ELSEVIER

Mutation Research 321 (1994) 253–264

MR
Genetic Toxicology

Mutagenicity of coal-dust and smokeless-tobacco extracts in *Salmonella typhimurium* strains with differing levels of *O*-acetyltransferase activities

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(Received 24 May 1993; revision received 14 January 1994; accepted 24 January 1994)

Abstract

Epidemiological studies have indicated an increased incidence of gastric neoplasia in coal miners. Because smokeless tobacco use is prevalent in the mining industry, nitrites or other components of these products may be etiologically associated with these gastric neoplasms. In this study both nitrosated and non-nitrosated coal-dust (from West Virginia and New Mexico) as well as smokeless-tobacco (snuff and chewing tobacco) extracts were examined for the presence of aromatic amines and nitroarenes by comparing the activities of these extracts in the pre-incubation variant of the Ames assay. Salmonella strains with differing *O*-acetyltransferase activities (TA98 and YG1024) were utilized in this investigation. The results of the examination of the coal-dust extracts indicated positive activity only in the nitrosated extracts. Both nitrosated extracts elicited an increased number of revertants (2–4-fold) on YG1024 without S9 in comparison to TA98, suggesting the presence of nitroarenes in these extracts. Additionally, the nitrosated West Virginia coal extract showed higher levels of activity on YG1024 with S9, indicating the possible presence of aromatic amines in this complex mixture. The non-nitrosated smokeless-tobacco extracts showed activity only on YG1024 in the presence of S9, with the highest amount of activity occurring in the snuff sample. Except for the chewing-tobacco extract on TA98 without S9, positive activity was found in both nitrosated tobacco extracts on YG1024 and TA98. As with the coal extracts, the presence of nitroarenes was inferred for these nitrosated materials. A comparative study of the non-nitrosated snuff extract across 5 tester strains with varying sensitivities to aromatic amines and nitroarenes (TA98NR, TA98/1,8-DNP$_6$, TA98, YG1021 and YG1024) indicated that aromatic amines were a probable source of the mutagenic activity. The curing process and/or the addition of certain flavorants are potential sources of the mutagenic aromatic amines suggested to be present in the non-nitrosated snuff extract. These findings are consistent with an etiologic role supplementary to the nitroso compounds for mutagenic nitroarenes and aromatic amines in the development of gastric neoplasia in coal miners.

Key words: Coal extracts; Tobacco extracts; Ames assay; Aromatic amines; Nitroarenes

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SSDI 0165-1218(94)00005-N

1. Introduction

Several epidemiological studies of American and British coal miners indicate a positive relationship between mining and the incidence of gastric neoplasia (Stocks, 1962; Matolo et al., 1972; Rockette, 1977; Falk and Jurgelski, 1979). Ong et al. (1983) suggests that coal dust inhaled by miners and cleared via mucociliary functions, undergoes nitrosation and/or other chemical interactions at low pH in the stomach resulting in carcinogen formation. The exposure of the gastric epithelial cells to these endogenously formed carcinogens then may result in neoplastic transformation. The source of the nitrites/nitrates responsible for the nitrosation reactions is thought to be dietary components such as preserved meats, vegetables or lifestyle factors such as the use of smokeless tobaccos.

Previous investigations by Whong and coworkers have shown that extracts of both nitrosated coal dusts (1983) and tobaccos (1984) display mutagenic activity in the Ames assay. These extracts also induce both sister-chromatid exchanges in human peripheral lymphocytes (Tucker et al., 1985) and the transformation of mouse fibroblasts (Wu et al., 1990; and Whong, personal communication). Because of a high incidence of smokeless tobacco use in the mining industry, especially for underground miners (Shapiro et al., 1981), and because synergistic interactions occur between tobacco and coal extracts (Whong et al., 1984), tobacco-related compounds may be particularly important in gastric carcinogenesis of miners.

Generally, nitroso compounds from nitrosation reactions are the chemical mutagens/carcinogens implicated in these studies as a possible source of the observed genotoxic activity of these extracts. However, other chemicals which may contribute to the genotoxicity either directly or indirectly as precursors/products of nitrosation reactions, need to be detailed in order to better understand the causal substances involved in the formation of these cancers.

Aromatic amines and nitroarenes are genotoxic compounds that are present in both coal-derived materials (Gray et al., 1988) and tobaccos (Schmeltz and Hoffmann, 1977). Although there is sufficient documentation of these agents in tobacco smoke, little information exists in regard to their presence in smokeless-tobacco products. The chemical detection of aromatic amines and nitroarenes in tobacco, which contains over 2500 compounds (IARC, 1985), is difficult due to their low concentrations and volatile natures (Schmeltz and Hoffmann, 1977). Likewise, examinations for the presence of these aromatic chemicals in coal and coal extracts are limited, although a great deal of information exists detailing their presence in coal-conversion materials. Direct chemical examination of coal for these genotoxic agents has been hindered by an inability to analyze this structurally complex organic material. However, biological assays in the form of Salmonella microbial mutagenicity assays (Ames and *umu* tests), with greatly enhanced sensitivities to these compounds, now exist (Watanabe et al., 1990; Yamazaki et al., 1992; Oda et al., 1992). These test systems utilize bacterial strains with multiple copies of the *O*-acetyltransferase (OAT) or the nitroreductase (NR) genes which are necessary for the conversion of aromatic amines and/or nitroarenes to their ultimately mutagenic nitrenium species (Weisburger, 1988; Aeschbacher and Turesky, 1991; Watanabe et al., 1990). Especially useful are the OAT over-producing strains which are capable of the sensitive detection of both aromatic amines and nitroarenes. Since nitroarenes are direct-acting chemical mutagens (upon bacterial conversion) and aromatic amines are indirect-acting agents which require additional activation by microsomal enzymes, the presence of these chemicals in complex mixtures can be ascertained biologically via comparison studies of strains with varying OAT activities in the presence and absence of the microsomal enzymes.

This study examined both nitrosated and non-nitrosated coal (from West Virginia and New Mexico) and smokeless-tobacco (chewing-tobacco and snuff) extracts for possible aromatic amine and nitroarene content by comparing the activities of the extracts in the Ames assay. Salmonella strains TA98 and YG1024 which possess differing *O*-acetyltransferase activities were utilized with all the extracts. Additionally, the non-nitrosated

snuff extract was subjected to mutagenic analyses using 5 tester strains — TA98NR, TA98/1,8-DNP_6, TA98, YG1021 and YG1024 — which possess an array of sensitivities to aromatic amines and nitroarenes.

2. Methods and materials

Coal samples

The coal-dust samples that were extracted and used in this study were obtained from the Coal Research Section of Pennsylvania State University, State College, PA. Sample No. PSOC-151 is a sub-bituminous coal that was collected in McKinley county, NM. Sample No. PSOC-816 is a bituminous coal that was collected in Harrison county, WV.

Tobacco samples

The tobacco samples were two of the most popular brands used in West Virginia, and were purchased at a local grocery store. They consisted of a ribbon-cut chewing tobacco and a moist snuff that has been manufactured in the United States since the early 1800's.

Chemicals

The organic solvents (dichloromethane, dimethyl sulfoxide, acetone and methanol) were purchased from Mallinckrodt, Paris, KY. The concentrated HCl and the sodium nitrite were obtained from Fisher Scientific, Fair Lawn, NJ. 2-Aminoanthracene (2AA), 1-nitropyrene (1NP), and aflatoxin B_1 (AfB_1), used as positive controls, were purchased from Aldrich Chemical Company, Milwaukee, WI. The SRC-II HD, which was used as a complex mixture positive control, is a heavy distillate from the Solvent Refined Coal-II coal liquefaction process (sample No. 2445, boiling point range 550–850°F). This coal-derived material was acquired from the Pittsburg and Midway Coal Mining Company, Fort Lewis, WA, and contains a variety of aromatic amines (Pelroy and Wilson, 1981).

Sample extraction and nitrosation

The coal and tobacco samples were extracted in a similar fashion; however, the moist tobacco samples were allowed to air dry at room temperature prior to being processed. Upon drying, the snuff sample was found to have contained 51% water by weight and the chewing tobacco sample 13% water by weight. Briefly, after being ground with a mortar and pestle, 100 g of coal dust or 65 g of tobacco was extracted with 250 ml of dichloromethane (DCM) for 48 h on a rotary shaker at 200 rpm. The pulverized starting material was separated from the DCM extract by filtration (using 3 sheets of Whatman No. 4 paper in a Buchner funnel), and washed once with 50 ml of fresh DCM in order to remove any traces of the DCM-soluble components. After being allowed to dry, the DCM-extracted, pulverized material was once again extracted — this time with 250 ml of 1:1 methanol plus acetone (M + A). Similar to the DCM extraction, the residual solid material was separated from the M + A extract by filtration, and washed once with 50 ml of fresh M + A. Each solvent extract was concentrated by evaporation of the solvent under streaming N_2 on a hot plate at 37°C until all traces of the DCM or M + A were removed, and the remaining extracts were solvated in 20 ml of dimethyl sulfoxide (DMSO). The final sample volumes were adjusted to 20 ml by lyophilization on a Virtis 12SL Freezemobile.

Prior to nitrosation, the DCM and M + A extracts of each material were combined, and each extract was split into two 20-ml samples. For each combined extract, sodium nitrite (30 mg/ml) in a 1:1 distilled water plus DMSO mixture was added to one sample portion to be nitrosated, and as a control, an equal amount of the mixture without nitrite was added to the other sample portion (non-nitrosated). All samples were adjusted to pH 3 using concentrated HCl and incubated at room temperature on a rotary shaker at 150 rpm for 4 h. After completion of the incubation period, the resultant samples were stored at −20°C until used. This procedure yielded (in relation to the mass of the starting materials) nitrosated and non-nitrosated coal and tobacco extracts with final concentrations of 1.25 g/ml and 812.5 mg/ml, respectively.

Mutagenicity assay

The pre-incubation modification of the Ames assay (Yagahi et al., 1975) was used with DMSO

serving as the sample solvent. All samples to be tested were sonicated for 3 min at a power setting of 15% using a Heat Systems XL probe-type sonicator. To achieve sample homogenization, each assay tube was vortexed prior to and after the 30-min period of pre-incubation used in these essays. The *Salmonella typhimurium* TA98 strain was obtained from Prof. Bruce N. Ames of the University of California, Berkeley. The OAT and NR over-producing derivatives of TA98 — YG1024 (pYG219) and YG1021 (pYG216) respectively — were acquired from Dr. Takehiko Nohmi of the National Institute of Hygenic Sciences, Tokyo, Japan. The TA98NR and TA98/1,8-DNP_6 (NR- and OAT-deficient strains) were donated upon request from Dr. Herbert Rosenkranz through strain curator Elaine McCoy of Case Western Reserve Institute, Cleveland,

Table 1
Mutagenic activity of non-nitrosated and nitrosated coal-dust extracts in *Salmonella typhimurium* TA98 and YG1024

Sample	Concentration (mg/plate)	Average number of revertant colonies [a]			
		TA98		YG1024	
		− S9	+S9	− S9	+S9
Non-nitrosated West Virginia coal extract	0.00 [b]	30 ± 6	54 ± 2	98 ± 11	206 ± 2
	1.85	35 ± 6	–	81 ± 14	–
	3.91	31 ± 3	61 ± 20	83 ± 14	180 ± 12
	7.81	24 ± 8	100 ± 7	77 ± 8	193 ± 3
	15.62	33 ± 3	91 ± 19	83 ± 4	188 ± 3
	31.25	–	105 ± 8	–	214 ± 9
Nitrosated West Virginia coal extract	0.00 [b]	30 ± 6	54 ± 2	98 ± 11	206 ± 2
	1.85	78 ± 2 [c]	–	318 ± 24 [c]	–
	3.91	114 ± 13 [c]	150 ± 16 [c]	452 ± 19 [c]	716 ± 46 [c]
	7.81	245 ± 9 [c]	263 ± 50 [c]	689 ± 23 [c]	1098 ± 153 [c]
	15.62	378 ± 29 [c]	348 ± 23 [c]	778 ± 51 [c]	1287 ± 177 [c]
	31.25	–	564 ± 56 [c]	–	2278 ± 340 [c]
Non-nitrosated New Mexico coal extract	0.00 [b]	30 ± 6	54 ± 2	98 ± 11	206 ± 2
	7.81	45 ± 8	51 ± 5	110 ± 5	181 ± 18
	15.62	38 ± 6	64 ± 1	114 ± 13	164 ± 24
	31.25	57 ± 3	54 ± 3	123 ± 19	154 ± 14
	62.50	54 ± 16	45 ± 3	170 ± 33	141 ± 4
Nitrosated New Mexico coal extract	0.00 [b]	30 ± 6	54 ± 2	98 ± 11	206 ± 2
	1.85	150 ± 19 [c]	–	274 ± 19 [c]	–
	3.91	218 ± 17 [c]	66 ± 10	425 ± 12 [c]	403 ± 16
	7.81	408 ± 12 [c]	106 ± 6	778 ± 50 [c]	623 ± 53 [c]
	15.62	–	146 ± 13 [c]	1220 ± 64 [c]	1184 ± 157 [c]
	31.25	–	234 ± 11 [c]	–	1948 ± 41 [c]
SRC-II HD [d]	0.00 [b]	30 ± 6	54 ± 2	98 ± 11	206 ± 2
	62.50	36 ± 4	266 ± 31 [c]	80 ± 3	2390 ± 216 [c]
2AA [d]	0.00 [b]	30 ± 6	54 ± 2	98 ± 11	206 ± 2
	0.25	–	–	171 ± 20	2264 ± 193 [c]
	2.50	33 ± 15	1002 ± 95 [c]	–	–

[a] Each datum reflects the mean of three plates.
[b] DMSO solvent control.
[c] Positive activity.
[d] (μg/plate) SRC-II HD is a coal-derived reference material; and 2AA (2-aminoanthracene) is a positive control.
–, not tested.

OH. Overnight cultures of TA98, TA98NR and TA98/1,8-DNP_6 were grown in Oxoid Nutrient Broth No. 2 in the presence of 25 μg/ml ampicillin. YG1021 and YG1024 were grown under similar conditions as the other strains; however, both ampicillin and 6.25 μg/ml of tetracycline

Table 2
Mutagenic activity of non-nitrosated and nitrosated smokeless tobacco extracts in *Salmonella typhimurium* TA98 and YG1024

Sample	Concentration (mg/plate)	Average number of revertant colonies [a]			
		TA98		YG1024	
		− S9	+S9	− S9	+S9
Non-nitrosated snuff extract	0.00 [b]	26 ± 4	44 ± 7	55 ± 6	111 ± 14
	2.54	24 ± 9	–	51 ± 2	–
	5.08	29 ± 2	41 ± 4	61 ± 6	256 ± 31 [b]
	10.15	27 ± 4	68 ± 2	64 ± 3	286 ± 38 [e]
	20.30	31 ± 3	66 ± 7	65 ± 10	327 ± 103 [e]
	40.60	–	63 ± 4	–	406 ± 73 [e]
	81.20	–	87 ± 10	–	705 ± 11 [e]
Nitrosated snuff extract	0.00 [b]	26 ± 4	44 ± 7	55 ± 6	111 ± 14
	1.27	40 ± 17	–	111 ± 10 [e]	–
	2.54	53 ± 8 [e]	–	174 ± 13 [e]	–
	5.08	80 ± 17 [e]	56 ± 16	302 ± 21 [e]	382 ± 45 [e]
	10.15	209 ± 11 [e]	123 ± 12 [e]	557 ± 30 [e]	746 ± 50 [e]
	20.30	–	165 ± 6 [e]	–	912 ± 118 [e]
	40.60	–	291 ± 14 [e]	–	874 ± 186 [e]
	81.20	–	87 ± 5 [c]	–	396 ± 95 [c,e]
Non-nitrosated chewing tobacco extract	0.00 [b]	26 ± 4	44 ± 7	55 ± 6	111 ± 14
	5.08	29 ± 7	31 ± 3	41 ± 9	169 ± 20
	10.15	34 ± 6	29 ± 6	54 ± 8	205 ± 1
	20.30	40 ± 2	37 ± 3	57 ± 10	202 ± 10
	40.60	28 ± 9	62 ± 13	59 ± 10	230 ± 29 [e]
	81.20	–	52 ± 14		277 ± 35 [e]
Nitrosated chewing tobacco extract	0.00 [b]	26 ± 4	44 ± 7	55 ± 6	111 ± 14
	2.54	23 ± 6	–	83 ± 17	–
	5.08	38 ± 4	34 ± 2	155 ± 6 [e]	208 ± 36
	10.15	39 ± 5	37 ± 12	80 ± 10	303 ± 48 [e]
	20.30	37 ± 8	51 ± 12	23 ± 6 [c]	541 ± 98 [e]
	40.60	–	91 ± 34 [e]	–	445 ± 102 [e]
	81.20	–	62 ± 5 [c]	–	268 ± 16 [c,e]
SRC-II HD [d]	0.00 [b]	26 ± 4	44 ± 7	55 ± 6	111 ± 14
	62.50	–	343 ± 20 [e]	–	2796 ± 203 [e]
2AA [d]	0.00 [b]	26 ± 4	44 ± 7	55 ± 6	111 ± 14
	0.25	32 ± 8	48 ± 6	124 ± 20 [e]	1043 ± 148 [e]
AfB_1 [d]	0.00 [b]	26 ± 4	44 ± 7	55 ± 6	111 ± 14
	0.03	29 ± 4	330 ± 113 [e]	58 ± 7	245 ± 36 [e]

[a] Each datum reflects the mean of three plates.
[b] DMSO solvent control.
[c] Cytotoxic.
[d] (μg/plate) SRC-II HD is a coal-derived reference material; 2AA (2-aminoanthracene) and AfB_1 (aflatoxin B_1) are positive controls.
–, not tested.
[e] positive activity.

Table 3
Differential mutagenic activity of non-nitrosated snuff extract in 5 *Salmonella typhimurium* strains

Sample	Conc. (mg/pl)	Average number of revertant colonies per plate [a]									
		TA98 DNP (NR+, OAT−)		TA98 NR (NR−, OAT+)		TA98 (NR+, OAT+)		YG1021 (NR++, OAT+)		YG1024 (NR+, OAT++)	
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Non-nitrosated snuff extract	0.00 [b]	8±3	12± 4	11±2	23± 6	23± 7	30± 5	68± 9	45± 9	48± 9	98± 20
	2.54	10±4	–	12±7	–	25± 4	–	56± 6	–	49± 10	–
	5.08	6±2	–	15±6	–	25± 8	–	58± 5	–	54± 5	–
	10.15	13±8	17± 6	26±5 [d]	34± 9	25± 6	37± 12	73±12	66±10	62± 4	202± 31 [d]
	20.30	8±4	20± 5	24±6 [d]	40±15	36±12	54± 13	72±12	85±10	60± 15	292± 55 [d]
	40.60	–	21± 6	–	63±18 [d]	–	73± 12 [d]	–	85±10	–	306± 56 [d]
	81.20	–	26±12 [d]	–	76±25 [d]	–	102± 2 [d]	–	106±19 [d]	–	422±106 [d]
AfB_1 [c]	0.00 [b]	8±3	12± 4	11±2	23± 6	23± 7	30± 5	68± 9	45± 9	48± 9	98± 20
	0.10	13±1	203±43 [d]	15±4	246±53 [d]	20± 3	400±131 [d]	54±11	173±48 [d]	52± 6	334± 87 [d]
2AA [c]	0.00 [b]	8±3	12± 4	11±2	23± 6	23± 7	30± 5	68± 9	45± 9	48± 9	98± 20
	0.25	11±2	15± 3	14±5	65±10 [d]	25± 4	175± 40 [d]	58±11	126±11 [d]	142± 33 [d]	1608±264 [d]
INP [c]	0.00 [b]	8±3	12± 4	11±2	23± 6	23± 7	30± 5	68± 9	45± 9	48± 9	98± 20
	0.003	13±3	9± 1	15±3	31± 7	30±13	34± 5	369±45 [d]	47± 5	248± 8 [d]	145± 5

[a] Each datum reflects the mean of two experiments each using three plates/concentration.
[b] DMSO solvent control.
[c] (μg/plate) AfB_1 (aflatoxin B_1); 2AA (2-aminoanthracene); and 1-NP (1-nitropyrene) are positive controls.
–, not tested.
[d] Positive activity.

were added to the media. The criteria used to determine a positive mutagenic response for the test materials follow those described by Ames et al. (1975). The assays were repeated at least once in order to confirm the reproducibility of the results, and adjustments were made in the concentrations of samples examined in subsequent tests. All the samples were tested with and without the $9000 \times g$ supernatant (S9) of liver homogenate from Aroclor 1254 pre-treated, male CD rats (500 mg/kg bw). Because the growth of the YG1021 and YG1024 strains is slower than that of the conventional Ames strains, the plates containing these bacteria were incubated 72 h instead of the 48 h used for TA98, TA98NR and TA98/1,8-DNP_6 (in order to reach comparable colony sizes as recommended by Dr. Nohmi [personal communication]). SRC-II HD and 2AA were positive controls used to demonstrate the improved sensitivity of YG1024 over TA98 in the detection of aromatic amines, and 1NP was utilized in a similar manner for showing the improved sensitivity of YG1021 and YG1024 over TA98 in the detection of nitroarenes. AfB_1 was a positive control that was used to examine the responses of the tester strains to a mutagen that was neither an aromatic amine nor a nitroarene.

3. Results

Table 1 shows that neither the WV nor the NM non-nitrosated coal extracts were mutagenic without nitrosation, but both extracts were mutagenic under all test conditions when nitrosated. The number of revertants generated were similar between extracts within strains, but between strains, YG1024 expressed 2–4-fold higher values than TA98 in the absence of the S9, and 4–5-fold or higher values in the presence of the S9 fraction. In contrast to tester strain YG1024, the mutagenic activity of the nitrosated WV extract on TA98 was similar with and without the S9. The sodium nitrite solution was assayed on both TA98 and YG1024 for mutagenic activity with and without S9, but failed to show any mutagenic activity (data not shown).

Table 2 shows the results of the nitrosated and non-nitrosated chewing-tobacco and snuff extracts on both tester strains in the presence and the absence of the S9 microsomal fraction. The non-nitrosated tobacco products showed positive mutagenic activity only when assayed on the YG1024 tester strain in this comparative study. This activity occurred in the presence of the S9 microsomal fraction with the snuff extract

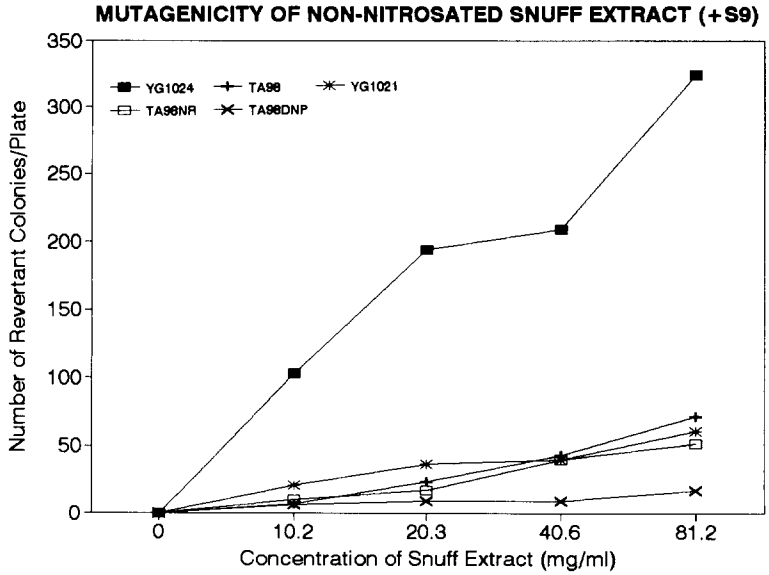

Fig. 1. Mutagenicity of non-nitrosated snuff extract (+S9).

demonstrating the highest amount of mutagenic response. However, some low but positive activity was indicated for the non-nitrosated snuff sample on TA98 with S9 in confirmatory assays. The nitrosated tobacco materials each showed positive mutagenic activity under all test conditions, except for the chewing-tobacco extract when assayed using TA98 without S9. The highest amount of mutagenic activity for each nitrosated extract occurred when YG1024 was used in the presence of the S9 fraction. This activity was 2–4-fold higher than the response for TA98 with S9. The nitrosated snuff extract with S9 demonstrated the highest amount of activity for the smokeless tobacco materials regardless of the tester strain utilized.

Table 3 shows the averaged data from two experiments examining the differential mutagenic activity of the non-nitrosated snuff extract across 5 tester strains with varying OAT and NR activities. Only TA98NR showed any positive response without S9 and the mutagenic activity was quite low. Conversely, in the presence of S9, the non-nitrosated snuff was mutagenic in at least one concentration in all tester strains. Fig. 1 illustrates that the S9-dependent mutagenicity (minus the solvent control values) was related to the activity of the OAT gene in the tester strain and not to the activity of the NR gene. The greatest mutagenicity was seen in YG1024 which contains multiple copies of the OAT gene and a single copy of the NR gene. Low amounts of mutagenic activity were seen in strains TA98NR, TA98 and YG1021, which contain single copies of the OAT gene and varying copy numbers of the NR gene. The least amount of mutagenic activity (marginally significant) was shown on TA98/1,8-DNP_6 which lacks the OAT gene and contains a single copy of the NR gene.

4. Discussion

The results of the Ames testing of the coal-dust extracts with TA98 (Table 1) confirmed the findings of Whong et al. (1983), which indicated that the acidified extracts were not mutagenic (or had very low mutagenicity) unless reacted with nitrite. Also, as previously detailed, the nitrosated extracts contain frameshift-inducing mutagens which expressed mutagenic activity in the absence of the S9 microsomal fraction. In the study reported here, we found that the mutagenic activity expressed on YG1024 without S9 was considerably higher (up to 4-fold) than the activity found using TA98. Since YG1024 shows enhanced activity to nitroaromatic compounds in the absence of the S9 (Watanabe et al., 1990), our results imply that nitroarenes may be one of the reaction products of nitrite with coal dust at low pH, and may in part contribute to the biological activity of nitrosated coal dusts. These findings are consistent with those of Pelroy and Stewart (1981), who suggested that the reaction of coal dust or coal fractions with nitrous acid may produce nitroaromatic compounds. While it is possible that YG1024 detected the presence of *N*-nitroso compounds with increased sensitivity, this is unlikely since these agents are generally base-pair substitution mutagens (Andrews et al., 1978). Einisto et al. (1991) indicated that of 3 nitroso compounds (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 1-nitrosopyrrolidine, and *p*-nitrosophenetole) assayed in comparative studies on TA100 and its OAT overproducing derivative (YG1029), only *p*-nitrosopyrrolidine demonstrated increased activity on the OAT tester strain. Thus, the results of our current study infer that it is improbable that *N*-nitroso compounds are involved in the observed genotoxicity of nitrosated coal dust extracts. Nitro-aromatic compounds, however, must be considered as likely candidates along with the *C*-nitroso compounds as suggested by Whong et al. (1983). Furthermore, because nitroarenes are converted into arylamines in vivo (Schemeltz and Hoffmann, 1977), the apparent presence of nitroarenes may imply in vivo exposure to arylamines as well.

The nitrosated WV coal extract elicited considerably higher mutagenic activity on YG1024 with S9 than without S9, whereas the NM extract showed slightly less activity in the presence of the S9 fraction for this tester strain. The increased amount of indirect-acting activity in conjunction with the greatly enhanced responses in the presence of the S9 for YG1024 versus TA98 may be

indicative of the presence of aromatic amines in the nitrosated WV extract, but not in the NM extract. Since it has been shown that the mutagenicity of nitro-aromatic compounds are generally reduced by the presence of the S9 fraction (Shah et al., 1991), the demonstration of increased mutagenicity in the presence of metabolic activation for the WV extract may further suggest the existence of aromatic amines in this coal-dust concentrate.

The Ames testing of smokeless-tobacco extracts also yielded interesting findings. In agreement with the *umu* assays of Shirname-Moré (1991), the acidified, non-nitrosated smokeless tobacco extracts failed to demonstrate significant mutagenic activity on TA98 with or without S9. However, low but significant mutagenic activity was detected in several confirmatory studies on the non-nitrosated snuff extract with S9. This slight variation in results between these two studies may be accounted for by the drying process, used in the current study prior to the extraction of the smokeless-tobacco materials, which could allow for more complete extraction of nonpolar plant matrix substances. Nonetheless, these results differ appreciably from those of Whong and co-workers (1987), who detected moderate amounts of mutagenic activity in the standard Ames plate-incorporation assay for acidified snuff extracts on TA98 with and without S9. These conflicting findings may be related to the use of pre-incubation in the assays of the current study and those of Shirname-Moré (1991). Alternatively, these data may indicate possible recent changes in the chemical composition of these tobacco products, such as a reduction in the nitrate levels which would lessen the chances for formation of mutagenic nitrogen containing compounds.

In contrast to the mutagenicity assays performed on TA98 with the acidified tobacco extracts, the comparative assays using these extracts on YG1024 indicated a positive response in the presence of the S9 for both tobacco products. This activity was marginal for the chewing-tobacco extract, but was moderately high for the snuff extract (Table 2). These findings are consistent with the need for both biometabolism (as indicated by the requirement for S9) and the need for OAT activity, which may implicate that aromatic amines play a role in the mutagenicity of the acidified tobacco extracts.

In the comparative study using 5 bacterial strains with varying OAT and NR activities, the acidified, non-nitrosated snuff was highly mutagenic in YG1024. The mutagenic activity corresponded directly with the activity of the OAT gene in the various tester strains (Fig. 1), further supporting the evidence for the presence of aromatic amines in these extracts. In contrast, the activity of the NR gene under these conditions (without S9) was unrelated to mutagenic activity and the presence of nitroarenes was not indicated. Aromatic amines such as aniline, aniline derivatives, and tyramine have been shown qualitatively by chemical analysis to be present in smokeless tobaccos (Hoffman and Schmeltz, 1977; IARC, 1985), however there is little known biological evidence that suggests the presence of aromatic amines in these materials. Furthermore, Pool-Zobel et al. (1991) have indicated that aromatic amines and nitrosamines may be responsible for the malignant tumors produced in humans upon exposure to tobacco and tobacco smoke-related products. Tobacco-specific nitrosamines (TSNAs) seem unlikely to account for the differential activities of the smokeless tobacco products between TA98 and YG1024, since these compounds are thought to be activated through hydroxylation of the α-carbon attached to the nitroso nitrogen (Hoffmann and Hecht, 1985).

The nitrosated smokeless tobacco samples generally demonstrated enhanced mutagenic activity over the non-nitrosated samples on both TA98 and YG1024 with and without the S9 fraction. Only the chewing-tobacco sample without S9, when tested on TA98, failed to show a positive response or trends of increased revertant numbers. The mutagenic activity of snuff was greater than that of chewing tobacco at all concentrations tested. Similar to the situation with the non-nitrosated tobacco, mutagenic activity on YG1024 was much greater than that on TA98. The nitrosated snuff extract was 3–4-fold more mutagenic on YG1024 than on TA98 without S9. These data may be indicative of the presence of

nitroarenes in the nitrosated extracts of the snuff material due to both the enhanced activity of YG1024 versus TA98 and the direct-acting nature of the mutagenic activity. Although the presence of nitroarenes has been documented for tobacco smoke (Schmeltz and Hoffmann, 1977), their presence in smokeless tobacco is incompletely investigated. However, their presence as reaction products of the nitrosation process is possible (Kato et al., 1991; Wang et al., 1984), and in the absence of further chemical identification cannot be ruled out (Whong et al., 1985). Also, the mutagenic activity with S9 was elevated in almost every case upon nitrosation, which may suggest the additive effect of pre- and post-nitrosatable materials on the overall mutagenic activity. Sasagawa et al. (1988) and Kato et al. (1991) have indicated that nitroarenes can be formed through the reaction of aromatic amines with nitrite, which may account for a portion of the increased direct-acting activity as detected with YG1024 in the nitrosated snuff extract.

Aromatic amines are thought to arise in tobacco from proteins and amino acids (notably phenylalanine or tyrosine) by decarboxylation as a result of enzymatic or microbial degradation (Schmeltz and Hoffmann, 1977). Differences in processing conditions allow more time for the conversion of proteinaceous substances into aromatic amines in the tobaccos used in manufacturing of snuffs, because snuffs are generally cured longer than chewing tobaccos (Shapiro, 1981; Rizio, 1984). The flue-curing process (used in many instances) is another possible source of aromatic amines in smokeless tobacco products. In this process, fires in the enclosed curing areas desiccate the tobacco material and terminate fermentative processes (IARC, 1985). Since incomplete combustion of organic fuels can cause the formation of soots containing aromatic amines (and nitroarenes), these fires may contribute to the mutagenic activity detected. Finally, the addition of flavorants is a potential source of mutagenic activity. Since these palatable ingredients are classified as proprietary information, we do not know their composition. Nitro-containing flavorants have been noted by Schmeltz and Hoffmann (1977) in cured tobaccos.

The findings of this study indicate the possible presence of nitroarenes as reaction products of the nitrosation processes for both coal and tobacco materials. Also, aromatic amines are likely components of an acidified snuff extract and may be present in the nitrosated extract of one coal (WV). Although these compounds may be in low concentrations (in comparison with nitroso compounds), they appear to be biologically active, and repeated exposure of miners to these compounds may in part contribute to the observed increases in gastric cancer in these individuals. Also, it is important to note that the carcinogenic potential of heterocyclic amines is enhanced by the presence of tumor promotors (Sugimura et al., 1982), which are potentially present within both smokeless tobacco and the working (particularly mining) environment. Aromatic amines and nitroarenes may also be etiologically associated with oral cancer in users of smokeless-tobacco products. Furthermore, this study may explain some of the ambiguous findings in regard to the examinations of tobacco materials for the presence of nitroso compounds in previous genotoxicity assays. As detailed by Bagwe and Bhisney (1991), Guttenplan et al. (1987), Hoffmann and Hecht (1985) and others, the genotoxicity of TSNAs often does not match the observed genotoxicity of tobacco products. Thus, the detailing of potentially bio-active compounds other than the nitrosamines may give a greater understanding of the overall genotoxicity of tobacco products. The findings presented in this study are not meant to de-emphasize the importance of the role of TSNAs in the genotoxicity of these chemically complex products, but rather to complement earlier studies and offer some alternatives that should be further examined for their importance.

It is somewhat unusual to use a biological assay system to examine samples for the detection of compounds that are either difficult to detect chemically or have not been subjected to chemical analysis. However, it may point to the importance of mutagen/carcinogen-specific as-

says that can rapidly detect genotoxic agents in select complex mixtures, which could then be subjected to more precise chemical analyses.

Acknowledgements

This study was conducted while the first author held a National Academy of Sciences — National Research Council Associateship. The authors of this paper would like to acknowledge Mr. John Stewart for his technical support and advice and Dr. Ann Hubbs for her editorial comments and contributions to the literature search.

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