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Monsanto



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MONSANTO POLYMER PRODUCTS CO.
800 N. Lindbergh Boulevard
St. Louis, Missouri 63167
Phone: (314) 894-1000

December 17, 1984

IR-432
MTS

Dr. Louis Borghi
Senior Scientist
Industrial Chemical Information Section
Dynamac Corporation
The Dynamac Building
11140 Rockville Pike
Rockville, MD 20852

Contains No CB

Dear Dr. Borghi:

Enclosed are the studies you requested on THIOTAX® accelerator (MTS, CAS number 120-78-5), including:

1. Health effects (Acute toxicity, skin and eye irritation, mouse lymphoma and microbial mutagenicity, and skin patch).
2. Environmental effects (acute toxicity to fathead minnow, bluegill, rainbow trout, algae).
3. A time-independent aquatic study using rainbow trout, which you did not request, but which may be of value.

We are glad to be able to help in this matter, Please call if you have any questions.

Sincerely,

Bernard J. Hill
Product Safety Manager
Rubber Chemicals

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94 JUL 14 AM 9:31

Enclosure

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15.341/SS.1

E C & G, Bionomics
 Aquatic Toxicology Laboratory
 790 Main Street
 Wareham, Massachusetts
 December, 1976

Test Material: Thiofide (NH07-013) CP-1612
 Description: Light yellow-colored powder
 Report No.: _____ Other Codes: BN-76-170
 Submitted by: Dr. James Laveglia, Monsanto Company, St. Louis, Missouri

**ACUTE (96-HOUR) TOXICITY OF THIOFIDE
 TO RAINBOW TROUT AND BLUEGILL**

PROCEDURES: Rainbow trout and bluegill toxicity testing procedures are on the reverse side.

SUMMARY: LC50* and 95% confidence interval (mg/l)

| <u>Rainbow trout</u> | | <u>Bluegill</u> | |
|----------------------|-------------|-----------------|---------------|
| 24-hour | 85 (69-105) | 24-hour | 199 (165-240) |
| 48-hour | 74 (54-101) | 48-hour | 123 (93-162) |
| 96-hour | 66 (53-82) | 96-hour | 82 (55-121) |

*LC50 = The concentration which is lethal to 50% of a population of test organisms during the specified time period.

RESULTS:

| <u>Nominal test concentration (mg/l)</u> | <u>Rainbow trout</u> Observed percentage mortality | | |
|--|--|----------------|----------------|
| | <u>24-hour</u> | <u>48-hour</u> | <u>96-hour</u> |
| | 140 | 100 | 100 |
| 100 | 70 | 100 | 100 |
| 75 | 40 | 40 | 40 |
| 56 | 0 | 0 | 0 |
| 42 | 0 | 0 | 10 |
| 32 | 0 | 0 | 0 |
| control (acetone) | 0 | 0 | 0 |
| control | 0 | 0 | 0 |

| <u>Nominal test concentration (mg/l)</u> | <u>Bluegill</u> Observed percentage mortality | | |
|--|---|----------------|----------------|
| | <u>24-hour</u> | <u>48-hour</u> | <u>96-hour</u> |
| | 320 | 100 | 100 |
| 240 | 90 | 100 | 100 |
| 180 | 40 | 70 | 100 |
| 140 | 0 | 10 | 50 |
| 100 | 0 | 10 | 10 |
| 75 | 0 | 10 | 30 |
| 56 | 0 | 10 | 10 |
| 42 | 0 | 0 | 10 |
| 32 | 0 | 0 | 20 |
| 24 | 0 | 0 | 10 |
| 18 | 0 | 0 | 0 |
| control (acetone) | 0 | 0 | 0 |
| control | 0 | 0 | 0 |

PROCEDURES:

Testing was conducted according to the protocol submitted to Monsanto Company in October, 1976.

Rainbow trout: The test material, in reagent-grade acetone, was introduced into 15 l of diluent water in all-glass vessels. To each test vessel, 10 rainbow trout (*Salmo gairdneri*, 3.7 cm standard length) were then added. Fish were not fed 48 hours prior to testing, nor during exposure. No aeration was provided during the test, and temperature was maintained at $12 \pm 1.0^{\circ}\text{C}$. Dissolved oxygen ranged from 9.9 mg/l (93% of saturation) to 2.0 mg/l (19% of saturation) at the beginning and end of exposure, respectively. pH values ranged from 7.5 initially to 6.8 at the end of the test. Observations and mortality counts were made every 24 hours during a 96-hour period following the initiation of exposure.

Bluegill: The test material, in reagent-grade acetone, was introduced into 15 l of diluent water in all-glass vessels. To each test vessel, 10 bluegill (*Lepomis macrochirus*, 3.8 cm standard length) were then added. Fish were not fed 48 hours prior to testing, nor during exposure. No aeration was provided during the test, and temperature was maintained at $22 \pm 1.0^{\circ}\text{C}$. Dissolved oxygen ranged from 9.2 mg/l (90% of saturation) to 0.2 mg/l (2% of saturation) at the beginning and end of exposure, respectively. pH values ranged from 7.4 initially to 6.7 at the end of the test. Observations and mortality counts were made every 24 hours during a 96-hour period following the initiation of exposure.

STATISTICS:

Test concentrations and observed percentage mortality were converted to logarithms and probits, respectively, and these values were utilized in a least squares regression analysis. The LC50's and the 95% confidence intervals were calculated from the regression equation.

Reported by:

Robert J. Succafusco

Robert J. Succafusco
Aquatic Biologist

Approved by:

Robert E. Bentley

Robert E. Bentley
Aquatic Toxicologist

PAF FISH TOXICITY -

Rubber Chemicals
Division

DEPT. OF MEDICINE & ENVIRONMENTAL HEALTH

J. Lavaglia, A2SC

DATE: February 10, 1977

cc:

SUBJECT: Results of acute aquatic toxicity studies
with ~~various~~ rubber chemicals

REFERENCE: BN-76-167 through BN-76-174
BN-76-252 through BN-76-261
BN-76-263 through BN-76-267
BN-76-269 through BN-76-270

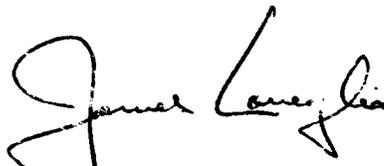
TO P.R. Graham *
B23L

The accompanying reports contain the results obtained from the acute screening studies with the following compounds:

| <u>Test Number</u> | <u>CP Number</u> | <u>Name</u> |
|--------------------|------------------|--------------------------------------|
| BN-76-167 | 1548 | Benzothiazole |
| " " 168 | 1601 | KaMBT, 50% |
| " " 169 | 1975 | Thiotax |
| " " 170 | 1612 | Thiofide |
| " " 171 | 118 | Santocure |
| " " 172 | 15255 | Santocure MOR |
| " " 173 | 6910 | Santocure NS |
| " " 174 | 26144 | NIBS |
| " " 252 | 29242 | Sanogard PVI |
| " " 253 | 4668 | Phthalimide |
| " " 254 | 25477 | Santoflex 77 |
| " " 255 | 20408 | Santoflex IF |
| " " 256 | 22423 | Santoflex 13 |
| " " 257 | 4060 | Santoflex AW |
| " " 258 | 26658 | Santoflex 14 |
| " " 259 | 17127 | Santoflex DD |
| " " 260 | 307 | Sulfasan R |
| " " 261 | 2181 | Santovar A |
| " " 263 | 27017 | Santowhite MK |
| " " 264 | 1815 | Santowhite crystals |
| " " 265 | FA 1915 | Santonox R crystals |
| " " 266 | 15575 | Vocol |
| " " 267 | 61957 | N-(cyclohexylthio)trimethylacetamide |
| " " 269 | 38849 | N-(cyclohexylthio)benzamide |
| " " 270 | 38852 | N-(cyclohexylthio)acetamide |

(cont.)

| <u>CP Number</u> | <u>Rainbow trout 96-hour LC50 (mg/l)</u> | <u>Bluegill 96-hour LC50 (mg/l)</u> | <u>Toxicity</u> |
|------------------|--|---|---|
| 1543 | 26 ¹⁶ | 18 ¹⁸ | relatively low order of toxicity |
| 1601 | 1.8 ¹¹ | 3.8 ¹² | mildly toxic |
| 1975 | 0.75 ⁷ | 1.5 ⁸ | relatively high order of toxicity to trout, mildly toxic to bluegill |
| 1612 | 66 ²¹ | 82 ²⁵ | relatively low order of toxicity |
| 118 | 5.4 ¹⁴ | 7.9 ¹⁶ | mildly toxic |
| 15255 | 1.3 ⁸ | 4.4 ¹⁴ | mildly toxic |
| 6910 | 1.6 ⁹ | 1.2 ⁷ | mildly toxic |
| 26144 | 7.4 | 5.6 ¹⁵ | mildly toxic |
| 29242 | 0.41 ⁵ | 1.2 ⁷ | relatively high order of toxicity to trout, mildly toxic to bluegill |
| 4668 | 51 ¹⁰ | 53 ²² | relatively low order of toxicity |
| 25477 | 32 ¹¹ | 182 ²⁷ | relatively low order of toxicity |
| 20408 | 0.34 ⁴ | 0.43 ⁴ | relatively high order of toxicity |
| 22423 | 0.14 ² | 0.40 ² | relatively high order of toxicity |
| 4060 | 5.7 ¹⁵ | 3.4 ¹¹ | mildly toxic |
| 26658 | 0.49 ⁶ | 0.42 ³ | relatively high order of toxicity |
| 17127 | 40 ¹⁴ | 38 ²¹ | relatively low order of toxicity |
| 307 | 1.8 ¹¹ | 1.6 ⁹ | mildly toxic |
| 2181 | 40 ¹⁴ | 34 ²⁰ | relatively low order of toxicity |
| 27017 | 2.4 ¹² | 4.2 ¹³ | mildly toxic |
| 1815 | 0.13 ¹ | 0.51 ⁵ | relatively high order of toxicity |
| FA 1815 | 0.16 ³ | 0.24 ¹ | relatively high order of toxicity |
| 15575 | 1.6 ⁹ | 32 ¹⁴ | mildly toxic to trout, relatively low order of toxicity to bluegill |
| 61957 | 43 ¹⁴ | 14 ¹⁷ | relatively low order of toxicity |
| 38849 | 2.8 ¹³ | 3.0 ¹⁰ | mildly toxic |
| 38852 | 1.7 ¹⁰ | 0.76 ⁶ | mildly toxic to trout, relatively high order of toxicity to bluegill |


 James Laveglia

/bkb

*rec. reports

Department of Medicine & Environmental Health - J. Laveglia, A2SC

DATE: December 16, 1976

cc:

SUBJECT: Microbial Mutagenicity Evaluation
of Rubber Chemicals

REFERENCE: BIO-76-175 through BIO-76-182

TO

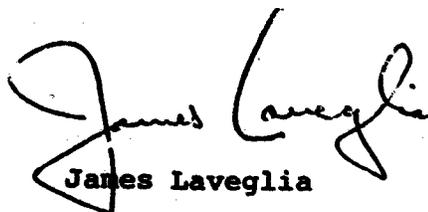
P. R. ...
B2BL

Microbial mutagenic plate assays were conducted with the following materials:

| <u>CP Number</u> | <u>Compound</u> | <u>Test Number</u> |
|------------------|-----------------|--------------------|
| 1548 | Benzothiazole | BIO-76-175 |
| 1601 | NAMBT-50% | BIO-76-176 |
| 1975 | Phiotax | BIO-76-177 |
| 1612 | Thiofide | BIO-76-178 |
| 118 | Santocure | BIO-76-179 |
| 15255 | Santocure MOR | BIO-76-180 |
| 6910 | Santocure NS | BIO-76-181 |
| 26144 | NIAS | BIO-76-182 |

Each material was evaluated at five dose levels by both nonactivation and rat liver microsome activation conditions. The test organisms included five strains of the bacteria Salmonella typhimurium (TA-98, TA-100, TA-1535, TA-1537, and TA-1538) and one strain of the yeast Saccharomyces cerevisiae (D4).

None of these compounds demonstrated mutagenic activity when tested by the standard plate assay technique.


James Laveglia

/bkp

*rec. reports (8)

Dr. James P. Laveglia, Public Health

October 6, 1976

Dr. David Brusick
Litton-Bionetics
5516 Nicholson Lane
Kensington, MT 20795

re: BIO-76-175: CP 1548 (Benzothiazole)
BIO-76-176: CP 1601 (MPT-505)
BIO-76-177: CP 1975 (THICTAX)
BIO-76-178: CP 1612 (THIOFIDE)
BIO-76-179: CP 118 (SANTOCURE)
BIO-76-180: CP 15255 (SANTOCURE MOR)
BIO-76-181: CP 6910 (SANTOCURE NS)
BIO-76-182: CF 26144 (NIBS)

Dear Dr. Brusick:

Under separate cover you will receive samples of the eight referenced materials. We would like you to conduct microbial plate assays with and without the addition of mammalian metabolic activation preparations using the five Salmonella strains and the yeast Saccharomyces.

The stated cost for these studies was \$300 each for a total of \$2400. Please prepare separate reports for each of the eight materials.

Very truly yours,

James Laveglia, PhD
Senior Toxicologist*

/bkp
cc: P. R. Graham
M. Thompson

MUTAGENICITY EVALUATION

OF

BIO-76-178
CPT6T2 THIOFIDE

11/24/74

FINAL REPORT

SUBMITTED TO

MONSANTO
800 N. LINDBERGH BOULEVARD
ST. LOUIS, MISSOURI 63166

SUBMITTED BY

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 2683

NOVEMBER 24, 1976



BIONETICS

Litton

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SPONSOR: Monsanto

MATERIAL: BIO-76-178 CP1612 Thiofide

SUBJECT: FINAL REPORT MUTAGENICITY PLATE ASSAY

1. OBJECTIVE

The objective of this study was to evaluate the test compound for genetic activity in microbial assays with and without the addition of mammalian metabolic activation preparations.

2. MATERIALS

A. Test Compound

1. Date Received: October 12, 1976
2. Description: Beige powder

B. Indicator Microorganisms

Salmonella typhimurium, strains: TA-1535 TA-98
TA-1537 TA-100
TA-1538

Saccharomyces cerevisiae, strain: D4

C. Activation System (Ames et al., Mutation Research 31:347, 1975)

1. Reaction Mixture

| <u>Component</u> | <u>Final Concentration/ml</u> |
|--|---|
| TPN | 4 μ moles |
| Glucose-6-phosphate | 5 μ moles |
| Sodium phosphate (diabasic) | 100 μ moles |
| MgCl ₂ | 8 μ moles |
| KCl | 33 μ moles |
| Homogenate fraction equivalent to 25 mg of wet tissue | 0.1-0.15 ml 9,000 x g supernatant of rat liver |

2. S-9 Homogenate

A 9,000 x g supernatant was prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 five days prior to kill.



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2. MATERIALS (Continued)

D. Positive Control Chemicals

Table 1 below lists the chemicals used for positive controls in the nonactivation and activation assays.

TABLE 1

| <u>ASSAY</u> | <u>CHEMICAL</u> ^a | <u>SOLVENT</u> | <u>PROBABLE MUTAGENIC SPECIFICITY</u> |
|---------------|-------------------------------|--------------------------------|---------------------------------------|
| Nonactivation | Methylnitrosoguanidine (MNNG) | Water or Saline | BPS ^b |
| | 2-Nitrofluorene (NF) | Dimethylsulfoxide ^c | FS ^b |
| | Quinacrine mustard (QM) | Water or saline | FS ^b |
| Activation | 2-Anthramine (ANTH) | Dimethylsulfoxide ^c | BPS ^b |
| | 2-Acetylaminofluorene (AAF) | Dimethylsulfoxide ^c | FS ^b |
| | 8-Aminoquinoline (AMQ) | Dimethylsulfoxide ^c | FS ^b |

^aConcentrations given in Results Section

^bBPS = Base-pair substitution

FS = Frameshift

^cPreviously shown to be nonmutagenic

E. Solvent

Either deionized water or dimethylsulfoxide (DMSO) was used to prepare stock solutions of solid materials. All dilutions of test materials were made in either deionized water or DMSO. The solvent employed and its concentration are recorded in the Results Section.

The material is...
the product...
but it is not...
source of...
in formal...
may be...
ment in St. Louis...
toxicity are to be...
ment in St. Louis for...



3. EXPERIMENTAL DESIGN

A. Plate Test (Overlay Method*)

Approximately 10^8 cells from an overnight culture of each indicator strain were added to separate test tubes containing 2.0 ml of molten agar supplemented with biotin and a trace of histidine. For non-activation tests, the four dose levels of the test compound were added to the contents of the appropriate tubes and poured over the surfaces of selective agar plates. In activation tests four dose levels of the test chemical were added to the appropriate tubes with cells. Just prior to pouring, an aliquot of reaction mixture (0.5 ml containing the 9,000 x g liver homogenate) was added to each of the activation overlay tubes, which were then mixed, and the contents poured over the surface of a minimal agar plate and allowed to solidify. The plates were incubated for 48 hours at 37C, and scored for the number of colonies growing on each plate. The concentrations of all chemicals are given in the Results Section. Positive and solvent controls using both directly active positive chemicals and those that require metabolic activation were run with each assay.

B. Recording and Presenting Data

The numbers of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and the solvent controls are provided as reference points. Other relevant data are provided on the computer printout.

*Certain classes of chemicals known to be mutagens and carcinogens do not produce detectable responses using the standard Ames overlay method. Some dialkyl nitrosamines and certain substituted hydrazines are mutagenic in suspension assays, but not in the plate assay. Chemicals of these classes should be screened in a suspension assay.



4a. SUMMARY OF PLATE TEST RESULTS

LITTON BIOMETICS, INC.

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: B10-76-170 CP 1612 THIOFIDE
 B. SOLVENT: DMSO
 C. TEST DATE: OCT. 27, 1976
 NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) PER PLATE.

| TEST | SPECIES | ISSUE | B E Y E R L A N D S P E R P L A T E | | | | | | | | | | | | |
|----------------------|---------|-------|-------------------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|-----|
| | | | 1A-1535 | 1A-1537 | 1A-1538 | 1A-1539 | 1A-1540 | 1A-1541 | 1A-1542 | 1A-1543 | 1A-1544 | 1A-1545 | 1A-1546 | 1A-1547 | |
| NONACTIVATION | | | | | | | | | | | | | | | |
| SOLVENT CONTROL | --- | --- | 24 | 20 | 12 | 52 | 226 | 11 | --- | --- | --- | --- | --- | --- | --- |
| POSITIVE CONTROL** | --- | --- | >1000 | >1000 | >1000 | >1000 | 510 | 12 | --- | --- | --- | --- | --- | --- | --- |
| TEST COMPOUND | --- | --- | 10 | 13 | 12 | 54 | 184 | 12 | --- | --- | --- | --- | --- | --- | --- |
| 0.10000 UG | --- | --- | 11 | 22 | 11 | 63 | 221 | 11 | --- | --- | --- | --- | --- | --- | --- |
| 1.00000 UG | --- | --- | 12 | 9 | 13 | 52 | 195 | 13 | --- | --- | --- | --- | --- | --- | --- |
| 10.00000 UG | --- | --- | 15 | 16 | 7 | 50 | 224 | 7 | --- | --- | --- | --- | --- | --- | --- |
| 100.00000 UG | --- | --- | 14 | 21 | 4 | 49 | 245 | 4 | --- | --- | --- | --- | --- | --- | --- |
| 500.00000 UG | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ACTIVATION | | | | | | | | | | | | | | | |
| SOLVENT CONTROL | RAT | LIVER | 27 | 32 | 17 | 66 | 284 | 17 | --- | --- | --- | --- | --- | --- | --- |
| POSITIVE CONTROL*** | RAT | LIVER | 312 | 555 | >1000 | >1000 | 904 | >1000 | --- | --- | --- | --- | --- | --- | --- |
| TEST COMPOUND | RAT | LIVER | 55 | 33 | 45 | 60 | 262 | 45 | --- | --- | --- | --- | --- | --- | --- |
| 0.10000 UG | RAT | LIVER | 27 | 20 | 25 | 64 | 276 | 25 | --- | --- | --- | --- | --- | --- | --- |
| 1.00000 UG | RAT | LIVER | 27 | 36 | 25 | 70 | 304 | 36 | --- | --- | --- | --- | --- | --- | --- |
| 10.00000 UG | RAT | LIVER | 25 | 28 | 20 | 77 | 275 | 28 | --- | --- | --- | --- | --- | --- | --- |
| 100.00000 UG | RAT | LIVER | 33 | 34 | 11 | 73 | 307 | 34 | --- | --- | --- | --- | --- | --- | --- |
| 500.00000 UG | RAT | LIVER | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |

* IBY. CONVERTANTS PER PLATE

| | | | | | |
|---------|------|--------------|---------|------|--------------|
| 1A-1535 | MWNG | 10 UG/PLATE | 1A-1535 | ANTH | 100 UG/PLATE |
| 1A-1537 | OH | 10 UG/PLATE | 1A-1537 | OH | 100 UG/PLATE |
| 1A-1538 | WF | 100 UG/PLATE | 1A-1538 | CAF | 100 UG/PLATE |
| 1A-1539 | WF | 100 UG/PLATE | 1A-1539 | CAF | 100 UG/PLATE |
| 1A-1540 | MWNG | 10 UG/PLATE | 1A-1540 | ANTH | 100 UG/PLATE |
| 1A-1541 | MWNG | 10 UG/PLATE | 1A-1541 | CAF | 100 UG/PLATE |
| 1A-1542 | MWNG | 10 UG/PLATE | 1A-1542 | CAF | 100 UG/PLATE |
| 1A-1543 | MWNG | 10 UG/PLATE | 1A-1543 | CAF | 100 UG/PLATE |
| 1A-1544 | MWNG | 10 UG/PLATE | 1A-1544 | CAF | 100 UG/PLATE |
| 1A-1545 | MWNG | 10 UG/PLATE | 1A-1545 | CAF | 100 UG/PLATE |
| 1A-1546 | MWNG | 10 UG/PLATE | 1A-1546 | CAF | 100 UG/PLATE |
| 1A-1547 | MWNG | 10 UG/PLATE | 1A-1547 | CAF | 100 UG/PLATE |

** SOLVENT: DMSO 2.5 U/PLATE

5. INTERPRETATION OF RESULTS AND CONCLUSIONS

The test compound was examined for mutagenic activity in a series of in vitro microbial assays employing Salmonella and Saccharomyces indicator organisms. The compound was tested directly and in the presence of liver microsomal enzyme preparations from Arochlor-induced rats. The following results were obtained:

A. Toxicity

The compound was tested over a series of concentrations such that there was either quantitative or qualitative evidence of some chemically induced physiological effects at the high dose level. The low dose in all cases was below a concentration that demonstrated any toxic effect. The dose range employed for the evaluation of this compound was from 0.1 μg to 500 μg per plate.

B. Nonactivation Test Results

The results of the tests conducted on the compound in the absence of a metabolic system were all negative.

C. Activation Test Results

The results of the tests conducted on the compound in the presence of the rat liver activation system were all negative.

D. Conclusions

The test compound, BIO-76-178 CP 1612 Thiofide, did not demonstrate mutagenic activity in any of the assays conducted in this evaluation and was considered not mutagenic under these test conditions.

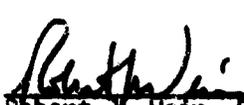
Submitted by:



David J. Brusick, Ph.D.
Director
Department of Genetics

Date

Reviewed by:



Robert J. Weir, Ph.D.
Vice President

11/24/76

Date



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6. EXPLANATION OF EVALUATION PROCEDURES FOR PLATE ASSAYS

Plate test data consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test chemical and the cells are incubated in the overlay for 2 to 3 days, and a few cell divisions occur during the incubation period, the test is semi-quantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replicating DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the compound and the cells in the overlay permits constant exposure of the indicator cells for 2 to 3 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test chemical, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol normally employs several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose Response Phenomena

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test chemical may kill any mutants that are induced, and the compound will not appear to be mutagenic.



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6. EXPLANATION OF EVALUATION PROCEDURES FOR PLATE ASSAYS (Continued)

C. Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays. Negative controls consist of the test compound solvent in the overlay agar together with the other essential components. The negative control plate for each strain gives a reference point to which the test data are compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

D. Evaluation Criteria for Ames Assay

Because the procedures used to evaluate the mutagenicity of the test chemical are semiquantitative, the criteria used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets are evaluated using the following criteria:

1. Strains TA-1535, TA-1537, and TA-1538

If the solvent control value is within the normal range, a chemical that produces a positive dose response over three concentrations with the lowest increase equal to twice the solvent control value is considered to be mutagenic.

2. Strains TA-98, TA-100, and D4

If the solvent control value is within the normal range, a chemical that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-100 and two to three times the solvent control value for strains TA-98 and D4 is considered to be mutagenic. For these strains, the dose response increase should start at approximately the solvent control value.

3. Pattern

Because TA-1535 and TA-100 were both derived from the same parental strain (G-46) and because TA-1538 and TA-98 were both derived from the same parental strain (E3052), there is a built-in redundancy in the microbial assay. In general the two strains of a set respond to the same mutagen and such a pattern is sought. It is also anticipated that if a

6. EVALUATION OF EVALUATION PROCEDURES FOR PLATE ASSAYS (Continued)

D. Evaluation Criteria for Ames Assay

3. Pattern

given strain, e.g. TA-1537, responds to a mutagen in nonactivation tests it will generally do so in activation tests. (The converse of this relationship is not expected.) While similar response patterns are not required for all mutagens, they can be used to enhance the reliability of an evaluation decision.

4. Reproducibility

If a chemical produces a response in a single test that cannot be reproduced in one or more additional runs, the initial positive test data loses significance.

The preceding criteria are not absolute and other extenuating factors may enter into a final evaluation decision. However, these criteria are applied to the majority of situations and are presented to aid those individuals not familiar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relationship Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/microsome test is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relationships have been demonstrated between these two end points. The results of comparative tests on 300 chemicals by McCann et al. (Proc. Nat. Acad. Sci. USA, 72:5135-5139, 1975) show an extremely good correlation between results of microbial mutagenesis tests and in vivo rodent carcinogenesis assays.

All evaluation and interpretation of the data presented in this report are based only on the demonstration of or lack of mutagenic activity.

STANDARD OPERATING PROCEDURES

To ensure an accurate and reliable mutagenicity testing program, LBI instituted the following procedures:

- The test compound was registered in a bound log book recording the date of receipt, complete client identification, physical description and LBI code number.
- Complete records of weights and dilutions associated with the testing of the submitted material were entered into a bound notebook.
- Raw data information was recorded on special printed forms that were dated and initialed by the individual performing the data collection at the time the observations were made. These forms were filed as permanent records.
- All animal tissue S-9 preparations used in the activation tests were taken from dated and pretested frozen lots identified by a unique number. The S-9 preparations were monitored for uniformity and the information recorded.



BIONETICS

DATE: April 11, 1979
SUBJECT: Mutagenicity Testing of THIOFIDE
REFERENCE: BO-78-239

TO: P. R. Graham *
B2SL

The referenced report has been received and reviewed.

In this study, THIOFIDE was evaluated for mutagenic activity in the mouse lymphoma assay. This assay evaluates materials for specific locus forward mutation induction in L5178Y TK (Thymidine Kinase) mouse lymphoma cells. THIOFIDE was tested both with and without a metabolic activation system in the assay.

The test material did not induce significant mutagenic activity at the TK locus. THIOFIDE was evaluated as being non-mutagenic under the conditions of this assay.

M.W. Stevens

/bks

*rec. report

To: Judy Luder

Judy - please note, record and file appropriately

YF

P26

LBI ASSAY NO. 3578

MUTAGENICITY EVALUATION OF

THIOFIDE 80-78-239

IN THE
MOUSE LYMPHOMA FORWARD
MUTATION ASSAY

2/7*

FINAL REPORT

SUBMITTED TO:

MONSANTO COMPANY
800 NORTH LINDBERGH BOULEVARD
ST. LOUIS, MISSOURI 63166

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 20989

REPORT DATE: MARCH 1979



BIONETICS

Litton

PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I-IX. Items I-IV provide sponsor and compound identification information, type of assay, and the protocol reference number. All protocol references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V provides the initiation and completion dates for the study, and Item VI provides identification of supervisory personnel. Item VII identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation itself is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report, entitled PROTOCOL, describes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices. The evaluation criteria are included to acquaint the sponsor with the methods used to develop and analyze the test results.

All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington Maryland, 20795.

Copies of raw data will be supplied to the sponsor upon request.



BIONETICS

- I. **SPONSOR:** Monsanto Company
- II. **MATERIAL (TEST COMPOUND):** LBI ASSAY NUMBER #3578
 - A. **Identification:** Thiofide B0-78-239
 - B. **Date Received:** September 6, 1978
 - C. **Physical Description:** Cream-colored powder
- III. **TYPE OF ASSAY:** Mouse Lymphoma Forward Mutation Assay
- IV. **PROTOCOL NUMBER:** 431 (DMT-106)
- V. **STUDY DATES:**
 - A. **Initiation:** October 12, 1978
 - B. **Completion:** February 20, 1979
- VI. **SUPERVISORY PERSONNEL:**
 - A. **Study Director:** Brian Myhr, Ph.D.
 - B. **Laboratory Supervisor:** Marie McKeon

VII. **RESULTS:**

The data are presented in Tables 1-4 on pages 4-7.

VIII. **INTERPRETATION OF RESULTS:**

The test material, Thiofide B0-78-239, was insoluble in water at high concentrations and was placed in DMSO at a concentration of 333 mg/ml for the preliminary cytotoxicity test. The material was not completely soluble and formed a fine, milky-white suspension even at the lower stock concentrations (5 mg/ml to 50 mg/ml) used for mutation experiments. Dilutions were performed with DMSO prior to final 1:100 dilutions into growth medium to obtain an applied concentration range of 3333 µg/ml to 6.5 µg/ml. Concentrations of 6.5 µg/ml and 13 µg/ml were toxic and 26 µg/ml was completely lethal (without activation) to the cells within 24 hours of treatment. Therefore, the mutation assay was initiated with a series of applied concentrations from 50 µg/ml to 0.1 µg/ml.

Five trials of the mutation assay were initiated because of technical deficiencies and variability in the toxicity of the test material to the mouse lymphoma cells. The results of four assays are presented in Tables 1 to 4, in sequence of performance. The unreported assay was invalidated by an unacceptably low cloning efficiency of 43% for the negative controls.

VIII. INTERPRETATION OF RESULTS: (continued)

Under nonactivation conditions, the mutant frequencies in the treated cultures were all comparable to the background frequency (average of the solvent and untreated negative control values) in the first trial (Table 1). A 2.5-fold increase over the background frequency is considered necessary to demonstrate mutagenic activity at any given dose level. This assay was marred by the lack of mutagenic activity for the EMS positive control (which appeared to be the result of hydrolytic decomposition, since little toxicity was observed), and the assay needed to be extended to more toxic treatments with the test material in order to adequately test for the absence of mutagenic activity.

In the nonactivation trials shown in Tables 2, 3, and 4, treatments causing high toxicity (minimum percent relative growth values of 5.3%, 2.6%, and 12.0%, respectively) did not induce mutant frequencies significantly elevated over the concurrent background frequencies. Several trials were performed because the toxicity in the second trial (Table 2) was much greater than in Trial 1; the additional trials showed further variability in the toxicity associated with a given dose. Within each trial the toxicity increased regularly with the applied concentration, so the variability between trials may represent a variable sensitivity of different stock cultures to the test material or an unknown variation in the actual applied concentrations (resulting from variable solubility in DMSO and growth medium). Thus, the applied concentrations causing about 2% - 5% relative growth varied from 50 $\mu\text{g/ml}$ (Table 3) to 12.5 $\mu\text{g/ml}$ (Table 2), and 1.56 $\mu\text{g/ml}$ was nontoxic in Table 4 but caused 38.1% relative growth in Table 2. Nevertheless, the test material was consistently inactive as a mutagen over the range of concentrations that caused a wide range of cytotoxicities.

In the presence of the S9 microsomal activation mix, the toxicity of the test material was reduced, and the first mutation assay (Table 1) showed no significant changes in the mutant frequencies for nontoxic treatments with 50 $\mu\text{g/ml}$ to 3.13 $\mu\text{g/ml}$. In the second trial (Table 2), 50 $\mu\text{g/ml}$ was toxic (34.8% relative growth). At 75 $\mu\text{g/ml}$, the percent relative growth was only 13%, but the mutant frequencies in all the treated cultures remained comparable to the background. In the two additional trials, highly toxic treatments corresponding to about 7% relative growth were obtained with 100 $\mu\text{g/ml}$ (Table 3) and 50 $\mu\text{g/ml}$ (Table 4). The mutant frequencies remained comparable to the concurrent backgrounds. [An elevated mutant frequency (6-fold increase) was observed in Table 3 for an extremely toxic treatment with 150 $\mu\text{g/ml}$ (0.6% relative growth). This result is of questionable reliability because the relative cloning efficiency of 13.8% was so low. Mutation assays are not normally completed for such extreme treatments--this was performed by technical oversight--so little or no significance should be attached to any mutagenic activity appearing under these conditions.]



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VIII. INTERPRETATION OF RESULTS. (continued)

The validity of the mutagenesis assays can be assessed by the results obtained for the positive and negative controls. The average cloning efficiencies for the solvent and untreated negative controls varied from 54% (Table 3) to 82% (Table 4) for nonactivation and from 59% (Table 3) to 88% (Table 4) for activation, which demonstrates acceptable to excellent culturing conditions for the assays. The background frequencies were all within the normal range, and the positive control compounds yielded frequencies greatly in excess of the backgrounds (except for EMS in Table 1). The EMS-induced mutant frequencies in Tables 2 and 3 and the DMN-induced frequency in Table 3 were all higher than the usual upper values of about 800×10^{-6} . The treatments were more toxic than usual, which would induce more mutants, and the low relative cloning efficiencies probably artificially inflated the observed frequencies.

IX. CONCLUSIONS:

The test material, Thiocide B0-78-239, did not induce an increase in mutations at the TK locus in L5178Y mouse lymphoma cells for applied concentrations of 0.39 $\mu\text{g/ml}$ to 50 $\mu\text{g/ml}$ without activation and for 3.13 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$ with microsomal activation. These concentration ranges included highly toxic treatments (less than 10% relative growth).

Therefore, the test material is considered to be inactive in the Mouse Lymphoma Forward Mutation Assay.

Submitted by:

Study Director

Brian Myhr 3-19-79
Brian Myhr, Ph.D. date
Section Chief
Mammalian Genetics
Department of Genetics
and Cell Biology

Reviewed by:

David J. Brusick 3/19/79
David J. Brusick, Ph.D. date
Director
Department of Genetics
and Cell Biology



BIONETICS

4. SUMMARY OF MOUSE LYMPHOMA INDUCTION RESULTS

TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: INTOPIDE RD-78-239
 B. CRT CODE #: 3578
 C. SOLVENT: DIMETHYL SULFOXIDE
 D. TEST DATE: 11/13/78

| TEST | SOURCE | ISSUE | DAILY COUNTS | | | RELATIVE SUSPENSION GROWTH % OF CONTROL | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES | RELATIVE CLONING EFFICIENCY (% OF CONTROL) | PERCENT RELATIVE GROUPS | MUTANT FREQUENCY ¹⁰⁰ X 10 ⁶ CELLS |
|-------------------|--------|-------|--------------|------|-------|---|---------------------|---------------------|--|-------------------------|---|
| | | | 1 | 2 | 3 | | | | | | |
| INDUCTION | | | | | | | | | | | |
| SOLVENT CONTROL | --- | --- | 13.4 | 8.0 | 100.0 | 20.0 | 156.0 | 100.0 | 100.0 | 12.8 | |
| SOLVENT CONTROL | --- | --- | 18.6 | 9.4 | 100.0 | 32.0 | 240.0 | 100.0 | 100.0 | 13.3 | |
| UNTREATED CONTROL | --- | --- | 17.0 | 13.7 | 139.4 | 23.0 | 131.0 | 66.2 | 92.2 | 17.6 | |
| EMS .5 UG/ML | --- | --- | 13.4 | 7.2 | 59.9 | 42.0 | 193.0 | 97.5 | 58.4 | 21.8 | |
| TEST COMPOUND | --- | --- | 12.0 | 8.2 | 61.1 | 14.0 + | 266.0 | 134.3 | 82.1 | 5.3 | |
| 1.260 UG/ML | --- | --- | 9.8 | 16.0 | 97.4 | 34.0 | 172.0 | 86.9 | 84.6 | 19.8 | |
| 3.130 UG/ML | --- | --- | 9.2 | 8.8 | 50.3 | 47.0 | 235.0 | 118.7 | 59.7 | 20.0 | |
| 6.250 UG/ML | --- | --- | 5.0 | 12.2 | 43.9 | 27.0 | 213.0 | 107.6 | 47.3 | 12.7 | |
| 12.500 UG/ML | --- | --- | 6.0 | 7.8 | 29.1 | 37.0 | 168.0 | 84.8 | 24.7 | 22.0 | |
| 25.000 UG/ML | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | |
| ACTIVATION | | | | | | | | | | | |
| SOLVENT CONTROL | RAT | LIVER | 10.6 | 6.0 | 100.0 | 49.0 | 306.0 | 100.0 | 100.0 | 16.0 | |
| SOLVENT CONTROL | RAT | LIVER | 6.2 | 11.2 | 100.0 | 41.0 | 222.0 | 100.0 | 100.0 | 18.5 | |
| UNTREATED CONTROL | RAT | LIVER | 17.6 | 13.2 | 369.2 | 36.0 | 164.0 | 62.1 | 217.0 | 22.0 | |
| DMN .3 UG/ML | RAT | LIVER | 11.0 | 10.6 | 188.0 | 528.0 + | 181.0 | 68.6 | 128.9 | 291.7 | |
| TEST COMPOUND | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | |
| 3.130 UG/ML | RAT | LIVER | 13.6 | 11.0 | 224.9 | 48.0 | 196.0 | 74.2 | 167.0 | 24.5 | |
| 6.250 UG/ML | RAT | LIVER | 13.6 | 9.4 | 192.2 | 41.0 | 261.0 | 91.3 | 175.4 | 17.0 | |
| 12.500 UG/ML | RAT | LIVER | 11.0 | 14.4 | 255.4 | 39.0 | 180.0 | 68.2 | 174.2 | 21.7 | |
| 25.000 UG/ML | RAT | LIVER | 14.4 | 7.4 | 160.2 | 42.0 | 285.0 | 108.0 | 172.9 | 14.7 | |
| 50.000 UG/ML | RAT | LIVER | 11.0 | 7.0 | 115.8 | 50.0 + | 206.0 | 78.0 | 90.3 | 24.3 | |

¹⁰⁰ RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY / 100
¹⁰⁰ THE RATIO OF CELLS SELED FOR MUTANT SELECTION TO CELLS SELED FOR CLONING EFFICIENCY IS 100+4.
 THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES) * 10E-4.
 THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.

+ = ONE PLATE CONTAMINATED; VALUE BASED ON REMAINING TWO PLATES.

1. SUMMARY OF MUTANT FREQUENCY RESULTS

TABLE 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: THIOFIDE BU-70-239
 W. LSI CODE: 0578
 C. SOLVENT: DIMETHYL SULFOXIDE
 D. TEST DATE: 12/05/78

1.1. SOURCE: ISSUES 1, 2, 3
 S-9 DAILY CONCENTRATIONS

| SUBJECT | TEST COMPOUND | SOURCE | ISSUE | CELLS/PLATE | DAILY CONCENTRATION | SUSPENSION GROWTH % OF CONTROL | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES (S.D. CONTROL) | RELATIVE CLONING EFFICIENCY (% OF CONTROL) | PERCENT RELATIVE GROWTH | MUTANT FREQUENCY (X 10 ⁻⁶) |
|---------|---------------|--------|-------|-------------|---------------------|--------------------------------|---------------------|------------------------------------|--|-------------------------|--|
| | | | | | | | | | | | |
| S-9 | CONTROL | --- | --- | 20.4 | 12.4 | 100.0 | 31.0 | 146.0 | 100.0 | 100.0 | 21.2 |
| | CONTROL | --- | --- | 18.0 | 13.0 | 100.0 | 41.0 | 131.0 | 100.0 | 100.0 | 31.3 |
| | CONTROL | --- | --- | 15.4 | 10.4 | 65.8 | 32.0 | 220.0 | 150.8 | 101.5 | 19.5 |
| | CONTROL | --- | --- | 10.0 | 9.2 | 37.8 | 444.0+ | 30.0 | 27.4 | 10.4 | 1100.4 |
| S-9 | 0.750 UG/ML | --- | --- | 8.4 | 7.0 | 24.1 | 36.0 | 252.0+ | 181.9 | 43.9 | 43.3 |
| | 1.500 UG/ML | --- | --- | 6.4 | 6.2 | 16.3 | 38.0 | 324.0+ | 231.9 | 30.1 | 11.7 |
| | 6.250 UG/ML | --- | --- | 4.2 | 2.6 | 5.2 | 17.0+ | 299.0 | 215.9 | 11.2 | 5.7 |
| | 12.500 UG/ML | --- | --- | 3.0++ | 0.6 | 4.7 | 25.0 | 156.0 | 112.6 | 5.3 | 16.0 |
| S-9 | CONTROL | RAT | LIVER | 6.2 | 18.2 | 100.0 | 63.0 | 194.0 | 100.0 | 100.0 | 32.5 |
| | | RAT | LIVER | 4.0 | 21.4 | 100.0 | 58.0 | 217.0 | 100.0 | 100.0 | 26.7 |
| | | RAT | LIVER | 6.2 | 19.4 | 122.5 | 50.0 | 200.0 | 97.3 | 119.2 | 25.0 |
| | | RAT | LIVER | 6.8 | 6.0 | 41.1 | 207.0 | 71.0 | 34.5 | 14.2 | 291.5 |
| | RAT | LIVER | 9.4 | 13.0 | 123.2 | 72.0 | 191.0 | 92.9 | 114.5 | 37.7 | |
| | RAT | LIVER | 6.6 | 17.4 | 117.1 | 67.0 | 146.0 | 70.1 | 92.0 | 46.5 | |
| | RAT | LIVER | 7.0 | 8.4 | 60.7 | 69.0 | 197.0 | 95.9 | 58.2 | 39.0 | |
| | RAT | LIVER | 3.6 | 12.0 | 36.3 | 86.0+ | 197.0 | 95.9 | 74.8 | 43.2 | |
| RAT | LIVER | 4.0 | 6.2 | 25.0 | 52.0 | 107.0 | 52.1 | 13.0 | 48.6 | | |

RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY / 100
 THE PATH OF CELLS SEEDING FOR MUTANT SELECTION TO CELLS SEEDING FOR CLONING EFFICIENCY IS 10F++.
 THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.

+ = ONE PLATE CONTAMINATED; VALUE BASED ON REMAINING TWO PLATES.

++ = DUE TO TOXICITY, CULTURE NOT SPLIT BACK TO 3.0 x 10⁶ CELLS; 380 CELLS RATHER THAN 300 CELLS USED FOR VIABLE CLONES -- VALUES FOR RELATIVE CLONING EFFICIENCY AND PERCENT RELATIVE GROWTH WERE ADJUSTED ACCORDINGLY.

5. SUBJURY UE MOUSE LYMPHOMA ASSAY RESULTS

TABLE 3

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: THIOFIDE 80-70-239
 B. LOT CODE #: 3578
 C. SOLVENT: DIMETHYL SULFOXIDE
 D. TEST DATE: 01/10/79

ICSI S-9 SOURCE ISSUE CELLS/ML X 10⁵ 1 2 3

| TEST COMPOUND | SOURCE | ISSUE | DAILY COUNTS | | | RELATIVE SUSPENSION GROWTH (% DE-CONTROL) | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES | RELATIVE CLONING EFFICIENCY (% DE-CONTROL) | PERCENT RELATIVE GROWTH | MUTANT FREQUENCY** |
|--------------------|--------|-------|--------------|-------|-------|---|---------------------|---------------------|--|-------------------------|--------------------|
| | | | 1 | 2 | 3 | | | | | | |
| BUNALIVIAID | | | | | | | | | | | |
| SOLVENT CONTROL | --- | --- | 16.4 | 6.4 | 100.0 | 46.0 | 196.0 | 100.0 | 100.0 | 23.5 | |
| SOLVENT CONTROL | --- | --- | 17.0 | 6.0 | 100.0 | 41.0 | 171.0 | 100.0 | 100.0 | 24.0 | |
| UNTREATED CONTROL | --- | --- | 14.0 | 8.8 | 141.5 | 45.0 | 119.0 | 64.9 | 91.7 | 37.8 | |
| EAS .5 UG/ML | --- | --- | 10.0 | 5.6 | 64.3 | 566.0 | 28.0 | 15.3 | 9.8 | 2021.4 | |
| TEST COMPOUND | --- | --- | 11.8 | 8.8 | 119.2 | 31.0 | 242.0 | 131.9 | 157.3 | 12.8 | |
| 0.300 UG/ML | --- | --- | 6.2 | 6.4 | 45.6 | 31.0 | 268.0 | 146.0 | 66.6 | 14.6 | |
| 3.130 UG/ML | --- | --- | 2.0 | 4.0 | 13.8 | 26.0 | 348.0 | 189.6 | 26.1 | 7.5 | |
| 12.500 UG/ML | --- | --- | 1.8 | 4.2 | 14.5 | 23.0 | 256.0 | 139.5 | 20.2 | 9.0 | |
| 25.000 UG/ML | --- | --- | 1.8 | 2.2++ | 7.6 | 17.0 | 64.0 | 34.9 | 2.6 | 26.6 | |
| 50.000 UG/ML | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | |
| ALIVIAID | | | | | | | | | | | |
| SOLVENT CONTROL | RAT | LIVER | 10.4 | 11.8 | 100.0 | 66.0 | 229.0 | 100.0 | 100.0 | 28.8 | |
| SOLVENT CONTROL | RAT | LIVER | 12.8 | 11.6 | 100.0 | 46.0 | 161.0 | 100.0 | 100.0 | 28.6 | |
| UNTREATED CONTROL | RAT | LIVER | 13.2 | 9.6 | 93.5 | 36.0 | 141.0 | 72.3 | 67.6 | 25.5 | |
| DMN .3 UG/ML | RAT | LIVER | 9.8 | 4.0 | 28.9 | 115.0 | 8.0 | 4.1 | 1.2 | 1437.5 | |
| TEST COMPOUND | RAT | LIVER | 9.6 | 13.0 | 92.0 | 55.0 | 166.0 | 85.1 | 78.3 | 33.1 | |
| 3.130 UG/ML | RAT | LIVER | 6.8 | 10.0 | 50.1 | 42.0 | 233.0 | 119.5 | 59.9 | 18.0 | |
| 25.000 UG/ML | RAT | LIVER | 4.0 | 6.0 | 17.7 | 66.0 | 224.0 | 114.9 | 20.3 | 29.5 | |
| 50.000 UG/ML | RAT | LIVER | 4.2 | 5.2 | 16.1 | 68.0 | 265.0 | 135.9 | 21.9 | 25.7 | |
| 100.000 UG/ML | RAT | LIVER | 2.4 | 4.8 | 10.6 | 37.0 | 124.0 | 63.6 | 6.8 | 29.8 | |
| 150.000 UG/ML | RAT | LIVER | 0.2 | 2.0++ | 4.4 | 45.0 | 27.0 | 13.8 | 0.6 | 166.7 | |

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
 ** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4.
 THE PICTURE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-4.
 THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.

++ = DUE TO TOXICITY, LESS THAN 3.0 X 10⁶ CELLS WERE AVAILABLE AT TIME OF CLONING; LESS THAN 300 CELLS WERE USED FOR VIABLE CLONES -- THE VALUES FOR RELATIVE CLONING EFFICIENCY AND PERCENT RELATIVE GROWTH WERE ADJUSTED ACCORDINGLY.

4. SUMMARY OF MOUSE LYMPHOMA-CELLS CULTURE RESULTS

TABLE 4

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: THIOFIDE BN-78-239
 B. LOT CODE #: 3578
 C. SOLVENT: DIMETHYL SULFOXIDE
 D. TEST DATE: 02/07/79

TEST 5-9 SOURCE TISSUE CELLS/DONOR X 10E5 1 2 3

DEGRADATION

| SOLVENT CONTROL | SOLVENT CONTROL | UNTHAWED CONTROL | THAWED CONTROL | TEST COMPOUND | 1 | 2 | 3 | RELATIVE SUSPENSION GROWTH % OF CONTROL | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES | RELATIVE CLONING EFFICIENCY % OF CONTROL | PERCENT RELATIVE CLONING | MUTANT FREQUENCY (10E-6) |
|-----------------|-----------------|------------------|----------------|---------------|------|------|-----|---|---------------------|---------------------|--|--------------------------|--------------------------|
| --- | --- | --- | --- | 1.560 UG/ML | 13.0 | 10.2 | --- | 100.0 | 117.0 | 244.0 | 100.0 | 100.0 | 40.0 |
| --- | --- | --- | --- | 3.130 UG/ML | 13.2 | 8.2 | --- | 100.0 | 44.0 | 223.0 | 100.0 | 100.0 | 19.7 |
| --- | --- | --- | --- | 6.250 UG/ML | 9.6 | 21.6 | --- | 173.3 | 56.0 | 272.0 | 116.5 | 201.0 | 20.6 |
| --- | --- | --- | --- | 12.500 UG/ML | 7.2 | 5.6 | --- | 37.6 | 671.0 | 104.0 | 44.5 | 16.7 | 643.2 |
| --- | --- | --- | --- | 25.000 UG/ML | 8.2 | 20.8 | --- | 159.1 | 47.0 | 245.0 | 104.9 | 166.9 | 19.2 |
| --- | --- | --- | --- | --- | 6.6 | 16.4 | --- | 101.0 | 51.0 | 175.0 | 74.9 | 75.7 | 29.1 |
| --- | --- | --- | --- | --- | 3.4 | 14.4 | --- | 40.3 | 45.0 | 276.0 | 118.2 | 47.6 | 18.1 |
| --- | --- | --- | --- | --- | 1.4 | 6.2 | --- | 17.3 | 70.0 | 167.0 | 71.5 | 12.4 | 44.9 |
| --- | --- | --- | --- | --- | 1.2 | 5.2 | --- | 14.5 | 57.0 | 192.0 | 82.2 | 12.0 | 21.7 |

ACTIVATION

| SOLVENT CONTROL | SOLVENT CONTROL | UNTHAWED CONTROL | THAWED CONTROL | TEST COMPOUND | 1 | 2 | 3 | RELATIVE SUSPENSION GROWTH % OF CONTROL | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES | RELATIVE CLONING EFFICIENCY % OF CONTROL | PERCENT RELATIVE CLONING | MUTANT FREQUENCY (10E-6) |
|-----------------|-----------------|------------------|----------------|---------------|------|------|-----|---|---------------------|---------------------|--|--------------------------|--------------------------|
| --- | --- | --- | --- | 3.130 UG/ML | 10.2 | 18.8 | --- | 100.0 | 58.0 | 273.0 | 100.0 | 100.0 | 21.2 |
| --- | --- | --- | --- | 6.250 UG/ML | 9.4 | 14.8 | --- | 100.0 | 80.0 | 309.0 | 100.0 | 100.0 | 25.9 |
| --- | --- | --- | --- | 12.500 UG/ML | 9.8 | 13.4 | --- | 79.4 | 65.0 | 207.0 | 71.1 | 56.5 | 31.4 |
| --- | --- | --- | --- | 25.000 UG/ML | 7.2 | 4.8 | --- | 20.9 | 353.0 | 100.0 | 34.4 | 7.2 | 353.0 |
| --- | --- | --- | --- | --- | 8.6 | 22.8 | --- | 118.5 | 69.0 | 303.0 | 104.1 | 123.4 | 24.8 |
| --- | --- | --- | --- | --- | 5.6 | 14.2 | --- | 48.1 | 83.0 | 308.0 | 105.8 | 50.9 | 28.9 |
| --- | --- | --- | --- | --- | 7.8 | 8.8 | --- | 41.5 | 66.0 | 254.0 | 87.3 | 36.2 | 26.0 |
| --- | --- | --- | --- | --- | 6.4 | 10.6 | --- | 41.0 | 75.0 | 147.0 | 50.5 | 20.7 | 51.0 |
| --- | --- | --- | --- | --- | 3.4 | 6.0 | --- | 10.9 | 114.0 | 196.0 | 67.4 | 7.1 | 60.7 |

* RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY / 100
 ** THE PERCENT OF CELLS SEEDING FOR MUTANT SELECTION TO CELLS SEEDING FOR CLONING EFFICIENCY IS 10E+4.
 *** THE PERCENT OF MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES) X 10E+4.
 THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.

PROTOCOL NO. 431

1. OBJECTIVE

The objective of this study is to evaluate the test material for its ability to induce forward mutation in the L5178Y TK+/- mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU).

2. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as BrdU is included in the growth medium, the analog will be phosphorylated via the TK pathway and be incorporated into DNA, eventually resulting in cellular death. Cells which are heterozygous at the TK locus (TK+/-) may undergo a single step forward mutation to the TK -/- genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by de novo synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK-/- mutants is the lack of any ability to utilize toxic analogs of thymidine, which enables only the TK-/- mutants to grow in the presence of BrdU. Cells which grow to form colonies in the presence of BrdU are therefore assumed to have mutated, either spontaneously or by the action of a test substance, to the TK-/- genotype.

3. MATERIALS

A. Indicator Cells

The mouse lymphoma cell line, L5178Y TK+/-, used in this assay is derived from the Fischer L5178Y line of Dr. Donald Clive. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK-/- mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK-/- phenotype (exposure to methotrexate) and are then returned to normal growth medium for three or more days before use.

B. Media

The cells are maintained in Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium is cloning medium containing 50 or 100 µg/ml of BrdU.



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3. MATERIALS (continued)

C. Control Compounds

1. Negative Controls

A negative control consisting of assay procedures performed on untreated cells is performed in all cases. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are also assayed as the solvent negative control to determine any effects on survival or mutation caused by the solvent alone. For test substances assayed with activation, the untreated and solvent negative controls will include the activation mixture.

2. Positive Controls

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and will be used at 0.5 $\mu\text{l/ml}$ as a positive control for nonactivation studies.

Dimethylnitrosamine (DMN) requires metabolic activation by microsomal enzymes to become mutagenic and will be used at 0.3 $\mu\text{l/ml}$ as a positive control for assays performed with activation.

D. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

4. EXPERIMENTAL DESIGN

A. Dosage Selection (Cytotoxicity testing)

The solubility of the test chemical in growth medium and/or DMSO is first determined. Then a wide range of chemical concentrations is tested for cytotoxicity, starting with a maximum applied dose of 10 mg/ml for test chemicals soluble in media or 1 mg/ml for solutions in organic solvents. After an exposure time of four hours, the cells are washed and a viable cell count is obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of untreated cells are used to select seven to ten doses that cover the range from 0 to 50-90% reduction in 24-hour growth. These selected doses are subsequently applied to cell cultures prepared for mutagenicity testing, but only four or five of the doses will be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of four or five doses spaced from 0 to 50-90% reduction in cell growth.



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B. Mutagenicity Testing

1. Nonactivation Assay

The procedure used is based on that reported by Clive and Spector (1975) and is summarized as follows. Cultures exposed to the test chemical for four hours at the preselected doses are washed and placed in growth medium for two or three days to allow recovery, growth and expression of the induced TK-/- phenotype. Cell counts are determined daily and appropriate dilutions are made to allow optimal growth rates.

At the end of the expression period, 3×10^6 cells for each selected dose are seeded in soft agar plates with selection medium and resistant (mutant) colonies are counted after 10 days incubation. To determine the actual number of cells capable of forming colonies, a portion of the cell suspension is also cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number is the mutant frequency.

A detailed flow diagram for the mutation assay is provided in Figure 1.

2. Activation Assay

The activation assay can be run concurrently with the nonactivation assay. The only difference is the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension are: 2.4 mg NADP/ml; 4.5 mg isocitric acid/ml; and 50 μ l S9/ml.

C. Preparation of 9,000 x g Supernatant (S9)

Fischer 344 male rats are normally used as the source of hepatic microsomes. Induction with Aroclor 1254 or other agents is performed by injections five days prior to sacrifice. After decapitation and bleeding, the liver is immediately dissected from the animal using aseptic technique and placed in ice cold 0.25M sucrose buffered with Tris at pH 7.4. When an adequate number of livers is obtained, the collection is washed twice with fresh buffered sucrose and completely homogenized. The homogenate is centrifuged for 10 minutes at 9,000 x g in a refrigerated centrifuge and the supernatant (S9) from this centrifuged sample is retained and frozen at -80°C until used in the activation system. The S9 fraction may be obtained from induced or noninduced rats or other species, as requested.



BIONETICS

EVALUATION CRITERIA

A compound is considered mutagenic in this assay if:

- A dose-response relationship is observed over 3 of the 5 dose levels employed.
- The minimum increase at the low level of the dose-response curve is at least 2.5 times greater than the solvent and/or negative control values.
- The solvent and negative control data are within the normal range of the spontaneous background for the TK locus.

All evaluations of mutagenic activity are based on consideration of the concurrent solvent and negative control values run with the experiment in question. Positive control values are not used as reference points, but are included to ensure that the current cell population responds to direct and promutagens under the appropriate treatment conditions.

Occasionally, a single point within a concentration range will show an increase 2.5 times greater than the spontaneous background. If the increase is at the high dose, is reproducible, and if an additional higher dose level is not feasible because of toxicity, the chemical can be considered mutagenic. If the increase is internal within the dose range and is not reproducible, the increase will normally be considered aberrant. If the internal increase is reproducible, several doses clustered around the positive concentration will be examined to either confirm or reject the reliability of the effect.

As the data base on the assay increases, the evaluation criteria can be expected to become more firmly established.

5. REPORT

The screened doses, cell counts, and mutant and viable colony counts will be entered into a computer program. The results are analyzed and printed.

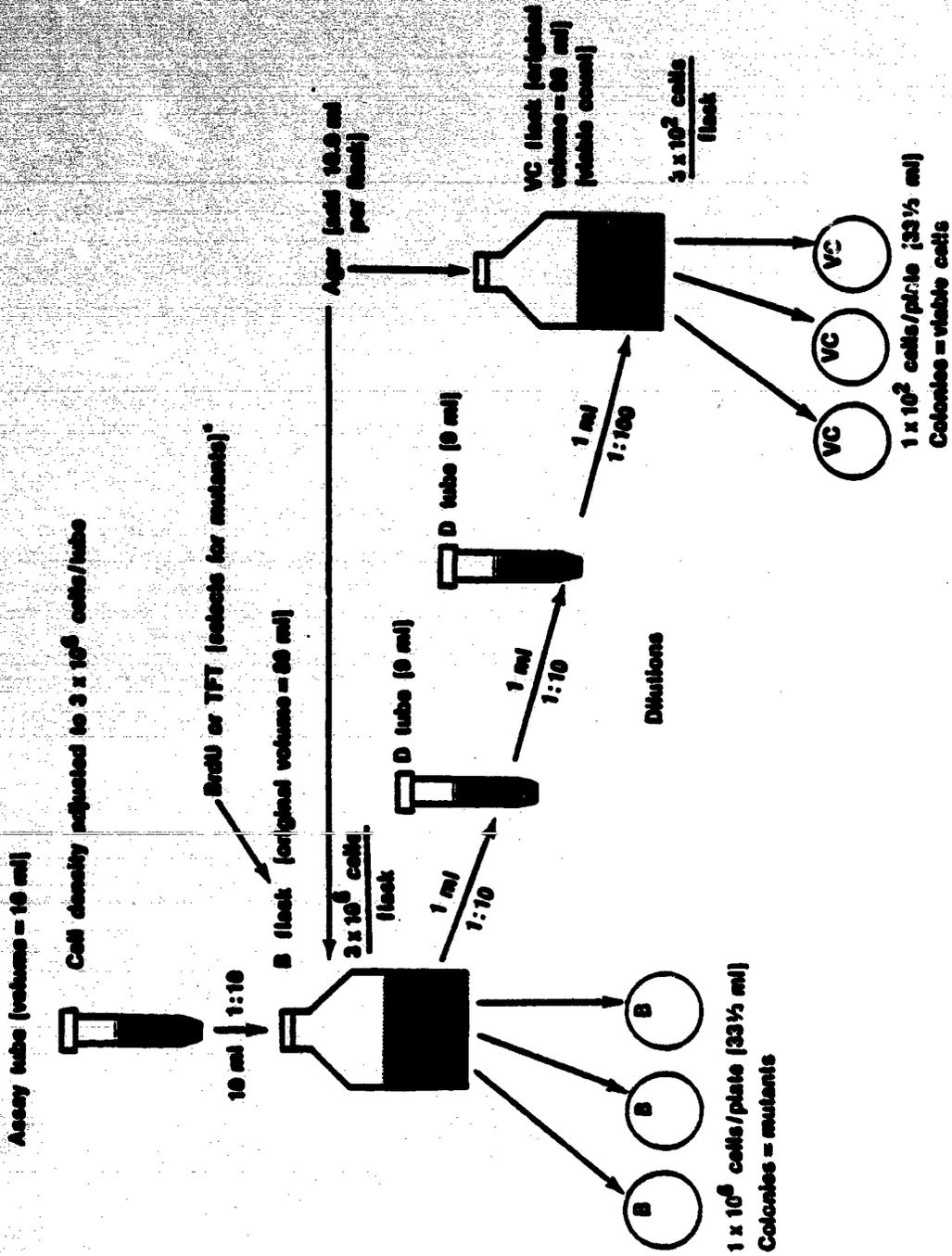
6. REFERENCE

Clive, D. and Spector, J.F.S.: Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. *Mutation Res.*, 31:17-29, 1975.



BIONETICS

Litton



*Added after removal of 1 ml for viable count dilutions.

FIGURE 1. LYMPHOMA CLONING FLOW CHART

Monsanto

FROM (NAME LOCATION PHONE)

G2WD

Dept. of Medicine & Environmental Health M.W. Stevens, 4-8822

PAF THIOFIDE TOXICITY FILE

DATE September 1, 1982

SUBJECT THIOFIDE® - Evaluation of Potential Hazards by Dermal Contact
SH-82-009

TO P. R. Graham

F. R. Johannsen
A. M. Spivey
A. Munn
Toxdata

The final report for the referenced study has been received and reviewed. A quality assurance review was conducted by the testing laboratory. A review of the data and an evaluation of the conclusions of this report are summarized below.

Methods

The potential for 2,2'-dithio-bis-benzothiazole (THIOFIDE®) to cause primary skin irritation and /or sensitization was assessed in 53 volunteer human subjects. This chemical was tested as a 70% preparation in petroleum. Using a specially prepared, occlusive adhesive bandage, 0.2 grams of test material was placed in contact with a selected site on the back of each subject for 24 hours. Following this exposure, the test material was removed and the reaction of the site of application was evaluated and graded (scores 0 through 4) by the clinical investigator. The test material was applied in this manner for four consecutive 24 hour periods each week (Monday through Friday) for three consecutive weeks (induction phase). Subjects were then allowed a two week rest period, during which no exposures to the test material occurred. During the sixth week of the study, the test material was applied to a new site on each subject for four consecutive 24 hour periods (challenge phase) with grading of the skin response again conducted following each 24 hour period. Minor differences in the above procedure occurred at the discretion of the investigator if individual subjects demonstrated a strong irritation response, or were absent, during the study or if holidays interfered with normally scheduled dates for the study.

Results

All 53 subjects completed the study. No evidence of irritation was observed in any subject at any time during the study. No primary or cumulative irritation was noted during the induction phase, and no response which would indicate a sensitization reaction was noted during the challenge phase.

Conclusion

There was no evidence that 2,2'-dithio-bis-benzothiazole, when tested as a 70% preparation in petrolatum, was a primary or cumulative irritant or a sensitizer in humans following repeated dermal contact.



M. W. Stevens

REPORT NUMBER FI-2400

**Evaluation of Potential Hazards
by Dermal Contact**

**Monsanto Company
800 N. Lindbergh Boulevard
St. Louis, Missouri 63166**

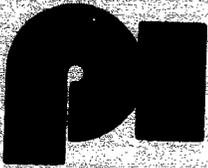
**Test Material: SH-82-009,
2, 2' - dithio-bis-benzothiazole**

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APPENDICES

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report

Report Number 2600

29 July 1982

Title: Evaluation to Determine Potential Hazards of Dermal Contact with SH-82-009, 2, 2' - dithio-bis-benzothiazole

Client: Monsanto Company
800 N. Lindbergh Boulevard
St. Louis, Missouri 63166

Test Material: Identification: SH-82-009, 2, 2' - dithio-bis-benzothiazole
Description: White Powder
Received: 12 April 1982
Tested as: 70% in petrolatum

Assay: Repeated Insult Patch Test

Method: Modified Shelanski (4 x 4 Method)

Authorized: 23 April 1982

Starting Date: 14 June 1982

Completion Date: 23 July 1982

- Purpose:**
1. To determine if the test material is capable of irritating the skin of humans under the rigorously controlled conditions of the procedure, and, if so
 2. To determine, on the basis of criteria which have been adopted as standards by Product Investigations, Inc.:
 - 2.1 Whether the responses may be attributed to the test material.
 - 2.2 Which mechanisms may be involved in the manifestation of these responses.
 - 2.3 What the responses mean in terms of hazards to the consumer population at risk.

Panel No.: 82-33; 53 individuals started; 53 individuals completed the study.

0 0 4 0

Report Number: 2600

Test Material: Supplied by Monsanto Company:

| <u>Monsanto Code No.</u> | <u>Description</u> | <u>PII No.</u> |
|--|--------------------|----------------|
| SH-82-009, 2, 2' - dithio-bis-benzothiazole | White Powder | 2600 |

Carrier: Supplied by Product Investigations, Inc.:

Parke-Davis Read-Bandages (not sterilized), Special Order, Lot Number WB6167, purchased from Parke-Davis & Company, Medical-Surgical Division, Detroit, Michigan.

Correspondent: M. W. Stevens, Ph.D.
Senior Product Toxicology Specialist
Monsanto Company
800 N. Lindbergh Boulevard
St. Louis, Missouri 63166

Monitor: M. W. Stevens, Ph.D.
Senior Product Toxicology Specialist
Monsanto Company
800 N. Lindbergh Boulevard
St. Louis, Missouri 63166

Report Number: 2600

Site of Investigations: Product Investigations, Inc.
151 East Tenth Avenue
Coshohocken, Pennsylvania 19428

Principal Investigator: Morris V. Shelanski, M.D., C.M., F.R.S.H.
Director

Associate Investigator: Glen Smith, M.D.
Associate Medical Director

Records Preparations: A. Kaufmann

Project Co-ordinator: C. Karras

Material Preparations: T. Levenson, Ph.D.
Chief Chemist

Quality Assurance: S. J. Charles III

Technicians:

Removal and Grading: S. Grobaker

Recording: M. Marine

Applications: M. McMonagle

Report Number: 2600

Rationale:

The conditions of this study are designed purposely to facilitate the elicitation of latent skin-damaging propensities, either primary irritation or sensitization, or both.

The propensity of the test material to produce irritation of the non-allergic type under the test conditions should, therefore, not be construed as a contra-indication to the use of this material. It should be understood that the propensity as manifested in the procedure exists as a potential only, and that it may require conditions as rigorous as those of the test to convert that potential into reality. The probability that the irritation may result from the use of the material would depend upon the conditions of actual use. Under conditions less stringent than those of the test, the propensity would, in all likelihood, diminish; and, theoretically, if the conditions of use are far enough removed, it may disappear altogether.

If, under the test conditions, a material is incapable of producing irritation, it would, therefore, qualify as very innocuous in actual use with regard to non-allergic irritation.

Minimal irritation under the conditions of this procedure is not indicative of a significant irritative capability, especially when the continuity of that irritation is interrupted by the apparent return of the skin to its normal condition. The appearance and disappearance of irritation may be a manifestation of the ability of the skin to adapt to the presence of the contactant, or it may be a phenomenon unrelated to the test material.

When minimal or moderate responses are observed as solitary incidents (i.e., without significant continuity), attribution to a deleterious effect of the test material is tenuous at best.

As the intensity and frequency of the responses increase, the likelihood that the test sample was responsible increases as well.

The attribution of irritation to a test material is strengthened when confirmation of irritation is obtained by subsequent applications on the same site. Attribution is strongest when confirmation of irritation is obtained on naive sites.

Report Number 2400

Selection of Panelists

Fifty-three (53) qualified individuals from the local population volunteered to participate in this evaluation. The criteria for qualification is referenced in Appendix I.

Application of Test Material

One technician was assigned solely to the task of applying the test material. This aided in minimizing the variables associated with the multitude of applications performed during the study.

Grading of Responses

One technician was assigned solely to the function of examining the contact sites and grading the condition of the skin at these sites. This helped to maintain a uniform evaluation of responses and consistent application of the scoring criteria throughout the study. The investigator confirmed the grades assigned to the visible responses throughout the evaluation.

Site Definitions

A site on the back of each individual was selected and identified as the intact contact site.

Patch Definitions

Specially prepared Parke-Davis Read-Bandages were used for patching in this study. Approximately 0.2 gm of the material, tested as a 70% preparation in petrolatum, was placed on the webril pad of a Parke-Davis Read-Bandage. The treated patch was then applied to a designated site. The adhesive was pressed all around to assure firm contact of the test material with the skin and to form a seal to retard loss of the test material.

Material Preparations

70 grams of the test material, as supplied, were mixed in a mortar with 30 grams of petrolatum to give a non-powdery, lumpy preparation.

Report Number: 2600

Procedure:

DESIGN: The procedure was a single-blind evaluation in a randomly selected group of panelists from the local population. The test material was one of several being evaluated concurrently on this panel. Each material was assigned a distinct and separate contact site for both the induction and challenge phases.

The procedure was not designed to evaluate the photosensitization capabilities of the test material.

OUTLINE:

Induction - Weeks #1, #2, and #3:

A series of twelve applications, each of twenty-four hours' duration was scheduled to be carried out during Weeks #1, #2, and #3.

Rest Period - Weeks #4 and #5

Challenge - Week #6:

A series of four applications on virgin sites was scheduled to be carried out during the challenge week.

Report Number 2400

Procedure: Induction Period

Week #1: Approximately 0.2 gm of the material, tested as a 70% preparation in petrolatum, was placed on the webril pad of a Parke-Davis Read-Bandage and positioned on its designated site.

Monday - The test material was applied to an intact site. After application, the participants were given these instructions:

1. If the test material could be tolerated without substantial difficulty, the panelists were to endeavor to keep the patch in place until the next visit to the clinic, so that it could be removed by the technician at the end of twenty-four hours.

2. If the test material caused substantial pain or discomfort, the panelists were instructed to:

a. remove the test material.

b. report immediately by phone to arrange for an examination by the investigator.

Tuesday - After twenty-four hours, the patch was removed and discarded. The contact site was examined and graded (cf. Criteria for Grading of Responses, Appendix II). If the reaction was a Grade 2 or less, a freshly prepared patch was applied to the same site. If responses of Grade 3 or 4 intensity were observed at this time, the investigator employed one or more of the options which were available to him for subsequent applications (cf. Alternate Procedures Available for Use by the Investigator During the Induction Period, Appendix III). The participants were instructed to return the next day and were dismissed.

Wednesday - Tuesday's procedure was repeated.

Thursday - Tuesday's procedure was repeated.

Friday - After twenty-four hours, the patch was removed and discarded. The contact site was examined and graded (cf. Criteria for Grading of Responses, Appendix II). The participants were dismissed for the weekend with instructions to examine the contact site daily and to notify the investigator immediately if irritation occurred.

Report Number: 2600

**Procedure: Induction Period (Cont'd)
(Cont'd)**

Week #2: Approximately 0.2 gm of the material, tested as a 70% preparation in petrolatum, was placed on the webril pad of a Parke-Davis Readi-Bandage and positioned on its designated site.

- Monday** - The test material was applied to the same site used during Week #1 unless responses of Grade 3 or 4 intensity had resulted from the previous week's applications (cf. Alternate Procedures Available for Use by the Investigator, Appendix III). After application, each participant was instructed as before (cf. Monday, Week #1, Page 7) and dismissed.
- Tuesday** - After twenty-four hours, the patch was removed and discarded. The contact site was examined and graded (cf. Criteria for Grading of Responses, Appendix II). If the reaction severity was a Grade 2 or less, a freshly prepared patch was applied to the same site. If responses of Grade 3 or 4 intensity were observed, the investigator employed one or more of the options available to him for subsequent applications (cf. Alternate Procedures Available for Use by the Investigator, Appendix III). The participants were instructed as before and dismissed.
- Wednesday** - Tuesday's procedure was repeated.
- Thursday** - Tuesday's procedure was repeated.
- Friday** - After twenty-four hours of contact, the patch was removed and discarded. The contact site was examined and graded (cf. Criteria for Grading of Responses, Appendix II). The participants were dismissed for the weekend with instructions to return to the laboratory on the following Monday and to examine the contact sites daily, notifying the investigator immediately if irritation occurred.

Report Number 2400

Procedure: Infection Period (Cont'd)
(Cont'd)

Week #3: Approximately 0.2 gm of the material, tested as a 70% preparation in petrolatum, was placed on the webbed pad of a Parke-Davis Readi-Bardage and positioned on its designated site.

- Monday** - The test material was applied to the same site used during Week #2 unless responses of Grade 3 or 4 intensity had resulted from the previous week's applications (cf. Alternate Procedures Available for Use by the Investigator, Appendix III). After application, each participant was instructed as before (cf. Monday, Week #1, Page 7) and dismissed.
- Tuesday** - After twenty-four hours, the patch was removed and discarded. The contact site was examined and graded (cf. Criteria for Grading of Responses, Appendix II). If the reaction severity was a Grade 2 or less, a freshly prepared patch was applied to the same site. If responses of Grade 3 or 4 intensity were observed, the investigator employed one or more of the options available to him for subsequent applications (cf. Alternate Procedures Available for Use by the Investigator, Appendix III). The participants were instructed as before and dismissed.
- Wednesday** - Tuesday's procedure was repeated.
- Thursday** - Tuesday's procedure was repeated.
- Friday** - After twenty-four hours of contact, the patch was removed and discarded. The contact site was examined and graded (cf. Criteria for Grading of Responses, Appendix II). The participants were dismissed with instructions to return to the laboratory on the third Monday following (19 July 1982) and to examine the contact site daily and notify the investigator immediately if irritation occurred.

Report Number: 2600

Procedures **Alternate Procedures used during the Induction Period:**

None were necessary.

Deviations in Procedure:

None were necessary.

Report Number: 2100

**Procedure
(Cont'd)**

**Rest Period: - Week #4: Entire Week
Week #5: Entire Week**

No patches of the test material were applied to the panelists during this period. This hiatus in applications provided a period of time for the skin to rest and recuperate from whatever trauma and injury the induction series had engendered.

The panelists were instructed to keep a close watch on the contact sites throughout the recess and to notify the investigator immediately if they observed any adverse changes in the contact sites.

Report Number: 2600

**Procedure: Challenge Period
(Cont'd)**

Week #6: The sixth week was scheduled for the challenge applications. Approximately 0.2 gm of the material, tested as a 70% preparation in petrolatum, was placed on the webril pad of a Parke-Davis Read-Bandage and positioned on its designated site.

- Monday** - The test material was applied to a Parke-Davis Read-Bandage, placed on virgin sites, and sealed. The participants were then dismissed with instructions to return to the laboratory in twenty-four hours.
- Tuesday** - After twenty-four hours, the patch was removed and discarded. The contact site was examined and graded (cf. Criteria for Grading of Responses, Appendix II). If the reaction severity was a Grade 2 or less, a freshly prepared patch was applied to the same site. If responses of Grade 3 or 4 intensity were observed, the investigator employed one or more of the options available to him for subsequent applications (cf. Alternate Procedures Available for Use by the Investigator, Appendix III). The participants were instructed to return the following day and were dismissed.
- Wednesday** - Tuesday's procedure was repeated.
- Thursday** - Tuesday's procedure was repeated.
- Friday** - The contact sites were examined and graded (cf. Criteria for Grading of Responses, Appendix II). This concluded the scheduled study. The panelists were dismissed with instruction to examine both the induction and challenge sites daily for the next week and to report to the investigator if any rash should appear at these sites.

Report Number: 2400

Results

PANEL 002-33

Summary: Number of Responses in Each Grade

| <u>Application Number</u> | <u>INDUCTION PHASE</u> | | | | | | | | | | | | <u>CHALLENGE PHASE</u> | | | |
|-------------------------------|------------------------|----------|----------|----------|----------|----------|----------|----------|----------|-----------|-----------|-----------|------------------------|-----------|-----------|-----------|
| | <u>1</u> | <u>2</u> | <u>3</u> | <u>4</u> | <u>5</u> | <u>6</u> | <u>7</u> | <u>8</u> | <u>9</u> | <u>10</u> | <u>11</u> | <u>12</u> | <u>13</u> | <u>14</u> | <u>15</u> | <u>16</u> |
| <u>Reaction Grade</u> | | | | | | | | | | | | | | | | |
| 0 | 53 | 51 | 53 | 53 | 53 | 53 | 52 | 52 | F1 | 52 | 52 | 52 | 53 | 53 | 53 | 53 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| NP | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| NPT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| A | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| NS | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 53 | 0 | 0 | 0 |

NOTE: Refer to Appendix II for definitions of symbols.

Report Number: 2600

Results:

PANEL #82-33

INDIVIDUAL REACTIONS

| Application Number | INDUCTION PHASE | | | | | | | | CHALLENGE PHASE | | | | | | | |
|--------------------|-----------------|---|---|---|---------|---|---|---|-----------------|----|----|----|---------|----|----|----|
| | Week #1 | | | | Week #2 | | | | Week #3 | | | | Week #6 | | | |
| Panelist Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | A | A | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 0 | A | 0 | 0 | 0 | 0 | A | A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Report Number 2600

Results

PANEL #62-33

INDIVIDUAL REACTIONS

| <u>Application Number</u> | <u>INDUCTION PHASE</u> | | | | | | | | <u>CHALLENGE PHASE</u> | | | | | | | |
|-------------------------------|------------------------|----------|----------|----------|----------------|----------|----------|----------|------------------------|-----------|-----------|-----------|----------------|-----------|-----------|-----------|
| | <u>Week #1</u> | | | | <u>Week #2</u> | | | | <u>Week #3</u> | | | | <u>Week #6</u> | | | |
| <u>Panelist Number</u> | <u>1</u> | <u>2</u> | <u>3</u> | <u>4</u> | <u>5</u> | <u>6</u> | <u>7</u> | <u>8</u> | <u>9</u> | <u>10</u> | <u>11</u> | <u>12</u> | <u>13</u> | <u>14</u> | <u>15</u> | <u>16</u> |
| 26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 29 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 31 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 33 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 34 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 35 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 37 | 0 | A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 42 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 43 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 44 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | A | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 46 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 48 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 49 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

port Number: 2600

Results:

PANEL #62-33

INDIVIDUAL REACTIONS

| <u>Application Number</u> | <u>INDUCTION PHASE</u> | | | | | | | | | | | | <u>CHALLENGE PHASE</u> | | | |
|-------------------------------|------------------------|----------|----------|----------|----------------|----------|----------|----------|----------------|-----------|-----------|-----------|------------------------|-----------|-----------|-----------|
| | <u>Week #1</u> | | | | <u>Week #2</u> | | | | <u>Week #3</u> | | | | <u>Week #6</u> | | | |
| | <u>1</u> | <u>2</u> | <u>3</u> | <u>4</u> | <u>5</u> | <u>6</u> | <u>7</u> | <u>8</u> | <u>9</u> | <u>10</u> | <u>11</u> | <u>12</u> | <u>13</u> | <u>14</u> | <u>15</u> | <u>16</u> |
| <u>Panelist Number</u> | | | | | | | | | | | | | | | | |
| 51 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 52 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 53 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Report Number: 2600

Interpretation of Results

Induction Phase:

Application No. 1:

The absence of visible irritation as a result of the first application indicated that Sample No. SK-62-009, 2, 2' - dithio-bis-benzothiazole, tested as a 70% preparation in petrolatum, was incapable of acting as a primary irritant of the immediate type in any of the individuals under test.

Application Nos. 2 through 12:

The absence of visible irritation as a result of two or more applications indicated that the material, tested as a 70% preparation in petrolatum, was incapable of acting as a primary irritant of the cumulative type in any of the individuals under test.

Challenge Phase:

The absence of visible irritation as a result of the challenge applications indicated that the material, tested as a 70% preparation in petrolatum, was incapable of acting as a primary irritant of either the immediate or the cumulative type in any of the individuals on the sites utilized for the challenge applications.

There was no evidence that the material was capable of sensitizing any of the individuals under test.

Report Number: 2600

Conclusions:

Monsanto Company's Sample No. SH-62-009, 2, 2' - dithio-bis-benzothiazole, was evaluated, as a 70% preparation in petrolatum, by the Modified Shelanski Repeated Insult Patch Test.

An estimation as to how well a general population will tolerate this product can be extrapolated from the results of the study within the limitations imposed on such extrapolation by the considerations discussed in Appendix VII.

Insofar as irritation of the non-allergic type, the results may be interpreted to indicate that the test material will be well tolerated by the majority of users if the recommended conditions of use are commensurate with or less stringent than those of the test procedure.

With regard to allergic responses, the hazard of sensitization should be exceedingly small even under occlusive conditions, e.g., based on the results of this study in which none of the 53 participants were sensitized under the test conditions, it is possible to state with 95% confidence that the material will be tolerated by no less than 93.27%, and by as much as 100% of the population at risk (cf. Appendix VIII).

The procedure did not evaluate the effect of UV light on the reactivity of the skin in the presence of the test material. Therefore, no comment concerning the risk of photosensitization can be made by the investigator on the basis of the results obtained in this study.

PRODUCT INVESTIGATIONS, INC.



MORRIS V. SHELANSKI, M.D., C.M., F.R.S.H.
Director

Reviewed by:



Samuel J. Charles III
Director, Quality Assurance

April 26, 1982

Thomson

Petrol SH-82-009 PI 2600

70% PI 2600

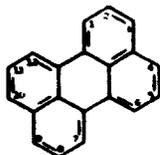
30% petrolatum

ground as above.

APPENDIX IX (Cont'd)

Peyonine

6977



Yellow to colorless crystals from toluene mp 273-274°. Sublimes 350-400°. d 1.35. Absorption spectrum: *Clar. Rev.* 68, 848 (1932). Freely sol in CS₂, chloroform, moderately sol in benzene; slightly in ether, alcohol, acetone; very sparingly sol in petr ether. Insol in water.

Mucopicate, C₂₈H₁₈N₂O₇, dark violet-blue needles, mp 223-225°.

6976. Petrolatum. Petroleum jelly; paraffin jelly; vasoliment. Vaseline; Sasoline; Cnoline. Purified mixture of saturated hydrocarbons, chiefly of the methane series of the general formula C_nH_{2n+2}. Actually, petrolatum is a colloidal system of nonstraight-chain solid hydrocarbons and high-boiling liq hydrocarbons, in which most of the liq hydrocarbons are held inside the micelles. Detailed historical account including chemistry and modern mg methods: *Schindler, Drug Cosmet. Ind.* 89, 36-37, 76, 78-80, 82 (1961).

Yellowish to light amber or white, amorphous, unctuous mass; practically odorless and tasteless. d₄ 0.820-0.865, mp 38-54°. n_D 1.460-1.474. White petrolatum is transparent in thin layers even at 0°. Practically insol in water, glycerol, alcohol, sol in benzene, chloroform, ether, petr ether, carbon disulfide, oils.

Primum white petrolatum, *Stanolone*.

USE: As ointment base and in cosmetics. Lubricating firearms and machinery, leather grease, shoe polish, rust preventives, modeling clays.

THERAP CAT: Pharmaceutical aid (ointment base).

6971. Petrolatum, Liquid. Liquid paraffin; mineral oil; white mineral oil; paraffin oil; Kaydol; Albolite; Nujol; Paroline; Sasol; Adepsine oil; Glymol. A mixture of liquid hydrocarbons from petrolatum.

Colorless, oily liquid, practically tasteless and odorless even when warmed. The density of the "light" oil is usually 0.83-0.860; the "heavy" 0.875-0.905. Surface tension at 25° slightly below 35 dynes/cm. Insol in water, alc. Sol in benzene, chloroform, ether, carbon disulfide, petr ether, oils.

THERAP CAT: Cathartic.

THERAP CAT (VET): Laxative, externally as a protectant, lubricant.

6972. Petroleum. Crude oil; mineral oil; rock oil; coal oil; seneca oil. Consists of a mixture of hydrocarbons from C₅H₁₂ and up—chiefly of the paraffins, cycloparaffins, or of cyclic aromatic hydrocarbons, with small amounts of benzene hydrocarbons, sulfur, and oxygenated compounds. Occurrence: U.S., Mexico, Iran, Russia, Roumania, Poland, Dutch East Indies, etc.

Dark yellow to brown or greenish-black, oily liquid. Insol in water and only a small portion of it may dissolve in alcohol; sol in benzene, chloroform, ether.

USE: Source of gasoline, petr ether, liq and solid petrolatum, fuel and lubricating oils, butane, isopropyl alcohol, etc.

6973. Petroleum Benzoin. Petroleum ether; benzoin; naphtha. Low boiling fractions of petroleum. Consists chiefly of hydrocarbons of the methane series—principally pentanes and hexanes. See also Lignin.

Clear, colorless, nonfluorescent, highly flammable, volatile liq, characteristic odor; does not solidify in the cold. The vapors mixed with air explode if ignited. d 0.625-0.660; bp between 35-80°. Flash pt about -40°. Insol in water; miscible with alc, benzene, chloroform, ether, carbon disulfide, carbon tetrachloride, and oils except castor oil. Caution: Keep tightly closed in a cool place and away from fire.

Human Toxicity: See Kerosine

THERAP CAT: Counterirritant, pharmaceutical aid (solvent).

6974. Petroselinic Acid. *cis-6-(tridecenoic acid; petroselinic acid; 5-heptadecylene-1-carboxylic acid; Δ¹-octadecyl-enoic acid.* C₁₈H₃₄O₂, mol wt 282.45. C 76.54%, H 12.13%, O

11.33%. CH₃(CH₂)₁₀CH=CH(CH₂)₇COOH. Isola from parsley seed oil, the oil extracted from dried ripe seed of *Petroselinum hortense* Hoffm., *Umbelliferae*: Fore et al., *J. Am. Oil Chem. Soc.* 37, 490 (1960).

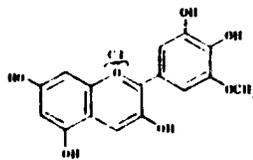
Leaflets from petr ether, mp 29.5-30.1°. bp₁₀ 237-238°; d₄ 0.8700, n_D 1.4533. Low temp solubilities: Heptane at -10° = 0.50 g/100 g solution; methanol at -20° = 0.48 g/100 g; ethyl acetate at -20° = 0.73 g/100 g; ether at -20° = 3.52 g/100 g. Ozonolysis yields 65% of adipic acid. Neutralization equivalent: 282.45; iodine value 89.87%.

Methyl ester, C₁₈H₃₄O₂, liq, d₄ 0.8767; n_D 1.4501; bp₁₀ 208-210°.

Glyceryl triester, C₅₄H₁₀₂O₆, glyceryl tripetroselinate, tripetroselin. Solidifies at 16.5°. n_D 1.4619.

Amide, C₁₈H₃₃NO, needles, mp 76°.

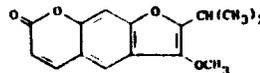
6975. Petroselin Chloride. 2-(3,4-Dihydroxy-5-methoxyphenyl)-3,5,7-trihydroxy-1-benzopyrylium chloride; 3,3',4',5,7-pentahydroxy-5'-methoxyflavylium chloride. C₁₅H₁₁ClO₇; mol wt 352.74. C 54.48%, H 3.71%, Cl 10.05%, O 31.75%. The aglucone of petunin: Willstätter, *Burdick, Ann.* 412, 217 (1917). Synthesis: Bradley et al., *J. Chem. Soc.* 1930, 793; Robinson, *Biochem. J.* 25, 1687 (1931). Chromatographic separation: Spaeth, Rosenblatt, *Anal. Chem.* 22, 1321 (1950).



Gray-brown leaflets or prisms from dil HCl.

3,5-Diglucoside, C₂₈H₂₈ClO₁₀, petunin. From *Petunia hybrida* Hort., *Solanaceae*: Willstätter, *Burdick, loc. cit.* Synthesis: Bell, Robinson, *J. Chem. Soc.* 1934, 1604. Violet plates with a coppery luster from dil HCl, mp about 178°. Absorption max (methanolic HCl): 540 nm.

6976. Peucedanis. 3-Methoxy-2-(1-methylthyl)-7H-furo[3,2-g][1]benzopyran-7-one; 6-hydroxy-2-isopropyl-3-methoxy-5-benzofuranacrylic acid β-lactone; 4-methoxy-5-isopropylfuro[2,3:6,7]coumarin; oreoselone methyl ether. C₂₀H₁₈O₅; mol wt 258.26. C 69.76%, H 5.46%, O 24.78%. From rhizome of *Peucedanum officinale* L., *Umbelliferae*: Schlatter, *Ann.* 5, 201 (1833); Hlasiwetz, *Waidl, ibid.* 174, 67 (1874); Jaesoy, *Mazsch, Arch. Pharm.* 236, 662 (1898); Popper, *Monatsh.* 19, 268 (1898). Structure: Späth et al., *Ber.* 64, 2203 (1931); Späth, Klager, *ibid.* 66, 749 (1933). Synthesis: Schmid, Ebnöther, *Helv. Chim. Acta* 34, 1982 (1951).



Yellowish crystals, polymorphous. mp 87 and 97°. uv max (methanol): 255, 295, 340 nm (log ε 4.40, 4.05, 3.70). Practically insol in water, freely sol in chloroform, carbon disulfide, sol in hot alcohol, ether, acetic acid; sparingly sol in benzene, petr ether. I.D.₅₀ orally in mice: 315 mg/kg. THERAP CAT: Exptl antineoplastic.

6977. Peyonine. 1-[2-(3,4,5-Trimethoxyphenyl)ethyl]-1H-pyrrole-2-carboxylic acid. C₁₄H₁₃NO₅; mol wt 305.32. C 62.94%, H 4.27%, N 4.59%, O 26.20%. A peyote alkaloid, isolated from *Lophophora williamsii* (Lem.) Coult. Isola and synthesis: Kapadia, Shah, *Lloydia* 30, 287 (1967). Structure studies: Kapadia, Highet, *J. Pharm. Sci.* 57, 191 (1968).

W. J. Adams - N1B (4-4407)

October 12, 1979

cc. W. E. Gledhill - N1B
B. B. Heidolph - N1B
J. P. Miere - N3A
M. W. Stevens - A2SC

THIOFIDE ACUTE AQUATIC TOXICITY

TO : P. R. Graham - B2SL

The acute toxicity tests scheduled to be conducted with Thiofide during 1979 have been completed. The results are summarized as follows:

| <u>Species</u> | <u>96-Hour LC50 (mg/l)</u> | <u>No Effect Conc. (mg/l)</u> |
|----------------------|---|-------------------------------|
| Selenastrum sp. | 0.7 (chlorophyll a) 0.6 (cell count) | <0.3 <0.3 |
| fathead minnow | >1000 mg/l | >1000 |
| | <u>48-Hour EC50 (mg/l)</u> | |
| <u>Daphnia magna</u> | 82 | 32 |

The data suggest that Thiofide is relatively non toxic to fathead minnows and Daphnia magna. However, this product appears to be highly toxic to the green alga, Selenastrum. Variation in the toxicity from one species to another is usually less than an order of magnitude. Excessive variation, as seen with Thiofide, is usually an indication the chemical was not truly in solution during the test. I believe this to be the case for Thiofide. This chemical is highly insoluble in water and no satisfactory solvent was found to use as a carrier. We did use dimethylformamide as a carrier solvent, however, it was not particularly effective. I suggest we rerun the fathead test using a water saturated solution of Thiofide.


W. J. Adams

/mas

Toxicity of Thiofide (BN-79-1384364-1e)
to the freshwater alga Selenastrum
capricornutum

Toxicity Test Report

Submitted to

Monsanto Industrial Chemicals Company

St. Louis, Missouri

Project Number H97-500

Report Number BP-79-7-105

EG&G, Bionomics
Marine Research Laboratory
Route 6, Box 1002
Pensacola, Florida 32507
July 1979

A phytotoxicity test was performed at Bionomics Marine Research Laboratory (BMRL), Pensacola, Florida, to determine the effect of Thiofide (BN-79-1384364-1a) on the freshwater alga Selenastrum capricornutum. Results of the test are reported as 24-, 48-, 72-, and 96-hour EC50's (the concentrations of the test material estimated to cause a 50% decrease of in vivo chlorophyll a in exposed cultures as compared to the control at the specified times). Cell numbers in exposed and control cultures were also determined after 96 hours of exposure and another 96-hour EC50 was calculated (the concentration of the test material estimated to cause a 50% decrease of cell numbers in exposed cultures as compared to the control). Confirmation of effect by measurement of two different growth factors is important, in our opinion, because of the various alga responses in the presence of toxicants (Hall, 1973).

All raw data related to this test are stored at BMRL.

MATERIALS AND METHODS

Test material

The sample was received at BMRL on 22 May 1979. The sample was contained in a clear-glass bottle labeled "Thiofide (lot NL02-023); BN-79-1384364-1a." The test material was a yellow white powder. Concentrations are reported here as milligrams (mg) of test material per liter (l) of algal growth medium or parts per million (ppm).

Test alga

The freshwater alga tested was the unicellular green alga, Selenastrum capricornutum. The culture was obtained from the U.S.

Environmental Protection Agency's Environmental Research Laboratory,
Corvallis, Oregon, and maintained in stock culture at BMRL.

Test conditions

Culture and test procedures followed those of U.S. Environmental Protection Agency (1971) except as noted. Beginning cell numbers in the test flasks were 20×10^3 cells/milliliter (ml). Cultures were incubated at 24 ± 1 degrees Celsius ($^{\circ}\text{C}$) under $\sim 3,800$ lux illumination. Triplicate cultures were employed for each of the test concentrations and the controls. Test containers were 125-ml flasks each containing 50 ml of test medium. Concentrations for the definitive test were based on the results of a 96-hour range-finding test. Test concentrations were prepared by adding appropriate volumes of a stock solution of test material to each flask. A primary stock solution was prepared by adding a weighed amount of test material to reagent grade dimethylformamide (DMF) and secondary stock solutions were prepared by serial dilution for the range-finding and definitive tests. A solvent control was also maintained to which was added 0.05 ml of DMF, the maximum volume added to a test flask for the definitive test.

Measurements of in vivo chlorophyll a in cultures were performed by using a Turner Model 111 fluorometer. Cell counts were made by using a hemacytometer and a Zeiss Standard 14 compound microscope.

The definitive test was conducted 5-9 June 1979.

Statistical analyses

Each test concentration was converted to a logarithm and the corresponding percentage decrease of in vivo chlorophyll a or cell numbers was converted to a probit (Finney, 1971). The 48-, 72-, and

0075

96-hour EC50's and 95% confidence limits were then calculated by linear regression.

To determine whether growth of the solvent control differed from that of the culture medium control, data were analyzed by "Student's" t-test (Steel and Torrie, 1960). Differences were considered significant at the 95% confidence level ($P \leq 0.05$).

RESULTS AND DISCUSSION

The toxicity of Thiofide to Selenastrum capricornutum appeared to reach maximum after 48 hours of exposure. Based on decrease of in vivo chlorophyll a , the calculated 48-hour EC50 was 0.7 ppm while the calculated 72- and 96-hour EC50's were both 0.6 ppm. The calculated 96-hour EC50, based on cell number decrease, was 0.7 ppm with 95% confidence limits of 0.1-3.4 ppm (Table 1). After 96 hours of exposure, decrease of in vivo chlorophyll a was from 15% in cultures exposed to 0.3 ppm to 91% in those exposed to 5.6 ppm. Decrease of cell numbers was from 12% in cultures exposed to 0.3 ppm to 90% in those exposed to 5.6 ppm after 96 hours. The pH was from 7.4-7.6 after 96 hours of exposure.

There was no significant difference between growth of the control and solvent control cultures after 96 hours of exposure, based on both in vivo chlorophyll a and cell numbers.

REFERENCES

- Finney, D.J. 1971. Probit Analysis. Cambridge University Press, London. 333 p.
- Hall, R.H. 1973. An Algal Toxicity Test Used in the Safety Assessment of Detergent Components. Presented before the Thirty-Sixth Annual Meeting of the American Society of Limnology and Oceanography, Inc., Salt Lake City, Utah. 23 p.
- Steel, R.G.D. and J.H. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill Book Company, Inc., New York. 481 p.
- U.S. Environmental Protection Agency. 1971. Algal Assay Procedure: Bottle Test. National Eutrophication Research Program, Pacific Northwest Water Laboratory, Corvallis, OR. 82 p.

TABLE 1. Estimated and calculated EC50's for the freshwater alga *Scenedesmus obliquus* exposed to Thiofide (BN-79-134364-1e). The criterion for effect was decrease of in vivo chlorophyll *a* in exposed cultures as compared to the control at 24, 48, 72, and 96 hours or decrease of cell numbers in exposed cultures as compared to the control at 96 hours. Calculations were based on nominal concentrations of Thiofide in algal growth medium.

| <u>Effect criterion</u> | <u>Hour</u> | <u>EC50 (mg/l;ppm)</u> | <u>95% confidence limits (mg/l;ppm)</u> |
|------------------------------|-------------|------------------------|---|
| <u>In vivo chlorophyll a</u> | 24 | >5.6 | --- |
| | 48 | 0.7 | 0.1-7.8 |
| | 72 | 0.6 | 0.1-4.5 |
| | 96 | 0.6 | 0.1-3.6 |
| ----- | | | |
| <u>Cell number</u> | 96 | 0.7 | 0.1-3.4 |

TABLE 2. Results of a 96-hour exposure of the freshwater alga *Selenastrum capricornutum* to Thiofide (BN-79-1384364-1e). Percentage change is increase or decrease of *in vivo* chlorophyll *a* in exposed cultures as compared to the control at 24, 48, 72, and 96 hours and decrease of cell numbers in exposed cultures as compared to the control at 96 hours.

| Nominal concentration (mg/l; ppm) | pH | | Percentage change | | | | Cell no. 96 h |
|---|-----|------|------------------------|------|------|------|------------------|
| | 0 h | 96 h | Chlorophyll <i>a</i> : | | | | |
| | | | 24 h | 48 h | 72 h | 96 h | |
| Control | 7.4 | 7.6 | --- | --- | --- | --- | --- |
| Sol. control | 7.4 | 7.6 | +4 | +3 | 0 | +4 | +1 |
| 0.3 | 7.4 | 7.6 | -12 | -21 | -17 | -15 | -12 |
| 0.6 | 7.4 | 7.6 | -38 | -58 | -59 | -60 | -54 |
| 1.0 | 7.4 | 7.6 | -46 | -73 | -79 | -81 | -84 |
| 3.2 | 7.4 | 7.5 | -50 | -77 | -84 | -87 | -87 |
| 5.6 | 7.3 | 7.4 | -48 | -81 | -88 | -91 | -90 |

PREPARED BY:

Terry Hollister

Terry A. Hollister
Study Director

AUDITED BY:

G. Scott Ward

G. Scott Ward
Quality Assurance Unit

REVIEWED BY:

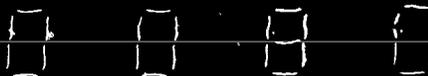
Peter Shuba, Ph.D.

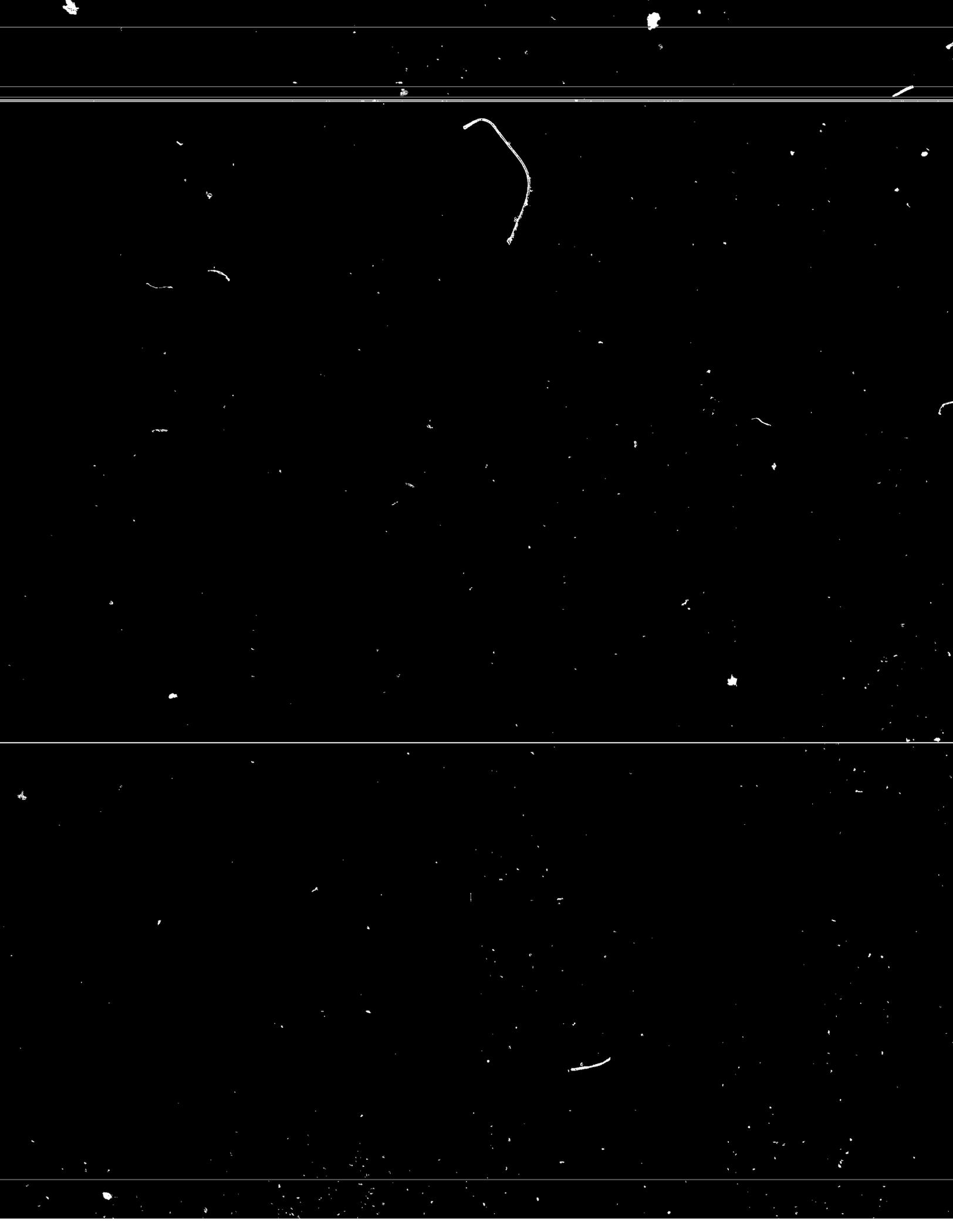
Peter Shuba
Project Coordinator

APPROVED BY:

Rod Parrish

Rod Parrish
Director





SRI International



Need new File #1

**TIME-INDEPENDENT TOXICITY STUDY ON THIOFIDE
USING RAINBOW TROUT AS THE TEST ORGANISM**

Final Report

8 September 1981

Submitted by:

**Howard C. Bailey, Director
Aquatic Toxicology Program**

Prepared for:

**MONSANTO INDUSTRIAL CHEMICALS COMPANY
800 N. Lindbergh Boulevard
St. Louis, Missouri 63166**

Attn: Ms. Barbara Heidolph

SRI Project LSC-1741

Monsanto Project No. SR-80-1803062-A1

Approved by:

David C. L. Jones
**David C. L. Jones, Director
Toxicology Laboratory**

W. A. Skinner
**W. A. Skinner, Vice President
Life Sciences Division**

THIOFIDE

Monsanto Project No. SR-80-1803062-A1

Introduction

At the request of Monsanto Industrial Chemicals Co., SRI performed a time-independent bioassay on Thiofide using rainbow trout as the test organism. Thiofide is a white powder that is supposedly soluble in water at 49-88 mg/L, depending on pH.

Methods

The test was performed in duplicate in 19-L glass aquaria under flow-through conditions using a Mount-Brungs diluter. Flow rate was set to provide 5 tank volumes per day. Juvenile rainbow trout were obtained from Mt. Lassen Trout Farm and maintained at SRI for 2 weeks prior to testing. At initiation of testing, fish were randomly selected and distributed 2 at a time among the test aquaria, for a total of 10 fish per replicate aquarium. Each day during the exposure period, they were fed frozen brine shrimp (Artemia salina) at a rate equal to 5% of their body weight. Because the range of toxicity needed to be established, fish were added only to the aquaria containing the controls and the lowest and highest concentrations.

Stock solutions were prepared initially by adding 1 g Thiofide to 100 mL acetone. Since Thiofide did not appear to be completely soluble in acetone at this concentration, the solvent was changed to N,N-dimethylformamide (DMF), which helped slightly. The material was added to the DMF, shaken, and allowed to settle for 20 minutes, after which the supernatant was decanted off and used to fill 30-mL gas-tight syringes. This solution was metered into the diluter by Sage syringe pumps. Since a 24-hour exposure to the 1 g Thiofide/100 mL DMF stock solution did not produce signs of toxicity, the stock concentration was increased to 10 g/100 mL. Initial pumping rate was 1.5 mL/hr, which was increased to 3.0 mL/hr after 48 hours of exposure, when

no signs of toxicity were apparent. A Mariotte bottle was used to deliver DMF to the solvent controls to obtain a nominal concentration of 150 µL/L. Nominal concentrations at the higher stock concentration and flow rate were: 0.0, 0.9, 1.9, 3.8, 7.5, and 15.0 mg/L in addition to the solvent control. The test was terminated after 13 days of exposure to these concentrations, during which no chemical-related mortality* occurred in the exposed fish. Dissolved oxygen (DO), pH, temperature, and chemical concentrations were monitored routinely in the tanks containing fish, alternating between the replicates. The pH was monitored with an Orion Model 407A Ionalyzer, and the DO with a YSI Model 54 dissolved oxygen meter. Temperature was monitored with a glass-mercury probe in addition to hourly readings taken on a Honeywell recording thermograph. Chemical concentrations were determined by reverse-phase liquid chromatography (Spectra-Physics 3500B) using the external standard technique under the following conditions:

Column: Waters Radial Pak A C₁₈
Mobile Phase: 50% acetonitrile, 50% water
Flow Rate: 2 mL/min
Detector Wavelength: 227 nm
Detector Sensitivity: 0.02 AUFs
Recorder Chart Speed: 0.2"/min
Injection Volume: 10-700 µL.

An effort was also made to estimate the precision and accuracy of the analytical method.

* Two fish jumped out of their tanks.

Results

Samples for chemical analysis were taken from the highest concentration (n = 6) and from the solvent stocks (n = 2). All samples from the highest concentration were below the level detectable by our techniques and probably contained less than 1 mg/L Thiofide. In contrast, the actual stock concentrations agreed very well with the nominal concentrations of 10 (actual 9.6) and 100 (actual 97) g/L. Since the syringe pumps and diluter were functioning properly, the toxicant must have entered the test system at close to nominal levels. This statement is verified by the slightly cloudy appearance of the water in the highest concentration. However, such cloudiness would indicate that the material was not in a soluble form. The octanol/water partition coefficient for Thiofide was estimated by SRI to be 4.5. This large a coefficient also suggests that the solubility of the chemical is less than 15 mg/L.

No mortalities related to the toxicant occurred in fish exposed to Thiofide at nominal concentrations of 0.9 and 15.0 mg/L. These results suggest that Thiofide is not acutely toxic within the range of concentrations tested and, based on the apparent suspension that occurred at a nominal concentration of 15.0 mg/L, is probably not acutely toxic up to its solubility limit. Data on water quality and fish length and weight are shown below.

| | <u>Mean</u> | <u>Standard Deviation</u> | <u>Range</u> | <u>n</u> |
|--------------------------------------|-------------|-------------------------------|--------------|----------|
| pH | 7.1 | 0.22 | 6.8-7.6 | 21 |
| DO (mg/L) | 8.9 | 1.60 | 3.2-12.0 | 21 |
| Temperature (°C) | 13.0 | 0 | -- | 21 |
| Conductivity (µmhos) | 81 | | | 1 |
| Hardness (mg/L CaCO ₃) | 28 | | | 1 |
| Alkalinity (mg/L CaCO ₃) | 28 | | | 1 |
| Acidity (mg/L CaCO ₃) | <5.0 | | | 1 |

| | |
|-----------------------------------|-----------------------------------|
| Fish Length (cm): \bar{x} : 8.2 | Fish Weight (g): \bar{x} : 3.56 |
| S.D.: 0.457 | S.D.: 0.619 |
| n: 10 | n: 10 |

We did not perform precision and accuracy studies on Thiofide because Thiofide appeared to be in equilibrium with other components also present in the test material and the equilibrium (or decomposition) appeared to be concentration-dependent. The problem is discussed below.

A high-pressure liquid chromatographic profile of Thiofide at 101 mg/L is shown in Figure 1. Of the five peaks observed, peak 3 had an identical retention volume as benzothiazole, and peak 4 was believed to be Thiofide.

Two experiments were performed, which were labeled A and B. Experiment A consisted of 15- to 90- μ L injections of 90.30 mg Thiofide/L. Experiment B consisted of 90- μ L injections of independently prepared standards ranging from 18.06-90.30 mg/L.

All injections were made under the same HPLC conditions as for other Thiofide analyses, and standards were prepared in CH_3CN with 2 mL CH_2Cl_2 , at a total volume of 50 mL (this proportion was found to be sufficient to keep Thiofide in solution).

For each experiment, we plotted mass injected versus peak area and the results are shown in Figures 2 through 5 for the five major peaks. Peaks 1 and 2 were not completely resolved and were treated as one peak. These graphs show that as the mass increases, the peak area increases, however at a faster rate in A than in B.

We then plotted mass injected versus percent of total peak areas for each peak in Figures 6 through 9 for Experiments A and B. For Experiment A the percent of total peak area remained constant. However, for Experiment B, the percent of total peak area increased for all peaks except for peak 4 (Thiofide), which decreased.

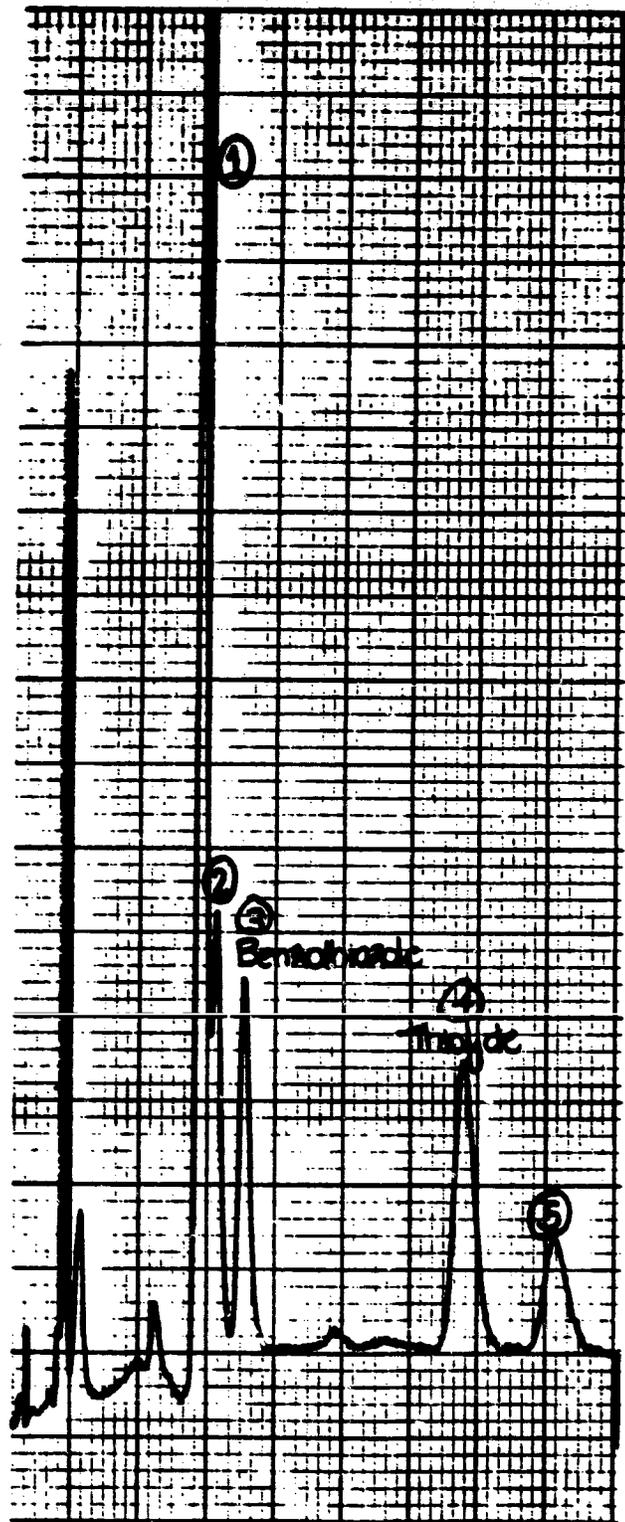
These results suggest that Thiofide is being converted to peaks 1, 2, 3, and 5, and the relative concentration of Thiofide to the other components decreases with increasing Thiofide concentrations. The variable relationship between peak area and concentration made it impossible to accurately assess Thiofide concentrations under test conditions. As a result, precision and accuracy studies were not performed because the values obtained could not be applied to unknown concentrations.

Copies of raw data associated with this report are appended.

FIGURES

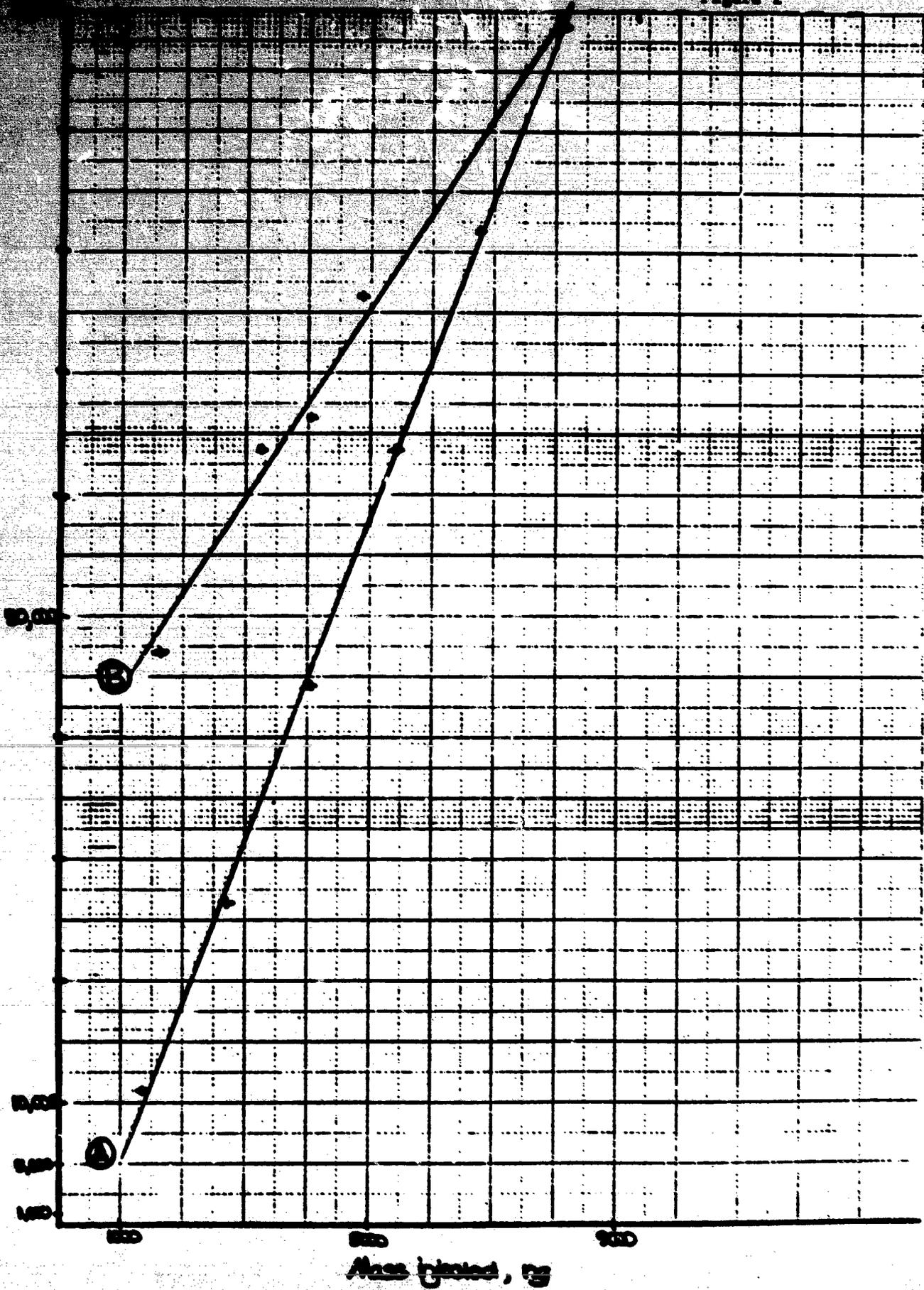
- Figure 1** High Pressure Liquid Chromatographic Profile of Thiofide at a Concentration of 101 mg/L
- Figure 2** Mass Injected Versus Peak Area for Peaks 1 and 2
- Figure 3** Mass Injected Versus Peak Area for Peak 3
- Figure 4** Mass Injected Versus Peak Area for Peak 4
- Figure 5** Mass Injected Versus Peak Area for Peak 5
- Figure 6** Mass Injected Versus Percent of Total Peak Area for Peaks 1 and 2
- Figure 7** Mass Injected Versus Percent of Total Peak Area for Peak 3
- Figure 8** Mass Injected Versus Percent of Total Peak Area for Peak 4
- Figure 9** Mass Injected Versus Percent of Total Peak Area for Peak 5

Figure 1



101.37 mg/l
60ul injection

Figure 2



Analysis

Figure 3

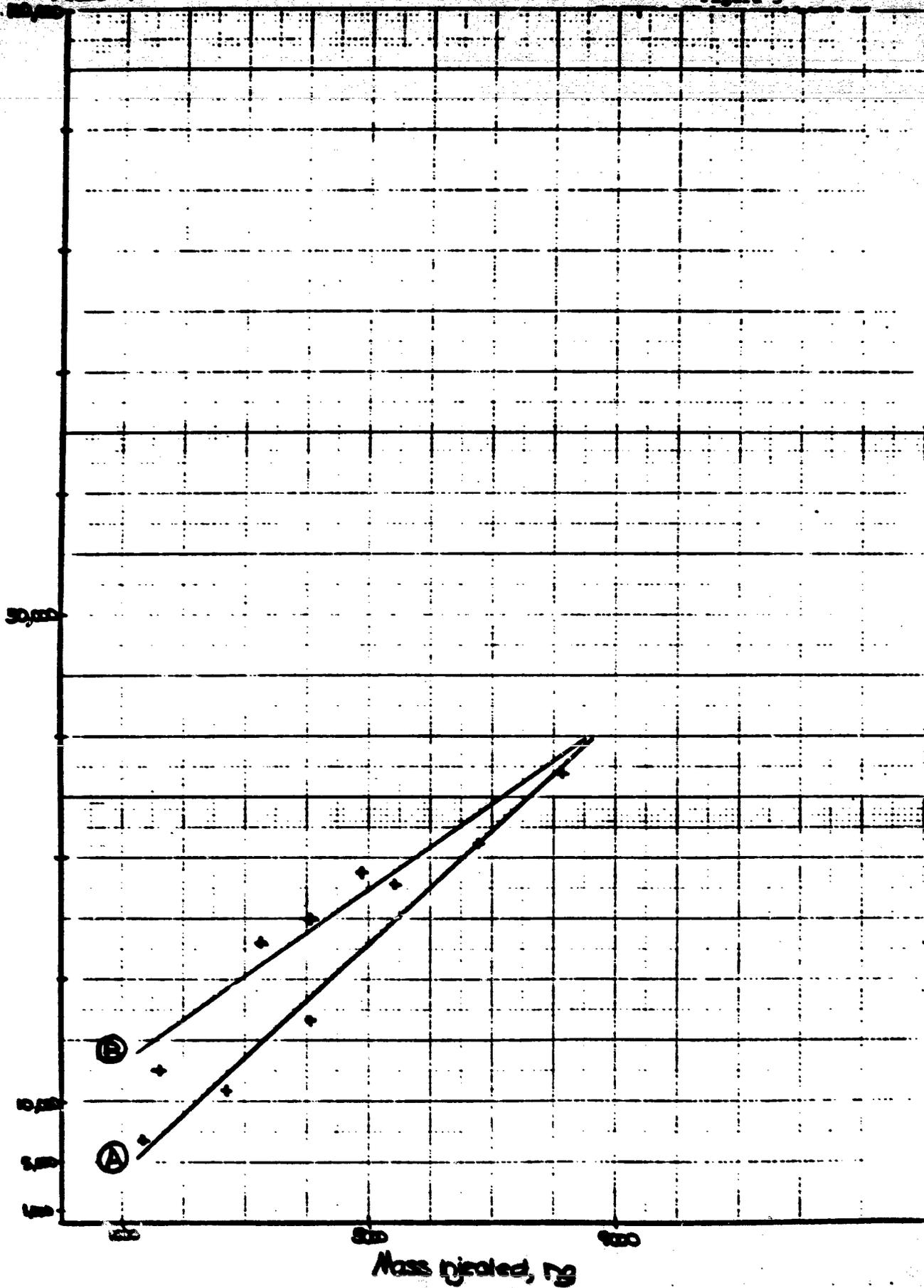
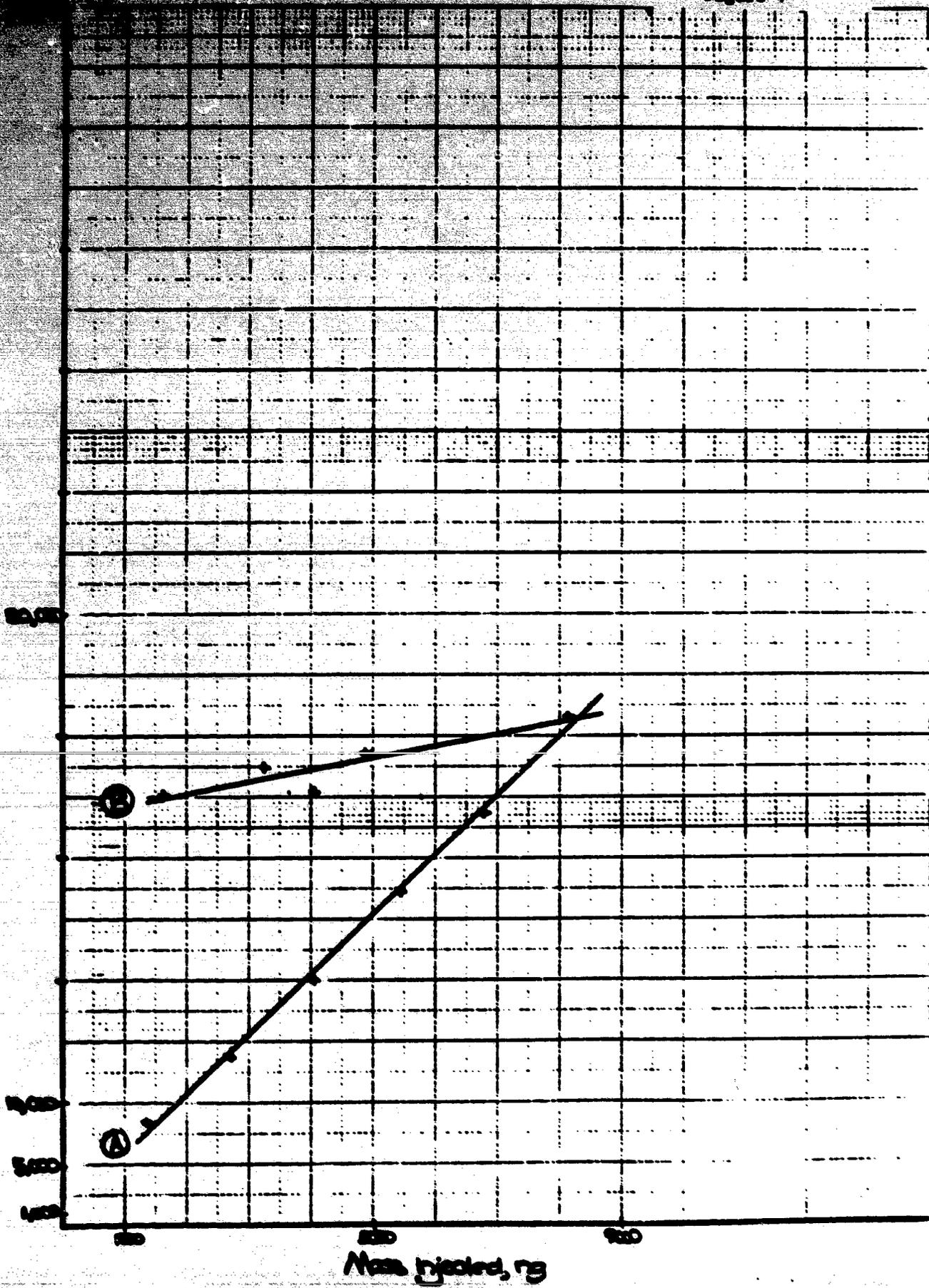
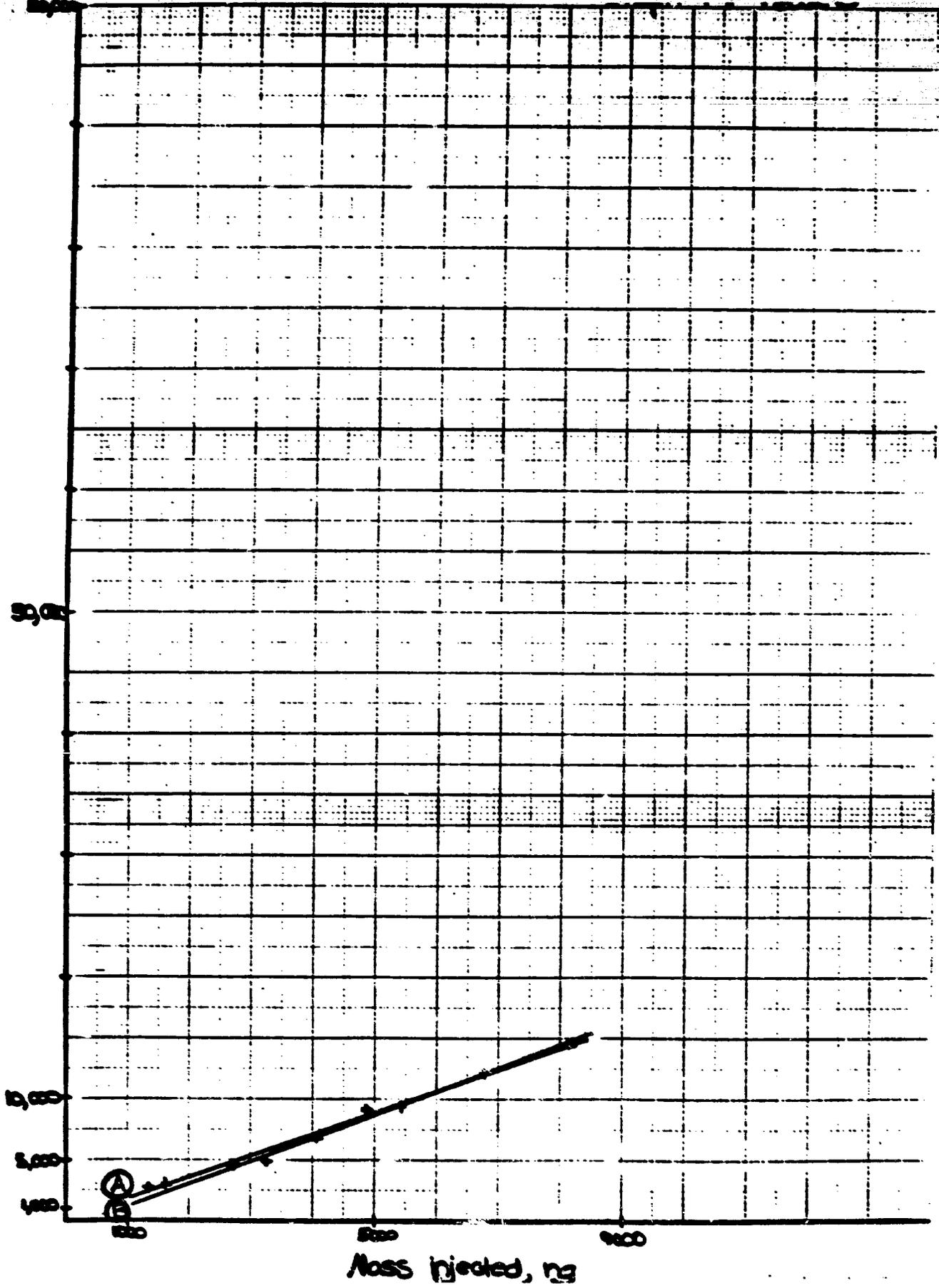


Figure 4



Analysis

Figure 5



SRI INTERNATIONAL
 Aquatic Toxicology Program
 TOXICANT ANALYSIS REQUEST FORM
 (Use one sheet per sample)

Project No. 174 Kind of Test 14 day Acute Tox
 Organism R. Trout Toxicant Thiofide
 Sample No. # 9 Tank No. stock Sampling Date 4-15-81
 Sampling Time 1:30 PM Day of Test 15 Sampler's Name JN
 Estimated Concentration 80.000 Units mg/l
 Measured Concentration 94 Units g/l
 Sample Submitted By Jane Math Date 4-15-81
 Sample Received By INAKUSUBA Date 4/15/81
 Sample Analyzed By INAKUSUBA Date 4/15/81
 Details recorded in Chemist's notebook no. 4291 Page 15

Instructions to Chemist 8 grams Thiofide + 100ml Methyl Dimethyl Formamide.
let stand & settle, stock decanted off top

(Yield 4 mg/l (nominal) high conc. - 4.85 actual)

Comments by Chemist _____

insarto

AUG 18 1983

FROM (NAME-LOCATION-PHONE) B. B. Heidolph - N1B - 4-4248

DATE : August 17, 1983

SUBJECT : TIME INDEPENDENT TOXICITY

REFERENCE :

TO : A. F. Werner - EISA

cc: W. J. Adams - N1B
W. E. Gledhill - N3A
B. J. Hill - EISA
DMEH Library - G2WF
(T. West)
J. P. Mieure - EISA

Attached is the final report from SRI International entitled "Time - Independent Toxicity Study on Thiofide using Rainbow Trout as the Test Organism", Project LSC-1741, No. SR-80-1803062-A1. The LC₅₀ values at 24, 96 and 336 hours were >1.0, >1.0, and >1.0 mg/L, respectively based upon measured exposure concentrations.

Analytical results suggest that extensive hydrolysis occurred during the study. 1.0 mg/L was the limit of detection for intact Thiofide.

B. B. Heidolph
B. B. Heidolph

Attachment

7.270/RNG.6



ANALYTICAL BIO CHEMISTRY LABORATORIES, INC.
P.O. Box 1097 Columbia, MO 65205 • (314) 474 9579

**Static Acute Bioassay Report
#23811**

Submitted To:

**Monsanto Chemical Company NIB
Attn: Dr. William J. Adams
800 N. Lindbergh Boulevard
St. Louis, Missouri 63166**

**Acute Toxicity of Thiofide (AB-79-1384364-1a)
to Fathead Minnow. (Pimephales promelas)**

August 27, 1979

Submitted By: Analytical BioChemistry Laboratories, Inc.
7200 East ABC Lane
P. O. Box 1097
Columbia, Missouri 65205

Prepared By:

Carl M. Thompson 8/30/79
Date
Carl M. Thompson
Aquatic Biologist

Alan D. Forbis 8/30/79
Date
Alan D. Forbis
Aquatic Supervisor

Approved By:

James A. Ault 8/30/79
Date
James A. Ault
Quality Assurance Officer

Lyle D. Johnson 8-30-79
Date
Lyle D. Johnson
Laboratory Manager

SUMMARY

The acute toxicity of Thiofide to fathead minnows (Pimephales promelas) was assessed using the methods outlined by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1). Water quality parameters of temperature, dissolved oxygen, pH and ammonia were measured throughout the test and were within acceptable limits.

As a quality check, the fathead minnows were challenged with a reference compound, Antimycin A. The observed 96 hour LC₅₀ and 95% confidence limits (C.I.) were within the 95% confidence limits reported in the literature (2), indicating that the fish were in good condition.

The results of the four day static fish toxicity study using fat-head minnows are summarized below.

| <u>Compound</u> | <u>96-hour LC₅₀ (95% C.I.)</u> |
|-----------------|---|
| Thiofide | >1,000 mg/l |
| Antimycin A | 0.000028 mg/l (0.000023-0.000034 mg/l) |

INTRODUCTION

This static bioassay was performed at the aquatic bioassay laboratory of Analytical BioChemistry Laboratories, Inc., Columbia, Missouri, for Monsanto Chemical Company, from August 23 to August 27, 1979, as authorized in a letter from Monsanto Chemical Company on February 1, 1979 (Appendix I). The purpose of this test was to determine the 24, 48 and 96 hour LC₅₀ levels for Thiofide to fathead minnows (Pimephales promelas). A preliminary range-finding test was conducted from August 7 to August 12, 1979, to determine the concentration range for the definitive bioassay. The study was performed following the procedures outlined in ABC Protocol Number 7601 (Appendix I) as approved by Dr. William J. Adams, Monsanto Chemical Company, on August 17, 1979.

METHODS AND MATERIALS

The procedures for static bioassay, as described in Standard Methods for Examination of Water and Wastewater (3) and Methods of Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians (1), were used in this experiment. The fathead minnows used in the test were obtained from Fattig Fish Hatchery in Brady, Nebraska. The fish were identified to species using the taxonomic keys developed by Eddy (4). All test fish were held in culture tanks on a 16 hour daylight photoperiod and observed for at least fourteen days prior to testing. Fish culture techniques used were basically those described by Brauhn et. al. (5). A daily record of fish observations during the holding period, along with prophylactic and therapeutic disease treatments, is included in Appendix I. During this period, the fish received a standard commercial fish food (Rangen's) daily until 48 hours prior to testing at which time feeding was discontinued. The fathead minnows used for this experiment had a mean weight of 0.23 g and a mean standard length of 22.5 mm. Weight and length measurements were made on the control group of fish at the termination of the test and are included in Appendix I.

The static fish bioassay was conducted in five gallon glass vessels containing 15 liters of laboratory well water with the characteristics shown in Table 1.

These vessels were kept in a water bath at 22°C (±1.0). The test fish were acclimated to the dilution water and test temperature and held without food 48 hours prior to testing.

A five day range-finding test was conducted to determine the concentration range for the definitive study. The preliminary test concentrations were set at 0.1, 1, 10, 100 and 560 mg/l. Based on the results of preliminary testing, the definitive test was conducted with a control versus duplicate test chambers at 1,000 mg/l. Ten fish per concentration were selected for definitive bioassay. The fish were added to the test chambers by random assignment within 30 minutes after addition of toxicant aliquots.

The Thiofide standard was received on August 22, 1979, in good condition. The sample upon receipt was observed to be a yellow powder

and was stored at 1°C. Sample purity was listed as 99+% active ingredient. Test concentrations were prepared based on the total compound. Before the test concentrations were prepared, the standard was allowed to warm to room temperature (22°C). The test concentrations were obtained by transferring appropriate weights of the test compound directly to the test chambers. The sample containers were rinsed with 10 ml of dimethylformamide (DMF) to effect a total delivery of the sample to the test chambers. The working solution for preliminary testing was prepared in DMF. The test material was observed to precipitate in the test chambers. It was noted that the concentration tested (1,000 mg/l) was above the solubility of Thiofide in water as reported in the protocol for this study (Appendix I). All standard weights and dilution values are listed in Appendix I.

RESULTS

Table 2 presents the predicted LC₅₀ values and 95% confidence intervals for Thiofide and the reference test against Antimycin A, a piscicide. These values were obtained by employing the statistical method described by Litchfield and Wilcoxon (6) or Stephan (9). Mortality rates, test concentrations and water quality data are presented in Table 3.

The dissolved oxygen concentration which stayed between 40% and 100% saturation was considered adequate for testing. The pH values remained consistent with the control throughout the study. The ammonia concentrations were below the toxic limit (7).

The study was conducted following the intent of the Good Laboratory Practice Regulations (8) and the final report was reviewed by Analytical BioChemistry Laboratories' Quality Assurance Unit. All original raw data was provided to Monsanto Chemical Company, with a copy retained at Analytical BioChemistry Laboratories.

TABLE 1

Chemical Characteristics of Well Water at
ABC's Aquatic Bioassay Laboratory

| <u>Parameter</u> | <u>Concentration</u> |
|----------------------------------|----------------------|
| Dissolved Oxygen | 9.3 ppm |
| pH | 8.2 |
| Hardness (CaCO ₃) | 255 ppm |
| Alkalinity (CaCO ₃) | 368 ppm |
| Conductivity | 50 μ mhos/cm |
| Total Ammonia (NH ₃) | <0.05 ppm |
| NO ₃ -N | 0.15 ppm |
| Ortho-Phosphate | 0.10 ppm |
| Aluminum | <0.01 ppm |
| Arsenic | <0.001 ppm |
| Cadmium | <0.001 ppm |
| Chromium | 0.001 ppm |
| Cobalt | <0.001 ppm |
| Copper | <0.01 ppm |
| Iron | 0.012 ppm |
| Lead | 0.009 ppm |
| Mercury | <0.0001 ppm |
| Nickel | 0.0157 ppm |
| Zinc | <0.01 ppm |
| DDVP | <40 ng/l |
| Diazinon | <20 ng/l |
| Disyston | <20 ng/l |
| Methyl Parathion | <80 ng/l |
| Malathion | <110 ng/l |
| Ethyl Parathion | <80 ng/l |

TABLE 2

The Acute Toxicity of Thiofide and Antimycin A
to Fathead Minnows (Pimephales promelas)

| Compound | LC ₅₀ in milligrams/liter (ppm) | | |
|----------------|--|-----------------------------------|-----------------------------------|
| | 24 hours | 48 hours | 96 hours |
| Thiofide | >1,000 | >1,000 | >1,000 |
| Antimycin A*** | 0.000048 (0.000040-0.000057)** | 0.000032 (0.000026-0.000039)** | 0.000022 (0.000023-0.000024)** |

*Bioassay as conducted at 22°C (±1.0), mean weight and length, 0.23 g and 22.5 mm.

**95% confidence interval.

***Antimycin A standard obtained from Sigma Chemical Company, Type III, crystalline, Lot 125C-0152.

TABLE 3

Mortality Rates and Water Quality Measurements During the Acute Toxicity Test of Thiofide to Fathead Minnows (Pimephales promelas)

| Concentration mg/l | Percent Mortality Hours | | Water Quality | | | | | | | | | | | |
|-----------------------|----------------------------|----|---------------|---------------|------|-------------|---------------|------|-------------|---------------|------|------|------|--|
| | 24 | 48 | 0 hours | | | 48 hours | | | 96 hours | | | | | |
| | | | Temp. °C | D.O.* mg/l | pH** | Temp. °C | D.O.* mg/l | pH** | Temp. °C | D.O.* mg/l | pH** | | | |
| Control | 0 | 0 | 22 | 9.6 | 8.2 | 22 | 8.4 | 8.0 | 22 | 7.3 | 8.2 | 0.21 | 0.21 | |
| 1,000 A | 0 | 0 | 22 | 9.4 | 8.2 | 22 | 8.2 | 8.2 | 22 | 6.6 | 8.2 | 0.20 | 0.20 | |
| 1,000 B | 0 | 10 | 22 | 9.4 | 8.2 | 22 | 8.2 | 8.3 | 22 | 6.6 | 8.2 | 0.20 | 0.20 | |

*Dissolved oxygen concentrations - Dissolved Oxygen Probe (Extech Model 8012) used with an Extech Model 671 pH and mV meter.

**pH - pH Probe (Fisher Model 13-639-108) used with an Extech Model 671 pH and mV meter.

***Total ammonia concentrations - Ammonia Probe (Extech Model 8002-8) used with an Extech Model 671 pH and mV meter.

LITERATURE CITED

- (1) Committee on Methods for Toxicity Tests with Aquatic Organisms (C. E. Stephan, Chairman). 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians. Environmental Protection Agency, Ecological Research Series EPA-660/3-75-009, April, 1975. 61 p.
- (2) Berger, B. L., R. E. Lennon and J. W. Hogan. 1969. Laboratory Studies on Antimycin A as a fish toxicant. U. S. Department of Interior, Investigations in Fish Control No. 26. 21 p.
- (3) American Public Health Association. 1975. Standard Methods for the Examination of Water and Wastewater. 14th ed. Washington, DC. 1193 p.
- (4) Eddy, Samuel. 1969. The Freshwater Fishes. 2nd ed. W. C. Brown Company, Dubuque, IA. 286 p.
- (5) Brauhn, J. L. and R. A. Schoettger. 1975. Acquisition and Culture of Research Fish: Rainbow Trout, Fathead Minnows, Channel Catfish and Bluegills. Environmental Protection Agency, Ecological Research Series EPA-660/3-75-011, May, 1975. 45 p.
- (6) Litchfield, J. T., Jr. and F. Wilcoxon. 1949. A Simplified Method of Evaluating Dose-Effect Experiments. Jour. Pharm. Exp. Ther. 96:99113.
- (7) National Academy of Sciences. 1971. Water Quality Criteria, 1972. U. S. Department of Commerce, PB-236 199. 592 p.
- (8) Food and Drug Administration. Regulations for Good Laboratory Practice. Federal Register, Vol. 43, No. 247, 59986-60025, December 22, 1978.
- (9) Stephan, C. 1977. Methods for calculating an LC50, p. 65-84. In F. L. Mayer and J. L. Hamelick (eds.). Aquatic Toxicology and Hazard Evaluation. ASTM Special Technical Publication 634. ASTM. Philadelphia.

Quality Assurance Statement for final report #23811 entitled, "Acute Toxicity of Thiofide to Fathead Minnows (Pimephales promelas)," for Dr. William J. Adams, Monsanto Chemical Company, St. Louis, Missouri.

In accordance with ABC Laboratories intent that all studies conducted at our facilities are designed and function in conformance with good laboratory practice regulations and the protocols for individual laboratory studies, an inspection of the final report for Thiofide was conducted and found to be in an acceptable form by a member of our Quality Assurance Unit. An inspection of the daily mortality rate of the test organisms prior to the initiation of the study indicated they were in good health and should not bias the observed mortality in the study. A final inspection of all data and records on August 28, 1979, indicated that the report submitted to you is an accurate reflection of the study as it was conducted by ABC Laboratories.

Should you have any questions relating to the information provided in this statement or the function of our Quality Assurance Unit, please contact me at your convenience.

James A. Ault
James A. Ault
Quality Assurance Officer

8/30/79
Date

APPENDIX I
RAW DATA

ANALYTICAL BIOCHEMISTRY LABS
Aquatic Toxicology Division
ACUTE TOXICITY BIOASSAY

Toxicant Thiophide Test Species Fathead Minnow (Lot #2779) Study No. 23811
 Date Initiated 8/23/79 Time 2:00 pm Date Terminated 8/27/79
 Dilution Water Well H₂O No./Vessel 10 Vessel Size 15.0

| Test Conc. mg/l (ppm) | MORTALITY AND BEHAVIORAL OBSERVATIONS | | | | | | | | | |
|--------------------------|---------------------------------------|------|--------|------|--------|------|--------|------|------|------|
| | 24 hr. | | 48 hr. | | 72 hr. | | 96 hr. | | Dead | Obs. |
| | Dead | Obs. | Dead | Obs. | Dead | Obs. | Dead | Obs. | | |
| Control | 0 | | 0 | | 0 | | 0 | | | |
| 1000 A | 0 | | 0 | | 0 | | 0 | | | |
| 1000 B | 0 | | 1 | | 1 | | 1 | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| Observer | CT | | WJH | | WJH | | CT | | | |
| Date | 8/24 | | 8-25 | | 8-26 | | 8/27 | | | |

Remarks: A direct wt. of 15g was added to each test chamber with the addition of 10ml of DMF. There was a formation of a white precipitate.

Prepared By: Jan Duffin Checked By: Carl H. Hesse

Monsanto

MONSANTO INDUSTRIAL CHEMICALS CO.
800 N. Lindbergh Boulevard
St. Louis, Missouri 63166
Phone: (314) 684-1000

February 1, 1979

Mr. Alan Forbis
ABC Laboratories
7200 East ABC Lane
P.O. Box 1097
Columbia, MO 65201

Dear Alan:

I would like you to conduct several aquatic toxicity tests as described in the attached toxicity agreement form.

These products should not present any special problem which would require special handling other than good hygienic practices. Information on the solubility of these materials will be provided at a later date. I suggest you use acetone whenever a solvent carrier is required. I prefer well water in place of reconstituted water. Please conduct the Daphnia magna studies using duplicate concentrations with 10 Daphnia per beaker. The required protocol for all studies is the EPA Methods For Acute Toxicity Tests With Fish, Macro-invertebrates and Amphibians (1972).

Our previous confidentiality agreement with ABC Laboratories will be considered binding.

Please send me four copies of the final report, all of which should contain copies of the raw data. Each report should contain the project number for the corresponding chemical and test as shown on the attached toxicity agreement form. The chemicals will be shipped in 1-3 weeks.

Please sign both copies of the toxicity agreement form and return the original to me. Please call me if you have any questions.

Sincerely,

William J. Adams
William J. Adams

1st

MONSANTO INDUSTRIAL CHEMICALS COMPANY
ENVIRONMENTAL SCIENCES SECTION

OUTSIDE TOXICITY
STUDIES AGREEMENT

Study Request

This is a request and the authorization for ABC Laboratories to conduct several aquatic toxicity studies as indicated below.

| <u>Chemical</u> | <u>Type of Test</u> | <u>Species</u> | <u>Project Number</u> | <u>Cost</u> |
|-----------------|---------------------|----------------------|-----------------------|-------------|
| 4-NPDA | Static Acute | fathead minnow | AB-79-1384357-1a | 300 |
| 4-NPDA | Static Acute | <u>Daphnia magna</u> | AB-79-1384357-1b | 300 |
| DPG | Static Acute | fathead minnow | AB-79-1384358-1a | 300 |
| DPG | Static Acute | rainbow trout | AB-79-1384358-1b | 300 |
| DPG | Static Acute | bluegill | AB-79-1384358-1c | 300 |
| DPG | Static Acute | <u>Daphnia magna</u> | AB-79-1384358-1d | 300 |
| Santocure | Static Acute | fathead minnow | AB-79-1384359-1a | 300 |
| Santocure | Static Acute | <u>Daphnia magna</u> | AB-79-1384359-1b | 300 |
| Cyclohexylamine | Static Acute | fathead minnow | AB-79-1384360-1a | 300 |
| Cyclohexylamine | Static Acute | rainbow trout | AB-79-1384360-1b | 300 |
| Cyclohexylamine | Static Acute | bluegill | AB-79-1384360-1c | 300 |
| Cyclohexylamine | Static Acute | <u>Daphnia magna</u> | AB-79-1384360-1d | 300 |
| Santoflex 77 | Static Acute | fathead minnow | AB-79-1384361-1a | 300 |
| Santoflex 77 | Static Acute | <u>Daphnia magna</u> | AB-79-1384361-1b | 300 |
| Santoflex DD | Static Acute | fathead minnow | AB-79-1384362-1a | 300 |
| Santoflex DD | Static Acute | <u>Daphnia magna</u> | AB-79-1384362-1b | 300 |
| Flectol Flakes | Static Acute | fathead minnow | AB-79-1384363-1a | 300 |
| Flectol Flakes | Static Acute | rainbow trout | AB-79-1384363-1b | 300 |
| Flectol Flakes | Static Acute | bluegill | AB-79-1384363-1c | 300 |
| Flectol Flakes | Static Acute | <u>Daphnia magna</u> | AB-79-1384363-1d | 300 |
| Thiofide ✓ | Static Acute | fathead minnow ✓ | AB-79-1384364-1a | 300 |
| Thiofide | Static Acute | <u>Daphnia magna</u> | AB-79-1384364-1b | 300 |

| <u>Material</u> | <u>Type of Test</u> | <u>Species</u> | <u>Project Number</u> | <u>Cost</u> |
|-----------------------|---------------------|----------------------|-----------------------|-------------|
| Carbazole Crystals | Static Acute | fathead minnow | AB-79-1384322-3b | 300 |
| Santocure IS | Static Acute | fathead minnow | AB-79-1384315-3a | 300 |
| Thiostat | Static Acute | fathead minnow | AB-79-1384365-1a | 300 |
| Thiostat | Static Acute | <u>Daphnia magna</u> | AB-79-1384365-1b | 300 |

To Be Performed By: ABC Laboratories

Assigned Project Number: See Above

Expected Date of Report: Studies to be completed as time allows. The testing sequence is from top to bottom. All tests should be completed by 8-1-79.

Cost:

Manner of Payment: Direct payment by check upon receipt of the final report.

William J. Adams 8-5-79
 William J. Adams
 Project Director
 Date

Paul R. Graham 2/21/79
 Paul R. Graham
 Manager, Product Acceptability
 Rubber Chemicals Division
 Date

Lyle Johnson 2-26-79
 Contract Laboratory Director
 Date



ANALYTICAL BIO CHEMISTRY LABORATORIES, INC.
P.O. Box 1097 • Columbia, MO 65205 • (314) 474-8579

ABC PROTOCOL NO. 7601
(Revised June 21, 1979)

STATIC BIOASSAY PROCEDURE FOR DETERMINING THE ACUTE TOXICITY
OF CHEMICAL SUBSTANCES TO FRESHWATER FISH

ABC Study Number 23811

Test Material Thiofide (Study No. AB-79-1384364-1a)

Test Species Fathead Minnows (Pimephales promelas)

1.0 INTRODUCTION

Aquatic toxicity tests have been used extensively in the assessment of the environmental effects of chemical substances. Indeed, aquatic bioassays are required by federal laws such as the Toxic Substances Control Act (1), FIFRA (2), and the Clean Water Act of 1977 (3). With the testing guidelines for these laws in mind, as well as EPA's Good Laboratory Practice Regulations (4) which complement them, Analytical BioChemistry Laboratories, Inc. has prepared the following protocol. The static bioassay method presented here was patterned after procedures that were formulated by the U. S. Environmental Protection Agency (5), American Public Health Association (6), and the American Society for Testing and Materials (7).

2.0 OBJECTIVES

The primary objective of the toxicity test described herein is to evaluate the acute toxicity of a chemical substance to freshwater fish under static conditions. This is achieved by determining LC₅₀ levels of the toxicant during a 96 hour exposure period. An LC₅₀ is the approximate concentration of the test material that produces 50 percent mortality of test fish after prescribed intervals. The method is designed to yield LC₅₀ values following 24, 48 and 96 hours of exposure.

3.0 TESTING FACILITY

The study will be conducted by the Aquatic Toxicology Division of Analytical BioChemistry Laboratories, Inc., 7200 East ABC Lane, P. O. Box 1097, Columbia, Missouri 65205.

4.0 RANGE-FINDING STUDY

4.1 General. For most chemical substances, the approximate toxic level to aquatic organisms is not known. Because this information is essential before a definitive toxicity test can be conducted, ABC routinely performs range-finding tests for static bioassays with fish. The information derived from this preliminary test will be used to set concentration levels for the definitive bioassay described in section 5.0.

4.2 Test Fish. The fish species to be used should be selected by the study sponsor from the list of recommended species in Table 1. The most common species used for toxicity testing includes bluegill sunfish (Lepomis macrochirus), rainbow trout (Salmo gairdneri), and fathead minnows (Pimephales promelas). Fish ranging in size from actively-feeding fry (~0.2 g) to 5.0 g will be used as test organisms. The fish standard length of the largest fish will be no more than twice that of the shortest fish. The fish will be obtained from an established commercial hatchery or in-house cultures. The particular source to be used is dependent upon the seasonal availability of test fish and will be listed at the time of protocol approval in the test-specific information of section 8.5, along with the species selected by the study sponsor.

The test fish lot will be from the same year class and will be identified to species using the taxonomic keys developed by Eddy (8) and cultured following the techniques described by Brauhn et al. (9). All test fish will be held on a 16-hour daylight photoperiod and observed for at least 14 days prior to testing. A daily record of fish observations during the holding period, along with prophylactic and therapeutic disease treatments, will be kept and included with the final report. During the holding period, they will receive a standard commercial fish food (Rangen's[®]) at a maintenance rate of 3 to 5 percent of their body weight per day. The test lot will be held without food and acclimated to the test temperature for 48 hours prior to testing. If mortality of the test lot exceeds 10 percent in the 48 hours previous to testing, the fish will not be used. Previous to or concurrent with the test, the fish lot will be challenged against a reference compound, Antimycin A*, to determine their general health and suitability as test organisms. The results of the Antimycin A test will be compared against published toxicity values (10).

4.3 Test System. The range finding test will be conducted in five gallon widemouth glass jars containing 15 liters of test solution and five fish per concentration. These test vessels will be immersed in a circulating water bath with temperatures maintained within $\pm 1^{\circ}\text{C}$ of the desired test temperature (Table 1). For temperature control in the water bath, thermostatically controlled heating elements will be used for warmwater tests ($22 \pm 1^{\circ}\text{C}$) and refrigeration units (MinoCool[®]) for coldwater bioassays ($12 \pm 1^{\circ}\text{C}$). The dilution water used will be a soft reconstituted water of the following makeup in deionized water: 48 mg/l NaHCO_3 , 30 mg/l $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 30 mg/l MgSO_4 , and 2.0 mg/l KCl . The water quality parameters of this dilution water are: pH: 7.2-7.6; total hardness: 40-48 mg/l CaCO_3 ; and total alkalinity: 30-35 mg/l CaCO_3 . Also available, at the sponsor's request, is an alternate dilution water from a deep well source with chemical and physical characteristics shown in Table 3.

4.4 Test Material. Specific information regarding the test material is to be supplied by the sponsor and will be addressed at the time of protocol approval in section 8.3. The test concentrations will be prepared on a weight/volume basis unless otherwise specified. A record of all sample weights and dilutions will be kept, checked by a second party, and furnished in the final report.

4.5 Test Procedure. The rangefinding procedure is as follows:

4.5.1 Test fish will be acclimated to the test temperature and dilution water for at least 48 hours prior to testing, during which time they will be held without food.

4.5.2 The range-finding test will be initiated by exposing groups of five fish to at least three toxicant concentrations

*Antimycin A standard obtained from Sigma Chemical Company, Type III, crystalline, Lot 125C-0152.

spaced by a factor of 10. The test fish will be placed in the test chambers by stratified random assignment within 30 minutes after solution preparations. The initial toxicant concentrations most often used are 1, 10 and 100 mg/l. Numerous static tests by ABC have shown that a significant percentage of the compounds tested have aquatic toxicities which fall within this range.

4.5.3 After 24 hours of exposure, the test chambers will be observed for mortality and/or adverse behavioral effects. Dead individuals will be removed at each observation and a record maintained of mortality and abnormal behavior. Dependent upon this observation, additional test concentrations may be added at levels above or below the initial concentrations. This procedure will be followed until a toxic range is determined. For example, if the 24 hour exposure results in total mortality, new solutions will be prepared at a factor of 10 below the lowest initial concentration until no mortality or partial mortality is reached. In the converse situation, if no mortality is observed after 24 hours, new solutions will be added at concentrations spaced by a factor of 10 above the highest initial level until mortality is noted. In this manner, a bracket is formed for the toxic range of the compound.

4.5.4 The preliminary test will be conducted for a period of 24 to 96 hours - the exact duration dependent upon the results of the initial concentrations tested. In most cases, a preliminary test for 48 hours at 3 toxicant concentrations is sufficient to determine the toxic range.

4.5.5 Results of the range-finding study will be used to set the concentration range of the definitive study described in section 5.0. At least five toxicant concentrations selected from the logarithmic scale presented in Table 2, which fall within the preliminary test range, have proven to be adequate in assessing most compounds.

5.0 DEFINITIVE STUDY

5.1 General. Following the preliminary range-finding study discussed in section 4.0, the definitive test will be conducted by the procedures described below. Test-specific information regarding the sponsor, test material, test fish, proposed study dates, study personnel and study approvals will be included in section 8.0 at the time of protocol approval.

5.2 Test Fish. Aspects concerning the acquisition, culture and acclimation of test fish will be the same as discussed in section 4.2.

5.3 Test System. For all definitive testing, the test vessel size will conform to the maximum loading limitation of 0.8 grams of fish per liter of solution (5). One of the following types of glass test chambers will be used: (a) 5 gallon glass jars containing 15 liters of solution, (b) 40 liter aquaria containing 30

liters of solution, or (c) 100 liter aquaria containing 75 liters of solution. ABC will select the type of chamber to be used. In most instances, the test chambers used will be 40 liter glass aquaria containing 30 liters of solution, which is a recommended test vessel for static bioassays (5). The water bath system described in section 4.3 will be used to control test temperatures. The test dilution water will be the same as discussed in section 4.3.

5.4 Test Material. Specific information regarding the test material is to be supplied by the sponsor and will be addressed in section 8.3 at the time of protocol approval.

5.5 Test Procedure-Biological. The basic test procedure for the definitive bioassay will be as follows:

5.5.1 The test fish will be acclimated to the test temperature and dilution water for at least 48 hours prior to testing, during which time they will be held without food.

5.5.2 The definitive test will be initiated by exposing groups of 10 organisms to at least 5 toxicant concentrations and a dilution water control. The test concentrations used will be based upon the results of the range-finding test and will be selected from one of the logarithmic series presented in Table 2. The exact concentrations to be used will be addressed in section 8.4 at the time of protocol approval. If a solvent is used in the preparation of test solutions, the control chamber will receive an aliquot of the solvent equivalent to the highest amount used in the test chambers. The test organisms will be placed in the test chambers by stratified random assignment within 30 minutes after solution preparations.

5.5.3 As an alternate test design, duplicate 5 gallon jars per concentration containing 15 liters of solution and 5 fish each may be used, if so authorized by the sponsor.

5.5.4 The test chambers will be observed for mortality and/or adverse behavioral effects every 24 hours. Dead individuals will be removed at each observation and a record maintained of mortality and abnormal behavior for each concentration tested.

5.5.5 Length and weight measurements will be made on either a representative group of the test fish prior to testing or on the control group at test termination.

5.6 Test Procedure-Chemical and Physical. Water quality parameters of temperature, dissolved oxygen, pH and ammonia will be monitored throughout the test. Measurements of temperature, dissolved oxygen and pH will be made at 0, 48 and 96 hours of testing in the control, low concentration and highest concentration with surviving fish. Ammonia levels will be determined in the same test chambers at 0 and 96 hours. If at any point in the study dissolved oxygen levels are observed to be below or approaching 40 percent saturation, ABC will contact the study director for authorization

to artificially aerate the test chambers with compressed air for the duration of the study. The authorization procedure used will be that described in section 7.0.

5.7 Analysis of Results. The results of the definitive study will be statistically analyzed for 24, 48 and 96 hour LC₅₀ values and their corresponding 95 percent confidence limits. For data sets with one or more partial mortalities, in addition to 0 and 100 percent response levels, the statistical method described by Litchfield and Wilcoxon (11) will be employed. If no partial mortalities are represented, i.e. if the test yields only 0 and 100 percent mortalities at any observation period, a binomial test (12) will be applied for the LC₅₀ estimation.

5.8 Report. A final report of the definitive study will be submitted to the study sponsor and will include the following. A draft of the final report will be submitted for sponsor review if so requested at an additional charge.

5.8.1 Study dates of both preliminary and definitive phases.

5.8.2 Objectives and test methods.

5.8.3 Reference to the statistical methods used for data analysis.

5.8.4 Description of test material (date of receipt, storage conditions, purity, physical characteristics, and method of preparing test concentrations).

5.8.5 Description of test design.

5.8.6 Summary of the data analysis, mortality observations and test water quality.

5.8.7 Location of raw data.

5.8.8 List and signatures of study personnel.

5.8.9 Statement by ABC's Quality Assurance Unit.

5.8.10 The report appendix will contain the original raw data for mortality observations and water quality, results of the Antimycin A reference test, records of fish size and daily holding log, letter of test authorization, letters of authorized protocol changes, and a copy of the approved protocol.

5.9 Data Retention. All original raw data generated in the preliminary and definitive studies will be provided to the study sponsor in the appendix to the final report. A copy of the data will be retained in ABC's archives.

6.0 PROTOCOL CHANGES

In the event that modifications of this protocol are deemed necessary, a written statement of any changes and reason(s) proposed by the study sponsor or ABC will be submitted to the other party. All agreed changes will be expressed in writing, signed and dated by the sponsor's study director. The signed changes will be appended to the protocol and included with the final report.

7.0 SPONSOR AUTHORIZATIONS DURING THE STUDY

Should a problem develop while the study is in progress, ABC will notify the study director within 24 hours. The problem and suggested test modifications will be discussed by telephone. ABC will proceed with the changes felt necessary upon the verbal authorization of the study director. A letter for written authorization will then be submitted by ABC to the study director and handled in the same manner discussed in section 6.0.

8.0 TEST-SPECIFIC INFORMATION

8.1 General. The following items will be addressed for each static bioassay. This information is necessary to be in compliance with Good Laboratory Practice Regulations (4). Sections 8.2 and 8.3 are to be completed by the study sponsor. Sections 8.4, 8.5, 8.6 and 8.7 will be completed by ABC.

8.2 Study Sponsor:

8.2.1 Company Monsanto Chemical Company

8.2.2 Address 800 N. Lindbergh Boulevard
St. Louis, Missouri 63166

8.2.3 Study Director (Coordinator)

| <u>Name</u> | <u>Title</u> |
|-----------------------------|-----------------------|
| <u>Dr. William J. Adams</u> | <u>Study Director</u> |

8.3 Test Material:

8.3.1 Name Thiofide (Study No. AB-79-1384364-1a)

8.3.2 Code Number AB-79-1384364-1a

8.3.3 Physical Description yellow powder

8.3.4 Purity 99.7% active Thiofide

8.3.5 Stability Stable to heat & light

8.3.6 Recommended Solvent acetone

8.3.7 Water Solubility ~ 5ppm

8.3.8 Handling Precautions No special handling required

8.4 Test Concentrations:

8.4.1 Definitive Concentrations 1,000 mg/l (duplicate)

8.5 Test Fish:

8.5.1 Species Fathead Minnows (Pimephales promelas)

8.5.2 Supplier Fattig Fish Hatchery
Brady, Nebraska

8.6 Study Dates:

8.6.1 Proposed starting date of definitive study 8/22/79

8.6.2 Proposed completion date of definitive study 8/26/79

8.7 ABC Study Personnel:

8.7.1 Study Director

Carl M. Thompson Aquatic Biologist
Name Title

8.7.2 Principle Investigator

Jerry R. Griffen Biologist
Name Title

8.7.3 Quality Assurance Officer

James A. Ault Quality Assurance Officer
Name Title

8.8 Protocol Approvals. The following is to be signed by the appropriate study personnel:

8.8.1 Sponsor's Study Director

William J. Adams S. Res. Biologist 8-17-79
Name Title Date

8.8.2 ABC's Study Director

Carl M. Thompson Study Director 8/15/79
Name Title Date

8.8.3 ABC's Laboratory Director

[Signature] Lab Manager 8/15/79
Name Title Date

TABLE 1: RECOMMENDED SPECIES AND TEST TEMPERATURES^a

| Recommended Species | Recommended Test Temperature (°C) |
|---|-----------------------------------|
| Rainbow Trout, <u>Salmo gairdneri</u> | 12 |
| Brook trout, <u>Salvelinus fontinalis</u> | 12 |
| Bluegill, <u>Lepomis macrochirus</u> | 22 |
| Fathead minnow, <u>Pimephales promelas</u> | 22 |
| Channel catfish, <u>Ictalurus punctatus</u> | 22 |
| Carp, <u>Cyprinus carpio</u> ^b | 22 |

^aAdapted from (5).

^bRecommended for compounds used in Japan.

TABLE 2: Guide to selection of experimental concentrations based on progressive bisection of intervals on logarithmic scale.

| <u>Col. 1</u> | <u>Col. 2</u> | <u>Col. 3</u> | <u>Col. 4</u> | <u>Col. 5</u> |
|---------------|---------------|---------------|---------------|---------------|
| 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| | | | 7.5 | 8.7 |
| | | | | 7.5 |
| | | 5.6 | 5.6 | 6.5 |
| | | | 4.2 | 5.6 |
| | | | | 4.9 |
| | | | 3.2 | 4.2 |
| 3.2 | 3.2 | 3.2 | 3.2 | 3.7 |
| | | | | 3.2 |
| | | | 2.4 | 2.8 |
| | | | | 2.4 |
| | | 1.8 | 1.8 | 2.1 |
| | | | | 1.8 |
| | | | 1.35 | 1.55 |
| | | | | 1.35 |
| 1.0 | 1.0 | 1.0 | 1.0 | 1.15 |
| | | | | 1.0 |

TABLE 3: Chemical characteristics of well water at ABC's Aquatic Bioassay Laboratory.

| <u>Parameters</u> | <u>Concentration</u> |
|----------------------------------|----------------------|
| Dissolved Oxygen | 9.3 ppm |
| pH | 8.2 |
| Hardness (CaCO ₃) | 255 ppm |
| Alkalinity (CaCO ₃) | 368 ppm |
| Conductivity | 50 umhos/cm |
| Total Ammonia (NH ₃) | <0.05 ppm |
| NO ₃ -N | 0.15 ppm |
| Ortho-Phosphate | 0.10 ppm |
| Aluminum | <0.01 ppm |
| Arsenic | <0.001 ppm |
| Cadmium | <0.001 ppm |
| Chromium | 0.001 ppm |
| Cobalt | <0.001 ppm |
| Copper | <0.01 ppm |
| Iron | 0.012 ppm |
| Lead | 0.009 ppm |
| Mercury | <0.0001 ppm |
| Nickel | 0.0157 ppm |
| Zinc | <0.01 ppm |
| DDVP | <40 ng/l |
| Diazinon | <20 ng/l |
| Disyston | <20 ng/l |
| Methyl Parathion | <80 ng/l |
| Malathion | <110 ng/l |
| Ethyl Parathion | <80 ng/l |

9.0 REFERENCES

- (1) U.S. Congress. 1976. Toxic Substances Control Act. Public Law 94-469. Federal Register, October 11, 1976. 2003-2051.
- (2) U.S. Environmental Protection Agency. 1978. Registration of pesticides in the United States, proposed guidelines. Federal Register, July 10, 1978: 29696-29741.
- (3) U.S. Congress. 1977. Clean Water Act of 1977. Public Law 95-217. Federal Register, December 27, 1977: 1566-1611.
- (4) Food and Drug Administration. 1978. Regulations for Good Laboratory Practice. Federal Register 43(247), December 22, 1978: 59986-60025.
- (5) Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians. Environmental Protection Agency, Ecological Research Series EPA-660/3-75-009, April, 1975. 61 p.
- (6) American Public Health Association. 1975. Standard Methods for the Examination of Water and Wastewater. 14th ed. Washington, D.C. 1193 p.
- (7) American Society for Testing and Materials. 1978. Proposed standard practices for conducting basic acute toxicity tests with fish, macroinvertebrates and amphibians. Draft No. 7, April 27, 1978, ASTM Committee E-35.21. 54 p.
- (8) Eddy, Samuel. 1969. The Freshwater Fishes. 2nd ed. W. C. Brown Company, Dubuque, IA. 286 p.
- (9) Drauhn, J. L. and R. A. Schoettger. 1975. Acquisition and Culture of Research Fish: Rainbow Trout, Fathead Minnows, Channel Catfish and Bluegills. Environmental Protection Agency, Ecological Research Series EPA-660/3-75-011, May, 1975. 45 p.
- (10) Berger, B. L., R. E. Lennon and J. W. Hogan. 1969. Laboratory Studies on Antimycin A as a fish toxicant. U.S. Department of Interior, Investigations in Fish Control No. 26. 21 p.
- (11) Litchfield, J. T., Jr. and F. Wilcoxon. 1949. A Simplified Method of Evaluating Dose-Effect Experiments. Jour. Pharm. Exp. Ther. 96:99-113.
- (12) American Society for Testing and Materials. 1977. Aquatic Toxicology and Hazard Evaluation. ASTM-STP 634. 315 p.

LABORATORIES

ST. LOUIS, MISSOURI
 63102

Technology Investigation of **TRIFLURIDE**
 GP 1612
 Lot Number **MG 05-001**
 Date **November 16, 1973**

TO: **MONSANTO COMPANY**
 St. Louis, Missouri
 Project No. **Y-73-190**

ACUTE ORAL TOXICITY Species: **Sprague-Dawley Albino Rats**

LD₅₀ **> 7,940** Mg/Kg 95% Confidence Limits ----- Mg/Kg
 CONDITIONS **Single Oral Dose - 25.0% suspension in corn oil**

Average

| Dose Mg/Kg | Initial Weight | | Mortality/Deceas | | | Time of Mortality |
|---------------|----------------|--------|------------------|--------|----------|-------------------|
| | Male | Female | Male | Female | Combined | |
| 1 3160 | --- | 220 | --- | 0/1 | 0/1 | |
| 2 5010 | 220 | --- | 0/1 | --- | 0/1 | |
| 3 7940 | 225 | 220 | 0/2 | 0/3 | 0/5 | |
| 4 | | | | | | |
| 5 | | | | | | |
| 6 | | | | | | |

Signs of Intoxication **Reduced appetite and activity one to three days.**

Gross Autopsy
 Decents

Survivors (7 Days) **Viscera appeared normal.**

ACUTE DERMAL TOXICITY Species: **New Zealand Albino Rabbits**

LD₅₀ **> 7,940** Mg/Kg 95% Confidence Limits ----- Mg/Kg
 CONDITIONS **Applied as a 40.0% suspension in corn oil - 24-hours exposure**

| Dose Mg/Kg | Initial Weight | | Mortality/Deceas | | | Time of Mortality |
|---------------|----------------|--------|------------------|--------|----------|-------------------|
| | Male | Female | Male | Female | Combined | |
| 1 5010 | 2.1 | --- | 0/1 | --- | 0/1 | |
| 2 7940 | 2.4 | 2.0 | 0/1 | 0/1 | 0/2 | |
| 3 | | | | | | |
| 4 | | | | | | |
| 5 | | | | | | |
| 6 | | | | | | |

Signs of Intoxication **Reduced appetite and activity one to two days.**

Gross Autopsy
 Decents

Survivors (14 Days) **Viscera appeared normal.**

The material in this report is to be used in development of the product and may be given to responsible unit contacts, but it is not to be used by them in advertising copy. The names of this material is not to be changed until it appears in formal publications. The analysis is in the established rules may be made without the approval of the Medical Department in St. Louis. Customer inquiries regarding matters of liability are to be referred as before to the

TBET MATERIAL
 LOT NUMBER
 PROJECT NO.

CP 1612
 NC 05-001
 Y-73-190

ACUTE EYE IRRITATION

Species New Zealand Albino Rabbits

Irritation Score (24, 48, 72 Hour Avg.)

0.6

Classification

Slight *

Dose and Conditions

100.0 Mg. applied as finely ground powder / F.H.S.A. / 24-hours exposure

* F.H.S.A.: NOT classed as an eye irritant

| Time of Reading Hours | STRUCTURE | SCORES | | | | | | Mean Score (X/110) | Comments |
|-----------------------|----------------|---------------|---|---|---|---|---|--------------------|--|
| | | ANIMAL NUMBER | | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | | |
| 24 | - CORNEA | 0 | 0 | 0 | 0 | 0 | 0 | 2.0 | Immediate: Slight discomfort 10 Min.: Slight erythema, moderate discharge 1 Hr.: Slight erythema, moderate discharge 24 Hr.: Slight erythema 48 Hr.: All scored zero |
| | - IRIS | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | - CONJUNCTIVAE | 2 | 2 | 2 | 2 | 2 | 2 | | |
| 48 | - CORNEA | 0 | 0 | 0 | 0 | 0 | 0 | 0.0 | |
| | - IRIS | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | - CONJUNCTIVAE | 0 | 0 | 0 | 0 | 0 | 0 | | |
| 72 | - CORNEA | 0 | 0 | 0 | 0 | 0 | 0 | 0.0 | |
| | - IRIS | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | - CONJUNCTIVAE | 0 | 0 | 0 | 0 | 0 | 0 | | |
| 168 | - CORNEA | 0 | 0 | 0 | 0 | 0 | 0 | 0.0 | |
| | - IRIS | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | - CONJUNCTIVAE | 0 | 0 | 0 | 0 | 0 | 0 | | |

PRIMARY SKIN IRRITATION

Species New Zealand Albino Rabbits

Irritation Score (24, 72 Hour Avg.)

0.0

Classification Non-Irritating *

Dose and Conditions

0.5 Gram applied as finely ground powder moistened with water / F.H.S.A. /

* F.H.S.A.: NOT classed as a primary skin irritant

24-hours exposure

| Animal Number | HOUR SKIN | ERYTHEMIA | | | | | | | | | | EDEMA | | | | | | | | | |
|---------------|-----------|-----------|---|----|---|----|---|----|---|-----|---|-------|---|----|---|----|---|----|---|-----|--|
| | | 4 | | 24 | | 48 | | 72 | | 168 | | 4 | | 24 | | 48 | | 72 | | 168 | |
| | | I | A | I | A | I | A | I | A | I | A | I | A | I | A | I | A | I | A | | |
| 1 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 2 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 3 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 4 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 5 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 6 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |

Comments

INHALATION TOXICITY

Species

Exposure period

Mortality

Vapor concn.

Avg. chamber temp

°C.

Avg. chamber rel. humidity

%

Chamber capacity

L.

Air flow rate

L./min.

Initial sample size

Recovered sample size

Sample vaporized

Recovered from condenser

Signs of Intoxication

Gross Autopsy

Decedents

Survivors (

Days)

April 11, 1979

Mutagenicity Testing of THIOFIDE

DO-78-239

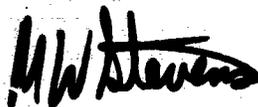
TO

F.R. Graham *
B26L

The referenced report has been received and reviewed.

In this study, THIOFIDE was evaluated for mutagenic activity in the mouse lymphoma assay. This assay evaluates materials for specific locus forward mutation induction in L5178Y TK (Thymidine Kinase) mouse lymphoma cells. THIOFIDE was tested both with and without a metabolic activation system in the assay.

The test material did not induce significant mutagenic activity at the TK locus. THIOFIDE was evaluated as being non-mutagenic under the conditions of this assay.



M.W. Stevens

/bks

*rec. report

TO: Judy Luder

Judy - please note, record and file
appropriately

VF
P26

LBI ASSAY NO. 3578

MUTAGENICITY EVALUATION OF

THIOFIDE 80-78-239

IN THE
MOUSE LYMPHOMA FORWARD
MUTATION ASSAY

FINAL REPORT

Thiofax

CAS No. { MBTS
120-78-5

SUBMITTED TO:

MONSANTO COMPANY
800 NORTH LINDBERGH BOULEVARD
ST. LOUIS, MISSOURI 63166

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 20989

REPORT DATE: MARCH 1979



BIONETICS

PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I-IX. Items I-IV provide sponsor and compound identification information, type of assay, and the protocol reference number. All protocol references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V provides the initiation and completion dates for the study, and Item VI provides identification of supervisory personnel. Item VII identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation itself is in Item VII. Item IX provides the conclusion and evaluation.

The second part of the report, entitled PROTOCOL, describes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices. The evaluation criteria are included to acquaint the sponsor with the methods used to develop and analyze the test results.

All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington Maryland, 20795.

Copies of raw data will be supplied to the sponsor upon request.



BIONETICS

- I. SPONSOR: Monsanto Company
- II. MATERIAL (TEST COMPOUND): LBI ASSAY NUMBER #3578
 - A. Identification: Thiofide 80-78-239
 - B. Date Received: September 6, 1978
 - C. Physical Description: Cream-colored powder
- III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay
- IV. PROTOCOL NUMBER: 431 (DMT-106)
- V. STUDY DATES:
 - A. Initiation: October 12, 1978
 - B. Completion: February 20, 1979
- VI. SUPERVISORY PERSONNEL:
 - A. Study Director: Brian Myhr, Ph.D.
 - B. Laboratory Supervisor: Marie McKeon
- VII. RESULTS:

The data are presented in Tables 1-4 on pages 4-7.

VIII. INTERPRETATION OF RESULTS:

The test material, Thiofide 80-78-239, was insoluble in water at high concentrations and was placed in DMSO at a concentration of 333 mg/ml for the preliminary cytotoxicity test. The material was not completely soluble and formed a fine, milky-white suspension even at the lower stock concentrations (5 mg/ml to 50 mg/ml) used for mutation experiments. Dilutions were performed with DMSO prior to final 1:100 dilutions into growth medium to obtain an applied concentration range of 3333 µg/ml to 6.5 µg/ml. Concentrations of 6.5 µg/ml and 13 µg/ml were toxic and 26 µg/ml was completely lethal (without activation) to the cells within 24 hours of treatment. Therefore, the mutation assay was initiated with a series of applied concentrations from 50 µg/ml to 0.1 µg/ml.

Five trials of the mutation assay were initiated because of technical deficiencies and variability in the toxicity of the test material to the mouse lymphoma cells. The results of four assays are presented in Tables 1 to 4, in sequence of performance. The unreported assay was invalidated by an unacceptably low cloning efficiency of 43% for the negative controls.



BIONETICS

VIII. INTERPRETATION OF RESULTS: (continued)

Under nonactivation conditions, the mutant frequencies in the treated cultures were all comparable to the background frequency (average of the solvent and untreated negative control values) in the first trial (Table 1). A 2.5-fold increase over the background frequency is considered necessary to demonstrate mutagenic activity at any given dose level. This assay was marred by the lack of mutagenic activity for the EMS positive control (which appeared to be the result of hydrolytic decomposition, since little toxicity was observed), and the assay needed to be extended to more toxic treatments with the test material in order to adequately test for the absence of mutagenic activity.

In the nonactivation trials shown in Tables 2, 3, and 4, treatments causing high toxicity (minimum percent relative growth values of 5.3%, 2.6%, and 12.0%, respectively) did not induce mutant frequencies significantly elevated over the concurrent background frequencies. Several trials were performed because the toxicity in the second trial (Table 2) was much greater than in Trial 1; the additional trials showed further variability in the toxicity associated with a given dose. Within each trial the toxicity increased regularly with the applied concentration, so the variability between trials may represent a variable sensitivity of different stock cultures to the test material or an unknown variation in the actual applied concentrations (resulting from variable solubility in DMSO and growth medium). Thus, the applied concentrations causing about 2% - 5% relative growth varied from 50 $\mu\text{g/ml}$ (Table 3) to 12.5 $\mu\text{g/ml}$ (Table 2), and 1.56 $\mu\text{g/ml}$ was nontoxic in Table 4 but caused 38.1% relative growth in Table 2. Nevertheless, the test material was consistently inactive as a mutagen over the range of concentrations that caused a wide range of cytotoxicities.

In the presence of the S9 microsomal activation mix, the toxicity of the test material was reduced, and the first mutation assay (Table 1) showed no significant changes in the mutant frequencies for nontoxic treatments with 50 $\mu\text{g/ml}$ to 3.13 $\mu\text{g/ml}$. In the second trial (Table 2), 50 $\mu\text{g/ml}$ was toxic (34.8% relative growth). At 75 $\mu\text{g/ml}$, the percent relative growth was only 13%, but the mutant frequencies in all the treated cultures remained comparable to the background. In the two additional trials, highly toxic treatments corresponding to about 7% relative growth were obtained with 100 $\mu\text{g/ml}$ (Table 3) and 50 $\mu\text{g/ml}$ (Table 4). The mutant frequencies remained comparable to the concurrent backgrounds. [An elevated mutant frequency (6-fold increase) was observed in Table 3 for an extremely toxic treatment with 150 $\mu\text{g/ml}$ (0.6% relative growth). This result is of questionable reliability because the relative cloning efficiency of 13.8% was so low. Mutation assays are not normally completed for such extreme treatments--this was performed by technical oversight--so little or no significance should be attached to any mutagenic activity appearing under these conditions.]



VIII. INTERPRETATION OF RESULTS: (continued)

The validity of the mutagenesis assays can be assessed by the results obtained for the positive and negative controls. The average cloning efficiencies for the solvent and untreated negative controls varied from 54% (Table 3) to 82% (Table 4) for nonactivation and from 59% (Table 3) to 88% (Table 4) for activation, which demonstrates acceptable to excellent culturing conditions for the assays. The background frequencies were all within the normal range, and the positive control compounds yielded frequencies greatly in excess of the backgrounds (except for EMS in Table 1). The EMS-induced mutant frequencies in Tables 2 and 3 and the DMN-induced frequency in Table 3 were all higher than the usual upper values of about 800×10^{-6} . The treatments were more toxic than usual, which would induce more mutants, and the low relative cloning efficiencies probably artificially inflated the observed frequencies.

IX. CONCLUSIONS:

The test material, Thiofide 80-78-239, did not induce an increase in mutations at the TK locus in L5178Y mouse lymphoma cells for applied concentrations of 0.39 $\mu\text{g/ml}$ to 50 $\mu\text{g/ml}$ without activation and for 3.13 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$ with microsomal activation. These concentration ranges included highly toxic treatments (less than 10% relative growth).

Therefore, the test material is considered to be inactive in the Mouse Lymphoma Forward Mutation Assay.

Submitted by:

Study Director

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3-19-79
date

Reviewed by:

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David J. Brusick, Ph.D.
Director
Department of Genetics
and Cell Biology

3/19/79
date



BIONETICS

3. SUMMARY OF MUTATION INDUCED BY DESMETHYL

TABLE 1

A. NAME OF CLONAL DESIGNATION ON THE TEST COMPASS: THOUFIDE NO-70-239
 B. LOT COMP: #3 3578
 C. STRAIN: DIPHYL SURFICOL
 D. TEST DATE: 11/11/70

TEST SOURCE ISSUE -1-1051-1-1051-2-1051-3

| TEST | SOURCE | ISSUE | 1 | DAILY GROWTH | 2 | 3 | RELATIVE SUSPENSION GROWTH % OF CONTROL | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES | RELATIVE CLONING EFFICIENCY % OF CONTROL | RELATIVE MUTANT FREQUENCY | |
|----------------|------------------|-------|-------|--------------|-------|-------|---|---------------------|---------------------|--|---------------------------|-------|
| QUANTIFICATION | SIV VENT CONTROL | --- | 15.4 | 0.0 | 100.0 | 20.0 | 150.0 | 100.0 | 100.0 | 100.0 | 100.0 | |
| | | | 18.6 | 9.4 | 100.0 | 32.0 | 240.0 | 100.0 | 100.0 | 100.0 | 100.0 | |
| | | | 17.0 | 11.7 | 139.4 | 23.0 | 131.0 | 92.2 | 92.2 | 92.2 | 92.2 | 92.2 |
| | | | 13.4 | 7.2 | 59.4 | 42.0 | 193.0 | 50.5 | 50.5 | 50.5 | 50.5 | 50.5 |
| | | | 12.0 | 0.2 | 61.1 | 16.0 | 266.0 | 174.3 | 174.3 | 174.3 | 174.3 | 174.3 |
| | | | 9.0 | 16.0 | 97.4 | 34.0 | 172.0 | 66.9 | 66.9 | 66.9 | 66.9 | 66.9 |
| | | | 9.2 | 0.8 | 50.3 | 47.0 | 235.0 | 118.7 | 118.7 | 118.7 | 118.7 | 118.7 |
| | | | 5.8 | 12.2 | 43.9 | 27.0 | 213.0 | 107.6 | 107.6 | 107.6 | 107.6 | 107.6 |
| | | | 6.0 | 7.8 | 29.1 | 37.0 | 160.0 | 84.8 | 84.8 | 84.8 | 84.8 | 84.8 |
| | | | 6.0 | 6.0 | 100.0 | 49.0 | 306.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| AGYBATION | SIV VENT CONTROL | KAT | LIVER | 10.6 | 6.0 | 100.0 | 49.0 | 306.0 | 100.0 | 100.0 | 100.0 | |
| | | | LIVER | 6.2 | 11.2 | 100.0 | 51.0 | 222.0 | 100.0 | 100.0 | 100.0 | |
| | | | LIVER | 17.6 | 11.2 | 349.2 | 36.0 | 166.0 | 62.1 | 62.1 | 62.1 | |
| | | | LIVER | 11.8 | 10.6 | 188.0 | 520.0 | 181.0 | 68.6 | 68.6 | 68.6 | |
| | | | LIVER | 13.0 | 11.0 | 224.9 | 48.0 | 196.0 | 74.2 | 74.2 | 74.2 | |
| | | | LIVER | 13.6 | 9.4 | 192.2 | 41.0 | 241.0 | 91.3 | 91.3 | 91.3 | |
| | | | LIVER | 11.8 | 16.4 | 255.4 | 39.0 | 180.0 | 68.2 | 68.2 | 68.2 | |
| | | | LIVER | 14.4 | 7.4 | 160.2 | 62.0 | 285.0 | 100.0 | 100.0 | 100.0 | |
| | | | LIVER | 11.0 | 7.0 | 115.8 | 50.0 | 206.0 | 78.0 | 78.0 | 78.0 | |
| | | | LIVER | 11.0 | 7.0 | 115.8 | 50.0 | 206.0 | 78.0 | 78.0 | 78.0 | |

* RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY / 100
 ** THE RATIO OF CELLS SELECTED FOR MUTANT SELECTION TO CELLS SELECTED FOR CLONING EFFICIENCY IS 101-6.
 THE RATIO OF MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES) - 101-6.
 THE MUTANT FREQUENCY IS GIVEN IN BRACKETS OF 101-6.

† - ONE PLATE CONTAMINATED; VALUE BASED ON REMAINING TWO PLATES.

4. SUMMARY OF MUTAGENESIS RESULTS

TABLE 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPUND: THIOFENE UD-78-259
 B. LOT CODE #: 1578
 C. SOLVENT: DIMETHYL SULFOXIDE
 D. TEST DATE: 12/05/78

| TEST | SUSPENSION CONC. (UG/ML) | SOURCE TISSUE | DAILY DOSE (UG/KG) | DAILY COURSE | RELATIVE SUSPENSION GROWTH (%) | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES | RELATIVE CLONING EFFICIENCY (%) | PERCENT RELATIVE GROWTH | MUTANT FREQUENCY (10 ⁻⁶) |
|-----------------|--------------------------|---------------|--------------------|--------------|--------------------------------|---------------------|---------------------|---------------------------------|-------------------------|--------------------------------------|
| | | | | | | | | | | |
| SOLVENT CONTROL | 0.780 | --- | 12.4 | 20.4 | 100.0 | 31.0 | 146.0 | 100.0 | 100.0 | 21.2 |
| | 1.560 | --- | 13.0 | 18.0 | 100.0 | 41.0 | 131.0 | 100.0 | 100.0 | 31.3 |
| | 6.250 | --- | 10.4 | 15.4 | 65.8 | 32.0 | 220.0 | 150.0 | 101.5 | 14.5 |
| | 12.500 | --- | 9.2 | 10.0 | 37.0 | 444.0+ | 30.0 | 27.4 | 10.4 | 1168.4 |
| MUTATED CONTROL | 0.780 | --- | 7.0 | 8.4 | 24.1 | 36.0 | 252.0+ | 181.9 | 43.9 | 14.3 |
| | 1.560 | --- | 6.2 | 6.4 | 16.3 | 18.0 | 324.0+ | 231.9 | 38.1 | 11.7 |
| | 6.250 | --- | 2.6 | 4.2 | 5.2 | 17.0+ | 299.0 | 215.9 | 11.2 | 5.7 |
| | 12.500 | --- | 0.6 | 3.8** | 4.7 | 25.0 | 156.0 | 117.6 | 5.3 | 16.0 |
| TEST COMPUND | 0.780 | RAT | 6.2 | 13.2 | 100.0 | 61.0 | 194.0 | 100.0 | 100.0 | 32.5 |
| | 1.560 | RAT | 4.0 | 21.4 | 100.0 | 58.0 | 217.0 | 100.0 | 100.0 | 26.7 |
| | 6.250 | RAT | 6.2 | 12.6 | 122.5 | 50.0 | 200.0 | 97.3 | 119.2 | 25.0 |
| | 12.500 | RAT | 6.8 | 6.0 | 41.1 | 207.0 | 71.0 | 34.5 | 14.2 | 291.5 |
| | 0.780 | RAT | 9.4 | 13.0 | 123.2 | 72.0 | 191.0 | 92.9 | 114.5 | 37.7 |
| | 1.560 | RAT | 6.6 | 17.6 | 117.1 | 67.0 | 144.0 | 70.1 | 92.0 | 46.5 |
| | 6.250 | RAT | 7.0 | 6.6 | 60.7 | 69.0 | 197.0 | 95.9 | 58.2 | 15.0 |
| | 12.500 | RAT | 3.6 | 12.0 | 36.1 | 86.0+ | 197.0 | 95.9 | 34.8 | 43.7 |

RELATIVE SUSPENSION GROWTH X PERCENT CLONING EFFICIENCY / 100
 THE PAIR OF CELLS SEEDED FOR MUTAGENESIS TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10F64.
 THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10⁻⁶.

+ = ONE PLATE CONTAMINATED; VALUE BASED ON REMAINING TWO PLATES.

** = DUE TO TOXICITY, CULTURE NOT SPLIT BACK TO 3.0 X 10⁶ CELLS; 300 CELLS RATHER THAN 3000 CELLS USED FOR VIABLE CLONES -- VALUES FOR RELATIVE CLONING EFFICIENCY AND PERCENT RELATIVE GROWTH WERE ADJUSTED ACCORDINGLY.

5. SUMMARY OF MOUSE LYMPHOMA CELL LINE RESULTS

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: DDT (CODE NO-78-239)

B. LSI CODE: 3578

C. SOLVENT: DIMETHYL SULFOXIDE

D. TEST DATE: 01/10/79

TEST SOURCE: ISSUE - (CELLS) 2, 3

| CONCENTRATION | SOLVENT CONTROL | SOLVENT CONTROL | UNIT MEAN ± SD CONTROL | EAS ± 5 UL/ML | TEST COMPOUND | DAILY COUNTS | | RELATIVE SUSPENSION GROWTH (% CONTROL) | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES (± SE CONTROL) | RELATIVE CLONING EFFICIENCY (% OF CONTROL) | PERCENT RELATIVE GROWTH ± SE CONTROL | MUTANT FREQUENCY ± SE CONTROL |
|---------------|-----------------|-----------------|------------------------|---------------|---------------|--------------|-------|--|---------------------|------------------------------------|--|--------------------------------------|-------------------------------|
| | | | | | | 1 | 2 | | | | | | |
| 0.130 UG/ML | --- | 16.4 | 4.4 | 100.0 | --- | 46.0 | 190.0 | 100.0 | 190.0 | 100.0 | 100.0 | 23.3 | |
| 1.130 UG/ML | --- | 17.0 | 6.0 | 100.0 | --- | 41.0 | 171.0 | 100.0 | 171.0 | 100.0 | 100.0 | 24.0 | |
| 12.500 UG/ML | --- | 14.0 | 8.0 | 141.5 | --- | 45.0 | 119.0 | 141.5 | 119.0 | 64.9 | 91.7 | 31.0 | |
| 25.000 UG/ML | --- | 10.0 | 5.6 | 64.3 | --- | 566.0 | 20.0 | 64.3 | 20.0 | 17.3 | 9.0 | 2821.4 | |
| 50.000 UG/ML | --- | 11.0 | 8.0 | 119.2 | --- | 91.0 | 242.0 | 119.2 | 242.0 | 131.9 | 137.3 | 12.0 | |
| | --- | 6.2 | 6.4 | 45.6 | --- | 31.0 | 208.0 | 45.6 | 208.0 | 140.0 | 66.0 | 11.0 | |
| | --- | 2.0 | 4.0 | 13.0 | --- | 26.0 | 348.0 | 13.0 | 348.0 | 107.0 | 26.1 | 1.3 | |
| | --- | 1.0 | 4.0 | 14.5 | --- | 23.0 | 256.0 | 14.5 | 256.0 | 179.5 | 20.2 | 9.0 | |
| | --- | 1.0 | 2.2++ | 7.6 | --- | 17.0 | 64.0 | 7.6 | 64.0 | 34.9 | 2.6 | 24.0 | |

| CONCENTRATION | SOLVENT CONTROL | SOLVENT CONTROL | UNIT MEAN ± SD CONTROL | EAS ± 5 UL/ML | TEST COMPOUND | SOURCE | DAILY COUNTS | RELATIVE SUSPENSION GROWTH (% CONTROL) | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES (± SE CONTROL) | RELATIVE CLONING EFFICIENCY (% OF CONTROL) | PERCENT RELATIVE GROWTH ± SE CONTROL | MUTANT FREQUENCY ± SE CONTROL |
|---------------|-----------------|-----------------|------------------------|---------------|---------------|--------|--------------|--|---------------------|------------------------------------|--|--------------------------------------|-------------------------------|
| | | | | | | | | | | | | | |
| 3.130 UG/ML | --- | 10.4 | 11.0 | 100.0 | --- | 66.0 | 279.0 | 100.0 | 279.0 | 100.0 | 100.0 | 20.0 | |
| 25.000 UG/ML | --- | 12.0 | 11.0 | 100.0 | --- | 46.0 | 161.0 | 100.0 | 161.0 | 100.0 | 100.0 | 20.0 | |
| 50.000 UG/ML | --- | 13.2 | 9.0 | 93.5 | --- | 36.0 | 0.0 | 93.5 | 0.0 | 77.3 | 67.6 | 24.5 | |
| 100.000 UG/ML | --- | 9.0 | 4.0 | 28.9 | --- | 115.0 | 0.0 | 28.9 | 0.0 | 4.1 | 1.2 | 1431.5 | |
| 3.130 UG/ML | --- | 7.6 | 13.0 | 92.0 | --- | 55.0 | 166.0 | 92.0 | 166.0 | 85.1 | 70.3 | 37.1 | |
| 25.000 UG/ML | --- | 6.0 | 10.0 | 50.1 | --- | 42.0 | 233.0 | 50.1 | 233.0 | 119.5 | 99.9 | 30.0 | |
| 50.000 UG/ML | --- | 4.0 | 6.0 | 17.7 | --- | 66.0 | 276.0 | 17.7 | 276.0 | 114.9 | 20.3 | 24.5 | |
| 100.000 UG/ML | --- | 4.2 | 5.2 | 16.1 | --- | 68.0 | 285.0 | 16.1 | 285.0 | 135.9 | 21.9 | 29.7 | |
| 100.000 UG/ML | --- | 2.4 | 4.0 | 10.6 | --- | 37.0 | 124.0 | 10.6 | 124.0 | 63.6 | 6.0 | 29.0 | |
| 150.000 UG/ML | --- | 0.7 | 2.0++ | 4.4 | --- | 45.0 | 27.0 | 4.4 | 27.0 | 13.0 | 6.0 | 166.7 | |

* RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY / 100
 ** THE RATIO OF CELLS SEDED FOR MUTANT SELECTION TO CELLS SEDED FOR CLONING EFFICIENCY IS 100:1
 *** PLEASE NOTE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES) x 10⁶ - 4
 **** MUTANT FREQUENCY IS GIVEN IN UNITS OF 10⁶ - 6.

** - DUE TO TOXICITY, LESS THAN 3.0 x 10⁶ CELLS WERE AVAILABLE AT TIME OF CLONING; LESS THAN 300 CELLS WERE USED FOR VIABLE CLONES --- THE VALUES FOR RELATIVE CLONING EFFICIENCY AND PERCENT RELATIVE GROWTH WERE ADJUSTED ACCORDINGLY.

3. SUMMARY OF MUTAGENICITY TEST RESULTS

TABLE 4

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: THIOFIDE NO-78-239
 B. CAS CODE #1: 5578
 C. SOLVENT: DIMETHYL SULFOXIDE
 D. TEST DATE: 02/07/79

| TEST IDENTIFICATION | SOURCE | DAILY COUNTS | | | RELATIVE SUSPENSION GROWTH OF CONTROL | TOTAL MUTANT CLONES | TOTAL VIABLE CLONES | RELATIVE CLONING EFFICIENCY (% OF CONTROL) | PERCENT RELATIVE GROWTH | MUTANT FREQUENCY (X 10 ⁻⁶) |
|---------------------|--------|--------------|------|-------|---------------------------------------|---------------------|---------------------|--|-------------------------|--|
| | | 1 | 2 | 3 | | | | | | |
| S-9 THIOFIDE | --- | 13.0 | 10.2 | 100.0 | 117.0 | 244.0 | 100.0 | 100.0 | 49.0 | |
| | --- | 13.2 | 6.2 | 100.0 | 44.0 | 223.0 | 100.0 | 100.0 | 19.7 | |
| | --- | 8.6 | 21.5 | 173.3 | 56.0 | 272.0 | 116.5 | 201.8 | 20.6 | |
| | --- | 7.2 | 9.5 | 37.6 | 671.0 | 104.0 | 44.5 | 16.7 | 645.2 | |
| | --- | 8.2 | 20.0 | 159.1 | 47.0 | 245.0 | 104.9 | 166.9 | 19.2 | |
| | --- | 6.6 | 16.4 | 101.0 | 51.0 | 175.0 | 74.9 | 75.7 | 29.1 | |
| | --- | 3.4 | 14.4 | 40.3 | 45.0 | 276.0 | 118.2 | 47.6 | 16.1 | |
| | --- | 1.4 | 6.2 | 17.3 | 70.0 | 167.0 | 71.5 | 12.4 | 41.9 | |
| | --- | 1.2 | 5.2 | 14.5 | 57.0 | 192.0 | 82.2 | 32.0 | 79.7 | |
| | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ACETAMINOPHEN | PAT | 10.2 | 10.0 | 100.0 | 50.0 | 273.0 | 100.0 | 100.0 | 21.2 | |
| | RAT | 9.5 | 14.0 | 100.0 | 80.0 | 309.0 | 100.0 | 100.0 | 25.9 | |
| | MAT | 9.8 | 13.4 | 79.4 | 65.0 | 207.0 | 71.1 | 56.5 | 31.4 | |
| | MAT | 7.2 | 4.8 | 20.9 | 353.0 | 100.0 | 14.4 | 7.2 | 393.0 | |
| | KAT | 8.6 | 22.8 | 118.5 | 69.0 | 303.0 | 104.1 | 123.4 | 22.8 | |
| | KAT | 5.6 | 14.2 | 48.1 | 87.0 | 308.0 | 105.8 | 50.9 | 26.4 | |
| | KAT | 7.8 | 8.8 | 41.5 | 66.0 | 254.0 | 87.3 | 34.2 | 28.0 | |
| | KAT | 6.4 | 10.6 | 41.0 | 75.0 | 147.0 | 50.5 | 20.7 | 51.0 | |
| | KAT | 3.4 | 6.0 | 10.9 | 114.0 | 196.0 | 67.4 | 7.3 | 60.7 | |
| | KAT | --- | --- | --- | --- | --- | --- | --- | --- | --- |

RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY / 100
 OR THE RATIO OF CELLS SEEDING FOR MUTANT SELECTION TO CELLS SEEDING FOR CLONING EFFICIENCY IS 100%
 THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10⁻⁶
 THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10⁻⁶

PROTOCOL NO. 431

1. OBJECTIVE

The objective of this study is to evaluate the test material for its ability to induce forward mutation in the L5178Y TK⁺/₋ mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU).

2. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as BrdU is included in the growth medium, the analog will be phosphorylated via the TK pathway and be incorporated into DNA, eventually resulting in cellular death. Cells which are heterozygous at the TK locus (TK⁺/₋) may undergo a single step forward mutation to the TK⁻/₋ genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by *de novo* synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK⁻/₋ mutants is the lack of any ability to utilize toxic analogs of thymidine, which enables only the TK⁻/₋ mutants to grow in the presence of BrdU. Cells which grow to form colonies in the presence of BrdU are therefore assumed to have mutated, either spontaneously or by the action of a test substance, to the TK⁻/₋ genotype.

3. MATERIALS

A. Indicator Cells

The mouse lymphoma cell line, L5178Y TK⁺/₋, used in this assay is derived from the Fischer L5178Y line of Dr. Donald Clive. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK⁻/₋ mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK⁻/₋ phenotype (exposure to methotrexate) and are then returned to normal growth medium for three or more days before use.

B. Media

The cells are maintained in Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium is cloning medium containing 50 or 100 µg/ml of BrdU.



BIONETICS

3. MATERIALS (continued)

C. Control Compounds

1. Negative Controls

A negative control consisting of assay procedures performed on untreated cells is performed in all cases. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are also assayed as the solvent negative control to determine any effects on survival or mutation caused by the solvent alone. For test substances assayed with activation, the untreated and solvent negative controls will include the activation mixture.

2. Positive Controls

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and will be used at 0.5 $\mu\text{l/ml}$ as a positive control for nonactivation studies.

Dimethylnitrosamine (DMN) requires metabolic activation by microsomal enzymes to become mutagenic and will be used at 0.3 $\mu\text{l/ml}$ as a positive control for assays performed with activation.

D. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

4. EXPERIMENTAL DESIGN

A. Dosage Selection (Cytotoxicity testing)

The solubility of the test chemical in growth medium and/or DMSO is first determined. Then a wide range of chemical concentrations is tested for cytotoxicity, starting with a maximum applied dose of 10 mg/ml for test chemicals soluble in media or 1 mg/ml for solutions in organic solvents. After an exposure time of four hours, the cells are washed and a viable cell count is obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of untreated cells are used to select seven to ten doses that cover the range from 0 to 50-90% reduction in 24-hour growth. These selected doses are subsequently applied to cell cultures prepared for mutagenicity testing, but only four or five of the doses will be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of four or five doses spaced from 0 to 50-90% reduction in cell growth.



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B. Mutability Testing

1. Nonactivation Assay

The procedure used is based on that reported by Clive and Spector (1975) and is summarized as follows. Cultures exposed to the test chemical for four hours at the preselected doses are washed and placed in growth medium for two or three days to allow recovery, growth and expression of the induced TK^{-/-} phenotype. Cell counts are determined daily and appropriate dilutions are made to allow optimal growth rates.

At the end of the expression period, 3×10^6 cells for each selected dose are seeded in soft agar plates with selection medium and resistant (mutant) colonies are counted after 10 days incubation. To determine the actual number of cells capable of forming colonies, a portion of the cell suspension is also cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number is the mutant frequency.

A detailed flow diagram for the mutation assay is provided in Figure 1.

2. Activation Assay

The activation assay can be run concurrently with the nonactivation assay. The only difference is the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension are: 2.4 mg NADP/ml; 4.5 mg isocitric acid/ml; and 50 μ l S9/ml.

C. Preparation of 9,000 x g Supernatant (S9)

Fischer 344 male rats are normally used as the source of hepatic microsomes. Induction with Aroclor 1254 or other agents is performed by injections five days prior to sacrifice. After decapitation and bleeding, the liver is immediately dissected from the animal using aseptic technique and placed in ice cold 0.25M sucrose buffered with Tris at pH 7.4. When an adequate number of livers is obtained, the collection is washed twice with fresh buffered sucrose and completely homogenized. The homogenate is centrifuged for 10 minutes at 9,000 x g in a refrigerated centrifuge and the supernatant (S9) from this centrifuged sample is retained and frozen at -80°C until used in the activation system. The S9 fraction may be obtained from induced or noninduced rats or other species, as requested.



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EVALUATION CRITERIA

A compound is considered mutagenic in this assay if:

- A dose-response relationship is observed over 3 of the 5 dose levels employed.
- The minimum increase at the low level of the dose-response curve is at least 2.5 times greater than the solvent and/or negative control values.
- The solvent and negative control data are within the normal range of the spontaneous background for the TK locus.

All evaluations of mutagenic activity are based on consideration of the concurrent solvent and negative control values run with the experiment in question. Positive control values are not used as reference points, but are included to ensure that the current cell population responds to direct and promutagens under the appropriate treatment conditions.

Occasionally, a single point within a concentration range will show an increase 2.5 times greater than the spontaneous background. If the increase is at the high dose, is reproducible, and if an additional higher dose level is not feasible because of toxicity, the chemical can be considered mutagenic. If the increase is internal within the dose range and is not reproducible, the increase will normally be considered aberrant. If the internal increase is reproducible, several doses clustered around the positive concentration will be examined to either confirm or reject the reliability of the effect.

As the data base on the assay increases, the evaluation criteria can be expected to become more firmly established.

5. **REPORT**

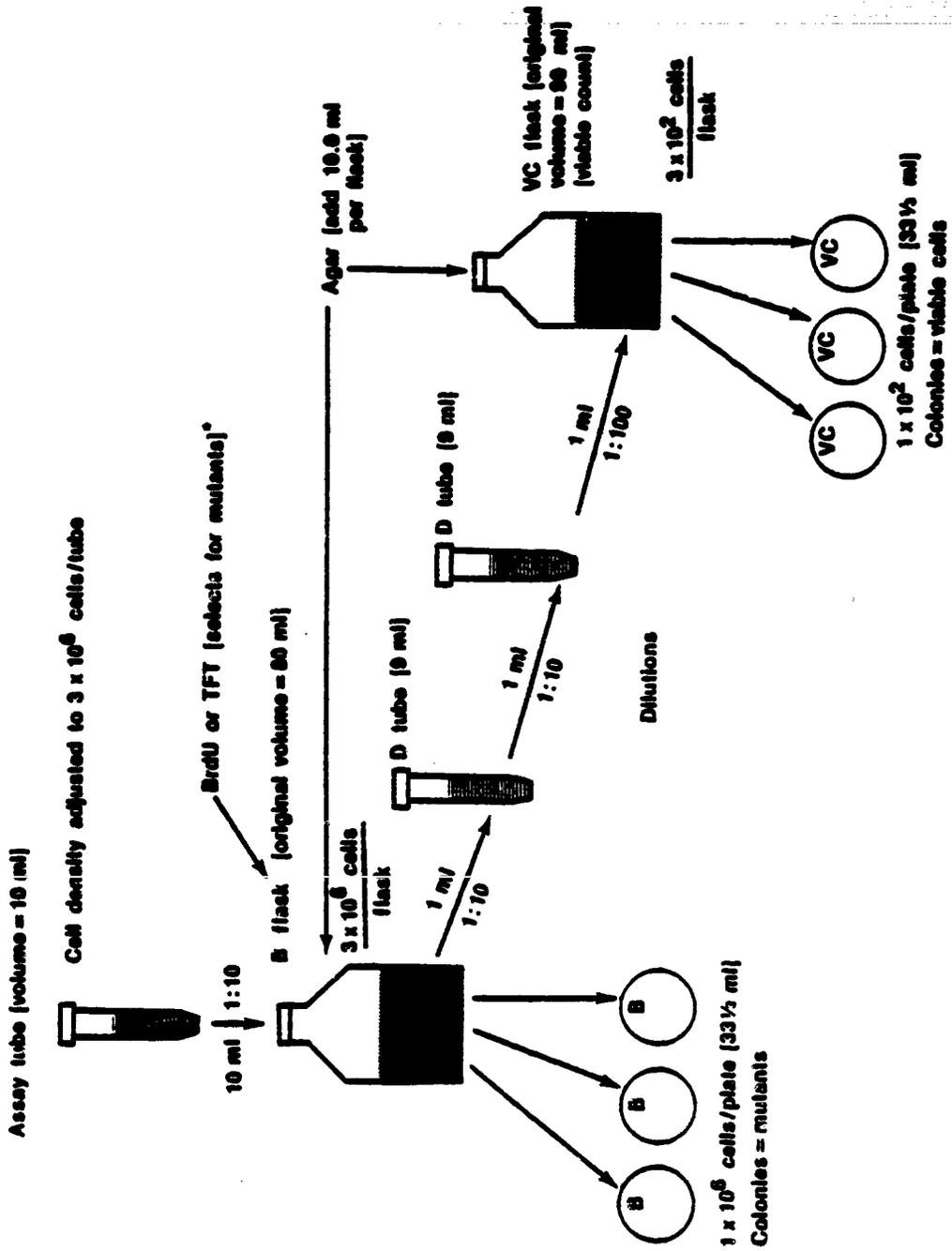
The screened doses, cell counts, and mutant and viable colony counts will be entered into a computer program. The results are analyzed and printed.

6. **REFERENCE**

Clive, D. and Spector, J.F.S.: Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. *Mutation Res.* 31:17-29, 1975.



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*Added after removal of 1 ml for viable count dilutions.

FIGURE 1. LYMPHOMA CLONING FLOW CHART