distillation in the presence of magnesium oxide and Devarda alloy.[4]

Fraction 2a.—The aqueous acidic solution of fraction 2 was concentrated on a water-bath to *ca.* 200 ml., adjusted to *p*H 10 with aqueous sodium hydroxide, and then shaken out with *t*-amyl alcohol[17] (previously saturated with water) 12 times, using 80 ml. of alcohol for each extraction. After every third extraction the *p*H of the aqueous phase was re-adjusted to 10 with sodium hydroxide. The combined extracts constitute fraction 2a.

Fraction 2b.—The aqueous residue of the *t*-amyl alcohol extraction, rich in oxynicotine, was made 1 *N* with hydrochloric acid and shaken out with ethyl ether (to remove the *t*-amyl alcohol). The aqueous residue was adjusted to *p*H 10 with sodium hydroxide and extracted with ethyl ether in a liquid–liquid extraction apparatus. The aqueous residue then was adjusted to *p*H 3 and re-extracted in a liquid–liquid apparatus with ethyl ether. All the ether extracts were discarded The residue of the third ether extraction (at *p*H 3) was fraction 2b.

Purification of Oxynicotine in Fraction 2b.—After neutralizing fraction 2b, it was heated to boiling; 4 g. of bone charcoal was added, the mixture shaken, and the charcoal was filtered off. The dark yellow filtrate was adjusted to *p*H 2 with hydrochloric acid, and a 12% solution of silicotungstic acid (STA) slowly added until no further precipitation occurred. After standing at 6° overnight, the precipitate was filtered off, and washed with hydrochloric acid (0.01 *N*).

The STA precipitate was suspended in water and 0.5 *N* sodium hydroxide was added until solution occurred. An excess of solid barium hydroxide was added and the mixture agitated intermittently for 6 to 7 hr. After the solids had settled, a few drops of supernatant liquid were tested with hydrochloric acid. The absence of a precipitate in this test indicates complete removal of the STA as the Ba salt. If a precipitate formed, the shaking with barium hydroxide was continued. When the test was negative, the solids were filtered off and discarded.

The excess barium was removed from the filtrate with sulfuric acid. The filtrate was precipitated once more with STA and the STA precipitate decomposed with barium hydroxide as already described. The final filtrate from this second purification *via* STA and barium hydroxide was neutralized and evaporated to dryness *in vacuo* at 40–50°. The solid residue was extracted with ethyl acetate.

Isolation of Oxynicotine as the Dipicrate.—The ethyl acetate extract was mixed with a saturated aqueous picric acid solution and ethyl acetate was allowed to evaporate off at room temperature, leaving yellow crystals in the aqueous phase. After standing for at least 24 hr. at 6°, these crystals were collected and recrystallized twice from boiling water, m.p. 167–168°, mixed m.p. 157–160°.

Anal. Calcd. for $C_{22}H_{20}O_{15}N_8$: C, 41.51; H, 3.17; N, 17.61. Found: C, 41.76; H, 3.24; N, 17.64.

Decomposition of Oxynicotine Dipicrate into its Component Parts.—Six hundred and fifty-one mg. of picrate was dissolved in hydrochloric acid (2.5 *N*) and extracted quantitatively with ethyl ether in a liquid–liquid extraction apparatus. The picric acid was extracted from the ether by aqueous sodium hydroxide and its concentration in this aqueous solution after acidification was determined spectrophotometrically. The ultraviolet absorption spectrum of the aqueous acidic residue of the liquid–liquid ether extraction was measured. In agreement with the spectrum of authentic oxynicotine, it showed a maximum at 258 mμ, and a minimum at 227 mμ. From the weight of the original picrate taken and the picric acid recovered, the weight of oxynicotine in this residue was computed. From this data the absorptivity at the absorption maximum of 258 mμ of the isolated oxynicotine was determined: calcd. from measurements of authentic oxynicotine: 31.4; found, 29.8.

The composition of the picrate was also computed: Calcd. for oxynicotine dipicrate, picric acid: 72.0%; found, picric acid, 72.4% (average of 2 determinations).

Reduction of Oxynicotine to Nicotine.—An aliquot containing 138 mg. of oxynicotine, evaluated spectrophotometrically, was steam distilled in the presence of magnesium oxide and Devarda alloy.[4] The nicotine in the distillate was determined spectrophotometrically.[10]

Calcd. for 100% reducibility of oxynicotine: 125 mg. of nicotine; found: 121 mg. of nicotine.

A picrate of the alkaloid which appeared in the distillate was prepared, m.p. 220–221°. A mixed m.p. with authentic nicotine dipicrate produced no depression.

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(17) The use of this solvent for this purpose was called to the authors' attention by Dr. A. Eisner, Eastern Utilization Research Branch, U. S. Department of Agriculture, Philadelphia 18, Pa.

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

The Chemistry of Tobacco Fermentation. I. Conversion of the Alkaloids. C. The Formation of 3-Pyridyl Propyl Ketone, Nicotinamide and N-Methylnicotinamide

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Additional nicotine degradation products formed during the fermentation of cigar filler tobacco are 3-pyridyl propyl ketone, nicotinamide and N-methylnicotinamide. An additional and major degradation product of nicotine was detected by paper chromatography, but not identified.

The chloroform fraction 2 obtained during the successive solvent extractions of fermented cigar tobacco leaves[1] (Pennsylvania Seedleaf, U. S. Type 41) contains a large proportion of the degradation products of nicotine formed during the fermentation[2] process. From the absorbance at *ca.* 260 mμ of the aqueous solution of this fraction 2, it is estimated that its content of these newly formed pyridine compounds can be as high as 30% of the nicotine content present in the leaves prior to fermentation.[1,2]

The chloroform fraction 2 is fractionated into two subfractions 2a and 2b.[2,3] Oxynicotine (I), a major component of fraction 2, is found enriched in the subfraction 2b. This paper deals with the further

(1) W. G. Frankenburg, A. M. Gottscho, E. W. Mayaud and T. C. Tso, THIS JOURNAL, **74**, 4309 (1952).

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

(3) W. G. Frankenburg and A. M. Gottscho, THIS JOURNAL, **77**, 5728 (1955).

fractionation of subfraction 2a and with the identification of the pyridine bases contained in it.

In the petroleum ether extract, fraction α, made from subfraction 2a, there are present, besides small amounts of nicotine,[4] some weakly basic pyridine derivatives. The latter are separated from nicotine, yielding fraction γ. Compound II, **3-pyridyl propyl ketone** was identified as the main component of fraction γ.

In fraction β, obtained by extracting the residue of the petroleum ether extract with ethyl ether, two pyridine compounds III and IV predominate, IV being the most abundant component. Compound III was isolated in pure form, and proved to be **N-methylnicotinamide.** By means of paper chromatography with three different solvent systems, **nicotinamide** (V), **3-pyridyl methyl ketone** (VI) and **nicotinic acid** (VII)[5] were identified as minor components of fraction β. The chemical structure of the main component IV in fraction β has not yet been clarified completely. It polymerizes rapidly with a concurrent loss of solubility in organic solvents,[6] and, at higher states of polymerization, in water.[7]

Identification of 3-Pyridyl Propyl Ketone (II) and of Minor Components in Fraction γ.—The ultraviolet spectra of fraction γ in aqueous acid and alkali showed the features characteristic for 3-pyridyl alkyl ketones marked by the appearance of a high absorption maximum at *ca.* 230 mμ in alkali. An aliquot of γ yields, on paper chromatography with three different solvent systems, predominant spots at the R_f positions listed in Table I for component II. An authentic specimen of 3-pyridyl propyl ketone yielded on chromatography with the same solvent systems, the same three R_f values. Furthermore, a mixture of fraction γ with authentic 3-pyridyl propyl ketone gave the identical chromatogram, except that the spots at the R_f positions for compound II were intensified. Weaker spots appearing on the chromatograms at other R_f positions indicated that traces of 3-pyridyl methyl ketone, N-methylnicotinamide and substance IV (see Table I) were present in fraction γ.

Investigation of Fraction β.—Tests showed that ether extract β contains no unsaturated compounds but a positive Tollens test indicated the presence of reducing groups. The ultraviolet spectra of this fraction in aqueous acid and alkali showed maxima at 261 mμ and minima at *ca.* 238 mμ. Inconsistencies of the absorbance ratios of the maxima to the minima of these spectra observed on different samples of fraction β, as well as their infrared spectra, proved that β contains a mixture of related pyridine derivatives rather than one single compound.

(4) Nicotine is found here in spite of the fact that it had been quantitatively removed from the tobacco in fraction 1.[2] Apparently this nicotine is an artefact formed during the working up of these fractions.

(5) Nicotinic acid is not soluble in chloroform. Its presence in this and other fractions derived from fraction 2 is ascribed to hydrolytic action on the amides and possibly to slow oxidation of some of the other nicotine degradation products.

(6) Because of this property, polymers of IV remain in the aqueous phase of all the successive extractions applied to the chloroform extract, fraction 2.

(7) The water-insoluble pyridine compounds found in fermented cigar tobacco leaves are probably highly polymerized products of IV.

Chromatograms of fraction β and of the oil fraction δ (obtained from β by removal of the solvent) with the three solvents, showed very strong spots for the unknown substance IV (Table I), and fairly strong spots for III. Appreciably weaker spots appeared for V, VI and VII. On adding an authentic sample of any one of these four components to an aliquot of fraction δ, and chromatographing the mixture using the solvents listed in Table I, the spots on the chromatograms appeared unchanged except that the spot corresponding to the substance added was intensified. No model substance investigated produced spots at the R_f positions found for substance IV.

TABLE I

R_f, VALUES[a] OF VARIOUS PYRIDINE COMPOUNDS IN THREE SOLVENT SYSTEMS

Compound	Solvents: Butanol–ethanol–sodium acetate–acetic acid buffer (pH 5.6) (50:10:40)	Butyl acetate–methanol–0.25% ammonia (95:5:25)[b]	Benzene–methanol–sodium acetate–acetic acid buffer (pH 5.6) (60:15:25)
Nicotinic acid[c]	0.41 ± 0.02	0.00	0.00
Compound VII	.40 ± .02	.00	.00
Nicotinamide[c]	.64 ± .03	.23 ± 0.01	.00
Compound V	.64 ± .02	.23 ± .02	.00
N-Methylnicotinamide	.77 ± .03	.36 ± .02	.12 ± 0.01
Compound III	.76 ± .01	.36 ± .01	.12 ± .02
3-Pyridyl methyl ketone[c]	.80 ± .01	.59 ± .03	.30 ± .01
Compound VI	.81 ± .03	.58 ± .03	.29 ± .02
3-Pyridyl propyl ketone[d]	.86 ± .01	.81 ± .01	.71 ± .00
Compound II	.86 ± .01	.80 ± .01	.71 ± .01
Unknown substance IV	.81 ± .02	.36 ± .03	.48 ± .01

[a] The values listed are averages of five determinations. [b] W. L. Porter, J. Naghski and A. Eisner, *Arch. Biochem.*, **24**, 461 (1949). [c] Commercially available preparations. [d] Kindly supplied by Dr. R. L. Frank, University of Illinois, Urbana, Ill.

Inasmuch as substance IV and N-methylnicotinamide are the predominant components in fraction β, their isolation was attempted. Substance IV was isolated by column chromatography and work aiming at its identification is in progress. The presence of N-methylnicotinamide was confirmed by acid hydrolysis of the oil (fraction δ) yielding nicotinic acid and methylamine as hydrolysis products. Vacuum sublimation of this oil yielded colorless needles of III. The melting point and the ultraviolet spectra in aqueous acid and alkali solutions of these needles confirmed that III is N-methylnicotinamide.

Discussion

The identification of 3-pyridyl propyl ketone (II) as a degradation product of nicotine during the fermentation of cigar filler tobacco substantiates further the proposed pathways[1] of the nicotine breakdown *via* either N-methylmyosmine (VIII) or myosmine (VIIIa) (Fig. 1). In the conversion VIII or VIIIa → II → VI → VII the pyrroline ring of VIII is opened at the 1,2-bond.

To fit into this scheme, the formation of N-methylnicotinamide (III) and nicotinamide (V) has to be explained by secondary reactions of nicotinic

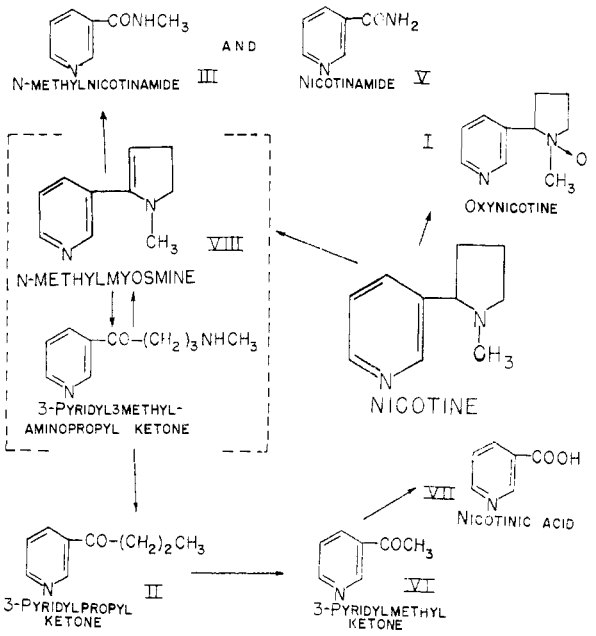

Fig. 1.—Proposed pathway for the formation of degradation products of nicotine in fermented Pennsylvania Seedleaf cigar tobacco.

acid (VII) with methylamine and with ammonia, respectively. However, the fact that the amounts of N-methylnicotinamide found in the fermented leaves exceed by far those of nicotinamide, whereas the quantities of methylamine (derived from the reaction VIII → II) available in the leaf tissues are much smaller than those of ammonia, strongly indicates that both amides are not formed by way of secondary reactions.

Rather, it appears necessary to postulate a direct formation of III and of V from VIII and VIIIa by an alternate pathway such as a simultaneous opening of the pyrroline rings of VIII and VIIIa at the 5,1- and the 2,3-positions.

In any case, there is no doubt that VIII is an important intermediate in the degradation of nicotine, as *in vitro* experiments with VIII and hydrogen peroxide have shown. Among the reaction products of these experiments, a major component was III, with II and VII being formed in smaller amounts.

Experimental[8-10]

Preparation of Fractions α and γ.—The *t*-amyl alcohol extract[1,3] of fraction 2a was distilled off *in vacuo* from 1 *N* sulfuric acid, yielding a brown aqueous solution. The last traces of the alcohol were removed from this solution by shaking it out with ethyl ether. The aqueous acid solution was adjusted to *p*H 9 with sodium hydroxide and extracted (LL)[11] with petroleum ether for 40 hr. The aqueous residue of this extraction was used for the preparation of fraction β. The petroleum ether extract is fraction α.

The petroleum ether, fraction α, was shaken with 0.1 *N* hydrochloric acid and evaporated off from the aqueous phase. The latter was adjusted to *p*H 3.5 with sodium hydroxide and extracted (LL) for 80 hr. with ether. The ether extract, fraction γ, after drying over anhydrous sodium sulfate and evaporation of the solvent, yielded a yellowish oil, having only partial solubility in water. This oil in aqueous acid solution displayed the characteristic ultraviolet spectra for 3-pyridyl alkyl ketones. A solution of the oil, *ca.* 1 mg. per ml., in 95% ethanol was used for chromatography.

Preparation of Fractions β and δ.—The aqueous residue obtained from the petroleum ether extract, fraction α, was adjusted to *p*H 10 with sodium hydroxide and extracted (LL) for 40 hr. with ethyl ether. Drying the ether extract, fraction β, over anhydrous sodium sulfate and evaporation of the ether yields a yellow viscous oil, fraction δ. This oil is easily soluble in water, but only partially soluble in ether.[6] Aqueous solutions of fraction δ having a concentration of *ca.* 1 mg. per ml. were used for chromatographic separations to show the presence of the major components N-methylnicotinamide (III), the unknown substance IV and of the minor components, nicotinamide (V), 3-pyridyl methyl ketone (VI) and nicotinic acid (VII).[4]

Acid Hydrolysis of Fraction δ.—An aliquot (135 mg.) of the oil (fraction δ) was dissolved in 30 ml. of 3 *N* hydrochloric acid and refluxed for 6 hr. After alkalinization with 6 *N* sodium hydroxide the sample was steam distilled into dil. hydrochloric acid. From the distillate 16 mg. of an amine hydrochloride, m.p. 226°, was isolated. The amine showed all the characteristic reactions of a primary amine and a mixed melting point of this hydrochloride with methylamine hydrochloride showed no depression.

The residue of the steam distillation was adjusted to *p*H 3 and extracted (LL) with ethyl ether for 40 hr. The ether extract yielded after drying and evaporating the solvent, 28 mg. of nicotinic acid, as shown by its melting point and by its ultraviolet absorption spectrum.[12] The yield of nicotinic acid indicates that N-methylnicotinamide comprises *ca.* 25% of fraction δ.

Sublimation of Fraction δ under Reduced Pressure.—An aliquot (100 mg.) of fraction δ was sublimed at 140° and 4 mm. pressure. The sublimate (35 mg.), an almost colorless oil, yielded long needles when stored at 3°. After trituration and recrystallization from benzene *ca.* 5 mg. of colorless needles, m.p. 102°, were obtained. A mixed melting point with authentic N-methylnicotinamide[13] gave no depression. The ultraviolet absorption spectrum of III is identical with that of N-methylnicotinamide (in acidic aqueous solution: max. at 263 mμ, ε 35.51; min. at 242 mμ, ε 21.43. In alkaline aqueous solution: max. at 262 mμ, ε 23.85; min. at 250 mμ, ε 21.54).

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(8) Spectrophotometric measurements were made in a Beckman model DU spectrophotometer.[1] All chromatograms were run on Whatman No. 1 paper, using the ascending technique.

(9) Chromatograms were developed by using the reaction described by W. König, *J. prakt. Chem.*, **69**, 105 (1904).

(10) After spraying with *p*-aminobenzoic acid, the chromatograms were exposed to cyanogen bromide vapors using the technique of E. Kodicek and K. K. Reddi, *Nature*, **168**, 475 (1951).

(11) Extraction in a liquid–liquid extraction apparatus.

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(13) A. Pictet and G. Sussdorf, *Chem. Zentr.*, **69**, I, 677 (1898), Beilstein's *Handbuch der Org. Chemie*, Vol. XXII, Springer Verlag, Berlin (1935) p. 40.