THE INCORPORATION OF CARBON DIOXIDE AND ACETATE INTO NICOTINE

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ABSTRACT

THE INCORPORATION OF CARBON DIOXIDE AND ACETATE INTO NICOTINE

by Horst Ronald Zielke

Glutamate, a proposed precursor of the pyrrolidine ring of nicotine, was isolated and degraded after metabolism of $^{14}CO_2$ for 6 hours by N. rustica and N. glutinosa and after metabolism of acetate-2-^{14}C for 2 hours by N. rustica. The ^{14}C distribution in glutamate from N. glutinosa after 6 hour $^{14}CO_2$ metabolism was 20.2%, 14.3%, 13.9%, 24.4% and 26.6%, in C-1 through C-5, respectively. The ^{14}C distribution in nicotine under the same conditions was pyridine ring, 77.8%; C-2', 4.3%; C-3', 4.4%; C-4', 4.7%; C-5', 4.1% and methyl carbon, 5.0%. The labeling pattern of the pyrrolidine ring is in agreement with the symmetrical intermediate pathway proposed for the formation of the pyrrolidine ring of nicotine from glutamate. Similar results were obtained after 6 hour $^{14}CO_2$ incorporation into nicotine by N. rustica. After 3 hour $^{14}CO_2$ metabolism by N. glutinosa, 90.4% of the radioactivity was in pyridine ring, 5.4% in the methyl group and the remaining radioactivity was distributed equally among the carbons of the pyrrolidine ring.

Acetate-2-^{14}C labeled glutamate in N. rustica as anticipated if acetate were metabolized via the tricarboxylic acid cycle: 1.5%, 5.4%, 4.6%, 81.0%, 2.8% in C-1 through C-5, respectively. The distribution of ^{14}C in nicotine was pyridine ring, 64.4%; C-2', 2.6%; C-3', 16.4%; C-4', 15.6%;

C-5', 1.8% and methyl carbon, 1.6%. These results support the symmetrical intermediate hypothesis for the formation of the pyrrolidine ring of nicotine proposed on the basis of previous precursor experiments.

The ^{14}C distribution in the pyridine ring of nicotine after 3 hour $^{14}CO_2$ metabolism by N. glutinosa was 10.0%, 9.2%, 25.0%, 24.2% and 26.7% in carbons 2 through 6, respectively. After 6 hour $^{14}CO_2$ metabolism by N. rustica, the ^{14}C distribution in the pyridine ring of nicotine was 13.1%, 14.0%, 23.4%, 21.7% and 23.0% in carbons 2 through 6, respectively. It is postulated that the above labeling pattern is the result of unequal labeling of glyceraldehyde and aspartic acid, the proposed precursors of the 5-carbon chain of the pyridine ring. It is proposed that glyceraldehyde and aspartic acid are labeled unequally due to different dilution rates and/or slower incorporation of label into the internal carbons of aspartic acid. After 2 hour metabolism of acetate-2-^{14}C by N. rustica, carbons 2 and 3 of the pyridine ring are labeled equally and contain approximately 88% of the total radioactivity of the pyridine ring. This labeling pattern is consistent with the proposal that acetate-2-^{14}C is incorporated into the pyridine ring of nicotine via aspartic acid.

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By

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INTRODUCTION

The Pyrrolidine Ring of Nicotine

The biosynthetic pathway for the alkaloid nicotine has been extensively studied through precursor experiments. These studies have shown that glutamic acid-2-^{14}C (1, 2) and ornithine-2-^{14}C (3, 4) are incorporated into the pyrrolidine ring of nicotine via a symmetrical intermediate in Nicotiana rustica var. humilus and Nicotiana tabacum. Wu, Griffith and Byerrum (5) and Wu and Byerrum (6) using ^{14}C labeled acetate, glycerol, propionate and aspartate demonstrated that the distribution of ^{14}C in the pyrrolidine ring of nicotine was in general agreement with the expected labeling if these compounds were converted to glutamate by way of the tricarboxylic acid cycle. Leete (7) postulated that ornithine-2-^{14}C (I) was incorporated into the pyrrolidine ring of nicotine (II) via glutamic semialdehyde-2-^{14}C (III), Δ^1-pyrroline-5-carboxylic acid-5-^{14}C (IV), Δ^1-pyrroline-5-carboxylate anion-5-^{14}C (V), mesomeric anion of Δ^1-pyrroline-2-^{14}C (VI) and Δ^1-pyrroline-2-^{14}C (VII) as shown in Figure 1.

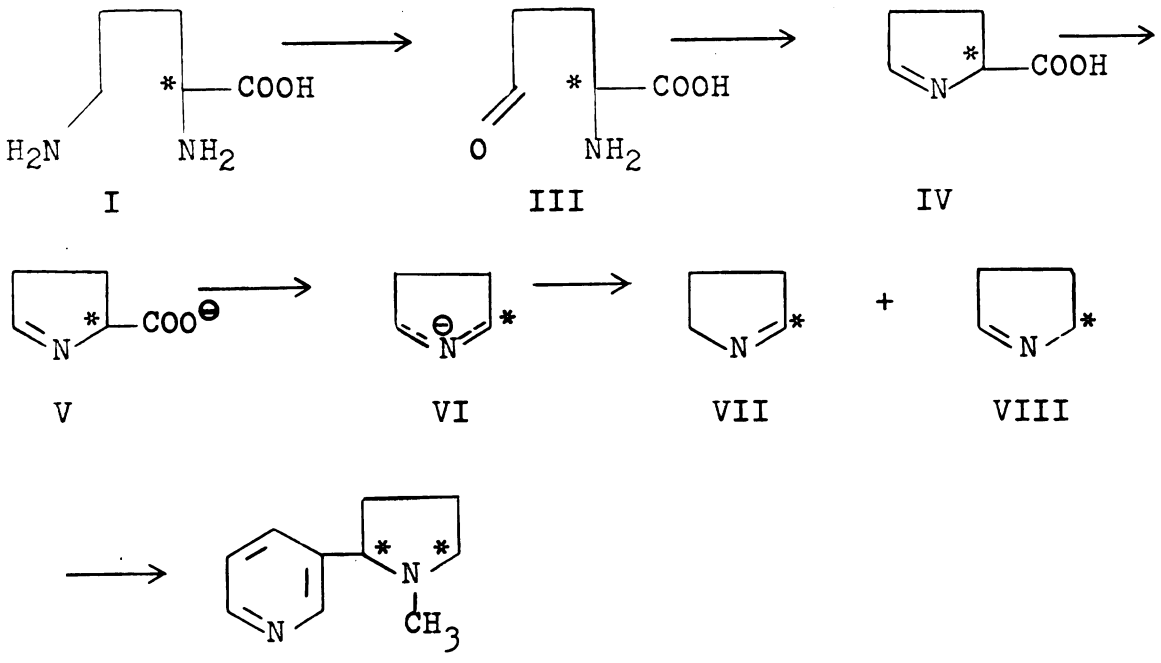


FIGURE 1

This hypothesis is in agreement with Krampl (8) and Krampl, Zielke and Byerrum (9) who observed that Δ^1-pyrroline-5-carboxylic acid-5-^{14}C labeled equally carbons 2' and 5' of the pyrrolidine ring. However, recent work has indicated that a different biosynthetic pathway is operative. Leete, Gros and Gilbertson (10) observed with sterile root cultures of N. tabacum that only the δ-amino group of ^{15}N-labeled ornithine-2-^{14}C was incorporated into the pyrrolidine ring. This eliminated glutamic semialdehyde and its cyclic form Δ^1-pyrroline-5-carboxylic acid, as an intermediate between ornithine and the pyrrolidine ring. The participation of other intermediates shown in Figure 1 was further questioned by the experiments of Mizusaki et al (11) and Leete (12). 4-(N-methyl)-aminobutyraldehyde (N-methyl pyrroline), a

compound rapidly labeled after metabolism of ornithine-2-^{14}C by aseptic root cultures of N. rustica, was found to be an efficient precursor of nicotine (11). 4-(N-methyl)-amino-butyraldehyde was also shown to be rapidly labeled by putrescine-1,4-^{14}C or methionine-$^{14}CH_3$ (11). Leete (12) observed after feeding N-methyl Δ^1-pyrrolinium-2-^{14}C chloride to N. tabacum that 18% of the N-methyl- Δ^1-pyrrolinium-2-^{14}C chloride was incorporated into the pyrrolidine ring of nicotine without undergoing tautomerism. Only carbon 2' of the pyrrolidine ring was labeled. The pathway in Figure 2 represents the hypothetical biosynthetic pathway proposed by Leete (12) and Mizusaki et al (11).

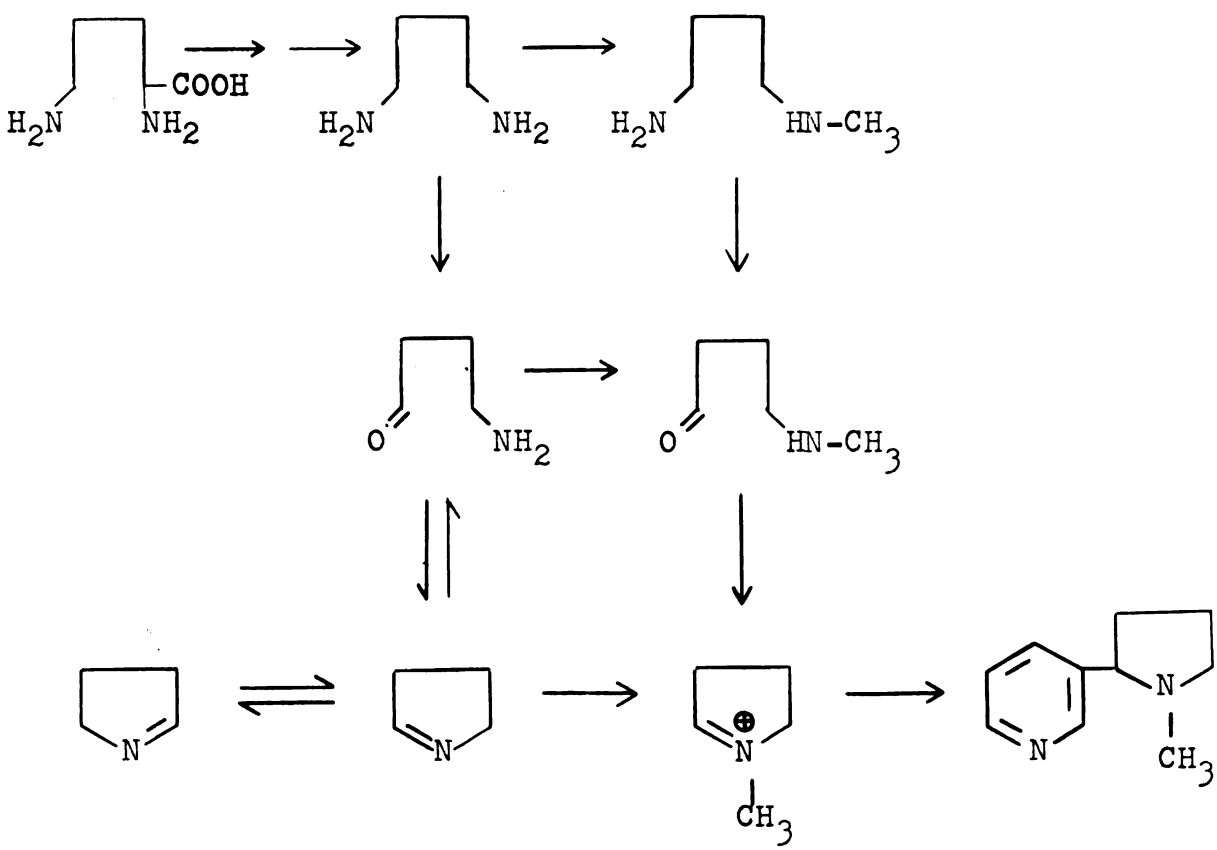
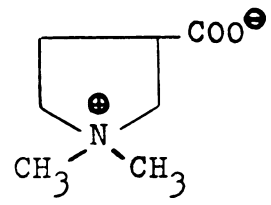


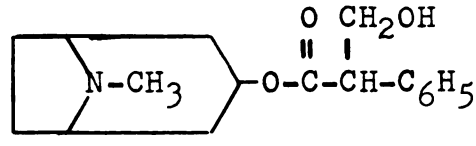
FIGURE 2

At presence no evidence is available to determine at which point methylation occurs in the sequence shown in Figure 2.

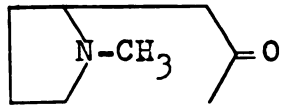
Although ornithine is incorporated symmetrically into the pyrrolidine ring of nicotine, present evidence is to the contrary for several other alkaloids that contain the N-methyl pyrrolidine ring.



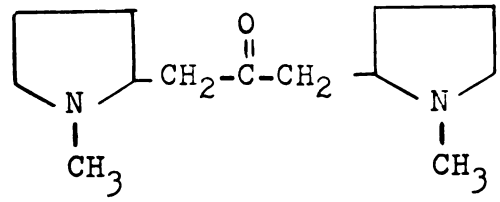
Stachydrine



Hyoscyamine



Hygrine



Cuscohygrine

Essery et al (13), using ornithine-2-^{14}C, obtained stachydrine from alfalfa plants labeled only at carbon 2. Robertson and Marion (14) observed that carboxyl-^{14}C proline was incorporated into stachydrine, labeling only the carboxyl group. Presumably proline is an immediate precursor of stachydrine. Hyoscyamine, a tropane alkaloid from Datura stramonium (Jimson weed) incorporated label from ornithine-2-^{14}C into only one of the bridgehead carbons next to the nitrogen atom (15). Unpublished data from Liebish, Shütte and Mothes quoted by Mothes and Shütte (16) indicates that putrescine-1,4-^{14}C labeled both bridgehead carbons. However, since ornithine is incorporated unsymmetrically, it is unlikely that putrescine

is a normal intermediate of the pathway through which ornithine is incorporated.

Hygrine, which is structurally related to the tropane skeleton, has not been studied with ^{14}C-labeled precursors, however, Anet et al (17) have been able to increase the amount of hygrine synthesized by administrating 4-(N-methyl)-amino-butyraldehyde. This has led Mothes and Shütte (16) to the opinion that the biosynthetic pathway for hygrine is similar to that for tropane alkaloids:

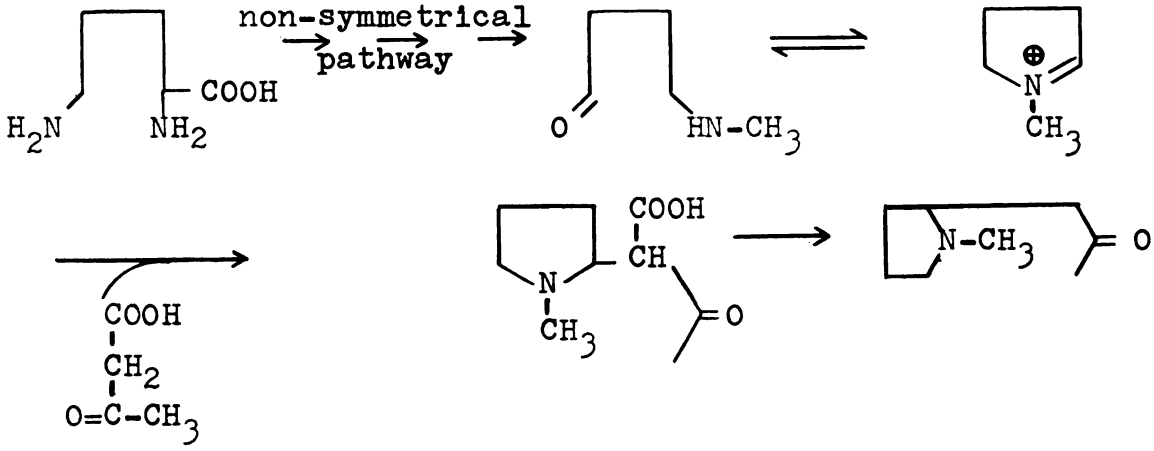


FIGURE 3

Clarke and Mann (18) using diamine oxidase isolated from pea cotyledons, coupled with catalase, observed that norhygrine was formed after the addition of putrescine and acetoacetate. Tuppy and Faltaous (19) observed hygrine and cuscohygrine formation after adding N-methylputrescine and acetoacetate or acetonedicarboxylic acid to diamine oxidase and catalase preparations.

Before discussing the mechanisms by which glutamate or ornithine may be incorporated either symmetrically or non-

symmetrically into the pyrrolidine ring, it is necessary to discuss the pathway for putrescine biosynthesis in plants. Smith and Garraway (20), using barley seedlings, obtained evidence which indicated that ornithine was converted to putrescine via citrulline, arginosuccinic acid, arginine, agmatine and N-carbamyl putrescine. Direct conversion of ornithine to putrescine does not proceed at a measurable rate under their conditions. This is unlike the multiple pathways of putrescine biosynthesis studied by Morris and Pardee in E. coli (21). Morris and Pardee observed direct conversion of ornithine to putrescine as well as conversion of ornithine to putrescine via arginine and agmatine.

In theory, glutamate or ornithine could be incorporated into the alkaloids mentioned previously via 4-(N-methyl)-aminobutyraldehyde by a symmetrical or non-symmetrical pathway depending upon the site of methylation or oxidation of the precursors for 4-(N-methyl)-aminobutyraldehyde. If ornithine is converted to 4-(N-methyl)-aminobutyraldehyde via putrescine, the labeling pattern in the alkaloid will show symmetry. If, however, methylation or oxidation occurs prior to the step in which putrescine forms, non-symmetry will result. Another unsymmetrical pathways for ornithine incorporation can be proposed if ornithine is incorporated via Δ^1-pyrroline-5-carboxylic acid and the attachment of the side chain occurs before decarboxylation of the Δ^1-pyrroline-5-carboxylic acid yields the mesomeric anion (22). These possibilities have not yet been explored for alkaloids in

which unsymmetrical ornithine incorporation occurs.

Recently the experiments leading to the pathway proposed in Figure 2 for the symmetrical labeling of the pyrrolidine ring of nicotine from ornithine have been criticized by Liebman *et al* (23). An important assumption in the precursor experiments is that the exogenously supplied metabolites are incorporated in a manner indistinguishable from endogenous metabolites. These authors initiated a series of experiments (23-26) in which nicotine was isolated and degraded after $^{14}CO_2$ incorporation by *N. glutinosa*. Liebman *et al* observed that carbon 2' of the pyrrolidine ring contained less radioactivity after 6 hour $^{14}CO_2$ incorporation than either carbons 3', 4' or 5'. Ornithine-2-^{14}C, unlike $^{14}CO_2$, was incorporated via a symmetrical intermediate into the pyrrolidine ring of nicotine by *N. glutinosa* (23). From these observations Liebman *et al* postulated that the symmetrical labeling pattern observed from precursor feedings may be the result of a minor or aberrant pathway since the $^{14}CO_2$ exposures represent normal growth conditions.

The Pyridine Ring of Nicotine

The biosynthesis of the pyridine ring of nicotine has been extensively studied. Previous work in this laboratory has indicated that carbons 4, 5 and 6 of the pyridine ring of nicotine are derived from glyceraldehyde or glyceraldehyde-3-phosphate while carbons 2 and 3 of the pyridine ring are derived from carbons 2 and 3 of aspartic acid. After 4 hour

aspartic acid-3-^{14}C incorporation by N. rustica, 57% of the pyridine ring label was in carbon 3 and 38% in carbon 2 (27). In a similar 4 hour glyceraldehyde-3-^{14}C incorporation study, 55% of the radioactivity of the pyridine ring was in carbon 4 (28). Fleeker and Byerrum (28) postulated a Schiff base formation between glyceraldehyde and aspartic acid as an early step in the pyridine ring formation. Further incorporation experiments with acetate-2-^{14}C, succinate-2,3-^{14}C and, malate-3-^{14}C (27) and glycerol-^{14}C (30) support this hypothesis. In the above studies with radioactive acetate, succinate and malate, carbons 2 and 3 of the pyridine ring of nicotine contained a large proportion of the pyridine ring label. This labeling pattern is explained if these compounds are incorporated into the pyridine ring of nicotine via aspartic acid. Fleeker and Byerrum (30), using glycerol-2-^{14}C and glycerol-1,3-^{14}C, observed that the carbon atoms of glycerol were incorporated *in toto* into positions 4, 5 and 6 of the pyridine ring of nicotine. Over 98% of the ^{14}C in the pyridine ring derived from glycerol-2-^{14}C was in carbon 5 of the pyridine ring after a 4 hour metabolic period. Glycerol-1,3-^{14}C labeled equally carbons 4 and 6 of the pyridine ring. Carbons 4 plus 6 contained 68% of the pyridine ring label, whereas carbon 5 had about 2% of the radioactivity. Since subsequent studies indicated that glyceraldehyde is incorporated with less dilution than glycerol (28), it is assumed that glycerol is incorporated into the pyridine ring of nicotine after oxidation to glyceraldehyde.

Similar mechanisms are indicated for the formation of the pyridine or pyridone rings of anabasine (31, 32) and ricinine (33-35) in other higher plants. Trypthophan is not a precursor of the pyridine ring of nicotine (36). The biosynthesis of nicotinic acid by Escherichia coli (37) and Mycobacterium tuberculosis (38) also proceeds via a 3-carbon compound condensing with aspartic acid. However Isquith and Moat (39), using partially purified extracts of Clostridium butylicum, concluded that acetate and formate, rather than glycerol, participated in the formation of the pyridine ring of nicotinic acid in their system. Scott and Mattey (40) observed that formate-^{14}C was readily incorporated into the pyridine ring by Cl. butylicum and that 90% of the label was located in carbon 6. Using extracts of Cl. butylicum Scott et al (41) observed formation of radioactive N-formyl-L-aspartate from aspartate and formate-^{14}C with more than 90% of the radioactivity located in the formyl carbon. When formate and aspartate in their incubation mixture were replaced by N-formyl-L-aspartate, the amount of nicotinic acid synthesized was increased three fold. Previous studies in this laboratory indicated that when formate-^{14}C was metabolized by N. rustica, over 90% of the radioactivity incorporated into the nicotine resided in the methyl group (42). This indicates that formate has different roles in these two systems.

The mechanism by which the precursors of the pyrrolidine and pyridine rings combine to form nicotine is not known.

However, Dawson *et al* (43) observed that label from nicotinic acid tritiated at carbon 6 was less readily incorporated into nicotine than tritium at the other carbons. This implies that a 1,6-dihydropyridine intermediate may be involved in the condensation reaction.

The work described in this thesis was initiated to determine the validity of precursor experiments by comparing the labeling pattern of the pyrrolidine and pyridine rings of nicotine after $^{14}CO_2$ and acetate-2-^{14}C incorporation by *N. rustica* and *N. glutinosa*. In addition glutamate, a precursor of the pyrrolidine ring, and aspartate, a precursor of the pyridine ring, were isolated in the same experiments and the labeling pattern in each determined. A hypothetical non-symmetrical pathway for the biosynthesis of the pyrrolidine ring of nicotine involving sarcasine (N-methyl glycine) was evaluated and rejected. The degradation of the amino acids was performed with the cooperation of Robert M. O'Neal, Linda C. Burns and Roger E. Koeppe from the Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma.

EXPERIMENTAL PROCEDURE

Plants were grown in a greenhouse under conditions of controlled light and temperature. For studies of acetate-2-^{14}C incorporation, 337 N. rustica plants with hydroponically regenerated roots were used as previously described (42). The acetate, purchased from Tracerlab Corporation, had a specific activity of 8.56 mC/mmole. Purity was determined by paper chromatography and radioautography. Ten to 40 μc of sodium acetate-2-^{14}C in 1 ml of water was administered per plant and additional water was then added as required. Light of 300 foot-candles intensity was supplied by two 30-watt fluorescent bulbs and two 150-watt incandescent bulbs. After 2 hours the roots were rinsed with distilled water, the plants cut into pieces with scissors and blended for 1 minute with boiling water in a large stainless steel blender. The nicotine was isolated from the blended material as the dipicrate (42). The nicotine dipicrate was transferred to a steam distillation apparatus and the solution was made basic and steam distilled. The nicotine was extracted from the steam distillate with ether. After drying, the ether was removed by evaporation to yield free nicotine.

When the isolation of glutamate and aspartate was desired, the plants were blended in 90% boiling ethanol. The blended material was refluxed on a steam bath for 30

minutes and the mixture was filtered, refluxed for 1 hour with fresh 80% ethanol and filtered again. The volume of the filtrate was reduced, the solution made strongly alkaline with KOH and extracted 4 times with ether. Water and HCl were added to the ether and the mixture was flash evaporated to remove the ether. The nicotine was then isolated from this solution. The alkaline aqueous solution containing the amino acids was neutralized with $HClO_4$, the mixture was cooled overnight, and the $KClO_4$ which formed was removed by centrifugation. The supernatent was treated with charcoal to remove colored substances. Aspartate and glutamate were isolated, degraded and assayed for ^{14}C as previously described (44).

Plants used for $^{14}CO_2$ experiments were grown in flats until 2 weeks prior to the feeding study. At this time, 3 to 5 plants ranging in height from 20 to 25 cm were transplanted to round enamel pans containing soil. Five days before the feeding experiment, the plants were topped. The $^{14}CO_2$ was administered to the plants in a 21.5 liter desiccator in which the $^{14}CO_2$ was released by adding 50% lactic acid to $Ba^{14}CO_3$. Two mC of $^{14}CO_2$ was generated per plant. The $Ba^{14}CO_3$ was purchased from New England Nuclear Corporation. After the $^{14}CO_2$ had been generated, the plants were allowed to metabolize the labeled gas in the sealed desiccator for one hour after which time air was flushed through the desiccator into saturated $Ba(OH)_2$. Essentially all the $^{14}CO_2$ had been fixed by the plants since nearly all the

$BaCO_3$ formed resulted from $^{12}CO_2$ in the air used to flush the feeding chamber. The desiccator top was removed after 1/2 hour of flushing and the plants maintained at normal CO_2 tension for the remainder of the experiment. The temperature in the desiccator rose from 25° to 27° during the first 1½ hours of the experiment. The feeding chamber was illuminated with water-cooled light from two 300-watt Ken-Rad flood lights. The flood lights provided a light intensity of 2400 foot-candles when measured with a Western illumination meter, Model 756. Total metabolic periods lasted 3 or 6 hours at which time the experiments were terminated and the amino acids and nicotine isolated as described previously. Nicotine from N. glutinosa was further purified by distillation through a Widmer column as described by Smith (45). Three N. rustica plants were used for the 6-hour $^{14}CO_2$ experiment. Eight N. glutinosa plants were used for the 6-hour experiment and 13 plants for the 3-hour experiment.

The degradation of the pyrrolidine ring to obtain carbons 3', 4', 5' and the methyl carbon was accomplished by the method of Liebman, Mundy and Rapoport (23), with minor modifications. The pyridine ring of nicotinic acid, obtained as a byproduct from the Liebman et al (23) degradation of nicotine, was degraded by the method of Jackanicz and Byerrum (27) with one modification. The N-methyl-2-pyridone, dissolved in 100 ml of 95% ethanol, was reduced to N-methyl-2-piperidone with 250 mg of 10% palladium on charcoal in a Paar low-pressure hydrogenation apparatus under 50 psi of

hydrogen for 6 hours at 75° (46). Nicotinic acid was decarboxylated to yield C-2' of the pyrrolidine ring. Decarboxylation was accomplished by heating 100 mg of nicotinic acid mixed with 375 mg of freshly prepared copper chromite catalyst (47) in a Wood's metal bath preheated to 270°. Two traps were connected to the decarboxylation apparatus. The first contained 4% $HClO_4$ in isopropyl alcohol and the second saturated $Ba(OH)_2$. CO_2 free-nitrogen was used to flush the pyridine and CO_2 from the decarboxylation apparatus. The reaction was usually completed in 1 hour yielding 50 mg of pyridine perchlorate and 120 mg of $BaCO_3$. The pyridine perchlorate was recrystallized from ethanol and ether. Scott recently published a similar method for the decarboxylation of nicotinic acid (48).

Dimethyl glycine labeled with ^{14}C in carbon 1 or 2 or the methyl group was synthesized by the method of Bowman and Stroud (49) using glycine-1-^{14}C, glycine-2-^{14}C or formaldehyde-^{14}C. The formaldehyde-^{14}C was obtained by heating ^{14}C-paraformaldehyde in aqueous solution at 110° overnight.

In determining the specific activity of a compound by either scintillation or planchet counter, an average of 5 replicate samples were counted per compound. All compounds, except $BaCO_3$, were recrystallized to constant specific activity and 2 to 8 mg were counted on a Packard Tri-Carb Scintillation Spectrometer, Model 3310. All counts were corrected for background and efficiency of the counter. The efficiency of the counter was approximately 92% as determined with a

benzoic acid-7-^{14}C standard purchased from New England Nuclear Corporation. Carbon dioxide from carbons 2' and 4' of the pyrrolidine ring was counted as $BaCO_3$ on a Nuclear Chicago Model C115 low background automatic sample changer with a Model 8703 decade scaler. All counts were corrected for background, self-absorption and efficiency of the counter. $BaCO_3$ standards for the low background counter were prepared by total oxidation of a compound of known specific activity to CO_2 (19), trapping the CO_2 in saturated $Ba(OH)_2$ and counting the $BaCO_3$. The efficiency of the low background counter was approximately 12%.

RESULTS AND DISCUSSION

Specificity of the Degradation:

The main obstacle to overcome in the study of the pyrrolidine ring of nicotine is the degradation procedure. At present there are 4 general degradative schemes in common use (1, 6, 12, 23). The procedure of Lamberts and Byerrum (1) required large amounts of starting material while that of Wu and Byerrum (6) and Leete (12) failed to yield the individual carbons of the pyrrolidine ring in a unique manner. The procedure of Liebman *et al* (23) required the least quantity of starting material and yielded the individual carbons of the pyrrolidine ring. The degradative scheme is shown in Figure 4. After exploring 3 of the above degradative schemes (1, 6, 23), the scheme of Liebman *et al* was decided upon. The specificity of the oxidation of dimethyl glycine by lead tetraacetate to CO_2, formaldehyde and dimethyl amine was tested by degrading ^{14}C-dimethyl glycine labeled in the various carbon atoms. The results are listed in Table 1. When dimethyl glycine was labeled either in carbon 1 or 2, more than 99% of the radioactivity was recovered in the expected degradation product. About 0.1% was found as a contaminant in other products. The degradation of dimethyl glycine-methyl-^{14}C indicated that the compound was not uniquely labeled. Only 90.5% of the

FIGURE 4. Pyrrolidine Ring Degradation Scheme from Liebman *et al* (23)

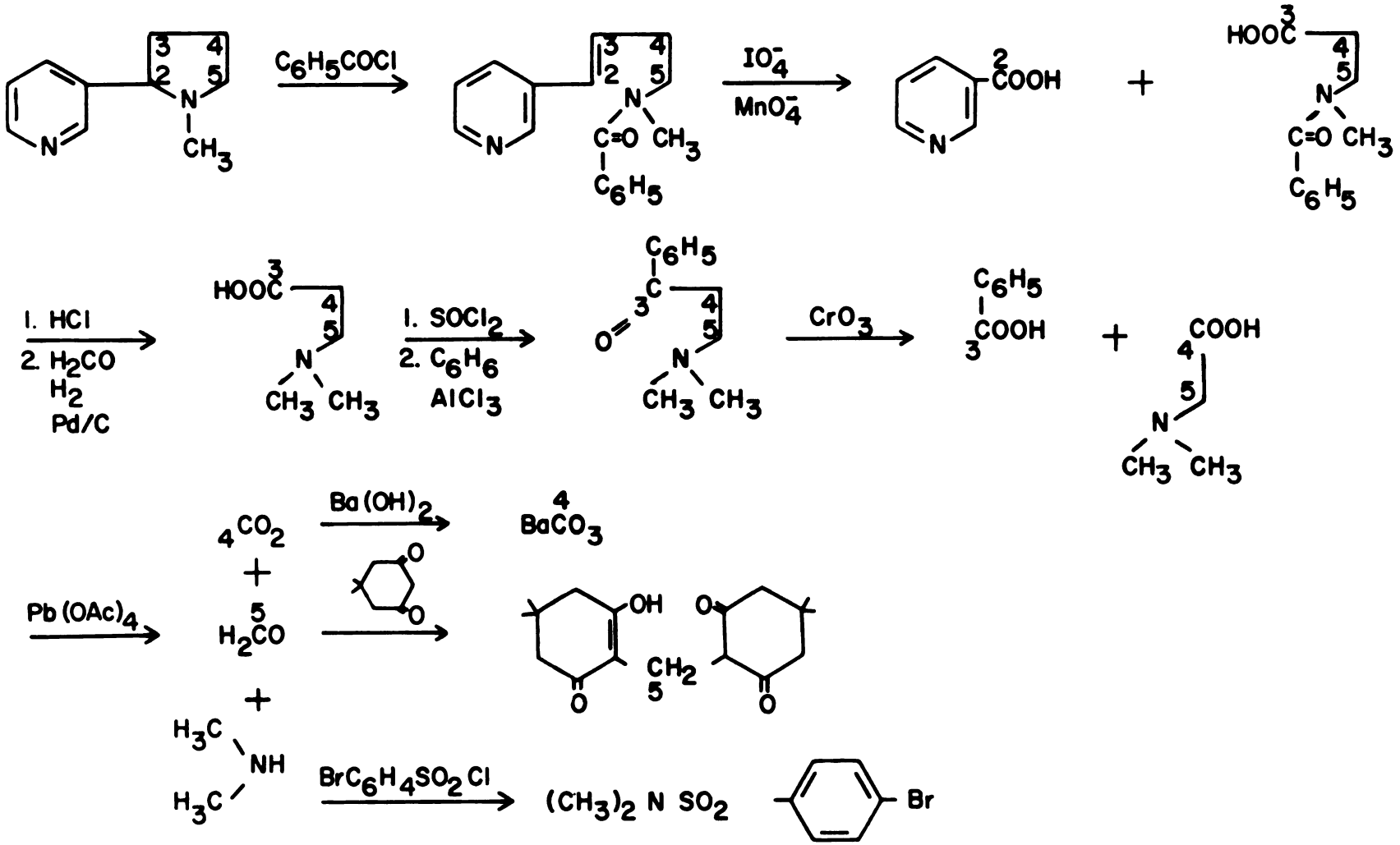

C6H5COCl
IO4-
MnO4-
1. HCl
2. H2CO
H2
Pd/C
1. SOCl2
2. C6H6
AlCl3
CrO3
Pb(OAc)4
Ba(OH)2
BaCO3
CO2
H2CO
BrC6H4SO2Cl
(CH3)2 N SO2
Br
OH
CH2
NH
COOH

TABLE 1. Degradation of Specifically Labeled Glycine

Position of Label	Starting Specific Activity (dpm/mM)	Radioactivity in the Individual Carbons					
		HOOC-		$-CH_2-$		$-N(CH_3)_2$	
		dpm/mM	%	dpm/mM	%	dpm/mM	%
$HOOC^{*}-$	650,100	646,360	99.4	35	0.05	290	0.04
$-CH_2^{*}-$	742,000	950	0.13	740,064	99.7	1,215	0.16
$-N(^{*}CH_3)(^{*}CH_3)$	259,600	3,510	1.3	15,120	5.9	234,050	90.5

radioactivity was recovered in the N-methyl derivative while 7.2% of the label was found in the other two derivatives. If the label in the latter two derivatives had been due to contamination from ^{14}C in the N-methyl groups, the label in the N-methyl derivative would have equalled 100% since specific activity is expressed as dpm per mmole. The total percent of radioactivity recovered in the degradation products of dimethyl glycine-methyl-^{14}C was 97.7%. It was concluded from this study that the degradation of dimethyl glycine was highly specific.

Since the work of Liebman et al (23) indicated low incorporation of label into C-2' from $^{14}CO_2$, the accurate determination of radioactivity in C-2' was critical. Previous methods for decarboxylation of nicotinic acid involved heating nicotinic acid with calcium oxide at 390° or higher. However, previous studies have shown that this results in breakdown of the pyridine ring to yield CO_2 (8, 25). When nicotinic acid with 98% of the radioactivity located in the carboxyl group was decarboxylated with CaO, only 75 to 85% of the total radioactivity was recovered in the $BaCO_3$ (8). After several decarboxylation methods proved to be ineffectual, the decarboxylation of nicotinic acid was successfully accomplished by heating nicotinic acid with copper chromite.

Nicotinic acid-7-^{14}C purchased from New England Nuclear Corporation and nicotinic acid-6-^{14}C purchased from Nuclear Chicago Corporation was decarboxylated with copper

chromite to verfity that the $BaCO_3$ formed was solely due to the carboxyl group of nicotinic acid. In each case 7 successive $BaCO_3$ samples were collected and counted. $BaCO_3$ samples 2 through 6 from nicotinic acid-7-^{14}C contained from 94 to 98% of the expected radioactivity while sample 1 contained 69% and sample 7 contained 84%. Pyridine perchlorate contained 0.1%. Pyridine perchlorate from nicotinic acid-6-^{14}C contained 99.6% of the expected radioactivity. $BaCO_3$ samples 2 through 6 contained 0.6 to 1.3% of the original radioactivity in nicotinic acid-6-^{14}C. Samples 1 and 7 contained 7.8 and 7.2% respectively. The first and last samples, which contained a limited amount of CO_2 from breakdown of the pyridine ring, were not used in determining the specific activity of carbon 2'. Since the pyridine ring contains proportionally a greater amount of radioactivity than the pyrrolidine ring, the experimental specific activity obtained for C-2' is the upper limit of the true value.

Incorporation of Label into Glutamate and Aspartate:

The data obtained in the degradation of glutamate and aspartate from N. rustica and N. glutinosa fed $^{14}CO_2$, acetate-1-^{14}C and acetate-2-^{14}C are presented in Table 2. Glutamate and aspartate were not isolated in the 3 hour $^{14}CO_2$ experiment. After 6 hour $^{14}CO_2$ incorporation by both species, carbons 2 and 3, as well as carbons 4 and 5, of glutamate were labeled equally. Approximately 30% of the label was found in carbons 2 and 3 and about 50% in carbons

TABLE 2. Distribution of ^{14}C in Glutamate and Aspartate

Species	Compound Fed	Time	Glutamate						Aspartate
			% ^{14}C Distribution					% Glutamate ^{14}C Recovered	% Aspartate ^{14}C in C-1 and C-4
			C-1	C-2	C-3	C-4	C-5		
N. rustica	$^{14}CO_2$	6 hrs	14.5	14.6	15.6	25.9	27.8	98.4	46.7
N. glutinosa	$^{14}CO_2$	6	20.2	14.3	13.9	24.4	26.6	99.4	53.6
N. rustica	Acetate-2-^{14}C	1	1.2	3.8	3.5	90.2	1.9	100.6	6.8
N. rustica	Acetate-2-^{14}C	2	1.5	5.4	4.6	81.0	2.8	95.3	25.8
N. rustica	Acetate-1-^{14}C	6	19.1	0	0	0	77.4	96.5	100.0

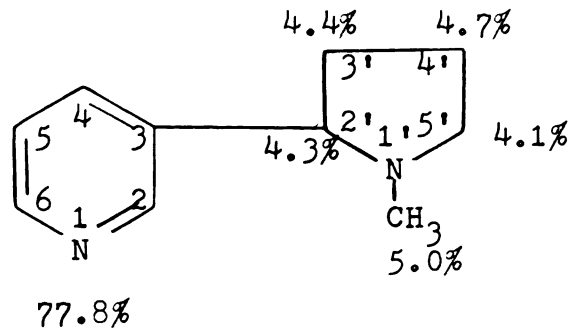
4 and 5. This labeling pattern corresponds well with that obtained by Burns *et al* (50) for glutamate isolated from *N. rustica* leaves exposed to $^{14}CO_2$ for 3 and 18 minutes. These authors found that after the 3 minute $^{14}CO_2$ exposure, carbon 1 of glutamate contained about 9%, carbons 2 and 3 each contained about 2% and carbons 4 and 5 each contained about 43% of the radioactivity. After 18 minutes the difference in radioactivity of the glutamate carbons diminished. Burns *et al* postulated that glyoxylate condensed with oxalacetate to form oxalmalate, γ-hydroxy-α-ketoglutarate, α-ketoglutarate and finally glutamic acid. This is the same pathway proposed by Sekizawa *et al* (51) for *Acetobacter suboxydans*, an organism with a largely nonfunctional tricarboxylic acid cycle. Whether this pathway is operative in higher plants is questionable. However, γ-hydroxy-α-ketoglutarate seems to be present in plants (23). In addition, the labeling pattern observed by Burns *et al* is in agreement with the proposed pathway for glutamate biosynthesis. If carbons 4 and 5 of glutamate are derived from glycolate via glyoxylate it is expected that these carbons would be rapidly and equally labeled (54). Carbons 2 and 3 of glutamate would originate from the internal carbons of oxalacetate and, therefore, would also be equally labeled. In short term experiments carbon 1 of glutamate, derived from carbon 1 of oxalacetate, would be more radioactive than carbons 2 and 3 of glutamate if the carboxyl groups of oxalacetate are more heavily labeled. Thus Burns *et al* (50) observed that after

a 3 minute $^{14}CO_2$ exposure, 80% of the radioactivity of aspartic acid was located in the two carboxyl groups. In Table 2 it is observed that the percent of radioactivity of aspartate in the carboxyl group after 6 hour $^{14}CO_2$ metabolism is approximately 50%. This would indicate nearly uniform labeling of aspartate and, presumably, oxalacetate. Although glutamate isolated from N. rustica after $^{14}CO_2$ incorporation shows equal labeling of carbons 1, 2 and 3, this is not the case for N. glutinosa where carbon 1 of glutamate contained 20% and carbons 2 and 3 about 14% each. This difference is not great enough to rule out the hypothesis of Burns et al.

After 1 and 2 hours of acetate-2-^{14}C metabolism by N. rustica carbon 4 of glutamate contained 90.2% and 81.0% of the total ^{14}C, respectively. Aspartate carboxyl groups contained 6.8% after 1 hour and 25.8% after a 2-hour metabolism period. Carbon 5 of glutamate contained 77.4% and carbon 1 contained 19.9% of the label after N. rustica metabolized acetate-1-^{14}C for 6 hours. Aspartate in this experiment had 100.0% of the radioactivity in the carboxyl groups. Glutamate and aspartate were labeled as expected, if it is assumed that acetate-1-^{14}C and acetate-2-^{14}C are metabolized to form these amino acids via the tricarboxylic acid cycle. Relatively little randomization of ^{14}C is apparent.

Incorporation of Label into the Pyrrolidine Ring of Nicotine:

Table 3 shows the distribution of ^{14}C in the pyrrolidine ring of nicotine after $^{14}CO_2$ incorporation. The numbering systems for the pyridine and pyrrolidine rings of nicotine and the ^{14}C-distribution pattern for nicotine from N. glutinosa after 6 hours of $^{14}CO_2$ incorporation is shown below.



Nearly uniform labeling of the pyrrolidine ring was observed in all $^{14}CO_2$ experiments. If glutamate were incorporated into the pyrrolidine ring of nicotine via the pathway involving a symmetrical intermediate proposed by Leete (Figure 2), carbon 1 would be lost and the remaining carbons randomized as shown in Figure 5. From the figure it is apparent that when carbons 4 and 5, as well as carbons 2 and 3 of glutamate are labeled equally, all the carbons of the pyrrolidine ring will be labeled equally. When this prodiction is compared with the results shown in Table 3, it is observed that there is no essential difference in labeling among any of the carbons of the pyrrolidine ring after $^{14}CO_2$ incorporated for 3 or 6 hours by either N. rustica or N. glutinosa.

Liebman, Mundy and Rapoport obtained ratios of C-5'

TABLE 3. Distribution of ^{14}C in the Pyrrolidine Ring of Nicotine After $^{14}CO_2$ Incorporation

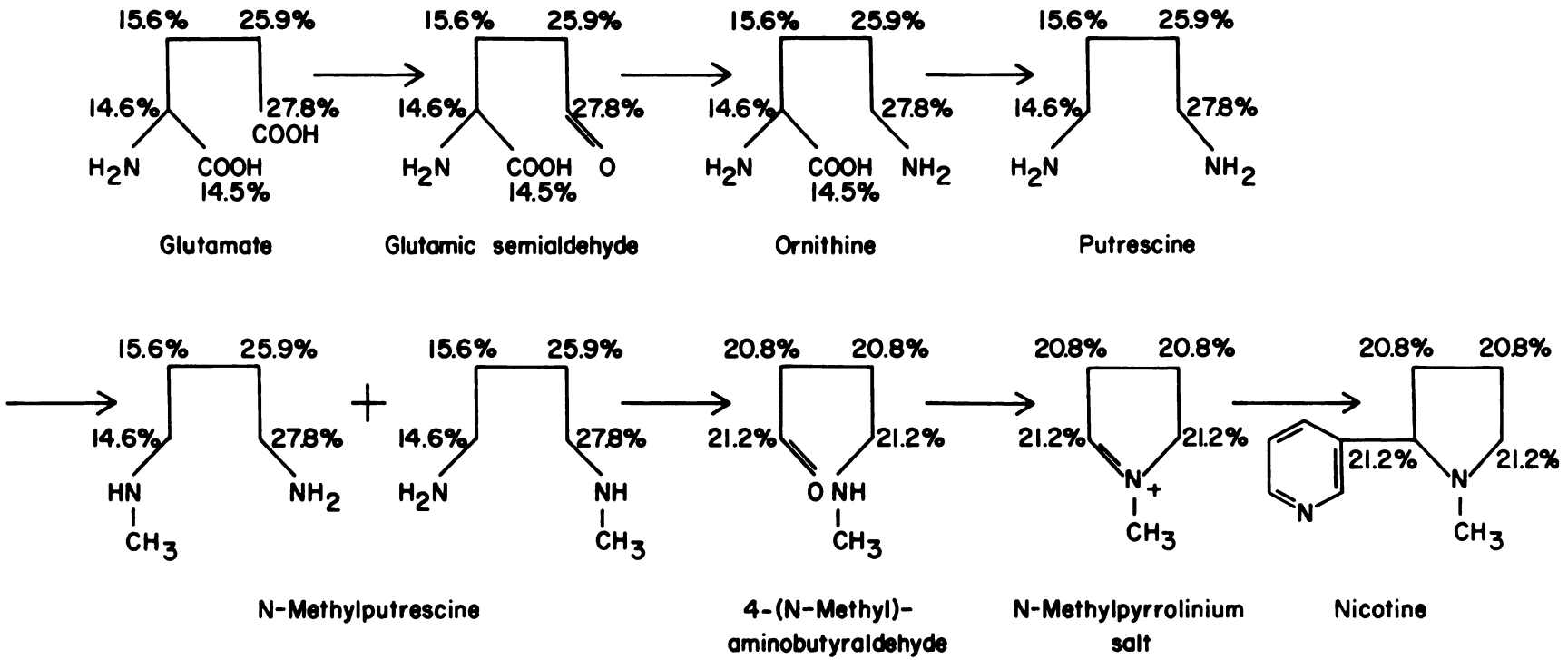
Samples	Carbons	*N. rustica* 6 hrs.		*N. glutinosa* 6 hrs.		*N. glutinosa* 3 hrs.	
		dpm/mmole	%	dpm/mmole	%	dpm/mmole	%
N-Benzoylmetanicotine	Pyr[a],2',3',4',5',CH_3	397,030 ± 1850[b]	100.0	435,080 ± 2610	100.0	93,220 ± 440	100.0
Nicotinic acid	Pyr,2'	315,800 ± 1890	79.5	361,330 ± 2290	83.0	86,090 ± 140	92.3
$BaCO_3$	2'	17,480 ± 360	4.4	18,610 ± 800	4.3	1,480 ± 30	1.6
Pyridine perchlorate	Pyr	296,060 ± 1900	74.6	338,350 ± 3320	77.8	84,300 ± 1920	90.4
N-Benzoyl-N-methyl-β-alanine	3',4',5',CH_3	83,330 ± 690	21.0	80,140 ± 810	18.4	7,220 ± 70	7.7
N,N-Dimethyl-β-alanine·HCl	3',4',5',CH_3	81,430 ± 560	20.5	79,510 ± 1020	18.3	7,960 ± 110	8.3
N,N-Dimethylglycine·HCl	4',5',CH_3	60,590 ± 1370	15.3	57,390 ± 830	13.2	6,830 ± 50	7.3

Benzoic acid	3'	19,040 ± 610	4.8	19,010 ± 130	4.4	1,570 ± 20	1.7
$BaCO_3$	4'	18,500 ± 320	4.7	20,350 ± 220	4.7	1,330 ± 60	1.4
Formaldehyde dimedone	5'	18,180 ± 300	4.6	17,950 ± 250	4.1	1,390 ± 20	1.5
N,N-Dimethyl-p-bromobenzene-sulfonamide	CH_3	27,500 ± 560	6.9	21,620 ± 520	5.0	4,990 ± 160	5.4

[a]The abbreviation used is Pyr, pyridine.

[b]Standard deviation from the mean as determined from the mean of a series of replicate samples.

FIGURE 5. Proposed randomization of glutamate in the formation of the pyrrolidine ring of nicotine after 6 hours of $^{14}CO_2$ metabolism by N. rustica.


15.6%
25.9%
14.6%
27.8%
COOH
H_2N
COOH
14.5%
Glutamate
Glutamic semialdehyde
Ornithine
Putrescine
HN
CH_3
NH_2
NH
O
20.8%
21.2%
N+
N
N-Methylputrescine
4-(N-Methyl)-
aminobutyraldehyde
N-Methylpyrrolinium
salt
Nicotine

label to C-2' label of 3.1, 4.3 and 1.6 from three 6 hour $^{14}CO_2$ incorporation experiments with N. glutinosa (23). The ratios of C-5' radioactivity to C-2' radioactivity in the present study with N. glutinosa were 0.95 and 0.94 after 6 and 3 hour periods, respectively. With N. rustica the ratio was 1.05 after 6 hour $^{14}CO_2$ incorporation. These values are essentially equal to unity. If an unsymmetrical pathway existed, non-symmetry should be more evident in a shorter period of metabolism after the initial uptake of a labeled precursor because of less randomization of the ^{14}C. However, the ratios of C-5' label to C-2' label after 3 and 6 hour metabolism of $^{14}CO_2$ by N. glutinosa were the same. At shorter times a greater percentage of the ^{14}C was found in the pyridine ring as previously observed (24).

The distribution of ^{14}C in the pyrrolidine ring of nicotine as N. rustica fed acetate-2-^{14}C for 2 hours is shown in Table 4. The label in C-2' and C-5' as well as in C-3' and C-4' is the same within experimental error; 2.6% and 1.8% of the ^{14}C in the nicotine molecule were found in C-2' and C-5' respectively. C-3' contained 16.4% and C-4' 15.6% of the total radioactivity in nicotine. Good correlation existed among the counting data for N-Benzoyl-N-methyl-β-alanine, N,N-dimethyl-β-alanine·HCl, N,N-dimethylglycine·HCl and the sum of the individual carbons composing these compounds.

If the glutamate from the 2 hour acetate-2-^{14}C experiment were randomized in the manner shown in Figure 5 before

TABLE 4. Distribution of ^{14}C in the Pyrrolidine Ring of Nicotine From N. rustica After 2 Hours of Acetate-2-^{14}C Metabolism

Sample	Carbons	dpm/mmole	%
N-Benzoylmetanicotine	Pyr[a],2',3',4',5',CH_3	35,870 ± 210[b]	100.0
Nicotinic acid	Pyr,2'	23,910 ± 470	66.7
$BaCO_3$	2'	950 ± 110	2.6
Pyridine perchlorate	Pyr	23,210 ± 420	64.4
N-Benzoyl-N-methyl-β-alanine	3',4',5',CH_3	12,300 ± 190	34.3
N,N-Dimethyl-β-alanine·HCl	3',4',5',CH_3	12,200 ± 180	34.0
N,N-Dimethylglycine·HCl	4',5',CH_3	6,990 ± 220	19.5
Benzoic acid	3'	5,880 ± 60	16.4
$BaCO_3$	4'	5,580 ± 630	15.6
Formaldehyde dimedone	5'	640 ± 20	1.8
N,N-Dimethyl-p-bromobenzene-sulfonamide	CH_3	590 ± 20	1.6

[a]The abbreviation used is Pyr, pyridine.

[b]Standard deviation from the mean as determined from the mean of a series of replicate samples.

incorporation into the pyrrolidine ring, C-2' label would equal C-5' label and C-3' would equal C-4'. The observed ratio of C-5' radioactivity to C-2' radioactivity shown in Table 4 was 0.69. However, because of the lower specific activity of nicotine synthesized in this experiment and the low incorporation of ^{14}C into the pyrrolidine ring, these ratios are not as accurate as in the previous experiment. The ratio of C-4' label to C-3' label is more reliable since C-3' and C-4' had a higher incorporation of ^{14}C. This ratio equaled 0.95. Wu and Byerrum observed equal labeling of C-2' and C-5' as well as C-3' and C-4' after 48 hour acetate-2-^{14}C incorporation by N. rustica (6).

It is predicted from the 2 hour acetate-2-^{14}C incorporation into glutamate that the ratio of C-3' label to C-2' label or the C-4' to C-5' ratios should equal 10.4. Since only 95.3% of the radioactivity of glutamate was recovered after the glutamate degradation, the value of 10.4 may not be entirely accurate. The experimental ratios obtained from the degradation data in Table 4 for the C-3' label to C-2' label and the C-4' to C-5' and 6.3 and 8.7 respectively. The ratio of C-3' label to C-5' label is probably the most accurate since both C-3' and C-5' were determined by scintillation counting and did not involve possible errors due to decarboxylation or planchet counting. This ratio is 9.1 and is in good agreement with the predicted value of 10.4.

The results in this study are consistent with a pathway involving a symmetrical intermediate in the biosynthesis

of the pyrrolidine ring of nicotine and with previous data obtained from precursor experiments. Species differences were not observed. The reason for the discrepancy between these results and those obtained by Liebman et al are not apparent unless they are related to the relatively low specific activity of nicotine degraded by those investigators. Although the scheme presented in Figure 2 for the biosynthesis of the pyrrolidine ring of nicotine is in agreement with the majority of data now available, the possibility of modifications cannot be eliminated.

Study of a Hypothetical Non-Symmetrical Pathway:

In initially considering various alternatives for the biosynthesis of the pyrrolidine ring, a pathway was proposed that would allow non-symmetrical labeling of the pyrrolidine ring. The most consistent feature of the earlier $^{14}CO_2$ incorporation studies by Rapoport's group with N. glutinosa (23-26) was the equal labeling of carbons 4' and 5' and the low label in carbon 2'. Carbon 3' had a specific activity similar to C-4' and C-5'. The proposed pathway was designed to meet several requirements: (1) it should explain the labeling pattern observed by Liebman et al (23), (2) the reactions should utilize physiological compounds via probable reaction mechanisms, (3) the pathway should involve relatively few and simple compounds, and (4) it should be feasible to test the hypothetical pathway. On this basis it was proposed that acetate and sarcosine (N-methyl glycine)

condensed to form the N-methyl pyrrolidine ring of nicotine. The hypothetical pathway is shown in Figure 6.

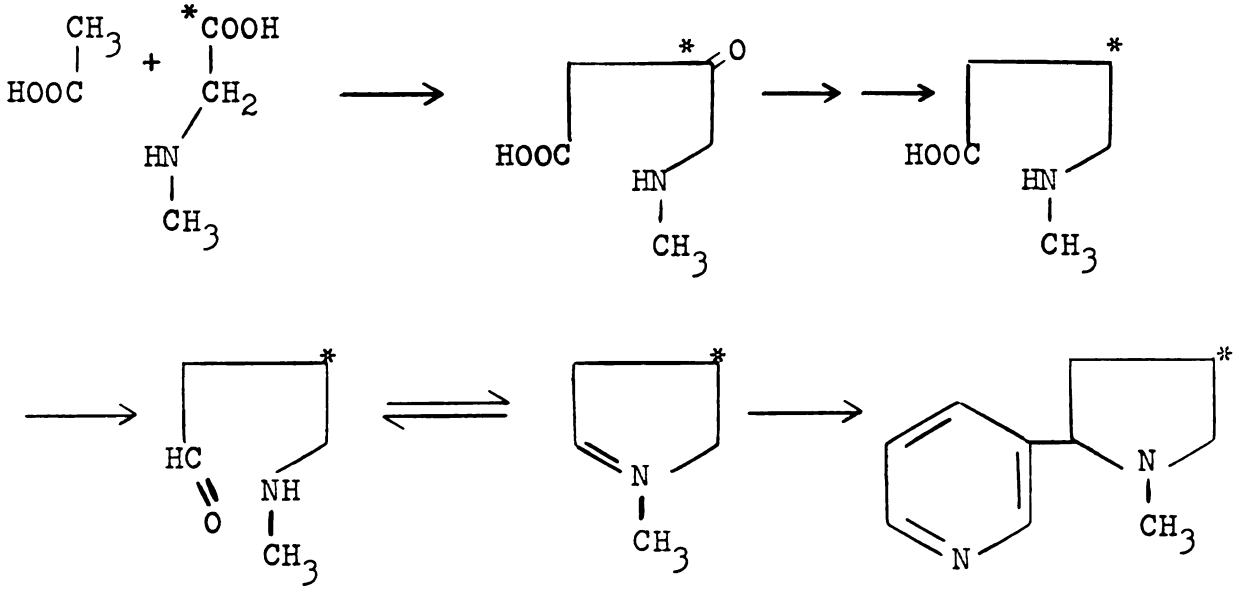


FIGURE 6

Hess and Tolbert (54) have shown that serine, a precursor of choline and therefore sarcosine, is equally labeled in all three carbon atoms 11 seconds after exposure of N. tabacum leaves to $^{14}CO_2$. This observation would explain equal labeling in carbon atoms 1 and 2 of sarcosine. Byerrum, Sato and Ball (22) observed the formation of labeled nicotine, betaine and dimethyl glycine after feeding choline-methyl-^{14}C to N. rustica. Although no mention was made of labeled sarcosine, the possibility existed that the methyl group labeling the nicotine was part of sarcosine rather than an "active C-1 fragment."

In order to explain the low labeling of C-2' observed by Liebman et al (23), the carboxyl group of acetate has to be less rapidly labeled by $^{14}CO_2$ than the methyl group.

Unfortunately the biosynthesis of acetate from $^{14}CO_2$ in higher plants has not yet been studied in detail. If acetate arises from phosphoenolpyruvic acid or from a phosphoclastic cleavage of thiaminepyrophosphate glycolaldehyde in the photosynthetic carbon reduction cycle (55), it is difficult to envision unequal labeling of the acetate carbons from $^{14}CO_2$. However, in the present hypothesis, it is assumed that acetate, or perhaps some other compound condensing with sarcosine, may possess the necessary labeling pattern. It was postulated that the reaction of the methyl group of acetate and the carboxyl group of sarcosine was analogous to the reaction of acetyl-CoA with the carbonyl group of oxalacetate.

To test the proposed pathway, the dilution factors (the specific activity of the compound fed divided by the specific activity of nicotine) for acetate-1 and 2-^{14}C, choline-1,2-^{14}C and sarcosine-1 and methyl-^{14}C were compared. Nicotine samples were partially degraded if they contained sufficient radioactivity. The results are shown in Table 5. Seventy-eight percent of the radioactivity incorporated into nicotine after 6 hours of choline-1,2-^{14}C metabolism was located in the pyrrolidine ring and the N-methyl group. The label in carbon 2' was 7%. This indicates that the majority of the label is located in carbons 3', 4' and 5' plus the N-methyl group. This is in agreement with the proposed pathway. However, Byerrum, Hamill and Ball (56) observed that carbon atom 2 of glycine almost exclusively labeled the N-methyl group of nicotine. Therefore, the possibility

TABLE 5. Incorporation of Small Metabolites Into Nicotine

Compound Fed	Time	S.A. of Compound Fed	S.A. of Nicotine	Dilution Factor	Nicotinic Acid	2'	Pyridine Ring	CH_3
		dpm/mmole	dpm/mmole		dpm/mmole	dpm/mmole	dpm/mmole	dpm/mmole
Acetate-2-^{14}C	2 hrs.	1.90×10^{10}	35,800	5.31×10^{5}	23,910	950	23,210	590
Acetate-1-^{14}C	6	1.575×10^{10}	15,000	10.5×10^{5}	---	---	---	---
Choline-1,2-^{14}C	6	4.63×10^{9}	5,720	8.12×10^{5}	1,600	400	1,280	---
Sarcosine-1-^{14}C	6	1.02×10^{10}	3,630	28.1×10^{5}	---	---	---	---
Sarcosine-methyl-^{14}C	2	5.41×10^{9}	8,320	6.51×10^{5}	---	---	---	4230

[a]Abbreviations used is S.A., specific activity.

exists that choline was metabolized to glycine and then incorporated into the methyl group. Byerrum et al (56) observed no measurable amount of glycine-1-^{14}C incorporation after a 7 day metabolic period. Unfortunately, low incorporation of choline-1,2-^{14}C into nicotine prevented the isolation of the N-methyl group.

In addition, the significance of the above proposed pathway is doubious due to the low incorporation (high dilution factor) of choline-1,2-^{14}C into nicotine. If sarcosine is a direct precursor of the pyrrolidine ring, it is expected that the dilution factors for choline and sarcosine would be lower than dilution factors for compounds, like acetate, which are more distantly related. Acetate-1-^{14}C, like choline-1,2-^{14}C, labeled mainly the pyrrolidine ring of nicotine (5), however its dilution factor is similar to that of choline-1,2-^{14}C. The dilution factor for sarcosine-1-^{14}C is three times as great as that for choline-1,2-^{14}C. In other words, choline-1,2-^{14}C was incorporated more efficiently than sarcosine-1-^{14}C. This observation, and those of Byerrum et al (56) on glycine-1 and 2-^{14}C incorporation, indicate that the label from choline-1,2-^{14}C and sarcosine-1-^{14}C may have been incorporated via glycine.

The incorporation of sarcosine-methyl-^{14}C for a 2 hour metabolic period is similar to that for acetate-2-^{14}C. However, sarcosine-1-^{14}C after 6 hours has a dilution factor 4 times as large as sarcosine-methyl-^{14}C after 2 hours. If sarcosine was incorporated in toto into the pyrrolidine ring

of nicotine, the dilution factor for sarcosine-1-^{14}C and for sarcosine-methyl-^{14}C should be equal at the end of any metabolic period. Since this was not observed and since the overall incorporation was low, it seems likely that the proposed pathway for sarcosine incorporation contributes little, if anything, to the normal biosynthetic pathway for the pyrrolidine ring of nicotine. It is probable that the label from sarcosine-methyl-^{14}C was incorporated via transmethylation. The participation of acetate in the biosynthesis of the pyrrolidine ring as envisioned above has also been eliminated by the data in the previous section which indicated that acetate-2-^{14}C was incorporated symmetrically.

Leete (12) has also postulated a non-symmetrical pathway for the biosynthesis of the pyrrolidine ring of nicotine. In Leete's pathway glycolic aldehyde condenses with acetate to yield 3,4-dihydroxybutyric acid. Dehydration followed by transamination and N-methylation would yield 4-(N-methyl)-aminobutyraldehyde. In order for unsymmetrical labeling to occur, the labeling pattern from $^{14}CO_2$ incorporation into acetate has to differ from the labeling pattern in glycolic aldehyde. This mechanism, as well as the mechanism proposed earlier, fail to explain unequal labeling of acetate. If the $^{14}CO_2$ incorporation studies in the previous section are correct, neither of these non-symmetrical pathways are necessary to explain the observed data.

Incorporation of Label into the Pyridine Ring of Nicotine:

The distribution of ^{14}C in the pyridine ring of nicotine after $^{14}CO_2$ incorporation by N. rustica and N. glutinosa is shown in Table 6. After a 3 hour metabolic period, the labeling pattern from $^{14}CO_2$ in the pyridine ring of nicotine from N. glutinosa is striking. Table 6 shows that the specific activity of carbons 4, 5 or 6 is $2\frac{1}{2}$ times as great as that of carbons 2 or 3. After a 6 hour metabolic period with N. rustica, $^{14}CO_2$ labeled the pyridine ring in a similar manner, however, greater equilibration had occurred among the carbons.

Since the evidence presented in the Introduction indicated that aspartate and glyceraldehyde condense to form the pyridine ring, the most obvious explanation is to assign the two groups of equally labeled carbons in the present experiments to two different precursor molecules. The two precursor molecules would have to meet two requirements. First, the carbons within each precursor that forms the pyridine ring would have to be equally labeled. Second, the specific activity of the two precursors has to differ at the time of pyridine ring synthesis. The first requirement is met by aspartate and 3-phosphoglycerate, which is closely related to the 3-carbon precursor. The two center carbons of aspartic acid, which give rise to carbons 2 and 3 of the pyridine ring, are randomized by cycling through the tricarboxylic acid cycle. Hess and Tolbert (54) have shown that 3-phosphoglycerate is essentially uniformly labeled in

TABLE 6. Distribution of ^{14}C in the Pyridine Ring of Nicotine After $^{14}CO_2$ Incorporation

Compound	Carbons	*N. glutinosa* 3 hrs.		*N. rustica* 6 hrs.	
		dpm/mmole	%*	dpm/mmole	%*
N-Methyl-5-amino-pentanoic acid	2,3,4,5,6	23,640	100.0	150,120	100.0
Sodium propionate	2,3,4	10,650	45.0	79,200	52.8
Sodium acetate	5,6	12,600	53.4	63,820	42.5
Barium carbonate	2	2,360	10.0	19,700	13.1
Barium carbonate	3	2,170	9.2	21,080	14.0
Barium carbonate	4	5,910	25.0	35,070	23.4
Barium carbonate	5	5,710	24.2	32,510	21.7
Barium carbonate	6	6,300	26.7	34,480	23.0

*Percentage of radioactivity as compared to *N*-Methyl-5-aminopentanoic acid.

leaves from N. tabacum after 1 minute photosynthesis in $^{14}CO_2$. The conversion of 3-phosphoglycerate to 3-phosphoglyceraldehyde would explain the uniform labeling of carbons 4, 5, and 6 of the pyridine ring.

The second requirement, that the two precursors have different specific activities at the time of pyridine ring synthesis, is met if the labeled aspartic acid is diluted out rapidly before incorporation into the pyridine ring due to pool size or turnover rate and/or if the rate of incorporation of label from $^{14}CO_2$ into the internal carbons of aspartic acid is slower than into glyceraldehyde or a related 3-carbon compound. Since the synthesis of nicotine is slow (24) and the central position of aspartic acid in metabolism results in a high turnover rate, it is anticipated that the carbons of the pyridine ring derived from aspartic acid would be relatively cold. Like 3-phosphoglycerate, aspartic acid is rapidly labeled by $^{14}CO_2$ (57). However, Burns et al (50) have demonstrated that aspartic acid is not uniformly labeled in N. rustica leaves after 3 or 18 minute exposure to $^{14}CO_2$. After an 18 minute exposure, more than 70% of the label was in the two carboxyl groups of aspartic acid. Thus the rapid labeling of aspartic acid does not truly reflect the rate of labeling of the carbons that form carbons 2 and 3 of the pyridine ring of nicotine. Although these considerations support the hypothesis that glyceraldehyde and aspartic acid condense to form the pyridine ring of nicotine, it does not eliminate the possibility that one, two or more compounds

with unequal internal labeling may be responsible for the observed labeling pattern of the pyridine ring.

The labeling pattern of the pyridine ring of nicotine from N. rustica after 2 hours of acetate-2-^{14}C metabolism shown in Table 7 corresponds well with that observed by Griffith and Byerrum (29). Using a partial degradation of the pyridine ring, they observed that more than 70% of the total radioactivity in the pyridine ring was equally distributed between carbons 2 and 3 after 6 hour acetate-2-^{14}C incorporation into nicotine by N. rustica. Anabasine, an analogue of nicotine, was similarily labeled in the pyridine ring after administering acetate-2-^{14}C to N. glauca (31). This labeling pattern is explained by assuming that acetate-2-^{14}C is incorporated into the pyridine ring of nicotine via aspartic acid. This would also explain the low incorporation of acetate-1-^{14}C into the pyridine ring (58).

The present $^{14}CO_2$ and acetate-2-^{14}C experiments further collaborate the pathway for pyridine ring biosynthesis previously proposed on the basis of feeding chemically synthesized intermediates.

TABLE 7. Distribution of ^{14}C in the Pyridine Ring of Nicotine From N. rustica After 2 Hours of Acetate-2-^{14}C Metabolism

Compound	Carbons	dpm/mmole	%*
N-Methyl-5-amino-pentanoic acid	2,3,4,5,6	11,170	100.0
Sodium acetate	5,6	1,350	12.1
Sodium propionate	2,3,4	9,630	91.3
Barium carbonate	2	4,990	44.7
Barium carbonate	3	4,780	42.8
Barium carbonate	4	460	4.1
Barium carbonate	5	790	7.1
Barium carbonate	6	530	4.7

*Percentage of radioactivity as compared to N-Methyl-5-amino-pentanoic acid.

SUMMARY

This study supports the biosynthetic pathways for the pyrrolidine and pyridine rings of nicotine previously proposed from incorporation experiments with chemically synthesized intermediates. The present evidence indicates that the chemically synthesized intermediates are incorporated into nicotine through normal physiological pathways. The $^{14}CO_2$ and acetate-2-^{14}C data demonstrate that a symmetrical intermediate is involved in the biosynthesis of the pyrrolidine ring of nicotine.

No evidence was found for a proposed non-symmetrical pathway involving sarcosine for the biosynthesis of the pyrrolidine ring.

The $^{14}CO_2$ and acetate-2-^{14}C data is in agreement with the theory that two compounds condense to form the pyridine ring of nicotine. The significance of long term $^{14}CO_2$ incorporation experiments is apparent from this study. In conjunction with chemically synthesized precursors, $^{14}CO_2$ incorporation under normal physiological conditions can provide supporting evidence regarding the probable minimum number of precursors forming the final product. However, the immediate precursors giving rise to a complex product cannot be identified solely from long term $^{14}CO_2$ incorporation.

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APPENDIX

The formula used in correcting the observed $BaCO_3$ counts to infinite thickness was (59, 60):

$$A = \frac{N_s}{FN_k} \qquad \text{where } N_k = \frac{N_{std}}{N_{th}\, F_{std}}$$

A = dpm/mg $BaCO_3$

N_s = cpm of sample minus background

F = Absorption factor for $BaCO_3$ sample

N_k = Efficiency factor for counter

F_{std} = Absorption factor for $BaCO_3$ standard

N_{th} = dpm/mg $BaCO_3$ (Calibrated standard)

N_{std} = cpm of standard $BaCO_3$ minus background