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BIOSYNTHESIS OF THE PYRROLIDINE RING OF NICOTINE IN *NICOTIANA GLUTINOSA*

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Abstract The biosynthesis of the pyrrolidine ring of nicotine has been studied using short-term steady-state exposures of *Nicotiana glutinosa* seedlings to $^{14}CO_2$. The pyrrolidine ring of the labeled nicotine has been degraded in a systematic manner to ascertain the radioactivity at each carbon, and a new method has been developed for obtaining C-2′ with complete radiochemical integrity. Some of the labeling patterns obtained were symmetrical while others were clearly unsymmetrical. The duality of the labeling patterns found in these $^{14}CO_2$ biosyntheses together with other data on pyrrolidine ring biosynthesis which are critically examined, is best rationalized by postulating two biosynthetic pathways for formation of the pyrrolidine ring, one involving a symmetrical precursor and the other an unsymmetrical one.

INTRODUCTION

THE BIOSYNTHETIC pathway forming the pyrrolidine ring of nicotine (**1**) in *Nicotiana* has been the subject of intensive study [1–4]. Based upon precursor feeding experiments alone, a reasonable pathway has been developed. Both glutamate-2-^{14}C[5,6] and ornithine-2-^{14}C[7–9] are incorporated only into the pyrrolidine ring of nicotine, C-2′ and C-5′ contained all the radioactivity and were equally labeled, requiring the intervention of a symmetrical intermediate. A logical intermediate between ornithine and glutamic acid in the biosynthetic sequence is glutamic γ-semialdehyde,[10,11] and the incorporation of 1-pyrroline-5-carboxylate, the cyclized imine of glutamic γ-semialdehyde, has been reported [12].

From ornithine, the next step in the biosynthesis of the pyrrolidine ring presumably involves decarboxylation to give putrescine, an established precursor of the pyrrolidine ring [13–15]. Subsequent methylation would give *N*-methylputrescine, which was found to be incorporated unsymmetrically as expected [16]. Transamination of *N*-methylputrescine

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would give 4-methylaminobutanal which would be in equilibrium with its cyclized iminium form, a 1-methyl-1-pyrrolinium salt, the 1-methyl-1-pyrrolinium salt has been shown to be a metabolite of ornithine and to be an efficient precursor[14,15,17,18] of the pyrrolidine ring of nicotine The remaining steps in the biosynthesis presumably involve condensation of the 1-methyl-1-pyrrolinium salt with a 1 6-dihydronicotinic acid derivative[19] followed by oxidative decarboxylation to give nicotine

In addition to the above precursors of the pyrrolidine ring of nicotine the incorporation of a large number of less immediate precursors has been examined[11,20–24] The majority of these latter precursors have been either tricarboxylic acid cycle or glycolysis intermediates In general the labeling pattern in the pyrrolidine ring of nicotine after the administration of these compounds indicated an incorporation sequence involving the Embden–Meyerhof glycolytic pathway the tricarboxylic acid cycle and glutamic acid However there were unexplained inconsistencies which have been noted[25,26] the most striking of which was in the incorporation of acetate-2-^{14}C as a function of time The labeling patterns in the pyrrolidine ring from acetate-2-^{14}C feedings were not always consistent with metabolism via the TC A cycle to give glutamic acid This result indicated either an alternate biosynthetic pathway for formation of glutamate or another precursor of the pyrrolidine ring

A distinction between a major, minor or aberrant pathway may be difficult to ascertain from precursor feeding experiments alone For example most of the TCA cycle or glycolysis intermediates gave incorporations into the pyrrolidine ring of nicotine consistent with metabolism via the TCA cycle to α-ketoglutarate which could be transaminated to the established precursor, glutamic acid An examination[27] of the labeling pattern of glutamic acid isolated from *Nicotiana rustica* after exposure to $^{14}CO_2$ however indicates that the major normal biosynthetic pathway of formation is not via the intermediacy of the TCA cycle The labeling patterns were difficult to rationalize with an α-ketoglutarate precursor formed via the TCA cycle and the formation of glutamate from α-ketoglutarate via glycolate, glyoxylate, oxalmalate and γ-hydroxy-α-ketoglutarate was indicated

Additional doubt has been cast on the exact interpretation of precursor feeding experiments with *Nicotiana* as a result of numerous contradictory reports On the basis of feeding experiments with γ- and δ-^{15}N-labeled ornithines the δ-nitrogen was reported[28] to be utilized in the biosynthesis of the pyrrolidine ring, and the α-nitrogen of ornithine was not incorporated In other work however the γ-nitrogen of ornithine-α-^{15}N was

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utilized in the biosynthesis of the pyrrolidine ring [29] Another contradiction has arisen with regard to the mode of incorporation of α- and δ-*N*-methylornithines [30,31] α-*N*-Methylornithine-*N*-$^{14}CH_3$ was incorporated more efficiently into the pyrrolidine ring than was δ-*N*-methylornithine-*N*-$^{14}CH_3$, in addition, the *N*-methyl group of nicotine resulting from α-*N*-methylornithine had four times greater radioactivity than from δ-*N*-methylornithine [30] In other experiments,[31] α-*N*-methylornithine was a less efficient precursor than δ-*N*-methylornithine A double-label experiment with the *N*-methylornithines indicated that α-*N*-methylornithine was not incorporated intact, while the δ-compound was incorporated intact, but in an unsymmetrical manner In addition to these contrasting results, the incorporation of α- and δ-*N*-methyl ornithines represent the incorporation of compounds generally considered not to be normal biosynthetic precursors of nicotine [31] The observed symmetrical incorporation of these precursors might have been the result of minor or aberrant pathways

The biosynthesis of the pyrrolidine ring of nicotine has also been studied with $^{14}CO_2$, [9,32] which is a normal precursor Exposure of *N. glutinosa* to $^{14}CO_2$, followed by degradation of the radioactive nicotine gave a clearly unsymmetrical labeling pattern in contradiction to the observed incorporation of intact precursors Other incorporation experiments[24] with $^{14}CO_2$ gave a symmetrical (uniform) labeling pattern in the pyrrolidine ring The labeling patterns found in the pyrrolidine ring were correlated with the labeling patterns found in glutamic acid, however such a correlation may be fortuitous since these exposures were performed under non-steady-state conditions

TABLE 1 THE CONDITIONS UTILIZED FOR EXPOSURES OF *Nicotiana glutinosa* SEEDLINGS TO $^{14}CO_2$

Expt	Length of exposure (hr)	Light Intensity (lx)*	% CO_2 (^{14}C + ^{12}C) Start	End
1†	2 0	6200‡	0 18	0 02
2	0 5	17 000§	0 03	0 02
3	2 0	17 000§	0 04	0 05
4	0 5	17 000§	0 04	0 05
5	2 0	5500‡	0 05	0 05
6	2 0	5500‡	0 22	0 22

* The light intensity was measured with a Weston Illuminator Meter (Model 756)
† Expt 1 was carried out using a modified steady-state apparatus [44]
‡ The light source was a bank of Nu-life Ultra Lux fluorescent bulbs
§ The light source was a bank of Sylvania Gro-Lux fluorescent bulbs

The present series of steady-state exposures was undertaken in an attempt to clarify this paradoxical status of the biosynthesis of the pyrrolidine ring *Nicotiana glutinosa* seedlings (6-day-old) were used throughout since the seedlings could be grown under reproducible, controlled conditions in a growth chamber In order to minimize interpretative difficulties, the exposures of the seedlings to $^{14}CO_2$ was planned so that the CO_2 concentration and light intensity could be precisely controlled The specific activity of $^{14}CO_2$ was kept constant (i e, steady-state conditions)[33] throughout the total exposure period in all the experiments so that the labeling patterns obtained would unambiguously reflect the labeling pattern of the pyrrolidine precursors (Table 1)

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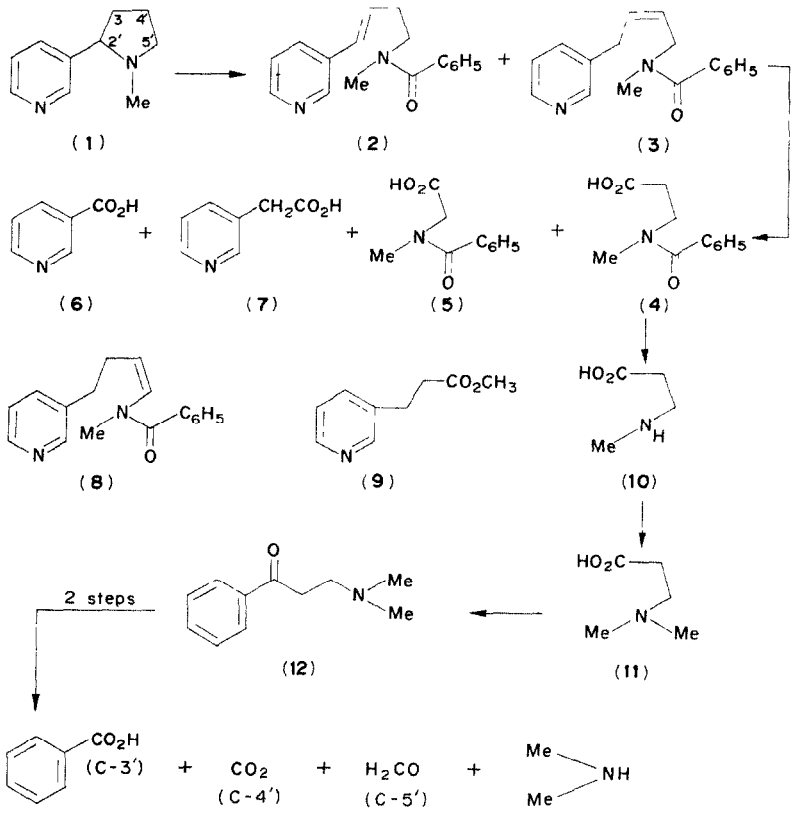
RESULTS

Degradation of radioactive nicotine

The radioactive nicotine (**1**), purified by preparative GLC, was diluted with carrier nicotine and systematically degraded as shown in Scheme 1, to obtain the labeling pattern in the pyrrolidine ring. The established procedure used without modification in degrading the radioactive nicotine from expt 1 (Table 3) was partially modified for the degradation of nicotines from expts 2–6 in order to expedite the degradation and to improve the radiochemical integrity. However, the nature and magnitude of the improvements in radiochemical integrity are not sufficient to change any previous results or interpretations.



SCHEME 1. Degradation of the pyrrolidine ring of nicotine for carbons 3, 4, and 5.

The nicotine (**1**) was heated with benzoyl chloride to give *N*-benzoylmetanicotine (**2**) and its unconjugated isomer **3** in 83% yield; the presence of **3** does not interfere with the utilization of the mixture of **2** and **3** as the base compound for scintillation counting. The unconjugated isomer **3** was present to the extent of approximately 5% as shown by subsequent degradation of the mixture.

Periodate–permanganate cleavage of **2** and **3** gave *N*-benzoyl-*N*-methyl-β-alanine (**4**) and *N*-benzoylsarcosine (**5**) and, by ion exchange chromatography, nicotinic acid (**6**) and 3-pyridylacetic acid (**7**) The presence of pyridylacetic acid in the nicotinic acid fraction was established by GLC of the corresponding methyl esters, prepared by acid-catalyzed esterification with methanol Comparisons with known GLC standards established that **7** was present to the extent of 4 6% in **6** The unconjugated isomer **3** probably arises by acid catalyzed isomerization of **2** The possible presence of **8** the other isomer of **2**, was eliminated by the absence of methyl 3-(3-pyridyl)-propionate (**9**) in the GLC of the mixture of methyl esters of **6** and **7**

The presence of *N*-benzoylsarcosine in the *N*-benzoyl-*N*-methyl-β-alanine fraction was established by hydrolysis to sarcosine and *N*-methyl-β-alanine hydrochloride (**10**), respectively TLC on kieselgel revealed the presence of sarcosine Prior to carrying out further degradation of the nicotine, the sarcosine, isolated in 6% yield, was separated from **10** by ion exchange chromatography For scintillation counting, a small portion of *N*-methyl-β-alanine hydrochloride was converted to the *p*-bromobenzenesulfonamide derivative

The pyridylacetic acid was quantitatively removed from the nicotinic acid by oxidation with alkaline permanganate Conversion of an aliquot of oxidized **6** to the methyl ester followed by GLC analysis showed a single peak and the complete absence of methyl 3-pyridylacetate

The *N*-methyl-β-alanine hydrochloride (**10**) was degraded essentially as previously described [9] Reductive methylation of **10** gave *N*,*N*-dimethyl-β-alanine hydrochloride (**11**) which was converted to acid chloride and used in Friedel–Crafts acylation of benzene to give the Mannich base, β-dimethylaminopropiophenone (**12**) After converting **12** to the sulfate, oxidative cleavage with chromium trioxide in acetic acid–trifluoroacetic acid gave benzoic acid (C-3′) and *N*,*N*-dimethyl glycine hydrochloride The yield of *N*,*N*-dimethylglycine hydrochloride was improved to 57% from the reported[9] 30% by the above modification Lead tetraacetate oxidation of *N*,*N*-dimethylglycine hydrochloride[34] gave CO_2 (C-4′), formaldehyde (C-5′), and dimethylamine (*N*-Me) which were isolated as $BaCO_3$, the dimedone derivative, and *N*,*N*-dimethyl-*p*-bromobenzenesulfonamide, respectively

Nicotinic acid hydrochloride was degraded by a combination of methods in order to obtain C-2′ of the pyrrolidine ring and the pyridine ring Thermal decarboxylation of nicotinic acid in CaO gave pyridine which was isolated as the perchlorate The classical procedure for isolating C-2′ of nicotine has been by regeneration of the decarboxylation carbon dioxide from the CaO to give $BaCO_3$ To test this procedure, the decarboxylation of a sample of nicotinic acid-7-^{14}C (50 660 dpm/mmol) was carried out and gave $BaCO_3$ of 48 130 dpm/mmol, indicating a lack of radiochemical integrity in this mode of decarboxylation due to partial decomposition of the pyridine ring, as has been found by others [24,35] Alternatively, the decarboxylation of nicotinic acid has been reported[24] using a copper chromite catalyst, however, this latter method also lacks complete radiochemical integrity As a consequence, an alternate completely reliable method for obtaining C-2′ was needed and was developed

The method for obtaining C-2′ of nicotine (i e the carboxyl of nicotinic acid) is shown in Scheme 2 Nicotinic acid hydrochloride was converted to the acid chloride, the acid chloride was then converted by Friedel-Crafts acylation of benzene to 3-benzoylpyridine

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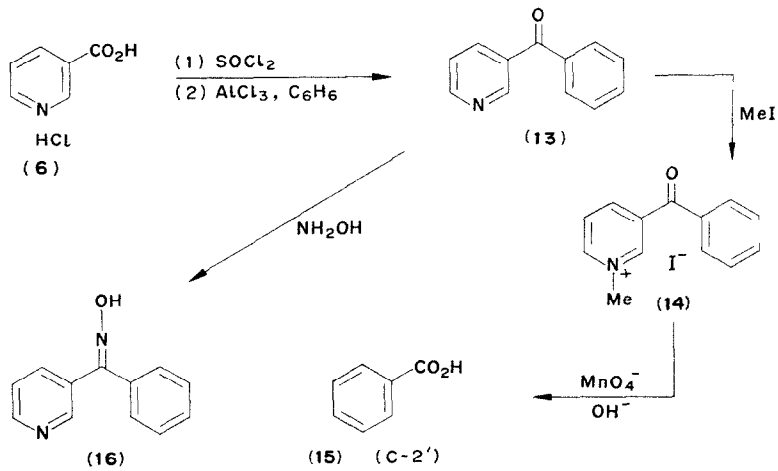
(**13**), and the quaternary pyridinium salt **14** was obtained quantitatively from **13** and methyl iodide Permanganate oxidation of **14** gave benzoic acid (**15**) in an over-all yield of 50%. The oxime **16** was prepared[36] as a solid derivative of **13** for scintillation counting In addition, Beckmann rearrangement of **16** with thionyl chloride in methylene chloride gave benzoic acid (38%) and nicotinic acid (57%) corresponding yields of aniline and 3-aminopyridine were also obtained The reported[36] stereospecificity in the preparation of the two possible oximes of **16** would appear to be lacking



SCHEME 2 Determination of C-2 of nicotine

To check the radiochemical integrity of the degradation of nicotinic acid, two different samples of nicotinic acid-7-^{14}C were degraded TLC and radioautography were used to verify the homogeneity of the radiochemicals The results of the two degradations are shown in Table 2 Clearly, the method for obtaining the carboxyl of nicotinic acid as benzoic acid proceeds with complete radiochemical integrity In addition, benzoic acid is a more suitable derivative for scintillation counting than $BaCO_3$

TABLE 2 DEGRADATION OF NICOTINIC ACID-7-^{14}C HCl (**6**)

Compound	Sp act (dpm mmol)	% of VI
Sample 1		
Nicotinic acid-7-^{14}C HCl	93 600	100 0
Phenyl 3-pyridyl ketoxime	94 500	101 1
Benzoic acid	94 650	101 1
Sample 2		
Nicotinic acid-7-^{14}C HCl	48 550	100 0
Phenyl 3-pyridyl ketoxime	47 250	97 3
Benzoic acid	48 270	99 5

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Labeling patterns in the pyrrolidine ring

The labeling patterns in the pyrrolidine ring of nicotine from six different $^{14}CO_2$ exposures are shown in Table 3 Both symmetrical and unsymmetrical labeling patterns were obtained as a result of steady-state biosynthesis with $^{14}CO_2$

DISCUSSION

The most striking support for the formation of the pyrrolidine ring of nicotine via an unsymmetrical intermediate is evident in the labeling pattern obtained in expt 1 Carbon-2′ of nicotine had 19 2% of the total activity while C-5′ had only 2 0% Asymmetry is also evident when the labeling of C-3′ and C-4′ is examined, C-3 and C-4′ had 2 8 and 0 8%, respectively Clearly, in the case of expt 1, the major pathway of biosynthesis of the pyrrolidine ring of nicotine was via a pathway in which the carbon skeleton was not symmetrized Experiments 2–6 also contain small degrees of asymmetry, however, the asymmetry found in expts 3–6 may be within experimental error

As a consequence, expt 3–6 may be consistent with formation of the pyrrolidine ring via a symmetrical precursor, C-2′, C-3′, C-4′ and C-5′ are all nearly equally labeled in each case For example, C-2′, C-3′, C-4′ and C-5′ had 3 0, 3 4, 3 1 and 3 2% of total activity of nicotine in expt 4 The observed labeling patterns in expts 3–6 can be correlated with the steady-state labeling pattern obtained previously[27] for glutamic acid A similar correlation has been made[24] based upon non-steady-state labeling patterns in the pyrrolidine ring of nicotine, this latter correlation is not definitive since the possible kinetic effects of an incorporation of $^{12}CO_2$ after $^{14}CO_2$ apparently were not considered

Although labeling patterns in glutamic acid after a non-steady-state exposure[24] were similar to those obtained under steady-state conditions,[27] several significant differences can be noted For example the trend[27] of increasing label in C-1 relative to C-4 and C-5 of glutamate as a function of time was not observed [24] The labeling pattern in glutamate from *N glutinosa* also differs significantly from the glutamate pattern observed for *N rustica*, in particular, the % ^{14}C in C-1 relative to C-2 and C-3 was greater in the former while less in the latter These discrepancies may reflect species differences and/or the kinetic effects due to non-steady-state exposures with $^{14}CO_2$ in the one case

The possibility exists that uniform labeling, resulting from saturation of all pyrrolidine ring metabolic precursors with C-14, has been misinterpreted[24] as symmetrical labeling, however the variety of conditions utilized and the low percentage of the total C-14 in the pyrrolidine ring in each of the present experiments makes this interpretation unlikely The possibility that the difference in the two types of labeling patterns observed is due to degradative errors also seems highly unlikely in view of the many examples of both symmetrical and non-symmetrical labeling in the pyrrolidine ring of nicotine, excellent activity balances have been obtained in all the reported degradations with the exception of expt 1 which contains a minor discrepancy due to the lack of complete integrity in the decarboxylation of nicotinic acid

The effect of CO_2 concentration was examined in expt 6 (% $^{14}CO_2$ = 0 22) since the possibility existed that the very high incorporation of label into C-2′ of nicotine in expt 1 was the result of a direct fixation of $^{14}CO_2$ into an immediate precursor of the pyrrolidine ring Although no effect other than kinetic was observed in expt 6 due to the high concentration of $^{14}CO_2$, the possibility that an unsymmetrical biogenetic pathway involving direct fixation of CO_2 exists in *Nicotiana* is an attractive explanation for the labeling

Table 3. Degradation of nicotine biosynthesized under steady-state

Compound	Sp act (dpm mmol)	% of act in 2	Compound
Expt 1			Expt 2
N-Benzoylmethylnicotinic (2)	51 720	100.0	N-Benzoylmethylnicotinic (2)
Methyl nicotinate	42 110	81.4	Nicotinic acid hydrochloride
Pyridine	34 590	66.9	Pyridine perchlorate
$BaCO_3$ (C-2)	9930	19.2	Benzoic acid (C-2)
N-Benzoyl-N-methyl-β-alanine	10 050	19.4	N-*p*-Bromobenzenesulfonyl-N-methyl-β-alanine
Benzoic acid (C-3)	1460	2.8	Benzoic acid (C-3)
$BaCO_3$ (C-4)	489	0.8	$BaCO_3$ (C-4)
Formaldehyde dimedone (C-5)	1080	2.0	Formaldehyde dimedone (C-5)
N-N-Dimethyl-*p*-bromobenzenesulfonamide ($N\text{-}CH_3$)	6120	11.8	N-N-Dimethyl-*p*-bromobenzenesulfonamide ($N\text{-}CH_3$)

in expt 1. Previously,[9] nonsymmetrical labeling was obtained in the pyrrolidine ring using lower levels and a pulsed exposure to $^{14}CO_2$ followed by $^{12}CO_2$ as chaser.

The effect of either $^{14}CO_2$ concentration or light intensity on the biosynthesis of the pyrrolidine ring of nicotine appears to be mainly kinetic. The labeling pattern in each of steady-state expts. 3, 5 and 6 was essentially the same. Each exposure was carried out for 2 hr, only the light intensity and $^{14}CO_2$ concentration were varied. Utilizing the fact that C-14 from $^{14}CO_2$ is incorporated more rapidly into the pyridine ring than into the pyrrolidine ring of nicotine,[25] the effect of these two variables upon the rate of biosynthesis becomes evident. Decreasing the measured light intensity from 17 000 to 5 500 lx gave 76.0 and 90.2% of the activity in the pyridine ring, respectively. Clearly a greater fraction of the activity was incorporated into the pyrrolidine ring in the same period of time at the higher intensity, the amount decreased from 16.5 to 5.6% on decreasing the light intensity.

Similar decreases in the amount of CO_2 fixed and the activity in both the total alkaloid fraction and nicotine also were observed with a decrease in light intensity using $^{14}CO_2$ of the same specific activity. At a light intensity of 5 500 lx from the same light source, the pyrrolidine ring contained 5.6 and 7.5% of the total activity after biosynthesis at a $^{14}CO_2$ concentration of 0.05 and 0.22%, respectively, the pyridine ring had 90.2 and 86.4%, respectively, of the activity. Thus a change in light intensity has a much greater effect upon the rate of biosynthesis in *Nicotiana* than a change in CO_2 concentration.

The fact that the pyridine ring of nicotine is biosynthesized more rapidly than the pyrrolidine ring is consistent with the hypothesis that the pyridine ring of nicotine is formed from 3-phosphoglyceraldehyde and aspartic acid.[37] 3-Phosphoglyceric acid, closely related to 3-phosphoglyceraldehyde has been shown[38] to be essentially uniformly labeled in the leaves of *N. tabacum* after 1 min of photosynthesis in $^{14}CO_2$. Accordingly it seems reasonable that the pyridine ring should incorporate C-14 from $^{14}CO_2$ more rapidly than the pyrrolidine ring as is evident from the distribution of C-14 in expts. 3 and 4. At the same light intensity and percentage of $^{14}CO_2$ the pyridine ring had 82 and 76% of the total activity after 0.5 and 2 hr, respectively.

Two areas of potential experimental and interpretative difficulty deserve comment. First, since experiments have been carried out with several different species of *Nicotiana* (*glutinosa*, *tabacum* and *rustica*), some of the observed contradictions in the precursor and $^{14}CO_2$ experiments may result from a species difference. A species difference however has

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CONDITIONS LABELLING PATTERNS IN THE PYRROLIDINE RING

Sp act (dpm/mmol)	% of act in 2	Sp act (dpm/mmol)	% of act in 2	Sp act (dpm/mmol)	% of act in 2	Sp act (dpm/mmol)	% of act in 2	Sp act (dpm/mmol)	% of act in 2
		Expt 3		Expt 4		Expt 5		Expt 6	
55 180	100 0	178 400	100 0	47 000	100 0	163 300	100 0	161 000	100 0
49 710	90 1	144 400	81 0	39 700	84 5	149 200	91 5	142 500	88 6
48 600	88 2	135 500	76 0	38 540	82 0	147 200	90 2	139 500	86 4
475	0 9	7560	4 2	1425	3 0	2290	1 4	2760	1 7
6770	12 3	33 260	18 6	7150	15 2	12 800	7 8	17 620	11 0
446	0 8	7750	4 4	1580	3 4	2350	1 4	3050	1 9
420	0 8	7210	4 1	1460	3 1	2190	1 3	2940	1 8
797	1 5	6650	3 8	1510	3 2	2520	1 5	3420	2 1
5260	9 5	10 660	6 0	2655	5 6	5610	3 4	8470	5 3

not yet been definitely detected The incorporation of ornithine-2-^{14}C into all three species studied has been found to give equal labeling at C-2′ and C-5′ of the pyrrolidine ring [7–9] As mentioned above, however, a species differences in *N glutinosa* and *N rustica* may have been observed[24] when differences in the labeling patterns of glutamate were obtained Another difficulty, in attempting to correlate the multitude of biosynthetic experiments concerned with the formation of the pyrrolidine ring of nicotine involves the variety of *Nicotiana* plant materials used by various workers Precursor feeding and $^{14}CO_2$ exposures have been carried out with root cultures, de-rooted plants, seedlings, intact plants, topped plants, and excised rooted leaves In general, there seems to be little experimental justification for the variety of modified plant materials used, the modifications merely introduce other variables into an already complex situation This would appear to be particularly true if the potential influence on the enzymology of a plant is considered For example, topping or partial defoliation of *Nicotiana* has been used by several workers[24,37] prior to $^{14}CO_2$ exposures Using several different plants, it has been established[39] that partial defoliation leads to an increased photosynthetic rate under varying light intensities and carbon dioxide concentrations, in addition, a corresponding increase in the level of carboxylating enzymes was also noted Potentially other enzymic and metabolic changes might also occur and influence the experimental result of biosynthetic experiments

The multitude of data and the resultant paradox implies the existence of two different pathways for the biosynthesis of the pyrrolidine ring, one symmetrical, the other unsymmetrical There are several cases in which two different biosynthetic pathways are known for the biosynthesis of a natural product, in fact, th *Nicotiana* alkaloids anabasine[13,40,41] and nornicotine[42] are cases in point A much greater understanding of the system in *Nicotiana* will be required before the inconsistencies obtained in precursor feedings and $^{14}CO_2$ exposures are resolved

EXPERIMENTAL

Growth of Nicotiana glutinosa *seedlings* The seedlings were grown in a modified Petri dish, half of which was fitted first with a circular wick of Whatman No 1 filter paper and then with a circular piece of Whatman seed test paper On each seed paper was spread evenly 300 mg of *N glutinosa* seeds The seeds (8 g) had been previously washed in a fritted filter with 500 ml 3% H_2O_2 and 250 ml dist H_2O subsequently the seeds were dried on the filter by drawing sterile air through for 18 hr The wick assembly was then placed on he other

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half of the Petri dish which was filled with dist H_2O. The seeds were germinated in the dark in a Percival growth chamber for 2 days with a temp cycle of 21° for 12 hr and 27° for 12 hr; the humidity was 50%. After 2 days, the H_2O was replaced with half-strength nutrient soln[43] and the seedlings were grown for 4 days with alternating 12 hr periods of light and dark. After 4 days, 15 dishes of the best seedlings were selected from a total of 25 dishes and exposed to $^{14}CO_2$.

Exposure of seedlings to $^{14}CO_2$. Steady-state exposures (except expt 1) of *N. glutinosa* seedlings to $^{14}CO_2$ were carried out in a specially designed seedling biosynthetic chamber as described.[33] Expt 1 was carried out using a modification of a previously described steady-state apparatus.[44] Directly above the chamber was a bank of either Sylvania Gro-Lux or Nu-lite Ultra-Lux fluorescent bulbs. The light intensity was measured with a Weston Illumination Meter (Model 756). A closed system containing a circulating pump, an IR CO_2 analyzer and a vibrating reed electrometer was used to monitor the atmosphere. The CO_2 analyzer was calibrated with standards obtained from the Pacific Oxygen Company containing 0.04, 0.08 and 0.18% CO_2. The chamber was first purged of CO_2 by circulating its atmosphere through a tube of ascarite. The $^{14}CO_2$ was then admitted into the exposure chamber and the concentration of CO_2 and C-14 was maintained[33] in the presence of the seedlings. After the desired exposure time, the seedlings were removed to a glove box and frozen in liq. N_2 within 3–4 min of removal. The conditions used for each exposure experiment are given in Table 1.

Isolation of radioactive nicotine. The extraction and isolation of the radioactive nicotine from the frozen seedlings were carried out as previously described.[5]

N-Benzoylmetanicotine (**2**). The previous[9] procedure was modified as follows. To 1.62 g (10 mmol) nicotine was added 6.4 ml benzoyl chloride; the mixture was heated at 150° for 12 hr, after which 50 ml 4 N NaOH was added and the resulting mixture was extracted (6 × 20 ml) with CH_2Cl_2. The combined extracts were extracted (7 × 20 ml) with 10% HCl; the aq. acidic extracts were taken to pH 6 and extracted (7 × 20 ml) with CH_2Cl_2 and the aq. residual soln was then adjusted to pH 7 and extracted again. The pH 6 and 7 extracts were combined and evaporated to give impure **2** which, after digestion with cyclohexane, was dissolved in C_6H_6 and chromatographed on a column of 250 g of Merck acid washed alumina eluting with 500 ml C_6H_6, 250 ml 50% $CHCl_3$ in C_6H_6, and $CHCl_3$. After two recrystallizations from cyclohexane, **2** melted at 77–78° [lit.[9] m.p. 78–79°].

Isolation of nicotinic acid hydrochloride (**6**). The aq. soln from the periodate–permanganate oxidation[9] of N-benzoyl-metanicotine remaining after the basic and acidic continuous extractions with CH_2Cl_2 was acidified to pH 2 with 10% HCl. This soln was placed on 3 l. of AG-50W-X8 (cation exchange resin, 200–400 mesh, H⁺ form). The column was washed with dist. H_2O until neutral, then with 2 N aq. NH_3. The first 2 l. of alkaline eluate was evaporated at 40–50° until the solution had a pH of 8 and then was applied to 300 ml of AG-1-X8 (200–400 mesh, OH⁻ form). Washing with dist. H_2O until the effluent was neutral was followed with 2 N HCl; the first 500 ml of the acidic eluate, giving impure nicotinic acid hydrochloride which was sublimed at 140–300°/μ to yield 1.60 g (66%) of **6** containing *ca.* 5 mol% of 3-pyridylacetic acid (**7**). To 1.52 g (9.5 mmol) of **6** and 87 mg (0.5 mmol) of **7** was added 50 ml H_2O, 20 mmol (2.77 g) K_2CO_3 and 9 mmol $KMnO_4$. After heating at reflux for 5 hr an additional 4 mmol $KMnO_4$ was added and this was repeated at the end of 10 hr. After 20 hr of reflux the MnO_2 was removed by centrifugation, the pH was adjusted to 8, and the aq. soln was applied to a cation exchange resin (AG-1-X8, OH⁻ form, 200–400 mesh, 300 ml resin), washing the column with H_2O until neutral and then eluting with 2 N HCl. The first 600 ml acidic eluate was collected and evaporated to dryness on a rotary evaporator. Sublimation at 130–20° gave 1.28 g (80%) of nicotinic acid hydrochloride, m.p. 265–267°.

3-Benzoylpyridine (**8**). Nicotinic acid hydrochloride (230 mg, 1.43 mmol) and 10 ml $SOCl_2$ was heated until all the nicotinic acid hydrochloride had dissolved. Excess $SOCl_2$ was removed *in vacuo*, 20 ml C_6H_6 was added followed by 480 mg (3.6 mmol) of $AlCl_3$, and the mixture was boiled for 2.5 hr, followed by cooling and pouring onto 100 g ice containing 10 ml conc. HCl. After extraction with Et_2O (2 × 15 ml), the soln was adjusted to pH 11 with 6 N NaOH and then extracted with CH_2Cl_2 (7 × 20 ml). Drying the CH_2Cl_2 extract over $MgSO_4$ and filtering, followed by removal of solvent and distillation at 110–2.5 mm gave 192 mg (72%) of 3-benzoylpyridine. IR (thin film) 1700 cm^{-1} (C=O). UV$_{max}$ (95% EtOH) 255 nm. NMR (CCl_4) δ 8.93 (s, 1H), 8.71 (*d*, 1H) and 7.76 (*m*, 7H). (*Anal.* Calcd. for $C_{12}H_9NO$: C, 78.7; H, 5.0; N, 7.6. Found: C, 78.6; H, 5.1; N, 7.5%.)

3-Benzoyl-N-methylpyridinium iodide (**14**). 3-Benzoylpyridine (5 mmol, 916 mg) and 15 ml MeI were stirred at 20° in the dark for 16 hr. Evaporation of the excess MeI gave 1.63 g (100%) of XIV, m.p. 146–148° after recrystallization from Et_2O–MeOH. NMR (CD_3OD) δ 9.28 (*m*, 2H), 8.92 (*m*, 1H), 8.37 (*m*, 1H), 7.87 (*m*, 2H), 7.68 (*m*, 3H) and 4.55 (*s*, 3H). (*Anal.* Calcd. for $C_{13}H_{12}INO$: C, 48.1; H, 3.8; I, 39.0; N, 4.3. Found: C, 48.1; H, 4.0; I, 38.8; N, 4.3%.)

Oxidation of 3-benzoyl-N-methylpyridinium iodide (**14**). A soln of 150 mg (0.46 mmol) of **14** in 10 ml 10% NaOH was treated with 1.02 g (6.5 mmol) $KMnO_4$ and the reaction mixture was heated at reflux for 24 hr. After

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cooling, sodium bisulfite was added to decompose any excess MnO_2, the MnO_2 was removed by centrifuging, and the aq soln was acidified with conc HCl The aq soln was then extracted with CH_2Cl_2 (8 × 20 ml) and the CH_2Cl_2 extract dried over $MgSO_4$ Filtration, removal of the solvent and sublimation gave 40 mg (71%) of pure benzoic acid, m p 119–120°

Chromic acid oxidation of β-dimethylaminopropiophenone hydrochloride (**12**) β-Dimethylaminopropiophenone hydrochloride[9] (1 02 g, 4 75 mmol), 50 ml H_2O, and 2 ml conc H_2SO_4 were mixed and the soln was evaporated to a viscous liquid *in vacuo* The remainder of the oxidation and isolation was carried out as previously described [9]

Ion exchange chromatography of sarcosine and N-*methyl-β-alanine* (**10**) The ion exchange column of AG-50W-X8 (sulfonic acid resin, H^+ form, 200–400 mesh, 5 × 58 cm) was washed first with 20 l 6 N HCl, then 1 l dist H_2O, and finally 4 0 15 N HCl The crystalline mixture of 89 mg sarcosine (1 mmol) and 2 20 g (15 mmol) *N*-methyl-β-alanine hydrochloride was dissolved in 50 ml 1 5 N HCl and applied to the column Using a flow rate of 200 ml/hr and collecting 50 ml fractions, the column was eluted with 0 15 N HCl (2 l), 0 25 N HCl (3 l), 0 40 N HCl (3 l) and 0 75 N HCl Each fraction was analyzed by TLC spraying with ninhydrin solution and heated at 105° for a few min Evaporation of fractions 114–125 gave sarcosine hydrochloride, and evaporation of fractions 130–145 gave *N*-methyl-β-alanine hydrochloride, completely separated from one another

Methyl 3-(3-*pyridyl*)-*propionate* (**9**) Ethyl 3-(3-pyridyl)-acrylate dissolved in EtOAc was hydrogenated at 3 kg/cm^2 over Pd–C The theoretical amount of H_2 was absorbed in 2 hr the soln was filtered, the solvent was evaporated and the residue of ethyl 3-(3-pyridyl)-propionate was dissolved in MeOH and heated at reflux for 96 hr after adding 130 mol % *p*-toluenesulfonic acid Evaporation of the MeOH and addition of satd $NaHCO_3$ soln was followed by extraction with CH_2Cl_2 The dried solvent was evaporated and the product purified by molecular distillation at 100–120°/2 mm to give methyl ester UV max (95% EtOH) 262 nm NMR (CCl_4) δ 8 50 (*m* 2H), 7 42 (*m* 1H) 7 20 (*m*, 1H), 3 58 (*s*, 3H), and 2 71 (*m* 4H) An analytical sample was prepared by GLC using 30% QF-1 on Chromosorb P (AW) (2 4 m × 13 mm column temp 156 flow rate 130 ml/min, R_t 22 4 min) (*Anal* Calcd for $C_9H_{11}NO_2$ C, 65 4, H 6 7 N, 8 5 Found C 65 4, H 6 7, N 8 4%)

N-p-*Bromobenzenesulfonyl*-N-*methyl-β-alanine* In a centrifuge tube was placed 0 28 mmol of *N*-methyl-β-alanine hydrochloride dissolved in 2 ml H_2O then 150 mg *p*-bromobenzenesulfonyl chloride was added along with 1 ml 6 N NaOH and the resulting aq slurry was sonicated at 20° for 1 hr The solid was removed by centrifugation and the liquid was decanted into another centrifuge tube acidified to Congo red with 10% HCl, and cooled After centrifugation, the liquid was decanted, and the resulting ppt was recrystallized from aq EtOH to give 15 mg of XI m p 107–109° (*Anal* Calcd for $C_{10}H_{12}BrNO_4S$ C 37 3, H, 3 8 Found C, 37 3, H, 3 7%)

Phenyl 3-*pyridyl ketoxime* (**16**) 3-Benzoylpyridine (3 mmol, 549 mg) was dissolved in 20 ml EtOH, after which 1 25 g (18 mmol) NH_2OH hydrochloride and 1 55 g (18 mmol) $NaHCO_3$ were added After heating at reflux under N_2 for 5 hr, the reaction mixture was cooled and 60 ml ice was added The ppt was removed by filtration, dried, and recrystallized from EtOH to give 500 mg, 84%, of **16** m p 145–148° [lit[36] m p 141–143°, 161–162°]

Beckmann rearrangement of phenyl 3-*pyridyl ketoxime* (**16**) To 100 ml CH_2Cl_2 and 5 96 g (30 mmol) phenyl 3-pyridyl ketoxime was slowly added 8 ml $SOCl_2$ and the mixture was heated at reflux for 18 hr Solvent and excess $SOCl_2$ were removed *in vacuo* 80 ml 6 N HCl was added and the mixture was heated at reflux for 6 5 hr After cooling the pH was adjusted to 11 with 10% NaOH and the aq soln continuously extracted with CH_2Cl_2 for 60 hr Evaporation of the CH_2Cl_2 gave 3 06 g of a liquid GLC examination of this liquid on a column of 30% QF-1 on Chromosorb P (3 m × 16 mm, column temp 186 , flow rate 100 ml/min, aniline R_t 4 5 min, 3-aminopyridine R_t 11 3 min) and comparison with known standards showed the presence of 1 74 g (57%) aniline and 1 32 g (43%) 3-aminopyridine The pH of the aq soln was then adjusted to 1 with 10% HCl and continuously extracted with CH_2Cl_2 for 48 hr Removal of the solvent gave 1 38 g (37 8%) benzoic acid Ion exchange chromatography of the aq soln as described previously gave, after sublimation, 2 75 g (57%) nicotinic acid hydrochloride

C-14 *determinations* The degradation compounds were assayed for by liquid scintillation employing a Nuclear-Chicago Mark I counter Sample quenching was determined with the external standard attachment for this instrument All sample counts were corrected for background When multiple counts were performed repetitive counts agreed within 2% in all cases When counting samples of low sp act sufficient sample was counted so that each scintillation sample was at least 50 cpm above background Each compound regardless of sp act was also subjected to liquid scintillation counting until a minimum of 20 000 total counts had been amassed, as a consequence, the sp act obtained has a 99% probability of being with 2% of the actual value

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