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OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

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879708

SUBJECT: ID. No. 063503-000862 - Mutagenicity Studies for
Petroleum Distillates, Oils, Solvents, or Hydrocarbons

Tox. Chem. No.: 646
DP Barcode No.: D195956
Record No.: S451162

TO: Kathryn Davis, Product Manager, PM Team 52
Bonnie Adler, Reviewer, PM Team 52
Accelerated Reregistration Branch
Special Review and Reregistration Division (7508W)

FROM: Sheryl K. Reilly, Ph.D.
Review Section II, Toxicology Branch I
Health Effects Division (7509C)

Sheryl K. Reilly 3-21-94

THRU: Joycelyn E. Stewart, Ph.D.
Section Head
Review Section II, Toxicology Branch I
Health Effects Division (7509C)

Joy Stewart 3/21/94

KB 3/22/94

CONCLUSIONS: Three mutagenicity studies, all submitted under
MRID 416853-23, in support of the reregistration of paraffinic
oil 78-9-70 SUS/100°F have been reviewed and the following
conclusions have been made for the product:

84-2 Mutagenicity: In an Ames reverse mutation assay, with
or without a preincubation/suspension step and with or without
metabolic activation, the test substance showed no toxicity nor
did it increase the number of histidine revertants in Salmonella
strains TA 1535, TA 1537, TA 98 and TA 100 at concentrations of
240,000, 160,000, 80,000, 45,000 and 40,000 µg/plate. Moderate
to heavy oil droplets observed in each of the plates indicated
that the paraffinic oil was insoluble in the cell culture medium.
Therefore, the toxicity and mutagenicity of paraffin oil in this
test system cannot be interpreted and the study is **Unacceptable**.
However, since it is doubtful that the solubility problems could
be resolved even at lower doses, the study does not need to be
repeated for regulatory purposes.

84-2 Mutagenicity - In a mouse lymphoma forward mutation assay, L5178Y heterozygous TK⁺/⁻ cells were exposed to concentrations of paraffinic oil (78-9-70) equal to 8,670, 17,340, 34,680, 52,020, 69,360, 86,700, 104,040, and 121,380 µg/ml, with and without metabolic activation with rat liver microsomal fraction S-9. The test compound could not be effectively removed from the cells after exposure, but was nontoxic at any dose even though it remained in contact with the cells throughout the study. This indicated that the substance was insoluble in the aqueous media, and therefore an evaluation of the toxicity and mutagenicity of paraffinic oil in this test system is not possible, and this study is **Unacceptable**. However, it is unlikely that the solubility problems could be overcome in this test system, and the study does not need to be repeated for regulatory purposes.

84-2 Mutagenicity - In vivo mammalian cytogenetics in rat bone marrow. Under the conditions of this study, paraffinic oil 78-9-70 SUS/100°F did not appear to be clastogenic (i.e., did not induce structural chromosome aberrations) in bone marrow metaphase cells) of albino rats at the oral concentrations tested (500, 1,000, and 2,000 mg/kg/day for 5 days). However, the highest dose tested was not toxic, and a limit dose (5,000 mg/kg) was not tested. Therefore, this study is **Unacceptable**, and must be repeated up to the limit dose, together with the submission of evidence that the test substance is absorbed from the gastrointestinal tract and transports to the target tissue (bone marrow cells) in effective concentrations. Alternatively, the test substance could be injected intraperitoneally at (only) the highest dosage feasible up to the limit dose, in order to assure the greatest potential for exposure and response (cytotoxic and/or clastogenic) of the target tissue, as prevailed for triethylene melamine (TEM), the positive control in this study.

Copies of the DERs are provided for your reference.

cc: Irving Mauer, Ph.D. OK!



Reviewed by: Sheryl K. Reilly, Ph.D.
 Section II, Tox. Branch I (7509C)
 Secondary Reviewer: Irving Mauer, Ph.D.
 Tox Branch I (7509C)

SKR 3-21-84
Irving Mauer
 3/21/84

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity Study - Reverse Mutation Assay
 in Salmonella typhimurium

GUIDELINE #: 84-2

TOX. CHEM. #: 646

MRID #: 416853-23

TEST MATERIAL: Paraffinic Oil (78-9-70, SUS/100°F)

SYNONYMS: 78-9-70

STUDY NUMBERS: 596-111

SPONSOR: American Petroleum Institute

TESTING FACILITY: Hazleton Laboratories America, Inc.
 9200 Leesburg Turnpike
 Vienna, Virginia 22180

TITLE OF REPORT: In Vivo and In Vitro Mutagenicity Studies
 with Paraffinic Oil 78-9-70 SUS/100°F:
Salmonella typhimurium Mammalian Microsome
 Plate Incorporation and Suspension Assays

AUTHOR: Hazleton Laboratories America, Inc.

REPORT ISSUED: June 19, 1981

EXECUTIVE SUMMARY: In an Ames reverse mutation assay, with or without a preincubation/suspension step, Salmonella typhimurium strains TA1535, TA1537, TA1538, TA98, and TA100 were exposed to concentrations of 240,000, 160,000, 80,000, 45,000 and 40,000 µg/plate paraffinic oil (78-9-70), with and without metabolic activation with rat liver microsomal fraction S-9. No treatment related increases in the number of revertants to histidine prototrophy were observed in either the plate incorporation assay or the liquid suspension assay.

The study is **Unacceptable** under § 84-2 for a Salmonella typhimurium reverse mutation assay, because the presence of moderate to heavy oil droplets indicates that the compound was not soluble in the medium at any of the concentrations used, thus, toxicity and mutagenicity cannot be evaluated. This test

does not need to be repeated for regulatory purposes, however, because it is unlikely that the solubility problems could be overcome in this test system. (MRID 416853-23).

QUALITY ASSURANCE: A statement concerning compliance with good laboratory practices was included in the submission. The study was performed prior to the publication of the requirements of 40 CFR 160, but was conducted under the provisions of 21 CFR 58.

MATERIALS:

1. Test Strains: Salmonella strains TA1535, TA1537, TA1538, TA98 and TA100 were obtained from the laboratory of Bruce Ames, Ph.D. Strains TA1535 and TA100 are used to detect base pair substitutions. Strains TA1537, TA1538, and TA98 are used for the detection of frameshift mutagens. TA98 and TA100 have plasmids that convey resistance to ampicillin, and are more effective in detecting some classes of mutagens than their parent strains (TA1538 and TA1535, respectively).

2. Test Compound: Paraffinic oil, a viscous clear, yellow liquid was the test compound; the purity of the compound was 100%.

3. Vehicle: DMSO

4. Controls:

a. Negative Controls: media, solvent (DMSO)

b. Positive Controls:

1) With S-9 Activation: 2-aminoanthracene (for all strains)

2) Without Metabolic Activation: MNNG (methylnitronitrosoguanidine) for TA1535, TA100; 9-aminoacridine for TA1537; 2-nitrofluorene for TA1538, TA98

METHODS:

1. Bacterial Stock Cultures: Each of the 5 strains of bacteria were inoculated into 5 ml of nutrient broth, and incubated overnight at $37 \pm 0.5^{\circ}\text{C}$. An 0.8 ml portion of these cultures was mixed with 0.07 ml DMSO in 2 ml sterile glass screw-capped vials, and frozen.

2. Verification of Tester Strains:

a. Spontaneous Reversion: The spontaneous reversion to histidine prototrophy was determined in each experiment. To determine the rate of spontaneous reversion, 0.1 ml of each of the overnight broth cultures was inoculated onto a nutrient agar plate, and incubated at $37 \pm 0.5^\circ\text{C}$ for 18-24 hours. Isolated colonies were transferred to fresh nutrient broth. These bacterial suspensions were inoculated onto nutrient agar plates of minimal agar medium and Vogel-Bonner Medium E (VBE), with and without 0.1M L-histidine HCl, and with 0.5 mM biotin, and the rate of spontaneous reversion was determined. Revertants to histidine prototrophy were counted with a New Brunswick Scientific electronic colony counter.

b. Genetic Characterization: Salmonella strains TA1535, TA1537, TA1538, TA100 and TA98 were tested for having deep rough characteristics by inoculation into a medium containing 10 μl of 2 mg/ml crystal violet. Strains TA100 and TA98 were tested for the presence of the ampicillin R factor by streaking the surface of a nutrient agar plate with ampicillin solution (8 mg/ml), then counterstreaking the tester strain across the ampicillin streak. Those cultures with the R factor showed no zone of inhibition in the area of the ampicillin streak.

3. S-9 Preparation: The metabolic activator, liver homogenate S-9, was prepared from adult male Sprague-Dawley rat livers. The rats had been given single intraperitoneal injections of 200 mg Aroclor 1254/ml corn oil, at dosages of 500 mg/kg, 5 days prior to sacrifice. The livers were removed from the sacrificed rats, weighed, and washed with 150 mM KCl solution, cut into pieces and homogenized in 3 volumes of sterile KCl solution. After centrifugation at $9000 \times g$ at 4°C , portions of the supernatant (S-9 fraction) were collected and immediately deep-frozen in dry ice, then stored in a freezer. Just before use, the S-9 was thawed and mixed (0.04-0.15 ml/ml mixture) with cofactors (8 moles MgCl_2 ; 33 moles KCl; 5 moles glucose-6-phosphate; 4 moles NADP; and 100 moles of sodium phosphate, buffer, pH 7.4).

4. Test of Toxicity:

a. Plate Incorporation Assay: The test substance was dissolved in 2 different solvents, as follows: 785 mg of the concentrate was dissolved in 0.78 ml ethyl acetate, and 762 mg was dissolved in 0.76 ml DMSO. Two tenfold dilutions of each was prepared using the appropriate solvent. To each 2 ml of complete top agar containing 0.1 ml of an overnight broth culture of bacterial strain TA100, 0.2, 0.1, and 0.15 ml volumes of concentrated test compound or 0.1 ml of each of the dilutions of

the test compound were added, mixed, inverted and the cultures allowed to harden, and incubated 48 h at 37 °C. These were used to establish the 50% survival level, but no toxicity was evident in the TA100 strain at any of the test levels. DMSO was chosen by the sponsor to be used as the solvent in one concentration (45,000 µg/plate) in the subsequent mutational assays.

b. Preincubation Suspension Assay: In the present study, the sponsor decided to perform the mutational assay without a separate toxicity assay, because toxicity studies with similar paraffinic oils demonstrated that concentrations of approximately 160,000 µg/plate caused no reduction in revertant colonies of TA100 following 20 and 40 minute exposures to the test compound, with or without S-9 activation.

5. Test of Mutagenicity:

a. Plate Incorporation Assay: Five concentrations of 78-9-70 were tested against the 5 Salmonella strains, with and without S-9 activation. Approximately 3 ml of the test substance (2460 mg) were suspended in 2.46 ml of DMSO to achieve approximately 45,000 µg/0.1 ml. This and increasing volumes of the concentrated test compound were used to develop a 5-point dose response curve. To each 2 ml of complete top agar containing 0.1 ml of an overnight broth culture of each bacterial strain, 0.3, 0.2, 0.1 or 0.05 ml of the concentrated compound (corresponding to approximately 240,000, 160,000, 80,000 and 40,000 µg/plate) or 0.1 ml (45,000 µg) of the 30% DMSO solution were added to the reaction mixture. The activated tests included 0.5 ml of the S-9 mixture. After thorough mixing, the contents of each tube were poured onto minimal agar plates in triplicate. Solvent and positive controls were treated similarly. After solidifying, the plates were incubated in a 37°C incubator for 48 hours, and the revertant colonies in the test and control plates counted.

b. Suspension Assay: Three ml (2,477 mg) of the concentrated test substance were suspended in 2.5 ml of 30% DMSO. 0.1 ml (45,000 µg) of this solution was added to overnight broth cultures of the 5 tester strains in 13 x 100 mm test tubes. In addition, 0.3, 0.2, 0.1, or 0.05 ml of concentrated 78-9-70 (corresponding to approximately 240,000, 160,000, 80,000 and 40,000 µg) were added to overnight broth cultures of the 5 Salmonella strains in 13 x 100 mm test tubes. For the activated tests, 0.5 ml of S-9 was added to the bacterial suspension. The tubes were incubated at 37°C for 20 minutes. Two ml of complete top agar was then added to each tube, mixed and poured onto minimal agar plates; each was plated in triplicate. After 48 hours of incubation, the revertants to histidine prototrophy in the test groups were counted and compared with controls.

6. Evaluation Criteria: The Ames assay is a test for

rate at which the compound causes strains of Salmonella histidine auxotrophs to revert to prototrophy. The numbers of histidine revertants in the test groups are compared with the spontaneous mutation rate of the negative controls to determine if the compound causes a dose-dependent increase in the numbers of colonies of the revertant bacteria. At least 3 of the doses tested should be below the level at which toxicity is evident, and the positive controls should fall within their expected ranges.

The mutagenicity test must meet 3 criteria in order to be valid:

- a) The toxicity of the chemical should be demonstrated, unless solubility is limited
- b) Solvent controls must be within the normal range
- c) The test results must be reproducible

If the chemical meets the above criteria, and exhibits a positive dose response over three concentrations with the baseline increase equal to twice the solvent control, then it is considered mutagenic in this system.

RESULTS: Heavy to moderate oil droplets were noted at all concentrations of the test substance, and no toxicity nor mutagenicity was observed in either kind of assay. Although bacterial toxicity was not demonstrated in this study, the limits of solubility of the test compound were reached. The results of the plate incorporation and preincubation/suspension assays, without and with S-9 activation, are summarized in attachment 1 (Tables 1A, 2A, 3A, and 4A, from pages 16-19 of the submission).

DISCUSSION: Although the results of the Ames plate incorporation and liquid suspension assays for potential mutagenic effects by paraffinic oil 78-9-70 indicated that the substance was not mutagenic in the Salmonella strains tested, with or without metabolic activation, the compound was insoluble in the medium at the doses tested (all of which were much greater than the limit dose of 5,000 μ g/plate); therefore, the study is unacceptable. However, it is doubtful that the solubility problems could be overcome with this test compound even at lower doses; therefore, the study does not need to be repeated for regulatory purposes.

ATTACHMENT 1

Table 1A
 Number of revertants/plate following exposure to graded doses
 of 78-9-70 without metabolic activation
Salmonella typhimurium Mammalian Microsome Plate Incorporation

| train | Dose (µg/plate) of 78-9-70 | | | | | | | | | | | | | | |
|-----------|----------------------------|-----|-----|----------------------|-----|-----|---------------------|------|------|---------------------|-----|-----|----------------------|-----|-----|
| | 240,000 ^a | | | 160,000 ^a | | | 80,000 ^a | | | 40,000 ^a | | | 45,000 ^{ab} | | |
| 535 | 24 | 23 | 36 | 33 | 28 | 34 | 26 | 27 | 26 | 28 | 30 | 29 | 29 | 26 | 28 |
| 537 | 6 | 9 | 7 | 4 | 5 | 10 | 4 | 5 | 8 | 9 | 5 | 8 | 8 | 4 | 9 |
| 538 | 14 | 17 | 16 | 18 | 15 | 17 | 9 | 12 | 11 | 8 | 15 | 13 | 11 | 15 | 16 |
| A98 | 27 | 19 | 26 | 24 | 25 | 36 | 22 | 23 | 29 | 30 | 29 | 34 | 34 | 24 | 30 |
| A100 | 224 | 220 | 192 | 213 | 204 | 183 | 190 | 203 | 213 | 189 | 205 | 211 | 191 | 200 | 213 |
| controls: | | | | | | | | | | | | | | | |
| Negative | | | | | | | | | | | | | | | |
| train | Organism | | | DMSO | | | MNNG 5 | | | 2-NF 50 | | | 9-AA 75 | | |
| 535 | 31 | 38 | 32 | 26 | 39 | 39 | 2247 | 2268 | 2334 | | | | | | |
| 537 | 10 | 5 | 6 | 5 | 7 | 8 | | | | 610 | | | 550 525 | | |
| 538 | 14 | 13 | 10 | 12 | 12 | 18 | | | | 1576 1740 1557 | | | | | |
| A98 | 23 | 29 | 19 | 18 | 17 | 17 | | | | 1777 1886 1862 | | | | | |
| A100 | 192 | 179 | 198 | 176 | 187 | 145 | 2912 | 2781 | 2967 | | | | | | |

heavy to moderate oil droplets
 prepared in DMSO

Table 2A
 Number of revertants/plate following exposure to graded doses
 of 78-9-70 with metabolic activation
Salmonella typhimurium Mammalian Microsome Plate Incorporation

| Strain | Dose (µg/plate) of 78-9-70 | | | | | | | | | | | | | | |
|--------|----------------------------|----------------------|---------------------|---------------------|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 240,000 ^a | 160,000 ^a | 80,000 ^a | 40,000 ^a | 45,000ab | | | | | | | | | | |
| 1535 | 17 | 22 | 19 | 20 | 21 | 26 | 24 | 23 | 28 | 26 | 29 | 26 | 23 | 22 | 24 |
| 1537 | 3 | 9 | 6 | 9 | 9 | 6 | 7 | 11 | 9 | 7 | 4 | 8 | 11 | 7 | 9 |
| 1538 | 25 | 28 | 29 | 27 | 22 | 24 | 29 | 28 | 27 | 24 | 22 | 30 | 31 | 30 | 24 |
| TA98 | 28 | 39 | 36 | 51 | 48 | 41 | 45 | 49 | 41 | 38 | 40 | 33 | 38 | 45 | 43 |
| TA100 | 146 | 150 | 158 | 175 | 171 | 146 | 158 | 163 | 181 | 195 | 167 | 168 | 187 | 162 | 175 |

| Strain | Negative | | Positive (µg/plate) | | | | | | | | | |
|--------|----------|---------------|---------------------|--------|-----|-----|-----|-----|-----|------|------|------|
| | Organism | Organism +S-9 | DMSO | 2-AA 5 | | | | | | | | |
| 1535 | 31 | 38 | 32 | 25 | 27 | 27 | 24 | 23 | 26 | 460 | 476 | 460 |
| 1537 | 10 | 5 | 6 | 12 | 9 | 11 | 13 | 14 | 13 | 207 | 174 | 242 |
| 1538 | 14 | 13 | 10 | 28 | 33 | 25 | 25 | 19 | 15 | 2545 | 2468 | 2485 |
| TA98 | 23 | 29 | 19 | 42 | 40 | 31 | 23 | 31 | 24 | 2755 | 2666 | 2511 |
| TA100 | 192 | 179 | 198 | 170 | 179 | 158 | 193 | 169 | 156 | 2428 | 2673 | 2367 |

^a heavy to moderate oil droplets
^b prepared in DMSO

Table 3A
 Number of revertants/plate following 20 minutes preincubation with graded doses
 of 78-9-70 without metabolic activation
Salmonella typhimurium Mammalian Microsome Suspension Assay

| Strain | Dose (µg/plate) of 78-9-70 | | | | | | | | | | | | | | |
|-----------|----------------------------|----------------------|---------------------|---------------------|----------------------|---------------------|------|------|------|-----|-----|------|------|------|-----|
| | 240,000 ^a | 160,000 ^a | 80,000 ^a | 40,000 ^a | 45,000 ^{ab} | | | | | | | | | | |
| 1535 | 23 | 26 | 24 | 16 | 30 | 19 | 20 | 16 | 17 | 23 | 19 | 26 | 22 | 18 | 13 |
| 1537 | 3 | 7 | 3 | 3 | 4 | 8 | 8 | 11 | 4 | 8 | 5 | 6 | 7 | 8 | 7 |
| 1538 | 22 | 12 | 12 | 15 | 17 | 13 | 13 | 17 | 16 | 11 | 15 | 14 | 16 | 16 | 9 |
| TA98 | 24 | 29 | 26 | 24 | 29 | 22 | 20 | 24 | 20 | 19 | 23 | 16 | 18 | 22 | 22 |
| TA100 | 159 | 167 | 192 | 169 | 181 | 214 | 183 | 186 | 208 | 183 | 176 | 181 | 126 | 141 | 130 |
| Controls: | | | | | | | | | | | | | | | |
| Strain | Negative | | | | | Positive (µg/plate) | | | | | | | | | |
| | Organism | 30% DMSO | MNNG 1.25 | 4 NPA 100 | 9-AA 75 | | | | | | | | | | |
| 1535 | 33 | 26 | 24 | 16 | 24 | 18 | 2019 | 2030 | 2135 | | | | | | |
| 1537 | 4 | 7 | 8 | 11 | 6 | 4 | | | | | | 642 | 557 | 541 | |
| 1538 | 13 | 13 | 11 | 11 | 13 | 11 | | | | | | 1616 | 1534 | 1482 | |
| TA98 | 22 | 20 | 22 | 18 | 21 | 18 | | | | | | 1825 | 1663 | 1534 | |
| TA100 | 151 | 171 | 180 | 141 | 135 | 137 | 2484 | 2452 | 2401 | | | | | | |

a heavy to moderate oil droplets
 b prepared in DMSO

Table 4A
 Number of revertants/plate following 20 minutes preincubation with graded doses
 of 78-9-70 in presence of metabolic activation
Salmonella typhimurium Mammalian Microsome Suspension Assay

| Strain | Dose (µg/plate) of 78-9-70 | | | | | | | | | | | | | | |
|--------|----------------------------|----------------------|---------------------|---------------------|----------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 240,000 ^a | 160,000 ^a | 80,000 ^a | 40,000 ^a | 45,000 ^{ab} | | | | | | | | | | |
| 1535 | 12 | 8 | 17 | 15 | 12 | 13 | 12 | 11 | 17 | 12 | 17 | 13 | 16 | 13 | 16 |
| 1537 | 9 | 10 | 9 | 11 | 7 | 8 | 6 | 4 | 5 | 10 | 7 | 10 | 6 | 6 | 4 |
| 1538 | 23 | 35 | 20 | 22 | 26 | 24 | 27 | 22 | 17 | 31 | 31 | 31 | 33 | 29 | 25 |
| TA98 | 35 | 42 | 46 | 46 | 36 | 33 | 35 | 30 | 37 | 29 | 28 | 33 | 45 | 48 | 40 |
| TA100 | 174 | 163 | 168 | 148 | 150 | 173 | 181 | 190 | 174 | 167 | 178 | 153 | 158 | 163 | 133 |

Controls:

| Strain | Negative | | | Positive (µg/plate) | | | | | | | | |
|--------|----------|----------------|----------|---------------------|-------|-----|-----|-----|-----|-----|-----|-----|
| | Organism | Organism + S-9 | 30% DMSO | 2-AA | 0.625 | | | | | | | |
| 1535 | 33 | 26 | 24 | 10 | 14 | 15 | 12 | 19 | 13 | 104 | 99 | 82 |
| 1537 | 4 | 7 | 8 | 3 | 8 | 8 | 9 | 6 | 7 | 52 | 46 | 41 |
| 1538 | 13 | 13 | 11 | 25 | 18 | 21 | 24 | 20 | 20 | 924 | 818 | 911 |
| TA98 | 22 | 20 | 22 | 41 | 36 | 36 | 34 | 29 | 46 | 739 | 729 | 754 |
| TA100 | 151 | 171 | 180 | 139 | 137 | 137 | 130 | 140 | 118 | 843 | 819 | 700 |

^a heavy to moderate oil droplets
^b prepared in DMSO

Reviewed by: Sheryl K. Reilly, Ph.D. *SI42 3-21-94*
 Section II, Tox. Branch I (7509C)
 Secondary Reviewer: Irving Mauer, Ph.D. *J. Mauer*
 Tox Branch I (7509C) *03/21/94*

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity - Mouse Lymphoma L5178Y Forward Mutation Assay

GUIDELINE #: 84-2

TOX. CHEM. #: 646

MRID #: 416853-23

TEST MATERIAL: Paraffinic Oil (78-9-70, SUS/100°F)

SYNONYMS: 78-9-70

STUDY NUMBERS: 596-112

SPONSOR: American Petroleum Institute

TESTING FACILITY: Hazleton Laboratories America, Inc.
 9200 Leesburg Turnpike
 Vienna, Virginia 22180

TITLE OF REPORT: In Vivo and In Vitro Mutagenicity Studies with Paraffinic Oil 78-9-70 SUS/100°F: Mouse Lymphoma Forward Mutational Test

AUTHOR: Hazleton Laboratories America, Inc.

REPORT ISSUED: June 19, 1981

EXECUTIVE SUMMARY: In a mouse lymphoma forward mutation assay, L5178Y heterozygous TK^{+/-} cells were exposed to concentrations of paraffinic oil (78-9-70) equal to 8,670, 17,340, 34,680, 52,020, 69,360, 86,700, 104,040, and 121,380 µg/ml, with and without metabolic activation with rat liver microsomal fraction S-9.

The study is **Unacceptable** under § 84-2 for a mammalian cells in culture forward mutation assay, because although the mutation frequencies increased approximately two-fold at all doses of the test compound in the S-9 activated cultures, there was no dose-relationship and it is not clear if the results were statistically significant, because a statistical test was not described. Further, the study discloses that the test compound could not be effectively removed from the cells after exposure, and thus remained in contact with the cells throughout the rest

of the incubation period. This also indicated that the substance was insoluble in aqueous media; therefore, an assessment of the mutagenicity of paraffinic oil under the conditions used in this test system is not possible. However, it is unlikely that the solubility problems could be overcome, even at lower doses. Therefore, the study does not need to be repeated for regulatory purposes. (MRID 416853-23).

QUALITY ASSURANCE

A statement concerning compliance with good laboratory practices was included in the submission. The study was performed prior to the publication of the requirements of 40 CFR 160, but was conducted under the provisions of 21 CFR 58.

MATERIALS

1. Test Compound: Paraffinic oil, a viscous clear, yellow liquid was the test compound; the purity of the compound was 100%.

2. Cells: L5178Y heterozygous TK^{+/-} mouse lymphoma cells, subline 3.7.2 C, received from Research Triangle Park, N.C. Mutated cells are designated TK^{-/-}.

3. Cell Media:

a. Growth Medium (F₁₀P): Fischer's Medium for Leukemic Cells of Mice, made up to a 1x concentration with sterile deionized, glass distilled water, supplemented with 10% (v/v) horse serum, sodium pyruvate, pluronic F68, and penicillin-streptomycin.

b. Selective Cloning Medium: F₁₀P supplemented with approximately 20% (v/v) horse serum. Warm molten noble agar is added to a final concentration of 0.32% (v/v), and a selective agent (trifluorothymidine, TFT) is incorporated into the medium at a concentration of 2 µg/ml.

c. Nonselective Cloning Medium: as in 3. b), without TFT.

4. Controls:

a. Positive Control, without S-9 activation: Ethyl methane sulfonate (EMS), dissolved in water to a concentration of 100 mg/ml.

b. Positive Control, with S-9 activation: 2-acetylaminofluorene (N-2-fluorenylacetamide; 2-AAF).

c. Negative Control: F₁₀P.

METHODS

1. S-9 Preparation: The metabolic activator, liver homogenate S-9, was prepared from adult male Sprague-Dawley rat livers. The rats had been given single intraperitoneal injections of 200 mg Aroclor 1254/ml corn oil, at dosages of 500 mg/kg, 5 days prior to sacrifice. The livers were removed at sacrifice, weighed, washed with 150 mM KCl solution, and homogenized in 3 volumes of sterile KCl solution. After centrifugation at 9000 x g at 4°C, portions of the supernatant (S-9) were collected and immediately deep-frozen in dry ice, then stored in a freezer. Just before use, 25 ml of the S-9 liver fraction was thawed and mixed with Fischer's Medium (75 ml), NADP (600 mg) and isocitric acid (1125 mg). The optimum S-9 liver fraction concentration was 1:10, diluted with Fischer's medium, based on standardization experiments with 2-AAF.

2. Test for Toxicity: Concentrated 78-9-70 was tested with and without metabolic activation in 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml volumes added to 50 ml centrifuge tubes containing 6×10^6 TK⁺/- cells in 6 ml of F10P medium, with an additional 4 ml of F₁₀P (non-activated cultures) or 4 ml S-9 mix (activated cultures). This resulted in final test compound concentrations of 8,830, 17,600, 35,320, 52,980, 70,640 and 88,300 µg/ml in both activated and non-activated cultures. For the controls, 0.1 ml of F₁₀P (with and without S-9 activation), EMS (without activation), or AAF (with S-9 activation) was added in place of the test compound. Each tube was mixed, gassed with a mixture of CO₂ and air, sealed and incubated 4 hours at 37°C on a roller drum to keep the test compound uniformly dispersed in the medium. Following the 4 hour exposure time, the cells were centrifuged, the treatment solutions decanted, the cells washed twice and resuspended with 20 ml of F₁₀P, gassed and reincubated for 2 days. Cell counts and viability were performed on both days of incubation, using a hemocytometer and trypan blue exclusion. After the first day, all cultures exceeding a viable cell count of 3×10^5 were adjusted to that count, and after 48 hours of incubation, the percent total suspension growth was determined according to the following formula:

$$\frac{\text{Total Cell Growth in Treated Cultures}}{\text{Total Cell Growth in Solvent Control}} \times 100$$

where Total Cell Growth =

$$\frac{\text{Day 2 Cell counts (viable cells/ml)} \times \text{Day 1 cell count}}{\text{Day 1 Cell counts } (\leq 3 \times 10^5)}$$

3. Forward Mutation Assay: Eight increasing volumes (0.1 ml to 1.4 ml) of the concentrated test substance were evaluated in duplicate with and without metabolic activation, resulting in

final test concentrations ranging from 8,670 $\mu\text{g/ml}$ to 121,380 $\mu\text{g/ml}$. The procedure was the same as for the toxicity assay.

After the 2-day incubation period, the cells were centrifuged and resuspended in F_{10}P at a concentration of 5×10^5 viable cells/ml. One ml of this cell suspension was cultured in triplicate on selective medium plates (containing 2 $\mu\text{g/ml}$ TFT), and 100 cells were cloned on 3 nonselective plates for each concentration of the test substance and controls. The cells were cultured for 11 days, and the mutant colonies ($\text{TK}^{-/-}$) were counted on the selective plates, and the survivors ($\text{TK}^{+/-}$ and $\text{TK}^{-/}$) were counted on the nonselective plates.

The mutation frequency was calculated as the number of $\text{TK}^{-/-}$ mutants per 10^5 colony forming cells (CFC) plated onto selective cloning medium/ 1×10^5 . (The number of CFCs plated onto the selective medium is the mean number of colonies on the nonselective cloning plate multiplied by 10^4 .) The mutation frequency of the treated culture was divided by the mutation frequency of the solvent controls to determine the fold-increase of mutations.

4. Statistical Analysis: Not described in the submission.

5. Criteria for Evaluation of a Mouse Lymphoma Assay: A chemical was considered positive if a dose-related response was observed over at least two test concentrations, in which mutation frequencies of the test compound were 2 or 3-fold higher than the mutation frequency of the solvent control. These mutation frequencies would be valued only at levels of 10% or greater total survival of the cells.

RESULTS

The test compound did not appear to be toxic at any of the concentrations tested, except at the 17,660 $\mu\text{g/ml}$ concentration with S-9 activation. The reduction in survival to only 43% at that concentration was not dose-related. A summary of the toxicity results is found in attachment 2 (Table 1B, from page 31 of the submission).

In the forward mutation assay, there was no apparent increase in mutation of L5178Y cells at the TK locus in the non-activated cultures. The mutation frequency in the S-9 activated cultures increased approximately 2 fold (up to 2.7 at the lowest dose) at all concentrations of the test substance, but there was not a dose-related increase in mutation frequency. The percent total survival, mutation frequencies, and fold-increases of the forward mutation test results are summarized in attachment 2 (Table 3B, from page 33 of the submission).

DISCUSSION

Although the mutation frequencies increased approximately 2-fold or greater at all doses of the test compound in the S-9 activated cultures, it is not clear if the small increases in mutation frequencies (compared with the non-activated cultures and the negative controls) observed are statistically significant, particularly if compared with the fold increases observed in the positive (74.2 for EMS, 95 for 2-AAF + S-9) controls, because it does not appear that a statistical test was used in this study. These increases did not appear to be dose-related.

It was noted in the study was that it was not possible to remove all residues of the test compound after the initial 4-hour exposure period, and thus remained in contact with the cells throughout the rest of the incubation period. This indicated that the substance was insoluble in aqueous media; therefore, an assessment of the mutagenicity of paraffinic oil under the conditions used in this test system is not possible. However, it is unlikely that the solubility of the test compound could be improved, even at lower doses; therefore, the study is unacceptable, but does not need to be repeated for registration purposes.

ATTACHMENT 2

Table 1B

Toxicity Results: % Suspension Growth
Compound 78-9-70

| <u>Tube Number</u> | <u>Contents</u> | <u>Percent Suspension Growth</u> |
|--------------------|--|----------------------------------|
| 1 | Media Control | 100% |
| 2 | EMS 620 µg/ml | 22% |
| 3 | 78-9-70 8,830 µg/ml ^a | 111% |
| 4 | 78-9-70 17,660 µg/ml | 121% |
| 5 | 78-9-70 35,320 µg/ml | 110% |
| 6 | 78-9-70 52,980 µg/ml | 122% |
| 7 | 78-9-70 70,640 µg/ml | 97% |
| 8 | 78-9-70 88,300 µg/ml | 79% |
| 9 | Solvent + S-9 | 100% |
| 10 | 2AAF + S-9 50 µg/ml | 56% |
| 11 | 78-9-70 + S-9 8,830 µg/ml ^a | 120% |
| 12 | 78-9-70 + S-9 17,660 µg/ml | 43% |
| 13 | 78-9-70 + S-9 35,320 µg/ml | b |
| 14 | 78-9-70 + S-9 52,980 µg/ml | 97% |
| 15 | 78-9-70 + S-9 70,640 µg/ml | 89% |
| 16 | 78-9-70 + S-9 88,300 µg/ml | 76% |

a = Estimated doses delivered with a glass syringe

b = Pellet lost in wash - no cells

Table 3B

Summary of Results:
House Lymphoma Forward Mutation Assay
Compound: 78-9-70

| Tube No. | Contents | Percent Total Survival | Mutation Frequency | Fold Increase |
|----------|--|------------------------|--------------------|---------------|
| 1 | Solvent | 100% | 6.2 | - |
| 2 | EMS 620 µg/ml | 1% | 46.0 | 74.2 |
| 3 | 78-9-70 8,670 µg/ml ^a | 110% | 5.4 | 0 |
| 4 | 78-9-70 17,340 µg/ml | 134% | 6.6 | 1.1 |
| 5 | 78-9-70 34,680 µg/ml | 102% | 10.1 | 1.6 |
| 6 | 78-9-70 52,020 µg/ml | 83% | 7.9 | 1.3 |
| 7 | 78-9-70 69,360 µg/ml | 93% | 6.3 | 1.0 |
| 8 | 78-9-70 86,700 µg/ml | 85% | 9.5 | 1.5 |
| 9 | 78-9-70 104,040 µg/ml | 81% | 9.4 | 1.5 |
| 10 | 78-9-70 121,380 µg/ml | 87% | 9.6 | 1.5 |
| 11 | Solvent + S-9 | 100% | 6.0 | -- |
| 12 | 2AAF + S-9 50 µg/ml | 13% | 57.0 | 95.0 |
| 13 | 78-9-70 + S-9 8,670 µg/ml ^a | 55% | 16.1 | 2.7 |
| 14 | 78-9-70 + S-9 17,340 µg/ml | 53% | 11.5 | 1.9 |
| 15 | 78-9-70 + S-9 34,680 µg/ml | 52% | 13.3 | 2.2 |
| 16 | 78-8-70 + S-9 52,020 µg/ml | 64% | 10.3 | 1.7 |
| 17 | 78-9-70 + S-9 69,360 µg/ml | 57% | 14.1 | 2.4 |
| 18 | 78-9-70 + S-9 86,700 µg/ml | 53% | 10.6 | - |
| 19 | 78-9-70 + S-9 104,040 µg/ml | 49% | 12.8 | 2.1 |
| 20 | 78-9-70 + S-9 121,380 µg/ml | 70% | 10.1 | 1.7 |
| 21 | Media Control + S-9 | 100% | 8.1 | - |
| 22 | Media Control | 100% | 6.9 | - |

^a = Estimated doses delivered with a glass syringe

Reviewed by: Sheryl K. Reilly, Ph.D.
 Section II, Tox. Branch I (7509C)
 Secondary Reviewer: Irving Mauer, Ph.D.
 Tox Branch I (7509C)

SKR 3-21-94
 J. Lawrence
 03/21/94

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity Study - In Vivo Mammalian
 Cytogenetics - Bone Marrow

GUIDELINE #: 84-2

TOX. CHEM. #: 646

MRID #: 416853-23

TEST MATERIAL: Paraffinic Oil (78-9-70, SUS/100°F)

SYNONYMS: 78-9-70

STUDY NUMBERS: 596-113

SPONSOR: American Petroleum Institute

TESTING FACILITY: Hazleton Laboratories America, Inc.
 9200 Leesburg Turnpike
 Vienna, Virginia 22180

TITLE OF REPORT: In Vivo and In Vitro Mutagenicity Studies
 with Paraffinic Oil 78-9-70 SUS/100°F: In
 vivo Bone Marrow Cytogenetic Assay

AUTHOR: Hazleton Laboratories America, Inc.

REPORT ISSUED: June 19, 1981

EXECUTIVE SUMMARY: In an in vivo, mammalian bone marrow cytogenicity assay, Sprague-Dawley rats of both sexes were exposed to oral doses of paraffinic oil (78-9-70) prepared daily in corn oil at concentrations of 500, 1000 or 2000 mg/kg. The test animals (5 rats of each sex per test and control group) were given a single daily dose of 78-9-90 by gavage for a period of 5 days; the negative controls received corn oil only. The positive control animals received a single intraperitoneal injection of TEM (0.4 mg/kg) on day 5. Colchicine was injected intraperitoneally on day 6 of the study (2 mg/kg) to arrest bone marrow cells in metaphase. The rats were sacrificed 2-3 hours after colchicine was administered, and bone marrow cells collected and prepared for chromosome analysis.

The test compound did not cause clinically toxic effects at any dose tested. There were no statistically significant

differences in weight gain, aberrant cells, or aberrations per cell between the test groups and the negative control animals. The mitotic indices of the test groups were similar to the negative control.

Under the conditions of this study, paraffinic oil 78-9-70 SUS/100°F did not appear to be mutagenic in the albino rat. However, the highest dose tested was not toxic, and a limit dose (5,000 mg/kg) was not tested.

The study is therefore **Unacceptable** under § 84-2 for an in vivo, mammalian bone marrow cytogenicity assay, and must be repeated up to the limit dose (5,000 mg/kg). (MRID 416853-23).

QUALITY ASSURANCE: A statement concerning compliance with good laboratory practices was included in the submission. The study was performed prior to the publication of the requirements of 40 CFR 160, but was conducted under the provisions of 21 CFR 58.

MATERIALS:

1. Test Animals: Sprague-Dawley, CD rats (30/sex), weighing approximately 180-290 g at the start of the experiment, obtained from Charles River Breeding, Inc., Wilmington, MA.
2. Test Compound: Paraffinic oil, a viscous clear, yellow liquid was the test compound; the purity of the compound was 100%.
3. Vehicle: Corn oil.
4. Positive Control: Triethylene melamine (TEM, 0.4 mg/kg).

METHODS:

1. Compound Preparation and Dosing: The test material was prepared daily in corn oil at concentrations of 500, 1000 or 2000 mg/kg. The test animals (5 each sex per group) were given a single daily dose of 78-9-90 by oral gavage for a period of 5 days; the negative controls received corn oil only. The positive control animals received a single intraperitoneal injection of TEM (0.4 mg/kg) on day 5. Colchicine in Hank's Balanced Salt Solution (pH 7.4) was injected intraperitoneally on day 6 of the study at a dose of 2 mg/kg body weight to arrest bone marrow cells in metaphase. The rats were sacrificed via CO₂ asphyxiation 2-3 hours after colchicine was administered.
2. Clinical Observations: The general appearance, behavior, toxicity and pharmacological effects were recorded daily. Body weights were measured on Day 1 and Day 6.

3. Preparation of Slides: Bone marrow cells were collected from both femurs of each rat immediately after sacrifice, by aspirating the cells into a 12 ml syringe filled with 5 ml of warm (37°C) Hank's Balanced Salt Solution. The cells were transferred to a 10 ml vacutainer tube and centrifuged 5 minutes (1200-1500 rpm). The supernatant was discarded, and 5 ml of warm 0.75M KCl added to the pellet. Carnoy's fixative (methanol:acetic acid, 3:1) was added 25 minutes later, the tubes capped and the contents mixed then centrifuged 5 minutes (1200-1500 rpm). The supernatant was discarded and 5 ml of Carnoy's fixative added slowly down the sides of each tube. The cells were resuspended and centrifuged 5 minutes. This fixing process was repeated twice, then the tubes sealed and refrigerated overnight (4°C).

After centrifuging 5 minutes, the cells were suspended in 1-3 ml fresh Carnoy's fixative, and 3 drops of the fixed cells placed on clean glass microscope slides and allowed to air dry. Three slides were prepared in this manner per rat. The slides were stained with Giemsa, coverslipped and examined by a person not involved in the preparation of the slides.

Fifty metaphase-stage cells were examined from each rat for cytogenetic abnormalities. The mitotic index was recorded for each animal as the number of cells in mitosis/100 cells counted.

4. Classification of Cytogenetic Aberrations: The metaphase chromosomes were examined microscopically for chromatid breaks, chromosome breaks, and markers (dicentric, exchanges, rings, deletions, fragments, minutes and acentric fragments). Those cells with greater than 10 aberrations were classified as severely damaged.

5. Statistical Analysis: The mean changes in pre- and postexposure body weights and the mean mitotic index were analyzed using Bartlett's test for equality of variance, the one-way ANOVA, Scheffe's multiple F test procedure, and the Games and Howell procedure for comparison of group means. The total number of aberrant cells and total aberrations per animals for each test group were compared using Wilcoxon's nonparametric test of group means. Significance was established at $p \leq 0.05$.

RESULTS

1. Clinical Observations, Mortality and Body Weight: There were no clinical signs of toxicity or fatalities in this study. There were reportedly no significant differences in weight gain or loss between the test groups and the negative control (ANOVA, $p > 0.05$). Positive controls were not included in this analysis, since they were treated under different conditions (i.p. administration of TEM on day 5). Mean initial and terminal body weights are presented below.

| Test Group | Initial Body Weight (g) | Terminal Body Weight (g) | Change in Body Weight (g) |
|------------------|-------------------------|--------------------------|---------------------------|
| Negative Control | 245.7 | 285.0 | 9.3 |
| 500 mg/kg | 267.7 | 281.2 | 13.6 |
| 1000 mg/kg | 269.1 | 282.4 | 13.3 |
| 2000 mg/kg | 279.3 | 289.2 | 9.9 |

2. Cytogenic Analysis: Bone marrow samples from at least one animals in groups 1, 2, and 4 were not considered for evaluation, due to technical problems in sampling. The numbers of cells/sex/group analyzed were as follows:

| Test Group | # Cells Counted/# males | # Cells Counted/ # females |
|------------------|-------------------------|----------------------------|
| Negative Control | 250/5 | 150/3 |
| Positive Control | 200/4 | 200/4 |
| 500 mg/kg | 250/5 | 250/5 |
| 1000 mg/kg | 200/4 | 250/5 |
| 2000 mg/kg | 250/5 | 250/5 |

The test substance did not significantly increase the number of aberrant cells and aberrations/cell at any concentration tested in this system (Wilcoxon test to compare group means of total aberrant cells and total aberrations per animal). A comparison of the frequencies of aberrations and percentage of aberrant cells according to group is summarized in attachment 3 (Tables 7C, 8C and 9C, from pages 61-63 of the submission).

Formulas used in calculations:

$$\text{percentage of aberrant cells/group} = \frac{\text{Total \# aberrant cells per group}}{\text{Total \# cells observed}}$$

$$\text{average number of aberrations per cell} = \frac{\text{Total \# aberrations}}{\text{total \# cells observed}}$$

$$\text{fold-increase in aberrations/cell} =$$

$$\frac{\text{Avg. \# Aberrations/cell (test group)}}{\text{Avg. \# Aberrations/cell (negative control)}}$$

3. Mitotic Index: The mean mitotic index ± s.d. for each group was calculated at the number of cells undergoing mitosis/100 cells counted. When analyzed by ANOVA, the

differences between the test groups and negative control were not significant. The positive control (TEM) group had significantly lower mitotic indices. These values are shown in the following table:

| Test Group | Mean Mitotic Index \pm standard deviation |
|------------------|---|
| Negative Control | 4.38 \pm 1.92 |
| Positive Control | 2.88 \pm 1.24 |
| 500 mg/kg | 3.40 \pm 1.78 |
| 1000 mg/kg | 3.33 \pm 1.50 |
| 2000 mg/kg | 3.20 \pm 1.69 |

4. Photographic Documentation: a minimum of 10 photographs of bone marrow cells were taken from each animal that yielded cells which could be analyzed. Only 4 representative photographs of each group were submitted. From these photographs, a single chromatid break was observed in the 500 and 2,000 mg/kg groups. Multiple aberrations were observed in all of the photographs of the positive control, and none were observed in the negative control.

DISCUSSION

The test compound did not cause toxic effects at oral doses of 500, 1,000 or 2,000 mg/kg given to rats of both sexes each day for 5 days. There was no statistically significant differences in weight gain, aberrant cells, or aberrations per cell between the test groups and the negative control animals. The mitotic indices of the test groups were similar to the negative control. Under the conditions of this study, paraffinic oil 78-9-70 SUS/100°F did not appear to be mutagenic in the albino rat at the concentrations tested. However, the highest dose tested was not toxic, and a limit dose (5,000 mg/kg) was not tested. Therefore, this study is unacceptable, and must be repeated up to the limit dose.

As an alternative to repeating the study by oral administration, the test substance could be injected (e.g., intraperitoneally) at (only) the highest dosage feasible, in order to assure exposure of the target tissue, and its possible response (cytotoxic and/or clastogenic), as prevailed for the mutagen, TEM, employed as the positive control in this study.

ATTACHMENT 3

Table 7C

Comparison of Frequencies of Chromosomal Aberrations in Bone Marrow Cells from Groups of Animals Treated With 78-9-70 and Controls

| Groups | Total No. Of Cells Analyzed | % Aberrant Cells ^a | Average No. Of Aberrations/Cell ^b | Fold Increase of Aberrations/Cell ^c |
|------------------------------------|-----------------------------|-------------------------------|--|--|
| 1) Negative Control (Corn Oil) | 400 | 4.3 | 0.045 | - |
| 2) Positive Control (.4 mg/kg TEM) | 400 | 31.3 | 1.44 | 32 |
| 3) Low Dose 78-9-70 500 mg/kg | 500 | 3.8 | 0.048 | 1.1 |
| 4) Mid Dose 78-9-70 1000 mg/kg | 450 | 2.0 | 0.024 | < 1 |
| 5) High Dose 78-9-70 2000 mg/kg | 450 | 2.8 | 0.032 | < 1 |

^a This number represents the total number of aberrant cells/group divided by the total number of cells analyzed per group x 100.

^b This number represents the total number of aberrations/group divided by the total number of cells analyzed/group.

^c This number represents the average aberrations/cell for the test groups divided by the value obtained for the negative control (0.045 ab./cell).

Table 8C

Wilcoxon Nonparametric Comparison of Group Means of the Total Number of Aberrant Cells from Bone Marrow of Rats Treated With 78-9-70, Positive or Negative Control Substances

| Groups Compared* | Average Rank | Calculated Test Statistic | Tabled Value | Results |
|--------------------|--------------|---------------------------|--------------|-----------------|
| 1 and 2 | 4.5, 12.5 | T = 36.0 | 49 | Significant |
| 1 and 3 | 10.2, 9.0 | T = 70.5 | 53 | Not Significant |
| 1 and 4 | 11.2, 7.1 | T = 54.5 | 51 | Not Significant |
| 1 and 5 | 10.9, 8.4 | T = 65.0 | 53 | Not Significant |

- * 1 = Negative Control (Corn Oil)
 2 = Positive Control (TEM)
 3 = 500 mg/kg 78-9-70
 4 = 1000 mg/kg 78-9-70
 5 = 2000 mg/kg 78-9-70

Table 9C

Wilcoxon Nonparametric Comparison of Group Means of the Total
Number of Aberrations from Bone Marrow of Rats Treated With
78-9-70, Positive or Negative Control Substances

| Groups Compared* | Average Rank | Calculated Test Statistic | Tabled Value | Results |
|------------------|--------------|---------------------------|--------------|-----------------|
| 1 and 2 | 4.5, 12.5 | T = 36 | 49 | Significant |
| 1 and 3 | 9.8, 9.3 | T = 74.0 | 53 | Not Significant |
| 1 and 4 | 10.9, 7.3 | T = 57.0 | 51 | Not Significant |
| 1 and 5 | 11.1, 8.3 | T = 63.5 | 53 | Not Significant |

* 1 = Negative Control (Corn Oil)

2 = Positive Control (TEM)

3 = 500 mg/kg 78-9-70

4 = 1000 mg/kg 78-9-70

5 = 2000 mg/kg 78-9-70