and 0.5 cc of water solution of methylene blue containing 0.1 mg was placed in the side arm. After the vessel was filled with oxygen and the dye dumped into the main chamber, the oxygen uptake was measured in the usual fashion at the desired water-bath temperature. A rapid oxygen uptake took place which stopped sharply after about 4 hrs at 40°, when an equimolar amount of oxygen had been taken up. The optimum pH for the oxygen uptake was about 9. There was no CO_2 evolution.

Irradiation experiments under anaerobic condition in Thunberg tubes, to prevent reoxidation of the leuco dye, showed that the bleaching time is practically not affected at temperatures of 30°, 40°, 50°, 60°, and 70°, thus indicating a true photochemical reaction in this phase. The reaction appeared to be reversible, although it proceeded slowly, as in the thionine-iron system (*2, 4, 5*). The aerobic photo-oxidation of nicotine, however, is strongly accelerated by increasing temperature, which would indicate the participation of a dark reaction in addition to the previously observed light-sensitive reaction.

The N—CH_3 group of the pyrrolidine ring seems to be important in the reaction, since under similar conditions *d,l*-nornicotine[2] acts sluggishly. Pyridine, β-picoline, β-vinylpyridine, nicotinic acid, and nicotinamide are unreactive. N—CH_3-pyrrolidine, however, which is a part of the nicotine molecule, is photo-oxidized in the presence of the dye at the same rate and to the same extent as nicotine itself. These experiments indicate that the chemical changes due to the irradiation are confined to the N—CH_3-pyrrolidine ring.

The product (obtained by larger-scale experiments) is a yellow viscous oil, not volatile in steam, practically insoluble in ether, but soluble in chloroform, in polar solvents, and in water. It is optically active ($\alpha_D^{20} = -35.2°$), indicating that the asymmetric carbon atom is not involved in the reaction. During the irradiation, the pH of the reaction mixture dropped from 10.4 to 6.8, suggesting that the strongly basic pyrrolidine nitrogen is involved in the reaction by forming, for example, an amine oxide. The elementary analysis of the ether-insoluble irradiation product of nicotine was: Calculated for $C_{10}H_{14}N_2O_2$: C, 61.85%; H, 7.21%; N, 14.43%; Found: C, 62.69%; H, 7.21%; N, 14.08%. Upon reduction with Zn and acetic acid in alcoholic solution, it was to a large part reconverted into nicotine (85–100%). Qualitative tests for aldehyde, keto, peroxide, and alcoholic groups were negative. The compound is not identical with oxy-nicotine. Further work is in progress to identify the product, but in the meantime, the following over-all equations are proposed for the reaction:

$$\text{(I)}\quad C_{10}H_{14}N_2 + M\text{—}B^{*} \xrightarrow{\text{light}} C_{10}H_{12}N_2 + M\text{—}B(H_2)^{\dagger}$$

$$\text{(II)}\quad M\text{—}B(H_2) + O_2 \longrightarrow M\text{—}B + H_2O_2$$

$$\text{(III)}\quad C_{10}H_{12}N_2 + H_2O_2 \longrightarrow C_{10}H_{14}N_2O_2$$

* Methylene blue

† Leuco methylene blue

Reaction I would represent the light reactions; II and III, the dark ones. An attempt to demonstrate the intermediate formation of H_2O_2, as required by reaction II, was not successful. Preliminary work indicated that the failure might be due to the high reactivity of certain tertiary amines with nascent H_2O_2.

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[2] Kindly supplied by P. G. Haines, of this laboratory.

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Transformation Products of Nicotine in Fermented Tobacco

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One of the chemical changes concurring with the fermentation of cigar leaf tobacco[1] is a considerable decrease of the nicotine contained in the leaf tissues. Table 1 shows the average nicotine contents (30 samples each) of four crops of Pennsylvania Seedleaf tobacco, before and after fermentation. For the individual samples, the nicotine decreases range from 6 to 94% of the amount present before fermentation. A definite parallelism exists between the degree to which each individual sample has been fermented and the extent of its nicotine decrease. To the author's knowledge, no systematic studies have been published concerning the fate of the disappearing nicotine, except that occasionally volatilization of the alkaloid has been assumed without experimental proof.

TABLE 1

Decrease of Nicotine Contents of Pennsylvania Cigar Tobacco for Various Crops

(Averages are based on analyses of 30 samples for each crop.)

Crop Year	Avg. nicotine contents (% of dry weights)*		Avg. decrease of nicotine due to fermentation (%)	Decrease of nicotine for individual samples	
	Before fermentation (BF)	After fermentation (AF)		Minimum (%)	Maximum (%)
1936	3.08	1.51	51	6	94
1938	2.82	1.76	38	7	72
1939	2.26	0.98	57	18	89
1941	3.85	2.04	47	27	83

* All values based on dry weights before fermentation.

In this laboratory, analytical studies have been conducted for several years on the nitrogenous components and particularly on the alkaloids and related substances in the leaf tissues of Pennsylvania tobacco. Specifically, we investigated the changes of these compounds resulting from fermentation.

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[1] The fermentation of Pennsylvania tobacco has been decribed briefly by the writer (*Arch. Biochem.*, 1947, **14**, 157–181).

The principle analytical methods used for the study of the alkaloids and related pyridine compounds were:

(1) Distillations under varying conditions of alkalinity.

(2) Distillations from alkaline solutions in presence of nascent hydrogen (Devarda alloy).

(3) Precipitations with silicotungstic acid. Of these precipitates, the following values were determined: (a) the weights after drying at 110° C, (b) the nitrogen contents (by Kjeldahl), and (c) the losses of weight on ignition.

(4) Determinations of pyridine-nitrogen by the color reaction with 2,4-dinitrochlorobenzene (DCB), based on a modification of the method of Karrer and Keller (*1*).

(5) Total nitrogen determinations of the various fractions (Kjeldahl).

(6) Analytical determinations of specific tobacco alkaloids, e.g. treatments of fractions with nitrous acid, followed by distillation with weak alkali [for detection of secondary nitrogen in the pyrrolidine- (pyrroline-, pyrrol-) ring], preparation of picrates, etc.

Preliminary studies repeatedly gave indications of the presence, in the tissues of fermented leaves, of substances which are chemically related to, but not identical with, the tobacco alkaloids. These substances, in common with the tobacco alkaloids, are pyridine derivatives and form precipitates with silicotungstic acid[2] and colored products with dinitrochlorobenzene. Contrary to the alkaloids, they are not distillable from alkaline solutions, at least not without partial decomposition, and are not extractable from alkalinized leaf tissues or from alkalinized aqueous leaf extracts by the usual alkaloid solvents, such as ethyl ether, or petrol ether.

Finally, a fractionated extraction method was developed, leading to a fairly satisfactory separation of the various pyridine compounds in tobacco leaves. This method involves the following steps: Tobacco powder is alkalinized by wetting with NaOH-solution or by mixing with wet magnesium oxide. Fraction A is obtained by extraction with petrol ether; Fraction B, by extracting the residue of A with chloroform; and Fraction C, by extracting the residue of B with alcohol.

With respect to fractions A and B, it was concluded that:

(1) Pennsylvania Seedleaf tobacco contains, besides its main alkaloid nicotine, small quantities of other tobacco alkaloids (''secondary'' alkaloids). These are all contained in Fraction A.

The nicotine of Fraction A is separated from the remaining ''secondary'' alkaloids by treatment with nitrous acid followed by a weak alkali distillation. Preliminary studies indicate that the ''secondary'' alkaloids in Pennsylvana tobacco consist almost exclusively of compounds containing a secondary N-atom in their 5-membered rings, probably of nornicotine with possibly smaller amounts of myosmine.

(2) The leaf tissues of the *fermented* samples contain, as mentioned above, considerably less alkaloids, but appreciable amounts of the nonalkaloid-pyridine compounds. A part of these compounds is insoluble in petrol ether but soluble in chloroform. Accordingly, it is obtained in Fraction B. *The pyridine compounds in this fraction are closely related to, or identical with, the oxidation product of nicotine, containing two oxygen atoms, which has been obtained by Weil (2) via photo-oxidation of nicotine in the presence of methylene blue.*

The common properties of the compound (or compounds) obtained by Weil (I) and of the substances (II) extracted with chloroform (Fraction B) from fermented tobacco which was previously extracted with petrol ether are as follows:

(a) In precipitation with silicotungstic acid, the precipitates of (I) and (II) have the same spongy, grayish-white appearance and show after drying at 110° C the same nitrogen content as well as the same percentage loss of weight on ignition.

(b) On reduction with granulated zinc in 10% acetic acid, or with Devarda alloy in 5% NaOH, a considerable part (50–70%) is converted into nicotine which can be isolated by ether extraction or by distillation.

(c) (I) and (II), if reacted with dinitrochlorobenzene, form a reaction product which develops a purple color on addition of small amounts of KOH. The absorption spectrum of these colored solutions differs from that of the DCB-reaction products obtained with the known tobacco alkaloids. It is practically identical for (I) and (II). The bleaching of the colored solutions obtained from (I) and (II) on standing is considerably slower than that of the colored solutions obtained from the tobacco alkaloids and from nicotinic acid.

(3) *An appreciable part of the initial nicotine of the unfermented leaves is converted during the fermentation into the nonalkaloid compounds to be found in Fraction B.*

The amount of substance II in the fermented leaves is considerably larger than the amount present in the same sample before its fermentation. The increase corresponds, per sample, to 30–40% of the nicotine which disappeared during the fermentation.

Of the pyridine compounds present in Fraction C, a small part consists of nicotinic acid; the remainder, of a compound or compounds which, according to their color reaction with DCB, seem to be related to nicotinic acid. Further work is required to investigate the latter substances. Similar to compounds of Fraction B, the amounts of the C-compounds increase as a result of fermentation. This increase is estimated to correspond to 20–30% of the nicotine loss during the fermentation.

Accordingly, 50–70% of the disappearing nicotine can be found in fermented tobacco leaves as pyridine compounds of a nonalkaloid nature. Their production is obviously due to catalytic or enzymic factors which start to operate if the leaves are subjected to the fermentation process.

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[2] This does not apply for nicotinic acid, which was also found as a minor component among the alkaloid transformation products.