

Kodak

November 27, 2006

RECEIVED
OPPT UNIT

06 NOV 28 PM 2:48

Document Control Office (DCO) (7407M)
Office of Pollution Prevention and Toxics (OPPT)
Environmental Protection Agency (EPA)
1200 Pennsylvania Ave. NW
Washington, DC 20004-0001

ATTN: 8(d) Health and Safety Reporting Rule

Subject: CAS # 142-73-4

Studies submitted by: Eastman Kodak Company
343 State Street
Rochester, NY 14650
1-800-698-3324

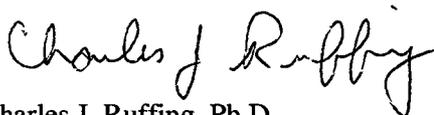
CONTAIN NO CBI

Eastman Kodak Company (Kodak) is submitting the following study (studies) in accordance with EPA's final rule on August 16, 2006, requiring submission of unpublished health and safety studies. To assist EPA, Kodak elected to submit all studies that met the reporting criteria, regardless of Kodak's manufacture or import history.

<u>Study name</u>	<u>Report Date</u>	<u>Results</u>
In vitro Transformation of balb/3T3 Cells Assay	January 1987	Positive
Mouse Lymphoma Mutagenesis Assay	2/15/1983	Negative in the presence and absence of metabolic activation
CHO HGPRT Forward Mutation Assay	March, 1983	Negative in the presence and absence of metabolic activation
In vitro Transformation of balb/3T3 Cells Assay	6/14/1984	Positive

Questions regarding this submission should be directed to: Judith M. Van Norstrand
1100 Ridgeway Avenue
Rochester, NY 14652-6267
(585) 588-6062
judith.vannorstrand@kodak.com

Sincerely,



Charles J. Ruffing, Ph.D
Director, HSE Product Stewardship
(585) 722-1311



CJV:JVN

Enc (4)

File: TSCA 8(d) Report - 2006

300474

CAS 142-73-4

RECEIVED
OPT. 6708

05 NOV 28 PM 2:49

82-0184
911551

EVALUATION OF
EK-82-0184 DI
IN THE
IN VITRO TRANSFORMATION
OF BALB/3T3 CELLS ASSAY

FINAL REPORT

SUBMITTED TO:

DR. R.L. RALEIGH, DIRECTOR
HEALTH, SAFETY AND HUMAN FACTORS LABORATORY
EASTMAN KODAK COMPANY
BUILDING 20, KODAK PARK
ROCHESTER, NEW YORK 14650

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO.: 20992

REPORT DATE: JANUARY 1987



BIONETICS

PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains Items I-IX. Items I-IV provide sponsor and test article identification information, type of assay, and the protocol reference number. Item V provides the initiation and completion dates of the study. Item VI identifies the supervisory personnel. Item VII indicates the tables and/or figures containing the test results. The interpretation of the results is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report describes the study design, which includes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices.

All test and control results presented in this report are supported by raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895.

Copies of the raw data will be supplied to the sponsor upon request.

The described study was performed in accordance with Good Laboratory Practice regulations except if noted to the contrary. To the best of the signer's knowledge there were no significant deviations from the Good Laboratory Practice regulations which affected the quality or integrity of the study.



BIONETICS

- I. SPONSOR: Eastman Kodak Company
- II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 6576
- A. Identification: EK-82-0184 DI
 - B. Date Received: August 30, 1982
 - C. Physical Description: White solid
- III. TYPE OF ASSAY: In Vitro Transformation of Balb/3T3 Cells Assay
- IV. ASSAY DESIGN NUMBER: 441
- V. STUDY DATES:
- A. Initiation: September 16, 1982
 - B. Completion: November 4, 1982
- VI. SUPERVISORY PERSONNEL:
- A. Study Director: John O. Rundell, Ph.D.
 - B. Laboratory Supervisor: Edwin J. Matthews, Ph.D.
- VII. RESULTS:

The results of the assay are presented in Tables 1, 2, and 3 on pages 4, 5 and 6.

VIII. INTERPRETATION OF RESULTS:

The test material, EK-82-0184 DI, was partially soluble in culture medium forming an acidic, fine particulate suspension at the maximum tested concentration of 10 mg/ml. Complete solubilization at this concentration was obtained by pH neutralization with 5M NaOH. Therefore, a series of dilutions of the neutralized test material in culture medium, ranging from 15610.0* to 0.953 µg/ml in two-fold dilution steps, was prepared for use in the preliminary cytotoxicity test. The cytotoxicity test measures the effect of the test material on the ability of 3T3 cells to form colonies after 72-hour exposures and is used to select concentrations for use in the transformation assay. The results of the preliminary cytotoxicity test are shown in Table 1. The treatments resulted in survivals ranging from 4.8% at 15610.0 µg/ml to approximately 89 to 106% over the 1951.0 to 0.953 µg/ml range.

*This solution concentration was derived by addition of 0.55 ml 5M NaOH to 22.31 ml of a 16.0 mg/ml acidic suspension.

VIII. INTERPRETATION OF RESULTS (Continued):

The transformation assay is normally applied to concentrations that cause survivals in the 10% to 100% range and is considered to be most sensitive near 10-20% survival, since maximum transformation frequencies for a series of model carcinogens were obtained for treatments that resulted in survivals over this range. Therefore, the concentrations of 15625.0 to 2778.0 $\mu\text{g/ml}$, corresponding to relative survivals of approximately 5% to nearly 90% (estimated graphically from the data reported in Table 1), were chosen for the assay.

The results of the transformation assay are shown in Table 2 and Table 3.

The negative control measures the frequency of the appearance of spontaneously transformed foci in the current assay. As shown in Table 2, one transformed focus was observed among the 26 negative control dishes for an average of 0.04 focus/dish. While this spontaneous transformation frequency is within the expected range of 0.0 to 0.5 focus/dish, negative control dishes with high numbers of transformed foci (>10 foci in a single dish) have been observed in other assays forming the historical negative control data base. In the present assay, dishes with high numbers of foci were observed in the positive control set; e.g., see dishes 19 and 29 (see also dish 12, 12.5 mg/ml test material). The appearance of large numbers of foci in a single dish, compared to the other dishes in a set, is considered to be caused by the respreading of one or a small number of foci by mechanical disruption during the refeeding process that occurs twice weekly for the incubation period. The assumption that the appearance of dishes with high numbers of foci results from a technical rather than a biological cause is supported by the finding that such dishes appear to occur randomly in all experimental data, in treated dishes as well as in negative controls. If such data is included in the analysis of experiments, a marked skewing of the average frequency of foci occurs that is inappropriate for application of parametric tests for statistical significances. Analyses of the distribution patterns of the transformation data showed that the data closely fit a log-normal distribution curve. Conversion of the raw data to the \log_{10} results in a normal distribution, and the presence of a few dishes with high numbers of foci does not disturb this relationship.

Bailey's modifications of Students' t-test can then be used to avoid the assumptions (intrinsic in many tests of statistical significance) of equal variances and equal numbers of entries in the treatment and control data sets. The results of these analyses of the current transformation assay data are shown in Table 4.

The positive control treatments with 5.0 $\mu\text{g/ml}$ MCA induced a total of at least 207 foci among the 29 positive control dishes (Table 2). \log_{10} analysis of these data (Table 3) showed that the positive control frequency of 4.11 foci/dish was highly significant ($p < .01$)



VIII. INTERPRETATION OF RESULTS (Continued):

compared to the negative control value of 0.03 focus/dish, and therefore the sensitivity of the assay appeared to be normal.

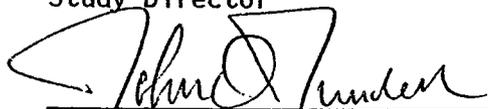
The effect of the test material treatments on 3T3 cell transformation is also shown in Tables 2 and 3. After \log_{10} analysis, the average number of foci/dish ranged from 1.22 at 12.5 mg/ml to 0.05 at 2.8 mg/ml. Compared to the negative control value, the frequency of transformed foci observed for the 12.5 mg/ml test material treatment achieved the 95% confidence level of being significantly altered. Evidence of a dose-related response was also observed over the tested dose range; and therefore, the test material was evaluated as being transforming to 3T3 cells.

IX. CONCLUSIONS:

The test material, EK-82-0184 DE, induced the appearance of a significant number of transformed foci for the 12.5 mg/ml treatment. Evidence of a dose-response was also observed over the 15.6 to 2.8 mg/ml concentration range. This concentration range corresponded to approximately 8% to nearly 90% survival in the preliminary cytotoxicity test. Therefore, the test material is considered to be active in the Balb/3T3 In Vitro Transformation Assay.

Submitted by:

Study Director



John O. Rundell, Ph.D.

Study Director

Section Chief

In Vitro Carcinogenesis

Department of Molecular Toxicology

11/17/82
Date



BIONETICS

TABLE 1

RAW DATA AND DATA SUMMARY FROM PRELIMINARY CYTOTOXICITY TESTTEST MATERIAL CODE: EK-82-0184 DELBI ASSAY NO.: 6576SOLVENT: MediumDATE SCORED: September 27, 1982

TEST MATERIAL DOSE ($\mu\text{g}/\text{ml}$)	DISH			AVERAGE NO. OF COLONIES/DISH	RELATIVE SURVIVAL (%)
	1	2	3		
15610.0	4	2	4	3.3	4.8
7805.0	31	43	30	34.7	50.4
3902.5	55	53	61	56.3	81.9
1951.3	65	60	66	63.7	92.5
975.63	63	66	55	61.3	89.1
487.81	71	71	66	69.3	100.8
243.91	63	68	72	67.7	98.4
121.95	59	72	60	63.7	92.5
60.977	63	65	56	61.3	89.1
30.488	71	61	66	66.0	95.9
15.244	80	55	65	66.7	96.9
7.622	61	62	64	62.3	90.6
3.811	69	68	93	76.7	111.4
1.906	72	78	65	71.7	104.2
0.953	76	81	61	72.7	105.6
Media Control	67	73	64	68.8	100.0
	74	80	55		



BIONETICS

TABLE 2

SUMMARY OF RAW DATA FROM TRANSFORMATION ASSAY

TEST MATERIAL CODE: EK-82-0184 DI LBI ASSAY NO. 6576 SOLVENT: Medium DATE SCORED: November 4, 1982

DOSES	NUMBER OF FOCI PER DISH SCORED																				TOTAL	NO. OF FOCI/DISH ABSOLUTE/LOG ₁₀ *
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
Negative Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	1	0.04/0.03
	0	0	0	0	1	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	1	
Positive Control	6	2	2	1	5	5	6	2	5	2	3	4	5	5	1	4	2	2	>53	20		
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	>207	~7.14/4.11
	4	4	7	4	1	0	5	4	43	C	C	C	C	C	C	C	C	C	C	C		
Test Material	15625.0	12500.0	8333.0	5556.0	2778.0	μg/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0.27/0.20
	0	0	0	0	1	3	2	1	2	1	0	50	C	C	C	C	C	C	C	C	60	5.0/1.22
	0	0	0	0	0	0	0	0	8	7	2	C	C	C	C	C	C	C	C	C	17	1.55/0.63
	0	0	0	0	0	0	0	0	0	0	0	0	1	14	C	C	C	C	C	C	16	1.07/0.32
	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	C	C	C	C	C	1	0.07/0.05

C = Contaminated
*Calculated as the antilog of the logarithmic mean (Table 3) minus one.

TABLE 3

STATISTICAL ANALYSIS OF TRANSFORMATION ACTIVITY

CLIENT: Eastman Kodak GENETICS ASSAY NO. 6576 EVALUATION DATE: November 4, 1982
 COMPOUND CODE: EK-82-0184 DI SOLVENT: Medium

Treatment Condition	Log ₁₀ Analysis* of Foci/Flask				t Statistic**	p Values**
	Mean ±	SD	n	SE		
Media Control	0.012	0.059	26	0.012	Control	Control
3-MCA, 5.0 µg/ml	0.708	0.365	29	0.068	+10.116	p<.01
<u>Test Material</u>						
15625.0 µg/ml	0.080	0.138	15	0.036	+1.837	.05<p<.1
12500.0 µg/ml	0.347	0.482	12	0.139	+2.401	.02<p<.05
8333.0 µg/ml	0.212	0.382	11	0.115	+1.734	.05<p<.1
5556.0 µg/ml	0.119	0.311	15	0.080	+1.318	.1<p<.2
2778.0 µg/ml	0.020	0.078	15	0.020	+0.368	.7<p<.8

*NOTE: Each raw data point was increased by 1.0 and converted to log₁₀ before statistical analysis was applied.

**NOTE: t-test equations:

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{\sqrt{(SE_1)^2 + (SE_2)^2}}, \text{ and}$$

$$\text{degrees of freedom} = df = \frac{1}{\frac{\mu^2}{n_1 - 1} + \frac{(1 - \mu)^2}{n_2 - 1}}$$

$$\text{where } \mu = \frac{(SD_1)^2/n_1}{(SD_1)^2/n_1 + (SD_2)^2/n_2} = \frac{(SE_1)^2}{(SE_1)^2 + (SE_2)^2}$$

SD = Standard Deviation

SE = Standard Error

NS = Not Significant

**Bailey, Norman, T.J.: Statistical Methods in Biology; Wiley and Sons, Inc., N.Y.; pg. 50, 1959.



BIONETICS

ASSAY DESIGN (NO 441)

1. OBJECTIVE

This assay evaluated the morphological transforming potential of the test material using mouse Balb/3T3 cells in culture. The objective of this semi-quantitative assay was to evaluate the test material for its ability to induce foci of transformed cells, recognized by dense, piled-up colonies on a monolayer of normal cells.

2. RATIONALE

Balb/c-3T3, 1-13 mouse cells multiply in culture until a monolayer is achieved and then cease further division. These cells, when injected into immunosuppressed, syngeneic host animals, did not produce neoplastic tumors. However, cells treated in vitro with chemical carcinogens gave rise to foci of cellular growth super-imposed on the cell monolayer. When these foci were picked from cultures, grown to larger numbers and injected into animals, a malignant tumor was obtained in most cases. Thus, the appearance of piled-up colonies in treated cell cultures at a higher frequency than in control cultures was highly correlated with malignant transformation.

3. MATERIALS

A. Indicator Cells

Clone 1-13 of Balb/3T3 mouse cells was obtained from Dr. Takeo Kakunaga. A subclone, C-14, selected for its low spontaneous frequency of foci formation, was used for the assay. Stocks were cryopreserved in liquid nitrogen and laboratory cultures were checked periodically to ensure the absence of mycoplasma contamination. Cultures were grown and passaged in Eagle's Minimum Essential Medium (EMEM) supplemented with fetal bovine serum, L-glutamine, penicillin and streptomycin.

B. Control Compounds

1. Negative Controls

A negative control consisting of assays performed on untreated cells was performed. If the test compound was not soluble in growth medium, an organic solvent was used; the final concentration of solvent in the growth medium was 1% or less or as was previously determined in solvent cytotoxicity and activity control experiments. Cells exposed to solvent in the medium were assayed as the solvent negative control to determine any effects on survival or transformation caused by the solvent alone. At least twenty dishes of the appropriate type of negative control were prepared for each assay.



BIONETICS

Litton

3. MATERIALS (Continued)

2. Positive Control

3-methylcholanthrene (MCA) is a known carcinogen and was used as a positive control for the transformation of 3T3 cells. At least 20 dishes were treated with between 2.5 and 10 µg MCA per milliliter for each assay.

C. Sample Forms

Solid materials were dissolved in growth medium, if possible, or in a compatible organic solvent. Liquids were tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

4. EXPERIMENTAL DESIGN

A. Dosage Selection

The solubility of the test chemical in growth medium or other solvent was first determined. Fifteen dose levels of the test compound were then chosen, normally starting with a maximum applied dose of 1 mg/ml for solid compounds or 1 µl/ml for liquid samples and decreasing in two-fold-dilution steps. (Note: Higher concentrations may have been tested as was required to meet the dose selection criteria.) Each dose was applied to three culture dishes seeded 24 hours earlier with 200 cells per dish. After an exposure period of 72 hours, the cells were washed and incubated in growth medium for an additional 3-5 days. The surviving colonies were stained and counted and a relative survival for each dose was obtained by comparing the number of colonies surviving treatment to the colony counts in negative control dishes. The highest dose chosen for subsequent transformation assays should normally have caused no more than an 90% reduction in colony forming ability. Four lower doses (usually including one dose with little apparent toxicity) were also selected for the transformation assay.

B. Transformation Assay

The procedure used at LBI was based on that reported by Kakunaga (1973). Twenty-four hours prior to treatment, a series of 60 mm dishes were seeded with 10^4 cells/dish and incubated. At least 20 dishes were then treated for each of the following conditions: Five preselected doses of test chemical; positive control; and solvent negative control, if applicable. The dishes were incubated for a 72 hour exposure period; the cells were then washed and incubation was continued for approximately four weeks with refeeding twice a week. The assay was terminated by fixing the cell monolayers with methanol and staining with Giemsa. The stained dishes were examined by eye and by microscope to determine the number of foci of transformed cells.



BIONETICS

Litton

5. SCORING OF TRANSFORMED FOCI

At the end of the four-week incubation period, cultures of normal cells yielded a uniformly stained monolayer of round, closely-packed cells. Transformed cells form a dense mass (focus or colony) that stained deeply (usually blue) and were superimposed on the surrounding monolayer of normal cells. The foci were variable in size.

Scored foci had several variations in morphological features. Most scored foci consisted of a dense piling-up of cells and exhibited a random, criss-cross orientation of fibroblastic cells at the periphery of the focus and extensive invasiveness into the contiguous monolayer. Other scored foci were composed of 1) more rounded cells with little criss-crossing at the periphery but with necrosis at the center caused by dense piling-up of a large number of cells, or 2) foci without the necrotic center and large number of cells but which exhibited the criss-cross pattern of overlapping cells throughout most of the colony. Foci that had these characteristics and exceeded 2 mm in diameter were scored +++ and those <2mm in diameter were designated ++.

Some densely stained areas were not scored as transformed foci. These included focal areas where some piling-up of rounded cells had occurred but the random orientation of fibroblastic cells was not observed (+). Microscopic examination was employed for scored foci and in the final judgement of transformed character for any marginal foci.

6. REFERENCES

Kakunaga, T.: A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB/3T3. *Int. J. Cancer*, 12:463-473, 1973.

Bailey, Norman T.J.: *Statistical Methods in Biology*, Wiley and Sons, Inc., NY, page 50, 1959.

7. ASSAY ACCEPTANCE CRITERIA

The assay was considered acceptable for evaluation of the test results because the following criteria were met:

1. The negative control dishes consisted of a contiguous monolayer of cells which may or may not have contained transformed foci. The lack of a contiguous sheet of cells would indicate growth conditions too poor to allow the reliable detection of weak transforming agents.
2. The negative control transformation frequency did not exceed an average of about 2-3 foci/dish after \log_{10} analysis. Attempts have been made to isolate and maintain cell stocks with a very low spontaneous frequency of transformation (e.g., subclone C-14 of Balb/c-3T3 1-13).



BIONETICS

7. ASSAY ACCEPTANCE CRITERIA (Continued)

3. The positive control yielded an average number of foci/dish that was significantly different from the negative control at the 99% confidence level.
4. A minimum of 10 dishes per test condition were available for analysis. At least 3 dose levels of test substance were assayed.
5. The dose range of test substance assayed fell within the 10-100% survival range as determined by the preliminary toxicity test, which measure relative cloning efficiencies.

8. EVALUATION CRITERIA

Historical experience with the C-14 subclone of 1-13 cells showed that the distribution of transformed foci was usually symmetric for any given treatment condition. However, large numbers of foci were observed to appear at random in one or more dishes in a set resulting in skewing of the mean number of foci in that set. This skewing appeared to be due to a technical rather than a biological cause and this conclusion was supported by the finding that dishes with high numbers of foci occurred randomly in all experimental data, in treated dishes as well as in negative controls. The appearance of large numbers of foci in a dish, compared to other dishes in a set, was considered to be caused by mechanical disruption during the refeeding process that occurred twice weekly during the 4-week assay period. Recent analyses of the historical negative control results showed that when the data was converted to logarithmic form (base 10) a normal distribution was obtained and a few dishes with abnormally high numbers of foci (e.g.: >10) did not disturb this relationship. With the transformation assay data in a normal distribution, a t-test could be applied for determining statistical significance.

Bailey's modification of Student's t-test (Statistical Methods in Biology, Wiley and Sons, Inc., NY, page 50, 1959) was used to determine whether the results for each treatment condition was significantly different from the experimental negative control ($\sim p \leq .05$ or $\sim p \leq .01$). The Study Director evaluated the results of each treatment condition in relation to the observed activities of model compounds and exercised scientific judgement in the evaluation of the test article. In general, a response at only one dose level just attaining the 95% confidence level was not considered sufficient evidence for activity in the assay. However, responses at one or more treatment levels attaining the 95% confidence level and exhibiting evidence of dose dependency were considered as positive evidence of transforming activity and responses achieving the 99% confidence level over one or more test material treatments were similarly interpreted.



BIONETICS

CAS 142-73-4

RECEIVED
OF 77 0110

03 NOV 23 PM 2:49

KAN 911551

L5178Y TK+/- MOUSE LYMPHOMA
MUTAGENESIS ASSAY

EASTMAN KODAK COMPANY

TEST ARTICLE EK 82-0184, DI



MICROBIOLOGICAL ASSOCIATES

Microbiological Associates
A Unit of Whittaker Corporation
5221 River Road
Bethesda, Maryland 20016
(301) 654-3400
Telex No. 90-8793

Whittaker

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

Sponsor: Eastman Kodak Company
Building 320, Kodak Park
Rochester, New York 14650

Testing Facility: 1530 East Jefferson Street
Rockville, Maryland 20852

Study No.: T1817.702

Test Article I.D.: EK 82-0184, DI

Test Article Lot No.: None Provided

Test Article Description: White Powder

Storage Conditions: Room Temperature

Date Received: 9/1/82

Date Study Started: 10/11/82

Date Study Completed: 2/4/83

Report Date: 2/15/83

Study Coordinator: Eugene D. Barber, Ph.D.
Eastman Kodak Company

Study Director: Paul E. Kirby, Ph.D.
Microbiological Associates

<u>Paul E. Kirby</u> 2/4/83 Paul E. Kirby, Ph.D. Date Study Director	<u>Rose F. Pizzarello</u> 2/4/82 Rose F. Pizzarello Date Laboratory Manager
<u>Andrea M. Rogers-Back</u> 2/4/83 Andrea M. Rogers-Back, Ph.D. Date Staff Scientist	<u>Alan Cohen</u> 2/10/83 Alan Cohen Date Group Leader
<u>Jane J. Clarke</u> 2/4/83 Jane J. Clarke Date Group Leader	<u>Ava P. Budacz</u> 2/14/83 Ava P. Budacz Date Biologist
<u>Leah C. Griswold</u> 2/4/83 Leah C. Griswold Date Biologist	<u>Janine L. Johnson</u> 2-4-83 Janine L. Johnson Date Biologist
<u>Reginald A. Voglezon</u> 2/4/83 Reginald A. Voglezon Date Laboratory Assistant	

Summary

Eastman Kodak Company's test article EK 82-0184, DI (MA #T1817) 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 53%* to 72% Total Growth. The S-9 activated cultures were cloned over a range of concentrations which produced from 27% to 94% Total Growth.

None of the cultures that were cloned (nonactivated or S-9 activated) exhibited mutant frequencies that were significantly different from the mean mutant frequency of the solvent controls. There was no indication of a dose response relationship.

The results indicate that under the conditions of this test, test article EK 82-0184, DI produced a negative response in the presence and absence of exogenous metabolic activation.

*Culture 9996 µg/ml B had contaminated TFT plates. The suspension growth of this culture was low in comparison to its duplicate and the suspension growth of the remaining cultures. It is probable that this culture was contaminated prior to cloning, and therefore the results for this culture will not be discussed in this report.

Introduction

Eastman Kodak Company's test article EK 82-0184, DI (MA #T1817) was received on September 1, 1982 for testing in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay with and without exogenous metabolic activation by Aroclor induced rat liver microsomes.

Objective

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

Materials and Methods

The experimental protocol (see Appendix) is 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 1×10^6 cells per ml. The solubility of the test article was determined in Acetone, Ethanol and H₂O, and H₂O was selected as the solvent of choice. The test article was solubilized and diluted for testing at 5000, 1000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1, and 0.05 µg/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 S-9 activation mixture or 4 ml of medium was added to each tube depending on whether or not they received activation. Each tube was gassed with 5% CO₂ 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 x g for 10 minutes and decanting the supernatant. The cells were washed twice in 10 ml of F₁₀P, re-suspended in 20 ml of F₁₀P, gassed with 5% CO₂ 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 1ml 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 with programs labeled "Cell Culture Adjustment" and "Initial Toxicity".

B. 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.¹ Three ml of THMG stock solution was added to a 100 ml cell suspension containing 0.1×10^6 cells per ml. The culture was gassed with 5% CO₂ 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 F₁₀P and reinstated in culture at

¹Clive, 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.

3×10^4 cells per ml in 100 ml of F₁₀P 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 1.0×10^6 cells per ml was prepared, and 6 ml aliquots were dispensed in Corning polypropylene centrifuge tubes.

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 diluted to produce evenly spaced dose levels which decreased approximately 10-fold from highest to lowest dose. The test article was added to duplicate cultures labeled with the test article T No., test concentration (A or B) and NA or S-9, in amounts at which the final solvent concentration was nontoxic to the cells. The compound was tested with and without S-9 activation. Four ml of S-9 activation mixture was added to half of the tubes and 4 ml of F₀P was added to the other half. This yielded a final cell suspension of 0.6×10^6 cells per ml.

Two control tubes received solvent only and the positive controls were treated with EMS (1.0 and 0.5 µl/ml) and 7,12-DMBA (7.5 and 5.0 µg/ml). All tubes were gassed with 5% CO₂ 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 F₁₀P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells were resuspended in 20 ml of F₁₀P, gassed with 5% CO₂ 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 0.3×10^6 cells per ml.

4. Cloning

At the end of the expression period, the cells were placed in cloning medium (C.M.) containing 0.35% Noble agar. TFT at a final concentration of 3 µg/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 or not 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 pre-warmed 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 (A or B), whether or not activation was used, 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 3×10^6 cells. This volume was removed, the remainder of the cells were discarded, and the 3×10^6 cells were replaced in the centrifuge tube. The cells were centrifuged at $1000 \times g$ 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 2×10^{-4} dilution was carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml of $F_{10}P$, 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 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 $37^{\circ}C$.

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 ($4^{\circ}C$) for 20 minutes. The plates were removed and incubated at $37^{\circ}C$ in a humidified 5% CO_2 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 frequency and toxicity data calculations were performed using a Texas Instruments TI-59 calculator with programs labeled "IMF" and "Total Compound Toxicity".

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

Control Articles:

Ethyl Methanesulfonate	$\text{CH}_3\text{SO}_3\text{C}_2\text{H}_5$
CAS Registry Number	62-50-0
Assay (by GLC)	98% min
7,12-Dimethylbenz(a)anthracene	
CAS Registry Number	57-97-6
Assay (by UV-VIS)	95% min
Melting Range	2°C range including 123°C

Solvents:

Acetone	CH_3COCH_3
CAS Registry Number	67-64-1
Assay (CH_3COCH_3)	Not less than 99.5%
Isopropyl Alcohol [$(\text{CH}_3)_2\text{CHOH}$]	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	$56.1 \pm 0.1^\circ\text{C}$
Residue after Evaporation	0.001%
Solubility in Water	Clear
Acidity (CH_3COOH)	0.002%
Alkalinity (as NH_3)	0.001%
Aldehyde (HCHO)	0.002%
Methanol (CH_3OH) (by G.C.)	0.05%
Subs Reducing KMnO_4 (Color)	Pass test
Water (H_2O)	0.5%
DMSO	$(\text{CH}_3)_2\text{SO}$
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)	15

Ethanol	C ₂ H ₅ 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 Rockville, 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.

Results

The Initial Toxicity Test (Table 1) conducted on test article EK 82-0184, DI (MA #T1817) indicated a threshold level of complete toxicity at concentrations greater than 5000 µg/ml for both the nonactivated and the S-9 activated cultures. Based on these data, the test article was tested in the mutagenesis assay over a range of concentrations from 9996 µg/ml to 1000 µg/ml for both the non-activated and the S-9 activated assays.* In both the nonactivated and the S-9 activated assays the test article was not soluble at concentrations greater than 1000 µg/ml. Cultures were treated with a suspension of test article. All treated cultures required the addition of 1 N NaOH to correct the pH.

After a two day expression period, ten nonactivated and twelve S-9 activated cultures were cloned based on their degree of toxicity. The nonactivated cultures were cloned at 9996,** 8711, 7426, 6140 and 4855 µg/ml. These concentrations produced a range of Suspension Growth from 49% to 71%. The S-9 activated cultures were cloned at 8711, 7426, 6140, 4855, 3570 and 2285 µg/ml. These concentrations produced a range in Suspension Growth from 26% to 92%. The Cloning Data are presented in Tables 2, 3 and 4 and the Total Compound Toxicity Data are presented in Tables 5, 6 and 7. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1 (without activation) and Figure 2 (with S-9 activation).

*Three S-9 activated assays were performed. Two were lost due to contamination. The results of the third assay are discussed in this report.

**Culture 9996 µg/ml B had contaminated TFT plates. The suspension growth of this culture was low in comparison to its duplicate and the suspension growth of the remaining cultures. It is probable that this culture was contaminated prior to cloning, and therefore the results for this culture will not be discussed in this report.

None of the cultures that were cloned, whether treated in the presence or absence of metabolic activation, exhibited mutant frequencies which were significantly different from the mean mutant frequency of the solvent controls. The % Total Growth of the cultures ranged from 53% to 72% for the nonactivated cultures and from 27% to 94% for the S-9 activated cultures.

Study No. 1 T1817 702

Table 1

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

1817-702 Study Number W. E. Kirby Study Director 5000 µg/d to 0.05 µg/d Dose Range
ZK 82-0189 DI (T1817) Test Article Identity Stoule H₂O / WASH solvent
deionized distilled

	Test Article Concentration	Cell Concentration (x10 ⁶)		Suspension Growth	
		Day 1	Day 2	Total	% of Control
Without Activation	5000 µg/d	0.987	1.316	19.4	81%
	1000 µg/d	1.121	1.252	15.6	88%
	500 µg/d	1.203	1.573	21.0	118%
	100 µg/d	1.240	1.344	18.5	104%
	50 µg/d	1.241	*		
	10 µg/d	0.853	*		
	5.0 µg/d	1.226	*		
	1.0 µg/d	1.175	*		
	0.5 µg/d	1.240	*		
	0.1 µg/d	1.223	*		
	0.05 µg/d	1.226	*		
	Solvent 1	1.150	1.350	17.3	17.8
	Solvent 2	1.240	1.328	18.3	
	With S-9 Activation	5000 µg/d	0.685	1.420	10.8
1000 µg/d		0.811	1.277	12.4	85%
500 µg/d		0.931	1.588	14.4	99%
100 µg/d		0.957	1.367	14.5	100%
50 µg/d		0.950	*		
10 µg/d		1.013	*		
5.0 µg/d		0.978	*		
1.0 µg/d		1.015	*		
0.5 µg/d		0.961	*		
0.1 µg/d		0.962	*		
0.05 µg/d		1.001	*		
Solvent 1		0.973	1.329	14.8	14.5
Solvent 2		0.958	1.335	14.2	

* Culture Lost

Table Prepared By W. E. Kirby Signature 10-13-82 Date

Form No. WL-233 Workbook Page No. 13 Report Page No. _____
2/25/82 * These cultures were not counted on the second day. ARB 12/13/82

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

Test Article Conc.	No. of Colonies/TFT Plate			Avg. #/ Plate	No. of Colonies/V.C. Plate			Avg. #/ Plate	Mutant* Frequency	Induced Mutant* Frequency		
	1	2	3		1	2	3					
Solvent 1	53	44	51	49	154	139	178	157	0.6	0.7		
Solvent 2	56	59	+	58	148	143	159	150	0.8	0.7		
9996 A	55	49	67	57	175	159	171	168	0.7	0.0		
9996 B	+	+	+		143	163	171	159				
8711 A	51	49	31	44	+	+	+					
8711 B	37	35	48	40	182	190	188	189	0.4	-0.3		
7426 A	62	52	47	54	162	162	149	158	0.7	0.0		
7426 B	35	29	46	37	170	173	141	161	0.5	-0.2		
6140 A	68	68	51	62	210	210	190	203	0.6	-0.1		
6140 B	48	53	38	46	161	160	143	155	0.6	-0.1		
4855 A	90	35	59	35	163	170	167	167	0.4	-0.3		
4855 B	+	+	+		7	+	+					
Test Article Identity												
Study Number	T1817.702			Study Director			Paul E. Kirby			Experiment Number		
Test Article Identity	EK82-0184DI (T1817)			Sterile, deionized distilled H ₂ O			Solvent			None		
Metabolic Activation												
Plates Counted By: <i>David Clarke</i> 6/2/82												
Calculations Performed By: <i>John N. Hayes - B</i>												
(Signature & Date) (11/3/82)												

* Per 10⁴ surviving cells

+ Culture Lost

Study No. 11817.702

~~11817.702~~ ~~11/3/82~~ ~~11/3/82~~
* incorrect stamp.

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

Test Article Conc.	No. of Colonies/TFT Plate			Avg. #/ Plate	No. of Colonies/V.C. Plate			Avg. #/ Plate	Mutant* Frequency	Induced Mutant* Frequency
	1	2	3		1	2	3			
Solvent 1	46	46	34	42	225	189	223	212	0.43	0.5
Solvent 2	42	48	58	49	151	187	165	168	0.6	
8711 ⁴⁹ / _m /A	+	+	+		238	210	210	219		
8711 ⁴⁹ / _m /B	48	23	30	34	208	202	200	203	0.3	-0.2
7426 ⁴⁹ / _m /A	48	60	58	55	224	182	187	198	0.6	0.1
7426 ⁴⁹ / _m /B	++	++	++		++	++	++			
6140 ⁴⁹ / _m /A	42	45	50	46	212	+	223	218	0.4	-0.1
6140 ⁴⁹ / _m /B	+	+	+		+	+	+			
4855 ⁴⁹ / _m /A	45	48	52	48	208	199	233	213	0.5	0.0
4855 ⁴⁹ / _m /B	38	40	37	38	219	164	172	185	0.4	-0.1
3570 ⁴⁹ / _m /A	65	60	61	62	175	189	210	191	0.6	0.1
3570 ⁴⁹ / _m /B	60	52	61	58	213	199	212	208	0.6	0.1
2285 ⁴⁹ / _m /A	62	49	54	55	200	215	198	204	0.5	0.0
2285 ⁴⁹ / _m /B	48	65	40	51	201	187	197	195	0.5	0.0

71817.702 Study Number
 Paul E. Kirby Study Director
 181784 Experiment Number

EK 82-0184 DI (1817) Test Article Identity
 Sterile Deionized Distilled H₂O Solvent
 Induced Rat Liver S-9
 Metabolic Activation

Plates Counted By: Karen M. Campbell 12/6/82 Calculations Performed By: Karen M. Campbell 12/6/82
 (Signature & Date) (Signature & Date)

* Per 10⁴ surviving cells
 ++ too toxic to clone
 + Culture lost

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

Study Number		Study Director				Experiment Number				
T1817.702		Paul E. Kirby				1817B7				
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)										
1.0ul/ml	3	4	2	3	0	0	0	0		
0.5ul/ml	203	182	196	194	13	23	15	17	22.8	22.1
SOLVENT 1	41	49	56	49	126	127	176	141	0.7	0.7
SOLVENT 2	38	56	45	46	135	133	130	133	0.7	0.7
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1										
SOLVENT 2										
Plates Counted By: <u>Jane Flauder 11/2/82</u> Calculations Performed By: <u>John A. Hayes - Beale 11/3/82</u> (Signature & Date) (Signature & Date)										

*Per 10⁴ surviving cells
+ Culture Lost

Table 4 Cont.

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

Study Number		Paul E. Kirby		Study Director		Experiment Number		1817B4	
Test Article Conc.	No. of Colonies/ TFT Plate			Ave. #/ Plate	No. of Colonies/V.C. Plate			Ave. #/ Plate	Induced * Mutant Frequency
	1	2	3		1	2	3		
Ethyl Methanesulfonate (No Activation)									
1.0µl/ml									
0.5µl/ml									
SOLVENT 1									
SOLVENT 2									
7,12 Dimethylbenz(a)anthracene (With Activation)									
7.5 µg/ml	179	171	189	180	27	49	29	35	9.8
5.0 µg/ml	250	232	231	238	149	112	131	131	3.6
SOLVENT 1	39	38	36	38	191	202	203	199	0.4, 0.5
SOLVENT 2	38	46	53	46	200	184	203	196	0.5
Plates Counted by: Karen M. Campbell 12/6/82 Calculations Performed By: Karen M. Campbell 12/6/82 (Signature & Date) (Signature & Date)									

Study No. 1817 702

*Per 10⁶ surviving cells

1 Culture Tank

Table 5

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

Study Number		Study Director		Experiment Number		
<u>T1817.702</u>		<u>Paul E. Kirby</u>		<u>1817B1</u>		
Test Article Identity		Solvent		Metabolic Activation		
<u>EK82-0184 DI (T1817)</u>		<u>H₂O / NaOH</u>		None		
Test Article Concentration	Cell Concentration X 10 ⁶ cells/ml Day 1	Cell Concentration X 10 ⁶ cells/ml Day 2	Suspension Growth Total	Suspension Growth % Control	Cloning Growth Avg. V. C. & Control	% Total Growth
SOLVENT 1	1.300	1.586	22.9	22.4	157	154
SOLVENT 2	1.328	1.485	21.9	22.4	150	154
9996 ¹ A	0.619	1.595	* 11.0	4.1%	168	109% * 33%
9996 B	0.188	1.050	3.5	16%	159	103%
8711 ¹ A	0.664	1.605	11.8	53%	+	16%
8711 B	0.630	1.619	11.3	51%	189	123%
7426 ¹ A	0.771	1.603	13.7	61%	158	103%
7426 B	0.676	1.572	11.8	53%	161	105%
6140 ¹ A	0.625	1.536	10.7	48%	203	132%
6140 B	0.983	1.462	16.0	71%	155	101%
4855 ¹ A	0.828	1.548	14.2	64%	167	108%
4855 B	0.498	1.575	8.7	39%	+	69%
A						
B						
A						
B						
A						
B						

Tables prepared and calculations performed by: Shirley M. Mason - Bunk

* Survey 10/1/67

+ Culture Lost

Table 6

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

Test Article Concentration	Cell Concentration Day 1	Cell Concentration X 10 ⁶ cells/ml Day 2	Suspension Growth % Control		Cloning Growth Avg. V. C. & Control		% Total Growth
			Total	% Control	Avg. V. C.	% Control	
SOLVENT 1	1.031	1.600	18.33	18.5	2122	190	
SOLVENT 2	1.034	1.622	18.6		1685		
9996 μ g/ml A	0.0	0.043	0.0	0%	+	+	
9996 μ g/ml B	0.291	0.089	0.0	0%	+	+	
8711 μ g/ml A	0.666	1.470	10.9	59%	219	115%	68%
8711 μ g/ml B	0.374	1.140	4.7	26%	203	107%	27%
7426 μ g/ml A	0.621	1.449	10.0	54%	198	104%	56%
7426 μ g/ml B	0.182	0.154	0.0	0%	+	+	
6140 μ g/ml A	0.629	1.514	10.6	57%	218	115%	66%
6140 μ g/ml B	0.649	1.464	10.6	57%	+	+	
4855 μ g/ml A	0.664	1.535	11.3	61%	213	112%	69%
4855 μ g/ml B	0.749	1.541	12.8	69%	185	97%	67%
3570 μ g/ml A	0.844	1.566	14.7	79%	191	101%	80%
3570 μ g/ml B	0.805	1.564	14.0	76%	208	109%	83%
2285 μ g/ml A	0.876	1.603	15.6	84%	204	107%	91%
2285 μ g/ml B	0.961	1.592	17.0	92%	195	103%	94%
A							
B							

Study Number: 71817.702
 Study Director: Paul E. Kirby
 Test Article Identity: EK 82-0184 DTC (1817)
 Solvent: Sterile Endotoxin Free H₂O
 Experiment Number: 1817B4
 Induced Rat Liver S-9 Metabolic Activation

Tables prepared and

Table 6

Table 7

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

Study Number		Study Director		Experiment No.	
T1817.702		Paul E. Kirby		1817B1	
Test Article Concentration	Cell Concentration x 10 ⁶ cells/ml		Suspension Growth Total	Cloning Growth Ave. V.C.	% Total Growth
	Day 1	Day 2			
Ethyl Methanesulfonate (No Activation)					
1.0 µl/ml	0.413	0.372	1.7	0	12%
0.5 µl/ml	0.619	0.703	4.8	17	34%
SOLVENT 1	1.080	1.235	14.8	141	137
SOLVENT 2	1.040	1.206	13.9	133	4%
7,12 Dimethylbenz(a)anthracene (With Activation)					
7.5 µg/ml					
5.0 µg/ml					
SOLVENT 1					
SOLVENT 2					

Tables prepared and calculations performed by: Andrea M. Rogers-Bull
 (Signature & Date) (1/8/82)

+ Culture Lost
 ++ Too Toxic to Clone

Table 7 Cont.

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

Study No. 1817 702

Study Number		<u>T1817.702</u>		Study Director		<u>Paul E. Kirby</u>		Experiment No.		<u>1817B4</u>	
Test Article Concentration	Cell Concentration x 10 ⁶ cells/ml		Suspension Growth Total	Cloning Growth Ave. V.C.	% Control	% Total Growth					
	Day 1	Day 2									
Ethyl Methanesulfonate (No Activation)											
1.0 µl/ml											
0.5 µl/ml											
SOLVENT 1	A.A		K.C		12/6/82						
SOLVENT 2											
7,12 Dimethylbenz(a)anthracene (With Activation)											
7.5 µg/ml	0.469	0.655	3.4	22%	35	18%					
5.0 µg/ml	0.632	1.401	9.8	63%	131	66%					
SOLVENT 1	0.920	1.452	14.8	15.5	199	198					
SOLVENT 2	0.951	1.529	16.2		196						

Tables prepared and calculations performed by: Karen M. Campbell/12/82
(Signature & Date)

+ Culture Lost
++ Too Toxic to Clone

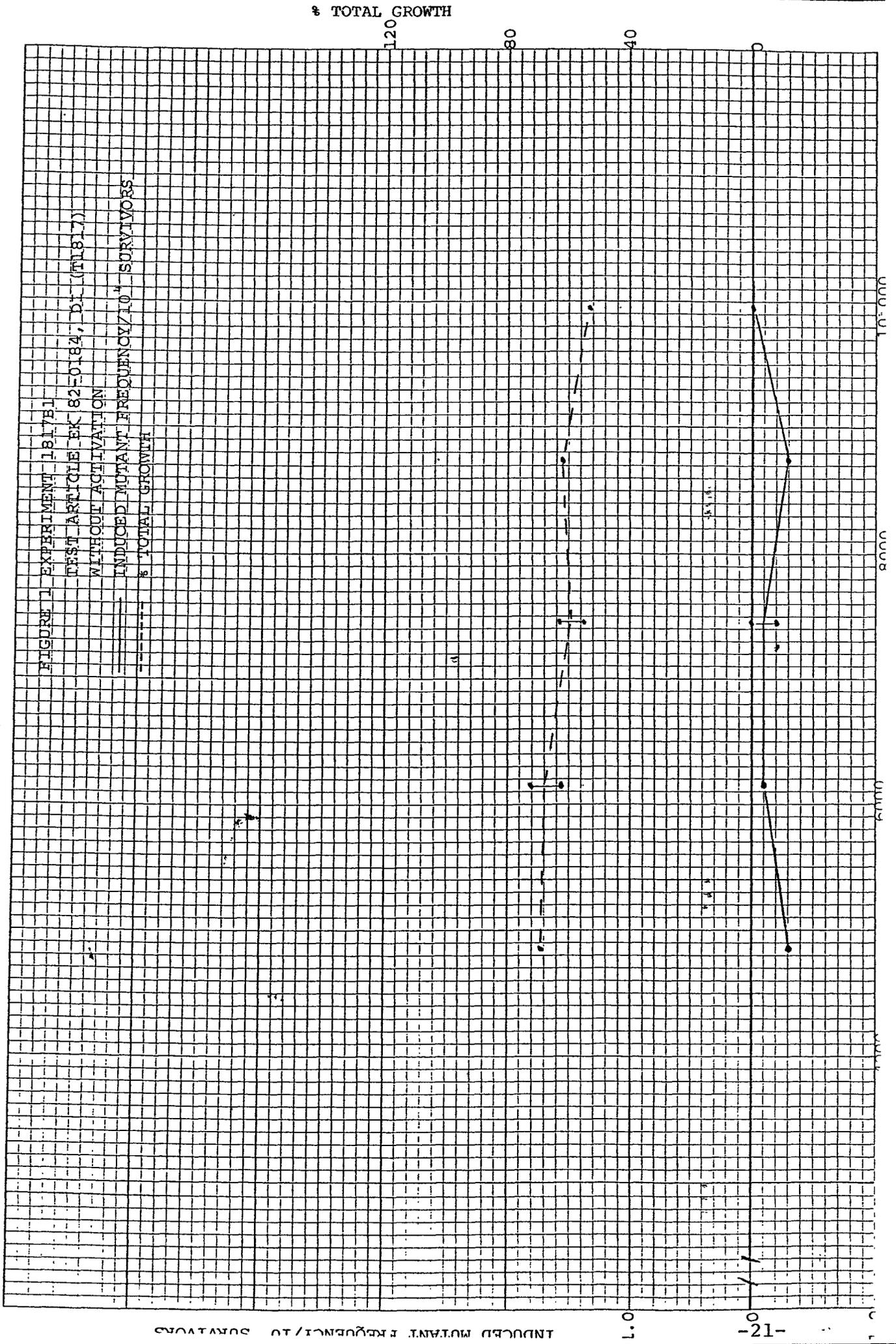
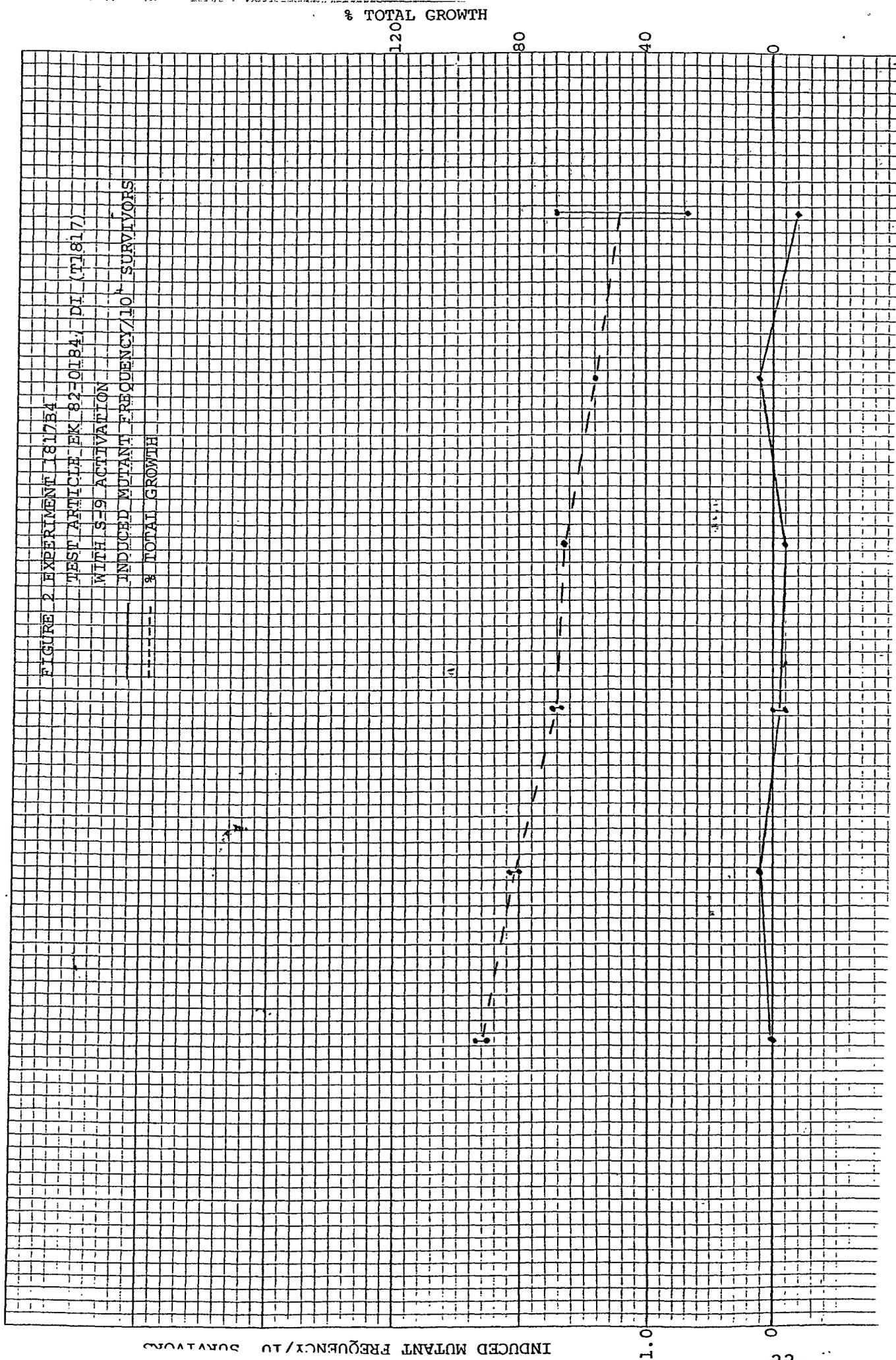


FIGURE 2 EXPERIMENT 18117B4
TEST ARTICLE EK 82-0184, DI (T1817)
WITH S-9 ACTIVATION
INDUCED MUTANT FREQUENCY/10⁸ SURVIVORS
----- % TOTAL GROWTH



Conclusion

Eastman Kodak Company's test article EK 82-0184, DI (MA #T1817) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. None of the cultures that were cloned, whether treated in the presence or absence of metabolic activation, exhibited mutant frequencies which were significantly different from the mean mutant frequency of the solvent controls and there was no indication of a dose response relationship. In addition, the test article was not very toxic to L5178Y cells.

The results indicate that under the conditions of this test, test article EK 82-0184, DI produced a negative response in the presence and absence of exogenous metabolic activation.

APPENDIX

*Rec'd. by RA/DA 9/27/82.
N.K./jrc*

REC'D AUG 27 1982

 **MICROBIOLOGICAL
ASSOCIATES**

Microbiological Associates
A Unit of Whittaker Corporation
5221 River Road
Bethesda, Maryland 20816
(301) 654-3400
Telex No. 90-8793

Whittaker

Protocol 1507.702

L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay

Sponsor: EASTMAN KODAK COMPANY

Testing Facility: Microbiological Associates
1530 East Jefferson Street
Rockville, Maryland 20852

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

Reason for Choice: The L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay is a sensitive indicator of mutagenic activity of a broad range of chemical classes.

Route of Administration: In vitro by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation.

Reason for Choice: This route of administration has been demonstrated to be useful in the detection of chemical mutagens in this system.

Test Article I.D.*: EK 82-0184, DI

*This test article has been adequately characterized by the sponsor.

Proposed Starting Date: *October 11, 1982* *OK 12/9/82*
November 15, 1982

Proposed Completion Date: *December 22, 1982*

The stipulations of this protocol will be implemented in conformance with Good Laboratory Practice Regulations (21 CFR, Part 58) for nonclinical laboratory studies.

Will this study be submitted to a regulatory agency? don't know
If so, to which agency or agencies? _____

If this study is to be submitted to the Food and Drug Administration, test substance characterization, determination of the stability of the test article and analytical measurement of each test or control article that is mixed with a solvent must be made. Microbiological Associates does not perform these determinations as part of this protocol.

The sponsor does does not (check one) request that samples of the test article solutions be returned.

Eugene D. Barber
Sponsor's Study Coordinator

August 24, 1982
Date Protocol Approved by Sponsor

Paul E. Kirby 9/27/82
Study Director Date

IN VITRO MAMMALIAN MUTAGENESIS ASSAY
(L5178Y TK+/- MOUSE LYMPHOMA ASSAY)

Microbiological Associates offers an In Vitro Mammalian Mutagenesis Assay which detects mutations at the thymidine kinase (TK) locus of L5178Y mouse lymphoma cells. A discussion on the theoretical basis of this assay has been presented by Clive and Spector.¹

L5178Y mouse lymphoma cells, which are heterozygous at the TK locus for the enzyme thymidine kinase, are exposed to potential mutagens, and the forward mutation rate to a TK deficient state (TK-/-) is determined.

The mammalian mutagenesis assay offered by Microbiological Associates consists of an initial toxicity test, the mutagenesis test, and preparation of the final report. The L5178Y TK+/- mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay at Microbiological Associates were obtained directly from Dr. Donald Clive, Burroughs Wellcome, Research Triangle Park, North Carolina. Details of the experimental procedures and the method of reporting the resulting data are described below.

A. DETERMINATION OF TOXICITY

In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and without S-9 activation is conducted. Cultures are 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 1×10^6 cells per ml. The test article is solubilized and diluted in an appropriate solvent. Unless solubility limits are exceeded, solid test

¹Clive, 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.

articles are tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 $\mu\text{g/ml}$, and liquid test articles are tested at 50, 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01 and 0.005 $\mu\text{l/ml}$. The test article is added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is non-toxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium is added to each tube depending on whether or not they receive activation. Each tube is gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at approximately 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions are prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article is removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells are washed twice in 10 ml of F_{10}P , resuspended in 20 ml of F_{10}P , gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Test article toxicity is determined by comparing the cell population growth at each dose level with that of the solvent controls. Cell population density is 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.

The stability of the test article under the actual experimental conditions used in this study is not determined by Microbiological Associates. All test article stock solutions are freshly prepared immediately before their use in each procedure.

B. TESTING FOR MUTAGENIC ACTIVITY

1. Cell Preparation

Prior to use in the assay, L5178Y cells which are actively growing in culture are cleansed as described by Clive, et al.¹, to reduce the frequency of spontaneously occurring TK-/- cells. Three ml of THMG stock solution is added to a 100 ml cell suspension containing 0.1×10^6 cells per ml. The culture is gassed with 5% CO₂ in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours the THMG is removed by pelletizing the cells and decanting the supernatant. The cells are rinsed in 20 ml of F₁₀P and reinstated in culture at 3×10^4 cells per ml in 100 ml of F₁₀P plus 1 ml of THG stock solution. The cells are ready for use after 72 hours incubation. Fresh cultures are periodically started from the cryopreserved stock.

The cell population density of the prepared cultures is 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 1.0×10^5 cells per ml is prepared, and 6 ml aliquots are dispensed in Corning polypropylene centrifuge tubes.

2. Test Compound Preparation

Based on the data derived from the toxicity test, the test article is prepared so that the highest concentration is 100% toxic. The test article is solubilized and diluted to produce evenly spaced dose levels which will decrease approximately 10-fold from highest to lowest dose. The test article is added to duplicate cultures labeled with the test article T no., test concentration A or B and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The

¹
Ibid.

compound is tested with and without S-9 activation. Four ml of S-9 activation mixture is added to half of the tubes and 4 ml of F₀P is added to the other half. This yields a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of TK-/- colonies, four control tubes receive solvent only. Two concentrations of EMS and 7,12-DMBA are used as positive controls for direct acting mutagens and promutagens, respectively. All tubes are gassed with 5% CO₂ in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article is carried out under amber lighting and the cells are incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells are washed twice in 10 ml of F₁₀P by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells are resuspended in 20 ml of F₁₀P, gassed with 5% CO₂ in air, and replaced on the roller drum apparatus at 37°C.

3. Expression Time

In order for induced mutations to be expressed, the cells must undergo several divisions. This period has been designated as the expression time. After the initial exposure to the test article, the cells are incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment is made by taking daily cell counts and then replacing a volume of cells with fresh medium which will yield a cell population density of 0.3×10^6 cells per ml.

4. Cloning

At the end of the expression period, the cells must 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 µg/ml).

The C.M. contains 0.35% Noble agar which maintains the cells in suspension and allows them to form discrete colonies of TK-/ τ cells.

a. General Preparation

For cloning, the test article dose levels which exhibit toxicity from approximately 5% to 95% growth inhibition during the expression period are selected. Two Florence flasks per culture to be cloned are labeled with the compound concentration and whether or not they received S-9 activation. For each pair of flasks one is labeled TFT and one is labeled V.C. (viable count). Each flask is 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 are labeled with the concentration (A or B), whether or not activation was used, and the experiment number. Three of the six are labeled TFT, and three are labeled V.C.

b. Cell Plating (See Figure 1)

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

A 2×10^{-4} dilution is carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of F₁₀P, adding 1.0 ml of this to 9 ml of F₁₀P, 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

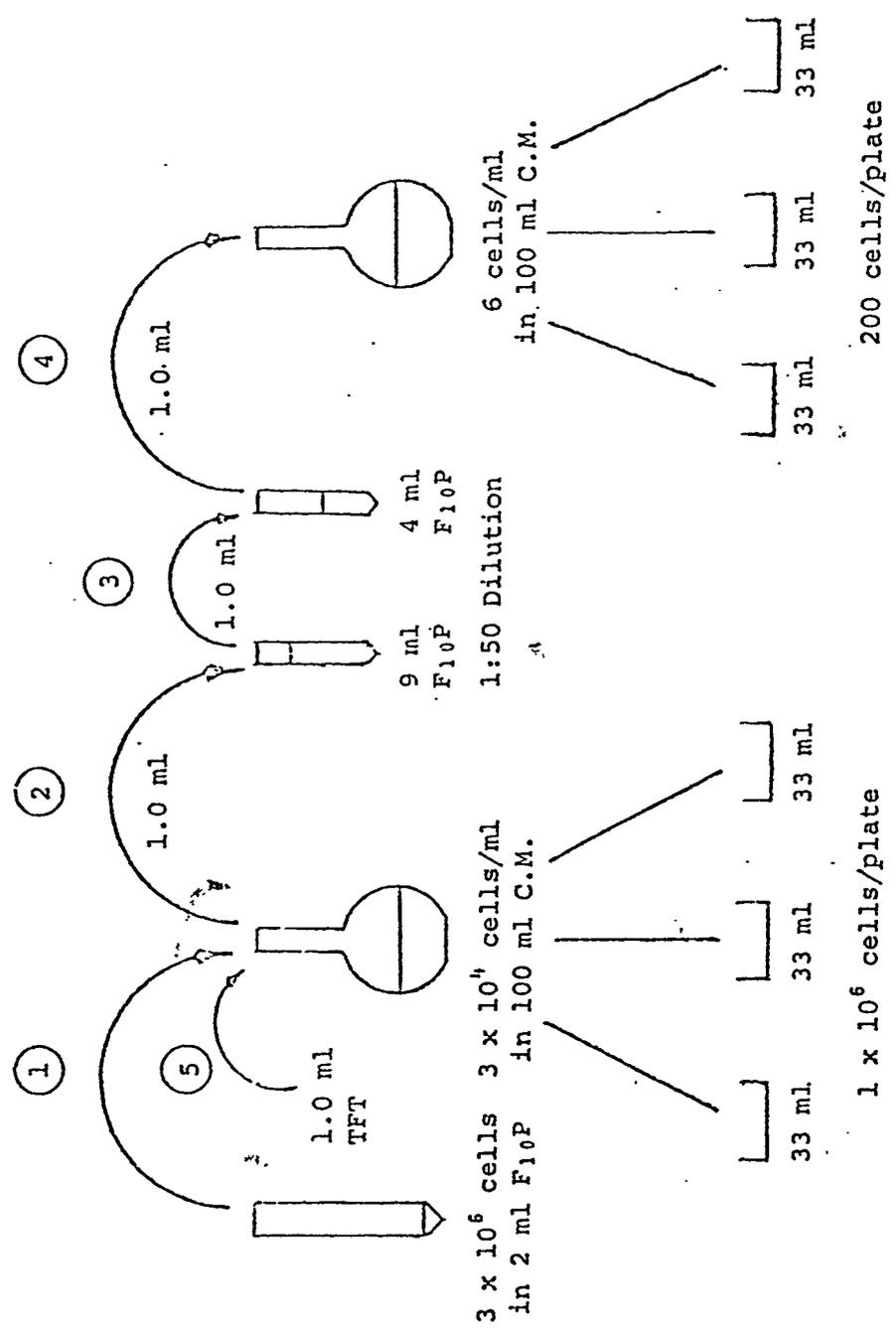


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

restrictive agent is added to the TFT flask, and both this flask and the V.C. flask are placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks are removed one at a time, and 33 ml of the cell suspension is pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates are placed in cold storage (4°C) for 20 minutes. The plates are removed and incubated at 37°C in a humidified 5% CO₂ atmosphere for 10-12 days.

5. Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates are scored for the total number of colonies per plate. Three counts per plate are made on an automatic colony counter, and the median count is recorded. In some instances the plates will be counted by hand. The mutation frequency is 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.

C. FINAL REPORT

Microbiological Associates provides a report which includes the following information:

1. The data from the toxicity test.
2. The data generated by the mutagenesis assay which includes:
 - a. The % total growth at each dose level which reflects test article toxicity.
 - b. The number of TK-/- colonies per TFT plate for the test article and the controls.
 - c. The number of colonies per V.C. plate for the test article and the controls.

- d. The mutation frequency of each dose level of the test article and the controls.
- e. The induced mutation frequency of each dose level of the test article and the positive controls.

The following criteria are 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.

Abbreviations Used in L5178Y Mouse
Lymphoma Mutagenesis Assay

C.M. : Cloning Medium

DMBA : 7,12 Dimethylbenz(a)Anthracene

EMS : Ethyl Methanesulfonate

F₀P : Fisher's Media for Leukemic Cells of Mice w/0.1%
Pluronic

F₁₀P : F₀P 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

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

1. Initial Toxicity Daily Counts (Form No. WL-180) and Final Counts (Form No. WL-179).

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

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

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

2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Initial Compound Toxicity Test (Form No. WL-233).

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

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

Cultures containing less than 0.3×10^6 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. WL-182) and L5178Y TK+/- Assay - Daily Counts (Positive Controls) (Form No. WL-139).

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

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-183, WL-184 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-230, WL-231 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 0.3×10^6 cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

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

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

CAS 142-73-4

GENETICS ASSAY NO. 6576

LBI SAFETY NO. 8027

RECEIVED
OPPE CRIC

06 NOV 28 PM 2:49

KAN 911551

MUTAGENICITY EVALUATION OF

EK 82-0184, DI

IN THE
CHO HGPRT FORWARD
MUTATION ASSAY

FINAL REPORT

SUBMITTED TO:

HEALTH, SAFETY AND HUMAN FACTORS LABORATORY
EASTMAN KODAK COMPANY
BUILDING 320, KODAK PARK
ROCHESTER, NEW YORK 14650

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO. 20989

REPORT DATE: MARCH, 1983



BIONETICS

PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains Items I-IX. Items I-IV provide sponsor and test article identification information, type of assay, and the protocol reference number. Item V provides the initiation and completion dates of the study. Item VI identifies the supervisory personnel. Item VII indicates the tables and/or figures containing the test results. The interpretation of the results is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report describes the study design, which includes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices.

All test and control results presented in this report are supported by raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895.

Copies of the raw data will be supplied to the sponsor upon request.

The described study was performed in accordance with Good Laboratory Practice regulations except if noted to the contrary. To the best of the signer's knowledge there were no significant deviations from the Good Laboratory Practice regulations which affected the quality or integrity of the study.



BIONETICS

Litton

- I. SPONSOR: Eastman Kodak Company
- II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NO.: 6576
 - A. Identification: EK 82-0184, DI
 - B. Date Received: August 30, 1982
 - C. Physical Description: White, crystalline powder
- III. TYPE OF ASSAY: CHO HGPRT Forward Mutation Assay
- IV. ASSAY DESIGN NUMBER: 435, Edition 4
- V. STUDY DATES:
 - A. Initiation: September 2, 1982
 - B. Completion: December 30, 1982
- VI. SUPERVISORY PERSONNEL:
 - A. Study Director: Brian C. Myhr, Ph.D.
 - B. Laboratory Supervisor: Robert Young

VII. RESULTS:

The data are presented in Figure 1 on page 5 and Tables 1-6 on pages 6-11.

VIII. INTERPRETATION OF RESULTS:

The test material, EK 82-0184, DI, was soluble in F12 culture medium at a concentration as high as 20 mg/ml, so this medium was chosen as the vehicle for the study. Later, in the second trial of the S9 activation mutation assay, a stock solution was prepared at 40 mg/ml in DMSO. The initial stock solutions were acidic, as indicated by the yellow color of the phenol red component of the medium. Neutralization was not performed for the preliminary cytotoxicity testing, and the pH was observed to return to near-neutrality after dilution to 1 mg/ml in F12 medium. For the mutation assays, the stock solutions were neutralized with sodium hydroxide prior to performing serial dilutions with F12 medium. All stocks were prepared just prior to use, and the F12 medium contained 10% fetal bovine serum for the nonactivation studies and 5% serum for the S9 activation studies. The treatments were initiated by replacing the media on the cell cultures with the media containing the desired concentrations of the test material.

The preliminary toxicity tests showed that increasing amounts of test material in the 0.01 mg/ml to 0.6 mg/ml concentration range caused virtually no increases in the toxicity. The measured survivals were consistently somewhat below the negative controls and



VIII. INTERPRETATION OF RESULTS: (continued)

ranged from 76% with S9 activation to 84% without activation at 0.6 mg/ml (Figure 1, Tables 1 and 2). At 1 mg/ml, however, the survival with activation dropped to 58% while the nonactivation survival remained unaffected. The next increment in dose (to 3 mg/ml) caused complete lethality under both test conditions. On the basis of these results, seven treatments ranging from 0.1 mg/ml to 3 mg/ml were selected for the mutation assays. It is not uncommon for sharp survival curves to shift slightly between trials, so four of the selected treatments were placed in the 1 mg/ml to 3 mg/ml range in an attempt to achieve toxic treatments without excessive lethality.

Two trials of the mutation assay were performed. The results of the two nonactivation test conditions are presented in Tables 3 and 4, and the S9 activation results are given in Tables 5 and 6.

Under nonactivation test conditions, the treatments with the test material caused no significant changes in the mutant frequency. The mutant frequencies varied randomly over the 0.1 to 3.0 mg/ml concentration range and provided no evidence for any mutagenic activity by the test material. Unexpectedly, the high doses were only slightly toxic. This shift to less toxicity, relative to the preliminary testing, could have been caused by the neutralization of the test chemical and/or the treatment of a much larger sample of cells in the mutation assay. The assay could therefore be extended to concentrations closer to the standard arbitrary limit of 10 mg/ml to provide greater confidence in the evaluation. Also, the results from a 3T3 cell transformation assay performed in this laboratory suggested that the upper concentration limit should be extended to 20 mg/ml to provide a definitive test.

In the second nonactivation trial, six treatments from 1 mg/ml to 20 mg/ml were assayed for mutagenic activity. As shown in Table 4, the test material remained only weakly toxic, resulting in survivals ranging from 78.5% to 97.4%. The mutant frequencies in the treated cultures remained very similar to the vehicle control and showed no evidence for a dose-related response. In contrast, the positive control treatment with BrdU (65.6% survival) clearly induced a mutant frequency that was about 10 times the vehicle control value. These results, obtained for excessive concentrations of the test material, were considered to provide conclusive evidence for the lack of mutagenicity under nonactivation conditions in the CHO/HGPRT assay.

In the presence of the S9 metabolic activation system, the test material did not exhibit any different behavior in regard to toxicity and mutagenicity. Concentrations from 0.1 mg/ml to 3 mg/ml in the first trial (Table 5) resulted in mutant frequencies typical of vehicle control cultures and did not yield a dose-related response. A statistical method for evaluating these results could not be used because no mutant colonies were obtained in the vehicle control cultures in this particular trial. Instead, past experience showing



VIII. INTERPRETATION OF RESULTS: (continued)

that negative (vehicle) control mutant frequencies usually vary from 0 to 10×10^{-6} and sometimes reach 15×10^{-6} was used to evaluate the test material as nonmutagenic in the presence of S9 activation mix in this trial.

In the second trial (Table 6), the concentration range were extended to 20 mg/ml in order to provide a more definitive test. The results were analogous to the nonactivation assay results for these high concentrations of test material. Only weak toxicity was observed (survival range of 73.0 to 102.2%), and the mutant frequencies remained very similar to the vehicle control. There was no evidence for a dose-related increase in mutant frequency, either as a function of test material concentration or a function of induced toxicity. The activity of the S9 activation system was confirmed (in both trials) by the high mutant frequency induced by the 3-MCA treatments, which caused about 68% survival. Thus, for excessive concentrations of the test material, there was no evidence obtained for metabolic activation by an active S9 mix, and the test material was conclusively evaluated as non-mutagenic under these test conditions.

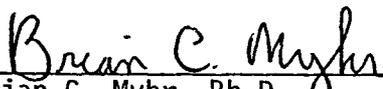


IX. CONCLUSIONS:

The test material, EK 82-0184, DI, was evaluated for mutagenic activity in two trials that spanned a concentration range of 0.1 mg/ml to 20 mg/ml with and without an S9 metabolic activation system. The survival at 20 mg/ml varied from 86.5% without S9 to 75.3% with S9, which contrasted with expectations of excessive toxicity above 3 mg/ml as shown in the preliminary toxicity studies. The mutant frequencies in the treated cultures remained within the usual range of variation for negative (vehicle) control cultures, and dose-related responses were not observed in the two trials. Therefore, for concentrations up to the excessive level of 20 mg/ml, the test material was evaluated as inactive in the CHO/HGPRT Forward Mutation Assay.

SUBMITTED BY:

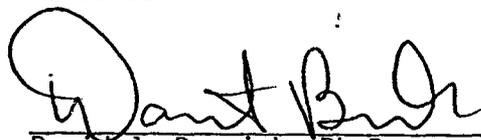
Study Director:



Brian C. Myhr, Ph.D.
Section Chief
Mammalian Genetics
Department of Molecular Toxicology

3-14-83
Date

REVIEWED BY:



David J. Brusick, Ph.D.
Director
Department of Molecular Toxicology

3/15/83
Date



BIONETICS

FIGURE 1. PLOT OF CLONAL CYTOTOXICITY RESULTS

EK 82-0184,DI

LBI # 6576

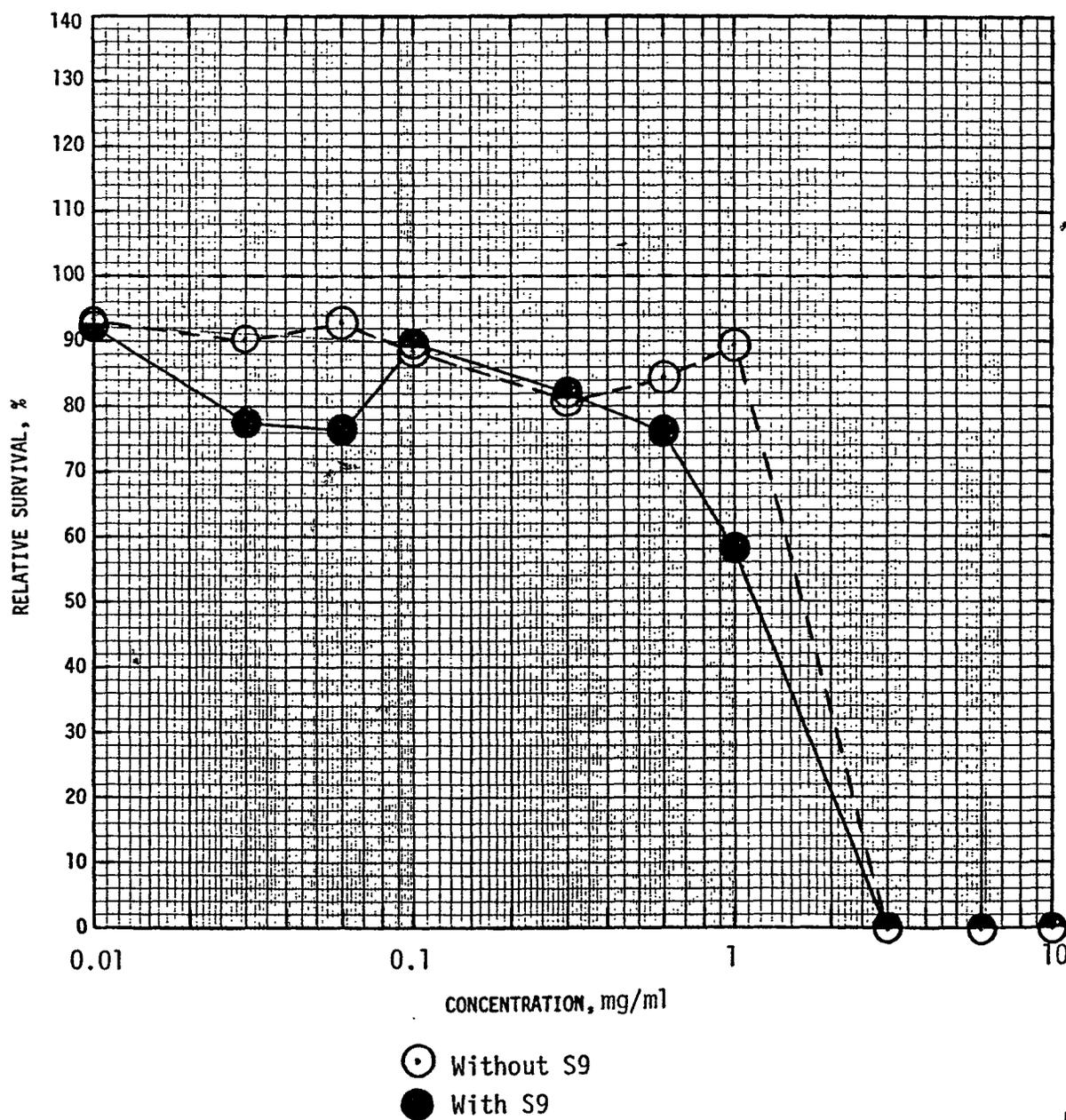


TABLE 1

CLONAL CYTOTOXICITY ASSAY

SAMPLE IDENTITY: EK 82-0184, DI

DESCRIPTION OF SAMPLE: WHITE, CRYSTALLINE
POWDER

LBI ASSAY NO. 6576 W/O S9

DATE RECEIVED: AUGUST 30, 1982

TEST DATE: SEPTEMBER 2, 1982

VEHICLE: F12 CULTURE MEDIUM

CELL TYPE: CHO-K1-BH4

CELLS SEEDED PER DISH: 200

PH ALTERATIONS: SAMPLE ACIDIC

COMMENTS ON TREATMENT: SAMPLE ACIDIC, PRIMARY STOCK
NOT PH ADJUSTED.

COLONY COUNTS

SAMPLE	APPLIED CONCENTRATION MG/ML	COLONY COUNTS			AVERAGE COUNT	RELATIVE SURVIVAL* (PERCENT)	CLONING EFFICIENCY (PERCENT)
		DISH #1	DISH #2	DISH #3			
NC	---	150	141	142	144.3	100.0	72.2
TEST	0.01	120	143	140	134.3	93.1	
TEST	0.03	130	131	130	130.3	90.3	
TEST	0.06	134	139	129	134.0	92.9	
TEST	0.1	120	130	135	128.3	88.9	
TEST	0.3	126	115	110	117.0	81.1	
TEST	0.6	121	120	124	121.7	84.3	
TEST	1.0	130	128	130	129.3	89.6	
TEST	3.0	0	0	0	0.0	0.0	
TEST	6.0	0	0	0	0.0	0.0	
TEST	10.0	0	0	0	0.0	0.0	

NC = NEGATIVE CONTROL, F12 MEDIUM

*RELATIVE TO NEGATIVE CONTROL

TABLE 2
CLONAL CYTOTOXICITY ASSAY

SAMPLE IDENTITY: EK 82-0184,DI

DESCRIPTION OF SAMPLE: WHITE, CRYSTALLINE
POWDER

LBI ASSAY NO. 6576 W/ S9

DATE RECEIVED: AUGUST 30, 1982

TEST DATES: SEPTEMBER 2, 1982

VEHICLE: F12 CULTURE MEDIUM

CELL TYPE: CHO-K1-BH4

CELLS SEEDED PER DISH: 200

PH ALTERATIONS: SAMPLE ACIDIC

COMMENTS ON TREATMENT: SAMPLE ACIDIC, PRIMARY STOCK
NOT PH ADJUSTED.

COLONY COUNTS

SAMPLE	APPLIED CONCENTRATION MG/ML	DISH			AVERAGE COUNT	RELATIVE SURVIVAL* (PERCENT)	CLONING EFFICIENCY (PERCENT)
		#1	#2	#3			
NC	---	149	140	142	143.7	100.0	71.9
TEST	0.01	115	145	137	132.3	92.1	
TEST	0.03	113	111	110	111.3	77.5	
TEST	0.06	105	113	112	110.0	76.5	
TEST	0.1	122	125	140	129.0	89.8	
TEST	0.3	122	126	106	118.0	82.1	
TEST	0.6	102	119	107	109.3	76.1	
TEST	1.0	92	86	73	83.7	58.2	
TEST	3.0	0	0	0	0.0	0.0	
TEST	6.0	0	0	0	0.0	0.0	
TEST	10.0	0	0	0	0.0	0.0	

NC = NEGATIVE CONTROL, F12 MEDIUM

*RELATIVE TO NEGATIVE CONTROL

TABLE 3

CHO/HGPRT MUTAGENESIS ASSAY RESULTS

CLIENT: Eastman Kodak TEST ARTICLE: EK 82-0184, DI ASSAY NO.: 6576 TEST DATE: September 17, 1982 (Trial 1)
 VEHICLE: F12 Medium Selective Agent: 10 µg/ml 6-thioguanine Cells seeded for analysis: 200/dish for C.E.,
 Expression Time: 7 days Expression Time: 2 x 10⁵/dish for mutants

TEST CONDITION	SURVIVAL TO TREATMENT		REL. POPULATION GROWTH (% OF CONTROL)	MUTANT COLONIES DISH NUMBER												TOTAL MUTANT COLONIES	ABSOLUTE C.E.±S.D. (%)	MUTANT FREQ. IN 10 ⁻⁶ UNITS	
	MEAN COLONY NUMBER ± S.D.	PERCENT NEG. CONTROL		1	2	3	4	5	6	7	8	9	10	11	12				
NONACTIVATION:																			
Vehicle Control	158.7±4.0	100.0	100.0	0	1	0	0	0	0	1	0	0	1	0	0	0	3	90.8±8.4	1.4
Positive Control (50 µg/ml BrdU)	72.3±6.5	45.6	19.5	2	1	4	5	2	3	5	4	3	1	0	2	32	85.0±2.2	15.7**	
TEST ARTICLE:																			
0.1 mg/ml	136.3±12.3	85.9	86.9	1	0	0	0	0	0	0	0	0	0	0	0	1	84.2±1.6	0.5	
0.3 mg/ml	146.7±8.5	92.4	32.5	0	0	0	0	1	0	0	0	0	0	0	0	1	77.8±4.0	0.5	
0.6 mg/ml	147.7±3.2	93.1	76.5	0	0	1	0	0	1	0	0	0	0	0	0	2	75.8±4.0	1.1	
1.0 mg/ml	152.3±2.5	96.0	105.3	0	0	3	0	0	0	1	0	1	0	0	0	5	82.5±7.1	2.5	
1.5 mg/ml	153.0±11.3	96.4	93.5	0	1	0	0	0	0	2	0	1	0	0	C	4	76.3±1.5	2.4	
2.0 mg/ml	148.7±13.1	93.7	74.4	0	0	0	0	0	0	0	0	1	0	0	0	1	81.7±7.2	0.5	
3.0 mg/ml	127.7±9.3	80.5	47.7	0	0	0	1	0	0	0	0	0	0	1	0	2	72.7±2.1	1.1	

BrdU = 5-Bromo-2'-deoxyuridine.
 C = Contaminated
 ∞ Mutant Frequency = Total mutant clones/(No. of dishes x 2 x 10⁵ x abs. C.E.)
 **Significant increase, p < 0.01.

TABLE 4

CHO/HGPRT MUTAGENESIS ASSAY RESULTS

CLIENT: Eastman Kodak TEST ARTICLE: EK 82-0184, DI ASSAY NO.: 6576 TEST DATE: December 9, 1982 (Trial 2)

VEHICLE: F12 Medium

Selective Agent: 10 µg/ml 6-thioguanine Cells seeded for analysis: 200/dish for C.E.,
 Expression Time: 7 days 2 x 10⁵/dish for mutants

TEST CONDITION	SURVIVAL TO TREATMENT MEAN COLONY NUMBER ± S.D.	NEG. CONTROL PERCENT	REL. POPULATION GROWTH (% OF CONTROL)	MUTANT COLONIES DISH NUMBER												TOTAL MUTANT COLONIES	ABSOLUTE C.E.±S.D. (%)	MUTANT FREQ. IN 10 ⁻⁶ UNITS
				1	2	3	4	5	6	7	8	9	10	11	12			
NONACTIVATION:																		
Vehicle Control	167.7±10.8	100.0	100.0	0	0	2	0	0	1	0	1	0	2	1	7	76.5±3.3	3.8	
Positive Control (50 µg/ml BrdU)	110.0±11.0	65.6	18.6	1	6	8	5	7	4	6	8	5	6	6	68	61.0±1.3	46.4**	
TEST ARTICLE:																		
1.0 mg/ml	146.0±5.6	87.1	37.4	2	1	0	1	0	0	0	0	0	0	0	4	65.5±8.3	2.5	
3.0 mg/ml	159.3±8.5	95.0	26.5	0	2	1	0	0	1	1	0	2	1	0	8	62.0±7.8	5.4	
6.0 mg/ml	131.7±9.1	78.5	33.7	0	0	0	0	0	0	0	0	0	0	0	0	67.3±2.8	0.0	
10.0 mg/ml	156.7±7.1	93.4	58.9	0	0	0	0	0	0	0	0	0	0	0	0	61.7±2.4	0.0	
15.0 mg/ml	163.3±3.2	97.4	39.5	0	1	1	0	0	0	0	0	0	2	3	7	65.7±0.6	4.8	
20.0 mg/ml	145.0±6.1	86.5	42.4	1	0	0	0	1	1	4	0	0	1	0	8	61.0±3.6	5.5	

BrdU = 5-Bromo-2'-deoxyuridine

C = Contaminated

Mutant Frequency = Total mutant clones/(No. of dishes x 2 x 10⁵ x abs. C.E.)

**Significant increase, p ≤ 0.01.

TABLE 5

CHO/HGPRT MUTAGENESIS ASSAY RESULTS

CLIENT: Eastman Kodak TEST ARTICLE: EK 82-0184, DI ASSAY NO.: 6576 TEST DATE: September 10, 1982 (Trial 1)

VEHICLE: F12 Medium Selective Agent: 10 µg/ml 6-thioguanine Cells seeded for analysis: 200/dish for C.E.,
 Expression Time: 7 days 2 x 10⁵/dish for mutants

TEST CONDITION	SURVIVAL TO TREATMENT MEAN COLONY NUMBER ± S.D.	NEG. CONTROL PERCENT	REV. POPULATION GROWTH (% OF CONTROL)	MUTANT COLONIES DISH NUMBER												TOTAL MUTANT COLONIES	ABSOLUTE C.E. ± S.D. (%)	MUTANT FREQ. IN 10 ⁻⁶ UNITS
				1	2	3	4	5	6	7	8	9	10	11	12			
<u>S9 ACTIVATION:</u>																		
Vehicle Control	183.3±4.7	100.0	100.0	0	0	0	0	0	0	0	0	0	0	0	0	0	96.3±8.1	0
Positive Control (5 µg/ml 3-MCA)	125.3±4.2	68.4	27.6	63	60	66	58	54	67	65	63	59	56	54	56	721	65.0±2.6	462.2**
<u>TEST ARTICLE:</u>																		
0.1 mg/ml	130.0±7.2	70.9	59.4	2	1	0	0	0	0	2	0	0	0	0	0	5	80.3±1.3	2.8
0.3 mg/ml	150.7±7.6	82.2	181.1	0	1	0	3	0	0	0	0	1	2	0	2	9	85.5±4.0	4.4
0.6 mg/ml	150.0±2.0	81.8	161.1	1	0	0	1	0	0	0	1	0	0	0	0	3	68.8±4.5	1.8
1.0 mg/ml	143.0±8.5	78.0	131.1	1	0	0	2	0	0	0	1	0	0	1	0	5	83.7±3.3	2.5
1.5 mg/ml	156.3±3.8	85.3	180.7	0	1	0	0	1	0	0	0	0	0	0	0	2	78.0±10.6 ^a	1.8
2.0 mg/ml	153.3±3.1	83.6	147.9	3	2	1	0	3	1	0	0	1	0	0	0	11	102.5±5.3	4.5
3.0 mg/ml	150.0±7.0	81.8	96.8	0	1	1	0	1	0	1	0	0	0	0	0	4	80.5±2.6	2.1

^a Calculated from two dishes; the third dish was lost to contamination.

3-MCA = 3-Methylcholanthrene

C = Contaminated

Mutant Frequency = Total mutant clones / (No. of dishes x 2 x 10⁵ x abs. C.E.)

** Significant increase, p < 0.01.

TABLE 6

CHO/HGPRT MUTAGENESIS ASSAY RESULTS

CLIENT: Eastman Kodak TEST ARTICLE: EK 82-0184, DI ASSAY NO.: 6576 TEST DATE: December 14, 1982 (Trial 2)

VEHICLE: F12 Medium

Selective Agent: 10 µg/ml 6-thioguanine Cells seeded for analysis: 200/dish for C.E.,
 Expression Time: 7 days 2 x 10⁵/dish for mutants

TEST CONDITION	SURVIVAL TO TREATMENT		REL. POPULA- TION GROWTH (% OF CONTROL)	MUTANT COLONIES DISH NUMBER												TOTAL MUTANT COLONIES	ABSOLUTE C.E.±S.D. (%)	MUTANT FREQ. IN 10 ⁻⁶ UNITS	
	MEAN COLONY NUMBER ± S.D.	PERCENT NEG. CONTROL		1	2	3	4	5	6	7	8	9	10	11	12				
S9 ACTIVATION:																			
Vehicle Control	178.0±10.6	100.0	100.0	1	0	0	1	0	0	0	0	0	0	0	0	0	2	78.2±3.0	1.1
Positive Control (5 µg/ml 3-MCA)	120.7±9.5	67.8	50.1	54	58	65	60	66	60	46	43	50	71	60	56	689	45.5±3.5	631.0**	
TEST ARTICLE:																			
1.0 mg/ml	157.0±9.6	88.2	97.6	0	0	0	0	0	0	0	0	0	0	0	0	0	63.2±1.9	0.0	
3.0 mg/ml	143.7±9.3	80.7	84.5	0	2	0	0	0	0	1	0	1	0	1	0	5	63.8±3.4	3.6	
6.0 mg/ml	182.0±5.3	102.2	59.0	0	0	0	0	0	1	C	C	C	C	C	1	63.8±5.4	1.3		
10.0 mg/ml	130.0±8.9	73.0	69.6	0	1	0	0	0	0	0	0	0	0	0	1	53.2±4.3	0.8		
15.0 mg/ml	133.7±3.8	75.1	65.3	0	0	0	0	0	0	0	0	0	0	1	0	1	57.0±1.8	0.7	
20.0 mg/ml	134.0±12.1	75.3	73.7	0	0	0	0	0	0	1	0	0	1	0	0	2	56.5±1.8	1.5	

3-MCA = 3-Methylcholanthrene

C = Contaminated

Mutant Frequency = Total mutant clones/(No. of dishes x 2 x 10⁵ x abs. C.E.)

**Significant increase, p ≤ 0.01.

ASSAY DESIGN (NO. 435)

1. OBJECTIVE

The objective of this study was to evaluate the test article for its ability to induce forward mutation at the HGPRT locus in the CHO-K1-BH₄ Chinese hamster cell line, as assessed by colony growth in the presence of 6-thioguanine (TG).

2. RATIONALE

Hypoxanthine guanine phosphoribosyl transferase (HGPRT) is a cellular enzyme that allows cells to salvage hypoxanthine and guanine from the surrounding medium for use in DNA synthesis. If a purine analog such as TG is included in the growth medium, the analog will be phosphorylated via the HGPRT pathway and incorporated into nucleic acids, eventually resulting in cellular death. The HGPRT locus is located on the X chromosome. Since only one of the two X chromosomes is functional in the female CHO cells, a single step forward mutation from HGPRT+ to HGPRT- in the functional X chromosome will render the cell unable to utilize hypoxanthine, guanine, or TG supplied in the culture medium. Such mutants are as viable as wild-type cells in normal medium because DNA synthesis may still proceed by de novo synthetic pathways that do not involve hypoxanthine or guanine as intermediates. The basis for the selection of HGPRT- mutants is the loss in their ability to utilize toxic purine analogs (e.g., TG), which enables only the HGPRT- mutants to grow in the presence of TG. Cells which grow to form colonies in the presence of TG are therefore assumed to have mutated, either spontaneously or by the action of the test article, to the HGPRT- genotype.

3. MATERIALS

A. Indicator Cells

The hypodiploid CHO cell line was originally derived from the ovary of a female Chinese hamster (Cricetulus griseus). The particular clone used in this assay was CHO-K1-BH₄. The CHO-K1 cell line was originally selected by Dr. T.T. Puck (University of Colorado Medical Center, Denver, Colorado) for high clonability (approximately 85%) and rapid doubling time (11-14 hours). The BH₄ subclone of CHO-K1 cells, isolated by Dr. A.W. Hsie (Oak Ridge National Laboratory, Oak Ridge, Tennessee), has been demonstrated to be sensitive to many chemical mutagens.

CHO-K1-BH₄ stocks were maintained in liquid nitrogen. Laboratory cultures were maintained as monolayers at 37±2°C in a humidified atmosphere containing approximately 5% CO₂. Laboratory cultures were periodically checked for karyotype stability and for the



3. MATERIALS (Continued)

absence of mycoplasma contamination. To reduce the negative control frequency (spontaneous frequency) of HGPRT- mutants to as low a level as possible, the cell cultures were exposed to conditions which selected against the HGPRT- phenotype and were subsequently returned to normal growth medium for three or more days prior to use in the assay.

B. Media

The cells used during experimental studies were maintained in Ham's Nutrient Mixture F12 supplemented with L-glutamine, penicillin G, streptomycin sulfate, and fetal bovine serum (10% by volume), hereafter referred to as culture medium. Medium used for reducing the spontaneous frequency of HGPRT- mutants prior to experimental studies consisted of culture medium supplemented with 5.0×10^{-6} M thymidine, 1.0×10^{-5} M hypoxanthine, 2.0×10^{-4} M glycine, and 3.2×10^{-6} M aminopterin. The selection medium for mutants was hypoxanthine-free F12 medium containing 10 mg/ml of TG and the fetal bovine serum component reduced to 5% by volume.

C. Control Articles

1. Negative controls

A negative control was performed for each portion of the assay by carrying cells unexposed to the test article through all of the assay operations. In the activation portion of the assay, the negative control cultures were exposed to the S9 activation mix. If a solvent or vehicle other than the culture medium was used, the negative control was exposed to the same concentration of test article carrier as the treated cultures. In such cases, the negative control is referred to as the solvent control or vehicle control. Normally, the final concentration of an organic solvent, such as dimethylsulfoxide (DMSO), in the medium is 1% or less.

2. Positive control articles

5-Bromodeoxyuridine (BrdU) is a stable chemical that is reproducibly and highly mutagenic to CHO-K1 cells. This chemical was used at a concentration of 50 μ g/ml as a positive control article for nonactivation studies.

3-Methylcholanthrene (3-MCA) requires metabolic activation by microsomal enzymes to become mutagenic to CHO-K1-BH₄ cells and was used at 5 μ g/ml as a positive control article for assays performed with S9 activation.



4. EXPERIMENTAL DESIGN

A. Dose Selection

After the selection of a suitable solvent or vehicle, a wide range of test article concentrations was tested for cytotoxicity, starting with a maximum applied dose of 10 mg/ml for solid test articles, 10 μ l/ml for nonaqueous liquid test articles, or 600 μ l/ml for aqueous samples. Ten concentrations spanning a 3-log concentration range were used.

The cells were quantitatively seeded at 200 cells/dish, allowed to attach for 16 to 18 hours, then exposed to approximately 10 dilutions of the test article for four hours. The cells were then washed and incubated in F12 culture medium (or F10 medium for aqueous test articles) for 7 days to allow colony development. Colonies were counted by eye, excluding those with approximately 50 cells or less. Cytotoxicity was expressed as a comparison of colony counts in treated cultures versus control cultures and was used to select 6 to 8 doses for the mutation assay covering the range from approximately 0% to 90% reduction in colony-forming ability. This dose selection procedure was performed both with and without S9 metabolic activation.

B. Mutagenicity Testing

1. Nonactivation assay

The assay procedure was based on that reported by Hsie, et. al. (1975) with modifications suggested by Myhr and DiPaolo (1978). The assay was initiated by exposing about 4×10^6 cells in a 250-mm flask to each concentration of the test article for 4 hours at $37 \pm 2^\circ\text{C}$. After treatment, the cell monolayers were washed, trypsinized and suspended in culture medium. The cell suspension from each dose level was counted by hemacytometer and replated at about 1.5×10^6 cells into each of two 150-mm dishes and at 200 cells into each of three 60-mm dishes. The small dishes were incubated for 7-8 days to permit colony development and the determination of the cytotoxicity associated with each treatment. The large dishes were incubated to permit growth and expression of induced mutations and were subcultured on day 2 or 3 and again on day 5 to allow for additional growth and expression time. At each subculture the two cultures for each dose level were combined and reseeded at 1.5×10^6 cells into each of two 150-mm dishes.

The assay treatment conditions consisted of one vehicle control culture, one positive control, and six to eight treatment levels. Several of the treated cultures may be eliminated during the expression period as long as 5 dose levels are left for analysis of mutant induction.



BIONETICS

4. EXPERIMENTAL DESIGN (Continued)

At the end of the expression period (6 to 7 days), each culture was reseeded at 2×10^5 cells per 100-mm dish (12 dishes total) in mutant selection medium. Also, three 60-mm dishes were seeded at 200 cells each in culture medium to determine the cloning efficiency of each culture. After incubation for 7 to 10 days, at $37^\circ \pm 2^\circ\text{C}$ in a humidified atmosphere with about 5% CO_2 , the colonies were fixed with alcohol, stained with Giemsa and counted to determine the number of TG-resistant colonies in the mutant selection dishes and the number of colonies in the cloning efficiency dishes. The colonies were counted by eye, excluding those with approximately 50 cells or less.

2. Activation assay

The activation assay was performed independently with its own set of negative and positive controls. The procedure was identical to the nonactivation assay except for the addition of the S9 fraction of rat liver homogenate and necessary cofactors during the four-hour treatment period. The fetal bovine serum content of the medium was reduced to 5% by volume. The cofactors consisted of nicotinamide adenine dinucleotide phosphate (NADP, sodium salt), glucose-6-phosphate, calcium chloride, potassium chloride and magnesium chloride all in a pH 7.8 sodium phosphate buffer.

3. S9 homogenate

Sprague-Dawley male rats, induced by Aroclor 1254, were used as the source of hepatic microsomes. A 9,000 x g supernatant in 0.25 M sucrose buffered with phosphate at pH 7.4 was prepared by Bionetics Laboratory Products, Litton Bionetics, Inc. and used in this assay.

5. DATA PRESENTATION

The collected data was used to calculate several assay parameters, and these results are presented in tabular form along with the mutant colony count data. The chosen combination of raw data and calculated data allows a complete description of events for each treatment condition. The significance of each calculated parameter and its method of calculation are listed below:

A. Relative Survival to Treatment

This parameter gives the toxicity of each treatment by showing what percentage of the cells are able to form colonies immediately after the treatment period. The average number of colonies in three dishes (seeded at 200 cells each) is determined for each treatment condition.



BIONETICS

5. DATA PRESENTATION (Continued)

Relative Survival (%) = [Average no. of colonies per treated culture/Average no. of colonies per negative control dish] x 100%

B. Relative Population Growth

This parameter shows the cumulative growth of the treated cell population, relative to the negative control growth, over the entire expression period and prior to mutant selection. In general, highly toxic treatments will reduce the growth rate as well as the survival. Values less than 100% indicate growth inhibition. For example, 50% and 25% relative growth values would indicate treated cell populations that were one and two population doublings behind the negative control culture. Treated populations that are more than 2 or 3 doublings behind the control might not achieve maximum expression of the TG-resistant phenotype. The relative population growth is calculated from cell count data not presented in this report and is intended to provide only an approximate indication of growth during the expression period, since cells are easily lost or not completely released by trypsin during the subculture procedures.

Relative population Growth (%) = [Treated culture population increase over the expression period/Negative control population increase over the expression period] x 100%

C. Absolute Cloning Efficiency

The ability of the cells to form colonies at the time of mutant selection is measured by the absolute cloning efficiency (CE). This parameter is used as the best estimate of the cloning efficiency of the mutant cells in the selection dishes. Thus, the observed frequency of mutant colonies can be converted to the frequency of mutant cells in the treated population.

Absolute CE (%) = [Average no. of viable colonies per dish/200] x 100%

D. Mutant Frequency

The mutant frequency is the endpoint of the assay. It is calculated as the ratio of colonies found in thioguanine-selection medium to the total number of cells seeded, adjusted by the absolute C.E. The frequency is expressed in units of 10^{-6} , e.g. the number of mutants per one million cells.

Mutant Frequency = Total mutant clones/[No. of dishes x 2×10^6 x abs. C.E.]



6. REFERENCES

1. Puck, T.T. and Kao, F.T.: Genetics of somatic mammalian cells, V. Treatment with 5-bromodeoxyuridine and visible light for isolation of nutritionally deficient mutants. Proc. Natl. Acad. Sci. (U.S.A.), 58:1227-1234, 1967.
2. Hsie, A.W., et. al.: Doseresponse relationship for ethylmethanesulfonate-induced mutations at the hypoxanthineguanine phosphoribosyl transferase locus in Chinese hamster ovary cells. Somat. Cell Genet., 1:247-261, 1975.
3. Myhr, B.C. and DiPaolo, J.A.: Mutagenesis of Chinese hamster cells in vitro by combination treatments with methyl methanesulfonate and N-acetoxy-2-acetylaminofluorene. Cancer Res., 38:2539-2543, 1978.
4. Kastenbaum, M.A. and Bowman, K.O.: Tables for determining the statistical significance of mutation frequencies. Mutation Res., 9:527-549, 1970.



BIONETICS

ASSAY ACCEPTANCE CRITERIA

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

1. The average absolute cloning efficiency of the negative controls should be between 70 and 115%. A value greater than 100% is possible because of errors in cell counts (usually $\pm 10\%$) and dilutions during cloning. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50 to 70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range will be conditionally acceptable and dependent on the scientific judgment of the Study Director. All assays below 50% cloning efficiency will be unacceptable.
2. The background mutant frequency (average of the negative controls) is calculated separately for the activation and nonactivation assays, even though the same population of cells may be used for concurrent assays. The activation negative controls contain the S9 activation mix and may have a somewhat different mutant frequency than the nonactivation negative controls. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is about 1×10^{-6} to 15×10^{-6} . Assays with backgrounds greater than 15×10^{-6} are not necessarily invalid but are not used as primary evidence for evaluation of a test article. These assays can provide supporting evidence.
3. A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria. If the test article appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency that is clearly in excess of the background.
4. For test articles with little or no mutagenic activity, an assay should include applied concentrations that reduce the survival to approximately 10% to 15% of the average negative control or reach the maximum applied concentrations given in the evaluation criteria. A reasonable limit to testing for the presence of mutagenic action is about 85 to 90% killing of cells. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.



5. An experimental mutant frequency is considered acceptable for evaluation only if the cloning efficiency is 10% or greater. This limit avoids factors larger than ten in the adjustment of the observed number of mutant clones to a unit number of cells (10^6) able to form colonies.
6. Mutant frequencies are normally derived from sets of twelve dishes for the mutant colony count and three dishes for the viable colony count. To allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of five mutant selection dishes and two cloning efficiency dishes.
7. The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions in order to accept a single assay for evaluation of the test article.



BIONETICS

Litton

ASSAY EVALUATION CRITERIA

Mutation assays are initiated by exposing cell cultures to about six to eight concentrations of test article that are expected, on the basis of preliminary toxicity studies, to span a range of cellular responses from no observed toxicity to about 10% survival. Then five dose levels are usually selected for completion of the mutation assay. These doses should cover a range of toxicities with emphasis placed on the most toxic doses. An assay may need to be repeated with different concentrations to properly evaluate a test article.

The statistical tables provided by Kastenbaum and Bowman (1970) are used to determine whether the results at each dose level are significantly different from the negative control at the 95% or 99% confidence levels. This test compares variables distributed according to Poissonian expectations by summing up the probabilities in the tails of two binomial distributions. The 95% confidence level must be met as the minimum criterion for considering the test article to be active at a particular dose level.

The observation of a mutant frequency that meets the minimum criterion for a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test article as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions:

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses, but this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears.
- An increase in mutant frequency may be followed by only small or no further increases at higher concentrations or toxicities. However, a decrease in mutant frequency to values below the minimum criterion in a single assay is not acceptable for classifying the test article as a mutagen. If the mutagenic activity at the lower concentrations or toxicities is large, a repeat assay is performed to confirm the mutagenic response pattern.
- If an increase greater than about two times the minimum criterion is observed for a single dose near the highest testable toxicity, the test article is considered mutagenic. Smaller increases at a single dose near the highest testable toxicity requires confirmation by a repeat assay.



BIONETICS

For some test articles, the correlation between toxicity and applied concentration is poor. The proportion of the applied article that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent survival), can be used to establish whether the mutagenic activity is related to an increase in effective treatment. A negative correlation with dose is acceptable only if a positive correlation with toxicity exists. An apparent increase in mutagenic activity as a function of decreasing toxicity is not acceptable evidence for mutagenicity.

A test article is evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to toxicity causing about 10% to 15% survival. If the test article is relatively nontoxic, the maximum applied concentrations are normally 10 mg/ml (or 10 μ l/ml) for water-soluble materials or 1 mg/ml (or 1 μ l/ml) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response as discussed above, the test article is evaluated as nonmutagenic in this assay system.

The ASSAY ACCEPTANCE and EVALUATION CRITERIA are presented to acquaint the Sponsor with the considerations used by the Study Director to determine assay validity and mutagenic activity of the test article. This presentation may not encompass all test situations, and the Study Director may use other criteria to arrive at a conclusion, especially when data from several repeat assays are available. The interpretation of the results (Section VIII) provides the reasoning involved when departures from the above descriptions occur.



BIONETICS

Q.A. Inspection Statement
(reference 21 CFR 58.35(b)(7))

PROJECT 20989

LBI Assay No. 6576

TYPE of STUDY CHO HGPRT Forward Mutation assay

This final study report was reviewed by the LBI Quality Assurance Unit on 1-18-85. A report of findings was submitted to the Study Director and to Management on 1-18-85.

The short-term nature of this study precluded inspection while it was in process. The Quality Assurance Unit inspects an in-process study of this type approximately once per month to assure that no significant problems exist that are likely to affect the integrity of this type of study.

Marshall T. Hanson
Auditor, Quality Assurance Unit



BIONETICS

Utton

CAS 142-73-4

911551

188410P

BC-84-22

06 NOV 28 PM 2:49

RECEIVED
OPTICENT

Evaluation of Iminodiacetic Acid (IDA)
In the In Vitro Balb/c3T3 Cell Transformation Assay

Angelo P. Andrese
Joseph J. Beilman
Lynda M. Stankavage

Genetic Toxicology Group
Toxicological Sciences
Health and Environment Laboratories

April, 1984

HEALTH AND ENVIRONMENT LABORATORIES
EASTMAN KODAK COMPANY, Rochester, N.Y. 14650

NOT FOR PUBLICATION

Introduction

This report contains a summary of the data compiled during the evaluation of iminodiacetic acid (IDA), (EK Accession No. 911551), in the in vitro Balb/c3T3 cell transformation assay.

The procedure used in this study is based on a report by Kakunaga (Int. J. Cancer, 12, 463-473, 1973). He described a quantitative assay to assess the in vitro transforming potential of various chemical compounds. Known animal carcinogens, 3-methylcholanthrene and N-methyl-N'-nitro-N-nitrosoguanidine, were found to be potent in vitro transforming agents.

The cells used in this study, Balb/c3T3 clone A31-1-13, were obtained from Litton Bionetics Inc., Kensington, Md. and stored in liquid nitrogen. The cells for each test were derived from a single frozen ampoule. Cells were grown and maintained in Eagle's minimal essential medium plus 5% heat-inactivated (56°C for 30 minutes) fetal bovine serum. Liquid medium was purchased from Grand Island Biological Co. (GIBCO), Grand Island, N.Y. Fetal bovine serum was purchased from GIBCO or Reheis Chemical Co., Phoenix, Arizona. Plastic culture vessels, 60 mm dishes and 25 and 75 cm² flasks, were purchased from Corning Co., Corning, N.Y.

Results

IDA, a white solid, was completely soluble in culture medium at a concentration of 40 mg/mL. This extremely acidic solution (pH 1-2) was neutralized with 5N NaOH before use in cell cultures. Beginning with a neutralized concentration of 40 mg/mL, eleven dilutions were prepared in complete growth medium for use in a preliminary cytotoxicity assay. This assay measures the effect of IDA on the cloning ability of 3T3 cells in growth medium after 72 hours exposure. The results of this assay are shown in Table 1 and graphically represented in Figure 1. The dose levels induced relative cell survivals ranging from 0% at 40 mg/mL to 104.2% at 0.25 mg/mL. From this cytotoxicity data, five dose levels in the range of 16 mg/mL to 8 mg/mL were selected for the transformation assay. The relative cell survival range covered by these doses was 10% to 80%.

The results of the transformation assay are shown in Table 2.

The spontaneous frequency of foci in the negative control culture medium flasks was 0.53 foci/flask. This frequency compared favorably with our historical control value of 0.69 foci per flask and was well below the maximum acceptable level of 1 to 2 foci per flask. The cytotoxicities of each applied dose level used in the transformation assay, Table 2 and Figure 1, ranged from a relative cell survival of 1.1% at 16 mg/mL to 70.9% at 8 mg/mL.

Of the 15 flasks in the positive control group treated with 0.5 µg/mL of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), 12 of 15 flasks had a total 34 foci for an average of 2.27 foci per flask. This positive control transformation frequency was highly significant ($p < .01$) compared to the negative control frequency of 0.53 foci/flask. Therefore, the sensitivity of the transformation assay was judged to be adequate.

The effect of the 5 treatment doses on Balb/c3T3 cells is shown in Table 2. All five dose levels induced a significant ($p < .01$) increase in the number of foci per flask compared to the negative control cultures. Although the 1.1% relative cell survival observed in the 16 mg/mL dose exceeded the maximum acceptable level of 90% cell toxicity, it induced a significant ($p < .01$) increase in the mean number of foci compared to the negative control. The lower frequency of foci per flask observed in the group of flasks treated with 16 mg/mL of IDA was probably due to increased cytotoxicity at this dose level. The data strongly suggests a dose-related response.

Conclusion

IDA induced a significant ($p < .01$) increase in the number of transformed foci per flask compared to the negative control. Evidence of a dose-related response was also observed. The cytotoxicity of the applied doses in the transformation assay ranged between 1.1% and 70.9%. IDA is therefore considered active in the in vitro Balb/c3T3 cell transformation assay.

AP Andrese
Angelo P. Andrese, Ph.D.
Genetic Toxicology Group

June 14, 1984
date

Eugene D. Barber
Eugene D. Barber, Ph.D., Group Leader
Genetic Toxicology Group

6-14-84.
date

C J Terhaar
C. J. Terhaar, Ph.D., Supervisor
Toxicological Sciences Section

15 June 84
date

Thomas S. Ely
Thomas S. Ely, M.D., Director
Occupational Health Laboratory

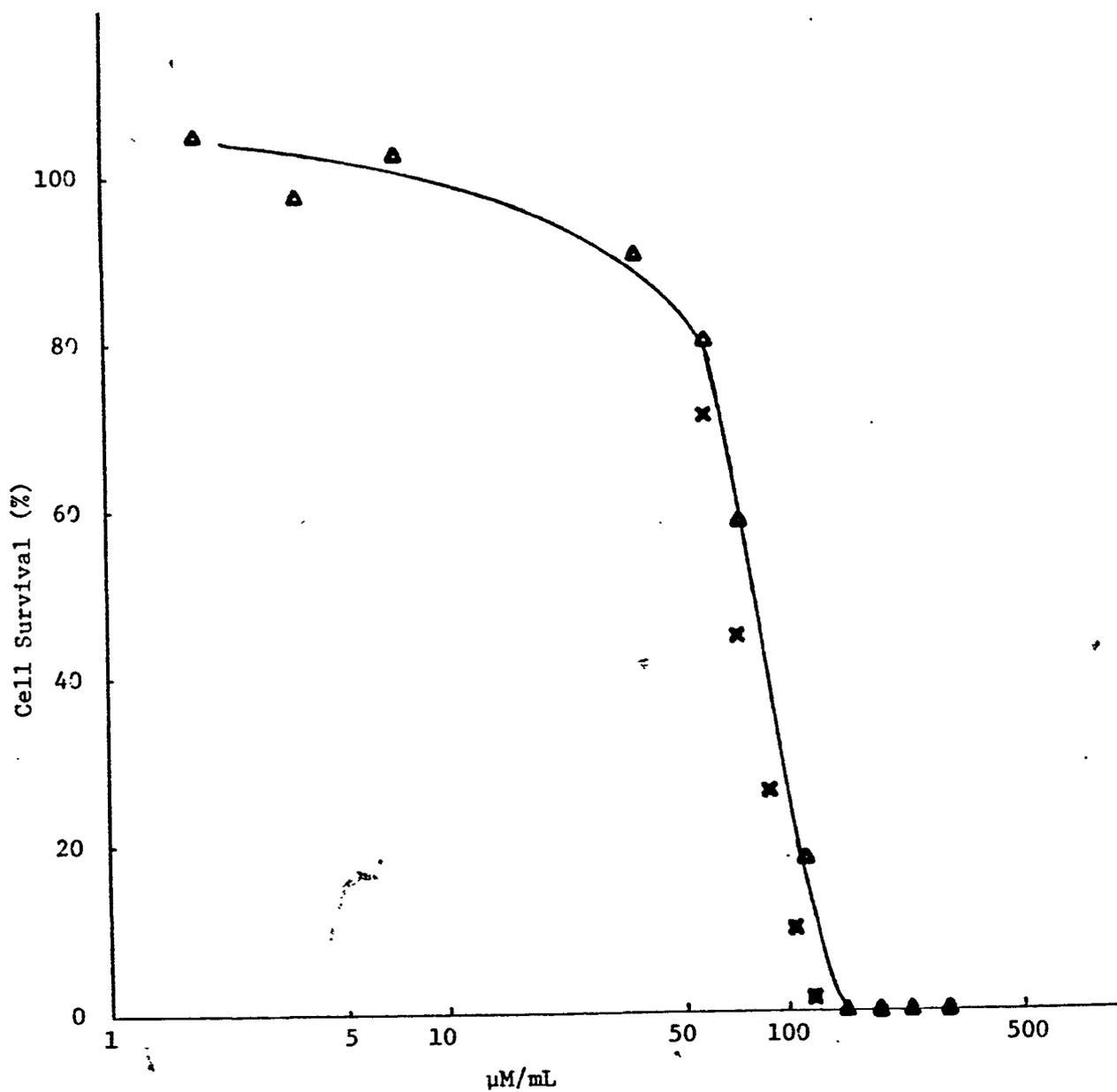
1984 JUN 17
date

TABLE 1

PRELIMINARY CYTOTOXICITY DATA FOR IDA

TEST MATERIAL DOSE (mg/mL)	FLASK			AVERAGE NO. OF COLONIES/FLASK	RELATIVE CELL SURVIVAL (%)
	1	2	3		
Culture Medium	98	87	75	86.7	100.0
40.0	0	0	0	0.0	0.0
30.0	0	0	0	0.0	0.0
25.0	0	0	0	0.0	0.0
20.0	0	0	0	0.0	0.0
15.0	19	13	16	16.0	18.4
10.0	52	53	47	50.7	58.3
8.0	49	86	73	69.3	79.9
5.0	82	69	83	78.0	89.9
1.0	90	85	92	89.0	102.5
0.5	98	76	80	84.7	97.7
0.25	91	92	88	90.3	104.2

Figure 1. IDA Clonal Cytotoxicity



Δ - Initial cytotoxicity curve.

\times - Transformation assay - cytotoxicity of applied doses.

TABLE 2

SUMMARY OF DATA FROM TRANSFORMATION ASSAY

IDA

	NUMBER OF FOCI PER FLASK SCORED															TOTAL NO. OF FOCI	NO. OF FOCI/FLASK	FLASKS WITH FOCI/TOTAL NO. FLASKS
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
Negative Control (Culture Medium)	0	1	1	0	1	0	0	1	0	0	1	2	1	0	0	8	0.53	7/15
Positive Control (B(a)P) 0.5 µg/mL	8	3	1	4	2	3	0	3	1	1	3	3	2	0	0	34	2.27	12/15
<u>Test Material</u>																		
16 mg/mL (1.1%)**	2	3	1	0	0	3	3	0	2	0	1	2	2	2	0	21	1.40	10/15
14 mg/mL (9.5%)**	1	1	0	1	4	3	5	2	3	3	2	2	5	2	3	37	2.47	14/15
12 mg/mL (26.0%)**	3	1	7	3	5	1	5	7	1	5	2	2	4	2	1	49	3.27	15/15
10 mg/mL (44.6%)**	4	2	2	3	2	2	7	3	1	2	3	1	3	3	5	43	2.87	15/15
8 mg/mL (70.9%)**	0	0	1	4	1	3	2	5	2	3	3	2	2	1	2	31	2.07	13/15

** = percent cell survival relative to solvent.

DISTRIBUTION

Eugene D. Barber, Ph.D.

R. Hays Bell, Ph.D.

George D. DiVincenzo, Ph.D.

Robert L. Raleigh, M.D.

C. J. Terhaar, Ph.D.

Douglas C. Topping, Ph.D.

Dept. of Information Services (3)

