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## MUTAGENICITY OF TOBACCO SNUFF: POSSIBLE HEALTH IMPLICATIONS FOR COAL MINERS

Wen-Zong Whong, Richard G. Ames, Tong-man Ong

Division of Respiratory Disease Studies, National  
Institute for Occupational Safety and Health,  
Morgantown, West Virginia

*Mutagenicity of tobacco snuff extracts was studied using the Ames Salmonella/microsome assay system. No mutagenic activity was found for tobacco snuff extracts without S9 activation. However, mutagenic substances were formed from tobacco snuff extracts in an acidic environment. The mutagenic substances induced predominantly frameshift mutations and were direct-acting mutagens. Mutagenic activity of tobacco snuff extracts was enhanced in the presence of coal-dust extracts at low pH. Since tobacco snuff has been used by some coal miners to substitute for cigarettes, a possible risk for gastric cancer induction among coal miners is proposed.*

### INTRODUCTION

Several epidemiological studies have reported an elevated risk of gastric cancer mortality among coal miners (Stocks, 1962; Enterline, 1972; Rockette, 1980). A systematic series of hypotheses was advanced to explain this elevated risk (Ames, 1982). In a study related to those hypotheses, coal-mine dust was found to be a gastric cancer risk in long-term cigarette smokers (Ames, 1983). Laboratory studies showed that coal-dust extracts were mutagenic after nitrosation with nitrite under acidic conditions (Whong et al., 1983). Based on these findings, it was hypothesized that intragastric nitrosation of chemicals associated with coal dust or interaction of chemicals from coal dust with exogenous substances may be responsible for the elevated risk of gastric cancer in coal miners (Ong et al., 1983). Since dipping tobacco snuff is common among coal miners, studies were performed to determine whether tobacco snuff posed any potential genetic hazards to the users and whether tobacco snuff interacted with coal dust as regards mutagenicity. In this report, results from these studies are presented.

Requests for reprints should be sent to Wen-Zong Whong, National Institute for Occupational Safety and Health, 944 Chestnut Ridge Road, Morgantown, West Virginia 26505.

## MATERIALS AND METHODS

Three strains (TA1535, TA98, and TA100) of *Salmonella typhimurium*, kindly provided by Professor B. N. Ames (University of California, Berkeley), were used for this study.

Two leading brands of tobacco snuff were purchased from a local store. Subbituminous coal dust was from Wyoming. Organic solvents (dichloromethane, acetone, and methanol) were obtained from Mallinckrodt, Inc. (St. Louis, Mo.).

### Sample Extraction

Tobacco snuff (34 g) or coal dust (50 g) was extracted first with 250 ml dichloromethane (DCM) and then extracted a second time with 250 ml of 1:1 mixture of acetone plus methanol (A+M) for 16 h at room temperature with shaking (250 rpm). The extracts were filtered and evaporated to dryness with a rotary evaporator. Then 10 ml of 75% dimethyl sulfoxide (DMSO) was used to redissolve each extract. For the extraction with H<sub>2</sub>O, 15 ml distilled H<sub>2</sub>O was added to 15 g tobacco snuff. The tobacco snuff was ground for 5 min in a porcelain mortar and then centrifuged. The supernatant was saved and served as the H<sub>2</sub>O extract.

### Treatment of Extracts

The DCM and A+M extracts of snuff or coal dust were combined. The combined extracts of snuff were mixed with an equal volume of 75% DMSO or the coal dust extract. The pH of some of the mixtures were adjusted to ~3.0 with 12 N hydrochloric acid. The original extracts of snuff and coal dust were pH ~8.2 and ~7.8, respectively. All samples were incubated at 37°C for 2 h with slow shaking (resembling foods retained in the stomach). At the end of incubation, samples were tested for mutagenic activity. The same procedures were used to study H<sub>2</sub>O extracts of snuff. The original pH of snuff H<sub>2</sub>O extracts was 7.5.

### Mutagenicity Test

Mutagenic activity was measured with the Ames *Salmonella*/microsome assay systems (Ames et al., 1975). Mutations were scored from histidine dependence to histidine independence. The plate-incorporation test with and without S9 activation in TA98, TA100, and/or TA1535 was conducted. The liver homogenate of male CD rats pretreated with Aroclor-1254 (500 mg/kg body weight) was prepared according to Ames et al. (1975). A minimum of two times the number of spontaneous revertants with a dose response was considered a positive result.

## RESULTS AND DISCUSSION

No mutagenic activity was observed for extracts of either brand of tobacco snuff without pH adjustment (Table 1). However, under the con-

TABLE 1. Effect of Low pH and Coal-Dust Extracts on the Mutagenicity of Tobacco Snuff in TA98 without S9 Activation<sup>a</sup>

Samples	pH adjustment <sup>b</sup>	Revertants/plate (mean $\pm$ SE) at snuff levels of			
		0 mg	21.2 mg	42.5 mg	85 mg
Snuff A	—	18 $\pm$ 1	21 $\pm$ 3	23 $\pm$ 2	20 $\pm$ 1
	+	18 $\pm$ 1	82 $\pm$ 9	175 $\pm$ 25	315 $\pm$ 22
Snuff B	—	18 $\pm$ 1	23 $\pm$ 2	22 $\pm$ 5	26 $\pm$ 1
	+	18 $\pm$ 1	29 $\pm$ 4	43 $\pm$ 3	105 $\pm$ 9
Coal dust	—	18 $\pm$ 1	21 $\pm$ 4	19 $\pm$ 2	16 $\pm$ 3
	+	18 $\pm$ 1	19 $\pm$ 5	22 $\pm$ 5	16 $\pm$ 2
Snuff A + coal dust	—	18 $\pm$ 1	19 $\pm$ 3	19 $\pm$ 3	21 $\pm$ 2
	+	18 $\pm$ 1	196 $\pm$ 18	332 $\pm$ 32	561 $\pm$ 26
Snuff B + coal dust	—	18 $\pm$ 1	27 $\pm$ 3	16 $\pm$ 4	22 $\pm$ 3
	+	18 $\pm$ 1	71 $\pm$ 2	134 $\pm$ 16	204 $\pm$ 45

<sup>a</sup>Results are averages of two independent experiments in duplicate; 0.05  $\mu$ g trinitrofluorenone/plate was used as the positive control ( $\sim$ 1651 revertants/plate).

<sup>b</sup>Minus, without pH adjustment (original extracts were pH  $\sim$ 8.2); plus, pH adjusted to  $\sim$ 3.0.

centrations studied, mutagenic activity was found when the extracts were adjusted to pH 3.0. A negative response was noted for coal-dust extracts regardless of pH. The mutagenic potency of tobacco snuff extracts at the low pH was highly enhanced in the presence of coal-dust extract.

For a further understanding of the mutagenicity of tobacco snuff, DCM (relatively nonpolar) and A+M (polar) extracts of tobacco snuff were studied. The results showed that only the polar extract of snuff at low pH was mutagenic (Fig. 1a). The enhancement of snuff mutagenicity by coal-dust extract was observed only for the polar extract of snuff. Further

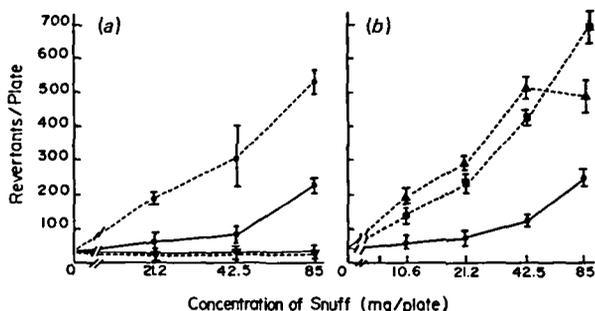


FIGURE 1. Mutagenicity of organic solvent extracts of tobacco snuff in TA98 without S9 activation. The pH of extracts was adjusted to  $\sim$ 3.0 (a) Mutagenic activity of polar (●) and nonpolar (▼) extracts of snuff in the presence (---) and absence (—) of coal dust extracts. (b) Mutagenic activity of polar extracts of snuff in the absence (●) and presence of polar (■) or nonpolar (▲) extract of coal dust. Bars are SE.

studies were conducted to determine the fraction of coal-dust extracts that exerted an enhancement of snuff mutagenicity. Polar extracts of tobacco snuff were treated separately with DCM and A+M extracts of coal dust at low pH. Results indicate that both extracts (polar and nonpolar) of coal dust exhibited an enhancement of snuff mutagenicity (Fig. 1*b*), although the effect was slightly higher with DCM extracts than with A+M extracts (based on the number of revertants).

Since the polar organic solvent extract of tobacco snuff, at low pH, is mutagenic, we questioned whether the mutagenic materials were also water-extractable. The findings of the water extraction, showing a dependence on a low pH for the mutagenic activity of snuff (Fig. 2*a*), were consistent with that of organic solvent extractions. Addition of coal-dust extract to the water extract of snuff at low pH resulted in an obvious enhancement of the mutagenic activity of snuff (Fig. 2*b*).

To determine the effect of pH on the mutagenic activity of tobacco snuff, the mutagenicity of snuff after treatment at different pHs was examined. As shown in Fig. 3, the mutagenic activity of snuff extracts was totally dependent on low pH, and the mutagenicity decreased as pH values increased.

As shown in Table 2, tobacco snuff extracts at low pH were mutagenic in both TA98 and TA100 with and without S9 activation, indicating that the mutagenic substances are direct-acting mutagens. A much higher mutagenic response was found in TA98 (a frameshift mutation tester) than TA1535 (a base-pair substitution mutation tester). This suggested that snuff extracts induced predominately frameshift mutations and a low frequency of base-pair substitution mutations.

Tobacco snuff dipping is not as popular as cigarette smoking, but it is a common substitute for smoking in several occupational groups, especially in coal miners. There is considerable literature regarding the possible health effects of the smokeless tobacco in general users (Christen, 1980; Hoffmann and Adams, 1981; Winn et al., 1981; Sundstrom et al.,

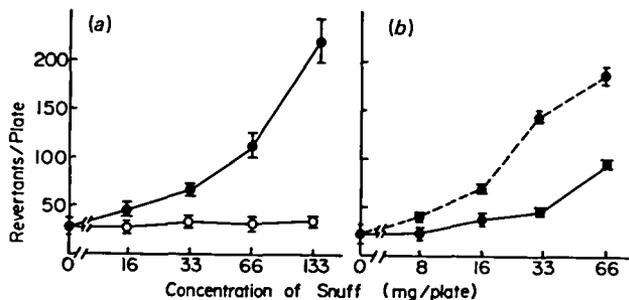


FIGURE 2. Mutagenicity of H<sub>2</sub>O extracts of tobacco snuff in TA98 without S9 activation. (a) Mutagenic activity of snuff at pH ~8.2 (○) and at pH ~3.0 (●). (b) Mutagenic activity of snuff extract (pH ~3.0) in the presence (---) and absence (—) of coal-dust extracts. Bars are SE.

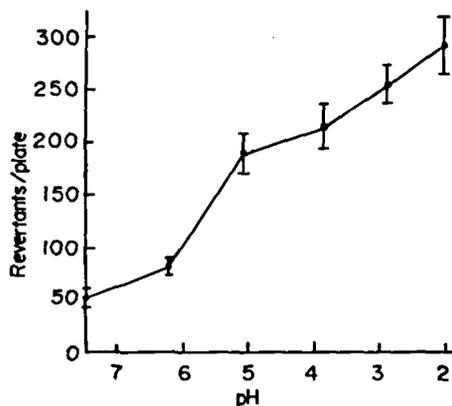


FIGURE 3. Effect of pH on the mutagenic activity of organic solvent extracts of tobacco snuff in TA98 without S9 activation. Bars show SE.

1982). However, no direct information is available on the possible health impact of tobacco snuff on the coal miner. In a case-control study of U.S. underground coal miners, coal-mine dust exposure was found to pose a gastric cancer risk in long-term cigarette smokers (Ames, 1983). Since nicotine is an addicting drug, it is conceivable that there is a combined use of snuff dipping (at work) and cigarette smoking (not at work) by the smoker. Tobacco snuff is commonly taken by dipping between the gum and lower lip or cheeks. Although most of snuff is eventually spit out, a considerable amount of saliva-soluble snuff materials may be swallowed. It is possible that the formation of mutagens from snuff at acidic conditions and the enhancement of the mutagenic activity of snuff by coal dust found in the *in vitro* studies may occur *in vivo* in the stomach. In the acidic stomach environment, mutagenic substances of snuff could

TABLE 2. Mutagenicity of Snuff Extracts after pH Adjustment<sup>a</sup>

Concentration of snuff (mg/plate)	Revertants/plate					
	TA98		TA100		TA1535	
	-S9	+S9	-S9	+S9	-S9	+S9
0	28	36	124	140	29	34
21.2	88	101	163	156	40	32
42.5	116	111	203	217	53	45
85.0	277	196	309	291	68	71

<sup>a</sup>Results are averages of two independent experiments. Organic solvent extracts (DCM and A+M) of snuff were adjusted to pH ~3.0; 2.5 µg 2-aminoanthracene per plate with S9 activation was used as the positive control (TA98, 2147 revertants/plate; TA100, 2496 revertants/plate; TA1535, 274 revertants/plate).

be generated. Coal dust inhaled by coal miners can be introduced into the stomach, via lung clearance (Task Group on Lung Dynamics, 1966). In the stomach, an enhancing effect of coal dust on the mutagenicity of snuff may occur. Since there is a high correlation between carcinogenicity and mutagenicity (McCann et al., 1975), the mutagens formed from snuff may be carcinogenic. It is conceivable that the etiology of gastric cancer in coal miners may involve a number of factors. Results from this study imply that tobacco snuff may also play a causal role in an elevated risk of gastric cancer in coal miners. This hypothesis is based on the assumptions that formation of mutagenic substances under an acidic condition observed in the *in vitro* bacterial mutagenicity experiment may occur in the stomach and the mutagenic substances are carcinogenic to humans. Further studies on the mutagenicity of snuff in mammalian mutagenicity assay systems and the mechanism of snuff mutagenesis at the low pH are in progress.

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