Autoxidation of Nicotine. II. Products and Proposed Mechanism

F. Paul Gavin[a] **and Robert H. Linnell**[b]

Department of Chemistry, University of Vermont, Burlington, Vermont, U.S.A.

Introduction

In our earlier work (1) we have shown that the oxygen uptake of nicotine near room temperature is a typical autoxidation with an over-all activation energy of the uninitiated reaction of 6.81 kcal. We have now investigated the products formed in the early phases of this oxidation.

Wada (2) reported ammonia, methyl amine, nicotinic acid, myosmine, cotinine, nicotyrine, and unidentified products in air oxidation of nicotine at 30°. However, his oxidations were carried out over several weeks and no kinetic data was obtained. Frankenburg (3) isolated cotinine from nicotine that had been stored in the laboratory under air. The work of Weil (4) on the photochemical oxidation of nicotine in the presence of methylene blue has shown that one oxygen molecule is taken up for each nicotine, that hydrogen peroxide is involved, and that the reaction centers around the tertiary pyrrolidine amino group.

A good deal of biochemical work has been reported on nicotine conversion, and both the pyrrolidine and pyridine ring systems seem to be involved. Thus dilute aqueous solutions of nicotine (pH 7) were degraded *in vitro* by microorganisms from tobacco seeds, and the first products were believed to be γ-methylaminopropyl-(3-pyridyl) ketone and 6-hydroxynicotine (5). Recent work on the bacterial oxidation of nicotine by Rittenberg and co-workers (6) indicates nicotine oxidation via 6-hydroxynicotine, 6-hydroxypseudooxynicotine, and 2,6-dihydroxypseudooxynicotine, and finally to 2,6-dihydroxy-N-methylmyosmine. Hucker and co-workers (7) report that cotinine is a major product of rabbit liver oxidation of nicotine, and McKennis and co-workers (8) found cotinine and γ-(3-pyridyl)-γ-(methylamino) butyric acid in urine from dogs receiving nicotine. Human urine from subjects ingesting nicotine or following smoking was found to contain cotinine and perhaps hydroxynicotine and desmethylcotinine as well as unidentified Koenig-positive compounds (9).

In view of the great interest and importance of nicotine degradation it was decided to investigate the initial products of the autoxidation of nicotine. In this work infrared spectroscopy has been used for quantitative analysis for cotinine, Karl Fisher reagent for water, and a paper chromatography method for oxynicotine.

Table 1—Experimental R_f Values[a]

Compound	Buffer A	Buffer B
Nicotine	0.48	0.92 (0.79)[ref. in b]
Cotinine	0.79 (0.79)[3]	0.72 (0.37)[3, b]
Oxynicotine	0.40	0.23
γ-(3-pyridyl)-γ-methylamino-butyric acid	0.20	0.07

[a] *Whatman No. 1, descending chromatography, 25 ± 3°C. Literature values inclosed in parentheses.*
[b] *This literature value seems to have come from Porter, W. L., Naghski, J., and Eisner, A., Arch. Biochem. 24: 461 (1949). We believe our higher experimental value is correct.*

Experimental

Nicotine was vacuum distilled at 10 mm. (97-98°) and stored under prepurified N_2 and at dry ice temperature. The low temperature storage appears to indefinitely preserve the O_2 uptake rate of the nicotine; storage in air rapidly decreases the O_2 uptake rate (1). Kinetic runs were made as previously reported (1).

Cotinine, 6-hydroxynicotine, and γ-(3-pyridyl)-γ-methylaminobutyric acid were supplied by Drs. McKennis and Bowman (Dept. of Pharmacology, Medical College of Virginia, Richmond). Since the latter compound was found to contain cotinine (paper chromatogram) the pure material was prepared by heating cotinine with barium hydroxide under N_2 (8). Oxynicotine was supplied by Dr. C. H. Rayburn (Research Laboratories, American Tobacco Co., Richmond, Va.) and was also synthesized by reaction of nicotine and H_2O_2 (10). 2-methyl-6-(3-pyridyl)-tetrahydro-1 2-oxazine dihydrochloride was also supplied by Dr. Rayburn.

Myosmine was vacuum distilled on a spinning band column from an old sample supplied by Dr. Eisner.

Pyrrolidine was supplied by Eastman Organic Chemicals and was purified by distillation at atmospheric pressure under N_2, b.p. 88°, and stored at −10° under N_2. The N-methyl pyrrolidine was supplied by K. and K. Laboratories and was purified in the same manner, b.p. 83°, and stored in the same manner as the pyrrolidine.

All other reagents were analytical grade and were used as received.

[a] *Second Lt., U. S. Army Reserve.*
[b] *To whom inquiries should be sent. Present address: Scott Research Laboratories, Perkasie, Pennsylvania.*

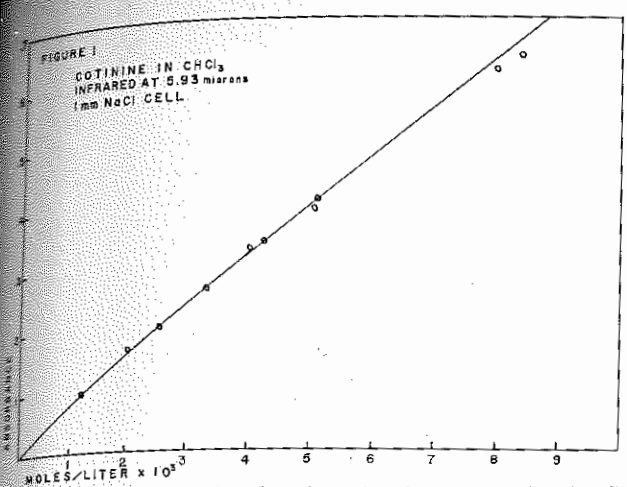


Figure 1. Cotinine in $CHCl_3$, Infrared at 5.93 microns, 1 mm NaCl Cell.

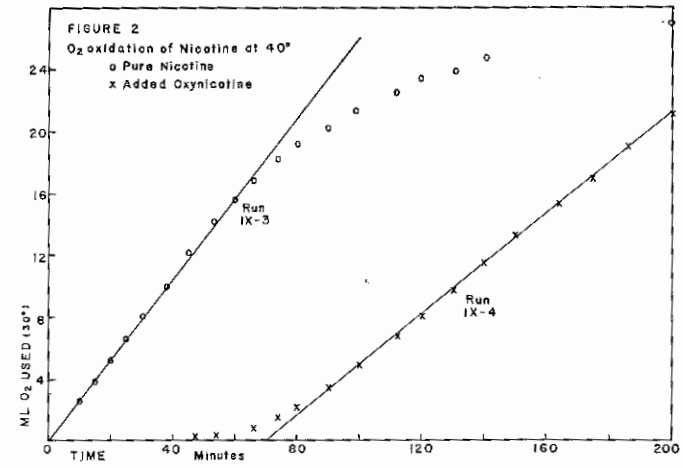


Figure 2. O_2 Oxidation of Nicotine at 40°. o = Pure Nicotine; x = Added Oxynicotine.

Karl Fisher reagent and diluent were supplied by Fisher Scientific Company.

Analytical Methods. Cotinine was determined by infrared spectroscopy using NaCl optics on a Perkin-Elmer Model 112 instrument. The strong 6 micron carbonyl band was used. **Figure 1** shows the absorbancy vs. concentration curve used in this work. $CHCl_3$ was used as solvent since nicotine-CCl_4 solutions were found to be unstable. One milliliter of nicotine was diluted with $CHCl_3$ to 25 ml. and the absorbancy measured at the peak of the 6.0 micron band (11), using an 85 micron slit width and a 1.0 mm. NaCl cavity cell. Water bands were eliminated by flushing with dry air. Knowns containing cotinine, nicotine, and oxynicotine were used, and it was shown that the large excess of nicotine and the oxynicotine do not interfere. Cotinine knowns in nicotine-$CHCl_3$ were analyzed from time to time and checked to within 5% of the amount present. We noted that in CCl_4 solvent the cotinine carbonyl maximum was at 5.86 microns, whereas in $CHCl_3$ the maximum was shifted to 5.93 microns. We suspect a weak H-bond complex formed between cotinine carbonyl and $CHCl_3$.

Water was determined by Karl Fisher reagent in a Beckman Aquameter (12) using a diluted reagent corresponding to about 2 mg. H_2O per ml. reagent. The reagent was standardized both with wet methanol containing known weights of water and with known weights of hydrated sodium tartrate. The agreement between methods was good and differences were less than 1%. It was assumed that all the water from a kinetic run on nicotine oxidation was in the liquid nicotine since attempts to flush ont the oxygen burette system with dry N_2 through a weighed water-absorption tower yielded low and erratic results. It was shown that Karl Fisher reagent did not react with nicotine. In the actual procedure, 1.00 ml. of oxidized nicotine was quickly added to methanol solvent (previously titrated for H_2O), and the titration was then carried out.

Paper chromatography was used both for qualitative and for quantitative analyses. Descending chromatograms were run in all cases. For most of our work, two buffer systems were used: A. butanol-ethanol-acetate (50-10-40 ml. each component); B. butyl acetate-methanol-0.25% ammonia (95-5-25 ml. each component). The experimental R_f values found in this work are given in **Table 1.** Some chromatograms were run under prepurified nitrogen but in no case was there any evidence of differences from chromatograms run in air. We conclude that no oxidation takes place during the chromatographic separation.

For qualitative work the solvent system also contained a trace of p-aminobenzoic acid or its ethyl ester so that development of Koenig positive spots with CNBr vapor would be facilitated. We thank Dr. E. R. Bowman for this helpful suggestion. For the qualitative identification work, oxidized nicotine containing 0.04 mole or less of 0_2 per mole nicotine was used. Cotinine and oxynicotine were always found in oxidized nicotine samples; in Buffer A, a low R_f spot, 0.1-0.2, was observed but not identified. The low R_f spot appeared less at higher temperature (40°) and might be a nicotine hydroperoxide. Nicotinic acid, nicotinamide, γ-(3-pyridyl)-γ-methylaminobutyric acid, methyl amine, cotinine, oxynicotine, myosmine, and 6-hydroxynicotine were all used as reference compounds in identifying the chromatograms.

The 6.05 micron infrared band of oxynicotine has a much lower absorbancy index than the 5.93 micron band of cotinine, and the small quantities of oxynicotine present in our oxidized nioctine could not be detected by infrared. Because Buffer B offers wider separation in R_f values, a technique was developed using heavy Whatman No. 3 paper and cutting out the oxynicotine and eluting in 0.05 N HCl (HCl gives better elution and higher ultraviolet absorbancy). The absorbancy of the eluate was then measured at 258.5 millimicrons (the experimental maximum) on a Beckman DU. The technique was worked out with knowns containing oxynicotine, cotinine, and nicotine in large excess. Although checks with knowns agreed to ± 10% of the amount of oxynicotine present, we do not feel confident of this method. The large samples that must be spotted to obtain sufficient oxynicotine for u.v. measurements are a major problem. Although the cotinine and nicotine are clearly separated in developed chromatograms, streaking from R_f 0.0-0.2 makes it difficult to be sure that some other compound is not present with the oxynicotine. In the dilute solutions run on Whatman No. 1 we do not find this problem.

Some experiments were done in which nicotine was treated with H_2O_2. An amount of H_2O_2 was added to provide roughly the same amount of O_2 as used in a typical kinetic experiment (*i.e.*, 2 ml. nicotine and 200 lambda of 30% by wt. concentrated H_2O_2 solution, equivalent to about 20 ml. O_2 at S.T.P.). Two hours at 0° C showed no reaction, but at 45 ° C both oxynicotine and cotinine were found.

Paper chromatograms showed coti-

Table 2—Linear Rates of O_2 Absorbed by Liquid Nicotine at 40° and Product Analytical Data

Run	Rate O_2[a]	ml. O_2 (STP)[b]	Time[c]	Co-tinine[d]	Water[d]	Oxy-nicotine[d]	Comments
II-3	2.27	20.8	125	—	—	—	——
VI-4	2.36	18.1	165	—	—	—	——
VIII-1	2.29	26.6	200	13.8	—	—	——
VIII-2	2.08	27.6	200	—	—	—	100 mg. cotinine added
VIII-3	~0[f]	0.7	200	—	—	—	11.2 mg. oxynicotine added[e]
VIII-4	2.03	18.1	84	—	—	—	Nicotine stored 21 days under N_2 at −10° C.
VIII-5	2.06	18.5	84	—	—	—	Same nicotine as VIII-4 + 8.2 mg. cotinine
VIII-6	1.95	17.6	84	—	—	—	Same nicotine as VIII-4 + 1.5 mg. oxynicotine
IX-3	2.17	27.6	248	17.4	—	—	——
IX-4	1.43	22.6	250	16.0	—	—	6.2 mg. oxynicotine added
IX-5	1.50	25.0	250	16.7	—	—	4.5 mg. oxynicotine added
XI-2	~0[f]	12.9	600	—	—	—	32.3 mg. oxynicotine added
XI-3	~0[f]	18.8	525	—	—	—	14.4 mg. oxynicotine added
XV-2	2.44	22.7	156	23.4	31.3	19.3	——
XVI-1	2.48	22.2	100	18.4	26.4	19.8	——
XVI-2	2.43	22.3	100	17.0	26.0	14.7	——
XVI-3	2.38	22.7	100	19.1	30.6	24.2	——
XXVI-4	2.25	22.7	100	19.1	33.2	24.6	——
XX-3	0	0	180	—	—	—	40 mg. pyrrolidine added

[a] *ml. O_2(STP) × 10^3 × sec^{-1} × ml. $nicotine^{-1}$.*
[b] *Total O_2 uptake for 2 ml. nicotine.*
[c] *Oxidation time in minutes.*
[d] *Expressed as percent of total oxygen used up.*
[e] *One mg. oxynicotine corresponds to 0.25 ml O_2 at STP.*
[f] *No O_2 uptake for first 60 minutes, then slow uptake rate.*

nine, oxynicotine, and γ-(3-pyridyl)-γ-methylamino butyric acid stable to traces of H_2O_2 at room temperature. At 50° all three compounds showed some conversion, the oxynicotine being most stable.

The amino acid, γ-(3-pyridyl)-γ-methylamino butyric acid, might be a precursor to the closed ring lactam, cotinine, in H_2O_2 reaction with nicotine. Since the amino acid form is reportedly stable in alkaline solution (8), we treated an aqueous ammonia solution of nicotine with H_2O_2 but found only cotinine and oxynicotine.

The actual oxidation runs were performed as described in our previous work (1). The apparatus was similar to a Warburg. A 50 ml. reaction flask shaken in a water thermostat was connected by ⅛″ rubber tubing to a gas burette with Hg containing liquid. Oxygen uptake rates were independent of shaking.

Lithium pyruvate was added to nicotine before a typical oxidation experiment at 40°. A control oxidation experiment was run simultaneously. The chromatogram for the control showed a much darker oxynicotine spot than the pyruvate experiment. A drop of mercury added to the nicotine before an oxidation experiment, turns black. This effect was observed even in the presence of the pyruvate.

Several kinetic experiments were performed on the initiated autoxidation of cumene inhibited by various amines (13). Although this work is to be published later, it is significant here that the stoichiometric factor "n"* is 1.0 for pyrrolidine and 2.6 for nicotine. Both compounds were good inhibitors. Pyrrolidine itself did not take up O_2 at temperatures up to 50°. N-methylpyrrolidine also did not take up O_2 at temperatures up to 50°. We had expected some autoxidation with the N-methyl pyrrolidine, and when this was not found we suspected some pyrrolidine was present which inhibited the oxidation. The presence of appreciable N-H in the infrared did show this impurity in the N-methyl compound.

Paper chromatography was used to investigate the hydrolysis of cotinine to the free amino acid, γ-(3-pyridyl)-γ-methylamine butyric acid. McKennis and co-workers (8) report that no amino acid is formed from cotinine at room temperature over the pH range 2.2-8.92; cotinine was formed from free amino acid in one hour at pH 4.25 (22°) but not at pH 2.2. In our work the free amino acid at pH 2.2 (room temperature) in citric acid-phosphate buffer was chromatogrammed after ½, 1, 2, 3, 4, and 5 hours, and no cotinine was found in any case. At pH 10.4 (room temperature) in NH_3 solution the free amino acid was stable for some time although we did find cotinine after four months.

Results and Discussion

The experimental results are tabulated in **Table 2.** The data from a typical nicotine oxidation run and from a run with added oxynicotine are shown in **Figure 2.** A number of conclusions can be drawn from the experimental results:

1. In the uninitiated autoxidation of nicotine with oxygen, near room temperature, oxynicotine, cotinine, and H_2O are major products in the early phases of the oxidation (*e.g.* mole ratio O_2/nicotine not over 0.10).

2. Cotinine does not strongly inhibit the nicotine autoxidation but oxynicotine is a strong inhibitor.

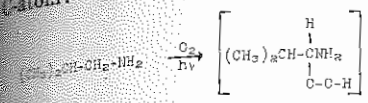
3. Since the rate of nicotine autoxidation is greatly increased by a free-radical source (AIBN) and reduced to zero by a well-known radical trap (BHT), the reaction is probably free-radical in nature (1).

4. Peroxide or hydroperoxide formation is probably involved in nicotine oxidation. Decrease in oxynicotine formation when pyruvate is present indicates H_2O_2 involvement in oxynicotine formation (4). The Hg blackening definitely indicates peroxide or hydroperoxide (14).

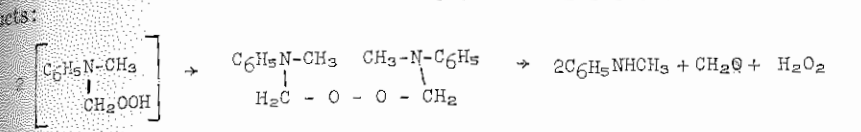
5. Pyrrolidine strongly inhibits nicotine autoxidation. Impurity pyrrolidine in N-methylpyrrolidine probably prevents rapid autoxidation of the latter compound.

* *n = free radicals used up/inhibitor molecules. For this work the initiator was 2,2′-Azobis (2-methyl propionitrile) for which we use the abbreviation AIBN.*

Very few studies have been reported on the autoxidation of amines. Photosensitized oxidation of an aliphatic amine has been reported by Schenck (15) to take place via hydroperoxide formation on the alpha C-atom:



but the unstable hydroperoxide was itself not isolated. Horner and Knapp (16) proposed unstable hydroperoxide from the autoxidation of dimethylaniline and isolated the diperoxide and its decomposition products:



This literature evidence does support our view of nicotine hydroperoxide formation of the alpha-C in the N-methylpyrrolidine ring. A suggested oxidation scheme is shown in Figure 3. Further questions remain unanswered. The nicotine-H_2O_2 reaction needs further investigation to determine the cotinine and oxynicotine formed as a function of reaction conditions. It does appear that near room temperature about 20% of the oxygen taken up by nicotine is converted to cotinine (which in turn should produce H_2O equal to 20% of the oxygen), about 20% of the oxygen goes into oxynicotine (which also produces an equivalent amount of H_2O, 20%), and about 30% appears as H_2O. Since the sum of cotinine and oxynicotine should appear as H_2O, our H_2O results are somewhat low (20 + 20 = 40% expected vs. 30% found). We believe the cotinine and H_2O analytical data are reliable but suspect the oxynicotine results are high. Probably the balance of the O_2 reacts in a complex manner producing higher molecular weight materials. Since tars are produced in the reaction, involvement of O_2 and the proposed diradical seems a plausible explanation.

Acknowledgments

The authors appreciate financial support of the Tobacco Industry Research Committee which made this work possible.

Thanks are due to T. James Hallee for assistance in the paper chromatography. We also appreciate the help of Kenneth R. McCormick in making some kinetic runs.

Literature Cited

1. Linnell, R. H., *Tobacco Science* 4: 89 (1960).
2. Wada, E., T. Kisaki and K. Saito, *Arch. Biochem. and Biophys.* 79: 124 (1959).
3. Frankenburg, W. G., and A. A. Vaitekunas, *J. Am. Chem. Soc.* 79: 149 (1957).
4. Weil, L., *Science* 107: 426 (1948); L. Weil and J. Mahar, *Arch. Biochem.* 29: 241 (1950).
5. Frankenburg, W. G., and A. A. Vaitekunas, *Arch. Biochem. and Biophys.* 58: 509 (1955).
6. Richardson, S. H., and S. C. Rittenburg, *J. Biol. Chem.* 236: 964 (1961). This paper contains earlier references.
7. Hucker, H. B., J. R. Gillette and B. B. Brodie, *Nature* 183: 47 (1959); *J. Pharm. and Exp. Therapeutics* 129: 94 (1960).
8. McKennis, H., L. B. Turnbull, H. N. Wingfield and L. J. Dewey, *J. Am. Chem. Soc.* 80: 1635, 6599 (1958).
9. Bowman, E. R., L. B. Turnbull and H. McKennis, *J. Pharm. and Exp. Therapeutics* 127: 92 (1959).
10. The explicit synthetic procedure was kindly supplied by Dr. C. H. Rayburn, Dept. of Research and Development, American Tobacco Co., Richmond, Va.
11. For infrared of nicotine and derivatives see C. R. Eddy and A. Eisner, *Anal. Chem.* 26: 1428 (1954). The 2-15 micron spectrum of cotinine will be found in reference 3.
12. Mitchell, J., and D. M. Smith, *Aquametry,* Interscience Publishers, Inc., New York (1948).
13. Boozer, C. E., George S. Hammond, C. E. Hamilton and J. N. Sen, *J. Am. Chem. Soc.* 77: 3233 (1955).
14. Summerbell, R. K. and D. Kareu, *J. Org. Chem.* 25: 1809 (1960).
15. Schenck, G. O., *Angew. Chem.* 69: 579 (1957).
16. Horner, L. and K. H. Knapp, *Liebigs Ann. Chem.* 622: 79 (1959).

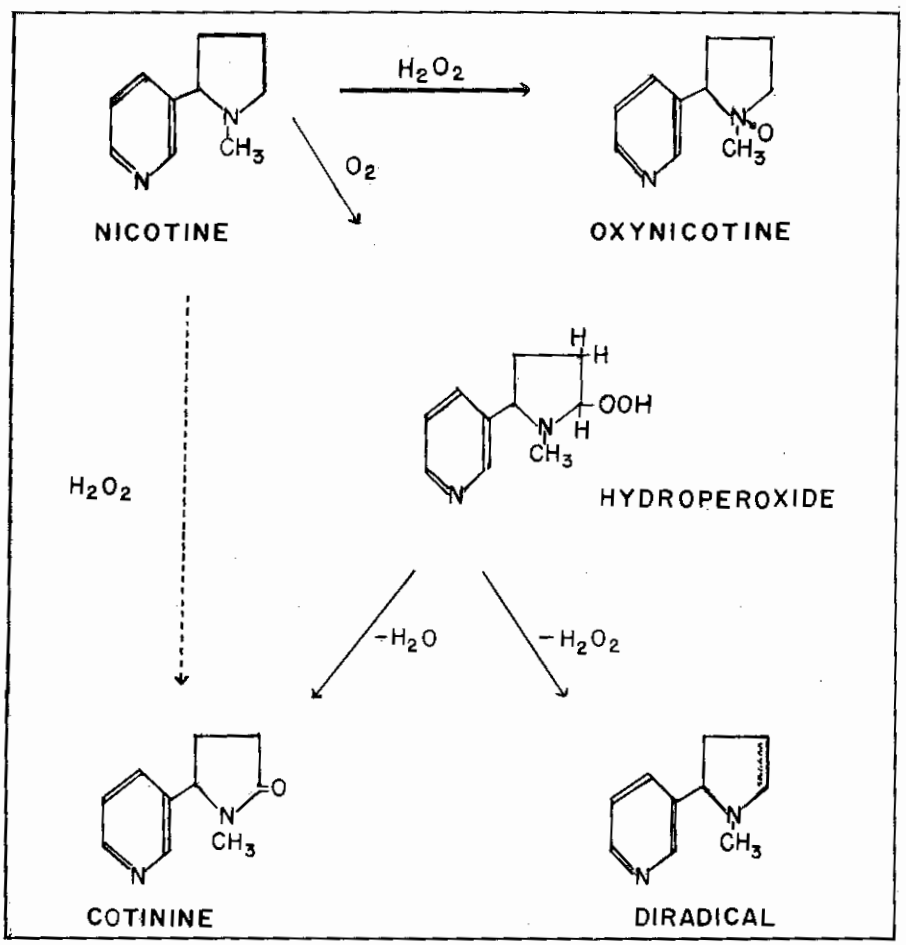


Figure 3. O_2 Oxidation of Nicotine near room temperature.