Usual chemical procedures[17] employ acylation of hydrazides, diacylation of hydrazine, thermal decomposition of hydrazides and oxidation of hydrazides with iodine. Among the uses of diacylhydrazines have been investigations of dipole moments[18] and apparent energies of N–N bonds.[19] Still another interesting usage of an acylated hydrazine has been the oxidation of N^{α},N^{α}-diphenyl-N^{β}-picrylhydrazine, which forms N^{α},N^{α}-diphenyl-N^{β}-picrylhydrazyl free radicals rather than the anticipated $N^{\alpha},N^{\alpha},N^{\delta},N^{\delta}$-tetraphenyl-$N^{\beta},N^{\gamma}$-dipicryltetrazane. This purple sold free radical is employed as a standard of measurement for magnetic moments.[20,21] Acylation of N^{α},N^{α}-diphenylhydrazine with picryl chloride yields the starting material for the synthesis of this free radical.

Acknowledgments.—Appreciation is expressed for grants-in-aid from the Society of the Sigma Xi and RESA, which took care of costs of some of the analyses and important chemicals. The Rudolph high-precision polarimeter was purchased through a grant-in-aid from the Research Corporation. Generous grants from the Fresno County Heart Association and the California Heart Association provided for an undergraduate research fellowship for Mr. Marvin Kientz, the purchase of equipment and chemicals, the assistance of Messrs. Ronald and Rodney Johnson in synthesizing a few important intermediates and the expense of many of the analyses. Professor John J. Sampson of the University of California Medical School in San Francisco and Chairman of the Research Committee of the California Heart Association and Dr. Robert Maybury of the University of Redlands and Chairman of the Undergraduate Research Sub-committee of the California Heart Association gave invaluable time and assistance with regard to securing this financial aid. Dr. Robert D. Beech, Chairman of the Research Committee of the Fresno County Heart Association, worked patiently in fostering the financial aid provided. Mrs. Joyce Richardson, Executive Director of the Fresno County Heart Association, and Miss Phillis Hecker, Program Consultant of the California Heart Association, provided much help in enabling the grants to be secured. Appropriate systems of nomenclature were outlined by Professor Charles D. Hurd of Northwestern University. Professor Carl Niemann of the California Institute of Technology read through the major portion of the research data and made important comments before the final organization into this paper. Professor Ennis B. Womack, Chairman of the Chemistry Department of Fresno State College, gave valuable assistance in providing certain items of equipment and space for carrying out the project. Student help, other than that of the major research workers, was given by Mr. Calvin Johnson, Mr. Jerome Blank and Mr. Leon Yengoyan. Most of the papain used in this project was supplied generously by the Wallerstein Laboratories of New York City. Some of the papain had been donated previously by the Schwarz Laboratories of Mount Vernon, N. Y. Nitrogen analyses were all performed by the Schwarzkopf Microanalytical Laboratory of Woodside, N. Y.

FRESNO 26, CALIF.

(17)(a) T. W. J. Taylor and W. Baker, "Sidgwick's Organic Chemistry of Nitrogen," Oxford University Press, London, 1937, p. 398; (b) C. D. Hurd, in H. Gilman, "Organic Chemistry, An Advanced Treatise," Vol. 1, first edition, John Wiley and Sons, Inc., New York, 1938, p. 682.

(18) P. R. Frey and E. C. Gilbert, THIS JOURNAL, **59**, 1345 (1937).

(19) C. M. Anderson and E. C. Gilbert, *ibid.*, **64**, 2369 (1942).

(20) E. Muller, L. Muller-Rodloff and W. Bunge, *Ann.*, **520**, 235 (1935).

(21) F. L. Allen and S. Sugden, *J. Chem. Soc.*, 440 (1936).

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA]

The Biogenesis of Morphine[1]

BY EDWARD LEETE

RECEIVED JANUARY 5, 1959

Radioactive morphine was obtained when DL-phenylalanine-2-C^{14} or DL-tyrosine-2-C^{14} was fed to *Papaver somniferum* plants. Systematic degradation of the morphine derived from the tyrosine yielded compounds whose activities were compatible with the hypothesis that morphine is formed from two molecules of tyrosine *via* norlaudanosine.

In 1925,[2] Gulland and Robinson suggested that the morphine skeleton is formed in the plant by the cyclization of norlaudanosine (III). The norlaudanosine is produced by a Mannich reaction, in which decarboxylation takes place between 3,4-dihydroxyphenylalanine (I) and 3,4-dihydroxyphenylacetaldehyde (II) which arises by the oxidative decarboxylation of a second molecule of I. Rotation of ring A of III through 180° gives rise to the equivalent structure IV. It is then immediately apparent that two cyclizations and reduction and dehydration of ring A will give rise to the morphine skeleton. This final conversion of IV to morphine (V) has been the subject of considerable discussion.[3] However, it seemed desirable to check the basic biogenetic scheme before becoming too excited about intimate details of this hypothesis. If this scheme is correct, the feeding of 3,4-dihydroxyphenylalanine-2-C^{14} (I) to opium poppies should result in the labeling of morphine on C-9 and C-16 as indicated in Fig. 1.

(1) A preliminary account of part of this work has appeared as a communication: E. Leete, *Chemistry & Industry*, 977 (1958). This investigation was supported by research grants M-2075 and M-2662, from the National Institute of Mental Health, Public Health Service.

(2) J. M. Gulland and R. Robinson, *Mem. Proc. Manchester Lit. and Phil. Soc.*, **69**, 79 (1925).

(3) R. Robinson, "The Structural Relations of Natural Products," Clarendon Press, Oxford, 1955, p. 82; C. Schöpf, *Naturwissenschaften*, **39**, 241 (1952); K. W. Bentley, *Experientia*, **12**, 251 (1956); T. Cohen, *Chemistry & Industry*, 1391 (1956).

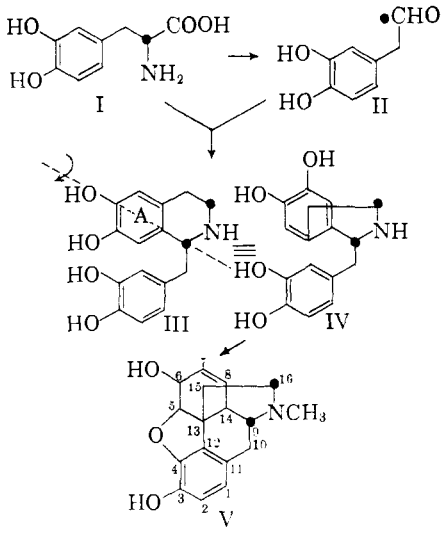


Fig. 1.—● position of the C^{14} label.

Radioactive dihydroxyphenylalanine was not readily available so we used phenylalanine-2-C^{14} and tyrosine-2-C^{14} in our feeding experiments since it is well established that phenylalanine is a precursor of tyrosine[4] which, in turn, is a precursor of dihydroxyphenylalanine.[5] In our original experiments[1] the tracers were administered at one time, by addition to the inorganic nutrient solution in which the roots of the opium poppies were growing. The morphine was isolated from the whole plants, without dilution, after two weeks. In a later experiment, a larger amount of tyrosine-2-C^{14} was fed to the roots during a period of three weeks and the morphine isolated at the end of the fourth week. This method of administration to the plant did not appreciably increase the percentage incorporation of tracer into the alkaloid. Phenylalanine was not as efficient a precursor of morphine as tyrosine and this presumably indicates that the phenylalanine must be converted to tyrosine before incorporation into morphine.

The radioactive morphine obtained from the second tyrosine feeding experiment (expt. 3, Table I) was degraded[6] by the sequence shown in Fig. 2. The first step was the conversion of the morphine to codeine methiodide (VI) by dissolving the morphine in ethanolic sodium ethoxide and the refluxing with methyl iodide.[7] The Hofmann elimination occurred readily on refluxing the codeine methiodide with aqueous sodium hydroxide giving a good yield of α-methylmorphimethine (VII).[7] Initial attempts to convert this methine base to 4-acetoxy-3-methoxyphenanthrene (acetylmethylmorphol) (VIII) by refluxing with acetic anhydride gave poor yields.[8] However, the yield was improved by carrying out the reaction in a sealed tube at 180° in the presence of a trace of hydrochloric acid. The trace of mineral acid may well have facilitated

(4) J. Massicot and L. Marion, *Can J. Chem.*, **35**, 1 (1957).

(5) A. Meister, "Biochemistry of the Amino Acids," Academic Press, Inc., New York, N. Y., 1957, p. 354.

(6) In the preliminary work[1] the low yield of VIII did not permit us to proceed beyond this compound in the degradation.

(7) L. Knorr, *Ber.*, **27**, 1144 (1894).

(8) O. Fischer and E. v. Gerichten, *Ber.*, **19**, 792 (1886).

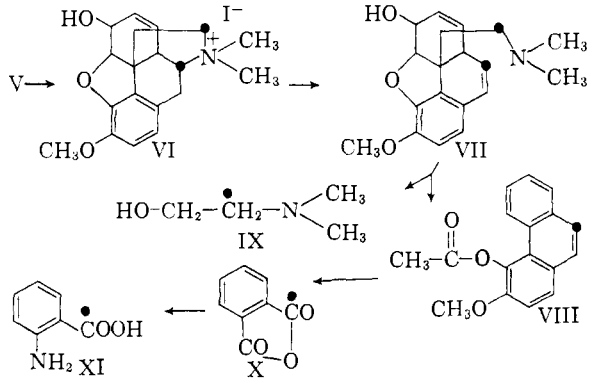


Fig. 2.—Degradation of the radioactive morphine; ●, position of the C^{14}-label.

the production of acylium ions which Stork[9] consideres to be the initial attacking species in this interesting elimination of the ethanamine side chain. The non-aromatic fragment of this reaction is O-acetyl-N,N-dimethyl-β-ethanolamine. This was not isolated as such, but was hydrolyzed by refluxing with sodium hydroxide to give N,N-dimethyl-β-ethanolamine which was isolated as the aurichloride. The morphine, codeine methiodide and the methine base all had essentially the same specific activity indicating that the morphine was free of any radioactive contaminants. In agreement with previous results[1] the phenanthrene derivative VIII had half the specific activity of the morphine. The other half was found in the ethanolamine derivative IX. It was not necessary to proceed any further with the degradation of IX since Battersby and Harper,[10] in independent work, showed that tyrosine-2-C^{14} yielded morphine which had half its activity at C-16. They also showed that the N-methyl group[11] was inactive. We can thus conclude that C-15 was also inactive. The phenanthrene derivative was hydrolyzed with sodium hydroxide and then oxidized with potassium permanganate to give phthalic acid which was sublimed yielding phthalic anhydride (X), which had the same specific activity as VIII. Thus C-1,2,3,4,10 and 11 of the morphine were inactive. Attempts to decarboxylate phthalic acid in boiling quinoline in the presence of copper chromite catalyst were unsuccessful, presumably because of the ready formation of phthalic anhydride. The Schmidt reaction on the active phthalic anhydride at 110° gave anthranilic acid (XI)[12] as the sole organic product. This anthranilic acid had half the specific activity of the phthalic anhydride. This result indicated that all the activity of the phthalic anhydride was located on one or both carbonyl groups. If any activity had resided in the benzene ring we would have expected the anthranilic acid to have more than half the specific activity of the phthalic anhydride. There were thus three possibilities for the distri-

(9) G. Stork, "The Alkaloids," Vol. II, ed. by R. H. F. Manske and H. L. Holmes, Academic Press, Inc., New York, N. Y., 1952, p. 189.

(10) A. R. Battersby and B. J. T. Harper, *Chemistry & Industry*, 364 (1958).

(11) Methionine has been shown to be a precursor of the N-methyl group of morphine: A. R. Battersby and B. J. T. Harper, *ibid.*, 365 (1958).

(12) G. Caronna, *Gazz. chim. ital.*, **71**, 189 (1941).

bution of activity in the phenanthrene skeleton of the morphine: (a) all the activity was at C-9, (b) all the activity was at C-12 or (c) the activity was distributed in any ratio between C-9 and C-12. Since it is impossible to conceive of any biogenetic scheme whereby tyrosine-2-C^{14} would lead to morphine labeled specifically at C-12, we feel confident in eliminating possibilities (b) and (c).[13] Thus our work, together with that of Battersby and Harper, has proved the 34-year old hypothesis of Sir Robert Robinson.

Experimental

Cultivation of the Opium Poppies[14] and Administration of the Tracers.—Yuma poppy, *Papaver somniferum* L. var. *alba* DC, U.S.D.A. No. 17, was used in all our experiments. The seeds[15] were germinated in vermiculite and successfully transplanted into soil when they were about 3 cm. tall. The plants were grown under a combination of tubular fluorescent lamps and sunlight. It was essential to subject the plants to several "long days" (about 18 hours each) to induce flowering of the poppies. About three months after germination the plants began to produce flower buds. Selected plants were then transferred to a hydroponics set up, where the roots were immersed in an aerated nutrient solution of the same composition as that used in our work with tobacco.[16] The poppies remained healthy and many new roots were produced. Three poppy plants were used in each feeding experiment. In experiments 1 and 2 the DL-phenylalanine-2-C^{14} and DL-tyrosine-2-C^{14}[17] were added to the nutrient solutions at one time and the morphine isolated 2 weeks later. In experiment 3 the tyrosine was administered to the plants three times, at intervals of a week, and the morphine isolated 4 weeks after the initial feeding. The amounts fed are shown in Table I.

TABLE I

Expt.	Tracer fed	Wt., mg.	Activity,[18] c.p.m.	Total activity in the morphine, c.p.m.	Incorporation, %
1	Phenylalanine-2-C^{14}	44.7	2.70×10^8	0.51×10^4	0.002
2	Tyrosine-2-C^{14}	46.5	2.33×10^8	3.12×10^4	.013
3	Tyrosine-2-C^{14}	138.5	8.63×10^8	1.47×10^5	.017

Isolation of the Morphine.—The following procedure is a simplification of that used by Achor and Geiling.[19] The fresh poppies from experiment 1 (57 g.) were macerated in a Waring Blendor with a 1:1 mixture of 1-butanol–benzene (200 ml.) and 10% aqueous sodium carbonate (40 ml.). After standing for 48 hr. the mixture was filtered and the marc washed with a further 50 ml. of the organic solvent. The combined organic layer was washed several times with 0.5 *N* sulfuric acid (150 ml.). The acid extract was made definitely alkaline by the addition of a 2% aqueous solution of potassium hydroxide saturated with barium hydroxide. The barium sulfate was filtered off and the filtrate was added to the top of a column (20 × 1.5 cm.) of Nalcite SAR[20] ion exchange resin in the hydroxide form. The column was then washed with 50% aqueous methanol (200 ml.). The morphine was eluted from the column with 0.1 *N* hydrochloric acid. Morphine appeared in the eluate as soon as the *p*H decreased to 2. When 50 ml. of such eluate had been collected it was evaporated to dryness *in vacuo*. The residue was dissolved in a minimum of water, filtered and made alkaline with a drop of sodium carbonate solution. The morphine crystallized out and was dried at 100° (17 mg.). The yields of morphine in experiments 2 and 3 were 15 and 16 mg., respectively. The activities of the morphine are shown in Table I.

Degradation of the Morphine.—The radioactive morphine from experiment 3 was diluted with about 1 g. of inactive morphine. The specific activities reported in Table II are for carrier-free material.

Codeine Methiodide (VI).[7]—The diluted morphine (1.088 g.) was dissolved in a 2.4% solution of sodium in ethanol[21] (3.65 ml.). Methyl iodide (3 ml.) was added and the mixture refluxed for one hour. Codeine methiodide crystallized out of the boiling solution and it was filtered off after prolonged cooling of the mixture (1.47 g., 91%), m.p. 267° dec.

α-Methylmorphimethine (VII).[7]—The codeine methiodide (1.46 g.) was dissolved in boiling water (7.5 ml.) and 25% aqueous sodium hydroxide (2 ml.) was added to the refluxing solution which was protected from the air with nitrogen. After 5 minutes the solution was cooled in ice and the aqueous supernatant liquid decanted from the product which was washed several times with cold water. It was then triturated with ether (10 ml.) when colorless prismatic needles of α-methylmorphimethine separated (883 mg., 88%), m.p. 118–119°.

TABLE II

ACTIVITIES OF MORPHINE AND ITS DEGRADATION PRODUCTS

Compound	Specific activity c.p.m./mM. × 10^{-6}
Morphine	2.62
Codeine methiodide	2.54
α-Methylmorphimethine	2.53
4-Acetoxy-3-methoxyphenanthrene	1.32
N,N-Dimethyl-β-ethanolamine aurichloride	1.25
N-Phenylphthalimide	1.29
Anthranilic acid	0.63

4-Acetoxy-3-methoxyphenanthrene (VIII).—The α-methylmorphimethine (876 mg.) was heated with acetic anhydride (2 ml.) and one drop of concentrated hydrochloric acid in a sealed tube at 180° for 16 hr. The brown contents of the tube were then poured into cold water (10 ml.) when the crude phenanthrene derivative separated (247 mg.). The material was sublimed (160°, 0.01 mm.) and the sublimate crystallized from a mixture of ethanol and petroleum ether (b.p. 50–60°) yielding fine colorless needles (139 mg., 18%) of 4-acetoxy-3-methoxyphenanthrene, m.p. 130–131°. The aqueous filtrate obtained after removal of the crude phenanthrene derivative was made basic with 10% sodium hydroxide solution (30 ml.) and the mixture distilled in a slow current of nitrogen into 2 *N* hydrochloric acid (25 ml.). When 25 ml. of liquid had distilled the distillate was evaporated to dryness. The residue was dissolved in a small volume of water and added to a solution of gold chloride ($HAuCl_4.3H_2O$) (0.2 g.) in 2 *N* hydrochloric acid. Small yellow plates (11 mg.) of **N,N-dimethyl-β-ethanolamine aurichloride** separated, m.p. 202° dec.

Phthalic Anhydride (X).—The 4-acetoxy-3-methoxyphenanthrene (100 mg.) was refluxed in water (40 ml.) with sodium hydroxide (200 mg.) for 15 minutes when most of the material had dissolved. Potassium permanganate (756 mg.) was added and the mixture refluxed on a metal-

(13) Tyrosine-3-C^{14} would give rise to morphine labeled at C-10 and C-15. A similar degradation should yield completely inactive phthalic anhydride removing any remaining doubt regarding the validity of Robinson's hypothesis.

(14) E. S. Mika, *Botan. Gaz.*, **116**, 323 (1955), describes, in considerable detail, the conditions required for the optimum production of morphine under laboratory conditions.

(15) The author thanks Dr. E. S. Mika, Dept. of Pharmacology, University of Chicago, and the U. S. Dept. of Agriculture, Beltsville, Md., for a supply of opium poppy seeds. Permission to grow opium poppies, under adequate safeguards, was obtained from the Commissioner of Narcotics, U. S. Treasury Dept., Washington, D. C.

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

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

(18) Counts were carried out in a Nuclear Chicago Model D 47 Q gas flow counter using a "Micromil" window. Determinations were carried out on samples of finite thickness, making corrections for efficiency and self absorption.

(19) L. B. Achor and E. M. K. Geiling, *Anal. Chem.*, **26**, 1061 (1954).

(20) This resin, obtainable from National Aluminate Corp., Chicago, Ill., is a strong cationic resin which forms a salt with the phenolic group of morphine. Codeine and other non phenolic alkaloids are thus not retained by the column.

(21) Knorr[7] used methanol in this reaction, but a better yield and readier crystallization was obtained with ethanol.

bath for 12 hr. The manganese dioxide was centrifuged off and the alkaline supernatant liquid extracted with ether. The aqueous layer was then evaporated to small bulk and acidified with a few drops of concentrated hydrochloric acid and extracted many times with ether (500 ml.). The dried ether extract was evaporated and the crystalline residue sublimed *in vacuo* to yield phthalic anhydride (24.2 mg., 42%), m.p. 130°. N-Phenylphthalimide was obtained by refluxing a few mg. of the anhydride with a drop of aniline and several drops of acetic acid. Crystallization from ethanol gave fine colorless needles, m.p. 212°, not depressed on admixture with authentic material.

Anthranilic Acid (XI).[12]—The phthalic anhydride (15 mg.) was dissolved in concentrated sulfuric acid (0.2 ml.), cooled and sodium azide (30 mg.) added. The mixture was warmed at 110° on a metal-bath for 30 minutes and then allowed to cool overnight. Ice was then added and the solution brought to a *p*H of about 5 by the addition of sodium hydroxide. Extraction of this solution with ether yielded anthranilic acid, purified by sublimation (10.2 mg., 73%), m.p. 145–146°, not depressed on admixture with authentic material.

MINNEAPOLIS 14, MINN.

[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, MEDICAL COLLEGE OF VIRGINIA]

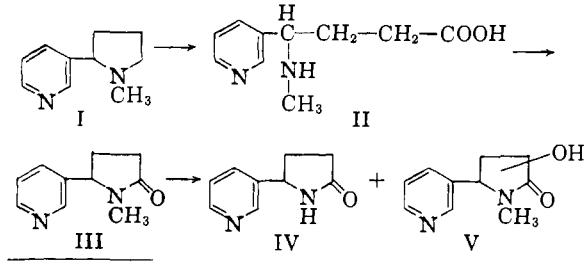
Demethylation of Cotinine *in Vivo*[1]

BY HERBERT MCKENNIS, JR.,[2] LENNOX B. TURNBULL, EDWARD R. BOWMAN[3] AND EINOSUKE WADA[4]

RECEIVED FEBRUARY 5, 1959

Administration of (−)-cotinine, an intermediate in the metabolism of (−)-nicotine, leads to the excretion of (−)-desmethylcotinine, hydroxycotinine and other Koenig positive material in the urine of the dog. Reductive amination of γ-(3-pyridyl)-γ-oxobutyric acid in the presence of Raney nickel yielded racemic desmethylcotinine which was resolved to give (−)-desmethylcotinine corresponding to the metabolic product.

Following the administration of (−)-nicotine the cat,[5] the dog[6–7] and the human[8,9] excrete in the urine a variety of Koenig positive materials which are not found in control urine. One of the several Koenig positive zones from paper chromatography has been found to contain cotinine.[6–9] The latter has been identified[10] chemically with the isolation of cotinine picrate,[6–9] and another has been found to contain γ-(3-pyridyl)-γ-methylaminobutyric acid,[6,7] identified after thermal cyclization into the lactam cotinine.[6,7] This cyclization to cotinine takes place spontaneously at physiological *p*H. Cotinine, through its limited toxicity, is a convenient intermediate, therefore, for exploration of nicotine metabolism. The isolation of two new nicotine metabolites now permits representation of the cotinine pathway of nicotine metabolism in the abbreviated form



(1) Presented in part at the IV International Congress of Biochemistry, Vienna, September 1–6, 1958.

(2) The authors are grateful to The American Tobacco Company and The Tobacco Industry Research Committee for generous support.

(3) Public Health Research Fellow of the National Heart Institute.

(4) American Tobacco Company Research Fellow.

(5) F. B. Owen, Jr., and P. S. Larson, *Arch. int. pharmacodyn.*, **115**, 402 (1958).

(6) H. McKennis, Jr., L. B. Turnbull and E. R. Bowman, THIS JOURNAL, **79**, 6342 (1957).

(7) H. McKennis, Jr., L. B. Turnbull and E. R. Bowman, *ibid.*, **80**, 6597 (1958).

(8) E. R. Bowman, L. B. Turnbull and H. McKennis, Jr., *Virg. J. Sci.*, **9**, 438 (1958).

(9) E. R. Bowman, L. B. Turnbull and H. McKennis, Jr., *J. Pharmacol. Exp. Therap.*, in press.

(10) Paper chromatographic and ultraviolet absorption data have led to the suggestion (F. E. Guthrie, R. L. Ringer and T. G. Bowery, *J. Econ. Entom.*, **50**, 822 (1957), that cotinine arises from the metabolism of nicotine in insects.

For the current studies attention was focused solely on the chloroform-soluble metabolites of nicotine (I) and cotinine in dog urine. (−)-Cotinine (III) was administered intravenously to male mongrel dogs under anesthesia. The urine was adjusted to *p*H 8–9 by addition of ammonia water and extracted continuously with chloroform. Samples from evaporation of the chloroform yielded Koenig positive zones at R_f 0.61 and R_f 0.74 upon paper chromatography with ammonia–ethanol–butanol.[11] The latter corresponded in R_f value to known cotinine upon cochromatography and the former corresponded in R_f value to material obtained from both dogs[6,7] and humans[8,9] following administration of (−)-nicotine (I).

The aqueous solution of the residue from evaporation of the chloroform was placed on Dowex 50 (H^+). The Koenig positive material was eluted with *N* ammonia water and further purified by passage through Dowex 1 ($O\bar{H}$) which served to remove some Koenig negative solids as well as a component with R_f 0.75. The latter has an R_f value virtually indistinguishable from cotinine in many solvent systems but differs from it by an apparent amphoteric behavior displayed on ion-exchange resins. Investigations on this component are in progress.

The effluent from the Dowex 1 ($O\bar{H}$) resin containing Koenig positive bases was reprocessed on Dowex 50 (H^+) for further purification. The solids from the latter were dissolved in chloroform and chromatographed on alumina and eluted with ether containing methanol in concentration increasing from 10–75% (v./v.). The 10% methanolic eluates contained material with R_f 0.74 corresponding to cotinine. The fractions removed with 11–75% methanol, although composed of both oil and crystals showed only a positive Koenig zone at R_f 0.61. Following treatment with acetic anhydride–pyridine the foregoing mixture was chromatographed on alumina and eluted with methanolic

(11) H. McKennis, Jr., L. B. Turnbull, H. N. Wingfield, Jr., and L. J. Dewey, THIS JOURNAL, **80**, 1634 (1958).