TRADE SECRET

Study Title

H-22762: Mutagenicity Testing in the
Salmonella typhimurium Plate Incorporation Assay

LABORATORY PROJECT ID: HL-1997-00676

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STUDY COMPLETED ON: August 26, 1997

PERFORMING LABORATORY: E. I. du Pont de Nemours and Company
Haskell Laboratory for Toxicology and Industrial Medicine
Elkton Road, P.O. Box 50
Newark, Delaware 19714-0050

MEDICAL RESEARCH NO.: [redacted]

GENERAL INFORMATION

Substance Tested: [redacted]

Synonyms/Codes: [redacted]

Haskell Number: 22762

Purity: [redacted]

Known Impurities: [redacted]

Physical Characteristics: [redacted]

Stability: Test substance appeared to be stable under the conditions of the study; no evidence of instability was observed

Sponsor: E. I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

Study Initiated/Completed: July 16, 1997 / (see report cover page)

In-Life Initiated/Completed: July 16, 1997 / July 25, 1997

All study records, raw data, and the final report are retained at Haskell Laboratory or at Iron Mountain, 200 Todds Lane, Wilmington, Delaware 19802 (formerly known as the E. I. du Pont de Nemours and Company Records Management Center).

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SUMMARY

The test substance, H-22762, was evaluated for mutagenicity in *Salmonella typhimurium* strains TA100, TA1535, TA97a, and TA98 with and without an exogenous metabolic activation system (S9). Concentrations of 10, 50, 100, 500, 1000, 2500, and 5000 µg/plate were evaluated in comparison to negative (solvent) controls.

Evidence for test substance toxicity to the bacteria was observed and documented in all *Salmonella typhimurium* strains, with and without S9, in concentrations as low as 1000 µg/plate. Under the conditions of this study, no evidence of mutagenic activity was detected in any *Salmonella typhimurium* strain in the absence or presence of the exogenous metabolic activation system. The test substance was negative.

Reported and Approved for Issue by Study Director: N. Larry Gladnick 8/26/97

N. Lawrence Gladnick, B.A.
Toxicology Associate

Date

STUDY PERSONNEL

Manager: Matthew S. Bogdanffy, Ph.D., DABT

The following individuals were responsible for conducting the in-life portion of the assay:

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Study Director: N. Lawrence Gladnick, B.A.

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INTRODUCTION

This study evaluated the mutagenic potential of the test substance, H-22762, in *Salmonella typhimurium* strains TA100, TA1535, TA97a, and TA98. The *Salmonella* strains are unable to synthesize histidine, an essential amino acid, because of mutations in the genes coding for histidine biosynthetic enzymes. Additional mutations in the defective genes can result in individual *Salmonella* bacteria regaining the ability to synthesize histidine.[1] A trace of histidine in the top agar permits several generations of auxotrophic cell division to fix pro-mutagenic lesions. This results in the formation of a microscopic "lawn" of bacteria. However, only those bacteria that revert to a prototrophic state can continue to divide and form macroscopic colonies. These revertants can be scored by their ability to grow on agar plates deficient in histidine. By comparing the number of chemically induced revertants to the number of spontaneous revertants, the mutagenicity of the test substance can be assessed.

MATERIALS AND METHODS

A. STUDY PROTOCOL

The protocol consisted of the Short Term Study Protocol Sheet and the Haskell General Testing Procedure [redacted] "Bacterial Mutagenicity Testing of Solids, Liquids, and Gases", effective 5/1/97).

B. TEST MATERIALS

1. Test Substance and Negative Control

The test substance is H-22762. Additional information regarding the test substance is found on the general information page of this report. The test substance was assumed to be stable during this assay and no evidence of instability was observed.

Based on information supplied by the sponsor, dimethyl sulfoxide (DMSO) was chosen as the test substance solvent and as the negative control. Preparation of the stock solution of the test substance at 50 mg/mL required sonication to maintain homogeneity and solubility. Additional dilutions of the test substance were performed at room temperature in DMSO. The negative control was assumed to be stable during this study, and no evidence of instability was observed. Any impurities were not expected to have interfered with the study.

2. Positive Controls

Positive controls included the following: 2-aminoanthracene (2AA), 2-nitrofluorene (2NF), sodium azide (NAAZ), ICR 191 Acridine (ICR 191). Deionized water was the solvent for NAAZ and ICR 191. The solvent for the other positive controls was DMSO. The positive controls were assumed to be stable in this study and no evidence of instability was observed. Any impurities were not expected to have interfered with the study.

C. TESTER STRAIN CHARACTERIZATION, STORAGE, AND CULTURE

1. Tester Strain Characterization

S. typhimurium tester strains were obtained from Dr. Bruce Ames, Berkeley, CA. The phenotypic characteristics and mutational sensitivities of the *S. typhimurium* strains are as follows:

Strain	Histidine Gene Locus Affected	Additional Characteristics			
		DNA Repair	LPS	R-factor Plasmid	Mutational Sensitivity
TA100	*his*G46	Δ*uvrB*	*rfa*	Present	Base pair substitution
TA1535	*his*G46	Δ*uvrB*	*rfa*	Absent	Base pair substitution
TA97a	*his*D6610	Δ*uvrB*	*rfa*	Present	Frameshift
TA98	*his*D3052	Δ*uvrB*	*rfa*	Present	Frameshift

The deletion (Δ) in *uvrB* (a gene which codes for DNA excision repair) increases the bacterial sensitivity to mutagens.[3] The *uvrB* trait is confirmed by demonstrating an increased bacterial sensitivity to ultraviolet light. Because this deletion also extends through a gene needed for biotin biosynthesis, the bacteria require exogenous biotin for growth. The *rfa* mutation causes a partial loss in the integrity of the lipopolysaccharide (LPS) cell wall so that permeability to large molecules is increased (Ames *et al.*, 1973). The presence of the R-factor plasmid (plasmid pKM101 which carries ampicillin resistance as a marker gene) results in an enhancement of an error-prone DNA repair system which is endogenous to these bacteria.[4]

Although the testing guidelines cited require *S. typhimurium* strain TA1537, the more recently developed TA97a strain was used. TA97 was the recommended replacement for TA1537 and has been demonstrated to be more sensitive to frameshift mutagens.[1,5] However, the reconstructed strain, TA97a, is now routinely used in place of TA97 due to its improved growth properties (personal communication with Bruce Ames and associates).

2. Tester Strain Storage and Culture

All bacterial strains were stored frozen in 8% DMSO in Oxoid nutrient broth at approximately -70°C. Overnight cultures were prepared by inoculating 20 mL of Oxoid nutrient broth with 0.1 mL of thawed bacterial suspension and incubating at 37°C with shaking. Overnight cultures were then stored on ice until used for mutagenesis assays.

D. METABOLIC ACTIVATION SYSTEM

Because the tester strains lack many of the enzymes required to convert various promutagens to a reactive state, the assay was performed with and without a rat liver homogenate activation system (S9 Mix) similar to the method of Maron and Ames (1983). The S9 Mix consisted of the following:

8 mM $MgCl_2$	4 mM $NADP^+$
33 mM KCl	100 mM sodium phosphate pH 7.4
5 mM glucose-6-phosphate	1.6 mg S9 protein /1.0 mL S9 Mix

The S9 (purchased from MOLTOX™) was the 9000 x g supernatant of liver homogenate (1 g wet liver: 3.0 mL KCl). Livers were from young male Sprague Dawley rats injected i.p. with Aroclor 1254® (500 mg/kg) five days before sacrifice.

E. DOSE SELECTION

In accordance with EPA and OECD test guidelines, the highest concentration evaluated in this study was a nominal concentration of 5000 µg/plate. Solubility information was confirmed empirically as needed during this study. Concentrations were calculated with the assumption that addition of the test substance to the solvent did not change the volume of the resulting solution.

F. STABILITY AND CONCENTRATION VERIFICATION

Solutions of the test substance were prepared immediately prior to treatment and were presumed to be stable under the conditions of the study. Treatment and control dosing solutions were not analyzed for concentration, uniformity, or stability. Top agar was not assayed for stability or concentration of the test substance or control articles since this assessment was not considered necessary to achieve the objectives of the study.

G. BACTERIAL MUTAGENICITY ASSAYS

This study consisted of one trial with and without activation. Two replicates were plated for each tester strain, test concentration, and condition. Positive and negative controls were run concurrently for all assays. Treatments with activation were conducted by adding 0.1 mL of negative control, positive control, or test substance solution, 0.5 mL of S9 mix, and 0.1 mL of an overnight culture containing approximately 1 $x10^8$ bacteria, to 2 mL of top agar [0.6% agar (w/v) and 0.6% NaCl (w/v)] supplemented with 0.05 mM L-histidine and 0.05 mM D-biotin. These components were mixed and poured onto a minimal glucose agar plate (approximately 25 mL, purchased from MOLTOX™). Treatments without activation were the same as those with activation with the exception that the S9 mix was replaced with 0.5 mL of sterile phosphate buffered saline. Revertant colonies were counted after the individually labeled plates were incubated at approximately 37°C for about 48 hours. When necessary, plates were refrigerated prior to counting.

H. STATISTICAL ANALYSIS

For each tester strain, the average number of revertants and the standard deviation at each concentration with and without S9 activation were calculated.

I. ACCEPTABILITY CRITERIA

An individual trial must have included at least five test concentrations (of which at least four must have been acceptable), a negative control, and a positive indicator for each selected tester strain. Data acceptability criteria were as follows:

- A single data point may have been rejected if contamination or excessive toxicity was seen on a treatment plate. A single data point may also have been rejected if excessive precipitate on the plate prevented accurate colony counting.
- A negative control data point may have been rejected if it fell outside the acceptable spontaneous mutation range.
- A concentration level was rejected if there were less than two data points at the treatment level or if the data point values were judged by the study director to be too divergent.
- A trial for the affected strain was rejected if the negative control was rejected or if there was no evidence of mutagenic activity on any positive indicator plate.
- Only those trials that met the criteria of acceptability were included in this report.

J. CLASSIFICATION GUIDELINES

A test substance was classified as POSITIVE when: (1) the average number of revertants in any strain at any test substance concentration studied was at least two times greater than the average number of revertants in the negative control; and (2) there was a positive dose-response relationship in that same strain.

A test substance was classified as NEGATIVE when either: (1) there were no test substance concentrations with an average number of revertants which was at least two times greater than the average number of revertants in the negative control; and (2) there was no positive dose-response relationship.

Results not meeting these criteria for positive or negative assessments were evaluated using scientific judgment.

RESULTS AND CONCLUSION

RESULTS

The test substance, H-22762, was evaluated for mutagenicity in *Salmonella typhimurium* strains TA100, TA1535, TA97a, and TA98 with and without an exogenous metabolic activation system (S9). Concentrations of 10, 50, 100, 500, 1000, 2500, and 5000 µg/plate were evaluated in comparison to negative (solvent) controls.

CONCLUSION

Under the conditions of this study, no evidence of mutagenic activity was detected in any *Salmonella typhimurium* strain in the absence or presence of the exogenous metabolic activation system. The test substance was negative.

REFERENCES

1. Maron, D. M. and B. N. Ames (1983). Revised methods for the Salmonella mutagenicity test. Mutation Research **113**, 173-215.

2. Ames, B. N., F. D. Lee, and W. E. Durston (1973). An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc. Natl. Acad. Sci. USA **70**, 782-786.

3. McCann, J., N. E. Springarn, J. Kobori, and B. N. Ames (1975). Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. Proc. Natl. Acad. Sci. USA **72**, 979-983.

4. Levin, D. E., E. Yamasaki, and B. N. Ames (1982). A new Salmonella tester strain, TA97a, for the detection of frameshift mutagens. A run of cytosines as a mutational hot spot. Mutation Research **94**, 315-330.

ABBREVIATIONS FOR TABLES

Evidence for test substance toxicity to the bacteria was documented by recording the appearance of the plates and background lawn using the following key:

T0 **Normal**, background microcolony lawn appears normal.
T1 **Slightly reduced**, background microcolony lawn is noticeably thinner.
T2 **Moderately reduced**, background lawn is markedly thinner resulting in an increase in the size of microcolonies compared to the vehicle control plate(s).
T3 **Severely reduced**, background lawn is distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate(s). Microcolonies may be seen readily by the unaided eye and are greatly enlarged relative to controls.
T4 **Absent**, plate(s) are distinguished by a complete lack of any microcolony lawn over a majority of the area of the plate(s)

Formation of a precipitate by the test substance will be documented using the following key:

P0 **No precipitate,** no precipitate observed
P1 **Microscopic precipitate**, precipitate present which does not interfere with background lawn evaluation or automated colony counting
P2 **Non-interfering precipitate,** precipitate present that is visible to the naked eye which does not interfere with automated colony counting
P3 **Interfering precipitate,** precipitate present which requires plate to be counted by hand
P4 **Heavy interfering precipitate**, precipitate present that prevents accurate colony counting and obscures the background lawn requiring plate rejection

Other appropriate observations relevant to the appearance of the plates:

N None
R Plate rejected

TABLE 1

**MUTAGENIC ACTIVITY OF H-22762
IN STRAIN TA100**

Concentration H-22762 (µg/plate)	Revertants Plate 1	Plate 2	Average (S.D.)	Observations
A. WITHOUT ACTIVATION				
0.0	101	110	106 (6)	T0,P0
10.0	112	112	112 (0)	T0,P0
50.0	111	112	112 (1)	T0,P0
100.0	113	124	119 (8)	T0,P0
500.0	102	80	91 (16)	T0,P0
1000.0	110	123	117 (9)	T1,P0
2500.0	91	111	101 (14)	T1,P0
5000.0	70	46	58 (17)	T2,P0
NAAZ				
2 µg/plate	915	872	894 (30)	N
B. WITH ACTIVATION				
0.0	134	134	134 (0)	T0,P0
10.0	115	126	121 (8)	T0,P0
50.0	118	115	117 (2)	T0,P0
100.0	117	137	127 (14)	T0,P0
500.0	124	113	119 (8)	T0,P0
1000.0	133	124	129 (6)	T0,P0
2500.0	128	116	122 (8)	T1,P0
5000.0	30	11	21 (13)	T3,P0
2AA				
1 µg/plate	449	457	453 (6)	N

TABLE 2

**MUTAGENIC ACTIVITY OF H-22762
IN STRAIN TA1535**

Concentration H-22762 (µg/plate)	Revertants Plate 1	Plate 2	Average (S.D.)	Observations
A. WITHOUT ACTIVATION				
0.0	18	15	17 (2)	T0,P0
10.0	17	14	16 (2)	T0,P0
50.0	19	17	18 (1)	T0,P0
100.0	15	14	15 (1)	T0,P0
500.0	14	15	15 (1)	T0,P0
1000.0	16	13	15 (2)	T1,P0
2500.0	11	13	12 (1)	T1,P0
5000.0	7	9	8 (1)	T2,P0
NAAZ				
2 µg/plate	632	593	613 (28)	N
B. WITH ACTIVATION				
0.0	21	16	19 (4)	T0,P0
10.0	16	22	19 (4)	T0,P0
50.0	17	23	20 (4)	T0,P0
100.0	21	20	21 (1)	T0,P0
500.0	17	15	16 (1)	T0,P0
1000.0	15	19	17 (3)	T0,P0
2500.0	16	21	19 (4)	T1,P0
5000.0	8	5	7 (2)	T2,P0
2AA				
2 µg/plate	626	582	604 (31)	N

TABLE 3

**MUTAGENIC ACTIVITY OF H-22762
IN STRAIN TA97a**

Concentration H-22762 (µg/plate)	Revertants Plate 1	Plate 2	Average (S.D.)	Observations
A. WITHOUT ACTIVATION				
0.0	100	107	104 (5)	T0,P0
10.0	103	107	105 (3)	T0,P0
50.0	113	102	108 (8)	T0,P0
100.0	104	96	100 (6)	T0,P0
500.0	122	115	119 (5)	T0,P0
1000.0	102	99	101 (2)	T0,P0
2500.0	78	68	73 (7)	T1,P0
5000.0	0	0	0 (0)	T2,P0
ICR 191				
2 µg/plate	2493	2536	2515 (30)	N
B. WITH ACTIVATION				
0.0	102	112	107 (7)	T0,P0
10.0	126	131	129 (4)	T0,P0
50.0	123	135	129 (8)	T0,P0
100.0	129	131	130 (1)	T0,P0
500.0	125	123	124 (1)	T0,P0
1000.0	120	128	124 (6)	T1,P0
2500.0	103	100	102 (2)	T1,P0
5000.0	113	103	108 (7)	T1,P0
2AA				
1 µg/plate	509	653	581 (102)	N

TABLE 4

**MUTAGENIC ACTIVITY OF H-22762
IN STRAIN TA98**

Concentration H-22762 (µg/plate)	Revertants Plate 1	Plate 2	Average (S.D.)	Observations
A. WITHOUT ACTIVATION				
0.0	26	20	23 (4)	T0,P0
10.0	25	25	25 (0)	T0,P0
50.0	25	29	27 (3)	T0,P0
100.0	27	23	25 (3)	T0,P0
500.0	22	22	22 (0)	T0,P0
1000.0	20	14	17 (4)	T0,P0
2500.0	16	16	16 (0)	T1,P0
5000.0	18	13	16 (4)	T1,P0
2NF				
25 µg/plate	1614	1411	1513 (144)	N
B. WITH ACTIVATION				
0.0	18	25	22 (5)	T0,P0
10.0	28	25	27 (2)	T0,P0
50.0	28	24	26 (3)	T0,P0
100.0	30	23	27 (5)	T0,P0
500.0	31	27	29 (3)	T0,P0
1000.0	28	28	28 (0)	T0,P0
2500.0	21	23	22 (1)	T1,P0
5000.0	15	20	18 (4)	T1,P0
2AA				
2 µg/plate	1404	1325	1365 (56)	N