

RECEIVED
OPPT CBIC

95 SEP -6 AM 8:02

50314

CORNING Hazleton

MUTAGENICITY TEST WITH
DIMETHYL ACRYLAMIDE (DMACM)
IN THE *SALMONELLA* - *ESCHERICHIA COLI*/MAMMALIAN-MICROSOME REVERSE
MUTATION ASSAY

FINAL REPORT

AUTHOR

Timothy E. Lawler, M.A.

PERFORMING LABORATORY

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

LABORATORY PROJECT ID

CHV Study No.: 16849-0-409

3M Study No.: 0795UK0165

SUBMITTED TO

3M Center
St. Paul, MN 55144-1000

STUDY COMPLETION DATE

June 14, 1995

QUALITY ASSURANCE STATEMENT

STUDY TITLE: *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay

STUDY NO.: 16849-0-409

PROTOCOL NO.: 409, Edition 4 Modified for 3M

Quality Assurance inspections of the study and the review of the 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>
Plating - 05/03/95	05/03/95	S. Ballenger
Draft Report Review - 06/05/95	06/05/95	S. Ballenger
Final Report Review - 06/14/95	06/14/95	S. Ballenger



Quality Assurance Unit 6/14/95
Date Released

COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Food and Drug Administration, 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 significant 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.

SUBMITTED BY:

Study Director:



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

6-14-95

Study Completion
Date

TABLE OF CONTENTS

	Page No.
I. SUMMARY	5
II. STUDY INFORMATION	7
III. MATERIALS AND METHODS	9
IV. RESULTS AND CONCLUSIONS	23
V. DATA TABLES	26

SECTION I. SUMMARY

INTRODUCTION AND CONCLUSIONS

SUMMARY

A. Introduction

At the request of Corning Hazleton Inc. investigated Dimethyl Acrylamide (DMACM) for mutagenic activity in the *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay. 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 and at the tryptophan locus in an *Escherichia coli* tester strain both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9).

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strains TA100 and WP2uvrA and ten doses of test article ranging from 5,000 to 6.67 µg per plate, one plate per dose, both in the presence and absence of S9 mix.

The tester strains used in the mutagenicity assay were *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* tester strain WP2uvrA. The assay was conducted with five doses of test article in both the presence and absence of S9 mix along with concurrent vehicle and positive controls using three plates per dose. The doses tested were 5,000, 3,330, 1,000, 333, and 100 µg per plate in both the presence and absence of S9 mix.

B. Conclusions

The results of the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay indicate that, under the conditions of this study, test article, Dimethyl Acrylamide (DMACM), did not cause a positive increase in the number of revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

SECTION II. STUDY INFORMATION

STUDY INFORMATION

- A. Sponsor: 3M
- B. Test Article: **Dimethyl Acrylamide (DMACM)**
Lot No.: 09430EW 11-0003-8010-2
 - 1. Physical Description: **clear colorless liquid**
 - 2. Date Received: **04/24/95**
- C. Type of Assay: *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay
 - 1. Protocol Number: CHV Protocol 409, Edition 4
Modified for 3M
 - 2. CHV Study Number: 16849-0-409
- D. Study Dates
 - 1. Study Initiation Date: **04/05/95**
 - 2. Experimental Start Date: **04/28/95**
 - 3. Experimental Termination Date: **05/08/95**
- E. Study Supervisory Personnel
 - Study Director: Timothy E. Lawlor, M.A.
 - Laboratory Supervisor: Michael S. Mecchi, 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.

SECTION III. MATERIALS AND METHODS

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames *et al* (1975) and Green and Muriel (1976).

MATERIALS

A. Tester Strains1. *Salmonella typhimurium*

The tester strains used were 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 I.

TABLE I. 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 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.

2. *Escherichia coli*

The tester strain used was the tryptophan auxotroph WP2*uvrA* as described by Green and Muriel (1976).

In addition to a mutation in the tryptophan operon, the tester strain contains a *uvrA* DNA repair deficiency which enhances its sensitivity to some mutagenic compounds. This deficiency allows the strain to show enhanced mutability since the *uvrA* repair system would normally act to remove the damaged part of the DNA molecule and accurately repair it afterwards.

Tester strain WP2*uvrA* is reverted from tryptophan dependence (auxotrophy) to tryptophan independence (prototrophy) by base substitution mutagens.

3. Source of Tester Strains

a. *Salmonella typhimurium*

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

b. *Escherichia coli*

The tester strain, WP2*uvrA*, in use at CHV was received from the National Collection of Industrial Bacteria, Torrey Research Station, Scotland (United Kingdom).

4. Storage of the Tester Strains

a. Frozen Permanent Stocks

Frozen permanent stocks were 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 were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with 1) for *Salmonella typhimurium*, an excess of histidine, and biotin, and for tester strains TA 98 and TA100, ampicillin (25 µg/ml), to ensure the stable maintenance of the pKM101 plasmid; and 2) for *Escherichia coli*, an excess of tryptophan. Tester strain master plates were stored at $5 \pm 3^\circ\text{C}$.

5. Preparation of Overnight Cultures

a. Inoculation

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

b. Harvest

To ensure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring of culture turbidity. Cultures were harvested once a predetermined turbidity was 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 were removed from incubation when the target %T was reached and were placed at $5 \pm 3^\circ\text{C}$.

6. Confirmation of Tester Strain Genotypes

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

a. *Salmonella typhimurium*

1) *rfa* Wall Mutation

The presence of the *rfa* wall mutation was confirmed by demonstration of the sensitivity of the culture to crystal violet. An aliquot of an overnight

culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

2) pKM101 Plasmid R-factor

The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

3) Characteristic Number of Spontaneous Revertants

The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 µl aliquots of the culture along with the appropriate vehicle on selective media.

b. *Escherichia coli*

1) Characteristic Number of Spontaneous Revertants

The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 µl aliquots of the WP2uvrA culture along with the appropriate vehicle on selective media.

7. Tester Strain Media

a. Culturing Broth

The broth used to grow overnight cultures of the tester strains was Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

b. Agar Plates

Bottom agar (25 ml per 15 x 100 mm petri dish) was 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 Revertants

Overlay (top) agar was prepared with 0.7% agar (w/v) and 0.5% NaCl (w/v) and was supplemented with 10 ml of 1) 0.5 mM histidine/biotin solution per 100 ml agar for selection of histidine revertants, or 2) 0.5 mM tryptophan solution per 100 ml of agar for selection of tryptophan revertants. When S9 mix was required, 2.0 ml of the supplemented top agar was used in the overlay. However, when S9 mix was not required, water was added to the supplemented top agar (0.5 ml of water per 2 ml of supplemented top agar) and the resulting 2.5 ml of diluted supplemented top agar was used for the overlay. This dilution ensured that the final top agar and amino acid supplement concentrations remained 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) were purchased from Molecular Toxicology, Inc., Annapolis, MD 21401, Batch 0568 (38.8 mg of protein per ml) and Batch 0564 (39.3 mg of protein per ml). The homogenate was prepared from male Sprague-Dawley rats that had been injected (i.p.) with Aroclor™ 1254 (200 mg per ml in corn oil) at 500 mg/kg as described by Ames *et al*, 1975.

2. S9 Mix

The S9 mix was prepared immediately prior to its use in any experimental procedure. The S9 mix contained the components indicated in Table II.

TABLE II. S9 MIX COMPONENTS

H ₂ O	0.70 ml
1M NaH ₂ PO ₄ /Na ₂ HPO ₄ , pH7.4	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

Vehicle controls were plated for all tester strains both in the presence and absence of S9 mix. The vehicle control was plated, using a 50 µl aliquot of vehicle (equal to the maximum aliquot of test article dilution plated), along with a 100 µl aliquot of the appropriate tester strain and a 500 µl aliquot of S9 mix (when necessary), on selective agar.

2. Positive Controls

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

TABLE III. POSITIVE CONTROLS

Tester Strain	S9 Mix	Positive Control	Conc per plate
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
WP2uvrA	+	2-aminoanthracene	25.0 µg
WP2uvrA	-	4-nitroquinoline-N-oxide	10.0 µg

a. Source and Grade of Positive Control Articles

2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., purity 97.5%; 2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., purity 98%; sodium azide (CAS #26628-22-8), Sigma Chemical Co., purity >98%; ICR-191 (CAS #1707-45-0), Polysciences Inc., purity >95%; 4-nitroquinoline-N-oxide (CAS #56-57-5), Sigma Chemical Co., purity >99%.

3. Sterility Controls

a. Test Article

The most concentrated test article dilution was checked for sterility by plating a 50 µl aliquot (the same volume used in the assay) on selective agar.

b. S9 Mix

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

METHODS

A. Dose Rangefinding Study

The growth inhibitory effect (cytotoxicity) of the test article to the test system was determined in order to allow the selection of appropriate doses to be tested in the mutagenicity assay.

1. Design

The dose rangefinding study was performed using tester strains TA100 and WP2uvrA both in the presence and absence of S9 mix. Ten doses of test article were tested at one plate per dose. The test article was checked for cytotoxicity up to a maximum concentration of 5 mg per plate.

a. Rationale

The cytotoxicity of the test article observed on tester strain TA100 is generally representative of that observed on the other tester strains and because of TA100's comparatively high number of spontaneous revertants per plate, gradations of cytotoxicity can be readily discerned from routine experimental variation. The *Escherichia coli* tester strain WP2uvrA does not possess the *rfa* wall mutation that the *Salmonella typhimurium* strains have and thus, a different range of cytotoxicity may be observed. Also, the cytotoxicity induced by a test article in the presence of S9 mix may vary greatly from that observed in the absence of S9 mix. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the S9 mix.

2. Evaluation of the Dose Ranging Study

Cytotoxicity is detectable as a decrease in the number of revertant colonies per plate and/or by a thinning or disappearance of the bacterial background lawn.

3. Selection of the Maximum Dose for the Mutagenicity Assay

a. No Cytotoxicity Observed

Since no cytotoxicity was observed in the dose ranging study, the highest dose of test article used in the mutagenicity assay was the same as that tested in the ranging study.

B. Mutagenicity Assay

1. Design

The assay was performed using tester strains TA98, TA100, TA1535, TA1537, and WP2uvrA both in the presence and absence of S9 mix. Five doses of test article were tested along with the appropriate vehicle and positive controls. The doses of test article were selected based on the results of the dose ranging study.

2. Frequency and Route of Administration

The tester strains were 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) were combined in molten agar which was overlaid onto a minimal agar plate. Following incubation at $37 \pm 2^\circ\text{C}$ for 48 ± 8 hr, revertant colonies were counted. All doses of the test article, the vehicle controls and the positive controls were plated in triplicate.

C. Plating Procedures

These procedures were used in both the dose ranging study and the mutagenicity assay.

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

When S9 mix was not required, 100 µl of tester strain and 50 µl of vehicle or test article dose was added to 2.5 ml of molten selective top agar (maintained at $45 \pm 2^\circ\text{C}$). When S9 mix was required, 500 µl of S9 mix, 100 µl of tester strain and 50 µl of vehicle or test article dose was added to 2.0 ml of molten selective top agar. After the required components had been added, the mixture was 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 had solidified, the plates were inverted and incubated for 48 ± 8 hr at $37 \pm 2^\circ\text{C}$. Positive control articles were plated using a 50 µl plating aliquot.

D. Scoring the Plates

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

1. Bacterial Background Lawn Evaluation

The condition of the bacterial background lawn was evaluated for evidence of cytotoxicity and test article precipitate. Evidence of cytotoxicity was scored relative to the vehicle control plate and was recorded along with the revertant counts for all plates at that dose on the data tables using the code system presented at the end of the Materials and Methods Section.

2. Counting Revertant Colonies

The number of revertant colonies per plate for the vehicle controls and all plates containing test article were counted manually. The number of revertant colonies per plate for the positive controls were counted by automated colony counter.

E. Analysis of Data

For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The results of these calculations are presented in tabular form in the Data Tables Section of this report.

EVALUATION OF TEST RESULTS

Before assay data were evaluated, the criteria for a valid assay had to be met.

A. Criteria For A Valid Assay

The following criteria were used to determine a valid assay:

1. Tester Strain Integrity : *Salmonella typhimurium*

a. *rfa* Wall Mutation

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

b. pKM101 Plasmid

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

c. Characteristic Number of Spontaneous Revertants

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

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

2. Tester Strain Integrity : *Escherichia coli*

a. Characteristic Number of Spontaneous Revertants

To demonstrate the requirement for tryptophan, the tester strain culture exhibited a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable range for WP2*uvrA* was 5 to 40 revertants per plate.

3. Tester Strain Culture Density

To demonstrate that appropriate numbers of bacteria are plated, the density of tester strain cultures were greater than or equal to 0.5×10^9 bacteria per ml and/or had 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.

4. Positive Control Values

a. Positive Control Values in the Absence of S9 Mix

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

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

To demonstrate that the S9 mix was 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 exhibited 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 was evaluated as having demonstrated both the integrity of the S9 mix and the ability of the tester strain to detect a mutagen.

2. Cytotoxicity

A minimum of three non-toxic doses were required to evaluate assay data.

B. Criteria For A Positive Response

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

1. Tester Strains TA98, TA100, and WP2uvrA

For a test article to be considered positive, it had to 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 had to 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 had to 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 had to be accompanied by a dose response to increasing concentrations of the test article.

RECORDS TO BE MAINTAINED

All raw data, documentation, records, the protocol, and the final report generated as a result of this study will be archived in the storage facilities of Corning Hazleton 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 Corning Hazleton Inc., for an additional period of time or sent to a storage facility designated by the Sponsor.

REFERENCES

- Ames, B.N., J. McCann and E. Yamasaki. Methods for detecting carcinogens and mutagens with the *Salmonella*/Mammalian-Microsome Mutagenicity Test. *Mutation Research* 31:347-364 (1975).
- Brusick, D.J., Simmon, V.F., Rosenkranz, H. S., Ray, V.A., and Stafford, R.S.
An evaluation of the *Escherichia coli* WP2 and WP2uvrA reverse mutation assay. *Mutation Research* 76:169-190 (1980).
- Green, M.H.L. and Muriel, W.J.. Mutagen testing using *trp*⁺ reversion in *Escherichia coli*. *Mutation Research* 38:3-32 (1976).
- Maron, D.M., and Ames, B. Revised Methods for the *Salmonella* Mutagenicity Test. *Mutation Research* 113:173-215 (1983).
- Vogel, H.J., and D.M. Bonner. Acetylornithinase of *E. coli*: Partial purification and some properties. *J. Biol. Chem.* 218:97-106 (1956).

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.

SECTION IV. RESULTS AND CONCLUSIONS

RESULTS

A. Test Article Handling

The test article, Dimethyl Acrylamide (DMACM), was stored at room temperature and was protected from light. Deionized water (CHV Lot 315) was used as the vehicle. At 100 mg per ml, which was the most concentrated stock dilution prepared, the test article formed a clear colorless solution. The test article remained a solution in all succeeding dilutions prepared for the mutagenicity assay.

B. Dose Rangefinding Study

Doses to be tested in the mutagenicity assay were selected based on the results of the dose rangefinding study conducted on the test article using tester strains TA100 and WP2uvrA in both the presence and absence of S9 mix (one plate per dose). Ten doses of test article, from 5,000 to 6.67 µg per plate, were tested and the results are presented in Tables 1 and 2. These data were generated in Experiment 16849-A1. No cytotoxicity was observed in either the presence or absence of S9 mix as evidenced by a normal background lawn and no decrease in the number of revertants per plate.

C. Mutagenicity Assay

The mutagenicity assay results for Dimethyl Acrylamide (DMACM) are presented in Tables 3 and 4. These data were generated in Experiment 16849-B1. The data are presented as mean revertants per plate ± standard deviation for each treatment and control group (Table 4) and as individual plate counts (Table 3).

The results of the dose rangefinding study were used to select five doses to be tested in the mutagenicity assay. The doses tested were 5,000, 3,330, 1,000, 333, and 100 µg per plate in both the presence and absence of S9 mix.

In Experiment 16849-B1 (Tables 3 and 4), all data were acceptable and no positive increases in the number of revertants per plate were observed with any of the tester strains either in the presence or absence of S9 mix.

All criteria for a valid study were met.

CONCLUSIONS

The results of the *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay indicate that under the conditions of this study, test article, Dimethyl Acrylamide (DMACM), did not cause a positive increase in the number of revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

SECTION V. DATA TABLES

CORNING Hazleton

TABLE 1

DOSE RANGE FINDING STUDY

TEST ARTICLE ID: Dimethyl Acrylamide (DMACM)

EXPERIMENT ID: 16849-A1

DATE PLATED: 28-Apr-95

VEHICLE: Deionized water

DATE COUNTED: 02-May-95

µg/PLATE	TA100 REVERTANTS PER PLATE			
	WITH S9		WITHOUT S9	
	REVERTANTS PER PLATE	BACKGROUND LAWN EVALUATION*	REVERTANTS PER PLATE	BACKGROUND LAWN EVALUATION*
0.00 (Vehicle) (50 µl)	95	1	91	1
Test Article 6.67	116	1	95	1
10.0	94	1	95	1
33.3	105	1	93	1
66.7	92	1	73	1
100	101	1	85	1
333	113	1	61	1
667	92	1	87	1
1000	117	1	82	1
3330	109	1	89	1
5000	89	1	88	1

* 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)

TABLE 2

DOSE RANGE FINDING STUDY

TEST ARTICLE ID: Dimethyl Acrylamide (DMACM)

EXPERIMENT ID: 16849-A1

DATE PLATED: 28-Apr-95

VEHICLE: Deionized water

DATE COUNTED: 02-May-95

µg/PLATE	WP2uvrA REVERTANTS PER PLATE			
	WITH S9		WITHOUT S9	
	REVERTANTS PER PLATE	BACKGROUND LAWN EVALUATION*	REVERTANTS PER PLATE	BACKGROUND LAWN EVALUATION*
0.00 (Vehicle) (50 µl)	11	1	12	1
Test Article 6.67	16	1	11	1
10.0	21	1	12	1
33.3	17	1	17	1
66.7	24	1	17	1
100	10	1	13	1
333	14	1	15	1
667	21	1	11	1
1000	20	1	11	1
3330	11	1	18	1
5000	11	1	14	1

* Background Lawn Evaluation Codes:

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

One hundred cells from each replicate culture at four dose levels of the test article and from each of the negative and solvent control cultures were analyzed for the different types of chromosomal aberrations (Evans, 1962; See Section XV.). At least 25 cells were analyzed for chromosomal aberrations from one of the positive control cultures. For control of bias, all slides except for the positive controls were coded prior to analysis. Cells with aberrations were recorded on the data sheets by the microscope stage location.

The following factors were taken into account in the evaluation of the chromosomal aberrations data:

1. The overall chromosomal aberration frequencies.
2. The percentage of cells with any aberrations.
3. The percentage of cells with more than one aberration.
4. Any evidence for increasing amounts of damage with increasing dose, i.e., a positive dose response.

Chromatid and isochromatid gaps, if observed, were noted in the raw data and were tabulated. They were not, however, considered in the evaluation of the ability of the test article to induce chromosomal aberrations since they may not represent true chromosomal breaks and may possibly be induced by toxicity. Historical control data are provided in Table 4.

A cell classified as "GT" is considered to contain 10 aberrations for statistical purposes but a ">" is also included in the tables for this classification to indicate that it is a minimum number.

Statistical analysis employed the Fisher's Exact Test with an adjustment for multiple comparisons (Sokal and Rohlf, 1981) to compare the percentage of cells with aberrations in each treatment group with the results from the pooled solvent and negative controls. If a positive Fisher's test was obtained at any test article dose level, the Cochran-Armitage test was used to measure dose-responsiveness at $p \leq 0.05$ (Armitage, 1971). Test article significance was established where $p < 0.01$. All factors as stated previously were taken into account and the final evaluation of the test article was based upon scientific judgement.

XI. RESULTS:

A. Solubility, Stability, and Dose Determination

Solubility was evaluated in RPMI culture medium. A light violet, translucent solution was obtained at a concentration of 427 mg/ml. McCoy's 5a culture medium (JRH Biosciences, Lot No.: 4K3761) was the solvent of choice. A stock concentration of 499 mg/ml was prepared for the dose rangefinding assay. Concentrations of 0.166, 0.499, 1.66, 4.99, 16.6, 49.9, 166, 499, 1660, and 4990 µg/ml were tested in rangefinding assays with and without metabolic activation. The stability of the test article under the dosing conditions of this assay is the responsibility of the Sponsor.

B. Rangefinding Assay Without Metabolic Activation

Floating dead cells and debris, <5% cell monolayer confluence, and no visible mitotic cells were observed in the culture treated with 4990 µg/ml. A slightly unhealthy cell monolayer, severe reductions in visible mitotic cells, and ~30% reduction in the cell monolayer confluence were observed in the culture treated with 1660 µg/ml. A slightly unhealthy cell monolayer, slight reductions in visible mitotic cells, and ~15% reduction in the cell monolayer confluence were observed in the culture treated with 499 µg/ml. Cell cycle kinetics were evaluated in the cultures treated with 49.9, 166, 499, and 1660 µg/ml (Table 1). A severe cell cycle delay was observed in the culture treated with 1660 µg/ml, with cell cycle delay persisting in the culture treated with 499 µg/ml. Based on these results, a 20.1 hour harvest was conducted testing concentrations of 458, 610, 915, 1220, 1520, 2270, 3020, and 4020 µg/ml in the nonactivation aberrations assay.

C. Rangefinding Assay With Metabolic Activation

No visual signs of toxicity were observed in any of the test cultures. Cell cycle kinetics were evaluated in the cultures treated with 499, 1660, and 4990 µg/ml (Table 1). Cell cycle delay was observed in the culture treated with 4990 µg/ml, with a slight cell cycle delay evident in the culture treated with 1660 µg/ml. Based on these results, a 20.1 hour harvest was conducted testing concentrations of 2020, 2770, 3520, 4270, and 5020 µg/ml in the aberrations assay with metabolic activation.

D. Chromosomal Aberrations Assay Without Metabolic Activation

Unhealthy cell monolayers, floating dead cells and debris, severe reductions in the numbers of visible mitotic cells, and ~50% reduction in the cell monolayer confluence were observed in the

cultures treated with 4020 µg/ml. Unhealthy cell monolayers, floating dead cells and debris, severe reductions in the numbers of visible mitotic cells, and ~40% reduction in the cell monolayer confluence were observed in the cultures treated with 3020 µg/ml. Slightly unhealthy cell monolayers, floating dead cells and debris, slight reductions in the numbers of visible mitotic cells, and ~25% reduction in the cell monolayer confluence were observed in the cultures treated with 1520 and 2270 µg/ml. Floating debris, slight reductions in the numbers of visible mitotic cells, and ~15% reduction in the cell monolayer confluence were observed in the cultures treated with 1220 µg/ml. Slight reductions in the numbers of visible mitotic cells were observed in the cultures treated with 915 µg/ml. Toxicity was manifested on the slides prepared from the cultures treated with 4020 and 3020 µg/ml by the extremely sparse numbers of metaphases of poor morphology available for analysis. Chromosomal aberrations were evaluated from the cultures treated with 915, 1220, 1520, and 2270 µg/ml (Table 2). Significant increases in cells with chromosomal aberrations were observed in the cultures treated with 1520 and 2270 µg/ml.

The sensitivity of the cell culture for induction of chromosomal aberrations is shown by the increased frequency of aberrations in the cells exposed to mitomycin C, the positive control agent. The test article is considered positive for inducing chromosomal aberrations under nonactivation conditions.

E. Chromosomal Aberrations Assay With Metabolic Activation

Floating debris and ~15% reduction in the cell monolayer confluence were observed in the cultures treated with 5020 µg/ml. Reductions of ~15% in the cell monolayer confluence were observed in the cultures dosed with 4270 µg/ml. Chromosomal aberrations were evaluated from the cultures treated with 2770, 3520, 4270, and 5020 µg/ml (Table 3). A weakly significant increase in cells with chromosomal aberrations was observed in the cultures treated with 5020 µg/ml.

The successful activation of the metabolic system is illustrated by the increased incidence of cells with chromosomal aberrations in the cultures induced with cyclophosphamide, the positive control agent. The test article is considered weakly positive for inducing chromosomal aberrations under conditions of metabolic activation.

XII. CONCLUSION:

The test article, Dimethyl Acrylamide (DMACM), was considered positive for inducing chromosomal aberrations in Chinese hamster ovary cells under nonactivation conditions and weakly positive under the activation conditions of this assay.

XIII. REFERENCES:

Armitage, P. Statistical Methods in Medical Research, John Wiley & Sons, Inc., New York, NY, 1971.

Evans, H.J.: Chromosomal aberrations produced by ionizing radiation. International Review of Cytology, 13:221-321, 1962.

Goto, K., Maeda, S., Kano, Y., and Sugiyama, T.: Factors involved in differential Giemsa-staining of sister chromatids. Chromosoma, 66:351-359, 1978.

Perry, P. and Wolff, S.: New Giemsa method for the differential staining of sister chromatids. Nature, 251:156-158, 1974.

Sokal, R.R., and Rohlf, F.J.: Biometry, Ed. 2, W.H. Freeman and Company, New York, 1981.

Terasima, T. and Tolmach, L.J.: Changes in X-ray sensitivity of HeLa cells during the division cycle. Nature, 190:1210-1211, 1961.

XIV. DEVIATION FROM THE SIGNED PROTOCOL

The following deviation was made from the protocol:

Due to a technical oversight, vernier stage location was not recorded for one cell with chromosomal aberrations from the culture treated with 2270 µg/ml without metabolic activation. This deviation does not negate the validity of the observation and does not impact on the integrity of this study.

XV. EXPERIMENTAL DATA TABLES

TABLE 1

RANGEFINDING ASSAY FOR ASSESSING TOXICITY

Compound: Dimethyl acrylamide (DMACM)
 Assay No.: 16849

Metabolic Activation: -S9 Lab No.: CY5095 Trial No.: I

Treatment		M1	M1+	M2	M2+	Confluence ^a % Solvent Control
NEGATIVE CONTROL	McCoy's 5a	0	5	92	3	100
SOLVENT CONTROL	McCoy's 5a 10.0 µl/ml	0	4	93	3	100
POSITIVE CONTROL	MMC 0.25 µg/ml	29	57	14	0	100
TEST ARTICLE	49.9 µg/ml	0	11	89	0	100
	166 µg/ml	0	6	94	0	100
	499 µg/ml	2	31	67	0	86
	1660 µg/ml	55	45	0	0	71
	4990 µg/ml*					<7

Metabolic Activation: +S9 Lab No.: CY5095 Trial No.: I

Treatment		M1	M1+	M2	M2+	Confluence ^a % Solvent Control
NEGATIVE CONTROL	McCoy's 5a	0	4	96	0	100
SOLVENT CONTROL	McCoy's 5a 10.0 µl/ml	0	5	95	0	100
POSITIVE CONTROL	CP 15.0 µg/ml	55	44	1	0	100
TEST ARTICLE	499 µg/ml	0	1	97	2	100
	1660 µg/ml	0	14	86	0	100
	4990 µg/ml	4	45	51	0	100

^aThis endpoint is based upon visual observations which are made prior to the harvest of the metaphase cells. Actual cell counts are not taken and any hypertrophy of the attached cells cannot be evaluated. At the time of the confluence observation the flasks are also evaluated for the appearance of floating mitotic cells and dead cells.

*Toxic dose level.

TABLE 2

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
Cells Fixed 20.1 Hours After Treatment

Assay No.: 16849 Trial #: 1 Lab #: CY5155 Metabolic Activation: -S9
Compound: Dimethyl acrylamide (DMACM) Date: 05/16/95

NUMBER AND TYPE OF ABERRATION

CELLS SCORED	NOT COMPUTED			SIMPLE			COMPLEX										# OF ABERRATIONS PER CELL	% CELLS WITH >1 ABERRATIONS
	TG	SG	UC	TB	SB	UC	TR	QR	CR	D	R	CI	DF	GT				
CONTROLS																		
McCoy's 5a	100	4				1										0.02	2.0	0.0
McCoy's 5a	100	4				1										0.01	1.0	0.0
Solvent: Negative + Solvent																		
MMC	25					1	1	1	1							0.02	1.5	0.0
Solvent: 0.05µg/ml																		
TEST ARTICLE																		
915µg/ml A	100	1	1			3										0.03	3.0	0.0
915µg/ml B	100	10	1			1	3		2							0.06	5.0	1.0
Solvent: A+B																		
1220µg/ml A	200	11	2			1	6		2							0.05	4.0	0.5
1220µg/ml B	100	6				1	3	1								0.04	4.0	0.0
Solvent: A+B																		
1520µg/ml A	200	8	2			1	4	1	1	1						0.05	5.0	0.0
1520µg/ml B	75**	20	4			3	77	19	4	2	1	1		1		>1.53	54.7	33.3
Solvent: A+B																		
2270µg/ml A	100	21	2			2	15	4	5	1	1					0.26	21.0	4.0
2270µg/ml B	175	41	6			5	92	23	9	3	1	2		1		>0.81	35.4*	16.6*
Solvent: A+B																		
50**	50**	2				2	56	9	2	1						13	3.96	66.0
50**	50**	12	3			2	40	15	1	1						20	5.16	74.0
Solvent: A+B																		
100	100	14	3			4	96	24	1	2	1	2				33	4.56	70.0*

* Significantly greater than the pooled negative and solvent controls, P<0.01.
** Fewer than 100 metaphases analyzed due to extremely positive response.

TABLE 3
 CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
 Cells Fixed 20.1 Hours After Treatment

Assay No. : 16849	Trial #: 1	Lab #: CY5155	Metabolic Activation: +S9	NUMBER AND TYPE OF ABERRATION																# OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS
				CELLS SCORED		NOT COMPUTED		SIMPLE		COMPLEX						OTHER ABERRATIONS						
				TG	SG	UC	TB	SB	ID	TR	QR	CR	D	R	CI	DF	GT					
CONTROLS																						
NEGATIVE:	McCoy's 5a	100	3	1															0.00	0.0	0.0	
SOLVENT:	McCoy's 5a	100	3	1						1	1								0.02	2.0	0.0	
	Negative + Solvent	200	6	2						1	1								0.01	1.0	0.0	
POSITIVE:	CP	25	7	2						4	2	2	4						1	>1.28	52.0*	28.0*
TEST ARTICLE																						
	2770µg/ml	A	100	4	1					2										0.04	3.0	1.0
		B	100	4						1										0.02	2.0	0.0
		A+B	200	8	1					2	1	2								0.03	2.5	0.5
	3520µg/ml	A	100	4	1															0.01	1.0	0.0
		B	100	5								1								0.03	3.0	0.0
		A+B	200	9	1							1								0.02	2.0	0.0
	4270µg/ml	A	100	4	1					1										0.02	2.0	0.0
		B	100	6	2					1	1									0.02	2.0	0.0
		A+B	200	10	3					2	1	2								0.02	2.0	0.0
	5020µg/ml	A	100	12	2					6										0.09	9.0	0.0
		B	100	4	2							1								0.02	2.0	0.0
		A+B	200	16	2					6	3	1								0.06	5.5**	0.0

* Significantly greater than the pooled negative and solvent controls, p<0.01.
 ** Significantly greater than the pooled negative and solvent controls, p<0.05.

TABLE 4

CONTROL DATA OF CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS 02-95 TO 05-95

	Activation		# Of Aberrations Per Cell	% Of Cells With Aberrations	% Of Cells With >1 Aberrations
Negative Control	Without	MIN	0.00	0.0	0.0
		MAX	0.04	4.0	1.0
		AVG	0.01	1.2	0.1
		N	20	20	20
Solvent Control McCoy's 5a	Without	MIN	0.00	0.0	0.0
		MAX	0.04	4.0	1.0
		AVG	0.01	1.3	0.1
		N	20	20	20
Positive Control Mitomycin C	Without	MIN	0.20	20.0	0.0
		MAX	0.84	52.0	20.0
		AVG	0.40	30.0	4.8
		N	18	18	18
Negative Control	With	MIN	0.00	0.0	0.0
		MAX	0.04	4.0	0.5
		AVG	0.02	1.7	0.1
		N	17	17	17
Solvent Control McCoy's 5a	With	MIN	0.00	0.0	0.0
		MAX	0.03	2.5	1.0
		AVG	0.01	1.1	0.1
		N	17	17	17
Positive Control Cyclophosphamide	With	MIN	0.32	32.0	0.0
		MAX	2.56	88.0	68.0
		AVG	1.03	46.9	21.3
		N	15	15	15

XVI. DEFINITIONS OF CHROMOSOME ABERRATIONS FOR GIEMSA STAINED CELLS

NOT COMPUTED

- TG Chromatid Gap: ("tid gap"). An achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations as they may not all be true breaks.
- SG Chromosome gap: ("isochromatid gap, IG"). Same as chromatid gap but at the same locus in both sister chromatids.
- UC Uncoiled chromosome: Failure of chromatin packing. Probably not a true aberration.
- PP Polyploid cell: A cell containing multiple copies of the haploid number (n) of chromosomes. Only indexed if very common. Not counted in the cells scored for aberrations.
- E Endoreduplication: 4n cell in which separation of chromosome pairs has failed. Only indexed if very common. Not counted in the cells scored for aberrations.

SIMPLE

- TB Chromatid break: An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced.
- SB Chromosome break: Chromosome has a clear break, forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated.
- DM "Double Minute" Fragment: These are small double dots, some of which are terminal deletions and some interstitial deletions and probably small rings. Their origins are not distinguishable.

COMPLEX

- ID Interstitial Deletion: Length of chromatid "cut out" from midregion of a chromatid resulting in a small fragment or ring lying beside a shortened chromatid or a gap in the chromatid.
- TR Triradial: An exchange between two chromosomes, or one chromosome and an acentric fragment, which results in a three-armed configuration.
- QR Quadriradial: As triradial, but resulting in a four-armed configuration.
- CR Complex Rearrangement: An exchange among more than two chromosomes or fragments which is the result of several breaks.
- D Dicentric: An exchange between two chromosomes which results in a chromosome with two centromeres. This is often associated with an acentric fragment in which case it is classified as DF.
- DF Dicentric with fragment.
- TC Tricentric: An exchange involving three chromosomes and resulting in a chromosome with three centromeres. Often associated with two to three AF. Such exchanges can involve many chromosomes and are named as follows:
- QC Quadricentric: four centromeres, up to four AF
PC Pentacentric: five centromeres, up to five AF
HC Hexacentric: six centromeres, up to six AF

- R Ring: A chromosome which forms a circle containing a centromere. This is often associated with an acentric fragment in which case it is classed as RF.
- RC Ring Chromatid: Single chromatid ring (acentric).
- RF Ring with associated acentric fragment.
- CI Chromosome Intrachange: Exchange within a chromosome; e.g., a ring that does not include the entire chromosome.
- T Translocation: Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes. When identifiable, scored as "T" not "2AB."
- AB Abnormal monocentric chromosome. This is a chromosome whose morphology is abnormal for the karyotype, and often the result of a translocation, pericentric inversion, etc. Classification used if abnormality cannot be ascribed to; e.g., a reciprocal translocation.
- OTHER
- GT A cell which contains more than 10 aberrations. A heavily damaged cell should be analyzed to identify the types of aberrations, since they may not actually have >10, e.g., multiple fragments such as those found associated with a tricentric.

Best Available Copy