Formation of 4-(Methylnitrosamino)-4-(3-pyridyl)butyric Acid in Vitro and in Mainstream Cigarette Smoke†

Mirjana V. Djordjevic,* Claire W. Sigountos, Klaus D. Brunnemann, and Dietrich Hoffmann

Division of Environmental Carcinogenesis, American Health Foundation, Valhalla, New York 10595

During tobacco growing, processing, and smoking, nicotine, a major tobacco alkaloid, gives rise to five *N*-nitrosamines including 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC). In this study we have shown that iso-NNAC was not detected in the mainstream smoke (MS) of a blended U.S. cigarette (<1 ng/cigarette) but it occurs in minute amounts (3 ng/cigarette) in the MS of a French dark tobacco cigarette which is rich in nitrate and other nicotine-derived *N*-nitrosamines. In the sidestream smoke (SS) of the French cigarette, the biologically highly active tobacco-specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone was present in significantly higher concentration than in MS, as was the case with iso-NNAC, amounting to 7 ng/cigarette in comparison to 3 ng in the MS. Spiking of cigarettes with nicotine (10 mg/cigarette), nornicotine (1 mg), cotinine (2 mg), and cotinine acid (0.05 mg) did not significantly increase iso-NNAC in MS. The transfer rate for iso-NNAC, determined by spiking each of the blended U.S. cigarettes and the French cigarettes with 0.1 mg of this synthetic nitrosamino acid, was between 0.8 and 1.0%. These findings support the hypothesis that the presence of iso-NNAC in physiological fluids of cigarette smokers is indicative of endogenous formation of nicotine-derived tobacco-specific *N*-nitrosamines. Accordingly, the compound may serve as a biomarker of endogenous *N*-nitrosamine formation. In vitro nitrosation of nicotine at pH 7 and 37 °C does not give rise to iso-NNAC, while its major metabolites, cotinine and cotinine acid, do.

Nicotine gives rise to specific *N*-nitrosamines during tobacco growing, curing, processing, and aging. (Hecht and Hoffmann, 1988; Burton et al., 1989a; Djordjevic et al., 1989b; Andersen et al., 1989). Three of the tobacco-specific nitrosamines (TSNA) derived from nicotine (Figure 1), namely, *N'*-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), are highly carcinogenic in mice, rats, and hamsters; 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC) is not carcinogenic, and it is not known whether 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL) is carcinogenic (Hecht and Hoffmann, 1988; Rivenson et al., 1988, 1989). However, Brunnemann et al. (1987) found that iso-NNAL is genotoxic in primary rat hepatocytes. Nicotine is also a precursor for TSNA which are formed during tobacco chewing and snuff dipping (Wenke et al., 1984; Nair et al., 1985; Carmella et al., 1990). Compared to nonsmokers, cigarette smokers have an increased potential for the endogenous N-nitrosation of amines as was shown by an increased urinary excretion of *N*-nitrosoproline and *N*-nitrosothioproline (Hoffmann and Brunnemann, 1983; Ladd et al., 1984; Tsuda et al., 1986; Scherer and Adlkofer, 1986). However, there are no data which demonstrate unambiguously that endogenous nitrosation of nicotine, inhaled with tobacco smoke, does actually occur. Recently, we have identified the noncarcinogenic iso-NNAC in processed tobacco, though not in the mainstream smoke (MS) of blended cigarettes (Djordjevic et al., 1989a). This finding leads to the hypothesis that urinary excretion of iso-NNAC by cigarette smokers may be an indicator of endogenous formation of TSNA from inhaled nicotine. Iso-NNAC could be formed endogenously by direct oxidative nitrosation of nicotine via 4-(methylnitrosamino)4-(3-pyridyl)butanal (NNA, Figure 1) and/or by nitrosation of the major nicotine metabolite, cotinine, and its hydrolysis product, 4-(methylamino)-4-(3-pyridyl)butyric acid (cotinine acid).

Before exploring the endogenous formation of TSNA in cigarette smokers with iso-NNAC as a marker, we studied (i) the in vitro formation of iso-NNAC from nicotine, from cotinine, and from cotinine acid with sodium nitrite at pH 7, 37 °C; (ii) the occurrence of TSNA, including iso-NNAC, in tobacco, MS, and sidestream smoke (SS) of commercially available nonfilter cigarettes; and (iii) the transfer rate of iso-NNAC from tobacco into MS and its formation during smoking. For the latter test, we employed nonfilter cigarettes spiked with synthetic iso-NNAC and with the potential alkaloid precursors, respectively.

MATERIALS AND METHODS

Apparatus. TSNA and iso-NNAC were separated and quantified on a Hewlett-Packard Model 5890 gas chromatograph interfaced with a Model 610 thermal energy analyzer (TEA) (Thermo Electron Corp., Waltham, MA) with a modification described earlier (Brunnemann and Hoffmann, 1981) and a Model 3390A integrator (Hewlett-Packard, Paramus, NJ). Tobacco alkaloids were analyzed on a Hewlett-Packard Model 5890 gas chromatograph equipped with a thermionic N-P-specific detector (NPD) and interfaced with a Hewlett-Packard Model 3390A integrator. Nitrate and nitrite nitrogen analyses were done by using a Technicon Auto-Analyzer System II with a 50-mm flow cell in the colorimeter (Crutchfield and Burton, 1989). MS was generated with a Borgwaldt RM 20/CS 20-port smoking machine with rotating head (Heinrich Borgwaldt, Hamburg, FRG) modified as described previously (Hoffmann et al., 1983). The total particulate matter (TPM) of the MS was collected on 92-mm Cambridge filters (CF) (Djordjevic et al., 1989a). SS was collected on 44-mm CF by using the device described by Neurath and Ehmke (1964) and modified by Brunnemann and Hoffmann (1974), in conjunction with a single-port piston-type smoking machine (Borgwaldt). A purge flow rate of 1.5 L of air/min guaranteed that MS deliveries of tar were equivalent to those generated when the cigarette is smoked freely without the containment device required for the collection of SS. The Dub-

† Presented in part at the 43rd Tobacco Chemists' Research Conference, Oct 2–5, 1989, Richmond, VA, Abstract 27.

0021-8561/91/1439-0209$02.50/0

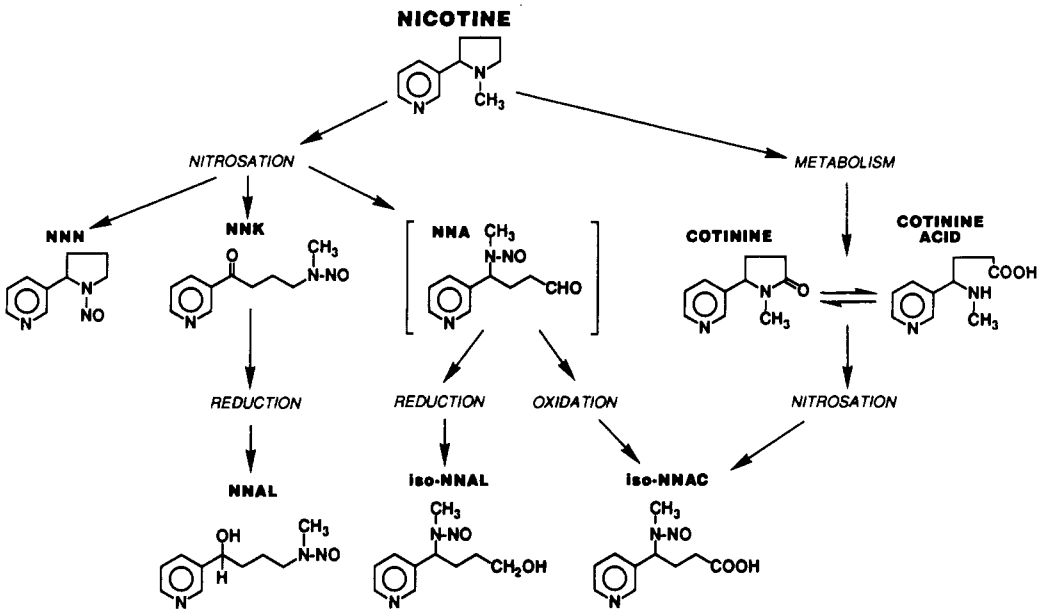

Figure 1. N'-Nitrosation products of nicotine.

Table I. In Vitro Formation of Iso-NNAC from Nicotine and Its Major Metabolites and Sodium Nitrite

A. conditions	
buffer system (pH 7)	0.2 M NaH_2PO_4:0.2 M Na_2HPO_4
temperature, °C	37
time	24 h (COT, NIC) 20 min (COTAC)
$NaNO_2$:substrate	~1.5
B. reactants	iso-NNAC yield,[a] %
NIC	0
NIC + 0.5 mM NaSCN	0
COT	0.001
COT + 0.5 mM NaSCN	0.001
COTAC	4.9
COTAC + 0.5 mM NaSCN	100

[a] Determined by GC–TEA.

Table II. Alkaloids TSNA and Nitrate and Nitrite Nitrogen in Cigarette Tobacco

	U.S. blended NF[a] cigarette	French dark tobacco NF cigarette
A. alkaloids, mg/g dwt		
nicotine	11.3	10.7
nornicotine	1.8	0.4
anabasine	0.2	0.1
anatabine	1.0	0.2
cotinine	0.04	0.09
total	14.3	11.4
B. TSNA,[b] μg/g dwt		
NAT	0.85	1.24
NAB	ND[c]	ND
NNN	1.58	5.95
NNK	0.19	0.35
iso-NNAC	ND	0.05
total	2.62	7.59
C. pH	5.1	6.3
D. nitrogen,[d] μg/g dwt		
nitrate	2675	3575
nitrite	2.65	2.60

[a] NF, nonfilter. [b] TSNA values for French cigarette tobacco are the averages of three analyses. [c] ND, not detected. [d] Nitrate and nitrite nitrogen values are the averages of two analyses.

noff metabolic shaking incubator (GCA/Precision Corp., Chicago, IL) was used for in vitro nitrosation.

Materials. An 85-mm U.S. blended nonfilter cigarette and a 70-mm French dark tobacco nonfilter cigarette were chosen for this study. These two cigarettes differ in tobacco composition and as such in chemical composition. While the U.S. blended cigarette is made of flue-cured (~50%) and air-cured burley (~30%) and sun-cured oriental (~10%) tobacco, the French cigarette used in this study is made exclusively of fermented dark tobaccos. The cigarettes were purchased on the open market in Westchester County, New York, in 1989. All samples were stored in a cold room (4 °C) and were opened immediately prior to analysis. Aqueous solutions (50 and 38 μL, respectively) of nicotine, nornicotine, cotinine, cotinine acid, and iso-NNAC were injected with an automatic microsyringe (Hoffmann et al., 1977; Adams et al., 1984) into a 62- or 47-mm segment of the tobacco column that was to be smoked.

Reagents. TSNA, cotinine acid, iso-NNAC, and *N*-nitrosoguvacoline (an internal standard for GC-TEA analysis of *N'*-nitrosamines) were synthesized as described earlier (Djordjevic et al., 1989a). The purity of the synthesized compounds (>99%) was verified by capillary GC with flame ionization detector (FID). Nicotine and nornicotine were purchased from ROTH-Atomergic Chemetals Corp., Farmingdale, NY. Cotinine and quinoline (an internal standard for GC-NPD analysis of tobacco alkaloids), as well as 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), the methylating agent, were purchased from Aldrich Chemical Co. Inc., Milwaukee, WI. Three percent XE-60 on GCQ (100/120 mesh), which was used for gas chromatographic analysis of TSNA, was purchased from Alltech/Applied Science, Deerfield, IL. The GB-5 fused silica capillary column for alkaloid determination or for verifying the purity of reference compounds was obtained from On-Site Instruments, Columbus, OH.

Methods. *In Vitro Nitrosation of Tobacco Alkaloids.* One hundred milligrams (0.62 mM) of nicotine, 100 mg (0.57 mM) of cotinine, and 2 mg (0.01 mM) of cotinine acid were placed in separate 50-mL round-bottom flasks and dissolved in 15 (nicotine and cotinine) or 5 mL (cotinine acid) of pH 7 buffer solution (prepared from 0.2 M monobasic sodium phosphate and dibasic sodium phosphate solutions). The solutions were heated to 37 °C in a water bath under magnetic stirring. A 1.5-fold molar excess of sodium nitrite was added slowly with continuous stirring. The flasks were stoppered, transferred into a metabolic incubator, and kept there at 37 °C for 24 h (nicotine and cotinine) or 20 min (cotinine acid). The reaction mixtures were processed as described in the quantitative analysis of iso-NNAC in tobacco (Djordjevic et al., 1989a). The second set of in vitro nitrosation experiments of nicotine, cotinine, and cotinine acid was carried out as described above except that sodium thiocyanate was added as a catalyst (0.6, 0.5, and 0.2 mM, respectively).

Smoke Generation and Collection. Cigarettes were smoked under standard conditions (Pillsbury et al., 1969). Prior to

Table III. TSNA and a Nicotine-Derived N'-Nitrosamino Acid in Mainstream Smoke (Nanograms per Cigarette)

	NAT	NAB	NNN	NNK	iso-NNAC	total
A. U.S. blended cigarette						
control	156	12	162	49	ND[b]	378
+nicotine[a] (10 mg)	124	11	134	43	ND	312
+nornicotine (1 mg)	140	4	207	47	ND	397
+cotinine (2 mg)	142	ND	185	49	2	378
+COTAC (0.05 mg)	162	ND	188	70	8	428
+iso-NNAC (0.1 mg)	155	10	186	58	951[c]	1360
B. French dark tobacco cigarette[d]						
control/SS[e]	162	57	348	180	7	754
control	177	15	527	69	3	791
+nicotine[f] (10 mg)	151	ND	500	97	3	751
+cotinine (2 mg)	167	ND	538	84	5	794
+COTAC (0.2 mg)	173	ND	435	88	5	701
+iso-NNAC (0.1 mg)	201	ND	471	91	822[g]	1585

[a] mg/cig, applied in 50 μL of water. [b] ND, not detected. [c] iso-NNAC transfer rate from U.S. blended tobacco into MS smoke, 0.95%. [d] TSNA values for the MS of French cigarette are the averages of three analyses. [e] SS, sidestream smoke of unspiked control. [f] mg/cig, applied in 38 μL of water. [g] iso-NNAC transfer rate from French dark tobacco into MS smoke, 0.82%.

Table IV. TSNA in the MS of an American Blend Nonfilter Cigarette (Nanograms per Cigarette)

sample		NAT	NNN	NNK	total
A[c]	CF[a]	147	163	41	351
	Aq[b]	0	0	0	0
	sum	**147**	**163**	**41**	**351**
B[d]	Aq	74	90	26	190
	CF	76	52	18	146
	sum	**150**	**142**	**44**	**336**
C[e]	Aq	129	133	39	301
	CF	38	27	10	75
	sum	**167**	**160**	**49**	**376**

[a] CF, Cambridge filter. [b] Aq, aqueous trap. [c] TSNA collected on CF placed between smoking machine and an aqueous trap (Djordjevic et al., 1989a). [d] TSNA collected in an aqueous trap and CF. [e] TSNA collected in an aqueous trap and CF treated with 50 mg of ascorbic acid in 10 mL of MeOH.

smoking, the cigarettes were kept for 24 h at relative humidity of 60 ± 2% and at 22 ± 2 °C. The MS of 100 cigarettes of each type was collected and processed as described earlier (Djordjevic et al., 1989a). For the determination of iso-NNAC in SS, 50 French dark tobacco cigarettes were smoked.

In addition, two sets of 50 U.S. blended cigarettes were smoked to determine whether the pretreatment of the Cambridge filters (CF) with ascorbic acid (50 mg/CF) prevents the artifactual formation of TSNA on the filter as was suggested by Caldwell and Conner (1989). In this experiment, cigarette MS was collected in an aqueous trap (four consecutive impingers, each containing 15 mL of water to which 0.5 mL of 20% ammonium sulfamate in 3.6 N H_2SO_4 was added) placed in line between the smoking machine and the CF. Results from an untreated and a treated 90-mm CF were compared.

Analysis of TSNA in Tobacco. Alkaloids, TSNA, including iso-NNAC, and nitrate and nitrite nitrogen were analyzed in tobacco according to published methods (Djordjevic et al., 1989a; Lowe and Gillespie, 1975; Crutchfield and Burton, 1989).

RESULTS AND DISCUSSION

In Vitro Nitrosation. As presented in Figure 1, cotinine, the major metabolite of nicotine, is in dynamic equilibrium with cotinine acid (McKennis et al., 1964). In vitro nitrosation at pH 7 of both nicotine metabolites gave rise to iso-NNAC (Table I). The yield of iso-NNAC, obtained from the reaction with cotinine, a tertiary amide, was rather low (0.001%). The nitrosation of the secondary amine, cotinine acid, was quantitative in the presence of thiocyanate as a catalyst (Boyland et al., 1971). In vitro nitrosation of nicotine did not form iso-NNAC, suggesting that, at pH 7, formation of this nitrosamino acid proceeds via cotinine rather than via NNA as was originally hypothesized (Djordjevic et al., 1989a). We are currently studying the formation of iso-NNAC by nitrosation of nicotine, cotinine, and cotinine acid at pH levels corresponding to those in selected biological fluids.

TSNA in Tobacco and MS and SS. To determine whether iso-NNAC occurs in tobacco smoke and, if it does, whether it originates from tobacco or from nitrosation of alkaloids during smoking, tobacco and MS of two commercial cigarettes were analyzed. As shown in Table II, there is no significant difference in nicotine and the total alkaloid content of these two cigarettes. On the other hand, a dark tobacco cigarette contained lower amounts of the minor alkaloids except cotinine, as described earlier (Piade and Hoffmann, 1980). However, TSNA content of the tobacco of the tested French cigarette was significantly higher (Table II), probably due to higher pH and nitrate content of dark tobaccos (Burton et al., 1989b; Chamberlain et al., 1986) and because these cigarettes were processed and manufactured with different technology. Iso-NNAC was found only in the tobacco of the French cigarette.

The average weights of the U.S. blended and the French dark tobacco cigarette used in this study were 1.12 and 1.08 g, respectively. TPM yields in MS were 24.0 and 22.0 mg/cigarette, respectively. Iso-NNAC was not detected in the MS of the U.S. blended cigarette or in the smoke of cigarettes spiked with nicotine and nornicotine (Table III). The transfer rate of iso-NNAC from tobacco into MS, as determined by smoking 100 U.S. blended cigarettes that were spiked with the authentic reference compound (0.1 mg/cigarette), was found to be about 1%. Iso-NNAC formation occurred only during smoking of the U.S. blended cigarettes enriched with cotinine and cotinine acid. Doubling the amount of nicotine in cigarette tobacco did not increase NNN in the MS. On the other hand, a 50% increase of nornicotine in the tobacco column resulted in a 30% increase of NNN in MS (Table III). The latter finding, which is not in agreement with data reported earlier (Hoffmann et al., 1974), suggests that secondary amine alkaloids play a dominant role as precursor in the formation of a related nitrosamine during smoking, although tertiary amine tobacco alkaloids should not be disregarded as indirect precursors for TSNA. The role of secondary amine alkaloids in the formation of TSNA during tobacco growing and curing is well established (Djordjevic et al., 1989b).

Quantities of individual and total TSNA in the MS of the French cigarette were significantly higher than those in the MS of the U.S. blended cigarette. This observation

is in agreement with earlier findings (Ruehl et al., 1980; Fischer et al., 1989). Interestingly, SS of the dark tobacco cigarette contained greater amounts of NAB and NNK (Table III) than MS of the corresponding cigarette. This finding is in line with earlier data for the U.S. blended cigarette (Adams et al., 1987).

A recent study suggests that TSNA may be artifactually formed during the trapping of MS (Caldwell et al., 1989). Our results, presented in Table IV, reveal no significant differences in the amounts of individual or total TSNA in cigarette MS with different methods of trapping and confirm that the collection system used in our laboratories for TSNA assays excludes artifactual TSNA formation.

If endogenous formation of iso-NNAC can be proven, the absence of this nitrosamine in the MS of cigarettes and its lack of genotoxic and biological activity (Djordjevic et al., 1989a; Rivenson et al., 1989) add qualifying attributes to its utility as a marker of endogenous formation of TSNA in active and passive smokers.

ABBREVIATIONS USED

TSNA, tobacco-specific *N*-nitrosamines; NIC, nicotine; NNN, *N'*-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNA, 4-(methylnitrosamino)-4-(3-pyridyl)butanal; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; iso-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; COT, cotinine; COTAC (cotinine acid), 4-(methylamino)-4-(3-pyridyl)butyric acid; iso-NNAC, 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid; NAT, *N'*-nitrosoanatabine; NAB, *N'*-nitrosoanabasine.

ACKNOWLEDGMENT

We thank Jonathan Cox for preparing the smoke samples for analysis and Mrs. Ilse Hoffmann for editorial assistance. This study is No. LXXXIX of the series "Chemical Studies on Tobacco Smoke". It was supported by Grant CA-29580 from the National Cancer Institute.

LITERATURE CITED

Adams, J. D.; Lee, S. J.; Hoffmann, D. Carcinogenic agents in cigarette smoke and influence of nitrite on their formation. *Carcinogenesis* **1984**, *5*, 221–222.

Adams, J. D.; O'Mara-Adams, K. J.; Hoffmann, D. Toxic and carcinogenic agents in undiluted mainstream smoke and sidestream smoke of different types of cigarettes. *Carcinogenesis* **1987**, *8*, 729–731.

Andersen, R. A.; Burton, H. R.; Fleming, P. D.; Hamilton-Kemp, T. R. Effect of storage conditions on nitrosated, acylated and oxidized pyridine alkaloid derivatives in smokeless tobacco products. *Cancer Res.* **1989**, *49*, 5895–5900.

Boyland, E.; Nice, E.; Williams, K. The catalysis of nitrosation by thiocyanate from saliva. *Food Cosmet. Toxicol.* **1971**, *9*, 639–643.

Brunnemann, K. D.; Hoffmann, D. The pH of tobacco smoke. *Food Cosmet. Toxicol.* **1974**, *12*, 115–124.

Brunnemann, K. D.; Hoffmann, D. Assessment of the carcinogenic N-nitrosodiethanolamine in tobacco products and tobacco smoke. *Carcinogenesis* **1981**, *2*, 1123–1127.

Brunnemann, K. D.; Genoble, L.; Hoffmann, D. Identification and analysis of a new tobacco-specific N-Nitrosamine, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol. *Carcinogenesis* **1987**, *8*, 465–469.

Burton, H. R.; Childs, G. H., Jr.; Andersen, R. A.; Fleming, P. D. Changes in Chemical Composition of burley tobacco during senescence and curing. 3. Tobacco-specific nitrosamines. *J. Agric. Food Chem.* **1989a**, *37*, 426–430.

Burton, H. R.; Bush, L. P.; Djordjevic, M. V. Influence of temperature and humidity on the accumulation of tobacco-specific nitrosamines in stored burley tobacco. *J. Agric. Food Chem.* **1989b**, *37*, 1372–1377.

Caldwell, W. S.; Conner, J. M. Artifact formation during smoke trapping. An improved method for the determination of N-nitrosamines in cigarette smoke. Presented at the 43rd Tobacco Chemists' Research Conference, Oct 2–5, 1989, Richmond, VA; Abstract 45.

Carmella, S. G.; Kagan, S. S.; Kagan, M.; Foiles, P. G.; Palladino, G.; Quart, A. M.; Quart, E.; Hecht, S. S. Mass spectrometric analysis of tobacco-specific nitrosamine hemoglobin adducts in snuff dippers, smokers and non-smokers. *Cancer Res.* **1990**, *50*, 5438–5445.

Chamberlain, W. J.; Chortyk, O. T.; Legg, P. D. Relationship of dark tobacco to nitrosamine content and use in smokeless products. Presented at the 38th ASC Southeast Regional Meeting, Nov 4, 1986, Louisville, KY.

Crutchfield, J.; Burton, H. R. Modified automated colorimetric procedure for quantification of nitrite in agricultural products. *Anal. Lett.* **1989**, *22*, 555–571.

Djordjevic, M. V.; Brunnemann, K. D.; Hoffmann, D. Identification and analysis of a nicotine-derived N-nitrosamino acid and other nitrosamino acids in tobacco. *Carcinogenesis* **1989a**, *10*, 1725–1731.

Djordjevic, M. V.; Gay, S. L.; Bush, L. P.; Chaplin, J. F. Tobacco-specific nitrosamine accumulation and distribution in flue-cured tobacco alkaloid isolines. *J. Agric. Food Chem.* **1989b**, *37*, 752–756.

Fischer, S.; Spiegelhalder, B.; Preussmann, R. Tobacco-specific nitrosamines in mainstream smoke of West German cigarettes—tar alone is not a sufficient index for the carcinogenic potential of cigarette smoke. *Carcinogenesis* **1989**, *10*, 169–173.

Hecht, S. S.; Hoffmann, D. Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. *Carcinogenesis* **1988**, *9*, 875–884.

Hoffmann, D.; Brunnemann, K. D. Endogenous formation of N-nitrosoproline in cigarette smokers. *Cancer Res.* **1983**, *43*, 5570–5574.

Hoffmann, D.; Rathkamp, G.; Liu, Y. Y. Chemical studies on tobacco smoke. XXVI. On the isolation and identification of volatile and nonvolatile N-nitrosamines and hydrazines in cigarette smoke. In *N-Nitroso Compounds in the Environment*; Bogovski, P., Walker, E. A., Eds.; IARC Scientific Publications 9; International Agency for Research on Cancer: Lyon, France, 1974; pp 159–165.

Hoffmann, D.; Dong, M.; Hecht, S. S. Origin in tobacco smoke of N'-nitrosonornicotine, a tobacco-specific carcinogen: brief communication. *J. Natl. Cancer Inst.* **1977**, *58*, 1841–1844.

Hoffmann, D.; Brunnemann, K. D.; Webb, K. S. Volatile nitrosamines in tobacco and mainstream and sidestream smoke and indoor environments. In *Environmental Carcinogens Selected Methods of Analysis*; Preussmann, R., O'Neill, I. K., Eisenbrand, G., Spiegelhalder, B., Bartsch, H., Eds.; IARC Scientific Publications 45; International Agency for Research on Cancer: Lyon, France, 1983; pp 69–85.

Ladd, K. F.; Newmark, H. L.; Archer, M. C. N-Nitrosation of proline in smokers and nonsmokers. *J. Natl. Cancer Inst.* **1984**, *73*, 83–87.

Lowe, R. H.; Gillespie, M. An *Escherichia coli* strain for use in nitrate analyses. *J. Agric. Food Chem.* **1975**, *23*, 783–787.

McKenis, H., Jr.; Schwartz, S. L.; Bowman, E. R. Alternate routes in the metabolic degradation of the pyrrolidine ring of nicotine. *J. Biol. Chem.* **1964**, *239*, 3390–3396.

Nair, J.; Ohshima, H.; Friesen, M.; Croisy, A.; Bhide, S. V.; Bartsch, H. Tobacco-specific and betel nut-specific N-nitroso compounds: occurrence in saliva and urine of betel quid chewers and formation *in vitro* by nitrosation of betel quid. *Carcinogenesis* **1985**, *6*, 295–303.

Neurath, G.; Ehmke, H. Apparatus zur Bestimmung des Nebenstrom rauches. *Beitr. Tabakforsch.* **1964**, *2*, 117–121.

Piade, J. J.; Hoffmann, D. Chemical studies on tobacco smoke. LXVII. Quantitative determination of alkaloids in tobacco by liquid chromatography. *J. Liq. Chromatogr.* **1980**, *3*, 1505–1515.

Pillsbury, H. C.; Bright, C. C.; O'Connor, K. J.; Irish, F. W. Tar and nicotine in cigarette smoke. *J. Assoc. Off. Anal. Chem.* **1969**, *52*, 458–462.

Rivenson, A.; Hoffmann, D.; Prokopczyk, B.; Amin, S.; Hecht, S. S. Induction of lung and exocrine pancreas tumors in F344 rats by tobacco-specific and Areca-derived N-nitrosamines. *Cancer Res.* **1988**, *48*, 6912–6917.

Rivenson, A.; Djordjevic, M. V.; Amin, S.; Hoffmann, D. A Study of Tobacco Carcinogenesis XLIV. Bioassay in A/J mice for some nitrosamines. *Cancer Lett.* **1989**, *47*, 111–114.

Ruehl, C. Chemical Studies on Tobacco Smoke LXVI. Comparative assessment of volatile and tobacco-specific N-nitrosamines in the smoke of selected cigarettes from the U.S.A., West Germany, and France. *J. Anal. Toxicol.* **1980**, *4*, 255–259.

Scherer, G.; Adlkofer, F. Endogenous formation of N-nitrosoproline in smokers and nonsmokers. In *Mechanisms in Tobacco Carcinogenesis*; Hoffmann, D., Harris, C., Eds.; Banbury Report 23; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1986; pp 137–148.

Tsuda, M.; Nutsuma, J.; Sato, S.; Hirayama, F.; Kakizoe, T.; Sugimura, T. Increase in the levels of N-nitrosoproline, N-nitrosothioproline, and N-nitroso-2-methylthioproline in human urine by cigarette smoking. *Cancer Lett.* **1986**, *30*, 117–124.

Wenke, G.; Brunnemann, K. D.; Hoffmann, D.; Bhide, S. V. A study of Betel Quid Carcinogenesis. IV. Analysis of the saliva of betel chewers. A preliminary report. *J. Cancer Res. Clin. Oncol.* **1984**, *108*, 110–113.

Received for review April 19, 1990. Accepted July 6, 1990.

Registry No. NNA, 123743-84-0; NNK, 64091-91-4; NIC, 54-11-5; COT, 486-56-6; COTAC, 15569-99-0; NAT, 71267-22-6; NAB, 1133-64-8; NNN, 16543-55-8; nitrite, 14797-65-0; nornicotine, 494-97-3; anabasine, 494-52-0; anatabine, 581-49-7; nitrate, 14797-55-8.