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**Whittaker**

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

Sponsor: Chemical Manufacturers Association  
2501 M Street, N.W.  
Washington, D.C. 20037

Testing Facility: Microbiological Associates  
5221 River Road  
Bethesda, Maryland 20816

Study No.'s: See Page I-2

Test Article I.D.'s: Group 1: T1851-T1857, T1866-T1867  
Group 2: T1856, T2253-T2256  
See Page I-2

Test Article Lot No.'s: Group 1: None Provided  
Group 2: See Page I-2

Test Article Descriptions: See Page I-2

Storage Conditions: Group 1: Room Temperature  
Group 2: See Page I-2

Dates Received: See Page I-2

Dates Studies Started: See Page I-2

Date Study Completed: 3/14/84

Report Date: 8/14/84

Study Coordinator: Carol R. Stack, Ph.D.  
Chemical Manufacturers Association

Study Director: Andrea M. Rogers-Back, Ph.D.  
Microbiological Associates

Andrea M. Rogers-Back 8/14/84 Jane J. Clarke 8/10/84  
Andrea M. Rogers-Back, Ph.D. Date Jane J. Clarke  
Study Director Group Leader

Janine J. Leffert 8-14-84 Dennis A. Sorrells 8-14-84  
Janine J. Leffert Date Dennis A. Sorrells  
Group Leader Biologist

QUALITY ASSURANCE STATEMENT

Study Title: L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay

Study Number: T1856.701006, T1866.701006, T1854.701006, T1857.701006,  
T1852.701006, T1855.701006, T1851.701006, T1867.701006,  
T1853.701006, T2253.701012, T2254.701012, T2255.701012  
and T2256.701012

Study Director: Andrea M. Rogers-Back, Ph.D.

Initiation Date: See Page I-2

Review Completed Date:

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc. are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice Regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

<u>DATE OF INSPECTION</u>	<u>PHASE INSPECTED</u>	<u>REPORT SUBMITTED TO STUDY DIRECTOR</u>	<u>MANAGEMENT</u>
<u>T1856.701006:</u>			
12/8/82	Protocol review	12/8/82	12/8/82
1/12/83	Initial toxicity: Test article removal and culture rinsing	1/13/83	1/13/83
2/21/83	Counting the plates	2/21/83	2/22/83
2/23/83	Initial toxicity: Preparation of S-9 mix	2/23/83	2/23/83
3/1/83	Dilution of test article and positive controls	3/1/83	3/14/83
3/3/83	Cloning medium preparation	3/3/83	3/14/83
4/4/83	Raw data audit	4/4/84	4/20/83
6/7/83	Draft report	6/7/83	6/8/83
9/7/83	Preparation of cultures Treatment of cultures	9/7/83	9/7/83
10/5/83	Preparation of cultures	10/5/83	10/5/83
10/5/83	Preparation of S-9 mix	10/5/83	10/5/83

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QUALITY ASSURANCE STATEMENT CONTINUED

<u>DATE OF INSPECTION</u>	<u>PHASE INSPECTED</u>	<u>REPORT SUBMITTED TO</u>	
		<u>STUDY DIRECTOR</u>	<u>MANAGEMENT</u>
<u>T1866.701006:</u>			
12/20/82	Protocol review	12/20/82	12/20/82
1/18/83	Initial toxicity: Preparation of cultures	1/18/83	1/19/83
1/25/83	Preparation of cultures Test article removal and culture rinsing	1/25/83	1/25/83
2/23/83	Initial toxicity: Preparation of S-9 mix	2/23/83	2/23/83
4/5/83	Raw data audit	4/5/83	4/20/83
6/7/83	Draft report	6/7/83	6/8/83
<u>T1854.701006:</u>			
12/6/82	Protocol review	12/8/82	12/8/82
1/11/83	Initial toxicity: Dilution of test article	1/11/83	1/11/83
1/28/83	Cloning medium preparation	1/28/83	1/28/83
2/10/83	Treatment of cultures	2/10/83	2/10/83
3/15/83	Daily cell counts and readjustments	3/15/83	3/15/83
4/15/83	Raw data audit	4/15/83	5/2/83
6/7/83	Draft report	6/7/83	6/8/82
<u>T1857.701006:</u>			
12/8/82	Protocol review	12/8/82	12/8/82
1/4/83	Initial toxicity: Dilution of test article	1/4/83	1/5/83
1/26/83	Test article removal and culture rinsing	1/26/83	1/26/83
1/28/83	Cloning medium preparation	1/28/83	1/28/83
2/10/83	Dilution of test article and positive control	2/10/83	2/10/83
3/1/83	Initial toxicity: Test article removal and culture rinsing	3/1/83	3/14/83
3/14/83	Preparation of S-9 mix	3/14/83	3/14/83
4/15/83	Raw data audit	4/15/83	4/18/83
6/7/83	Draft report	6/7/83	6/8/83

QUALITY ASSURANCE STATEMENT CONTINUED

<u>DATE OF INSPECTION</u>	<u>PHASE INSPECTED</u>	<u>REPORT SUBMITTED TO</u> <u>STUDY DIRECTOR      MANAGEMENT</u>	
<u>T1852.701006:</u>			
12/8/82	Protocol review	12/8/82	12/8/82
12/16/82	Initial toxicity: Preparation of cultures	12/16/82	12/17/82
1/18/83	Dilution of test article and positive controls	1/18/83	1/19/83
2/7/83	Initial toxicity: Preparation of S-9 mix	2/7/83	2/7/83
2/23/83	Preparation of S-9 mix	2/23/83	2/23/83
3/22/83	Dilution of test article and positive control Treatment of cultures	3/22/83	3/23/83
4/28/83	Raw data audit	4/28/83	5/4/83
6/7/83	Draft report	6/7/83	6/8/83
<u>T1855.701006:</u>			
12/8/82	Protocol review	12/8/82	12/8/82
1/17/83	Dilution of test article and positive control Treatment of cultures	1/17/83	1/17/83
1/19/83	Cell dilution and TFT addition to cloning flasks	1/19/83	1/20/83
3/22/83	Dilution of test article and positive control	3/22/83	3/23/83
4/15/83	Raw data audit	4/15/83	4/22/83
6/7/83	Draft report	6/7/83	6/8/83
<u>T1851.701006:</u>			
12/8/82	Protocol review	12/8/82	12/8/82
12/16/82	Initial toxicity: Preparation of cultures	12/16/82	12/17/82
12/17/82	Initial toxicity: Daily cell counts and readjustments	12/17/82	12/17/82
1/11/83	Dilution of test article and positive controls	1/11/83	1/11/83
2/9/83	Test article removal and culture rinsing	2/9/83	2/10/83
5/4/83	Raw data audit	5/4/83	5/11/83
6/7/83	Draft report	6/7/83	6/8/83

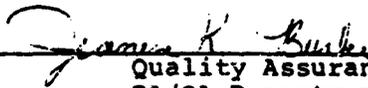
QUALITY ASSURANCE STATEMENT CONTINUED

<u>DATE OF INSPECTION</u>	<u>PHASE INSPECTED</u>	<u>REPORT SUBMITTED TO STUDY DIRECTOR</u>	<u>MANAGEMENT</u>
<u>T1867.701006:</u>			
12/20/82	Protocol review	12/20/82	12/20/82
1/27/83	Cell preparation and addition to cloning flasks Cell dilution and TFT addition to cloning flasks	1/28/83	1/28/83
4/5/83	Treatment of cultures	4/5/83	4/5/83
5/5/83	Raw data audit	5/5/83	5/11/83
6/7/83	Draft report	6/7/83	6/8/83
<u>T1853.701006:</u>			
12/8/82	Protocol review	12/8/82	12/8/82
1/17/83	Test article removal and culture rinsing	1/17/83	1/17/83
1/19/83	Cloning medium preparation	1/19/83	1/19/83
2/9/83	Initial toxicity: Test article removal and culture rinsing	2/9/83	2/10/83
2/16/83	Preparation of S-9 mix	2/16/83	2/16/83
4/13/83	Raw data audit	4/13/83	4/15/83
6/7/83	Draft report	6/7/83	6/8/83
<u>T2253.701012, T2254.701012, T2255.701012 and T2256.701012:</u>			
1/16/84	Protocol review	1/16/84	1/16/84
<u>T2253.701012:</u>			
1/23/84	Initial toxicity: Test article removal and culture rinsing	1/24/84	1/26/84
2/7/84	Preparation of S-9 mix Preparation of cultures	2/7/84	2/7/84
3/28/84	Removal of test article and culture rinsing	3/29/84	3/29/84
<u>T2254.701012:</u>			
1/23/84	Initial toxicity: Test article removal and culture rinsing	1/24/84	1/27/84
2/7/84	Preparation of S-9 mix Preparation of cultures	2/7/84	2/7/84

QUALITY ASSURANCE STATEMENT CONTINUED

<u>DATE OF INSPECTION</u>	<u>PHASE INSPECTED</u>	<u>REPORT SUBMITTED TO STUDY DIRECTOR MANAGEMENT</u>	
<u>T2254.701012 Cont.:</u>			
2/9/83	Cell preparation and addition to the cloning flasks	2/9/84	2/10/84
<u>T2255.701012:</u>			
2/8/84	Test article removal and culture rinsing	2/8/84	2/9/84
2/10/84	Cell preparation and addition to cloning flasks Cell dilution and TFT addition to the cloning flasks	2/10/84	2/10/84
<u>T2256.701012:</u>			
1/25/84	Initial toxicity: Cell counts and readjustments	1/25/84	1/26/84
2/8/84	Test article removal and culture rinsing	2/8/84	2/9/84
2/10/84	Cell preparation and addition to cloning flasks	2/10/84	2/10/84
<u>T1856.701006, T2253.701012, T2254.701012, T2255.701012 and T2256.701012:</u>			
5/22/84	Draft report	5/24/84	5/31/84
<u>T1851.701006 - T1857.701006, T1866.701006 - T1867.701006, T2253.701012 - T2256.701012:</u>			
8/13/84	Draft to final report	8/13/84	8/14/84

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

  
Quality Assurance  
RA/QA Department

TEST ARTICLE INFORMATION

GROUP 1

Study #	T1856.701006	T1866.701006	T1854.701006	T1857.701006
Test Article I.D.	CMA-102	CMA-105	CMA-106	CMA-107
Test Article Description	Clear, Slightly Viscous Liquid	Yellow, Slightly Viscous Liquid	Clear, Viscous Liquid	Clear Amber, Slightly Viscous Liquid
Date Received	11/24/82	12/20/82	11/24/82	11/24/82
Date Study Started	1/12/83	1/18/83	1/11/83	1/4/83

Study #	T1852.701006	T1855.701006	T1851.701006	T1867.701006	T1853.701006
Test Article I.D.	CMA-108	CMA-109	CMA-110	CMA-111	CMA-112
Test Article Description	Light Brown Viscous Liquid	Clear Amber, Slightly Viscous Liquid	Clear, Dark Amber, Viscous Liquid	Dark Amber, Slightly Viscous Liquid	Clear, Dark Amber, Viscous Liquid
Date Received	11/24/82	11/24/82	11/24/82	12/10/82	11/24/82
Date Study Started	12/16/82	12/20/82	12/16/82	1/18/83	12/20/82

GROUP 2

Test Article I.D.	Zinc Dialkyl Dithiophosphate (CMA-102 & CMA-102B)	Calcium Dialkyl Dithiophosphate	Zinc Oleate	Zinc Chloride
Test Article Lot No.	None Provided	None Provided	34495-10	KTJY
Study No.	T1856.701006 T2254.701012	12253.701012	T2256.701012	T2255.701012
Test Article Description	T1856: Clear, Slightly Viscous Liquid T2254: Yellow, Slightly Viscous Liquid	Light Green Viscous Liquid	Off-white Solids	White Granules
Storage Conditions	Room Temperature Protected from Light	Room Temperature Protected from Light	Room Temperature with Desiccation Protected from Light	Room Temperature with Desiccation Protected from Light
Date Received	T1856: 11/24/82 T2254: 12/12/83	12/12/83	12/12/83	12/12/83
Date Study Started	T1856: 9/7/83 T2254: 1/23/84	1/23/84	1/24/84	1/24/84

Note: All test articles in Group I are Zinc Dialkyl Dithiophosphates

Summary

Chemical Manufacturers Association's test articles CMA-102 (MA #'s T1856 and T2254), CMA-105 (MA #T1866), CMA-106 (MA #T1854), CMA-107 (MA #T1857), CMA-108 (MA #T1852), CMA-109 (MA #T1855), CMA-110 (MA #T1851), CMA-111 (MA #T1867) and CMA-112 (MA #T1853) were tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. All the test articles were Zinc Dialkyl Dithiophosphates. In addition, Calcium Dialkyl Dithiophosphate (MA #T2253), Zinc Oleate, Lot No. 34495-10 (MA #T2256) and Zinc Chloride, Lot No. KTJY (MA #T2255) were also tested in the presence of Aroclor induced rat liver S-9. The results are summarized in Summary Table 1.

Detailed results for each test article are presented in separate sections distinguished by a blue tab with the test article I.D.

SUMMARY TABLE 1

	<u>CMA #</u>	<u>MA #</u>	<u>Threshold Toxicity Level (µl/ml)</u>		<u>Mutation Assay Results</u>		
			<u>- S-9</u>	<u>+ S-9</u>	<u>- S-9</u>	<u>+ S-9</u>	
Zinc Dialkyl Dithiophosphates	102 *	T1856	0.05	0.05	-	± (+) *	
	102(B)	T2254	nt	0.05	nt	±	
	105	T1866	0.05	0.05	-	±	
	106	T1854	0.05	0.05	-	+	
	107	T1857	0.1	0.1	-	+	
	108	T1852	0.1	0.05	-	±	
	109	T1855	0.1	0.05	-	-	
	110	T1851	0.1	0.05	-	±	
	111	T1867	0.05	0.1	-	±	
	112	T1853	0.1	0.05	-	-	
	Calcium Dialkyl Dithiophosphate		T2253	nt	0.1	nt	-
	Zinc Chloride		T2255	nt	50 µg/ml	nt	+
Zinc Oleate		T2256	nt	100 µg/ml	nt	-	

nt = not tested

\*Second series of experiments

## Introduction

Ten Zinc Dialkyl Dithiophosphate test articles, CMA-102 (MA #T1856 and T2254), CMA-105 (MA #T1866), CMA-106 (MA #T1854), CMA-107 (MA #T1857), CMA-108 (MA #T1852), CMA-109 (MA #T1855), CMA-110 (MA #T1851), CMA-111 (MA #T1867) and CMA-112 (MA #T1853); were tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay with and without exogenous metabolic activation by Aroclor induced rat liver microsomes. In addition, Calcium Dialkyl Dithiophosphate (MA #T2253), Zinc Oleate, Lot No. 34495-10 (MA #T2256) and Zinc Chloride, Lot No. KTJY (MA #T2255) were tested in the presence of S-9.

## Objective

To evaluate the mutagenic potential of the test article using the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay.

## Materials and Methods

The experimental protocols (see Appendices) are based on that described by Clive, D. and Spector, J. F. S. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y Mouse Lymphoma cells. Mutation Research 31:17-29, 1975.

### A. DETERMINATION OF TOXICITY

A preliminary toxicity test with and without S-9 activation was conducted. Tube cultures were initiated by seeding one Corning polypropylene centrifuge tube per dose level and two per solvent control with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells per ml. The solubility of the test articles was determined in Acetone, DMSO, Ethanol and H<sub>2</sub>O, and Acetone was selected as the solvent of choice. Test articles CMA-102 and CMA-105 through CMA-112 were tested in the presence and absence of S-9. In the presence of S-9, test articles T1851 through T1867 were solubilized and diluted for testing at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001  $\mu$ l/ml. In the absence of S-9, test articles CMA-102, CMA-105, CMA-106 and CMA-111 were solubilized and diluted for testing at 100, 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001  $\mu$ l/ml. Test articles CMA-107, CMA-108, CMA-109, CMA-110 and CMA-112 were solubilized and diluted for testing at 100, 10, 1.0, 0.1, 0.01 and 0.001  $\mu$ l/ml.

A preliminary toxicity test with S-9 activation was conducted on test articles T2253, T2254, T2255 and T2256. Acetone was selected as the solvent of choice for T2254, T2253 and T2256. Water was selected as the solvent for T2255. Test articles T2254 and T2253 were solubilized and diluted for testing at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001  $\mu\text{l/ml}$ . Test article T2255 was solubilized and diluted for testing at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05  $\mu\text{g/ml}$ . Test article T2256 was solubilized and diluted for testing at 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05  $\mu\text{g/ml}$ .

The test article was added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration was nontoxic to the cell suspension. Four ml of F<sub>0</sub>P or S-9 activation mixture was added to each tube. The tube was gassed with 5% CO<sub>2</sub> in air and placed on a Bellco roller drum apparatus at approximately 25 rpm for a 4-hour exposure period. The test solutions were prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article was removed by centrifuging the cells at 1000 rpm for 10 minutes and decanting the supernatant. The cells were washed twice in 10 ml of F<sub>10</sub>P, re-suspended in 20 ml of F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus.

Test article toxicity was determined by comparing the cell population growth at each dose level with that of the solvent controls. Cell population density was determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes, and counting the samples with an electronic cell counter.

All calculations were performed using a Texas Instruments TI-59 calculator and/or a Hewlett Packard 86 Minicomputer with programs labeled "Cell Culture Adjustment" and "Initial Toxicity".

### 3. TESTING FOR MUTAGENIC ACTIVITY

#### 1. Cell Preparation

Prior to use in the assay, L5178Y cells which were actively growing in culture were cleansed as described by Clive, et al.<sup>1</sup> Three ml of THMG stock solution was added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells per ml. The culture was gassed with 5% CO<sub>2</sub> in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours the THMG was removed by pelletizing the cells and decanting the supernatant. The cells were rinsed in 20 ml of F12P and reinstated in culture at  $3 \times 10^6$  cells per ml in 100 ml of F12P plus 1 ml of THG stock solution.

The cell population density of the prepared cultures was determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml was prepared, and 6 ml aliquots were dispensed in Corning polypropylene centrifuges.

#### 2. Test Compound Preparation

Based on the data derived from the toxicity test, the test article was prepared so that the highest concentration was 100% toxic. The test article was solubilized and 15 serial eighth log dilutions were carried out. This produced 16 dose levels decreasing approximately 75-fold from highest to lowest. In some experiments, 8 evenly spaced dose levels, which decreased approximately 10-fold from highest to lowest dose were used. The test article was added to duplicate cultures labelled with the T# and A or B. For T1856 and T2253 (Trial II), the test article was solubilized

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<sup>1</sup>Clive, D. and Spector, J. F. S. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res. 31:17-29, 1975.

and 12 serial dilutions were carried out. Fourteen dose levels were produced and cultures were treated in triplicate. The test article was added to each tube, labelled with the test article T No., test concentration and NA or S-9, where appropriate, in amounts at which the final solvent concentration was nontoxic to the cells. Four ml of S-9 activation mixture or 4 ml F<sub>0</sub>P was added to the tubes. This yielded a final cell suspension of  $0.6 \times 10^6$  cells per ml.

Two control tubes received solvent only and the positive controls were treated with 7,12-DMBA (7.5 and 5.0  $\mu\text{g/ml}$ ) or EMS (1.0 and 0.5  $\mu\text{l/ml}$ ). All tubes were gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article was carried out under amber lighting and the cells were incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells were washed twice in 10 ml of F<sub>0</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells were resuspended in 20 ml of F<sub>0</sub>S, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

### 3. Expression Time

After the initial exposure to the test article, the cells were incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment was made by taking daily cell counts and then replacing a volume of cells with fresh medium which yielded a cell population density of  $0.3 \times 10^6$  cells per ml.

### 4. Cloning

At the end of the expression period, the cells were placed in cloning medium (C.M.) containing 0.34% Noble agar. TFT at a final concentration of 3  $\mu\text{g/ml}$  was used as the restrictive agent.

#### a. General Preparation

Two Florence flasks per culture to be cloned were labeled with the compound concentration and whether they received S-9

activation. For each pair of flasks one was labeled TFT and one was labeled V.C. (viable count). Each flask was prewarmed to 37°C, filled with 100 ml of C.M., and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per culture were labeled with the concentration, and the experiment number. Three of the six were labeled TFT and three were labeled V.C.

b. Cell Plating

Cell counts were made for each tube to determine the volume of each cell population which would yield  $3 \times 10^5$  cells. This volume was removed, the remainder of the cells were discarded, and the  $3 \times 10^6$  cells were replaced in the centrifuge tube. The cells were centrifuged at 1000 rpm for 10 minutes, and the supernatant, except for 2 ml, was removed by pipetting. The cells were resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article.

A  $2 \times 10^{-4}$  dilution was carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of F<sub>11</sub>P, adding 1.0 ml of this to 9 ml of F<sub>11</sub>P, and adding 1.0 ml of that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution 1 ml of stock solution of TFT was added to the TFT flask, and both this flask and the V.C. flask were placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks were removed one at a time, and 33 ml of the cell suspension was pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates were placed in cold storage (4°C) for 20 minutes. The plates were removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

### 5. Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates were scored for the total number of colonies per plate. Three counts per plate were made on an automatic colony counter, and the median count was recorded. The mutation frequency was determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. All mutant frequencies and toxicity data calculations were performed using either a Texas Instruments TI-59 calculator with programs labelled "Mutant Frequency" or "Total Compound Toxicity" or a Hewlett-Packard microcomputer with programs labelled "Final Report Generation".

Chemical information on control articles and solvents used for this study:

#### Control Article:

7,12-Dimethylbenz(a)anthracene	57-97-6
CAS Registry Number	95% min
Assay (by UV-VIS)	2°C range including 123°C
Melting Range	

Ethyl Methanesulfonate	CH <sub>3</sub> SO <sub>3</sub> C <sub>2</sub> H <sub>5</sub>
CAS Registry Number	62-50-0
Assay (by GLC)	98% min

#### Solvents:

Acetone	CH <sub>3</sub> COCH <sub>3</sub>
CAS Registry Number	67-64-1
Assay (CH <sub>3</sub> COCH <sub>3</sub> )	Not less than 99.5%
Isopropyl Alcohol [(CH <sub>3</sub> ) <sub>2</sub> CHOH]	0.05%
Color (APHA)	10
Density (g/ml) at 25°C	Not above 0.7857
Boiling Range	Not more than 0.8°C
Boiling Point	56.1 ± 0.1°C
Residue after Evaporation	0.001%
Solubility in Water	Clear
Acidity (CH <sub>3</sub> COOH)	0.002%
Alkalinity (as NH <sub>3</sub> )	0.001%
Aldehyde (HCHO)	0.002%
Methanol (CH <sub>3</sub> OH) (by G.C.)	0.05%
Subs Reducing KMnO <sub>4</sub> (Color)	Pass test
Water (H <sub>2</sub> O)	0.5%

DMSO	(CH <sub>3</sub> ) <sub>2</sub> SO
CAS Registry Number	67-68-5
Appearance	Clear, Colorless liquid
Density (gms./ml) at 25°C	1.095 min
Freezing Point	18.0°C min
Residue after Evaporation	0.01%
Color (APHA)	13
Ethanol	C <sub>2</sub> H <sub>5</sub> OH
Brand Name	Pharmco
Bottled by	Publicker Industries Co. Linfield, Pa.
Assay	190 Proof
Molecular Weight	46.07
Density (gms/ml) at 20°C	0.789
Boiling Point	78.5°C
Melting Point	-114.1°C
Solidifies below	-130°C
Flash Point	9-11°C

The following criteria were used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the scientist's evaluation must be the final endpoint.

**Positive** - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

**Equivocal** - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

**Negative** - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

- Note:
1. Some of the numbers generated by the test data, whether it is Toxicity, Mutant Frequency, etc., are computed using non-rounded numbers. This may, in some instances, cause what appear to be errors in calculation if only the rounded numbers are used when checking the data.
  2. All of the raw data generated by the assay and the original final report will be maintained in Microbiological Associates' archives located in our Bethesda, Maryland facilities.

3. The stability of the test article under the actual experimental conditions used in this study was not determined by Microbiological Associates.

4. All test article stock solutions were freshly prepared immediately before their use in each procedure.

PROTOCOL AMENDMENT

Date: August 7, 1984

Sponsor: Chemical Manufacturers Association

Sponsor's Test Article Designation: 1) CMA-110 5) CMA-109  
2) CMA-108 6) CMA-102  
3) CMA-112 7) CMA-107  
4) CMA-106 8) CMA-105  
9) CMA-111

Study No.'s: 1) T1851.701006 5) T1855.701006  
2) T1852.701006 6) T1856.701006  
3) T1853.701006 7) T1857.701006  
4) T1854.701006 8) T1866.701006  
9) T1867.701006

Protocol No.: SPGT701006 1082

Protocol Title: L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay

1. Section 4.4 Study Director, should be changed to Andrea M. Rogers-Back, Ph.D.

Reason for Amendment:

After July 13, 1984, Paul E. Kirby, Ph.D. was no longer in the employ of Microbiological Associates.

APPROVAL:

Andrea M. Rogers-Back 8/16/84  
Andrea M. Rogers-Back, Ph.D. Date  
Study Director

Carol R. Stack 8/16/84  
Carol R. Stack, Ph.D. Date  
Study Coordinator

PROTOCOL AMENDMENT

Date: August 7, 1984

Sponsor: Chemical Manufacturers Association

Sponsor's Test Article Designation: 1) Calcium Dialkyl Dithiophosphate,  
2) Zinc Dialkyl Dithiophosphate,  
CMA-102B  
3) Zinc Chloride  
4) Zinc Oleate

Study No.'s: 1) T2253.701012 3) T2255.701012  
2) T2254.701012 4) T2256.701012

Protocol No.: SPGT701012 112383

Protocol Title: I5178Y TK+/- Mouse Lymphoma Mutagenesis Assay  
(With S-9 Activation)

1. Section 4.4 Study Director, should be changed to Andrea M. Rogers-Back, Ph.D.

Reason for Amendment:

After July 13, 1984, Paul E. Kirby, Ph.D. was no longer in the employ of Microbiological Associates.

APPROVAL:

Andrea M. Rogers-Back 8/14/84  
Andrea M. Rogers-Back, Ph.D. Date  
Study Director

Carol R. Stack 8/16/84  
Carol R. Stack, Ph.D. Date  
Study Coordinator

### Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-102 (MA #T1856) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of concentrations which produced from 35% to 98% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 22% to 117% Total Growth.

The highest test article concentration cloned in the S-9 activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. None of the nonactivated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicated that, under the conditions of this test, test article CMA-102 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

Three assays were conducted on the test article in the presence of S-9 as follow-up studies to that reported above. In the first assay, the cultures that were cloned were treated with a range of test article concentrations which produced from 3% to 71% Total Growth. There was some contamination in this assay and complete results were obtained for eleven of the eighteen cultures that were cloned. However, all eleven cultures exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The assay was repeated due to the contamination and the erratic dose-response relationship in toxicity in the treated cultures. Previous studies with the test article had also demonstrated a precipitous toxic response. In the second experiment, the cultures that were cloned were treated with a range of concentrations which produced from 3% to 44% Total Growth. Seven of seven cultures that were cloned exhibited mutant frequencies

which were significantly greater than the mean mutant frequency of the solvent controls. In a third experiment, conducted concurrently with an assay on Calcium Dialkyl Dithiophosphate (MA #T2253) and with a second sample of test article (T2254), the cultures that were cloned were treated with a range of concentrations which produced from 27% to 96% Total Growth. None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. However, a dose-dependent response was noted.

## Results

The Initial Toxicity Test (Table 1) performed on test article CMA-102 (MA #T1856) in the absence of S-9 indicated a threshold level of complete toxicity at 0.05  $\mu$ l/ml. Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.05  $\mu$ l/ml to 0.00067  $\mu$ l/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The Cloning Data indicated a low cloning efficiency which was unacceptable. The data for this assay will not be reported here. A repeat assay was performed over the same dose range.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, 0.0012, 0.00089 or 0.00067  $\mu$ l/ml test article. These concentrations produced a range in Suspension Growth of 37% to 91%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 35% to 98%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-102 (MA #T1856). The results indicated a threshold level of complete toxicity at 0.05  $\mu$ l/ml. Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05  $\mu$ l/ml to 0.00067  $\mu$ l/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.021, 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, or 0.0016  $\mu$ l/ml. These concentrations produced a

range in Suspension Growth of 25% to 97%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2. One culture (0.021  $\mu$ l/ml) that was cloned exhibited a mutant frequency which was 2.3 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 22%.

None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 91% to 117%.

Two mutagenesis assays in the presence of S-9 were conducted as follow-up studies to the original assays. In the first assay, the cultures were treated in triplicate with a range of test article concentrations from 0.030  $\mu$ l/ml to 0.017  $\mu$ l/ml. After a two day expression period, eighteen cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.022, 0.021, 0.020, 0.019, 0.018 or 0.017  $\mu$ l/ml. These concentrations produced a range in Suspension Growth of 7% to 76%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 11 and 12. These data are also presented graphically in Figure 3. The Cloning Data and Total Compound Toxicity Data for the positive control are presented in Table 13.

All the cultures that were cloned for which complete results were obtained exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The increases in mutant frequency ranged from 9.4 to 2.2 times the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 3% to 71%.

The assay was repeated due to some contamination and an erratic toxic response. The repeat assay was conducted over the same dose range as described above. After a two day expression

period, seven cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.021, 0.018 or 0.017  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 7% to 47%. The Cloning Data are presented in Tables 14 and 15 and the Total Compound Toxicity Data are presented in Tables 16 and 17. These data are also presented graphically in Figure 4.

All the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The increases in mutant frequency ranged from 11.3 to 3.1 times the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 3% to 44%.

A second sample of the test article was received. The Initial Toxicity Test (Table 18) indicated a complete toxicity in the presence of S-9 at 0.05  $\mu\text{l/ml}$ .<sup>\*</sup> Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05  $\mu\text{l/ml}$  to 0.00067  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.021, 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021 or 0.0016  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 31% to 100%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 19 and 20. These data are also presented graphically in Figure 5. The data for the positive control are presented in Table 21.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 27% to 96%. However, a dose-dependent increase in mutant frequency was noted. The increase ranged from 1.0 to 1.9 times the mean mutant frequency of the solvent controls.

<sup>\*</sup>Cultures treated with 10  $\mu\text{l/ml}$  and 5  $\mu\text{l/ml}$  appeared to have cell growth. Upon microscopic examination of the cultures, there were few viable cells present and a large quantity of compound precipitate and cell debris.

Study No. T1856.701006

Table 1

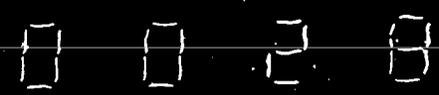
702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY  
INITIAL TOXICITY TEST

T1856.701006 Study Number      Paul E. Kirby Study Director      100  $\mu$ l to 0.001  $\mu$ l Dose Range  
CMA-102 (T1856) Test Article Identity      Acetone Solvent

	Test Article Concentration	Cell Concentration (x10 <sup>6</sup> )		Suspension Growth	
		Day 1	Day 2	Total	% of Control
Without Activation	100 $\mu$ l	2.519*	3.537*	0.0	0%
	10 $\mu$ l	1.094*	1.294*	0.0	0%
	5.0 $\mu$ l	0.204	0.284	0.0	0%
	1.0 $\mu$ l	0.006	0.007	0.0	0%
	0.5 $\mu$ l	0.000	0.000	0.0	0%
	0.1 $\mu$ l	0.000	0.000	0.0	0%
	0.05 $\mu$ l	0.002	0.001	0.0	0%
	0.01 $\mu$ l	0.577	0.714	4.6	30%
	0.005 $\mu$ l	1.102	1.247	15.3	100%
	0.001 $\mu$ l	1.129	1.262	15.8	104%
		Solvent 1	1.097	1.258	15.3/15.2
	Solvent 2	1.104	1.232	15.1	
With S-9 Activation					
		Solvent 1			
	Solvent 2				

*not applicable ATKB 1/14/83*

+ Culture Lost: \* counts indicated debris, not cells 9/11/83  
 Table Prepared By: James [Signature] 1-14-83 Date  
 Signature: [Signature]  
 Form No. WZ-233 Workbook Page No. 15 Report Page No. II-6



Study No. 1856 701004

Table 2  
 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY  
 CLONING DATA

Test Article Conc.	No. of Colonies/TFT Plate			Ave. #/ Plate	No. of Colonies/V.C. Plate			Ave. #/ Plate	Mutant Frequency	Induced Mutant Frequency
	1	2	3		1	2	3			
0.0059%	64	62	98	75	187	186	174	182	0.8	0.3
0.0067%	62	55	65	61	104	180	225	192	0.6	0.1
0.0050%	46	57	51	51	209	174	190	191	0.5	0.0
0.0058%	+	+	40	46	170	186	200	192	0.5	0.0
0.0028%	65	56	69	63	183	177	161	174	0.7	0.2
0.0021%	+	44	49	47	149	+	215	182	0.5	0.0
0.0016%	29	40	29	33	196	194	219	203	0.3	0.2
0.0012%	52	57	51	53	201	200	200	200	0.5	0.0
0.0008%	64	53	52	56	154	179	179	171	0.7	0.2
0.00067%	63	54	67	61	218	170	212	202	0.6	0.1
Solvent 1	52	48	47	49	189	187	177	184	0.57	0.5
Solvent 2	47	48	55	50	189	188	199	192	0.55	

Study Number 1856 701006  
 Study Director Paul E. Kirby  
 Experiment Number 185662

Test Article Identity CMA 102 (1856)  
 Solvent Acetone  
 Metabolic Activation None

Plates Counted by: Karen Gouffon 2/21/83 Calculations Performed By: Karen Gouffon 2/21/83  
 (Signature & Date) (Signature & Date)

\* Per 10<sup>6</sup> surviving cells + Culture Lost

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

Study No. T1856 701006

Study Number		Study Director		Experiment Number		
<u>T1856 701006</u>		<u>Paul S. Kuby</u>		<u>1856B2</u>		
Test Article Conc.	No. of Colonies/ TPT Plate	Ave. #/ Plate	No. of Colonies/V.C. Plate	Ave. #/ Plate	Mutant Frequency	Induced Mutant Frequency
	1 2 3		1 2 3			
Ethyl Methanesulfonate (No Activation)						
1.0µl/ml	237 231 196	221	20 15 +	18	25.1	23.9
0.5µl/ml	310 305 311	309	136 123 116	125	9.9	3.7
SOLVENT 1	97 88 93	93	171 158 187	172	1.1	1.2
SOLVENT 2	98 89 98	95	173 169 151	163	1.2	1.2
7,12 Dimethylbenz(a)anthracene (With Activation)						
7.5 µg/ml						
5.0 µg/ml						
SOLVENT 1						
SOLVENT 2						
Plates Counted By: <u>[Signature]</u>		Calculations Performed By: <u>Karen Joan Form</u>		Date: <u>2/21/83</u>		
				(Signature & Date)		

\*Per 10<sup>6</sup> surviving cells  
+ Culture Lost

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY  
TOTAL COMPOUND TOXICITY DATA

Test Article Concentration	Cell Concentration x 10 <sup>6</sup> cells/ml		Suspension Growth Total	Cloning Growth Av. V. C.	Total Growth*
	Day 1	Day 2			
0.016 <sup>11/20</sup>	0.061	0.070	0.0	++	0.0 %
0.012 <sup>10/20</sup>	0.036	0.030	0.0	++	0.0 %
0.0089 <sup>11/10</sup>	0.608	0.921	6.2	182	97 %
0.0067 <sup>11/10</sup>	0.776	1.119	9.6	192	102 %
0.0050 <sup>11/10</sup>	0.836	1.201	11.2	191	102 %
0.0038 <sup>11/10</sup>	0.900	1.333	14.0	192	102 %
0.0028 <sup>11/10</sup>	0.992	1.333	14.7	174	93 %
0.0021 <sup>11/10</sup>	1.001	1.335	14.8	182	97 %
<del>0.0016</del> <sup>11/10</sup>	0.990	1.406	15.5	203	108 %
0.0012 <sup>11/10</sup>	0.862	1.398	13.4	200	106 %
0.00089 <sup>11/10</sup>	0.959	1.440	15.3	171	91 %
0.00067 <sup>11/10</sup>	0.995	1.399	15.5	202	107 %
SOLVENT 1	0.989	1.576	17.3	184	188
SOLVENT 2	1.061	1.410	16.6	192	188

Study Number: 185662  
Study Director: Paul Z. Kidy  
Solvent: Acetone  
Metabolic Activation: None

Workbook Page No. 52

Report Page No. II-9

Study No. 1856 70'006

Tables prepared and calculations performed by: Karen Janssens 2/22/82  
(Signature & Date)

\* Total Growth = (Suspension Growth) / (Cloning Growth) x 100  
+ Culture Lost  
++ Too Toxic to Clone  
erratic error KSF 2/22/82

Table 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY  
TOTAL COMPOUND TOXICITY DATA

Study No. 1856 701006

Test Article Concentration	Cell Concentration x 10 <sup>6</sup> cells/ml		Suspension Growth Total	Cloning Growth Ave. V.C.	% Total Growth
	Day 1	Day 2			
Ethylmethanesulfonate (No Activation)					
1.0 µl/ml	0.603	0.701	4.7	18	3%
0.5 µl/ml	0.860	1.155	11.0	125	57%
SOLVENT 1	1.075	1.220	14.6	172	} 168
SOLVENT 2	1.071	1.189	14.2	163	
7.12 Dimethylbenz(a)anthracene (With Activation)					
7.5 µg/ml					
5.0 µg/ml					
SOLVENT 1		Not Applicable			
SOLVENT 2					

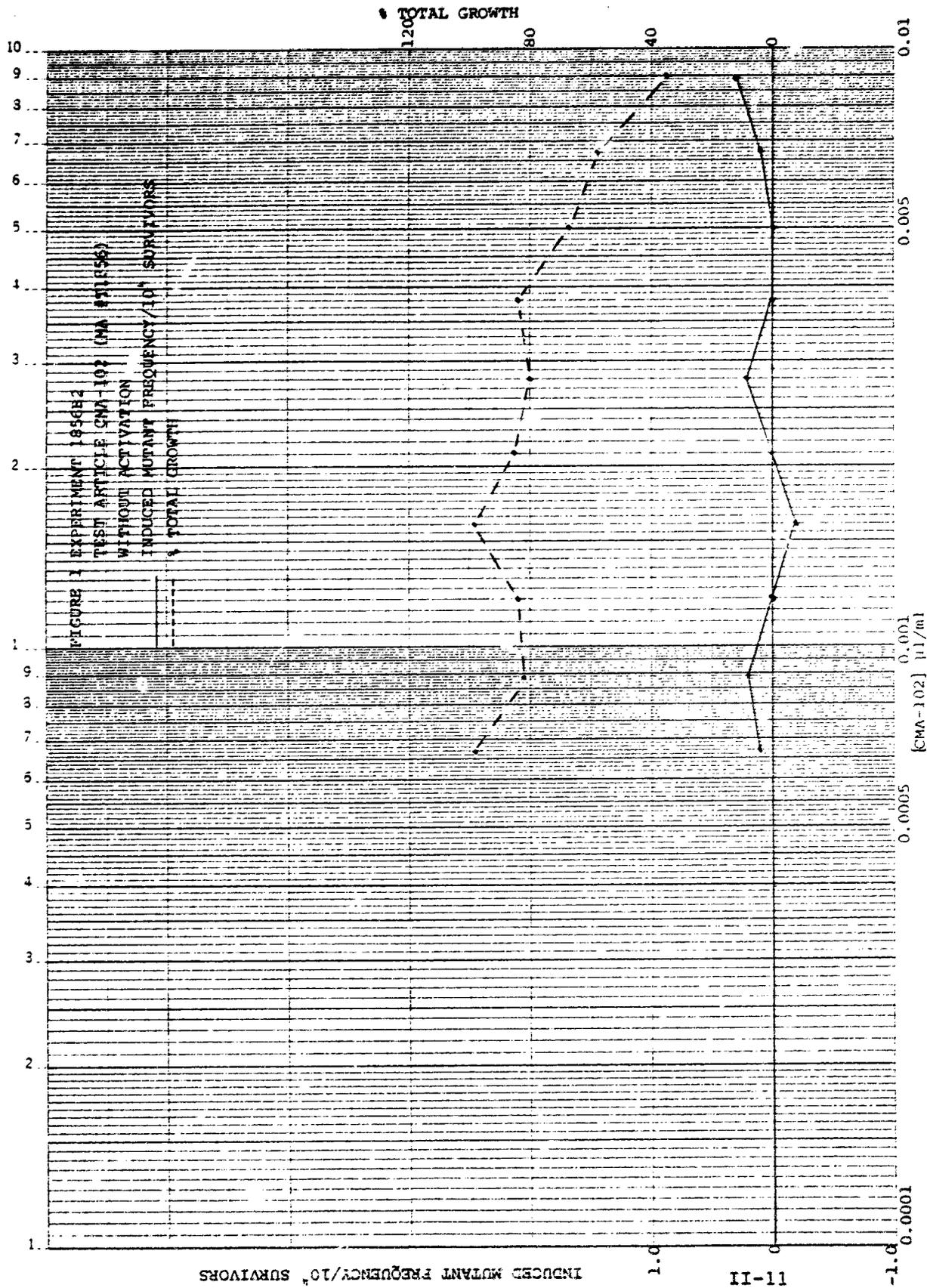
1856701006  
Study Number

Paul S. Kirby  
Study Director

185662  
Experiment No.

Tables prepared and calculations performed by: Karen Jan Bms 2/25/82  
(Signature & Date)

+ Culture Lost  
++ Too Toxic to Clone



00000

Table 6

Study No. T1856 701006

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY  
INITIAL TOXICITY TEST

T1856 701006 Study Number      Paul E. Kirby Study Director      10.0  $\mu$ g/ml to 0.001  $\mu$ g/ml Dose Range  
CMA-102 (T1856) Test Article Identity      Acetone Solvent

	Test Article Concentration	Cell Concentration ( $\times 10^6$ )		Suspension Growth	
		Day 1	Day 2	Total	% of Control
Without Activation					
With S-9 Activation	10 $\mu$ g/ml	0.491	0.806	*0.0	0%
	5.0 $\mu$ g/ml	0.199	0.285	0.0	0%
	1.0 $\mu$ g/ml	0.010	0.015	0.0	0%
	0.5 $\mu$ g/ml	0.007	0.025	0.0	0%
	0.1 $\mu$ g/ml	0.012	0.070	0.0	0%
	0.05 $\mu$ g/ml	0.021	0.017	0.0	0%
	0.01 $\mu$ g/ml	0.886	1.440	14.2	99%
	0.005 $\mu$ g/ml	0.906	1.400	14.1	99%
	0.001 $\mu$ g/ml	0.957	1.437	15.3	107%
	Solvent 1				
Solvent 2					
Solvent 1	0.847	1.446	13.6343		
Solvent 2	0.927	1.451	14.9		

*Not Applicable - Same M. Control*

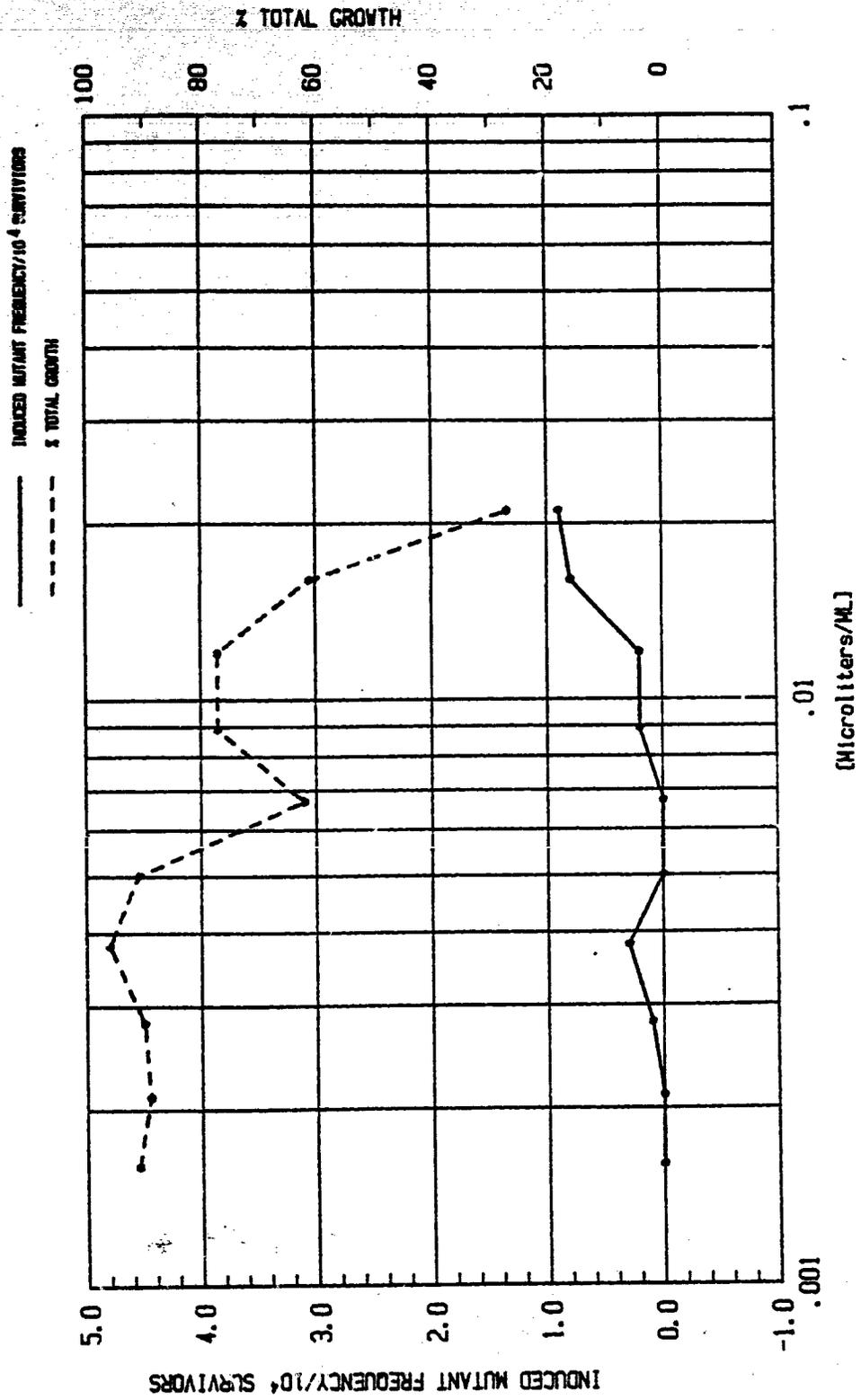
*2+5-83*

+ Culture Lost

Table Prepared By K counts indicated debris, not cells, 2/15/83  
Richard Howard Signature      2/15/83 Date



FIGURE 5  
 Study No. T2254.701012 (Trial 1)  
 Test Article CMA 1028  
 With S-9 Activation



154

### Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-102 (MA #T1856) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest test article concentration cloned in the S-9 activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. None of the nonactivated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-102 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of exogenous metabolic activation.

Three follow-up assays were conducted on test article Zinc Dialkyl Dithiophosphate (CMA-102 & CMA-102B) (MA #'s T1856 and T2254). In the first assay, eleven of eighteen cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The assay was repeated due to some contamination and an erratic dose-response in toxicity in the treated cultures. All the cultures that were cloned exhibited mutant frequencies which were more than twice the mean mutant frequency of the solvent controls. A second sample (MA #T2254) of the test article was tested concurrently with Calcium Dialkyl Dithiophosphate (MA #T2253). None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. However, a dose-related response was noted.

The results of all assays indicate that Zinc Dialkyl Dithiophosphate (MA #'s T1856 and T2254) produced a positive response in the presence of exogenous metabolic activation and a negative response in the absence of metabolic activation.

APPENDIX

II-34

11056

RECEIVED OCT 12 1983

PROTOCOL AMENDMENT

Date: October 6, 1983

Sponsor: Chemical Manufacturers Association

Test Article Designation: CMA-102

Study No.: T1856.701006

Protocol No.: SPGT701006

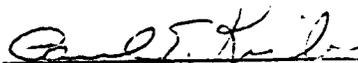
Protocol Title: L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay

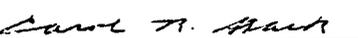
1. Section 6.1 Dosages, sentence 5 should read, "The test article will be solubilized and 14 doses in triplicate will be selected for treatment".

Reason for Amendment:

A previous study had shown a broad range in toxicity. Fourteen doses in triplicate were chosen to narrow the range.

APPROVAL:

 10/13/83  
Paul E. Kirby, Ph.D. Date  
Study Director

 10/13/83  
Carol R. Stack, Ph.D. Date  
Study Coordinator

0057

RECEIVED OCT 3 5 1982

*Spec'd by  
RA/DA 12/8/82  
N.K./j.k.*

**L5178Y TK<sup>+</sup>/- MOUSE LYMPHOMA MUTAGENESIS ASSAY**

**1.0 PURPOSE**

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK<sup>+</sup>/- Mouse Lymphoma Mutagenesis Assay.

**2.0 TEST ARTICLE**

2.1 Identification: CMA-102

2.2 Analysis:

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

**3.0 SPONSOR**

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.  
Washington, D.C.

3.3 Authorized Representative: Carol Stack

**4.0 TESTING FACILITY**

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

II-36

SGT701006 1082 1 of 16

0-058

## 5.0 TEST SYSTEM

L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells Clone J.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:  
\$2,650

Total charge for assay with metabolic activation:  
\$2,650

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

## 6.2 Route and Frequency of Administration

Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

## 6.3 Exogenous Metabolic Activation

### 6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

#### 6.3.1.1 Species, Strain, Sex and Inducer

Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

#### 6.3.1.2 Homogenate Preparation

Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

#### 6.3.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

#### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

#### 6.4 Controls

##### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

##### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

### 7.0 METHODS

#### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5%  $\text{CO}_2$  in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at  $1000 \times g$  for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of  $\text{F}_{10}\text{P}$ , resuspended in 20 ml of  $\text{F}_{10}\text{P}$ , gassed with 5%  $\text{CO}_2$  in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at  $37^\circ\text{C}$  for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, L5178Y  $\text{TK}^{+/-}$  cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring  $\text{TK}^{-/-}$  cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5%  $\text{CO}_2$  in air and placed on an environmental incubator shaker at 125 rpm and  $37^\circ\text{C}$ . After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of  $\text{F}_{10}\text{P}$  and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of  $\text{F}_{10}\text{P}$  plus 1 ml of THG stock solution.

7062

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

#### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F<sub>0</sub>P will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>-/-</sup> colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

#### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

4 0 6 3

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

#### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the  $TK^{-/-}$  cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of  $TK^{-/-}$  cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to  $37^{\circ}C$ , filled with 100 ml of C.M. and placed on an incubator shaker at  $37^{\circ}C$  until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^6$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at  $1000 \times g$  for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of  $F_{10}P$ , adding 1.0 ml of this to 9 ml  $F_{10}P$ , and adding 1.0 ml of

0054

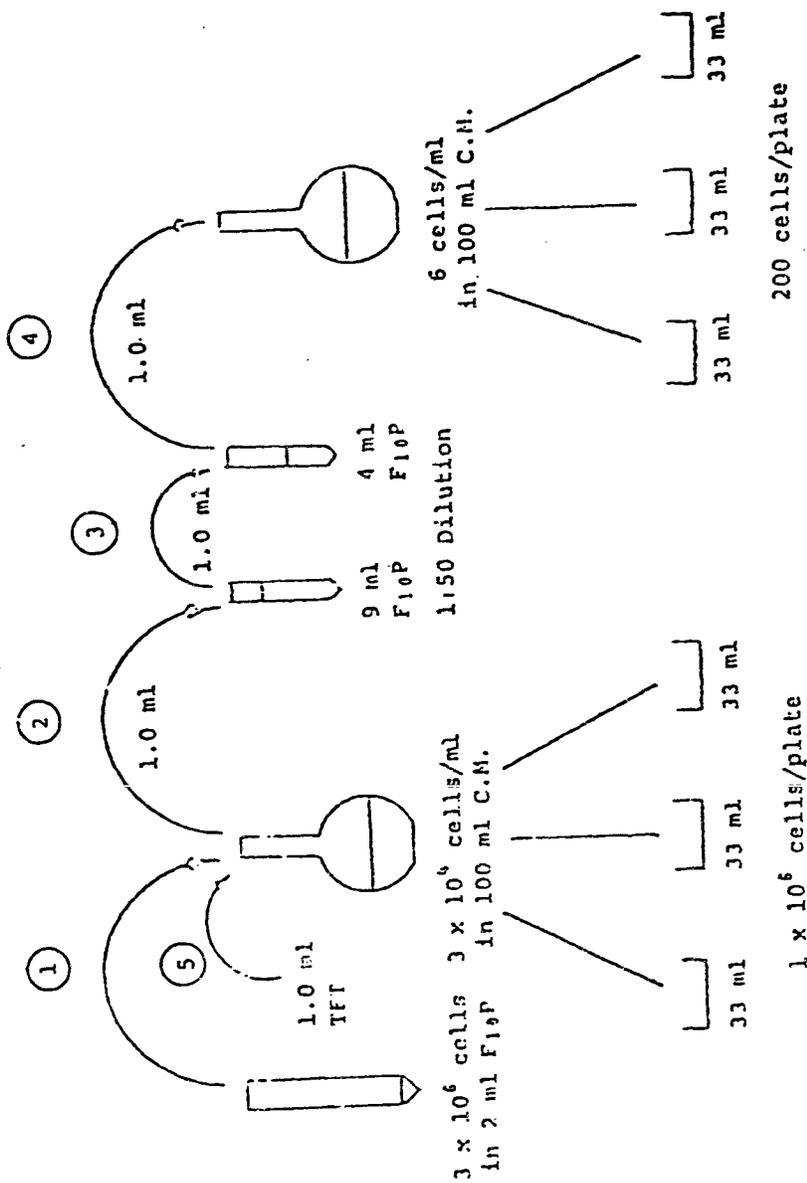


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

## 8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

- 8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.
- 8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

- 9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.
- 9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.
- 9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

- 10.1 \_\_\_\_\_ copies of the draft final report (with \_\_\_\_\_ copies of all raw data) will be sent to the Study Monitor within one month of termination. \_\_\_\_\_ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.
- 10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:
  - 10.2.1 The data from the toxicity test.
  - 10.2.2 The data generated by the mutagenesis assay which includes:
    - 10.2.2.1 The % total growth at each dose level which reflects test article toxicity.
    - 10.2.2.2 The number of TK<sup>-/-</sup> colonies per TFT plate for the test article and the controls.
    - 10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

### 11.0 RECORD AND TEST ARTICLE ARCHIVES

#### 11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

#### 11.2 Test Article

A Test Article sample will be held in storage in accordance with the Terms and Conditions.

### 12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? \_\_\_\_\_  
If so, to which agency or agencies? \_\_\_\_\_

Does the sponsor request that samples of the Test Article dosing solutions be returned? \_\_\_\_\_

### 13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: 11/17/82 PK 3/22/83

13.2 Proposed Initiation Date: December 20, 1982 PK 4/14/83

13.3 Scheduled Completion Date: February 18, 1983

13.4 Report Submission to Sponsor Date: February 18, 1983

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

*Carol Stark*

SPONSOR'S AUTHORIZED REPRESENTATIVE

*11/22/82*

DATE PROTOCOL APPROVED BY SPONSOR

*Paul E. Kirby*

STUDY DIRECTOR

*11/24/82*

DATE

0069

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F <sub>0</sub> P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
F <sub>10</sub> P	F <sub>0</sub> P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136)  
and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \frac{\text{Average Counts (corrected for coincidence)}}{\text{for coincidence}} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3 \times 10^6 \text{ cells/ml}) (20 \text{ ml})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = 20 \text{ ml} - \text{Volume of Cells Retained}$$

- 
2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay  
Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3 \times 10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\% \text{ of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than  $0.3 \times 10^5$  cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

- 
3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138)  
and L5178Y TK+/- Assay - Daily Counts (Positive Controls)  
(Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^5 = \frac{\text{Average Counts (corrected for coincidence)}}{\text{for coincidence}} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3 \times 10^6 \text{ Cells/ml}) (20 \text{ ml}^*)}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20 \text{ ml}^*) - (\text{Volume of Cells Retained})$$

\* The final volume is adjusted to 10 ml on the third day of expression.

4. Tables 2, 3, and 4 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^6 \text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left( \text{Mutant Frequency of Treated Cultures} \right) - \left( \text{Average Mutant Frequency of Solvent Controls} \right)$$

5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3 \times 10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\% \text{ Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than  $0.3 \times 10^6$  cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

$$\% \text{ Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\% \text{ Growth} = \frac{(\% \text{ Suspension Growth}) (\% \text{ Cloning Growth})}{100}$$

0073

Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-105 (MA #T1866) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 11% to 98% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 59% to 101% Total Growth.

The highest test article concentration cloned in the S-9 activated cultures exhibited a mutant frequency which was twice the mean mutant frequency of the solvent controls. None of the nonactivated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that under the conditions of this test, the test article CMA-105 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

## Results

The Initial Toxicity Test (Table 1) performed on test article CMA-105 (MA #T1866) in the absence of S-9 indicated a threshold level of complete toxicity at 0.05  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.05  $\mu\text{l/ml}$  to 0.00067  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, 0.0012, 0.00089 or 0.00067  $\mu\text{l/ml}$  test article. These concentrations produced a range in Suspension Growth of 21% to 98%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 11% to 98%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-105 (MA #T1866). The results indicated a threshold level of complete toxicity at 0.05  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05  $\mu\text{l/ml}$  to 0.00067  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.021, 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021 or 0.0016  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 68% to 97%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. The Cloning

Data and Total Compound Toxicity Data are also presented graphically in Figure 2. One culture (0.021  $\mu$ l/ml) that was cloned exhibited a mutant frequency which was 2.0 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 59%.

None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 78% to 101%.

Study No. T1866-701903

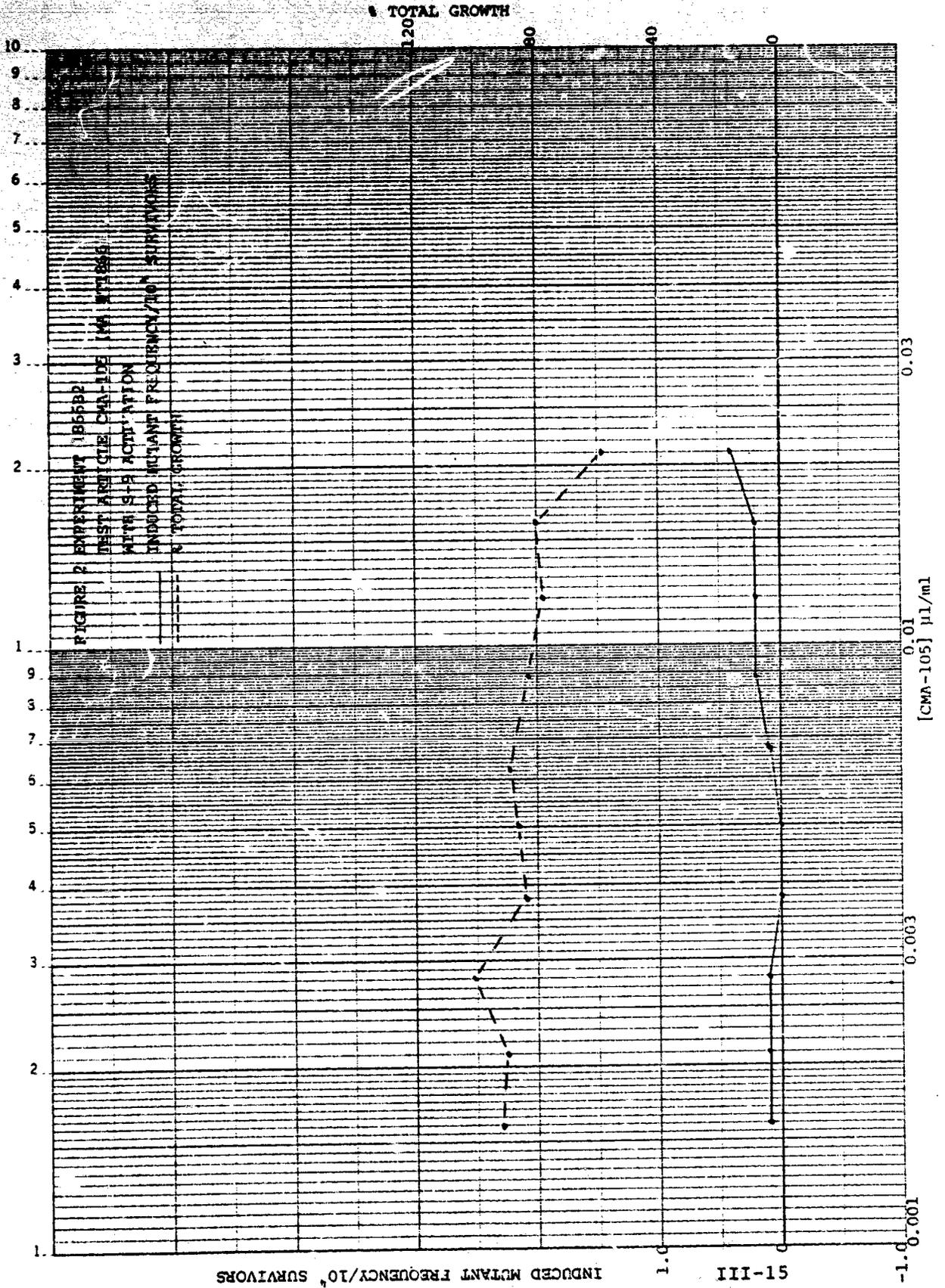
Table 1

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY  
INITIAL TOXICITY TEST

T1866-701006 Study Number      Paul F. Kirby Study Director      100<sup>μ</sup>l/d to 0.001<sup>μ</sup>l/d Dose Range  
CMF-105 (T1866) Test Article Identity      Acetone Solvent

	Test Article Concentration	Cell Concentration (x10 <sup>6</sup> )		Suspension Growth	
		Day 1	Day 2	Total	% of Control
Without Activation	100 <sup>μ</sup> l/d	0.807 *	0.191	0.0	0%
	10 <sup>μ</sup> l/d	0.440 *	0.093	0.0	0%
	5.0 <sup>μ</sup> l/d	0.132	0.161	0.0	0%
	1.0 <sup>μ</sup> l/d	0.009	0.009	0.0	0%
	0.5 <sup>μ</sup> l/d	0.009	-0.001	0.0	0%
	0.1 <sup>μ</sup> l/d	0.011	0.016	0.0	0%
	0.05 <sup>μ</sup> l/d	0.030	0.028	0.0	0%
	0.01 <sup>μ</sup> l/d	0.631	0.915	6.4	45%
	0.005 <sup>μ</sup> l/d	0.989	1.169	12.8	91%
	0.001 <sup>μ</sup> l/d	0.957	1.312	14.0	99%
	Solvent 1	0.902	1.283	13.7	14.1
	Solvent 2	1.004	1.289	14.4	
With S-9 Activation					
		Solvent 1			
	Solvent 2				

+ Culture Lost      \* observation showed no cells present 1-21-83  
 Table Prepared By Jean Blanche 1-21-83  
 Signature Jean Blanche Date  
 Form No. WL-233      Workbook Page No. 13      Report Page No. III-4  
 2/25/82



### Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-105 (MA #T1866) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest concentration cloned in the S-9 activated cultures exhibited a mutant frequency which was twice the mean mutant frequency of the solvent controls. None of the nonactivated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-105 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of exogenous metabolic activation.

APPENDIX

RECEIVED OCT 9 1982

Rec'd. by  
RA/SH 12/15/82  
N.K. /rc

L5178Y TK<sup>+</sup>/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK<sup>+</sup>/- Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-105

2.2 Analysis:  
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.  
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

III-18

SPGT701006 1087 1 of 16

5.1 TEST SYSTEM

L5178Y TK<sup>+</sup> mouse lymphoma cells are a sensitive indicator of mutagenic range of chemical classes.

5.1.1 Source

The L5178Y TK<sup>+</sup> mouse lymphoma cells used in the mutagenesis assay were directly from Dr. David Olive, a Calgary, Alberta, Canada, and the cells were cryopreserved and stored from reconstructed cells.

5.2 EXPERIMENTAL DESIGN

Refrigeration (optimal) equipment without metabolic activation to determine levels. Test material will then be tested activity without metabolic activation range (giving no more than 100 and no suspension growth) during this test the addition of three dose levels within the growth inhibition, with at least one dose then or equal to 10% total suspension test conditions produce positive results no further testing is to be done. If a positive or equivocal result is obtained the Sponsor is to be contacted. Data from the Sponsor, the test material(s) or equipment results will be tested without. Cloning data with activation for a minimum of three dose levels with to 10% total suspension growth, with a yielding total suspension growth of less than 10%.

Final change for assay without metabolic activation

Final change for assay with metabolic activation

6.1 Doses

For the initial toxicity test, cell deaths are recorded, viable test material is tested at 5,000, 1,000, 500, 100, 0.5, 0.1 and 0.05 mg/ml, and they will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05 and 0.01 mg/ml. Test materiality will be determined by population growth at each dose level.

SPGT701006 1087 1 of 16 III-18

## 5.0 TEST SYSTEM

L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:  
\$2,650

Total charge for assay with metabolic activation:  
\$2,650

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration  
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

### 6.3 Exogenous Metabolic Activation

#### 6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer  
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation  
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

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#### 6.3.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

#### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

#### 6.4 Controls

##### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

##### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

#### 7.0 METHODS

##### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

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Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5%  $\text{CO}_2$  in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at  $1000 \times g$  for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of  $\text{F}_{10}\text{P}$ , resuspended in 20 ml of  $\text{F}_{10}\text{P}$ , gassed with 5%  $\text{CO}_2$  in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at  $37^\circ\text{C}$  for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, LS178Y TK<sup>+</sup>/<sup>-</sup> cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring TK<sup>-</sup>/<sup>-</sup> cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5%  $\text{CO}_2$  in air and placed on an environmental incubator shaker at 125 rpm and  $37^\circ\text{C}$ . After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of  $\text{F}_{10}\text{P}$  and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of  $\text{F}_{10}\text{P}$  plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

#### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F<sub>10</sub>P will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>-/-</sup> colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

#### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 1.0 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

#### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the  $TK^{-/-}$  cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of  $TK^{-/-}$  cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^6$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at  $1000 \times g$  for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of  $F_{10}P$ , adding 1.0 ml of this to 9 ml  $F_{10}P$ , and adding 1.0 ml of

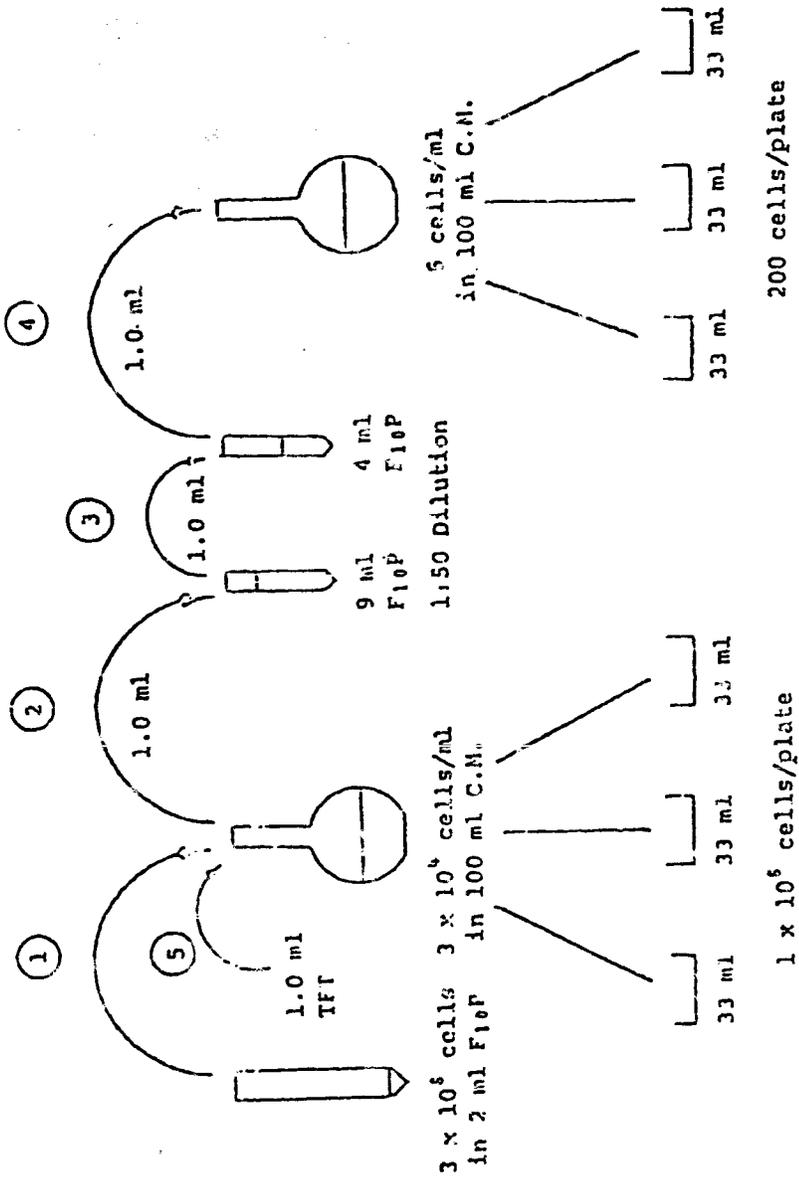


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

#### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

### 8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

- 8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

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8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

#### 9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

#### 10.0 FINAL REPORT

10.1 \_\_\_\_\_ copies of the draft final report (with \_\_\_\_\_ copies of all raw data) will be sent to the Study Monitor within one month of termination. \_\_\_\_\_ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of TK<sup>-/-</sup> colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article

A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? \_\_\_\_\_  
If so, to which agency or agencies? \_\_\_\_\_

Does the sponsor request that samples of the Test Article dosing solutions be returned? \_\_\_\_\_

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: 12/10/82

13.2 Proposed Initiation Date: 1/18/83

13.3 Scheduled Completion Date: 2/18/83

13.4 Report Submission to Sponsor Date: 2/18/83

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

*Carol Stark*

\_\_\_\_\_  
SPONSOR'S AUTHORIZED REPRESENTATIVE

*11/22/82*

\_\_\_\_\_  
DATE PROTOCOL APPROVED BY SPONSOR

*Paul E. Kiehl*

\_\_\_\_\_  
STUDY DIRECTOR

*11/24/82*  
\_\_\_\_\_  
DATE

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F <sub>0</sub> P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
F <sub>10</sub> P	F <sub>0</sub> P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE  
L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136)  
and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \frac{\text{Average Counts (corrected for coincidence)}}{\text{for coincidence}} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3 \times 10^6 \text{ cells/ml}) (20 \text{ ml})}{\text{(No. of Cells/ml)}}$$

$$\text{Volume of Media to Add} = 20 \text{ ml} - \text{Volume of Cells Retained}$$

- 
2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay  
Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3 \times 10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\% \text{ of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than  $0.3 \times 10^6$  cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

- 
3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138)  
and L5178Y TK+/- Assay - Daily Counts (Positive Controls)  
(Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^6 = \frac{\text{Average Counts (corrected for coincidence)}}{\text{for coincidence}} \times 20$$

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3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3 \times 10^5 \text{ Cells/ml}) (20 \text{ ml}^*)}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20 \text{ ml}^*) - (\text{Volume of Cells Retained})$$

\* The final volume is adjusted to 10 ml on the third day of expression.

- 
4. Tables 2, 3, and 4 - LS178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^5 \text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left( \text{Mutant Frequency of Treated Cultures} \right) - \left( \text{Average Mutant Frequency of Solvent Controls} \right)$$

- 
5. Tables 5, 6, and 7 - LS178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3 \times 10^5 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\% \text{ Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than  $0.3 \times 10^5$  cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

$$\% \text{ Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\% \text{ Growth} = \frac{(\% \text{ Suspension Growth}) (\% \text{ Cloning Growth})}{100}$$

### Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-106 (MA #T1854) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures (Experiment 1854B2) were cloned over a range of test article concentrations which produced from 6% to 101% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 1% to 88% Total Growth.

The highest test article concentration cloned in the non-activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. The result is not considered significant since mutant frequencies observed at such highly toxic levels may be due to epigenetic events. Four S-9 activated cultures exhibited mutant frequencies which were significantly different from the mean mutant frequency of the solvent controls.

The results indicate that under the conditions of this test, the test article CMA-106 produced a negative response in the absence of exogenous metabolic activation and a positive response in the presence of exogenous metabolic activation.

## Results

The Initial Toxicity Test (Table 1) performed on test article CMA-106 (MA #T1854) in the absence of S-9 indicated a threshold level of complete toxicity at 0.05  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.01  $\mu\text{l/ml}$  to 0.00013  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.01, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0016, 0.0010, 0.00075 or 0.00056  $\mu\text{l/ml}$  test article. These concentrations produced a range in Suspension Growth of 2% to 109%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 17% to 120%. However, the toxic response at the higher doses was erratic and an acceptable range in toxicity was not achieved. Therefore the assay was repeated over a range of concentrations from 0.05  $\mu\text{l/ml}$  to 0.00067  $\mu\text{l/ml}$ .

After a two day expression period, nine cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, 0.0012, 0.00089 or 0.00067  $\mu\text{l/ml}$  test article. These concentrations produced a range in Suspension Growth of 13% to 100%. The Cloning Data are presented in Tables 6 and 7 and the Total Compound Toxicity Data are presented in Tables 8 and 9. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2.

One culture that was cloned exhibited a mutant frequency which was 2.3 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 22% to 101%.

An Initial Toxicity Test (Table 10) was conducted in the presence of S-9 on test article CMA-106 (MA #T1854). The results indicated a threshold level of complete toxicity at 0.05  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05  $\mu\text{l/ml}$  to 0.005  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.031, 0.024, 0.018, 0.011 or 0.005  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 6% to 99%. The Cloning Data are presented in Tables 11 and 12 and the Total Compound Toxicity Data are presented in Tables 13 and 14. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 3. Cultures treated with 0.031, 0.024, or 0.018  $\mu\text{l/ml}$  test article exhibited mutant frequencies which ranged from 16.3 to 2.0 times the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 1% to 72%.

None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 78% to 88%.

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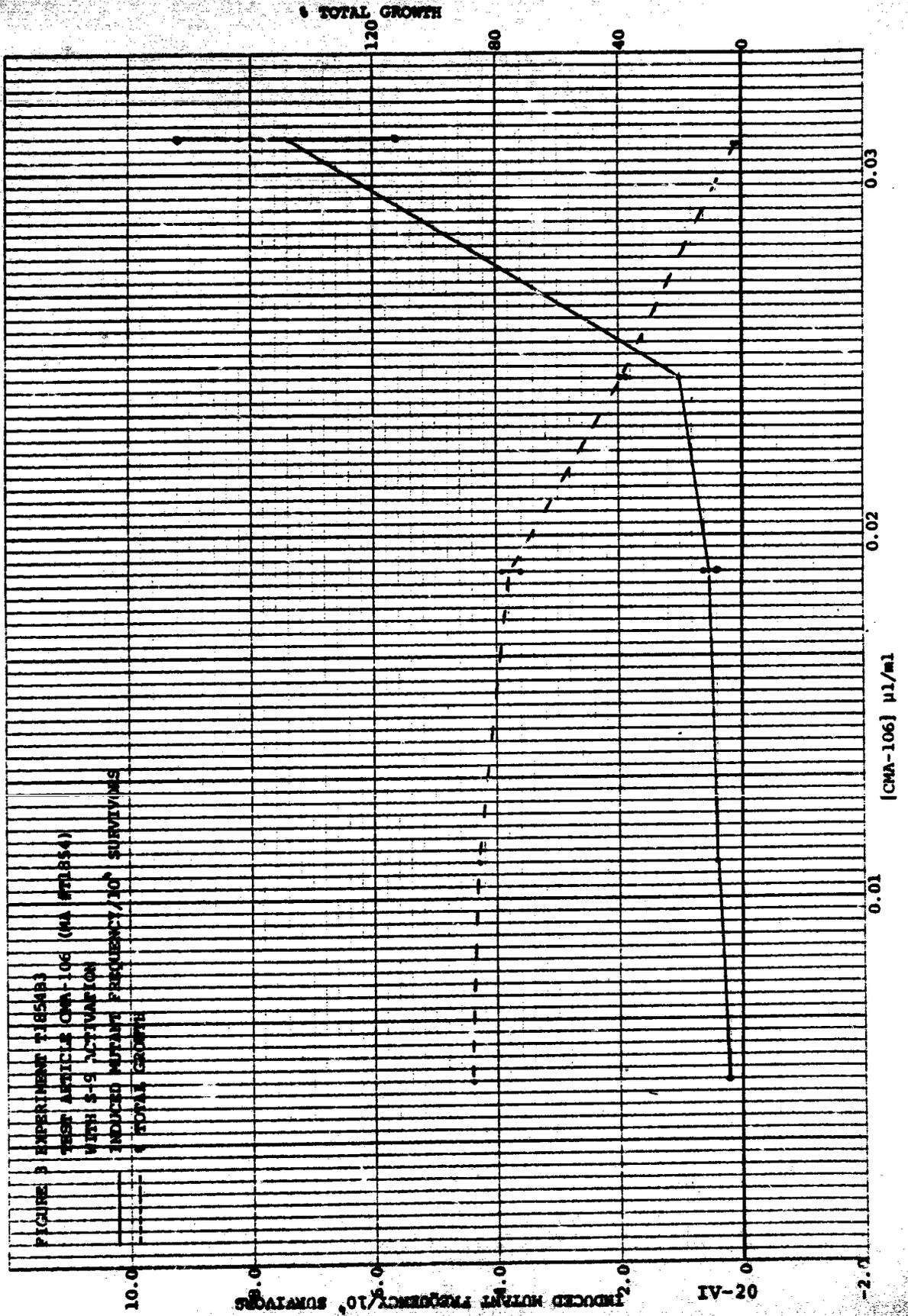
K-2 10 X 10 TO THE INCMO / A IN MUMS  
KUB FEL & SHER CO MADE IN U.S.A.

FIGURE 3 EXPERIMENT T1654B3  
TEST ARTICLE CMA-106 (MA #71854)

WITH S-9 ACTIVATION

INDUCED MUTANT FREQUENCY/ $10^6$  SURVIVINGS

○ TOTAL GROWTH



5-2-68

### Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-106 (MA #T1854) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest concentration cloned in the nonactivated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. The result is not considered significant since mutant frequencies observed at such highly toxic doses may be due to epigenetic events. Four S-9 activated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-106 produced a negative response in the absence of exogenous metabolic activation and a positive response in the presence of exogenous metabolic activation.

APPENDIX

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*Rec'd by  
RA/QA 10/1/82  
N.K./jpc*

L5178Y TK<sup>+/-</sup> MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK<sup>+/-</sup> Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-106

2.2 Analysis:

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.  
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

1129

## 5.0 TEST SYSTEM

L5178Y TK<sup>+</sup>/<sub>-</sub> mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The L5178Y TK<sup>+</sup>/<sub>-</sub> mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:  
\$2,650

Total charge for assay with metabolic activation:  
\$2,650

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

#### 6.2 Route and Frequency of Administration

Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

#### 6.3 Exogenous Metabolic Activation

##### 6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

###### 6.3.1.1 Species, Strain, Sex and Inducer

Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

###### 6.3.1.2 Homogenate Preparation

Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

#### 6.3.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

#### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

#### 6.4 Controls

##### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

##### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

#### 7.0 METHODS

##### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5%  $\text{CO}_2$  in air and placed on a Bellico roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of  $\text{F}_{10}\text{P}$ , resuspended in 20 ml of  $\text{F}_{10}\text{P}$ , gassed with 5%  $\text{CO}_2$  in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at  $37^\circ\text{C}$  for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, L5178Y TK<sup>+</sup>/<sub>-</sub> cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring TK<sup>-</sup>/<sub>-</sub> cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5%  $\text{CO}_2$  in air and placed on an environmental incubator shaker at 125 rpm and  $37^\circ\text{C}$ . After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of  $\text{F}_{10}\text{P}$  and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of  $\text{F}_{10}\text{P}$  plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

#### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F<sub>0</sub>P will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>-/-</sup> colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

#### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

#### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the TK<sup>-/-</sup> cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of TK<sup>-/-</sup> cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^5$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of F<sub>10</sub>P, adding 1.0 ml of this to 9 ml F<sub>10</sub>P, and adding 1.0 ml of

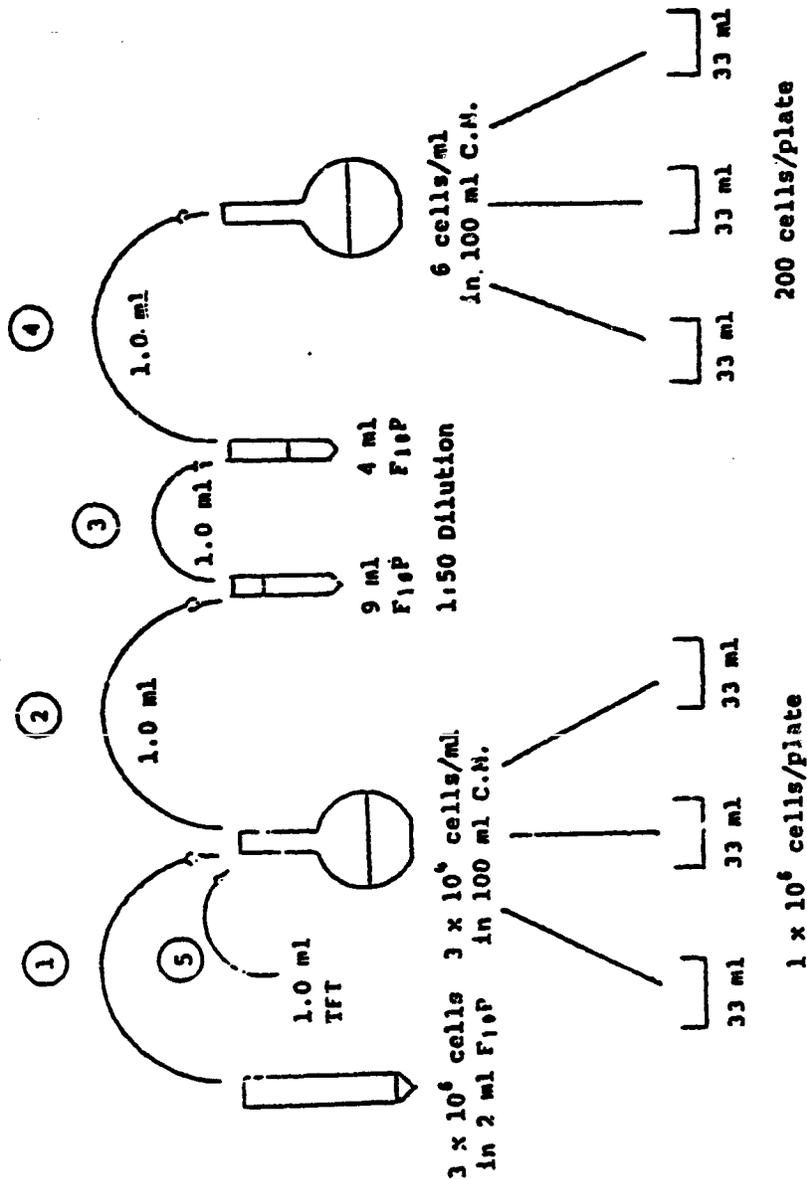


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

#### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

### 8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

- 8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

- 8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.
- 8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

#### 9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

- 9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.
- 9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.
- 9.3 The plating efficiency of the solvent controls must be greater than 50%.

#### 10.0 FINAL REPORT

- 10.1 \_\_\_\_\_ copies of the draft final report (with \_\_\_\_\_ copies of all raw data) will be sent to the Study Monitor within one month of termination. \_\_\_\_\_ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.
- 10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:
- 10.2.1 The data from the toxicity test.
- 10.2.2 The data generated by the mutagenesis assay which includes:
- 10.2.2.1 The % total growth at each dose level which reflects test article toxicity.
- 10.2.2.2 The number of TK<sup>-/-</sup> colonies per TFT plate for the test article and the controls.
- 10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

#### 11.0 RECORD AND TEST ARTICLE ARCHIVES

##### 11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

##### 11.2 Test Article

A Test Article sample will be held in storage in accordance with the Terms and Conditions.

#### 12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? \_\_\_\_\_  
If so, to which agency or agencies? \_\_\_\_\_

Does the sponsor request that samples of the Test Article dosing solutions be returned? \_\_\_\_\_

#### 13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: ~~11/24/82~~ <sup>1/11/83</sup> <sup>OK 4/20/83</sup>

13.2 Proposed Initiation Date: December 20, 1982

13.3 Scheduled Completion Date: February 11, 1983

13.4 Report Submission to Sponsor Date: February 11, 1983

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

*Carol Shank*

\_\_\_\_\_  
SPONSOR'S AUTHORIZED REPRESENTATIVE

*11/24/82*

\_\_\_\_\_  
DATE PROTOCOL APPROVED BY SPONSOR

*Paul E. Kirby*

\_\_\_\_\_  
STUDY DIRECTOR

*11/24/82*  
\_\_\_\_\_  
DATE

APPENDIX I

Abbreviations Used in the LS178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F <sub>0</sub> P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
F <sub>10</sub> P	F <sub>0</sub> P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

3. (Cont'd.)

$$\% \text{ Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\% \text{ Growth} = \frac{(\% \text{ Suspension Growth}) + (\% \text{ Cloning Growth})}{100}$$

0 1 4 4

Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-107 (MA #T1857) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 47% to 123% Total Growth in one assay and from 2% to 97% Total Growth in a second assay. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 8% to 95% Total Growth.

One nonactivated culture having less than 10% Total Growth exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant due to the high toxicity observed. Five S-9 activated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that under the conditions of this test, test article CMA-107 produced a negative response in the absence of exogenous metabolic activation and a positive response in the presence of metabolic activation.

## Results

The Initial Toxicity Test (Table 1) performed on test article CMA-107 (MA #T1857) in the absence of S-9 indicated a threshold level of complete toxicity at 0.1 ml/ml. Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.01  $\mu$ l/ml to 0.00013  $\mu$ l/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018, 0.0013, 0.0010 or 0.00075  $\mu$ l/ml test article. These concentrations produced a range in Suspension Growth of 47% to 105%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 47% to 123%. However, the toxicity observed in the treated cultures did not cover the optimum range (10% to 90% Total Growth). The assay was repeated over a concentration range of 0.1  $\mu$ l/ml to 0.0013  $\mu$ l/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.024, 0.018, 0.013, 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024 or 0.0018  $\mu$ l/ml test article. These concentrations produced a range in Suspension Growth of 8% to 96%. The Cloning Data are presented in Tables 6 and 7 and the Total Compound Toxicity Data are presented in Tables 8 and 9. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2.

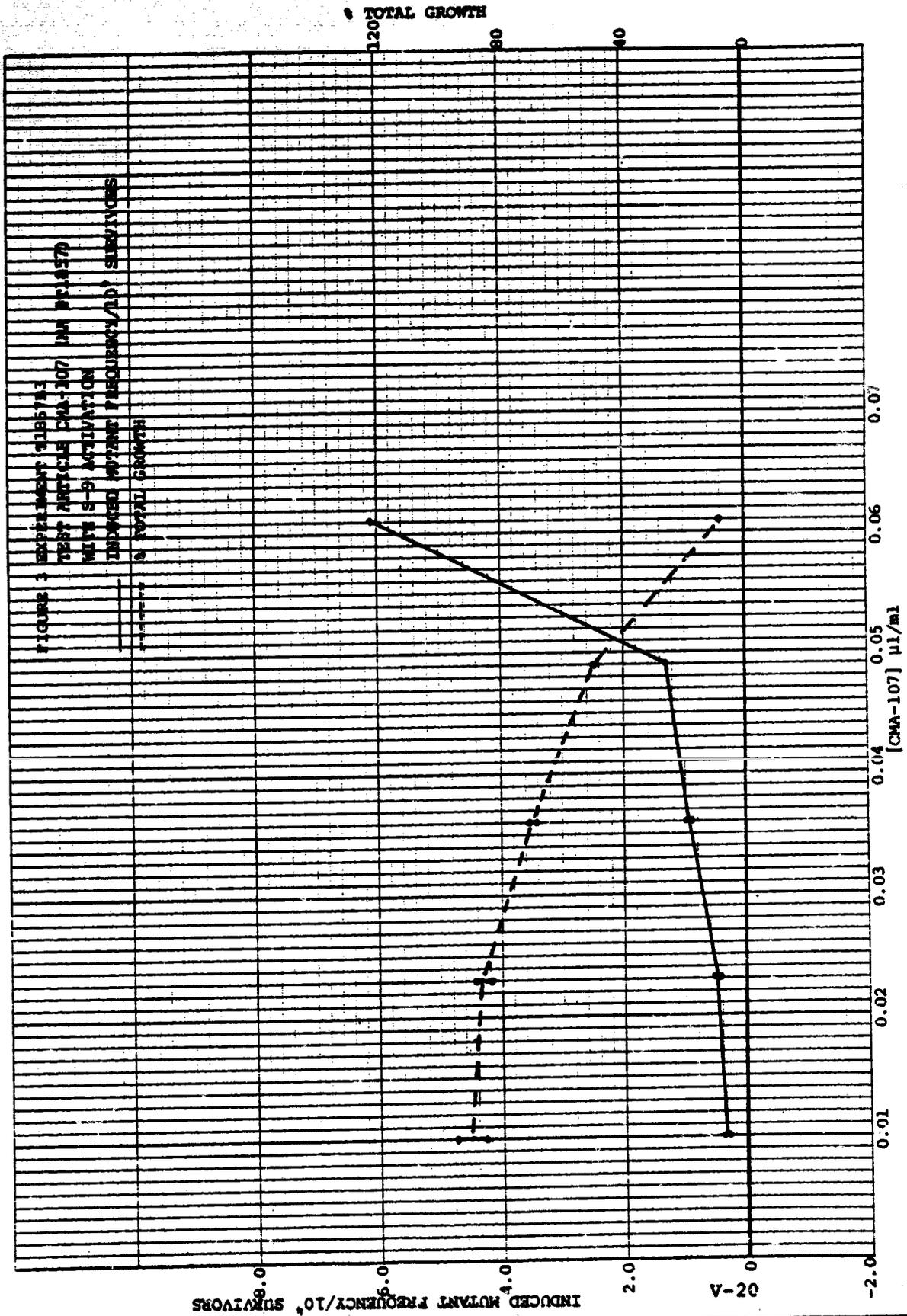
One of the cultures that were cloned (0.024  $\mu$ l/ml) exhibited a mutant frequency which was 2.8 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 2%.

None of the remaining cultures that were cloned exhibited mutant frequencies that were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 19% to 97%.

An Initial Toxicity Test (Table 10) was conducted in the presence of S-9 on test article CMA-107 (MA #T1857). The results indicated a threshold level of complete toxicity at 0.1  $\mu$ l/ml. Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.1  $\mu$ l/ml to 0.01  $\mu$ l/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.061, 0.049, 0.036, 0.023 or 0.01  $\mu$ l/ml. These concentrations produced a range in Suspension Growth of 17% to 94%. The Cloning Data are presented in Tables 11 and 12 and the Total Compound Toxicity Data are presented in Tables 13 and 14. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 3.

Five of the cultures that were cloned (0.061, 0.049, 0.036, 0.023  $\mu$ l/ml) exhibited mutant frequencies which were 13.2 to 2.0 times the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 8% to 84%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 86% to 95%.



### Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-107 (MA #T1857) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. One nonactivated culture having less than 10% Total Growth exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant due to the high toxicity observed. Five S-9 activated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-107 produced a negative response in the absence of exogenous metabolic activation and a positive response in the presence of metabolic activation.

APPENDIX

RECEIVED OCT 05 1982

*Rec'd. by  
RA/DA 12/1/82.  
N.K./JRC*

L5178Y TK<sup>+/-</sup> MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK<sup>+/-</sup> Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-107

2.2 Analysis:

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.  
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

## 5.0 TEST SYSTEM

L5178Y TK<sup>+</sup>/- mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The L5178Y TK<sup>+</sup>/- mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:  
\$2,650

Total charge for assay with metabolic activation:  
\$2,650

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration  
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

### 6.3 Exogenous Metabolic Activation

#### 6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer  
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation  
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

#### 6.3.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

#### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

#### 6.4 Controls

##### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

##### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

### 7.0 METHODS

#### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5%  $\text{CO}_2$  in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at  $1000 \times g$  for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of  $\text{F}_{10}\text{P}$ , resuspended in 20 ml of  $\text{F}_{10}\text{P}$ , gassed with 5%  $\text{CO}_2$  in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at  $37^\circ\text{C}$  for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, L5178Y TK<sup>+</sup>/<sup>-</sup> cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring TK<sup>-</sup>/<sup>-</sup> cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5%  $\text{CO}_2$  in air and placed on an environmental incubator shaker at 125 rpm and  $37^\circ\text{C}$ . After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of  $\text{F}_{10}\text{P}$  and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of  $\text{F}_{10}\text{P}$  plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

#### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F<sub>0</sub>P will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>-/-</sup> colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

#### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

#### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the TK<sup>-/-</sup> cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of TK<sup>-/-</sup> cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^6$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of F<sub>10</sub>P, adding 1.0 ml of this to 9 ml F<sub>10</sub>P, and adding 1.0 ml of

0 1 7 3

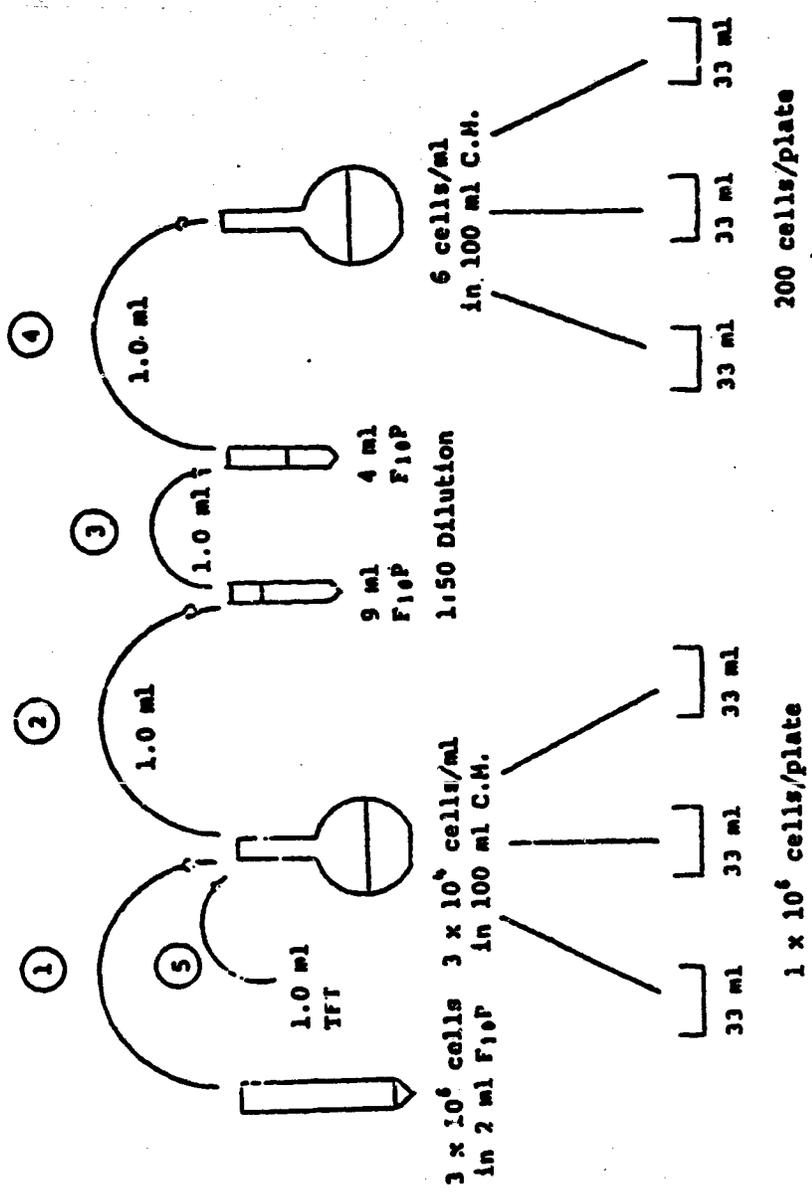


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

## 8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

- 8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.
- 8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

#### 9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

- 9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.
- 9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.
- 9.3 The plating efficiency of the solvent controls must be greater than 50%.

#### 10.0 FINAL REPORT

- 10.1        copies of the draft final report (with        copies of all raw data) will be sent to the Study Monitor within one month of termination.        copies of the final report will be sent after the draft report has been reviewed by the Sponsor.
- 10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:
- 10.2.1 The data from the toxicity test.
- 10.2.2 The data generated by the mutagenesis assay which includes:
- 10.2.2.1 The % total growth at each dose level which reflects test article toxicity.
- 10.2.2.2 The number of TK<sup>-/-</sup> colonies per TFT plate for the test article and the controls.
- 10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

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10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

#### 11.0 RECORD AND TEST ARTICLE ARCHIVES

##### 11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

##### 11.2 Test Article

A Test Article sample will be held in storage in accordance with the Terms and Conditions.

#### 12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? \_\_\_\_\_  
If so, to which agency or agencies? \_\_\_\_\_

Does the sponsor request that samples of the Test Article dosing solutions be returned? \_\_\_\_\_

#### 13.0 SCHEDULE OF EVENTS

- 13.1 Test Material Received Date: *11/24/82*
- 13.2 Proposed Initiation Date: *January 4, 1983*
- 13.3 Scheduled Completion Date: *February 25, 1983*
- 13.4 Report Submission to Sponsor Date: *February 25, 1983*

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

*Carol Hunt*

\_\_\_\_\_  
SPONSOR'S AUTHORIZED REPRESENTATIVE

*11/24/82*

\_\_\_\_\_  
DATE PROTOCOL APPROVED BY SPONSOR

*Paul E. Kirby*

\_\_\_\_\_  
STUDY DIRECTOR

*11/24/82*  
\_\_\_\_\_  
DATE

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APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F <sub>0</sub> P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
F <sub>10</sub> P	F <sub>0</sub> P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
TMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Coun.

0-1-79

APPENDIX II  
FORMULAS AND CALCULATIONS FOR THE

LS178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136)  
and Final Counts (Form No. WL-137).

$$\begin{aligned} \text{No. of Cells/ml} &= \frac{\text{Average Counts (corrected for coincidence)}}{\text{Volume of Cells to Retain}} \times 20 \\ \text{Volume of Cells to Retain} &= \frac{(0.3 \times 10^6 \text{ cells/ml}) (20 \text{ ml})}{(\text{No. of Cells/ml})} \\ \text{Volume of Media to Add} &= 20 \text{ ml} - \text{Volume of Cells Retained} \end{aligned}$$

2. Table 1 - LS178Y TK+/- Mouse Lymphoma Mutagenesis Assay  
Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3 \times 10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\% \text{ of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than  $0.3 \times 10^6$  cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

3. LS178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138)  
and LS178Y TK+/- Assay - Daily Counts (Positive Controls)  
(Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^6 = \frac{\text{Average Counts (corrected for coincidence)}}{\text{Volume of Cells to Retain}} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3 \times 10^6 \text{ Cells/ml}) (20 \text{ ml}^*)}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20 \text{ ml}^*) - (\text{Volume of Cells Retained})$$

\* The final volume is adjusted to 10 ml on the third day of expression.

4. Tables 2, 3, and 4 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^4 \text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left( \frac{\text{Mutant Frequency of Treated Cultures}}{\text{of Treated Cultures}} \right) - \left( \frac{\text{Average Mutant Frequency of Solvent Controls}}{\text{of Solvent Controls}} \right)$$

5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3 \times 10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\% \text{ Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than  $0.3 \times 10^6$  cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

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3. (Cont'd.)

$$\% \text{ Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\% \text{ Growth} = \frac{(\% \text{ Suspension Growth}) (\% \text{ Cloning Growth})}{100}$$

### Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-108 (MA #T1852) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 6% to 105% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 12% to 103% Total Growth (Experiment 1852B4).

The highest test article concentration cloned in the non-activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. The result is not considered significant since mutant frequencies observed at such highly toxic levels may be due to epigenetic events. One S-9 activated culture having greater than 10% Total Growth exhibited a mutant frequency which was significantly different from the mean mutant frequency of the solvent controls.

The results indicate that under the conditions of this test, the test article CMA-108 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

## Results

The Initial Toxicity Test (Table 1) performed on test article CMA-108 (MA #T1852) in the absence of S-9 indicated a threshold level of complete toxicity at 0.1  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.1  $\mu\text{l/ml}$  to 0.0013  $\mu\text{l/ml}$ .

After a two day expression period, seven cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018 or 0.0013  $\mu\text{l/ml}$  test article. These concentrations produced a range in Suspension Growth of 12% to 100%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

One of the cultures that was cloned (0.0075  $\mu\text{l/ml}$ ) exhibited a mutant frequency which was 3.8 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 30% to 105%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-108 (MA #T1852). The results indicated a threshold level of complete toxicity at 0.05  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05  $\mu\text{l/ml}$  to 0.00067  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016 or 0.0012  $\mu\text{l/ml}$ . These concentrations

produced a range in Suspension Growth of 67% to 100%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 70% to 109%.

The positive control cultures of the assay described above were lost to contamination. Therefore, the assay was repeated in the presence of S-9 over a range of concentrations from 0.05  $\mu\text{l/ml}$  to 0.00067  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. These cultures were treated with 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, 0.0012 or 0.00089  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 17% to 106%. The Cloning Data are presented in Tables 11 and 12 and the Total Compound Toxicity Data are presented in Tables 13 and 14. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 3.

One culture (0.012  $\mu\text{l/ml}$ ) that was cloned exhibited a mutant frequency that was 3.4 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 12%. None of the remaining cultures that were cloned exhibited mutant frequencies that were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 69% to 103%.



### Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-108 (MA #T1852) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest concentration cloned in the nonactivated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. The Total Growth of this culture was 63. The result is not considered significant since mutant frequencies observed at such highly toxic doses may be due to epigenetic events. One S-9 activated culture that was cloned exhibited a mutant frequency which was significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-108 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of exogenous metabolic activation.

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*Rec'd by  
RA/DA 12/18/82.  
N.K./RC*

LS178Y TK<sup>+/-</sup> MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the LS178Y TK<sup>+/-</sup> Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-108

2.2 Analysis:

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.  
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

VI-22

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## 5.0 TEST SYSTEM

15178V TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The 15178V TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells Clone 3.7.2C used in the mutagenicity assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:  
\$2,650

Total charge for assay with metabolic activation:  
\$2,650

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

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the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

**6.2 Route and Frequency of Administration**  
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

**6.3 Exogenous Metabolic Activation**

**6.3.1 Liver Microsomal Enzymes - S-9 Homogenate**

**6.3.1.1 Species, Strain, Sex and Inducer**  
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

**6.3.1.2 Homogenate Preparation**  
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

#### 6.3.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the pro-mutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

#### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

#### 6.4 Controls

##### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

##### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for pro-mutagens.

### 7.0 METHODS

#### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5%  $\text{CO}_2$  in air and placed on a Bellico roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at  $1000 \times g$  for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of  $\text{F}_{10}\text{P}$ , resuspended in 20 ml of  $\text{F}_{10}\text{P}$ , gassed with 5%  $\text{CO}_2$  in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at  $37^\circ\text{C}$  for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, LS178Y  $\text{TK}^{+/-}$  cells which are actively growing in culture will be cleaned as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring  $\text{TK}^{-/-}$  cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5%  $\text{CO}_2$  in air and placed on an environmental incubator shaker at 125 rpm and  $37^\circ\text{C}$ . After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of  $\text{F}_{10}\text{P}$  and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of  $\text{F}_{10}\text{P}$  plus 1 ml of THG stock solution.

00008

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

#### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F<sub>0</sub>P will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>+</sup> colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

#### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

#### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the TK<sup>-/-</sup> cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of TK<sup>-/-</sup> cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^6$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of F<sub>10</sub>P, adding 1.0 ml of this to 9 ml F<sub>10</sub>P, and adding 1.0 ml of

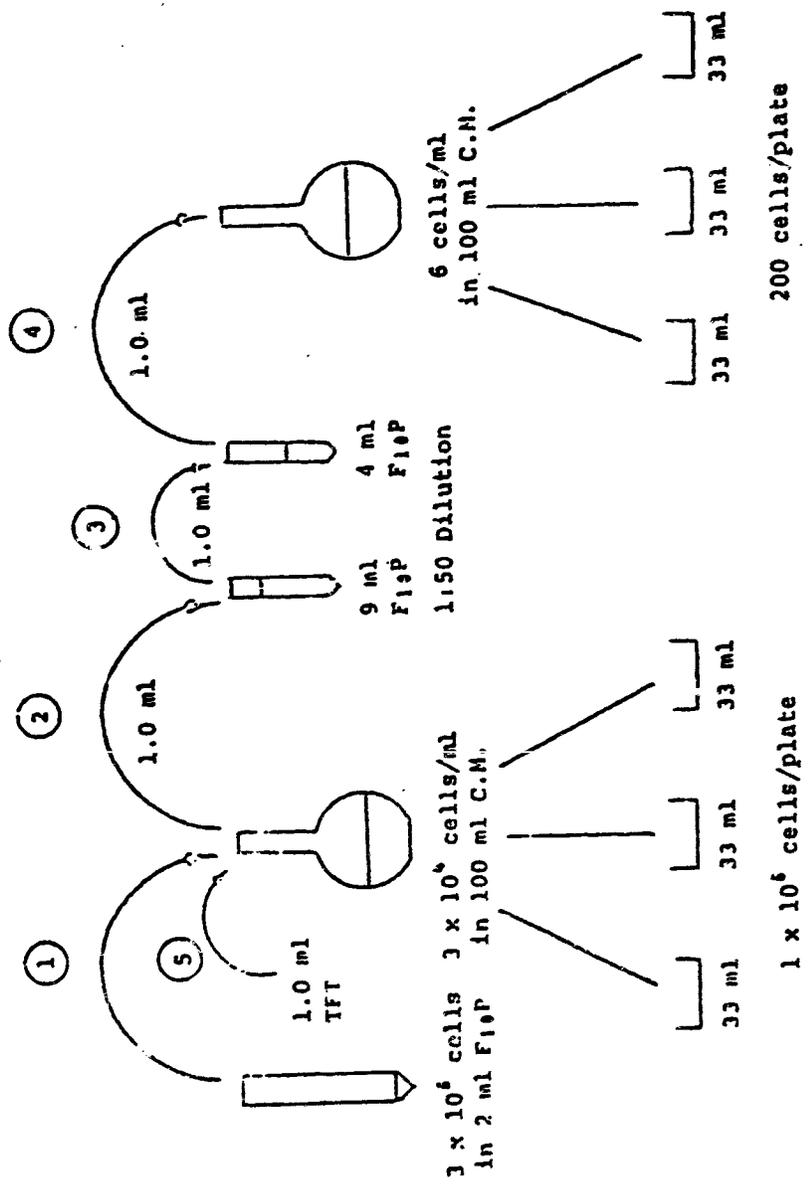


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

## 8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

- 8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.
- 8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

#### 9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

- 9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.
- 9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.
- 9.3 The plating efficiency of the solvent controls must be greater than 50%.

#### 10.0 FINAL REPORT

- 10.1 \_\_\_\_\_ copies of the draft final report (with \_\_\_\_\_ copies of all raw data) will be sent to the Study Monitor within one month of termination. \_\_\_\_\_ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.
- 10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:
- 10.2.1 The data from the toxicity test.
- 10.2.2 The data generated by the mutagenesis assay which includes:
- 10.2.2.1 The % total growth at each dose level which reflects test article toxicity.
- 10.2.2.2 The number of TK<sup>-/-</sup> colonies per TFT plate for the test article and the controls.
- 10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article

A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? \_\_\_\_\_  
If so, to which agency or agencies? \_\_\_\_\_

Does the sponsor request that samples of the Test Article dosing solutions be returned? \_\_\_\_\_

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: *11/24/82*

13.2 Proposed Initiation Date: *December 16, 1982*

13.3 Scheduled Completion Date: *January 29, 1983*

13.4 Report Submission to Sponsor Date: *January 29, 1983*

0214

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

*Carol Stuck*

\_\_\_\_\_  
SPONSOR'S AUTHORIZED REPRESENTATIVE

*11/24/82*

\_\_\_\_\_  
DATE PROTOCOL APPROVED BY SPONSOR

*Paul E. Kirby*

\_\_\_\_\_  
STUDY DIRECTOR

*11/24/82*

\_\_\_\_\_  
DATE

00215

### Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-109 (MA #T1855) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 2% to 92% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 6% to 125% Total Growth.

The highest test article concentration cloned in the S-9 activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. Two of the nonactivated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. These results are not considered significant as the Total Growth of these cultures was less than 10%. TFR resistance observed at these highly toxic levels may be due to epigenetic events.

The results indicate that under the conditions of this test, test article CMA-109 produced a negative response in the presence and absence of exogenous metabolic activation. In the presence of metabolic activation, the Total Growth of the treated cultures that were cloned did not cover the critical range of survival (10-40%). A precipitous toxic response was induced by the test article. The cultures treated with the two highest concentrations of test article had 6% and 77% Total Growth. It was felt that a repeat assay would not provide any additional information since the difference in concentration between the cultures having 6% and 77% Total Growth was only 0.006  $\mu$ l/ml.

## Results

The Initial Toxicity Test (Table 1) performed on test article CMA-109 (MA #T1855) in the absence of S-9 indicated a threshold level of complete toxicity at 0.1  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.1  $\mu\text{l/ml}$  to 0.0013  $\mu\text{l/ml}$ .

After a two day expression period, nine cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.013, 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018 or 0.0013  $\mu\text{l/ml}$  test article. These concentrations produced a range in Suspension Growth of 11% to 96%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

Two of the cultures (0.013 and 0.010  $\mu\text{l/ml}$ ) that were cloned exhibited mutant frequencies which were 10.8 and 2.5 times, respectively, the mean mutant frequency of the solvent controls. The Total Growth of these cultures were 2% and 4%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly different from the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 20% to 92%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-109 (MA #T1855). The results indicated a threshold level of complete toxicity at 0.05  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.1  $\mu\text{l/ml}$  to 0.0013  $\mu\text{l/ml}$ .\*

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\*Three S-9 activated assays were performed. Two of the assays were lost to contamination. The results of the third assay are reported here.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.024, 0.018, 0.013, 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024 or 0.0018  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 11% to 82%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2. One culture (0.024  $\mu\text{l/ml}$ ) that was cloned exhibited a mutant frequency which was 7.2 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%.

None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 77% to 125%.

Study No. T1855 701006

**Table 1**

**701 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY  
INITIAL COMPOUND TOXICITY TEST**

T1855 701006  
Study Number

Paul E. Kirby  
Study Director

100 cells to 0.001 cells  
Dose Range

CMA 100 (T1855)  
Test Article Identity

Acetone  
Solvent

	Test Article Concentration	Cell Concentration (X 10 <sup>6</sup> )		Suspension Growth	
		Day 1	Day 2	Total	% of Control
Without Activation	100 cells/ml	1.995	*		
	10 cells/ml	0.465	*		
	1.0 cells/ml	0.068	*		
	0.1 cells/ml	0.047	0.140	0.0	0%
	0.01 cells/ml	0.508	0.823	4.6	80%
	0.001 cells/ml	1.029	1.177	7.5**	129%
	Solvent 1	1.009	1.148	7.6**	5.8
Solvent 2	0.927	0.621	4.1**		
With S-9 Activation					
	Solvent 1				
	Solvent 2				

+ Culture Lost

Table Prepared By: Lucretia M. Rogers - Bueck 1/13/83  
Signature Date

\* Cultures not counted on the 2nd day.  
dkr KB 1/13/83

\*\* Values take into account incorrect adjustment on day 1. AMKB 1/13/83

Workbook Page No. 13 Report Page No. VII-4

0 2 2 3

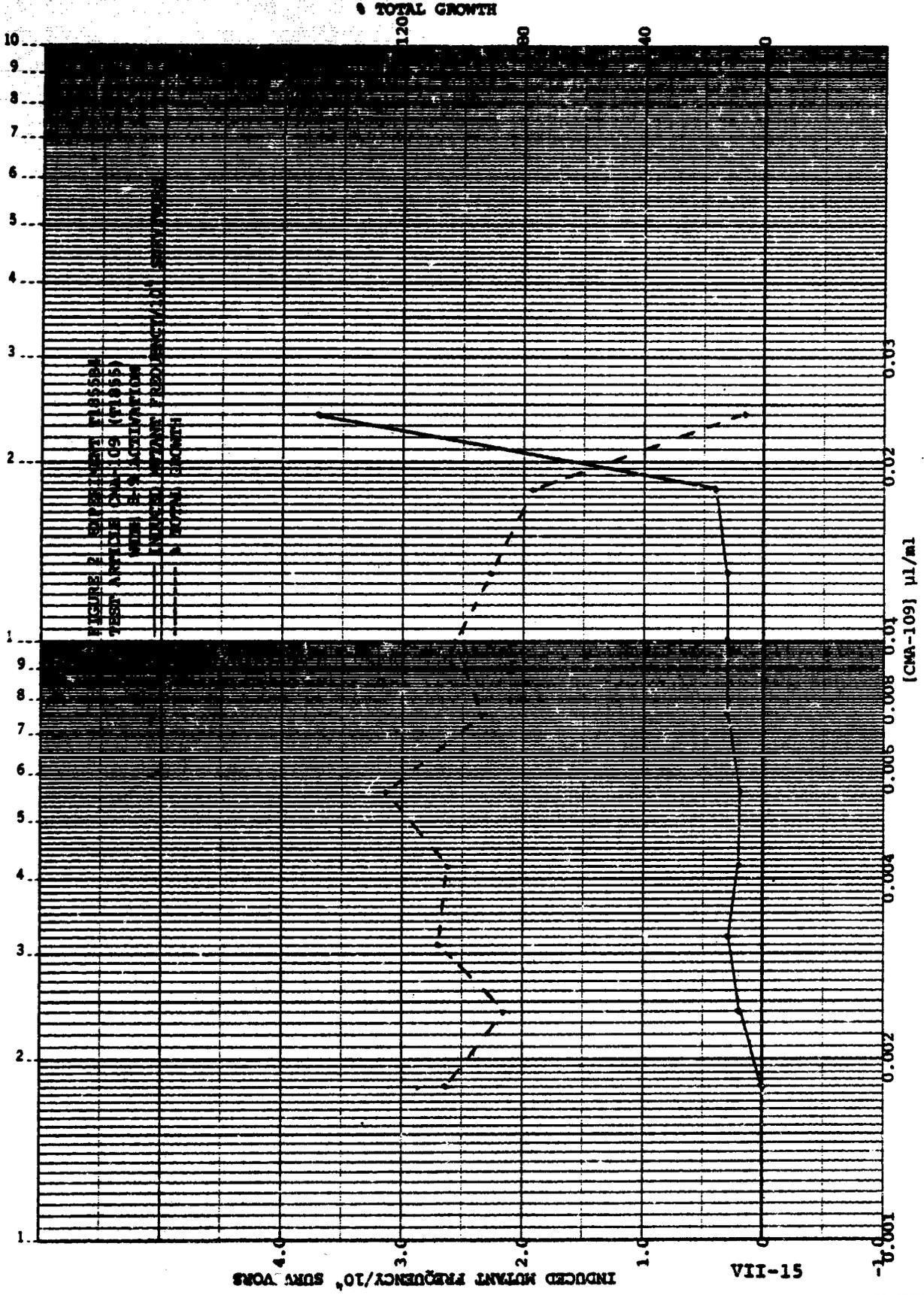


FIGURE 2. SUPPLEMENT FIGURE  
TEST ANTI-CMA-109 (1/1955)  
WITH S-S ACTIVATION  
INDUCED MUTANT FREQUENCY  
● TOTAL GROWTH

VII-15

5

### Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-109 (MA #T1855) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest test article concentration cloned in the S-9 activated cultures and the two highest test article concentrations cloned in the non-activated cultures exhibited mutant frequencies which were more than twice the mean mutant frequency of the solvent controls. These results are not considered significant as the Total Growth of these cultures was less than 10%. Mutations observed at these highly toxic levels may be due to epigenetic events. The results indicate that under the conditions of this test, test article CMA-109 produced a negative response in the presence and absence of exogenous metabolic activation. In the presence of metabolic activation, the Total Growth of the treated cultures that were cloned did not cover the critical range of survival (10-40%). A precipitous toxic response was induced by the test article. The cultures treated with the two highest concentrations of test article had 6% and 77% Total Growth. It was felt that a repeat assay would not provide any additional information since the difference in concentration between the cultures having 6% and 77% Total Growth was only 0.006  $\mu$ l/ml. Thus the results obtained for treatment in the presence of metabolic activation do not permit an accurate assessment of the compound's mutagenic potential.

RECEIVED OCT 3 5 1982

*Spec'd. by  
RA/9A 12/14/82  
N.K. /jkc*

LS178Y TK<sup>+/-</sup> MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the LS178Y TK<sup>+/-</sup> Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-109

2.2 Analysis:

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.  
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

## 5.0 TEST SYSTEM

L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:  
\$2,650

Total charge for assay with metabolic activation:  
\$2,650

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

**6.2 Route and Frequency of Administration**  
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

**6.3 Exogenous Metabolic Activation**

**6.3.1 Liver Microsomal Enzymes - S-9 Homogenate**

**6.3.1.1 Species, Strain, Sex and Inducer**  
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

**6.3.1.2 Homogenate Preparation**  
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

0 2 3 8

#### 6.3.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

#### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

#### 6.4 Controls

##### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

##### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

### 7.0 METHODS

#### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5%  $\text{CO}_2$  in air and placed on a Belco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at  $1000 \times g$  for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of  $\text{F}_{10}\text{P}$ , resuspended in 20 ml of  $\text{F}_{10}\text{P}$ , gassed with 5%  $\text{CO}_2$  in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at  $37^\circ\text{C}$  for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, L5178Y TK<sup>+</sup>/<sup>-</sup> cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring TK<sup>-</sup>/<sup>-</sup> cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5%  $\text{CO}_2$  in air and placed on an environmental incubator shaker at 125 rpm and  $37^\circ\text{C}$ . After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of  $\text{F}_{10}\text{P}$  and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of  $\text{F}_{10}\text{P}$  plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

#### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F<sub>0</sub>P will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>-/-</sup> colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

#### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

#### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the  $TK^{-/-}$  cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of  $TK^{-/-}$  cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to  $37^{\circ}C$ , filled with 100 ml of C.M. and placed on an incubator shaker at  $37^{\circ}C$  until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^6$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at  $1000 \times g$  for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of  $F_{10}P$ , adding 1.0 ml of this to 9 ml  $F_{10}P$ , and adding 1.0 ml of

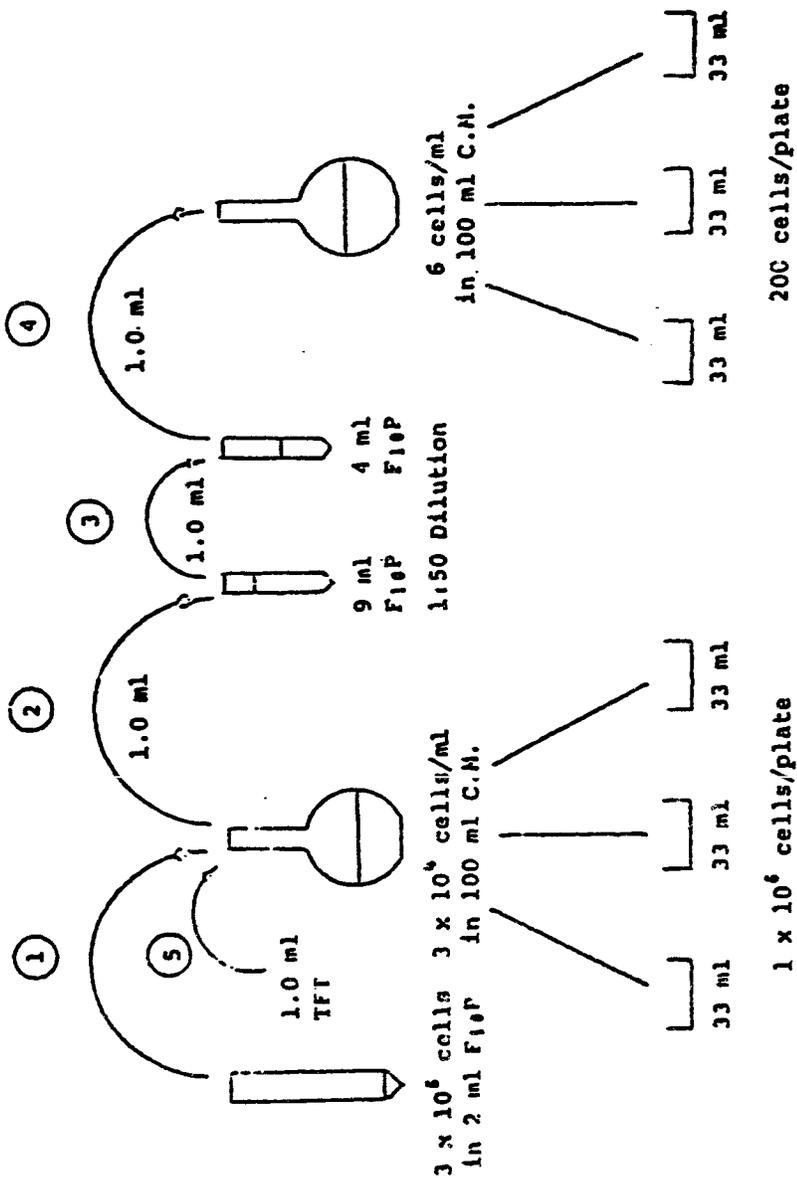


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

## 8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

- 8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.
- 8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

- 9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.
- 9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.
- 9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

- 10.1 \_\_\_\_\_ copies of the draft final report (with \_\_\_\_\_ copies of all raw data) will be sent to the Study Monitor within one month of termination. \_\_\_\_\_ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.
- 10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:
- 10.2.1 The data from the toxicity test.
- 10.2.2 The data generated by the mutagenesis assay which includes:
- 10.2.2.1 The % total growth at each dose level which reflects test article toxicity.
- 10.2.2.2 The number of TK<sup>-/-</sup> colonies per TFT plate for the test article and the controls.
- 10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

#### 11.0 RECORD AND TEST ARTICLE ARCHIVES

##### 11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

##### 11.2 Test Article

A Test Article sample will be held in storage in accordance with the Terms and Conditions.

#### 12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? \_\_\_\_\_  
If so, to which agency or agencies? \_\_\_\_\_

Does the sponsor request that samples of the Test Article dosing solutions be returned? \_\_\_\_\_

#### 13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: *11/24/82*

13.2 Proposed Initiation Date: *December 28, 1982*

13.3 Scheduled Completion Date: *February 18, 1983*

13.4 Report Submission to Sponsor Date: *February 18, 1983*

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14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

*Carol Stank*

\_\_\_\_\_  
SPONSOR'S AUTHORIZED REPRESENTATIVE

*11/22/82*

\_\_\_\_\_  
DATE PROTOCOL APPROVED BY SPONSOR

*Paul E. Kirby*

\_\_\_\_\_  
STUDY DIRECTOR

*11/24/82*  
\_\_\_\_\_  
DATE

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### Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-110 (MA #T1851) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 1% to 114% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 34% to 110% Total Growth in one experiment and from 11% to 86% Total Growth in a second experiment.

The highest test article concentration cloned in the non-activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant since the Total Growth of this culture was less than 10%. TFT resistance observed at these highly toxic levels may be due to epigenetic events. In the first experiment with S-9 activation, one culture exhibited a mutant frequency which was twice the mean mutant frequency of the solvent controls. There were some technical difficulties associated with the second experiment with S-9 activation. However, the results obtained correlated well with the results of the first experiment.

The results indicate that under the conditions of this test, the test article CMA-110 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

## Results

The Initial Toxicity Test (Table 1) performed on test article CMA-110 (MA #T1851) in the absence of S-9 indicated a threshold level of complete toxicity at 0.1  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.1  $\mu\text{l/ml}$  to 0.0013  $\mu\text{l/ml}$ .\*

After a two day expression period, nine cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.013, 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018 or 0.0013  $\mu\text{l/ml}$  test article. These concentrations produced a range in Suspension Growth of 1% to 99%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

One of the cultures that was cloned (0.013  $\mu\text{l/ml}$ ) exhibited a mutant frequency which was 5.0 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 1%. None of the remaining nonactivated cultures exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.\*\* The Total Growth of these cultures ranged from 6% to 114%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-110 (MA #T1851). The results indicated a threshold level of complete toxicity at 0.05  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05  $\mu\text{l/ml}$  to 0.00067  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned

\* Two nonactivated assays were performed. In the first assay the cloning efficiency was not optimum and the assay was repeated. Only the results of the second nonactivated assay will be presented here.

\*\* Culture 0.0056  $\mu\text{l/ml}$  exhibited a mutant frequency which was 4.0 times the mean mutant frequency of the solvent controls. This elevated mutant frequency is probably due to the low VC counts since there is no significant increase in the TFT counts for this culture. The suspension growth of the culture was 56%. Thus the increase in mutant frequency is not significant.

were treated with 0.038, 0.028, 0.021, 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038 or 0.0028  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 37% to 99%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2. One culture (0.038  $\mu\text{l/ml}$ ) that was cloned exhibited a mutant frequency which was 2.0 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 34%.

None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 78% to 110%.

In an attempt to obtain cultures having Total Growth in the range 11% to 30%, a repeat experiment was performed over a narrower dose range from 0.055  $\mu\text{l/ml}$  to 0.019  $\mu\text{l/ml}$ .<sup>\*\*\*</sup>

After a two day expression period, eleven cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.055, 0.050, 0.045, 0.039, 0.035 or 0.029  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 15% to 83%. The Cloning Data are presented in Tables 11 and 12 and the Total Compound Toxicity Data are presented in Tables 13 and 14. The data are also presented graphically in Figure 3.

Due to contamination, no solvent control mutant frequencies were obtained from the assay. However, the solvent control mutant frequencies for the positive control compound were obtained. The solvent used for the test article and the positive control compound were the same (Acetone). Applying these solvent control mutants, the induced mutant frequencies for the test article can be calculated (shown on Table 11 in parenthesis). Six cultures

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<sup>\*\*\*</sup> Two repeat experiments were performed. In the first assay there was considerable contamination. However, the results are presented in this report as a comparison with the results of the initial experiment in the presence of S-9. In the second repeat assay, some difficulty was experienced in the toxic response of the test article and only two doses were cloned. The results will not be presented here.

exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 11% to 83%. None of the remaining cultures exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 57% to 86%.



### Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-110 (MA #T1851) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest concentration cloned in the nonactivated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant since the Total Growth of this culture was less than 10%. TFT resistance observed at these highly toxic levels may be due to epigenetic events. In the first experiment with S-9 activation, one culture exhibited a mutant frequency which was twice the mean mutant frequency of the solvent controls. There were some technical difficulties associated with the second experiment with S-9 activation. However, the results obtained correlated well with the results of the first experiment. The results indicate that under the conditions of this test, test article CMA-110 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of exogenous metabolic activation.

APPENDIX

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*Rec'd. by  
RA/DA 12/8/82.  
N.K./jbc*

L5178Y TK<sup>+/-</sup> MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK<sup>+/-</sup> Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-110

2.2 Analysis:

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.  
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

## 5.0 TEST SYSTEM

L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:  
\$2,650

Total charge for assay with metabolic activation:  
\$2,650

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

#### 6.2 Route and Frequency of Administration

Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

#### 6.3 Exogenous Metabolic Activation

##### 6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

###### 6.3.1.1 Species, Strain, Sex and Inducer

Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

###### 6.3.1.2 Homogenate Preparation

Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

#### 6.3.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

#### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

#### 6.4 Controls

##### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

##### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

### 7.0 METHODS

#### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

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Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5%  $\text{CO}_2$  in air and placed on a Bellico roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at  $1000 \times g$  for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of  $\text{F}_{10}\text{P}$ , resuspended in 20 ml of  $\text{F}_{10}\text{P}$ , gassed with 5%  $\text{CO}_2$  in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at  $37^\circ\text{C}$  for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, L5178Y TK<sup>+</sup>/<sup>-</sup> cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring TK<sup>-</sup>/<sup>-</sup> cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5%  $\text{CO}_2$  in air and placed on an environmental incubator shaker at 125 rpm and  $37^\circ\text{C}$ . After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of  $\text{F}_{10}\text{P}$  and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of  $\text{F}_{10}\text{P}$  plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

#### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F<sub>0</sub>P will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>+</sup> colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

#### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

#### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the TK<sup>-/-</sup> cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of TK<sup>-/-</sup> cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

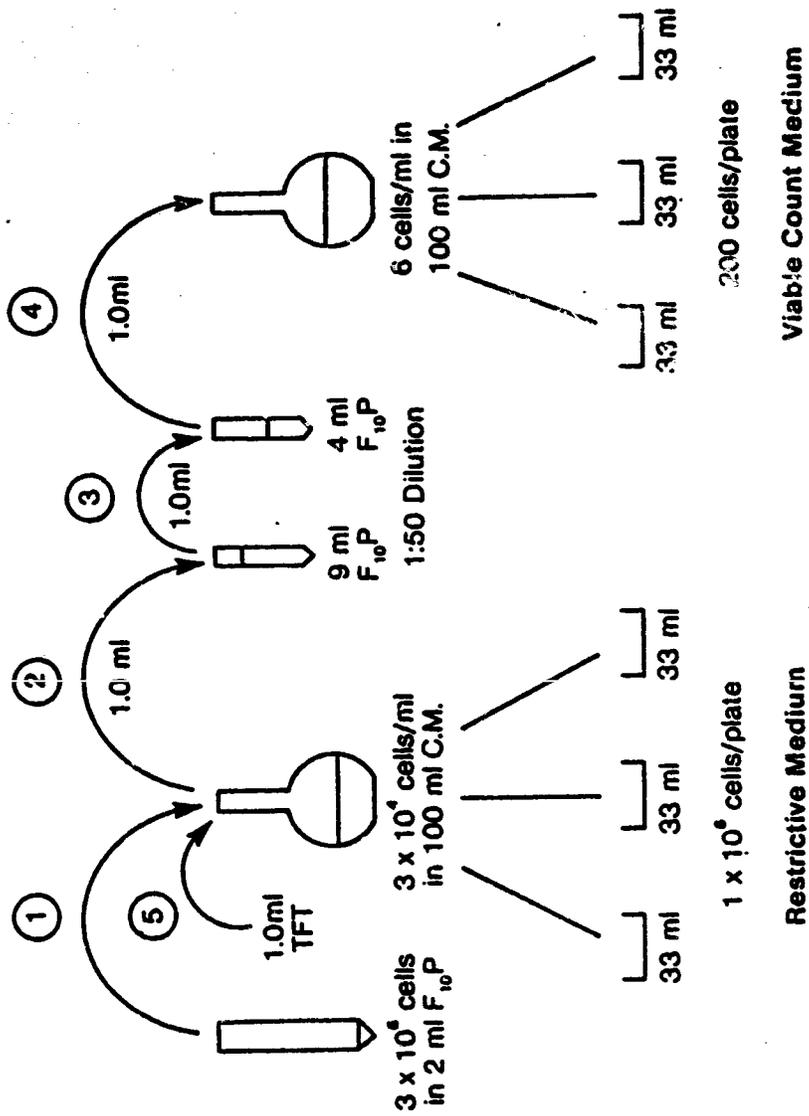
Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^6$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of F<sub>10</sub>P, adding 1.0 ml of this to 9 ml F<sub>10</sub>P, and adding 1.0 ml of

**ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS**



**FIGURE 1**

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

## 8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

#### 9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

#### 10.0 FINAL REPORT

10.1 \_\_\_\_\_ copies of the draft final report (with \_\_\_\_\_ copies of all raw data) will be sent to the Study Monitor within one month of termination. \_\_\_\_\_ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of TK<sup>-/-</sup> colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

#### 11.0 RECORD AND TEST ARTICLE ARCHIVES

##### 11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

##### 11.2 Test Article

A Test Article sample will be held in storage in accordance with the Terms and Conditions.

#### 12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? \_\_\_\_\_  
If so, to which agency or agencies? \_\_\_\_\_

Does the sponsor request that samples of the Test Article dosing solutions be returned? \_\_\_\_\_

#### 13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: *11/24/82*

13.2 Proposed Initiation Date: *December 16, 1982*

13.3 Scheduled Completion Date: *January 29, 1983*

13.4 Report Submission to Sponsor Date: *January 29, 1983*

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

*Carole Stark*

\_\_\_\_\_  
SPONSOR'S AUTHORIZED REPRESENTATIVE

*11/20/82*

\_\_\_\_\_  
DATE PROTOCOL APPROVED BY SPONSOR

*Paul E. Kelly*

\_\_\_\_\_  
STUDY DIRECTOR

*11/24/82*

\_\_\_\_\_  
DATE

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F <sub>0</sub> P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
F <sub>10</sub> P	F <sub>0</sub> P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	rifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

0 2 8 8

5. (Cont'd.)

$$\% \text{ Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\% \text{ Growth} = \frac{(\% \text{ Suspension Growth}) (\% \text{ Cloning Growth})}{100}$$

### Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-111 (MA #T1867) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures that were cloned were treated with a range of test article concentrations which produced from 2% to 116% Total Growth. The S-9 activated cultures that were cloned were treated with a range of test article concentrations which produced from 27% to 111% Total Growth.

One nonactivated culture that was cloned exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant since the Total Growth of this culture was 2%. TFT resistance observed at these highly toxic levels may be due to epigenetic events. One S-9 activated culture that was cloned exhibited a mutant frequency which was significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that, under the conditions of this test, test article CMA-111 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

## Results

The Initial Toxicity Test (Table 1) conducted on test article CMA-111 (MA #T1867) in the absence of S-9 indicated a threshold level of complete toxicity at 0.05  $\mu\text{l/ml}$ . Based on these data, the test article was tested in the mutagenesis assay in the absence of S-9 over a range of concentrations from 0.05  $\mu\text{l/ml}$  to 0.00067  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, or 0.0012  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 11% to 100%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. These data are also presented graphically in Figure 1.

One nonactivated culture that was cloned (0.016  $\mu\text{l/ml}$ ) exhibited a mutant frequency which was 3.4 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 2%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 8% to 116%.

The Initial Toxicity Test (Table 6) conducted on test article CMA-111 (MA #T1867) in the presence of S-9 indicated a threshold level of complete toxicity at 0.1  $\mu\text{l/ml}$ . Based on these data, the test article was tested in the mutagenesis assay over a range of concentrations from 0.1  $\mu\text{l/ml}$  to 0.0013  $\mu\text{l/ml}$ .\*

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.024, 0.018, 0.013, 0.010, 0.0075, 0.0056, 0.0042.

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\*Three S-9 activated assays were performed. Considerable contamination was experienced in two assays and the results of these assays will not be reported. The results of the third assay are discussed in this report.

0.0032, 0.0024 or 0.0018  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 22% to 93%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. These data are also presented graphically in Figure 2.

One culture that was cloned (0.024  $\mu\text{l/ml}$ ) exhibited a mutant frequency which was 2.0 times the mean mutant frequency of the solvent control. The Total Growth of this culture was 27%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly different from the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 43% to 111%.

### Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-111 (MA #T1867) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. One nonactivated culture that was cloned exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant since the Total Growth of this culture was less than 10%. TFT resistance observed at these highly toxic levels may be due to epigenetic events. One S-9 activated culture that was cloned exhibited a mutant frequency which was significantly greater than the mean mutant frequency of the solvent controls. The results indicate that, under the conditions of this test, test article CMA-111 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

APPENDIX

RECEIVED OCT 3 5 1982

*Rec'd by  
CA/DA 12/15/82  
N.K./jlec*

**LS178Y TK<sup>+</sup>/- MOUSE LYMPHOMA MUTAGENESIS ASSAY**

**1.0 PURPOSE**

The purpose of this study is to evaluate the mutagenic potential of the test article using the LS178Y TK<sup>+</sup>/- Mouse Lymphoma Mutagenesis Assay.

**2.0 TEST ARTICLE**

2.1 Identification: CMA-111

2.2 Analysis:

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

**3.0 SPONSOR**

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.  
Washington, D.C.

3.3 Authorized Representative: Carol Stack

**4.0 TESTING FACILITY**

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

TX-18

## 5.0 TEST SYSTEM

L5178Y TK<sup>+</sup>/- mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The L5178Y TK<sup>+</sup>/- mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 5.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:  
\$2,650

Total charge for assay with metabolic activation:  
\$2,650

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

**6.2 Route and Frequency of Administration**  
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

**6.3 Exogenous Metabolic Activation**

**6.3.1 Liver Microsomal Enzymes - S-9 Homogenate**

**6.3.1.1 Species, Strain, Sex and Inducer**  
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

**6.3.1.2 Homogenate Preparation**  
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

#### 6.3.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

#### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

#### 6.4 Controls

##### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

##### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

#### 7.0 METHODS

##### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5%  $\text{CO}_2$  in air and placed on a Ballico roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at  $1000 \times g$  for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of  $\text{F}_{10}\text{P}$ , resuspended in 20 ml of  $\text{F}_{10}\text{P}$ , gassed with 5%  $\text{CO}_2$  in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at  $37^\circ\text{C}$  for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, LS178Y  $\text{TK}^{+/-}$  cells which are actively growing in culture will be cleaned as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring  $\text{TK}^{-/-}$  cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5%  $\text{CO}_2$  in air and placed on an environmental incubator shaker at 125 rpm and  $37^\circ\text{C}$ . After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of  $\text{F}_{10}\text{P}$  and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of  $\text{F}_{10}\text{P}$  plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

#### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F<sub>10</sub>P will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>+</sup> colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

#### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

#### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the TK<sup>-/-</sup> cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of TK<sup>-/-</sup> cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^6$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of F<sub>10</sub>P, adding 1.0 ml of this to 9 ml F<sub>10</sub>P, and adding 1.0 ml of

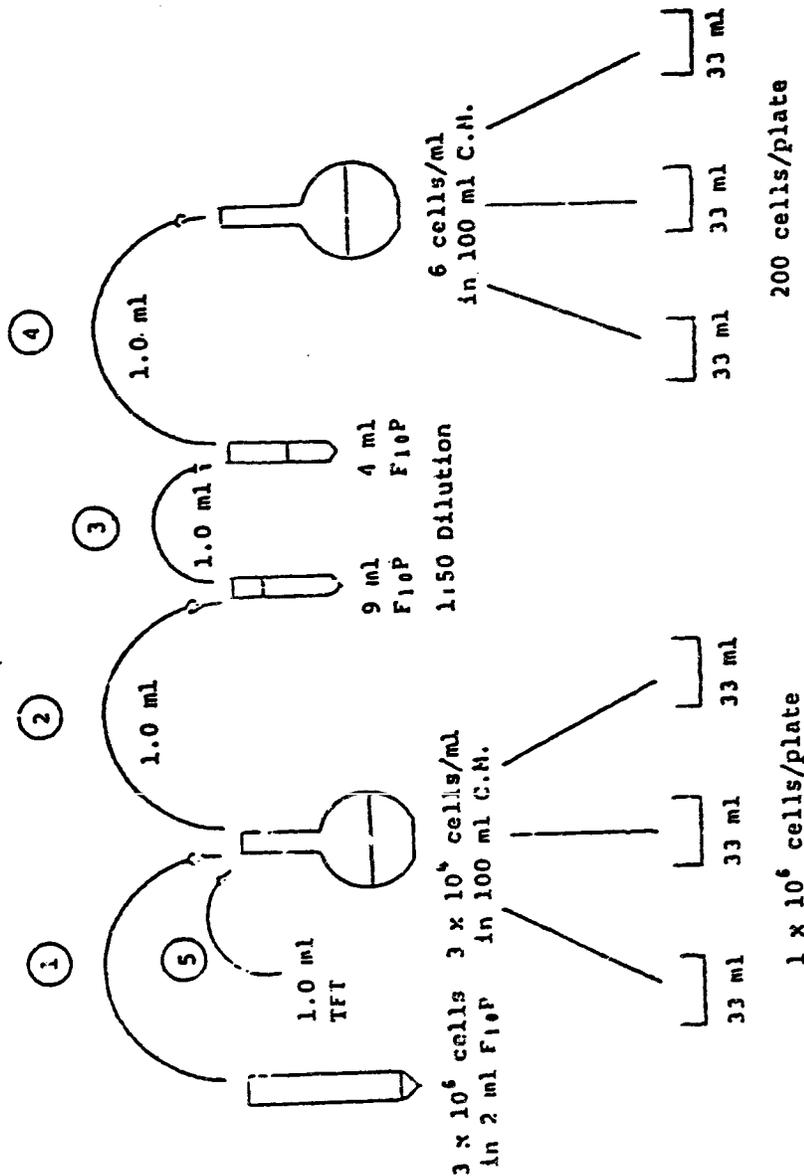


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

## 8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

- 8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.
- 8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

#### 9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

- 9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.
- 9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.
- 9.3 The plating efficiency of the solvent controls must be greater than 50%.

#### 10.0 FINAL REPORT

- 10.1 \_\_\_\_\_ copies of the draft final report (with \_\_\_\_\_ copies of all raw data) will be sent to the Study Monitor within one month of termination. \_\_\_\_\_ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.
- 10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:
  - 10.2.1 The data from the toxicity test.
  - 10.2.2 The data generated by the mutagenesis assay which includes:
    - 10.2.2.1 The % total growth at each dose level which reflects test article toxicity.
    - 10.2.2.2 The number of TK<sup>-/-</sup> colonies per TFT plate for the test article and the controls.
    - 10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

**11.0 RECORD AND TEST ARTICLE ARCHIVES**

**11.1 Records**

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

**11.2 Test Article**

A Test Article sample will be held in storage in accordance with the Terms and Conditions.

**12.0 GOOD LABORATORY PRACTICES**

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? \_\_\_\_\_  
If so, to which agency or agencies? \_\_\_\_\_

Does the sponsor request that samples of the Test Article dosing solutions be returned? \_\_\_\_\_

**13.0 SCHEDULE OF EVENTS**

13.1 Test Material Received Date: 12/10/82

13.2 Proposed Initiation Date: 1/4/83  
12/15/82

13.3 Scheduled Completion Date: 2/18/83

13.4 Report Submission to Sponsor Date: 2/18/83

0 3 1 8

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

*Carol Stuart*

\_\_\_\_\_  
SPONSOR'S AUTHORIZED REPRESENTATIVE

*11/24/82*

\_\_\_\_\_  
DATE PROTOCOL APPROVED BY SPONSOR

*Paul E. Kirby*

\_\_\_\_\_  
STUDY DIRECTOR

*11/24/82*

\_\_\_\_\_  
DATE

Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-112 (MA #T1853) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 4% to 93% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 22% to 103% Total Growth.

One nonactivated culture having less than 10% Total Growth exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant due to the high toxicity observed. None of the S-9 activated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that under the conditions of this test, test article CMA-112 produced a negative response in the presence and absence of exogenous metabolic activation.

## Results

The Initial Toxicity Test (Table 1) performed on test article CMA-112 (MA #T1853) in the absence of S-9 indicated a threshold level of complete toxicity at 0.1  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.1  $\mu\text{l/ml}$  to 0.0013  $\mu\text{l/ml}$ .

After a two day expression period, eight cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018 or 0.0013  $\mu\text{l/ml}$  test article. These concentrations produced a range in Suspension Growth of 18% to 98%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

One culture that was cloned exhibited a mutant frequency which was 2.7 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 4%. None of the remaining cultures exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 24% to 33%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-112 (MA #T1853). The results indicated a threshold level of complete toxicity at 0.05  $\mu\text{l/ml}$ . Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05  $\mu\text{l/ml}$  to 0.00067  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.016, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, 0.0012 or 0.00089  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 43% to 108%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data

are presented in Tables 9 and 10. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 22% to 103%.

Study No. T1853 701005

Table 1

701 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY  
INITIAL COMPOUND TOXICITY TEST

T1853.701006 Study Number      Paul S Kirby Study Director      100 µl/l - 0.001 µl/l Dose Range  
CHA 112 (T1853) Test Article Identity      Acetone Solvent

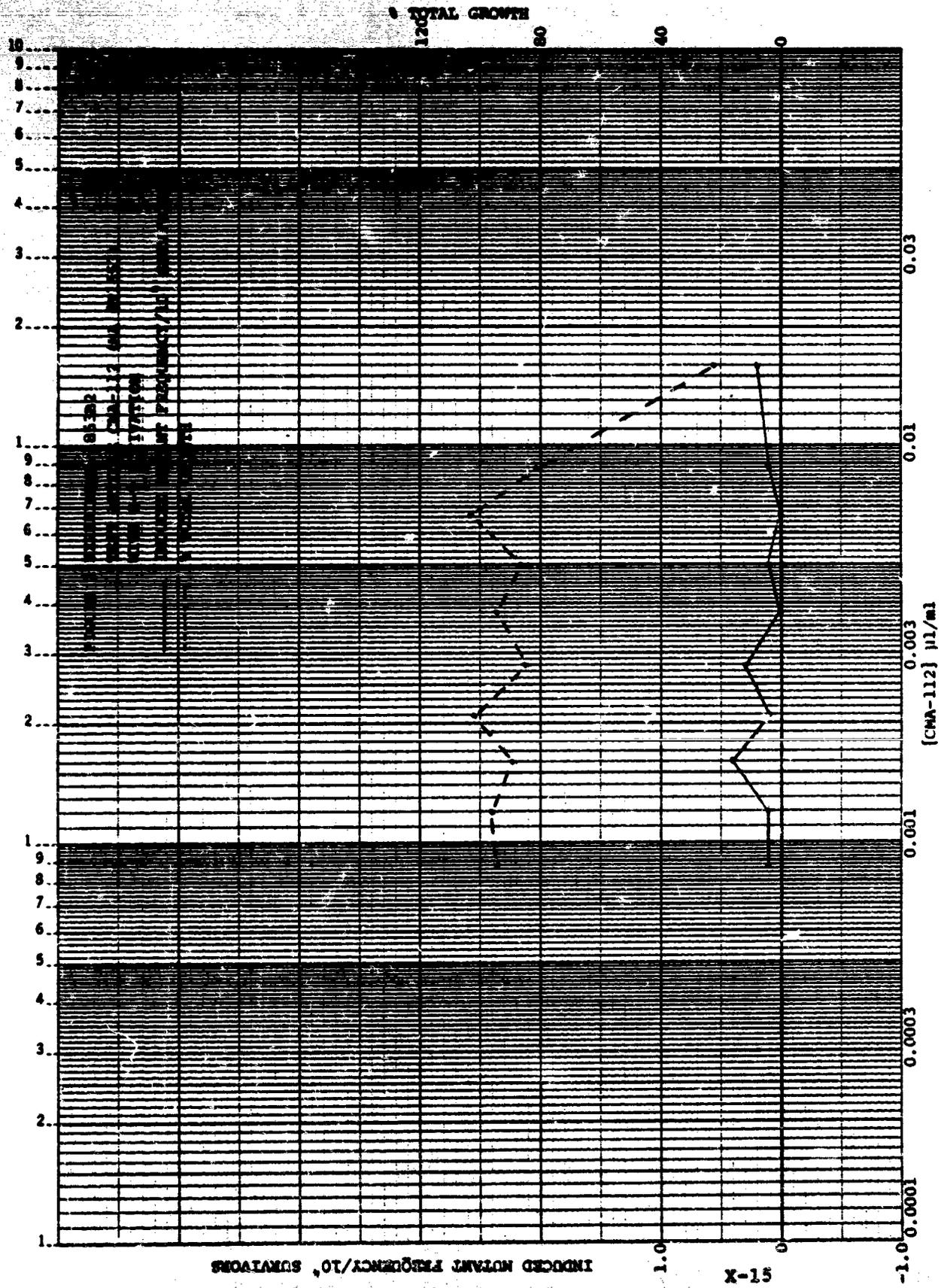
	Test Article Concentration	Cell Concentration (X 10 <sup>6</sup> )		Suspension Growth	
		Day 1	Day 2	Total	% of Control
Without Activation	100 µl/l	1.880**	*		
	10 µl/l	0.580**	*		
	1.0 µl/l	0.036	*		
	0.1 µl/l	0.041	0.055	0.0	0%
	0.01 µl/l	0.277	0.602	1.3	24%
	0.001 µl/l	0.576	0.876	5.6	102%
	Solvent 1	0.580	0.819	5.3	5.5
Solvent 2	0.597	0.848	5.6		
With S-9 Activation					
	Solvent 1				
	Solvent 2				

*not applicable* <sup>ATL (B)</sup>  
 12/22/82

+ Culture Lost

Table Prepared By: Antonia N Hoopes - Bando Signature      12/22/82 Date  
 \*\* data only no viable cells.      \* not counted on 2nd day  
 MRB 12/22/82      MRB 12/22/82

Workbook Page No. 13 Report Page No. X-4



### Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-112 (MA #T1853) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. One nonactivated culture having less than 10% Total Growth exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant due to the high toxicity observed. None of the S-9 activated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-112 produced a negative response in the presence and absence of exogenous metabolic activation.

**APPENDIX**

RECEIVED OCT 05 1982

*Rec'd. by  
RA/DA 12/8/82  
N.K./jko*

L5178Y TK<sup>+</sup>/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK<sup>+</sup>/- Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-112

2.2 Analysis:

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.  
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

X-18

## 5.0 TEST SYSTEM

L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:

\$2,650

Total charge for assay with metabolic activation:

\$2,650

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

- 6.2 Route and Frequency of Administration  
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.
- 6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer  
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation  
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

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#### 6.3.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

#### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

#### 6.4 Controls

##### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

##### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

#### 7.0 METHODS

##### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5%  $\text{CO}_2$  in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at  $1000 \times g$  for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of  $\text{F}_{10}\text{P}$ , resuspended in 20 ml of  $\text{F}_{10}\text{P}$ , gassed with 5%  $\text{CO}_2$  in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at  $37^\circ\text{C}$  for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, L5178Y TK<sup>+</sup>/<sup>-</sup> cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring TK<sup>-</sup>/<sup>-</sup> cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5%  $\text{CO}_2$  in air and placed on an environmental incubator shaker at 125 rpm and  $37^\circ\text{C}$ . After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of  $\text{F}_{10}\text{P}$  and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of  $\text{F}_{10}\text{P}$  plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

#### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F<sub>0</sub>P will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>-/-</sup> colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

#### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

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cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

#### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the  $TK^{-/-}$  cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of  $TK^{-/-}$  cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to  $37^{\circ}C$ , filled with 100 ml of C.M. and placed on an incubator shaker at  $37^{\circ}C$  until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^6$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at  $1000 \times g$  for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of  $F_{10}P$ , adding 1.0 ml of this to 9 ml  $F_{10}P$ , and adding 1.0 ml of

0343

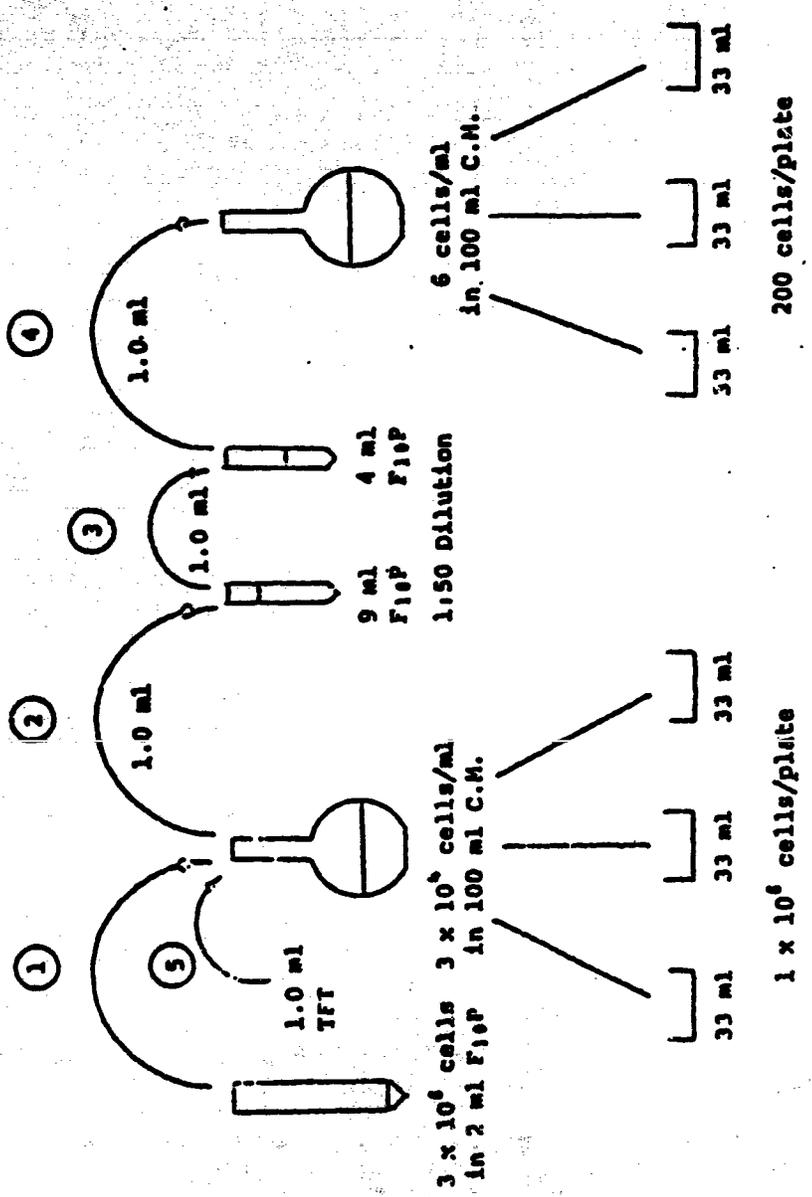


Figure 1  
 ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

## 8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

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8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

#### 9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

#### 10.0 FINAL REPORT

10.1 \_\_\_\_\_ copies of the draft final report (with \_\_\_\_\_ copies of all raw data) will be sent to the Study Monitor within one month of termination. \_\_\_\_\_ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of TK<sup>-/-</sup> colonies per TPT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

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10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article

A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? \_\_\_\_\_  
If so, to which agency or agencies? \_\_\_\_\_

Does the sponsor request that samples of the Test Article dosing solutions be returned? \_\_\_\_\_

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: *11/24/82*

13.2 Proposed Initiation Date: *December 20, 1982*

13.3 Scheduled Completion Date: *February 11, 1983*

13.4 Report Submission to Sponsor Date: *February 11, 1983*

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14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

*Carol Stuck*

\_\_\_\_\_  
SPONSOR'S AUTHORIZED REPRESENTATIVE

*11/29/82*

\_\_\_\_\_  
DATE PROTOCOL APPROVED BY SPONSOR

*Paul E. Kirby*

\_\_\_\_\_  
STUDY DIRECTOR

*11/29/82*  
\_\_\_\_\_  
DATE

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F <sub>0</sub> P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
F <sub>10</sub> P	F <sub>0</sub> P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
TEMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

5. (Cont'd.)

$$\% \text{ Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\% \text{ Growth} = \frac{(\% \text{ Suspension Growth}) (\% \text{ Cloning Growth})}{100}$$

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### Summary

Two mutagenesis assays in the presence of S-9 were conducted on test article Calcium Dialkyl Dithiophosphate (MA #T2253). In the first assay, the cultures that were cloned were treated with a range of test article concentrations which produced from 43% to 119% Total Growth. None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. A repeat study was conducted to generate cultures having between 10% and 40% Total Growth. In the repeat study, conducted over a narrower dose range and with triplicate cultures per dose, the cultures that were cloned exhibited 17% to 74% Total Growth. None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that, under the conditions of these tests, test article Calcium Dialkyl Dithiophosphate produced a negative response in the presence of exogenous metabolic activation.

## Results

The Initial Toxicity Test (Table 1) conducted on test article Calcium Dialkyl Dithiophosphate (MA #T2253) in the presence of S-9, indicated complete toxicity at 0.1  $\mu\text{l/ml}$ .<sup>\*</sup> Based on these data, the test article was tested in a mutagenesis assay over a range of concentrations from 0.1  $\mu\text{l/ml}$  to 0.0013  $\mu\text{l/ml}$ .

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.018, 0.013, 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018 or 0.0013  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 36% to 96%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 2 and 3. These data are also presented graphically in Figure 1. The Cloning Data and Total Compound Toxicity Data for the positive control are presented in Table 4.

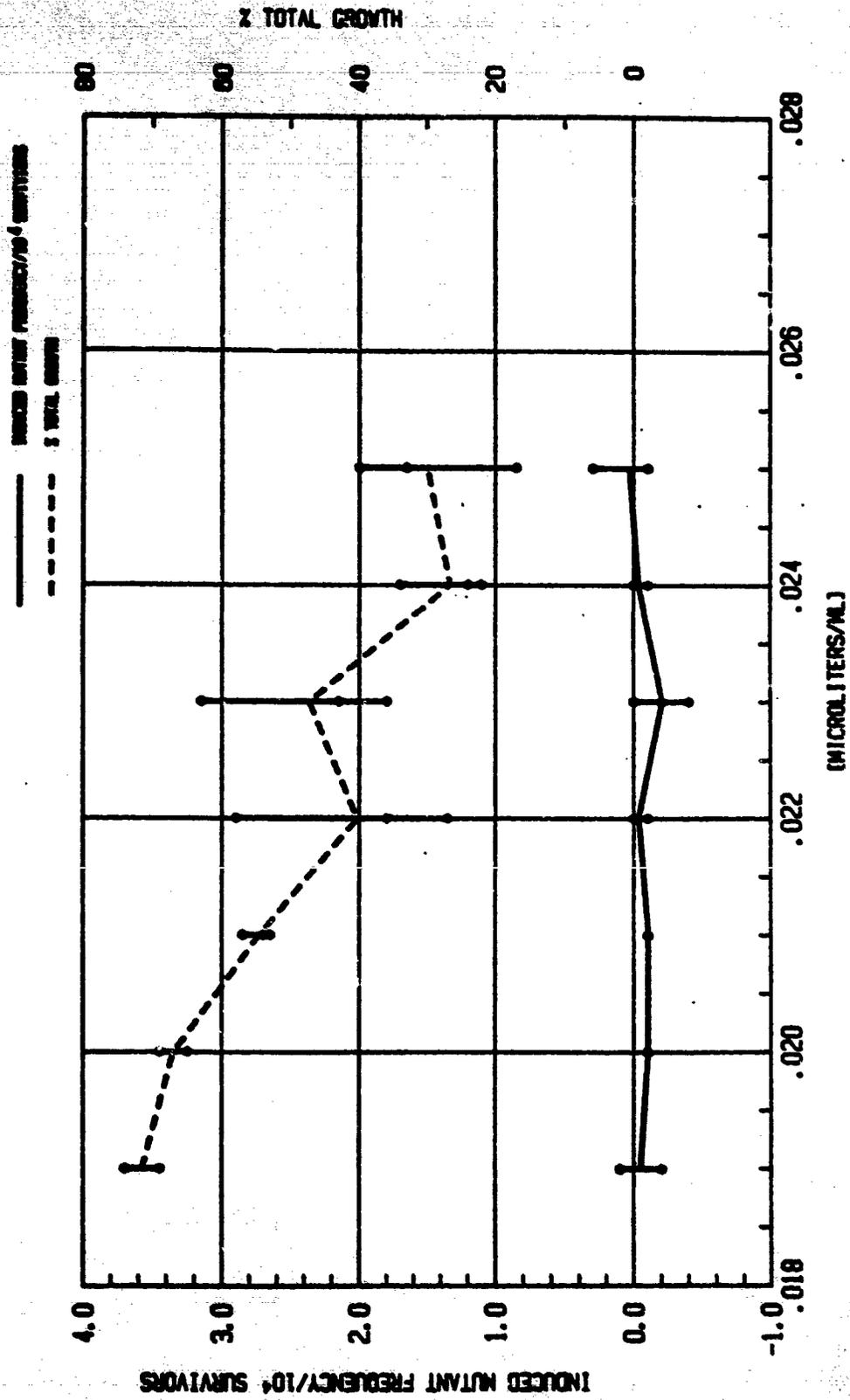
None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 43% to 119%.

A repeat assay was conducted in an attempt to generate cultures having between 10% and 40% Total Growth. The cultures were treated in triplicate in the presence of S-9 with a range of concentrations from 0.025  $\mu\text{l/ml}$  to 0.012  $\mu\text{l/ml}$ . After a two day expression period, twenty-one cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.025, 0.024, 0.023, 0.022, 0.021, 0.020 or 0.019  $\mu\text{l/ml}$ . These concentrations produced a range in Suspension Growth of 19% to 79%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 5 and 6. These data are also presented graphically in Figure 2. The Cloning Data and Total Compound Toxicity Data for the positive control are presented in Table 7.

<sup>\*</sup>The culture treated with 10  $\mu\text{l/ml}$  appeared to have cell growth. Upon microscopic examination of the culture, there were few viable cells present and a large quantity of compound precipitate and cell debris.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 17% to 74%.

FIGURE 2  
 Study No. T253.701012 (Trial 2)  
 Test Article CALCIUM DIALKYL DITHIOPHOSPHATE  
 With S-9 Activation



### Conclusion

Two mutagenesis assays were conducted on test article Calcium Dialkyl Dithiophosphate (MA #T2253). None of the cultures that were cloned in the first assay exhibited mutant frequencies which were more than twice the mean mutant frequency of the solvent controls. A repeat assay was conducted in an attempt to produce cultures having between 10% and 40% Total Growth. Cultures were treated in triplicate and none of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that, under the conditions of these tests, test article Calcium Dialkyl Dithiophosphate produced a negative response in the presence of exogenous metabolic activation.

Received by RA/QA 4/13/84  
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Study No. T2254 701012

Study No. T2253 701012

**L5178Y TK<sup>+/-</sup> MOUSE LYMPHOMA MUTAGENESIS ASSAY  
(with S-9 activation)**

**1.0 PURPOSE**

The purpose of this study is to evaluate the mutagenic potential of the test articles using the L5178Y TK<sup>+/-</sup> Mouse Lymphoma Mutagenesis Assay.

**2.0 TEST ARTICLE**

**2.1 Identification:** Zinc Dialkyl Dithiophosphate (CMA 102B)  
and Calcium Dialkyl Dithiophosphate

**2.2 Analysis:**

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test articles (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

**3.0 SPONSOR**

**3.1 Name:** Chemical Manufacturers Association

**3.2 Address:** 2501 M Street, N.W.  
Washington, D.C. 20037

**3.3 Authorized Representative:** Dr. Carol Stack

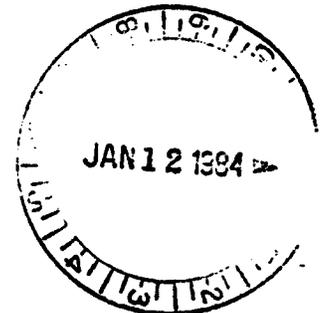
**4.0 TESTING FACILITY**

**4.1 Name:** Division of Genetic Toxicology  
Microbiological Associates

**4.2 Address:** 5221 River Road  
Bethesda, Maryland 20816

**4.3 Study Location:** Rockville Laboratory

**4.4 Study Director:** Paul E. Kirby, Ph.D.



## 5.0 TEST SYSTEM

L5178Y TK<sup>+/-</sup> mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The L5178Y TK<sup>+/-</sup> mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted with metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity with metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth.

Total charge for assay with metabolic activation:  
\$2,950 per test article

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, or additional information concerning the test article indicates alternative doses would be better, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

## 6.2 Route and Frequency of Administration

Cell cultures will be treated once by way of a vehicle compatible with the system, in the presence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

## 6.3 Exogenous Metabolic Activation

### 6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer  
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation  
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70 °C.

6.3.1.3 S-9 Characterization  
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45  $\mu$ m filter.

## 6.4 Controls

### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

## 7.0 METHODS

### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with S-9 activation will be conducted. Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture will be added to the tubes. Each tube will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus at 25 rpm for a

4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of F<sub>10</sub>P, resuspended in 20 ml of F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, L5178Y TK<sup>+</sup>/<sup>-</sup> cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring TK<sup>+</sup> cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5% CO<sub>2</sub> in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of F<sub>10</sub>P and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of F<sub>10</sub>P plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

0 3 7 4

### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with S-9 activation. Four ml of S-9 activation mixture will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>-/-</sup> colonies, two control tubes will receive solvent only. Two concentrations of 7,12-DMBA will be used as a positive control. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the TK<sup>-/-</sup> cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.34%) which maintains the cells in suspension and allows them to form discrete colonies of TK<sup>-/-</sup> cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

0 3 7 5

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and that they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^6$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of  $F_{10}P$ , adding 1.0 ml of this to 9 ml  $F_{10}P$ , and adding 1.0 ml of that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5%  $CO_2$  atmosphere for 10-12 days.

### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of

**ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS**

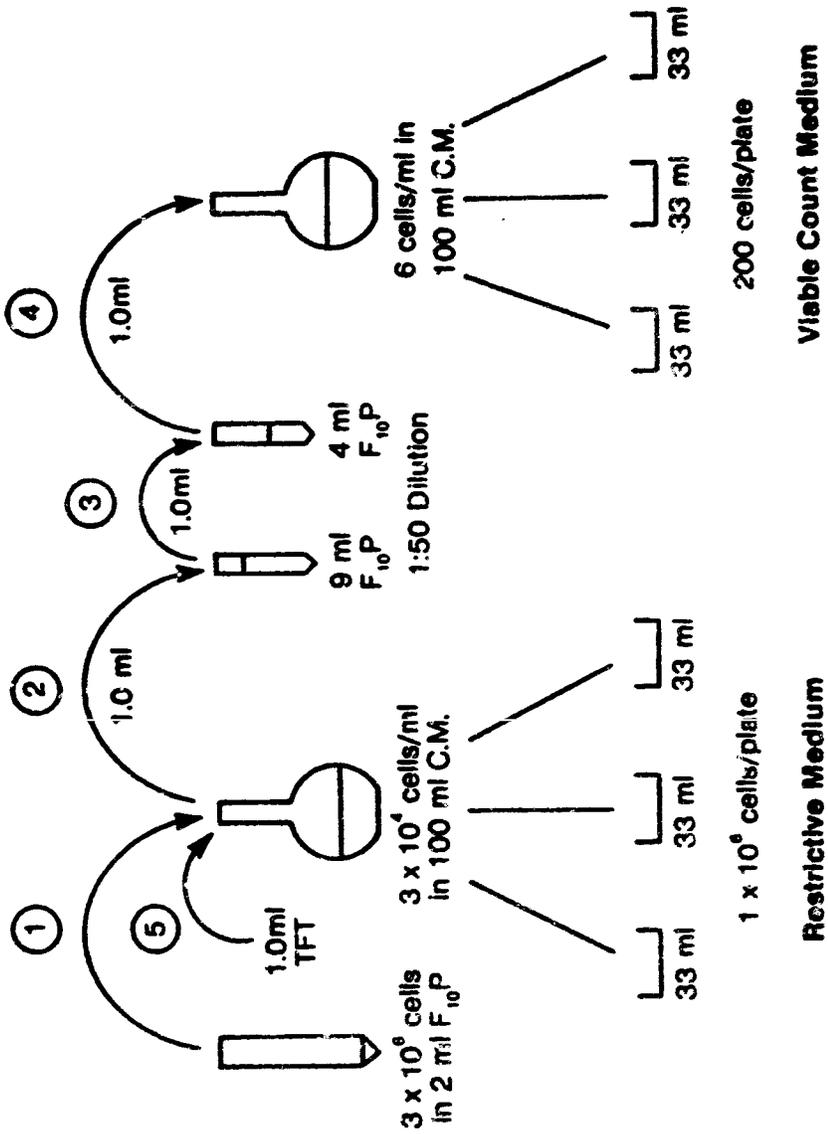


FIGURE 1

colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plate, the presence of a significant level of mutagenic activity can be detected.

#### **8.0 EVALUATION OF TEST RESULTS**

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

- 8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.
- 8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.
- 8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

#### **9.0 CRITERIA FOR DETERMINATION OF A VALID TEST**

- 9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.
- 9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.
- 9.3 The plating efficiency of the solvent controls must be greater than 50%.

## 10.0 FINAL REPORT

- 10.1 \_\_\_\_\_ copies of the draft final report (with \_\_\_\_\_ copies of all raw data) will be sent to the Study Monitor within one month of termination. \_\_\_\_\_ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.
- 10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:
- 10.2.1 The data from the toxicity test.
  - 10.2.2 The data generated by the mutagenesis assay which includes:
    - 10.2.2.1 The % total growth at each dose level which reflects test article toxicity.
    - 10.2.2.2 The number of TK<sup>-/-</sup> colonies per TFT plate for the test articles and the controls.
    - 10.2.2.3 The number of colonies per V.C. plate for the test articles and the controls.
    - 10.2.2.4 The mutation frequency of each dose level of the test articles and the controls.
    - 10.2.2.5 The induced mutation frequency of each dose level of the test articles and the positive controls.

## 11.0 RECORD AND TEST ARTICLE ARCHIVES

- 11.1 Records  
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.
- 11.2 Test Article  
Test Article samples will be held in storage in accordance with the Terms and Conditions.

**12.0 GOOD LABORATORY PRACTICES**

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? YES  
If so, to which agency or agencies? OSHA, EPA, NIOSH,  
NCI, NIEHS, CPSC, FDA

Does the Sponsor request that samples of the Test Article dosing solutions be returned? NO

**13.0 SCHEDULE OF EVENTS**

- 13.1 Test Material Received Date: December 12, 1983
- 13.2 Proposed Initiation Date: *January 23, 1984* <sup>3 PL 5/24/84</sup>
- 13.3 Scheduled Completion Date: *February 24, 1984*
- 13.4 Report Submission to Sponsor Date: *March 2, 1984*

**14.0 REFERENCES**

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

*Carol M. Hank*  
SPONSOR'S AUTHORIZED REPRESENTATIVE

*11/19/83*  
DATE PROTOCOL APPROVED BY SPONSOR

*Paul S. Kirby*  
STUDY DIRECTOR

*11/28/83*  
DATE

**Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay**

<b>C.M.</b>	<b>Cloning Medium</b>
<b>DMBA</b>	<b>7,12-Dimethylbenz(a)anthracene</b>
<b>EMS</b>	<b>Ethyl Methanesulfonate</b>
<b>F<sub>0</sub>P</b>	<b>Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic</b>
<b>F<sub>10</sub>P</b>	<b>F<sub>0</sub>P with 10% heat inactivated horse serum</b>
<b>NADP</b>	<b>Nicotinimide Adenine Dinucleotide Phosphate</b>
<b>S-9</b>	<b>1254, 1242 Aroclor-induced rat liver S-9</b>
<b>TFT</b>	<b>Trifluorothymidine</b>
<b>THMG</b>	<b>Thymidine, Hypoxanthine, Methotrexate and Glycine</b>
<b>V.C.</b>	<b>Viable Count</b>

B. (Cont'd.)

$$\% \text{ Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\% \text{ Growth} = \frac{(\% \text{ Suspension Growth}) (\text{Cloning Growth})}{100}$$

11784

Summary

Chemical Manufacturers Association's test article Zinc Oleate, Lot No. 34495-10 (MA #T2256) was tested in the L5178Y TK-/- Mouse Lymphoma Mutagenesis Assay in the presence of Aroclor induced rat liver S-9. Due to solubility problems with the test article, the cultures that were cloned exhibited a range in Total Growth of 74% to 107%. None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that, under the conditions of this test, test article Zinc Oleate produced a negative response in the presence of exogenous metabolic activation.

### Results

The Initial Toxicity Test (Table 1) conducted on test article Zinc Oleate, Lot No. 34495-10 (MA #12256) in the presence of S-9, indicated complete toxicity at 100 µg/ml. Based on these data, the test article was tested in the mutagenesis assay over a range of concentrations from 100 µg/ml to 1.3 µg/ml.

After a two day expression period, the first ten cultures were cloned. Due to solubility problems with the test article, the toxic response demonstrated in the Initial Toxicity Test was not reproduced. The cultures that were cloned were treated with 100, 75, 56, 42, 32, 24, 18, 13, 10 or 7.5 µg/ml test article. These concentrations produced a range in Suspension Growth of 85% to 109%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 2 and 3. These data are also presented graphically in Figure 1. The data for the positive controls are presented in Table 4.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 74% to 107%.

TABLE 1

LS178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY  
INITIAL COMPOUND TOXICITY TEST

Study Number : T2256.701012  
 Test Article : ZINC OLEATE  
 Activation : Induced Rat Liver S-9  
 Trial Number : 1  
 Dose Range : 1000 US/ML TO 0.02 US/ML  
 Solvent : Acetone  
 Operator : Terrence R. O'Keefe  
 Study Director : Paul E. Kirby, Ph.D.  
 Date & Time : 01/27/84 3:40 pm

Test Article Concentration (µg/ml)	Cell Concentration (X 10 <sup>6</sup> )		Suspension Growth	
	Day 1	Day 2	Total	% of Control

WITH S-9 ACTIVATION

1000	0.069	0.017	0.0	0
500	0.038	0.042	0.0	0
100	0.087	0.200	0.0	0
50	0.676	1.624	12.2	69
10	0.877	1.659	16.2	92
5	0.962	1.539	16.7	93
1	0.969	1.548	16.7	93
.5	1.006	1.613	18.1	103
.1	0.875	1.522	14.8	84
.05	1.040	1.618	18.7	106
Solvent 1	0.987	1.585	17.4	
Solvent 2	1.049	1.531	17.8	

+ Culture Lost

Table Prepared By: Terrence R. O'Keefe 1/27/84  
 Signature Date

Workbook No. 15 Report Page No. XII-3

### Conclusion

Chemical Manufacturers Association's test article Zinc Oleate, Lot No. 34495-10 (MA #T2256) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence of Aroclor induced rat liver S-9. Solubility difficulties were experienced with the test article and a Total Growth range between 10% and 50% was not achieved in the assay. None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that, under the conditions of this test, test article Zinc Oleate produced a negative response in the presence of exogenous metabolic activation.

Summary

Chemical Manufacturers Association's test article Zinc Chloride, Lot No. KTJY (MA #T2255) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence of Aroclor induced rat liver S-9. The cultures that were cloned were treated with a range of test article concentrations which produced from 2% to 77% Total Growth. Seven of ten cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that, under the conditions of this test, test article Zinc Chloride produced a positive response in the presence of exogenous metabolic activation.

0 3 4 3

## Results

The Initial Toxicity Test (Table 1) conducted on test article Zinc Chloride, Lot No. KTJY (MA #T2255) indicated complete toxicity at 50 µg/ml. The cultures were treated in the presence of 8-9. Based on these data, the test article was tested in the mutagenesis assay over a range of concentrations from 75 µg/ml to 1.0 µg/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 56, 42, 32, 24, 18, 13, 10, 7.5, 5.6 or 4.2 µg/ml. These concentrations produced a range in Suspension Growth of 10% to 95%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 2 and 3. These data are also presented graphically in Figure 1. The Cloning Data and Total Compound Toxicity Data for the positive control are presented in Table 4.

Seven cultures that were cloned exhibited mutant frequencies which ranged from 19.2 to 2.0 times the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 2% to 61%. The three remaining cultures that were cloned (7.5, 5.6 and 4.2 µg/ml) exhibited mutant frequencies which were not significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures was 62%, 77% and 57%, respectively.

**TABLE 1**

**LS178Y TR+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY  
INITIAL COMPOUND TOXICITY TEST**

Study Number : T2255.701012  
 Test Article : ZINC CHLORIDE  
 Activation : Induced Rat Liver S-9  
 Trial Number : 1  
 Dose Range : 5000 UG/ML TO 0.05 UG/ML  
 Solvent : Deionized, Distilled Water  
 Operator : Geri Breidenthal  
 Study Director : Paul E. Kirby, Ph.D.  
 Date & Time : 05/25/84 9:45 am

Test Article Concentration (µg/ml)	Cell Concentration (X 10 <sup>6</sup> )		Suspension Growth % of Control	
	Day 1	Day 2	Total	Control

**WITH S-9 ACTIVATION**

5000	0.253	0.248	0.0	0
1000	0.142	0.073	0.0	0
500	0.212	0.189	0.0	0
100	0.028	0.043	0.0	0
50	0.205	0.194	0.0	0
10	0.729	1.991	17.5	79
5	0.863	1.732	16.6	75
1	0.972	1.862	20.1	91
.5	1.061	1.865	22.0	99
.1	1.132	1.808	20.2	91
.05	1.119	1.765	21.9	99
Solvent 1	1.118	1.751	21.8	
Solvent 2	1.129	1.798	22.6	

\* Culture Lost

Table Prepared By: Geri Breidenthal 5/25/84

Workbook No. 15c Report Page No. XIII-3 Signature \_\_\_\_\_ Date \_\_\_\_\_

0 3 4 5

### Conclusion

Chemical Manufacturers Association's test article Zinc Chloride, Lot No. KTJY (MA #T2255) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence of Aroclor induced rat liver S-9. Seven of ten cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that, under the conditions of this test, test article Zinc Chloride produced a positive response in the presence of exogenous metabolic activation.

Received by RA/QA *1/13/74*  
*N.K./jkc*

Study No. T 2256. 701012  
T 2255. 701012.

L5178Y TK<sup>+/-</sup> MOUSE LYMPHOMA MUTAGENESIS ASSAY  
(with S-9 activation)

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test articles using the L5178Y TK<sup>+/-</sup> Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: Zinc Chloride and Zinc Oleate

2.2 Analysis:

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test articles (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M Street, N.W.  
Washington, D.C. 20037

3.3 Authorized Representative: Dr. Carol Stack

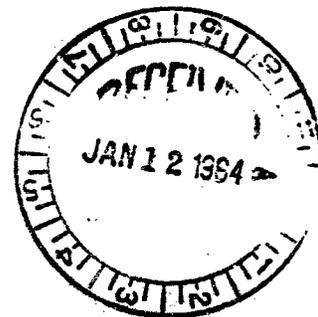
4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.



## 5.0 TEST SYSTEM

L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

### 5.1 Source

The L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

## 6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted with metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity with metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth.

Total charge for assay with metabolic activation:  
\$2,950 per test article

### 6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, or additional information concerning the test article indicates alternative doses would be better, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

## 6.2 Route and Frequency of Administration

Cell cultures will be treated once by way of a vehicle compatible with the system, in the presence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

## 6.3 Exogenous Metabolic Activation

### 6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

#### 6.3.1.1 Species, Strain, Sex and Inducer

Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

#### 6.3.1.2 Homogenate Preparation

Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

#### 6.3.1.3 S-9 Characterization

Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

0 4 0 3

### 6.3.2 S-9 Mix

The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F <sub>0</sub> P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F<sub>0</sub>P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

## 6.4 Controls

### 6.4.1 Negative Control

The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H<sub>2</sub>O, ethanol, acetone, and DMSO.

### 6.4.2 Positive Controls

Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

## 7.0 METHODS

### 7.1 Determination of Toxicity

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with S-9 activation will be conducted. Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing  $1 \times 10^6$  cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture will be added to the tubes. Each tube will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus at 25 rpm for a

4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of F<sub>10</sub>P, resuspended in 20 ml of F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

## 7.2 Testing for Mutagenic Activity

### 7.2.1 Cell Preparation

Prior to use in the assay, L5178Y TK<sup>+</sup>/<sup>-</sup> cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring TK<sup>-</sup> cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing  $0.1 \times 10^6$  cells/ml. The culture will be gassed with 5% CO<sub>2</sub> in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of F<sub>10</sub>P and reinstated in culture at  $3 \times 10^4$  cells per ml in 100 ml of F<sub>10</sub>P plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing  $1.0 \times 10^6$  cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

0405

### 7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with S-9 activation. Four ml of S-9 activation mixture will be added to the tubes. This will yield a final cell suspension of  $0.6 \times 10^6$  cells per ml.

To establish the background level of TK<sup>-/-</sup> colonies, two control tubes will receive solvent only. Two concentrations of 7,12-DMBA will be used as a positive control. All tubes will be gassed with 5% CO<sub>2</sub> in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of F<sub>10</sub>P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml F<sub>10</sub>P, gassed with 5% CO<sub>2</sub> in air, and replaced on the roller drum apparatus at 37°C.

### 7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of  $0.3 \times 10^6$  cells/ml.

### 7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the TK<sup>-/-</sup> cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.34%) which maintains the cells in suspension and allows them to form discrete colonies of TK<sup>-/-</sup> cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and that they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

...x 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield  $3 \times 10^6$  cells. This volume will be removed, the remainder of the cells discarded, and the  $3 \times 10^6$  cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A  $2 \times 10^{-4}$  dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of F<sub>10</sub>P, adding 1.0 ml of this to 9 ml F<sub>10</sub>P, and adding 1.0 ml of that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

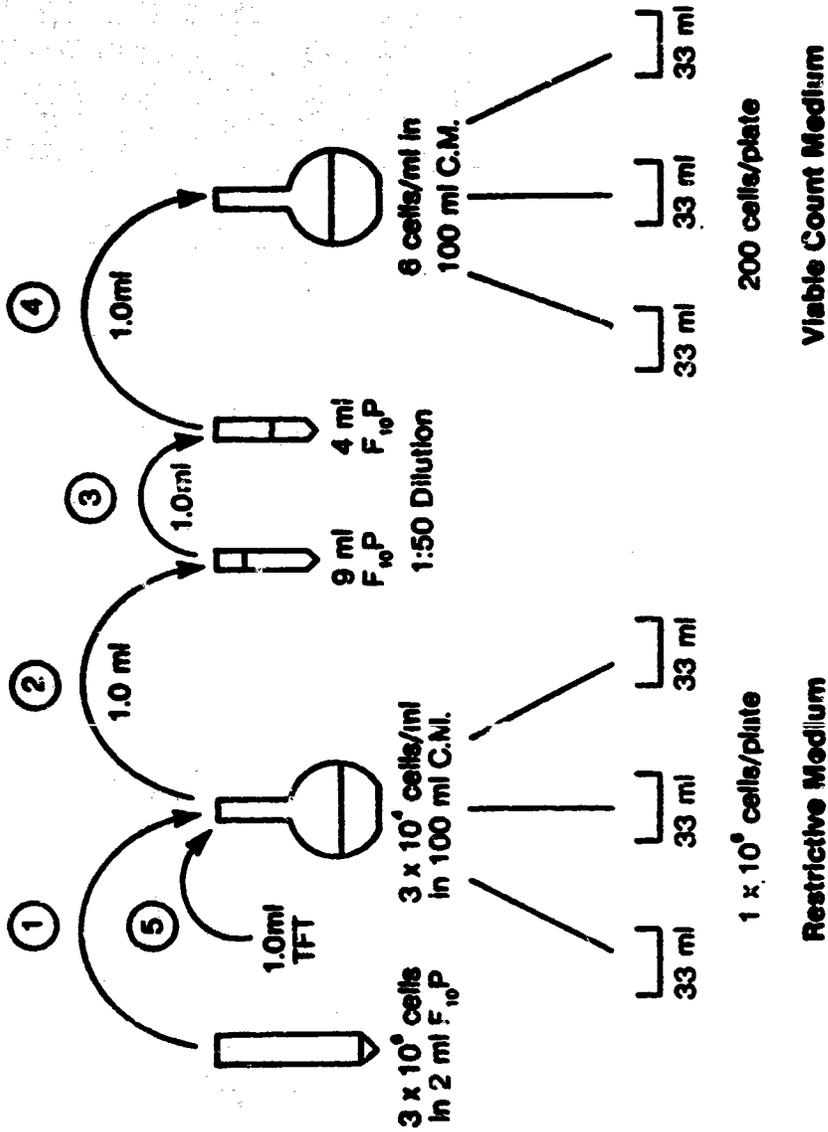
After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10-12 days.

### 7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of

0407

**ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS**



**FIGURE 1**

colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies  $\times 10^4$  in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

#### 8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

- 8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.
- 8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.
- 8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

#### 9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

- 9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.
- 9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per  $10^4$  surviving cells.
- 9.3 The plating efficiency of the solvent controls must be greater than 50%.

## 10.0 FINAL REPORT

10.1 \_\_\_\_\_ copies of the draft final report (with \_\_\_\_\_ copies of all raw data) will be sent to the Study Monitor within one month of termination. \_\_\_\_\_ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of TK<sup>-/-</sup> colonies per TFT plate for the test articles and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test articles and the controls.

10.2.2.4 The mutation frequency of each dose level of the test articles and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test articles and the positive controls.

## 11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records  
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article  
Test Article samples will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? YES  
If so, to which agency or agencies? OSHA, EPA, NIOSH,  
NCI, NIEHS, CPSC, FDA

Does the Sponsor request that samples of the Test Article dosing solutions be returned? NO

13.0 SCHEDULE OF EVENTS

- 13.1 Test Material Received Date: December 12, 1983
- 13.2 Proposed Initiation Date: January 28, 1984 <sup>PK 5/24/84</sup>
- 13.3 Scheduled Completion Date: February 24, 1984
- 13.4 Report Submission to Sponsor Date: March 2, 1984

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

Carol R. Hawk  
SPONSOR'S AUTHORIZED REPRESENTATIVE

12/19/83  
DATE PROTOCOL APPROVED BY SPONSOR

Paul E. Kirby 11/28/83  
STUDY DIRECTOR DATE

40411

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**Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay**

<b>C.M.</b>	<b>Cloning Medium</b>
<b>DMBA</b>	<b>7,12-Dimethylbenz(a)anthracene</b>
<b>EMS</b>	<b>Ethyl Methanesulfonate</b>
<b>F<sub>0</sub>P</b>	<b>Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic</b>
<b>F<sub>10</sub>P</b>	<b>F<sub>0</sub>P with 10% heat inactivated horse serum</b>
<b>NADP</b>	<b>Nicotinimide Adenine Dinucleotide Phosphate</b>
<b>S-9</b>	<b>1254, 1242 Aroclor-induced rat liver S-9</b>
<b>TFT</b>	<b>Trifluorothymidine</b>
<b>THMG</b>	<b>Thymidine, Hypoxanthine, Methotrexate and Glycine</b>
<b>V.C.</b>	<b>Viable Count</b>

FORMULAS AND CALCULATIONS FOR THE  
L5178Y TK<sup>+</sup>/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts and Final Counts

$$\text{No. of Cells/ml} = \frac{\text{Average Counts}}{(\text{corrected for coincidence})} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3 \times 10^6 \text{ cells/ml}) (20 \text{ ml})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = 20 \text{ ml} - \text{Volume of Cells Retained}$$

2. Table 1 - L5178Y TK<sup>+</sup>/- Mouse Lymphoma Mutagenesis Assay  
Initial Compound Toxicity Test

Total Suspension Growth =

Day 1 Cell Conc.      Day 2 Cell Conc.

$$\frac{0.3 \times 10^6 \text{ Cells/ml}}{\text{Day 1 Adjusted Cell Conc.}} \times \frac{\text{Day 2 Cell Conc.}}{\text{Day 1 Cell Conc.}}$$

% of Control Suspension Growth =

$$\frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than  $0.3 \times 10^6$  cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

3. L5178Y TK<sup>+</sup>/- Assay - Daily Counts (Test Article)  
and L5178Y TK<sup>+</sup>/- Assay - Daily Counts (Positive Controls)

$$\text{No. of Cells/ml} \times 10^6 = \frac{\text{Average Counts}}{(\text{corrected for coincidence})} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3 \times 10^6 \text{ Cells/ml}) (20 \text{ ml}^*)}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20 \text{ ml}^*) - (\text{Volume of Cells Retained})$$

\* The final volume is adjusted to 10 ml on the second day of expression.

4. Tables 2, 4, 6 and 7 - L5178Y TK<sup>+</sup>/<sub>-</sub> Mouse Lymphoma Mutagenesis Assay Cloning Data

$$\text{Mutant Frequency per } 10^4 \text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left( \text{Mutant Frequency of Treated Cultures} \right) - \left( \text{Average Mutant Frequency of Solvent Controls} \right)$$

5. Tables 3, 5, 6 and 7 - L5178Y TK<sup>+</sup>/<sub>-</sub> Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data

Total Suspension Growth =

$$\frac{\text{Day 1 Cell Conc.}}{0.3 \times 10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Conc.}}{\text{Day 1 Adjusted Cell Conc.}}$$

% Control Suspension Growth =

$$\frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than  $0.3 \times 10^6$  cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

$$\% \text{ Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\% \text{ Growth} = \frac{(\% \text{ Suspension Growth}) (\text{Cloning Growth})}{100}$$