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Role of Nitrosation in the Mutagenic Activity of Coal Dust: A Postulation for Gastric Carcinogenesis in Coal Miners

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The mutagenicity of coal dust solvent extracts with and without nitrosation was studied using the *Salmonella*/microsome assay system. Coal dust solvent extracts were either non-mutagenic or very weakly mutagenic with S9 activation. High mutagenic activities, however, were found when extracts of bituminous, subbituminous, and lignite coal dusts were reacted with nitrite under an acidic condition. Formation of mutagens from coal dust extracts by nitrosation was highest at pH 3.2 and decreased with increasing pH in the reaction mixture. Mutagenic activity appeared to be independent of metabolic activation. The mutagens formed from nitrosation of coal dust extracts induced frameshift mutations. The results reported here may have possible implications for the explanation of an elevated incidence of gastric cancer in coal miners.

INTRODUCTION

Results from epidemiological studies suggest that there is a lowered incidence of lung cancer mortality and an elevated incidence of gastric cancer mortality among coal miners (Goldman, 1965; Costello *et al.*, 1974; Falk and Jurgelski, 1979). An excess risk for gastric cancer in coal miners has been reported in both the United States and the United Kingdom (Stocks, 1962; Matolo *et al.*, 1972; Rockette, 1977). The actual etiology of gastric carcinogenesis in humans has not been elucidated. However, occupational exposure, life-style, and worker-susceptibility variables have all been postulated as possible risk factors for increased stomach cancer in coal miners (Ames, 1982). Coal mine dust exposure has been suggested as a possible gastric cancer risk (Falk and Jurgelski, 1979). A close link between coal dust exposure and gastric cancer has been drawn in a study by Jacobsen (1976), who reported a positive relationship between gastric cancer mortality and pneumoconiosis progression. Since pneumoconiosis progression is partly a function of prolonged coal dust exposure, Jacobsen's findings imply that an increase in stomach cancer mortality is likely to be associated with coal dust exposure.

Most inhaled coal dust can be cleared from the lung by mucociliary function; less than 2% enters the interstitium (Task group report, 1966; Gross, 1971). In the clearance function, coal dust is escalated, swallowed, and introduced into the stomach. Carcinogenic and/or mutagenic materials may be formed in the acidic

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environment of the stomach from the interaction of coal dust and nitrosating agents, such as nitrite. The formation of mutagenic and carcinogenic *N*-nitrosamines from the reaction of secondary amines and nitrite under an acidic condition has been well documented for *in vitro* and *in vivo* studies (Sen *et al.*, 1969; Lijinsky *et al.*, 1972; Mirvish, 1975; Whong *et al.*, 1979). The linkage of nitroso compounds to human gastric cancer has been suggested by Mirvish (1971). In this report, mutagenicity of coal dust extracts after treatment with sodium nitrite under an acidic condition was studied using the *Salmonella*/microsome test system. The effect of pH on the mutagenicity of coal dust extracts treated with nitrite was also examined. This study may provide information on one of the possible explanations for an elevated gastric cancer incidence among coal miners.

MATERIALS AND METHODS

Tester strains. Three strains (TA1535, TA98, and TA100) of *Salmonella typhimurium,* kindly provided by Professor Bruce Ames (University of California, Berkeley, Calif.), were used for this study (Ames *et al.*, 1975).

Chemical. Seven coal dust samples from different ranks of coal (degree of hardness) were used for this study. Coal dust samples and their sources were peat from North Carolina, lignite from Texas, subbituminous from New Mexico, bituminous-A from north central West Virginia, bituminous-B from northern West Virginia, bituminous-C from southwestern Pennsylvania, and anthracite from central Pennsylvania. Sodium nitrite was purchased from Mallinckrodt, Inc., Paris, Kentucky. NADP and glucose 6-phosphate used for S9 activation were obtained from Sigma Chemical Company, St. Louis, Missouri.

Sample extraction. Initially, each coal dust sample (100 g) was extracted with 250 ml of dichloromethane (DCM) for 16 hr at room temperature with vigorous shaking in a rotary shaker (250 rpm). The DCM extract was collected by filtration. The residue of coal dust 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 DCM extraction. Each extract was concentrated to 0.5 ml with a rotary evaporator. Then, 10 ml of dimethylsulfoxide (DMSO) was added to the extract, which was further concentrated under a nitrogen stream to 10 ml.

Nitrosation of extract from coal dust. Prior to nitrosation, equal amounts of DCM extract and M + A extract from the same coal dust sample were mixed together. An equal volume of sodium nitrite solution (30 mg/ml in DMSO) was then added to the mixed extracts. The mixture of coal dust extract and nitrite was adjusted to pH ~3.5 with hydrochloric acid (12 N). The coal dust extract and the sodium nitrite solution alone served as controls and were also adjusted to pH ~3.5 under the same condition. All the pH-adjusted solutions were incubated at 37°C for 3 hr with shaking (150 rpm) in a rotary shaker. At the completion of incubation, the solutions were either used immediately for mutagenicity testing or were kept at −20°C until used.

Mutagenicity assay. Mutagenic activity was detected using the standard *Salmonella*/microsome assay system (Ames *et al.*, 1975). Mutations were scored from histidine dependence to histidine independence. Determination of a positive

mutagenic response is based on criteria recommended by Ames *et al.* (1975). The plate-incorporation test with and without S9 (liver 9000*g* supernatant) activation in TA1535, TA100, and/or TA98 was conducted throughout the study. The experimental procedures followed the standard Ames assay (Ames *et al.*, 1975). The liver homogenate of Aroclor-1254 (500 mg/kg body wt)-pretreated male Syrian golden hamsters was prepared according to Ames *et al.* (1975).

RESULTS

Extracts of coal dust from five differently ranked coals were nitrosated with $NaNO_2$ under an acidic condition. With tester TA98, mutagenic activity was observed for nitrosated extracts from lignite, subbituminous, and bituminous coal dusts (Table 1). The mutagenic potency of these nitrosated extracts was in the order subbituminous > bituminous > lignite. The order of mutagenicity does not parallel the rank of the coal. Mutagenicity of the nitrosated extracts was found in the absence of S9 activation, and no obvious change in mutagenic activity was observed as activation was incorporated. No mutagenic activity was found with or without S9 activation for nitrosated extracts from peat or anthracite. $NaNO_2$ alone, used as a control, was inactive over the concentration range used. Without nitrosation there was no mutagenic activity for any of the coal extracts in the absence of S9 activation. In the presence of S9, a very weak mutagenicity was displayed for bituminous and subbituminous extracts without treatment with nitrite. The mutagenic nitrosated extracts from bituminous and subbituminous coal dusts were further tested with TA100 and TA1535. The mutagenic activity was substantially decreased in TA100, and no mutagenic activity was found for TA1535 (results not shown). The mutagenicity of extracts treated with nitrite from similarily ranked coal obtained from different sources has also been examined. A highly variable mutagenic potency was observed for different bituminous coal dusts (Table 1).

For further elaboration into the mutagenicity of coal dust, polar (M + A) and nonpolar (DCM) extracts from subbituminous coal dust were treated separately with nitrite and tested for mutagenic activities. The results are shown in Table 2. Both nitrosated polar and nonpolar extracts showed similar mutagenic potency. Figure 1 shows the mutagenic activity of extracts nitrosated with nitrite at different pH values. No mutagenic activity was observed for the extract treated with nitrite at pH 9.5. However, the mutagenicity of nitrosated extracts increased as the pH decreased. Within the pH range 3.2 to 9.5 neither extracts nor nitrite by themselves was mutagenic.

DISCUSSION

As shown under Results, reaction products of coal dust extracts and nitrite were mutagenic in TA98, but not in TA1535. These results indicate that the reaction products were frameshift mutagens. The formation of mutagenic substances increased as the pH of the coal dust extracts and $NaNO_2$ mixture decreased from pH 7.5 to 3.2 (Fig. 1). This pH dependence for mutagen formation via nitrosation by nitrite is a general phenomenon (Fan and Tannenbaum, 1973; Mirvish, 1975).

TABLE 1
MUTAGENIC ACTIVITY OF COAL DUST EXTRACTS NITROSATED WITH SODIUM NITRITE IN TA98

Sample	Concentration of sample		Revertants/plate[a]	
	$NaNO_2$ (μg/plate)	Coal dust (mg/plate)	− S9	+ S9
Negative control (DMSO)[b]			19	32
Positive control (2AA)[c]				2527
$NaNO_2$	23.4	0	14	31
	46.8	0	19	34
	93.7	0	22	32
	187.5	0	24	35
	375.0	0	20	47
Lignite	0	15.6	19	21
	0	31.2	36	47
	46.8	7.8	24	46
	93.7	15.6	33	50
	187.5	31.2	62	83
Subbituminous	0	7.8	19	42
	0	15.6	32	61
	11.7	1.9	141	139
	23.4	3.9	259	216
	46.8	7.8	404	294
	93.7	15.6	686	567
Bituminous-A	0	7.8	30	65
	0	15.6	22	67
	0	31.2	22	64
	23.4	3.9	35	73
	46.8	7.8	55	101
	93.7	15.6	89	135
	187.5	31.2	142	188
Bituminous-B	0	9.37	29	63
	0	18.75	29	60
	23.4	2.34	112	137
	46.8	4.68	199	204
	93.7	9.37	359	404
	187.5	18.75	671	628
Bituminous-C	0	37.50	29	58
	93.7	9.37	128	116
	187.5	18.75	222	159
	375.0	37.50	397	227

[a] Results are mean values with variation (range) less than 32% from three to four experiments in duplicate.
[b] 0.1 ml DMSO (dimethylsulfoxide)/plate.
[c] 2.5 μg 2-AA (2-aminoanthracene)/plate.

Since any reaction of nitrite is actually a reaction of nitrous acid, it is obvious that above pH 3.4 the nitrous acid is converted to nitrite, resulting in less reaction. The mutagenic activity of coal dust extracts after nitrosation did not require S9 activation (Table 1). This finding implies that the nitrosation products may be

TABLE 2
MUTAGENIC RESPONSE OF TA98 TO POLAR AND NONPOLAR EXTRACTS FROM SUBBITUMINOUS COAL DUST TREATED WITH SODIUM NITRITE

Sample	Concentration of sample		Revertants/plate[a]	
	$NaNO_2$ (μg/plate)	Coal dust (mg/plate)	−S9	+S9
Negative control (DMSO)[b]			17	24
Positive control (2AA)[c]				2653
$NaNO_2$	46.8	0	17	16
	93.7	0	16	25
Nonpolar Extract	0	15.6	20	46
	46.8	7.8	448	479
	93.7	15.6	622	624
Polar Extract	0	15.6	18	52
	46.8	7.8	423	316
	93.7	15.6	641	471

[a] Results are mean values with variation (range) less than 26% from two experiments in duplicate.
[b] 0.1 ml dimethylsulfoxide/plate.
[c] 2.5 μg 2-aminoanthracene/plate.

nitroso compounds other than *N*-nitrosamines, because *N*-nitrosamine mutagenesis generally is dependent upon S9 metabolic activation (McCann *et al.*, 1975; Andrews *et al.*, 1978). Formation of direct-acting mutagens from nitrosation of refined coal distillates with nitrite has been reported (Pelroy and Stewart, 1981).

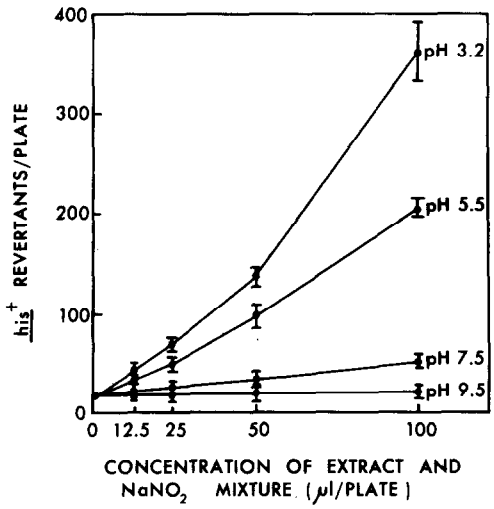

FIG. 1. Effect of pH on the mutagenic activity of extracts treated with nitrite. The polar extract of bituminous-B coal dust was treated with sodium nitrite at different pHs. The procedures for nitrosation were the same as those described under Materials and Methods. Bars are standard errors of the means derived from three separate experiments.

Since mutagenic materials from reaction of coal dust and nitrite were frameshift mutagens, they were probably not *N*-nitroso compounds but were more likely to be *C*-nitroso compounds. A highly variable mutagenic potency observed for different sources of nitrosated bituminous coal dust extracts indicates different types or amounts of nitrosatable materials among different bituminous coal samples. Therefore, the observation of nonmutagenic activity from an anthracite and a peat sample treated with nitrite in this study cannot rule out the possibility that there might be some other anthracite or peat samples showing mutagenic activity after nitrosation.

Meyer *et al.* (1980) hypothesized an important role for lung clearance in stomach carcinogenesis. An inverse temporal relationship between lung cancer and gastric cancer in the United States was postulated to be related to lung clearance. According to Meyer *et al.* (1980), given inhaled carcinogens, those persons with impaired clearance would develop lung cancer and those whose lung clearance was not impaired would become at risk of gastric cancer. Swallowing coal dust into the stomach following lung clearance, therefore, may play a role in the observed elevated incidence of gastric cancer in coal miners. However, since coal dust extracts alone are either very weak mutagens or not mutagenic at all, it is reasonable to postulate that carcinogenic substances contributing to stomach cancer are not present in coal dust per se, but are transformed products, generated through the nitrosation process.

Nitrite is one of the best known nitrosating agents present in the human environment. It is widely used in coloring and preservation of meats such as bacon, hot dogs, and ham. An excess level (>100 μg/ml) of salivary nitrite in humans resulting from nitrate ingested through eating certain vegetables has been reported by a number of investigators (Harada *et al.*, 1975; Tannenbaum *et al.*, 1976). Therefore, it is possible that simultaneous swallowing of coal dust from lung clearance and nitrite from saliva could result in nitrosation and production of mutagenic and/or carcinogenic materials in the acidic condition of the stomach. This process may be the possible factor contributing an elevated incidence of gastric cancer among coal miners. This postulation, of course, is based on the following two assumptions: (1) elevated incidence of gastric cancer in coal miners is indeed related to coal dust exposure; and (2) formation of mutagenic compounds by nitrosation observed in the *in vitro* experiment can occur in the stomach and are carcinogenic to humans.

Further studies on the nitrosation of coal dust with nitrite in experimental animals and the identification of nitrosated mutagenic constituents are in progress.

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