



FYI-94-001135
INIT 07/26/94

FYI-0794-1155

August 15, 1984

Contains No CBI

Mr. Louis Borghi
Staff Scientist
Dynamac Corporation
The Dynamac Corporatio
11140 Rockville Pike
Rockville, MD 20852



84940000227

Dear Mr. Borghi:

Your recent phone call to Dr. D. W. Kreh requested additional information for the following compounds:

1,8-dihydroxy-4,5-dinitroanthraquinone, CAS No. 81-55-0
N,N-dimethylcyclohexylamine, CAS No. 98-42-2

Specifically you requested the raw data and test protocols for the basic toxicity studies we submitted to Mr. Martin Creif on June 4, 1984.

Enclosed please find the raw data for these two studies. The acute aquatic effects data were reorganized and typed to better present the information. We are unable to provide the protocols since these studies were performed before we started writing formal protocols.

We are also enclosing two additional reports for the 1,8-dihydroxy-4,5-dinitroanthraquinone. These reports are:

Mouse Lymphoma Forward Mutation Assay, and
Unscheduled DNA Synthesis in Human W1-38 Cells Assay

Sincerely,

RL Raleigh

Robert L. Raleigh, M.D., Director
Health and Environment Laboratories
(716) 722-2879

RLR/JRH:drc
Enclosures

RECEIVED
AUG 26 1984
PM 3:31

LBI ASSAY NO. 2991

MUTAGENICITY EVALUATION OF

81-55-0

DNC COMPOUND 78-142

IN THE
HOUSE LYMPHOMA FORWARD
MUTATION ASSAY

SUBMITTED TO:

EASTMAN KODAK
1669 LAKE AVE.
ROCHESTER, NY 14650

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 20989

REPORT DATE: DECEMBER 1978



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-VIII. Items I-IV provide sponsor and compound identification information, type of assay, and the protocol reference number. All protocol references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V provides the initiation and completion dates for the study, and item VI identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation itself is in item VII. Item VIII provides the conclusion and evaluation.

The second part of the report, entitled PROTOCOL, describes, in detail, 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. The evaluation criteria are included to acquaint the sponsor with the methods used to develop and analyze the test results.

All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology. Copies of raw data will be supplied to the sponsor upon request.



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- I. SPONSOR: Eastman Kodak**
- II. MATERIAL (TEST COMPOUND): LBI ASSAY NO. 2991**
- A. Identification: DNC Compound 78-142**
- B. Date Received: March 17, 1978**
- C. Physical Description: Brown powder**
- III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay**
- IV. PROTOCOL NUMBER: 431 (DMT-106)**
- V. STUDY DATES:**
- A. Initiation: May 18, 1978**
- B. Completion: November 12, 1978**

VI. RESULTS:

The data are presented in Tables 1 and 2. The data show the concentrations of the test compound employed, surviving populations during the expression period, number of mutant clones obtained, and calculated mutation frequencies. All calculations are performed by computer program.

VII. INTERPRETATION OF RESULTS:

The test compound, DNC Compound 78-142, was suspended in water at a concentration of 500 to 1000 $\mu\text{g}/\text{ml}$. Upon ten-fold dilution into culture medium, only a slight amount of material remained in suspension.

Data from two trials of the mutation assay form the basis for evaluation of the test compound. The nonactivation assay was performed over the dose range of 0.625 $\mu\text{g}/\text{ml}$ to 35 $\mu\text{g}/\text{ml}$ and the activation assay from 0.625 $\mu\text{g}/\text{ml}$ to 25 $\mu\text{g}/\text{ml}$. Preliminary cytotoxicity and mutation assays showed severe toxicity to cell growth within 24 hours of treatment at 40 $\mu\text{g}/\text{ml}$ and higher doses without activation. With activation, variable toxicity was observed such that the maximum dose without excessive toxicity varied from 10 to 40 $\mu\text{g}/\text{ml}$. The doses chosen for the mutation assays were within the range of cytotoxicities where any mutagenic activity is normally observed. After the cells were cloned for mutant selection, the percent relative growth in the treated cultures was found to range from 10.7% to 83.1% (Table 1) and 2.6% to 130.6% (Table 2) without activation and from 36.5% to 80.3% (Table 1) and 58.6% to 141.2% (Table 2) for the activation assay.



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VII. INTERPRETATION OF RESULTS (continued)

The results of the mutation assays are presented in Tables 1 and 2.

The assay given in Table 1 showed the test compound to be mutagenic under nonactivation conditions and to be inactive in the presence of S9 metabolic activation. At four dose levels in the nonactivation assay, the mutant frequency exceeded the 2.5-fold minimum increase over the average background frequency (solvent and negative control average) considered necessary to establish a positive response. Furthermore, the mutation frequency was dose-related, increasing to 14.5 times the background frequency at 35 $\mu\text{g/ml}$. With activation, the mutant frequencies in the treated cultures were comparable to the solvent and negative control values and none exceeded the 2.5-fold minimum increase for a positive response.

The mutation assay was repeated because the average cloning efficiency of the solvent and negative controls was only about 57%. With good culturing conditions the cloning efficiency should exceed 70%. The cells used for this assay appeared to be in a healthy state and no reason is known for the low cloning efficiency. The positive control compounds yielded mutant frequencies in the normal range that are greatly in excess of the background frequency, which suggests the efficiency of mutant detection was normal. Nevertheless, a repeat assay with a normal cloning efficiency was considered desirable to confirm the results obtained.

The average cloning efficiency of the solvent and negative controls in the repeat assay (Table 2) was 77.8%, which demonstrated good culturing conditions for this trial. Without activation, the test compound was mutagenic at two dose levels; a 5-fold increase over the average background frequency was obtained at 20 $\mu\text{g/ml}$ and a 23-fold increase occurred at 30 $\mu\text{g/ml}$. These results compare well with the dose-response shown in Table 1. With S9 activation, the mutant frequencies in the treated cultures remained comparable to the average background frequency. Although the mutant frequency at 2.5 $\mu\text{g/ml}$ was nearly 2.5 times the background, the frequency at 5.0 $\mu\text{g/ml}$ was at the background level and no dose-related response was obtained. The test compound was excessively toxic at 25 $\mu\text{g/ml}$ and no mutant colonies were observed, presumably because the cells did not survive the cloning and selection conditions.



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VII. INTERPRETATION OF RESULTS (continued):

The EMS positive control in the nonactivation assay apparently decomposed prior to exposing the cells in this trial since no toxicity was observed (141.0% relative growth) and the mutant frequency was at the background level. The positive control for the activation assay gave a normal mutant frequency about fourteen times the background level. The background frequency was within the range of values normally obtained but was higher than in the preceding trial in Table 1. The background may vary from trial to trial, mainly because of differences in the efficiency of removal of pre-existing mutants prior to the use of cell cultures in the mutation assay.

VIII. CONCLUSIONS:

The test compound, DNC Compound 78-142, induced an increase in mutations at the TK locus in L5178Y mouse lymphoma cells only under nonactivation conditions and not in the presence of the S9 metabolic activation system. The compound was active in the 15 to 35 µg/ml applied concentration range.

Submitted by:

Study Director

Brian Myhr 12-22-78
Brian Myhr, Ph.D. Date

Section Chief
Mammalian Genetics
Department of Genetics
and Cell Biology

Reviewed by:

David J. Brusick 12/22/78
David J. Brusick, Ph.D. Date

Director
Department of Genetics
and Cell Biology



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9. SUMMARY OF HOUSE LYMPHOMA (LS170) RESULTS

TABLE I

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: 78-142 DMC
 B. LBI CODE #: 2991
 C. SOLVENT: WATER
 D. TEST DATE: 1 30/78

TEST	S-9 SOURCE	ISSUE	DAILY COUNTS			RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH	MUTANT FREQUENCY**
			1	2	3						
NONACTIVATION											
SOLVENT CONTROL	---	---	7.2	6.6	6.6	100.0	9.0	210.0	100.0	100.0	4.3
NEGATIVE CONTROL	---	---	11.2	6.8	7.0	100.0	7.0	119.0	100.0	100.0	5.9
EMS .5 UG/ML	---	---	10.6	9.0	9.0	163.0	17.0	155.0	102.0	166.3	11.0
TEST COMPOUND	---	---	7.4	6.2	6.2	78.4	346.0	72.0	47.4	37.2	480.6
2.500 UG/ML	---	---	7.4	6.4	6.4	80.9	13.0	156.0 +	102.7	83.1	8.3
10.000 UG/ML	---	---	5.6	8.4	8.4	80.4	19.0	136.0 +	90.8	73.0	13.8
25.000 UG/ML	---	---	5.8	6.6	6.6	67.4	22.0	106.0	69.8	45.6	20.8
30.000 UG/ML	---	---	5.2	4.4	4.4	39.1	22.0	116.0	76.4	29.9	19.0
35.000 UG/ML	---	---	2.6	6.0	6.0	30.6	53.0	75.0	49.4	15.2	84.0
	---	---	2.8	4.0	4.0	20.5	81.0	79.0	20.0	10.7	102.5
ACTIVATION											
SOLVENT CONTROL	RAT	LIVER	9.8	9.0	9.0	100.0	11.0	189.0	100.0	100.0	5.8
NEGATIVE CONTROL	RAT	LIVER	8.4	11.6	11.6	100.0	22.0	218.0	100.0	100.0	10.1
DMN .3 UG/ML	RAT	LIVER	8.2	10.4	10.4	163.0	27.0	137.0	67.7	140.3	19.7
TEST COMPOUND	RAT	LIVER	6.0	5.6	5.6	55.3	73.0	32.0	15.8	5.7	228.1
1.250 UG/ML	RAT	LIVER	7.2	11.0	11.0	85.5	26.0	165.0	81.5	69.7	15.8
2.500 UG/ML	RAT	LIVER	11.8	4.0	4.0	51.0	28.0	319.0	137.6	40.3	8.8
5.000 UG/ML	RAT	LIVER	5.0	9.6	9.6	82.9	21.0	171.0	84.5	70.1	12.3
10.000 UG/ML	RAT	LIVER	7.1	7.0	7.0	55.9	36.0	132.0	65.2	36.5	27.3

* RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY / 100
 ** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 100:4.
 THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES) * 10E-7.
 THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10⁷.

+ = ONE PLATE CONTAMINATED, VALUE BASED ON REMAINING TWO PLATES.

5-1000000-1121000-RESULTS

TABLE 2

A. NAME OF CODE DESIGNATION OF THE TEST COMPOUND: FR-142
 B. THE CODE OF 2591
 C. SOURCE: 1-2114
 D. TEST DATE: 2/13/74

TEST REACTIVITY	SOURCE	ISSUE	DAILY COUNTS		RELATIVE SUSPENSION GROWTH IN DL-CUBIC/ML	TOTAL MUTANT CLOUES	TOTAL VIABLE CLOUES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH	MUTANT FREQUENCY (10 ⁶ -1)
			1	2						
SOLVENT CONTROL	---	---	7.2	11.6	100.0	59.0	230.0	100.0	100.0	25.7
SOLVENT CONTROL	---	---	5.4	14.6	100.0	68.0	179.0	100.0	100.0	30.0
NEGATIVE CONTROL	---	---	10.2	8.0	100.6	67.0	215.0	100.8	107.4	31.2
145 25 UG/ML	---	---	7.0	14.6	138.5	62.0	205.0	101.4	141.0	30.2
TEST COMPOUND	---	---	7.0	12.6	114.1	57.0	217.0	107.8	127.3	26.3
1.250 UG/ML	---	---	8.2	10.6	105.1	75.0	250.0	124.2	130.6	30.0
2.500 UG/ML	---	---	5.0	7.0	73.1	96.0	215.0	106.8	46.1	44.7
10.000 UG/ML	---	---	4.0	5.2	25.6	23.04	145.0	72.0	18.5	140.7
15.000 UG/ML	---	---	4.0	3.6	17.8	220.0	30.0	14.9	2.6	773.3
ALIVE/ML										
SOLVENT CONTROL	PAT	LIVER	9.8	8.2	100.0	65.0	300.0	100.0	107.0	41.7
SOLVENT CONTROL	PAT	LIVER	8.6	9.2	100.0	70.0	250.0	100.0	100.0	28.0
NEGATIVE CONTROL	PAT	LIVER	6.2	14.2	110.4	82.0	227.0	83.2	91.9	36.1
145 25 UG/ML	PAT	LIVER	4.0	11.2	64.6	71.0	175.0	64.2	41.5	410.9
TEST COMPOUND	PAT	LIVER	14.2	9.4	167.4	96.0	230.0	84.3	141.7	40.9
0.625 UG/ML	PAT	LIVER	9.2	6.8	78.5	60.0	290.0	106.3	63.4	20.7
1.250 UG/ML	PAT	LIVER	9.6	10.2	122.8	124.0	175.0	64.2	78.8	70.9
2.500 UG/ML	PAT	LIVER	8.0	5.4	54.2	75.0	245.0	168.2	58.8	32.2
5.000 UG/ML	PAT	LIVER	5.0	2.6	3.8	0	9.0	3.3	0.3	-----

* RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY / 100
 ** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+6.
 *** THE RATIO OF MUTANT FREQUENCY TO TOTAL MUTANT CLOUES/TOTAL VIABLE CLOUES IS 10E-6.
 THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 1E-6.

+ = ONE PLATE CONTAMINATED, VALUE BASED ON REMAINING TWO PLATES.

PROTOCOL 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⁺/⁻ mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU).

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⁺/⁻) may undergo a single step forward mutation to the TK⁻/⁻ genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by de novo synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK⁻/⁻ mutants is the lack of any ability to utilize toxic analogs of thymidine, which enables only the TK⁻/⁻ 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⁻/⁻ genotype.

3. MATERIALS

A. Indicator Cells

The mouse lymphoma cell line, L5178Y TK⁺/⁻, 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⁻/⁻ mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK⁻/⁻ 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 50 or 100 µg/ml of BrdU.



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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 μ 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 μ 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 by 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, 3×10^6 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 μ l S9/ml.

C. Preparation of 9,000 x g Supernatant (S9)

Fischer 344 male rats are normally used as the source of hepatic microsomes. Induction with Aroclor 1254 or other agents is performed by injections five days prior to sacrifice. After decapitation and bleeding, the liver is immediately dissected from the animal using aseptic technique and placed in ice cold 0.25M sucrose buffered with Tris at pH 7.4. When an adequate number of livers is obtained, the collection is washed twice with fresh buffered sucrose and completely homogenized. The homogenate is centrifuged for 10 minutes at 9,000 x g in a refrigerated centrifuge and the supernatant (S9) from this centrifuged sample is retained and frozen at -80°C until used in the activation system. The S9 fraction may be obtained from induced or noninduced rats or other species, as requested.



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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.

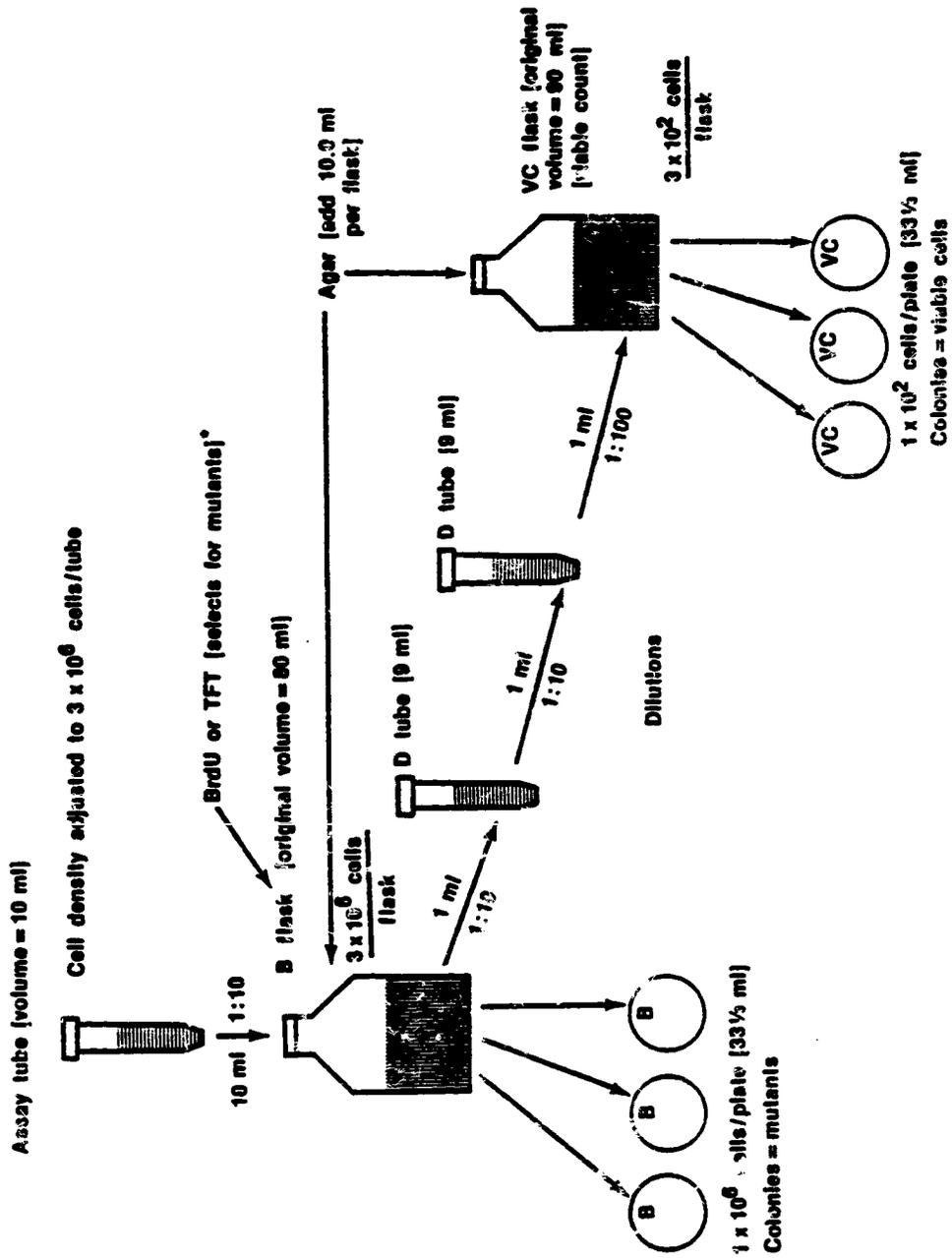
6. REFERENCE

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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urton



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

FIGURE 1. LYMPHOMA CLONING FLOW CHART

EVALUATION CRITERIA

A compound is considered mutagenic in this assay if it meets the following criteria:

- . A dose-response relationship is observed over 3 of the 5 dose levels tested.
- . The minimum increase at the low level of the dose-response curve is at least 2.5 times greater than the solvent and negative control average value.
- . The solvent and negative control data are within the normal range of the spontaneous background for the TK locus.

All evaluations of mutagenic activity are based on consideration of the concurrent solvent and negative control values run with the experiment in question. Positive control values are not used as reference points, but are included to ensure that the current cell population responds to direct and promutagens under the treatment conditions.

Occasionally, a single point within a concentration range will show an increase 2.5 times greater than the spontaneous background. If the increase is at the high dose, is reproducible, and if an additional higher dose level is not feasible because of toxicity, the compound can be considered mutagenic. If the increase in internal within the dose range and is not reproducible, the increase will normally be considered aberrant. If the internal increase is reproducible, several doses clustered around the positive concentration will be examined to either confirm or reject the reliability of the effect.

As the data base on the assay increases, the evaluation criteria can be expected to become more firmly established.



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LBI ASSAY NO. 2991

EVALUATION OF
DNC COMPOUND 78-142
IN THE
UNSCHEDULED DNA SYNTHESIS IN
HUMAN WI-38 CELLS ASSAY
FINAL REPORT

81-55-0

SUBMITTED TO:

EASTMAN KODAK
7669 LAKE AVENUE
ROCHESTER, NY 14650

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
BENNINGTON, MARYLAND 20795

LBI PROJECT NO. 20991

REPORT DATE: DECEMBER 1978



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-VIII. Items I-IV provide sponsor and compound identification information, type of assay, and the protocol reference number. All protocol references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V provides the initiation and completion dates for the study, and item VI identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation itself is in item VII. Item VIII provides the conclusion and evaluation.

The second part of the report, entitled PROTOCOL, describes, in detail, 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. The evaluation criteria are included to acquaint the sponsor with the methods used to develop and analyze the test results.

All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology. Copies of raw data will be supplied to the sponsor upon request.



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0 0 1 8

- I. SPONSOR: Eastman Kodak
- II. MATERIAL (TEST COMPOUND): LBI ASSAY NO. 2991
 - A. Identification: DNC Compound 78-142
 - B. Date Received: March 17, 1978
 - C. Physical Description: Brown powder
- III. TYPE OF ASSAY: Unscheduled DNA Synthesis in Human WI-38 Cells Assay
- IV. PROTOCOL NO.: 434 (DMT-108)
- V. STUDY DATES:
 - A. Initiation: June 15, 1978
 - B. Completion: October 17, 1978
- VI. RESULTS:

The results are presented in Tables 1 through 4.
- VII. INTERPRETATION OF RESULTS:

The test compound, DNC Compound 78-142, dissolved in DMSO at a concentration of 5.0 mg/ml to form a dark reddish solution. The compound remained soluble in the growth medium at the highest tested dose level of 50 µg/ml. A preliminary UDS assay showed that concentrations of 10⁰ µg/ml and above were toxic to the cells (reduced the DNA synthetic activity considerably below the negative control value) under both activation and nonactivation conditions. Several UDS assays were performed at applied doses ranging from 1 to 50 µg/ml without activation and from 0.1 to 50 µg/ml with activation. Data from these trials are presented in Tables 1-4, which are considered together in the evaluation of this test compound.

Table 1 presents unscheduled DNA synthesis (UDS) data for the applied concentration range of 1 µg/ml to 50 µg/ml. The test compound was toxic at 50 µg/ml, reducing the DNA synthetic activity below the negative controls. No evidence for UDS activity was obtained under activation conditions, but since the BaP positive control also did not cause any detectable UDS activity, this assay was not definitively negative. UDS was observed for two dose levels (1 µg/ml and 10 µg/ml) in the nonactivation assay; however, no response was obtained at the intermediate dose of 5 µg/ml. This



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VII. INTERPRETATION OF RESULTS (continued)

lack of a clear dose-related response suggested the assay be repeated. Also, the positive control (MNNG) apparently decomposed and did not result in UDS activity.

The repeat assay shown in Table 2 yielded UDS activity (206% of control) only at the dose level of 10 $\mu\text{g}/\text{ml}$ for the nonactivation conditions. The activation assay provided no evidence for UDS activity, although the lack of response to the positive control makes it uncertain whether good activation conditions were established. The MNNG positive control was very active in the nonactivation assay.

A repeat activation assay, using S9 from Aroclor 1254 induced rat liver, is shown in Table 3. The BaP positive control gave significant UDS activity in this trial; the UDS activity normally does not increase with BaP concentrations above 10 $\mu\text{g}/\text{ml}$, presumably because of saturation associated with insolubility. For the test compound, no evidence was obtained for UDS activity over the concentration range of 0.1 $\mu\text{g}/\text{ml}$ to 10 $\mu\text{g}/\text{ml}$. The DNA synthetic activity at all tested doses was comparable to the untreated (solvent) control.

Another trial of the nonactivation assay was performed to confirm the positive response found in earlier trials. Table 4 shows that the test compound does cause a dose-related UDS activity. At 25 $\mu\text{g}/\text{ml}$, the DNA synthetic activity was 234% of the untreated (negative) control; this activity decreased to 186% at 12.5 $\mu\text{g}/\text{ml}$ and to 121% at 10.0 $\mu\text{g}/\text{ml}$. The latter value is within the normal variability of the UDS assay. Thus, the UDS activity observed in earlier trials was confirmed, although the concentration level for the maximum UDS activity varies from one trial to the next.



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VIII. CONCLUSIONS

The test compound, DNC Compound 78-142, induced unscheduled DNA synthesis (UDS) in human WI-38 cells only under nonactivation conditions. Maximum UDS activity occurred in the 1 µg/ml to 25 µg/ml concentration range. Therefore, the test compound is considered active in the UDS assay.

Submitted by:

Study Director

Brian Myhr 12-22-78
Brian Myhr, Ph.D. Date
Section Chief
Mammalian Genetics
Department of Genetics
and Cell Biology

Reviewed by:

David Brusick 12/22/78
David Brusick, Ph.D. Date
Director
Department of Genetics
and Cell Biology

TABLE 1

UNSCHEDULED DNA SYNTHESIS IN HUMAN WI-38 CELLS ASSAYCLIENT: Eastman Kodak LBI ASSAY NO. 2991 DATE: August 8, 1978CLIENT'S COMPOUND CODE: DNC Compound 78-142 SOLVENT: DMSOACTIVATION SYSTEM: S9: Noninduced Mouse Liver

TEST	CONCENTRATION	$\mu\text{g DNA}$	DPM/ $\mu\text{g DNA}$	PERCENT OF CONTROL
<u>NONACTIVATION:</u>				
Negative Control	-----	21.45	35	100
Positive Control (MNNG)	5 $\mu\text{g/ml}$	27.56	35	100
Test Compound:				
DNC 78-142	1 $\mu\text{g/ml}$	22.77	61	174
DNC 78-142	5 $\mu\text{g/ml}$	25.90	35	100
DNC 78-142	10 $\mu\text{g/ml}$	22.94	66	189
DNC 78-142	50 $\mu\text{g/ml}$	10.73	20	57
<u>ACTIVATION:</u>				
Negative Control	----	35.80	29	100
Positive Control (BaP)	1 $\mu\text{g/ml}$	32.67	24	83
Test Compound:				
DNC 78-142	1 $\mu\text{g/ml}$	28.71	33	114
DNC 78-142	5 $\mu\text{g/ml}$	24.26	19	66
DNC 78-142	10 $\mu\text{g/ml}$	29.86	27	93
DNC 78-142	50 $\mu\text{g/ml}$	25.58	10	34

MNNG = N-methyl-N'-nitro-N-nitrosoguanidine

BaP = Benz (a)pyrene



BIONETICS

TABLE 2

UNSCHEDULED DNA SYNTHESIS IN HUMAN WI-38 CELLS ASSAYCLIENT: E-stman Kodak LBI ASSAY NO. 2991 DATE: August 21, 1978CLIENT'S COMPOUND CODE: DNC Compound 78-142 SOLVENT: DMSOACTIVATION SYSTEM: S9, Non-induced Mouse Liver

TEST	CONCENTRATION	$\mu\text{g DNA}$	DPM/ $\mu\text{g DNA}$	PERCENT OF CONTROL
NONACTIVATION:				
Negative Control	---	32.01	17	100%
Solvent Control	---	30.66	16	94%
Positive Control (MNNG)	5 $\mu\text{g/ml}$	33.73	86	506%
Test Compound:				
DNC, 78-142	1 $\mu\text{g/ml}$	33.07	20	118%
DNC, 78-142	5 $\mu\text{g/ml}$	34.91	20	118%
DNC, 78-142	10 $\mu\text{g/ml}$	33.56	35	206%
DNC, 78-142	50 $\mu\text{g/ml}$	29.57	22	129%
ACTIVATION:				
Negative Control	---	28.78	24	100%
Solvent Control	---	27.82	25	104%
Positive Control (BaP)	1 $\mu\text{g/ml}$	28.97	28	117%
Test Compound:				
DNC, 78-142	1 $\mu\text{g/ml}$	33.82	20	83%
DNC, 78-142	5 $\mu\text{g/ml}$	28.81	28	117%
DNC, 78-142	10 $\mu\text{g/ml}$	35.28	21	88%
DNC, 78-142	50 $\mu\text{g/ml}$	42.44	21	88%

MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
BaP = Benz (a)pyrene

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TABLE 3

UNSCHEDULED DNA SYNTHESIS IN HUMAN WI-38 CELLS ASSAY

CLIENT: Eastman Kodak LBI ASSAY NO. 2991 DATE: October 17, 1978

CLIENT'S COMPOUND CODE: DNC Compound 78-142 SOLVENT: DMSO

ACTIVATION SYSTEM: S9: Aroclor 1254 Induced Rat Liver

TEST	CONCENTRATION	µg DNA	DPM/µg DNA	PERCENT OF CONTROL
------	---------------	--------	------------	--------------------

NONACTIVATION:

Negative Control
Solvent Control
Positive Control
(MNNG)
Test Compound:

ACTIVATION:

Negative Control	----	21.02	14	100
Solvent Control	----	25.18	9	64
Positive Control (BaP)	10 µg/ml	21.48	30	214
Positive Control (BaP)	50 µg/ml	22.64	33	236

Test Compound:

DNC 78-142	0.1 µg/ml	22.51	14	100
DNC 78-142	0.5 µg/ml	21.78	12	86
DNC 78-142	1.0 µg/ml	21.25	12	86
DNC 78-142	5.0 µg/ml	22.21	10	71
DNC 78-142	10.0 µg/ml	25.15	15	107

BaP = Benz(a)pyrene



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TABLE 4

UNSCHEMULED DNA SYNTHESIS IN HUMAN WI-38 CELLS ASSAYCLIENT: Eastman Kodak LBI ASSAY NO. 2957 DATE: November 22, 1978CLIENT'S COMPOUND CODE: DNC compound 78-142 SOLVENT: DMSOACTIVATION SYSTEM: Non-activation

TEST	CONCENTRATION	$\mu\text{g DNA}$	DPM/ $\mu\text{g DNA}$	PERCENT OF CONTROL
<u>NONACTIVATION:</u>				
Solvent Control	-----	13.70	80	100%
Positive Control (MNNG)	10 $\mu\text{g/ml}$	10.43	570	712%
Test Compound:				
DNC, 78-142	0.50 $\mu\text{g/ml}$	18.91	71	89%
DNC, 78-142	1.00 $\mu\text{g/ml}$	16.17	66	82%
DNC, 78-142	2.50 $\mu\text{g/ml}$	11.35	97	121%
DNC, 78-142	5.00 $\mu\text{g/ml}$	10.30	102	128%
DNC, 78-142	10.00 $\mu\text{g/ml}$	12.24	97	121%
DNC, 78-142	12.50 $\mu\text{g/ml}$	22.24	149	186%
DNC, 78-142	25.00 $\mu\text{g/ml}$	9.21	187	234%
DNC, 78-142	50.00 $\mu\text{g/ml}$	11.68	32	40%

MNNG = N-methyl-N'-nitro-N-nitrosoguanidine



BIONETICS

PROTOCOL NO. 434

1. OBJECTIVE

The objective of this assay is to evaluate the test material for its ability to induce unscheduled DNA synthesis (UDS) in WI-38 human cells as determined by the amount of radioactive thymidine incorporated per microgram of DNA.

2. RATIONALE

WI-38 cells attach to a surface and multiply until contact inhibition imposed by a confluent monolayer causes complete growth arrest. Cultures thus arrested are composed primarily of cells which are not actively synthesizing DNA. A further block of DNA synthesis is achieved by inclusion of hydroxyurea in the medium. If tritiated thymidine ($^3\text{H-TdR}$) is introduced in the medium in the presence of hydroxyurea, little or no label will be incorporated into DNA. Addition of a test substance that interacts with the DNA usually 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 nondividing cells is known as UDS and can be measured by determining the amount of $^3\text{H-TdR}$ incorporated into DNA. Exposure of WI-38 cells to various forms of radiation or to chemicals known to be mutagenic or carcinogenic has resulted in the observation of UDS.

3. MATERIALS

A. Indicator Cells

The WI-38 normal human diploid cell strain is obtained directly from ATCC, Rockville, MD, at the lowest passage available. Laboratory cultures are grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 100 units penicillin and 100 ug streptomycin per milliliter.

B. Control Compounds

1. Negative Controls

A negative control consisting of assay procedures on untreated cells is utilized 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 UDS caused by solvent alone. For test substances assayed with activation, untreated and solvent negative controls will include the activation mixture.



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2. Positive Controls

N-methyl-n'-nitro-n-nitrosoguanidine (MNNG) is highly mutagenic via alkylation of DNA and is used at a final concentration of 5 to 10 $\mu\text{g/ml}$ as a positive control for assays performed without activation. Benz(a)pyrene requires metabolic activation to react with DNA and is used at 1 to 20 $\mu\text{g/ml}$ as a positive control for activation conditions.

C. 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

An assay is performed with at least four doses of test compound starting generally with a stock solution of 5 mg/ml (or 5 $\mu\text{l/ml}$ for a liquid) and diluting, alternating 5-fold and 2-fold dilutions, to 0.1 mg/ml (or 0.1 $\mu\text{l/ml}$). Doses which result in excess toxicity will be recognized by UDS activities less than control values. If necessary, the assay will be repeated with at least four doses spaced below the excess toxicity level.

B. UDS Assay

1. Nonactivation Assay

This assay is based on the procedure given by Stich and Laishes (1973). WI-38 cells are seeded at 2.5×10^5 cells per 100 mm dish and are grown to confluency. The serum concentration is then reduced from 10% to 0.5% for another five days to help ensure synchronization of the cells into a state of no DNA synthesis. To avoid a spurt of DNA synthesis when the medium is changed prior to treatment, hydroxurea is added to the medium one-half hour before the addition of test chemical. Selected doses of the test compound and a positive compound are applied (two dishes/dose), and additional dishes are treated with solvent, if appropriate. $^3\text{H-TdR}$ is added to all the cultures simultaneously with the test substance. After an exposure period of 1.5 hours at 37°C , the dishes are washed free of chemical and label and are stored frozen until DNA extraction is performed.



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2. Activation Assay

The activation assay can be run separately or concurrently with the nonactivation assay. The only difference is the addition of an activation mixture to the cells immediately before the test chemical. The activation mixture consists of S9 fraction of rat liver homogenate and the cofactor mixture, NADP and isocitric acid.

C. Extraction of DNA

The cells from the frozen stored dishes are removed by scraping in 0.5% sodium lauryl sulfate (SLS) in 0.017M disodium citrate and are placed into centrifuge tubes. The two dishes for each dose of test substance are combined in this procedure. DNA is precipitated by the addition of at least two volumes of 95% ethanol. The mixture is centrifuged and the pellets are washed with 95% ethanol and resuspended in absolute ethanol:ethyl ether (2:1). The tubes are incubated at 70°C for 3 minutes to solubilize nonpolymeric contaminants, then are centrifuged and the pellets washed with 95% ethanol. The pellets are next dissolved in 0.3N NaOH and incubated at 70°C for 20 minutes to hydrolyze any RNA contaminant. The DNA is then reprecipitated by chilling and the addition of an equal volume of 2N perchloric acid in an ice bath. After centrifugation and a 0.2N perchloric acid wash, the DNA pellet is dissolved in 1N perchloric acid by heating at 70°C. Any remaining insoluble material is removed by centrifugation and the supernatant is analyzed for DNA and radiolabel.

D. DNA Analysis

Samples from each tube are removed for determination of absorbance at 260 nm, 280 nm and 320 nm. The micrograms of DNA are then calculated from the 260 nm absorbance reading based on a standard curve of hydrolyzed DNA. The other absorbance values are used as indicators of purity (280 nm) and turbidity (320 nm) in the assayed solutions of DNA.

Samples (0.5 ml) from each tube are also placed into 10 ml scintillation fluid and the amount of ³H label counted by a liquid scintillation spectrometer. The counts are converted into disintegrations per minute (dpm).

The results for each sample are expressed as dpm per microgram of DNA. These values are compared to the UDS activity of the appropriate negative control.



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E. Preparation of 9,000 x g Supernatant (S9)

Fischer 344 male rats are normally used as the source of hepatic microsomes. After decapitation and bleeding, the liver is immediately dissected from the animal using aseptic techniques and placed into ice-cold 0.25M sucrose, buffered with Tris at pH 7.4. When an adequate number of livers has been obtained, the collection is washed twice with fresh buffered sucrose and completely homogenized. The homogenate is centrifuged for 10 minutes at 9,000 x g in a refrigerated centrifuge and the supernatant (S9) from this centrifuged sample is retained and frozen at -80°C until used in the activation system. The S9 fraction may also be obtained from induced rats or from other species, as requested.

5. REFERENCE

Stich, H.F. and Laishes, B.A.: DNA repair and chemical carcinogens. Pathobiology Annual, 3:341-376, 1973.



BIONETICS

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EVALUATION CRITERIA

Several criteria have been established which, if met, provide a basis for declaring a material genetically active in the UDS assay. These criteria are derived from a historical data base and are helpful in maintaining uniformity in evaluations from material to material and run to run. While these criteria are reasonably objective, a certain amount of flexibility may be required in making the final evaluations since absolute criteria may not be applicable to all biological data.

A compound is considered active in the UDS assay if:

- a. A dose-response relationship is observed over two consecutive dose levels.
- b. The minimum increase at the high level of the dose response is approximately 150% of the control value. The positive control data for a large sampling of 5 µg/ml of MNNG tests was found to be 206% of the control and for 10 µg/ml of Benz(a)pyrene it was 162% of the control.

All evaluations of UDS activity are based on the concurrent solvent control value run with the experiment in question. Positive control values are not used as reference points to measure activity, but rather to demonstrate that the cell population employed was responsive to chemicals known to induce repair synthesis under the appropriate test conditions.

As the data base for the UDS assay increases, the evaluation criteria will be more firmly established.



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The acute aquatic effects of the subject chemicals were determined under the following conditions.

1,8-Dihydroxy-4,5-dinitroanthraquinone

Test type: 96-hour static exposure (range finding)

Test solution preparation: 2g, 0.2g and 0.02g of test chemical each were dissolved in 20mL of acetone. Each acetone solution subsequently was added to 20L of diluent water to give nominal test chemical concentrations of 100, 10 and 1 mg/L.

Control solution: diluent water

Water quality parameters: Temperature 17-19°C; pH, 7.6-8.1; dissolved oxygen, 9.6-5.1 ppm

Test start dates: 12/1/75 - 100 mg/L exposure
12/15/75 - 10 mg/L exposure
1/5/76 - 1 mg/L exposure

N,N-Dimethylcyclohexylamine

Test type: 96-hour static exposure (range finding)

Test solution preparation: 2 mL of test article was dissolved in 10 mL of acetone and this acetone solution was added to 20 L of diluent water. The pH of the resulting aqueous solution was adjusted from 9.5 to 7.0 by the addition of 10% H₂SO₄.

Control Solution: 10 mL acetone/20 L diluent water

Water Quality Parameters: Temperature, 17-20°C; pH, 7.0-7.4; dissolved oxygen 10.0-3.8 ppm.

Test start date: 11/14/77

The present survivals of the aquatic organisms observed at selected times during the tests are presented in Tables I and II. In the test with the cyclohexylamine compound, oxygen depletion occurred in both the test and control solutions to the same extent; however, there was no mortality among the controls.

Table I

Data from Acute Aquatic Effects Testing of
1,6-Dihydroxy-4,5-dinitroanthroquinone

Species	Normal Test Chemical Concentration (mg/L)	% Survival After X Hours of Exposure					
		X= 0	6	24	48	72	96
Fathead minnow (<u>Pimephales promelas</u>)	100	100	100	0	--	--	--
	10	100	100	10	0	--	--
	1	100	100	100	100	100	100
Water flea (<u>Daphnia magna</u>)	100	100	100	0	--	--	--
	10	100	100	100	100	100	70
Flatworm (<u>Dugesia tigrina</u>)	100	100	100	0	--	--	--
	10	100	100	17	0	--	--
	1	100	100	100	100	100	83
snail (<u>Helisoma trivolvis</u>)	100	100	100	100	100	100	83

0 0 3 2

Compound 73-285

Summary and Tabulation of Histopathological Evaluation

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0 0 6 8

Compound 75-285

A focal unilateral spermatogonial degeneration with tubular atrophy was present in 2 test (IX) and no control (0%) rats (Table 1). This was interpreted as a senile degeneration unrelated to treatment.

Multifocal interstitial pneumonia, usually mild, was present in 2 test and 1 control rats. These lesions were interpreted as manifestations of Mycoplasma pulmonis infection (Chronic Murine Pneumonia).

A mild lymphocytic interstitial nephritis was present in 1 test and 1 control rat. These were felt to be incidental lesions common to the rat.

Multiple clear vacuoles were present in white matter tracts of the brains of test and control rats. The exact nature of these clear spaces was not determined, however, they have been encountered in normal rats in other studies and represent a "normal" histologic vagary for this specie.

A coagulum composed of eosinophilic amorphous material and partially lysed erythrocytes was present in the urinary bladder of 2 control and 1 test rats. No lesions were present in the bladder or kidneys of these animals and the nature and source of the coagulum was not determined.

No treatment-related lesions were detected with this compound. The blue discolored tissue found grossly in the test group had no histopathological counterpart.

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CA 1001

0 0 6 9

Table 1. Study 75-205. Summary of Lesions

Group	Lesion		
	Lung, Focal Interstitial Pneumonia	Testis, Spermatogenic Degeneration with Tubular Atrophy, Unilateral	Bladder, Amorphous Eosinophilic Coagulum
12	2/5	2/5	1/5
02	1/5	0/5	2/5

* = Number affected/total

Kidney, Multifocal
Lymphocytic
Interstitial
Nephritis

1/5

1/5

1780

INDUSTRIAL LABORATORY REQUEST FOR ANALYSIS

SPONSOR NUMBER FOR SUBMITTER'S USE ONLY **59350**

TO: Industrial Lab

BLDG. 34 DATE OF REQUEST 11-4-75 3518

MATERIAL, SAMPLE DESCRIPTION, OR PROJECT TITLE
 1) 75-274 2) 75-277 3) 75-278 4) 75-279
 5) 75-280 6) 75-281 7) 75-282 8) 75-283
 9) 75-284 10) 75-285 11) 75-286

CHARGE INFORMATION - FILL IN ONE

E.W.O. 700 9930

DEPT. CHARGE 9100

DEPT. AND EXPENSE CODE

OTHER KODAK PLANTS 000 0100

SYL CHEM. CHG. 000

FACILITIES SHOP ORDER

HANDLING AND SAFETY PRECAUTIONS

REPORT RESULTS TO:

NAME: C.J. Tarhara DEPT. NO.: 549

BLDG.: 306 PHONE:

DEPT.: Health & Safety

SENDER: NO.:

USE THIS SECTION FOR SPECIFICATION AND STD. TEST REQUESTS

SPEC. OR STD. TEST NO.	VENDOR	PURCHASE ORDER NO.
1.		
2.		
3.		
4.		
5.		

USE THIS SECTION FOR ALL OTHER REQUESTS

COMPLAINT OR IDENTIFICATION NO.

DESCRIBE PROBLEM AND ACTION YOU REQUEST. ATTACH SEPARATE SHEET IF NECESSARY.

BOD₅, BOD₂₀, TOD, COD

NO. OF SAMPLES SUBMITTED WITH THIS REQUEST 11

FOR INDUSTRIAL LAB USE ONLY

JOB FILE NO.	DATE RECEIVED	ASSIGNED TO	REG. NO.	GRP. NO.	NO. OF SLIP CARDS	REPORT NUMBER
<u>IL4005</u>	<u>11/4</u>	<u>G MORS</u>	<u>9437</u>	<u>40</u>	<u>-</u>	<u>3518</u>

REPORT OF RESULTS AS FOLLOWS:

** All results are expressed in grams O₂ per gram sample*

Sample	BOD ₅	BOD ₂₀	COD	TOD
75-274	•	0.017	1.46	*
75-277	+	+	†	†
75-278	0.034	0.057	1.24	1.06
75-279	•	•	1.44	*
75-280	0.020	0.098	0.873	1.06
75-281	•	0.010	†	0.62
75-282	0.012	0.016	1.71	1.0
75-283	•	0.010	1.57	1.0
75-284	I.R.	I.R.	1.43	1.0
75-285	•	0.015	1.35	1.0
75-286	0.015	0.015	1.56	*

* = Due to the consistency of the sample in solution TOD could not be run.
 • = no apparent BOD
 † = Sample could not be observed and would not run
 ‡ = Sample not amenable to COD method. Consistent duplicate values of uncorrected COD not in correlation
 I.R. = Insufficient sample for testing
 I.R. = Inconsistent result

REQUESTED BY: R.C. [Signature] 11/11/75

Acc. No. 908018

AGRIC. SOCIETY

93-42-2

908018

DATE: 1/14/27

Chemical: NH₄ liquid fertilizer

Diluent: 6.000

Dilution: _____

Feed

Feed withheld

Species: Chick House CP Sex: M F Route: FO IP IT Date: 1/14/27

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
	3	3								3			

Weight	Feeds	Vol	Time	Remarks
141	153 222	200	.612	
142	161 290	200	.611	
143	182 25 ⁹	200	.63	
144	148 26 ⁷	200	.63	
145	188 25 ⁹	400	.615	
146	147 -	400	.66	
147	195 215	400	.658	not accurate
148	159 -	400	.656	
149	155 -	800	.12	
150	154 ✓	800	.12	
151	152 ✓	800	.11	
152	144 ✓	800	.11	
153	154 -	1600	.21	not accurate
154	157 -	1600	.25	not accurate
155	153 -	1600	.24	not accurate
156	191 ✓	1600	.25	not accurate
157	197 210	3200	.49	
158	146 27	3200	.47	
159	147 275	3200	.49	
160	176 265	3200	.47	

30

Signed: _____

No. 73

Date 1/10/60

Injection - Suspension 07.1.60

TEST RESULTS

Sl. No.	Batch No.	Route	Dose mg/kg	Days	Remarks
412	107325	PO	200	10	
413	107325	PO	200	10	
414	107325	PO	400	10	
415	107325	PO	400	10	
416	107325	PO	800	10	
417	107325	PO	800	10	
418	107325	PO	1600	10	
419	107325	PO	1600	10	
420	107325	PO	3200	10	
421	107325	PO	3200	10	

10.0073

Compound 75-285

Summary and Tabulation of Histopathological Evaluation

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6832

Compound 73-293

A focal unilateral spermatogonial degeneration with tubular atrophy was present in 2 test (IX) and no control (OX) rats (Table 1). This was interpreted as a senile degeneration unrelated to treatment.

Multifocal interstitial pneumonia, usually mild, was present in 2 test and 1 control rats. These lesions were interpreted as manifestations of Mycoplasma pulmonis infection (Chronic Murine Pneumonia).

A mild lymphocytic interstitial nephritis was present in 1 test and 1 control rat. These were felt to be incidental lesions common to the rat.

Multiple clear vacuoles were present in white matter tracts of the brains of test and control rats. The exact nature of these clear spaces was not determined, however, they have been encountered in normal rats in other studies and represent a "normal" histologic vagary for this specie.

A coagulum composed of eosinophilic amorphous material and partially lysed erythrocytes was present in the urinary bladder of 2 control and 1 test rats. No lesions were present in the bladder or kidneys of these animals and the nature and source of the coagulum was not determined.

No treatment-related lesions were detected with this compound. The blue discolored tissue found grossly in the test group had no histopathological counterpart.

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Table 1. Study 75-285. Summary of Lesions

Group	Lesion			
	Lung, Focal Interstitial Pneumonia	Testis, Spermatogonial Degeneration with Tubular Atrophy, Unilateral	Bladder, Amorphous Eosin. phillc Coagulum	Kidney, Multifocal Lymphocytic Interstitial Nephritis
1X	2/5	2/5	1/5	1/5
0X	1/5	0/5	2/5	1/5

* = Number affected/total

1780