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Mutagenicity of Tween 80-Solvated Mild Gasification Products in the Ames Salmonella Microsomal Assay System

INTRODUCTION

Mild gasification is an emerging coal-conversion technology that is currently being developed by the United States Department of Energy (DOE) and private industry to help meet our future energy needs. With each passing year this technology is becoming more refined and accepted as a viable alternative to our present use of coal; especially for the clean generation of electricity. Since the commercialization of the mild gasification process is likely to occur in the near future, efforts are now being intensified in the area of studies detailing the possible toxic effects of the products of this process. For example, the National Institute of Occupational Safety and Health (NIOSH), through an interagency agreement with DOE, is actively examining these products to assess the extent of their genotoxic activity.

To date, NIOSH has examined the mutagenic activity of six mild gasification samples from various sources and processing conditions in the Ames Salmonella microsomal assay (Maron and Ames, 1983). The Ames assay is a widely used and accepted short-term, lower tier, test for the detection of genotoxic agents and potential carcinogens. This assay system determines the mutagenic activity of test articles by following the reversion of bacterial strains dependent on the amino acid histidine to the condition of histidine independence. Since it is generally accepted that mutation is involved in the initiation of cancer, assays such as the Ames test can be valuable in assessing the risks of long-term health effects of genetic origin.

Dimethylsulfoxide (DMSO) is a predominant solvent vehicle used in mutagenicity studies in order to dissolve samples in a way that is compatible for their interaction with test organisms. This solvent was chosen for use in the initial mutagenicity testing of the mild gasification products due to its: 1) ability to dissolve high concentrations of the samples (up to 100mg/ml), 2) previous usage in synfuel-related and many other studies, 3) low volatility and 4) ease and rapidity of sample preparation. The results from the testing of the six DMSO-solvated mild gasification samples in the Ames assay revealed that although some marginal activity and dose-related responses were noted at times in several samples (especially the composite materials), no significant or repeatable activity was detected (as previously reported in "Genotoxicity Studies of Six DMSO-Solvated Mild Gasification Products in the Ames Salmonella Microsomal Assay System", which was included in the September monthly report).

Recent studies have indicated the possibility of solvent/mutagen interactions occurring during the testing of chemical mutagens

which decrease or increase their response values in genotoxicity assay systems (Demarini et al., 1991; Maron et al., 1981; and Mori et al., 1985). While awaiting the delivery of new samples, it was decided that follow-up Ames testing of the mild gasification samples using a different solvent vehicle would be of interest. Tween 80 (polyoxyethylene-sorbitan mono-oleate) is a non-ionic detergent that when mixed with water forms an emulsion which is compatible for the dissolution of both polar and nonpolar samples for mutagenicity testing. This solvent system has very low cytotoxicity to bacterial cells and is of a nonvolatile nature. Tween 80 has also been used successfully in the examination of the mutagenicity of both coal-derived and petroleum samples (Ma et al., 1983). Hence, the six mild gasification samples, which were previously solvated and tested in DMSO, were submitted for testing using Tween 80 as an alternative solvent vehicle. This paper reports the results of these studies and their possible significance in the future testing of complex mixtures with similar structure.

METHODS AND MATERIALS

Sample descriptions

Mild gasification samples:

PSIS #820331 is a low-temperature coal tar which was obtained from Shell Oil Company, Houston, Texas.

MG-122 (IBP-420°F), MG-122 (420-720°F), and MG-122 (720°F+) are samples with different boiling point ranges derived from the same production/coal source. MG-122 (IBP-420°F) and MG-122 (420-720°F) are liquid samples, whereas MG-122 (720°F+) is a solid, but brittle residue material. These samples were obtained from Western Research Institute (WRI), Laramie, Wyoming.

MG-119 and MG-120 are composite materials that have a liquid/tar consistency. These samples contain materials from a wide range of boiling points and were also obtained from WRI.

Reference material:

SRC-II HD is a heavy distillate from the Solvent Refined Coal-II coal-liquefaction process (sample #2445, boiling point range 550-850°F). This material was obtained from the Pittsburg and Midway Coal Mining Company, Fort Lewis, Washington.

Chemicals

Positive controls:

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

Solvent control:

Tween 80 (polyoxyethylene-sorbatan mono-oleate), was used in the assay system in a 4% w/v solution suspended in triple distilled, deionized water, and was obtained from Sigma Chemical Company, St. Louis, Missouri.

Sample preparation

The samples were prepared for mutagenicity testing using methodologies similar to those described by Ma et al. (1983). Approximately 100mg of each sample was placed in a sterile vial (20ml capacity) and approximately 400mg of Tween 80 was added. This mixture was then warmed to 50°C and sonicated in a Branson model 3200 water bath/sonicator for 30 minutes in order to facilitate homogenization. Distilled water (5 ml) was then added dropwise and the mixture was again warmed, sonicated, and vortexed until a homogeneous emulsion was obtained. Distilled water was then added to the emulsified sample until a final sample concentration of 20mg/ml (4% Tween 80) was reached. Lower concentrations of sample utilized in testing were obtained by serial dilution of the original 20mg/ml emulsion in sterile, 4% Tween 80. The solid sample, MG-122 (720°F), required further sonication using a probe type sonicator (Heat Systems XL at a power setting of 10-15% for 3 minutes) in order to break up the small particles present in this sample. The positive controls and the reference material were prepared in a similar manner as the coal-derived materials in order to insure consistent experimental design.

Ames Salmonella microsomal assay

The mutagenicity of all test materials was determined using the pre-incubation variant of the Ames Salmonella microsomal assay system (Maron and Ames, 1983), which is often more sensitive than the standard protocol. The treatment started with 0.1 ml aliquots of overnight bacterial culture (TA98 or TA100) being added to two test tubes, each containing 0.1 ml of Tween 80-solvated sample, which were then vortexed. In order to assess the effects of metabolic activation, which is

required for indirect-acting chemical mutagens, 0.5 ml of S9 metabolic activation mixture (from liver preparations of Aroclor 1254 treated male Wistar rats) was added to one tube (+S9) and to the other tube 0.5 ml of physiological saline (-S9). This mixture was then vortexed and incubated on a rotary shaker at 37°C for 30 minutes prior to plating. Finally, 2.5 ml of molten (45°C) soft agar containing trace amounts of biotin and histidine (required for initial bacterial growth) was pipetted into each tube. This solution was vortexed at low speed to prevent bubble formation; then poured onto plates containing minimal medium (bottom agar). After solidification, the plates were inverted and incubated at 37°C for two days, and bacterial colonies (his+ revertants) were scored on an automatic colony counter. For each concentration of sample tested, three replicate plates were used in order to get statistically valid results. Positive (2AA and/or TNF) and solvent (Tween 80) controls and the SRC-II HD reference material were plated out for direct comparisons to the response of the treatment plates. In this assay it was found to be impractical to increase the sample concentrations above 20mg/ml using the 4% final concentration of Tween 80 since most of the samples rapidly became insoluble. The criteria for positive test results consisted of obtaining revertant counts that numbered at least twice those of the solvent control value, accompanied by dose-related increases in activity. The bacterial strains utilized in our studies were kindly provided upon request from Dr. B.N. Ames of the University of California at Berkeley.

RESULTS AND DISCUSSION

The data from the Tween 80-solvated Ames testing of the six mild gasification samples can be viewed in Tables I through IV (attached). Testing of the mild gasification samples was initiated using the two samples which demonstrated the most suspect activity in the DMSO-solvated assays, composite samples MG-119 and MG-120. The results of the first test (Table I) showed significant mutagenic activity in both samples on tester strain TA98 in the presence of the S9 metabolic fraction. No mutagenic activity was indicated on TA100 in either metabolic treatment, or on TA98 without S9 metabolic activation. Since TA98 detects frameshift mutagens and TA100 detects base-pair substitution mutagens, the activity expressed in this assay (and subsequent ones) appeared to be that of an indirect-acting, frameshift mutagen(s).

Since significant mutagenic activity was found in the suspect composite samples, it was necessary to also re-examine the mutagenicity of the other four "non-suspect" mild gasification samples using Tween 80 as a solvent. Although dose-related trends in the number of revertants were indicated in all of the nonsuspect samples, only MG-122 (420-720°F) and MG-122 (720°F+) indicated mutagenic activity on TA98 with S9. That activity, however, was marginal, at best. MG-122 (420-720°F) displayed at least a doubling in the number of revertants over the solvent control

values at only one concentration (1000 μg/plate); whereas MG-122 (720°F+) was tested at two concentrations (100 and 1000 μg/plate) where the number of revertants at least doubled. Again, no significant mutagenic activity was found on TA100 or on TA98 without metabolic activation.

The results of a follow-up, dose adjusted, confirmatory assay (Table III) on all six mild gasification samples failed to verify any marginal mutagenic activity in the nonsuspect samples; however, MG-122 (420-720°F) did show a partial dose-related response and MG-122 (720°F+) had a doubling of the number of revertants on TA98 without S9 (60 at 2000 μg/plate). This assay confirmed the results of the initial Tween 80-solvated testing of the suspect composite materials, where significant mutagenic activity was detected. A final confirmatory test using TA98 with S9 was run on samples MG-122 (420-720°F), MG-122 (720°F+), and the composite materials MG-119 and MG-120 (Table IV). The results of this second confirmatory experiment again failed to demonstrate any significant mutagenic activity in the two non-suspect samples, which is in sharp contrast to the significant and repeatable activity found in the two composite materials that were examined in the same experiment.

Several interesting points can be raised when contrasting the data from the Tween 80-solvated versus the DMSO-solvated Ames testing of the mild gasification samples. First, it is clear that all six of the mild gasification samples examined to date can be tested at higher concentrations without cytotoxicity in Tween 80 than in DMSO. Although DMSO is capable of solvating higher concentrations of these samples than Tween 80 (100mg/ml as compaired to 20 mg/ml respectively), the higher DMSO-solvated sample concentrations can not be tested due to their cytotoxicity to the bacterial test organism. For example, the composite materials (MG-119 and MG-120) could only be tested to 400μg/plate without cytotoxic effects when solvated in DMSO whereas, these samples could be tested at up to 2000μg/plate when solvated in Tween 80. Since the mutagenic activity of these mild gasification samples are low, the inability to test the highest, most mutagenic concentrations with DMSO as a solvent vehicle may limit the full range of genotoxic activities from being evident.

Second, the possibility exists that solvent/mutagen interactions are occurring during the testing of these samples when solvated in DMSO that reduce the mutagenic response values. In our experiments with coal-derived materials, the mutagenic response values (treatment/solvent) are often higher for the Tween 80-solvated samples than for those solvated in DMSO when compared to the solvent control values. For example, the average number of revertants/plate for the final confirmatory studies on MG-120 yielded 67 at the 100μg/plate concentration for the DMSO-solvated sample and 66 at the 125μg/plate concentration for the Tween 80-solvated sample. Because the solvent control values were 55 and 30 respectively, the Tween 80 response value denotes a significant increase (greater than two-fold) over the spontaneous mutational background, whereas the DMSO response value does not. Solvent/mutagen interactions are known to exist, and more information is becoming available about this type of interference with various genotoxicity assay systems. In regard to DMSO, Mori

et al. (1985) found that this solvent eliminated the mutagenic potential of four N-nitrosamines and severely reduced the activity of five others in the Ames assay. The findings for one N-nitrosamine (DMN) were confirmed when tested by Brodberg et al. (1987) in the Drosophila melanogaster recessive sex-linked lethal mutation assay. Also, Demarini et al., (1991) has found that both DMSO and methanol reduce the genotoxic potencies of 2-Aminoanthracene (2AA) and 2-Nitrofluorene (2NF) in the E. coli WP2s Microscreen assay. This may be of significance in the testing of coal-derived samples in that primary aromatic amines (such as 2AA) and nitroarenes have been demonstrated to be major mutagenic components of these materials (Gray et al., 1988). The reduced mutagenic response values sometimes noted for DMSO may be related to its ability to quench the free radical forms of chemical mutagens which can directly bind to DNA (Hrelia, 1990). Alternatively, DMSO has been shown to give higher mutagenic response values than acetone for 2AA in the Ames assay (Anderson, et al., 1980), and DMSO has been shown to increase the activity of hexachloroacetones when compared to other solvents in this test system as well (Nestman, er al., 1980). Furthermore, in our laboratory, we have noticed two-fold or greater increases in the Ames test response values for 2AA when solvated in Tween 80 as compared to DMSO. The effects of other solvents on the mutagenic response of test articles have been noted as well (Maron, et al., 1981 and others). Thus, the use of two different solvent systems may be of great value when analyzing the mutagenic activity of compounds, especially when they are complex mixtures with undefined chemical properties (such as the mild gasification samples).

Finally, the only Ames study which details the comparative mutagenic responses of coal-derived and petroleum samples solvated in Tween 80 or DMSO was conducted by Ma et al. (1983). The results of solvent effects in this investigation were somewhat ambiguous as far as coal-derived liquids were concerned. For example, coal oil CRM1 was found to have greater activity when solvated in DMSO than Tween 80. However, the other coal-derived oil use in this study (591E) showed similar or higher mutagenic responses when solvated in Tween 80 versus DMSO. One problem with this study was that the DMSO-solvat samples were not the original, intact samples, but had been either dewaxed using cyclohexane or chemically fractionated prior to use in the assay. The mutagenic response values for the fractionated samples solvated and tested in DMSO used the summation of the mutagenic activity in each fraction, whereas the Tween 80-solvated samples were tested on the intact coal liquids without prior chemical treatment. Thus, a comparison of the mutagenic activity between the two solvent systems utilized in this study is problematic.

CONCLUSIONS

The results of the Tween 80-solvated Ames testing of six mild gasification samples indicate significant mutagenic activity only in the composite materials (MG-119 and MG-120), previously suspected from the DMSO-solvated assays, which had shown some variable but ultimately insignificant mutagenic responses. The activity of these samples from the Tween 80-solvated assays was quite low when compared to either the positive controls or the SRC-II HD coal-liquefaction reference material. The class of mutagenic activity expressed by these samples solvated in Tween 80 was that of an indirect-acting, frameshift mutagen(s) since significant activity was found only on tester strain TA98 in the presence of the metabolic activation fraction (S9). Because DMSO and other solvents have been shown to affect the mutagenic activity of certain pure chemicals, the possibility of solvent/mutagen interactions in complex mixtures such as coal-derived liquids exists. Thus, the testing of the genotoxic activity of undefined, chemically complex compounds may require the use of at least two solvent systems to reduce the possibility of artifactual findings.

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TABLE I

Ames Test --- Summary Table

Initial Tween 80-Solvated Testing of Suspect Mild Gasification Samples, MG-119 and MG-120

Test conditions: Pre-incubation Assay

Sample	Concentration μg/plate	Average Number of Revertant Colonies per Plate			
		TA98		TA100	
		- S9	+ S9	- S9	+S9
MG-119 COMPOSITE	0^	25	33	123	112
	10	21	43	125	101
	100	32	80	99	128
	1000	23	109	134	139
MG-120 COMPOSITE	0^	25	33	123	112
	10	28	38	108	102
	100	27	84	132	133
	1000	23	92	101	137
SRC-II HD	0^	25	33	123	112
	62.5	29	313	114	200
2AA	0^	25	33	123	112
	2.5	32	447	118	671

^ Tween 80 solvent control

TABLE II

Ames Test --- Summary Table

Initial Tween 80-Solvated Testing of Non-suspect Mild Gasification Samples

Test Conditions: Pre-incubation Assay

Sample	Concentration µg/plate	Average Number of Revertant Colonies per Plate			
		TA98		TA100	
		- S9	+ S9	- S9	+S9
Shell Oil	0[A]	17	27	107	129
	10	33	32	154	107
	100	27	46	157	160
	1000	24	51	194	154
MG-122 IBP-420°F	0[A]	17	27	107	129
	10	24	26	200	119
	100	40	35	166	126
	1000	32	42	120	95
MG-122 420°F-720°F	0[A]	17	27	107	129
	10	29	33	182	125
	100	26	46	131	159
	1000	28[B]	61	90[B]	144
MG-122 720°F+	0[A]	17	27	107	129
	10	29	40	125	161
	100	30	59	153	173
	1000	36	62	149	158
SRC II HD	0[A]	17	27	107	129
	125	36	299	136	336
	250	34	518	135	286
2AA	0[A]	17	27	107	129
	2.5	29	954	137	1370

[A] Tween 80 solvent control [B] Too much cytotoxicity - Not tested

TABLE III

Ames Test --- Summary Table

Dose Adjusted Confirmatory Tween 80-Solvated Testing of All Mild Gasification Samples

Test Conditions: Pre-incubation Assay

Sample	Concentration μg/plate	Average Number of Revertant Colonies per Plate			
		TA98		TA100	
		- S9	+ S9	- S9	+S9
Shell Oil	0[A]	32	26	100	104
	250	33	54	119	125
	500	45	46	111	140
	1000	45	54	125	113
	2000	28[B]	49	105	117
MG-122 IBP-420°F	0[A]	32	26	100	104
	250	33	32	126	115
	500	36	34	134	111
	1000	30	51	106	113
	2000	35[B]	39[B]	63[B]	135[B]
MG-122 420°F-720°F	0[A]	32	26	100	104
	250	29[B]	45	144	124
	500	24[B]	53	121	150
	1000	18[B]	35	71[B]	123
	2000	-	25[B]	-	59[B]
MG-122 720°F+	0[A]	32	26	100	104
	250	37	39	115	101
	500	26	39	148	118
	1000	44	34	126	104
	2000	62	60	131	126
MG-119 COMPOSITE	0[A]	-	26	-	-
	250	-	48	-	-
	500	-	61	-	-
	1000	-	73	-	-
	2000	-	94	-	-

TABLE III (cont.)
Ames Test --- Summary Table
Dose Adjusted Confirmatory Tween 80-Solvated Testing of All Mild Gasification Samples
Test Conditions: Pre-incubation Assay

Sample	Concentration μg/plate	Average Number of Revertant Colonies per Plate			
		TA98		TA100	
		- S9	+ S9	- S9	+S9
MG-120 COMPOSITE	0[A]	-	26	-	-
	250	-	55	-	-
	500	-	61	-	-
	1000	-	91	-	-
	2000	-	103	-	-
SRC-II HD	0[A]	32	26	100	104
	125	36	192	127	207
2AA	0[A]	32	26	100	104
	2.5	31	980	117	1464

[A] Tween 80 solvent control [B] Too much cytotoxicity - Not tested

TABLE IV
Ames Test --- Summary Table
Dose Adjusted Confirmatory Tween 80-Solvated Testing
of Mild Gasification Samples
Test Conditions: Pre-incubation

Sample	Concentration µg/plate	Average Number of Revertant Colonies per Plate	
		TA98	
		- S9	+ S9
MG-122 420°F - 720°F	0^	31	30
	62.5	-	-
	125	-	44
	250	-	29
	500	-	33
	1000	-	33
	2000	-	14[a]
MG-122 720° F+	0^	31	30
	62.5	-	-
	125	-	49
	250	-	48
	500	-	55
	1000	-	55
	2000	-	44
MG-119 COMPOSITE	0^	31	30
	62.5	-	-
	125	-	44
	250	-	58
	500	-	63
	1000	-	75
	2000	-	77

TABLE IV (cont.)
Ames Test --- Summary Table
Dose Adjusted Confirmatory Tween 80-Solvated Testing
of Mild Gasification Samples
Test Conditions: Pre-incubation

Sample	Concentration μg/plate	Average Number of Revertant Colonies per Plate	
		TA98	
		- S9	+ S9
MG-120 COMPOSITE	0[A]	31	30
	62.5	-	-
	125	-	66
	250	-	71
	500	-	67
	1000	-	92
	2000	-	113
SRC-II HD	0[A]	31	30
	62.5	-	146
	125	-	283
	250	-	275
	500	-	412
2AA	0[A]	31	30
	2.5	39	1383

[A] Tween 80 solvent control [B] Too much cytotoxicity - Not tested

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