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(A)

CYTEC

Toxicology & Product Safety Dept.
5 Garret Mountain Plaza
West Paterson, NJ 07424

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**CERTIFIED MAIL
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February 10, 1997

Document Control Office (7407)
Attn: TSCA Section 8(e) Coordinator
Office of Pollution Prevention and Toxics (OPPT)
U.S. Environmental Protection Agency
401 M Street, S.W.
Washington, DC 20460

Attention: TSCA SECTION 8(E) COORDINATOR

REFERENCE: 8EHQ-0197-13858

Contains No CBI

Dear Sir/Madam:

As a follow-up to our previous 8(e) submission dated January 13, 1997 for a chemical identified as 3,5-diamino-4'-phenylethynylbenzophenone, I am enclosing a copy of the final report entitled "Mutagenicity Test With CT-605-96 In The *Salmonella*/Mammalian-Microsome Reverse Mutation Screening Assay (Ames Test)". This report was received by CYTEC on February 10, 1997. This report **does not** contain confidential business information.

If you have any questions please contact me at (201) 357-3375.

Sincerely,



Patricia Ann Vernon
Toxicology Program & Product
Registration Specialist

cc: K. E. Koster - CY3
S. J. Sherman - CY3



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MUTAGENICITY TEST WITH

CT-605-96

IN THE *SALMONELLA*/MAMMALIAN-MICROSOME REVERSE MUTATION
SCREENING ASSAY (AMES TEST)

FINAL REPORT

RECEIVED

FEB 10 1997

P. A. VERNON

AUTHOR

Timothy E. Lawlor, M.A.

PERFORMING LABORATORY

Corning Hazleton, Inc. (CHV)
9200 Leesburg Pike
Vienna, Virginia 22182

LABORATORY PROJECT ID

CHV Study No.: 18057-0-401SC

SUBMITTED TO

Cytec Industries
Five Garret Mountain Plaza
West Paterson, NJ 07424

STUDY COMPLETION DATE

February 6, 1997

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QUALITY ASSURANCE STATEMENT

CORNING Hazleton

STUDY TITLE: *Salmonella*/Mammalian-Microsome Reverse Mutation
Screening Assay (Ames Test)

ASSAY NO.: 18057-0-401SC

PROTOCOL NO.: 401SC, Edition 17, Modified for Cytec Industries

Quality Assurance review of the draft and final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

<u>Inspection/Date</u>	<u>Findings Reported</u>	<u>Auditor</u>
Draft Report Review - 12/23/96	12/23/96	S. Ballenger
Final Report Review - 02/06/97	02/06/97	S. Ballenger



Quality Assurance Unit 2-6-97 Date Released

STUDY COMPLIANCE AND CERTIFICATION

The study was conducted in the spirit of compliance with the Good Laboratory Practice regulations as set forth by the Food and Drug Administration (FDA) in Title 21 of the U.S. Code of Federal Regulations Part 58, issued December 22, 1978, (effective June 20, 1979) with any applicable amendments. There were no deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

Study Director:



Timothy E. Lawlor, M.A.
Bacterial Mutagenesis
Genetic and Cellular Toxicology

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2.6.97
Study Completion Date

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SECTION I. STUDY INFORMATION

STUDY INFORMATION

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- A Sponsor: **Cytec Industries**
- B. Test Article: **CT-605-96 , Lot #2**
 - 1. Physical Description: **greenish-yellow solid**
 - 2. Date Received: **10/17/96**
- C. Type of Assay: *Salmonella*/Mammalian-Microsome Reverse Mutation Screening Assay (Ames Test)
 - 1. Protocol Number: CHV Protocol 401SC, Edition 17
Modified for Cytec Industries
 - 2. CHV Study Number: **18057-0-401SC**
- D. Study Dates
 - 1. Study Initiation Date: **04/09/93**
 - 2. Experimental Start Date: **11/11/96**
 - 3. Experimental Termination Date: **11/18/96**
- E. Study Supervisory Personnel
 - Study Director: Timothy E. Lawlor, M.A.
 - Associate Study Director: Michael S. Mecchi, B.S.
 - Laboratory Supervisor: Carlos E. Orantes, B.S.

NOTE: As of April 1, 1995, the company name, Hazleton Washington, Inc. (HWA), was legally changed to Corning Hazleton Inc. (CHV). Modifications are currently underway to reflect the company name change. Both designations for the company (HWA and CHV) may be used in this report.

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SECTION II. RESULTS AND CONCLUSIONS

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RESULTS

A. Introduction

At the request of Cytec Industries, Corning Hazleton Inc. investigated **CT-605-96** for mutagenic activity in the *Salmonella*/Mammalian-Microsome Reverse Mutation Screening Assay (Ames Test). This assay evaluated the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9).

The tester strains used in the mutagenicity assay were TA98, TA100, TA1535, and TA1537. The assay was conducted with eight doses of test article in both the presence and absence of S9 mix. All doses of test article and positive controls were plated at one plate per dose. The vehicle controls were plated in triplicate. In the mutagenicity assay, doses of 5,000, 3,330, 1,000, 333, 100, 33.3, 10.0, and 3.33 µg per plate were tested.

B. Test Article Handling

The test article, **CT-605-96**, was stored at room temperature and protected from light. Dimethylsulfoxide (DMSO, CAS# 67-68-5), Sigma Chemical Co., Lot 26H08681) was used as the vehicle. At 100 mg per ml, which was the most concentrated stock dilution prepared, the test article formed an opaque, dark brown solution. The test article remained a solution in all subsequent dilutions prepared for the mutagenicity assay.

C. Mutagenicity Assay

The mutagenicity assay results for **CT-605-96** are presented in Table 1. These data were generated in Experiments 18057-B1. The data are presented as individual plate counts along with a mean and standard deviation where appropriate.

The doses for the mutagenicity assay were 5,000, 3,330, 1,000, 333, 100, 33.3, 10.0, and 3.33 µg per plate both in the presence and absence of S9 mix (S9 homogenate purchased from Molecular Toxicology, Inc., Batch 0673, 37.8 mg of protein per ml).

In Experiment 18057-B1 (Table 1), all data generated with tester strains TA98, TA100, TA1535, and TA1537 in the presence of S9 mix were acceptable and positive increases in the number of histidine revertants per plate were observed with tester strains TA98 (83.3-fold) and TA1537 (40.0-fold) in the presence of S9 mix. No positive increases were observed with tester strains TA100 or TA1535 in the presence of S9 mix. Cytotoxicity was observed in the presence of S9

mix at 333 µg per plate and above as evidenced by a slight reduction in the bacterial background lawn. Slight precipitate was observed on the plates at 1,000 µg per plate and moderate precipitate was observed on the plates at 3,330 and 5,000 µg per plate.

In the absence of S9 mix, only two non-cytotoxic doses were observed. For this reason, these data could not be evaluated. Cytotoxicity was observed in the absence of S9 mix at 33.3 µg per plate and above as evidenced by a decrease in the number of revertants per plate and/or a thinning of the bacterial background lawn. Slight precipitate was observed on the plates at 333 and 1,000 µg per plate and moderate precipitate was observed on the plates at 3,330 and 5,000 µg per plate.

CONCLUSIONS

The results of the *Salmonella*/Mammalian-Microsome Reverse Mutation Screening Assay (Ames Test) indicate that under the conditions of this study, Cytec Industries' test article, **CT-605-96**, did cause positive increases in the numbers of histidine revertants per plate with tester strains TA98 (83.3-fold) and TA1537 (40.0-fold) in the presence of S9 mix. No positive increases in the number of revertants per plate were observed with tester strains TA100 or TA1535 in the presence of S9 mix.

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SECTION III. DATA TABLE

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TABLE 1
SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION SCREENING ASSAY RESULTS

TEST ARTICLE ID: CT-605-96
DATE PLATED: 11-Nov-96
DATE COUNTED: 15-Nov-96

EXPERIMENT ID: 18057-B1
VEHICLE: Dimethylsulfoxide
PLATING ALIQUOT: 50 µl

DOSE/PLATE	REVERTANTS PER PLATE				BACKGROUND LAWN*
	TA98	TA100	TA1535	TA1537	
MICROSOMES: Rat Liver					
VEHICLE CONTROL \oplus	13. 14. 23 17 \pm 6	107. 82. 101 97 \pm 1	6. 14. 7 9 \pm 4	8. 2. 3 4 \pm 3	1
TEST ARTICLE					
3.33 µg	18	80	8	5	1
10.0 µg	33	77	13	8	1
33.3 µg	47	94	16	4	1
100 µg	113	89	11	8	1
333 µg	214	54	9	16	2
1000 µg	592	82	5	67	2sp
3330 µg	1416	105	14	160	2mp
5000 µg	1283	82	11	61	2mp
POSITIVE CONTROL **	1033	1096	195	140	1
MICROSOMES: None					
VEHICLE CONTROL \oplus	9. 6. 11 9 \pm 3	75. 65. 87 76 \pm 1	7. 10. 11 9 \pm 2	4. 4. 2 3 \pm 1	1
TEST ARTICLE					
3.33 µg	11	86	8	9	1
10.0 µg	11	48	10	4	1
33.3 µg	3	18	10	2	3
100 µg	0	0	2	0	4
333 µg	0	0	0	0	4sp
1000 µg	0	0	0	0	4sp
3330 µg	0	0	0	0	4mp
5000 µg	0	0	0	0	4mp
POSITIVE CONTROL **	172	653	662	895	1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate

* Background Lawn Evaluation Codes:

1 = normal	2 = slightly reduced	3 = moderately reduced
4 = extremely reduced	5 = absent	6 = obscured by precipitate
sp = slight precipitate	mp = moderate precipitate (requires hand count)	hp = heavy precipitate (requires hand count)

• Vehicle control plated in triplicate. individual plate counts along with mean \pm standard deviation are indicated.

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE DEFINITION	<u>CHARACTERISTICS OF BACKGROUND LAWN</u>
1 Normal	A healthy microcolony lawn.
2 Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3 Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4 Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5 Absent	A complete lack of any microcolony lawn.
6 Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

sp Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
mp Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus requiring the plate to be hand counted.
hp Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4mp would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

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SECTION IV. APPENDIX: STUDY PROTOCOL AND AMENDMENT

SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION SCREENING ASSAY
(AMES TEST)

Hazleton Washington, Inc. (HWA) will conduct this study in the spirit of compliance with EPA and FDA Good Laboratory Practice (GLP) Guidelines. The final report will be subject to audit by Quality Assurance in accordance with SOPs at Hazleton Washington, Inc. This study will be conducted by HWA at 9200 Leesburg Pike, Vienna, VA 22182.

PART 1. SPONSOR INFORMATION AND APPROVALS

I. SPONSOR IDENTIFICATION: Cyttec Industries
Five Garret Mountain Plaza
West Paterson, NJ 07424

II. TEST ARTICLE IDENTIFICATION: CT-605-96

III. TEST ARTICLE ANALYSIS

Determination of the test article stability and the test article characteristics as defined in the GLP regulations of FDA (21 CFR 58.105), EPA-TSCA (40 CFR 792.105), and EPA-FIFRA (40 CFR 160.105) is the responsibility of the Sponsor.

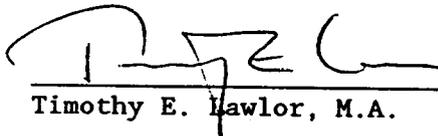
IV. STUDY DATES

Proposed Experimental Start Date: November, 1996

Proposed Experimental Termination Date: December, 1996

V. APPROVAL OF STUDY PROTOCOL

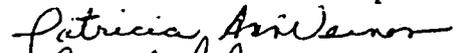
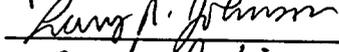
Study Director:



Timothy E. Lawlor, M.A.

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Date: 1/9/93

Sponsor's Authorized Representatives:


1/6/93
1/11/93
Date: _____
1/20/93
Date: _____

PART 2. STUDY PROTOCOL

SALMONELLA/MAMMALIAN-MICROSOME REVERSE MUTATION SCREENING ASSAY
(AMES TEST)

I. OBJECTIVE

The objective of this study is to evaluate the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of several strains of Salmonella typhimurium in the presence and absence of mammalian microsomal enzymes.

II. TEST SYSTEM

The Salmonella/Mammalian-microsome reverse mutation assay (Ames Test) detects point mutations, both frameshifts and/or base pair substitutions, in bacteria. The strains of Salmonella typhimurium used in this assay are histidine auxotrophs by virtue of conditionally lethal mutations in their histidine operon. When these histidine-dependent cells (his-) are exposed to the test article and grown under selective conditions (minimal media with a trace amount of histidine) only those cells which revert to histidine independence (his+) are able to form colonies. The trace amount of histidine in the media allows all the plated bacteria to undergo a few cell divisions which is essential for mutagenesis to be fully expressed. The his+ revertants are readily discernable as colonies against the limited background growth of the his- cells. By utilizing several different tester strains, both base pair substitution mutations and frameshift mutations can be detected. In addition, the utilization of a mammalian microsomal enzyme preparation (S9 mix), also allows detection of mutagenic metabolites of the test article. The Ames Test has been shown to be a sensitive, rapid and accurate indicator of the mutagenic activity of many materials covering a wide range of chemical classes.

III. MATERIALS

A. Tester Strains

The tester strains to be used will be the Salmonella typhimurium histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames et al (1975). The specific genotypes of these strains are shown in Table 1.

TABLE 1. TESTER STRAIN GENOTYPES					
Histidine Mutation			Additional Mutations		
<u>hisG46</u>	<u>hisC3076</u>	<u>hisD3052</u>	LPS	Repair	R Factor
TA1535	TA1537		<u>rfa</u>	<u>uvrB</u>	-
TA100		TA98	<u>rfa</u>	<u>uvrB</u>	+R

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide (LPS) barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the uvrB gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA1535 is reverted by base substitution mutagens and TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

1. Source of Tester Strains

The tester strains in use at HWA were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

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2. Storage of Tester Strains

a. Frozen Permanent Stocks

Frozen permanent stocks will be prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing small aliquots (0.5 - 1.5 ml) at $\leq -70^{\circ}\text{C}$.

b. Master Plates

Master plates will be prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 μM), biotin (3 μM), and for strains containing the R-factor, ampicillin (25 $\mu\text{g/ml}$). Tester strain master plates will be stored at $5 \pm 3^{\circ}\text{C}$.

3. Preparation of Overnight Cultures

a. Inoculation

Overnight cultures for use in all testing procedures will be inoculated by transferring a colony from the appropriate master plate to a flask containing culture medium. Inoculated flasks will be placed in a shaker/incubator which will be programmed to begin operation (shaking, 125 ± 25 rpm; incubation, $37 \pm 2^{\circ}\text{C}$) so that the overnight cultures are in log phase or late log phase when turbidity monitoring begins.

b. Harvest

To ensure that cultures are harvested in late log phase, the length of incubation will be determined by spectrophotometric monitoring of culture turbidity. Cultures will be harvested once a predetermined turbidity is reached as determined by a percent transmittance (%T) reading on a spectrophotometer. This target turbidity ensures that cultures have reached a density of at least 0.5×10^9 cells per ml and that the cultures have not overgrown. Overgrown (stationary) cultures may exhibit decreased sensitivity to some mutagens. Cultures will be removed from incubation when the target %T is reached and placed at $5 \pm 3^{\circ}\text{C}$.

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4. Confirmation of Tester Strain Genotypes

Tester strain cultures will be checked for the following genetic markers on the day of their use in the mutagenicity assay:

a. rfa Wall Mutation

The presence of the rfa wall mutation will be confirmed by demonstration of the cultures sensitivity to crystal violet.

b. pKM101 Plasmid

The presence of the R factor plasmid, pKM101, will be confirmed for cultures of tester strains TA98 and TA100 by demonstration of resistance to ampicillin.

c. Characteristic Number of Spontaneous Revertants

The number of spontaneous revertants per plate in the vehicle controls that is characteristic of the respective strains will be demonstrated by plating aliquots of each culture along with the appropriate vehicle on selective medium.

5. Tester Strain Media

a. Culturing Broth

The broth used to grow overnight cultures of the tester strains will be Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (W/V) Oxoid Nutrient Broth #2 (dry powder).

b. Agar Plates

Bottom agar (25 ml per 15 x 100 mm petri dish) will be Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) supplemented with 1.5% (W/V) agar and 0.2% (W/V) glucose.

c. Overlay Agar for Selection of Histidine Revertants

Overlay (top) agar will be prepared with 0.7% agar (W/V) and 0.5% NaCl (W/V) and will be supplemented with 10 ml of 0.5 mM histidine/biotin solution per 100 ml agar.

When S9 mix is required, 2.0 ml of the supplemented top agar is used for the overlay. However, when S9 is not required, water is added to the supplemented top agar (0.5 ml of water per 2 ml of supplemented top agar) and 2.5 ml of the diluted supplemented top agar is used for the overlay. This dilution ensures that the final top agar and amino acid supplement concentrations remain the same both in the presence and absence of S9 mix.

B. Liver Microsomal Enzyme Reaction Mixture (S9 Mix)

1. S9 Homogenate

Liver microsomal enzymes (S9 homogenate) will be purchased commercially and will be prepared from male Sprague-Dawley rats that have been injected (i.p.) with Aroclor™ 1254 (200 mg/ml in corn oil) at 500 mg/kg as described by Ames et al, 1975.

2. S9 Mix

The S9 mix will be prepared immediately prior to its use in any experimental procedure. The S9 mix will contain the components indicated in Table 2.

TABLE 2. S9 MIX COMPONENTS

H ₂ O	0.70 ml
1M NaH ₂ PO ₄ /Na ₂ HPO ₄ , pH 7.4	0.10 ml
0.25M Glucose-6-phosphate	0.02 ml
0.10M NADP	0.04 ml
0.825M KCl/0.2M MgCl ₂	0.04 ml
S9 Homogenate	<u>0.10 ml</u>
	1.00 ml

C. Controls

1. Vehicle Controls

Appropriate vehicle controls will be plated for all strains in the presence and absence of S9 mix. Vehicles compatible with this test system include but will not be limited to: Deionized H₂O, dimethylsulfoxide (CAS #67-68-5), ethanol (CAS #64-17-5), and dimethylformamide (CAS #68-12-2).

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2. Positive Controls

The combinations of positive controls, activation condition and tester strains plated concurrently with the assay are indicated in Table 3.

<u>TABLE 3. POSITIVE CONTROLS</u>			
<u>Tester Strain</u>	<u>S9 Mix</u>	<u>Positive Control</u>	<u>Conc. per Plate</u>
TA98	+	2-aminoanthracene	2.5 µg
TA98	-	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	-	sodium azide	2.0 µg
TA1535	+	2-aminoanthracene	2.5 µg
TA1535	-	sodium azide	2.0 µg
TA1537	+	2-aminoanthracene	2.5 µg
TA1537	-	ICR-191	2.0 µg

 3. Sterility Controls

 a. Test Article

The most concentrated test article dilution will be checked for sterility by plating an aliquot of the same volume used in the assay on selective agar.

 b. S9 Mix

The S9 mix will be checked for sterility by plating 0.5 ml on selective agar.

 IV. METHODS

 A. Mutagenicity Assay

 1. Design

The assay will be performed using tester strains TA98, TA100, TA1535 and TA1537, both in the presence and absence of S9 mix. Eight dose levels of test article will be tested along with the appropriate vehicle and positive controls. Unless the Sponsor specifies dose levels to be tested, the test article will be tested up to a maximum concentration of 5,000 µg per plate if solubility/miscibility permits. If the test article exhibits limited solubility/miscibility, it

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will be tested up to the maximum workable concentration attainable in the vehicle of choice. When solubility/miscibility of the test article is not limiting, the following doses will be tested: 5,000, 3,330, 1,000, 333, 100, 33.3, 10.0 and 3.33 μg per plate.

2. Frequency and Route of Administration

The tester strains will be exposed to the test article via the plate incorporation methodology originally described by Ames et al (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. In the plate incorporation methodology, the test article, the tester strain and the S9 mix (where appropriate) are combined in molten agar which is overlaid onto a minimal agar plate. Following incubation at $37 \pm 2^\circ\text{C}$ for 48 ± 8 hr, revertant colonies will be counted. All dose levels of test article and positive controls will be plated in singlet. The vehicle controls will be plated in triplicate.

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C. Plating Procedures

Each plate will be labeled with a code which identifies the test article, test phase, tester strain, activation condition and dose level. The S9 mix and dilutions of the test article will be prepared immediately prior to their use.

When S9 mix is not required, 100 μl of tester strain and 50 μl of vehicle or test article dose will be added to 2.5 ml of molten selective top agar (maintained at $45 \pm 2^\circ\text{C}$). When S9 mix is required, 500 μl of S9 mix, 100 μl of tester strain and 50 μl of vehicle or test article dose will be added to 2.0 ml of molten selective top agar. After the required components have been added, the mixture will be vortexed and overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay has solidified, the plates will be inverted and incubated for 48 ± 8 hr at $37 \pm 2^\circ\text{C}$. When necessary, plating aliquots of other than 50 μl of test article/vehicle will be used. Positive control articles will be plated using a 50 μl plating aliquot.

D. Scoring the Plates

Plates which are not evaluated immediately following the incubation period will be held at $5 \pm 3^\circ\text{C}$ until such time that colony counting and bacterial background lawn evaluation can take place.

1. Bacterial Background Lawn Evaluation

The condition of the bacterial background lawn will be evaluated for evidence of cytotoxicity and test article precipitate. Evidence of cytotoxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate.

2. Counting Revertant Colonies

Revertant colonies for a specific tester strain within a given test article dilution series will be counted either entirely by automated colony counter or entirely by hand. If there is sufficient test article precipitate on the plates at any dose level which interferes with automated colony counting, then the plates at all dose levels for that specific strain and activation condition will be counted by hand.

E. Analysis of Data

For all replicate platings, the mean revertants per plate and the standard deviation will be calculated.

EVALUATION OF TEST RESULTS

Before assay data can be evaluated, the criteria for a valid assay must be met.

A. Criteria For A Valid Assay

The following criteria will be used to determine a valid assay:

1. Tester Strain Integrity

a. rfa Wall Mutation

To demonstrate the presence of the rfa wall mutation, tester strain cultures must exhibit sensitivity to crystal violet.

b. -pKM101 Plasmid

To demonstrate the presence of the R-factor plasmid, pKM101, cultures of tester strains TA98 and TA100 must exhibit resistance to ampicillin.

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c. Characteristic Number of Spontaneous Revertants

To demonstrate the requirement for histidine, the tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable ranges for the mean vehicle controls are as follows:

TA98	8 - 60
TA100	60 - 240
TA1535	4 - 45
TA1537	2 - 25

d. Tester Strain Culture Density

To demonstrate that appropriate numbers of bacteria are plated, the density of tester strain cultures must be greater than or equal to 0.5×10^9 bacteria per ml and/or have reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 0.5×10^9 bacteria per ml.

e. Positive Control Values

(1) Positive Control Values in the Absence of S9 Mix

To demonstrate that the tester strains are capable of identifying a mutagen, the mean value of a positive control for a respective tester strain must exhibit at least a 3-fold increase over the mean value of the vehicle control for that strain.

(2) Positive Control Values in the Presence of S9 Mix (S9 Mix Integrity)

To demonstrate that the S9 mix is capable of metabolizing a promutagen to its mutagenic form(s), the mean value of the positive control for a respective tester strain in the presence of the S9 mix must exhibit at least a 3-fold increase over the mean value of the vehicle control for that strain.

An acceptable positive control in the presence of S9 for a specific strain will be evaluated as having demonstrated both the integrity of the S9 Mix and the ability of the tester strain to detect a mutagen.

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2. Cytotoxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data.

B. Criteria For A Positive Response

Once the criteria for a valid assay have been met, responses observed in the assay are evaluated as follows:

1. Tester Strains TA98 and TA100

For a test article to be considered positive, it must produce at least a 2-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

2. Tester Strains TA1535 and TA1537

For a test article to be considered positive, it must produce at least a 3-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

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VI. REPORTING THE RESULTS

A report of the results of this study will be prepared by HWA and will include a data table with:

- the identity of the bacterial tester strains used in the assay
- dose levels at which the test article was tested
- individual plate counts for all treated, positive and vehicle control plates
- calculated mean and standard deviation for all replicate plate counts

The report will also include an evaluation of the results as defined in the protocol. A copy of this protocol will be attached to the report as an appendix.



VII. CHANGES AND REVISIONS

Any changes or revisions of this approved protocol will be documented, signed by the Study Director, dated, and maintained with this protocol. The Sponsor will be notified of any changes or revisions.

VIII. RECORDS TO BE MAINTAINED

All raw data, documentation, records, protocols, and final reports generated as a result of this study will be archived in the storage facilities of Hazleton Washington, Inc., for at least one year following submission of the final report to the sponsor. After the one year period, the sponsor may elect to have the aforementioned materials retained in the storage facilities of Hazleton Washington, Inc., for an additional period of time or sent to a storage facility designated by the sponsor.

IX. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Maron, D.M., and Ames B. (1983). Revised Methods for the Salmonella Mutagenicity Test. Mutation Research 113:173-215.

Vogel, H.J. and Bonner, D.M. (1956). Acetylornithinase of Escherichia coli: Partial purification and some properties. J. Biol Chem. 218:97-106.

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AMENDMENT TO THE STUDY PROTOCOL

STUDY TITLE: *Salmonella*/Mammalian-Microsome Reverse Mutation Screening Assay
(Ames Test)

PROTOCOL NO.: 401, Edition 17

STUDY NO.: 18057-0-401

Amendment #1.

1. Testing Laboratory Identification.

The testing laboratory identification is changed from Hazleton Washington, Inc. (HWA) to Corning Hazleton Inc. (CHV).

Reason:

As of April 1, 1995, the name of the testing laboratory was legally changed to Corning Hazleton Inc., which is abbreviated as CHV.

NOTE: Modifications are currently underway to reflect the company name change. Both designations for the company (HWA and CHV) may appear in the raw data and/or the report.

2. Protocol Number.

In the protocol number, 401 will be changed to 401SC.

Reason:

To clarify the protocol number.

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Study Director:



Timothy E. Lawlor, M.A.
Bacterial Mutagenesis
Genetic and Cellular Toxicology

12-23-96
Date

TRIAGE of 8(e) Submissions

Date sent to triage: _____

NON-CAP

CAP

Submission number: 13887 A

TSCA Inventory: Y N D

STUDY TYPE (circle appropriate):

Ernest Falke (E605C)

ATOX SBTOX SEN CARC

Gordon Cash (E425)

ECO AQUATO

Katherine Anitole (E613B)

RTOX/DTOX

Daljit Sawhney (E611A)

CTOX STOX

Deborah Norris (E606)

NEUR

Elizabeth Margosches (E613C)

EPI

~~**Michael Cimino (E611D)**~~

~~GTOX~~

Leonard Keifer (E611C)

Metabolism/Pharmacokinetics

OTHER: _____

NOTES:

CECATS DATA:
 Submission # BEHO. 0297-13887 SEQ A

TYPE: INT SUPP FLWP

SUBMITTER NAME: CYTEC Industries

INFORMATION REQUESTED: FLWP DATE:
 6591 NO INFO REQUESTED
 6592 INFO REQUESTED (TECH)
 6593 INFO REQUESTED (VOL ACTIONS)
 6594 INFO REQUESTED (REPORTING RATIONALE)
 DISPOSITION:
 REFER TO CHEMICAL SCREENING
 CAP NOTICES

VOL UNITARY ACTIONS:
 INI ACTION (ALTERED)
 6601 STUDIES PLANNED (MIN MAX)
 6602 INTERACTION (MIN MAX)
 6603 LABELS (TANKS)
 6604 PROCESS (MIN MAX)
 6605 APP ASE DISCONTINUED
 6607 PRODUCTION DISCONTINUED
 6608 CONFIDENTIAL

SUB DATE: 2-10-97 RECD DATE: 2-19-97 GRAD DATE: 5-8-97

CHEMICAL NAME: 3,5-diamino-4'-phenylethylylbenzophenone CASE UNKNOWN

INFORMATION TYPE:	P F C	INFORMATION TYPE:	P F C	INFORMATION TYPE:	P F C
0201 ONCO (HUMAN)	01 02 04	0216 EPICLIN	01 02 04	0241 BAMBURNO (ANIMAL)	01 02 04
0202 ONCO (ANIMAL)	01 02 04	0217 HUMAN EXPOS (PROD CONTAM)	01 02 04	0242 BAMBURNO (HUMAN)	01 02 04
0203 CELL TRANS (IN VITRO)	01 02 04	0218 HUMAN EXPOS (ACCIDENTAL)	01 02 04	CHEM/PHYS PROP	01 02 04
<input checked="" type="radio"/> 0204 MUTA (IN VITRO)	01 02 04	0219 HUMAN EXPOS (MONITORING)	01 02 04	CLASTO (IN VITRO)	01 02 04
0205 MUTA (IN VIVO)	01 02 04	0220 BOWAQUA TOX	01 02 04	CLASTO (ANIMAL)	01 02 04
0206 REPRO/TERATO (HUMAN)	01 02 04	0221 ENV OCCUR/EXPOS	01 02 04	CLASTO (HUMAN)	01 02 04
0207 REPRO/TERATO (ANIMAL)	01 02 04	0222 ENV EXPOS OF ENV CONTAM	01 02 04	DNA DAMAGE/REPAIR	01 02 04
0208 NEURO (HUMAN)	01 02 04	0223 RESPONSE REQUEST DELAY	01 02 04	PROD/USE/PROC	01 02 04
0209 NEURO (ANIMAL)	01 02 04	0224 PROD/CON/CHIEF ID	01 02 04	MSDS	01 02 04
0210 ACUTE TOX (HUMAN)	01 02 04	0225 REPORTING RATIONALE	01 02 04	OTHER	01 02 04
0211 CHR TOX (HUMAN)	01 02 04	0226 CONFIDENTIAL	01 02 04		
0212 ACUTE TOX (ANIMAL)	01 02 04	0227 ALLELO (HUMAN)	01 02 04		
0213 SUB ACUTE TOX (ANIMAL)	01 02 04	0228 ALLELO (ANIMAL)	01 02 04		
0214 SUB CHRONIC TOX (ANIMAL)	01 02 04	0229 METAB/PHARMACO (ANIMAL)	01 02 04		
0215 CHRONIC TOX (ANIMAL)	01 02 04	0230 METAB/PHARMACO (HUMAN)	01 02 04		

TRIAL/DATA: NON-CELL INVENTORY ONGOING REVIEW SPECIES TOXICOLOGICAL CONCERN: USE: PRODUCTION:

CAS SR YES NO IN N AMIN

YES (DROPPABLE) Salmomella LOW MED HIGH

UNCLASSIFIED

“13887A”=“____”=“MUTAGENICITY OF CT-605-96 (CAS NO- UNKNOWN) WAS EVALUATED IN THE TA98, TA100, TA1535, TA1537, AND TA1538 STRAINS OF SALMONELLA TYPHIMURIUM AT CONCENTRATION LEVELS OF 0, 3.33, 10.0, 33.3, 100, 333, 1000, 3330, AND 5000 UG/PLATE, IN THE PRESENCE AND ABSENCE OF AROCLOR-INDUCED RAT LIVER S-9 METABOLIC ACTIVATION. CYTOTOXICITY WAS OBSERVED IN THE PRESENCE OF S9 MIX AT CONCENTRATION LEVELS OF 333 UG/PLATE AND ABOVE AND IN THE ABSENCE OF S9 MIX AT 33.3 UG/PLATE AND ABOVE. THE TEST SUBSTANCE INCREASED THE FREQUENCY OF HIS+ REVERTANTS IN THE TA1537 AND TA98 STRAINS WITH METABOLIC ACTIVATION BUT NOT THE TA100 OR TA1535 STRAINS WITH ACTIVATION. DATA FROM THE TESTS DONE IN THE ABSENCE OF S9 MIX COULD NOT BE EVALUATED DUE TO THE HIGH LEVELS OF CYTOTOXICITY.”