was concentrated to give the oily free amine, and 4.0 ml. of formalin was added to form the Schiff base with evolution of heat. The mixture was warmed on the steam-bath for 20 minutes with trituration and extracted with benzene. The benzene solution was concentrated to the free Schiff base to which 12 ml. of 1:1 hydrochloric acid was added at room temperature. Heat was evolved and crystals of the hydrochloride formed in a few minutes. After standing overnight, the crystals were removed by filtration, 3.48 g. (64%), m.p. 215–230° dec. A second crop of crystals precipitated from the mother liquor, 0.396 g. (7%), m.p. 222–228° dec. The latter crop contained a second isomer that was not purified or further investigated. The material from the first crop was recrystallized three times from methanol–ether to give fine needles, m.p. 240–242° dec.

Anal. Calcd. for $C_{14}H_{18}NOCl$: C, 66.79; H, 7.21; N, 5.57. Found: C, 66.87; H, 7.21; N, 5.68.

The phenylthiourea of VI was prepared by the method of Shriner and Fuson[14] in 81% yield. This compound showed polymorphism; it melted at 158°, resolidified, and melted again at 176–178°. Two recrystallizations from methanol gave fine needles, m.p. 176–178°.

Anal. Calcd. for $C_{21}H_{22}N_2OS$: C, 71.96; H, 6.33; S, 9.15. Found: C, 72.18; H, 6.33; S, 8.86.

6-Methoxy-10-methyl-1,4,4a,9,10,10a-hexahydrophenanthridine (VII).—This compound was prepared by the N-methylation procedure of Icke, Wisegarver and Alles.[15] A solution of 1.33 g. (5.3 mmoles) of 6-methoxy-1,4,4a,9,10,10a-hexahydrophenanthridine hydrochloride in water was made basic with 5% sodium hydroxide solution and extracted with ether. The ether extracts were concentrated to give the free amine. To this amine was added 1.5 ml. of 88% formic acid and 1.5 ml. of formalin. The reaction mixture was heated under reflux. During the first 15 minutes strong evolution of gas was noted. After 19 hours of heating, 2 ml. of 1:1 hydrochloric acid was added and the mixture was concentrated to a sirupy oil. Crystallization of the hydrochloride was induced by trituration with acetone, 1.36 g. (96%) of white powder, m.p. 199–207° dec. Two recrystallizations from isopropyl alcohol and one from methanol–ether gave colorless prisms, m.p. 211.5–213° dec.

Anal. Calcd. for $C_{15}H_{20}NOCl$: C, 67.78; H, 7.59; N, 5.27. Found: C, 67.72; H, 7.47; N, 5.29.

Acknowledgment.—This work was made possible by a grant to Princeton University from the Eugene Higgins Fund.

PRINCETON, NEW JERSEY

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[14] R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds," 3rd ed , John Wiley and Sons, Inc., New York, N. Y., 1948, p. 179.

[15] R. N. Icke, B. B. Wisegarver and G. A. Alles, *Org. Syntheses*, **25**, 89 (1945).

[CONTRIBUTION FROM THE CENTRAL RESEARCH INSTITUTE OF THE JAPAN MONOPOLY CORPORATION]

Degradation of Nicotine by Soil Bacteria[1]

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Pseudoöxynicotine (III) and the previously unknown 3-nicotinoylpropionic acid (IV) were isolated from microbial degradation products of a medium containing nicotine.

In connection with studies of tobacco alkaloids, several investigations have been made on the biochemical and chemical decomposition of nicotine.[2]

Frankenburg and co-workers[3–5] have shown that considerable amounts of the nicotine contained in cigar tobacco leaves are converted, during fermentation of the leaves, into other 3-substituted pyridine compounds such as oxynicotine, 3-pyridyl methyl ketone, 2,3′-dipyridyl and nicotinic acid.

Wenusch[6] isolated N-methylmyosmine and a purple crystalline substance from a nicotine phosphate medium as a degradation product of nicotine by bacteria. Bucherer[7] also reported the isolation of a purple crystalline substance from a nicotine-containing culture medium after incubation with three species of bacteria: *Bacterium nicotinophagum*, *B. nicotinovorum* and *B. nicotinobacter*.

Neither Wenusch nor Bucherer, however, supplied exact details concerning the substances obtained.

In studying the decomposition of nicotine by livers of rabbits and guinea pigs, etc., Werle, *et al.*,[8] considered that N-methylmyosmine and nicotyrine may be intermediate products of nicotine degradation, and that they may be decomposed further.

In our research the microbial degradation of nicotine, employing a species of bacteria isolated from the soil was examined. The present paper deals with some investigation on the mechanism of degradation of nicotine with special reference to transformation products produced by bacteria.

We have isolated the previously unknown 3-nicotinoylpropionic acid (IV), pseudoöxynicotine (III) and a purple crystalline substance as decomposition products of nicotine. No evidence of the presence of nicotinic acid was found.

Although Frankenburg and Gottscho have isolated nicotinic acid from processed cigar tobacco,[5] the authors of this paper are unaware of any other evidence for the presence of nicotinic acid among the degradation products of pure nicotine in biological systems. In a study on dogs, Larson,[9,10] however, noted that following nicotine administration the urine contained a compound that yielded a red color with cyanogen bromide and further noted that the compound was not extractable with ether from alkaline urine, thus suggesting the existence of a carboxyl group at the end of the side chain.

Haines and Eisner[11] prepared pseudoöxynicotine

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[1] A preliminary note on this topic was published in *Science*, **117**, 152 (1953).

[1a] Department of Botany, Columbia University, N. Y.

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[8] E. Werle and K. Koekbe, *Ann.*, **562**, 60 (1949).

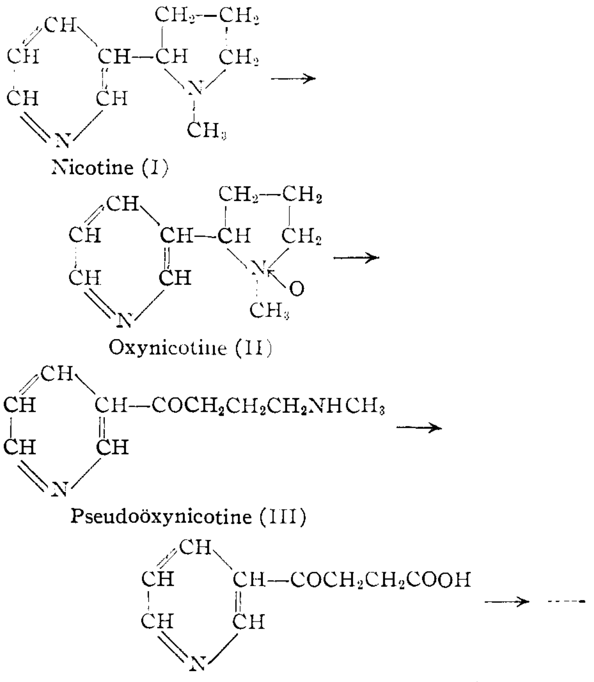
[9] P. S. Larson, *Ind. Eng. Chem.*, **44**, 279 (1952).

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

[11] P. G. Haines and A. Eisner, THIS JOURNAL, **72**, 1720 (1950).

by heating oxynicotine with hydrochloric acid in a sealed tube at 140° and identified pseudoöxynicotine as a 3-pyridyl-3-methylaminopropyl ketone. On the other hand, Pinner and Wolfenstein[12] obtained oxynicotine by moderate oxidation of nicotine at room temperature.

From these and our own results, we may assume the process of the decomposition of nicotine by the microörganisms to be



Nicotine (I)

Oxynicotine (II)

Pseudoöxynicotine (III)

3-Nicotinoylpropionic acid (IV)

Nicotine is believed to be oxidized first to oxynicotine by the organism, or merely by the action of aeration, and then to be converted to pseudooxynicotine (III). The presence of 3-nicotinoylpropionic acid (IV) is believed to be the result of the utilization of the nitrogen at the side chain of pseudoöxynicotine (III) as a nitrogen source by the microörganism.

Organism and Fermentation Process.—Many bacteria which can utilize nicotine as a carbon source as well as a nitrogen source were obtained from soil. One of these bacteria was temporarily named No. 41 and belongs most probably to *Pseudomonas*. This strain was employed in our investigation. When these bacteria were incubated into media containing both nicotine and such fermentable substances as glucose, however, they lost their ability to ferment nicotine and used only glucose or similar substances. On the basis of this fact, it is believed that the ability of these bacteria to ferment nicotine is a result of adaptation.

A synthetic medium, described in the Experimental part, was used. In 12–24 hours after incubation, the nicotine content sharply decreased, the number of the organisms increased, and the medium suddenly became muddy. The timing and extent of this effect varies to some extent with the initial nicotine content of the medium. Simultaneously with the decrease of nicotine, the *p*H of the solution was sharply reduced from 6.4 to 4.6, probably due to the production of 3-nicotinoylpropionic acid (IV). With this *p*H decrease, the multiplication of the bacteria and the consumption of nicotine ceased. About 35% of the original nicotine remained unchanged.

Properties and Identification of Degradation Products.—Two crystalline substances and an oil identified as pseudoöxynicotine were obtained from the culture medium. One of the crystallline substances was acidic and was obtained in form of colorless needles which melted at 161–162°. When dissolved in water (1% solution), the compound gave a solution of *p*H 4.4 and when a small quantity of the compound was added to sodium bicarbonate solution, carbon dioxide was evolved. It gave a red coloration with sodium nitroprusside,[13] showing the presence of a carbonyl group, and it gave also a strong 2,4-dinitrochlorobenzene reaction, indicating a pyridine nucleus. It gave a semicarbazone which decomposed at 202–203°. Nicotinic acid was isolated from its oxidation with permanganate. In the Beckmann rearrangement, the compound gave succinic acid and 3-aminopyridine, and was thus identified as 3-nicotinoylpropionic acid (IV).

The ultraviolet absorption spectra yielded the following characteristics. In 95% ethanol, maxima occurred at 228.5 (E_M 8632) and at 267 mμ (E_M 2971) and a minimum at 250 mμ. In 0.1 N HCl, maxima were found at 222 (E_M 4933) and 263 mμ (E_M 4019) with minima at 211 and 243 mμ. In 0.1 N NaOH the maxima were located at 230 (E_M 9550) and 267 mμ (E_M 3678) with a minimum at 251 mμ.

The second crystalline compound was purple in color and resembled closely the substances obtained by Wenusch[6] and Bucherer.[7] This compound has not yet been identified.

The oily pseudoöxynicotine was vacuum distilled at 91–93° under 2 mm. It formed a picrate melting at 154–156°. The ultraviolet absorption spectrum of the substance showed maxima at 223 mμ (E_M 6480) and 263 mμ (E_M 5580) and minima at 211 mμ and 241 mμ in 0.1 N hydrochloric acid. In 0.1 N NaOH maxima occurred at 261 (E_M 3843) and at 225.5 mμ (E_M 6190) with a minimum at 250 mμ.

The substance gave a 2,4-dinitrophenylhydrazone melting at 224–225°. According to Haines and Eisner,[11] the picrate of pseudoöxynicotine melts at 128–130° when recrystallized from water and at 158–160° from absolute methanol. Their ultraviolet absorption spectra of pseudoöxynicotine showed maxima at 223 and 263 mμ and minima at 211 and 241 mμ.

Experimental[14]

Fermentation Procedure.—Strain No. 41 was employed in our experiments. The synthetic medium consisted of 0.1% KH_2PO_4, 0.05% $MgSO_4$; $FeSO_4$ (1% solution) 0.3 ml./l., $CaCl_2$ (1% solution) 0.3 ml./l.; $MnSO_4$ trace. To the basic medium of this composition was added 2 g. of nicotine per liter, the *p*H being adjusted to 6.4 with hydrochloric acid. The organism was cultured in a 10-l. vessel (containing 5 l. of medium) equipped with an aeration at-

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(12) A. Pinner and R. Wolfenstein, *Ber.*, **25**, 1428 (1892).

(13) V. Bitot, *Ann.*, **267**, 372 (1892).

(14) All melting points are uncorrected.

tachment at 30° for 5 days. The vessel and medium were sterilized at 100° for an hour. A half-liter of a 2-day culture of the organism in the same medium was used as an inoculum.

Nicotine Analysis.—During incubation the nicotine content of the culture medium was determined by means of the silicotungstic acid method.[15] Aliquots of the solution were taken at regular intervals, made strongly alkaline and distilled. Due to the volatility of pseudoöxynicotine and to its insolubility as the silicotungstate, the assay values for residual nicotine so obtained were somewhat high. The exact magnitude of this error cannot be ascertained with methods presently available.

Isolation of the Degradation Products.—The broth (5 l.) was thoroughly mixed with Celite Filter Aid and a precipitate was removed by filtration. The filtrate was concentrated to about 500 ml. under reduced pressure. A purple crystalline substance precipitated, and was removed by filtration. The filtrate, after adjustment to *p*H 4.4, was extracted with ether for about 80 hours. The ethereal extract was evaporated, leaving a white crystalline substance as a residue (IV) (yield 2.24 g.). The aqueous extraction residue was then adjusted to a *p*H between 8 and 9, extracted with ether 2–3 times, thus removing almost all of the unchanged nicotine. The remaining aqueous phase was nearly saturated with sodium chloride, and after addition of enough sodium hydroxide to make it strongly alkaline, again extracted with ether. The ethereal extract was dried with anhydrous sodium carbonate overnight, and the ether removed. The oil residue distilled at 91–93° under a pressure of 2 mm. There was 1.3 g. of a pale yellow distillate (III), a small amount of nicotine in the first distillate and a fairly large non-distillable residue which solidified to a black solid upon cooling.

Identification of 3-Nicotinoylpropionic Acid (IV).—The colorless crystalline substance IV, 3-nicotinoylpropionic acid, obtained as described above, was recrystallized from 60% methanol 3 times and melted at 161–162°.

Anal. Calcd. for $C_9H_9O_3N$: C, 60.33; H, 5.03; N, 7.84. Found: C, 60.08; H, 5.00; N, 7.44.

Semicarbazone of 3-Nicotinoylpropionic Acid (IV).—A mixture of 0.1 g. of the substance (IV), 0.35 g. of semicarbazide hydrochloride, 0.35 g. of potassium acetate, 1.0 ml. of water and 20 ml. of methanol was refluxed for an hour on a water-bath. After standing overnight, a colorless needle-like crystalline substance was precipitated. After recrystallization from 60% methanol, the semicarbazone melted at 228–229° (yield 0.28 g.).

Anal. Calcd. for $C_{10}H_{12}O_3N_4$: C, 50.84; H, 5.08; N, 23.72. Found: C, 51.00; H, 4.95; N, 23.25.

Beckmann Rearrangement of 3-Nicotinoylpropionic Acid (IV).—For esterification 3 g. of the substance were dissolved in 30 ml. of absolute ethanol and to this solution was added 9 g. of concentrated sulfuric acid. This solution was refluxed for 7 hours on a water-bath. After the alcohol was distilled off, the mixture was diluted with 50 ml. of water and was made alkaline. The alkaline solution was extracted with ether and the ethereal solution was dried over potassium carbonate for one day. After evaporation of the ether, the ester was obtained as a residue. A mixture of 1 g. of the ester, 1 g. of hydroxylamine hydrochloride, 5 ml. of pyridine and 5 ml. of absolute ethanol was refluxed for 2 hours on a water-bath. After the solvents were removed by evaporation, the residue was brought to dryness under reduced pressure. The residue was triturated thoroughly with 30 ml. of cold water and the mixture was filtered. The oxime was obtained almost quantitatively in the form of aggregated needles (m.p. 72–73°).

Anal. Calcd. for $C_{11}H_{14}N_2O_3$: C, 59.46; H, 6.30; N, 12.61. Found: C, 59.83; H, 6.01; N, 12.87.

To this ketoxime (*ca.* 1 g., *ca.* 0.0045 mole) in chloroform (30 ml.) was added slowly a slight excess of phosphorus oxychloride (*ca.* 0.8 g., *ca.* 0.0052 mole). Exothermal reaction occurred with evolution of gas. Evaporation of the resultant yellow solution under reduced pressure gave a yellow solid. On hydrolysis of this solid by refluxing for 2.5 hours with 3% hydrochloric acid (20 ml.) on a water-bath, succinic acid (yield 0.18 g., 52.1%, m.p. 181–183°) and 3-aminopyridine (yield 0.21 g., 74.4%, m.p. 61–63°) were obtained. These products showed no depression of melting points when mixed with authentic succinic acid and with synthetic 3-aminopyridine[16] (m.p. 63–65°), respectively.

Identification of Pseudoöxynicotine (III).—The oily substance III, pseudoöxynicotine, obtained as described above, was vacuum distilled at 91–93° and 2 mm. Its picrate melted at 127–130° when recrystallized from water and melted at 154–156°, when recrystallized from absolute methanol.

Anal. Calcd. for $C_{22}H_{20}N_2O_{15}$: C, 41.51; H, 3.15; N, 17.61. Found: C, 42.14; H, 3.92; N, 18.23.

Some Derivatives of Pseudoöxynicotine (III).—These derivatives were obtained according to the methods described by Haines, *et al.*[11]

0.638 g. of 2,4-dinitrophenylhydrazone was obtained from 0.4 g. of pseudoöxynicotine (III), and melted at 224–225° (lit.[11] reports 224–225°).

After recrystallization from absolute ethanol, pseudooxynicotine dihydrochloride melted at 195–197° (lit.[11] reports 196–198°).

Addition of an aqueous solution of pseudoöxynicotine dihydrochloride (III) to excess aqueous solution of mercuric chloride gave white crystals melting at 210–213° (lit.[11] reports 211–213°).

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TOKYO, JAPAN

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[15] C. V. Bowen and W. F. Berthel, *Ind. Eng. Chem., Anal. Ed.*, **15**, 740 (1943).

[16] This was kindly supplied by Mr. T. Hirose, National Institute of Hygiene in Tokyo.