**Ames Test Mutagenicity Studies of the Subfractions of the
Mild Gasification Composite Material, CTC#11** **RECEIVED**

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INTRODUCTION**OSTI**

Mild gasification of coal is a technology being developed by the United States Department of Energy (DOE) and private industry with the hope that a cleaner method of coal use can help meet future energy needs. As the technology develops and its commercial use becomes a more viable possibility, efforts are being made to study the safety and possible toxicity of the mild gasification products. DOE and the National Institute for Occupational Safety and Health (NIOSH) are cooperating through an interagency agreement to examine some of these products for their genotoxic potential.

NIOSH has studied the mutagenicity of seven mild gasification product samples using the Ames Salmonella/microsomal assay (Maron and Ames, 1983). The Ames assay is widely used as a short-term test for the detection of possible genotoxic agents and potential carcinogens. Bacterial tester strains used in the Ames assay contain specific mutations (frameshift or base-pair substitution) that cause the bacteria to be dependent on growth medium containing the amino acid histidine. The genotoxic activity of a test substance is measured by the number of colonies demonstrating a mutation reversing the histidine requirement. It is generally accepted that DNA mutation is involved in the initiation stage of carcinogenesis; therefore, the Ames assay has often been used as the first tier of testing in the evaluation of long-term health risks associated with exposure to certain chemicals. A coal liquid sample from Coal Technology Corporation, CTC#11, has been examined in the Ames assay.

The assay was performed using two solvents with quite different properties, dimethylsulfoxide (DMSO) and Tween 80 (polyoxyethylene-sorbitan mono-oleate). The two solvents were used because there has been evidence to suggest that solvent/mutagen interactions can occur during testing and cause falsely decreased or elevated mutagenic activity. As reported earlier in "Mutagenicity of CTC#11 in the Ames Salmonella/Microsomal Assay" which was included in the May 1992 monthly report, CTC#11 displayed genotoxic activity when tested in the Ames assay as a whole sample. The activity was apparent under all conditions tested. The results of testing on TA98 indicated the presence of potent, indirect-acting and moderate, direct-acting, frameshift mutagens. Mutagenic activity on TA100, which indicates base-pair substitutions, was moderate with microsomal activation and weak without. Similar results were found for both solvents used.

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Since the mild gasification products are complex mixtures, interactions between various components are likely to occur. Gray, et al. (1988) and Petrilli et al. (1980) have proposed that the interactions between various components of complex mixtures may increase or decrease genotoxic activity in short-term assays like the Ames test. Although all synergistic interactions may not be detailed, the separate analysis of those components in several classes gives a more accurate view of the genotoxicity of each component and better allows for chemical characterization of the mutagen(s) in the mixture. NIOSH has performed mutagenicity studies on the subfractions of CTC#11. The results of those studies are detailed in this report.

METHODS AND MATERIALS

Sample description

CTC#11 is a mild gasification product with a liquid/tar consistency. It is a composite, containing materials from a wide range of boiling points. This sample was obtained from Coal Technology Corporation, Bristol, Virginia.

Chemicals

Positive controls:

2-aminoanthracene (2AA), a chemical mutagen which requires metabolic activation (+S9), and trinitrofluorenone (TNF), a direct acting mutagen (-S9), were used in the assay system at final concentrations of 2.5µg/plate and 0.5µg/plate, respectively. Both were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin.

Solvent control:

Dimethylsulfoxide (sterile, spectrophotometric grade) was purchased from EM Science, Cherry Hill, New Jersey. Tween 80 (cell culture grade) was purchased from Sigma Chemical Company, St. Louis, Missouri.

Sample preparation

The fractionation of CTC#11 was performed by Dr. Joseph Ma of the West Virginia University School of Pharmacology. Briefly, the sample was dissolved in ethyl acetate and extracted with sodium hydroxide (NaOH). The NaOH layer was neutralized with HCl, and the precipitate (the acidic fraction) was collected. The ethyl acetate layer was extracted again; this time with HCl. The acidic layer was separated and neutralized with sodium bicarbonate. The precipitate, the basic fraction, was collected. The remaining ethyl acetate layer then contained only the neutral fractions of the sample. Crude silica gel was added to the solution. After stirring, the solvent, ethyl acetate, was removed under reduced pressure. An affinity column was prepared and eluted with hexane and cyclohexane to

collect the nonpolar neutral fraction. The column was then eluted with cyclohexane/ethyl acetate to collect the polar neutral fraction. The solvents were removed from each fraction before testing was done. The fractions were redissolved in DMSO or Tween 80 for the Ames assay. Care was taken to insure that homogeneous mixtures were used while preparing dilutions and during testing.

Microsuspension assay

The microsuspension assay is a variant of the Ames Salmonella microsomal assay system (Maron and Ames, 1983) that can be used when the amount of sample is very small. This test may be more sensitive than the standard protocol (Kado et al., 1983), due to the fact that a more concentrated solution of bacteria is used. Tester strains TA98 and TA100 (kindly provided by Dr. B.N. Ames of the University of California at Berkeley) were grown and centrifuged to a density of approximately 4×10^9 cells/ml nutrient broth. A 25 μ l aliquot of the bacterial solution was added to each test tube used in the assay. To determine the metabolic activation requirements of the sample, each dose was tested both with and without S9 metabolic activation mixture (prepared from liver homogenates of Aroclor 1254-treated male Wistar rats). The +S9 treatments received 65 μ l aliquots of the S9 mix, while -S9 treatments received 65 μ l physiological saline. 10 μ l of chemical solution was added to the test tubes which were then incubated at 37°C for 30 min on a rotary shaker. Molten top agar was added to each tube after the incubation and poured onto plates, which were then incubated at 37°C for 48 h. The colonies that survived and grew were mutated, histidine-independent revertants. Two or three replicates of each treatment were scored on an automatic colony counter. The criteria for positive test results consists of revertant counts at least twice that of the solvent control in one or more concentrations and a clear dose-related increase in mutagenic activity.

RESULTS AND DISCUSSION

All of the four subfractions of CTC#11 displayed positive genotoxic activity in the microsuspension Ames assay. The strongest activity was shown in the basic fraction on TA98 with S9 activation. That fraction's activity on TA98 without S9 was moderate, but significant. These results indicate the presence of a strong, indirect-acting and a moderate, direct-acting, frameshift mutagen. The basic fraction also contains — as indicated by the genotoxicity shown on TA100 with, but not without, activation — a moderate, indirect-acting, basepair substitution mutagen. The acidic and polar neutral fractions displayed significant mutagenic activation only on TA98 with S9, indicating only the presence of an indirect-acting, frame-shift mutagen in each of those fractions. The

activity was moderate in the acidic fraction and weak in the polar neutral fraction. The nonpolar neutral fraction, on the other hand, displayed moderate to strong activity in every condition tested. The activity appeared stronger on TA98 than TA100 and stronger without than with S9. In this fraction, results suggest the presence of strong, direct-acting, frameshift and basepair substitution mutagens as well as moderate, indirect-acting, mutagens of both classes. It is possible, although unlikely, that the fractionation procedure, with acid/base treatments, may alter the chemical structure of compounds in the mixture; thereby activating or inactivating them.

CONCLUSION

The results of the Ames testing of the CTC#11 subfractions indicates significant genotoxic activity in each of the four fractions. CTC#11 is quite genotoxic to bacteria and apparently contains a wide variety of mutagens. Since mutation is generally accepted as one of the first steps in carcinogenesis, CTC#11 has displayed potential carcinogenicity. The evidence collected from this study and testing of the whole sample is sufficient to suggest further testing of CTC#11 as a possible carcinogen.

REFERENCES

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TABLE 1
Ames Test --- Summary Table
Results of Mutagenicity Tests of Fractionated CTC#11
Test Conditions: Microsuspension Assay

Sample	Concentration μg/plate	Average Number of Revertant Colonies per Plate			
		TA98		TA100	
		- S9	+ S9	- S9	+S9
BASIC	0.00 ^A	26	29	115	99
	4.70	56	565	160	277
	9.40	90	685	181	361
	18.75	113	642	162	367
	37.50	120	923	195	423
ACIDIC	0.00 ^A	26	29	115	99
	37.50	37	57	161	122
	75.00	46	74	174	166
	150.00	68	200	173	200
	300.00	69	274	190	234
NONPOLAR NEUTRAL	0.00 ^A	26	29	115	99
	9.40	152	147	-	-
	18.75	227	243	639	442
	37.50	345	293	686	545
	75.00	520	430	823	619
	150.00	-	-	913	717
POLAR NEUTRAL	0.00 ^A	26	29	115	99
	1.20	32	48	-	-
	2.40	28	53	158	164
	4.70	38	72	180	167
	9.40	152	147	188	193
	18.75	-	-	224	186
2AA	2.50	26	1030	100	1129
TNF	0.50	523	-	-	-

^A DMSO solvent control

- Not tested

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