Glycerol-2- C^{14} and aspartic acid-3- C^{14} both contributed relatively large quantities of radioactive carbon to the pyridine ring, whereas propionate-3- C^{14} did not. Glycerol-2- C^{14} was incorporated into the pyridine ring to about the same extent as glycerol-1,3- C^{14} . Partial degradation of the pyridine ring of nicotine from plants fed aspartic acid-3- C^{14} revealed that aspartic acid was not converted directly to the ring, since C^{14} was located in more than a single position in the ring. The three labeled compounds all contributed C^{14} to the pyrrolidine ring of nicotine. The pattern of labeling in the pyrrolidine ring suggested that propionate was converted to acetate before utilization for nicotine biosynthesis.

It has recently been shown that several metabolites which are either constituents of, or closely related to, the glycolytic pathway or the tricarboxylic acid cycle contribute carbon to some pyridine ring-containing compounds synthesized by several plants. For instance, glycerol-1,3- C^{14} , acetate-2- C^{14} , and propionate-2- C^{14} contribute radioactive carbon to the pyridine ring of nicotine in the metabolic processes in tobacco plants (*Nicotiana rustica*) (Griffith *et al.*, 1960). In addition, these same precursors along with succinate are incorporated into the 2-pyridone ring of ricinine by castor plants (Anwar *et al.*, 1961; Waller and Henderson, 1961a, b), and Ortega and Brown (1959) have demonstrated that succinate and glycerol are utilized by *Escherichia coli* for the synthesis of the pyridine ring of nicotinic acid.

In the studies of incorporation of various metabolites into nicotine, degradations of the alkaloid from plants fed acetate-2- C^{14} or propionate-2- C^{14} have revealed that about half of the C^{14} of the pyridine ring was located in carbon-3. In addition, the extent of incorporation and distribution of C^{14} in nicotine after feeding of labeled glycerol was different than after feeding either acetate or propionate (Griffith *et al.*, 1960). These findings suggested that glycerol contributed carbon to one part of the pyridine ring and acetate or propionate to another part.

These three precursors were incorporated into nicotine with a relatively low dilution of C^{14} , and it appeared, therefore, that they were all closely related to the precursors which were utilized directly for pyridine ring synthesis. However, the processes leading from glycerol, acetate, and propionate to the pyridine ring are not well understood, and the purpose of the present study, therefore, was to provide further information about these processes. Evidence is presented which indicates that carbon

2 of glycerol was incorporated into the pyridine ring of nicotine to about the same extent as the 1 and 3 carbons.

Propionate-3- C^{14} , on the other hand, was not utilized for pyridine ring synthesis, and it therefore appeared that propionate was converted to acetate before incorporation into nicotine. Aspartic acid was also demonstrated to be a precursor of the pyridine ring, but randomization of carbon occurred in the reactions involved in the conversion.

EXPERIMENTAL PROCEDURE

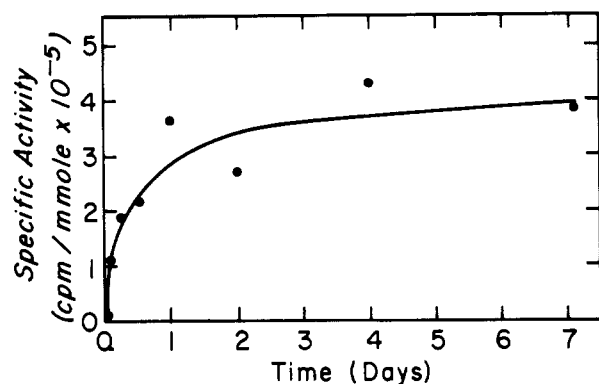
Nicotiana rustica plants were grown from seed in a greenhouse to a height of 12 to 15 cm and were then treated and administered radioactive compounds as described previously (Henderson *et al.*, 1959). When these techniques were employed, about 75% of a given radioactive compound was absorbed through the roots of each plant in a 2-hour period. Each plant was fed 1 ml of a solution containing 2.44×10^{-5} moles of a given compound having 5 μ c of C^{14} .

After a designated period of metabolism, the plants were harvested, cut into pieces with scissors, and placed under heat lamps to dry. Within 15 minutes after harvest the temperature of the plant material was 80°. Nicotine was isolated and purified as the dipicrate derivative (Brown and Byerrum, 1952).

Degradation of Radioactive Nicotine.—Three procedures were used to partially degrade nicotine to ascertain C^{14} in various positions of the molecule. These procedures have been described in detail elsewhere. Nonradioactive nicotine was usually added to the sample isolated from the experimental plants to insure a sufficient quantity of nicotine for the degradations.

The first degradation of nicotine led to the isolation of the pyridine ring and carbon 2 of the pyrrolidine ring (Lamberts and Byerrum, 1958). Nicotine was treated with neutral potassium permanganate, yielding potassium nicotinate and potassium bicarbonate from the *N*-methyl carbon

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Fig. 1.—Rate of incorporation of acetate-2-C¹⁴ into nicotine.

and carbons 3, 4, and 5 of the pyrrolidine ring. Nicotinic acid was isolated and purified. The bicarbonate was decomposed by addition of dilute nitric acid, and the resulting carbon dioxide was collected as barium carbonate. Nicotinic acid was decarboxylated to yield pyridine, which was isolated as the picrate derivative, and carbon dioxide. The carbon dioxide was collected as barium carbonate. This sample of barium carbonate represented carbon 2 of the pyrrolidine ring.

The second degradation resulted in isolation of carbon 3 of the pyridine ring (Griffith *et al.*, 1960). The pyridine nitrogen of nicotine hydriodide was methylated with methyl iodide, and the methiodide was oxidized with potassium ferricyanide in alkaline solution. The products of this reaction were treated with chromic acid to yield hygrinic acid (*N*-methyl-pyrrolidine-2-carboxylic acid). Hygrinic acid was decarboxylated, and the resulting carbon dioxide, which represented carbon 3 of the pyridine ring, was isolated as barium carbonate.

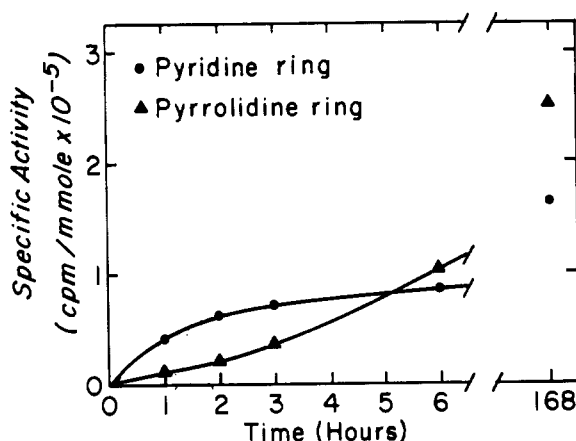
In the third degradation the *N*-methyl group was isolated by treatment of nicotine with hydriodic acid (Brown and Byerrum, 1952). Methyl iodide formed by this reaction was recovered as methyltriethylammonium iodide.

In most cases compounds were converted to barium carbonate for measurement of C¹⁴ content.

All assays for C¹⁴ were performed with a Nuclear Chicago 192 scaler and D-47 proportional flow counter (Nuclear Chicago Corporation, Chicago, Ill.). All samples were corrected for self-absorption. Radiochemicals were purchased from Research Specialties Company, Richmond, Calif.

RESULTS

Incorporation of Acetate-2-C¹⁴ into Nicotine.—Sodium acetate-2-C¹⁴ was fed to tobacco plants to study its rate of incorporation into nicotine and to investigate differences in the rate of incorporation of C¹⁴ into the pyrrolidine and pyridine rings. Periods of metabolism of 1 hour to 7 days were used. The data in Figure 1 show that C¹⁴ was incorporated into nicotine rapidly and that, with periods of metabolism longer than one day, little change in the specific activity of nicotine occurred.

Fig. 2.—Rate of biosynthesis of the pyridine and pyrrolidine rings of nicotine from acetate-2-C¹⁴.

The observed rate of introduction of C¹⁴ into nicotine does not necessarily represent the true rate of nicotine synthesis, since the solution of radioactive precursor was absorbed by the roots during a 2 to 3 hour period.

Radioactive nicotine from plants administered sodium acetate-2-C¹⁴ for several different metabolism periods was degraded to ascertain changes of labeling in the pyridine and pyrrolidine rings with time. These data are shown in Figure 2. In the 1, 2, or 3 hour experiments, about 66% of the C¹⁴ nicotine was associated with the pyridine ring. In the 6-hour experiment, the C¹⁴ was distributed equally between the two rings, and with a metabolism period of 7 days the pyridine ring then contained about 40% of the C¹⁴ of the nicotine.

Incorporation of C¹⁴-Labeled Glycerol, Propionate, and Aspartic Acid into Nicotine.—A comparison of the extent of incorporation of C¹⁴ from glycerol-2-C¹⁴, aspartic acid-3-C¹⁴, and sodium propionate-3-C¹⁴ is shown in Table I. Dilutions were calculated by dividing the specific activity of the precursor by the specific activity of the isolated nicotine. Of the three compounds, glycerol was converted to nicotine with the least dilution, followed by aspartic acid and then propionate. It is interesting to note that glycerol-1,3-C¹⁴ was shown previously to be incorporated into nicotine with a dilution of about 140, whereas propionate-2-C¹⁴ and propionate-1-C¹⁴ were incorporated with dilutions of about 200 and 9000 respectively (Griffith *et al.*, 1961).

TABLE I
INCORPORATION OF C¹⁴-LABELED PRECURSORS INTO NICOTINE IN TOBACCO PLANTS

Precursor	Specific Activity (cpm/mmole × 10 ⁵)	Dilution
Aspartic acid-3-C ¹⁴	11.4	360
Glycerol-2-C ¹⁴	18.7	219
Propionate-3-C ¹⁴	5.2	795

Incorporation of Glycerol-2-C¹⁴ into Nicotine.—Previous experiments had shown that nicotine isolated from plants fed glycerol-1,3-C¹⁴ for a 7-day period contained 56% of the C¹⁴ in the pyridine

ring (Griffith *et al.*, 1960). The results of degradations of nicotine from plants fed glycerol-2- C^{14} , reported in Table II, reveal that in a 7-day experiment 57% of the C^{14} was in the pyridine ring. In contrast, when the glycerol-2- C^{14} was fed for only 2 hours, essentially all of the C^{14} in the molecule was in the pyridine ring. The precise location of the C^{14} in the pyridine ring is under study.

TABLE II
DISTRIBUTION OF C^{14} IN NICOTINE FROM PLANTS FED GLYCEROL-2- C^{14}

Compound	Maximal Specific Activity	
	7-day Exper. (cpm/mmole $\times 10^{-3}$)	2-hour Exper. (cpm/mmole $\times 10^{-3}$)
Nicotine dipicrate	12.6	8.8
Barium carbonate ^a	4.0	0.4
Nicotinic acid	7.5	8.5
Barium carbonate ^b	1.9	0.3
Pyridine picrate	7.2	9.0
Methyltriethylammonium iodide	0.8	

^a From the 3,4,5, and *N*-methyl carbons of the pyrrolidine ring. The specific activity was multiplied by 4. ^b From the carboxyl carbon of nicotinic acid (the 2 carbon of the pyrrolidine ring).

In the 7-day experiment, carbon 2 of the pyrrolidine ring contained 15% of the C^{14} of nicotine. If the fact is taken into account that a precursor of the pyrrolidine ring is a symmetrical compound (Lamberts and Byerrum, 1958; Leete, 1958; Wu, Griffith, and Byerrum, unpublished) an equal quantity of C^{14} would be located in position 5. Since the *N*-methyl carbon contains 6% of the C^{14} , the remaining 11% would be associated with carbons 3 and 4. The small quantity of C^{14} associated with the pyrrolidine ring in the 2-hour experiment was located in carbons 2 and 5.

Incorporation of Propionate-3- C^{14} into Nicotine.—It has been shown that the 2 carbon, but not the carboxyl carbon of propionate, was introduced to a large extent into the pyridine ring of nicotine (Griffith *et al.*, 1960). It seemed possible that the 3 carbon of propionate might contribute to the pyridine ring, perhaps without scission of the carbon-carbon bond between the 2 and 3 positions. Table III shows the results of degradation of

TABLE III
DISTRIBUTION OF C^{14} IN NICOTINE FROM PLANTS FED PROPIONATE-3- C^{14}

Compound	Maximal Specific Activity (cpm/mmole $\times 10^{-4}$)
Nicotine dipicrate	2.80
Barium carbonate ^a	1.19
Nicotinic acid	1.43
Barium carbonate ^b	1.09
Pyridine picrate	0.06

^a From carbon 3,4,5, and the *N*-methyl group of the pyrrolidine ring. The specific activity was multiplied by 4. ^b From the carboxyl carbon of nicotinic acid (the 2 carbon of the pyrrolidine ring).

radioactive nicotine from plants fed propionate-3- C^{14} . The pyrrolidine ring contained most of the radioactive carbon in the molecule, and this was

divided almost equally between positions 2 and 5. The pyridine ring contained only about 3% of the total C^{14} of the molecule.

Synthesis of Nicotine from Aspartic Acid-3- C^{14} .—Since aspartic acid-3- C^{14} was found to be incorporated extensively into nicotine (Table I), additional studies with this compound were undertaken. The labeled amino acid was fed to plants for 7 days in an initial experiment and for 2 hours in a second experiment. The radioactive nicotine was isolated and partially degraded as described earlier. The results of these degradations are shown in Table IV.

TABLE IV
DISTRIBUTION OF C^{14} IN NICOTINE FROM PLANTS FED ASPARTIC ACID-3- C^{14}

Compound	Maximal Specific Activity	
	7-day Exper. (cpm/mmole $\times 10^{-3}$)	2-hour Exper. (cpm/mmole $\times 10^{-3}$)
Nicotine dipicrate	5.7	4.3
Barium carbonate ^a	2.1	0.5
Pyridine picrate	2.4	2.7
Barium carbonate ^b	0.6	0.5
Hygrinic acid	3.9	2.8
Barium carbonate ^c	1.0	1.2

^a From carbons 3,4,5, and the *N*-methyl group of the pyrrolidine ring. The specific activity was multiplied by 4. ^b From the carboxyl carbon of nicotinic acid (the 2 carbon of the pyrrolidine ring). ^c From the carboxyl carbon of hygrinic acid (the 3 carbon of the pyridine ring).

In the 7-day experiment approximately 42% of the radioactive carbon resided in the pyridine ring, and of this approximately half was in carbon 3. In the pyrrolidine ring, carbon 2 contained 11% of the C^{14} in the whole molecule. Since a symmetrical intermediate is involved between aspartate and the pyrrolidine ring, carbon 5 would also have 11% of the C^{14} and carbons 3 and 4 would each contain 13%.

In comparison, in the 2-hour experiment approximately 63% of the radioactive carbon in nicotine was in the pyridine ring and, of this, half again resided in carbon 3. In the pyrrolidine ring, 12% of the C^{14} of the molecule was in position 2 with the remainder, then, in position 5.

DISCUSSION

The data presented here show that the incorporation of acetate-2- C^{14} into nicotine occurred rapidly with about one-fourth as much C^{14} entering nicotine in a 2-hour feeding period as in a 7-day experiment. This result is in contrast to the incorporation of C^{14} from the methyl group of methionine into the *N*-methyl group of nicotine (Brown and Byerrum, 1952). When quantities of C^{14} similar to those employed in the present study were fed tobacco plants, nicotine contained detectible radioactivity only after a day or two, and the radioactivity then increased about ten times during a 5 or 6 day period. Although different feeding techniques were used in these studies, the great difference in the rate of incorporation of C^{14} into nicotine cannot be attributed to this factor alone.

It has been suggested previously that acetate is incorporated into the pyrrolidine ring of nicotine by a different route than into the pyridine ring (Griffith and Byerrum, 1959). The fact that acetate is introduced into these two rings at different rates supports this hypothesis.

Radioactive carbon from glycerol-2- C^{14} was incorporated into both the pyridine and pyrrolidine rings of nicotine. In the 7-day feeding experiment some 57% of the C^{14} in nicotine was in the pyridine ring, whereas in the 2-hour feeding experiment over 90% of the C^{14} in the molecule was present in the pyridine ring. The distribution of C^{14} in the pyrrolidine ring in the 7-day experiment is consistent with known metabolic reactions if it is assumed that glycerol is metabolized to α -ketoglutarate. The α -ketoglutarate could arise from glycerol either through glycolysis and acetate to the tricarboxylic acid cycle, or through a carboxylation of pyruvate or phosphoenol-pyruvate to the tricarboxylic acid cycle (Mazelis and Vennesland, 1957). In the 2-hour experiment a small amount of radioactive carbon was found in the pyrrolidine ring. The distribution of radioactive carbon within the ring was the same as was obtained after feeding of acetate-1- C^{14} , and, therefore, the evidence suggests that the small amount of glycerol which was converted to the pyrrolidine ring of nicotine by way of the tricarboxylic acid cycle in 2 hours was almost completely metabolized through acetate.

The location of C^{14} in the pyridine ring of nicotine from plants fed glycerol-2- C^{14} was not ascertained. However, glycerol was converted to the pyridine ring by a pathway which excluded acetate or a compound of the tricarboxylic acid cycle. This conclusion was clear because glycerol-2- C^{14} when metabolized by way of glycolysis yielded acetate-1- C^{14} , as judged by the labeling in the pyrrolidine ring in both the 7-day and 2-hour experiments. Acetate-1- C^{14} was shown to contribute only small quantities of C^{14} to the pyridine ring in a previous study (Griffith and Byerrum, 1959).

Although the pathway for incorporation of glycerol into the pyridine ring is unknown, the data presented provide strong evidence that glycerol, or a closely related metabolite, is a major source of carbon for at least part of that ring.

Since the total incorporation of C^{14} into the pyridine ring of nicotine from glycerol-1,3- C^{14} and glycerol-2- C^{14} was similar, our results would indicate that all three carbons of glycerol participate in the synthesis of the pyridine moiety. This conclusion is in contrast to that of Dawson and Christman (1961), who have shown that, in nicotine produced by sterile tobacco root cultures, the radiochemical yield from glycerol-2- C^{14} was twice that from glycerol-1,3- C^{14} . Their data would indicate that glycerol is converted to a 2-carbon compound before incorporation into nicotine. Further experiments will be necessary to establish the metabolic pathway for incorporation of glycerol into the pyridine ring.

Previous experiments showed that carbon 2 of acetate and propionate were incorporated into the pyridine ring of nicotine, and it was suggested that these compounds might first be converted to an intermediate in the tricarboxylic acid cycle. The experimental basis for this suggestion was that after the feeding of either acetate-2- C^{14} or propionate-2- C^{14} the pyridine ring contained half of the C^{14} in the 3 position. If it is assumed that the remainder is in the 2 position, then the conversion of acetate to either succinate or fumarate could provide the observed labeling. Condensation of these acids or closely related metabolites with glycerol, or some compound similar to glycerol, could provide the carbon for nicotinic acid, which has been shown by Dawson to be a precursor of the pyridine ring of nicotine (Dawson *et al.*, 1956). Such a hypothesis is supported by evidence concerning the biogenesis of ricinine (Anwar *et al.*, 1961). It was found that C^{14} from acetate-1- C^{14} resided almost entirely in the nitrile carbon of ricinine, whereas the C^{14} from acetate-2- C^{14} and propionate-2- C^{14} was located to a large extent in the pyridone ring.

It was previously suggested that propionate was an immediate precursor of nicotinic acid and might be utilized for pyridine ring synthesis by way of β -alanine or some other 3-carbon compound with no scission of the carbon chain of propionate (Griffith *et al.*, 1960). The fact that propionate-3- C^{14} does not provide radioactive carbon for pyridine ring synthesis eliminates this possibility.

The labeling in the pyrrolidine ring of nicotine was similar after feeding of either propionate-3- C^{14} or acetate-1- C^{14} . It would appear, therefore, that propionate when it serves as a precursor for either the pyridine or pyrrolidine ring is first converted to acetate, perhaps by the mechanism suggested by Giovanelli and Stumpf (1957, 1958) in which the 3 carbon of propionate becomes the carboxyl carbon of acetate. A similar suggestion has been made by Waller and Henderson as a result of their studies on the biosynthesis of ricinine in the castor plant (Waller and Henderson, 1961a).

Additional evidence that an intermediate in the tricarboxylic acid cycle is an immediate precursor of the pyridine ring is provided by the experiments with aspartic acid-3- C^{14} . A possible route of pyridine ring synthesis might involve condensation of aspartic acid or a closely related compound with glycerol or some similar compound. The amino group of aspartic acid could become the nitrogen of the pyridine ring. If such a condensation occurred, the 3 carbon of aspartic acid would become the 3 carbon of the pyridine ring. In the experiment in which aspartic acid-3- C^{14} was fed for 7 days, about half the C^{14} of the pyridine ring was in position 3. It is possible that randomization of carbon could occur in this relatively long period of metabolism.

The labeling in the pyrrolidine ring after the feeding of aspartic acid-3- C^{14} can be explained by assuming that aspartic acid is transformed to

oxalacetate. This compound may yield α -keto-glutarate, by way of the tricarboxylic acid cycle, which is then converted to the pyrrolidine ring with glutamic acid as an intermediate. In the 2-hour feeding experiment all of the C^{14} in the pyrrolidine ring was in the 2 and 5 positions, as would be expected if the reactions described had occurred.

This result shows that little or no randomization of the C^{14} occurred during synthesis of the pyrrolidine ring in a 2-hour period. Thus during such a period, synthesis of the pyridine ring also should occur with limited randomization of the isotope. Therefore, if aspartic acid were converted directly to the pyridine ring, more than half of the C^{14} would be expected to reside in position 3. However, in the experiment in which labeled aspartic acid was fed for 2 hours, carbon 3 of the pyridine ring again contained about one-half the C^{14} . If aspartic acid were converted to either fumarate or succinate before incorporation into the pyridine ring the observed labeling could result.

Succinate has been shown to be a precursor of the pyridone ring of ricinine in castor plants (Waller and Henderson, 1961a) and of the pyridine ring of nicotinic acid formed by *E. coli* (Ortega and Brown, 1959).

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Hadacidin, a New Growth-Inhibitory Substance in Human Tumor Systems

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The fermentation broth of *Penicillium frequentans* Westling was found to inhibit the growth of the human adenocarcinoma-1 in the embryonated egg. The active substance in the fermentation broth, which was responsible for the inhibition of tumor growth, was designated hadacidin. It was isolated as a crystalline monosodium salt having the composition $C_3H_4NO_4Na$. The free dibasic acid was converted to a monomethylester. Hadacidin was degraded to formic acid and hydroxyaminoacetic acid. The latter was converted to glycine by hydrogenation, and was identical with a synthetic specimen of hydroxyaminoacetic acid. *N*-Formylation of synthetic hydroxyaminoacetic acid yielded hadacidin, *N*-formylhydroxyaminoacetic acid; the products were compared as monosodium salts. Hadacidin and 6-mercaptopurine show a similar degree of activity against the human adenocarcinoma-1 in the embryonated egg.

Transplantation of human tumors into the conditioned laboratory animal has become a useful laboratory tool, according to Toolan (1958). It is noteworthy that these neoplasms remain human in both their chromosomal (Levan, 1956) and antigenic (Korngold and Lipari, 1955) composition, even though some have been transplanted for several years.

After such human tumors have grown in the

conditioned animal, they can be passed serially in the embryonated egg (Dagg *et al.*, 1955; Harris, 1958). The implanted and embryonated egg offers a biological system which serves as a primary screen for new antitumor agents. At this stage, the embryonated egg does not require conditioning by x-irradiation or cortisone.

The use of human tumors offers a new methodology for seeking and evaluating broth filtrates