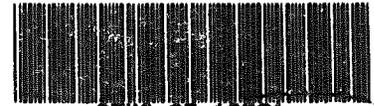


General Offices/3M

3M Center
St. Paul, Minnesota 55144-1000
612/733 1110



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ATTENTION: 8(e) Coordinator

SUBJECT: TSCA 8(E) Submission on N,N-Dimethylacrylamide CAS# 2680-03-7

Dear Sir:

3M has obtained toxicity information that may reasonably support a conclusion of substantial risk under TSCA section 8(e) for N,N-Dimethylacrylamide CAS# 2680-03-7. While 3M's activity is very limited and probably does not support a "substantial risk" conclusion, the material is a commercial product and may have other uses and exposures of concern.

We have conducted three in vitro mutagenicity assays on this chemical: An Ames test which was negative, a mouse lymphoma forward mutation assay which was positive and a CHO chromosomal aberration test which was positive (enclosed). This profile matches that of acrylamide. No information regarding mutagenicity is provided on the vendor MSDS.

3M purchases this material from an importing distributor, JARCHEM Industries. We currently use it for limited batch commercial production as an E-beam cured primer. We also are conducting R & D for miscellaneous other radiation cure applications. Total commercial and R & D use involves less than 1000kg/yr. Less than 10 production workers handle the chemical.

Workplace exposure can occur during coating above required limits for acrylamide. On the assumption that this material is similar to acrylamide, 3M has required our researchers and plant workers to wear respiratory protection when handling the chemical. We have distributed limited samples of uncured dimethylacrylamide containing product to customers. Such customers coat and cure the material in clean room facilities and also are advised to use respirators when handling the uncured coating.

We are ensuring that industrial hygiene measures appropriate for acrylamide are taken in our workplaces and will commercially distribute uncured material only to licensed customers. MSDS are being updated with this information. We are also informing our vendor, JARCHEM Industries, of this information.

Additional testing is underway and will be forwarded to EPA when available.



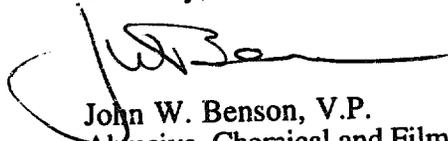
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Please contact Kurt Werner, Toxicology, at 612-733-8494, for further information about the status of the toxicity testing.

Correspondence regarding this notice should be sent to Georjean Adams, Regulatory Affairs Manager, Corporate Product Responsibility, 3M Center Building 290-04-01, St. Paul, MN 55144.

Sincerely,



John W. Benson, V.P.
Abrasive, Chemical and Film Products Group

Enclosures:

1. Mutagenicity Test on Dimethyl acrylamide (DMACM) in the L5178 TK+/- Mouse Lymphoma Forward Mutation Assay
2. Mutagenicity Test on Dimethyl acrylamide (DMACM) in the *Salmonella-escherichia coli* /Mammalian-Microsome Reverse Mutation Assay
3. Mutagenicity Test on Dimethyl acrylamide (DMACM) Measuring Chromosomal Aberrations in Chinese Hamster Ovary Cells

c: JARCHEM Industries Inc., 414 Wilson Ave, Newark, NJ 07105

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CORNING Hazleton

MUTAGENICITY TEST ON
DIMETHYL ACRYLAMIDE (DMACM)
IN THE L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

FINAL REPORT

AUTHOR

MARIA A. CIFONE, Ph.D.

PERFORMING LABORATORY

CORNING HAZLETON, INC. (CHV)
9200 LEESBURG PIKE
VIENNA, VIRGINIA 22182

LABORATORY PROJECT ID

CHV STUDY NO.: 16849-0-431
3M STUDY NO.: 0795UK0168

SUBMITTED TO

3M CENTER
ST. PAUL, MN 55144-1000

STUDY COMPLETION DATE

JULY 14, 1995

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: LS178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

CHV PROJECT NO.: 20989

ASSAY NO.: 16849

PROTOCOL NO.: 431 EDITION NO.: 14

Quality Assurance inspections of the study and 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> |
|---------------------------------------|--------------------------|-----------------------------|
| Viable cell count/ 5-3-95 | 5-3-95 | M. Murphy |
| Draft report review/ 6-27,28,29-95 | 6-30-95 | S. Ballenger/ K. Newland |
| Final report review/ 7-14-95 | 7-14-95 | S. Ballenger |


Quality Assurance Unit

7/14/95
Date Released

COMPLIANCE AND CERTIFICATION STATEMENT

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160). 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.

All raw data, documentation, records, protocol, and 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. Copies of the raw data will be supplied to the sponsor upon request.

SUBMITTED BY:

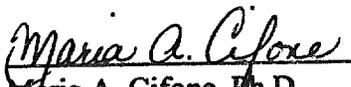
RESEARCH ASSISTANT:



Fariba Oveisitork, B.S.
Research Assistant

7/14/95
Date

STUDY DIRECTOR:



Maria A. Cifone, Ph.D.
Cell Biologist
Department of Genetic and
Cellular Toxicology

7-14-95
Study Completion
Date

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ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of dimethyl acrylamide (DMACM) to induce forward mutations at the thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line. The test material formed a clear colorless liquid in water at 50.0 mg/ml. In the preliminary cytotoxicity assay, cells were exposed to the test material at concentrations up to 5000 µg/ml for four hours in the presence and absence of rat liver S9 metabolic activation. The test material was moderately cytotoxic to noncytotoxic without metabolic activation and highly cytotoxic to noncytotoxic with activation. The mutation assays were initiated with treatments up to 5000 µg/ml.

A single mutation assay was performed for both nonactivation and S9 metabolic activation conditions. Nine treatments from 500 µg/ml to 5000 µg/ml were initiated with and without activation. Under nonactivation conditions, six treatments from 2000 µg/ml to 5000 µg/ml were chosen for mutant induction. The mutation assay was more cytotoxic than the rangefinding assay and several of the dose levels were excessively cytotoxic. However, all treatments including a moderately cytotoxic dose with 41.3% relative growth induced mutant frequencies that exceeded the minimum criterion for a positive response. Increases that exceeded the minimum criterion for a positive response were also induced in the presence of metabolic activation. The test article was also more cytotoxic with activation and only four treatments from 500 µg/ml to 2000 µg/ml were available for analysis. The four treatments induced weak to very high cytotoxicities and the two highly cytotoxic treatments (1500 µg/ml and 2000 µg/ml) induced increases in the mutant frequency that exceeded the minimum criterion for a positive response.

The test material, dimethyl acrylamide (DMACM), was therefore evaluated as positive for inducing forward mutations at the TK locus in L5178Y mouse lymphoma cells under the nonactivation and S9 metabolic activation conditions used in this study.

**L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY
WITH DIMETHYL ACRYLAMIDE (DMACM)**

I. STUDY IDENTIFICATION

A. Sponsor:

B. Test Article:

1. Sponsor Identification: Dimethyl acrylamide (DMACM)
(N,N-dimethyl acrylamide monomer, Aldrich lot 09430EW)
2. Physical Description: clear colorless liquid
3. Date Received: April 24, 1995
4. CHV Assay Number: 16849

C. Type of Assay: L5178Y TK+/- Mouse Lymphoma Forward Mutation Assay

D. CHV Protocol Number: 431, Edition 14 Modified for 3M

E. Study Dates:

1. Study Initiation Date: April 5, 1995
2. Experimental Start Date: April 27, 1995
3. Experimental Termination Date: May 17, 1995

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

F. Supervisory Personnel:

1. Research Assistant: Fariba Oveisitork, B.S.
2. Study Director: Maria A. Cifone, Ph.D.

II. OBJECTIVE

The objective of this study was to evaluate the ability of dimethyl acrylamide (DMACM) to induce forward mutations at the thymidine kinase (TK) locus in L5178Y TK^{+/-} mouse lymphoma cells as assayed by colony growth in the presence of 5-trifluorothymidine (TFT).

III. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If the thymidine analog TFT is included in the growth medium, the analog will be phosphorylated via the TK pathway and cause cell death by inhibiting DNA synthesis. Cells which are heterozygous at the TK locus (TK^{+/-}) may undergo a single-step forward mutation to the TK^{-/-} genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis may still proceed by *de novo* synthetic pathways that do not involve thymidine as an intermediate. TK^{-/-} mutants cannot utilize toxic analogs of thymidine. Cells which grow to form colonies in the presence of TFT are therefore assumed to have mutated, either spontaneously or by the action of the test substance, to the TK^{-/-} genotype.

IV. MATERIALS

A. Indicator Cells

The mouse lymphoma L5178Y cell line, heterozygous at the TK locus and designated as clone 3.7.2C was used for this assay. Stock cultures were obtained from Dr. Donald Clive (Burroughs Wellcome Company, Research Triangle Park, NC) and are stored in liquid nitrogen. All laboratory cultures were maintained in logarithmic growth by serial subculturing for up to 4 months and were then replaced by cells from the frozen stock. Cultures were grown in a shaker incubator at

approximately 37°C. A continuous cell log was kept to record growth, doubling times, and subculture operations. Laboratory cultures were periodically tested for mycoplasma contamination and karyotype. To reduce the frequency of spontaneous TK-/- mutants prior to use in the mutation assay, cell cultures were exposed to conditions that selected against the TK-/- phenotype (exposure to methotrexate). Cell cultures were maintained in cleansing medium for one day, placed in recovery medium for one day and then returned to normal growth medium for three to eight days before use.

B. Media

The medium used for this study was RPMI 1640 (Amacher et al., 1980; Clive et al., 1987) supplemented with Pluronic® F68, L-glutamine, sodium pyruvate, antibiotics, and heat-inactivated horse serum (10% by volume). Treatment medium was Fischers medium with the same media supplements used in the culture medium except that the horse serum concentration was reduced to 5% by volume. Cloning medium consisted of the preceding growth medium with up to 20% horse serum, without Pluronic® F68 and with the addition of BBL purified agar at a final concentration of 0.24 percent to achieve a semisolid state. Selection medium was cloning medium that contained 3 µg/ml of TFT.

C. Control Compounds

1. Untreated (negative) control articles

Untreated controls were performed for each cytotoxicity assay by carrying cells unexposed to the test article through all of the assay operations. In the activation portion of the assay, untreated control cultures were exposed to the S9 metabolic activation mix. A single culture was used in the cytotoxicity assays.

2. Vehicle controls

The test material was soluble in water; JRH BioSciences, lot number 4M3941, CAS number 7732-18-5). Primary 10X stocks of the test material were prepared using water as the diluent. The primary test material stocks were then diluted 1:10 into culture medium that resulted in varying test material concentrations that contained 10 percent water supplied as the vehicle. Therefore, concurrent vehicle controls were performed for each portion of the assay by exposing the cells to 10 percent water in culture medium. In the activation portion of the assays, the vehicle controls were also exposed to the

S9 metabolic activation mix. Single vehicle control cultures were initiated in the cytotoxicity assays and three vehicle control cultures were initiated in the mutation assays.

3. Positive Controls

Mandatory reference substances for use as positive control articles are not available (Clive et al., 1987). The positive control articles used were chosen because of the large data base available and because both chemicals detect small and large colonies (Young et al., 1991).

Methyl methanesulfonate (MMS) is mutagenic via alkylation of cellular DNA and is highly mutagenic to mouse lymphoma cells without S9 metabolic activation. MMS (Kodak Chemical Co., lot number C114119, CAS number 66-27-3) was used at 10 nl/ml and 15 nl/ml as a positive control for nonactivation mutation studies.

Methylcholanthrene (MCA) requires metabolic activation by microsomal enzymes to become mutagenic. MCA (Sigma Chemical Co., lot number 66F-3432, CAS number 56-49-5) was used at 2.0 µg/ml and 4.0 µg/ml as a positive control for assays performed with S9 metabolic activation.

D. S9 Metabolic Activation System

The *in vitro* metabolic activation system was comprised of rat liver enzymes (S9 fraction) and an energy producing system (CORE) comprised of nicotinamide adenine dinucleotide phosphate (NADP, sodium salt) and isocitrate. The enzymes were prepared commercially (Molecular Toxicology, Inc., Annapolis, MD) where the S9 was tested for sterility and enzyme activity. The enzymes were obtained from the 9000 x g supernatant of liver homogenate from male Sprague Dawley rats treated with 500 mg/kg of Aroclor 1254 five days prior to sacrifice. The treatment with Aroclor 1254 was used to induce mixed function oxidase enzymes capable of transforming chemicals to more active forms. The S9 and reaction mixture (CORE)

were retained frozen at about -80°C until used. The S9 fraction and CORE were thawed immediately before use and combined to form the activation system described below:

| <u>Component</u> | <u>Final Concentration in Cultures</u> |
|--------------------|--|
| NADP (sodium salt) | 3 mM |
| Isocitrate | 15 mM |
| S9 homogenate | 5 µl/ml |

The amount of S9 homogenate per culture depends upon the enzyme activity of individual lots. S9 at various concentrations was tested against a reference chemical (methylcholanthrene) that requires metabolic activation to become mutagenic to mouse lymphoma cells. The optimum S9 concentration was selected based upon induction of TK-/- mutants in mouse lymphoma cells, and this amount of S9 was used in all subsequent assays with that lot of S9. This allows the monitoring of activation in the positive controls (Clive et al., 1987).

V. EXPERIMENTAL DESIGN

A. Solubility and Test Material Handling

The maximum dose was determined by taking into account both solubility and any relevant cytotoxicity information available on the test article. The solvent of choice was water. The test material was prepared at 10-times the highest desired treatment concentration. Lower 10X primary stocks were prepared by further dilution with water. Dosing media that contained the test material at the final concentrations were prepared by making 1:10 dilutions of the primary stocks into treatment media. The volume of treatment medium diluent used in the metabolic activation studies was reduced to compensate for the volume of S9 reaction mix used. Treatment conditions consisted of 6.0×10^6 cells suspended in 10.0 ml of treatment medium. Preparations of test material in the vehicle were prepared fresh each day for biological testing. The stability of the test material under the conditions of testing is the responsibility of the Sponsor.

B. Dose Selection

A preliminary cytotoxicity experiment was performed both with and without S9 metabolic activation since substantial shifts in toxicity often occur for the two test conditions. A wide range of test article concentrations was tested for cytotoxicity,

starting with the maximum applied dose of 5000 µg/ml and followed by nine lower concentrations prepared in two-fold dilution steps. After an exposure time of four hours at approximately 37°C in an orbital shaker at 80 ± 10 orbits per minute, the cells were washed twice and resuspended in growth medium. The cells were then incubated overnight using procedures identical to those used for the mutation experiments. Cell counts were made after approximately twenty-four hours to measure the reduction in cell growth relative to the concurrent vehicle control cultures. The preliminary cytotoxicity information was then used to select dose levels for the mutation assay. Doses were selected using the following criteria:

- Concentrations were chosen in appropriate steps to cover a toxicity range from little or no survival to no apparent effect on growth compared to the vehicle control, or
- If little or no toxicity was observed and solubility was maintained, the mutation experiment was initiated with a maximum concentration of 5000 µg/ml.
- If precipitation of the test article occurred in the culture medium, then the maximum applied dose was 5000 µg/ml or approximately twice the apparent solubility limit in culture medium.
- For insoluble test materials, a range of concentrations that was practical was assayed.

Although nine doses were used to initiate a mutation experiment, the objective was to carry at least five doses through the entire experiment. This procedure compensates for normal variations in cellular toxicity and helps to ensure the choice of at least three doses appropriately spaced in the relative growth range that meets the criteria above.

C. Mutagenicity Testing

1. Nonactivation Assay

The assay procedure used was based on that reported by Clive and Spector (1975), Clive, et al. (1979), Amacher et al. (1980) and Clive et al. (1987). The cells were obtained from logarithmically growing laboratory stock cultures and

were seeded into a series of tubes at 6×10^6 cells per tube. The cells were pelleted by centrifugation, the culture medium removed, and the cells resuspended in a final volume of 10.0 ml of treatment medium. The dosed tubes were closed, vortexed and placed in an orbital shaker incubator at approximately 37°C at 80 ± 10 orbits per minute. After an exposure period of about four hours, the cells were washed twice, resuspended in 20.0 ml of growth medium and returned to the orbital shaker incubator as closed-tube cultures.

The assay conditions consisted of three vehicle controls, two positive controls and nine different test material dose levels using one culture per dose level. Several treated cultures may be eliminated during the expression period as long as five appropriate dose levels were left for analysis of mutant induction. If the dose range resulted in excessively toxic treatments, as few as three doses may be carried through the mutation assay. The appearance of the treated cultures was recorded both at the time of treatment and after the four hour treatment period.

A standard expression period of two days was used to allow recovery, growth and expression of the TK^{-/-} phenotype. Cell densities were determined on day one (about twenty-four hours after treatment) and were adjusted to 3×10^5 cells/ml in 20.0 ml of growth medium to maintain optimal growth rates. If the cells in a culture failed to multiply to a density of 4×10^5 on the first day after treatment, the culture was returned to the incubator without being subcultured. On day two, cell counts were again determined, and appropriate cultures were selected for cloning and mutant selection.

Four or six doses were selected for mutant analysis. If possible, doses were selected to include nontoxic to highly toxic (approximately ten to twenty percent relative growth) treatment conditions. Cultures with cell densities less than approximately 3×10^5 cells/ml were not considered for selection and analysis.

A total sample size of 3×10^6 cells was suspended in selection medium to selectively recover mutants. This sample was distributed into three 100 mm dishes so that each dish contained approximately 1×10^6 cells. The cloning efficiency was determined by serially diluting the cells and seeding each of three dishes with approximately 200 cells in cloning medium. All of the dishes were placed in a humidified incubator at approximately 37°C with approximately five percent CO_2 :95 percent air. After ten to fourteen days in the

incubator, the colonies were counted on an Artek Model 880 colony counter fitted with a ten turn potentiometer for discrimination of colony size. The smallest detectable colony was between 0.2 and 0.3 mm in diameter, depending on its position in the agar matrix.

The mutant frequency was calculated as the ratio of the total number of mutant colonies found in each set of three mutant selection dishes to the total number of cells seeded, adjusted by the absolute selection cloning efficiency. If one dish in either set was lost due to contamination or other cause, the colony count of the missing dish was determined by a proportion equation based upon the weights of the three dishes of the set and the colony counts in the two acceptable dishes. If a lost plate was not available for weighing, the colony count of the lost plate was determined from the average of the two remaining acceptable plates. A mutant frequency calculated by either method was identified by footnote in the data tables as a reminder of the reduced sample size in the event of a spurious variation.

The measurement of the toxicity of each treatment was the relative suspension growth of the cells over the two-day expression period multiplied by the relative cloning efficiency at the time of selection. Although not strictly a measure of cell survival, this parameter (called percent relative growth) provides a measure of the effectiveness of treatment and is used as the basis for selecting doses for any necessary repeat trials.

Selection plates from representative control and representative dose levels with elevated mutant frequencies were recounted to permit colony sizing.

2. Activation Assay

The activation assay is often run concurrently with the nonactivation assay, but it is an independent assay performed with its own set of vehicle and positive controls. The two assays were identical except for the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. The 10 ml volume during treatment included this S9 activation mix which was prepared just prior to use and kept on ice. CORE consisted of nicotinamide adenine dinucleotide phosphate (NADP, sodium salt) and isocitrate (isocitric acid). The S9 homogenate was commercially prepared (Molecular Toxicology, Inc., lot number 0501) and consisted of the 9000 x g supernatant from the homogenized livers of Aroclor 1254-induced adult male Sprague Dawley rats.

D. Data Presentation

The data were used to calculate several assay parameters. The reported raw data, analyzed data and the methods of calculation are listed below:

1. Test article identification, vehicle, selection agent and concentration, and study dates.
2. Daily cell densities in the individual cultures carried through the expression period and cloning.
3. The suspension growth for the vehicle and positive controls, calculated as (Day 1 cell density/ 3×10^5) x (Day 2 cell density/ 3×10^5 or Day 1 density if not split back).
4. Relative suspension growth for the test article-treated cultures. These values are expressed as percentages of the average vehicle control suspension growth.
5. Total mutant colonies - total number of mutant colony counts obtained from 3×10^6 cells sampled from one culture, seeded into selective medium, and divided among three culture dishes.
6. Total viable colonies - total number of colony counts obtained from 600 cells sampled from one culture, seeded into nonselective medium, and divided among three culture dishes.
7. Cloning efficiency of the vehicle and positive controls, calculated as (total number of viable colonies/600) x 100%.
8. Relative cloning efficiency of the test article-treated cultures. These values are expressed as percentages of the average vehicle control cloning efficiency.
9. Percent relative growth - an expression of the toxicity of a treatment obtained by multiplying the relative suspension growth by the relative cloning efficiency/100. The vehicle controls are by definition set to 100%.
10. The mutant frequency for each culture. The ratio of cells seeded for mutant selection to cells seeded for cloning efficiency is 0.5×10^4 . Therefore, the mutant frequency is: (total mutant colonies/total viable colonies) x 2×10^4 . The mutant frequency is given in units of 10^{-6} .

E. Assay Acceptance Criteria

An assay will be considered acceptable for evaluation of the test results only if all of the criteria given below are satisfied. The activation and nonactivation portions of the mutation assays are usually performed concurrently, but each portion is in fact an independent assay with its own positive and vehicle controls. The activation or nonactivation assays may be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

- The average absolute cloning efficiency of the vehicle controls should be between sixty percent and one hundred thirty percent. A value greater than one hundred percent is possible because of errors in cell counts (usually \pm ten percent) and variations in cell division during unavoidable delays between the counting and cloning. Cloning efficiencies below sixty percent do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies and still yield internally consistent and valid results. Assays with cloning efficiencies in the range of 50% and 60% are conditionally acceptable and dependent upon the scientific judgement of the study director. All assays below fifty percent cloning efficiency are unacceptable.
- The minimum value for the suspension growth of the average vehicle controls for two days is 8.0 fold increase from the original cell numbers. Lower values will render an assay unacceptable for evaluation because of poor cell growth.
- The background mutant frequency (average mutant frequency of the vehicle controls) is calculated separately for concurrent activation and nonactivation assays, even if the same population of cells was used for each assay. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is 30×10^{-6} to 120×10^{-6} . Assays with backgrounds outside this range are not necessarily invalid but will be used with caution.
- A positive control was included with each assay to provide confidence in the procedures used to detect mutagenic activity. The minimum acceptable mutant frequency induced by both the MMS positive control (nonactivation assay) and MCA positive control (activation assay) is 200×10^{-6} . An assay is acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria.

- For test articles with little or no mutagenic activity, an assay includes applied concentrations that reduce the relative growth to ten to twenty percent of the average vehicle controls or reach the maximum applied concentration given in the evaluation criteria. The relative growth represents a calculation of survival that is based on both relative suspension growth during the expression period and relative cloning efficiency at the time of plating. Because mutant frequencies generally increase as a function of toxicity, an attempt to obtain treatments in the range of ten to twenty percent relative growth must be made for an assay to be considered conclusive. This requirement is waived if the concentration of the highest assayed dose is at least 75 percent of an excessively toxic dose level or if the highest assayed dose is at least twice the solubility limit of the test material in culture medium. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.
- An experimental mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is ten percent or greater and the total number of viable clones in the selection plating efficiency plates exceeds about sixty. These limits avoid problems with the statistical distribution of colonies that can be scored among dishes.
- The mutant frequencies for five treated cultures are normally determined in each assay, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions to accept a single assay for evaluation of the test material.
- Mutant frequencies are normally derived from sets of three dishes for both the mutant colony count and the viable colony count. In order to allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set.

F. Assay Evaluation Criteria

Test articles are evaluated in the Mouse Lymphoma Forward Mutation Assay on the basis of a combination of a minimum increase in mutant frequency and a series of assay evaluation criteria. An assay may need to be repeated with different concentrations in order to properly evaluate a test material.

The minimum criterion considered necessary to demonstrate mutagenesis for any given treatment is a mutant frequency that is ≥ 2 times the concurrent background mutant frequency. The background mutant frequency is defined as the average mutant frequency of the vehicle control cultures.

A mutant frequency that meets the minimum criterion for a single treated culture is not sufficient evidence to evaluate a test material as a mutagen. The following test results must be obtained to reach this conclusion:

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses, but this depends upon the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears.
- If the mutant frequency obtained for a single dose at or near the highest testable toxicity is about two or more times the minimum criterion, the test material will be considered mutagenic in a single trial. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.
- For some test materials, the correlation between toxicity and applied concentration is poor. The proportion of the applied test material that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in frequency of induced mutants may occur with concentration changes that cause only small changes in observed toxicity. Therefore, either parameter, applied concentration or toxicity (percent relative growth), can be used to establish whether the increase in mutant frequency is related to an increase in effective treatment.
- Treatments that induce less than ten percent relative growth are included in the assay, but are not used as primary evidence for mutagenicity as it relates to risk assessment.

A test article is evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to toxicity causing ten to twenty percent relative growth or, in the case of relatively nontoxic materials, a range of applied concentrations extending to the maximum of 5000 $\mu\text{g/ml}$.

This presentation may not encompass all test situations, and the Study Director may use other criteria to arrive at a conclusion, especially when data from several repeat assays are available. The interpretation of the results in the Results and Discussion section provide the reasoning involved when departures from the above descriptions occur.

VI. RESULTS AND DISCUSSION

A. Test Material Handling

The test material, dimethyl acrylamide (DMACM), was received from 3M Pharmaceuticals on April 24, 1995. The test material was stored at room temperature and protected from light. The vehicle of choice was water. A solution was prepared in water at 10-times the highest desired concentration. The remaining 10X primary stocks were prepared by performing serial dilutions with water.

Treatment was initiated by making 1:10 dilutions of the primary stocks into cell cultures that contained 6×10^6 cells suspended in the appropriate volume of culture medium. All dosing was performed with freshly prepared test article stocks. The test material appeared to be in solution in culture medium from 9.77 $\mu\text{g}/\text{ml}$ to the testing limit of 5000 $\mu\text{g}/\text{ml}$. Based upon the color of the pH indicator in the culture medium and pH paper, the test material did not alter the pH of the treatment medium outside the range that is acceptable for this assay.

B. Rangefinding Cytotoxicity Assay

The test material, Dimethyl acrylamide (DMACM), was tested in the preliminary rangefinding cytotoxicity assay both with and without S9 metabolic activation. Ten dose levels were used in each case that ranged from 9.77 $\mu\text{g}/\text{ml}$ to 5000 $\mu\text{g}/\text{ml}$.

The test material was moderately cytotoxic to noncytotoxic without metabolic activation and highly cytotoxic to noncytotoxic with activation (Table 1). The mutation assays were therefore initiated with treatments up to 5000 $\mu\text{g}/\text{ml}$.

C. Mutation Assays

One nonactivation mutation assay was performed with the test material (Table 2). Nine dose levels were initiated that ranged from 500 $\mu\text{g}/\text{ml}$ to 5000 $\mu\text{g}/\text{ml}$. All treatments survived and the six highest concentrations were chosen for mutant analysis. With the cell survival observed on day 1, recovery usually occurs quickly.

This test article also induced delayed cytotoxicity and the relative growth (representing cell growth over the two day expression period and cloning efficiency at the time of selection) was very low at the highest concentrations.

In order for a culture to be evaluated as mutagenic in the nonactivation assay, a mutant frequency of greater than 96.2×10^{-6} was required. This threshold value was equal to twice the average mutant frequency of the concurrent vehicle controls. Four of the treatments had relative growths less than 10% and while all four were positive, the results should be viewed with caution. However, there were two remaining treatments (41.3% and 16.0% relative growths) that were also positive. The increases at 2000 $\mu\text{g/ml}$ and 3000 $\mu\text{g/ml}$ were 2.6-fold and 5.1-fold above the background mutant frequency. The results of the nonactivation mutation trial were therefore evaluated as positive for inducing forward mutation at the TK locus in mouse lymphoma cells under nonactivation test conditions.

One activation mutation assay was performed with the test material (Table 3). Nine dose levels were initiated that ranged from 500 $\mu\text{g/ml}$ to 5000 $\mu\text{g/ml}$. As was observed under nonactivation conditions, the test article was more cytotoxic in the assay than it was in the rangefinding cytotoxicity assay. Only four treatments from 500 $\mu\text{g/ml}$ to 2000 $\mu\text{g/ml}$ were available for analysis. A good range of cytotoxicities was induced (85.8% to 9.5% relative growths).

In the activation assay, the two highest concentrations of test material (1500 $\mu\text{g/ml}$ and 2000 $\mu\text{g/ml}$) induced mutant frequencies that exceeded the minimum criterion for a positive response, which was 119.3×10^{-6} in this assay. The increases were 2.1-fold and 3.5-fold above the background mutant frequency. The results of the activation mutation assay were therefore evaluated as positive for inducing forward mutation at the TK locus in mouse lymphoma cells.

The L5178Y TK+/- mutation assay produces a bimodal distribution of large and small mutant colonies. The origin of the bimodal distribution of mutant colony sizes is considered to reflect the scale of genetic damage, with the smaller colonies associated with gross, chromosomal damage and the larger colonies associated with changes within the gene. The increases in mutant frequency in the nonactivation and activation assays (Tables 2 and 3) were due to the presence of both small and large mutant colonies, with a slightly higher number of small colonies at the highest concentrations analyzed. The positive controls showed the characteristic bimodal response with increases in both small and large mutant colonies.

The average cloning efficiencies for the vehicle controls varied from 101.6% without activation to 83.9% with S9 metabolic activation which demonstrated good cloning conditions for the assays. The background mutant frequencies were within the historical range and the positive control cultures had mutant frequencies that were greatly in excess of the background and met assay acceptance criteria. Refer to Appendix A for historical control data.

VII. CONCLUSIONS

The test material, dimethyl acrylamide (DMACM), was positive in the mouse lymphoma forward mutation assay both with and without S9 metabolic activation under the conditions of testing.

VIII. REFERENCES

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IX. EXPERIMENTAL DATA TABLES

TABLE 1

CYTOTOXICITY ASSAY WITH Dimethyl acrylamide (DMACM)

SAMPLE IDENTITY: Dimethyl acrylamide (DMACM)

ASSAY NUMBER: 16849

TEST DATE: APRIL 27, 1995

SOLVENT: WATER

COMMENTS ON TREATMENT: FOUR-HOUR TREATMENT PERIOD

| APPLIED CONCENTRATION $\mu\text{g/ml}$ | WITHOUT S9 ACTIVATION | | WITH S9 ACTIVATION | |
|--|---|-----------------------------------|---|-----------------------------------|
| | CELL DENSITY/ML ($\times 10^5$) ^a | % VEHICLE CONTROL ^b | CELL DENSITY/ML ($\times 10^5$) ^a | % VEHICLE CONTROL ^b |
| NCC | 14.7 | 80.3 | 17.9 | 168.9 |
| VCd | 18.3 | 100.0 | 10.6 | 100.0 |
| 9.77 | NTC | --- | NTC | --- |
| 19.5 | NTC | --- | NTC | --- |
| 39.1 | NTC | --- | NTC | --- |
| 78.1 | NTC | --- | 16.2 | 152.8 |
| 156 | NTC | --- | 14.9 | 140.6 |
| 313 | 14.0 | 76.5 | 11.3 | 106.6 |
| 625 | 13.9 | 76.0 | 11.2 | 105.7 |
| 1250 | 16.3 | 89.1 | 7.6 | 71.7 |
| 2500 | 9.9 | 54.1 | 4.6 | 43.4 |
| 5000 | 5.6 | 30.6 | 0.7 | 6.6 |

^aCell density determined by hemocytometer approximately 24 hours after treatment initiation.^bRelative to vehicle control cell density for all treatments.

NCC = Negative (media) control.

VCd = Vehicle control, 10% water.

NTC = Not counted; not cytotoxic.

TABLE 2
MUTATION ASSAY WITHOUT ACTIVATION

A. TEST ARTICLE: DIMETHYL ACRYLAMIDE (DMACM)
 B. GENETICS ASSAY NO: 16849
 C. VEHICLE: WATER
 D. SELECTIVE AGENT: 3.0 µg/ml
 E. TEST DATE: 05/02/95

| TEST CONDITION: | DAILY CELL COUNTS (CELLS/ML.10E5 UNITS) | | SUSPENSION GROWTH* | TOTAL MUTANT COLONIES | TOTAL VIABLE COLONIES | CLONING EFFICIENCY* | RELATIVE GROWTH (%) ^c | MUTANT FREQUENCY (10E-6 UNITS) ^d |
|-------------------------------------|--|------|---------------------------------------|-----------------------------|-----------------------------|------------------------|---------------------------------------|---|
| | 1 | 2 | | | | | | |
| NONACTIVATION CONTROLS ^e | | | | | | | | |
| | | | AVG VEHICLE CONTROL | | | | AVG VEHICLE CONTROL | |
| VEHICLE CONTROL | 18.7 | 14.6 | 30.3 | 172 | 586 | | 97.7 | 100.0 |
| VEHICLE CONTROL | 15.0 | 20.3 | 33.8 | 141 | 630 | | 105.0 | 100.0 |
| VEHICLE CONTROL | 15.4 | 19.3 | 33.0 | 125 | 612 | 102.0 | 101.6 | 100.0 |
| MMS 10 n1/ml | 12.5 | 14.1 | 19.6 | 710 | 347 | 57.8 | 34.4 | 409.2 ^f |
| MMS 15 n1/ml | 11.3 | 15.1 | 19.0 | 656 | 255 | 42.5 | 24.5 | 514.5 ^f |
| TEST COMPOUND | | | | | | | | |
| | | | RELATIVE TO VEHICLE CONTROL (%) | | | | RELATIVE TO VEHICLE CONTROL (%) | |
| 2000 µg/ml | 7.9 | 18.0 | 48.8 | 323 | 516 | 84.6 | 41.3 | 125.2 ^f |
| 3000 µg/ml | 6.9 | 9.5 | 22.5 | 534 | 434 | 71.2 | 16.0 | 246.1 ^f |
| 3500 µg/ml | 3.9* | 12.2 | 12.6 | 621 | 392 | 64.3 | 8.1 | 315.8 ^f |
| 4000 µg/ml | 3.6* | 12.3 | 12.7 | 492 | 337 | 55.3 | 7.0 | 292.0 ^f |
| 4500 µg/ml | 3.7 | 10.9 | 13.8 | 409 | 203 | 33.3 | 4.6 | 403.0 ^f |
| 5000 µg/ml | 3.2 | 4.0 | 4.4 | 518 | 195 | 32.0 | 1.4 | 531.3 ^f |

*SUSPENSION GROWTH = (DAY 1 COUNT/3) * (DAY 2 COUNT)/(3 OR DAY 1 COUNT IF NOT SPLIT BACK)

*CLONING EFFICIENCY = TOTAL VIABLE COLONY COUNT/NUMBER OF CELLS SEEDED * 100

*RELATIVE GROWTH = (RELATIVE SUSPENSION GROWTH * RELATIVE CLONING EFFICIENCY) / 100

*MUTANT FREQUENCY = (TOTAL MUTANT COLONIES/TOTAL VIABLE COLONIES) X 2X10E-4. DECIMAL IS MOVED TO EXPRESS THE FREQUENCY IN UNITS OF 10E-6

*VEHICLE CONTROL = 10% WATER; MMS = METHYL METHANESULFONATE POSITIVE CONTROL

*MUTAGENIC, EXCEEDS MINIMUM CRITERION OF 96.2 X 10E-6

* NOT SPLIT BACK

TABLE 3
MUTATION ASSAY WITH ACTIVATION

A. TEST ARTICLE: DIMETHYL ACRYLAMIDE (DMACH)
 B. GENETICS ASSAY NO: 16849
 C. VEHICLE: WATER
 D. SELECTIVE AGENT: 3.0 µg/ml
 E. TEST DATE: 05/02/95

| TEST CONDITION: | DAILY CELL COUNTS (CELLS/ML. 10E5 UNITS) | | SUSPENSION GROWTH* | TOTAL MUTANT COLONIES | TOTAL VIABLE COLONIES | CLONING EFFICIENCY ^b | RELATIVE GROWTH (%) ^c | MUTANT FREQUENCY (10E-6 UNITS) ^d | | |
|-------------------------|---|------|---------------------------------------|-----------------------------|-----------------------------|---------------------------------------|-------------------------------------|---|-------|------|
| | 1 | 2 | | | | | | | | |
| NONACTIVATION CONTROLS* | S9 BATCH NO: 0501 | | AVG VEHICLE CONTROL | | AVG VEHICLE CONTROL | | | | | |
| VEHICLE CONTROL | 14.4 | 16.0 | 25.6 | 167 | 556 | 92.7 | 100.0 | 60.1 | | |
| VEHICLE CONTROL | 18.4 | 15.3 | 31.3 | 114 | 489 | 81.5 | 100.0 | 46.6 | | |
| VEHICLE CONTROL | 19.2 | 15.5 | 33.1 | 30.0 | 168 | 465 | 77.5 | 83.9 | 100.0 | 72.3 |
| MCA 2 µg/ml | 12.0 | 12.3 | 16.4 | 854 | 457 | 76.2 | 49.6 | 373.7 ^e | | |
| MCA 4 µg/ml | 12.3 | 13.0 | 17.8 | 800 | 406 | 67.7 | 47.9 | 394.1 ^e | | |
| TEST COMPOUND | | | RELATIVE TO VEHICLE CONTROL (%) | | | RELATIVE TO VEHICLE CONTROL (%) | | | | |
| 500 µg/ml | 12.8 | 16.9 | 80.1 | 155 | 539 | 107.1 | 85.8 | 57.5 | | |
| 1000 µg/ml | 8.0 | 15.8 | 46.8 | 224 | 479 | 95.2 | 44.6 | 93.5 | | |
| 1500 µg/ml | 4.7 | 15.7 | 27.3 | 266 | 417 | 82.8 | 22.6 | 127.6 ^e | | |
| 2000 µg/ml | 3.0* | 10.8 | 12.0 | 419 | 398 | 79.1 | 9.5 | 210.6 ^e | | |

*SUSPENSION GROWTH = (DAY 1 COUNT/3) * (DAY 2 COUNT)/(3 OR DAY 1 COUNT IF NOT SPLIT BACK)

^bCLONING EFFICIENCY = TOTAL VIABLE COLONY COUNT/NUMBER OF CELLS SEED * 100

^cRELATIVE GROWTH = (RELATIVE SUSPENSION GROWTH * RELATIVE CLONING EFFICIENCY) / 100

^dMUTANT FREQUENCY = (TOTAL MUTANT COLONIES/TOTAL VIABLE COLONIES) X 2X10E-4. DECIMAL IS MOVED TO EXPRESS THE FREQUENCY IN UNITS OF 10E-6

^eVEHICLE CONTROL = 10% WATER; MCA = METHYLCHOLANTHRENE POSITIVE CONTROL

^fMUTAGENIC. EXCEEDS MINIMUM CRITERION OF 119.3 X 10E-6

* NOT SPLIT BACK

Table 4
 Analysis of Colony Size
 with Dimethyl Acrylamide (DMACH)
 CHV Assay Number 16849

| Dose ^a ($\mu\text{g}/\text{ml}$) | S9 | Mutant Frequency* ($\times 10^{-6}$) | % Large Colonies | % Small Colonies | Ratio S/L ^a |
|--|----|--|---------------------|---------------------|---------------------------|
| VC ^b | - | 42.0 | 33.1 | 66.9 | 2.02 |
| PC ^c | - | 371.7 | 21.7 | 78.3 | 3.61 |
| 2000 | - | 107.6 | 30.0 | 70.0 | 2.34 |
| 3000 | - | 215.0 | 18.2 | 81.8 | 4.48 |
| VC ^b | + | 50.7 | 41.3 | 58.7 | 1.42 |
| PC ^d | + | 312.0 | 48.8 | 51.2 | 1.05 |
| 1500 | + | 108.0 | 39.6 | 60.4 | 1.53 |
| 2000 | + | 186.0 | 24.3 | 75.7 | 3.11 |

* ratio of small colonies to large colonies

VC^b - vehicle control (10% water)

PC^c - methyl methanesulfonate (10 ml/ml)

PC^d - 20-methylcholanthrene (2.0 $\mu\text{g}/\text{ml}$)

* Entire plate cannot be sized due to interference from sides of plate. Mutant frequencies will therefore appear different from Tables 2 and 3.

**APPENDIX A HISTORICAL MOUSE LYMPHOMA FORWARD MUTATION ASSAY
CONTROL MUTANT FREQUENCY DATA**

- A. Nonactivation Studies**
1. Pooled negative and vehicle controls

| | |
|-----------------------|----------------------------------|
| Mean (\pm SD) | 50.6 \pm 14.0 $\times 10^{-6}$ |
| Range | 24.9 to 104.7 $\times 10^{-6}$ |
| Number of experiments | 50 |
| Number of controls | 132 |
 2. Positive controls (10.0 nl/ml methyl methanesulfonate)

| | |
|-----------------------|------------------------------------|
| Mean (\pm SD) | 484.1 \pm 137.8 $\times 10^{-6}$ |
| Range | 202.6 to 933.3 $\times 10^{-6}$ |
| Number of experiments | 38 |
| Number of controls | 38 |
 3. Positive controls (15.0 nl/ml methyl methanesulfonate)

| | |
|-----------------------|------------------------------------|
| Mean (\pm SD) | 715.1 \pm 238.2 $\times 10^{-6}$ |
| Range | 206.5 to 1309.0 $\times 10^{-6}$ |
| Number of experiments | 31 |
| Number of controls | 31 |
- B. Activation Studies**
1. Pooled negative and vehicle controls

| | |
|-----------------------|----------------------------------|
| Mean (\pm SD) | 63.5 \pm 27.4 $\times 10^{-6}$ |
| Range | 24.3 to 167.1 $\times 10^{-6}$ |
| Number of experiments | 53 |
| Number of controls | 158 |
 2. Positive controls (2.0 μ g/ml 3-methylcholanthrene)

| | |
|-----------------------|------------------------------------|
| Mean (\pm SD) | 475.6 \pm 158.9 $\times 10^{-6}$ |
| Range | 283.8 to 1010.4 $\times 10^{-6}$ |
| Number of experiments | 17 |
| Number of controls | 17 |
 3. Positive controls (4.0 μ g/ml 3-methylcholanthrene)

| | |
|-----------------------|------------------------------------|
| Mean (\pm SD) | 587.6 \pm 379.6 $\times 10^{-6}$ |
| Range | 271.1 to 2961.9 $\times 10^{-6}$ |
| Number of experiments | 50 |
| Number of controls | 58 |

The historical control data was compiled from fifty experiments when possible. The mean (\pm one standard deviation) and the range of the mutant frequencies were reported for each control condition. Because some experiments contained multiple controls, the number of independent control cultures exceeded the number of experiments.