[CONTRIBUTION FROM THE RESEARCH LABORATORY, GENERAL CIGAR CO., INC.]

The Chemistry of Tobacco Fermentation. I. Conversion of the Alkaloids. D. Identification of Cotinine in Fermented Leaves

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Among the conversion products of nicotine present in fermented cigar tobacco leaves, cotinine (N-methyl-2-(3-pyridyl)-5-pyrrolidone) has been identified as a major component. Cotinine was also found among the autoxidation products formed from nicotine on standing.

As described in an earlier paper,[1] a subfraction δ was isolated from fermented Pennsylvania Seedleaf tobacco which contained a considerable amount of a then unidentified pyridine derivative I. This same substance has since been discovered in and isolated from, autoxidized nicotine, from nicotine treated with hydrogen peroxide and also from nicotine irradiated with ultraviolet light. In this report, it is shown that this substance (I) is cotinine, first described by Pinner,[2] who assigned to it the structure II on the basis of its monobasicity. Pinner's structure has been confirmed by spectral studies and by degradation of the pyridine moiety.

Confirmation of Cotinine Structure.—A strong infrared band at 6.0 μ, in agreement with structure II was exhibited (Fig. 2). The ultraviolet spectrum in aqueous acid showed a maximum at 262 and a minimum at 234 mμ. The ratio of the absorbances at these two wave lengths is 6.1 and the absorbancy index at 262 mμ is 31.6.

To confirm the lactam structure of cotinine its pyridine moiety was degraded by the series of reactions described by Karrer[3] for the oxidative destruction of the pyridine ring in nicotine, *via* its methiodide, oxidation of the latter to an N-methylpyridone and oxidative split of the pyridone ring, to leave the carboxylated pyrrolidine ring as the final product. Structure II should yield, by the same series of reactions, N-methyl-5-pyrrolidone-2-carboxylic acid (V). To prove this, an authentic specimen of V, prepared from N-methylglutamic acid, was compared to V obtained *via* the route shown in Fig. 1. Paper chromatography with three different solvent systems gave identical R_f values for V from the two sources. Both compounds were also reduced to hygrinic acid (VI), which was identified by chromatography and as its copper salt.

The identity of authentic cotinine, structure II, with substance I obtained from fermented tobacco leaves *via* subfraction δ, from autoxidized nicotine and from nicotine treated with hydrogen peroxide or irradiated with ultraviolet light was clearly shown by comparison of their infrared and ultraviolet spectra, R_f values in different systems and their chloroplatinates (see Experimental).

Occurrence in Tobacco Leaves.—As obtained from fermented tobacco leaves after partition chromatography of subfraction δ, cotinine is a yellowish very hygroscopic oil which gives an almost neutral solution in water. Aqueous solutions of authentic cotinine and the substance isolated from autoxidized nicotine are also non-basic. Oxidation of both authentic cotinine and the material from fermented tobacco with potassium permanganate or chromic acid gave nicotinic acid, oxalic acid and smaller amounts of malonic acid.

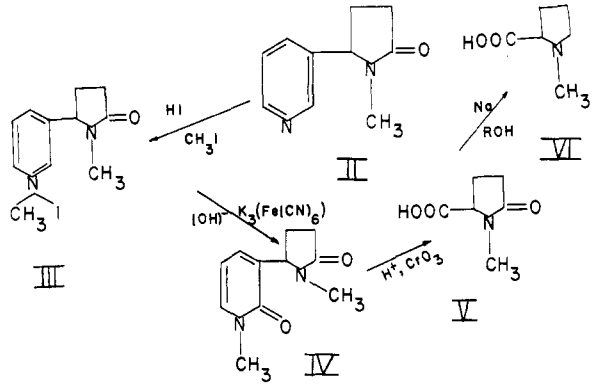

Fig. 1.—Conversion of cotinine to N-methyl-5-pyrrolidone-2-carboxylic acid and hygrinic acid by oxidative degradation of its pyridine ring.

There are indications that there is an additional substance present in the crude cotinine fraction from fermented tobacco, even after purification by column chromatography on cellulose. This substance may possibly be derived from cotinine. The ratio of maximum absorbance to minimum absorbance of the ultraviolet spectra of this crude cotinine and of cotinine prepared by the Pinner method differ (crude, 2.0 to 3.0; synthetic, 6.1). However, when synthetic cotinine is extracted from a slightly alkaline (*p*H 10) solution with ether, its spectrum becomes similar to that of the crude cotinine from fermented tobacco. The infrared spectrum of this crude cotinine also differs somewhat from that of the synthetic material in the 3500–3000 cm.$^{-1}$ region. In addition a second chloroplatinate having a different melting point was observed when cotinine chloroplatinate was prepared from the crude cotinine fraction of tobacco.

Discussion

According to the scheme proposed in a previous publication,[1] five of the six oxidation products of nicotine found in the fermented leaves are probably formed *via* N-methylmyosimine. Oxynicotine is the only exception. As to cotinine, separate *in vitro* experiments showed that the compound cannot be produced from N-methylmyosmine under the same conditions under which it is formed from nicotine. Accordingly, we may conclude that the formation of cotinine proceeds like that of

(1) W. G. Frankenburg, A. M. Gottscho, A. A. Vaitekunas and R. M. Zacharius, THIS JOURNAL, **77**, 5730 (1955).
(2) A. Pinner, *Arch. Pharm.*, **231**, 378 (1893).
(3) P. Karrer and R. Widmer, *Helv. Chim. Acta*, **8**, 364 (1925).

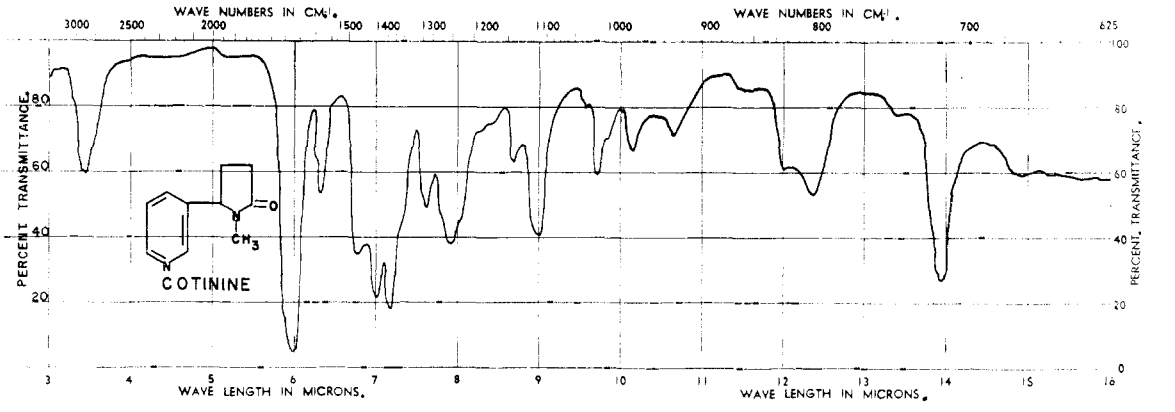

Fig. 2.—Infrared absorption spectrum of cotinine.

oxynicotine *via* a chemical pathway that does not include N-methylmyosmine as an intermediate.

Whether cotinine itself undergoes any further changes and is perhaps responsible for the formation of the water-insoluble pyridine compounds found to be present in the fermented leaves[4] is not known and has to be clarified by future investigations.

Experimental[5]

Isolation of Substance I from Subfraction δ.—The yellow oil δ¹ (100 mg.) was dissolved in 5 ml. of ethanol (95%) and this solution was impregnated on filter paper disks (Whatman No. 1, diameter 5 cm.). After air-drying, the disks were placed on top of a column (diameter 5 cm., length 52 cm.) of cellulose powder.[6] By passing 3 liters of a solvent consisting of 60 parts of benzene, 15 parts of methanol and 25 parts of sodium acetate buffer (*p*H 5.6) through the column, substance I was eluted from the column. The eluate was collected in test-tubes, using an automatic fraction collector.[7] The contents of these tubes were checked by color tests on paper strips, after spraying the strips with a 0.1% ethanolic solution of *p*-aminobenzoic acid and exposure to cyanogen bromide vapor. The eluate in all the test-tubes yielding a red color on the test paper strips was combined. The benzene and methanol were distilled off, leaving an aqueous solution which was alkalinized and extracted repeatedly with chloroform. The combined chloroform extracts, dried over anhydrous sodium sulfate, yielded after evaporation of the solvent a yellow oil which was extracted with ethyl ether. The yellow oil, containing I (30 mg.), obtained after evaporation of the ether was chromatographed on paper with the three solvent mixtures listed in Table I. The chromatograms developed with *p*-aminobenzoic acid and cyanogen bromide vapor, showed spots having R_f values identical with those of cotinine (II). Besides the spots corresponding to II, these chromatograms contained immobile spots which developed an orange color, gradually turning pink. The oil, "crude cotinine," is soluble in chloroform and sparingly soluble in ether. With water, it forms a partly colloidal solution of *p*H 6 to 7. Attempts to distil this oil *in vacuo* were unsuccessful because of its small quantity; it is hygroscopic and does not lend itself to satisfactory elementary analysis.

Isolation of Cotinine from Autoxidized Nicotine.—A commercial nicotine sample (100 g.) that had been standing in a glass bottle for several years and had turned brown was subjected to a distillation *in vacuo* to remove unreacted nicotine. The brown residue (*ca.* 3 g.) of this distillation was made strongly alkaline with sodium hydroxide and steam distilled until samples of the distillate showed no turbidity when tested with a few drops of silicotungstic acid and hydrochloric acid (2.5 *N*). The distillation residue was neutralized, made 3 *N* with hydrochloric acid and hydrolyzed for 6 hr. The solution was adjusted to *p*H 10, boiled for 5 minutes to remove methylamine,[8] then cooled and extracted with ethyl ether in a liquid–liquid extraction apparatus for 40 hr. The extract yielded, on evaporation of the solvent, a yellow oil (1.56 g.) which, as judged from paper chromatograms, consisted almost entirely of cotinine. This oil was distilled at 210–211° at 6 mm., yielding a colorless, viscous oil (350 mg.) which showed ultraviolet absorption and infrared absorption spectra identical with those of cotinine (II) prepared according to Pinner.[2] Further confirmation that this oil was cotinine was provided by paper chromatography (Table I).

TABLE I

R_f VALUES FOR COTININE

Cotinine	Butanol–ethanol–acetate buffer[a] (50–10–40)	Butyl acetate–methanol–ammonia 0.25%[b] (95–5–25)	Benzene–methanol–acetate buffer[a] (60–15–25)
From fermented tobacco	0.81 ± 0.01	0.37 ± 0.01	0.56 ± 0.02
From nicotine *in vitro*	.80 ± .01	.37 ± .01	.56 ± .02
Synthetic	.79 ± .02	.37 ± .01	.57 ± .03

[a] This buffer has a *p*H of 5.6 and consists of a mixture of 0.2 *M* acetic acid (9.5 ml.) and 0.2 *M* sodium acetate (90.5 ml.). [b] W. L. Porter, J. Naghski and A. Eisner, *Arch. Biochem.*, 24, 461 (1949).

When commercial nicotine, in aqueous solution, had been treated with hydrogen peroxide or had been subjected to irradiation by ultraviolet light, the unreacted nicotine was removed and cotinine isolated as described above.

Preparation of Chloroplatinates.—The chloroplatinate of cotinine synthesized by Pinner's method was prepared by dissolving 100 mg. of this compound in 2 ml. of 3 *N* hydrochloric acid. To this was added, dropwise with stirring, a solution of 95 mg. of chloroplatinic acid in 0.5 ml. of 1 *N* hydrochloric acid. The chloroplatinate separated in orange prisms, m.p. 220°, identical with the melting point reported by Pinner.[2] In a similar manner, the chloroplatinate of cotinine prepared from autoxidized nicotine, m.p. 220° dec., and of cotinine (I) from fermented tobacco leaves, m.p. 219–220° dec., were prepared; mixed m.p. of each of these chloroplatinates with the chloroplatinate of synthetic cotinine is 220° dec. For the chloroplatinate of I: *Anal.* Calcd. for $PtCl_4(C_{10}H_{12}N_2O\cdot HCl)_2$: C, 31.54; H, 3.44; N, 7.35; Pt, 25.61. Found: C, 31.60; H, 3.20; N, 7.05; Pt, 25.32.

During the preparation of the chloroplatinate of cotinine from fermented tobacco, two crops of crystals were obtained. The first crop was only partially crystalline, m.p. 290° dec.

(4) W. G. Frankenburg and A. M. Gottscho, *Ind. Eng. Chem.*, **44**, 301 (1952).

(5) Melting points are not corrected and were determined with a Fisher–John Apparatus. Ultraviolet spectra were measured with Beckman DU and DK-2 Spectrophotometers.

(6) "Coarse Grade," W. R. Ralston, Ltd., England. Before use, this powder was freed from interfering materials by an exhaustive extraction with hot benzene followed by washing, on the column with the eluting solvent described.

(7) Time operated, manufactured by Gilson Medical Electronics, Madison, Wis.

(8) Formed by the hydrolysis of N-methylnicotinamide.

The second crop yielded on recrystallization the derivative of m.p. 219–220° dec. described above.

Oxidation of Cotinine from Fermented Tobacco Leaves with Potassium Permanganate.—A solution of 39 mg. of the oil, purified *via* chromatography on a cellulose column, in 7 ml. of hot water and 0.3 ml. of 10% sodium hydroxide, was placed in a water-bath maintained at 60–70°. To this was added 17 ml. of 5% potassium permanganate solution. After 3 hr. digestion on the water-bath, the manganese dioxide was filtered off and the unreacted permanganate was reduced with 0.5 g. of sodium bisulfite. The solution was then adjusted to *p*H 3 and extracted in a liquid-liquid extraction apparatus with ethyl ether for 40 hr. After evaporation of the solvent, crystals were obtained which after recrystallization from ethanol melted at 233°; yield of nicotinic acid, 21 mg.; theoretical nicotinic acid from cotinine 27 mg. This shows that this substance contains one pyridine moiety per mole. Oxidation with chromic acid produced a similar result.

Preparation of N-Methyl-2-(3-N-methyl-2-pyridone)-5-pyrrolidone (IV) from Cotinine.—Hydriodic acid (sp. gr. 1.79) (2.6 g., 0.023 mole) was added to 25 ml. of an alcoholic solution of 2 g. (0.015 mole) of cotinine (prepared *via* the perbromide as described by Pinner). To this mixture, while cooling, was added slowly 3.2 g. (0.023 mole) of methyl iodide. After refluxing the reaction mixture for 12 hr., the solvent was removed under reduced pressure. The yellow sirupy residue (impure compound III) weighing 5 g. was dissolved in 12.5 ml. of water. To this were added 2.5 ml. of 0.021 *N* potassium hydroxide and 25 ml. of 0.009 *N* potassium ferricyanide. After 0.5 hr., the reaction mixture was saturated with potassium carbonate and then shaken several times with benzene. On evaporation of the benzene from the extract, a brown viscous oil weighing 0.5 g. was obtained. This is impure N-methyl-2-(3-N-methyl-2-pyridone)-5-pyrrolidone (IV).

N-Methyl-5-pyrrolidone-2-carboxylic Acid (V) from Cotinine.—Following further the method of Karrer,[3] one-half gram of impure IV (0.023 mole) was dissolved in 20 ml. of water, and to this was added 30 ml. of a solution containing 4.5 g. (0.046 mole) of chromic acid and 6.8 g. (0.068 mole) of sulfuric acid. After refluxing the reaction mixture for 5 hr., the unreacted chromic acid was reduced by sulfur dioxide; the latter was expelled by boiling. The chromium salts were precipitated with saturated barium hydroxide and filtered off. The filtrate, together with washings from a hot-water extraction of the precipitate, was evaporated to dryness under reduced pressure and the residue extracted with absolute ethanol. This alcoholic extract was passed through a charcoal column. After elution from the latter of the inorganic salts with water, the organic material was removed with 50% ethanol. Evaporation of the solvent yielded a sirupy material which formed a hydrochloride, m.p. 185–187°. For chromatographic data, see Table II.

N-Methyl-5-pyrrolidone-2-carboxylic Acid (V) from N-Methylglutamic Acid.—One gram (0.0068 mole) of N-methylglutamic acid prepared according to Knoop[9] was heated at 170–180° in a silicone oil-bath for 4 hr. The dark brown reaction mixture was dissolved in 3 ml. of water and put on an Amberlite-100 column (30 cm.). The unreacted N-methylglutamic acid and the N-methyl-5-pyrrolidone-2-carboxylic acid formed by dehydration and lactam formation were eluted from the column with three portions of 2 *N* hydrochloric acid, yielding three fractions. The second fraction (300 ml.) contained practically pure N-methyl-5-pyrrolidone-2-carboxylic acid (V) as shown by chromatographic tests (Table II). This aqueous solution, on evaporation to dryness, yielded 0.6 g. of a yellow, partly crystalline sirup.

Reduction of N-Methyl-5-pyrrolidone-2-carboxylic Acid (V) to Hygrinic Acid.—Substance V (0.1 g., 0.007 mole) was dissolved in 10 ml. of absolute ethanol. To this was added 0.5 g. (0.022 mole) of sodium. After the reaction subsided, the mixture was heated on a water-bath for 1.5 hr., cooled, neutralized with 3 *N* hydrochloric acid and finally boiled with an excess of cupric carbonate, suspended in the solution. The cupric hygrinate was isolated and purified as described by Karrer.[3]

Identical results were obtained with V from N-methylglutamic acid (copper salt, m.p. 207–208° dec.,[10] and with V from cotinine (copper salt, m.p. 208°)[10]). Admixture of the two cupric hygrinates produced no depression. The results of chromatography of the hygrinic acid obtained are shown in Table II.

TABLE II

R_f FACTORS FOR N-METHYL-5-PYRROLIDONE-2-CARBOXYLIC ACID (V) AND HYGRINIC ACID

	Ethanol: propanol ammonia: buffer[a,b] (60:10:30)	Ethanol:*t*-amyl alcohol: ammonia buffer (60:10:30)	Phenol: water: formic acid (90%)[c] (30:10:0.1)
V from cotinine	0.50 ± 0.02	0.47 ± 0.02	0.26 ± 0.02
V from N-methylglutamic acid	.48 ± .03	.45 ± .01	.25 ± .01
Hygrinic acid from cotinine *via* V	.54 ± .01	.51 ± .03	.69 ± .00
Hygrinic acid from N-methylglutamic acid *via* V	.58 ± .04	.54 ± .02	.70 ± .03

[a] A. J. Van Duuren, *Rec. trav. chim.*, **72**, 889 (1953). [b] Ammonia buffer consists of mixture of equal parts of 1.6 *N* ammonia and 1.6 *N* ammonium carbonate. [c] S. L. Ranson in *Modern Methods of Plant Analysis*, K. Paech and H. V. Tracey, Vol. II, Springer Verlag, Berlin, 1955, pp. 539–582.

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(9) Knoop and Oesterlin, *Z. physiol. Chem.*, **148**, 309 (1925); "Beilsteins Handbuch der organischen Chemie," 2nd Supplement, Vol. IV, Springer Verlag, Berlin, 1942, p. 901.

(10) R. Willstätter and Ettlinger, *Ann.*, **326**, 91 (1903), reported a melting point of 209–210° for this copper salt.