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Formation of bacterial mutagens from the reaction of chewing tobacco with nitrite

Wen-Zong Whong, John D. Stewart and Tong-man Ong

Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505 (U.S.A.)

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Summary

Using the Salmonella/microsome assay system, the mutagenicity of chewing tobacco extracts (CTE) treated with and without sodium nitrite under acidic conditions was examined. Mutagenic activity was found only for nitrite-treated CTE in both tester strains, TA98 and TA100, and was independent of metabolic activation. Formation of mutagenic substances from CTE by nitrite was dependent on acidic pHs (the highest at pH 2) and could be inhibited by ascorbate. The mutagenic potency of CTE plus nitrite was proportional to the content of nitroso compounds generated in the reaction mixture, indicating that the nitrosation process was involved. The possible in vivo nitrosation and the potential health effect are discussed.

Chewing tobacco (one of the smokeless tobaccos) is a common substitute for cigarette smoking in several occupational groups, especially in coal miners. Although tobacco chewing is not as popular as cigarette smoking, it is becoming increasingly so among young male athletes and high school and college students (Christen, 1980). The health hazard associated with smokeless tobacco (e.g., chewing tobacco) has been reported to be a potential for inducing cancer in the mouth and organs close to the oral cavity of the user (Christin, 1980; Joyant et al., 1977). Carcinogenic tobacco-specific *N*-nitrosamines [e.g., *N*-nitrosonornicotine and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone] and *N*-nitrosodiethanolamine, generated during tobacco processing, have been found in chewing tobacco (Hecht et al., 1975, 1978; Hoffmann et al., 1975, 1982). These nitrosamines are possible causative agents for the reported cancers (Hecht et al., 1978). However, to our knowledge, the information regarding the genotoxicity and other potential health hazards of chewing tobacco has been very limited.

In a previous study, we found that tobacco snuff (another smokeless tobacco) treated under acidic conditions is mutagenic in *Salmonella typhimurium* (Whong et al., 1984). In this present report, the mutagenic activity of chewing tobacco with or without nitrite treatment at acidic pH was investigated using the Ames Salmonella/microsome assay system (Maron and Ames, 1983). The relationship between nitrosation by nitrite and mutagen formation in the reaction mixture of CTE/nitrite was also determined.

Correspondence directed to: Wen-Zong Whong, National Institute for Occupational Safety and Health, 944 Chestnut Ridge Road, Morgantown, WV 26505-2888, U.S.A.

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Materials and methods

Chemicals

Two leading brands of American chewing tobacco tested in this study were purchased from a local supermarket. Sulfanilic acid, *N*-(1-naphthyl) ethylenediamine · 2HCl and ammonium sulfamate were obtained from Sigma Chemical Co. (St. Louis, MO). Hydrobromic acid (30 ~ 32%), sodium nitrite and ascorbate (sodium salt) were purchased from Fisher Scientific Co. (Fair Lawn, NJ), Mallinckrodt Inc. (Paris, KY) and Calbiochem-Behring Corp. (La Jolla, CA), respectively.

Extraction

One pack (63.7 or 85 g/pack) of chewing tobacco was extracted first with 250 ml of dichloromethane (DCM) for 16 h at room temperature (~ 25°C) with shaking, and then extracted a second time with 250 ml of a 1:1 mixture of acetone and methanol (A + M) under the same conditions. The extracts were then filtered and concentrated with a rotary evaporator. Each extract was redissolved in 20 ml of 50% dimethyl sulfoxide (DMSO). For the extraction with H_2O, 40 ml distilled H_2O was added to 20 g of chewing tobacco. The chewing tobacco was ground for 10 min in a porcelain mortar and then centrifuged (9000 × *g*, 15 min). The supernatant was further concentrated to 10 ml by freeze drying.

Treatment of extracts with nitrite

Prior to extract/nitrite reaction, equal volumes of DCM and A + M extracts from the same brand of chewing tobacco were mixed (original pH ~ 7.8). An equal volume of sodium nitrite solution (15 mg/ml in 50% DMSO) was added to the mixed extract. The mixture of chewing tobacco extract (CTE) and nitrite was then adjusted to pH ~ 3 with hydrochloric acid (12N). The controls (CTE and nitrite solution alone) were also adjusted to pH ~ 3.0 under the same condition. All the pH-adjusted solutions were incubated at 37°C for 2 h with shaking, and then were tested for mutagenic activity.

Treatment of extracts with nitrite and ascorbate

The mixture of CTE and sodium nitrite solution, as described above, was further mixed with different amounts of ascorbate. The reaction mixtures were subjected to pH adjustment and incubation the same as described for the extract/nitrite treatment.

Mutagenicity test

Mutagenic activity was determined using the standard Ames Salmonella assay system (Maron and Ames, 1983). Mutations were scored from histidine dependence to histidine independence using 2 tester strains (TA98 and TA100) with and without S9 activation. Determination of a positive mutagenic response was based on criteria recommended by Ames et al. (1975). The doses of test materials used were not based on the weight of extracted materials, but were based on that of original materials.

Determination of nitroso-compound formation

The concentration of nitroso compounds was measured using the method of Takeda and Kanaya (1982) with slight modification. The test sample (0.2 ml) was treated with 0.25 ml of ammonium sulfamate (30 mg/ml) for 15 min at 4°C with shaking. A small amount of the mixture (0.025 ml) was reacted with 1 ml of hydrobromic acid (1% in glacial acetic acid) for 10 min at 25°C. The reaction mixture was then treated with 2 ml of the Griess reagent [0.5% sulfanilic acid and 0.05% *N*-(1-naphthyl)ethylenediamine · 2HCl in 30% acetic acid] for 10 min. The absorbance of the reaction mixture was determined at 550 nm with a spectronic colorimeter.

Results

The mutagenic activity of CTE was observed only after being treated with nitrite under acidic conditions. The mutagenicity was detected in both TA98 and TA100 with and without S9 activation. No obvious effect of S9 on the mutagenic activity was observed. Fig. 1 shows the results of two brands of CTE with and without nitrite treatments in TA98 without S9 activation. The finding was consistent for both extracts of chewing tobacco. A similar observation was also found in TA100 (results not shown).

To determine further the mutagenicity of CTE plus nitrite, polar and non-polar extracts of chew-

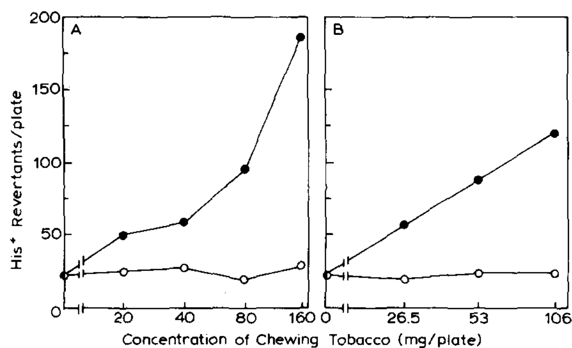

Fig. 1. Mutagenicity of chewing tobacco organic solvent extracts treated with (●) and without (○) nitrite in TA98 without S9 activation (A = brand A; B = brand B).

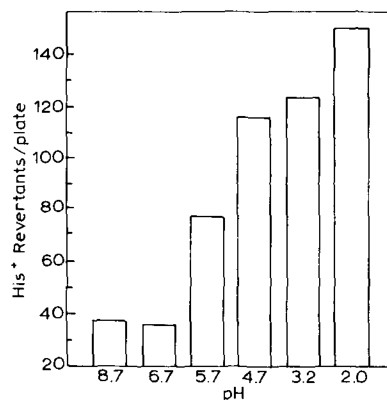

Fig. 3. Effect of pH on the mutagenicity of chewing tobacco organic solvent extracts treated with nitrite in TA98 without S9 activation.

ing tobacco were treated separately with nitrite at pH 3. Both fractions, polar and non-polar, showed mutagenic activity only with nitrite treatment (Fig. 2). The activities were similar with amounts up to 80 mg chewing tobacco/plate. At the highest dose tested, a decrease in mutagenicity of non-polar extract plus nitrite was found. The decrease probably was due to a toxic effect, because the bacterial background growth was considerably inhibited.

Fig. 3 shows the mutagenicity of CTE treated with nitrite at different pHs. Mutagenic activity was found only at acidic pHs and increased as the pH was descreased from 5.7 to 2.0.

Ascorbic acid was used to test whether the mutagenicity of CTE plus nitrite may be attributed to the nitrosation process. As shown in Fig. 4, a dose-related decrease in the mutagenicity of CTE plus nitrite by ascorbate was observed when it was added during the reaction between CTE and nitrite. At the dose of 50 mg ascorbate/plate, the formation of mutagenic substances from CTE plus nitrite was abolished.

By using the Griess reagent test, the detection of nitroso compounds formed in the mixture of CTE plus nitrite in the acidic condition was also conducted. Based on absorbances observed (Table 1), CTE and nitrite alone gave a slight and no indication of the presence of nitroso compounds,

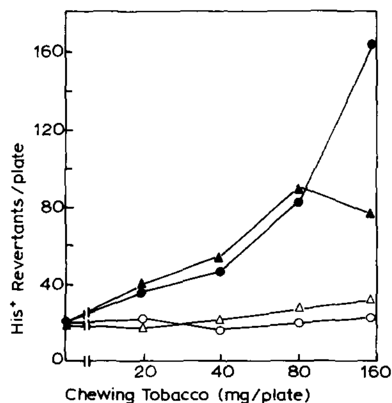

Fig. 2. Mutagenicity of polar (●, ○) and non-polar (▲, △) extracts of chewing tobacco treated with (●, ▲) and without (○, △) nitrite in TA98 without S9 activation.

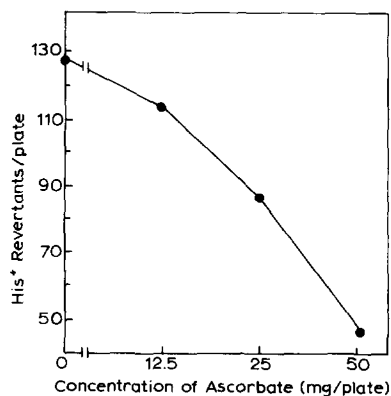

Fig. 4. Inhibitory effect of ascorbate on the mutagenicity of chewing tobacco organic solvent extracts during treatment with nitrite. Ascorbate was incorporated into the mixture of extract and nitrite and then adjusted to pH ~ 3. Mutagenic activity was tested in TA98 without S9 activation.

TABLE 1

RELATION BETWEEN MUTAGENICITY AND ABSORBANCES OF CHEWING TOBACCO H_2O EXTRACT UNDER DIFFERENT TREATMENT CONDITIONS [a]

Sample	Revertants/plate (mean ± S.E.)	Absorbance [b] (mean ± S.E.)
Nitrite	31 ± 3	0.01 ± 0.006
Extract	36 ± 5	0.04 ± 0.02
Extract + nitrite	125 ± 11	0.71 ± 0.09
Extract + nitrite + ascorbate (12.5 mg)	114 ± 9	0.69 ± 0.05
Extract + nitrite + ascorbate (25 mg)	85 ± 7	0.35 ± 0.03
Extract + nitrite + ascorbate (50 mg)	40 ± 2	0.04 ± 0.008
Control	32 ± 3	0.00

[a] The procedures for the treatment of chewing tobacco H_2O extract (106 mg from brand B) with nitrite and/or ascorbate were described in Materials and Methods. Mutagenic activity was assayed in TA98 without S9 activation. Results are from 3 Expts. with 2 plates for each dose in each experiment.

[b] The absorbance of each sample was determined after being treated with the Griess reagent.

respectively. However, a high concentration of nitroso compounds was found in CTE treated with nitrite under an acidic condition. A dose-related decrease in absorbances of CTE plus nitrite was noted after being treated with different doses of ascorbate. Furthermore, the absorbances observed were proportional to their corresponding mutagenic activities.

Results of the mutagenic activity of chewing tobacco H_2O extract (H_2O-CTE) with and without nitrite treatment are given in Table 2. As compared to chewing tobacco organic solvent extracts (CTE), a consistent result was also found for the H_2O-CTE, where only the extract plus nitrite showed mutagenic activity. Low and high amounts of nitroso substances were also detected for H_2O-CTE alone and H_2O-CTE plus nitrite, respectively, with the Griess reagent test.

Discussion

Results from the present study show that chewing tobacco alone was not mutagenic. This observation is comparable to that of Stich and Stich (1982), who found that saliva obtained from western-type tobacco chewers did not cause genotoxic effect (chromosomal aberrations) in Chinese hamster cells. However, it should be noted that the clastogenicity was found in betel and India tobacco chewers by the same group of investigators (Stich and Stich, 1982).

TABLE 2

MUTAGENICITY AND ABSORBANCE OF CHEWING TOBACCO H_2O EXTRACT TREATED WITH AND WITHOUT NITRITE [a]

Extract (mg)	Nitrite (μg)	Revertants/plate (mean ± S.E.)		Absorbance [b] (mean ± S.E.)
		− S9	+ S9	
Negative control		32 ± 2	43 ± 3	0
0	187.5	33 ± 3	42 ± 2	
0	375	33 ± 2	55 ± 5	
0	750	31 ± 4	53 ± 3	0.01 ± 0.002
25	0	28 ± 2	52 ± 3	
50	0	30 ± 3	52 ± 1	
100	0	32 ± 1	50 ± 2	0.03 ± 0.004
12.5	93.7	48 ± 1	50 ± 4	
25	187.5	63 ± 2	78 ± 2	
50	375	95 ± 4	91 ± 3	
100	750	137 ± 10	173 ± 9	0.78 ± 0.03

[a] 5 ml of the extract was treated with an equal amount of $NaNO_2$ solution (15 mg/ml in H_2O) at pH ~ 3 for 2 h at 37°C with shaking. Mutagenicity was assayed in TA98 without S9 activation. Results are from 3 Expts. with 2 plates for each dose in each experiment.

[b] The absorbance of each sample was determined after being treated with the Gries reagent.

The bacterial mutagenic activity observed in the reaction mixture of CTE plus nitrite under acidic conditions in the present study implied that certain chemical reactions, probably nitrosation, occurred resulting in the production of mutagenic substance. Mutagenic products were found only at acidic pH, and mutagenicity increased as pH was decreased (Fig. 3). This acidic-pH dependence for producing mutagens is the typical phenomenon of nitrosation by nitrite (Fan and Tannenbaum, 1973; Mirvish, 1975). Ascorbic acid is a well known inhibitor for nitrosation by nitrite (Archer et al., 1975; Mirvish et al., 1975). An inhibitory effect on the mutagenic activity of CTE plus nitrite (Fig. 4) seems to indicate that the nitrosation process is involved in the production of mutagenic substances. The Griess reagent test is a simple and specific method for detecting nitroso compounds after nitrite is decomposed by ammonium sulfamate and nitroso components are reacted with hydrobromic acid. With this method the amount of nitroso compounds is reflected by the amount of absorbance at 550 nm detected with a Spectronic colorimeter. The parallel finding between mutagenicity and the amount of absorbances (Tables 1 and 2) gave further evidence that mutagenic substances formed in the reaction mixture of CTE and nitrite are most likely nitroso compounds. However, without further chemical identification, a possibility of the formation of mutagenic nitro compounds contributing to the observed mutagenicity cannot be ruled out, because generation of mutagenic nitro compounds from the reaction with nitrite under an acidic condition has been reported (Ohta et al., 1983; Wang et al., 1984).

Chewing tobacco contains nitrosatable substances such as alkaloids (Hecht et al., 1975, 1978). Mutagenic activity was found in both nitrite-treated polar and non-polar CTE (Fig. 2), indicating at least two types of nitrosatable precursors (polar and non-polar) in this tobacco. It is well known that nitrite is one of the best known nitrosating agents present in our environment (Mirvish, 1983). It is used in coloring and preserving meats. An excess level (> 100 μg/ml) of salivary nitrite resulting from nitrate ingested by eating vegetables has been demonstrated (Harada et al., 1975; Tannenbaum et al., 1976). The nitrosation of dietary components and other sources of secondary and tertiary amines by nitrite has been described (McCarty, 1981). Tobacco chewers may also have higher salivary nitrite level than non-chewers (Murdia et al, 1982). Although the tobacco is eventually spit out by chewers, it is conceivable that a considerable amount of saliva-soluble substances from chewing tobacco may be swallowed. Therefore, an in vivo nitrosation of certain chewing tobacco substances by nitrite producing genotoxic nitroso compounds in the acidic environment of the stomach is possible.

The potential health effect of chewing tobacco on the oral cavity, pharynx, and larynx of the chewer has been documented (Christen, 1980; Joyant et al., 1977). A health hazard to tobacco chewers via an in vivo nitrosation has previously been suggested (Mirvish et al., 1977). The present study indicates that the potential health impact of in vivo-nitrosated smokeless tobacco on the gastrointestinal tract of the tobacco user cannot be ignored.

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