Tobacco-Specific N-Nitrosamines: Formation during Processing of Midrib and Lamina Fines

Charles T. MacKown,* Beth Douglass, Mirjana V. Djordjevic, and Lowell P. Bush

Changes in NO_3^-, NO_2^-, and tobacco-specific N-nitrosamine (TSNA) levels in air-cured Burley tobacco lamina and midrib tissue slurries incubated in aqueous solutions at different pH and temperatures were determined. After 24-h incubation, slurry NO_3^- decreased (2–19%) whereas NO_2^- increased in tobacco slurries incubated at 50 °C, but not at 25 or 75 °C. Incubation caused an increase in TSNA but was not related to the NO_2^- content of the tobacco slurries. For example, incubation (unbuffered pH 6.1; 25 or 50 °C) slurries of tobacco midrib tissue had levels of N'-nitrosonornicotine and N'-nitrosoanatabine more than 40-fold greater than those of midrib tissues incubated at a buffered pH 4.5 or 8.5. The NO_2^- content of these slurries, however, ranged from <0.1 (25 °C, pH 4.5) to 220 μmol/g of tobacco (50 °C, pH 8.5). Addition of ascorbic acid (5.0 mM) to tobacco midrib tissues during incubation effectively blocked formation of TSNA. These results indicate that tobacco slurries can be manipulated to reduce NO_3^- levels without accumulating NO_2^- and adversely increasing TSNA levels.

The average content of reconstituted tobacco sheet (RTS) in American blended cigarettes has increased from about 15–30% during the past 20 years (Wynder and Hoffmann, 1967; DeJong et al., 1975; Selke, 1980). Tobacco midrib and scrap leaf tissues not suitable for normal processing are manufactured into RTS using a two-step paper process (Selke, 1980). For the first step, tobacco tissues are pulped with water and extracted and the remaining insoluble fibers are formed into a base sheet on a paper-making type of machine. For the second step, the aqueous extract is concentrated and applied to the base sheet before drying and cutting the RTS into shreds for cigarette blending.

Tobacco alkaloids and NO_3^--derived nitrosating species are involved in the formation of carcinogenic tobacco-specific and volatile N-nitrosamines found in tobacco and tobacco smoke. It is generally accepted that decreased levels of N-nitrosamines and oxides of nitrogen present in tobacco products and smoke are desirable (U.S. Surgeon General, 1982). The RTS process provides an opportunity to favorably modify tobacco products, perhaps more easily achieved than through tobacco cultural practices or genetic modifications of the crop. Modification of the chemical composition of RTS can be achieved by combinations of biological, chemical, and physical treatments of the aqueous pulp and extract. Several patents for decreasing NO_3^- (Mattina and Selke, 1974; Gellatly et al., 1978; Kite et al., 1978; Selke, 1980; Keritsis, 1981; Mattina, 1981; Semp and Teng, 1986) and nicotine (Newton et al., 1975; Geiss et al., 1977) in tobacco materials have been developed.

The objectives of this study were to determine the effects of temperature, pH, and the presence of ascorbic acid, an inhibitor of nitrosation (Douglass et al., 1978), on the levels of NO_3^-, NO_2^-, and tobacco specific N-nitrosamines in incubated aqueous slurries of air-cured tobacco tissues. Burley tobacco tissues were used in these studies, since Burley tobacco in contrast to other tobacco types (e.g., flue-cured) contains markedly higher concentrations of NO_3^- due to differences in cultural practices among tobacco types (Akehurst, 1981; Miner and Sims, 1983).

U.S. Department of Agriculture—Agricultural Research Service (C.T.M.), and Department of Agronomy (C.T.M., B.D., M.V.D., L.P.B.), University of Kentucky, Lexington, Kentucky 40546-0091.

EXPERIMENTAL SECTION

Burley Tobacco Tissues. Lamina and midrib tissues from air-cured high-intermediate alkaloid Burley 21 alkaloid isoline grown at Spindletop Research Farm, Lexington, KY, in 1984 were used in the incubation studies. Recommended cultural practices were followed during the growing season (Atkinson et al., 1976), and the mature tobacco was harvested and air-cured in a manner conventional for Burley tobacco. Also, a sample of commercially manufactured reconstituted tobacco sheet (RTS) was obtained for chemical characterization. The RTS was manufactured from stem and lamina tissue remnants of burley and flue-cured tobaccos by the two-step paper process. The ratios of leaf tissue and tobacco types were representative of remnants used for RTS after manufacture of American Standard Blend cigarettes. All samples were ground to pass a 40-mesh screen, stored for later use in plastic bags, and kept in containers at room temperature in the dark. Moisture contents of stored samples were about 5–7% (w/w).

Incubation Experiments. Ground Burley lamina and midrib tissues (2.0 g) were weighed into 50-mL centrifuge tubes, and slurries were prepared with three different initial pH levels. Slurries were created by adding to each tube 18 mL of either a pH 4.5 citrate–phosphate buffer solution (Gomori, 1962) with 1 N HCl to offset the buffering capacity of the tobacco tissues, distilled deionized water only, or 200 mM TAPS [N-[tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid] buffer with 1 N NaOH to provide an initial pH 8.5. The tubes were then sealed with paraffin film and capped with aluminum foil before the tubes were incubated for 24 h at 25, 50, and 75 °C in a water bath. The incubated unbuffered tissue slurries of lamina increased from an initial pH 5.2 to about pH 5.5, and midribs increased from an initial pH 6.1 to about pH 6.4. The buffered tissue slurries changed less than 0.3 pH unit during the incubation experiments. The experimental design was randomized complete block with four replicates.

The effect of ascorbic acid on chemical composition and tobacco-specific N-nitrosamine (TSNA) formation in tobacco slurries was determined in a separate experiment. Unbuffered (pH 6.1) and pH 8.5 midrib tissue slurries were

0021-8561/88/1436-1031$01.50/0

Table I. Chemical Composition of Air-Cured High-Intermediate Alkaloid Burley 21 Isoline Lamina and Midrib Tissues and Commercially Produced Reconstituted Sheet

				alkaloids,[a] %				TSNA,[b] ppm		
sample	total N, %	NO_3^-, μmol/g	NO_2^-, μmol/g	NIC	NNIC	ANAB	ANAT	NNN	NAT	NNK
lamina	4.6	663	<0.1	3.56	0.09	0.02	0.09	4.3	13	0.34
midrib	4.7	2230	<0.1	0.55	0.04	<0.01	0.02	1.5	1.8	0.57
RTS	2.3	433	0.6	0.51	0.02	<0.01	0.01	3.4	2.5	0.49

[a] Alkaloid abbrevations: NIC, nicotine; NNIC, nornicotine; ANAB, anabasine; ANAT, anatabine. [b] TSNA abbreviations: NNN, *N'*-nitrosonornicotine; NAT, *N'*-nitrosoanatabine; NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone.

incubated at 50 °C for 24 h in the absence and presence of 5.0 mM ascorbic acid as described above. The unbuffered tobacco slurry treated with ascorbic acid remained at about pH 6.0 throughout the 24-h incubation.

At the end of the incubation period, the tissue slurries were centrifuged (1000*g*), the supernatant was filtered by vacuum through a 0.45-μm HA Millipore filter, and the pelleted tissue was resuspended in H_2O, centrifuged, and filtered an additional three times. Filtrates were stored frozen until analyzed. Tissue residues were frozen, freeze-dried, mixed, and stored at room temperature in a desiccator until analyzed.

TSNA Analyses. The general procedures described by Hecht et al. (1975) as modified by Andersen and Kemp (1985) were adapted. A 10-mL aliquot of the tissue–slurry filtrate was mixed with 10 mL of citrate–phosphate buffer (pH 4.5) plus ascorbic acid (20 mM) solution and adjusted to pH 5.0. This aqueous mixture was partitioned with three 30-mL portions of ethyl acetate. The combined ethyl acetate fractions were partitioned with three 5.0-mL portions of 1 N HCl and the combined HCl fractions containing the TSNA were adjusted at <4 °C to pH 5.0 with 10 N NaOH. This aqueous fraction was partitioned with three 5.0-mL portions of chloroform, and the filtered chloroform fractions were dried over anhydrous sodium sulfate. An aliquot of the chloroform fraction was evaporated to dryness with N_2 and diluted with acetone containing azobenzene as an internal standard. Tissue TSNA were extracted from 1-g samples with 12 mL of the citrate–phosphate buffer (pH 4.5) plus ascorbic acid (20 mM) by shaking at room temperature in a stoppered centrifuge tube on a wrist-action shaker overnight. The suspension was centrifuged and the supernatant decanted. The pellet was washed twice with 10-mL portions of the buffered extracting solution by suspending the pellet and centrifuging and decanting the wash. The aqueous extract and washes were combined and pH adjusted to 5.0 with 1 N NaOH before the TSNA were partitioned and prepared for GC analysis as described above.

A Varian 3700 GC with a 50-m WCOT GB-5 fused silica capillary column from Analab connected to a splitless injector and a thermionic-specific N–P detector was used (Djordjevic et al., 1986). The injector and detector were maintained at 280 °C, and the oven temperature was held at 150 °C for 3 min postinjection and then programmed to 225 °C at 2 °C/min and held for 5 min.

The GC quantitation of the isolated TSNA was achieved by internal standardization with azobenzene. Calibration of retention times and recovery–response factors of NNN (*N'*-nitrosonornicotine), NAT (*N'*-nitrosoanatabine), and NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) were performed with authentic TSNA.

Other Chemical Analyses. Alkaloids were determined by GC (Severson et al., 1981; Madsen et al., 1985) using the same gas chromatograph, injector, column, and detector used for TSNA. Injector and detector temperatures were 220 and 230 °C, respectively. Oven temperature was 110 °C for 3 min postinjection and then raised to 200 °C at 6 °C/min. Quantitation of nornicotine, anabasine, and anatabine were based on nicotine recovery–response factor.

Tissue total N (including NO_3^-) in Kjeldahl digest (Bradstreet, 1965) was measured spectrophotometrically following Berthelot reaction (Cataldo et al., 1974). Tissue NO_3^- in hot-water (97 °C) extracts, filtrate NO_3^- from incubated tobacco tissues, and tissue NO_2^- were determined by methods previously described (Lowe and Gillespie, 1975; MacKown et al., 1984). The NO_2^- present in hot-water (97 °C) extracts of residues and filtrates of incubated tobacco tissues were analyzed colorimetrically (Snell and Snell, 1949).

Reagents. The NNN was provided by Dr. K. Brunnemann, American Health Foundation, Naylor Dana Institute for Disease Prevention, Valhalla, NY; NAT was provided by Dr. R. Andersen, USDA—ARS, Department of Agronomy, University of Kentucky, Lexington, KY; NNK was purchased from Chemsyn Science Laboratories, Lenexa, KS. The chromatogram of each TSNA reference compound indicated high purity.

RESULTS AND DISCUSSION

The chemical compositions of lamina and midrib tissues used in the incubation studies and commercially produced RTS are presented in Table I. Consistent with previous reports (Phillips and Bacot, 1958; Neurath and Ehmke, 1964; Andersen and Litton, 1975; Djordjevic et al., 1985; MacKown and Jones, 1986), midrib tissue concentrations of alkaloids were lower and NO_3^- higher than lamina tissue. Comparisons of the same alkaloids in RTS and midrib tissue indicated similar levels, but the NO_3^- level of RTS was lower than the midrib tissue. The low NO_3^- concentration of the RTS probably reflects a dilution of Burley (high-NO_3^-) with flue-cured (low-NO_3^-) tobacco tissues used in manufacturing. In addition, methods to reduce NO_3^- levels during RTS processing may have been used. Midrib tissue concentrations of TSNA were lower (NNN, NAT) or slightly greater (NNK) than lamina TSNA. Differences in the alkaloid concentrations of lamina and midrib tissues could partially account for the differences in TSNA levels of these tissue (MacKown et al., 1984). Concentrations of TSNA in RTS were not greatly different from values in the air-cured tissues, indicating that the manufacturing methods apparently did not adversely alter the accumulation of TSNA during processing.

Incubation Experiments. Tissue residue NO_3^- and NO_2^- generally accounted for less than 5 and 2% of the total NO_3^- plus NO_2^- recovered from the slurries of lamina and midribs, respectively (data not shown). The NO_3^- and NO_2^- concentrations presented are based on total recoveries from the slurries and are expressed on a tissue weight basis for comparison with the initial chemical composition of the tobacco tissues. The TSNA values reported, however, represent TSNA recovered from the filtrate. Essentially all (>94%) of the TSNA were associated with the filtrate from the slurries (data not shown), reflecting effective separation of TSNA for the centrifuging, filtering, and washing methods used in this study.

Table II. Slurry NO_3^- and NO_2 Recovered after 24-h Incubation of Lamina and Midrib Tobacco Tissues

	temperature of lamina slurry, °C						temperature of midrib slurry, °C					
	NO_3^-, μmol/g			NO_2^-, μmol/g			NO_3^-, μmol/g			NO_2^-, μmol/g		
init pH[a]	25	50	75	25	50	75	25	50	75	25	50	75
4.5	647	612	596	0.1	3.4	0.5	2110	2090	2090	<0.1	1.0	<0.1
5.2/6.1	627	596	540	0.1	6.5	<0.1	2060	2060	2030	4.5	37	<0.1
8.5	642	595	587	0.1	19	0.4	2110	1900	2030	0.1	220	<0.1
LSD (0.05)[b]												
pH		ns[c]			ns			ns			28	
°C		48			ns			58			28	
pH × °C		ns			ns			100			50	

[a] Initial pH 4.5, citrate–phosphate buffered; 5.2/6.1, unbuffered lamina/midrib; 8.5, TAPS buffered. [b] LSD (0.05) = least significant difference ($p = 0.05$). [c] ns = not significant.

Table III. Tobacco-Specific *N*-Nitrosamines Recovered after 24-h Incubation of Lamina and Midrib Tissues

		lamina TSNA,[a] ppm			midrib TSNA, ppm		
temp, °C	init pH[b]	NNN	NAT	NNK	NNN	NAT	NNK
25	4.5	6.3	18	2.5	3.2	6.2	1.9
	5.2/6.1	8.1	18	0.63	530	580	3.7
	8.5	4.8	16	2.3	4.3	5.4	1.7
50	4.5	6.3	17	6.4	2.5	3.7	2.7
	5.2/6.1	6.8	20	9.7	410	460	3.9
	8.5	8.6	20	2.6	7.4	11	1.6
75	4.5	5.7	15	2.8	1.4	3.3	1.1
	5.2/6.1	6.1	16	3.5	2.1	3.6	1.5
	8.5	4.8	9.7	2.1	2.0	2.2	1.7
	LSD (0.05)[c]						
	pH	ns[d]	ns	ns	17	15	ns
	°C	ns	5.1	ns	17	15	ns
	pH × °C	ns	ns	ns	30	26	ns

[a] TSNA abbrevations: see footnote *b*, Table I. [b] Initial pH, see footnote *a*, Table II. [c] LSD (0.05) = least significant difference ($p = 0.05$). [d] ns = not significant.

Recoveries of lamina and midrib NO_3^- after 24-h incubation were consistently lower (2–19% for lamina, 5–15% for midrib) than the initial tissue NO_3^- contents (cf. Tables I and II). Temperature had a greater effect than pH on the net decrease in NO_3^- levels. For both tissues, generally more NO_3^- was recovered at 25 °C than at 50 or 75 °C. Incubations at 50 °C and pH 8.5 generally gave the greatest accumulations of NO_2^-.

Mixed populations of bacteria capable of respiratory reduction of NO_3^- are present in large numbers on tobacco leaves and clearly are responsible for NO_3^- reduction in uncured leaf homogenates and filtrates from cured tobacco leaves (Parsons et al., 1986). The microorganisms involved in respiratory reduction of NO_3^- form a biochemically and taxonomic diverse group, including thermophilic bacteria, that use *N*-oxides as terminal electron acceptors for energy metabolism in the absence of oxygen (Delwiche and Bryan, 1976; Payne, 1981; Knowles, 1982). This diversity among the NO_3^--respiring bacteria would likely elicit differential NO_3^- reduction and NO_2^- accumulation responses to the temperature and pH treatments imposed. Parsons et al. (1986) reported that uncured tobacco leaf homogenates incubated at about 21 °C exhibit a lag period of nearly 12 h before the onset of rapid population growth of NO_2^- accumulators and denitrifiers and the commencement of NO_3^- reduction. In the present experiments the incomplete reduction of NO_3^- is probably due to an insufficient population of bacteria.

The decrease in slurry NO_3^- was greater than NO_2^- accumulation. Denitrifying microorganisms associated with air-cured tobacco tissues are capable of reducing NO_3^- to N gases (N_2O, N_2) when leaf homogenates are incubated (Parsons et al., 1986). The occurrence of dissimilatory reduction (denitrification) may account for the difference between net NO_3^- loss and NO_2^- accumulation in the present experiments. However, chemical decomposition of NO_2^-, particularly at temperatures >50 °C and pH <5.0, cannot be excluded (Keeney et al., 1979).

Incubation of tobacco lamina tissues resulted in only slight increases in NNN and NAT, but an average 10-fold increase in NNK compared to the unincubated lamina tissue (cf. Tables I and III). The TSNA levels of incubated lamina were generally not significantly different among the temperature and pH treatments. In contrast, high levels of NNN and NAT were recovered from tobacco midrib tissues incubated at 25 and 50 °C in unbuffered (initial pH 6.1) solution (Table III). Nitrosation and demethylation of nicotine could account for the formation of NNN (Hecht et al., 1978) in excess of the apparent nornicotine level present in unincubated midrib tissue. Reasons for the formation of higher levels of NAT compared to the initial levels of anatabine are not clear, but may be related to an underestimate of anatabine, which was quantitated based on a nicotine recovery–response factor. The differences between lamina and midrib NNN and NAT levels after unbuffered incubation are unknown. Midrib NNK levels averaged about 4-fold greater than unincubated midrib tissue (cf. Tables I and III), but the NNK levels were not significantly different among the treatments. For the unbuffered incubated midrib tissue, NNN and NAT levels of the 25 °C treatment were significantly greater than 50 °C.

Despite the marked differences in midrib TSNA levels, there was no apparent association between NO_3^- loss, NO_2^- accumulation, and TSNA levels in these incubated tissues. At pH 8.5 and 50 °C the NO_2^- level was considerably greater than any other treatment (Table II), yet NNN and NAT levels for these conditions were more than 40-fold less than the unbuffered treatment (Table III; 7.4 and 11 versus 410 and 460 ppm, respectively). At high pH, the protonation of amines (tobacco alkaloids) and the formation of the nitrosating reactant N_2O_3 from NO_2^- would not

Table IV. Effect of Ascorbate (5.0 mM) on Slurry NO_3^- and NO_2^- and Tobacco-Specific *N*-Nitrosamines Recovered after 24-h Incubation of Midrib Tissues at 50 °C

treatment	init pH[a]	NO_3^-, μmol/g	NO_2^-, μmol/g	TSNA,[b] ppm NNN	NAT	NNK
control	6.1	2110	63	1100	430	6.0
	8.5	2100	170	30	13	3.9
ascorbate	6.1	2240	4.7	4.9	2.6	1.6
	8.5	2180	96	10	5.2	3.0
LSD (0.05)[c]						
treatment		ns[d]	58	320	170	ns
pH		57	58	320	170	1.3
treatment × pH		ns	ns	450	240	1.9

[a] Initial pH, see footnote *a*, Table II. [b] TSNA abbreviations: see footnote *b*, Table I. [c] LSD (0.05) = least significant difference (p = 0.05). [d] ns = not significant.

be favored (Mirvish, 1975). Incubation of tobacco tissues at high pH would not only facilitate NO_3^- and NO_2^- reduction (Parsons et al., 1986) but also minimize accumulation of NNN and NAT (Table III). The lack of accumulation of TSNA in tissues incubated at pH 4.5 is less clear but may be the consequence of insufficient NO_2^- due to denitrification or chemical decomposition.

Incubations of tobacco midrib tissue at 50 °C in the presence of 5.0 mM ascorbic acid markedly minimized the formation of TSNA (Table IV). Results of the control treatments of the experiment with ascorbic acid generally agreed well with the same treatments described previously. Compared to unbuffered pH 6.1 incubation, apparent reduction of NO_3^- and accumulation of NO_2^- were significantly greater, but accumulations of NNN and NAT were less for midrib tissues incubated at buffered pH 8.5 (Table IV). These results clearly indicate the effectiveness of the nitrosation inhibitor ascorbic acid (Douglass et al., 1978) in reducing the accumulation of TSNA during aqueous incubation of tobacco midrib tissue. Since ascorbic acid reacts with nitrite to form nitric oxide and dehydroascorbic acid under acidic conditions, a beneficial effect of ascorbic acid at alkaline pH would not be expected (Mirvish, 1975).

The low levels of TSNA present in the commercially produced RTS indicate that RTS can be produced with levels of TSNA comparable to those of air-cured Burley lamina [these results and those of MacKown et al. (1984)]. Tobacco slurries treated by either sterilization, with microbial inhibitors, with bases to increase pH, or with ascorbic acid would each probably be effective in decreasing the accumulation of TSNA during the production of RTS. But, decreasing NO_3^- content would be desirable also since NO_3^- levels are strongly correlated with NO formation and the levels of volatile and tobacco-specific *N*-nitrosamines in tobacco smoke (Tso et al., 1975; Sims et al., 1979; Brunnemann and Hoffmann, 1982). Our results indicate that formation of TSNA can be minimized and reduction, presumably dissimilatory, of NO_3^- and NO_2^- achieved with high NO_3^- tobacco tissues. The incubation of alkaline pH modified or ascorbic acid treated tobacco slurries inoculated with denitrifiers (Mattina and Selke, 1974; Mattina, 1981; Semp et al., 1986) would appear to be the most appropriate approach to rapidly decrease tissue NO_3^- levels and block accumulation of TSNA.

ACKNOWLEDGMENT

We extend our appreciation to Klaus D. Brunnemann of the American Health Foundation, Naylor Dana Institute for Disease Prevention, Valhalla, NY, for providing NNN and Roger A. Andersen, USDA—ARS, Department of Agronomy, University of Kentucky, Lexington, for providing NAT.

Registry No. NNN, 16543-55-8; NAT, 71267-22-6; NIC, 54-11-5; NNIC, 494-97-3; ANAB, 494-52-0; ANAT, 581-49-7; NNK, 64091-91-4; NO_3^-, 14797-55-8; NO_2^-, 14797-65-0; ascorbic acid, 50-81-7.

LITERATURE CITED

Akehurst, B. C. *Tobacco*, 2nd ed.; Longman: New York, 1981.
Andersen, R. A.; Litton, C. L. *Tob. Sci.* **1975**, *29*, 69.
Andersen, R. A.; Kemp, T. R. *Cancer Res.* **1985**, *45*, 1.
Atkinson, W. O.; Sims, J. L.; Calvert, J. R. *Tob. Sci.* **1976**, *20*, 32.
Bradstreet, R. B. *The Kjeldahl Method for Organic Nitrogen*; Academic: New York, 1965.
Brunnemann, K. D.; Hoffmann, D. *Recent Adv. Tob. Sci.* **1982**, *8*, 103.
Cataldo, D. A.; Schrader, L. E.; Youngs, Y. L. *Crop Sci.* **1974**, *14*, 854.
Delwiche, C. C.; Bryan, B. A. *Annu. Rev. Microbiol.* **1976**, *30*, 241.
DeJong, D. W.; Lam, J.; Lowe, R.; Yoder, E.: Tso, T. C. *Beitr. Tabakforsch.* **1975**, *8*, 93.
Djordjevic, M.; MacKown, C. T.; Bush, L. P. *39th Tob. Chem. Res. Conf.* **1985**, 22.
Djordjevic, M.; Gay, S. L.; Bush, L. P.; MacKown, C. T. *40th Tob. Chem. Conf.* **1986**, 6.
Douglass, M. L.; Kabacoff, B. L.; Anderson, G. A.; Cheng, M. C. *Soc. Cosmet. Chem.* **1978**, *29*, 581.
Geiss, V. L.; Gregory, C. F.; Newton, R. P.; Gravely, L. E. U.S. Patent 4 038 993, 1977.
Gellatly, G.; Uhl, R. G. U.S. Patent 4 131 118, 1978.
Gomori, G. *Methods Enzymol.* **1962**, *1*, 138.
Hecht, S. S.; Ornaf, R. M.; Hoffmann, D. *J. Natl. Cancer Inst. (U.S.)* **1975**, *54*, 1237.
Hecht, S. S.; Chen, C. B.; Hirota, N.; Ornaf, R. M.; Tso, T. C.; Hoffmann, D. *J. Natl. Cancer Inst. (U.S.)* **1978**, *60*, 819.
Keeney, D. R.; Fillery, I. R.; Marx, G. P. *Soil Sci. Soc. Am. J.* **1979**, *43*, 1124.
Keritsis, G. D. U.S. Patent 4 301 817, 1981.
Kite, G. F.; Gellatly, G.; Uhl, R. G. U.S. Patent 4 131 117, 1978.
Knowles, R. *Microbiol. Rev.* **1982**, *46*, 43.
Lowe, R. H.; Gillespie, M. C. *J. Agric. Food Chem.* **1975**, *23*, 783.
MacKown, C. T.; Jones, B. *Tob. Sci.* **1986**, *30*, 132.
MacKown, C. T.; Eivazi, F.; Sims, J. L.; Bush, L. P. *J. Agric. Food Chem.* **1984**, *32*, 1269.
Madsen, J. P.; Bush, L. P.; Gay, S. L. *J. Agric. Food Chem.* **1985**, *33*, 1182.
Mattina, C. F. U.S. Patent 4 308 877, 1981.
Mattina, C. F.; Selke, W. A. U.S. Patent 3 847 164, 1974.
Miner, G. S.; Sims, J. L. *Recent Adv. Tob. Sci.* **1983**, *9*, 4.
Mirvish, S. S. *Toxicol. Appl. Pharmacol.* **1975**, *31*, 325.
Neurath, G.; Ehmke, H. *Beitr. Tabakforsch.* **1964**, *2*, 333.
Newton, R. P.; Geiss, V. L.; Jewell, J. N.; Gravely, L. E. U.S. Patent 4 037 609, 1975.
Parsons, L. L.; Smith, M. S.; Hamilton, J. L.; MacKown, C. T. *Tob. Sci.* **1986**, *30*, 100.
Payne, W. J. *Denitrification*; Wiley: New York, 1981.
Phillips, M.; Bacot, A. M. *U.S. Dept. Agric. Tech. Bull.* **1958**, *No. 1186*.
Selke, W. A. "Reconstituted Tobacco Sheet". In Gori, G. B., Bock, F. G., Eds. *Banbury Report III. A Safe Cigarette*? Cold Spring Laboratory: Cold Spring Harbor, NY, 1980; pp 205–214.
Semp, B. A.; Teng, D. M. U.S. Patent 4 566 469, 1986.
Severson, R. F.; McDuffie, K. L.; Arrendale, R. F.; Gwynn, G. R.; Chaplin, J. F. *J. Chromatogr.* **1981**, *211*, 111.
Sims, J. L.; Atkinson, W. O.; Benner, J. F. *Tob. Sci.* **1979**, *23*, 11.
Snell, F. C.; Snell, C. T. *Colorimetric Methods of Analysis*, 3rd ed.; Van Nostrand: New York, 1949; Vol. II.
Tso, T. C.; Sims, J. L.; Johnson, D. E. *Beitr. Tabakforsch.* **1975**, *8*, 34.
U.S. Surgeon General. *The Health Consequences of Smoking-Cancer*; U.S. Surgeon General: Washington, DC; 82-50179, 1982; DHHS (PHS) pp 181–235.
Wynder, E. L.; Hoffmann, D. *Tobacco and Tobacco Smoke, Studies in Experimental Carcinogenesis*; Academic: New York, 1967.

Received for review May 26, 1987. Revised manuscript received November 9, 1987. Accepted March 29, 1988. Mention of a

trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture (USDA) and does not imply its approval to the exclusion of other products or vendors that may be suitable. This work was supported in part by USDA Tobacco Cooperative Agreement No. 58-43YK-5-0036 and represents a cooperative investigation by the USDA—ARS and the Kentucky Agricultural Experiment Station (KAES), Lexington. Paper No. 87-3-87 of the journal series of the KAES, Lexington, published with the approval of the Director.

Methylation of DNA of Maize and Wheat Grains during Fumigation with Methyl Bromide

Alvin N. Starratt* and Edwin J. Bond

The possibility that methylation of DNA occurs during fumigation of foodstuffs with methyl bromide was investigated in two grains, maize and wheat, using ^{14}C-labeled fumigant. 7-Methylguanine and 1-methyladenine were identified as major products along with lesser amounts of 3-methylcytosine and 3-methyladenine. 3-Methylguanine was probably also formed in minor amounts. Although less than 1% of the bound radioactivity was associated with the DNA isolated, the results indicated that 0.5–1% of the guanine residues in the DNA of these grains was methylated during treatment with 48 mg/L methyl bromide for 72 h.

Methyl bromide, long used as a fumigant to control insects, rapidly dissipates from foodstuffs after treatment and appears to offer little risk to the consumer (Thompson, 1966; Alexeeff and Kilgore, 1983). Some reaction, however, occurs during fumigation, resulting in methylation of food constituents and the concomitant formation of bromide ion. Although appreciable toxicological effects have not been observed in animals fed food treated with methyl bromide [see Winteringham (1955) and Alexeeff and Kilgore, 1983)], this remains a serious area for concern since so few studies have been conducted.

Proteins are the major site of methylation when commodities are treated with this fumigant; the amount of methylation as indicated by the level of inorganic bromide or of unrecoverable methyl bromide appears to be directly related to protein content (Lewis and Eccleston, 1946; Winteringham and Harrison, 1946; Cova et al., 1986). Studies on wool (Blackburn et al., 1941); wool, silk, collagen, and gelatin (Blackburn and Phillips, 1944); wheat flour (Winteringham et al., 1955; Bridges, 1955); and cocoa beans (Asante-Poku et al., 1974) have indicated that N-, O-, and S-methylation of proteins occurs.

Methyl bromide has been demonstrated to be mutagenic in several systems (Djalali-Behzad et al., 1981; Ehrenberg et al., 1974; Kramers et al., 1985). It also was reported to be carcinogenic in rats (Danse et al., 1984), but this conclusion was not substantiated when the evidence was reexamined (Anonymous, 1984), and it has not been confirmed by other work (Boorman et al., 1986; Reuzel et al., 1987). Identification of labeled 7-methylguanine as a component of DNA from mice exposed to [^{14}C]methyl bromide (Djalali-Behzad et al., 1981) indicates that this fumigant can react with nucleophilic sites of DNA in a manner similar to that observed for other alkylating agents (Singer and Kuśmierek, 1982; Jeffrey, 1985). The methylation of the DNA of foodstuffs during fumigation with methyl bromide, however, does not appear to have been investigated despite the possibility that methylated purines and/or pyrimidines, nucleotides, or other methylated fragments of the nucleic acids may be reincorporated [see Saffhill et al. (1985)] into the DNA of the food consumer, potentially leading to mispairing of the bases and mutation. The present study is concerned with the methylation of maize and wheat DNA during fumigation with [^{14}C]-methyl bromide.

Research Centre, Agriculture Canada, 1400 Western Road, London, Ontario N6G 2V4, Canada.

MATERIALS AND METHODS

Chemicals. [^{14}C]Methyl bromide (8.95 μCi/μmol) from Pathfinder Laboratories (St. Louis, MO) and unlabeled methyl bromide (99.5% pure) from Matheson Gas Products (Whitby, Ontario) were used for the treatments. Concentrations of methyl bromide were determined by gas chromatography using a 2 m × 3 mm (i.d.) nickel column packed with 120/140-mesh Chromosorb 102, in a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector.

3-Methylcytosine, 1-methyladenine, 3-methyladenine, and 7-methylguanine were obtained from Sigma Chemical Co. (St. Louis, MO), and 3-methylguanine and 7-methyladenine were purchased from Chemical Dynamics Corp. (South Plainfield, NJ). O^6-Methylguanine (6-methoxy-2-aminopurine) was prepared from 6-chloroguanine (Sigma) as described by Balsiger and Montgomery (1960).

Radioanalysis. A Beckman LS-9000 liquid scintillation spectrometer was used for quantitating radioactivity. Aliquots of methyl bromide, methylated DNA hydrolysis products, and chromatographic fractions were added to scintillation cocktail (Omnifluor; Dupont Canada, NEN Products, Lachine, PQ). For estimation of the radioactivity in treated maize and wheat, samples were combusted in an R. J. Harvey Instrument Corp. (Hillsdale, NJ) biological oxidizer, Model OX300, and the $^{14}CO_2$ was collected in 15 mL of carbon-14 cocktail (R. J. Harvey).

Methyl Bromide Treatments. Maize grain (30 g) was placed in a 290-mL flask fitted with adapters and septa. A mixture of labeled and unlabeled methyl bromide to give a final concentration of approximately 48 mg/L (calculated on total volume of empty flask) was introduced by means

0021-8561/88/1436-1035$01.50/0 Published 1988 by the American Chemical Society