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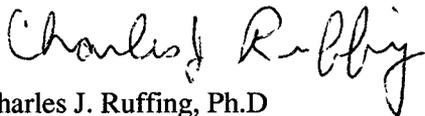
Studies submitted by: Eastman Kodak Company  
343 State Street  
Rochester, NY 14650  
1-800-698-3324

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the following study (studies) in accordance with EPA's final rule on August 16, 2006, requiring submission of unpublished health and safety studies.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>  |
|--|--------------------|---|
| Mouse lymphoma forward mutation assay                  | April, 1982        | Negative with and without metabolic activation.                     |
| Ames Salmonella/microsome mutagenesis assay            | July 1, 1986       | Negative in all five strains with and without metabolic activation. |
| Chromosomal aberrations in CHO cells                   | July 1, 1986       | Negative in the presence and absence of metabolic activation        |
| Primary rat hepatocyte unscheduled DNA synthesis assay | April, 1982        | Negative  |
| In vitro transformation of balb/3T3 cells assay        | July 1, 1986       | Negative  |

Questions regarding this submission should be directed to: Judith M. Van Norstrand  
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Rochester, NY 14652-6267  
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Sincerely,



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



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File: TSCA 8(d) Report - 2006

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CAS 62-56-6

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81-389

MUTAGENICITY EVALUATION OF

EK 81-0389

IN THE  
MOUSE LYMPHOMA 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: APRIL, 1982



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## 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.



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- I. SPONSOR: Eastman Kodak Company
- II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NO.: 6084
  - A. Identification: EK 81-0389
  - B. Date Received: December 18, 1981
  - C. Physical Description: White crystalline powder
- III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay
- IV. ASSAY DESIGN NUMBER: 431
- V. STUDY DATES:
  - A. Initiation: December 23, 1981
  - B. Completion: March 28, 1982
- VI. SUPERVISORY PERSONNEL:
  - A. Study Director: Maria A. Cifone, Ph.D.
  - B. Laboratory Supervisor: Jane Fisher

VII. RESULTS:

The data are presented in Tables 1 and 2 on pages 4 and 5.

VIII. INTERPRETATION OF RESULTS:

The test material, EK 81-0389, was soluble in deionized water at a concentration of 100 mg/ml after a few minutes of vortexing. Water was therefore used as the vehicle for this study, and stock solutions were prepared just prior to testing. The treatments were initiated by performing 1:10 dilutions of the aqueous stocks into culture medium containing the cells. The test material remained soluble in the medium, and no significant changes in pH were observed.

Two trials of the mutation assay were initiated, and the results are given in Tables 1 and 2. The second trial was performed with S9 metabolic activation only.

Under nonactivation conditions, no evidence for mutagenic activity was obtained for concentrations of EK 81-0389 up to 10 mg/ml. As shown in Table 1, five concentrations from 0.625 mg/ml to 10 mg/ml resulted in no significant changes in the mutant frequency away from the background frequency (average of the solvent and untreated negative control mutant frequencies). The treatments caused variable toxicity (unrelated to the concentration) that ranged from no apparent effect at 2.5 mg/ml to 27.3% relative growth (moderately toxic) at 1.25 mg/ml.



### VIII. INTERPRETATION OF RESULTS: (continued)

The reason for this variability appeared to be inconsistent cell growth and cloning efficiency, but the lack of mutant induction was clear for all of the treatments. Higher concentrations of test material were not assayed because 10 mg/ml (1% by weight) is the routine testing limit. The test material was therefore considered to be nonmutagenic without activation.

In the presence of S9 microsomal activation, increases in the mutant frequency were observed for treatments in the same concentration range (Table 1). The results were difficult to interpret, however, because the responses were inconsistent with respect to concentration and toxicity. Thus, two highly toxic treatments caused either no significant change in mutant frequency (at 0.625 mg/ml) or a sharp increase (at 1.25 mg/ml). Higher doses became less toxic and caused only marginal increases in mutant frequency that might be considered significant. Only two treatments (1.25 and 2.5 mg/ml) caused increases in the absolute numbers of mutant colonies. Therefore, some evidence for weak mutagenic activity was obtained, but a repeat assay was necessary to determine whether the unusual response pattern of this assay was yielding a reliable conclusion.

The second activation trial was performed with duplicate doses, and the cultures exposed to 4 mg/ml to 10 mg/ml of test material were assayed for their mutant frequencies. As shown in Table 2, the mutant frequencies remained relatively constant and were not significantly elevated over the background frequency. The minimum criterion for indicating mutagenesis in this trial was a mutant frequency exceeding  $42.6 \times 10^{-6}$ . Only one treatment (10 mg/ml) induced a mutant frequency that just exceeded this value, but the duplicate treatment caused no increase over the background. The isolated increase was therefore evaluated as a spurious event. Also, the lack of any dose-related response was consistent with the observed toxicities of the treatments. Since the results did not confirm the mutant frequency increases indicated by the first trial, the test material was considered to be inactive in the presence of S9 activation.

The average cloning efficiencies of the solvent and untreated negative controls were at the low end of the acceptable range for the first trial. However, consistently high cloning efficiencies for the second trial provided confidence in the evaluation. The negative control mutant frequencies were all in the normal range, and the positive control treatments yielded normal frequencies that were greatly in excess of the backgrounds.



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IX. CONCLUSIONS:

The test material, EK 81-0389, did not induce reproducible increases in the mutant frequency at the TK locus in L5178Y mouse lymphoma cells. No increases in mutant frequency were observed under non-activation conditions for concentrations up to the very high level of 10 mg/ml. With rat liver S9 metabolic activation, sporadic increases were observed that were not repeatable in a second trial. Thus, to the testing limit of 10 mg/ml (1% by weight), the test material was considered to be inactive in the Mouse Lymphoma Forward Mutation Assay.

SUBMITTED BY:

Maria Cifone  
Maria A. Cifone, Ph.D.  
Study Director  
Cell Biologist  
Department of Molecular Toxicology

5/6/82  
Date

REVIEWED BY:

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

5/6/82  
Date



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SUMMARY OF MOUSE LYMPHOMA (LS17AY) RESULTS

TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: EK 81-03R9  
 R. GENETICS ASSAY NO: 6084  
 C. SOLVENT: WATER  
 D. SELECTIVE AGENT: 100 UG/ML RRDU  
 E. TEST DATE: 02/22/82

| TEST CONDITION:                | DAILY CELL COUNTS<br>(CELLS/ML) (X 10 <sup>6</sup> ) | SUSPENSION<br>GROWTH            | TOTAL<br>MUTANT<br>COLONIES | TOTAL<br>VIABLE<br>COLONIES | CLONING<br>EFFICIENCY           | RELATIVE<br>GROWTH (%) | MUTANT<br>FREQUENC<br>(1.0E-6) UNI |
|--------------------------------|--|---------------------------------|-----------------------------|-----------------------------|---------------------------------|------------------------|------------------------------------|
| <u>NONACTIVATION</u>           |  |                                 |                             |                             |                                 |                        |                                    |
| SOLVENT CONTROL                | 11.9   | 12.2                            | 13.0                        | 148.0                       | 49.3                            | 100.0                  | 8.8                                |
| SOLVENT CONTROL                | 9.1  | 15.6                            | 23.0                        | 209.0                       | 69.7                            | 100.0                  | 11.0                               |
| UNTREATED CONTROL              | 10.8   | 12.2                            | 21.0**                      | 206.0                       | 68.7                            | 101.7                  | 10.2                               |
| EMS 0.5 UL/ML<br>TEST COMPOUND | 5.9  | 3.6                             | 227.0**                     | 45.0                        | 15.0                            | 6.5                    | 504.4                              |
|                                |  | RELATIVE TO<br>SOLV CONTROL (%) |                             |                             | RELATIVE TO<br>SOLV CONTROL (%) |                        |                                    |
| 0.6250 MG/ML                   | 11.7   | 54.2                            | 16.0                        | 138.0                       | 77.3                            | 41.9                   | 11.6                               |
| 1.2500 MG/ML                   | 8.0  | 52.4                            | 12.0                        | 93.0                        | 52.1                            | 27.3                   | 12.9                               |
| 2.5000 MG/ML                   | 13.5   | 170.5                           | 14.0                        | 136.0                       | 76.2                            | 129.9                  | 10.3                               |
| 5.0000 MG/ML                   | 10.5   | 68.8                            | 12.0                        | 123.0                       | 68.9                            | 47.4                   | 9.8                                |
| 10.0000 MG/ML                  | 8.6  | 55.7                            | 17.0                        | 151.0                       | 84.6                            | 47.1                   | 11.3                               |
| <u>S9 ACTIVATION</u>           |  |                                 |                             |                             |                                 |                        |                                    |
| SOLVENT CONTROL                | 8.8  | 16.3                            | 33.0**                      | 174.0                       | 58.0                            | 100.0                  | 19.0                               |
| SOLVENT CONTROL                | 8.7  | 14.0                            | 45.0                        | 171.0                       | 57.0                            | 100.0                  | 26.3                               |
| UNTREATED CONTROL              | 13.0   | 14.6                            | 42.0**                      | 199.0                       | 66.3                            | 110.8                  | 21.1                               |
| DMN 0.3 UL/ML<br>TEST COMPOUND | 10.7   | 7.6                             | 50.0                        | 22.0                        | 7.3                             | 6.4                    | 227.3                              |
|                                |  | RELATIVE TO<br>SOLV CONTROL (%) |                             |                             | RELATIVE TO<br>SOLV CONTROL (%) |                        |                                    |
| 0.6250 MG/ML                   | 3.8*   | 19.5                            | 38.0                        | 116.0                       | 67.3                            | 13.1                   | 32.8                               |
| 1.2500 MG/ML                   | 2.2*   | 17.1                            | 78.0                        | 73.0                        | 42.3                            | 7.2                    | 106.8                              |
| 2.5000 MG/ML                   | 8.1  | 38.5                            | 72.0                        | 123.0                       | 71.3                            | 27.5                   | 58.5                               |
| 5.0000 MG/ML                   | 7.8  | 61.6                            | 51.0                        | 123.0                       | 71.3                            | 43.9                   | 41.5                               |
| 10.0000 MG/ML                  | 5.5  | 51.1                            | 55.0                        | 120.0                       | 69.6                            | 35.6                   | 45.8                               |

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

MUTANT FREQUENCY = (TOTAL MUTANT COLONIES/TOTAL VIABLE COLONIES) X 10E-4. DECIMAL IS MOVFD TO EXPRESS THE

FREQUENCY IN UNITS OF 10E-6

CLONING EFFICIENCY = TOTAL VIABLE COLONY COUNT/NUMBER OF CELLS SEEDD

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

\* NOT SPLIT BACK

\*\*ONE PLATE CONTAMINATED; TOTAL CALCULATED USING ASSUMED VALUE FOR THE LOST PLATE, BASED ON AVERAGE OF TWO REMAINING PLATES

SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

TABLE 2

- A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: EK 81-0389  
 B. GENETICS ASSAY NO: 6084  
 C. SOLVENT: WATER  
 D. SELFCTIVE AGENT: 100 UG/ML BRDU  
 E. TEST DATE: 03/16/82

| TEST CONDITION:      | DAILY CELL COUNTS<br>(CELLS/ML $\times 10^5$ UNITS) |      | SUSPENSION<br>GROWTH               | TOTAL<br>MUTANT<br>COLONIES | TOTAL<br>VIABLE<br>COLONIES | CLONING<br>EFFICIENCY              | AVG SOLV<br>CONTROL | RELATIVE<br>GROWTH (%) | MUTANT<br>FREQUENC<br>( $10^6$ -6 UNI |
|----------------------|---|------|------------------------------------|-----------------------------|-----------------------------|------------------------------------|---------------------|------------------------|---------------------------------------|
|                      | 1   | 2    |                                    |                             |                             |                                    |                     |                        |                                       |
| <u>S9 ACTIVATION</u> |   |      |                                    |                             |                             |                                    |                     |                        |                                       |
| SOLVENT CONTROL      | 10.0  | 18.5 | 20.6                               | 57.0                        | 259.0                       | 86.3                               | 100.0               | 100.0                  | 22.0                                  |
| UNTREATED CONTROL    | 8.1   | 17.1 | 15.4                               | 70.0                        | 345.0                       | 115.0                              | 100.7               | 100.0                  | 20.3                                  |
| DMN 0.3 UL/ML        | 7.1   | 17.1 | 13.5                               | 57.0                        | 250.0                       | 83.3                               |                     | 62.0                   | 22.8                                  |
| DMN 0.3 UL/ML        | 6.0   | 8.7  | 5.8                                | 111.0                       | 37.0                        | 12.3                               |                     | 3.9                    | 300.0                                 |
| TEST COMPOUND        | 6.0   | 7.9  | 5.3                                | 163.0                       | 44.0                        | 14.7                               |                     | 4.3                    | 370.5                                 |
|                      |   |      | RELATIVE TO<br>SOLV CONTROL<br>(%) |                             |                             | RELATIVE TO<br>SOLV CONTROL<br>(%) |                     |                        |                                       |
| 4.0000 MG/ML         | 5.9   | 15.6 | 56.8                               | 57.0                        | 152.0                       | 50.3                               |                     | 28.6                   | 37.5                                  |
| 4.0000 MG/ML         | 5.1   | 20.0 | 63.0                               | 64.0                        | 170.0                       | 56.3                               |                     | 35.5                   | 37.6                                  |
| 6.0000 MG/ML         | 7.2   | 15.9 | 70.7                               | C                           | 157.0                       | 52.0                               |                     | 36.8                   |                                       |
| 6.0000 MG/ML         | 7.5   | 16.4 | 75.9                               | 68.0                        | 190.0                       | 62.9                               |                     | 47.7                   | 35.8                                  |
| 8.0000 MG/ML         | 5.3   | 10.0 | 32.7                               | 68.0                        | 205.0                       | 67.9                               |                     | 22.2                   | 33.2                                  |
| 8.0000 MG/ML         | 6.5   | 10.4 | 41.7                               | 85.0                        | 289.0                       | 95.7                               |                     | 39.9                   | 29.4                                  |
| 10.0000 MG/ML        | 6.7   | 11.8 | 48.8                               | 82.0                        | 190.0                       | 62.9                               |                     | 30.7                   | 43.2                                  |
| 10.0000 MG/ML        | 7.4   | 15.7 | 71.7                               | 51.0                        | 262.0                       | 86.7                               |                     | 62.2                   | 19.5                                  |

SUSPENSION GROWTH = (DAY 1 COUNT/3) \* (DAY 2 COUNT/3 OR DAY 1 COUNT IF NOT SPLIT BACK) \* (DAY 3 COUNT/3 OR DAY 2 COUNT IF NOT SPLIT BACK)  
 MUTANT FREQUENCY = (TOTAL MUTANT COLONIES/TOTAL VIABLE COLONIES) X  $10^6$ -4. DECIMAL IS MOVED TO EXPRESS THE FREQUENCY IN UNITS OF  $10^6$ -6  
 CLONING EFFICIENCY = TOTAL VIABLE COLONY COUNT/NUMBER OF CELLS SEEDED  
 RELATIVE GROWTH = (RELATIVE SUSPENSION GROWTH \* RELATIVE CLONING EFFICIENCY) / 100  
 CT C TWO OR MORE PLATES CONTAMINATED

ASSAY DESIGN NO. 431

CHANGE SHEET

1. The dosage selection portion of the Assay Design (Part 4.A) was not performed as a separate, preliminary cytotoxicity test as was usually done for previous studies. Instead, dose selection became an integral part of the mutation assay by the use of the range of concentrations normally employed in the preliminary test. More often than not, a suitable number of treatments will be available for mutant analysis; but if not, a second trial with an adjusted dose range for either activation test condition would then be initiated. This procedure appears to be more efficient in time and materials and is under serious consideration of becoming standard procedure.
2. In Trial 1, the concentration of Fischer 344 Aroclor-induced rat liver S9 microsomal activation mix was reduced from 0.5 ml per 10 ml assay to 0.3 ml per 10 ml. Due to the variable nature of different batches of rat liver S9, adjustment of the concentration is sometimes necessary. The reduced concentration resulted in positive control mutant frequencies that were in keeping with the historical data.
3. In Trial 2, the source of adult male rats for the preparation of the S9 homogenate, described in part 4B-3 of the Assay Design, was changed from Fischer 344 rats to Sprague-Dawley rats. The S9 was prepared by Litton Bionetics, Inc., using the same protocol that was used in the preparation of the Fischer S9 homogenate. The change was initiated because the Fischer S9 was temporarily unavailable, and Sprague-Dawley S9 was prepared and available for immediate use. S9 homogenate prepared from adult male Sprague-Dawley rats is used routinely for metabolic activation in many laboratories in the Mouse Lymphoma Forward Mutation Assay and other assays requiring a source of metabolic activation. The concentration used, 0.3 ml/10 ml was determined in a preliminary assay.



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## ASSAY DESIGN (NO. 431)

### 1. OBJECTIVE

The objective of this study is to evaluate the test material for its ability to induce forward mutation in the L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU) or 5-trifluorothymidine (TFT).

### 2. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as BrdU is included in the growth medium, the analog will be phosphorylated via the TK pathway and be incorporated into DNA, eventually resulting in cellular death. Cells which are heterozygous at the TK locus (TK<sup>+</sup>/<sup>-</sup>) may undergo a single step forward mutation to the TK<sup>-</sup>/<sup>-</sup> genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by de novo synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK<sup>-</sup>/<sup>-</sup> mutants is the lack of any ability to utilize toxic analogs of thymidine, which enables only the TK<sup>-</sup>/<sup>-</sup> mutants to grow in the presence of BrdU. Cells which grow to form colonies in the presence of BrdU are therefore assumed to have mutated, either spontaneously or by the action of a test substance, to the TK<sup>-</sup>/<sup>-</sup> genotype.

### 3. MATERIALS

#### A. Indicator Cells

The mouse lymphoma cell line, L5178Y TK<sup>+</sup>/<sup>-</sup>, used in this assay is derived from the Fischer L5178Y line of Dr. Donald Clive. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK<sup>-</sup>/<sup>-</sup> mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK<sup>-</sup>/<sup>-</sup> phenotype (exposure to methotrexate) and are then returned to normal growth medium for three or more days before use.

#### B. Media

The cells are maintained in Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium is cloning medium containing 100 µg/ml of BrdU or 3 µg/ml of TFT.



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### 3. MATERIALS (continued)

#### C. Control Compounds

##### 1. Negative Controls

A negative control consisting of assay procedures performed on untreated cells is performed in all cases. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are also assayed as the solvent negative control to determine any effects on survival or mutation caused by the solvent alone. For test substances assayed with activation, the untreated and solvent negative controls will include the activation mixture.

##### 2. Positive Controls

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and will be used at 0.5  $\mu$ l/ml as a positive control for nonactivation studies.

Dimethylnitrosamine (DMN) requires metabolic activation by microsomal enzymes to become mutagenic and will be used at 0.3  $\mu$ l/ml as a positive control for assays performed with activation.

#### D. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

### 4. EXPERIMENTAL DESIGN

#### A. Dosage Selection (Cytotoxicity testing)

The solubility of the test chemical in growth medium and/or DMSO is first determined. Then a wide range of chemical concentrations is tested for cytotoxicity, starting with a maximum applied dose of 10 mg/ml for test chemicals soluble in media or 1 mg/ml for solutions in organic solvents. After an exposure time of four hours, the cells are washed and a viable cell count is obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of untreated cells are used to select seven to ten doses that cover the range from 0 to 50-90% reduction in 24-hour growth. These selected doses are subsequently applied to cell cultures prepared for mutagenicity testing, but only four or five of the doses will be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of four or five doses spaced from 0 to 50-90% reduction in cell growth.



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## B. Mutagenicity Testing

### 1. Nonactivation Assay

The procedure used is based on that reported by Clive and Spector (1975) and is summarized as follows. Cultures exposed to the test chemical for four hours at the preselected doses are washed and placed in growth medium for two or three days to allow recovery, growth and expression of the induced TK-/- phenotype. Cell counts are determined daily and appropriate dilutions are made to allow optimal growth rates.

At the end of the expression period,  $3 \times 10^6$  cells for each selected dose are seeded in soft agar plates with selection medium and resistant (mutant) colonies are counted after 10 days incubation. To determine the actual number of cells capable of forming colonies, a portion of the cell suspension is also cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number is the mutant frequency.

A detailed flow diagram for the mutation assay is provided in Figure 1.

### 2. Activation Assay

The activation assay can be run concurrently with the nonactivation assay. The only difference is the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension are: 2.4 mg NADP/ml; 4.5 mg isocitric acid/ml; and 50  $\mu$ l S9/ml.

### 3. S9 Homogenate

A 9,000 x g supernatant prepared from Fischer 344 adult male rat liver induced by Aroclor 1254 (described by Ames et al., 1975) is purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. and used in this assay.



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## 5. REPORT

The screened doses, cell counts, and mutant and viable colony counts will be entered into a computer program. The results are analyzed and printed.

The suspension growth of each culture is calculated as (Day 1 Cell Count/3) x (Day 2 Cell Count/3) x (Day 3 Cell Count/3) when the cultures are split back to  $3 \times 10^5$  cells/ml after the daily count. If the cell count is less than  $4 \times 10^5$  cells/ml, the culture is not split back and the cell count is substituted for 3 in the denominator of the next daily count. In most assays, 3-day expressions are not used, so only the first two factors in the preceding calculation are used. The suspension growth is calculated for each solvent control and then averaged. Relative suspension growth values are derived by dividing the suspension growth values by the average solvent control value and multiplying by 100%.

The average cloning efficiency for the negative controls in an assay is the average number of viable colonies for the solvent and untreated controls, divided by 300 and multiplied by 100%. In the computer tables, the cloning efficiency of each culture is expressed relative to the average solvent control cloning efficiency. Whenever the number of cells seeded for viable colony counts differs from 300, the computer calculation of the relative cloning efficiency is adjusted by the factor (300/cells seeded).

A percent relative growth value is calculated as (relative suspension growth) x (relative cloning efficiency/100). Corrected values for the relative cloning efficiency are used in the cases where the number of cells seeded for viable colonies differs from 300.

The mutant frequency is calculated as the ratio of mutant colonies to viable colonies times  $10^{-4}$ . This calculation is unaffected by changes in the number of cells seeded for viable count because the number of cells seeded for mutant selection is changed by the same factor. Thus, as an example, if 250 cells are seeded for viable count,  $2.5 \times 10^6$  cells are seeded for mutant selection; the  $10^{-4}$  factor remains constant.

## 6. REFERENCE

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



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## ASSAY ACCEPTANCE CRITERIA

An assay will normally be considered acceptable for evaluation of the test results only if all of the criteria given below are satisfied. The activation and nonactivation portions of the mutation assays are usually performed concurrently, but each portion is in fact an independent assay with its own positive and 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 (average of the solvent and untreated controls) should be between 70% and 130%. A value greater than 100% is possible because of errors in cell counts (usually  $\pm 10\%$ ) and cell division during unavoidable delays between the counting and cloning of many cell cultures. 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 are conditionally acceptable and dependent on the scientific judgement of the study director. All assays below 50% cloning efficiency are unacceptable.

2) The solvent and untreated negative controls normally have the same growth rates and cloning efficiencies within experimental error. An unusual effect by the solvent therefore indicates an abnormal cell state or excessive amount of solvent in the growth medium. An assay will be unacceptable if the average percent relative growth of the solvent controls is less than about 70% of the untreated control value.

3) The minimum acceptable value for the suspension growth of the average negative control (average of the solvent and untreated control values) for two days is 8.0. Lower values will render an assay unacceptable for evaluation because of the high frequency of unreliable measurements for both the induced mutant frequency and toxicity of a given treatment. The value of 8 corresponds to three population doublings over the 2-day expression period. The most desirable range for the negative control suspension growth is 12 to 25, since the cells are capable of a 5-fold increase in number under optimal growth conditions for 24 hours.

4) The background mutant frequency (average frequency of the solvent and untreated negative controls) is calculated separately for concurrent activation and nonactivation assays, even though the same population of cells is used for each assay. The activation negative controls contain the S9 activation mix and typically have a somewhat higher mutant frequency than the nonactivation negative controls. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is  $5 \times 10^{-6}$  to  $50 \times 10^{-6}$ . Assays with backgrounds outside this range are not necessarily invalid but will not be used as primary evidence for the evaluation of a test material. These assays can provide supporting evidence.



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5) A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. The normal range of mutant frequencies induced by 0.5  $\mu\text{l/ml}$  EMS (nonactivation assay) is 300 to 800  $\times 10^{-6}$ ; for 0.3  $\mu\text{l/ml}$  DMN (activation assay) the normal range is 200 to 800  $\times 10^{-6}$ . The concurrent background frequencies have been subtracted from these values. These ranges are broad primarily because the effective treatment with these agents is variable between assays. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test material clearly shows mutagenic activity as described in the evaluation criteria. If the test material appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency above the lower limits of the normal range. Assays in which the normal range is exceeded may require further interpretation by the study director.

6) For test materials with little or no mutagenic activity, an assay must include applied concentrations that reduce the suspension growth to 5% to 10% of the average solvent control or reach the maximum applied concentrations given in the evaluation criteria. Suspension growth is a combined measure of cell death and reduced growth rates. A 5% relative suspension growth therefore could correspond to 90% killing, followed by growth of the survivors at one-half the normal rate for one day and normal growth for the second day. At the other extreme, this condition could be obtained by no killing and complete inhibition of growth for two days. A reasonable limit to testing for the presence of mutagenic action is about 80% to 90% killing of cells. Because of the uncertainty in the actual lethality of treatment in the assay and the fact that mutant frequencies increase as a function of lethality, an acceptable assay for the lack of mutagenic activity must extend to the 5% to 10% relative suspension growth range. There is no maximum toxicity requirement for test materials which clearly show mutagenic activity.

7) An experimental treatment that results in fewer than  $2.5 \times 10^6$  cells by the end of the two-day growth period will not be cloned for mutant analysis.

8) An experimental mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds about 20. These limits avoid problems with the statistical distribution of scoreable colonies among dishes and allows factors no larger than 10 in the adjustment of the observed number of mutant clones to a unit number of cells ( $10^6$ ) able to form colonies.



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9) Mutant frequencies are normally derived from sets of three dishes for both the mutant colony count and the viable colony count. In order to allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set if the colony numbers in the two dishes differ by no more than about 3-fold.

10) 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 to accept a single assay for evaluation of the test material.



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## ASSAY EVALUATION CRITERIA

Mutation assays are initiated by exposing cell cultures to a range of concentrations of test material that is expected, on the basis of preliminary toxicity studies, to span the cellular responses of no observed toxicity to growth to complete lethality within 24 hours of treatment. Then five dose levels are usually selected for completion of the mutation assay. The doses are selected to cover a range of toxicities to growth with emphasis on the most toxic doses. An assay may need to be repeated with different concentrations in order to properly evaluate a test material.

The minimum condition considered necessary to demonstrate mutagenesis for any given treatment is a mutant frequency that exceeds 150% of the concurrent background frequency by at least  $10 \times 10^{-6}$ . The background frequency is defined as the average mutant frequency of the solvent and untreated negative controls. The minimum increase is based on extensive experience which indicates that assay variability increases with higher backgrounds and the calculated minimum increase as defined above is often a repeatable result; statistical analysis for the confidence limits is not yet available.

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 material 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 is not acceptable in a single assay for classifying the test material as a mutagen. If the mutagenic activity at lower concentrations or toxicities was large, a repeat assay will be performed to confirm the mutagenic activity.
- If an increase of about two times the minimum criterion or greater is observed for a single dose near the highest testable toxicity, as defined in the Assay Acceptance Criteria, the test material will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.



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- For some test materials, the correlation between toxicity and applied concentration is poor. The proportion of the applied material 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 relative growth), 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 material will be 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 5% to 10% relative suspension growth. If the test material is relatively nontoxic, the maximum applied concentrations will normally be 10 mg/ml (or 10  $\mu$ l/ml) for water-soluble materials or 1 mg/ml (or 1  $\mu$ l/ml) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response, as discussed above, the test material will be 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 the mutagenic activity of the test material. This presentation may not encompass all test situations, and the study director may use other criteria, especially when data from several repeat assays are available, to arrive at a conclusion. The report will provide the reasoning involved when departures from the above descriptions occur.



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

PROJECT 20989

LBI Assay No. 6084

TYPE of STUDY Mouse Lymphoma Assay

This final study report was reviewed by the LBI Quality Assurance Unit on 5-5-82. A report of findings was submitted to the Study Director and to Management on 5-6-82.

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 S. Hyman  
Auditor, Quality Assurance Unit



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230851W  
TX-86-210

MUTAGENICITY EVALUATION OF  
COMPOUND 81-0389 (Thiourea)

IN THE  
MOUSE LYMPHOMA FORWARD  
MUTATION ASSAY

Accession No. 900497

FINAL REPORT

Submitted by: Dr. E.D. Barber

Genetic Toxicology Group  
Toxicological Sciences Section  
Health and Environment Laboratories  
Eastman Kodak Company  
Rochester, New York 14650

July 1, 1986

230851W  
TX-86-210

#900497  
Thiourea  
81-0389

MUTAGENICITY EVALUATION OF

EK 81-0389

IN THE  
MOUSE LYMPHOMA 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: APRIL, 1982



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## 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.



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- I. SPONSOR: Eastman Kodak Company
- II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NO.: 6084
  - A. Identification: EK 81-0389
  - B. Date Received: December 18, 1981
  - C. Physical Description: White crystalline powder
- III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay
- IV. ASSAY DESIGN NUMBER: 431
- V. STUDY DATES:
  - A. Initiation: December 23, 1981
  - B. Completion: March 28, 1982
- VI. SUPERVISORY PERSONNEL:
  - A. Study Director: Maria A. Cifone, Ph.D.
  - B. Laboratory Supervisor: Jane Fisher

VII. RESULTS:

The data are presented in Tables 1 and 2 on pages 4 and 5.

VIII. INTERPRETATION OF RESULTS:

The test material, EK 81-0389, was soluble in deionized water at a concentration of 100 mg/ml after a few minutes of vortexing. Water was therefore used as the vehicle for this study, and stock solutions were prepared just prior to testing. The treatments were initiated by performing 1:10 dilutions of the aqueous stocks into culture medium containing the cells. The test material remained soluble in the medium, and no significant changes in pH were observed.

Two trials of the mutation assay were initiated, and the results are given in Tables 1 and 2. The second trial was performed with S9 metabolic activation only.

Under nonactivation conditions, no evidence for mutagenic activity was obtained for concentrations of EK 81-0389 up to 10 mg/ml. As shown in Table 1, five concentrations from 0.625 mg/ml to 10 mg/ml resulted in no significant changes in the mutant frequency away from the background frequency (average of the solvent and untreated negative control mutant frequencies). The treatments caused variable toxicity (unrelated to the concentration) that ranged from no apparent effect at 2.5 mg/ml to 27.3% relative growth (moderately toxic) at 1.25 mg/ml.



### VIII. INTERPRETATION OF RESULTS: (continued)

The reason for this variability appeared to be inconsistent cell growth and cloning efficiency, but the lack of mutant induction was clear for all of the treatments. Higher concentrations of test material were not assayed because 10 mg/ml (1% by weight) is the routine testing limit. The test material was therefore considered to be nonmutagenic without activation.

In the presence of S9 microsomal activation, increases in the mutant frequency were observed for treatments in the same concentration range (Table 1). The results were difficult to interpret, however, because the responses were inconsistent with respect to concentration and toxicity. Thus, two highly toxic treatments caused either no significant change in mutant frequency (at 0.625 mg/ml) or a sharp increase (at 1.25 mg/ml). Higher doses became less toxic and caused only marginal increases in mutant frequency that might be considered significant. Only two treatments (1.25 and 2.5 mg/ml) caused increases in the absolute numbers of mutant colonies. Therefore, some evidence for weak mutagenic activity was obtained, but a repeat assay was necessary to determine whether the unusual response pattern of this assay was yielding a reliable conclusion.

The second activation trial was performed with duplicate doses, and the cultures exposed to 4 mg/ml to 10 mg/ml of test material were assayed for their mutant frequencies. As shown in Table 2, the mutant frequencies remained relatively constant and were not significantly elevated over the background frequency. The minimum criterion for indicating mutagenesis in this trial was a mutant frequency exceeding  $42.6 \times 10^{-6}$ . Only one treatment (10 mg/ml) induced a mutant frequency that just exceeded this value, but the duplicate treatment caused no increase over the background. The isolated increase was therefore evaluated as a spurious event. Also, the lack of any dose-related response was consistent with the observed toxicities of the treatments. Since the results did not confirm the mutant frequency increases indicated by the first trial, the test material was considered to be inactive in the presence of S9 activation.

The average cloning efficiencies of the solvent and untreated negative controls were at the low end of the acceptable range for the first trial. However, consistently high cloning efficiencies for the second trial provided confidence in the evaluation. The negative control mutant frequencies were all in the normal range, and the positive control treatments yielded normal frequencies that were greatly in excess of the backgrounds.



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IX. CONCLUSIONS:

The test material, EK 81-0389, did not induce reproducible increases in the mutant frequency at the TK locus in L5178Y mouse lymphoma cells. No increases in mutant frequency were observed under non-activation conditions for concentrations up to the very high level of 10 mg/ml. With rat liver S9 metabolic activation, sporadic increases were observed that were not repeatable in a second trial. Thus, to the testing limit of 10 mg/ml (1% by weight), the test material was considered to be inactive in the Mouse Lymphoma Forward Mutation Assay.

SUBMITTED BY:

Maria Cifone  
Maria A. Cifone, Ph.D.  
Study Director  
Cell Biologist  
Department of Molecular Toxicology

5/6/82  
Date

REVIEWED BY:

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

5/6/82  
Date



BIONETICS

SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: EK 81-0389  
 R. GENETICS ASSAY NO: 6084  
 C. SOLVENT: WATER  
 D. SELECTIVE AGENT: 100 UG/ML RRDU  
 E. TEST DATE: 02/22/82

| TEST CONDITION:      | DAILY CELL COUNTS<br>(CELLS/ML) <sup>1</sup> <sub>2</sub> | SUSPENSION<br>GROWTH         | TOTAL<br>MUTANT<br>COLONIES | TOTAL<br>VIABLE<br>COLONIES | CLONING<br>EFFICIENCY        | AVG SOLV<br>CONTROL | RELATIVE<br>GROWTH (%) | MUTANT<br>FREQUENCY<br>(10E-6 UNIT) |
|----------------------|---|------------------------------|-----------------------------|-----------------------------|------------------------------|---------------------|------------------------|-------------------------------------|
| <u>NONACTIVATION</u> |   |                              |                             |                             |                              |                     |                        |                                     |
| SOLVENT CONTROL      | 11.9  | 12.2                         | 13.0                        | 148.0                       | 49.3                         | AVG SOLV CONTROL    | 100.0                  | 8.8                                 |
| SOLVENT CONTROL      | 9.1   | 15.6                         | 23.0                        | 209.0                       | 69.7                         | 13.9                | 100.0                  | 11.0                                |
| UNTREATED CONTROL    | 10.8  | 12.2                         | 21.0**                      | 206.0                       | 68.7                         |                     | 101.7                  | 10.2                                |
| EMS 0.5 UL/ML        | 5.9   | 3.6                          | 227.0**                     | 45.0                        | 15.0                         |                     | 6.5                    | 504.4                               |
| TEST COMPOUND        |   | RELATIVE TO SOLV CONTROL (%) |                             |                             | RELATIVE TO SOLV CONTROL (%) |                     |                        |                                     |
| 0.6250 MG/ML         | 11.7  | 54.2                         | 16.0                        | 138.0                       | 77.3                         |                     | 41.9                   | 11.6                                |
| 1.2500 MG/ML         | 8.0   | 52.4                         | 12.0                        | 93.0                        | 52.1                         |                     | 27.3                   | 12.9                                |
| 2.5000 MG/ML         | 13.5  | 170.5                        | 14.0                        | 136.0                       | 76.2                         |                     | 129.9                  | 10.3                                |
| 5.0000 MG/ML         | 10.5  | 68.8                         | 12.0                        | 123.0                       | 68.9                         |                     | 47.4                   | 9.8                                 |
| 10.0000 MG/ML        | 8.6   | 55.7                         | 17.0                        | 151.0                       | 84.6                         |                     | 47.1                   | 11.3                                |
| <u>S9 ACTIVATION</u> |   |                              |                             |                             |                              |                     |                        |                                     |
| SOLVENT CONTROL      | 8.8   | 16.3                         | 33.0**                      | 174.0                       | 58.0                         | AVG SOLV CONTROL    | 100.0                  | 19.0                                |
| SOLVENT CONTROL      | 8.7   | 14.0                         | 45.0                        | 171.0                       | 57.0                         | 15.2                | 100.0                  | 24.3                                |
| UNTREATED CONTROL    | 13.0  | 14.6                         | 42.0**                      | 199.0                       | 66.3                         |                     | 110.8                  | 21.1                                |
| DMN 0.3 UL/ML        | 10.7  | 7.6                          | 50.0                        | 22.0                        | 7.3                          |                     | 6.4                    | 227.3                               |
| TEST COMPOUND        |   | RELATIVE TO SOLV CONTROL (%) |                             |                             | RELATIVE TO SOLV CONTROL (%) |                     |                        |                                     |
| 0.6250 MG/ML         | 3.8*  | 19.5                         | 38.0                        | 116.0                       | 67.3                         |                     | 13.1                   | 32.8                                |
| 1.2500 MG/ML         | 2.2*  | 17.1                         | 78.0                        | 73.0                        | 42.3                         |                     | 7.2                    | 106.8                               |
| 2.5000 MG/ML         | 8.1   | 38.5                         | 72.0                        | 123.0                       | 71.3                         |                     | 27.5                   | 58.5                                |
| 5.0000 MG/ML         | 7.8   | 61.6                         | 51.0                        | 123.0                       | 71.3                         |                     | 43.9                   | 41.5                                |
| 10.0000 MG/ML        | 5.5   | 51.1                         | 55.0                        | 120.0                       | 69.6                         |                     | 35.6                   | 45.8                                |

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

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

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

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

\* NOT SPLIT RACK

\*\*ONE PLATE CONTAMINATED; TOTAL CALCULATED USING ASSUMED VALUE FOR THE LOST PLATE, BASED ON AVERAGE OF TWO REMAINING PLATES

SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

TABLE 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: EK H1-0389  
 B. GENETICS ASSAY NO: 6084  
 C. SOLVENT: WATER  
 D. SELECTIVE AGENT: 100 UG/ML BRDU  
 E. TEST DATE: 03/16/82

| TEST CONDITION       | DAILY CELL COUNTS<br>(CELLS/ML $\times 10^5$ UNITS) |      | SUSPENSION<br>GROWTH               | TOTAL<br>MUTANT<br>COLONIES | TOTAL<br>VIABLE<br>COLONIES | CLONING<br>EFFICIENCY              | AVG SOLV<br>CONTROL | RELATIVE<br>GROWTH (X) | MUTANT<br>FREQUENCY<br>( $10E-6$ UNIT) |
|----------------------|---|------|------------------------------------|-----------------------------|-----------------------------|------------------------------------|---------------------|------------------------|--|
|                      | 1   | 2    |                                    |                             |                             |                                    |                     |                        |  |
| <u>S9 ACTIVATION</u> |   |      |                                    |                             |                             |                                    |                     |                        |  |
| SOLVENT CONTROL      | 10.0  | 18.5 | 20.6                               | 57.0                        | 259.0                       | 86.3                               | 100.0               | 100.0                  | 22.0                                   |
| SOLVENT CONTROL      | 8.1   | 17.1 | 15.4                               | 70.0                        | 345.0                       | 115.0                              | 100.0               | 100.0                  | 20.3                                   |
| UNTREATED CONTROL    | 7.1   | 17.1 | 13.5                               | 57.0                        | 250.0                       | 83.3                               | 100.0               | 62.0                   | 22.8                                   |
| DMN 0.3 UL/ML        | 6.0   | 8.7  | 5.8                                | 111.0                       | 37.0                        | 12.3                               | 3.9                 | 3.9                    | 300.0                                  |
| DMN 0.3 UL/ML        | 6.0   | 7.9  | 5.3                                | 163.0                       | 44.0                        | 14.7                               | 4.3                 | 4.3                    | 370.5                                  |
| TFST COMPOUND        |   |      | RELATIVE TO<br>SOLV CONTROL<br>(X) |                             |                             | RELATIVE TO<br>SOLV CONTROL<br>(X) |                     |                        |  |
| 4.0000 MG/ML         | 5.9   | 15.6 | 56.8                               | 57.0                        | 152.0                       | 50.3                               | 28.6                | 28.6                   | 37.5                                   |
| 4.0000 MG/ML         | 5.1   | 20.0 | 63.0                               | 64.0                        | 170.0                       | 50.3                               | 35.5                | 35.5                   | 37.6                                   |
| 6.0000 MG/ML         | 7.2   | 15.9 | 70.7                               | C                           | 157.0                       | 52.0                               | 36.8                | 36.8                   | ---                                    |
| 6.0000 MG/ML         | 7.5   | 16.4 | 75.9                               | 68.0                        | 190.0                       | 62.9                               | 47.7                | 47.7                   | 35.8                                   |
| 8.0000 MG/ML         | 5.3   | 10.0 | 32.7                               | 68.0                        | 205.0                       | 67.9                               | 22.2                | 22.2                   | 33.2                                   |
| 8.0000 MG/ML         | 6.5   | 10.4 | 41.7                               | 85.0                        | 289.0                       | 95.7                               | 39.9                | 39.9                   | 29.4                                   |
| 10.0000 MG/ML        | 6.7   | 11.8 | 48.8                               | 82.0                        | 190.0                       | 62.9                               | 30.7                | 30.7                   | 43.2                                   |
| 10.0000 MG/ML        | 7.4   | 15.7 | 71.7                               | 51.0                        | 262.0                       | 86.7                               | 62.2                | 62.2                   | 19.5                                   |

SUSPENSION GROWTH = (DAY 1 COUNT/3) \* (DAY 2 COUNT/3 OR DAY 1 COUNT IF NOT SPLIT BACK) \* (DAY 3 COUNT/3 OR DAY 2 COUNT IF NOT SPLIT BACK)  
 MUTANT FREQUENCY = (TOTAL MUTANT COLONIES/TOTAL VIABLE COLONIES) X  $10E-6$ . DECIMAL IS MOVED TO EXPRESS THE FREQUENCY IN UNITS OF  $10E-6$   
 CLONING EFFICIENCY = TOTAL VIABLE COLONY COUNT/NUMBER OF CELLS SEEDED  
 RELATIVE GROWTH = (RELATIVE SUSPENSION GROWTH \* RELATIVE CLONING EFFICIENCY) / 100  
 (C TWO OR MORE PLATES CONTAMINATED)

ASSAY DESIGN NO. 431

CHANGE SHEET

1. The dosage selection portion of the Assay Design (Part 4.A) was not performed as a separate, preliminary cytotoxicity test as was usually done for previous studies. Instead, dose selection became an integral part of the mutation assay by the use of the range of concentrations normally employed in the preliminary test. More often than not, a suitable number of treatments will be available for mutant analysis; but if not, a second trial with an adjusted dose range for either activation test condition would then be initiated. This procedure appears to be more efficient in time and materials and is under serious consideration of becoming standard procedure.
2. In Trial 1, the concentration of Fischer 344 Aroclor-induced rat liver S9 microsomal activation mix was reduced from 0.5 ml per 10 ml assay to 0.3 ml per 10 ml. Due to the variable nature of different batches of rat liver S9, adjustment of the concentration is sometimes necessary. The reduced concentration resulted in positive control mutant frequencies that were in keeping with the historical data.
3. In Trial 2, the source of adult male rats for the preparation of the S9 homogenate, described in part 4B-3 of the Assay Design, was changed from Fischer 344 rats to Sprague-Dawley rats. The S9 was prepared by Litton Bionetics, Inc., using the same protocol that was used in the preparation of the Fischer S9 homogenate. The change was initiated because the Fischer S9 was temporarily unavailable, and Sprague-Dawley S9 was prepared and available for immediate use. S9 homogenate prepared from adult male Sprague-Dawley rats is used routinely for metabolic activation in many laboratories in the Mouse Lymphoma Forward Mutation Assay and other assays requiring a source of metabolic activation. The concentration used, 0.3 ml/10 ml was determined in a preliminary assay.



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## ASSAY DESIGN (NO. 431)

### 1. OBJECTIVE

The objective of this study is to evaluate the test material for its ability to induce forward mutation in the L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU) or 5-trifluorothymidine (TFT).

### 2. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as BrdU is included in the growth medium, the analog will be phosphorylated via the TK pathway and be incorporated into DNA, eventually resulting in cellular death. Cells which are heterozygous at the TK locus (TK<sup>+</sup>/<sup>-</sup>) may undergo a single step forward mutation to the TK<sup>-</sup>/<sup>-</sup> genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by de novo synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK<sup>-</sup>/<sup>-</sup> mutants is the lack of any ability to utilize toxic analogs of thymidine, which enables only the TK<sup>-</sup>/<sup>-</sup> mutants to grow in the presence of BrdU. Cells which grow to form colonies in the presence of BrdU are therefore assumed to have mutated, either spontaneously or by the action of a test substance, to the TK<sup>-</sup>/<sup>-</sup> genotype.

### 3. MATERIALS

#### A. Indicator Cells

The mouse lymphoma cell line, L5178Y TK<sup>+</sup>/<sup>-</sup>, used in this assay is derived from the Fischer L5178Y line of Dr. Donald Clive. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK<sup>-</sup>/<sup>-</sup> mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK<sup>-</sup>/<sup>-</sup> phenotype (exposure to methotrexate) and are then returned to normal growth medium for three or more days before use.

#### B. Media

The cells are maintained in Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium is cloning medium containing 100 µg/ml of BrdU or 3 µg/ml of TFT.



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### 3. MATERIALS (continued)

#### C. Control Compounds

##### 1. Negative Controls

A negative control consisting of assay procedures performed on untreated cells is performed in all cases. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are also assayed as the solvent negative control to determine any effects on survival or mutation caused by the solvent alone. For test substances assayed with activation, the untreated and solvent negative controls will include the activation mixture.

##### 2. Positive Controls

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and will be used at 0.5  $\mu\text{l/ml}$  as a positive control for nonactivation studies.

Dimethylnitrosamine (DMN) requires metabolic activation by microsomal enzymes to become mutagenic and will be used at 0.3  $\mu\text{l/ml}$  as a positive control for assays performed with activation.

#### D. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

### 4. EXPERIMENTAL DESIGN

#### A. Dosage Selection (Cytotoxicity testing)

The solubility of the test chemical in growth medium and/or DMSO is first determined. Then a wide range of chemical concentrations is tested for cytotoxicity, starting with a maximum applied dose of 10 mg/ml for test chemicals soluble in media or 1 mg/ml for solutions in organic solvents. After an exposure time of four hours, the cells are washed and a viable cell count is obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of untreated cells are used to select seven to ten doses that cover the range from 0 to 50-90% reduction in 24-hour growth. These selected doses are subsequently applied to cell cultures prepared for mutagenicity testing, but only four or five of the doses will be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of four or five doses spaced from 0 to 50-90% reduction in cell growth.



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## B. Mutagenicity Testing

### 1. Nonactivation Assay

The procedure used is based on that reported by Clive and Spector (1975) and is summarized as follows. Cultures exposed to the test chemical for four hours at the preselected doses are washed and placed in growth medium for two or three days to allow recovery, growth and expression of the induced TK-/- phenotype. Cell counts are determined daily and appropriate dilutions are made to allow optimal growth rates.

At the end of the expression period,  $3 \times 10^6$  cells for each selected dose are seeded in soft agar plates with selection medium and resistant (mutant) colonies are counted after 10 days incubation. To determine the actual number of cells capable of forming colonies, a portion of the cell suspension is also cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number is the mutant frequency.

A detailed flow diagram for the mutation assay is provided in Figure 1.

### 2. Activation Assay

The activation assay can be run concurrently with the nonactivation assay. The only difference is the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension are: 2.4 mg NADP/ml; 4.5 mg isocitric acid/ml; and 50  $\mu$ l S9/ml.

### 3. S9 Homogenate

A 9,000 x g supernatant prepared from Fischer 344 adult male rat liver induced by Aroclor 1254 (described by Ames et al., 1975) is purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. and used in this assay.



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## 5. REPORT

The screened doses, cell counts, and mutant and viable colony counts will be entered into a computer program. The results are analyzed and printed.

The suspension growth of each culture is calculated as (Day 1 Cell Count/3) x (Day 2 Cell Count/3) x (Day 3 Cell Count/3) when the cultures are split back to  $3 \times 10^5$  cells/ml after the daily count. If the cell count is less than  $4 \times 10^5$  cells/ml, the culture is not split back and the cell count is substituted for 3 in the denominator of the next daily count. In most assays, 3-day expressions are not used, so only the first two factors in the preceding calculation are used. The suspension growth is calculated for each solvent control and then averaged. Relative suspension growth values are derived by dividing the suspension growth values by the average solvent control value and multiplying by 100%.

The average cloning efficiency for the negative controls in an assay is the average number of viable colonies for the solvent and untreated controls, divided by 300 and multiplied by 100%. In the computer tables, the cloning efficiency of each culture is expressed relative to the average solvent control cloning efficiency. Whenever the number of cells seeded for viable colony counts differs from 300, the computer calculation of the relative cloning efficiency is adjusted by the factor (300/cells seeded).

A percent relative growth value is calculated as (relative suspension growth) x (relative cloning efficiency/100). Corrected values for the relative cloning efficiency are used in the cases where the number of cells seeded for viable colonies differs from 300.

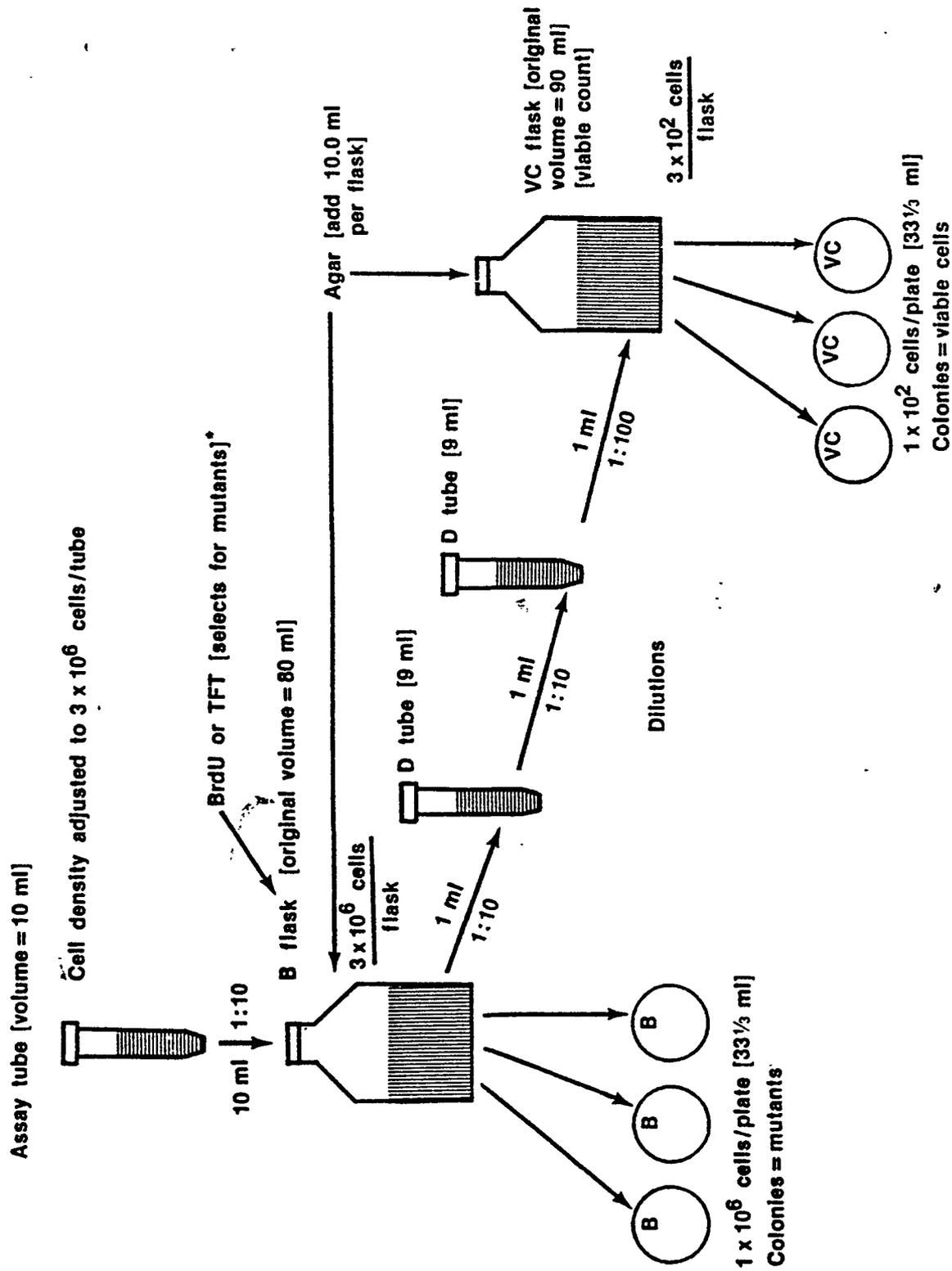
The mutant frequency is calculated as the ratio of mutant colonies to viable colonies times  $10^{-4}$ . This calculation is unaffected by changes in the number of cells seeded for viable count because the number of cells seeded for mutant selection is changed by the same factor. Thus, as an example, if 250 cells are seeded for viable count,  $2.5 \times 10^6$  cells are seeded for mutant selection; the  $10^{-4}$  factor remains constant.

## 6. REFERENCE

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



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\*Added after removal of 1 ml for viable count dilutions.

FIGURE 1. LYMPHOMA CLONING FLOW CHART

## ASSAY ACCEPTANCE CRITERIA

An assay will normally be considered acceptable for evaluation of the test results only if all of the criteria given below are satisfied. The activation and nonactivation portions of the mutation assays are usually performed concurrently, but each portion is in fact an independent assay with its own positive and 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 (average of the solvent and untreated controls) should be between 70% and 130%. A value greater than 100% is possible because of errors in cell counts (usually  $\pm 10\%$ ) and cell division during unavoidable delays between the counting and cloning of many cell cultures. 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 are conditionally acceptable and dependent on the scientific judgement of the study director. All assays below 50% cloning efficiency are unacceptable.
- 2) The solvent and untreated negative controls normally have the same growth rates and cloning efficiencies within experimental error. An unusual effect by the solvent therefore indicates an abnormal cell state or excessive amount of solvent in the growth medium. An assay will be unacceptable if the average percent relative growth of the solvent controls is less than about 70% of the untreated control value.
- 3) The minimum acceptable value for the suspension growth of the average negative control (average of the solvent and untreated control values) for two days is 8.0. Lower values will render an assay unacceptable for evaluation because of the high frequency of unreliable measurements for both the induced mutant frequency and toxicity of a given treatment. The value of 8 corresponds to three population doublings over the 2-day expression period. The most desirable range for the negative control suspension growth is 12 to 25, since the cells are capable of a 5-fold increase in number under optimal growth conditions for 24 hours.
- 4) The background mutant frequency (average frequency of the solvent and untreated negative controls) is calculated separately for concurrent activation and nonactivation assays, even though the same population of cells is used for each assay. The activation negative controls contain the S9 activation mix and typically have a somewhat higher mutant frequency than the nonactivation negative controls. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is  $5 \times 10^{-6}$  to  $50 \times 10^{-6}$ . Assays with backgrounds outside this range are not necessarily invalid but will not be used as primary evidence for the evaluation of a test material. These assays can provide supporting evidence.



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5) A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. The normal range of mutant frequencies induced by 0.5  $\mu\text{l/ml}$  EMS (nonactivation assay) is 300 to 800  $\times 10^{-6}$ ; for 0.3  $\mu\text{l/ml}$  DMN (activation assay) the normal range is 200 to 800  $\times 10^{-6}$ . The concurrent background frequencies have been subtracted from these values. These ranges are broad primarily because the effective treatment with these agents is variable between assays. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test material clearly shows mutagenic activity as described in the evaluation criteria. If the test material appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency above the lower limits of the normal range. Assays in which the normal range is exceeded may require further interpretation by the study director.

6) For test materials with little or no mutagenic activity, an assay must include applied concentrations that reduce the suspension growth to 5% to 10% of the average solvent control or reach the maximum applied concentrations given in the evaluation criteria. Suspension growth is a combined measure of cell death and reduced growth rates. A 5% relative suspension growth therefore could correspond to 90% killing, followed by growth of the survivors at one-half the normal rate for one day and normal growth for the second day. At the other extreme, this condition could be obtained by no killing and complete inhibition of growth for two days. A reasonable limit to testing for the presence of mutagenic action is about 80% to 90% killing of cells. Because of the uncertainty in the actual lethality of treatment in the assay and the fact that mutant frequencies increase as a function of lethality, an acceptable assay for the lack of mutagenic activity must extend to the 5% to 10% relative suspension growth range. There is no maximum toxicity requirement for test materials which clearly show mutagenic activity.

7) An experimental treatment that results in fewer than  $2.5 \times 10^6$  cells by the end of the two-day growth period will not be cloned for mutant analysis.

8) An experimental mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds about 20. These limits avoid problems with the statistical distribution of scoreable colonies among dishes and allows factors no larger than 10 in the adjustment of the observed number of mutant clones to a unit number of cells ( $10^6$ ) able to form colonies.



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9) Mutant frequencies are normally derived from sets of three dishes for both the mutant colony count and the viable colony count. In order to allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set if the colony numbers in the two dishes differ by no more than about 3-fold.

10) 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 to accept a single assay for evaluation of the test material.



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## ASSAY EVALUATION CRITERIA

Mutation assays are initiated by exposing cell cultures to a range of concentrations of test material that is expected, on the basis of preliminary toxicity studies, to span the cellular responses of no observed toxicity to growth to complete lethality within 24 hours of treatment. Then five dose levels are usually selected for completion of the mutation assay. The doses are selected to cover a range of toxicities to growth with emphasis on the most toxic doses. An assay may need to be repeated with different concentrations in order to properly evaluate a test material.

The minimum condition considered necessary to demonstrate mutagenesis for any given treatment is a mutant frequency that exceeds 150% of the concurrent background frequency by at least  $10 \times 10^{-6}$ . The background frequency is defined as the average mutant frequency of the solvent and untreated negative controls. The minimum increase is based on extensive experience which indicates that assay variability increases with higher backgrounds and the calculated minimum increase as defined above is often a repeatable result; statistical analysis for the confidence limits is not yet available.

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 material 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 is not acceptable in a single assay for classifying the test material as a mutagen. If the mutagenic activity at lower concentrations or toxicities was large, a repeat assay will be performed to confirm the mutagenic activity.
- If an increase of about two times the minimum criterion or greater is observed for a single dose near the highest testable toxicity, as defined in the Assay Acceptance Criteria, the test material will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.



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- For some test materials, the correlation between toxicity and applied concentration is poor. The proportion of the applied material 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 relative growth), 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 material will be 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 5% to 10% relative suspension growth. If the test material is relatively nontoxic, the maximum applied concentrations will normally be 10 mg/ml (or 10  $\mu$ l/ml) for water-soluble materials or 1 mg/ml (or 1  $\mu$ l/ml) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response, as discussed above, the test material will be 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 the mutagenic activity of the test material. This presentation may not encompass all test situations, and the study director may use other criteria, especially when data from several repeat assays are available, to arrive at a conclusion. The report will provide the reasoning involved when departures from the above descriptions occur.



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

PROJECT 2989

LBI Assay No. 6084

TYPE of STUDY Mouse Lymphoma Assay

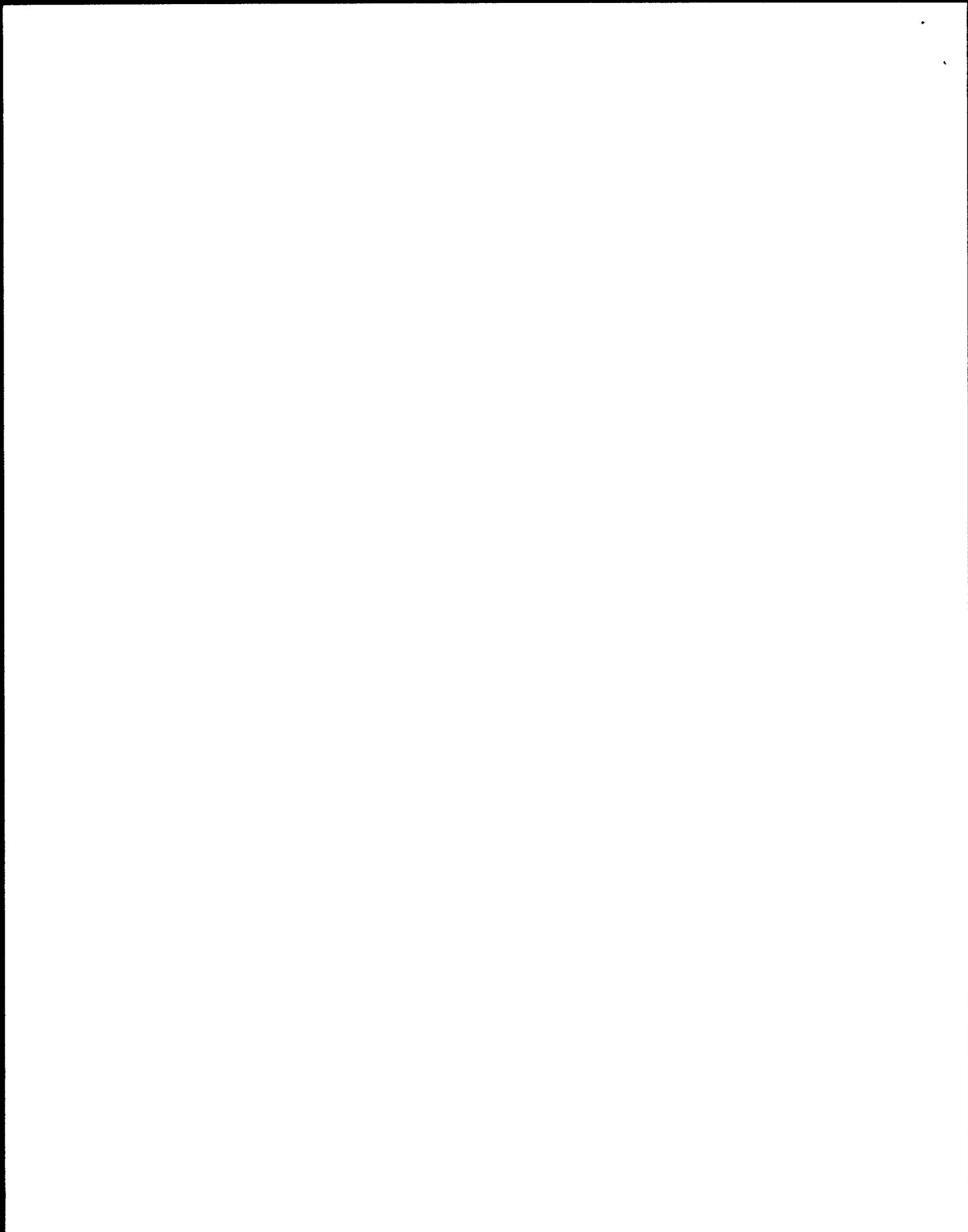
This final study report was reviewed by the LBI Quality Assurance Unit on 5-5-82. A report of findings was submitted to the Study Director and to Management on 5-6-82.

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 S. Hyman  
Auditor, Quality Assurance Unit



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CAS 62-56-6

230852X

TX-86-211

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MUTAGENICITY EVALUATION OF  
COMPOUND 81-0389 (Thiourea)  
IN THE  
AMES SALMONELLA/MICROSOME  
PLATE TEST

Accession No. 900497

FINAL REPORT

Submitted by: Dr. E.D. Barber

Genetic Toxicology Group  
Toxicological Sciences Section  
Health and Environment Laboratories  
Eastman Kodak Company  
Rochester, New York 14650

July 1, 1986

230852X  
TX-86-211

MUTAGENICITY EVALUATION OF

EK 81-0389

IN THE  
AMES SALMONELLA/MICROSOME  
PLATE TEST

FINAL REPORT

SUBMITTED TO:

EASTMAN KODAK  
BUILDING 320  
1669 LAKE AVENUE  
ROCHESTER, NEW YORK 14650

SUBMITTED BY:

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

LBI PROJECT NO. 20988

REPORT DATE: FEBRUARY, 1982



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## 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.



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- I. SPONSOR: EASTMAN KODAK
- II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 6084
- A. Identification: EK 81-0389
- B. Date Received: December 18, 1981
- C. Physical Description: white powder
- III. TYPE OF ASSAY: Ames Salmonella/microsome Mutagenesis Assay
- IV. PROTOCOL NUMBER: 401
- V. STUDY DATES:
- A. Initiation: January 7, 1982
- B. Completion: January 26, 1982
- VI. STUDY DIRECTOR: D.R. Jagannath, Ph.D.
- VII. RESULTS:

The results of this assay are presented in Tables 1, 2, and 3.

VIII. INTERPRETATION OF RESULTS:

The test compound was examined for mutagenic activity in a series of in vitro microbial assays employing Salmonella indicator organisms. The compound was tested directly and in the presence of liver microsomal enzyme preparations from Aroclor-induced rats.

A negative control consisting of the solvent used for preparing the stock solutions and subsequent dilutions of the test material and specific positive compounds were also assayed concurrently with the test material. The negative control data was used as the base for evaluating the results obtained with the test material.

DOSE RANGE

A preliminary toxicity study conducted on the test material at 14 doses of 1.22  $\mu\text{g}$  to 10,000.0  $\mu\text{g}$  per plate using the strain TA-100, did not exhibit significant toxicity at any of the doses (Table 1). As such, the mutagenicity assays were conducted at 8 doses of 1.0  $\mu\text{g}$  to 10,000.0  $\mu\text{g}$  per plate. The assays were conducted using one plate per dose initially and repeated later using three plates per dose level.



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VIII. INTERPRETATION OF RESULTS: (continued)

The results of the initial and repeat tests conducted on the test material in the absence of a metabolic activation system were negative.

The results of the initial and repeat tests conducted on the test material in the presence of a rat liver activation system were negative.

IX. CONCLUSIONS:

The test material EK 81-0389 did not exhibit genetic activity in any of the assays conducted in this evaluation and was considered not mutagenic under these test conditions according to our evaluation criteria.

Submitted by:

Study Director

*D.R. Jagannath* 2/18/82

D.R. Jagannath, Ph.D.  
Section Chief  
Submammalian Genetics  
Department of  
Molecular Toxicology

Date

Reviewed by:

*David J. Brusick* 2/18/82

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

Date



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TABLE 1  
TOXICITY TEST WITH TA-100

SPONSOR: EASTMAN KODAK

COMPOUND CODE: EK 81-0389

ASSAY NO.: 6084

SOLVENT: D.H<sub>2</sub>O

DATE INITIATED: January 7, 1982

DATE COMPLETED: January 11, 1982

| TEST COMPOUND<br>UG/PLATE | NUMBER OF<br>COLONIES/PLATE | % SURVIVAL<br>RELATIVE TO CONTROL |
|---------------------------|-----------------------------|-----------------------------------|
| 0 (control)*              | 222.5**                     | 100.00                            |
| 1.22                      | 251                         | 112.81                            |
| 2.44                      | 242                         | 108.76                            |
| 4.88                      | 304                         | 136.63                            |
| 9.77                      | 292                         | 131.24                            |
| 19.53                     | 269                         | 120.90                            |
| 39.06                     | 295                         | 132.58                            |
| 78.13                     | 252                         | 113.26                            |
| 156.25                    | 234                         | 105.17                            |
| 312.50                    | 301                         | 135.28                            |
| 625.00                    | 315                         | 141.57                            |
| 1250.00                   | 258                         | 115.96                            |
| 2500.00                   | 236                         | 106.07                            |
| 5000.00                   | 228                         | 102.47                            |
| 10,000.00                 | 268                         | 120.45                            |

\* Solvent Control (100  $\mu$ l/plate).

\*\* Average of two plates.



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RESULTS

TABLE 3

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: EK R1-03R9  
 B. SOLVENT: D.H<sub>2</sub>O  
 C. TEST INITIATION DATE: 01/21/R2  
 D. TEST COMPLETION DATE: 01/26/R2  
 E. S-9 LOT#: REG053  
 NOTE: CONCENTRATIONS ARE GIVEN IN MICROGRAMS PER PLATE

REVERTANTS PER PLATE

| TEST               | SPECIES TISSUE |     |     | TA-1537 |     |     | TA-1538 |      |      | TA-98 |      |     | TA-100 |      |      |
|--------------------|----------------|-----|-----|---------|-----|-----|---------|------|------|-------|------|-----|--------|------|------|
|                    | 1              | 2   | 3   | 1       | 2   | 3   | 1       | 2    | 3    | 1     | 2    | 3   | 1      | 2    | 3    |
| NONACTIVATION      |                |     |     |         |     |     |         |      |      |       |      |     |        |      |      |
| SOLVENT CONTROL    | ---            | --- | --- | ---     | --- | --- | ---     | ---  | ---  | ---   | ---  | --- | ---    | ---  | ---  |
| POSITIVE CONTROL** | 17             | 26  | 26  | 24      | 10  | 17  | 18      | 12   | 16   | 30    | 26   | 29  | 154    | 193  | 166  |
|                    | 957            | 812 | 984 | 668     | 677 | 550 | 1646    | 1775 | 1522 | 946   | 1040 | 880 | 1314   | 1239 | 1188 |
| TEST COMPOUND      |                |     |     |         |     |     |         |      |      |       |      |     |        |      |      |
| 1.000000 UG        | 24             | 27  | 30  | 14      | 14  | 16  | 21      | 10   | 18   | 24    | 24   | 28  | 190    | 139  | 182  |
| 10.000000 UG       | 33             | 35  | 33  | 19      | 21  | 23  | 24      | 17   | 19   | 26    | 24   | 23  | 192    | 149  | 195  |
| 100.000000 UG      | 36             | 25  | 34  | 18      | 15  | 16  | 11      | 11   | 17   | 16    | 36   | 30  | 205    | 145  | 163  |
| 500.000000 UG      | 32             | 27  | 25  | 18      | 25  | 15  | 18      | 14   | 17   | 42    | 23   | 34  | 197    | 163  | 195  |
| 1000.000000 UG     | 39             | 31  | 30  | 16      | 16  | 18  | 15      | 14   | 14   | 28    | 22   | 43  | 184    | 159  | 197  |
| 2500.000000 UG     | 35             | 31  | 30  | 18      | 16  | 18  | 11      | 19   | 21   | 31    | 27   | 19  | 175    | 154  | 156  |
| 5000.000000 UG     | 18             | 34  | 36  | 16      | 16  | 14  | 20      | 16   | 16   | 21    | 30   | 15  | 190    | 175  | 187  |
| 10000.000000 UG    | 22             | 31  | 34  | 16      | 18  | 15  | 12      | 16   | 14   | 26    | 36   | 29  | 191    | 159  | 162  |

ACTIVATION

| TEST                | SPECIES | TISSUE | RAT | LIVER | 24  | 27  | 22  | 15  | 23  | 304 | 294  | 2009 | 2015 | 1961 | 1228 | 1290 | 1435 | 2458 | 2442 | 2566 |
|---------------------|---------|--------|-----|-------|-----|-----|-----|-----|-----|-----|------|------|------|------|------|------|------|------|------|------|
|                     |         |        |     |       |     |     |     |     |     |     |      |      |      |      |      |      |      |      |      |      |
| SOLVENT CONTROL     | RAT     | LIVER  |     |       |     |     |     |     |     |     |      |      |      |      |      |      |      |      |      |      |
| POSITIVE CONTROL*** | RAT     | LIVER  | 417 | 415   | 412 | 412 | 412 | 273 | 304 | 294 | 2009 | 2015 | 1961 | 1228 | 1290 | 1435 | 2458 | 2442 | 2566 |      |
| TEST COMPOUND       |         |        |     |       |     |     |     |     |     |     |      |      |      |      |      |      |      |      |      |      |
| 1.000000 UG         | RAT     | LIVER  | 20  | 26    | 23  | 20  | 18  | 18  | 18  | 26  | 26   | 34   | 22   | 39   | 34   | 49   | 165  | 173  | 126  |      |
| 10.000000 UG        | RAT     | LIVER  | 25  | 25    | 21  | 17  | 12  | 24  | 18  | 18  | 18   | 18   | 20   | 40   | 55   | 47   | 129  | 184  | 140  |      |
| 100.000000 UG       | RAT     | LIVER  | 19  | 24    | 34  | 14  | 19  | 21  | 19  | 19  | 23   | 11   | 48   | 33   | 48   | 188  | 193  | 156  |      |      |
| 500.000000 UG       | RAT     | LIVER  | 22  | 27    | 24  | 20  | 20  | 19  | 20  | 20  | 21   | 23   | 50   | 29   | 37   | 170  | 201  | 130  |      |      |
| 1000.000000 UG      | RAT     | LIVER  | 29  | 21    | 31  | 14  | 17  | 17  | 21  | 21  | 22   | 31   | 31   | 41   | 44   | 173  | 175  | 142  |      |      |
| 2500.000000 UG      | RAT     | LIVER  | 28  | 30    | 31  | 19  | 24  | 17  | 24  | 24  | 23   | 23   | 41   | 58   | 44   | 176  | 196  | 149  |      |      |
| 5000.000000 UG      | RAT     | LIVER  | 21  | 24    | 19  | 21  | 19  | 20  | 22  | 22  | 22   | 23   | 51   | 46   | 40   | 176  | 179  | 161  |      |      |
| 10000.000000 UG     | RAT     | LIVER  | 22  | 28    | 12  | 20  | 15  | 21  | 23  | 30  | 18   | 14   | 36   | 33   | 51   | 131  | 184  | 139  |      |      |

\*\*\*

\*\* TA-1535 SODIUM AZIDE 10 UG/PLATE  
 TA-1537 9-AMINOACRIDINE 50 UG/PLATE  
 TA-1538 2-NITROFLUORENE 10 UG/PLATE  
 TA-98 2-NITROFLUORENE 10 UG/PLATE  
 TA-100 SODIUM AZIDE 10 UG/PLATE  
 SOLVENT 100 UL/PLATE

\*\*\* TA-1535 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-1537 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-1538 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-98 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-100 2-ANTHRAMINE 2.5 UG/PLATE

## AMES SALMONELLA/MICROSOME PLATE ASSAY

### 1. OBJECTIVE

The objective of this study is to evaluate a test material for mutagenic activity in a bacterial assay with and without a mammalian S9 activation system.

### 2. RATIONALE

The Salmonella typhimurium strains used at LBI are all histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown in a minimal media petri plate containing a trace of histidine, only those cells that revert to histidine independence (his+) are able to form colonies. The trace amount of histidine allows all the plated bacteria to undergo a few divisions; this growth is essential for mutagenesis to occur. The his+ revertants are easily scored as colonies against the slight background growth. The spontaneous mutation frequency of each strain is relatively constant, but when a mutagen is added to the agar the mutation frequency is increased 2- to 100-fold. Cells which grow to form colonies on the minimal media petri plates are therefore assumed to have reverted, either spontaneously or by the action of a test substance to his+ genotype.

### 3. MATERIALS

#### A. Indicator Microorganism

The Salmonella typhimurium strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley.<sup>1-5</sup> The following five strains are routinely used:

| Strain Designation | Gene Affected | Additional Mutations  |            |          | Mutation Type Detected |
|--------------------|---------------|-----------------------|------------|----------|------------------------|
|                    |               | Repair                | LPS        | R Factor |                        |
| TA-1535            | <u>his</u> G  | $\Delta$ <u>uvr</u> B | <u>rfa</u> | -        | Base-pair substitution |
| TA-1537            | <u>his</u> C  | $\Delta$ <u>uvr</u> B | <u>rfa</u> | -        | Frameshift             |
| TA-1538            | <u>his</u> D  | $\Delta$ <u>uvr</u> B | <u>rfa</u> | -        | Frameshift             |
| TA-98              | <u>his</u> D  | $\Delta$ <u>uvr</u> B | <u>rfa</u> | pKM101   | Frameshift             |
| TA-100             | <u>his</u> G  | $\Delta$ <u>uvr</u> B | <u>rfa</u> | pKM101   | Base-pair substitution |



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### 3. MATERIALS (Continued)

The aforementioned strains have, in addition to the mutation in the histidine operon, a mutation (rfa-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (bio-) and in the repair of ultraviolet (uv) - induced DNA damage (uvrB-). The rfa- mutation makes the strains more permeable to many large molecules. The uvrB- mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens<sup>5</sup>. In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

All indicator strains are kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine. The plates with plasmid-carrying strains contain in addition ampicillin (25 µg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (his, rfa, uvrB, bio) and for the presence of plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid CM67).

#### B. Media

The bacterial strains were cultured in Oxoid Media #2 (nutrient Broth). The selective medium was Vogel Bonner Medium E with 2% glucose<sup>7</sup>. The overlay agar consisted of 0.6% purified agar with 0.5 mM histidine, 0.05 mM biotin and 0.1 M NaCl according to the methods of Ames et. al.<sup>6</sup>

#### C. Activation System

##### (1) S9 Homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (described by Ames et. al.<sup>6</sup>) was purchased commercially and used in this assay.



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### 3. MATERIALS (Continued)

#### (2) S9 Mix

| Components                                       | Concentration Milliliter<br>S9 Mix |
|--|------------------------------------|
| NADP (sodium salt)                               | 4 $\mu$ moles                      |
| D-glucose-6-phosphate                            | 5 $\mu$ moles                      |
| MgCl <sub>2</sub>                                | 8 $\mu$ moles                      |
| KCl  | 33 $\mu$ moles                     |
| Sodium phosphate buffer<br>pH 7.4                | 100 $\mu$ moles                    |
| Organ homogenate from rat<br>liver (S9 fraction) | 100 $\mu$ liters                   |

### 4. EXPERIMENTAL DESIGN

#### A. Dosage Selection

Doses used in the mutagenicity assays were selected from a preliminary toxicity test performed on the strain JA-100. For preliminary toxicity test, 14 doses from 1.0  $\mu$ g to 10,000  $\mu$ g per plate for solids and 10 doses from 0.01  $\mu$ l to 150  $\mu$ l per plate for liquids were used. In the mutagenicity assays, at least six doses were used with the highest dose exhibiting a 50% toxicity. If the test material is not toxic, 8 doses of 1.0, 10, 100, 500, 1000, 2500, 5000 and 10,000  $\mu$ g per plate for solids and 0.1, 1, 5, 10, 25, 50, 100 and 150  $\mu$ l per plate for liquids are used.

If the sponsor specifies doses, no toxicity testing will be performed and the tests are run using the specified doses.

#### B. Toxicity Studies

To a sterile test tube containing 2.0 ml of overlay agar (placed in a 43°-45°C water bath) the following is added:

- 0.1 ml to 0.2 ml of a solution of the test material to give the appropriate dose.
- 0.2 ml of 10<sup>-6</sup> dilution of overnight culture.
- 0.5 ml of 0.2M phosphate buffer, pH 7.4.

This mixture is swirled gently and then poured on to nutrient agar plates. After the overlay agar was set, the plates are incubated at 37°C for approximately 24 hours. The number of colonies growing on the plates counted and recorded.



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#### 4. EXPERIMENTAL DESIGN (Continued)

##### C. Mutagenicity Testing

The procedure used is based on the paper published by Ames et al.<sup>6</sup> and is performed as follows:

###### (1) Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath the following is added in order:

- (a) 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.
- (b) 0.05 ml of a solution of the test chemical to give the appropriate dose.
- (c) 0.1 ml - 0.2 ml of indicator organism(s).
- (d) 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture is swirled gently and then poured onto minimal agar plates (see 3B, Media). After the top agar has set, the plates are incubated at 37°C for approximately 2 days. The number of his+ revertant colonies growing on the plates is counted and recorded.

###### (2) Activation Assay

The activation assay is run concurrently with the nonactivation assay. The only difference is the addition of 0.5 ml of S9 mix (see 3C:2, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which is added in nonactivation assays. All other details are similar to the procedure for nonactivation assays.

A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

##### D. Control Compounds

A negative control consisting of the solvent used for the test material is also assayed concurrently with the test material. For negative controls, step 'b' of Nonactivation Assays is replaced by 0.05 ml of the solvent. The negative controls are employed for each indicator strain and are performed in the absence and presence of S9 mix. The solvent used to prepare the stock solution of the test material is given in the Results section of this report. All dilutions of the test material are made using this solvent. The amount to solvent used is equal to the maximum volume used to give appropriate test dose.

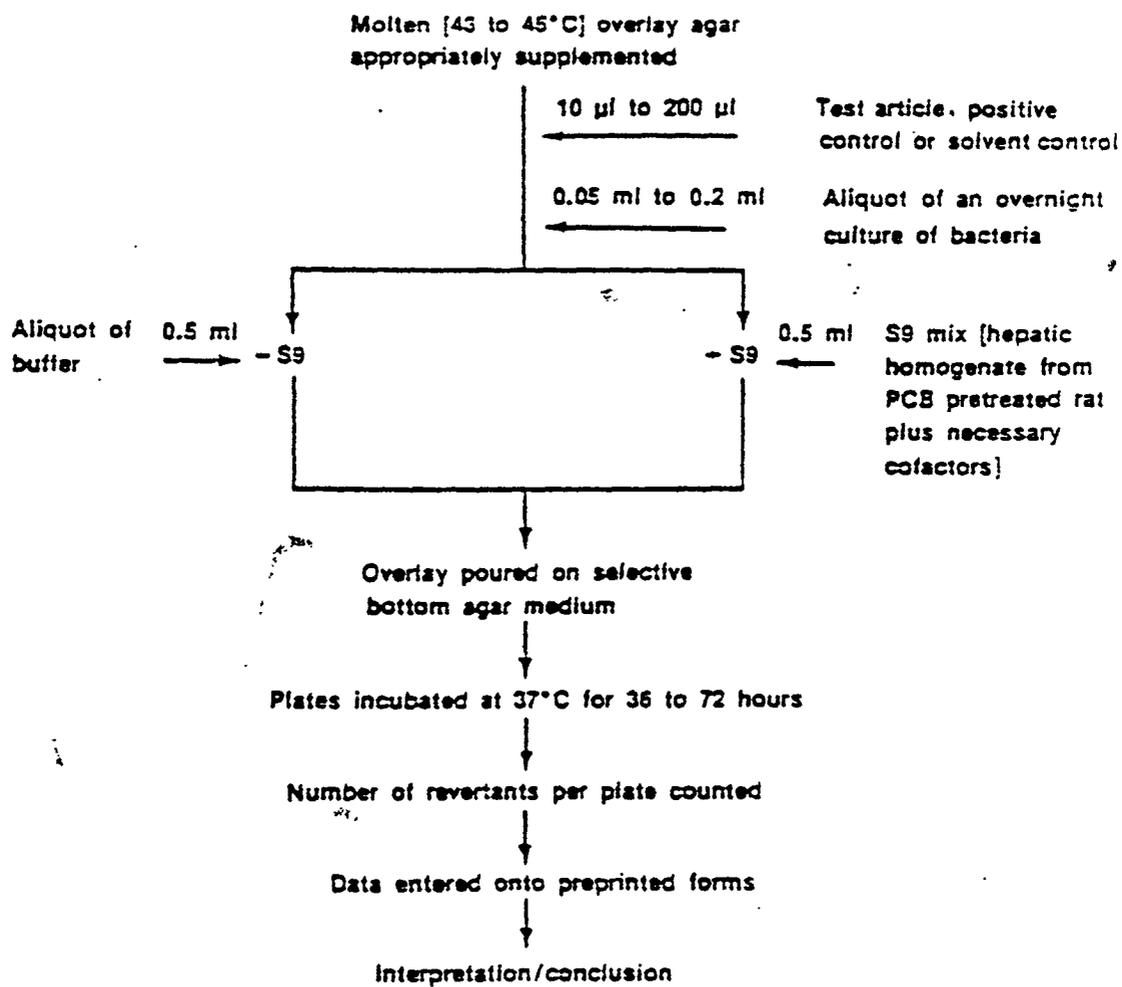
Specific positive control compounds known to revert each strain are also used and assayed concurrently with the test material. The concentrations and specificities of these compounds are given in the following table:



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FIGURE 1

REVERSE MUTATION ASSAY  
[Agar Incorporation Method]



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#### 4. EXPERIMENTAL DESIGN (Continued)

| Assay         | Chemical                 | Solvent                | Concentration<br>per plate<br>(µg) | <u>Salmonella</u><br><u>Strains</u> |
|---------------|--------------------------|------------------------|------------------------------------|-------------------------------------|
| Nonactivation | Sodium azide             | Water                  | 10.0                               | TA-1535, TA-100                     |
|               | 2-Nitrofluorene<br>(NF)  | Dimethyl-<br>sulfoxide | 10.0                               | TA-1538, TA-98                      |
|               | 9-aminoacridine<br>(9AA) | Ethanol                | 50.0                               | TA-1537                             |
| Activation    | 2-anthramine<br>(ANTH)   | Dimethyl-<br>sulfoxide | 2.5                                | For all strains                     |

#### 5. EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of result, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act in replicating DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test material and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

#### C. Evaluation Criteria for Ames Assay

Because the procedures used to evaluate the mutagenicity of the test material are semiquantitative, the criteria used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets are evaluated using the following criteria:

- (1) Strains TA-1535, TA-1537 and TA-1538

If the solvent control value is within the normal range, a test material producing a positive response equal to three times the solvent control value is considered mutagenic.



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5. EVALUATION CRITERIA (Continued)

(2) Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test material producing a positive response equal to twice the solvent control value for TA-98 and TA-100 is considered mutagenic.

The following normal range of revertants for solvent controls are generally considered acceptable:

|          |        |
|----------|--------|
| TA-1535: | 8-30   |
| TA-1537: | 4-30   |
| TA-1538: | 10-35  |
| TA-98 :  | 20-75  |
| TA-100 : | 80-250 |

(3) Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46) and because TA-1538 and TA-98 are both derived from the same parental strain (D3052), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests. Occasionally, exception to this pattern may also be seen.

B. Dose-Response Phenomena

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. Since, we employ several doses in the actual assay, a dose response would normally be seen with a mutagenic test material. Additional tests may be performed at narrower dose, if the mutagenic test material fails to exhibit a dose-response in the initial assay. However, occasionally it is difficult to generate a dose-response and the test material will be evaluated based on the available data.

C. Reproducibility

If a test material produces a response in a single test which cannot be reproduced in additional runs, the initial positive test data lose significance.

D. Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens requiring metabolic biotransformation in activation assays. Negative controls consist of the test material solvent in the overlay agar together with the other essential components. The negative control plate for each



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5. EVALUATION CRITERIA (Continued)

strain gives a reference point to which the test data is compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

E. Relation Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative test on 300 chemicals by McCann et al.<sup>1</sup> show an extremely good correlation between results of microbial mutagenesis tests and in vivo rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.



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

PROJECT 20988

LBI Assay No. 6084

TYPE of STUDY Ames Plate Test

This final study report was reviewed by the LBI Quality Assurance Unit on 2.16.82. A report of findings was submitted to the Study Director and to Management on 2.16.82.

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 Stinson  
Auditor, Quality Assurance Unit



BIONETICS

MUTAGENICITY EVALUATION OF

EK 81-0389

IN THE  
AMES SALMONELLA/MICROSOME  
PLATE TEST

FINAL REPORT

SUBMITTED TO:

EASTMAN KODAK  
BUILDING 320  
1669 LAKE AVENUE  
ROCHESTER, NEW YORK 14650

SUBMITTED BY:

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

LBI PROJECT NO. 20988

REPORT DATE: FEBRUARY, 1982



**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
- II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 6084
- A. Identification: EK 81-0389
- B. Date Received: December 18, 1981
- C. Physical Description: white powder
- III. TYPE OF ASSAY: Ames Salmonella/microsome Mutagenesis Assay
- IV. PROTOCOL NUMBER: 401
- V. STUDY DATES:
- A. Initiation: January 7, 1982
- B. Completion: January 26, 1982
- VI. STUDY DIRECTOR: D.R. Jagannath, Ph.D.
- VII. RESULTS:

The results of this assay are presented in Tables 1, 2, and 3.

VIII. INTERPRETATION OF RESULTS:

The test compound was examined for mutagenic activity in a series of in vitro microbial assays employing Salmonella indicator organisms. The compound was tested directly and in the presence of liver microsomal enzyme preparations from Aroclor-induced rats.

A negative control consisting of the solvent used for preparing the stock solutions and subsequent dilutions of the test material and specific positive compounds were also assayed concurrently with the test material. The negative control data was used as the base for evaluating the results obtained with the test material.

DOSE RANGE

A preliminary toxicity study conducted on the test material at 14 doses of 1.22  $\mu\text{g}$  to 10,000.0  $\mu\text{g}$  per plate using the strain TA-100, did not exhibit significant toxicity at any of the doses (Table 1). As such, the mutagenicity assays were conducted at 8 doses of 1.0  $\mu\text{g}$  to 10,000.0  $\mu\text{g}$  per plate. The assays were conducted using one plate per dose initially and repeated later using three plates per dose level.



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VIII. INTERPRETATION OF RESULTS: (continued)

The results of the initial and repeat tests conducted on the test material in the absence of a metabolic activation system were negative.

The results of the initial and repeat tests conducted on the test material in the presence of a rat liver activation system were negative.

IX. CONCLUSIONS:

The test material EK 81-0389 did not exhibit genetic activity in any of the assays conducted in this evaluation and was considered not mutagenic under these test conditions according to our evaluation criteria.

Submitted by:

Study Director

*D.R. Jagannath* 2/18/82

D.R. Jagannath, Ph.D.  
Section Chief  
Submammalian Genetics  
Department of  
Molecular Toxicology

Date

Reviewed by:

*David J. Brusick* 2/18/82

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

Date



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TABLE 1  
TOXICITY TEST WITH TA-100

SPONSOR: EASTMAN KODAK

COMPOUND CODE: EK 81-0389

ASSAY NO.: 6084

SOLVENT: D.H<sub>2</sub>O

DATE INITIATED: January 7, 1982

DATE COMPLETED: January 11, 1982

| TEST COMPOUND<br>UG/PLATE | NUMBER OF<br>COLONIES/PLATE | % SURVIVAL<br>RELATIVE TO CONTROL |
|---------------------------|-----------------------------|-----------------------------------|
| 0 (control)*              | 222.5**                     | 100.00                            |
| 1.22                      | 251                         | 112.81                            |
| 2.44                      | 242                         | 108.76                            |
| 4.88                      | 304                         | 136.63                            |
| 9.77                      | 292                         | 131.24                            |
| 19.53                     | 269                         | 120.90                            |
| 39.06                     | 295                         | 132.58                            |
| 78.13                     | 252                         | 113.26                            |
| 156.25                    | 234                         | 105.17                            |
| 312.50                    | 301                         | 135.28                            |
| 625.00                    | 315                         | 141.57                            |
| 1250.00                   | 258                         | 115.96                            |
| 2500.00                   | 236                         | 106.07                            |
| 5000.00                   | 228                         | 102.47                            |
| 10,000.00                 | 268                         | 120.45                            |

\* Solvent Control (100  $\mu$ l/plate).

\*\* Average of two plates.



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RESULTS

TABLE 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: EKR1-0389  
 B. SOLVENT: D-H2O  
 C. TEST INITIATION DATES: 01/13/82  
 D. TEST COMPLETION DATE: 01/18/82  
 E. S-9 LOT#: REG053  
 NOTE: CONCENTRATIONS ARE GIVEN IN MICROGRAMS PER PLATE

| TEST                | SPECIES | TISSUE | REVERTANTS PER PLATE |     |     |         |     |     |         |      |      |       |      |      |        |   |   |
|---------------------|---------|--------|----------------------|-----|-----|---------|-----|-----|---------|------|------|-------|------|------|--------|---|---|
|                     |         |        | TA-1535              |     |     | TA-1537 |     |     | TA-1538 |      |      | TA-98 |      |      | TA-100 |   |   |
|                     |         |        | 1                    | 2   | 3   | 1       | 2   | 3   | 1       | 2    | 3    | 1     | 2    | 3    | 1      | 2 | 3 |
| NONACTIVATION       |         |        |                      |     |     |         |     |     |         |      |      |       |      |      |        |   |   |
| SOLVENT CONTROL     | ---     | ---    | 15                   | 10  | 15  | 15      | 17  | 15  | 37      | 48   | 37   | 121   | 123  | 121  |        |   |   |
| SOLVENT CONTROL     | ---     | ---    | 13                   | 7   | 13  | 17      | 7   | 17  | 48      | 48   | 48   | 1280  | 1519 | 1280 |        |   |   |
| POSITIVE CONTROL**  | ---     | ---    | 889                  | 831 | 889 | 831     | 831 | 831 | 991     | 991  | 991  | 1519  | 1519 | 1519 |        |   |   |
| POSITIVE CONTROL**  | ---     | ---    | 729                  | 845 | 729 | 845     | 845 | 845 | 967     | 967  | 967  |       |      |      |        |   |   |
| TEST COMPOUND       | ---     | ---    | 19                   | 13  | 19  | 13      | 13  | 13  | 46      | 53   | 46   | 182   | 172  | 182  |        |   |   |
| 1.000000 UG         | ---     | ---    | 21                   | 6   | 21  | 6       | 6   | 6   | 53      | 44   | 53   | 187   | 187  | 187  |        |   |   |
| 10.000000 UG        | ---     | ---    | 20                   | 9   | 20  | 9       | 9   | 9   | 44      | 49   | 44   | 159   | 190  | 159  |        |   |   |
| 100.000000 UG       | ---     | ---    | 29                   | 7   | 29  | 7       | 7   | 7   | 49      | 50   | 49   | 157   | 154  | 157  |        |   |   |
| 500.000000 UG       | ---     | ---    | 30                   | 8   | 30  | 8       | 8   | 8   | 50      | 57   | 50   | 154   | 163  | 154  |        |   |   |
| 1000.000000 UG      | ---     | ---    | 19                   | 10  | 19  | 10      | 10  | 10  | 57      | 34   | 57   |       |      |      |        |   |   |
| 2500.000000 UG      | ---     | ---    | 20                   | 8   | 20  | 8       | 8   | 8   | 34      | 44   | 34   |       |      |      |        |   |   |
| 5000.000000 UG      | ---     | ---    | 20                   | 5   | 20  | 5       | 5   | 5   | 44      |      | 44   |       |      |      |        |   |   |
| 10000.000000 UG     | ---     | ---    |                      |     |     |         |     |     |         |      |      |       |      |      |        |   |   |
| ACTIVATION          |         |        |                      |     |     |         |     |     |         |      |      |       |      |      |        |   |   |
| SOLVENT CONTROL     | RAT     | LIVER  | 15                   | 7   | 15  | 19      | 17  | 19  | 49      | 48   | 49   | 174   | 144  | 174  |        |   |   |
| SOLVENT CONTROL     | RAT     | LIVER  | 19                   | 10  | 19  | 17      | 10  | 17  | 48      | 48   | 48   | 2121  | 2075 | 2121 |        |   |   |
| POSITIVE CONTROL*** | RAT     | LIVER  | 327                  | 368 | 327 | 368     | 368 | 368 | 1461    | 1461 | 1461 |       |      |      |        |   |   |
| POSITIVE CONTROL*** | RAT     | LIVER  | 309                  | 385 | 309 | 385     | 385 | 385 | 1617    | 1617 | 1617 |       |      |      |        |   |   |
| TEST COMPOUND       | ---     | ---    | 13                   | 6   | 13  | 6       | 6   | 6   | 51      | 55   | 51   | 184   | 152  | 184  |        |   |   |
| 1.000000 UG         | RAT     | LIVER  | 18                   | 5   | 18  | 5       | 5   | 5   | 55      | 45   | 55   | 157   | 175  | 157  |        |   |   |
| 10.000000 UG        | RAT     | LIVER  | 26                   | 14  | 26  | 14      | 14  | 14  | 45      | 51   | 45   | 173   | 194  | 173  |        |   |   |
| 100.000000 UG       | RAT     | LIVER  | 15                   | 11  | 15  | 11      | 11  | 11  | 51      | 57   | 51   | 152   | 162  | 152  |        |   |   |
| 500.000000 UG       | RAT     | LIVER  | 11                   | 6   | 11  | 6       | 6   | 6   | 57      | 45   | 57   |       |      |      |        |   |   |
| 1000.000000 UG      | RAT     | LIVER  | 13                   | 7   | 13  | 7       | 7   | 7   | 45      | 46   | 45   |       |      |      |        |   |   |
| 2500.000000 UG      | RAT     | LIVER  | 13                   | 4   | 13  | 4       | 4   | 4   | 46      | 45   | 46   |       |      |      |        |   |   |
| 5000.000000 UG      | RAT     | LIVER  | 13                   | 7   | 13  | 7       | 7   | 7   | 45      | 45   | 45   |       |      |      |        |   |   |
| 10000.000000 UG     | RAT     | LIVER  | 13                   |     | 13  |         |     |     | 45      |      | 45   |       |      |      |        |   |   |

\*\*\*

\*\* TA-1535 SODIUM AZIDE  
 TA-1537 9-AMINOACRIDINE  
 TA-1538 2-NITROFLUORENE  
 TA-98 2-NITROFLUORENE  
 TA-100 SODIUM AZIDE  
 SOLVENT 100UL/PLATE

TA-1535 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-1537 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-1538 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-98 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-100 2-ANTHRAMINE 2.5 UG/PLATE

RESULTS

TABLE 3

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: EK R1-0389  
 B. SOLVENT: D-H2O  
 C. TEST INITIATION DATES: 01/21/82  
 D. TEST COMPLETION DATE: 01/26/82  
 E. S-9 LOT#: REG053  
 NOTE: CONCENTRATIONS ARE GIVEN IN MICROGRAMS PER PLATE

| TEST                | SPECIES   | REVERTANTS PER PLATE |     |     |         |     |     |         |      |      |       |      |      |        |      |      |
|---------------------|-----------|----------------------|-----|-----|---------|-----|-----|---------|------|------|-------|------|------|--------|------|------|
|                     |           | TA-1535              |     |     | TA-1537 |     |     | TA-1538 |      |      | TA-98 |      |      | TA-100 |      |      |
|                     |           | 1                    | 2   | 3   | 1       | 2   | 3   | 1       | 2    | 3    | 1     | 2    | 3    | 1      | 2    | 3    |
| NONACTIVATION       |           |                      |     |     |         |     |     |         |      |      |       |      |      |        |      |      |
| SOLVENT CONTROL     | ---       | 17                   | 26  | 26  | 24      | 10  | 17  | 18      | 12   | 16   | 30    | 26   | 29   | 154    | 193  | 166  |
| POSITIVE CONTROL**  | ---       | 957                  | 812 | 984 | 668     | 677 | 550 | 1646    | 1775 | 1522 | 946   | 1040 | 880  | 1314   | 1239 | 1188 |
| TEST COMPOUND       |           |                      |     |     |         |     |     |         |      |      |       |      |      |        |      |      |
| 1.000000 UG         | ---       | 24                   | 27  | 30  | 14      | 14  | 16  | 21      | 10   | 18   | 24    | 24   | 28   | 190    | 139  | 182  |
| 10.000000 UG        | ---       | 33                   | 35  | 33  | 19      | 21  | 23  | 24      | 17   | 19   | 26    | 24   | 23   | 192    | 149  | 195  |
| 100.000000 UG       | ---       | 36                   | 25  | 34  | 18      | 15  | 16  | 11      | 11   | 17   | 16    | 36   | 30   | 205    | 145  | 163  |
| 500.000000 UG       | ---       | 32                   | 27  | 25  | 18      | 25  | 15  | 18      | 14   | 17   | 42    | 23   | 34   | 197    | 163  | 195  |
| 1000.000000 UG      | ---       | 39                   | 31  | 30  | 16      | 16  | 18  | 15      | 14   | 14   | 28    | 22   | 43   | 184    | 159  | 197  |
| 2500.000000 UG      | ---       | 35                   | 31  | 30  | 18      | 16  | 18  | 11      | 19   | 21   | 31    | 27   | 19   | 175    | 154  | 156  |
| 5000.000000 UG      | ---       | 18                   | 34  | 36  | 16      | 16  | 14  | 20      | 16   | 16   | 21    | 30   | 19   | 190    | 175  | 187  |
| 10000.000000 UG     | ---       | 22                   | 31  | 34  | 16      | 18  | 15  | 12      | 16   | 14   | 26    | 36   | 29   | 191    | 159  | 162  |
| ACTIVATION          |           |                      |     |     |         |     |     |         |      |      |       |      |      |        |      |      |
| SOLVENT CONTROL     | RAT LIVER | 24                   | 27  | 22  | 15      | 23  | 18  | 32      | 30   | 25   | 45    | 49   | 49   | 189    | 183  | 161  |
| POSITIVE CONTROL*** | RAT LIVER | 417                  | 415 | 412 | 273     | 304 | 294 | 2009    | 2015 | 1961 | 1228  | 1290 | 1435 | 2458   | 2442 | 2566 |
| TEST COMPOUND       |           |                      |     |     |         |     |     |         |      |      |       |      |      |        |      |      |
| 1.000000 UG         | RAT LIVER | 20                   | 26  | 23  | 20      | 18  | 18  | 26      | 34   | 22   | 39    | 34   | 49   | 165    | 173  | 126  |
| 10.000000 UG        | RAT LIVER | 25                   | 25  | 21  | 17      | 12  | 24  | 18      | 18   | 20   | 40    | 55   | 47   | 129    | 184  | 140  |
| 100.000000 UG       | RAT LIVER | 19                   | 24  | 34  | 14      | 19  | 21  | 19      | 23   | 11   | 48    | 33   | 48   | 188    | 193  | 156  |
| 500.000000 UG       | RAT LIVER | 22                   | 27  | 24  | 20      | 20  | 19  | 20      | 21   | 23   | 50    | 29   | 37   | 179    | 201  | 130  |
| 1000.000000 UG      | RAT LIVER | 29                   | 21  | 31  | 14      | 17  | 17  | 21      | 22   | 31   | 31    | 41   | 44   | 173    | 175  | 142  |
| 2500.000000 UG      | RAT LIVER | 28                   | 30  | 31  | 19      | 24  | 17  | 24      | 23   | 23   | 41    | 58   | 44   | 176    | 196  | 149  |
| 5000.000000 UG      | RAT LIVER | 21                   | 24  | 19  | 21      | 19  | 20  | 22      | 22   | 23   | 51    | 46   | 40   | 176    | 179  | 161  |
| 10000.000000 UG     | RAT LIVER | 22                   | 28  | 12  | 20      | 15  | 21  | 23      | 30   | 18   | 36    | 33   | 51   | 131    | 184  | 139  |

\*\* TA-1535 SODIUM AZIDE 10 UG/PLATE  
 TA-1537 9-AMINOACRIDINE 50 UG/PLATE  
 TA-1538 2-NITROFLUORENE 10 UG/PLATE  
 TA-98 2-NITROFLUORENE 10 UG/PLATE  
 TA-100 SODIUM AZIDE 10 UG/PLATE  
 SOLVENT 100 UL/PLATE

\*\*\* TA-1535 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-1537 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-1538 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-98 2-ANTHRAMINE 2.5 UG/PLATE  
 TA-100 2-ANTHRAMINE 2.5 UG/PLATE

## AMES SALMONELLA/MICROSOME PLATE ASSAY

### 1. OBJECTIVE

The objective of this study is to evaluate a test material for mutagenic activity in a bacterial assay with and without a mammalian S9 activation system.

### 2. RATIONALE

The Salmonella typhimurium strains used at LBI are all histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown in a minimal media petri plate containing a trace of histidine, only those cells that revert to histidine independence (his<sup>+</sup>) are able to form colonies. The trace amount of histidine allows all the plated bacteria to undergo a few divisions; this growth is essential for mutagenesis to occur. The his<sup>+</sup> revertants are easily scored as colonies against the slight background growth. The spontaneous mutation frequency of each strain is relatively constant, but when a mutagen is added to the agar the mutation frequency is increased 2- to 100-fold. Cells which grow to form colonies on the minimal media petri plates are therefore assumed to have reverted, either spontaneously or by the action of a test substance to his<sup>+</sup> genotype.

### 3. MATERIALS

#### A. Indicator Microorganism

The Salmonella typhimurium strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley.<sup>1-5</sup> The following five strains are routinely used:

| Strain Designation | Gene Affected | Additional Mutations  |            |          | Mutation Type Detected |
|--------------------|---------------|-----------------------|------------|----------|------------------------|
|                    |               | Repair                | LPS        | R Factor |                        |
| TA-1535            | <u>his</u> G  | $\Delta$ <u>uvr</u> B | <u>rfa</u> | -        | Base-pair substitution |
| TA-1537            | <u>his</u> C  | $\Delta$ <u>uvr</u> B | <u>rfa</u> | -        | Frameshift             |
| TA-1538            | <u>his</u> D  | $\Delta$ <u>uvr</u> B | <u>rfa</u> | -        | Frameshift             |
| TA-98              | <u>his</u> D  | $\Delta$ <u>uvr</u> B | <u>rfa</u> | pKM101   | Frameshift             |
| TA-100             | <u>his</u> G  | $\Delta$ <u>uvr</u> B | <u>rfa</u> | pKM101   | Base-pair substitution |



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### 3. MATERIALS (Continued)

The aforementioned strains have, in addition to the mutation in the histidine operon, a mutation (rfa-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (bio-) and in the repair of ultraviolet (uv) - induced DNA damage (uvrB-). The rfa- mutation makes the strains more permeable to many large molecules. The uvrB- mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens<sup>5</sup>. In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

All indicator strains are kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine. The plates with plasmid-carrying strains contain in addition ampicillin (25 µg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (his, rfa, uvrB, bio) and for the presence of plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid CM67).

#### B. Media

The bacterial strains were cultured in Oxoid Media #2 (nutrient Broth). The selective medium was Vogel Bonner Medium E with 2% glucose<sup>7</sup>. The overlay agar consisted of 0.6% purified agar with 0.5 mM histidine, 0.05 mM biotin and 0.1 M NaCl according to the methods of Ames et. al.<sup>6</sup>

#### C. Activation System

##### (1) S9 Homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (described by Ames et. al.<sup>6</sup>) was purchased commercially and used in this assay.



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### 3. MATERIALS (Continued)

#### (2) S9 Mix

| Components                                       | Concentration Milliliter<br>S9 Mix |
|--|------------------------------------|
| NADP (sodium salt)                               | 4 $\mu$ moles                      |
| D-glucose-6-phosphate                            | 5 $\mu$ moles                      |
| MgCl <sub>2</sub>                                | 8 $\mu$ moles                      |
| KCl  | 33 $\mu$ moles                     |
| Sodium phosphate buffer<br>pH 7.4                | 100 $\mu$ moles                    |
| Organ homogenate from rat<br>liver (S9 fraction) | 100 $\mu$ liters                   |

### 4. EXPERIMENTAL DESIGN

#### A. Dosage Selection

Doses used in the mutagenicity assays were selected from a preliminary toxicity test performed on the strain JA-100. For preliminary toxicity test, 14 doses from 1.0  $\mu$ g to 10,000  $\mu$ g per plate for solids and 10 doses from 0.01  $\mu$ l to 150  $\mu$ l per plate for liquids were used. In the mutagenicity assays, at least six doses were used with the highest dose exhibiting a 50% toxicity. If the test material is not toxic, 8 doses of 1.0, 10, 100, 500, 1000, 2500, 5000 and 10,000  $\mu$ g per plate for solids and 0.1, 1, 5, 10, 25, 50, 100 and 150  $\mu$ l per plate for liquids are used.

If the sponsor specifies doses, no toxicity testing will be performed and the tests are run using the specified doses.

#### B. Toxicity Studies

To a sterile test tube containing 2.0 ml of overlay agar (placed in a 43°-45°C water bath) the following is added:

- 0.1 ml to 0.2 ml of a solution of the test material to give the appropriate dose.
- 0.2 ml of 10<sup>-6</sup> dilution of overnight culture.
- 0.5 ml of 0.2M phosphate buffer, pH 7.4.

This mixture is swirled gently and then poured on to nutrient agar plates. After the overlay agar was set, the plates are incubated at 37°C for approximately 24 hours. The number of colonies growing on the plates counted and recorded.



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#### 4. EXPERIMENTAL DESIGN (Continued)

##### C. Mutagenicity Testing

The procedure used is based on the paper published by Ames et al.<sup>6</sup> and is performed as follows:

###### (1) Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath the following is added in order:

- (a) 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.
- (b) 0.05 ml of a solution of the test chemical to give the appropriate dose.
- (c) 0.1 ml - 0.2 ml of indicator organism(s).
- (d) 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture is swirled gently and then poured onto minimal agar plates (see 3B, Media). After the top agar has set, the plates are incubated at 37°C for approximately 2 days. The number of his+ revertant colonies growing on the plates is counted and recorded.

###### (2) Activation Assay

The activation assay is run concurrently with the nonactivation assay. The only difference is the addition of 0.5 ml of S9 mix (see 3C:2, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which is added in nonactivation assays. All other details are similar to the procedure for nonactivation assays.

A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

##### D. Control Compounds

A negative control consisting of the solvent used for the test material is also assayed concurrently with the test material. For negative controls, step 'b' of Nonactivation Assays is replaced by 0.05 ml of the solvent. The negative controls are employed for each indicator strain and are performed in the absence and presence of S9 mix. The solvent used to prepare the stock solution of the test material is given in the Results section of this report. All dilutions of the test material are made using this solvent. The amount to solvent used is equal to the maximum volume used to give appropriate test dose.

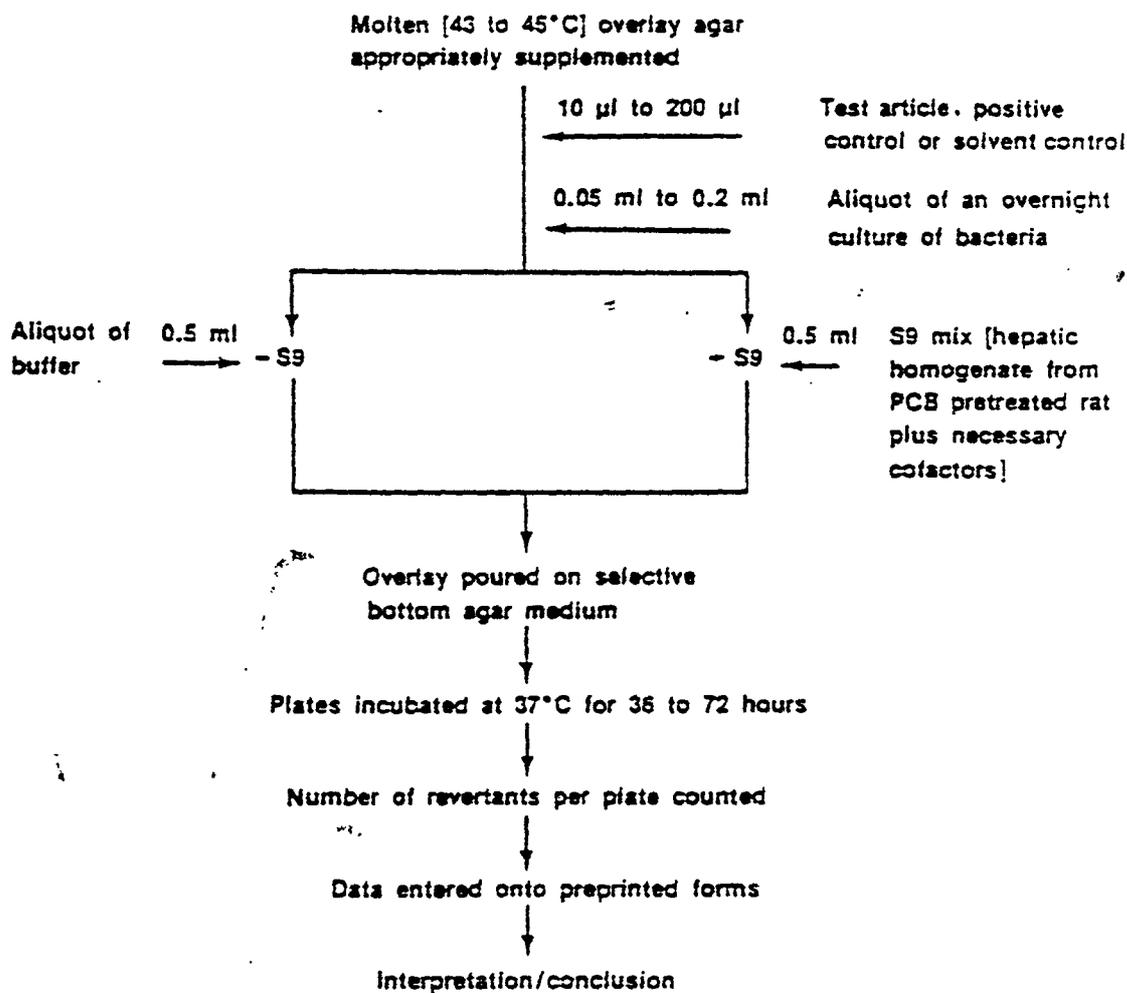
Specific positive control compounds known to revert each strain are also used and assayed concurrently with the test material. The concentrations and specificities of these compounds are given in the following table:



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FIGURE 1

REVERSE MUTATION ASSAY  
[Agar Incorporation Method]



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#### 4. EXPERIMENTAL DESIGN (Continued)

| Assay         | Chemical              | Solvent           | Concentration per plate ( $\mu\text{g}$ ) | Salmonella Strains |
|---------------|-----------------------|-------------------|---|--------------------|
| Nonactivation | Sodium azide          | Water             | 10.0                                      | TA-1535, TA-100    |
|               | 2-Nitrofluorene (NF)  | Dimethylsulfoxide | 10.0                                      | TA-1538, TA-98     |
|               | 9-aminoacridine (9AA) | Ethanol           | 50.0                                      | TA-1537            |
| Activation    | 2-anthramine (ANTH)   | Dimethylsulfoxide | 2.5                                       | For all strains    |

#### 5. EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of result, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act in replicating DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test material and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

#### C. Evaluation Criteria for Ames Assay

Because the procedures used to evaluate the mutagenicity of the test material are semiquantitative, the criteria used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets are evaluated using the following criteria:

- (1) Strains TA-1535, TA-1537 and TA-1538

If the solvent control value is within the normal range, a test material producing a positive response equal to three times the solvent control value is considered mutagenic.



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5. EVALUATION CRITERIA (Continued)

(2) Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test material producing a positive response equal to twice the solvent control value for TA-98 and TA-100 is considered mutagenic.

The following normal range of revertants for solvent controls are generally considered acceptable:

|          |        |
|----------|--------|
| TA-1535: | 8-30   |
| TA-1537: | 4-30   |
| TA-1538: | 10-35  |
| TA-98 :  | 20-75  |
| TA-100 : | 80-250 |

(3) Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46) and because TA-1538 and TA-98 are both derived from the same parental strain (D3052), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests. Occasionally, exception to this pattern may also be seen.

B. Dose-Response Phenomena

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. Since, we employ several doses in the actual assay, a dose response would normally be seen with a mutagenic test material. Additional tests may be performed at narrower dose, if the mutagenic test material fails to exhibit a dose-response in the initial assay. However, occasionally it is difficult to generate a dose-response and the test material will be evaluated based on the available data.

C. Reproducibility

If a test material produces a response in a single test which cannot be reproduced in additional runs, the initial positive test data lose significance.

D. Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens requiring metabolic biotransformation in activation assays. Negative controls consist of the test material solvent in the overlay agar together with the other essential components. The negative control plate for each



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5. EVALUATION CRITERIA (Continued)

strain gives a reference point to which the test data is compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

E. Relation Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative test on 300 chemicals by McCann et al.<sup>1</sup> show an extremely good correlation between results of microbial mutagenesis tests and in vivo rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.



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

PROJECT 20988

LBI Assay No. 6084

TYPE of STUDY Ames Plate Test

This final study report was reviewed by the LBI Quality Assurance Unit on 2-16-82. A report of findings was submitted to the Study Director and to Management on 2-16-82.

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 Stipan  
Auditor, Quality Assurance Unit



CAS 62-56-6

230853Y

TX-86-212

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MUTAGENICITY EVALUATION OF  
COMPOUND 81-0389 (Thiourea)

IN AN

IN VITRO CYTOGENETIC  
ASSAY MEASURING CHROMOSOME  
ABERRATION FREQUENCIES IN  
CHINESE HAMSTER OVARY (CHO)  
CELLS

Accession No. 900497

FINAL REPORT

Submitted by: Dr. E.D. Barber

Genetic Toxicology Group  
Toxicological Sciences Section  
Health and Environment Laboratories  
Eastman Kodak Company  
Rochester, New York 14650

July 1, 1986

LBI SAFETY NO.: 7452

230853Y

TX-86-212

#900497

Thiourea

81-0389

MUTAGENICITY EVALUATION OF

EK 81-0389

IN AN IN VITRO CYTOGENETIC  
ASSAY MEASURING CHROMOSOME  
ABERRATION FREQUENCIES IN  
CHINESE HAMSTER OVARY (CHO)  
CELLS

FINAL REPORT

SUBMITTED TO:

EASTMAN KODAK  
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1669 LAKE AVENUE  
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SUBMITTED BY:

LITTON BIONETICS, INC.  
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LBI PROJECT NO.: 20990

REPORT DATE: APRIL, 1982



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## CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS

### OBJECTIVE

The objective of this in vitro assay was to evaluate the ability of EK 81-0389 to induce chromosome aberrations in Chinese Hamster Ovary (CHO) cells, with and without metabolic activation.

### CONCLUSIONS

In conclusion, EK 81-0389, is considered negative in the aberrations test under the conditions of these tests.



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- I. SPONSOR: Eastman Kodak
- II. MATERIAL TESTED
- A. Client's Identification: EK 81-0389
  - B. Genetics Assay No.: 6084
  - C. Date Received: December 18, 1981
  - D. Physical Description: White crystalline powder
- III. TYPE OF ASSAY: In Vitro Cytogenetic Assay Measuring Chromosome Aberration Frequencies in Chinese Hamster Ovary (CHO) Cells
- IV. PROTOCOL NO.: 437, Edition 7
- V. STUDY DATES:
- A. Initiation Date: February 24, 1982
  - B. Completion Date: April 20, 1982
- VI. SUPERVISORY PERSONNEL
- A. Study Director: Sheila M. Galloway, Ph.D.
  - B. Laboratory Supervisor: Helen Lebowitz
- VII. RESULTS:
- The results are presented in Tables 1-4.
- VIII. INTERPRETATION OF RESULTS:
- i. Solubility and dose determination  
According to normal laboratory procedures, an attempt was made to prepare a stock solution of the test compound at 100 mg/ml; this stock solution is then diluted one hundred-fold into cultures to achieve a maximum dose of 1 mg/ml. The test compound, EK 81-0389, was soluble in culture medium at 100 mg/ml after vigorous pipetting to mix, and warming to about 37°C in a water bath. Due to the low toxicity observed in Trial I (see below) higher doses were used in the second test, by adding up to 0.5 ml of the 100 mg/ml stock solution to 9.5 ml of culture medium, to achieve a maximum top dose of 5 mg/ml.
  - ii. Chromosome aberration assay without metabolic activation  
In the first trial, a standard half-log series of doses from 33.3 ng/ml to 1 mg/ml of EK 81-0389 was tested. There was



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## VIII. INTERPRETATION OF RESULTS: (continued)

### ii. Chromosome aberration assay without metabolic activation (continued)

slight reduction in cell confluence at doses of 33.3  $\mu\text{g/ml}$  or more, and at 1 mg/ml there was also apparent suppression of cell division, with a reduction in available mitotic cells. The results from the first test are shown in Table 1. The frequencies of aberrations in the negative and solvent control cultures were in the normal background range for this laboratory and the positive control compound, Mitomycin C, caused a significant amount of chromosome damage. The culture exposed to MMC at 0.5  $\mu\text{g/ml}$  was not used for aberration analysis. Aberrations were scored on slides prepared from the top five dose levels of EK 81-0389, from 10  $\mu\text{g/ml}$  to 1 mg/ml. There were no statistically significant increases and no evidence for a positive dose relation. However, the finding of five dicentrics at one dose level, 10  $\mu\text{g/ml}$ , is not within normal background limits. Because of this one slightly elevated aberration-response, and in order to achieve results in the presence of more marked cytotoxicity, the test was repeated using a narrowly spaced dose range, from 250  $\mu\text{g/ml}$  to 5 mg/ml. There was a reduction in cell confluence at 750  $\mu\text{g/ml}$  or more, and a severe reduction in the frequency of dividing cells at 1 mg/ml or more, with fewer mitotic cells observed at 5 mg/ml. The aberration results for the top four doses are shown in Table 2. The negative and solvent control aberration frequencies were within normal limits for this laboratory, and there were no statistically significant increases in aberrations in the cultures exposed to EK 81-0389. The slight increase at the top dose, 5 mg/ml, is not significant and is due in part to the presence of three cells showing pulverization, which may be associated with toxicity, although pulverization is also noted at times in control cultures e.g., Table 4. There is no evidence for aberration induction by EK 81-0389.

### iii. Chromosome aberration test in the presence of the metabolic activation system (S9 mix)

In the first trial, a standard series of doses from 33.3 ng/ml to 1 mg/ml was tested. A reduction in cell confluence was noted only at the highest two doses tested, 333.3  $\mu\text{g/ml}$  and 1 mg/ml. There was, however, some suppression of mitosis at doses of 33.3  $\mu\text{g/ml}$  or more. The results are shown in Table 3. The negative and solvent control cultures contained normal background frequencies of aberrations, and the high frequency of damage in the positive control culture illustrates the effective activation of cyclophosphamide by the S9 mix. Because this result at 50  $\mu\text{g/ml}$  of CP was suitable the culture exposed to CP at 25  $\mu\text{g/ml}$  was not used for aberration scoring. The aberration results for cultures exposed to EK 81-0389 at 10  $\mu\text{g/ml}$  to 1 mg/ml (Table 3) show no evidence for an increase in aberrations, or for a positive dose relation. In parallel with the test without activation, the assay was repeated in an attempt to achieve results in the presence of more marked cytotoxicity. The



VIII. INTERPRETATION OF RESULTS: (continued)

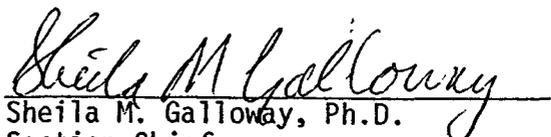
iii. Chromosome aberration test in the presence of the metabolic activation system (S9 Mix) (continued)

aberration results are shown in Table 4. The appearance of the cell monolayer at the top dose, 5 mg/ml, suggest cytotoxicity and there was some suppression of cell division at this dose level. The aberration results show negative and solvent control frequencies that are slightly higher than average but are within normal range for historical control values in our laboratory. The frequencies of damaged cells in the cultures exposed to EK 81-0389 were not significantly higher than the controls, and there was no evidence for induction of the chromatid breaks and rearrangements typical of chemical clastogenesis.

IX. CONCLUSIONS:

In conclusion, EK 81-0389, is considered negative in the aberrations test under the conditions of these tests.

SUBMITTED BY:

  
Sheila M. Galloway, Ph.D.  
Section Chief  
Mammalian Cytogenetics  
Department of Molecular Toxicology

5/3/82  
Date

REVIEWED BY:

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

5/3/82  
Date



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## STUDY DESIGN

### 1. MATERIALS

#### A. Indicator Cells

Chinese hamster ovary (CHO-WBI) cells used in this assay were obtained from Dr. S. Wolff's laboratory, University of California, San Francisco, and cloned in Dr. A. Bloom's laboratory, Columbia University, New York. This is a permanent cell line with an average cycle time of 12 to 14 hours.

#### B. Medium and Cell Cultures

CHO cells for this assay were grown in McCoy's 5a medium supplemented with 10% fetal calf serum, (FCS), L-glutamine, and antibiotics. Cultures were set up approximately 24 hours prior to treatment by seeding 6 to  $1.5 \times 10^6$  cells per 75 cm<sup>2</sup> plastic flask in 5 ml of fresh medium.

#### C. Test Compound and Control Articles

i. Immediately before use, a stock solution of the test compound<sup>1</sup> was prepared in serum-free serum at a final concentration of 100 mg/ml. Serial dilutions were performed in the same solvent to achieve the desired final concentrations by addition of 0.1 ml of the test solution to each 10 ml culture (Trial I) and up to 0.5 ml of the test solution per culture (Trial II).

#### ii. Negative and solvent Controls:

Nothing was added to the negative controls, which contained simply cells and culture medium. Solvent controls contained the solvent for the test article, serum-free medium at a final concentration of 1% (Trial I) and 5% (Trial II).

#### iii. Positive Controls:

Known mutagenic and chromosome breaking agents were used.

a. No metabolic activation required: Mitomycin C (MMC) was dissolved in water. The final concentrations were 0.5 and 1.0  $\mu$ g/ml.

b. Metabolic activation required: Many mutagens do not act directly but must be converted to active intermediates by enzymes found in microsomes. An example is cyclophosphamide (CP), used here as positive control to demonstrate activity of the metabolic activation system used. CP was dissolved in water and used at final concentrations of 25 and 50  $\mu$ g/ml.



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## 2. EXPERIMENTAL DESIGN

### A. Toxicity and Dose Determination

Cultures were exposed to the test compound in a series of doses covering four orders of magnitude. The top dose was limited by solubility and the lower doses formed a half-log series. Before fixation, cultures were examined for degree of confluence and presence of large, rounded (mitotic cells). Only those flasks expected to yield at least some dividing cells were fixed. Cells were read from the top five doses at which results were available.

### B. Cell Treatment

#### i. Assay without metabolic activation system

One day after culture initiation, approximately  $3 \times 10^6$  cells were treated with the test article at predetermined doses for 8 1/2 to 10 hours. The cultures were then washed with saline and fresh culture medium was added, with colcemid at a final concentration of 0.1  $\mu\text{g/ml}$ . Two to two-and-a-half hours after the addition of colcemid, metaphase cells were collected by mitotic shake-off (Terasima and Tolmach, 1961). The cells were swollen with 0.075M KCl hypotonic solution, then washed 3 times in fixative (methanol: acetic acid, 3:1), dropped onto slides and air-dried.

#### ii. Assay with the metabolic activation system

Cells were incubated at 37°C for 2 hours in the presence of the test article and the S9 reaction mixture, in growth medium without fetal calf serum (FCS). The short incubation time is used because prolonged exposure to the S9 mixture is toxic to cells; also enzyme activity is lost rapidly at 37°C. Serum is omitted to avoid the possible inactivation, by binding to serum proteins, of short-lived, highly reactive intermediates produced by S9 enzymes. After the 2-hour exposure period, cells were washed at least twice with buffered saline and normal growth medium containing 10% FCS was added. Incubation was continued for a further 8 to 10 h, with colcemid present during the last two to two-and-a-half hours of incubation. Thereafter, the procedure was that described above (B.,i.).



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C. The Metabolic Activation System

i. The in vitro metabolic activation system comprises rat liver enzymes and an energy-producing system necessary for their function (NADP and isocitric acid). The enzymes are contained in a preparation of liver microsomes (S9 fraction) from rats treated previously with an alkylating agent, Arochlor, to induce enzymes capable of transforming chemicals to more active forms.

ii. Preparation of S9 reaction mixture.

The S9 fraction in buffered sucrose, made from male Fisher rats "induced" with Arochlor 1254, was purchased from Litton Biological Products, Inc. The samples were kept frozen at -80°C and thawed immediately before use. The liver fraction was then added to a "core" reaction mixture to form the activation system described below:

---

| Component                | Final Concentration<br>per Milliliter |
|--------------------------|---------------------------------------|
| NADP (sodium salt)       | 2.4 mg                                |
| Isocitric acid           | 4.5 mg                                |
| Homogenate (S9 fraction) | 15 microliters                        |

---

### 3. STAINING AND SCORING OF SLIDES

Slides were stained with 5% Giemsa at pH 6.8 for subsequent scoring of chromosome aberration frequencies. One hundred cells were scored per dose. For control of bias, all slides were coded prior to scoring and scored "blind."

Standard forms were used to score and record gaps, breaks, fragments and reunion figures, as well as numerical aberrations such as polyploid cells. The aberrations scored include:

|                    |                             |
|--------------------|-----------------------------|
| chromatid gap      | pulverized chromosome       |
| chromatid break    | pulverized chromosomes      |
| chromosome gap     | pulverized cell             |
| chromosome break   | complex rearrangement       |
| chromatid deletion | ring chromosome             |
| chromatid fragment | dicentric chromosome        |
| acentric fragment  | minute chromosome           |
| triradial          | greater than 10 aberrations |
| quadriradial       | endoreduplication           |

### 4. EVALUATION CRITERIA

A number of general guidelines was established to serve as an aid in determining the meaning of CHO chromosomal aberrations.

#### A. General

The objective is to establish whether the test article or its metabolites can interact with cells to induce gross chromosomal breaks, or changes in chromosome numbers. Chemically induced lesions may result in breaks in chromatin that are either repaired by the cell in such a way as to be undetectable, or can result in visible damage. Aberrations are a consequence of failure or mistakes in repair processes that result in lack of rejoining of breaks, or rejoining in abnormal configurations (reviewed by Evans, 1962).

#### B. Data Interpretation

Data were summarized in tables and evaluated. Gaps were not counted as significant aberrations. Open breaks were considered indicators of genetic damage, as were configurations resulting from the abnormal repair of breaks such as multiradials, rings and multicentrics.

When the number of aberrations per cell is computed, pulverized cells and chromosomes cannot be used because the number of aberrations is unknown: this is denoted by a ">" sign before the total number of aberrations per cell in the tables.



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4. EVALUATION CRITERIA (Continued)

B. Data Interpretation

The following factors were taken into account in evaluation:

- i. The estimated number of breaks involved in production of the different types of aberrations observed.
- ii. The frequency of cells with more than one aberration.
- iii. Any evidence for increasing amounts of damage with increasing dose, i.e. a positive dose response.

Statistical analysis employed the Student t-test.

5. REFERENCES

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

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

6. RECORDS MAINTAINED

All raw data, protocols, modifications, test article weight and dispensation records and correspondence between LBI and the Sponsor are maintained in a central file within the Department of Genetics and Cell Biology. These records are filed under Departmental assay number and held up to 2 years following submission of the final report to the Sponsor. After 2 years they are transferred to the LBI Archives for permanent storage.



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## Definitions of Chromosome Aberrations for Giemsa Stained Cells

### CHROMATID TYPE

- TG Chromatid gap ("tid gap"). An achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations as they may not all be true breaks.
- TB Chromatid break An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced.
- TF Chromatid fragment A fragment of a single chromatid with no evident centromere, in a cell with no obvious "parent" chromosome with deleted material.
- IG Isochromatid gap ("Chromosome gap"). Same as chromatid gap but at the same locus in both sister chromatids. If the gap is large or chromosome fragment displaced, the break is included with acentric fragments.

### CHROMATID INTERCHANGES

- TR Triradial: An exchange between two chromosomes, or one chromosome and an acentric fragment, which results in a three-armed configuration.
- QR Quadriradial: As triradial, but resulting in a four-armed configuration.
- CR Complex Rearrangement: An exchange among more than two chromosomes or fragments which is the result of several breaks.

### CHROMOSOME-TYPE

- AF Acentric Fragments: Two parallel chromatids with no evident centromere. The fragment can be of any size greater than the width of a chromatid.
- DM "Double Minute Fragment": These are small double dots, some of which are terminal deletions and some interstitial deletions and probably small rings. Their origins are not distinguishable.
- D Dicentric An exchange between two chromosomes that results in a chromosome with two centromeres. This is often associated with an acentric fragment in which case it is classified as DF.



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- DF Dicentric with fragment.
- R Ring A chromosome which forms a circle containing a centromere. This is often associated with an acentric fragment in which case it is classed as RF.
- RF Ring with fragment.
- Ab Abnormal monocentric chromosome. This is a chromosome whose morphology is abnormal for the karyotype, and the result of a translocation.

Other

- \* PM Prematurely divided "medium" group chromosome. This resembles an acentric fragment the same size as one of the "medium" or C group sub-metacentric chromosomes. It may appear singly or in 3 or more copies, replacing or in addition to the normal complement of C group chromosomes, and has been shown to be a chromosome that has divided at the centromere before the rest of the complement. It is thus not a true acentric fragment.
- > Greater than 10 aberrations: A cell which contains more than 10 aberrations.
- PU Pulverized chromosome: A spread containing one fragmented or pulverized chromosome.
- P+ Pulverized chromosomes: A spread containing two or more fragmented or pulverized chromosomes, but with some intact chromosomes still remaining.
- PC Pulverized Cell: A cell in which all the chromosomes are totally fragmented.
- \* PP Polyploid Cell: A cell containing multiple copies of the haploid number (n) of chromosomes.
- E Endoreduplication = 4n cell in which separation of chromosome pairs has failed.
- \* HR Hyperdiploid: A cell with up to two chromosomes additional to the normal diploid number (2n) (for humans 2n is 46).
- \* HO Hypodiploid: A cell with up to 2 chromosomes missing from the normal diploid number; (cells with fewer chromosomes are thought to be broken and are discounted).

\* not scored in CHO cell line



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Page Three.

|     |                        |   |
|-----|------------------------|---|
| SB  | Chromosome break       | Chromosome has a clear break, forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated but apparently related. Designated "SB" and not an "Ab+AF" which implies two aberrations. |
| CI  | Chromosome Intrachange | e.g. a ring that does not include the entire chromosome.  |
| TI  | Chromatid Intrachange  |   |
| RC  | Ring Chromatid         |   |
| T   | Translocation          | Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes.   |
| ID  | Interstitial Deletion  | Length of chromatin "cut out" from mid-region of a chromatid resulting in a small fragment or ring lying beside a shortened chromatid or a gap in the chromatid.  |
| Unc | Uncoiled Chromosome    | Failure of chromatin packing.   |



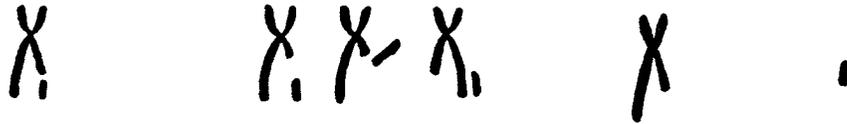
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EXAMPLES OF CHROMOSOME ABERRATIONS

I BREAKS

CHROMATID-TYPE



Chromatid Gap      Chromatid Break      Chromatid Deletion      Fragment

II EXCHANGES



Triradial

Quadriradial



Complex Rearrangement

I BREAKS

CHROMOSOME-TYPES



Chromosome Gap

Chromosome Break

Acentric Fragments

Minute Chromosomes

II EXCHANGES



Dicentric

Ring

Translocation

## CLASSIFICATION OF CHROMOSOME ABERRATIONS

| <u>Complex</u>                                     | <u>Simple</u>             | <u>Other</u>  | <u>Not Included<br/>In Total</u>  |
|--|---------------------------|---------------|-----------------------------------|
| TR, QR, CR<br>D, DF, R, RF,<br>AB, T, CI, TI<br>ID | TB, TF, AF,<br>DM, SB, RC | PU, P+,<br>PC | IG, TG, PM,<br>* PP, E, HR,<br>HO |

PP- Polyploid cells:- Aberrations are not read in polyploid cells. PP is itself scored as an aberration, and the cell is not counted towards the total of one hundred diploid cells counted, i.e. if one hundred cells plus one PP are read, there will be a total of 101 cells under "cells scored".

Aberration Totals for cells with >10 aberrations or pulverization.

| CODE              | ABERRATIONS PER CELL | % CELLS WITH MORE THAN ONE ABERRATIONS |
|-------------------|----------------------|--|
| ><br>PU, P+ or PC | >10<br>>1            | 1<br>1                                 |



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

PROJECT 2990

LBI Assay No. 6084

TYPE of STUDY Chromosome Aberration assay

This final study report was reviewed by the LBI Quality Assurance Unit on 4.29.82. A report of findings was submitted to the Study Director and to Management on 4.30.82.

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 J. Henson  
Auditor, Quality Assurance Unit



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MUTAGENICITY EVALUATION OF  
EK 81-0389  
IN AN IN VITRO CYTOGENETIC  
ASSAY MEASURING CHROMOSOME  
ABERRATION FREQUENCIES IN  
CHINESE HAMSTER OVARY (CHO)  
CELLS

FINAL REPORT

SUBMITTED TO:

EASTMAN KODAK  
BUILDING 320  
1669 LAKE AVENUE  
ROCHESTER, NY 14250

SUBMITTED BY:

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

LBI PROJECT NO.: 20990

REPORT DATE: APRIL, 1982



**BIONETICS**

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## CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS

### OBJECTIVE

The objective of this in vitro assay was to evaluate the ability of EK 81-0389 to induce chromosome aberrations in Chinese Hamster Ovary (CHO) cells, with and without metabolic activation.

### CONCLUSIONS

In conclusion, EK 81-0389, is considered negative in the aberrations test under the conditions of these tests.



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- I. SPONSOR: Eastman Kodak
- II. MATERIAL TESTED
- A. Client's Identification: EK 81-0389
  - B. Genetics Assay No.: 6084
  - C. Date Received: December 18, 1981
  - D. Physical Description: White crystalline powder
- III. TYPE OF ASSAY: In Vitro Cytogenetic Assay Measuring Chromosome Aberration Frequencies in Chinese Hamster Ovary (CHO) Cells
- IV. PROTOCOL NO.: 437, Edition 7
- V. STUDY DATES:
- A. Initiation Date: February 24, 1982
  - B. Completion Date: April 20, 1982
- VI. SUPERVISORY PERSONNEL
- A. Study Director: Sheila M. Galloway, Ph.D.
  - B. Laboratory Supervisor: Helen Lebowitz
- VII. RESULTS:
- The results are presented in Tables 1-4.
- VIII. INTERPRETATION OF RESULTS:
- i. Solubility and dose determination  
According to normal laboratory procedures, an attempt was made to prepare a stock solution of the test compound at 100 mg/ml; this stock solution is then diluted one hundred-fold into cultures to achieve a maximum dose of 1 mg/ml. The test compound, EK 81-0389, was soluble in culture medium at 100 mg/ml after vigorous pipetting to mix, and warming to about 37°C in a water bath. Due to the low toxicity observed in Trial I (see below) higher doses were used in the second test, by adding up to 0.5 ml of the 100 mg/ml stock solution to 9.5 ml of culture medium, to achieve a maximum top dose of 5 mg/ml.
  - ii. Chromosome aberration assay without metabolic activation  
In the first trial, a standard half-log series of doses from 33.3 ng/ml to 1 mg/ml of EK 81-0389 was tested. There was



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## VIII. INTERPRETATION OF RESULTS: (continued)

### ii. Chromosome aberration assay without metabolic activation (continued)

slight reduction in cell confluence at doses of 33.3  $\mu\text{g/ml}$  or more, and at 1 mg/ml there was also apparent suppression of cell division, with a reduction in available mitotic cells. The results from the first test are shown in Table 1. The frequencies of aberrations in the negative and solvent control cultures were in the normal background range for this laboratory and the positive control compound, Mitomycin C, caused a significant amount of chromosome damage. The culture exposed to MMC at 0.5  $\mu\text{g/ml}$  was not used for aberration analysis. Aberrations were scored on slides prepared from the top five dose levels of EK 81-0389, from 10  $\mu\text{g/ml}$  to 1 mg/ml. There were no statistically significant increases and no evidence for a positive dose relation. However, the finding of five dicentrics at one dose level, 10  $\mu\text{g/ml}$ , is not within normal background limits. Because of this one slightly elevated aberration-response, and in order to achieve results in the presence of more marked cytotoxicity, the test was repeated using a narrowly spaced dose range, from 250  $\mu\text{g/ml}$  to 5 mg/ml. There was a reduction in cell confluence at 750  $\mu\text{g/ml}$  or more, and a severe reduction in the frequency of dividing cells at 1 mg/ml or more, with fewer mitotic cells observed at 5 mg/ml. The aberration results for the top four doses are shown in Table 2. The negative and solvent control aberration frequencies were within normal limits for this laboratory, and there were no statistically significant increases in aberrations in the cultures exposed to EK 81-0389. The slight increase at the top dose, 5 mg/ml, is not significant and is due in part to the presence of three cells showing pulverization, which may be associated with toxicity, although pulverization is also noted at times in control cultures e.g., Table 4. There is no evidence for aberration induction by EK 81-0389.

### iii. Chromosome aberration test in the presence of the metabolic activation system (S9 mix)

In the first trial, a standard series of doses from 33.3 ng/ml to 1 mg/ml was tested. A reduction in cell confluence was noted only at the highest two doses tested, 333.3  $\mu\text{g/ml}$  and 1 mg/ml. There was, however, some suppression of mitosis at doses of 33.3  $\mu\text{g/ml}$  or more. The results are shown in Table 3. The negative and solvent control cultures contained normal background frequencies of aberrations, and the high frequency of damage in the positive control culture illustrates the effective activation of cyclophosphamide by the S9 mix. Because this result at 50  $\mu\text{g/ml}$  of CP was suitable the culture exposed to CP at 25  $\mu\text{g/ml}$  was not used for aberration scoring. The aberration results for cultures exposed to EK 81-0389 at 10  $\mu\text{g/ml}$  to 1 mg/ml (Table 3) show no evidence for an increase in aberrations, or for a positive dose relation. In parallel with the test without activation, the assay was repeated in an attempt to achieve results in the presence of more marked cytotoxicity. The



VIII. INTERPRETATION OF RESULTS: (continued)

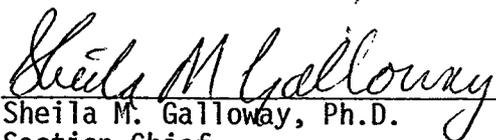
iii. Chromosome aberration test in the presence of the metabolic activation system (S9 Mix) (continued)

aberration results are shown in Table 4. The appearance of the cell monolayer at the top dose, 5 mg/ml, suggest cytotoxicity and there was some suppression of cell division at this dose level. The aberration results show negative and solvent control frequencies that are slightly higher than average but are within normal range for historical control values in our laboratory. The frequencies of damaged cells in the cultures exposed to EK 81-0389 were not significantly higher than the controls, and there was no evidence for induction of the chromatid breaks and rearrangements typical of chemical clastogenesis.

IX. CONCLUSIONS:

In conclusion, EK 81-0389, is considered negative in the aberrations test under the conditions of these tests.

SUBMITTED BY:



Sheila M. Galloway, Ph.D.  
Section Chief  
Mammalian Cytogenetics  
Department of Molecular Toxicology

5/3/82  
Date

REVIEWED BY:



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

5/3/82  
Date



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TABLE 4  
**CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS**

Table 4, Assay No.: 6084, Compound: EK 81-0389, Trial No.: II, Lab. Code Cy 3242, With: Activation Without:

| Treatment                             | Cells Scored | Number and Type of Aberration |    |   |    |    |    |            |    |   |   |    |    | No. of Aberrations Per Cell | % Cells with Aberrations | % Cells with >1 Aberration |            |
|---------------------------------------|--------------|-------------------------------|----|---|----|----|----|------------|----|---|---|----|----|-----------------------------|--------------------------|----------------------------|------------|
|                                       |              | Chromatid                     |    |   |    |    |    | Chromosome |    |   |   |    |    |                             |                          |                            |            |
|                                       |              | TB                            | TD | F | TR | QR | CR | SB         | AF | D | R | MT | PU |                             |                          |                            | E          |
| <b>Controls</b>                       |              |                               |    |   |    |    |    |            |    |   |   |    |    |                             |                          |                            |            |
| Negative: Medium                      | 100          |                               |    | 2 | 2  | 1  | 1  |            |    |   |   |    |    |                             |                          |                            |            |
| Solvent: Medium 5%                    | 100          |                               |    | 1 | 2  |    |    |            |    | 1 | 2 |    |    |                             |                          |                            | 1uc        |
| Positive: Cyclophosphamide 50.0 µg/ml | 100          |                               |    | 1 | 8  | 2  | 3  | 4          | 1  | 1 |   |    |    |                             |                          |                            | 2ID        |
| EK 81-0389                            |              |                               |    |   |    |    |    |            |    |   |   |    |    |                             |                          |                            |            |
| 1.0 mg/ml                             | 100          |                               |    |   |    |    |    |            |    | 1 | 3 |    |    |                             |                          |                            | 1uc        |
| 2.0 mg/ml                             | 100          |                               |    | 2 |    |    |    |            |    | 1 | 5 |    |    |                             |                          |                            | 1DF<br>1uc |
| 3.0 mg/ml                             | 100          |                               |    |   |    |    |    |            |    |   | 1 |    |    |                             |                          |                            |            |
| 4.0 mg/ml                             | 100          |                               |    |   |    |    |    |            |    |   | 2 |    |    |                             |                          |                            | 1uc        |
| 5.0 mg/ml                             | 100          |                               |    |   |    |    |    |            |    |   | 6 | 3  |    |                             |                          |                            |            |
|                                       |              |                               |    |   |    |    |    |            |    |   |   |    |    |                             |                          |                            |            |
|                                       |              |                               |    |   |    |    |    |            |    |   |   |    |    |                             |                          |                            |            |
|                                       |              |                               |    |   |    |    |    |            |    |   |   |    |    |                             |                          |                            |            |
|                                       |              |                               |    |   |    |    |    |            |    |   |   |    |    |                             |                          |                            |            |

\*\* Significantly greater than solvent control. p<0.01.

## STUDY DESIGN

### 1. MATERIALS

#### A. Indicator Cells

Chinese hamster ovary (CHO-WBI) cells used in this assay were obtained from Dr. S. Wolff's laboratory, University of California, San Francisco, and cloned in Dr. A. Bloom's laboratory, Columbia University, New York. This is a permanent cell line with an average cycle time of 12 to 14 hours.

#### B. Medium and Cell Cultures

CHO cells for this assay were grown in McCoy's 5a medium supplemented with 10% fetal calf serum, (FCS), L-glutamine, and antibiotics. Cultures were set up approximately 24 hours prior to treatment by seeding 6 to  $1.5 \times 10^6$  cells per 75 cm<sup>2</sup> plastic flask in 5 ml of fresh medium.

#### C. Test Compound and Control Articles

i. Immediately before use, a stock solution of the test compound was prepared in serum-free serum at a final concentration of 100 mg/ml. Serial dilutions were performed in the same solvent to achieve the desired final concentrations by addition of 0.1 ml of the test solution to each 10 ml culture (Trial I) and up to 0.5 ml of the test solution per culture (Trial II).

#### ii. Negative and solvent Controls:

Nothing was added to the negative controls, which contained simply cells and culture medium. Solvent controls contained the solvent for the test article, serum-free medium at a final concentration of 1% (Trial I) and 5% (Trial II).

#### iii. Positive Controls:

Known mutagenic and chromosome breaking agents were used.

a. No metabolic activation required: Mitomycin C (MMC) was dissolved in water. The final concentrations were 0.5 and 1.0  $\mu$ g/ml.

b. Metabolic activation required: Many mutagens do not act directly but must be converted to active intermediates by enzymes found in microsomes. An example is cyclophosphamide (CP), used here as positive control to demonstrate activity of the metabolic activation system used. CP was dissolved in water and used at final concentrations of 25 and 50  $\mu$ g/ml.



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## 2. EXPERIMENTAL DESIGN

### A. Toxicity and Dose Determination

Cultures were exposed to the test compound in a series of doses covering four orders of magnitude. The top dose was limited by solubility and the lower doses formed a half-log series. Before fixation, cultures were examined for degree of confluence and presence of large, rounded (mitotic cells). Only those flasks expected to yield at least some dividing cells were fixed. Cells were read from the top five doses at which results were available.

### B. Cell Treatment

#### i. Assay without metabolic activation system

One day after culture initiation, approximately  $3 \times 10^6$  cells were treated with the test article at predetermined doses for 8 1/2 to 10 hours. The cultures were then washed with saline and fresh culture medium was added, with colcemid at a final concentration of 0.1  $\mu\text{g/ml}$ . Two to two-and-a-half hours after the addition of colcemid, metaphase cells were collected by mitotic shake-off (Terasima and Tolmach, 1961). The cells were swollen with 0.075M KCl hypotonic solution, then washed 3 times in fixative (methanol: acetic acid, 3:1), dropped onto slides and air-dried.

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ii. Preparation of S9 reaction mixture.

The S9 fraction in buffered sucrose, made from male Fisher rats "induced" with Arochlor 1254, was purchased from Litton Biological Products, Inc. The samples were kept frozen at  $-80^{\circ}\text{C}$  and thawed immediately before use. The liver fraction was then added to a "core" reaction mixture to form the activation system described below:

| Component                | Final Concentration<br>per Milliliter |
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| NADP (sodium salt)       | 2.4 mg                                |
| Isocitric acid           | 4.5 mg                                |
| Homogenate (S9 fraction) | 15 microliters                        |



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### 3. STAINING AND SCORING OF SLIDES

Slides were stained with 5% Giemsa at pH 6.8 for subsequent scoring of chromosome aberration frequencies. One hundred cells were scored per dose. For control of bias, all slides were coded prior to scoring and scored "blind."

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4. EVALUATION CRITERIA (Continued)

B. Data Interpretation

The following factors were taken into account in evaluation:

- i. The estimated number of breaks involved in production of the different types of aberrations observed.
- ii. The frequency of cells with more than one aberration.
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Statistical analysis employed the Student t-test.

5. REFERENCES

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

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

6. RECORDS MAINTAINED

All raw data, protocols, modifications, test article weight and dispensation records and correspondence between LBI and the Sponsor are maintained in a central file within the Department of Genetics and Cell Biology. These records are filed under Departmental assay number and held up to 2 years following submission of the final report to the Sponsor. After 2 years they are transferred to the LBI Archives for permanent storage.



**BIONETICS**

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## Definitions of Chromosome Aberrations for Giemsa Stained Cells

### CHROMATID TYPE

- TG Chromatid gap ("tid gap"). An achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations as they may not all be true breaks.
- TB Chromatid break An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced.
- TF Chromatid fragment A fragment of a single chromatid with no evident centromere, in a cell with no obvious "parent" chromosome with deleted material.
- IG Isochromatid gap ("Chromosome gap"). Same as chromatid gap but at the same locus in both sister chromatids. If the gap is large or chromosome fragment displaced, the break is included with acentric fragments.

### CHROMATID INTERCHANGES

- TR Triradial: An exchange between two chromosomes, or one chromosome and an acentric fragment, which results in a three-armed configuration.
- QR Quadriradial: As triradial, but resulting in a four-armed configuration.
- CR Complex Rearrangement: An exchange among more than two chromosomes or fragments which is the result of several breaks.

### CHROMOSOME-TYPE

- AF Acentric Fragments: Two parallel chromatids with no evident centromere. The fragment can be of any size greater than the width of a chromatid.
- DM "Double Minute Fragment: These are small double dots, some of which are terminal deletions and some interstitial deletions and probably small rings. Their origins are not distinguishable.
- D Dicentric An exchange between two chromosomes that results in a chromosome with two centromeres. This is often associated with an acentric fragment in which case it is classified as DF.

- DF Dicentric with fragment.
- R Ring A chromosome which forms a circle containing a centromere. This is often associated with an acentric fragment in which case it is classed as RF.
- RF Ring with fragment.
- Ab Abnormal monocentric chromosome. This is a chromosome whose morphology is abnormal for the karyotype, and the result of a translocation.

Other

- \* PM Prematurely divided "medium" group chromosome. This resembles an acentric fragment the same size as one of the "medium" or C group sub-metacentric chromosomes. It may appear singly or in 3 or more copies, replacing or in addition to the normal complement of C group chromosomes, and has been shown to be a chromosome that has divided at the centromere before the rest of the complement. It is thus not a true acentric fragment.
- > Greater than 10 aberrations: A cell which contains more than 10 aberrations.
- PU Pulverized chromosome: A spread containing one fragmented or pulverized chromosome.
- P+ Pulverized chromosomes: A spread containing two or more fragmented or pulverized chromosomes, but with some intact chromosomes still remaining.
- PC Pulverized Cell: A cell in which all the chromosomes are totally fragmented.
- \* PP Polyploid Cell: A cell containing multiple copies of the haploid number (n) of chromosomes.
- E Endoreduplication = 4n cell in which separation of chromosome pairs has failed.
- \* HR Hyperdiploid: A cell with up to two chromosomes additional to the normal diploid number (2n) (for humans 2n is 46).
- \* HO Hypodiploid: A cell with up to 2 chromosomes missing from the normal diploid number; (cells with fewer chromosomes are thought to be broken and are discounted).

\* not scored in CHO cell line

Page Three.

|     |                        |   |
|-----|------------------------|---|
| SB  | Chromosome break       | Chromosome has a clear break, forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated but apparently related. Designated "SB" and not an "Ab+AF" which implies two aberrations. |
| CI  | Chromosome Intrachange | e.g. a ring that does not include the entire chromosome.  |
| TI  | Chromatid Intrachange  |   |
| RC  | Ring Chromatid         |   |
| T   | Translocation          | Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes.   |
| ID  | Interstitial Deletion  | Length of chromatin "cut out" from mid-region of a chromatid resulting in a small fragment or ring lying beside a shortened chromatid or a gap in the chromatid.  |
| Unc | Uncoiled Chromosome    | Failure of chromatin packing.   |

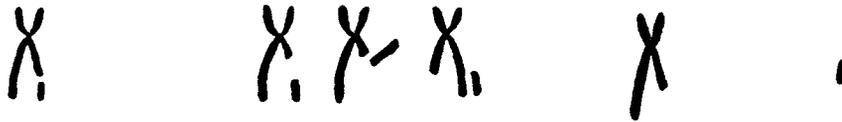


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EXAMPLES OF CHROMOSOME ABERRATIONS

CHROMATID-TYPE

I BREAKS



Chromatid Gap      Chromatid Break      Chromatid Deletion      Fragment

II EXCHANGES



Triradial

Quadriradial



Complex Rearrangement

I BREAKS

CHROMOSOME-TYPES



Chromosome Gap

Chromosome Break

Acentric Fragments

Minute Chromosomes

II EXCHANGES



Dicentric

Ring

Translocation

## CLASSIFICATION OF CHROMOSOME ABERRATIONS

| <u>Complex</u>                                     | <u>Simple</u>             | <u>Other</u>  | <u>Not Included<br/>In Total</u>  |
|--|---------------------------|---------------|-----------------------------------|
| TR, QR, CR<br>D, DF, R, RF,<br>AB, T, CI, TI<br>ID | TB, TF, AF,<br>DM, SB, RC | PU, P+,<br>PC | IG, TG, PM,<br>* PP, E, HR,<br>HO |

PP- Polyploid cells:- Aberrations are not read in polyploid cells. PP is itself scored as an aberration, and the cell is not counted towards the total of one hundred diploid cells counted, i.e. if one hundred cells plus one PP are read, there will be a total of 101 cells under "cells scored".

Aberration Totals for cells with >10 aberrations or pulverization.

| CODE         | ABERRATIONS PER CELL | % CELLS WITH MORE THAN ONE ABERRATIONS |
|--------------|----------------------|--|
| PU, P+ or PC | >10<br>>1            | 1<br>1                                 |



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

PROJECT 2970

LBI Assay No. 6084

TYPE of STUDY Chromosome Aberration assay

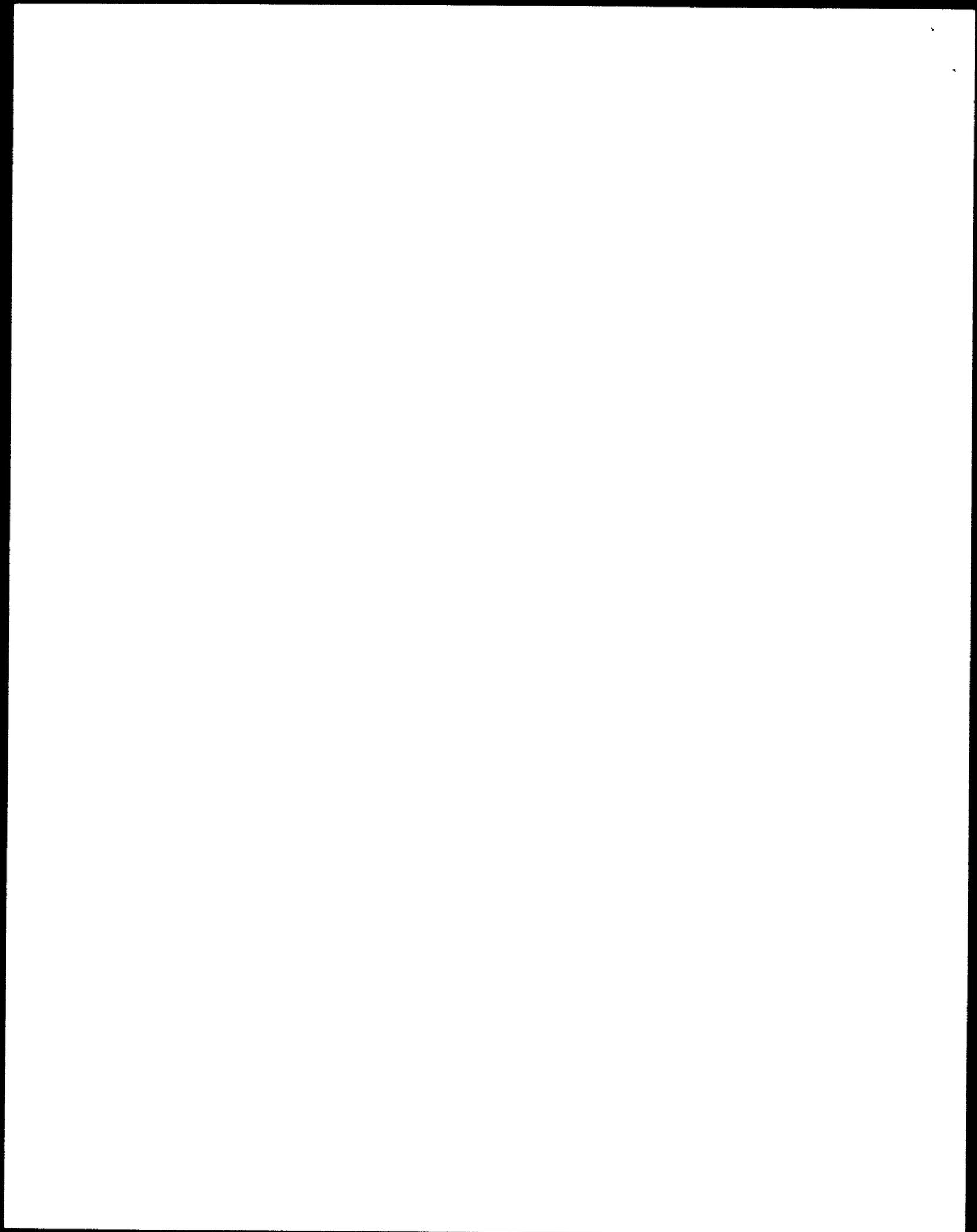
This final study report was reviewed by the LBI Quality Assurance Unit on 4.29.82. A report of findings was submitted to the Study Director and to Management on 4.30.82.

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



CAS 62-56-6

GENETICS ASSAY NO. 0007

LBI SAFETY NO. 7452

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81-0389

EVALUATION OF

EK 81-0389

IN THE  
PRIMARY RAT HEPATOCYTE  
UNSCHEDULED DNA SYNTHESIS ASSAY

FINAL REPORT

SUBMITTED TO:

EASTMAN KODAK  
BUILDING 320  
1669 LAKE AVENUE  
ROCHESTER, NEW YORK 14250

SUBMITTED BY:

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

LBI PROJECT NO. 20991

REPORT DATE: APRIL, 1982



BIONETICS

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## 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: 6084
  - A. Identification: EK 81-0389
  - B. Date Received: December 18, 1981
  - C. Physical Description: Fine, white crystalline powder
- III. TYPE OF ASSAY: Primary Rat Hepatocyte Unscheduled DNA Synthesis Assay
- IV. ASSAY DESIGN NUMBER: 447
- V. STUDY DATES:
  - A. Initiation: January 19, 1982
  - B. Completion: March 26, 1982
- VI. SUPERVISORY PERSONNEL:
  - A. Study Director: Brian C. Myhr, Ph.D.
  - B. Laboratory Supervisor: Marie McKeon
- VII. RESULTS:

The results of the assay are presented in Table 1 on page 4.
- VIII. INTERPRETATION OF RESULTS:

The test material, EK 81-0389, was soluble in WME culture medium (containing 1% fetal bovine serum) at a concentration of 10 mg/ml after brief sonication and warming to 37°C in a water bath. No significant change in pH was observed. This stock was serially diluted with WME (plus 1% serum) to obtain ten concentrations ranging from 10 mg/ml (10,000 µg/ml) to 10 µg/ml. This concentration range was chosen on the basis of the results of a first trial in which concentrations up to 1000 µg/ml were only slightly toxic to the hepatocytes. The treatments were initiated by replacing the media on the cultures with the media containing the different concentrations of test material.

The hepatocytes for the UDS assay were collected at approximately 87% viability (determined by trypan blue exclusion), and about 84% of the viable cells attached to the culture dishes during the 1.5-hour settling period. The treatments were initiated approximately two hours later with cell monolayers that were about 98% viable. After an additional 22 hours in culture (which encompassed the 18-hour treatment period), the average viable cell count in the negative control cultures was about 91% of the viable count at the beginning of the treatments. This stability in cell number, as well as the normal morphological appearance of the cells, indicated that the hepatocyte cultures were in good metabolic condition for the UDS assay.



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### VIII. INTERPRETATION OF RESULTS (continued)

The results of the UDS assay (second trial) are presented in Table 1. The slides from the first trial were not analyzed, since the highest concentration was 1000  $\mu\text{g/ml}$  and the second trial was adjusted to overlap and include higher concentrations.

The test material was moderately toxic at 10,000  $\mu\text{g/ml}$ , which is the maximum concentration applied in this assay and 10-fold higher than the usual high dose of 1000  $\mu\text{g/ml}$ . The number of viable cells was reduced to 35.8% of the number in the negative controls, and the survivors were generally not grouped into islands like normal cells. At 5000  $\mu\text{g/ml}$ , the test material was weakly toxic and the morphology of the cultures returned to normal. No reductions in cell numbers were observed for the treatments with 1000  $\mu\text{g/ml}$  and lower concentrations. Eight treatments from 10,000  $\mu\text{g/ml}$  to 50  $\mu\text{g/ml}$  were therefore selected for analysis of nuclear labeling, since this range more than covered an adequate range of toxic action to look for the possible presence of DNA repair synthesis (UDS). The positive control treatment of 0.10  $\mu\text{g/ml}$  of 2-AAF was weakly toxic and was chosen to demonstrate that overt toxicity is not a necessary condition for detecting induced UDS.

The minimum criteria for UDS in this trial were a mean net nuclear grain count exceeding 6.87, or at least 12.7% of the nuclei containing 6 or more net grains, or at least 2% of the nuclei containing 20 or more net grains. None of the treatments with test material induced changes that even approached these criteria. In fact, the labeling remained very similar to the negative control, and no evidence for a dose-related response was collected. In contrast, the 2-AAF treatment induced a large increase in nuclear labeling that greatly exceeded all three criteria. Since the cultures were clearly responsive to 2-AAF, the test was considered to provide conclusive evidence for the lack of UDS being induced by the test material, as detectable by this assay system.

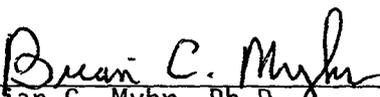
Heavily labeled nuclei (blackened with numerous grains) represent cells undergoing DNA replication rather than DNA repair. The number present in this assay was low, evenly distributed among all treatment groups, and did not interfere with the determination of repair labeling. Among 45,000 cells screened for heavy labeling, only 94 (or 0.63%) were so labeled.



IX. CONCLUSIONS: (continued)

The test material, EK 81-0389, did not induce detectable changes in the nuclear labeling of primary rat hepatocytes for an applied concentration range of 10,000  $\mu\text{g}/\text{ml}$  to 50  $\mu\text{g}/\text{ml}$ . Eight treatments in this range resulted in a cell survival range of 35.8% to 105.5%. None of the criteria used to indicate UDS were even approached by the treatments, and no dose-related response was observed. Therefore, the test material was evaluated as inactive in the Primary Rat Hepatocyte UDS Assay.

SUBMITTED BY:

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

5/4/82  
Date

REVIEWED BY:

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

5/4/82  
Date



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TABLE 1

## SUMMARY OF DATA FROM RAT HEPATOCYTE UDS ASSAY

CLIENT: Eastman Kodak LBI ASSAY NO. 6084 ASSAY INITIATION DATE: February 16, 1982 (Tr  
 CLIENT'S CODE: EK 81-0389 SOLVENT: WME Medium TECH: Marie McKeon

| Test Condition           | Concentration | UDS* grains/nucleus | Avg. † % nuclei with ≥ 6 grains | Avg. † % nuclei with ≥ 20 grains | Survival †† at 22 hrs % |
|--------------------------|---------------|---------------------|---------------------------------|----------------------------------|-------------------------|
| Negative Control         | -----         | 0.87                | 2.7                             | 0                                | 100.0                   |
| Positive Control (2-AAF) | 0.10 µg/ml    | 32.30               | 99.3                            | 80.7                             | 79.0                    |
| <u>Test Material</u>     |               |                     |                                 |                                  |                         |
| EK 81-0389               | 10,000 µg/ml  | 0.91                | 0                               | 0                                | 35.8                    |
| EK 81-0389               | 5,000 µg/ml   | 0.81                | 1.3                             | 0                                | 72.4                    |
| EK 81-0389               | 2,500 µg/ml   | 0.81                | 2.0                             | 0                                | 94.0                    |
| EK 81-0389               | 1,000 µg/ml   | 1.06                | 1.3                             | 0                                | 105.5                   |
| EK 81-0389               | 500 µg/ml     | 0.89                | 0.7                             | 0                                | 99.7                    |
| EK 81-0389               | 250 µg/ml     | 0.77                | 0                               | 0                                | 100.4                   |
| EK 81-0389               | 100 µg/ml     | 1.09                | 1.3                             | 0                                | ND                      |
| EK 81-0389               | 50 µg/ml      | 0.74                | 0                               | 0                                | ND                      |

\*UDS = Average of net nuclear grain counts on triplicate coverslips (150 total cells).

†Average values for triplicate coverslips.

††Survival = Number of viable cells per unit area relative to the negative control x 100%.

2-AAF = 2-acetyl aminofluorene

ND = Not determined

DEVIATIONS FROM THE SIGNED PROTOCOL

1. The 2-AAF concentration was increased from 0.05  $\mu\text{g}/\text{ml}$  to 0.1  $\mu\text{g}/\text{ml}$  in order to obtain weakly toxic treatments. Treatments with 0.05  $\mu\text{g}/\text{ml}$  have been essentially non-toxic in many assays, and the induced level of UDS, while clear-cut, did not always achieve all three criteria. Thus, in order to obtain more nuclei with 20 or more grains, the concentration of 2-AAF has been increased to 0.1  $\mu\text{g}/\text{ml}$  in most current assays.



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## ASSAY DESIGN (NO. 447)

### 1. OBJECTIVE

The objective of this assay is to detect DNA damage caused by the test material, or an active metabolite, by measuring UDS in primary rat hepatocytes in vitro. The existence and degree of DNA damage will be inferred from an increase in nuclear grain counts compared to untreated hepatocytes. The types of detectable DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including  $^3\text{H}$ -thymidine) into the DNA.

### 2. RATIONALE

Fresh hepatocytes obtained from rat liver will attach to a surface in culture and continue to metabolize for several days without undergoing cell division. Only a small percentage of the cells enter S phase (replicative DNA synthesis). Therefore, if  $^3\text{H}$ -thymidine is introduced in the culture medium, little or no label will be incorporated into nuclear DNA. The addition of a test material that interacts with the DNA often stimulates a repair response in which the altered portion of DNA is excised and the missing region replaced by DNA synthesis. This synthesis of DNA by non-dividing cells is known as UDS and can be measured by determining the amount of  $^3\text{H}$ -thymidine incorporated into DNA. In this assay, an autoradiographic technique is used to determine the number of grains per nucleus caused by  $^3\text{H}$ -thymidine incorporation. Cells involved in DNA replication are recognized by heavy labeling of the nuclei and are excluded from the evaluation of UDS activity. Autoradiographic UDS measurement of DNA repair is highly sensitive and appears to correlate very well with the known mutagenic or carcinogenic activities of chemicals (Williams, 1977). Furthermore, the use of primary hepatocytes has the advantage that these cells have sufficient metabolic activity to eliminate the need for the addition of a microsomal activation system.

### 3. MATERIALS

#### A. Indicator Cells

The indicator cells for this assay are hepatocytes obtained from adult male Fischer 344 rats (150-300 g), which are purchased from Charles River Breeding Laboratories, Inc. The animals are fed Purina Certified Rodent Chow (Formula 5002) and water ad libitum. One animal, identified by cage card, is used for the assay after a minimum quarantine period of five days.

The cells are obtained by perfusion of the liver in situ with a collagenase solution, as described in Experimental Design. Monolayer cultures are established on plastic coverslips in culture dishes and are used the same day for initiation of the UDS assay.



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### 3. MATERIALS (Continued)

#### B. Media

The cell cultures are established in Williams' Medium E supplemented with 5% fetal bovine serum, 2mM L-glutamine, 2.4µM dexamethasone, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, and 150 µg/ml gentamicin. After the establishment period, the dexamethasone and serum components are removed. This latter culture medium is referred to simply as WME.

#### C. Controls

##### 1. Negative control

A negative control consisting of assay procedures performed on cells exposed only to the test material solvent is performed in all cases. If the test material is not soluble in water, a stock solution in an organic solvent (normally dimethylsulfoxide; DMSO) is prepared; the final concentration of solvent in the growth medium will be 1% or less in the treated cultures and the negative (solvent) control.

##### 2. Positive control

The positive control compound is known to induce UDS in rat hepatocyte primary cell cultures. 2-Acetyl aminofluorene (2-AAF) at  $4.48 \times 10^{-7}M$  (0.10 µg/ml) was used as the positive control.

### 4. EXPERIMENTAL DESIGN

#### A. Dosing Procedure

The test material is dissolved, if possible, at the highest desired concentration in WME containing 1% serum, and lower concentrations are then prepared by serial dilution with WME plus 1% serum. If the test material is incompletely soluble in WME, dimethylsulfoxide (DMSO) is investigated as the solvent. A solution in DMSO is serially diluted with DMSO and each stock is then diluted 1:100 (or greater) into WME plus 1% serum to obtain the final desired concentrations of test material. If incomplete solubility is obtained in both WME and DMSO, the chosen vehicle will be the one giving the higher solubility and/or better dispersion characteristics. Fresh preparations of test material in the vehicle are used for any testing purpose. Treatments are initiated by replacing the medium on the cell cultures with WME (1% serum) containing the test material at the desired concentrations.

#### B. Dose Selection

The dose selection procedure is an integral part of the UDS assay in order to select appropriate doses for a particular, fresh primary culture of hepatocytes. A range of 15 concentrations is applied



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#### 4. EXPERIMENTAL DESIGN (Continued)

initially to the cells, starting at 1000 µg/ml (or 1000 nanoliters/ml) and diluting in approximately two-fold steps to about 0.025 µg/ml (or 0.025 nanoliters/ml). A viable cell count (trypan blue exclusion) is then obtained about 20-24 hours after initiation of the treatments. At least 6 concentrations are chosen for analysis of nuclear labeling, starting with the highest dose that results in a sufficient number of survivors with intact morphologies and proceeding to successively lower doses.

#### C. UDS Assay

This assay is based on the procedures described by Williams (1977, 1980). The hepatocytes are obtained by perfusion of livers *in situ* for about 4 minutes with Hanks' balanced salts (Ca<sup>++</sup> - Mg<sup>++</sup>-free) containing 0.5 mM ethyleneglycol-bis (β-aminoethyl ether)-N, N-tetraacetic acid (EGTA) and HEPES buffer at pH 7.0. Then WME with 70-100 units/ml of collagenase is perfused through the liver for about 10 minutes. The hepatocytes are obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WME culture medium and collagenase. The suspended tissue and cells are then filtered through sterile cheesecloth to remove cell clumps and debris. The filtrate is centrifuged, and the cell pellet resuspended in WME containing 5% serum and 2.4µM dexamethasone. After obtaining a viable cell count, a series of 35-mm culture dishes (each containing a 25-mm round, plastic coverslip) is inoculated with approximately 0.5 x 10<sup>6</sup> viable cells in 3 ml of WME plus dexamethasone and 5% serum per dish.

An attachment period of 1.5 to 2 hours at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> is used to establish the cell cultures. Unattached cells are then removed and the cultures are refed with WME. The UDS assay is initiated within 3 hours by replacing the media in the culture dishes with 2.5 ml WME containing 1% fetal bovine serum, 1 µCi/ml <sup>3</sup>H-thymidine, and the test material at the desired concentration. Each treatment, including the positive and negative controls, is performed on five cultures, two of which are used for cytotoxicity measurements. After treatment for 18-19 hours, the UDS assay is terminated by washing the cell monolayers twice with WME. Three of the cultures from each treatment are washed with WME containing 1 mM thymidine and are further processed as described below. The other two cultures used to monitor the toxicity of each treatment are refed with WME and returned to the incubator. At 20-24 hours after the initiation of the treatments, viable cell counts (trypan blue exclusion) are determined to estimate cell survival relative to the negative control.

The nuclei in the labeled cells are swollen by placement of the coverslips in 1% sodium citrate for 10 minutes, and then the cells are fixed in acetic acid:ethanol (1:3) and dried for at least 24 hours. The coverslips are mounted on glass slides (cells up); dipped in Kodak NTB2 emulsion, and dried. The coated slides are stored for



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#### 4. EXPERIMENTAL DESIGN (Continued)

7-10 days at 4°C in light-tight boxes containing packets of Drierite. The emulsions are then developed in D19, fixed, and stained with Williams' modified hematoxylin and eosin procedure.

The cells are examined microscopically at approximately 1500x magnification under oil immersion and the field is displayed on the video screen of an automatic counter. UDS is measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (background count). This value is referred to as the net nuclear grain count. The coverslips are coded to prevent bias in grain counting.

#### 5. EVALUATION CRITERIA

The net nuclear grain count is determined for 50 randomly selected cells on each coverslip. Only normally-appearing nuclei are scored, and any occasional nuclei blackened by grains too numerous to count are excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. If the actual count for any nucleus is less than zero (i.e., cytoplasmic count is greater than nuclear count), a net value of zero is used in the calculation of the mean value. The mean net nuclear grain count is determined from the triplicate coverslips (150 total nuclei) for each treatment condition. Occasionally, a coverslip is recounted at a later date or by a different technician. Since a different cell population will generally be scored, the average count for 50 cells is used in the calculation of the mean for the triplicate treatment.

Several criteria have been established which, if met, provide a basis for evaluation of a test material as active in the UDS assay. These criteria are formulated on the basis of published results and laboratory experience and are used in lieu of a statistical treatment at this time to indicate a positive response. While the criteria are arbitrary guidelines that may not be applicable to all assays and may need revision as the data base increases, they represent a reasonable approach to the evaluation of the test material.

The test material is considered active in the UDS assay at applied concentrations that cause:

- 1) An increase in the mean nuclear grain count to at least 6 grains/ nucleus in excess of the concurrent negative control value, and/or
- 2) The percent of nuclei with 6 or more grains to increase above 10% of the examined population, in excess of the concurrent negative control, and/or
- 3) The percent of nuclei with 20 or more grains to reach or exceed 2% of the examined population.



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## 5. EVALUATION CRITERIA

Generally, if the first condition is satisfied, the second and often the third conditions will also be met. However, satisfaction of only the second or third conditions can also indicate UDS activity. Different DNA-damaging agents can give a variety of nuclear labeling patterns, and weak agents may strongly affect only a small minority of the cells. Therefore, all three of the above conditions are considered in an evaluation. If the negative control shows an average of 6 grains/nucleus or 1% of the cells have 20 grains/nucleus, the assay will normally be considered invalid.

A dose-related increase in UDS for at least two consecutive applied concentrations is also desirable to evaluate a test material as active in this assay. In some cases, UDS can increase with dose and then decrease to near-zero with successively higher doses. If this behavior is associated with increased toxicity, the test material can be evaluated as active. If an isolated increase occurs for a treatment far removed from the toxic doses, the UDS will be considered spurious.

The test material is considered inactive in this assay if none of the above conditions are met and if the assay includes the maximum applied dose or other doses that are shown to be toxic by the survival measurements. If little or no toxicity is demonstrated for any of the applied doses and the test material remains soluble in the culture medium, the assay may be considered inconclusive and may be repeated with higher doses after consultation with the Sponsor.

The positive control nuclear labeling is not used as a reference point to estimate mutagenic or carcinogenic risks associated with the UDS activity of the test material. UDS elicited by test agents in this assay is probably more dependent on the type of DNA damage inflicted and the available repair mechanisms than on the potency of the test agent as a mutagen or carcinogen. Some forms of DNA damage are repaired without the incorporation of new nucleic acids. Thus, the positive controls are used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS.

## 6. REFERENCE

Williams, G.M.: Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell culture. *Cancer Res.*, 37:1845-1851, 1977.

Williams, G.M.: The detection of chemical mutagens-carcinogens by DNA repair and mutagenesis in liver cultures, In: Chemical Mutagens, Vol. 6, F. DeSerres and A. Hollaender, (eds.), Plenum Press, NY, 1980, pp. 61-79.



BIONETICS

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

PROJECT 20991

LBI Assay No. 6084

TYPE of STUDY Unscheduled DNA Synthesis

This final study report was reviewed by the LBI Quality Assurance Unit on 5.4.82. A report of findings was submitted to the Study Director and to Management on 5.4.82.

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 J. Hepson  
Auditor, Quality Assurance Unit



BIONETICS

230854Z

TX-86-213

MUTAGENICITY EVALUATION OF  
COMPOUND 81-0389 (Thiourea)  
IN THE  
PRIMARY RAT HEPATOCYTE  
UNSCHEDULED DNA SYNTHESIS ASSAY

Accession No. 900497

FINAL REPORT

Submitted by: Dr. E.D. Barber

Genetic Toxicology Group  
Toxicological Sciences Section  
Health and Environment Laboratories  
Eastman Kodak Company  
Rochester, New York 14650

July 1, 1986

GENETICS ASSAY NO. 6084

LBI SAFETY NO. 7452

#900497  
Thiourea  
81-0389

230854Z  
TX-86-013

EVALUATION OF

EK 81-0389

IN THE  
PRIMARY RAT HEPATOCYTE  
UNSCHEDULED DNA SYNTHESIS ASSAY

FINAL REPORT

SUBMITTED TO:

EASTMAN KODAK  
BUILDING 320  
1669 LAKE AVENUE  
ROCHESTER, NEW YORK 14250

SUBMITTED BY:

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

LBI PROJECT NO. 20991

REPORT DATE: APRIL, 1982



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## 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.



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- I. SPONSOR: Eastman Kodak Company
- II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 6084
  - A. Identification: EK 81-0389
  - B. Date Received: December 18, 1981
  - C. Physical Description: Fine, white crystalline powder
- III. TYPE OF ASSAY: Primary Rat Hepatocyte Unscheduled DNA Synthesis Assay
- IV. ASSAY DESIGN NUMBER: 447
- V. STUDY DATES:
  - A. Initiation: January 19, 1982
  - B. Completion: March 26, 1982
- VI. SUPERVISORY PERSONNEL:
  - A. Study Director: Brian C. Myhr, Ph.D.
  - B. Laboratory Supervisor: Marie McKeon
- VII. RESULTS:

The results of the assay are presented in Table 1 on page 4.
- VIII. INTERPRETATION OF RESULTS:

The test material, EK 81-0389, was soluble in WME culture medium (containing 1% fetal bovine serum) at a concentration of 10 mg/ml after brief sonication and warming to 37°C in a water bath. No significant change in pH was observed. This stock was serially diluted with WME (plus 1% serum) to obtain ten concentrations ranging from 10 mg/ml (10,000 µg/ml) to 10 µg/ml. This concentration range was chosen on the basis of the results of a first trial in which concentrations up to 1000 µg/ml were only slightly toxic to the hepatocytes. The treatments were initiated by replacing the media on the cultures with the media containing the different concentrations of test material.

The hepatocytes for the UDS assay were collected at approximately 87% viability (determined by trypan blue exclusion), and about 84% of the viable cells attached to the culture dishes during the 1.5-hour settling period. The treatments were initiated approximately two hours later with cell monolayers that were about 98% viable. After an additional 22 hours in culture (which encompassed the 18-hour treatment period), the average viable cell count in the negative control cultures was about 91% of the viable count at the beginning of the treatments. This stability in cell number, as well as the normal morphological appearance of the cells, indicated that the hepatocyte cultures were in good metabolic condition for the UDS assay.



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## VIII. INTERPRETATION OF RESULTS (continued)

The results of the UDS assay (second trial) are presented in Table 1. The slides from the first trial were not analyzed, since the highest concentration was 1000  $\mu\text{g/ml}$  and the second trial was adjusted to overlap and include higher concentrations.

The test material was moderately toxic at 10,000  $\mu\text{g/ml}$ , which is the maximum concentration applied in this assay and 10-fold higher than the usual high dose of 1000  $\mu\text{g/ml}$ . The number of viable cells was reduced to 35.8% of the number in the negative controls, and the survivors were generally not grouped into islands like normal cells. At 5000  $\mu\text{g/ml}$ , the test material was weakly toxic and the morphology of the cultures returned to normal. No reductions in cell numbers were observed for the treatments with 1000  $\mu\text{g/ml}$  and lower concentrations. Eight treatments from 10,000  $\mu\text{g/ml}$  to 50  $\mu\text{g/ml}$  were therefore selected for analysis of nuclear labeling, since this range more than covered an adequate range of toxic action to look for the possible presence of DNA repair synthesis (UDS). The positive control treatment of 0.10  $\mu\text{g/ml}$  of 2-AAF was weakly toxic and was chosen to demonstrate that overt toxicity is not a necessary condition for detecting induced UDS.

The minimum criteria for UDS in this trial were a mean net nuclear grain count exceeding 6.87, or at least 12.7% of the nuclei containing 6 or more net grains, or at least 2% of the nuclei containing 20 or more net grains. None of the treatments with test material induced changes that even approached these criteria. In fact, the labeling remained very similar to the negative control, and no evidence for a dose-related response was collected. In contrast, the 2-AAF treatment induced a large increase in nuclear labeling that greatly exceeded all three criteria. Since the cultures were clearly responsive to 2-AAF, the test was considered to provide conclusive evidence for the lack of UDS being induced by the test material, as detectable by this assay system.

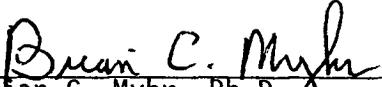
Heavily labeled nuclei (blackened with numerous grains) represent cells undergoing DNA replication rather than DNA repair. The number present in this assay was low, evenly distributed among all treatment groups, and did not interfere with the determination of repair labeling. Among 15,000 cells screened for heavy labeling, only 94 (or 0.63%) were so labeled.



IX. CONCLUSIONS: (continued)

The test material, EK 81-0389, did not induce detectable changes in the nuclear labeling of primary rat hepatocytes for an applied concentration range of 10,000  $\mu\text{g/ml}$  to 50  $\mu\text{g/ml}$ . Eight treatments in this range resulted in a cell survival range of 35.8% to 105.5%. None of the criteria used to indicate UDS were even approached by the treatments, and no dose-related response was observed. Therefore, the test material was evaluated as inactive in the Primary Rat Hepatocyte UDS Assay.

SUBMITTED BY:

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

5/4/82  
Date

REVIEWED BY:

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

5/4/82  
Date



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TABLE 1

## SUMMARY OF DATA FROM RAT HEPATOCYTE UDS ASSAY

CLIENT: Eastman Kodak LBI ASSAY NO. 6084 ASSAY INITIATION DATE: February 16, 1982 (Tri  
 CLIENT'S CODE: EK 81-0389 SOLVENT: WME Medium TECH: Marie McKeon

| Test Condition           | Concentration           | UDS* grains/nucleus | Avg.† % nuclei with ≥ 6 grains | Avg.† % nuclei with ≥ 20 grains | Survival†† at 22 hrs % |
|--------------------------|-------------------------|---------------------|--------------------------------|---------------------------------|------------------------|
| Negative Control         | -----                   | 0.87                | 2.7                            | 0                               | 100.0                  |
| Positive Control (2-AAF) | 0.10 <sup>3</sup> µg/ml | 32.30               | 99.3                           | 80.7                            | 79.0                   |
| <u>Test Material</u>     |                         |                     |                                |                                 |                        |
| EK 81-0389               | 10,000 µg/ml            | 0.91                | 0                              | 0                               | 35.8                   |
| EK 81-0389               | 5,000 µg/ml             | 0.81                | 1.3                            | 0                               | 72.4                   |
| EK 81-0389               | 2,500 µg/ml             | 0.81                | 2.0                            | 0                               | 94.0                   |
| EK 81-0389               | 1,000 µg/ml             | 1.06                | 1.3                            | 0                               | 105.5                  |
| EK 81-0389               | 500 µg/ml               | 0.89                | 0.7                            | 0                               | 99.7                   |
| EK 81-0389               | 250 µg/ml               | 0.77                | 0                              | 0                               | 100.4                  |
| EK 81-0389               | 100 µg/ml               | 1.09                | 1.3                            | 0                               | ND                     |
| EK 81-0389               | 50 µg/ml                | 0.74                | 0                              | 0                               | ND                     |

\*UDS = Average of net nuclear grain counts on triplicate coverslips (150 total cells).

†Average values for triplicate coverslips.

††Survival = Number of viable cells per unit area relative to the negative control x 100%.

2-AAF = 2-acetylaminofluorene

ND = Not determined

DEVIATIONS FROM THE SIGNED PROTOCOL

1. The 2-AAF concentration was increased from 0.05  $\mu\text{g/ml}$  to 0.1  $\mu\text{g/ml}$  in order to obtain weakly toxic treatments. Treatments with 0.05  $\mu\text{g/ml}$  have been essentially non-toxic in many assays, and the induced level of UDS, while clear-cut, did not always achieve all three criteria. Thus, in order to obtain more nuclei with 20 or more grains, the concentration of 2-AAF has been increased to 0.1  $\mu\text{g/ml}$  in most current assays.



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## ASSAY DESIGN (NO. 447)

### 1. OBJECTIVE

The objective of this assay is to detect DNA damage caused by the test material, or an active metabolite, by measuring UDS in primary rat hepatocytes in vitro. The existence and degree of DNA damage will be inferred from an increase in nuclear grain counts compared to untreated hepatocytes. The types of detectable DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including  $^3\text{H}$ -thymidine) into the DNA.

### 2. RATIONALE

Fresh hepatocytes obtained from rat liver will attach to a surface in culture and continue to metabolize for several days without undergoing cell division. Only a small percentage of the cells enter S phase (replicative DNA synthesis). Therefore, if  $^3\text{H}$ -thymidine is introduced in the culture medium, little or no label will be incorporated into nuclear DNA. The addition of a test material that interacts with the DNA often stimulates a repair response in which the altered portion of DNA is excised and the missing region replaced by DNA synthesis. This synthesis of DNA by non-dividing cells is known as UDS and can be measured by determining the amount of  $^3\text{H}$ -thymidine incorporated into DNA. In this assay, an autoradiographic technique is used to determine the number of grains per nucleus caused by  $^3\text{H}$ -thymidine incorporation. Cells involved in DNA replication are recognized by heavy labeling of the nuclei and are excluded from the evaluation of UDS activity. Autoradiographic UDS measurement of DNA repair is highly sensitive and appears to correlate very well with the known mutagenic or carcinogenic activities of chemicals (Williams, 1977). Furthermore, the use of primary hepatocytes has the advantage that these cells have sufficient metabolic activity to eliminate the need for the addition of a microsomal activation system.

### 3. MATERIALS

#### A. Indicator Cells

The indicator cells for this assay are hepatocytes obtained from adult male Fischer 344 rats (150-300 g), which are purchased from Charles River Breeding Laboratories, Inc. The animals are fed Purina Certified Rodent Chow (Formula 5002) and water ad libitum. One animal, identified by cage card, is used for the assay after a minimum quarantine period of five days.

The cells are obtained by perfusion of the liver in situ with a collagenase solution, as described in Experimental Design. Monolayer cultures are established on plastic coverslips in culture dishes and are used the same day for initiation of the UDS assay.



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### 3. MATERIALS (Continued)

#### B. Media

The cell cultures are established in Williams' Medium E supplemented with 5% fetal bovine serum, 2mM L-glutamine, 2.4µM dexamethasone, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, and 150 µg/ml gentamicin. After the establishment period, the dexamethasone and serum components are removed. This latter culture medium is referred to simply as WME.

#### C. Controls

##### 1. Negative control

A negative control consisting of assay procedures performed on cells exposed only to the test material solvent is performed in all cases. If the test material is not soluble in water, a stock solution in an organic solvent (normally dimethylsulfoxide; DMSO) is prepared; the final concentration of solvent in the growth medium will be 1% or less in the treated cultures and the negative (solvent) control.

##### 2. Positive control

The positive control compound is known to induce UDS in rat hepatocyte primary cell cultures. 2-Acetyl aminofluorene (2-AAF) at  $4.48 \times 10^{-7}$ M (0.10 µg/ml) was used as the positive control.

### 4. EXPERIMENTAL DESIGN

#### A. Dosing Procedure

The test material is dissolved, if possible, at the highest desired concentration in WME containing 1% serum, and lower concentrations are then prepared by serial dilution with WME plus 1% serum. If the test material is incompletely soluble in WME, dimethylsulfoxide (DMSO) is investigated as the solvent. A solution in DMSO is serially diluted with DMSO and each stock is then diluted 1:100 (or greater) into WME plus 1% serum to obtain the final desired concentrations of test material. If incomplete solubility is obtained in both WME and DMSO, the chosen vehicle will be the one giving the higher solubility and/or better dispersion characteristics. Fresh preparations of test material in the vehicle are used for any testing purpose. Treatments are initiated by replacing the medium on the cell cultures with WME (1% serum) containing the test material at the desired concentrations.

#### B. Dose Selection

The dose selection procedure is an integral part of the UDS assay in order to select appropriate doses for a particular, fresh primary culture of hepatocytes. A range of 15 concentrations is applied



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#### 4. EXPERIMENTAL DESIGN (Continued)

initially to the cells, starting at 1000 µg/ml (or 1000 nanoliters/ml) and diluting in approximately two-fold steps to about 0.025 µg/ml (or 0.025 nanoliters/ml). A viable cell count (trypan blue exclusion) is then obtained about 20-24 hours after initiation of the treatments. At least 6 concentrations are chosen for analysis of nuclear labeling, starting with the highest dose that results in a sufficient number of survivors with intact morphologies and proceeding to successively lower doses.

##### C. UDS Assay

This assay is based on the procedures described by Williams (1977, 1980). The hepatocytes are obtained by perfusion of livers *in situ* for about 4 minutes with Hanks' balanced salts (Ca<sup>++</sup> - Mg<sup>++</sup>-free) containing 0.5 mM ethyleneglycol-bis (β-aminoethyl ether)-N, N-tetraacetic acid (EGTA) and HEPES buffer at pH 7.0. Then WME with 70-100 units/ml of collagenase is perfused through the liver for about 10 minutes. The hepatocytes are obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WME culture medium and collagenase. The suspended tissue and cells are then filtered through sterile cheesecloth to remove cell clumps and debris. The filtrate is centrifuged and the cell pellet resuspended in WME containing 5% serum and 2.4µM dexamethasone. After obtaining a viable cell count, a series of 35-mm culture dishes (each containing a 25-mm round, plastic coverslip) is inoculated with approximately 0.5 x 10<sup>6</sup> viable cells in 3 ml of WME plus dexamethasone and 5% serum per dish.

An attachment period of 1.5 to 2 hours at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> is used to establish the cell cultures. Unattached cells are then removed and the cultures are refed with WME. The UDS assay is initiated within 3 hours by replacing the media in the culture dishes with 2.5 ml WME containing 1% fetal bovine serum, 1 µCi/ml <sup>3</sup>H-thymidine, and the test material at the desired concentration. Each treatment, including the positive and negative controls, is performed on five cultures, two of which are used for cytotoxicity measurements. After treatment for 18-19 hours, the UDS assay is terminated by washing the cell monolayers twice with WME. Three of the cultures from each treatment are washed with WME containing 1 mM thymidine and are further processed as described below. The other two cultures used to monitor the toxicity of each treatment are refed with WME and returned to the incubator. At 20-24 hours after the initiation of the treatments, viable cell counts (trypan blue exclusion) are determined to estimate cell survival relative to the negative control.

The nuclei in the labeled cells are swollen by placement of the coverslips in 1% sodium citrate for 10 minutes, and then the cells are fixed in acetic acid:ethanol (1:3) and dried for at least 24 hours. The coverslips are mounted on glass slides (cells up), dipped in Kodak NTB2 emulsion, and dried. The coated slides are stored for



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#### 4. EXPERIMENTAL DESIGN (Continued)

7-10 days at 4°C in light-tight boxes containing packets of Drierite. The emulsions are then developed in D19, fixed, and stained with 'Williams' modified hematoxylin and eosin procedure.

The cells are examined microscopically at approximately 1500x magnification under oil immersion and the field is displayed on the video screen of an automatic counter. UDS is measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (background count). This value is referred to as the net nuclear grain count. The coverslips are coded to prevent bias in grain counting.

#### 5. EVALUATION CRITERIA

The net nuclear grain count is determined for 50 randomly selected cells on each coverslip. Only normally-appearing nuclei are scored, and any occasional nuclei blackened by grains too numerous to count are excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. If the actual count for any nucleus is less than zero (i.e., cytoplasmic count is greater than nuclear count), a net value of zero is used in the calculation of the mean value. The mean net nuclear grain count is determined from the triplicate coverslips (150 total nuclei) for each treatment condition. Occasionally, a coverslip is recounted at a later date or by a different technician. Since a different cell population will generally be scored, the average count for 50 cells is used in the calculation of the mean for the triplicate treatment.

Several criteria have been established which, if met, provide a basis for evaluation of a test material as active in the UDS assay. These criteria are formulated on the basis of published results and laboratory experience and are used in lieu of a statistical treatment at this time to indicate a positive response. While the criteria are arbitrary guidelines that may not be applicable to all assays and may need revision as the data base increases, they represent a reasonable approach to the evaluation of the test material.

The test material is considered active in the UDS assay at applied concentrations that cause:

- 1) An increase in the mean nuclear grain count to at least 6 grains/ nucleus in excess of the concurrent negative control value, and/or
- 2) The percent of nuclei with 6 or more grains to increase above 10% of the examined population, in excess of the concurrent negative control, and/or
- 3) The percent of nuclei with 20 or more grains to reach or exceed 2% of the examined population.



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## 5. EVALUATION CRITERIA

Generally, if the first condition is satisfied, the second and often the third conditions will also be met. However, satisfaction of only the second or third conditions can also indicate UDS activity. Different DNA-damaging agents can give a variety of nuclear labeling patterns, and weak agents may strongly affect only a small minority of the cells. Therefore, all three of the above conditions are considered in an evaluation. If the negative control shows an average of 6 grains/nucleus or 1% of the cells have 20 grains/nucleus, the assay will normally be considered invalid.

A dose-related increase in UDS for at least two consecutive applied concentrations is also desirable to evaluate a test material as active in this assay. In some cases, UDS can increase with dose and then decrease to near-zero with successively higher doses. If this behavior is associated with increased toxicity, the test material can be evaluated as active. If an isolated increase occurs for a treatment far removed from the toxic doses, the UDS will be considered spurious.

The test material is considered inactive in this assay if none of the above conditions are met and if the assay includes the maximum applied dose or other doses that are shown to be toxic by the survival measurements. If little or no toxicity is demonstrated for any of the applied doses and the test material remains soluble in the culture medium, the assay may be considered inconclusive and may be repeated with higher doses after consultation with the Sponsor.

The positive control nuclear labeling is not used as a reference point to estimate mutagenic or carcinogenic risks associated with the UDS activity of the test material. UDS elicited by test agents in this assay is probably more dependent on the type of DNA damage inflicted and the available repair mechanisms than on the potency of the test agent as a mutagen or carcinogen. Some forms of DNA damage are repaired without the incorporation of new nucleic acids. Thus, the positive controls are used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS.

## 6. REFERENCE

Williams, G.M.: Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell culture. *Cancer Res.*, 37:1845-1851, 1977.

Williams, G.M.: The detection of chemical mutagens-carcinogens by DNA repair and mutagenesis in liver cultures, In: Chemical Mutagens, Vol. 6, F. DeSerres and A. Hollaender, (eds.), Plenum Press, NY, 1980, pp. 61-79.



**BIONETICS**

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

PROJECT 20991

LBI Assay No. 6084

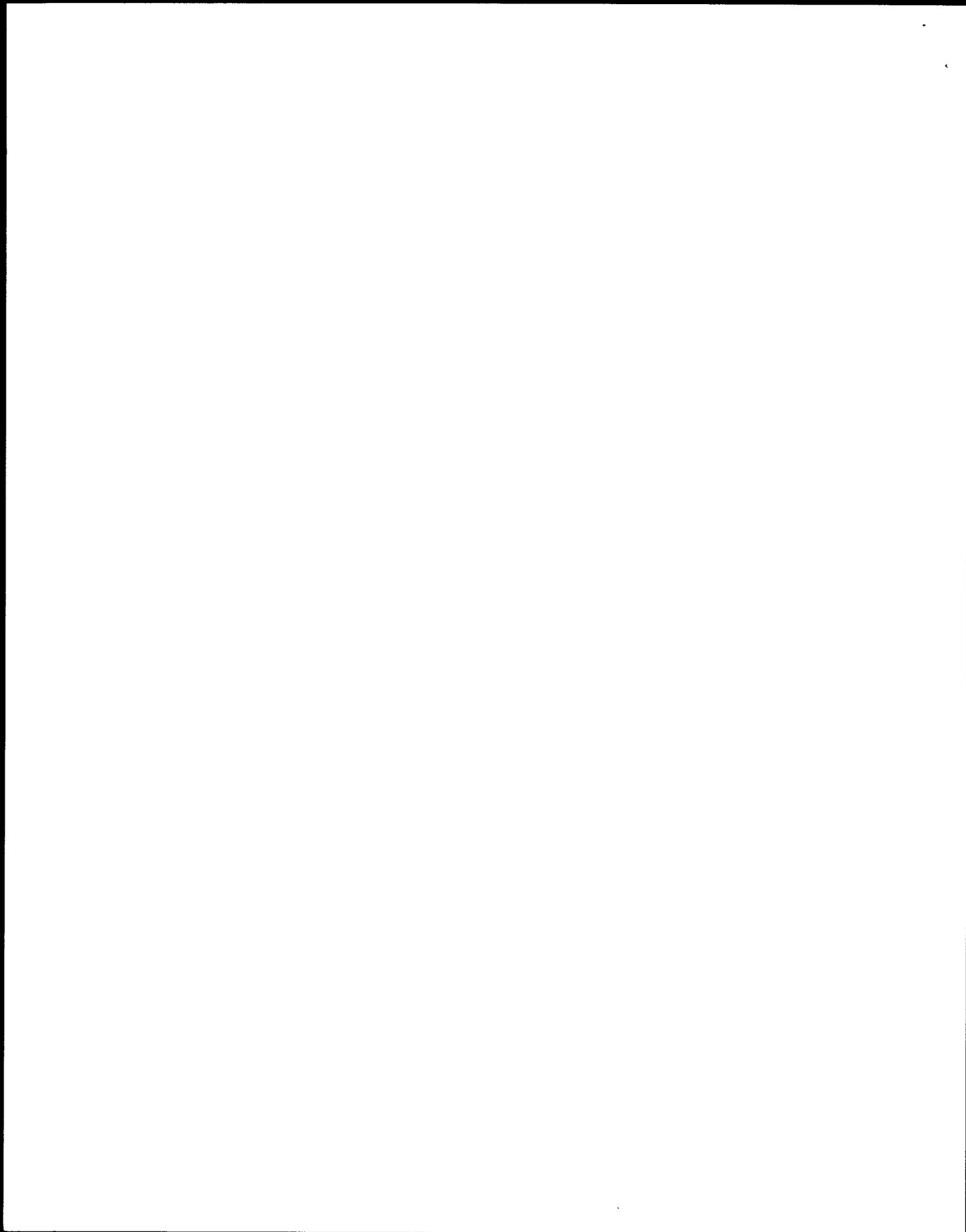
TYPE of STUDY Unscheduled DNA Synthesis

This final study report was reviewed by the LBI Quality Assurance Unit on 5.4.82. A report of findings was submitted to the Study Director and to Management on 5.4.82.

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 J. Hepson  
Auditor, Quality Assurance Unit





CAS 62-56-6

230855A

TX-86-214

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MUTAGENICITY EVALUATION OF  
COMPOUND 81-0389 (Thiourea)

IN THE

IN VITRO TRANSFORMATION  
OF BALB/3T3 CELLS ASSAY

Accession No. 900497

FINAL REPORT

Submitted by: Dr. E.D. Barber

Genetic Toxicology Group  
Toxicological Sciences Section  
Health and Environment Laboratories  
Eastman Kodak Company  
Rochester, New York 14650

July 1, 1986

GENETICS ASSAY NO. 6084

LBI SAFETY NO. 7452

# 900,497

Thiourea

81-0389

230855A

TX-86-214

EVALUATION OF

EK-81-0389

IN THE

IN VITRO TRANSFORMATION  
OF BALB/3T3 CELLS ASSAY

FINAL REPORT

SUBMITTED TO:

EASTMAN KODAK CO.  
BUILDING 320  
1669 LAKE AVENUE  
ROCHESTER, NY 14250

SUBMITTED BY:

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

LBI PROJECT NO.: 20992

REPORT DATE: APRIL, 1982



**BIONETICS**

## PREFACE

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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.



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Litton

- I. SPONSOR: Eastman Kodak Company
- II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 6084
  - A. Identification: EK-81-0389
  - B. Date Received: December 18, 1981
  - C. Physical Description: White powder
- III. TYPE OF ASSAY: In Vitro Transformation of Balb/3T3 Cells Assay
- IV. ASSAY DESIGN NUMBER: 441
- V. STUDY DATES:
  - A. Initiation: January 8, 1982
  - B. Completion: February 22, 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-81-0389, was completely soluble in culture medium at the maximum tested concentration of 20 mg/ml. A series of dilutions of the test material in culture medium, ranging from 4.0 mg/ml to 0.244 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 14% at 4.0 mg/ml to approximately 90% to 106% over the 62.5 to 0.244 µg/ml concentration range. 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 4.95 mg/ml to 206 µg/ml, corresponding to relative survivals of approximately 10% to 84% (estimated graphically from the data reported in Table 1), were chosen for the assay.

## VIII. INTERPRETATION OF RESULTS (Continued)

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, a total of two transformed foci were observed among 20 dishes for an average of 0.10 focus/dish. While this spontaneous transformation frequency is well 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, at least one dish with a high number of transformed foci was observed among the positive control treatments (e.g., dish #20). 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 2.5  $\mu\text{g}/\text{ml}$  MCA induced a total of 241 foci among the 20 positive control dishes (Table 2).  $\log_{10}$  analysis of these data (Table 3) showed that the positive control frequency of 9.5 foci/dish was highly significant ( $p < .01$ ) compared to the negative control value of 0.07 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 0.04 at 206 and 1650  $\mu\text{g}/\text{ml}$  to 0.14 at 4.95 mg/ml.

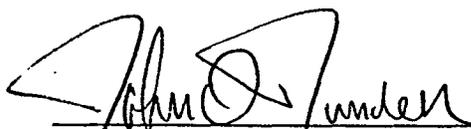
VIII. INTERPRETATION OF RESULTS (Continued)

Compared to the negative control value, none of the frequencies of transformed foci observed for the treatments achieved the 95% confidence level of being significantly altered. In addition, no evidence of a dose-related response was observed, and therefore concentrations of test material from 4.95 mg/ml to 206 µg/ml were evaluated as being nontransforming to 3T3 cells.

IX. CONCLUSIONS

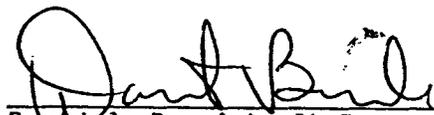
The test material, EK-81-0389, did not induce the appearance of a significant number of transformed foci over the concentration range of 206 µg/ml to 4.95 mg/ml. This concentration range corresponded to approximately 85% to near 10% survival in the preliminary cytotoxicity test. Therefore, the test material is considered to be inactive in the Balb/3T3 In Vitro Transformation Assay.

SUBMITTED BY:

  
\_\_\_\_\_  
John O. Rundell, Ph.D.  
Study Director  
Section Chief  
In Vitro Carcinogenesis  
Department of Molecular Toxicology

5/6/82  
Date

REVIEWED BY:

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

5/6/82  
Date

TABLE 1

TOXICITY TEST IN BALB 3T3 CELLSCLIENT: Eastman Kodak DEPT. ASSAY NO. 6084 DATE: January 18, 1982TEST MATERIAL CODE: EK 81-0389 SOLVENT: Culture Medium

| <u>TEST COMPOUND<br/>DOSES TESTED</u> ( $\mu\text{g/ml}$ ) | <u>AVERAGE NUMBER<br/>OF COLONIES/PLATE</u> | <u>% SURVIVAL<br/>RELATIVE TO CONTROL</u> |
|--|---|---|
| 1. 4000.0  | 10.0  | 14.0                                      |
| 2. 2000.0  | 26.3  | 37.0                                      |
| 3. 1000.0  | 44.0  | 61.8                                      |
| 4. 500.0   | 56.0  | 78.7                                      |
| 5. 250.0   | 58.7  | 82.4                                      |
| 6. 125.0   | 60.7  | 85.2                                      |
| 7. 62.5  | 64.3  | 90.4                                      |
| 8. 31.3  | 74.0  | 103.9                                     |
| 9. 15.6  | 74.3  | 104.4                                     |
| 10. 7.81   | 67.7  | 95.0                                      |
| 11. 3.91   | 72.3  | 101.6                                     |
| 12. 1.95   | 70.0  | 98.3                                      |
| 13. 0.977  | 69.7  | 97.8                                      |
| 14. 0.488  | 75.3  | 105.8                                     |
| 15. 0.244  | 68.7  | 96.4                                      |
| 16. Control*   | 71.2  | 100.0                                     |

\* The control consisted of a total of 12 dishes.



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TABLE 2

SUMMARY OF DATA FROM TRANSFORMATION ASSAY

CLIENT: Eastman Kodak      GENETICS ASSAY NO. 6084      TEST DATE: February 22, 1982  
 CLIENT'S COMPOUND CODE: EK 81-0389      SOLVENT: Medium      3T3 CLONE: 1-13, C-14

| TEST  | DOSES TESTED | NUMBER OF FOCI PER DISH SCORED |   |    |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |     |     | TOTAL NO. OF FOCI | NO. OF FOCI/DISH ABSOLUTE/LOG <sub>1</sub> |
|---|--------------|--------------------------------|---|----|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|-----|-----|-------------------|--|
|   |              | 1                              | 2 | 3  | 4 | 5 | 6 | 7 | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19  | 20  |                   |  |
| Negative Control <sup>2</sup><br>(Culture Medium) | 0            | 0                              | 0 | 0  | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1   | 2   | 0.10/0.07         |  |
| Positive Control<br>MCA 2.5 µg/ml                 | 22           | 16                             | 4 | 19 | 6 | 9 | 7 | 4 | 11 | 13 | 13 | 9  | 6  | 5  | 5  | 11 | 16 | 6  | 4  | >55 | 241 | 12.05/9.49        |  |
| Test Material                                     |              |                                |   |    |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |     |     |                   |  |
| 4950.0 µg/ml                                      | 0            | 0                              | 0 | 0  | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1  | 0  | 4  | C  | C   | 5   | 0.28/0.14         |  |
| 3300.0 µg/ml                                      | 0            | 0                              | 0 | 0  | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1  | C   | 1   | 0.05/0.04         |  |
| 1650.0 µg/ml                                      | 0            | 0                              | 0 | 0  | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1   | 1   | 0.05/0.04         |  |
| 825.0 µg/ml                                       | 0            | 0                              | 0 | 0  | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 2   | 2   | 0.10/0.06         |  |
| 206.0 µg/ml                                       | 0            | 0                              | 0 | 0  | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 1  | 0   | 1   | 0.05/0.04         |  |

C=Dish was contaminated and not scored.  
 \* Calculated as the antilog of the logarithmic mean.(Table 3) minus one.

TABLE 3

STATISTICAL ANALYSIS OF TRANSFORMATION ACTIVITYCLIENT: Eastman Kodak GENETICS ASSAY NO. 6084 DATE: March 4, 1982COMPOUND CODE: EK 81-0389 SOLVENT Culture Medium

| Treatment Condition | Log <sub>10</sub> Analysis* of Foci/Dish |    |       |  | t Statistic** | p Values** |
|---------------------|--|----|-------|--|---------------|------------|
|                     | Mean ± SD                                | n  | SE    |  |               |            |
| Media Control       | 0.030 0.093                              | 20 | 0.021 |  | Control       | Control    |
| MCA 2.5 µg/ml       | 1.021 0.270                              | 20 | 0.060 |  | +15.501       | p<.01      |
| Test Material       |  |    |       |  |               |            |
| 4950.0 µg/ml        | 0.056 0.176                              | 18 | 0.041 |  | + 0.551       | .5<p<.6    |
| 3300.0 µg/ml        | 0.016 0.069                              | 19 | 0.016 |  | - 0.548       | N.S.       |
| 1650.0 µg/ml        | 0.015 0.067                              | 20 | 0.015 |  | - 0.548       | N.S.       |
| 825.0 µg/ml         | 0.024 0.107                              | 20 | 0.024 |  | - 0.196       | N.S.       |
| 206.0 µg/ml         | 0.015 0.067                              | 20 | 0.015 |  | - 0.584       | N.S.       |

\*NOTE: Each raw data point was increased by 1.0 and converted to log<sub>10</sub> 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;  
NS = Not Significant

SE = Standard Error

\*\*

Bailey, Norman T.J.: Statistical Methods in Biology; Wiley & Sons, Inc., N.Y.; Pg. 50, 1959.



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## ASSAY DESIGN (NO 441)

### 1. OBJECTIVE

This assay evaluates the morphological transforming potential of test materials using mouse Balb/3T3 cells in culture. The objective of this semi-quantitative assay is 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/3T3 mouse cells will multiply in culture until a monolayer is achieved and will then cease further division. These cells, if injected into immunosuppressed, syngeneic host animals, will not produce neoplastic tumors. However, cells treated in vitro with chemical carcinogens will give rise to foci of cellular growth super-imposed on the cell monolayer. If these foci are picked from cultures, grown to larger numbers and injected into animals, a malignant tumor will be obtained in most cases. Thus, the appearance of piled-up colonies in treated cell cultures at a higher frequency than in control cultures is highly correlated with malignant transformation.

### 3. MATERIALS

#### A. Indicator Cells

Clone 1-13 of Balb/3T3 mouse cells was obtained from Dr. Takeo Kakunaga. Further subclones, selected for low spontaneous frequencies of foci formation, are used for assays. Stocks are cryopreserved in liquid nitrogen and laboratory cultures are checked periodically to ensure the absence of mycoplasma contamination. Cultures are 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 is performed. If the test compound is not soluble in growth medium, an organic solvent is used; the final concentration of solvent in the growth medium will be 1% or less or as previously determined in solvent cytotoxicity and activity control experiments. Cells exposed to solvent in the medium are 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 are prepared for each assay.



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### 3. MATERIALS (Continued)

#### 2. Positive Control

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

#### C. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in a compatible organic solvent. Liquids are 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 is first determined. Fifteen dose levels of the test compound are 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 be tested as required to meet the dose selection criteria.) Each dose is applied to three culture dishes seeded 24 hours earlier with 200 cells per dish. After an exposure period of 24 hours, the cells are washed and incubated in growth medium for an additional 5-7 days. The surviving colonies are stained and counted and a relative survival for each dose is 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 cause no more than an 90% reduction in colony forming ability. Four lower doses (usually including one dose with little apparent toxicity) are also selected for the transformation assay.

#### B. Transformation Assay

The procedure used at LBI is based on that reported by Kakunaga (1973). Twenty-four hours prior to treatment, a series of 60 mm dishes is seeded with  $10^4$  cells/flask and incubated. At least 20 dishes are then treated for each of the following conditions: Five preselected doses of test chemical; positive control; and solvent negative control, if applicable. The dishes are incubated for a 24 hours exposure period; the cells are then washed and incubation is continued for approximately four weeks with refeeding twice a week. The assay is terminated by fixing the cell monolayers with methanol and staining with Giemsa. The stained dishes are examined by eye and by microscope to determine the number of foci of transformed cells.



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## 5. SCORING OF TRANSFORMED FOCI

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

Scored foci have several variations in morphological features. Most scored foci consist of a dense piling-up of cells and exhibit a random, criss-cross orientation of fibroblastic cells at the periphery of the focus and extensive invasiveness into the contiguous monolayer. Other scored foci are 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 exhibit the criss-cross pattern of overlapping cells throughout most of the colony. Foci that have these characteristics and exceed 2 mm in diameter are scored +++ and those <2mm in diameter are designated ++.

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

## 6. CONFIRMATION OF TUMORIGENICITY OF TRANSFORMED CLONES

Most transformed clones will produce malignant tumors when collected from an unstained transformation plate, passaged to obtain sufficient cells, and injected into syngeneic host animals. Although not routinely performed, this confirmation step can be conducted at additional cost.

## 7. 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.



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## ASSAY ACCEPTANCE CRITERIA

The assay will be considered acceptable for evaluation of the test results if the following criteria are met:

1. The negative control dishes consist of a contiguous monolayer of cells which may or may not contain transformed foci. The lack of contiguous sheet of cells indicates growth conditions too poor to allow the reliable detection of weak transforming agents.
2. The negative control transformation frequency does not exceed an average of about 2-3 foci/dish after  $\log_{10}$  analysis. Attempts are made to isolate and maintain cell stocks (subclones of Balb/3T3 1-13) with a very low spontaneous frequency of transformation.
3. The positive control yields an average number of foci/dish that is significantly different from the negative control at the 95 or 99% confidence level.
4. A minimum of 10 flasks per test condition are available for analysis. At least 3 dose levels of test substance are assayed.
5. The dose range of test substance assayed falls within the 10-100% survival range as determined by the preliminary toxicity test, which measures relative cloning efficiencies.



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## EVALUATION CRITERIA

The appearance of transformed foci is usually symmetric for any given treatment condition. However, large numbers of foci may 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 appears to be due to a technical rather than a biological cause and this conclusion is supported by the finding that dishes with high numbers of foci occur 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, is considered to be caused by mechanical disruption during the refeeding process that occurs 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 can 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) will be 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 will evaluate the results of each treatment condition in relation to the observed activities of model compounds and will exercise scientific judgement in the evaluation of each test article. In general, a response at only one dose level just attaining the 95% confidence level will normally not be considered sufficient evidence for activity in this assay. However, responses at one or more treatment levels attaining the 95% confidence level and exhibiting evidence of dose dependency will be considered as positive evidence of transforming activity and responses achieving the 99% confidence level over one or more test material treatments will be similarly interpreted.



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Q.A. Inspection Statement  
(reference 2] CFR 58.35(b)(7))

PROJECT 20992

LBI Assay No. 6084

TYPE of STUDY Malignant Transformation assay

This final study report was reviewed by the LBI Quality Assurance Unit on 5.3.82. A report of findings was submitted to the Study Director and to Management on 5.4.82.

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 J. Hyman  
Auditor, Quality Assurance Unit

