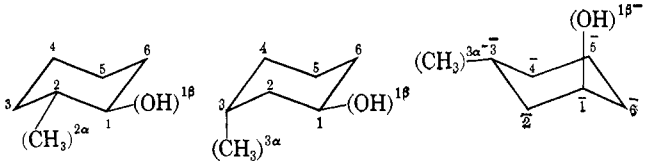

C1 C1 1C

Figure 1.

trans-2-methylcyclohexanol, $-43.9 \equiv \Sigma[\mu]_D{}^{20}{}_{obs}$ of (−)-*trans*-2-methylcyclohexanol = $(OH)^{1\beta} \Lambda (CH_3)^{2\alpha} = (OH)^{1\beta} \times (CH_3)^{2\alpha}\, \zeta_{OH}\zeta_{CH_3}\{(n^2 + 2)/3\}^{23} = -25.30\, \zeta_{OH}\zeta_{CH_3}\{(n^2 + 2)/3\}^{24}$

$$\therefore \zeta_{OH}\zeta_{CH_3} = 1.7352\{3/(n^2 + 2)\} \quad (7)$$

or

$$\zeta_{OH}\zeta_{CH_3}\{(n^2 + 2)/3\} = 1.7352 \quad (7')$$

The values of E, F, and G for $[\mu]_D{}^{20}{}_{obs}$ in Table II can be obtained by multiplying those for $[\mu]_D{}^{20}{}_{calcd} \cdot \{3/(n^2 + 2)\}$ by the value of $\zeta_{OH}\zeta_{CH_3}\{(n^2 + 2)/3\}$ given in eq. 7′.

(24) Table II is used.

Next, as seen in Table I, unit groups in (−)-*trans*-3-methylcyclohexanol of C1 conformation are $(OH)^{1\beta}$ and $(CH_3)^{3\alpha}$ (Fig. 1). Therefore, $\Sigma[\mu]_D{}^{20}{}_{obs}$ of (−)-*trans*-3-methylcyclohexanol of C1 conformation = $(OH)^{1\beta} \Lambda (CH_3)^{3\alpha} = -F = -7.3$.[24] Similarly, $\Sigma[\mu]_D{}^{20}{}_{obs}$ for all of the methylcyclohexanols are calculated and given in the fourth column of Table I. As shown in the fifth column of Table I, even in the cases of (−)-*trans*- and (+)-*cis*-3-methylcyclohexanols where there are no adjacent substituents in the molecules, the values of $[M]_D{}^{20}$ are not zero, and yet these observed values are nearly equal to the calculated values, $\Sigma[\mu]_D{}^{20}{}_{obs}$. The observed value of $[M]_D{}^{20}$, −15.3°, of (−)-*cis*-2-methylcyclohexanol indicates the presence of 32.6% of C1 conformation (an equatorial OH and an axial CH_3) and 67.4% of 1C conformation (an axial OH and an equatorial CH_3) in the equilibrium state.[25] This is compatible with the anticipation that, owing to a larger volume of the CH_3 group, the repulsion between a CH_3 group and the cyclohexane ring is stronger than the repulsion between an OH group and the cyclohexane ring. The facts may indicate the application of the PM method to methylcyclohexanol.

(25) By his own method of calculation of $[M]_D$, Brewster reached the same conclusion (*cf.* J. H. Brewster, *J. Am. Chem. Soc.*, **81**, 5483 (1959)).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND LAWRENCE RADIATION LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIF.]

The Biosynthesis of Nicotine in *Nicotiana glutinosa* from Carbon-14 Dioxide[1]

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RECEIVED SEPTEMBER 26, 1963

Plants of *Nicotiana glutinosa* were grown in an atmosphere containing $^{14}CO_2$ for periods varying from 2 hr., the shortest time at which incorporation of radioactivity into nicotine could be detected, to 12 hr. The nicotine, isolated separately from the root and aerial portions, was degraded, and the activity in the pyridine ring, the N-methyl group, and carbon-2′ of the pyrrolidine ring was determined. These data were correlated in terms of (1) the rate of incorporation of CO_2 into nicotine, (2) the site of nicotine syntheses, (3) the relative rate of N-methyl syntheses, and (4) the relative rate of syntheses of the pyridine and pyrrolidine rings. The conclusions thus reached have been compared with those in the literature derived from grafting experiments and from feeding precursors other than CO_2. Evidence is presented for independent nicotine synthesis in both root and aerial portions, and some questions are raised concerning the glutamate-symmetrical intermediate hypothesis for pyrrolidine ring biosynthesis.

Introduction

The biosynthesis of nicotine, and of related tobacco alkaloids, probably has been more extensively studied than that of any other alkaloid. The results and conclusions in this area have been summarized in three comprehensive reviews[3-5] that have appeared recently. These results have been obtained almost exclusively by the now familiar technique of feeding various *Nicotiana* species with potential precursors which are isotopically labeled. The nicotine isolated is degraded to determine the site, if any, of incorporated label. Interesting results have been obtained in particular by feeding nicotinic acid,[6] various amino acids related to glutamic acid,[7-9] the usual N-methyl precursors,[10] and glycerol and organic acids related to the Krebs cycle.[11-14] These results subsequently will be discussed in detail as they relate to our data.

Another approach to the study of alkaloid biosynthesis is by exposure of the intact plant to radioactive carbon dioxide. In this case, the role of carbon dioxide as precursor is obvious; the question becomes one of rate of incorporation. For this approach to be of any value, *de novo* alkaloid synthesis from carbon dioxide must be sufficiently rapid to allow a differential labeling pattern among the various carbon atoms of the alkaloid. At least in *Papaver somniferum* this has been the case[15,16]

(1) Sponsored in part by the United States Atomic Energy Commission.

(2) Public Health Service Predoctoral Research Fellow of the National Institute of General Medical Sciences.

(3) R. F. Dawson, *Am. Scientist*, **48**, 321 (1960).

(4) A. R. Battersby, *Quart. Rev.* (London), **15**, 259 (1961).

(5) K. Mothes and H. R. Schutte, *Angew. Chem.*, **75**, 265 (1963).

(6) R. F. Dawson, D. R. Christman, A. D'Adamo, M. L. Solt, and A. P. Wolf, *J. Am. Chem. Soc.*, **82**, 2628 (1960).

(7) L. J. Dewey, R. U. Byerrum, and C. D. Ball, *Biochim. Biophys. Acta*, **18**, 141 (1955).

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

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

(10) R. U. Byerrum, L. J. Dewey, R. L. Hamill, and C. D. Ball, *ibid.*, **219**, 345 (1956).

(11) T. Griffith, K. P. Hellman, and R. U. Byerrum, *ibid.*, **235**, 800 (1960).

(12) T. Griffith, K. P. Hellman, and R. U. Byerrum, *Biochemistry*, **1**, 336 (1962).

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

(14) D. R. Christman and R. F. Dawson, *Biochemistry*, **2**, 182 (1963).

and has led to findings difficult to obtain by other methods.

Encouraged by the results in *P. somniferum*, we have undertaken a detailed study of the biosynthesis of alkaloids of *Nicotiana glutinosa*[17] using $^{14}CO_2$. Previous studies of tobacco alkaloid biosynthesis with $^{14}CO_2$ have been sparse and limited[18-21] because long-term exposures were used and few degradations were made. In the present study of nicotine biosynthesis, growth periods varying from 1 to 12 hr. were employed, the aerial and root portions of the plant were examined separately, and a number of degradations were made. Data were obtained in four areas: (1) the rate of nicotine synthesis, (2) the site of nicotine synthesis, (3) the relative rate of synthesis of the N-methyl group and the ring system, and (4) the relative rate of synthesis of the pyridine and pyrrolidine rings. These data and their evaluation are presented in the Discussion section following a description of the methodology and a summary of the results.

Methods

Plant Growth.—All the plants used in this work were *Nicotiana glutinosa*.[22] The plants used in runs I, II, and III were grown in soil and were used for biosynthetic experiments when they had reached the stage of growth where buds were becoming evident. This occurred when the plants were about 2.5 months old, at which time they were about 20 cm. high and had about 10–12 major leaves. The leaf and stem portion of each plant weighed about 28 g. and the root portion which could be obtained from the soil weighed about 3 g. Removal of the roots of these plants from the soil was a tedious job; after the indicated end of the biosynthetic run, about 1 hr. was required before the roots were killed in liquid nitrogen. In order to shorten this "kill time," we turned to *N. glutinosa* grown hydroponically.

For hydroponic growth, 1 month old plants were removed from the soil, the roots were carefully rinsed, and the plants were transferred to a 46 × 18 × 7 cm. white enameled tray containing 11 l. of a nutrient solution.[23] For the first week, the nutrient solution was made up to only one-half the indicated strength. After 1 week, this solution was siphoned off and new, full strength solution added. The nutrient solution also was changed every week thereafter. Iron was added daily, 1 ml. of a 0.5% ferric nitrate solution for each liter of nutrient solution. Distilled water was also added when required to maintain a constant level, and the solution was continuously aerated. Five *N. glutinosa* plants were suspended into each tray through holes in a fiberboard lid and were held in place by means of cotton plugs. The plants were grown in a greenhouse with supplementary lighting provided from 6:00 A.M. to 6:00 P.M. by G.E. Power Groove fluorescent lights located 70 cm. above the plant trays.

The *N. glutinosa* plants growing in nutrient solution also were used for biosynthetic experiments when they reached the stage of growth where buds were becoming evident. This usually occurred 7–8 weeks after being placed in the nutrient solution, when the plants were about 3 months old. The plants were usually 30–40 cm. high and had 15–17 major leaves at this stage of growth. The leaf and stem portion of such plants weighed 100–150 g. and the root portion weighed about 16 g.

The four most uniform plants out of the group of five were utilized for the biosynthetic run. Directly before the run, the plants were removed from the tray and placed in 500-ml. erlenmeyer flasks which had been spray-painted with black and then with white paint to prevent illumination of the roots. The flasks were filled with freshly prepared nutrient solution and the mouths tightly plugged with cotton wads. The plants were then transferred to the biosynthesis chamber and supported in an upright position on ring stands.

Biosyntheses.—The biosynthesis chamber was the same as that described earlier for multiplant biosyntheses with opium poppies,[15] with a few modifications. The "daylight" fluorescent lights used to illuminate the chamber during the run have been replaced by Nu-Lite Ultra Lux fluorescent lights, the spectrum of which more closely approximates sunlight. The $^{14}CO_2$ was generated directly into the system by adding concentrated sulfuric acid to barium carbonate-^{14}C having a specific activity of about 110 μc./mg. The apparatus for generating $^{14}CO_2$ in this manner replaced the feed loop of the earlier description. It required about 6 min. from the first addition of acid until the maximum activity was recorded in the system by the vibrating reed electrometer. The time of this maximum was taken to be the starting point of the biosynthetic run.

The volume of the biosynthesis chamber was such that about 150 ml. of CO_2 was initially present. Generation of 30 mc. of $^{14}CO_2$ introduced an additional 30 ml., an increase of only about 20%. The initial uptake of the $^{14}CO_2$ was rapid. The plants growing in soil absorbed about 65% of the $^{14}CO_2$ during the first hour; the somewhat larger plants growing in nutrient solution absorbed about 90% of the $^{14}CO_2$ during this time. As the CO_2 in the chamber became depleted, the rate of $^{14}CO_2$ absorption decreased markedly. After 1.5 hr., over 95% of the $^{14}CO_2$ had been absorbed, and further absorption, as indicated by the recorded trace of the vibrating reed electrometer, had nearly ceased. Before each run, the infrared CO_2-analyzer was standardized with gas mixtures containing various concentrations of CO_2. In the region of interest from normal air (approximately 0.03% CO_2) down to less than 0.003% CO_2, the infrared analyzer was not very precise; however, the plot of values obtained indicated that the CO_2 concentration in the chamber was falling at a rate which paralleled the loss of radioactivity. After 1.5 hr., the infrared analyzer indicated that less than 10% of the initial CO_2 concentration remained in the chamber.

Two types of experiments were carried out. In the first series (I–VII), no additional CO_2 was added to the chamber. In the second series (VIII–X), after 1.5 hr. when 95% of the $^{14}CO_2$ had been taken up, additional $^{12}CO_2$ was added. The $^{12}CO_2$ was added from a cylinder, through a bubble flow meter, directly into the biosynthesis chamber at a rapid rate until the infrared analyzer indicated that a normal CO_2 concentration had been restored to the chamber, then the rate of $^{12}CO_2$ addition was decreased until the normal CO_2 concentration was just being maintained. A flow of 4–5 ml./min. was required for this purpose. In neither type of experiment did the plants display any ill effects after 12 hr. of illumination.

The biosynthesis chamber described in the earlier work also has been modified to contain "hot box" glove ports. At the end of the required biosynthesis period, the plants were separated into root (R, below ground) and aerial (A, leaf and stem, above ground) portions by using the glove ports. This required about 5 min. When the plant portions had been separated, simultaneously, the lights were turned off, evacuation of the chamber was begun, and liquid N_2 was poured into the dewars containing the plant material in the chamber. Within 15 min. after the indicated end of the biosynthetic period all the plant material had been frozen in liquid N_2 (except as noted above in runs I–III).

Alkaloid Isolation.—After the $^{14}CO_2$ had been fully evacuated from the chamber, the still frozen plant material was removed and the two portions blended separately in a Waring Blendor with 50% aqueous acetone, the aqueous content of the plant matter being included; *e.g.*, the root portion from four plants was blended with a solution of 325 ml. of water and 375 ml. of acetone. The total aerial portion was treated with a solution of 800 ml. of water and 1000 ml. of acetone. The blended plant material was allowed to stand in contact with the aqueous acetone for 24 hr. The suspension was filtered, the filtrate was acidified (pH 2) with phosphoric acid, and the acetone was removed at

(15) H. Rapoport, F. R. Stermitz, and D. R. Baker, *J. Am. Chem. Soc.*, **82**, 2765 (1960).

(16) H. Rapoport, N. Levy, and F. R. Stermitz, *ibid.*, **83**, 4298 (1961).

(17) A large variety of *Nicotiana* species have been used by others; usually the species selected has been a high producer of the specific alkaloid under study. We shall adhere to *N. glutinosa*, since this species gives a good distribution among the various alkaloids [E. Wada, T. Kisaki, and M. Ihida, *Arch. Biochem. Biophys.*, **80**, 258 (1959)] and adherence to a single species may better reveal alkaloidal interrelationships.

(18) S. Aronoff, *Plant Physiol.*, **31**, 355 (1957).

(19) A. M. Kuzin and V. I. Merenova, *Dokl. Akad. Nauk SSSR*, **85**, 393 (1952).

(20) T. C. Tso, R. N. Jeffrey, and T. P. Sorokin, *Arch. Biochem. Biophys.*, **92**, 241 (1961).

(21) T. C. Tso, *ibid.*, **92**, 248 (1961).

(22) We wish to thank Mr. Billy Roberts and his staff of the Virus Laboratory Greenhouse, University of California, Berkeley, for furnishing us with the *Nicotiana glutinosa* plants.

(23) D. R. Hoagland and D. I. Arnon, California Agricultural Experimental Station Circular 347, revised 1950, College of Agriculture, University of California, Berkeley.

30° on a rotary evaporator *in vacuo*. The resulting acidic solution, after being extracted twice with equal volumes of methylene chloride, was made strongly alkaline by the addition of 6 *N* sodium hydroxide and then extracted continuously with methylene chloride for 24 hr. The methylene chloride extract was dried over sodium sulfate and evaporated (rotary evaporator, *in vacuo*), leaving a brown oil which constituted the crude alkaloid fraction.

This crude alkaloid fraction next was fractionated by gas–liquid chromatography (g.l.c.). A 0.5-in. × 5-ft. stainless steel column packed with potassium hydroxide treated 60/80 firebrick coated with 10% by weight polybutylene glycol[24] was used for the fractionation. At 169° with a He flow of 300 ml./min., the following retention times were obtained: nicotine, 6 min. 15 sec.; nornicotine, 10 min. 15 sec.; anabasine, 13 min. 00 sec.; anatabine, 16 min. 00 sec.

The alkaloids corresponding to each of these peaks were collected by cooling the effluent in liquid nitrogen. Benzene was used as the injection solvent, and aliquots were added so that no more than 15–20 mg. of crude alkaloidal material was injected at any one time. The peak areas of the chromatogram were measured with a planimeter. By using a standard solution of nicotine in benzene, a plot of peak area *vs.* nicotine mass was prepared and was used to determine the amount of nicotine in each of the crude alkaloidal fractions. To prevent contamination, the g.l.c. column was replaced several times during the course of experiments I–X.

To check the purity of the various fractions, paper chromatography also was employed. Whatman No. 4 paper was buffered by dipping the sheets in a 0.2 *M* potassium hydrogen phosphate solution (pH 6.7) and allowing them to drip dry. *t*-Amyl alcohol saturated with water was used as the moving phase.[25] No prior equilibration time was allowed before beginning the chromatography. The chromatographs were allowed to run for about 10 hr. during which time the solvent front advanced about 30 cm. Using the descending technique, the R_f values observed were nornicotine, 0.18; anabasine, 0.31; anatabine, 0.48; and nicotine, 0.81. The König reaction[26] was used to locate the alkaloids, nornicotine giving an orange spot, anabasine a pink spot, anatabine a rose spot, and nicotine a gold spot. The radiochemical purity of the various alkaloid fractions was routinely checked by radioautography of the above paper chromatographs.

Determination of Radioactivity by G.l.c. Proportional Counting.—The nicotine that had been separated and purified by g.l.c. (as determined by reinjection, the total recovery was 80–90%) was utilized for the specific activity determination described below.

The apparatus used consists of a chromatograph (Aerograph, Wilkens Instrument and Research Co.), a dual pen recorder (Leeds and Northrup Speedomax-G), a scaler (Nuclear-Chicago Model 182), a ratemeter (Nuclear–Chicago Model 1620 B), and a printer (digital recorder Model 560 A, Hewlett–Packard). The flow-through proportional counter was designed by Mr. Irville Whittemore of the Lawrence Radiation Laboratory and is a modification of the counter described by Wolfgang and Rowland,[27] who were the first to use this technique for the counting of tritium and ^{14}C-labeled compounds.

The g.l.c. column used was a 0.25-in. × 5-ft. stainless steel column packed as above and held at about 198° with a He flow of 75 ml./min. The retention time of nicotine under these conditions was 6 min. 12 sec. The proportional counter tube was heated to 175–185° by means of heating tape. In order to provide a gas mixture that was suitable for ^{14}C counting, it was necessary to add a methane flow of about 35 ml./min. to the carrier gas just before it entered the counter tube.

After the high voltage supply had been adjusted so that the counter was operating in the center of its plateau region (about 200 v.), the proportional counter was standardized. Several injections of 5-μl. samples of hexane-^{14}C of known activity (1570 d.p.m./μl.) were made. The counts registered by the proportional counter were printed out at 20-sec. intervals by the digital recorder. The average background registered over several minutes was subtracted from the counting peaks corresponding to the samples of hexane-^{14}C to give the net counts registered per injection, and eq. 1 was used to calculate an efficiency–volume factor for the counter.

$$N = \frac{eVA}{f} \qquad (1)$$

where N = net number of counts per peak, e = counter efficiency in counts/disintegrations, V = effective counter volume in ml., f = measured flow rate through the counter in ml./min., A = absolute activity of hexane added in disintegrations/min. The efficiency–volume factor varied from 25–35 count-ml./disintegrations. Since the counter was designed to have an effective counting volume of roughly 60 ml., the actual counting efficiency was about 50%.

The next step in the determination of the specific activity of nicotine by this method was the standardization of the thermal conductivity detector of the g.l.c. apparatus toward nicotine. Several injections of a standard solution of nicotine in benzene were made, and a plot of peak area *vs.* mg. of nicotine prepared. This plot showed good linearity in the region of interest from about 50 μg. to 1.5 mg.

The two standardizations described above allowed the activity and mass of an unknown nicotine sample to be determined. The counts due to the unknown nicotine injection were calculated by subtracting the average background from the counting peak which corresponded to the nicotine mass peak. Use of the previously calculated eV factor in eq. 1 then allows the absolute activity in d.p.m. to be determined for the given injection. The mass of this nicotine peak was determined by comparison of the observed peak area with the standardization plot. The ratio of the absolute activity and mass determined in this way gave the specific activity of the unknown nicotine sample.

Except for the samples from run X, all nicotine samples were determined on at least two different occasions when the entire standardization procedure was performed independently. Two separate determinations were made of the nicotine samples from run X, but these were made on one occasion with the same set of standardization values. The reproducibility of the method is demonstrated by the values in Table I.

TABLE I

Sample[a]	Detmn. 1	Detmn. 2	Detmn. 3	Average
IX_6^R, μc./mmole	5.72, 6.21	5.2	6.73, 6.03	5.85
IX_6^A, μc./mmole	0.82, 0.70	0.98	0.71, 0.99	0.86

[a] The Roman numeral represents the experiment number, the subscript represents the duration of the experiment in hours, and the superscript, the portion of the plant.

In order to test the validity of this method of determining the activity of the nicotine, in certain cases the nicotine was collected on cotton as it left the proportional counter tube. The nicotine so obtained was eluted with ethanol, the concentration of the resulting solution established by ultraviolet absorption, and the activity determined by counting an aliquot of the solution in a scintillation counter. The values obtained by the two methods are compared in Table II.

TABLE II

	Activity, μc./mmole		
Sample	By g.l.c.	By scintillation counting	Error in g.l.c., %
III_{12}^A	40.7	47.4	−13
IX_6^R	5.85	6.23	− 8
IX_6^A	0.86	0.95	−11

Table II demonstrates that the specific activity of nicotine can be determined by the g.l.c. proportional counter method to an accuracy of better than 15%. The sample size used varied from 50 μg. to 1.5 mg., depending upon the specific activity. The lower limit of the method appeared to be about 0.1 μc./mmole. In order to determine the specific activity of the VII_2^{AG} nicotine sample, it was necessary to collect the nicotine as it was eluted from the 0.25-in. g.l.c. column and determine its activity by the scintillation–ultraviolet method described above.

The g.l.c. proportional counting method also was used to determine the specific activity of the N-methyl group of the nicotine. Samples which had been counted as described were collected on cotton as they were eluted from the counter tube.

(24) L. D. Quin and N. A. Pappas, *J. Agr. Food Chem.*, **10**, 79 (1962).
(25) T. C. Tso and R. N. Jeffrey, *Arch. Biochem. Biophys.*, **80**, 46 (1959).
(26) S. Aronoff, "Techniques of Radiobiochemistry," Iowa State University Press, Ames, Iowa, 1956, p. 165.
(27) R. Wolfgang and F. S. Rowland, *Anal. Chem.*, **30**, 903 (1958).

The nicotine was then removed and subjected to a Herzig–Meyer determination, collecting the methyl iodide generated in 250 ml. of toluene cooled in a Dry Ice–acetone bath. The solution of CH_3I in toluene could now be injected into the g.l.c. proportional counter apparatus and the mass and activity determined by the procedure described for nicotine.

For the methyl iodide determination, a 0.25-in. × 10-ft. stainless steel column of 7.5% Apiezon L, 7.5% polyamine 6 (Wilkens Instrument Co.) on 60/80 firebrick was used. In this case, it proved possible to use pure methane as the carrier gas for the g.l.c.; hence, it was not necessary to add methane for counting purposes. At 55° with a flow rate of 40 ml./min., the methyl iodide was eluted in about 4 min. Three injections could be made before the toluene was eluted. The thermal conductivity detector was standardized using a known solution of methyl iodide in toluene and showed good linear response in the region of interest from 10 to 200 μg.

To check the validity of the g.l.c. proportional counting method of determining the specific activity of $^{14}CH_3I$, some standard $^{14}CH_3I$ in toluene solutions were prepared. Specific activities were determined by (1) g.l.c. method and (2) titration and scintillation counting. The values obtained by the two independent methods are compared in Table III. They indicate that the g.l.c. proportional counting method is less precise when applied to $^{14}CH_3I$ than when applied to nicotine. This is probably due to quenching by methyl iodide. The limit of detection was about 0.1–0.2 μc./mmole, where, as listed below, the determined specific activity of the $^{14}CH_3I$ was low by a factor of 2. It required 150 μg. of $^{14}CH_3I$ of specific activity 0.13 μc./mmole for the activity to be detected by the g.l.c. proportional counting method. In instances where the specific activity of the N-methyl was high enough, a Herzig–Meyer analysis on 0.5 mg. of nicotine provided enough $^{14}CH_3I$ for several determinations by the g.l.c. method.

TABLE III

$^{14}CH_3I$ activity by g.l.c., μc./mmole	Activity by scintillation counting, μc./mmole	Error in g.l.c. detmn., %
1.5	2	−25
0.6	0.95	−37
.21	.385	−45
.062	.13	−52

To determine specific activities less than 0.1 μc./mmole, the methyl iodide generated in the Herzig–Meyer determination was collected as before, the concentration in the toluene solution was determined by g.l.c., and the activity was determined by scintillation counting. It should be noted that this modified method is less reliable than the previous method, since all the activity generated in the Herzig–Meyer is attributed to $^{14}CH_3I$. The specific activities determined in this way should, therefore, be considered an upper limit for the activity of the $^{14}CH_3I$.

Degradations. A. Conversion of Nicotine to Methyl Nicotinate.—The methods available for the conversion of nicotine to nicotinic acid involve oxidation with nitric acid,[28] potassium permanganate,[29] or chromic acid.[30] The nitric acid oxidation has been utilized[28] in a semimicro degradation of radioactive nicotine to nicotinic acid accompanied by a 4% yield of 3-nitro-5-(3-pyridyl)pyrazole. The method of purification involving copper salt precipitation and regeneration with hydrogen sulfide led to a 31% yield of nicotinic acid.[31] A similar purification *via* the silver salt was developed[29b] for use with the neutral permanganate oxidation and gave nicotinic acid in about 45% yield.

Our own experience with this conversion on the semimicro scale has indicated: (i) the nitric acid oxidation gave inconsistent results, (ii) the optimum molar ratio of potassium permanganate to nicotine is 8 to 1, (iii) the preferred method of isolation of nicotinic acid is as its methyl ester, and (iv) the preferred method of purification of methyl nicotinate is zone melting after an initial sublimation.

A typical degradation involved gradual addition of a solution of 1145 mg. (7.26 mmoles) of potassium permanganate in 100 ml. of water in 30 min. to a stirred solution of 147 mg. (0.91 mmole) of nicotine[32] in 100 ml. of water. The mixture was heated on the steam bath for 16 hr., treated with a few drops of ethanol to destroy excess permanganate, filtered through Celite, concentrated to dryness at reduced pressure, treated with 1 ml. of concentrated sulfuric acid and 10 ml. of anhydrous methanol, and heated at reflux for 2 hr. The volatile solvent was removed rapidly at reduced pressure, and the residue was treated with 10 ml. of water and excess sodium bicarbonate and extracted with four portions of methylene chloride. The extract was dried over magnesium sulfate and concentrated at reduced pressure, and the residue was sublimed at 35° (30 mm.) onto a cold finger maintained at 0°[33] yielding 90 mg. (72% of theoretical)[34] of crude methyl nicotinate, m.p. 32–36°. The radioactive samples were purified to constant specific activity (scintillation counting), melting point (39.0–40.8°), and ultraviolet absorption (λ_{max}^{EtOH} 262 mμ, ε 2780) by repeated sublimations with large losses or preferably by zone melting.[35]

B. **Conversion of Methyl Nicotinate to Pyridine and Barium Carbonate.**—The method of decarboxylation used here is a modification of that employed by Lamberts and Byerrum.[29b] In a typical example, a mixture of 5.5 mg. (0.04 mmole) of methyl nicotinate, 0.50 ml. of 6 *N* sodium hydroxide and approximately 1 ml. of water rinse was heated in a sealed tube at 105° for 45 min. The contents were cooled, transferred to a 250-ml. flask using a water rinse, treated with 1 ml. of 12 *N* hydrochloric acid, evaporated to dryness at 40° (10 mm.), and diluted with 1 g. of calcium oxide. The entire solid was transferred to a mechanical mixing device (Wig-L-Bug), treated with 2 drops of water, and shaken for 1 min.[36] Heating of the resulting mixture in a stream of nitrogen in a glass grease-free apparatus caused decarboxylation of the nicotinic acid. Heating was stopped after 5–10 min. when no more pyridine and water appeared to condense from the effluent vapors. The condensate was rinsed into a small flask with a minimum of 95% ethanol, and the solution, containing 1.17 mg. (37% yield) of pyridine as determined by ultraviolet absorption (λ_{max}^{EtOH} 257 mμ, ε 2650), was counted by liquid scintillation.

The carbon dioxide remained as calcium carbonate and was regenerated by the addition of a mixture of 2.0 ml. of 85% phosphoric acid and 2.0 ml. of water and recovered and counted[37] as barium carbonate (4.2 mg., 53% yield).

Results

The nicotine activities obtained in ten biosynthesis experiments are presented in Table IV. In each case where exposure to $^{14}CO_2$ was for 2 hr. or more, radioactive nicotine resulted. No additional carbon dioxide was added in the first seven experiments over that initially present plus the $^{14}CO_2$; in the last three experiments, $^{12}CO_2$ was added after 1.5 hr. to maintain a normal carbon dioxide concentration. Nicotine assay was carried out separately on the aerial and root plant sections in each experiment.

The various data of Table IV are presented graphically in Fig. 1 and 2. Figure 1 shows the change in specific activity of the nicotine in both portions of the plant as a function of time of exposure, and Fig. 2 shows the same function for the ratio of total activity in nicotine.

Distribution of radioactivity among various portions of the nicotine molecule is presented in Table V as a percentage of the total nicotine activity. The portions for which data are presented are the N-methyl group and the pyridine ring and carbon-2′, the latter being

(28) (a) S. M. McElvain, "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1941, p. 385; (b) E. Leete and K. J. Siegfried, *J. Am. Chem. Soc.*, **79**, 4529 (1957).

(29) (a) R. Laiblin, *Ann.*, **196**, 129 (1879); (b) B. L. Lamberts and R. U. Byerrum, *J. Biol. Chem.*, **233**, 939 (1958).

(30) S. Hoogewerff and W. A. van Dorp, *Rec. trav. chim.*, **1**, 107 (1882); R. Camps, *Arch. Pharm.*, **240**, 353 (1902).

(31) E. Leete, *J. Am. Chem. Soc.*, **78**, 3520 (1956).

(32) The radioactive nicotine as obtained above was repurified by gas–liquid chromatography, diluted with inactive nicotine, and counted by the scintillation technique; λ_{max}^{EtOH} 262 mμ, ε 2910.

(33) The cold finger must be allowed to equilibrate to room temperature in a dry atmosphere before attempting to remove the sublimate.

(34) Yields ranged as high as 92%.

(35) J. H. Beynon and R. A. Saunders, *Brit. J. Appl. Phys.*, **11**, 128 (1960).

(36) This procedure is necessary for reproducible and successful decarboxylation.

(37) F. H. Woeller, *Anal. Biochem.*, **2**, 508 (1961).

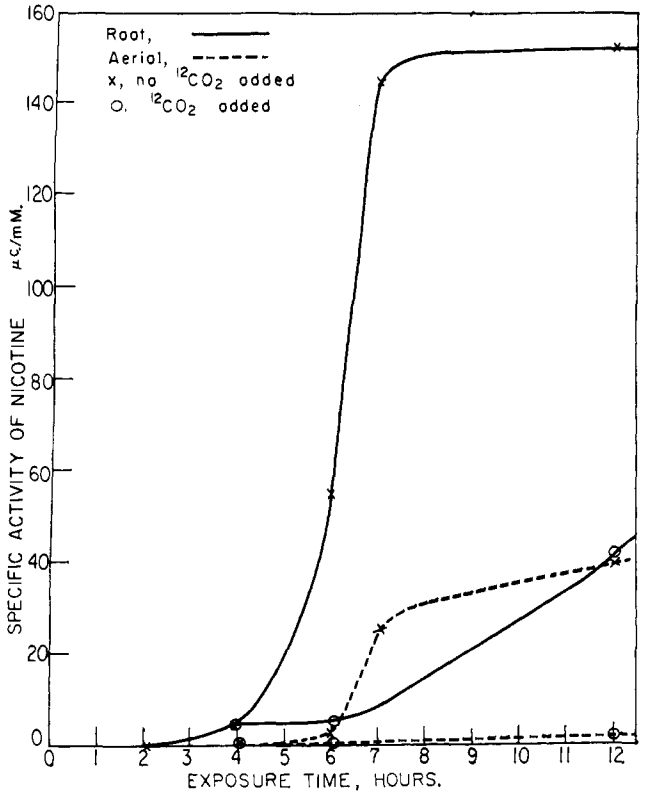

Fig. 1.—Incorporation of $^{14}CO_2$ into nicotine.

presented collectively as methyl nicotinate, and separately after decarboxylation.

The percentage of the total nicotine activity found in the pyridine ring is plotted in Fig. 3 as a function of exposure time. The value for the pyridine ring is the average of the pyridine value and the methyl nicotinate minus carbon-2′ value for each experiment, and each point should have an accuracy well within ±4% except experiment VII_2^R.

TABLE IV

BIOSYNTHESIS OF NICOTINE FROM $^{14}CO_2$

Expt.[a]	$^{14}CO_2$ (mc.) absorbed	Total exposure to $^{14}CO_2$, hr.	Nicotine: Sp. activity, μc./mmole	Sp. act. R / Sp. act. A	Mass A / Mass R	Total act. R / Total act. A
I_R^A	24.0	7	25.6 145	5.7	~6	~1
II_R^A	29.3	6	3.2 54.6	17.1	8.1	2.1
III_R^A	29.6	12	40.7 152	3.7	5.0	0.7
IV_R^A	30.4	1	0	...		
V_R^A	30.6	1	0	..		
VI_R^A	36.7	1	0	..		
VII_R^A	30.4	2	0.008 0.16	20	5.6	3.6
$VIII_R^A$	30.6	4[b]	0.14 4.9	35	6.7	5.2
IX_R^A	36.0	6[b]	0.86 5.85	6.9	12.8	0.5
X_R^A	29.8	12[b]	2.58 41.4	16.1	7.5	2.1

[a] Letters refer to plant portion: A refers to the aerial portion of the plant (leaves and stems); R, to the roots. [b] After 1.5 hr. $^{12}CO_2$ was added and maintained at normal atmospheric concentration.

Discussion

Rate of Incorporation of $^{14}CO_2$ into Nicotine.—In spite of the large number of experiments concerning the biosynthesis of nicotine, there are few studies in which the rate of incorporation of the various compounds into nicotine has been examined. This lack is particularly true for short-term experiments in which incorporation of a potential precursor into nicotine is examined during the first few hours after exposure; only recently has this type of experiment been carried out. Active nicotine was isolated[38] 1 hr. after feeding acetate-2-^{14}C; 2 hr. after feeding glycerol-2-^{14}C, propionate-2-^{14}C, or aspartate-3-^{14}C; and 3 hr. after feeding acetate-1-^{14}C to the roots of *N. rustica*. In the case of acetate-1-^{14}C, the incorporation rose by a factor of 6.5 as the exposure time increased from 3 to 6 hr. The incorporation of

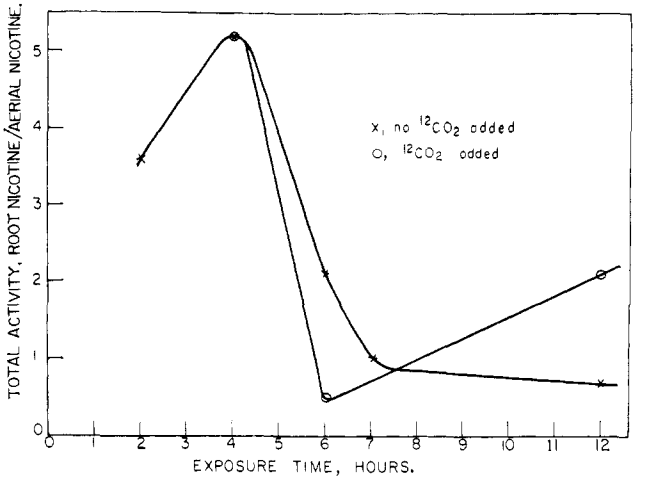

Fig. 2.—Ratio of total activity in root nicotine to aerial nicotine.

TABLE V

PERCENTAGE OF TOTAL NICOTINE ACTIVITY IN ITS VARIOUS DEGRADATION PRODUCTS

Expt.[a]	N-Methyl[b]	Methyl nicotinate[c]	Pyridine	Carbon-2′[d]
I_7^A	<1[e]	80	75	0.8
I_7^R	0.44	76	73	1.1
II_6^A	<8[e]	70	67	1.7
II_6^R	0.55	79	77	3.8
III_{12}^A	7.8	69	69	1.9
III_{12}^R	1.4	79	74	1.9
VII_2^R	6[f]	87	99	...
$VIII_4^A$	8[f]	73	75	0.4
$VIII_4^R$	3.4[f]	85	86	2.7
IX_6^A	4.7[f]	80	82	0.8
IX_6^R	2.7[f]	77	68[g]	0.3
X_{12}^A	8.2[f]	68	66	1.6
X_{12}^R	1.5	61	58	1.6

[a] The Roman number designates chronological sequence, the subscript designates the hours of metabolism from the beginning of $^{14}CO_2$ feeding, and the superscript refers to the aerial and root portion of the plant. [b] Determination of N-methyl activities was made with undiluted nicotine of specific activity as given in Table IV. [c] Obtained from a portion of the nicotine of specific activity as given in Table IV diluted with approximately 150 mg. of inactive nicotine to a final specific activity of 1–0.1 μc./mmole in most cases. [d] The carbonyl carbon of methyl nicotinate, counted as barium carbonate. [e] This arises from the experimental error in detecting low levels of methyl iodide activity. [f] The concentration of methyl iodide was determined by v.p.c.; determination of activity includes everything volatile that results from the action of hydrogen iodide on nicotine, since the quantity of methyl iodide was too small to isolate. Therefore, these values should be considered only as upper limits. [g] This represents a minimum value and is not used in any further computation.

(38) P. L. Wu, T. Griffith, and R. U. Byerrum, *J. Biol. Chem.*, **237**, 887 (1962).

acetate-2-^{14}C after 2 hr. was found[12] to be about 25% of that after 7 days and after 1 day was nearly equal to the 7-day level. There was a steady, nearly linear increase in the incorporation of acetate-2-^{14}C into nicotine as the incubation time increased from 1 to 6 hr., with the level at 6 hr. being about double that at 2 hr.

The most rapid incorporation reported[39] to date is the detection of tritium in the alkaloid fraction 30 min. after the roots of *N. rustica* were exposed to tritiated water for 1 min. However, this result is difficult to interpret in terms of *de novo* nicotine synthesis because of the possibility of facile tritium exchange with late precursors.

The data in Table IV show that activity could be detected in nicotine 2 hr. after exposure of intact *N. glutinosa* plants to $^{14}CO_2$. Figure 1 indicates that in those runs where no $^{12}CO_2$ is introduced into the sealed exposure chamber, the maximum incorporation into root nicotine takes place between 4 and 7 hr. after exposure to $^{14}CO_2$. In those runs where $^{12}CO_2$ was added after 1.5 hr. to maintain the CO_2 concentration at about 0.03%, Fig. 1 indicates two regions of rapid incorporation, the first occurring between 2 and 4 hr. and the second occurring between 6 and 12 hr. after exposure to $^{14}CO_2$. Since very little incorporation of ^{14}C has occurred by 2 hr. after exposure to $^{14}CO_2$, in the case of the 4-hr. run, only a small incorporation of ^{12}C can have occurred from the $^{12}CO_2$ added 2.5 hr. earlier. For purposes of the following discussions, therefore, the 4-hr. run (VIII) is considered to be both a $^{12}CO_2$ added and a $^{12}CO_2$ not added type experiment. In the runs where no additional $^{12}CO_2$ is introduced into the chamber, the metabolism of the plant, and thus the synthesis of nicotine, would be expected to come to a halt after a time, as the precursor pools become depleted. After the rapid rise in activity between 4 and 7 hr. in these runs, the specific activity curve becomes level, indicating little new incorporation into the root nicotine during the remaining hours of the experiment.

In those runs where the CO_2 concentration was maintained, the plant's metabolism would be expected to continue at its normal rate. Figure 1 indicates the first effect of the added $^{12}CO_2$ is to cause rapid incorporation of ^{14}C into root nicotine at 2–4 hr. *vs.* the 4–7 hr. in the runs where no $^{12}CO_2$ is added. However, as ^{12}C from the added $^{12}CO_2$ also begins to be incorporated into the nicotine, the period of rapid incorporation is not sustained for as long a time, and the activity does not rise to as high a level as when no $^{12}CO_2$ is added. The plateau from 4–6 hr. represents the combined effects of ^{12}C and ^{14}C incorporation into root nicotine. During the period from 6–12 hr., Fig. 1 shows that the specific activity of the root nicotine increased again. Figure 3 shows that during this period the percentage of activity in the pyridine ring of the root nicotine fell sharply, despite the increased incorporation. This second region of rapid incorporation is, therefore, consistent with the formation of new active nicotine in the root, where the ^{14}C enters the pyrrolidine ring of the nicotine molecule *via* different, slower responding, precursor pools. In the case where no $^{12}CO_2$ was added, this type of incorporation into nicotine was not observed, since the slowing down of the over-all metabolism prevented the ^{14}C from advancing through these slower responding pre-

(39) T. C. Tso and R. N. Jeffrey, *Arch. Biochem. Biophys.*, **97**, 4 (1962).

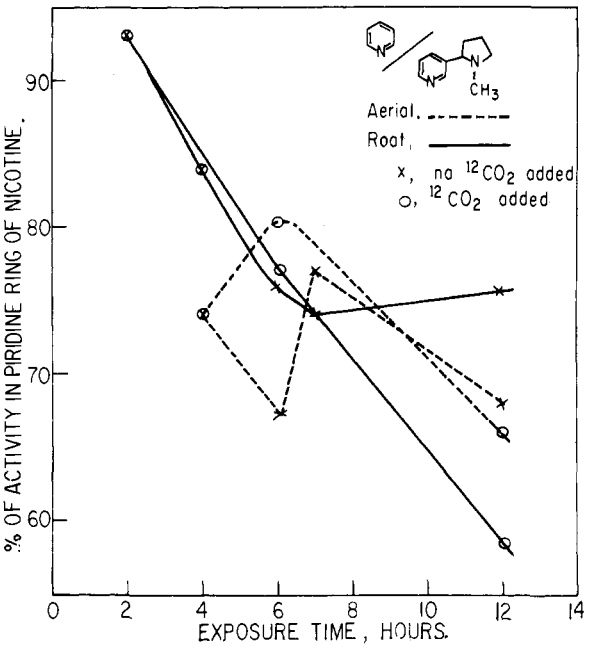

Fig. 3.—Percentage of total activity in the pyridine ring of nicotine.

cursor pools into nicotine within the period of the experiment. In all cases, the incorporation of ^{14}C into the aerial nicotine is significantly slower than into the root nicotine.

In their study on the incorporation of acetate-2-^{14}C, Griffith, Hellman, and Byerrum[12] found that incorporation after 1 hr. of incubation was largely (66%) into the pyridine ring. The incorporation into the pyridine ring continued to increase at a slow rate. Incorporation into the pyrrolidine ring from acetate-2-^{14}C, however, increased at a rapid rate, such that at 6 hr. the two rings were nearly equally labeled. Our data indicate that the relative rate of incorporation of ^{14}C into the pyridine ring *vs.* the pyrrolidine ring is much greater with $^{14}CO_2$ then it was with acetate-2-^{14}C. After 2-hr. exposure to $^{14}CO_2$, about 90% of the activity of the root nicotine is found in the pyridine ring, and after 12-hr. exposure, the pyridine ring still contains about 60% of the activity present, even when $^{12}CO_2$, which would be expected to incorporate rapidly into the pyridine ring, has been added.

The only previous work concerning the rate of $^{14}CO_2$ incorporation into nicotine is due to Tso, Jeffrey, and Sorokin.[20] Working with *N. rustica*, they found no activity 1 hr. after 0.5-hr. exposure to $^{14}CO_2$. Eight days later, however, they detected activity in nicotine. While the data we report here are in agreement with these results, the implication of the earlier work, namely, that it requires a long incubation period to obtain incorporation of $^{14}CO_2$ into nicotine, is not true. Our data show that activity can be detected in nicotine 2 hr. after exposure to $^{14}CO_2$, and that after 6 hr. of exposure, a substantial amount of activity can be found in the nicotine. We find that $^{14}CO_2$ incorporates into nicotine at a rate nearly comparable with that found for the incorporation of acetate; $^{14}CO_2$, however, has a greater tendency to incorporate into the pyridine ring of nicotine than does acetate-2-^{14}C.

If photosynthesis and metabolism proceed normally under the conditions of our experiments, the percentage

of the plant's total activity incorporated into nicotine would be expected, as a steady state is reached, to equal the percentage of the plant's total carbon which is present in nicotine. As a close approximation to this latter figure, we have used the percentage of nicotine in the plant's dry weight. The ratio of these two percentages (% activity as nicotine to % mass as nicotine) proceeds roughly from a low value of 0.004 after a 2-hr. exposure, when very little activity is found in the nicotine, to 0.1 after 4 hr., 0.2 after 6 hr., and 1.0 after 12 hr., which probably is at or near the steady state. These results are what would be expected for normal growth.

Site of Nicotine Synthesis in Intact *N. glutinosa*.— A series of early experiments in which *Nicotiana* plants were grafted onto *Solanaceae* indicated the decisive role of the root in nicotine formation. Due to an excessive consciousness of the importance of the green leaf in plant metabolism, some of the early results were misinterpreted, but by 1942, Dawson[40] and Hieke[41] had concluded that the tobacco root played the primary role in nicotine synthesis and that nicotine was translocated from the root to the stem and leaves. After the demonstration[42] of nicotine production in isolated root cultures, the remaining question was whether any independent nicotine synthesis was carried out in the aerial portions of *Nicotiana*. Careful examination of *N. rustica* scions on tomato stock revealed that traces of alkaloids were present.[43] When possible error arising from the alkaloid present in the scion at the time of the graft was eliminated by grafting young tobacco embryos onto tomato, a significant amount of nicotine was still found[44] in the tobacco scion. While these experiments certainly were indicative of synthesis of nicotine in the shoot, criticisms were raised which prevented them from being accepted as conclusive proof.

The tomato plant itself was found[45] to produce small amounts of nicotine; and while tobacco scions of tomato stock were found to contain much larger amounts of the alkaloid than the intact tomato plant, nevertheless, it was argued that this increase could be due to either a stimulated production of nicotine by the tomato root due to the graft or to a decreased rate of destruction of nicotine in the grafted plant compared to intact tomato. In addition, in grafting experiments, particularly in the embryo grafting, the scion frequently forms roots which may grow inside the stem of the stock and therefore escape detection.[46] Such roots would be a serious source of error.

Several additional reports have appeared which indicate independent nicotine synthesis in the shoot, although in most cases, the criticisms made of the earlier experiments are still valid. Tso and Jeffrey[47] found that the nicotine level of tobacco scions on tomato stock rose to about four times the level present in the scion at the time of grafting. By placing the roots in nutrient solution containing ^{15}N-enriched nitrate, they showed that the grafted plants could incorporate nitrogen from nitrate into nicotine. The amount of ^{15}N-enrichment of the isolated nicotine indicated, however, that more than one-half of the nitrogen found in the newly formed nicotine entered the plant either before the grafting or during the 6 weeks directly following the grafting, before the 4-week period spent in the ^{15}N-enriched nutrient.

Solt[48] also carried out a study on the nicotine production of tobacco scions grafted on *Solanaceae* stocks, finding that the amount of nicotine accumulated in the first segment of the scion adjacent to the graft union was far in excess of the total in the other segments, even though the dry weight increased in each segment in a nearly uniform manner. This suggests that the graft itself has a profound effect on the production of nicotine. Also, *N. tabacum* scions from tomato root stocks formed radioactive nicotine when supplied with recoil-tritium-labeled nicotinic acid through their cut stems for 8 days. This seems to be the strongest evidence reported to date for nicotine synthesis in the shoot. On the other hand,[49] no active nicotine was formed when rootless shoots of *N. tabacum* were grown in $NaH^{14}CO_3$ solution for 7 days. Three tobacco shoots which started new roots during the experiment produced active nicotine.

The data presented in Table IV indicate that the primary site of nicotine synthesis is the root. Though the ^{14}C in our experiments must enter the plant through the aerial portions by $^{14}CO_2$ reduction and then be translocated to the root portions, in every case the nicotine isolated from the root had a higher specific activity than that from the aerial portion. Figure 4, where the ratio of the total activity in the root nicotine to the total activity in the aerial nicotine is plotted, leads to the same conclusion. Even when the much larger nicotine pool present in the aerial portion is considered, the roots contain more activity in the form of nicotine-^{14}C during the first 6 to 7 hr. after exposure to $^{14}CO_2$. The R/A ratio is maximum at 4 hr. and then falls steadily until it becomes unity at about 7 hr. This is the general trend that would be expected if the nicotine were synthesized in the root and translocated into the stem and leaves. The subsequent rise in R/A ratio in those experiments where the CO_2 level was maintained is consistent with the formation of new active nicotine in the root where the ^{14}C that enters the pyrrolidine ring of the nicotine molecule comes *via* slower responding precursor pools, as discussed above.

In Fig. 3, we find compelling evidence for independent nicotine synthesis in the aerial portions of intact *N. glutinosa*. The percentage of ^{14}C in the pyridine ring of nicotine in the root clearly decreases as a function of time; after 2 hr., it is over 90%, and after 4 hr., it is about 84%. The nicotine obtained from the aerial portions after 4 hr. however, contains only 74% of its activity in the pyridine ring. It is not possible to alter the proportion of activity in the two rings of the nicotine molecule merely by translocation. Therefore, the active nicotine obtained from the aerial parts after 4 hr. of exposure must be unique compared to the nicotine that was obtained from the root after either 2- or 4-hr. exposure to $^{14}CO_2$. Consequently, the *N. glutinosa* plant must have the capacity to synthesize nicotine in its aerial portions, where different pool sizes or perhaps

(40) R. F. Dawson, *Am. J. Botany*, **29**, 66 (1942).
(41) K. Hieke, *Planta*, **33**, 185 (1942).
(42) R. F. Dawson, *Am. J. Botany*, **29**, 813 (1942).
(43) K. Mothes and A. Romeike *Biol. Zentr.*, **70**, 97 (1951).
(44) M. F. Mashkovtsev and A. A. Sirotenko, *Dokl. Akad. Nauk SSSR*, **79**, 487 (1951).
(45) R. Wahl, *Tabak-Forsch.*, **10**, 3 (1953).
(46) K. Mothes, *Ann. Rev. Plant Physiol.*, **6**, 393 (1955).
(47) T. C. Tso and R. N. Jeffrey, *Plant Physiol.*, **32**, 86 (1957).
(48) M. L. Solt, *ibid.*, **32**, 484 (1957).
(49) G. S. Iljin, *Abhandl. Deut. Akad. Wiss. Berlin, Kl. Chem. Geol. Biol.*, **7**, 111 (1956).

slightly different pathways result in a different labeling pattern than is found in the nicotine synthesized in the root.

The R/A ratio plotted in Fig. 2 is also indicative of an independent nicotine synthesis in the aerial portion. As the exposure time becomes shorter and shorter, it should be possible nearly to eliminate the translocation of active nicotine. If nicotine were formed only in the root, the R/A ratio should go to infinity as the exposure time approached zero. After only 2 hr., when activity was first detected in the root nicotine, it was also possible to detect a small amount of activity in nicotine from the aerial portions. From Fig. 2, it can be seen that the R/A ratio has its maximum value after 4 hr. and decreases slightly as the exposure time is shortened to 2 hr. This behavior of the R/A ratio at the shorter exposure times is most readily explained by assuming independent synthesis in the aerial portions. The ^{14}C enters the aerial portion originally and must be translocated to the root before active nicotine can be formed there. Though the root is the major site of nicotine synthesis in the plant, after 2 hr., the site of aerial synthesis has been in contact with ^{14}C-containing precursors for a longer time, and, therefore, the more limited aerial synthesis has incorporated a considerable amount of ^{14}C into nicotine relative to the root. After 4 hr. of exposure, enough ^{14}C has been translocated into the root to overcome this initial concentration advantage afforded the site of aerial synthesis, and the R/A ratio rises. When exposure times become longer than 4 hr., the R/A ratio falls rapidly, reflecting the translocation of nicotine from the root to the stem as discussed earlier.

If we assume that the activity found in the aerial portions 4 hr. after exposure is due to independent synthesis (We have not studied the rate of translocation of the nicotine directly, but we do know that after 4 hr., not enough nicotine has been translocated from the root to the aerial portions to eliminate the difference in labeling pattern discussed above.), and further that the ^{14}C is incorporated by a similar mechanism throughout the plant, the R/A ratio of 5.2 indicates that the root has formed five times as much nicotine as the leaf and stem during the 4-hr. period. This would mean that about 16% of the nicotine in *N. glutinosa* is synthesized in the aerial portions. If, instead of the 4-hr. value, one takes the R/A ratio of 3.8 after 2 hr. and subtracts 1-hr. transport time from the apparent exposure time of the root to ^{14}C, the computation indicates about 12% of the nicotine in *N. glutinosa* is synthesized in the aerial portions of the plant.

As was anticipated from previous work, the $^{14}CO_2$ experiments confirm the roots as the major site of nicotine biosynthesis. However, we were able to detect a marked difference in labeling pattern in root nicotine and aerial nicotine which establishes, for the first time in intact *Nicotiana*, independent synthesis of nicotine in the leaf and stem of the plant. Finally, by making some assumptions about the rate of transport of ^{14}C precursors to the root and the translocation of nicotine from the root, we can estimate that independent nicotine synthesis in the aerial portions accounts for 12–16% of the total nicotine in *N. glutinosa*.

Relative Rate of Synthesis of the N-Methyl Group.—Although the N-methyl of nicotine has received considerable study, its specific origin and function in the intact plant remain unknown. Byerrum and coworkers, using *Nicotiana rustica*, have reported the incorporations of a myriad of methyl precursors: formic acid,[10,50] formaldehyde,[51] serine,[51] methionine,[50,52] choline,[53] betaine,[54] glycolic acid,[10] and glycine.[55] Since all of these substances were incorporated into the N-methyl of nicotine, although admittedly at different rates, it may be concluded that more than one path can be utilized in this methyl synthesis. It should also be noted that all the known methyl precursors have not yet been tested.[5]

The evidence that the N-methyl of nicotine can come from so many sources, the demonstration[56] that the N-dealkylation of nicotine and various analogs is quite nonspecific, the evidence[57] that the N-methyl group can be transferred to choline, and the widespread occurrence of nicotine[58] in plants leads to the possible consideration that nicotine itself might be involved in a fundamental type of one-carbon transfer or transmethylation in plant systems.

In the present experiments, feeding of $^{14}CO_2$ has led to radioactive nicotine with a relatively low ratio of ^{14}C appearing in the N-methyl, significantly below the uniform statistical distribution of 1:10. This is in direct contrast to the results obtained with *P. somniferum*,[15] wherein the N-methyl of thebaine, codeine, and morphine, isolated after short-term $^{14}CO_2$ feeding, contained far more activity than the statistical value.

It is clear from the data that the rate of incorporation of ^{14}C into the N-methyl increases with time from 2–12 hr., since the total activity of nicotine is increasing rapidly while the relative activity of the N-methyl is increasing slightly. Another interesting correlation, derived from the data in Table V, is that the activity of the pyrrolidine ring, as a function of exposure time, parallels that of the N-methyl group. Whether this indicates a parallel incorporation cannot be said without more information.

The present data are consistent with a mechanism whereby the N-methyl arises from precursors, such as amino acids, which become labeled slowly relative to more rapidly labeled precursors of the pyridine ring.

Relative Rate of Synthesis of the Pyridine and Pyrrolidine Rings.—The currently accepted concept which emerges from the large mass of accumulated data is that the pyrrolidine ring is derived from an amino acid such as glutamic acid[8,9] or the related amino acids ornithine,[7,8,28b] and proline.[8] Further, it has been demonstrated that putrescine, a potential intermediate from ornithine or glutamic acid, also could be incorporated.[8]

The pyridine ring can arise, *via* nicotinic acid,[6] from a number of two-, three-, and four-carbon metabolites, *viz.*, acetate, propionate, succinate, and aspartate[11,13] and glycerol.[12,14]

(50) S. A. Brown and R. U. Byerrum, *J. Am. Chem. Soc.*, **74**, 1523 (1952).
(51) R. U. Byerrum, R. L. Ringler, R. L. Hamill, and C. D. Ball, *J. Biol. Chem.*, **216**, 371 (1955).
(52) L. J. Dewey, R. U. Byerrum, and C. D. Ball, *J. Am. Chem. Soc.*, **76**, 3997 (1954).
(53) R. U. Byerrum and R. E. Wing, *J. Biol. Chem.*, **205**, 637 (1953).
(54) R. U. Byerrum, C. S. Sato, and C. D. Ball, *Plant Physiol.*, **31**, 374 (1956).
(55) R. U. Byerrum, R. L. Hamill, and C. D. Ball, *J. Biol. Chem.*, **210**, 645 (1954).
(56) R. F. Dawson, *J. Am. Chem. Soc.*, **73**, 4218 (1951).
(57) E. Leete and V. M. Bell, *ibid.*, **81**, 4358 (1959).
(58) K. Mothes, *J. Pharm. Pharmacol.*, **11**, 193 (1959).

Our present results from the $^{14}CO_2$ feedings to *N. glutinosa* show an early and high activity in the pyridine portion of nicotine (Fig. 3). This rapid appearance of label in the pyridine ring certainly is consistent with the hypothesis that the pyridine ring arises from the simple precursors mentioned above. These compounds, with origins close to the fixation of carbon dioxide, would acquire an early and high label which would be reflected in the pyridine ring.

The relatively slow incorporation of carbon into the pyrrolidine ring is consistent with entry of carbon *via* an amino acid, since amino acids would be expected to become labeled more slowly than the simpler precursors of the pyridine ring. However, it is very interesting to note the consistently low activity of carbon-2′ (Table V) and to attempt to reconcile these data with the currently accepted hypothesis for biosynthesis of the pyrrolidine ring. This hypothesis[8,9,28b,38] invokes glutamic acid through several possible pathways, all involving a symmetrical intermediate. Applying the glutamate-symmetrical intermediate hypothesis to our data would require equal labeling in C-2′ and C-5′. As a result, by difference, C-3′ and C-4′ would contain significantly larger amounts of activity. Such a requirement, that the methylene carbons of glutamate be the more highly labeled, is contrary to all current ideas on the biosynthesis of glutamate.[59-62] Therefore, either the glutamate-symmetrical intermediate hypothesis does not apply in the present case or a new, as yet unsuspected, mechanism exists for glutamate biosynthesis.

(59) R. B. Roberts, D. B. Cowie, R. Britten, E. Bolton, and P. H. Abelson, *Proc. Natl. Acad. Sci. U. S.*, **39**, 1013 (1953).
(60) N. Tomlinson, *J. Biol. Chem.*, **209**, 605 (1954).
(61) V. Moses, O. Holm-Hansen, J. A. Bassham, and M. Calvin, *J. Mol. Biol.*, **1**, 21 (1959).
(62) D. S. Hoare, *Biochem. J.*, **87**, 284 (1963).

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ρ–σ–π Analysis. A Method for the Correlation of Biological Activity and Chemical Structure

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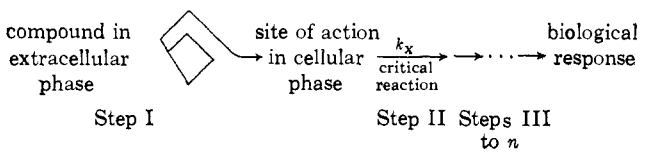
RECEIVED AUGUST 19, 1963

Using the substituent constant, σ, and a substituent constant, π, defined as $\pi = \log P_X - \log P_H$ (P_H is the partition coefficient of a parent compound and P_X that of a derivative), regression analyses have been made of the effect of substituents on the biological activity of benzoic acids on mosquito larvae, phenols on gram-positive and gram-negative bacteria, phenyl ethyl phosphate insecticides on houseflies, thyroxine derivatives on rodents, diethylaminoethyl benzoates on guinea pigs, and carcinogenic compounds on mice.

Recently[2,3] we have shown the advantage of using partition coefficients in connection with the Hammett equation to rationalize the substituent effect on the growth-promoting activity of the phenoxyacetic acids and the bactericidal action of chloromycetin derivatives on various bacteria. In particular, it was found that a substituent constant, π, patterned after the Hammett σ-constant was useful in evaluating the lipo–hydrophilic character of a molecule upon which biological activity is highly dependent. π is defined as: $\pi = \log (P_X/P_H)$ where P_H is the partition coefficient of a parent compound and P_X is the value for a derivative. The reference system is octanol–water, and all of the work reported in this paper refers to this pair of solvents. The purpose of this report is to show that our previously employed expression appears to have general applicability.

We have assumed that the rate-limiting conditions for many biological responses to chemicals can be defined in the simplest and most general way as follows.



The first step in the above reaction scheme is pictured as a random walk process in which the molecule in question makes its way from a very dilute solution outside the cell to a particular site in the cell which may be within an organelle. This is visualized as being a relatively slow process, the rate of which is highly dependent on the molecular structure of the compound in question. It is assumed, as a first approximation, for many types of biologically active molecules there will be one key rate-controlling reaction at the active sites. This could be formulated as in eq. 1. A is the probability of a molecule reaching a site of action in a given time interval and C is the extracellular molar concentration of the compound being tested. The product AC represents the "effective" concentration at the sites of action. The constant k_X might be either an equilibrium or rate constant. It is assumed that a relatively large number of reaction sites are available so that these remain essentially constant during the test interval. In certain instances, it may be that new sites are being constantly generated. It also is considered that the many reactions which may occur subsequently to the one critical one (Steps III to n) before the visible response is elicited can be neglected for a first approximation. Thus the two parameters A and k_X will be the important determinates governing the relative effectiveness of the members of a given series of biologically active compounds. Our model, then, is a steady-state one in contrast to the equilibrium model of Ferguson.[4,5]

$$\text{rate of biological response} = \frac{\text{d(response)}}{\text{d}t} = ACk_X \quad (1)$$

(1) On leave from Kyoto University, Kyoto, Japan.
(2) C. Hansch, P. P. Maloney, T. Fujita, and R. M. Muir, *Nature*, **194**, 178 (1962).
(3) C. Hansch, R. M. Muir, T. Fujita, P. P. Maloney, C. F. Geiger, and M. J. Streich, *J. Am. Chem. Soc.*, **85**, 2817 (1963).
(4) A. Burger, "Medicinal Chemistry," 2nd Ed., Interscience Publishers, Inc., New York, N. Y., 1960, p. 49.
(5) J. Ferguson, *Proc. Roy. Soc.* (London), **127B**, 387 (1939).