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Forward mutation of *S. typhimurium* by smokeless tobacco extracts

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Summary

Mutagenicity of 4 popular brands of smokeless tobaccos was studied using a *S. typhimurium* forward mutation assay. Aqueous extracts of 4 brands and dichloromethane and methanol extracts of 1 of the 4 brands of smokeless tobacco's did not induce significant mutagenicity either in the presence or absence of metabolic activation. Aqueous and organic extracts were however mutagenic when treated with physiological levels of sodium nitrite (0.25 mM) at acidic pH and without metabolic activation. The results indicate that smokeless tobacco contain polar and non-polar chemicals which become mutagenic to *S. typhimurium* under nitrosation conditions.

The habit of chewing smokeless tobacco is world-wide and chewing tobacco has gained increased popularity in the U.S.A. as a substitute for cigarette smoking (IARC, 1985). The habit has been causatively associated with cancer of the oral cavity and pharynx (Christen, 1980). Three major carcinogens have been identified in smokeless tobacco, namely polonium 210, benzo[*a*]pyrene and *N*-nitrosamines (Hoffmann, 1986). Of the 19 nitrosamines identified in smokeless tobacco, the carcinogenic nitrosamines are *N*-nitrosonornicotine (NNN) and 1-(methylnitrosamino)-l-(3-pyridyl)-1-butanone (NNK) (Hoffmann, 1981; Hecht et al., 1986). Snuff contains 1.6–135 mg/kg of NNN and 0.1–14 mg/kg of NNK. For comparison, U.S. foods and beverages may not contain more than 0.01 mg/kg of nitrosamines (MacMahon et al., 1988). Benzo[*a*]pyrene is present in trace amounts (0.1–63 ppb) and concentration of polonium 210 ranges from 0.16 to 1.22 pCi/g (Hoffmann et al., 1986). Smokeless tobacco has been reported to be carcinogenic (Hirsch et al., 1981, 1984; Park et al., 1985) and mutagenic (Whong et al., 1984, 1985, 1987; Tucker et al., 1985; Paulson et al., 1989; Chen, 1989 Guttenplan, 1987). It was the aim of this study to examine the bacterial mutagenicity of popular brands of smokeless tobacco under conditions of extraction similar to mastication in human saliva. Since tobacco contains nitrosable susbtances (Hecht et al., 1976) and saliva contains nitrite (Tannenbaum et al., 1974), a known nitrosating agent (Mirvish, 1975), the effect of physiological levels of nitrite (Bos et al., 1988; Granli et al., 1989; Tannenbaum et al., 1974) on smokeless tobacco extracts under neutral and acidic conditions (to simulate conditions in mouth and stomach respectively) was

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examined. In fact smokeless tobacco extracts have been shown to be mutagenic in the presence of acid alone and acidic nitrite using the Ames test and the increase in mutagenicity correlated with the increase in the formation of *N*-nitroso compounds (Whong et al., 1985, 1987). The concentration of nitrite used was much higher (0.22 M) than the physiological range. In this work an attempt has been made to study the effect of low concentration of nitrite (0.25 mM) on the mutagenicity of smokeless tobacco extracts using a forward mutation assay.

Materials and methods

Chemicals. 4 leading brands of American smokeless tobacco were purchased from the local supermarket. Sodium nitrite was purchased from Calbiochem-Behring Corp. (La Jolla, CA).

Extraction. Aqueous extracts of smokeless tobacco were prepared by mixing 35 g of tobacco with three times its weight of double distilled water, shaking it overnight at room temperature and filtering the extract under vacuum. Organic extracts were prepared by extracting 35 g of tobacco with 500 ml of dichloromethane (DCM) and methanol (MeOH) seperately for 12 h in a Soxhlet apparatus. The organic extracts were concentrated to 50 ml using a Kuderna Danish evaporator.

Weight of solids in aqueous and organic extracts of smokeless tobacco. Samples of extracts (0.1 ml) were applied to pre-weighed planchets and allowed to dry in vacuum dessicator overnight. The weight of residual dark brown powder was determined on a micro balance. A range of 40–45 mg and 350 mg 'solids'/ml extract was found for aqueous and organic extracts respectively. Organic extracts were exchanged with equal volume of dimethyl sulphoxide under nitrogen. All extracts were stored at −70 °C.

Nitrite treatment. 10 ml aqueous and organic extracts of tobacco were reacted with 1 ml of a 2.0 mM solution of sodium nitrite. The extracts originally contained 0.05 mM nitrite which was determined using Greiss reagent test (data not shown). The final nitrite concentration of the extracts was therefore 0.25 mM. The extracts (original pH 7.0) were adjusted to pH 3.0 with HCl (12 N). The control tobacco extracts and nitrite solutions were also adjusted to appropriate pH under the same conditions. All extracts were incubated at 37 °C for 2 h in dark before testing for mutagenicity.

Mutation assay. Mutagenic activity was tested using the *S. typhimurium* TM677 forward mutation assay (Skopek et al., 1978) with and without postmitochondrial supernatant (PMS) from Aroclor-induced rats (Moltox). This system scores mutations that render the bacterial cells resistant to 8-azaguanine. 0.2 ml and 10 μl of appropriate dilutions of aqueous and organic extracts of smokeless tobacco respectively were incubated in 1 ml of liquid medium containing 10 exponentially growing *S. typhimurium* TM677 cell, exposed for 2 h at 37 °C, diluted and plated on selective media containing 8-azaguanine and further diluted and plated on non-selective media in order to measure

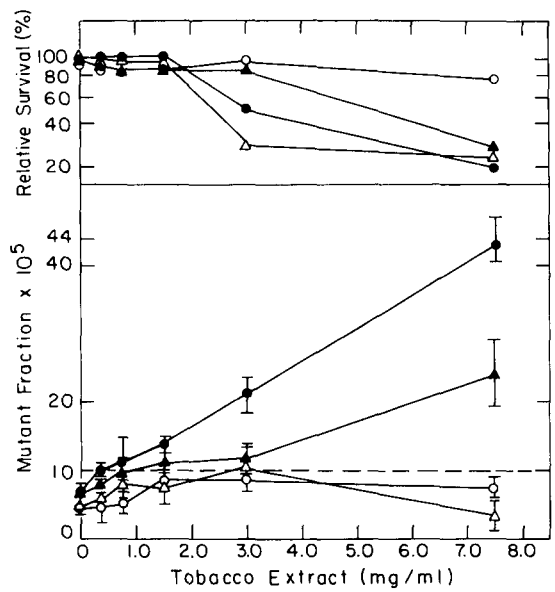

Fig. 1. Mutagenicity of aqueous extracts (Brand A ▲ △; Brand B ● ○) treated with (▲ △) and without (△ ○) nitrite at pH 3.0 and in the absence of metabolic activation. The results are mean of 4 replicates ± SD. The dashed line is the 95% upper confidence limit for historical negative control.

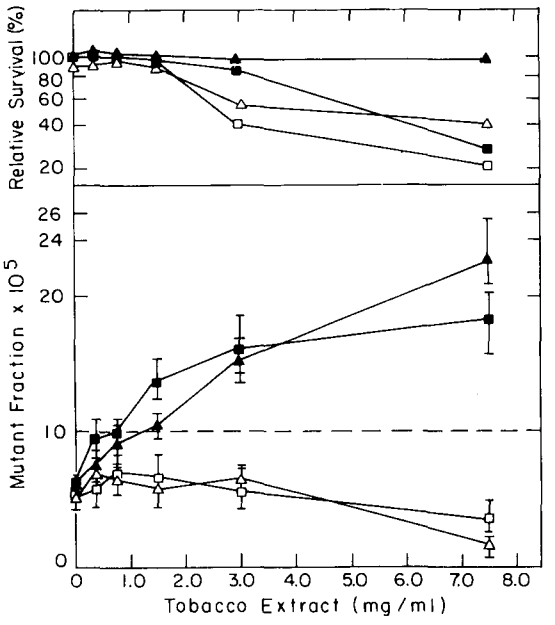

Fig. 2. Mutagenicity of aqueous extracts (Brands C ■ □; D ▲ △) treated with (■ ▲) and without (□ △) nitrite at pH 3.0 and without metabolic activation. The results are mean of 4 replicates ± SD. The dashed line is the 95% upper confidence limit for historical negative control.

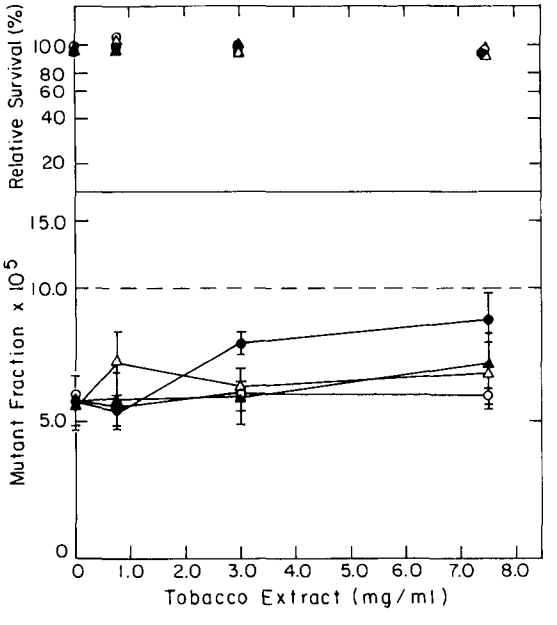

Fig. 3. Mutagenicity of aqueous extracts (Brands: A ▲ △; B ● ○) treated with (▲ ●) and without (△ ○) nitrite at pH 3.0 and with metabolic activation. The results are mean of 4 replicates ± SD. The dashed line is the 95% upper confidence limit for historical negative control.

survival following treatment. After a 40-h incubation at 37 °C the plates were counted and mutation frequency was determined by dividing the number of colonies on selective media by the number of colonies on non-selective media and correcting for the dilution between selective and non-selective plates. The concentration was expressed as mg extractable tobacco solids/ml of liquid medium containing 10^8 bacterial cells in the presence and absence of metabolic activation. Significant mutagenic activity was judged to be present in the extracts if treatment increased the mutant fraction both above the concurrent controls and above the laboratory historical 95% upper confidence limit for untreated controls.

Results

Figs. 1 and 2 summarize the results with the 4 sample extracts tested with or without nitrite treatment and in the absence of PMS. Without

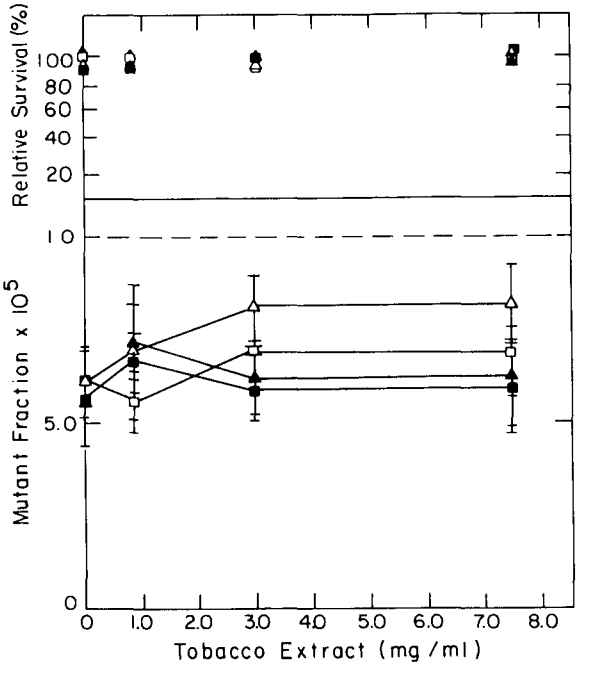

Fig. 4. Mutagenicity of aqueous extracts (Brands: C ■ □; D ▲ △) treated with (■ ▲) and without (□ △) nitrite at pH 3.0 and with metabolic activation. The results are mean of 4 replicates ± SD. The dashed line is the 95% confidence limit for historical negative control.

nitrite treatment extracts of brands A, C and D were markedly toxic at 3.0 mg/ml. Extract B did not show any toxicity. None of the extracts however, induced significant mutagenicity without nitrite treatment at acidic pH. Treatment of the extracts with nitrite under acidic conditions altered the bacterial toxicity of extracts B and D, but not of extracts A and C; increasing the toxicity of extract of brand B and decreasing the toxicity of extract of brand D. Mutagenicity of all 4 extracts was markedly increased by acidic nitrite treatment so that all extracts displayed significant mutagenicity at or above 1.5 mg/ml.

Figs. 3 and 4 show that the toxicity and mutagenicity of each of the extracts treated with acidic nitrite is eliminated when tested in the presence of PMS.

Fig. 5 shows that extraction of the tobaccos brand B with dichloromethane to obtain nonpolar components or with methanol to obtain polar organic compounds produces samples which are neither toxic nor mutagenic when treated without PMS at acidic pH. Treatment of either extract with acid nitrite solution produced samples which were markedly toxic and mutagenic only in the absence of PMS.

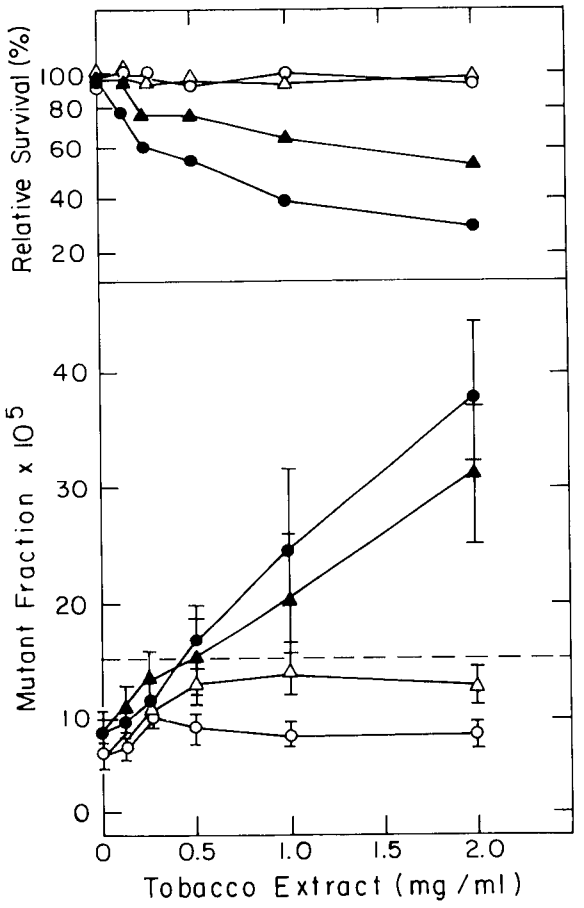

Fig. 5. Mutagenicity of DCM extract (▲ △) and MeOH extract (● ○) treated with (▲ ●) and without (△ ○) nitrite at pH 3.0 and without metabolic activation. The results are mean of 4 replicates ± SD. The dashed line is the 95% upper confidence limit for historical negative control.

Discussion

This study indicates that smokeless tobacco extracts contain bacterial mutagens which are probably precursors of *N*-nitroso compounds. Aqueous and organic extracts were mutagenic only in the presence of acidic–nitrite (0.25 mM) (Figs. 1 and 2). These results are in agreement with the findings of Whong et al. (1985) who found acidic–nitrite mediated mutagenicity of aqueous and organic extracts of smokeless tobacco using the Ames test. They also found that this mutagenic effect was pH-dependent (highest at pH 2.0). However, the concentration of nitrite used by Whong et al. was much higher than the physiological level (Bos et al., 1988; Granli et al., 1989; Tannenbaum et al., 1974) and it was felt necessary in this work to test the effect of physiological levels of nitrite.

It is interesting to note that presence of nitrite had different effects on the toxicity of smokeless tobacco extracts; while nitrite treatment at acidic pH did not alter the toxicity of aqueous brands C and A, it decreased the toxicity significantly for brand D and increased the toxicity significantly of brand B (Figs. 1 and 2).

The significant mutagenicity of the extracts at acidic pH and not at neutral pH (data not shown) when treated with nitrite is probably due to rapid nitrosation at acidic pH than at neutral pH (Mirvish, 1975). Formation of *N*-nitroso compounds in the neutral environment of the mouth has been found in betelnut chewers (Nair, 1985) and snuff dippers (Brunnemann et al., 1987). The absence of mutagenicity of the extracts in the presence of acid alone is in contrast with the

results of Whong et al. (1987). They attributed the acid-mediated mutagenicity of the tobacco extracts to the presence of nitrite in the snuff which is formed by reduction of nitrate by bacteria (both of which are present in the snuff) to nitrite. The concentration of nitrite found in snuff extracts was 1.9–5.4 mg/g of snuff. In the present study the concentration of nitrite found in the aqueous extracts was 0.09 mg/g snuff as determined by the Greiss reagent test (data not shown). The low concentration of nitrite may be responsible for the lack of acid-mediated mutagenicity of the extracts in the present study. The drastic-reduction of mutagenicity in the presence of PMS may be due to detoxification of the active compound(s) by the microsomal enzymes or due to non-specific reaction of active intermediates with proteins in the PMS. Whong et al. (1985) however, found that the presence of S9 did not alter the acidic-nitrite mediated mutagenicity. The present assay involves a liquid preincubation step before the cells are plated. During the preincubation step, the bacteria are exposed to higher concentrations of the test chemical, PMS, and cofactors than exist at any time during the standard plate assay (Prival et al., 1979). This concentration effect may facilitate the inactivation of the mutagenic compound(s) discussed.

Mutagenicity of dichloromethane and methanol extracts in the presence of acidic-nitrite treatment indicates that both non-polar and polar compounds are involved and these results are in agreement with the results of Whong et al. (1985).

The present study indicates that aqueous extracts of smokeless tobacco contain precursor(s) of *N*-nitroso compound(s) which do not require metabolic activation and that both polar and non-polar precursors are involved. These results confirm the findings of Whong et al. (1987) who also found that the concentration of nitroso compounds in snuff extracts correlated with the mutagenic activity and that this activity could be inhibited by vitamin C treatment.

Tobacco is a complex mixture and more than 2550 compounds have been identified in this mixture (IARC, 1985). Polyphenols, amines and amides and are among the many compounds present in smokeless tobacco.

Amines can be nitrosated under acidic conditions to give nitrosamines (Mirvish, 1975) and mutagenic nitrosamines have been isolated from snuff (Hoffmann et al., 1976). It is unlikely that the *N*-nitroso compound(s) is one of the tobacco specific nitrosamines since they require metabolic activation to mutagenic form (Hoffmann, 1985). Among the aromatic amine present in tobacco is tyramine (IARC, 1985). 3-Diazotyramine, a reaction product of tyramine with acidic-nitrite, has been identified as a compound responsible for the cytochrome P450 independent mutagenicity of soy sauce; it has also been reported to induce tumors of the oral cavity in rats fed with a solution of the compound in drinking water (Fujita et al., 1987).

Secondary amides present in smokeless tobacco can give rise to *N*-nitrosamides but have not been detected as yet in smokeless tobacco (IARC, 1985) and that *N*-nitrosamides may also be formed in human stomach (Mirvish, 1975). *N*-nitrosamide, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) which is mutagenic in the absence of metabolic activation produced adenocarcinoma of glandular stomach that resembled human gastric cancer (Sugimura et al., 1967). In a recent study a number of polyphenols treated with acidic-nitrite, exhibited genotoxicity in the absence of metabolic activation using SOS chromotest (Oshima et al., 1989).

It thus appears that the smokeless tobacco extracts contain a number of precursors for the formation of *N*-nitroso compounds which are mutagenic in the absence of metabolic activation. These findings are very important to smokeless tobacco chewers since smokeless tobacco compounds can react with salivary nitrites and form mutagenic compounds in the acidic environment of the stomach and possibly in the neutral environment of the mouth. Of course *S. typhimurium* may be a poor predictor of human cell responses to this complex nitrosated mixture. Experiments using human cell mutation assay directly are in progress.

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