removed by filtration and the filtrate was again stirred 12 hours with 7.0 g. of mercuric oxide. After filtration the acetone was distilled under reduced pressure and the residue was purified by distillation at 80° (0.05 mm.), yield 3.5 g. (76%).

Anal. Calcd. for $C_9H_{17}N_3O$: C, 58.98; H, 9.35; N, 22.93. Found: C, 59.11; H, 9.34; N, 23.10.

1-Ethyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide Metho-*p*-toluenesulfonate (III).—A mixture of 2.0 g. (0.011 mole) of methyl *p*-toluenesulfonate and 2.0 g. (0.11 mole) of carbodiimide II was heated on a steam-bath for 10 minutes. On trituration with benzene the mixture crystallized; 3.6 g. (90%), m.p. 86–88°. Recrystallization from an acetone–benzene mixture raised the m.p. to 91–93°.

Anal. Calcd. for $C_{17}H_{27}N_3O_4S$: C, 55.27; H, 7.37; N, 11.38. Found: C, 55.41; H, 7.50; N, 11.50.

Reaction of Gelatin[7] with an Excess of 1-Ethyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide Metho-*p*-toluenesulfonate (III). Experiment 1.—To a solution of 2.0 g. of gelatin[7] in 25.0 ml. of water was added 1.5 g. of III. The solution gelled almost immediately (30 seconds), but liquefied on storage at room temperature for 16 hours. The resulting solution was dialyzed (using $^8/_{32}$″ cellulose casing) for 4 days. The solution was freeze-dried to a white powder; weight 2.0 g. A Sorensen titration[5] on 0.4 g. required 0.25 ml. of 0.103 *N* sodium hydroxide. An untreated gelatin sample (0.4 g.) required 2.6 ml. A Van Slyke analysis yielded 0.19% nitrogen, while untreated gelatin yielded 0.64% nitrogen.

Experiment 2.—A solution of gelatin in 50.0 ml. of water was gelled with 2.25 g. of carbodiimide III. The gel was immediately freeze-dried, followed by trituration with 500 ml. of water. The resulting material after drying weighed 3.0 g. The wash water on evaporation yielded 2.3 g. of urea IV.

A 0.5-g. sample of the above-treated gelatin was dissolved in 20 ml. of a 50% calcium chloride solution by heating on a steam-bath for 1 hour. A Sorensen titration[5] required 0.65 ml. of 0.103 *N* sodium hydroxide.

When 0.1 g. of the gelatin prepared above was suspended in 2.0 ml. of water containing 75 mg. of urea IV, no liquefaction occurred even after 9 days. If, however, carbodiimide was substituted for IV, liquefaction occurred.

When conductometric amino titrations[8] were carried out on the gelatins prepared in both runs 1 and 2, a decrease in amino content as compared to untreated gelatin was observed.

Experiment 3.—A solution of 0.2 g. of gelatin, 0.45 g. of urea and 0.15 g. of carbodiimide III in 4.0 ml. of water gelled after 20 seconds. On storage overnight the reaction mixture liquefied.

Reaction of Carbodiimide III and Benzoyl Gelatin.[4]—To a solution of 0.1 g. of benzoyl gelatin[4] in 2 ml. of water was added 0.1 g. of III. On stirring no sudden gelation occurred as with the control gelatin.

(7) The gelatin was obtained from the Atlantic Gelatin Co., Division of General Foods, Lot #6200-X.

(8) S. Ellis and J. Parkhurst, *Biochem. J.*, **52**, 350 (1952).

CAMBRIDGE, MASSACHUSETTS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, LOS ANGELES]

The Biogenesis of Nicotine. III. Further Observations on the Incorporation of Ornithine into the Pyrrolidine Ring[1]

BY EDWARD LEETE AND KENNETH J. SIEGFRIED

RECEIVED MARCH 6, 1957

Radioactive nicotine was isolated from two groups of *Nicotiana tabacum* plants, which were harvested one and three weeks after the administration of equal counts of ornithine-2-C^{14}. The nicotine from the two experiments had almost the same specific activity, indicating that little or no metabolic breakdown of nicotine had occurred during the last two weeks of the second experiment. The nicotine from both experiments was degraded unambiguously and all the activity in the alkaloid was shown to be equally divided between the two α-carbons of the pyrrolidine ring.

Introduction

It has been shown that radioactive nicotine is obtained when ornithine-2-C^{14} is fed intact *N. tabacum*[1a] or *N. rustica*[2] plants, or to sterile roots[3] of the former species. Degradation of the radioactive nicotine[1a,2] indicated that half the activity was located on C-2 of the nicotine molecule I. This result could be explained by postulating that ornithine was metabolized to a symmetrical compound before incorporation into nicotine. In the biogenetic scheme suggested by the author,[1b] the mesomeric anion of Δ^1-pyrroline functioned as the symmetrical intermediate. Putrescine (1,4-diaminobutane) is another plausible intermediate, which would result from ornithine on decarboxylation. However the feeding of radioactive putrescine to *Datura stramonium* plants did not yield radioactive hyoscyamine,[4] whereas radioactive ornithine was incorporated into the pyrrolidine ring of the hyoscyamine.[5] Furthermore in other biological systems the pyrrolidine ring has been shown to arise from ornithine *via* glutamic-γ-aldehyde, putrescine not being an intermediate.[6]

It was considered of interest to investigate changes in the activity of the nicotine after feeding ornithine-2-C^{14} for varying lengths of time. In the present work radioactive ornithine was administered to two groups of *N. tabacum* plants growing in inorganic nutrient solution. The first group was harvested after 7 days and the second after 21 days. The degradative steps which were used to locate the positions of activity in the radioactive nicotine are shown in Fig. 1. Treatment of nicotine with hydrogen iodide yielded methyl iodide which was absorbed in triethylamine affording methyltriethylammonium iodide (II). Oxidation of nicotine with concentrated nitric acid yielded

(1) Parts I and II of this series are considered to be: (a) E. Leete, *Chemistry & Industry*, 537 (1955), and (b) E. Leete, THIS JOURNAL, **78**, 3520 (1956), respectively. This work has been supported in part by a grant from the Research Corporation, New York.

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

(3) R. F. Dawson, private communication.

(4) D. G. M. Diaper, S. Kirkwood and L. Marion, *Can. J. Chem.*, **29**, 964 (1951).

(5) (a) E. Leete, L. Marion and I. D. Spenser, *ibid.*, **32**, 1116 (1954); (b) *Nature*, **174**, 650 (1954).

(6) M. R. Stetten in "Amino Acid Metabolism," edited by W. D. McElroy and H. B. Glass, John Hopkins Press, Baltimore, Md., 1955, pp. 277–290.

nicotinic acid (III) and 3-nitro-5-(3′-pyridyl)-pyrazole (V). This pyrazole derivative was first isolated by Gough and King[7] who suggested that it was 4-nitro-5-(3′-pyridyl)-pyrazole. The assigned position of the nitro group was shown to be incorrect by Lund[8] and shown to be actually on the 3-position by Clemo and Holmes.[9] The mechanism of the formation of the pyrazole is being investigated. It is only obtained in about 5% yield from nicotine, but it is separated readily from nicotinic acid by adjustment of the *p*H of the oxidation mixture. The pyrazole derivative contains all the carbons of nicotine except C-5 and the N-methyl group. The nicotinic acid was decarboxylated in boiling quinoline, the evolved carbon dioxide being absorbed in barium hydroxide.

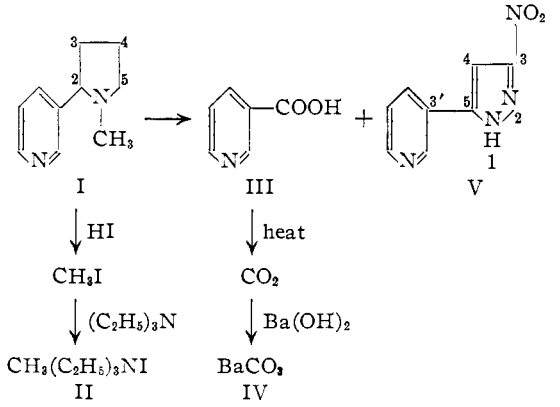

Fig. 1.—Degradation of radioactive nicotine.

Experimental[10]

Administration of the Ornithine-2-C^{14} to the Tobacco Plants and Isolation of the Nicotine.—The *N. tabacum* (variety Turkish Samsun) was grown as previously described[1b] in soil and then in inorganic nutrient. When they were about 4 months old, eight healthy plants (about 30 inches tall) were selected and ornithine-2-C^{14} monohydrochloride[11] (490 mg.) with an activity of 7.56×10^7 c.p.m./m*M*[12] (total activity, 2.22×10^8) was divided equally between their nutrient solutions. After 7 days four of the plants were harvested. The method of isolation of the nicotine was identical with that used for the isolation of anabasine from *N. glauca*,[1b] and was less tedious than the method previously used. In brief, the method involved digestion of the fresh macerated plant with chloroform and aqueous ammonia. The chloroform was extracted with 2 *N* sulfuric acid, which was then made basic with ammonia and again extracted with chloroform yielding crude nicotine after drying and evaporation. The crude alkaloid[13] was distilled *in vacuo* and converted to the diperchlorate. After 21 days the other four plants were harvested and the nicotine similarly isolated. Activity and weights of plant material from the two experiments are shown in Table I.

TABLE I

TOTAL ACTIVITIES IN C.P.M.

	7 day	21 day
Fresh wt. of plants at time of harvesting, g.	810	860
Dried wt. of plant after extraction with $CHCl_3$ and NH_3, g.	41	51
Wt. of distilled nicotine, mg.	96	115
Activity of:		
Chloroform extract	1.0×10^6	1.1×10^6
Ammoniacal sap	1.5×10^6	0.8×10^6
Dried residue[14]	7.1×10^5	4.2×10^6
Crude nicotine (not distilled)	0.6×10^6	0.7×10^6

Degradation of the Nicotine. (a) Activity in the N-Methyl Group.—About 70 mg. of the undiluted nicotine diperchlorate was dissolved in water, made alkaline with sodium hydroxide and the solution extracted with ether. The dried ether solution water evaporated and the residue distilled *in vacuo* (120° (0.01 mm.)). The distilled nicotine was demethylated according to the procedure of Brown and Byerrum.[15]

(b) **Oxidation with Nitric Acid.**—The active nicotine diperchlorate (100 mg.) was diluted with inactive perchlorate (1.900 g.) and recrystallized from ethanol. Diluted nicotine diperchlorate (1.50 g.) was dissolved in 20 ml. of concentrated nitric acid and heated at 100° for 18 hours. The solution was then evaporated to dryness *in vacuo* and the residue redissolved in 10 ml. of water. The pale yellow solution was brought to *p*H 5 by the addition of 10% sodium hydroxide. At this *p*H a yellow crystalline precipitate of 3-nitro-5-(3′-pyridyl)-pyrazole (V) (32.6 mg., 4.1%) separated out. It was sublimed (200° (0.001 mm.)) and recrystallized from glacial acetic yielding long colorless needles, m.p. 277–278°.

Anal. Calcd. for $C_8H_6N_4O_2$: C, 50.53; H, 3.18. Found: C, 50.76; H, 2.98.

The filtrate obtained after removal of the pyrazole derivative contained nicotinic acid which was isolated as its cupric salt and degraded as previously described.[1b] The activities of nicotine and its degradation products from the two feeding experiments are shown in Table II. The activities are for carrier free material.

TABLE II

	Specific activities (c.p.m./m*M*) 7 day expt.	21 day expt.
Ornithine-2-C^{14} fed	7.56×10^7	7.56×10^7
Nicotine diperchlorate	9.0×10^5	8.4×10^5
Nicotine dipicrate	8.9×10^5	8.2×10^5
3-Nitro-5-(3′-pyridyl)-pyrazole (V)	4.6×10^5	4.4×10^5
Nicotinic acid (III)	4.3×10^5	4.1×10^5
Nicotinic acid hydrochloride	4.4×10^5	4.0×10^5
Methyltriethylammonium iodide (II)	0	0
Barium carbonate (IV)	4.2×10^5	3.9×10^5

Discussion

An examination of Table I shows that the loss of activity from the plants was surprisingly large. Thus after 7 days only 9% (9.6×10^6 c.p.m.) of the radioactive carbon administered in the form of ornithine remained in the plant. After 21 days the activity in the plant had been reduced to 5% (6.1×10^6). The radioactive carbon was presumably ex-

(7) G. A. C. Gough and H. King, *J. Chem. Soc.*, 2968 (1931).

(8) H. Lund, *ibid.*, 686 (1933).

(9) G. R. Clemo and T. Holmes, *ibid.*, 1739 (1934).

(10) All melting points are corrected and analyses were performed by Miss Heather King of this department.

(11) The ornithine-2-C^{14} was synthesized from ethyl acetamidocyanoacetate-2-C^{14} (purchased from Tracerlab, Inc., Boston, Mass.) by the method of M. Fields, D. E. Walz and S. Rothchild, THIS JOURNAL, **73**, 1000 (1951).

(12) All counts were determined using a windowless G. M. counter (Nuclear Instrument and Chemical Co. Model D-46-A) using "Q gas" as the quenching gas. Determinations of activity were carried out in duplicate, making corrections for the geometry of the instrument and self absorption. The preparation of the samples was standardized and results reproducible to 5% were obtained.

(13) Paper chromatography of the crude alkaloid did not indicate the presence of any alkaloids other than nicotine.

(14) Activity in the residual plant material was determined by the method of D. D. Van Slyke and J. Folch, *J. Biol. Chem.*, **136**, 509 (1940).

(15) S. S. Brown and R. U. Byerrum, THIS JOURNAL, **74**, 1523 (1952).

pired as carbon dioxide since the activity of the nutrient solution was negligible. Although the total activity of the plants in the 21 day experiment was less than in the 7 day experiment, the activity of the crude alkaloid fraction had increased slightly.

It is considered that there was an initial rapid uptake of radioactive ornithine which was incorporated into proteins and a certain fraction into nicotine. Inactive ornithine was then synthesized by normal routes from inactive precursors. This inactive ornithine apparently exchanged with combined radioactive ornithine in proteins or other non-alkaloid plant components reducing the activity in the residual plant material. Some of the released radioactive ornithine would be incorporated into nicotine and some would be oxidized to carbon dioxide which on expiration would reduce the over-all activity in the plant. Since the nicotine fraction had a slightly higher activity after 21 days it appears that nicotine does not re-enter the general metabolic pool of the plant once it has been formed. Work is proceeding to see whether the nicotine remains indefinitely in the plant without undergoing metabolic change.

The activities of nicotine and its degradation products in the two experiments are almost identical and they can be discussed together. No activity was found in the N-methyl group. This result is in agreement with that previously obtained.[1a] Dewey, *et al.*,[2] detected a small amount of activity in the N-methyl group after feeding ornithine-2-C^{14} to tobacco plants. The plants used by these workers were younger than ours and possibly had not as large a photosynthetic capacity. Thus the plants were possibly deficient in carbon and would utilize some of the breakdown products of ornithine for the synthesis of N-methyl groups, albeit inefficiently. Our plants were grown in a strong draught, expired radioactive carbon dioxide being rapidly swept away. It has been reported[16] that excised tobacco leaves utilize carbon dioxide for the synthesis of the N-methyl group of nicotine.

The activity of the barium carbonate represents the activity of C-2 of nicotine. Since the activity of the nicotinic acid and its hydrochloride are almost identical with this, there must be negligible activity in the pyridine ring. Since no activity was found in the N-methyl group, the difference in the activity of the nicotine and the pyrazole (V) represents the activity of C-5. The difference in the activity of the pyrazole and nicotinic acid represents the activity of C-3 and C-4. This is negligible, thus all the activity in the nicotine is located on C-2 and C-5 and is equally divided between them. This confirms our previous hypothesis[1a] that ornithine is metabolized to a symmetrical four carbon compound before incorporation into the nicotine molecule.

Acknowledgment.—The authors are indebted to Dr. S. G. Wildman and Dr. A. Lang of the Department of Botany of this University for help in the cultivation of the tobacco plants.

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

LOS ANGELES 24, CAL.

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE]

The Veratrum Alkaloids. XLI.[1] The Position of the Second Hydroxyl in Rubijervine and the Identity of Certain Dehydrogenation Products

BY S. WILLIAM PELLETIER AND DAVID M. LOCKE

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The second hydroxyl of rubijervine is shown to be at position 12 by infrared and optical rotatory dispersion studies on rubijervone-12. Reduction of this ketone with sodium-in-propanol gives 12-epirubijervine (IIIa), showing that the 12-hydroxyl in rubijervine (Ia) is axial. Molecular rotation data for rubijervine and 12-epirubijervine derivatives are also in agreement with assignment of the 12α- and 12β-configurations, respectively, to these compounds. The hydrocarbon $C_{18}H_{16}$ from the dehydrogenation of rubijervine is shown to be 1′-methyl-1,2-cyclopentenophenanthrene (VIII). The phenol $C_{18}H_{16}O$ obtained in the same dehydrogenation has been converted to 1′-methyl-1,2-cyclopentenophenanthrene (VIII) by reduction of its diethyl phosphate ester with sodium in liquid ammonia. Its infrared spectrum leads to the conclusion that it is 1′-methyl-1,2-cyclopentenophenanthrol-3 (X).

The various species of *Veratrum* elaborate a series of complex alkaloids, some of which have found use in combatting hypertensive disorders. The alkaloids fall into two broad classes.[2,3] The first includes a series of highly oxygenated alkamines of the formula $C_{27}H_{43}NO_{7-9}$, which occur in nature as esters of relatively simple organic acids. The second comprises a group of C_{27}-bases of low oxygen content which occur free or as glycosides. Rubijervine,[3,4] an example of the latter class, was first discovered by Wright and Luff[5] in *Veratrum album*[6] and has since been found in *Veratrum viride*.[7,8] Studies by Jacobs and Craig showed this alkaloid to have the molecular formula $C_{27}H_{43}NO_2$[9] and to be a hexacyclic tertiary steroidal base containing a 3β-hydroxy-Δ^5-stenol system and a second hydroxy

(1) A preliminary account of a portion of this work is outlined in Paper XL, D. M. Locke and S. W. Pelletier, *Chemistry & Industry*, 1049 (1956).

(2) V. Prelog and O. Jeger, in 'The Alkaloids, Chemistry and Physiology," edited by R. H. F. Manske and H. L. Holmes, Vol. III, Academic Press, Inc., New York, N. Y., 1953, p. 270.

(3) J. McKenna, *Quart. Revs.*, **7**, 231 (1953).

(4) Reference 2, pp. 277–280.

(5) C. R. A. Wright and A. P. Luff, *J. Chem. Soc.*, **35**, 405, 421 (1879).

(6) G. Salzberger, *Arch. Pharm.*, **228**, 462 (1890).

(7) E. J. Seiferle, I. B. Johns and C. H. Richardson, *J. Econ. Entomol.*, **35**, 35 (1942).

(8) W. A. Jacobs and L. C. Craig, *J. Biol. Chem.*, **160**, 555 (1945).

(9) W. A. Jacobs and L. C. Craig, *ibid.*, **148**, 41 (1943).