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Acid-mediated mutagenicity of tobacco snuff: its possible mechanism

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Summary

Polar solvent extracts of tobacco snuff under acidic conditions were mutagenic in *Salmonella typhimirium*. Using the Griess reagent test, nitrite ranging from ~ 1.8 to 5.4 mg/g of snuff was found in the polar fraction of extracts. After acid treatment, nitroso compounds in the amount corresponding to the nitrite concentration were detected. The mutagenic potency of the acid-treated extracts was consistent with the content of nitroso compounds generated. Formation of nitroso compounds and the mutagenic activity under acidic conditions was inhibited by ascorbic acid. The results indicate that a nitrosation process was involved in snuff extracts during acid treatment. Studies related to the source of nitrite in tobacco snuff demonstrated that snuff contained bacteria which were able to reduce nitrate to nitrite and that the amount of nitrite in snuff extracts could be further increased by incubation of the extracts with the bacteria. Since snuff contains a considerable amount of nitrate, it seems that (1) reduction of nitrate in snuff to nitrite by bacteria, and (2) nitrosation of certain constituents in snuff by nitrite under acidic conditions to form mutagenic nitroso compounds are possible mechanisms responsible for the acid-mediated mutagenicity of snuff extracts.

Tobacco snuff, one form of smokeless tobacco, is commonly used as a substitute for smoking by several occupational groups, e.g., coal miners. In the general population, snuff dipping is becoming increasingly popular among young male athletes and students in high school and college (Christen, 1980). Generally, snuff is taken by dipping between the gum and lower lip or cheeks. During the dipping, most of snuff is spit out by the user; however, a considerable amount of saliva-soluble substances may be swallowed. The potential health impact of tobacco snuff to the user has been well documented (Christen, 1980; Hecht et al., 1975; Sundstrom et al., 1982; Winn et al., 1981). It has been reported that snuff dipping poses potential risks for cancers in the oral cavity, pharynx, larynx, and esophagus (Christen, 1980). Furthermore, carcinogenic tobacco-specific *N*-nitrosamines generated during processing have been detected in tobacco snuff (Hoffmann and Adams, 1981).

In a case-control study of U.S. underground coal miners, coal mine dust was found to be a gastric cancer risk in long-term cigarette smokers (Ames, 1983). Since tobacco snuff is a common

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substitute for cigarette smoking during work and saliva-soluble substances can be swallowed into the acidic environment of the stomach, it is of importance to know the effect of acidic conditions on the mutagenic activity of tobacco snuff. In a previous study, we found that crude solvent extracts of tobacco snuff under acidic conditions were mutagenic in *Salmonella typhimurium* (Whong et al., 1984). The mutagenic activity was observed only in the polar solvent extracts. The mutagenic substances induced predominantly frameshift mutations and were direct-acting mutagens (Whong et al., 1984). In this report, studies were extended to determine the possible mechanism contributing to the acid-mediated mutagenicity of tobacco snuff.

Materials and methods

Samples and chemicals. Two leading brands of American tobacco snuff tested in this study were purchased from a local supermarket. Ammonium sulfamate, *N*-(1-naphthyl) ethylenediamine · 2HCl, and sulfanilic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Ascorbate (sodium salt) and hydrobromic acid (30–32%), were purchased from Calbiochem-Behring Corp. (La Jolla, CA) and Fisher Scientific Co. (Fair Lawn, NJ), respectively. Sodium nitrite, potassium nitrate and organic solvents (dichloromethane, acetone, and methanol) were obtained from Mallinckrodt, Inc. (St. Louis, MO).

Sample extraction. One can (~ 34 g) of tobacco snuff was extracted first with 250 ml of dichloromethane (DCM) for 16 h at room temperature (~ 25°C) with shaking (250 rpm). The DCM extract was collected by filtration and the residue was extracted a second time with 250 ml of 1 : 1 mixture of methanol plus acetone (M + A) under the same condition as used for the DCM extraction. Each extract was evaporated to dryness with a rotary evaporator and was redissolved in 10 ml of 75% dimethyl sulfoxide (DMSO). For the extraction with H_2O, 15 ml of distilled H_2O were added to 10 g of tobacco snuff which was then ground in a porcelain mortar at ~25°C for 15 min and centrifuged. The supernatant of tobacco snuff served as the H_2O extract.

Acid treatment of extracts. The combined (DCM and M + A) or individual extract was adjusted to pH 3.0 with 12 N hydrochloric acid. The original extracts (pH ~ 8.2) without acid adjustment served as control. All extracts, with and without acid treatment, were incubated at 37°C for 2 h with slow shaking (100 rpm). At the end of incubation, extracts were assayed for mutagenicity, nitrite content and nitroso compound formation.

Mutagenicity test. Mutagenic activity of tobacco snuff extracts was determined using the standard Ames Salmonella assay system (Ames et al., 1975). Reverse mutations were scored from histidine dependence (His^-) to histidine independence (His^+) using tester strain TA98 without metabolic activation. The doses of test extracts as shown in tables were based on the weight of original materials. A 2-fold or greater number of spontaneous revertants than the control with a dose-dependent response was considered a positive mutagenic response.

Determination of nitrite content. An aliquot (0.025 ml) of original or properly diluted (2–16 times dilution) extract was treated with the Griess reagent as follows: (1) 1 ml of 0.5% sulfanilic acid for 5 min and then (2) 1 ml of 0.05% *N*-(1-naphthyl) ethylenediamine · 2HCl (in 30% acetic acid) for 10 more min. The absorbance (OD) of the reaction mixture was determined at 550 nm with a spectronic colorimeter (Takada and Kanaya, 1982). The OD of reaction mixtures was converted into actual nitrite content from a standard curve of ODs determined with known amounts of nitrite.

Determination of nitroso-compound formation. The formation of nitroso compounds in snuff samples was measured using the method of Takeda and Kanaya (1982) with slight modification. Each test sample (0.2 ml) from snuff extracts was treated with 0.25 ml of ammonium sulfamate (30 mg/ml in 30% acetic acid) for 15 min at 4°C with shaking (200 rpm). This treatment was used to eliminate nitrite in the sample. A small amount of the above mixture (0.025 ml) was further reacted with 1 ml of hydrobromic acid (1% in glacial acetic acid) for 10 min at 25°C and then treated with the same Griess reagent as that used for determining nitrite content.

Treatment of extracts with ascorbate. A sample (0.5 ml) of snuff extracts was mixed with an equal

amount of ascorbate solutions (12.5, 25, 50 mg/ml in H_2O). The reaction mixture was adjusted to pH ~ 3.0 with HCl and then incubated at 37°C for 2 h with shaking (100 rpm). The mixtures were further used for determining mutagenic activity and nitroso compound formation.

Determination of bacterial contamination. 1 g of snuff was added to 10 ml of sterile H_2O and the mixtures were vigorously vortexed for 5 min. After being filtered (through paper filters), samples of filtrates were plated onto nutrient agar plates. After 2-day incubation, colonies occurring on the plates were scored. Bacterial colonies were identified by their response to different antibiotics (i.e., streptomycin, ampicillin, chloramphenicol, and cycloheximide).

Results

The effect of acidic pH on the mutagenic activity of tobacco snuff extracts is shown in Fig. 1. Only acid-treated polar solvent extracts showed mutagenic activity. Using the Griess reagent test, high amounts of nitrite (1.8–5.4 mg/g of snuff) were detected in H_2O and polar solvent extracts, whereas trace amounts of nitrite were found in non-polar extracts (Table 1). The result of spectrophotometric determination showed a high concentration of nitroso compounds in acid-treated snuff polar and H_2O extracts. Very low absorbances for nitroso compounds were found, however, in snuff polar and H_2O extracts without acid treatment (Table 2). The amounts of nitroso

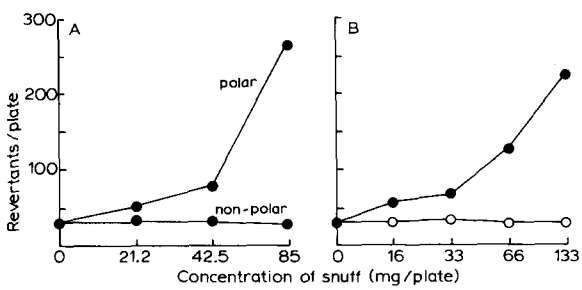

Fig. 1. Mutagenicity of tobacco snuff extracts in TA98 without S9 activation. (A) Organic solvent extracts with pH adjustment to 3. The mutagenic activity of extracts without pH adjustment was similar to that of non-polar extract with pH adjustment. (B) H_2O extracts with (●) and without (○) pH adjustment to 3.

TABLE 1

NITRITE CONTENT IN TOBACCO SNUFF EXTRACTS [a]

Sample	Nitrite	
	mg/ml of extract (mean ± SE)	mg/g of snuff (mean ± SE)
Snuff A		
Polar (M + A) extract [b]	13.6 ± 3.2	4.0 ± 0.9
Non-polar (DCM) extract [c]	0.08 ± 0.02	0.02 ± 0.005
H_2O extract	3.6 ± 0.5	5.4 ± 0.75
Snuff B		
Polar (M + A) extract	6.2 ± 0.9	1.8 ± 0.26
Non-polar (DCM) extract	0.03 ± 0.005	0.01 ± 0.001
H_2O extract	1.5 ± 0.1	2.2 ± 0.14

[a] 34 g of tobacco snuff was first extracted with DCM and then extracted second time with M + A. The final volume was 10 ml for each extract. For extracting with H_2O, 10 g of snuff was ground in 15-ml distilled H_2O. Results are from 3 Expts. in duplicate.

[b] M + A is methanol plus acetone.

[c] DCM is dichloromethane.

compounds in the extracts corresponded to their mutagenic potencies. The acid-mediated mutagenic activity of snuff extracts, however, was inhibited in the presence of ascorbic acid during the acid treatment (Table 3). The degree of inhibition corresponded to an increase in the concentration of ascorbic acid and a decrease in the absorbances

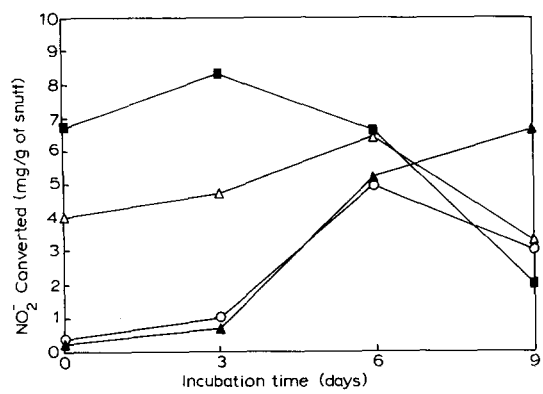

Fig. 2. Nitrite content in the extract of tobacco snuff as a function of incubation time. Snuff (1 g) was added into 15 ml of sterile H_2O, vortexed for 5 min and incubated at room temperature (~ 25°C). At designated incubation times, samples were taken from the snuff/water mixture and nitrite concentration was determined with the Griess reagent. 4 different batches of snuff were assayed.

TABLE 2

MUTAGENICITY AND NITROSO COMPOUND CONTENT OF TOCABBO SNUFF EXTRACTS WITH AND WITHOUT pH ADJUSTMENT [a]

Sample	pH adjustment to 3	Revertants/plate [c] (mean ± SE)	Absorbance for [b] nitroso compound formation (mean ± SE)
Snuff A			
Polar (M + A) extract [d]	−	18 ± 4	0.07 ± 0.01
	+	296 ± 32	0.65 ± 0.09
Non-polar (DCM) extract [e]	−	22 ± 4	0.06 ± 0.01
	+	18 ± 3	0.07 ± 0.008
H_2O extract	−	22 ± 5	0.06 ± 0.01
	+	184 ± 18	0.48 ± 0.04
Snuff B			
Polar (M + A) extract	−	20 ± 5	0.06 ± 0.007
	+	135 ± 12	0.28 ± 0.04
Non-polar (DCM) extract	−	17 ± 3	0.04 ± 0.01
	+	23 ± 4	0.06 ± 0.005
H_2O extract	−	18 ± 5	0.05 ± 0.01
	+	82 ± 10	0.20 ± 0.02

[a] Mutagenic activity was assayed using TA98 without S9 activation. The spontaneous reversion of TA98 was 20 revertants/plate. Results are from 3 Expts.

[b] Absorbance of each sample was determined after being treated with ammonium sulfamate and hydrobromic acid and then reacted with the Griess reagent.

[c] 85 mg of extract was added onto each plate.

[d] M + A is methanol plus acetone.

[e] DCM is dichloromethane.

TABLE 3

EFFECT OF ASCORBATE ON THE MUTAGENICITY AND NITROSO COMPOUND FORMATION OF TOBACCO SNUFF EXTRACTS UNDER ACIDIC CONDITIONS [a]

Snuff extract (mg/plate)	Concentration of ascorbate (mg/plate)	Revertants/plates (mean ± SE)	Absorbance [b] for nitroso compound formation (mean ± SE)
0	0	19 ± 5	0
0	5	22 ± 4	0.01 ± 0.002
85	0	287 ± 23	0.60 ± 0.010
85	1.25	238 ± 18	0.43 ± 0.010
85	2.5	43 ± 5	0.09 ± 0.005
85	5	30 ± 6	0.07 ± 0.007

[a] Mutagenic activity was determined in TA98 without S9 activation. Results are from 3 Expts. in duplicate.

[b] Absorbance was measured after being treated with ammonium sulfamate and hydrobromic acid and then reacted with the Griess reagents.

TABLE 4

CONVERSION OF SUPPLEMENTED NITRATE TO NITRITE IN CULTURE BY BACTERIA ISOLATED FROM TOBACCO SNUFF [a]

Incubation time (h)	NO_2^- absorbance (mean ± SE)	Amount of NO_2^- converted (mean ± SE)	% Conversion (mean ± SE)
0	0	0	0
2	0.01 ± 0.005	0	0
4	0.08 ± 0.01	24 ± 2.9	0.16 ± 0.02
6	0.20 ± 0.01	56 ± 2.7	0.37 ± 0.01
8	0.31 ± 0.02	88 ± 5.5	0.58 ± 0.03
16	0.66 ± 0.03	172 ± 7.9	1.14 ± 0.05
24	1.75 ± 0.05	498 ± 13.9	3.32 ± 0.09

[a] The culture, containing 3 ml KNO_3 (20 mg/ml), 1 ml nutrient broth, and isolated bacteria (5×10^7 cells/ml), was incubated at room temperature (~ 25°C). Amounts of NO_2^- were determined with the Griess reagent method. Results are from 4 Expts. in duplicate.

for nitroso compound formation in snuff extracts.

Attempts were made to determine the possible source of nitrite found in tobacco snuff. Approximately $1.1–2.3 \times 10^6$ bacteria/g of snuff were observed among 8 different batches. According to colony characteristics, at least 5–8 different types of bacteria were present. For determining whether the bacteria were responsible for nitrite formation in snuff, nitrate was supplemented to a culture liquid medium in which bacteria isolated from snuff were inoculated. Conversion of supplemented nitrate to nitrite in culture by the bacteria started after 4-h incubation at room temperature ($\sim 25°C$) and further increased as a function of incubation time (Table 4).

Fig. 2 shows the profile of nitrite content in snuff extracts containing pre-existing bacteria during 9-day incubation. In the study, 4 batches of snuff manufactured at different dates were extracted with H_2O. A considerable difference in nitrite ($\sim$ 0.3–6.7 mg/g of snuff) was found among these batches at day 0. Nitrite contents increased to between 5 and 8.2 mg/g of snuff after 3- or 6-day incubation and then (except for one sample) decreased with further incubation.

Discussion

A high amount of nitrite was found in the polar solvent extracts of snuff (H_2O or M + A), but not in their non-polar solvent (DCM) extracts. This is because nitrite salts can readily dissolve in polar inorganic solvents (e.g., H_2O), and also dissolve at some degree in polar organic solvents such as methanol and acetone (especially when they contain water). Nitrite has been shown to be weakly mutagenic and to induce predominantly base-pair substitution mutation in the Ames assay (McCann et al., 1975). In this study, snuff polar extracts containing nitrite were not mutagenic without pH adjustment. The reason for the negative response is probably due to a frameshift mutant tester (TA98) used, which was revertible by frameshift mutagens, and/or an insufficient amount of nitrite in the extracts tested.

It has been well documented that nitrosation of nitrosatable substances by nitrite readily occur under acidic conditions (McCarty, 1981). Since snuff contains nitrosatable precursors (Hecht et al., 1975, 1978), the mutagenic activity of snuff extract observed under acidic conditions could be due to the production of mutagenic nitroso compounds through nitrosation by nitrite. This speculation was substantiated by the detection of nitroso-compound formation using colorimetric determination, and by the correlation of mutagenic activities and the amount of nitroso-compounds generated (Table 2). Ascorbic acid is an effective inhibitor of nitrosation by nitrite (Fan and Tannenbaum, 1973; Mirvish, 1975). An obvious inhibitory effect of ascorbic acid on the mutagenic activity of snuff extracts under acidic conditions, coupled with a decrease in nitroso-compound absorbances, was demonstrated (Table 3). This finding gave further evidence that the acid-mediated mutagenicity of snuff extract is, at least in part, due to nitrosation reaction between certain constituents of snuff and nitrite at the low pH. Although *N*-nitroso compounds almost exclusively induce base-pair substitution mutations with (e.g., for *N*-nitrosamines) and/or without (e.g., for *N*-nitrosamides) metabolic activations, direct-acting nitroso mutagens cause frameshift mutations have also been demonstrated (Rice et al., 1981; Tannenbaum, 1981). The snuff mutagens of a direct-acting frameshift activity observed in our studies might belong to that type of mutagenic nitroso compounds. However, it has been reported that mutagenic nitro compounds can be generated from a reaction of chemicals with nitrite under acidic conditions (Ohta et al., 1983; Wang et al., 1984). Therefore, a possibility of generating mutagenic nitro compounds responsible for the acid-mediated mutagenicity of snuff cannot be excluded.

Mutagenic tobacco-specific *N*-nitrosamines (e.g., *N*-nitrosonornicotine) and other volatile *N*-nitrosamines have been found in tobacco snuff at ppm levels (Brunnemann et al., 1982; Hecht et al., 1975; Hoffmann and Adams, 1981). In the present studies, a trace amount of nitroso compounds detected in snuff extracts without acid treatment might represent *N*-nitrosamines generated during snuff processing. Since tobacco snuff is of neutral or slight basic pH, the occurrence of nitrosation by nitrite during storage may not be significant, because the nitrosation by nitrite is dependent on acidic conditions. However, a high degree of the

nitrosation of swallowed salica-soluble snuff substances by nitrite in the acidic environment of the stomach in users is conceivable.

For further insight into the source of nitrite found in tobacco snuff the possibility of reducing nitrate to nitrite during the processing had been the first concern, because tobacco leaves contained a considerable amount of nitrate. The nitrate contents in tobacco range from ~ 0.1 to 2.48% (dry weight), depending on the type of tobacco and cultivation conditions (Wynder and Hoffmann, 1967). Moreover, a remarkably high number of viable bacteria ($\sim 1.1-2.3 \times 10^6$ cells/g of snuff) were found in different batches of snuff. The ability of the bacteria to convert nitrate to nitrite using supplemented substrate (KNO_3) and snuff extracts has been demonstrated (Table 4 and Fig. 2). The observation of an increase and then a decrease in nitrite levels in snuff extracts during incubation with pre-existing bacteria (Fig. 2) might be due to a continuous reduction process, where nitrate was first reduced to nitrite and nitrite was then decomposed by further reduction. Based on the obtained result, it seems that nitrite formed in tobacco snuff may be derived from pre-existing nitrate by the reduction action of bacteria.

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