the methods were applied to the hemoglobin mixtures that are known to occur in certain pathological conditions, it probably would be possible to determine whether entirely different components are present or whether components normally present in minor amount have simply been increased proportionately.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, MEDICAL COLLEGE OF VIRGINIA AND RESEARCH LABORATORY, THE AMERICAN TOBACCO COMPANY]

Metabolites of Nicotine and a Synthesis of Nornicotine

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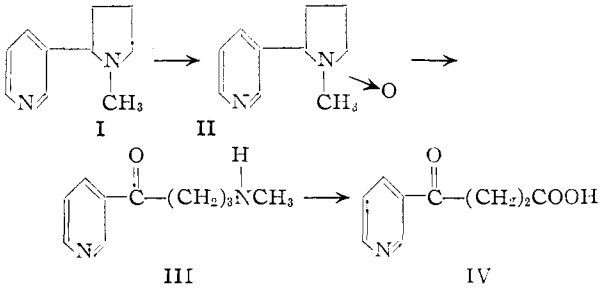
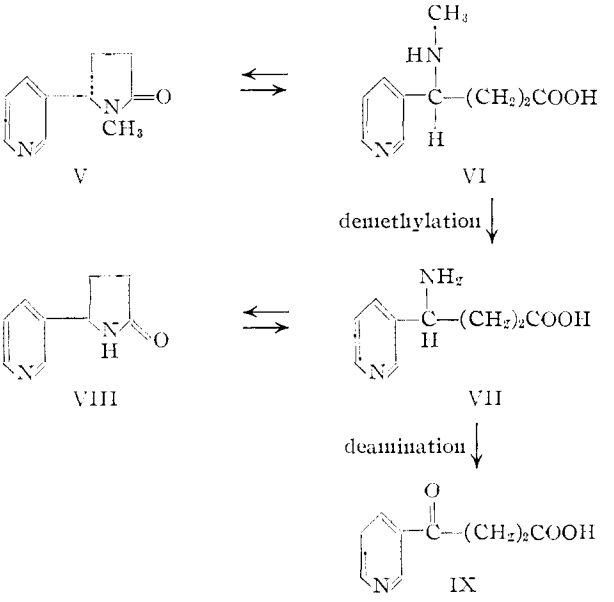
Cotinine is hydrolyzed by the action of strong barium hydroxide to γ-(3-pyridyl)-γ-methylaminobutyric acid, a possible intermediate in the biological degradation of nicotine. Zinc dust reduction of γ-(3-pyridyl)-γ-oximinobutyric acid gives a mixture from which γ-(3-pyridyl)-γ-aminobutyric acid and desmethylcotinine were obtained. The latter yields the corresponding amino acid upon hydrolysis. Nornicotine is obtained by reduction of desmethylcotinine with lithium aluminum hydride.

The demonstration by Wada and Yamasaki[2] that soil bacteria can degrade nicotine to γ-(3-pyridyl)-γ-oxobutyric acid has given rise to a number of concepts concerning the mechanism of metabolic degradation of the pyrrolidine ring of nicotine. Wada and Yamasaki[2] believed that nicotine (I) was first oxidized to oxynicotine (II) by the microbial organism, "or merely by the action of aeration." Subsequent steps, according to these authors, involved the formation of pseudo oxynicotine (III) and γ-(3-pyridyl)-γ-oxobutyric acid (IV).



As a result of the isolation of cotinine[3-5] from fermented leaves of tobacco, Frankenburg, Gottscho and Vaitekunas[3,5] have suggested γ-3-pyridyl-γ-methylaminobutyrylaldehyde as a precursor of cotinine, and Werle, *et al.*,[6] considered γ-3-pyridyl-γ-methylaminobutyric acid a possible precursor of the keto acid IV. Since cotinine itself has been obtained[4] from samples of autoxidized nicotine as well as from fermented tobacco, cotinine may play a key role in the metabolism of nicotine according to the scheme



This study was designed to provide synthetic compounds which would facilitate the acceptance or rejection of such an hypothesis. In addition the suggestion of Larson and Haag[7,8] that a compound such as γ-(3-pyridyl)-γ-methylaminobutyric acid might occur in urine of dogs following administration of nicotine gave further impetus to our investigation.

Using nicotine as a starting material, Pinner[9,10] many years ago obtained cotinine from the zinc dust reduction of dibromocotinine. γ-(3-Pyridyl)-

(1) Presented in part at the 131st meeting of the American Chemical Society, Miami, Florida, April 11, 1957. Aided by a grant from the Tobacco Industry Research Committee.

(2) E. Wada and K. Yamasaki, THIS JOURNAL, **76**, 155 (1954).

(3) W. G. Frankenburg, A. M. Gottscho and A. A. Vaitekunas, *Proc. First Internat. Sci. Cong. on Tobacco*, **2**, 419 (1955).

(4) W. G. Frankenburg and A. A. Vaitekunas, THIS JOURNAL, **79**, 149 (1957).

(5) W. G. Frankenburg, A. M. Gottscho and A. A. Vaitekunas, Abstract of Papers, Tobacco Chemists Research Conference, Oct. 6–7, 1955, Raleigh, N. C.

(6) K. Werle, H. Schievelbein and D. Spieth, *Arzneimittel-Forsch.*, **6**, 322 (1956).

(7) P. S. Larson and H. B. Haag, *J. Pharmacol. Exptl. Therap.*, **76**, 240 (1942).

(8) P. S. Larson, H. B. Haag and J. K. Finnegan, *ibid.*, **86**, 239 (1946).

(9) A. Pinner, *Ber.*, **26**, 292 (1893).

(10) A. Pinner, *Arch. Pharm.*, **231**, 378 (1893).

γ-oxobutyric acid (IX), another of the metabolites of the scheme, has been synthesized by Castle and Burger[11] and also by Sugasawa, Tatsuno and Kamiya.[12] With the use of cotinine and the keto acid as intermediates all of the compounds of the hypothetical metabolic scheme (V-IX) have now been synthesized and some of their properties have been studied.

γ-(3-Pyridyl)-γ-methylaminobutyric acid (VI) was prepared by heating cotinine with strong barium hydroxide. After removal of barium as barium carbonate, the methylamino acid was obtained as a monohydrate which readily lost water of crystallization when dried *in vacuo*. Pinner[9] reported that cotinine was stable to the action of barium hydroxide. If such were the case the pyrrolidone structure of cotinine which he enunciated would appear to be in considerable doubt. Frankenburg and Vaitekunas[4] have degraded cotinine to N-methyl-5-pyrrolidone-2-carboxylic acid. This, as well as hydrolysis to the methylamino acid, affords confirmation of the γ-lactam (pyrrolidone) structure of cotinine.

The reason for Pinner's assertion that cotinine is stable to the action of barium hydroxide is not certain since his experimental details were not reported in the literature. In limited studies on the stability of γ-(3-pyridyl)-γ-methylaminobutyric acid spontaneous reformation of the lactam, cotinine, was observed to take place in aqueous solutions at room temperature. This instability of the amino acid may explain Pinner's failure to observe hydrolysis at the time he treated cotinine with barium hydroxide.

Although reactions analogous to those employed in the synthesis of γ-(3-pyridyl)-γ-methylaminobutyric acid, using nornicotine instead of nicotine, could presumably be used for the synthesis of γ-(3-pyridyl)-γ-aminobutyric acid, other methods were sought. Castle and Burger[11] prepared γ-(3-pyridyl)-γ-oximinobutyric acid. These authors reported that all attempts to reduce the oximino compound to the amino acid VII or the corresponding lactam VIII were unsuccessful. Similar failure attended their attempts to prepare the amino compound by reduction of the keto acid in the presence of ammonia. Although others[12] succeeded in an analogous catalytic reduction by preparing DL-cotinine from the keto acid and methylamine, means were sought in our study for chemical reduction of the oximino compound.

γ-(3-Pyridyl)-γ-oximinobutyric acid was reduced in the presence of zinc dust and acetic acid. The product was a glassy solid, shown to be a mixture by paper chromatography. After thermal dehydration the reaction mixture yielded desmethylcotinine, the lactam of the desired amino acid. Upon hydrolysis in barium hydroxide the lactam yielded γ-(3-pyridyl)-γ-aminobutyric acid. Proof of structure for this acid and its lactam was obtained by reduction of the lactam to nornicotine.

Desmethylcotinine upon treatment with lithium aluminum hydride according to the method employed to reduce cotinine to nicotine[12] yielded DL-nornicotine, identified by infrared spectra of the acetyl derivative and the picrate of the N-acetyl compound which were compared with derivatives of authentic L-nornicotine. In addition to affording a synthetic proof of structure, the reduction of desmethylcotinine provides a new synthesis of nornicotine[13] and consequently nicotine[13a] through an additional methylation reaction.[14-16] Biological studies on the compounds will be described in subsequent reports.

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Experimental[17]

γ-(3-Pyridyl)-γ-methylaminobutyric Acid.—A solution of barium hydroxide (15 g.) in 30 ml. of water containing 2 g. of cotinine was heated under reflux for 20 hr. After cooling to room temperature, the mixture was diluted with 70 ml. of water. Excess barium hydroxide was removed by filtration. The filtrate was extracted with six portions of chloroform (100 ml. each). Unhydrolyzed cotinine (320 mg.) was recovered upon evaporation of the chloroform extracts. The aqueous phase was saturated with carbon dioxide, and barium carbonate was removed by filtration. The filtrate was concentrated at 10° to a glassy residue which was treated with methanol. The methanol solution was filtered to remove traces of barium carbonate and then concentrated to 15 ml. Acetone was added to induce crystallization. The product (1.20 g.) melted at 132–133°. It was chromatographed on Whatman No. 1 paper with ammoniacal alcohol (1 vol. *N*/2 ammonium hydroxide, 1 vol. 95% ethanol, 4 vol. *n*-butanol) to yield a single transient red to orange spot (R_f 0.14) of the Koenig reaction when treated with a *p*-aminobenzoic acid spray (2% in ethanol) which was followed by gaseous cyanogen bromide.[18] For analysis, the compound was air-dried to give a monohydrate.

Anal. Calcd. for $C_{10}H_{14}N_2O_2 \cdot H_2O$: C, 56.58; H, 7.60; N, 13.20. Found: C, 56.84; H, 7.77; N, 13.35.

The latter lost a mole of water when dried at 1 mm. over potassium hydroxide.

Anal. Calcd. for $C_{10}N_{14}N_2O_2$: C, 61.83; H, 7.27; N, 14.42. Found: C, 61.77; H, 7.18; N, 14.48.

The acid showed optical activity, $[\alpha]^{22}D$ +17.2° (*c* 17.44, water). The optical purity of the compound, however, has not been ascertained. On fusion, the acid yields cotinine with rotation of opposite sign.

γ-(3-Pyridyl)-γ-oxobutyric acid was prepared by modification of previous methods. To a solution of 24.6 ml. (0.42 mole) of absolute ethanol and 100 ml. of dry benzene was added 9.68 g. (0.42 mole) of sodium in small pieces. After 15 minutes of stirring a solution of freshly distilled diethyl succinate (69.7 g., 0.4 mole) and 33.3 g. (0.2 mole) of ethyl nicotinate were added in one portion. The mixture was stirred under reflux for 1 hr. and then cooled. Concentrated hydrochloric acid (34.4 ml.) in 100 ml. of water was added to the brown mixture. The resulting two phase system was saturated with sodium bicarbonate and then extracted five times with 100-ml. portions of 5% hydrochloric acid. After neutralization with sodium bicarbonate the aqueous solution was extracted three times with 200-ml. portions of ether. The ethereal extract was dried over anhydrous sodium sulfate and concentrated to a brown oil. This residue was distilled to give 9.8 g. of ethyl nicotinate (b.p. 75–78°, 0.75 mm.) and 16.5 g. (38%) of diethyl α-nicotinylsuccinate boiling at 155–165° and 0.75 mm. The

(11) R. N. Castle and A. Burger, *J. Amer. Pharm. Assn. Sci. Ed.*, **43**, 163 (1954).

(12) S. Sugasawa, T. Tatsuno and T. Kamiya, *Pharm. Bull. (Japan)*, **2**, [1], 39 (1954).

(13) (a) Previous syntheses have been reviewed by L. Marion in R. H. F. Manske and H. L. Holmes, "The Alkaloids," Vol. I, Academic Press, Inc., New York, N. Y., p. 165. (b) P. G. Haines, A. Eisner and C. F. Woodward, THIS JOURNAL, **67**, 1258 (1945).

(14) L. C. Craig, *ibid.*, **55**, 2854 (1933).

(15) E. Späth and P. Kainrath, *Ber.*, **71**, 1276 (1938).

(16) L. N. Markwood, *J. Assoc. Off. Agr. Chemists*, **26**, 283 (1943).

(17) Microanalyses by Spang Microanalytical Laboratory.

(18) E. Kodicek and K. K. Reddi, *Nature*, **168**, 475 (1951).

ester was hydrolyzed and decarboxylated in 35 ml. of *N* sulfuric acid heated under reflux for 24 hr. The hydrolysate was adjusted to *p*H 4.3 with concentrated ammonia water to yield 9.45 g. (87%) of crystalline γ-(3-pyridyl)-γ-oxobutyric acid, m.p. 161–164°. The product was sufficiently pure for preparation of the oxime. Castle and Burger reported a melting point of 161.5–163° for the keto acid.

γ-(3-Pyridyl)-γ-oximinobutyric acid was prepared essentially according to Castle and Burger. The product, m.p. 163–166° dec., was sufficiently pure for reduction; λ_{max} 250 mμ, ε 8510 (95% ethanol).

DL-Desmethylcotinine.—γ-(3-Pyridyl)-γ-oximinobutyric acid (5.0 g.) was dissolved in a mixture of 95% ethanol (125 ml.) and glacial acetic acid (35 ml.). Zinc dust (30 g.) was added portionwise with stirring over a 2 hr. period. After filtration the solution was concentrated to a thick sirup *in vacuo* at room temperature. Water (200 ml.) was added, and the solution was saturated with hydrogen sulfide. Zinc sulfide was removed by filtration with the aid of Celite. The filtrate was concentrated *in vacuo* at room temperature to a clear glassy solid. The ultraviolet absorption spectrum of this glass showed one clear maximum at 262 mμ, characteristic of pyridine absorption.[19] Paper chromatography (ammonia–ethanol–butanol) showed the material to be a mixture with a major Koenig positive spot (R_f 0.16) and another minor spot (R_f 0.31). Components giving a Koenig positive reaction were disclosed as described under γ-(3-pyridyl)-γ-methylaminobutyric acid. The solid was heated at 200° under nitrogen until no more water was evolved to give a mixture (4.2 g.) with major component of R_f 0.75 and minor component of R_f 0.90. The mixture was dissolved in ether and chromatographed on acid-washed alumina. Elution with ether containing 10–15% methanol by volume gave fractions exhibiting only the R_f 0.75 spot upon chromatography with the ammonia–butanol–ethanol system. On standing, these fractions yielded 2.00 g. of crystals. These were combined and recrystallized to give desmethylcotinine, m.p. 65–68°. The air-dried sample gave analytical values for a monohydrate.

Anal. Calcd. for $C_9H_{12}N_2O_2$: C, 59.98; H, 6.71; N, 15.55. Found: C, 60.22; H, 6.61; N, 15.62.

The monohydrate was sublimed at 0.5 mm. and 80° to give an anhydrous lactam, m.p. 113–116°.

Anal. Calcd. for $C_9H_{10}N_2O$: C, 66.65; H, 6.22; N, 17.27. Found: C, 66.75; H, 6.21; N, 17.31.

A solution of the compound in alcohol yielded a monopicrate which was recrystallized from alcohol and dried at 70° and 1 mm. (m.p. 162–164°).

Anal. Calcd. for $C_{15}H_{13}N_5O_8$: C, 46.04; H, 3.35; N, 17.90. Found: C, 46.04; H, 3.12; N, 17.83.

(19) M. L. Swain, A. Eisner, C. F. Woodward and B. A. Brice, THIS JOURNAL, **71**, 1341 (1949).

γ-(3-Pyridyl)-γ-aminobutyric Acid.—A solution containing 410 mg. of DL-desmethylcotinine hydrate and 5 g. of barium hydroxide in 30 ml. of water was boiled under reflux overnight. After cooling to room temperature, excess barium hydroxide was removed by filtration, and the solution was saturated with carbon dioxide. Barium carbonate was removed by filtration, and unhydrolyzed lactam was extracted with six portions (100 ml. each) of chloroform. The aqueous solution was concentrated to dryness *in vacuo* at room temperature to a glassy solid. The latter was dissolved in ethanol. Traces of barium carbonate were removed by filtration. Upon concentration at room temperature the filtrate deposited colorless crystals. These were recrystallized from water–acetone to give 100 mg. of amino acid monohydrate, m.p. 166–167°, air dried.

Anal. Calcd. for $C_9H_{14}N_2O_3$: C, 54.53; H, 7.12; N, 14.13. Found: C, 54.70; H, 7.01; N, 14.26.

When chromatographed in the ammonia–butanol–ethanol system the compound gave a single spot (R_f 0.16) on paper.

Acetyl-DL-nornicotine.—A solution of 4.0 g. of DL-desmethylcotinine in 100 ml. of dry tetrahydrofuran containing 3 g. of lithium aluminum hydride was heated under reflux for 40 hours.[12] The mixture was treated with dilute sodium hydroxide and then extracted with chloroform. The chloroform extract upon evaporation yielded 1.4 g. of nornicotine as a brown oil (R_f 0.73 with diffuse impurities by paper chromatography). The oil was dissolved in 12 ml. of dry pyridine and treated with 2 ml. of acetic anhydride. The solvent was evaporated to give a brown oil. The latter was treated with methanolic picric acid and yielded yellow crystals of acetyl-DL-nornicotine picrate. After recrystallization from methanol, a sample, m.p. 157–160°, was dried at 70° and 1 mm.

Anal. Calcd. for $C_{17}H_{17}N_5O_8$: C, 48.69; H, 4.09; N, 16.70. Found: C, 48.29; H, 4.13; N, 16.83.

The infrared absorption spectrum of this compound in a KBr pellet was identical with that of authentic acetyl-L-nornicotine picrate, m.p. 158–161°, which was recrystallized from methanol. Von Braun and Weissbach[20] reported m.p. 151° for this compound. Others[13b] reported m.p. 158.5–159.5°.

The above DL-picrate (900 mg.) was dissolved in 10 ml. of 6 *N* hydrochloric acid and repeatedly extracted with ether to remove picric acid. The aqueous phase was then made alkaline with sodium carbonate and extracted with chloroform. The chloroform extract yielded, upon evaporation, a colorless oil, giving a single, clearly defined R_f 0.73. Authentic acetyl-L-nornicotine had an identical R_f when chromatographed in the previously described system.

(20) J. von Braun and K. Weissbach, *Ber.*, **63**, 2018 (1930).

RICHMOND, VIRGINIA

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF HOFFMANN-LA ROCHE, INC.]

Isolation of Antibiotic X-465A and its Identification with Chartreusin

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A crystalline antibiotic, X-465A, was isolated from cultures of two streptomyces. Its empirical formula is $C_{32}H_{34-36}O_{14}$. The atoxic antibiotic is active *in vitro* against certain gram-positive bacteria, bacteriophages, mycobacteria and streptomyces, and inactive *in vivo* against bacterial, fungal, protozoan and viral infections. The identity of this antibiotic with chartreusin is demonstrated.

In the course of our search for new antibiotics, a gray, sometimes bluish-green sporulating *Streptomyces* sp., designated X-465, was isolated in our laboratory from a soil sample of Salem, Va. Another, possibly identical *Streptomyces* sp., designated X-3988, was isolated from a soil sample of Sao Paulo, Brazil. These two cultures look similar and produce at least one antibiotic in common (referred to as antibiotic X-465A), which has been isolated in crystalline form from the broths in which each organism was grown.[2]

(1) Presented in part by M. W. G. at the XIVth International Congress of Pure and Applied Chemistry in Zurich (1955); Congress Handbook, p. 233.

(2) Some strains of *Streptomyces* sp. X-465 produce a second antibiotic, referred to as antibiotic X-465B. Non-crystalline concentrates