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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Centers for Disease Control
National Institute for Occupational
Safety and Health – ALOSH
944 Chestnut Ridge Road
Morgantown, WV 26505–2888

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METC Contractor Reports Receipt Coordinator
Department of Energy
Morgantown Energy Technology Center
P.O. Box 880
Morgantown, WV 26507-0880

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Dear Coordinator:

This is the quarterly report of 3Q92, for the Interagency Agreement #DE-AI21-89MC26018, Task D entitled "Toxicity Studies of Mild Gasification Products".

We received a sample of mild gasification product during 2Q92 from Mr. Glenn O'Neal of the Coal Technology Corporation, Bristol, Virginia and have spent much of the 3Q92 testing it. The sample is a composite, containing materials from a wide range of boiling points. It has a liquid/tar consistency so solvation at the highest doses required care to ensure that the test solution was homogenous. The sample was tested in each of two solvents, Tween 80 and DMSO. Solvents may interact with a test chemical in such a way as to cause false positives or negatives. The use of two solvents with different chemical properties may allow us to recognize that phenomenon, if it occurs. When a chemical enters the body, it is usually metabolized in some way, often by liver enzymes. Therefore, the Ames test (bacterial mutagenicity test) assays each chemical with and without the addition of S9 preparation (made from rat livers) to determine if a nonmutagenic chemical is metabolized into a mutagenic one.

The Ames mutagenicity assay of the whole sample, CTC#11 was completed in May. The results show that the highest response was on TA98 with metabolic activation, indicating the presence of a potent, indirect-acting, frameshift mutagen. Moderate significant activity was also noted on TA98 without metabolic activation and on TA100 with metabolic activation. Weak, but significant mutagenic activity was shown on TA100 without S9. Therefore, direct-acting frameshift mutagen(s) and direct- and indirect-acting base-pair substitution mutagens are all indicated. The patterns of mutagenicity were similar using either solvent. A report was submitted in June; for your convenience a copy is also enclosed. CTC#11 has been chemically fractionated, and the Ames assay for the mutagenicity of those subfractions is underway.

This quarter, we were informed that Mr. Lan would be taking over as project manager, and during June my colleagues and I had the opportunity to meet with him to discuss progress on the project. A brief outline of NIOSH efforts to date was available at the meeting; a copy is enclosed. As a result of the discussion, we prioritized the projects we are currently working on or will start as per requested. The list, in order, is as follows:

1) Complete testing on the subfractions of sample CTC#11
2) Test the subfractions of the sample Shell PSIS#830331
3) Complete the mammalian cell assays on sample MG-120
4) Test CTC#11 in the mammalian cell assays
5) Consider testing Shell PSIS#830331 in the mammalian cell assays.

Mammalian cell assays, the second tier of testing, have been started on an earlier sample, MG-120, which was found to be mutagenic to bacteria when Tween-80 was used as solvent. These tests will allow us to determine if the coal liquid samples that are mutagenic to bacteria are also mutagenic/genotoxic to mammalian cells. The battery of tests in the mammalian cell assay system includes gene mutation, micronucleus formation, and sister chromatid exchange. The tests are performed on cultures of Chinese hamster lung fibroblasts (V79 cells). Progress on these tests is good, but it will take approximately 2 more months to complete all the assays.

Sincerely,

Tong-man Ong

Tong-man Ong, Ph.D.
Chief, Microbiology Section
Division of Respiratory Disease Studies

Enclosure

# Mutagenicity of CTC#11 in the Ames Salmonella/Microsomal Assay

## INTRODUCTION

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 results of tests on six of those samples have been previously reported in "Genotoxicity Studies of Six DMSO-Solvated Mild Gasification Products in the Ames Salmonella Microsomal Assay System" and "Mutagenicity of Tween 80-Solvated Mild Gasification Products in the Ames Salmonella Microsomal Assay System". 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 mutagenic activity of a test substance is measured by the number of reverse mutations eliminating the histidine requirement. DNA mutation is generally accepted to be 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.

The assay was performed on CTC#11 using two solvents with quite different properties, DMSO (dimethylsulfoxide) 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. Previous testing in this project has demonstrated that these interactions can occur in mild gasification products, thereby confirming the importance of testing in at least two different solvents.

## METHODS AND MATERIALS

### Sample description

CTC#11 is a mild gasification product with a liquid/tar consistency. It is a composite material 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 trinitroflourenone (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

Tween 80 Solvation:

CTC#11 was prepared for mutagenicity testing using methodologies similar to those described by Ma et al. (1983). Approximately 100 mg of the sample was placed in a sterile vial; approximately 400 mg Tween 80 was added. The mixture was heated to 50°C and sonicated in a Branson model 3200 water bath/sonicator for 30 minutes to facilitate homogenization. Distilled water was added dropwise (5 ml) and the mixture was warmed, sonicated, and vortexed until a homogeneous emulsion was obtained. Distilled water was again added to the emulsion until a final sample concentration of 20 mg/ml (4% Tween 80) was reached. Lower concentrations of CTC#11 were obtained by serial dilution (using 4% Tween 80) of the original emulsion. The positive controls were prepared in a manner similar to the sample to ensure consistent experimental design.

DMSO Solvation:

The sample was dissolved in pure DMSO to the desired concentrations. The mixture was heated, sonicated, and vortexed in a manner similar to the Tween 80 preparation to ensure a homogeneous solution. Again, the controls were prepared the same way to ensure consistency.

## Ames Salmonella/microsomal Assay

The mutagenicity of CTC#11 was determined using the pre-incubation variant of the Ames Salmonella/microsomal assay system (Maron and Ames, 1983), which is frequently more sensitive than the standard protocol. The sample was tested on TA98 and TA100 bacterial tester strains. Each concentration on each tester was tested with and without metabolic activation from S9, a preparation made from the livers of Aroclor 1254-treated male Wistar rats. Each of these

treatments was tested in triplicate. In a test tube, 0.1 ml of the sample or a control chemical was combined with 0.1 ml overnight bacterial culture and 0.5 ml S9 preparation or 0.5 ml physiological saline. Each test tube was vortexed and incubated on a rotary shaker at 37°C for 30 minutes prior to adding 2.5 ml molten (45°C) top agar and pouring the mixture onto a petri dish. After the top agar solidified, the dishes were inverted and incubated for 48 hours at 37°C. The top agar contained trace amounts of biotin and histidine which are required for initial bacterial growth. Within several hours after plating, the biotin and histidine present were depleted. Only those bacteria that had mutated to be biotin/histidine-independent continued to grow and form a colony. Those bacteria are called revertants because they have reverted (in that trait only) back to the wild-type Salmonella. The revertant colonies were scored on an automatic colony counter. The criteria for positive mutagenic activity in the Ames assay is a doubling of the negative control in the number of revertants, accompanied by a dose-related increase in revertant numbers. The positive controls allow us to be certain that the bacteria grew and the S9 worked properly.

## RESULTS AND DISCUSSION

CTC#11 displayed significant mutagenic activity in all conditions tested. Results of the testing can be found in Tables I and II. Confirmatory tests gave similar results. The highest response was noted on TA98 with microsomal activation. Although both solvents allowed a strong response to be evident, the mutagenic activity was highest when DMSO was used as the solvent. Significant response under these conditions indicates the presence of potent, indirect-acting, frameshift mutagen(s). Moderate significant mutagenic activity was also noted on TA98 without microsomal activation indicating the presence of a direct-acting frameshift mutagen. In this case, the response was slightly higher when Tween 80 was used as the solvent. 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.

## REFERENCES

Ma, C.Y., C.H. Ho, R.B. Quincy, M.R. Guerin, T.K. Rao, B.E. Allen, and J.L. Epler (1983) Preparation of oils for bacterial testing, Mutat. Res., 118:15-24.

Maron, D.M., and B.N. Ames, 1983. Revised methods for the *Salmonella* mutagenicity test. Mutat Res, 113:173-215.

TABLE I
Mutagenicity Testing of CTC#11 Solvated in DMSO

| Sample | Concentration µg/pl | Average Number of Revertant Colonies per Plate | | | |
|---|---|---|---|---|---|
| | | TA 98 | | TA 100 | |
| | | - S9 | + S9 | - S9 | + S9 |
| CTC#11 | 0[A] | 22 | 27 | 101 | 86 |
| | 12.5 | 73 | 347 | 171 | 196 |
| | 25 | 55 | 663 | 183 | 328 |
| | 50 | 138 | 853 | 239 | 321 |
| | 100 | 87[B] | 1035 | 218 | 363 |
| | 200 | 53 | 1145 | 194 | 560 |
| | | | | | |
| 2AA | 0[A] | 22 | 27 | 101 | 86 |
| | 2.5 | 41 | 1223 | 99 | 954 |
| | | | | | |
| TNF | 0[A] | 22 | 27 | 101 | 86 |
| | 0.5 | 1148 | - | 224 | - |

[A] DMSO solvent control [B] Too much cytotoxicity

TABLE II
Mutagenicity Testing of CTC#11 Solvated in Tween 80

| Sample | Concentration µg/pl | Average Number of Revertant Colonies per Plate | | | |
|---|---|---|---|---|---|
| | | TA 98 | | TA 100 | |
| | | - S9 | + S9 | - S9 | + S9 |
| CTC#11 | 0[A] | 18 | 19 | 98 | 83 |
| | 25 | 35 | 59 | 184 | 172 |
| | 50 | 46 | 71 | 225 | 234 |
| | 100 | 54 | 101 | 254 | 252 |
| | 200 | 105 | 171 | 244 | 304 |
| | 400 | 173 | 228 | 224 | 301 |
| | | | | | |
| 2AA | 0[A] | 18 | 19 | 98 | 83 |
| | 2.5 | 27 | 508 | 125 | 911 |

[A] DMSO solvent control [B] Too much cytotoxicity

Toxicity Studies of Mild Gasification Products
(Outline)

Samples Received to Date:

PSIS #830331 is a low-temperature coal tar which was sent by Shell Oil Company, Houston, Texas.

MG-122 (IBP-420°F) and MG-122 (420-720°F) are liquid samples with different boiling point ranges derived from the same production/coal source. MG-122 (720°F+) is from the same source, but is a brittle solid residue. These samples were sent by 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 the entire range of boiling points and were also obtained from WRI.

CTC#11 is another composite material with a liquid/tar consistency. It was sent by Coal Technology Corporation in Bristol, Virginia.

Dr. Joseph Ma of the West Virginia University School of Pharmacology has chemically fractionated samples MG-119 and MG-120 into four subfractions: acidic, basic, nonpolar neutral, and polar neutral.

Solvents used in testing:
- Dimethylsulfoxide (DMSO)
- Tween 80

Ames Tests and modifications run on the samples using bacterial tester strains TA98 and TA100.
- Standard plate incorporation assay
- Pre-incubation assay
- Microsuspension assay (used only on the subfractions)

Genotoxicity Tests on Mammalian Cell Cultures - these tests have been started, but the results are not yet available.
- Gene mutation
- Micronucleus induction
- Sister Chromatid Exchange

Results - summarized on the attached tables.

The standard plate incorporation assay was used only to test DMSO-solvated samples. Those tested with this assay include: PSIS #830331, all MG-122 samples, MG-119 and MG-120. No repeatable, significant mutagenic activity was detected in these samples with this protocol.

The pre-incubation assay has been used to test all samples using both DMSO and Tween 80 as solvents. When the samples were solvated in DMSO, significant mutagenic activity was detected only in CTC#11. However, due to recent reports in the professional literature about solvent/mutagen interactions, we proposed the use of another solvent, Tween 80. The results are as follows.

PSIS #830331 - no significant mutagenic activity detected.

MG-122 (IBP-420°F) - no significant mutagenic activity detected.

MG-122 (420-720°F) - no significant mutagenic activity detected.

MG-122 (720°F+) - no significant mutagenic activity detected.

MG-119 - significant mutagenic activity detected on TA98 (indicating a frameshift mutagen) with S9 metabolic activation (indicating an indirect acting mutagen).

MG-120 - significant mutagenic activity detected on TA98 with S9 metabolic activation.

CTC#11 - displayed significant mutagenic activity under every condition tested. DMSO gave a stronger response than Tween 80, but both were significant. The response was stronger on TA98 with S9 activation, but the significant response on TA100 and without S9 also indicate, respectively, the presence of base-pair substitution and direct-acting mutagens.

The microsuspension assay was used in testing the subfractions of MG-119 and MG-120. In both samples, only the nonpolar neutral fraction was found to have significant mutagenic activity. Both were mutagenic to TA98 and TA100. MG-119 was mutagenic only with S9 activation; MG-120 was mutagenic with and without activation. The inhibition of mutagens by nonmutagenic components of complex mixtures, a phenomenon known as chemical masking, may be responsible for the lack of activity of the whole samples on TA100. It was for this purpose that the subfraction tests were proposed. The subfractions of CTC#11 have just recently arrived; testing will begin soon on those.

TABLE I

Results of Pre-incubation Ames Testing of Whole Samples on Salmonella Tester Strains

| Sample | Solvent used | Mutagenic activity |
|---|---|---|
| PSIS #830331 | DMSO<br>Tween 80 | none<br>none |
| MG-122(IBP-420°F) | DMSO<br>Tween 80 | none<br>none |
| MG-122 (420-720°F) | DMSO<br>Tween 80 | none<br>none |
| MG-122 (720°F+) | DMSO<br>Tween 80 | none<br>none |
| MG-119 | DMSO<br>Tween 80 | none<br>weak |
| MG-120 | DMSO<br>Tween 80 | none<br>weak |
| CTC#11 | DMSO<br>Tween 80 | strong<br>strong |

TABLE II

Results of Microsuspension Ames Testing of Subfractions on Salmonella Tester Strains

| Sample | Fraction | Mutagenic Activity |
|---|---|---|
| MG-119 | acidic | none |
| | basic | none |
| | nonpolar neutral | weak |
| | polar neutral | none |
| | | |
| MG-120 | acidic | none |
| | basic | none |
| | nonpolar neutral | weak/moderate |
| | polar neutral | none |
| | | |
| CTC#11 | acidic | [these tests have not yet been performed] |
| | basic | |
| | nonpolar neutral | |
| | polar neutral | |

TABLE III

Genotoxicity Tests on Mammalian Cell Cultures

| Sample | Gene Mutation | Micronuclei Induction | SCE |
|---|---|---|---|
| MG-120 | Results are not yet available for these tests | | |
| CTC#11 | This sample has not yet been assayed by these tests | | |

# END

# DATE FILMED

4/12/93