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The Biogenesis of Nicotine. V. New Precursors of the Pyrrolidine Ring[1]

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Nicotiana tabacum plants were allowed to grow for varying lengths of time, up to 9 weeks, after feeding ornithine-2-C^{14} to the roots of the plant. The activity of the nicotine reached a maximum after 3 weeks and then slowly decreased. Glutamic acid-2-C^{14}, proline (uniformly labeled with C^{14}) and putrescine-1,4-C^{14} were fed to the tobacco plant, and radioactive nicotine was obtained in each case. Degradation of the nicotine from these experiments showed that all the activity was present in the pyrrolidine ring. Under similar conditions the incorporation of ornithine, putrescine, proline and glutamic acid into nicotine was 0.48, 0.12, 0.032 and 0.0078%, respectively. The significance of these results is discussed.

Introduction

We have shown previously[2] that ornithine-2-C^{14} is incorporated into nicotine, equal labeling occurring on C-2 and C-5. In order to investigate the metabolic turnover of nicotine in the living plant, we have extended earlier experiments[2] and allowed tobacco plants to grow for 3 days, 1, 3, 5, 7 and 9 weeks after adding ornithine-2-C^{14} to the inorganic nutrient solution in which the plants were growing. The total activity of the nicotine, the aqueous sap and the plant residue was determined in each case. The roots, stems and leaves of the plants allowed to grow for 9 weeks after feeding the ornithine were extracted separately. The nicotine obtained from the leaves of these plants was degraded systematically[2] to locate positions of activity.

The metabolic relationship between ornithine, glutamic acid and proline is well established in some microörganisms and animals.[3,4] This relationship has received little study in higher plants and it was of interest to see whether glutamic acid and proline would serve as precursors of the pyrrolidine ring of nicotine. The incorporation of putrescine (1,4-diaminobutane) also was investigated since this compound is a plausible intermediate between ornithine and nicotine. The radioactive compounds fed to the tobacco were *dl*-glutamic acid-2-C^{14},[5] uniformly labeled C^{14}-*l*-proline[6] and putrescine-1,4-C^{14} dihydrochloride.[7]

Experimental

Feeding of Ornithine-2-C^{14} to Tobacco Plants for Various Times.—The tobacco used in these experiments was *N. tabacum* var. Maryland Mammoth, a vegetative variety which does not flower if subjected to long days. The plants were grown as previously described,[8] except that they were exposed to light for 20 hr. each day. Eight 4-month old plants of approximately equal size (36″ high) were selected, and 66 mg. of ornithine-2-C^{14} hydrochloride having a total activity of 4.8×10^7 c.p.m.[9] was added to the nutrient solution of each of the plants. The roots rapidly absorbed the ornithine, and after 48 hr. the nutrient solutions had only 2% of their original activity. The plants were harvested in turn at the times indicated in Table I. The nicotine was extracted from the whole plant by shaking the macerated fresh plant with a mixture of chloroform and aqueous ammonia. The nicotine was isolated from the chloroform by established methods.[2,8] The dried plant residue and the ammoniacal aqueous sap were assayed for radioactivity. The results are recorded in Table I. Nine weeks after administration of the ornithine the lower leaves of the three remaining plants had become yellow. These old leaves were discarded and the roots, stems and healthy leaves of these plants were worked up separately. The nicotine obtained from the leaves of these plants was degraded as previously described,[2] the results being recorded in Table III.

TABLE I

Time of harvesting	Wt., mg.	Nicotine Specific activity $\times 10^{-6}$ c.p.m./m.	Nicotine Total activity $\times 10^{-6}$ *M*c.p.m.	Plant residue Wt., g.	Plant residue Activity $\times 10^{-6}$ c.p.m.	Aqueous sap Activity $\times 10^{-6}$ c.p.m.
3 d.	41	0.95	2.4	28	3.8	1.4
1 wk.	42	2.01	5.2	29	2.9	1.0
3 wk.	54	1.61	5.3	34	1.8	0.5
5 wk.	66	1.10	4.5	37	1.6	.4
7 wk.	70	1.07	4.6	40	1.9	.5
9 wk. (3 plants)						
leaves	104	0.57	..	79	1.1	.07
stems	54	.35	..	73	0.4	.17
roots	11	.13	..	30	4.2	1.5

Putrescine-1,4-C^{14} Dihydrochloride.—Potassium cyanide-C^{14} (0.65 g., 0.01 mole) with an activity of 1.15×10^9 c.p.m. was dissolved in a mixture of 3 ml. of ethanol and 1 ml. of water containing 0.05 g. of potassium iodide. 1,2-Dibromoethane (0.45 ml., 0.052 mole) was added, and the mixture was refluxed for 75 minutes. The reaction mixture was diluted with 50 ml. of water and extracted once with 150 ml. of ether. The aqueous solution was acidified with 20 ml. of 4 *N* sulfuric acid and extracted with chloroform in a continuous extractor for 24 hr. The chloroform solution was evaporated, and the residue of 1,2-dicyanoethane was dissolved in a mixture of 50 ml. of methanol and 5 ml. of concentrated hydrochloric acid and hydrogenated in the presence of 0.5 g. of Adams catalyst at 40 lb./sq. in. for 3 hr. The coagulated catalyst was filtered off and the solution was evaporated to dryness *in vacuo*. The residue was crystallized twice from a mixture of methanol and ether, yielding fine colorless needles of putrescine-1,4-C^{14} dihydrochloride (0.37 g., 45% yield) with an activity of 1.43×10^6 c.p.m./mg.

Feeding of Radioactive Putrescine, Proline and Glutamic Acid to the Tobacco Plants.—The plants used in these experiments were 3-month old *N. tabacum* var. Turkish Samsun. The uptake of the radioactive compounds was rapid in each case. With glutamic acid-2-C^{14} only 2.5% of the original activity remained in the nutrient solution 24 hr. after administration. Putrescine hydrochloride had a beneficial effect on root growth. The plants were

(1) Part IV: E. Leete, *Chemistry & Industry*, 1270 (1957). Presented in part at the 132nd meeting of the American Chemical Society, New York, N. Y., Sept., 1957. This work has been supported by a grant from the Research Corporation, New York.

(2) E. Leete and K. J. Siegfried, THIS JOURNAL, **79**, 4529 (1957).

(3) M. R. Stetten, p. 277, and H. J. Vogel, p. 335, in "Amino Acid Metabolism," Ed. by W. D. McElroy and H. B. Glass, Johns Hopkins Press, Baltimore, Md., 1955.

(4) H. J. Strecker, *J. Biol. Chem.*, **225**, 825 (1957).

(5) Purchased from Tracerlab, Inc., Waltham, Mass.

(6) Purchased from Schwarz Laboratories, Inc., Mt. Vernon, N. Y.

(7) Prepared from C^{14}-potassium cyanide in 45% yield by a modification of the method described by D. G. M. Diaper, S. Kirkwood and L. Marion, *Can. J. Chem.*, **29**, 964 (1951).

(8) E. Leete, THIS JOURNAL, **78**, 3520 (1956).

(9) All counts were carried out in a windowless flow G. M. counter (Nuclear-Chicago Co. Model D-46 A) using "Q gas" as the quencher. Determinations were carried out on the organic compounds making corrections for geometry and self absorption.

harvested after 10 days. The results of these experiments are summarized in Table II. The percentage incorporation

TABLE II

Tracer	Putrescine-1,4-C^{14}	*l*-Proline-uniform-C^{14}	*dl*-Glutamic acid-2-C^{14}
Amount, m*M*	0.75	0.75	0.75
Activity (c.p.m.)	1.73×10^8	8.03×10^7	1.67×10^8
Nicotine			
Wt., mg.	81	81	69
Specific activity (c.p.m./m*M*)	4.26×10^5	5.10×10^4	3.08×10^4
% Incorporation[10]	0.12	0.032	0.0078
Plant residue			
Wt., g.	51	40	45
Activity (c.p.m.)	6.7×10^8	4.5×10^8	5.7×10^8
Aqueous sap			
Activity (c.p.m.)	2.4×10^8	1.8×10^8	2.5×10^8

was calculated by dividing the total activity found in the pure nicotine by the total activity fed. The nicotine from these experiments was degraded by established methods.[8] In the present work the nicotinic acid, obtained by the nitric acid oxidation of nicotine, was decarboxylated by heating with calcium hydroxide[11] the evolved pyridine being absorbed in dilute hydrochloric acid. The pyridine hydrochloride obtained by the evaporation of this solution was converted to the picrate by the addition of methanolic picric acid. The results of the degradations are summarized in Table III.

allowed to grow for 9 weeks after feeding the ornithine-2-C^{14} indicated that the bulk of the radioactivity was located at C-2 and C-5 with little activity on other carbons and certainly none in the pyridine ring. Hence our remarks on the metabolic stability of nicotine in the healthy plant apply only to the C^{14}-labeled pyrrolidine ring. However, it seems improbable that the pyrrolidine–pyridine bond would be a labile one. The possibility that the N-methyl group of nicotine is in mobile equilibrium with methyl acceptors such as homocysteine is much more likely and is being investigated. The observed stability of nicotine in the plant is in accord with the work of Dawson[13] and Mothes[14] who found that nicotine would not serve as a nitrogen source for protein synthesis in the tobacco plant. It is of interest to note that Tso and Jeffrey[15] have reported on the disappearance of nicotine from *N. glutinosa*, presumably by conversion to nornicotine. In excised or fermented tobacco leaves the nicotine is metabolized to a variety of compounds.[16–19] The rather low specific activity of the nicotine from the leaves of the tobacco allowed to grow for 9 weeks after administration of the ornithine is possibly due to the fact that the old

TABLE III

DEGRADATION OF THE NICOTINE FROM VARIOUS SOURCES (ACTIVITIES IN C.P.M./m*M*)

Precursor	Ornithine-2-C^{14} (nicotine from "9 wk. leaves")	Putrescine-1,4-C^{14}	Proline uniform-C^{14}	Glutamic acid 2-C^{14}
Nicotine diperchlorate	5.73×10^5	4.26×10^5	5.1×10^4	3.0×10^4
Nicotine dipicrate	5.70×10^5	4.14×10^5	4.9×10^4	2.8×10^4
3-Nitro-5-(3′-pyridyl)-pyrazole	3.10×10^5	2.15×10^5	3.9×10^4	.....[12]
Nicotinic acid	2.88×10^5	2.01×10^5	1.4×10^4	1.5×10^4
Nicotinic acid hydrochloride	2.84×10^5	2.04×10^5	1.3×10^4	1.4×10^4
Pyridine picrate	0	0	0	0
Methyltriethylammonium iodide	0	0	.....[12]	.....[12]

Discussion

In all our work on the biogenesis of alkaloids we have allowed the roots of a plant to grow in an aqueous solution to which has been added a radioactive compound. In none of our experiments have we recovered more than 20% of the administered tracer from the whole plant. The organic compounds are, in general, rapidly absorbed by the roots and then are apparently oxidatively degraded at one or more sites in the plant, the radioactive carbon being expired as carbon dioxide. In the present work we found that only 11% of the activity remained in the tobacco plant 3 days after feeding the ornithine-2-C^{14}. However after 7 weeks, 6% of the activity still remained in the plant. Thus, although there is a rapid loss of much of the administered tracer, some of it is incorporated into compounds which undergo slow metabolic turnover. Nicotine is such a compound. The decrease in the total activity of the nicotine during 7 weeks was small and could be due in part to volatilization from the leaves. Degradation of the nicotine obtained from the leaves of plants yellow leaves were not included in the extraction. The difference in the specific activity of the nicotine, obtained from the roots, stems and leaves in this 9 week experiment, is consistent with the findings of Dawson[20] who considers that the roots are the main site of nicotine synthesis. Thus in our experiments there will be an initial rapid synthesis of radioactive nicotine from the active ornithine. The bulk of this radioactive nicotine will be translocated to the leaves and replaced in the roots by nicotine synthesized from inactive precursors, resulting in the lower specific activity of the nicotine from the roots.

The results recorded in Tables II and III indicate that proline, glutamic acid and putrescine are all precursors of the pyrrolidine ring of nicotine, putrescine being the most efficient and glutamic acid the least. The nicotine obtained after feeding putrescine was labeled equally at C-2 and C-5.

(10) Under almost identical conditions[8] (7 days contact with the radioactive compound instead of 10) the incorporation of *dl*-ornithine-2-C^{14} was 0.48%.

(11) H. Weidel, *Ann.*, **165**, 328 (1873).

(12) The nicotine obtained after feeding glutamic acid and proline to the tobacco plant was not sufficiently active to enable us to carry out a complete degradation.

(13) R. F. Dawson, *Am. J. Bot.*, **27**, 190 (1940).

(14) K. Mothes, *Planta*, **5**, 563 (1928).

(15) T. C. Tso and R. N. Jeffrey, *Plant Physiol.*, **31**, 433 (1956).

(16) T. C. Tso and R. N. Jeffrey, *Arch. Biochem. Biophys.*, **43**, 269 (1953).

(17) W. G. Frankenburg, A. M. Gottscho, E. W. Mayaud and T. C. Tso, THIS JOURNAL, **74**, 4309 (1952).

(18) W. G. Frankenburg, A. M. Gottscho, A. A. Vaitekunas and R. M. Zacharius, *ibid.*, **77**, 5730 (1955).

(19) W. G. Frankenburg and A. A. Vaitekunas, *ibid.*, **79**, 149 (1957).

(20) R. F. Dawson, *Advances in Enzymol.*, **8**, 203 (1948).

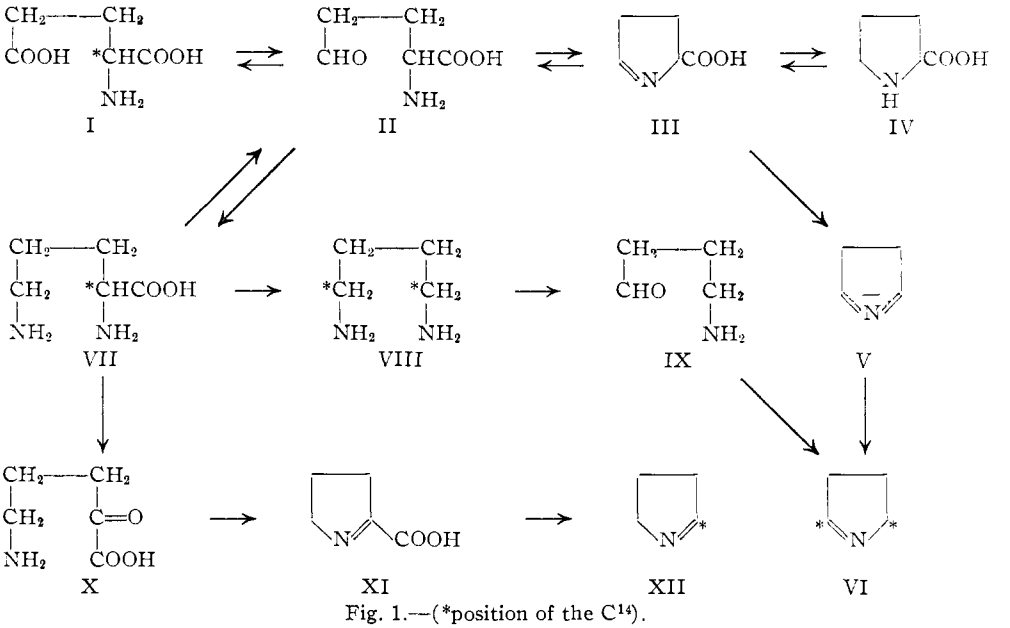

Fig. 1.—(*position of the C^{14}).

The activities of the degradation products from the nicotine produced after feeding the proline were those expected from nicotine labeled equally at C-2, 3, 4 and 5. The nicotine obtained after feeding the glutamic acid 2-C^{14} had half its activity at C-2, and it seems probable that the other half is located at C-5. It has been shown[21] that nicotinic acid is a precursor of the pyridine ring of nicotine, and as a working hypothesis we consider that the carbon skeleton of nicotine is formed by a condensation between reduced diphosphopyridine nucleotide and Δ^1-pyrroline. The metabolic routes whereby Δ^1-pyrroline can arise from glutamic acid (I), proline (IV), ornithine (VII) and putrescine (VIII) are shown in Fig. 1. The metabolic pathways connecting ornithine, glutamic acid and proline are those which probably exist in animals and microörganisms.[3,4] One route whereby glutamic acid and ornithine-2-C^{14} could yield Δ^1-pyrroline, labeled equally at C-2 and C-5, is *via* glutamic-γ-aldehyde (II) and Δ^1-pyrroline-5-carboxylic acid (III). Decarboxylation of III *via* the mesomeric anion V yields the pyrroline (VI). Another route to VI is *via* putrescine and 4-aminobutanal (IX).[22] A third route to Δ^1-pyrroline is *via* α-keto-δ-aminovaleric acid (X). Ring closure yields Δ^1-pyrroline-2-carboxylic acid (XI). However, the decarboxylation of XI does not yield a mesomeric anion and Δ^1-pyrroline-2-C^{14} (XII) would be obtained. This metabolic route would seem to be ruled out. It is rather strange that the higher homolog of ornithine, namely, lysine, apparently is metabolized by this latter route.[23,24] At this time it is not possible to decide between the first two metabolic routes. Although putrescine was a less efficient precursor than ornithine in our experiments, this does not eliminate it as an intermediate between ornithine and nicotine. There are unknown factors such as cell permeability which could favor the transport of ornithine to the site of nicotine synthesis. The significantly lower incorporation of glutamic acid could be due to preferential oxidation to α-ketoglutaric acid rather than reduction to II. Dilution with inactive glutamic acid, which is abundant in *N. tabacum*,[25,26] may be a factor contributing to the low incorporation. The incorporation of proline was intermediate between glutamic acid and putrescine, and it seems probable that proline is related to ornithine and glutamic acid by the same metabolic routes as exist in microörganisms and animals.

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(21) R. F. Dawson, D. R. Christman, R. C. Anderson, M. L. Solt, A. F. D'Adamo and U. Weiss, THIS JOURNAL, **78**, 2645 (1956).

(22) P. J. G. Mann and W. R. Smithies, *Biochem. J.*, **61**, 89 (1955), showed that the oxidation of putrescine catalyzed by a plant amine oxidase resulted in the formation of Δ^1-pyrroline

(23) The formation of pipecolic acid from lysine has been shown to proceed *via* α-keto-ϵ-aminocaproic; *cf.* R. S. Schweet, J. T. Holden and P. H. Lowy, ref. 3, p. 496, and A. Meister and S. D. Buckley, *Biochim. Biophys. Acta*, **23**, 202 (1957).

(24) Lysine-2-C^{14} was incorporated into anabasine (2-(3-pyridyl)-piperidine) labeled only at C-2 of the piperidine ring.[8]

(25) E. A. H. Roberts and D. J. Wood, *Arch. Biochem. Biophys.*, **33**, 299 (1951).

(26) H. Michl and H. Kuhn, *Fachliche Mitt. österr. Tabakregie*, **1**, 10 (1954); *C. A.*, **49**, 4947 (1955).