Biosynthetic Studies with $^{13}CO_2$ of Secondary Plant Metabolites. *Nicotiana* Alkaloids. 1. Initial Experiments

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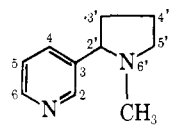
Abstract: The results of an investigation to assess the utility of highly enriched $^{13}CO_2$ as a biosynthetic probe of secondary plant metabolites are described wherein the biosynthesis of nicotine (**1**) in *Nicotiana tabacum* and *Nicotiana glutinosa* is reinvestigated using 97 atom % $^{13}CO_2$ coupled with ^{13}C NMR analysis of ^{13}C-enriched **1.** It is shown that the incorporation of ^{13}C into **1** as a time function is reflected in the sequence of the appearance of satellite resonances due to $^{1}J_{cc}$ in the proton noise decoupled ^{13}C NMR spectrum of the alkaloid. Thereby it is possible to identify the portions of **1**'s carbon skeleton that arise from different biosynthetic precursors. Unsymmetrical labeling of the *N*-methylpyrrolidine ring of **1** is observed corroborating earlier findings from $^{14}CO_2$ based biosynthetic experiments.

The applicability of ^{13}C-labeled compounds to biosynthetic studies has been well documented;[1] however, the successful use of ^{13}C-labeled precursors for biosynthetic studies in intact plants has been reported in few instances[2] largely because of the prohibitively high precursor dilutions that frequently result therein. The use of $^{13}CO_2$ appeared to us to offer one way of circumventing this dilution problem, as well as the tediousness of label localization by chemical degradation characteristic of $^{14}CO_2$ based experiments. Yet at the outset we did not know if any meaningful biosynthetic information would be obtainable via $^{13}CO_2$ feeding experiments, although in view of results from similar experiments using $^{14}CO_2$[3] we anticipated that the ^{13}C-labeling pattern of the secondary metabolites would not be uniform and might therefore be reflective of the different units that had been assembled in vivo during the metabolite's biosynthesis. We have completed some initial experiments designed to assess the sensibility of using 97 atom % $^{13}CO_2$ as a biosynthetic probe of plant alkaloids and are able to report that quite meaningful results can be obtained when the intramolecular ^{13}C distribution of the natural products is analyzed directly by ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy.

The biosynthesis of nicotine (**1**) has been investigated extensively.[4] It is generally believed that **1** is formed in vivo from



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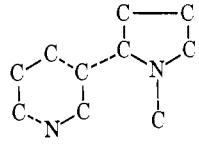

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four principal biochemical compounds as depicted by **2.** The majority of the experimental results obtained by the use of specifically labeled organic compounds and $^{14}CO_2$ have been interpreted as supporting the biosynthetic pathway adumbrated in Scheme I. Importantly, the equal labeling of C-2′ and C-5′ of **1** by [2-^{14}C]ornithine (F) in all cases but one[5] and the positive incorporation of putrescine (G) into **1**[6] have been accepted as sound evidence that a symmetrical compound (G) is an intermediate of the biosynthetic pathway to **1.** In fact, Japanese workers[7] have isolated and partially purified enzymes from *Nicotiana tabacum* that in vitro catalyze the reactions giving G, H, and I from F, although this is not sufficient evidence alone that these enzymes are involved in the biosynthesis of **1** in vivo.

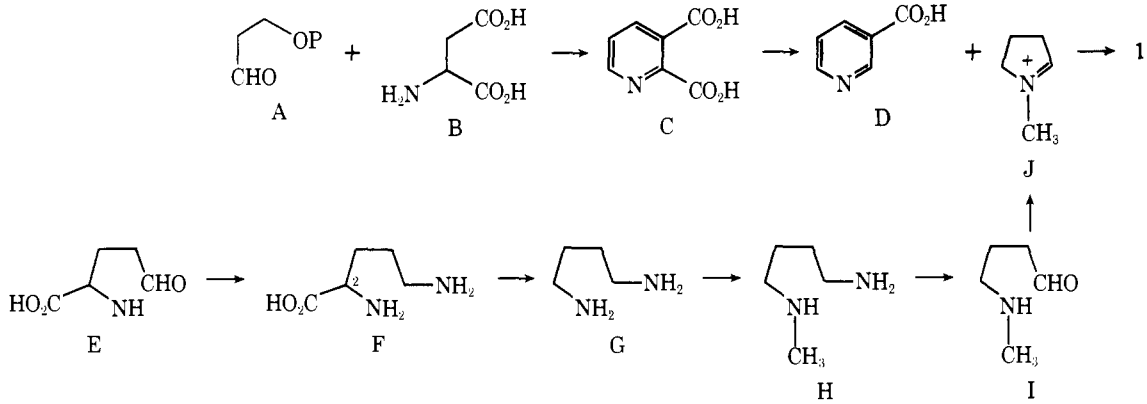
Results from biosynthetic studies of **1** obtained using $^{14}CO_2$ wherein the distribution of ^{14}C label in **1** was ascertained by chemical degradation *occasionally*[8] seem to be discordant with those obtained using specifically labeled C and N precursors. Beginning in 1964 Rapoport and co-workers have presented evidence in a set of four major papers[9] that in *Nicotiana glutinosa* label from $^{14}CO_2$ does not always appear in carbons 2′, 3′, 4′, and 5′ of **1** in a manner consistent with a symmetrical biosynthetic intermediate. They did observe that as a function of time (a) the pyridine ring of **1** was more heavily ^{14}C labeled than the *N*-methylpyrrolidine ring and (b) the specific radioactivity of the *N*-methyl of **1** usually[8] was similar to that of the pyrrolidine ring's carbons.

These experiments have been challenged independently by two groups. Byerrum and co-workers[10] carried out $^{14}CO_2$ feedings using *Nicotiana rustica* and *N. glutinosa* wherein symmetrical ^{14}C labeling of the *N*-methylpyrrolidine ring of **1** was observed in three separate experiments. They also reported that label from [2-^{14}C]acetate was incorporated into **1**[10a] consistent with the presence of a symmetrical intermediate in the biosynthetic pathway. Leete[11] showed that one of the chemical degradations used in Rapoport's work may have been ambiguous and thus could have led to erroneous results in the determination of the ^{14}C-labeling pattern of carbons 4′, 5′, and *N*-CH_3 of **1.** However, this disclaimer subsequently was refuted by Rapoport[12] and in later $^{14}CO_2$ experiments[9e] a modified degradative sequence of **1** was used by this group apparently to avoid the purported ambiguity in data collection.[13] One outcome of the work conducted by Rapoport's group has been the espousal that "the labeling pattern (of **1**) via a symmetrical intermediate produced from precursor feedings (other than CO_2) might result from a minor or aberrant pathway".[9c]

Since this particular controversy has been stated to have its genesis in a chemical degradation ambiguity,[11] we chose to reexamine the biosynthesis of **1** in *N. tabacum* and *N. glutinosa* via $^{13}CO_2$ in which the label distribution would be determined spectroscopically without recourse to degradative techniques. Additionally, such a well-studied, not necessarily well-understood, system offered a good test of the validity and predictability of $^{13}CO_2$ based biosynthetic experiments, which we felt to be necessary if such a technique is to be accepted into the realm of possible ways to study biosynthetic processes in higher plants. It should be obvious that for $^{13}CO_2$ to become as useful as $^{14}CO_2$ has been, or to exceed the latter's applicability in such research, the most difficult hurdle to overcome is the apparent lack of quantifiability of ^{13}C NMR spectral analyses. We address ourselves to this and other questions in the following discussion.

Methodology for $^{13}CO_2$ Feedings. All CO_2 feeding experiments were done in a sealed growth chamber similar to that described by Rapoport et al.[14] Provision was made for plant removal during feeding experiments by a simple two-door chamber affixed to the front of the growth chamber to minimize loss of labeled CO_2. Humidity levels inside the growth

Scheme I. Generally Accepted Biosynthetic Pathway to Nicotine (1)



chamber rose to saturating levels soon after feeding experiments were commenced and remained there during CO_2 feedings. The internal temperature varied between 18 and 23 °C. Illumination levels were from 350 μEinsteins M^{-2} s^{-1} (top) to 100 μEinsteins M^{-2} s^{-1} (bottom) of photosynthetically active radiation, which were not saturating for the plants used.

In our initial experiments *N. tabacum* (two experiments) or *N. glutinosa* (one experiment) plants, 3–5 months old and just prior to their flowering stage, were fed a mixture of 97 atom % $^{13}CO_2$ and a tracer quantity of $^{14}CO_2$ for 12–14 h. During the CO_2 feeding period the atmospheric CO_2 concentration (0.03%) and specific radioactivity (usually 1.1×10^8 dpm/mmol) were held constant by continuous replacement of the photosynthetically fixed CO_2, as monitored by a differential gas flow ir analyzer and a vibrating reed electrometer. At the end of the CO_2 feeding period the growth lights were turned off and subsequent plant metabolism was maintained in normal atmospheric CO_2 at a 14-h day/10-h night cycle under the same illumination levels.

Aliquot groups of plant tops were frozen in liquid N_2 upon removal from the growth chamber and chromatographically pure [^{13}C,^{14}C]-**1** was isolated by standard methods. Its ^{13}C content was measured by EI mass spectrometry by which the intramolecular ^{13}C distribution of the *N*-methylpyrrolidine ring could be accurately measured from the *N*-methylpyrrolinium fragment ion at *m/e* 84,[15] but not that of the molecular ion at *m/e* 163 due to an intense $M^+ - 1$ peak at all voltages tried. Initial determination of the specific radioactivity of [^{13}C,^{14}C]-**1** was done to give a rough idea of its ^{13}C enrichment by percentage specific incorporation of ^{14}C; subsequently, after its ^{13}C NMR analysis had been done, accurate determination of its specific radioactivity was done by quantitative dilution with radioinactive **1** and recrystallization of its diperchlorate to constant specific radioactivity. The ^{13}C NMR spectral determinations were done using the N_b-monoethanesulfonate of [^{13}C,^{14}C]-**1** prepared by titration of the free base in aqueous methanol with 0.05 N aqueous ethanesulfonic acid to pH 5.5 at which only the pyrrolidine nitrogen is protonated.[16] It was found to be absolutely necessary to extract the NMR samples as solutions in D_2O with a dilute solution of dithiazone in CCl_4 to remove paramagnetic metal impurities.[17] Otherwise, line broadening and low-signal intensities resulted, particularly for C-2′, C-5, and C-3 of **1**.

Results and Discussion

The technical manipulations associated with the CO_2 feeding experiments are simple and expedient, once a growth chamber is constructed and the requisite CO_2 monitoring instruments are obtained. However, this requires more capital expense than is characteristic of ^{13}C-based biosynthetic experiments using bacterial and fungal systems. The size of our initial growth chamber limited the amount of plants that could be used in CO_2 feeding experiments, which can be a serious limitation in view of the quantity of pure alkaloid needed for ^{13}C NMR spectral determinations in a reasonable period of time. For the latter reason the first feeding experiments were done using rather large plants, which is not the optimal way to carry out such feedings. As with bacterial and fungal systems it is desirable to add ^{13}C-labeled precursors, whose incorporation is to be determined spectrally, to the system at a time when endogenous precursor and product pools are of minimal size,[18] thus reducing the dilution of added precursor, minimizing the resultant time needed to obtain a satisfactory S/N ratio in ^{13}C NMR spectral measurements, and maximizing the sensitivity of the experimental approach. In our case the dilution of the ^{13}C label incorporated into **1** during the course of exposure to $^{13}CO_2$ and subsequent metabolism in $^{12}CO_2$ was high as reflected by the percentage specific incorporation of $^{14}CO_2$, which increased uniformly from ca. 0.1% after 4 h of the plants' exposure to $^{13,14}CO_2$ to 10.2% at 240-h total metabolism time.[19]

Since the percentage specific incorporation of $^{14}CO_2$ into **1** represents the statistical average of all nicotine molecules randomly labeled with ^{14}C, a comparable value that can be obtained from the ^{13}C NMR spectrum of **1** is the increase in the total signal intensity per carbon summed over all its ten carbons relative to a natural ^{13}C abundance standard. After examining several derivatives of nicotine we chose to use its N_b-monoethanesulfonate because of the resultant carbon chemical shift dispersion and resolution of the spectra determined in D_2O. The appropriate spectral data are shown in Table I and typically represented by Figure 1. In order to maximize the reproducibility of the ^{13}C enrichment determined on the basis of total signal intensity enhancement we measured the ratio of the areas of the individual carbon resonances of natural ^{13}C abundance **1** to that of either[20] the methyl or methylene of the ethanesulfonate internal reference and compared this ratio to the corresponding one obtained from samples of [^{13}C,^{14}C]-**1** as their natural ^{13}C abundance ethanesulfonates. This method should minimize inherent differences in the relaxation rates of individual carbons and instrumental error in the precision of the ^{13}C NMR spectral determinations. Rather good precision (footnote *e*, Table I) among the ratios was obtained in this way, except for the quaternary carbon (C-3) of **1** whose S/N ratio under our spectrometer parameters was very low. The product of these ratios times 1.1% represents the statistical ^{13}C enrichment at any carbon in [^{13}C,^{14}C]-**1** due to the presence in the ^{13}C NMR analysis sample of a random mixture of molecules intermolecularly ^{13}C labeled at single positions or intramolecularly ^{13}C labeled at multiple positions, i.e., at either C-2,3; C-4,5; C-4,5,6; C-2′,3′; C-4′,5′; or *N*-CH_3. Thus a value of 1.1 in columns 4–6 of Table I represents no ^{13}C enrichment over natural ^{13}C abundance. The percentage total ^{13}C specific incorporation of $^{13}CO_2$ into

Table I. ^{13}C-Labeling Pattern of [^{13}C,^{14}C]Nicotine Isolated from $^{13}CO_2$ Feeding to *N. tabacum*

Carbon	Chemical shift,[a] ppm	$^1J_{cc}$,[b] Hz	Relative ^{13}C enrichment, % By N_b-monoethanesulfonate standard[c,e] 52 h[f]	122 h	240 h	By ratio of total area of satellites to singlet area[d,e] 52 h	122 h	240 h
2	150.2	55	1.2	1.1	2.7	*h*	*h*	41
3	130.7	52	*g*	*g*	2.8	*h*	*h*	34[j,k]
4	138.9	54	1.5	1.7	2.9	29	47	62[j]
5	126.4	d 54, q 52	1.5	1.5	2.3	62	64	57
6	151.5	58	1.3	1.7	2.4	29	47	62
2′	71	33	1.1	1.1	1.8	*h*	*i*	62[k]
3′	31.5	33	1.1	1.2	1.9	*h*	*i*	65[l]
4′	22.8	33	1.3	1.2	2.2	*h*	*i*	58[l]
5′	57.4	33	1.1	1.1	1.9	*h*	*i*	49
N-CH_3	39.5		1.1	1.1	2.3	*h*	*h*	*h*

[a] Assigned relative to Me_4Si as external standard according to W. O. Crain, Jr., W. C. Wildman, and J. D. Roberts, *J. Am. Chem. Soc.*, **93**, 990 (1971), but reversing their assignment for carbons 2 and 6 based on (a) the carbon shift assignments of **1** derived by Birdsall analysis [B. Birdsall, N. J. M. Birdsall, and J. Feeney, *J. Chem. Soc., Chem. Commun.*, 316 (1972)] and (b) the ^{13}C labeling of these carbons as a function of time. [b] Accurate to ±3 Hz for the aromatic carbons and ±1 Hz for the aliphatic carbons. [c] Ratio times 1.1% of the normalized signal area ratios of N_b-monoethanesulfonate CH_3- or $-CH_2-$ of [^{13}C,^{14}C]-**1** to corresponding ratios of natural ^{13}C abundance **1**. [d] Calculated by eq 4, text. [e] All peak areas were measured by cutting and weighing of 2× expanded ^{13}C NMR spectra wherein the data precision was C-2 (±1.9), C-3 (±21.6), C-4 (±1.1), C-5 (±5.3), C-6 (±3.0), C-2′ (±1.4), C-3′ (±4.2), C-4′ (±1.9), C-5′ (±5.3), and N-CH_3 (±1.7), expressed as percent standard deviation of three replicate ^{13}C NMR spectral determinations. [f] Exposure time to 97 atom % $^{13}CO_2$ (14 h) plus subsequent metabolism in normal CO_2. [g] S/N ratio too low for highly accurate area measurement. [h] No observable satellite signals at comparable S/N ratio. [i] Doublet satellites observable but signal intensity suspected to be inaccurate. [j] Assumed no $^1J_{3,4}$. [k] Assumed no $^1J_{2',3}$. [l] Assumed no $^1J_{3',4'}$.

nicotine is calculated by summing the values in columns 4–6, Table I, that are greater than 1.1 (the values of C-3 in the 52- and 122-h samples were approximated as equal to those of C-2) and then multiplying this sum by 100/97. The specific incorporation of ^{13}C calculated in this way agrees rather well with the specific incorporation of ^{14}C into **1** for the 52-h (1.4 vs. 1.4%) and the 240-h (12.6 vs. 10.2%) samples, but that of the 122-h sample (1.9 vs. 2.7%) does not, which we feel is due to paramagnetic contamination.[21]

The relative percentage ^{13}C enrichment of the individual carbons of nicotine as a time function should be reflective of the portions of **1** that are derived from biosynthetically distinct precursors. By comparison of these values for the three different samples of [^{13}C,^{14}C]-**1** (columns 4–6, Table I), it appears that C-4, C-5, and C-6 become ^{13}C enriched before the other carbons of **1** in the 52- and 122-h samples, which is consistent with their origin from A. However, in the 240-h sample this distinction is less clear-cut; in fact, now the ^{13}C labeling of carbons 2–6 is of similar magnitude and distinctly larger than that of carbons 2′–5′ and the N-CH_3. Since it had been reported that label from $^{14}CO_2$ rapidly enters carbons 2,3 and 4,5,6 of **1** uniformly, although not equally between these two units,[10a] we anticipated that each of these carbon sets would be equally ^{13}C enriched in the [^{13}C,^{14}C]-**1** samples. That this does not appear to be the case from the N_b-monoethanesulfonate derived ^{13}C enrichment data may be a valid observation *or* may be due to the experimental error inherent in this method. The only independent assessment we can make of the accuracy of this method is to note that the *m/e* 84 fragment in the mass spectrum of the 240-h sample of [^{13}C,^{14}C]-**1** had a 2.1% ^{13}C enrichment in its M + 1 peak, which is similar to the N-CH_3 ^{13}C enrichment value (2.3%) calculated from the ^{13}C NMR spectral data. Until we complete a synthesis of [2′,3′,N-CH_3-$^{13}C_3$]-**1**, of a known intramolecular ^{13}C distribution, which is presently underway, the question of the accuracy of this method will have to remain unanswered. Presently, we emphasize that this method is at best only indicative of the relative ^{13}C enrichment of the nicotine samples and does not seem to permit distinction quantitatively between singly and multiply labeled molecules present in the alkaloid samples. Thus, these ^{13}C enirchment values for [^{13}C,^{14}C]-**1** are not strictly comparable to those derived by the satellite method described below.

The most striking feature of the ^{13}C NMR spectrum of the 240-h sample of [^{13}C,^{14}C]-nicotine (Figure 1) is that all resonances, except those of C-5, the N-CH_3, and, of course, the internal standard, are flanked by only doublets due to $^1J_{cc}$ indicative that a random mixture of essentially doubly ^{13}C labeled molecules is present. This is corroborated by the absence of mass spectral peaks at *m/e* 87 and higher mass values for the N-methylpyrrolinium ion of [^{13}C,^{14}C]-**1**. On the other hand, the C-5 resonance, a doublet and a quartet of ca. equal total intensity, is evidence for a ca. 1:1 mixture of molecules labeled at C-4,5 or C-5,6 and C-4,5,6 present in the sample. Since all of the satellite intensities are much greater than expected based on an ca. 2% statistical single carbon ^{13}C enrichment, a mixture of natural ^{13}C abundance **1** and highly ^{13}C-enriched **1** must be present.[22] Analogous to the methods thoroughly delineated by London et al.,[23] an equation can be derived that permits calculation of the fractional concentrations of natural abundance and highly ^{13}C-enriched material present in the ^{13}C NMR analysis sample. (We are indebted to Dr. A. G. McInnes for pointing this out to us.) Thus for two directly bonded carbons, $C^{\bullet}$-$C^{\blacktriangle}$

$$\frac{I_D^{\bullet}}{I_S^{\bullet}} = \frac{f(^{13}C_{NA}^{\bullet} - {}^{13}C_{NA}^{\blacktriangle}) + (1 - f)(^{13}C_E^{\bullet} - {}^{13}C_E^{\blacktriangle})}{f(^{13}C_{NA}^{\bullet} - {}^{12}C_{NA}^{\blacktriangle}) + (1 - f)(^{13}C_E^{\bullet} - {}^{12}C_E^{\blacktriangle})} \quad (1)$$

where I_D is the summed spectral satellite intensities of $C^{\bullet}$, I_S is the singlet intensity of $C^{\bullet}$, C_{NA} is a natural ^{13}C (or ^{12}C) abundance carbon, and C_E is a ^{13}C-enriched carbon. Using the value shown in column 9, Table I, for C-2′ and the appropriate signal intensities of C-2′ measured from Figure 1 in eq 1, it is calculable that the 240-h ^{13}C NMR sample must consist of 98.99% natural ^{13}C abundance **1** and 1.01% of **1**, 62–65% ^{13}C enriched at C-2′ and C-3′. This is corroborated by a 0.66% ^{13}C enrichment in the M + 2 ion of the N-methylpyrrolinium fragment of the 240-h sample of [^{13}C,^{14}C]-**1**.

The second-order spin–spin coupling effects shown by the inset of Figure 1, wherein the satellite resonances of coupled atoms bear a mirror image intensity relationship,[24] are sug-

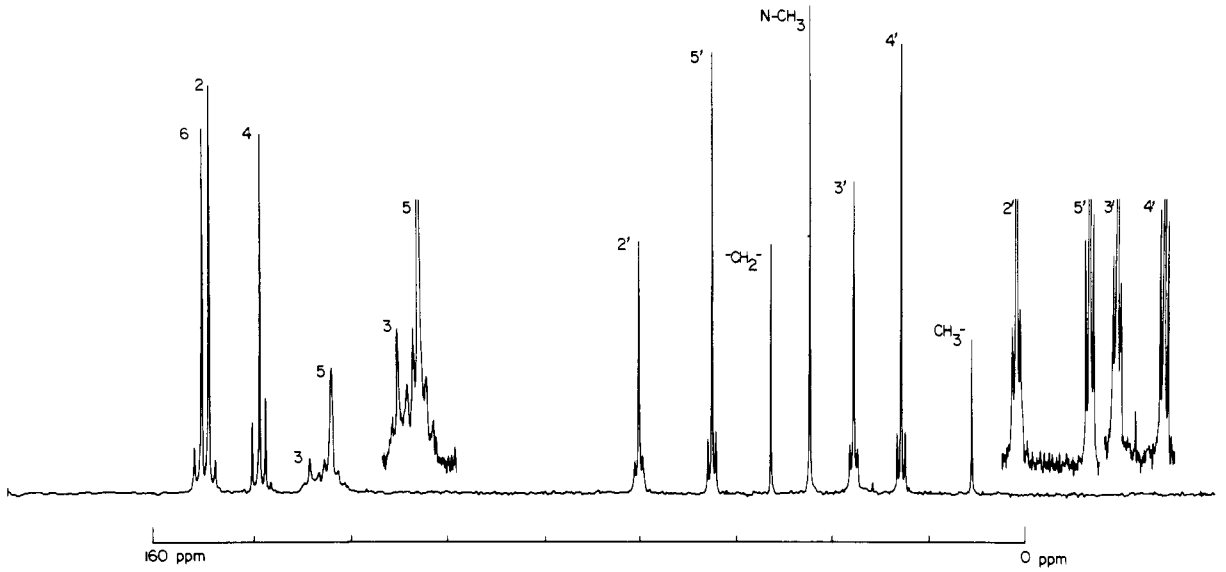


Figure 1. Proton noise decoupled ^{13}C NMR spectrum of the N_b-monoethanesulfonate of [^{13}C,^{14}C]-**1** from the 240-h isolation. Insets showing second-order effects in satellite resonances are 4× vertical scale. The spectrum was determined as a D_2O solution of the chromatographically pure alkaloid (12.9 mg) in a 125-μl cylindrical microcell at 37 °C on a Bruker HX-90E NMR spectrometer at 22.63 MHz, 30° pulse angle, 0.82-s pulse repetition rate, 204K scans using 8K time domain points over 5K Hz (data resolution of ±1.2 Hz).

gestive evidence that sets of coupled carbons in [^{13}C,^{14}C]-**1** are C-4,5,6 (and C-4,5 + C-5,6); C-2,3; C-2′,3′; and C-4′,5′. This will have to be proven securely by homonuclear carbon decoupling, which we were not equipped to do at the time these initial experiments were done. (The differences in the $^1J_{cc}$ values of carbons 2–6 or 2′–5′ are not large enough to permit distinction in this way.[25])

If the above assumption is valid for the ^{13}C NMR spectra of the three nicotine samples, then the quantitative relationship between the sum of the areas of the satellite resonances and the center singlet's area, as has been recently demonstrated by London et al.,[23] can be used to calculate the relative percentage ^{13}C enrichment of intramolecularly multiply ^{13}C-labeled molecules present in these samples. (Again we are indebted to Dr. A. G. McInnes for clarifying how this can be done.) For two (or more) directly bonded carbons, C$^\bullet$–C*, eq 4, representing the percentage of ^{13}C enrichment at C$^\bullet$ can be derived as follows. Let

$$\frac{I_D^*}{I_S^*} = \frac{(^{13}C_*)(^{13}C_\bullet)}{(^{13}C_*)(^{12}C_\bullet)} \qquad (2)$$

where I_D is the summed satellite areas of C*,[26a] I_S is the singlet area of C* corrected for the presence of any natural ^{13}C abundance compound,[26b] and $^{13}C_*$ is the concentration of ^{13}C at carbon C*, etc. Since these concentrations can be expressed as the probability of ^{13}C labeling at any carbon, eq 2 can be reexpressed as eq 3.

$$\frac{I_D^*}{I_S^*} = \frac{P^*P^\cdot}{(1 - P^\cdot)P^*} = \frac{P^\cdot}{(1 - P^\cdot)} \qquad (3)$$

In other words, the satellite to singlet area ratio at C* depends on the ^{13}C concentration or labeling probability at C$^\bullet$. After appropriate rearrangement, eq 4 results, which allows one to calculate the desired ^{13}C concentrations.

$$P^\cdot = I_D/(I_S^* + I_D^*) \qquad (4)$$

It is important to note that the values for C-5 shown in columns 7 and 8 of Table I calculated according to eq 4 appear to show that this carbon is ^{13}C enriched markedly greater than carbons 4 and 6. This assuredly is an artifact of the calculation method. Since the resonance of C-5 in the 52- and 122-h samples appears only as a doublet, it is highly likely that the majority of molecules present are labeled at *either* C-4,5 *or* C-5,6 but not equally at C-4,5,6.[10a] Consequently, if these molecules were ca. equally doubly labeled and present in ca. equal amounts, it would appear (according to eq 4 where a three contigious carbon case is used), for example, that C-5 contained ca. twice the ^{13}C content of C-4 or C-6 in the 52-h sample. Since we do not have an independent assessment of the relative amount of doubly and triply labeled nicotine present in these two ^{13}C NMR analysis samples, this rationalization should be considered only as one which seems sensible. For the 240-h sample from the appearance of the C-5 resonance (vide supra) it seems valid to conclude directly that C-4, C-5, and C-6 of [^{13}C,^{14}C]-**1** are equally ^{13}C enriched.

The relative ^{13}C enrichment of carbons 2′–5′ of nicotine could not be calculated according to eq 4 for the 122-h sample because the relevant signal intensities were too low, which we ascribe to paramagnetic contamination.[21] Those of the 240-h sample shown in column 9 of Table I seem to be reflective of *unequal* ^{13}C labeling of C-4′ and C-5′, particularly, in comparison to C-2′ and C-3′. However, the absolute differences among these four carbons are not great, which casts a shadow of doubt on their significance since we have not independently proven the accuracy of the satellite method used to calculate the ^{13}C enrichments. It has been demonstrated, however, that the satellite method has an accuracy of ≤±1.4% for uniformly or nonuniformly ^{13}C-labeled acetate.[27] On this basis and the good agreement between ^{13}C enrichment derived from ^{13}C NMR and MS data (vide supra) we *tentatively* believe that these numbers are significantly different. Thus our results seem to be a corroboration of some of the observations reported by Rapoport and co-workers.[28,32] It possibly is also significant that the difference in the ^{13}C enrichment of C-2 vs. C-3 and C-3′ vs. C-4′ of the 240-h sample of [^{13}C,^{14}C]-**1** is of similar magnitude, ca. 7%. In both pairs, one of each of these carbons represents the CH_3– of an acetate molecule, which has been shown to unequally label aspartate[29] or glutamate.[10b]

The most informative deduction about the biosynthesis of nicotine that can result from the use of $^{13}CO_2$ is that the appearance of satellite resonances as a time function occurs in discrete portions of **1**, indicating that carbons 4–6 are labeled differently than carbons 2′–5′ and carbons 2 and 3. This is consistent with the known derivation of these sets of carbons from A, B, and E (Scheme I) but, of course, is not proof of this. *If* true steady-state $^{13}CO_2$ exposure conditions had been used,[9e,14] then we could validly state that the ^{13}C enrichment pattern of **1** as reflected in the satellites' appearance as a time

function develops in a way consistent with the flow of ^{13}C through the pool(s) of A then E (→F, etc.) then B.[29] We assume to have approximated such conditions in view of our results. To a lesser extent the differential labeling of these portions of **1** is reflected in the more quantitative percentage ^{13}C enrichments shown in columns 7–9 of Table I and to the least extent by those of columns 4–6. In fact, there appears to be contradictions between these latter two sets of data as to the relative ^{13}C labeling of **1** as a time function. At our present level of experimental refinement we prefer to accept the validity of the satellite method data, as it is applicable here, but to reserve judgement on the N_b-monoethanesulfonate method data until further work is done. If ^{13}C NMR spectra are determined with comparable S/N ratios, then it does seem clear that the time dependence of the appearance of satellite resonances is a valid reflection of those portions of **1** that arise from biosynthetically distinct precursors.

The results of the two experiments using *N. tabacum* and the one using *N. glutinosa* were consistent as far as the appearance of satellite resonances as a time function is concerned. We could not determine the percentage ^{13}C enrichment of the carbons of nicotine isolated from *N. glutinosa* by either of our methods due to small sample sizes and sample losses encountered in preliminary ^{13}C NMR analyses, wherein it had been necessary to convert the dihydrochloride of **1** to its N_b-monoethanesulfonate.

One disadvantageous outcome of our present experiments is the high dilution of [^{13}C]-**1** produced by the incorporation of $^{13}CO_2$ with endogenous natural ^{13}C abundance **1.** Thus the magnitude of the differential labeling of **1** is much less than that characteristic of $^{14}CO_2$ based work.[3] It appears from the results of one of the *N. tabacum* experiments,[30] in which smaller plants had been used than in the other feeding experiment, and more recent feeding experiments that this dilution problem can be circumvented by using many very young, small plants. Should this turn out to be true generally, then we will easily be able to carry out true steady-state $^{13}CO_2$ feeding experiments, to analyze the ^{13}C NMR data without the problem of excessive product dilution, and to shorten the feeding periods' length so the experiments become more comparable to those based on $^{14}CO_2$.

Conclusions

It appears from the results described herein that by using highly enriched $^{13}CO_2$ dynamic information can be obtained about the biosynthesis of secondary plant products. Thus, it clearly is possible to deduce which portions of **1** arise from different biosynthetic precursors. The method also should be applicable to deducing carbon migrations subsequent to the formation of a primary carbon skeleton similar to the use of [1,2-$^{13}C_2$]acetate in biosynthetic experiments.[31]

Additional experiments must be done to refine the quantitative aspects of the use of $^{13}CO_2$ as a biosynthetic probe of secondary natural products. For this and other reasons related to the general lack of detailed knowledge about the biochemistry of plant alkaloids, it would be foolhardy to state that our results and those of Rapoport are evidence of a biosynthetic pathway to nicotine alternative to that shown in Scheme I. The exciting fact is that it now should be sensible and expedient to use $^{13}CO_2$, along with other techniques, to attempt to better understand what is happening within the "black box" of secondary natural product plant metabolism in this case and others.

Experimental Section

CO_2 Feeding. A plant growth chamber with the outside dimensions of ca. 48 × 22 × 16 in. constructed of 0.25 in. thick lucite was used to administer labeled CO_2 to intact plants. This had two 8-in. glove ports on the front between which was a two-door chamber (inner door could be slid to the right to open and the seal preventing rapid CO_2 loss depended on the close contact of three parallel pieces of lucite; outer door was hinged to swing upward and an airtight seal was maintained by an O-ring and pressure clamps on three sides of the closed door), 6 × 12 × 6 in., large enough to accommodate an average sized plant plus its pot. This airlock chamber could be flushed with N_2 independently of the growth chamber during removal of aliquot plants. An external bank of four 48-in. 40-W Westinghouse Agro-lites 1 in. above the growth chamber's top provided the illumination. An inlet and outlet (0.25 in. o.d.) for external atmosphere circulation were provided at diagonally opposite corners of the front of the growth chamber, while the internal atmosphere was circulated by a small electrically driven fan.

The atmosphere in the system was swept from the growth chamber through a suction flask chilled externally with dry ice to remove excess H_2O, then into a Beckmann Model 864 infrared differential gas flow analyzer, and subsequently through a 500-ml Cary-Tolbert spherical ionization chamber attached to a Varian 401 vibrating reed electrometer, the outlet of which was connected to a flow meter, then a small diaphragm pump, before returning to the growth chamber. A dual pen recorder was used to visually record and monitor the $^{13}CO_2$ concentration and $^{14}CO_2$ specific radioactivity. Tygon tubing (0.25 in. i.d.) was used to connect all devices in the closed atmosphere circulation loop.

The mixture of $^{13}CO_2$ and $^{14}CO_2$ was generated by decomposition of labeled $BaCO_3$ with concentrated H_2SO_4 in an evacuated system and trapping the CO_2 in a liquid N_2 cooled gas lecture bottle filled with clean sand. A two-stage gas regulator and micrometer needle valve was affixed to the top of the cold cylinder before it was allowed to warm to ambient temperature. This gas mixture was introduced into the growth chamber's atmosphere circulation loop between the diaphragm pump and the growth chamber gas inlet. At the usual atmospheric recirculation rate of 1200–1500 ml/min^{-1}, a pen response was seen within 1 min of the addition of $^{13,14}CO_2$. The concentration and specific radioactivity of CO_2 in the internal atmosphere could be kept exactly parallel on the recorder chart by occasional fine adjustment of the micrometer needle valve on the gas cylinder.

In a typical run intact plants, from stock germinated and grown under artificial lighting comparable to that used during the CO_2 feeding experiment, were placed in the growth chamber the day before CO_2 feeding was to be commenced. The next morning the growth chamber would be sealed and atmosphere circulation begun. Immediately on turning on the lights, addition of labeled CO_2 was commenced to balance the uptake of CO_2 due to photosynthesis while maintaining a ca. 0.03% concentration of CO_2 inside the system. Twice during the CO_2 feeding period an aliquot of $^{13,14}CO_2$ sample was removed through a sampling port by bubbling the atmosphere through a half-saturated aqueous solution of $Ba(OH)_2$. The resulting [^{13}C,^{14}C]$BaCO_3$ was washed with H_2O, EtOH, and then Et_2O and dried, and its specific radioactivity was determined by liquid scintillation counting of a Cab-O-Sil gel. Since the specific radioactivity of the $^{14}CO_2$ inside the growth chamber reached a stable value within 5 min after the lights were turned on, the average specific radioactivity of these $BaCO_3$ samples was taken as the base value for calculating the percentage specific incorporations of $^{14}CO_2$ into **1.** At appropriate time intervals not less than four plant tops per aliquot were removed via the airlock and immediately frozen in liquid N_2. After 12–14 h the lights were turned off, the addition of labeled CO_2 was ceased, and the labeled CO_2 remaining in the growth chamber was flushed into a vented fume hood as rapidly as possible. Then the growth chamber was left open to the normal room atmosphere (glove ports open; airlock open) for subsequent plant metabolism.

Isolation and Purification of Nicotine (1). The frozen plant tops were macerated for 2 min at high speed in a 1-gal stainless steel Waring blender with CH_2Cl_2 and NH_4OH (900:100). The resulting pulpy mass was suction filtered through two layers of clean cotton cloth and filter paper, and the marc was washed with a mixture of CH_2Cl_2 and NH_4OH and then H_2O and pressed dry by suction using a rubber sheet fitted tightly over the funnel's top. The entire aqueous layer was washed twice with CH_2Cl_2, the latter solutions were combined with the rest of the organic extract, and this solution was evaporated in vacuo on a Rotavap at ≤40 °C by continuous addition beneath a layer of 15% aqueous H_2SO_4. The resulting acidic solution was suction filtered through a Celite pad to remove insoluble gummy material. The clear amber filtrate was cooled to an ice-bath temperature and made basic (pH ~12) by the addition of 40% aqueous NaOH while

stirring magnetically. The resulting dark solution was extracted four times with CH_2Cl_2, the combined organic extracts were dried (Na_2SO_4), and then they were evaporated in vacuo on a Rotavap without external warming to a light brown oil. This oil was chromatographed on thick silica gel PF_{254} plates in $CHCl_3$–MeOH–NH_4OH (180:20:1), the uv absorbing band ($R_f \sim 0.4$) corresponding to nicotine was removed, and the nicotine was eluted with a solution of 20% MeOH in CH_2Cl_2. After the addition of a little H_2O to the resulting nicotine solution, most of the organic solvents were removed on a Rotavap without external warming to give a single-phase solution. This solution was titrated with 0.05 N aqueous ethanesulfonic acid to pH 5.5 using a pH meter and the nicotine N_b-monoethanesulfonate was obtained as a pale yellow oil by evaporation in vacuo (≤ 0.1 mmHg).

The specific radioactivity determinations were done by quantitating the amount of [^{14}C]nicotine in an aliquot sample using its uv absorbance at 260 nm in ethanol by comparison to a Beer's law plot prepared from five standard samples of the free base. The sample's pH was adjusted to 2.95 with 0.1 N aqueous $HClO_4$, the solvents were removed in vacuo, and the resulting hot diperchlorate was diluted 5–20 times with radioinactive nicotine diperchlorate. Recrystallization of the diperchlorate from absolute ethanol to a constant specific radioactivity was done wherein the dried samples were weighed on a Cahn microbalance and their radioactivity was determined by liquid scintillation counting in 7 ml of a solution of dioxane (500 ml), naphthalene (50 g), PPO (3.5 g), and POPOP (0.25 g) on a Packard Model 3002 liquid scintillation counter at ambient temperatures. Internal standardization with [^{14}C]-*n*-hexadecane was used to determine counting efficiencies. Samples were assayed in duplicate, collecting not <10 000 counts per sample.

Mass spectral measurements were done with an AEI MS-9 mass spectrometer at 25 °C, 70 eV, in duplicate whence natural ^{13}C abundance reference samples of **1** were run just before ^{13}C-enriched samples.

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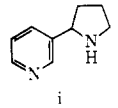

i

(6) (a) E. Leete, *Chem. Ind. (London)*, 537 (1955); (b) E. Leete, *J. Am. Chem. Soc.*, **80**, 2162 (1958).

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(8) It must be emphasized that repetition of the $^{14}CO_2$ experiments by Rapoport's group itself has led to erratic results wherein unsymmetrical ^{14}C labeling of the *N*-methylpyrrolidine ring of **1** was observed in only about 50% of the experiments, the remaining being reflective of symmetrical labeling. Likewise the specific radioactivity of the *N*-methyl group as a percentage of the specific radioactivity of **1** has been observed to vary considerably.

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(13) Yet the determination of the relative specific radioactivity of C-4′, C-5′, and the *N*-CH_3 of **1** was done as had been described earlier by Rapoport; only the degradation for C-2′ was altered. It is the former degrative reaction that Leete has shown possibly to be ambiguous.

(14) (a) H. Rapoport, F. R. Stermitz, and D. R. Baker, *J. Am. Chem. Soc.*, **82**, 2765 (1960); (b) H. L. Parker, G. Blaschke, and H. Rapoport, *ibid.*, **94**, 1276 (1972).

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(16) The accuracy of this technique was determined to be within ±2% of the true value by titration of standard samples of **1**. ^{1}H NMR analysis of the salt of [^{13}C,^{14}C]-**1**, prior to ^{13}C NMR spectral measurement, was done to show that within the usual 5% error limitation of integration a 1:1 ratio of alkaloid to acid was present.

(17) Cf. H. Pearson, D. Gust, I. M. Armitage, H. Huber, J. D. Roberts, R. E. Stark, R. R. Vold, and R. L. Vold, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1599 (1975); K. Tanaka, I. M. Armitage, H. S. Ramsdell, Y. E. Hsia, S. R. Lipsky, and L. E. Rosenburg, *ibid.*, **72**, 3692 (1975).

(18) Cf. T. J. Simpson, *Chem. Soc. Rev.*, **4**, 497 (1975).

(19) It is remarkable that the specific and total incorporation of radioactivity into **1** steadily rose throughout the metabolism period even though $^{14}CO_2$ was fed to the plants for only 14 h. This is, of course, due in part to the slow catabolism of **1** by the plant, but we had expected that the specific radioactivity of **1** would decline soon after the feeding of $^{13,14}CO_2$ was terminated.

(20) For nicotine the use of either group as an internal reference gave ratios with good precision and internal agreement.

(21) This contamination most likely was introduced via an improperly cleaned microcell after the D_2O solution of [^{13}C,^{14}C]-**1** had been extracted with dithiazone.

(22) This is the explanation for the appearance of "extra" satellite resonances occasionally seen from the incorporation of ^{13}C-labeled acetate into bacterial and fungal metabolites: cf. R. C. Paulik, M. L. Casey, D. F. Hillenbrand, and H. W. Whitlock, Jr., *J. Am. Chem. Soc.*, **97**, 5305 (1975).

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(25) F. J. Weigert and J. D. Roberts, *J. Am. Chem. Soc.*, **94**, 6021 (1972).

(26) (a) For resonances in the ^{13}C NMR spectra of [^{13}C,^{14}C]-**1** in which one of the satellites was obscured by an overlapping signal, e.g., that of C-2 and C-6, it was assumed that an AB spin system was present and thus the intensity of the obscured satellite signal was calculated according to ref 24, pp 135–138. This assumption seems valid since close agreement between calculated and observed satellite signal intensities was obtained for directly measurable resonances in the ^{13}C NMR spectra. (b) This was done by multiplying the ratio of the internal standard's methyl or methylene signal area to that of the desired carbon's resonance obtained from completely natural ^{13}C abundance ^{13}C NMR spectra of **1** times the absolute area of the corresponding internal standard carbon's resonance in the ^{13}C NMR spectra of [^{13}C,^{14}C]-**1**, then subtracting this value from the singlet resonance's area for the desired carbon of [^{13}C,^{14}C]-**1**.

(27) N. A. Matwiyoff and B. F. Burnham, *Ann. N.Y. Acad. Sci.*, **206**, 365, 368–369 (1973).

(28) However, Rapoport's group has observed greater ^{14}C labeling of C-4′/5′ than of C-2′/3′[9b-d] as well as lesser labeling.[9e]

(29) J. Bonner and J. E. Varner, "Plant Biochemistry", Academic Press, New York, N.Y., 1965.

(30) In this instance [^{13}C,^{14}C]-**1** was obtained after only 37-h metabolism with a specific incorporation of ^{13}C about 50% greater than that of the present 240-h sample.

(31) This possibility has been substantiated by the results of our preliminary experiments on the biosynthesis of indole alkaloids in *Catharanthus roseus* using $^{13}CO_2$.

(32) Professor E. Leete, University of Minnesota, has degraded the 240-h [^{13,14}C]nicotine sample by a new procedure (*J. Org. Chem.*, in press). The resulting relative percentage of ^{14}C labeling of **1** was shown to be C-2–C-6, 59.0%; C-2′, 6.7%; and C-5′, 6.9%. These values are in good agreement with the comparable ^{13}C NMR derived values (Table I, column 6): C-2–C-6, 62.3%; C-2′, 5.7%; and C-5′, 6.5%. Consequently, when *all* labeled species of **1** are taken into consideration, C-2′ and C-5′ appear to be equally labeled; yet, these new results are not inconsistent with the possibility of unequal *intramolecular* ^{13}C labeling of C-2′ and C-5′ as shown in Table I, column 9.