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MUTAGENICITY EVALUATION OF AMMONIUM PICRATE IN THE AMES SALMONE--ETC(U)
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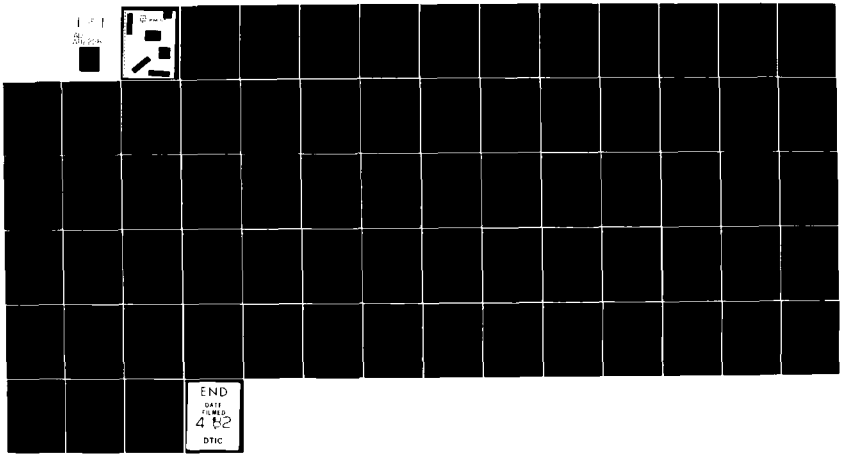
UNCLASSIFIED GENETICS ASSAY-3737 NL



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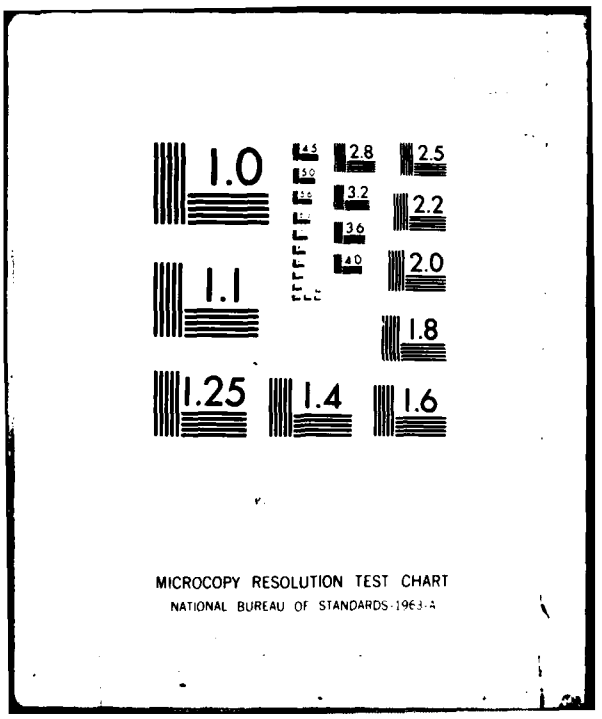
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MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

Litton BIONETICS

5516 Nicholson Lane
Kensington Maryland
20795

GENETICS ASSAY # 3737

(1)

MUTAGENICITY EVALUATION OF

AMMONIUM PICRATE

IN THE AMES SALMONELLA/MICROSOME PLATE TEST

SEGMENT REPORT

SUBMITTED TO:

DEPARTMENT OF THE NAVY
800 N. QUINCY STREET
ARLINGTON, VA. 22217

N00014-79-C-0112

SUBMITTED BY:

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

LBI PROJECT NO. 20988

FEBRUARY 1979

Litton BIONETICS

I. SPONSOR: Department of the Navy

II. MATERIAL

A. Identification: Ammonium Picrate

B. Date Received: November 7, 1978

C. Physical Description: Yellow crystals

III. TYPE OF ASSAY: Ames Salmonella/Microsome Plate Test

IV. PROTOCOL NO.: DMT-100

V. RESULTS

The results of the assay are presented in Table 1.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

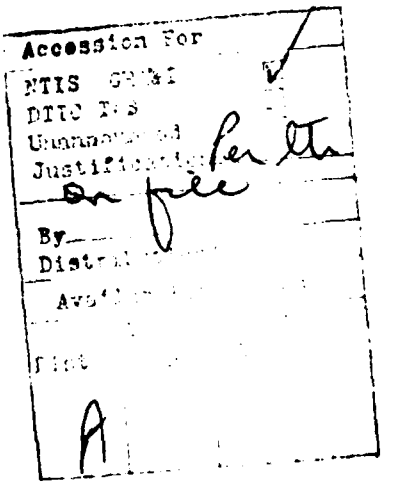
The test compound was examined for mutagenic activity in a series of in vitro microbial assays employing Salmonella and Saccharomyces indicator organisms. The compound was tested directly and in the presence of liver microsomal enzyme preparations from Aroclor-induced rats.

The compound was tested over a series of concentrations such that there was either quantitative or qualitative evidence of some chemically-induced physiological effects at the high dose level. The low dose in all cases was below a concentration that demonstrated any toxic effect. The dose range employed for the evaluation of this compound was from 0.5 µg to 1000 µg per plate.

The results of the tests conducted on the compound in the absence of a metabolic system were all negative.

The results of the tests conducted on the compound in the presence of the rat liver activation system were all negative.

The test with TA-1538 was repeated because of contamination in the initial activation and nonactivation assays.



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VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

The test compound, Ammonium Picrate, did not demonstrate mutagenic activity in any of the assays conducted in this evaluation and was considered not mutagenic under these test conditions.

Submitted by:

Study Director

D.R. Jagannath 2.14.79

D.R. Jagannath, Ph.D. Date
Section Chief
Submammalian Genetics
Department of Genetics
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Reviewed by:

David J. Brusick 2/14/79

David J. Brusick, Ph.D. Date
Director
Department of Genetics
and Cell Biology

V. RESULTS

TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: AMMONIUM PICRATE
B. SOLVENT: DIST WATER
C. TEST INITIATION DATE: 01/23/79
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) PER PLATE.

TEST	SPECIES	TISSUE	REVERTANTS PER PLATE TA-1535		TA-1537		TA-1538		TA-98		TA-100		04*	
			1	2	1	2	1	2	1	2	1	2	1	2
NONACTIVATION														
SOLVENT CONTROL	---	---	22		22		C	18	26		199		104	
POSITIVE CONTROL**	---	---	1157		611		C	992	1205		1367		502	
TEST COMPOUND														
0.500000 UG	---	---	25		22		C	14	14		191		89	
1.000000 UG	---	---	25		32		C	17	16		166		108	
10.000000 UG	---	---	25		17		C	13	25		192		92	
100.000000 UG	---	---	26		20		C	11	18		223		78	
500.000000 UG	---	---	18		15		C	11	14		181		93	
1000.000000 UG	---	---	31		22		C	7	25		191		87	
ACTIVATION														
SOLVENT CONTROL	RAT	LIVER	21		23		C	15	37		197		105	
POSITIVE CONTROL***	RAT	LIVER	521		112		C	1920	2146		1749		118*	
TEST COMPOUND														
0.500000 UG	RAT	LIVER	12		11		C	13	40		113		122	
1.000000 UG	RAT	LIVER	25		20		C	19	31		151		108	
10.000000 UG	RAT	LIVER	26		24		C	21	36		170		129	
100.000000 UG	RAT	LIVER	29		25		C	22	38		183		114	
500.000000 UG	RAT	LIVER	20		21		C	24	37		179		139	
1000.000000 UG	RAT	LIVER	23		22		C	15	38		172		108	

* TRY* CONVERTANTS PER PLATE

** TA-1535 SODIUM AZIDE 1 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 1 UG/PLATE
04 N-METHYL,N-NITRO,N-NITROSOGUANIDINE 10 UG/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
04 2-ANTHRAMINE 2.5 UG/PLATE

* THERE IS NO KNOWN POSITIVE CONTROL COMPOUND THAT WORKS CONSISTENTLY WITH THIS STRAIN IN THE ACTIVATION PLATE ASSAYS.

SOLVENT 50 UL/PLATE

C= CONTAMINATION

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for genetic activity in a microbial assay with and without the addition of mammalian metabolic activation preparations.

2. MATERIALS

A. Indicator Microorganisms

Salmonella typhimurium	TA-1535 TA-1537 TA-1538 TA-98 TA-100
Saccharomyces cerevisiae	D4

B. Activation System

1. Reaction Mixture

Component	Final Concentration/ml
TPN (Sodium salt)	4 µmol
Glucose-6-phosphate	5 µmol
Sodium phosphate (dibasic)	100 µmol
$MgCl_2$	8 µmol
KCl	33 µmol
Homogenate S9 fraction	0.1 ± 0.05 ml

2. S9 Homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 lot #BIO-82 was purchased from Biological Products, Litton Bionetics, Inc. and was used in these assays.

2. MATERIALS (Continued)

C. Positive Control Chemicals

The chemicals used for positive controls in the nonactivation and activation assays are given in Table 1 of Section V. Results.

D. Solvent

The solvent employed to prepare the stock solution of the test chemical is given in Table 1 of Section V. Results.
All dilutions of the test chemical were made using this solvent.

3. EXPERIMENTAL DESIGN

A. Plate Test (Agar Incorporation)

Approximately 10^8 cells from an overnight culture of each indicator strain were added to separate test tubes containing 2.0 ml molten agar supplemented with biotin and a trace of histidine. For non-activation tests, at least 4 dose levels of the test compound were added to the contents of the appropriate tubes and poured over the surfaces of selective agar plates. In activation tests, at least 4 dose levels of the test chemical were added to the appropriate tubes with cells. Just prior to pouring, an aliquot of reaction mixture (0.5 ml containing the 9,000 x g liver homogenate) was added to each of the activation overlay tubes, which were then mixed, and the contents poured over the surface of a minimal agar plate and allowed to solidify. The plates were incubated for 48 hrs at 37°C and scored for the number of colonies growing on each plate. D4 yeast plates were incubated at 30°C for 3-5 days and then scored. The concentrations of all chemicals are given in Table 1 of Section V. Results. Positive and solvent controls using both directly active positive chemicals and those that require metabolic activation were run with each assay.

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* Certain classes of chemicals known to be mutagens and carcinogens do not produce detectable responses using the standard Ames agar incorporation method. Some dialkyl nitrosamines and certain substituted hydrazines are mutagenic in suspension assays, but not in the plate assay. Chemicals of these classes should be screened in a suspension assay.

3. EXPERIMENTAL DESIGN (Continued)

B. Recording and Presenting Data

The numbers of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants (or convertants for D4) per plate for each indicator strain employed in the assay. The positive and solvent controls are provided as reference points. Other relevant data are provided on the computer printout.

4. EVALUATION CRITERIA

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 chemical and the cells are incubated in the overlay for 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 results, they provide certain advantages not contained in a quantitative suspension test:

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

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test chemical, the surviving population on the treatment plates is essentially the same as that on the negative control plates. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol normally employs several doses ranging over 2 or 3 log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose Response Phenomena

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected.

4. EVALUATION CRITERIA (Continued)

B. Dose-Response Phenomena

Conversely, if the lowest dose employed is highly cytotoxic, the test chemical may kill any mutants that are induced, and the compound will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays. Negative controls consist of the test compound solvent in the overlay agar together with the other essential components. The negative control plate for each strain gives a reference point to which the test data are compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

D. Evaluation Criteria for Ames Assay

Because the procedures used to evaluate the mutagenicity of the test chemical 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 chemical that produces a positive dose response over three concentrations with the lowest increase equal to twice the solvent control value is considered to be mutagenic.

2. Strains TA-98, TA-100 and D4

   If the solvent control value is within the normal range, a chemical that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-100 and 2-3 times the solvent control value for strains TA-98 and D4 is considered to be mutagenic. For these strains, the dose-response increase should start at approximately the solvent control value.

4. EVALUATION CRITERIA (Continued)

D. Evaluation Criteria for Ames Assay

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), 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. It is also anticipated that if a given strain, e.g., TA-1537, responds to a mutagen in nonactivation tests, it will generally do so in activation tests (the converse of this relationship is not expected). While similar response patterns are not required for all mutagens, they can be used to enhance the reliability of an evaluation decision.

4. Reproducibility

If a chemical produces a response in a single test that cannot be reproduced in one or more additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria are applied to the majority of situations and are presented to aid those individuals not familiar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relationship Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/Microsome Plate Test is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relationships have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann et al. (1975) show an extremely good correlation between results of microbial mutagenesis tests and in vivo rodent carcinogenesis assays.

All evaluations and interpretation of the data presented in this report are based only on the demonstration, or lack, of mutagenic activity.

REFERENCES

Ames, B.N., McCann, J. and Yamasake, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian-microsome mutagenicity test. Mutation Res. 31, 347-364.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Nat. Acad. Sci. 72, 5135-5139.

LBI ASSAY NO. 3737

LBI SAFETY NO. 3303

MUTAGENICITY EVALUATION OF

AMMONIUM PICRATE

IN THE
MOUSE LYMPHOMA FORWARD
MUTATION ASSAY

FINAL REPORT

SUBMITTED TO:

DEPARTMENT OF THE NAVY
800 N. QUINCY STREET
ARLINGTON, VA. 22217

SUBMITTED BY:

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

LBI PROJECT NO. 20989

REPORT DATE: FEBRUARY 1979

Litton 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 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 provides identification of supervisory personnel. Item VII 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 VIII. Item IX provides the conclusion and evaluation.

The second part of the report, entitled PROTOCOL, describes 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 or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington Maryland, 20795.

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

I. SPONSOR: Department of the Navy

II. MATERIAL (TEST COMPOUND): LBI ASSAY NUMBER 3737

A. Identification: Ammonium Picrate

B. Date Received: November 7, 1978

C. Physical Description: Irregular bright yellow crystals

III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay

IV. PROTOCOL NUMBER: 431 (DMT-106)

V. STUDY DATES:

A. Initiation: December 6, 1978

B. Completion: January 22, 1979

VI. SUPERVISORY PERSONNEL:

A. Study Director: Brian Myhr, Ph.D.

B. Laboratory Supervisor: Marie McKeon

VII. RESULTS:

The data are presented in Table 1 on page 4.

VIII. INTERPRETATION OF RESULTS:

The test compound, Ammonium Picrate, was dissolved in deionized water to give a yellow stock solution at 10 mg/ml. Dilutions were performed with water prior to final 1:10 dilutions into growth medium to obtain an applied concentration range of 1000 µg/ml to 0.488 µg/ml. Higher concentrations (2500, 5000, and 10,000 µg/ml) were obtained by placing the weighed test compound directly into growth medium; the compound was not completely soluble at these concentrations and was tested as a suspension. The test concentrations of 5000 and 10,000 µg/ml in the nonactivation assay and 1000 µg/ml and higher with activation were completely lethal to the mouse lymphoma cells within 24 hours of treatment. The mutation assay was initiated with this wide concentration range in order to eliminate additional handling associated with a preliminary cytotoxicity test. However, a large number of mutant selection and viability culture dishes were contaminated, making it necessary to repeat the mutation assay.

In the repeat assay, the applied dose range was 4000 µg/ml to 250 µg/ml (nonactivation) and 1000 µg/ml to 31.3 µg/ml (activation).

VIII. INTERPRETATION OF RESULTS (continued):

Again, concentrations above 1000 µg/ml were obtained by placing weighed compound directly into growth medium; suspended material was present at the beginning of the 4 hour exposure period, but appeared to dissolve during this time. At 4000 µg/ml (nonactivation) all the cells were destroyed within 24 hours of treatment. With activation, the 1000 µg/ml applied concentration was extremely toxic so that only 2.2×10^6 cells were available for mutant analysis instead of the usual sample of 3×10^6 cells. The six dose levels chosen for completion of the nonactivation and activation assays were selected to span the observed range of toxicity to growth with emphasis on the most toxic doses. After the cells were seeded for mutant selection and measurement of viability, the percent relative growth in the treated cultures was found to range from 36.0% to 12.8% (nonactivation) and from 78.2% to 6.1% (activation).

The results of the second trial of the mutation assay are presented in Table 1. The first trial yielded no evidence for mutagenic activity and, because of the high contamination rate, was not contributory to this evaluation.

With and without activation, the test compound did not induce a significant increase in mutant frequency at any of the tested concentrations (Table 1). A 2.5-fold increase over the background frequency (average of the solvent and untreated negative control values) is considered necessary to demonstrate mutagenesis at any given dose level. Even at the highly toxic doses of 3000 µg/ml without activation (12.8% relative growth) and 1000 µg/ml with activation (6.1% relative growth), the mutant frequencies were comparable to the background frequencies.

The validity of the mutagenesis assay can be assessed by the results obtained for the positive and negative controls. The cloning efficiencies for the solvent and untreated negative controls varied from 88% without activation to 101% with activation, which demonstrates excellent culturing conditions for the assay. The negative (solvent and untreated) control mutant frequencies are all within the normal ranges for the nonactivation and activation tests, and the positive control compounds yielded frequencies in the normal range that are greatly in excess of the negative control values.

IX. CONCLUSIONS

The test compound, Ammonium Picrate, did not induce an increase in mutations at the TK locus in L5178Y mouse lymphoma cells at applied concentrations of 500 to 3000 µg/ml without activation and 31.3 to 1000 µg/ml with microsomal activation. These concentration ranges included highly toxic treatments.

Therefore, the test compound is considered to be inactive in the Mouse Lymphoma Forward Mutation Assay.

Submitted by:

Study Director

Brian C. Myhr 2-13-79

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Section Chief
Mammalian Genetics
Department of Genetics
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Reviewed by:

David J. Brusick 2/13/79

David J. Brusick, Ph.D. date
Director
Department of Genetics
and Cell Biology

Litton BIONETICS

a. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: AMMONIUM PICRATE
B. LRI CODE #: 3737
C. SOLVENT: WATER
D. TEST DATE: 01/09/79

TEST	S-9 SOURCE	S-9 TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY** (X 10E+6)
NONACTIVATION											
SOLVENT CONTROL	---	---	8.4	12.0		100.0	51.0	292.0	100.0	100.0	17.5
SOLVENT CONTROL	---	---	11.0	10.8		100.0	45.0+	256.0	100.0	100.0	17.6
UNTREATED CONTROL	---	---	12.2	11.6		128.9	45.0	243.0	89.7	114.3	18.5
EMS .5 UL/ML	---	---	6.6	9.0		54.1	752.0	90.0	32.8	17.8	835.6
TEST COMPOUND											
500.000 UG/ML	---	---	6.6	8.2		49.3	54.0	180.0	65.7	32.4	30.0
1000.000 UG/ML	---	---	5.8	8.2		43.3	40.0	228.0	83.2	36.0	17.5
1500.000 UG/ML	---	---	5.0	10.6		48.3	42.0+	194.0	70.8	34.2	21.6
2000.000 UG/ML	---	---	4.4	9.6		38.5	44.0	159.0	58.0	22.3	27.7
2500.000 UG/ML	---	---	4.4	10.0		40.1	47.0	242.0	88.0	35.2	19.4
3000.000 UG/ML	---	---	3.5	8.2		22.4	42.0	156.0	56.9	12.8	26.9
ACTIVATION											
SOLVENT CONTROL	RAT	LIVER	8.4	13.8		100.0	64.0	255.0	100.0	100.0	25.1
SOLVENT CONTROL	RAT	LIVER	6.4	13.2		100.0	63.0	375.0	100.0	100.0	16.8
UNTREATED CONTROL	RAT	LIVER	6.6	12.0		79.0	66.0	281.0	89.2	70.5	23.5
DMN .3 UL/ML	RAT	LIVER	6.0	4.8		28.7	374.0	95.0	30.2	8.7	394.7
TEST COMPOUND											
31.300 UG/ML	RAT	LIVER	5.2	12.4		64.4	65.0	383.0+	121.6	78.2	17.0
125.000 UG/ML	RAT	LIVER	8.0	9.4		75.0	50.0	255.0	81.0	60.8	19.6
300.000 UG/ML	RAT	LIVER	5.0	14.0		69.9	47.0	241.0	76.5	53.4	19.5
400.000 UG/ML	RAT	LIVER	3.8	14.0		41.9	92.0	282.0	89.5	37.5	32.6
500.000 UG/ML	RAT	LIVER	2.0	11.4		34.1	56.0	154.0	48.9	16.7	36.4
1000.000 UG/ML	RAT	LIVER	0.4	2.2 ++		6.6	56.0	213.0	92.2	6.1	26.3

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

** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4. THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-4. THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.

\+ = ONE PLATE CONTAMINATED

++ = ONLY 2.2 x 10^6 CELLS WERE AVAILABLE FOR MUTANT ANALYSIS. THUS, 220 CELLS WERE SCORED FOR VIABLE CLONE COUNT RATHER THAN THE USUAL 300.

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.

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 µl/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 µl/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.

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 µl 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.

PROTOCOL NO. 431 CHANGE SHEET

Part 4 Experimental Design

A. Dosage Selection

Because of the explosive properties of the test compound, a separate cytotoxicity test was not performed. Instead, a wide range of concentrations was employed for the initial mutation assay. Since it was necessary to perform a repeat mutation assay, the initial assay in effect served as the cytotoxicity test.

Six dose levels were chosen for mutant analysis in order to increase the probability of successful completion of at least four dose levels.

B. Mutagenicity Testing

At the highest tested dose level in the activation assay (1000 µg/ml), an insufficient number of cells was available to analyze 3×10^6 cells for mutants. Therefore, the entire available population of 2.2×10^6 cells was analyzed and 220 cells were seeded among three culture dishes for the viability measurement. This change does not affect the validity of the assay, but the decreased sample size can increase the variability of the results.

EVALUATION CRITERIA

A compound is considered mutagenic in this assay if:

- A dose-response relationship is observed over 3 of the 5 dose levels employed.
- The minimum increase at the low level of the dose-response curve is at least 2.5 times greater than the solvent and/or negative control values.
- 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 appropriate 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 chemical can be considered mutagenic. If the increase is 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.

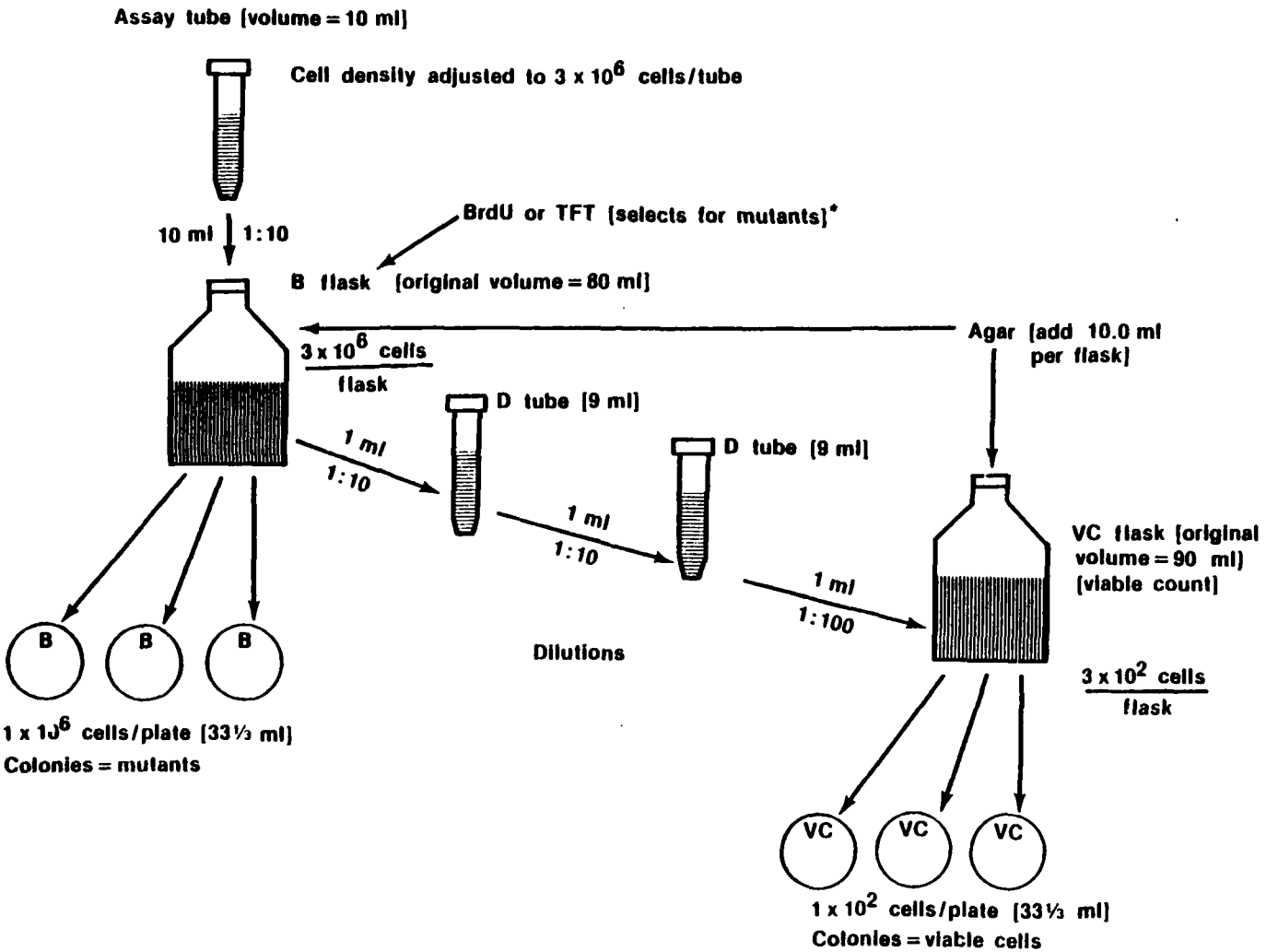
As the data base on the assay increases, the evaluation criteria can be expected to become more firmly established.

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.



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

FIGURE 1. LYMPHOMA CLONING FLOW CHART

ASSAY NO. 3737

MUTAGENICITY EVALUATION OF

AMMONIUM PICRATE

IN THE
SISTER CHROMATID EXCHANGE ASSAY
IN L5178Y MOUSE LYMPHOMA CELLS

SEGMENT REPORT

SUBMITTED TO:

U.S. NAVY
CAMERON STREET
ALEXANDRIA, VA. 22314

SUBMITTED BY:

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

LBI PROJECT NO: 20990

FEBRUARY, 1979

Litton 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-VI. 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 identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation of the results and conclusions are in Item VI.

The second part of the report, entitled PROTOCOL, describes the materials and procedures employed inconducting 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 Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland, 20795.

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

I. **SPONSOR:** U.S. Navy

II. **MATERIAL**

A. **Identification:** Ammonium Picrate

B. **Date Received:** November 7, 1978

C. **Physical Description:** Irregular bright yellow crystals

III. **TYPE OF ASSAY:** **Sister Chromatid Exchange Assay ***

IV. **PROTOCOL NO.:** **DMT-116**

V. **RESULTS**

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

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

This compound was evaluated for its ability to induce SCE's directly and also in the presence of a metabolic activation system that contained liver microsomal enzymes from Aroclor-induced rats (the S9 activation mixture).

When used directly this compound induced significant increases in SCE frequency (compared to solvent control) at all dose levels (Table 1). Linear regression analysis indicates a good correlation between SCE frequency and dose:

$$\text{SCE/chromosome} = 0.0006\ D + 0.303,$$

where D = dose in µg/ ml, and the correlation factor (r) = 0.88. Therefore, ammonium picrate clearly induced dose-related increases in SCE frequency when used directly in this assay.

With activation the solvent control frequency was somewhat elevated, and the test compound failed to induce significant increases in SCE frequency at all doses (Table 2) but linear regression suggests a fair correlation between SCE frequency and dose,

$$\text{SCE/chromosome} = 0.0014\ D + 0.333,$$

as r = 0.68. It should be noted that the compound was far more toxic following activation, because cultures exposed to doses above 31.3 µg/ml did not contain scorable, second division metaphase cells. Within the limited dose range employed, the SCE frequency actually increased more rapidly with dose than it did in the non-activation series

*Initiation Date: 1-15-79 Completion Date:1-30-79

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

(based on a comparison of the slopes of the linear regressions, 0.0006 vs, 0.0014). Thus in one sense ammonium picrate appears to be a more potent inducer of SCE following metabolic activation, despite the absence of statistically significant increases in SCE frequency; but the actual increases are so small (less than 21%) that this compound must be considered negative in this assay.

In conclusion, ammonium picrate induces SCE's when used directly but fails to produce clearly positive results when used with the S9 activation mixture.

Submitted by:

Study Director

Daniel Stetka — Mar 19, 1979

Daniel Stetka, Ph.D. — **Date**
Section Leader
Animal Genetics and
Cytogenetics
Department of Genetics
and Cell Biology

Reviewed by:

3/20/79

David J. Brusick, Ph.D. — **Date**
Director
Department of Genetics
and Cell Biology

TABLE 1

SCE FREQUENCIES IN CELLS EXPOSED TO

AMMONIUM PICRATE WITHOUT METABOLIC ACTIVATION

Treatment	Dose	No. of Chromosomes+	No. of SCE's	SCE/Chromosome ±SE	SCE/Cell
Negative Control (medium)	--	742 (20)	228	0.307 ± 0.020	12.29
Positive Control (EMS)	0.5 µl/ml	760 (20)	829	1.091 ± 0.038*	43.63*
Solvent Control (H_2O)	1.0 ml/tube	735 (20)	181	0.246 ± 0.018	9.85
Ammonium picrate	15.6 µg/ml	758 (20)	252	0.332 ± 0.021*	13.30*
	31.3 µg/ml	762 (20)	255	0.334 ± 0.021*	13.38*
	62.5 µg/ml	730 (20)	280	0.384 ± 0.023*	15.34*
	125.0 µg/ml	761 (20)	290	0.381 ± 0.022*	15.24*
	250.0 µg/ml	749 (20)	339	0.453 ± 0.025**	18.10**

+No. of cells scored are indicated in parentheses ( )

*Significantly greater than solvent control, $P < 0.01$ by t-test

**Significantly greater than both solvent and negative control at $P < 0.01$, t-test

TABLE 2

SCE FREQUENCIES IN CELLS EXPOSED TO

AMMONIUM PICRATE WITH METABOLIC ACTIVATION

Treatment	Dose	No. of Chromosomes+	No. of SCE's	SCE/Chromosome ±SE	SCE/Cell
Negative Control (medium)	--	740 (20)	237	0.320 ± 0.021	12.81
Positive Control (DMN)	0.3 µl/ml	756 (20)	746	0.987 ± 0.036*	39.47*
Solvent Control (H_2O)	1 ml/tube --	727 (20)	233	0.320 ± 0.021	12.82
Ammonium picrate	1.00 µg/ml	756 (20)	250	0.331 ± 0.021	13.23
	2.00 µg/ml	751 (20)	260	0.346 ± 0.021	13.85
	3.90 µg/ml	746 (20)	269	0.361 ± 0.022	14.42
	7.80 µg/ml	756 (20)	259	0.343 ± 0.021	13.70
	15.60 µg/ml	738 (20)	241	0.327 ± 0.021	13.06
	31.30 µg/ml	741 (20)	286	0.386 ± 0.023	15.44

+No. of cells scored are indicated in parentheses ( )

*Significantly greater than solvent control, $P < 0.01$ by t-test

PROTOCOL

1. OBJECTIVE

The objective of this study was to evaluate Ammonium Picrate for Sister Chromatid Exchange (SCE) induction in L5178Y mouse lymphoma cells.

2. MATERIALS AND METHODS

A. Toxicity

The solubility, toxicity, and doses for all chemicals were determined prior to screening. The effect of each chemical on the survival of the indicator cells was determined by exposing the cells to a wide range of chemical concentrations in complete growth medium. Toxicity was measured as loss in growth potential of the cells induced by a four-hour exposure to the chemical followed by a 24-hour expression period in growth medium. A minimum of four doses was selected from the range of concentrations by using the highest dose that showed no loss in growth potential as the penultimate dose and by bracketing this with one higher dose and at least two lower doses.

B. Indicator Cells

The cells used in this study were derived from Fischer mouse lymphoma cell line L5178Y. The cells are heterozygous for a specific autosomal mutation at the TK locus and are bromodeoxyuridine (BrdU) sensitive.

C. Media

The cells were maintained in Fischer's medium for leukemic cells of mice with 10% horse serum and sodium pyruvate.

D. Control Compounds

1. Negative Control

The solvent in which the test compound was prepared was used as the solvent or vehicle control and is designated as solvent control in the data table. The actual solvent is listed in Table 1 of Section V. Results. A negative control consisting of cells exposed to media only is also used in the assay.

2. MATERIALS AND METHODS (continued)

D. Control Compounds

2. Positive Control

Ethylmethanesulfonate (EMS), which induces mutation by base-pair substitution, was dissolved in culture medium and used as a positive control for the nonactivation studies at a final concentration of 0.5 µl/ml.

Dimethylnitrosamine (DMN), which induces mutation by base-pair substitution and requires metabolic biotransformation by microsomal enzymes, was used as a positive control substance for activation studies at a final concentration of 0.3 µl/ml.

E. Cell Treatment

Mouse lymphoma cells (L5178Y) were treated as described below.* The test compound was added to aliquots of 3 million cells in growth medium at the predetermined doses with or without an S-9 activation mixture and incubated at 37°C for 4 hours on a rocker. The incubation period was terminated by washing the cells twice with growth medium. BrdU (0.1 mM final concentration) was then added to the culture tubes and incubation was continued in the dark for 20 hours or two cell cycles. This permits BrdU to be incorporated into the DNA through two replication cycles so that sister chromatid exchanges may be detected.

Colchincine was added to a concentration of 5×10^{-5} mg/ml during the last 3 hours of incubation, and metaphase cells collected by centrifugation. Treated cells were harvested in 0.075 M KCL fixed in Carnoy's fixative and air-dried onto microscope slides.

Sister chromatid exchanges were visualized by staining with techniques described in Stetka et al (Mutat. Res. 51, 1978).

F. Activation System

1. S9 Mixture

Component	Final Concentration/ml
NADP (Sodium salt)	2.4 mg
Isocitric acid	4.5 mg
Homogenate S9 fraction	15 µl

*Stock solution of test compound prepared in distilled H_2O at 10 mg/ml. Serial dilutions performed in same solvent prior to dosing with $\leq$ 1.0 ml of solution per culture.

2. S9 Homogenate

A 9,000 x g supernatant was prepared from Fischer 344 adult male rat liver induced by Aroclor 1254 five days prior to kill according to the procedure of Ames et al. (1975). S9 samples were coded by lot number and assayed for milligrams protein per milliliter and relative P448/ P450 activity by methods described in LBI Technical Data on Rat Liver S9 Product.

3. RESULTS

The data presented in Tables 1A and 1B show the concentrations of the test compound employed and the number of SCE's per cell.

Interpretation of data is based on the relative increase in SCE with respect to dose compared to the spontaneous level. Statistical analysis of the data is made by a t-statistic.

REFERENCES

Ames, B.N., McCann, J. and Yamasake, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian-microsome mutagenicity test. Mutation Res. 31, 347-364.

#3737

MUTAGENICITY EVALUATION OF

AMMONIUM PICRATE

IN THE
MOUSE BONE MARROW
CYTOGENETIC ANALYSIS

SEGMENT REPORT

SUBMITTED TO:

CODE 4444
U.S. NAVY
OFFICE OF NAVAL RESEARCH
ARLINGTON, VIRGINIA
22217

SUBMITTED BY:

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

LBI PROJECT NO. 21022

MAY 1979

Litton BIONETICS

I. SPONSOR: U.S. Navy

II. MATERIAL

A. Identification: Ammonium Picrate

B. Date Received: November 7, 1978

C. Physical Description: Yellow crystals

III. TYPE OF ASSAY: Mouse Bone Marrow Cytogenetic Analysis*

IV. PROTOCOL NO.: 451

V. RESULTS

The toxicology and dosage selection results are presented in Table 1. The acute and subchronic test results have been collected from raw data sheets and tabulated in summary form in Table 2.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

Ammonium Picrate is evaluated here in terms of its ability to cause chromosome breakage in bone marrow cells of mice following oral exposure (per os). Both acute and subchronic dosing schedules were employed in this assay.

Results presented in Table 2 indicate that this compound failed to induce chromosome aberrations at all 3 dose levels. Only the positive control article (TEM) caused significant increases in the frequency of structural aberrations and in the percentage of cells with aberrations. Furthermore, in over 4000 total cells scored from animals exposed to Ammonium Picrate, only one two-break type aberration was observed (1t at 48 hours, low dose). This is well within the normal control range.

*Initiation Date: Feb. 26, 1979 Completion Date: April 11, 1979

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

The test compound was clearly negative in this assay.

Submitted by:

Study Director

Helen Stetka (for) 5/7/79

Daniel Stetka, Ph.D. Date
Section Leader
Animal Genetics
and Cytogenetics
Department of Genetics
and Cell Biology

Reviewed by:

David J. Brusick 5/7/79

David J. Brusick, Ph.D. Date
Director
Department of Genetics
and Cell Biology

Table 1

Sponsor U.S. Navy **Study ID** Mouse Bone Marrow **Initiation Date** February 26, 1979

Project No. 21022 **Strain/Species** Ha ICR Mice **Termination Date** March 2, 1979

Compound Ammonium Picrate **Breeder** Cumberland **Location** Kensington

Assay No. 3737 **Purchase Order No.** 87836 **Room Nos.** 6X 8X

Treatment	Vehicle	Dosage	Route of Administration	Volume/ Animal [ml]	Number of Administrations	Animal Numbers
AMMONIUM PICRATE	Deionized Water	2.23 mg/kg	PO	0.07	Acute-1 SC-5	
AMMONIUM PICRATE	Deionized Water	7.43 mg/kg	PO	0.25	Acute-1 SC-5	
AMMONIUM PICRATE	Deionized Water	22.3 mg/kg	PO	0.08	Acute-1 SC-5	
DEIONIZED WATER	---	7.0 ml/kg	PO	0.25	Acute-1 SC-5	
TEM	0.85% Saline	1.0 mg/kg	IP	0.32	1	

Supervisory Personnel: Gary Roy

A Dosage levels based on client provided information.
B Dosage levels based on LD_{50} determination.
C Toxic signs noted:

TABLE 2

A SUMMARY OF THE CYTOGENETIC ANALYSIS OF AMMONIUM PICRATE

Treatment	Dose	Kill Time (Hrs)[a]	No. of Animals[b]	Total No. of Cells	Type and Number( ) of Aberrations Structural	Numerical	Structural[c] Aberration Frequency	No. of Cells with one or more Aberrations	Percent Cells with Aberrations
CONTROLS									
Negative Control H_2O	0.25 mls Per Mouse S.C.	6	7	312	-- (0)	3h (3)	0.000	3	1.0
		24	7	291	1tb (1)	-- (0)	0.003	1	0.3
		48	8	391	2f (2)	5h 1pp (6)	0.005	6	1.5
		6	7	322	1f (1)	-- (0)	0.003	1	0.3
Positive Control TEM	1.0 mg/kg	24	7	162	8tb 3td 29f 14 af 7t 7tr 3pu+ 5 puc 1qr 2cr 1d 14> (94)	7h (7)	0.580**	59	36.4**

[a]Time after final exposure when bone marrow was harvested

[b]Includes only those animals from which at least 5 scorable metaphases were obtained

[c]No. of Aberration/No. of Cells

*P<0.05 by t-test, two-tailed

** P<0.01 by t-test, two-tailed

TABLE 2

A SUMMARY OF THE CYTOGENETIC ANALYSIS OF AMMONIUM PICRATE

Treatment	Dose	Kill Time (Hrs)[a]	No. of Animals[b]	Total No. of Cells	Type and Number( ) of Aberrations Structural	Numerical	Structural[c] Aberration Frequency	No. of Cells with one or more Aberrations	Percent Cells with Aberrations
Ammonium Picrate	2.23 ml/kg	6	7	319	1f (1)	1h 1pp (2)	0.003	3	0.9
		24	8	331	-- (0)	6h (6)	0.000	6	1.8
		48	7	350	1tb 1td 2f 2af 1t (7)	1h (1)	0.020	8	2.3
	S.C.	6	7	325	1f (1)	2h (2)	0.003	3	0.9
Ammonium Picrate	7.43 ml/kg	6	8	373	1f 1tb (2)	1h 1pp (2)	0.005	4	1.1
		24	8	352	2f (2)	1h (1)	0.006	3	1.0
		48	6	300	4f (4)	4h 1pp (5)	0.013	8	2.7
	S.C.	6	8	306	2f (2)	3h (3)	0.007	5	1.6
Ammonium Picrate	22.30 ml/kg	6	8	370	-- (0)	5h (5)	0.000	5	1.4
		24	8	347	1tb(1)	-- (0)	0.003	1	0.3
		48	→7	282	1sb 2f 3af (6)	3h (3)	0.021	7	2.5
	S.C.	6	†7	350	1f (1)	1h (1)	0.003	2	0.6

[a] Time after final exposure when bone marrow was harvested

[b] Includes only those animals from which at least 5 scorable metaphases were obtained

*P<0.05 by t-test, two-tailed

** P<0.01 by t-test, two-tailed

[c] No. of Aberration/No. of Cells

→One animal has been deleted from this study (#9767), due to dosing mix up.

†One animal lost #9666

DEFINITIONS OF ABERRATIONS

tg = Chromatid gap: An achromatic region in one chromatid, the size of which is equal to or smaller than the width of the chromatid.

tb = Chromatid break: An achromatic region in one chromatid larger than the width of the chromatid. It may either be aligned or unaligned.

sg = Chromosome gap: Same as tg only in both chromatids.

sb = Chromosome break: Same as tb only in both chromatids.

td = Chromatid deletion: Deleted material at the end of one chromatid.

f = Fragment: A single chromatid without an evident centromere.

af = Acentric fragment: Two aligned (parallel) chromatids without an evident centromere.

t = Translocation: Obvious transfer of material between two or more chromosomes.

tr = Triradial: An abnormal arrangement of paired chromatids resulting in a triarmed configuration.

qr = Quadriradial: An abnormal arrangement of paired chromatids resulting in a four armed configuration.

pu = Pulverized chromosome: A spread containing one fragmented or pulverized chromosome.

pu+ = Pulverized chromosomes: A spread containing two or more fragmented or pulverized chromosomes, but with some intact chromosomes still remaining.

puc = Pulverized cell: A cell in which all the chromosomes are totally fragmented.

cr = Complex rearrangement: An abnormal translocation figure which involves many chromosomes and is the result of several of breaks and mispairing chromatids.

r = Ring: A chromosome which as a result of telomeric deletions at both ends of the chromosome and the subsequent joining of the ends of the two chromosome arms.

min = Minute: A small chromosome which contains a centromere and does not belong in the karyotype.

DEFINITIONS OF ABERRATIONS (Continued)

pp = Polyploid: A cell in which the chromosome number is an even multiple of the haploid number, or n, and is greater than 2 n.

*h = Hyperdiploid: A cell in which the chromosome number is greater than 2 n + 1 but is not an even multiple of n.

d = Dicentric: A chromosome containing two centromeres.

> = Greater than 10 aberrations: A cell which contains more than 10 aberrations.

*Scored as "AP" on raw data sheet.

Chromosome Gap

Chromatid Gap

Chromatid Break

Chromatid Break

Acentric Fragments

Fragment

Minutes

Chromatid Deletion

Ring

Dicentric

Chromosome Break

Translocation

Translocation

Triradial

Quadriradial

Complex Rearrangement

PROTOCOL

1. PURPOSE

The purpose of this study was to determine the potential genetic activity of a chemical administered to mice by examination of cells arrested at metaphase of mitosis for structural changes and rearrangements of their chromosomes.

2. MATERIALS

A. Animals

Adult, male mice (HA/ICR) purchased from Cumberland Labs were used in this cytogenetic study.

B. Control Chemicals

Triethylenemelamine (TEM) was used as the positive control compound. The negative control consisted of the solvent or vehicle used for the test compound. The concentrations and routes of administration are given in Table 1.

3. EXPERIMENTAL DESIGN

A. Animal Husbandry

The animals were group housed according to LBI standard operating procedures and offered a commercial diet (Purina) and water ad libitum unless contraindicated by the particular experimental design.

The animals were randomly assigned to experimental groups. Prior to study initiation, 10% of the animals were weighed and a mean body weight was determined for the group. Dose levels were established using this mean unless there was significant variation among individuals, in which case individual weighings and calculations were performed. Animals were identified by ear tag and cage number.

3. EXPERIMENTAL DESIGN (continued)

B. Dosage Determination

Dosage information was calculated on the basis of range finding studies using 6 groups of 6 rats each. The high dose level was selected from these data. One-third and one-tenth of the high dose were used as the intermediate and low dose levels, respectively. For nontoxic compounds a maximum high dose level of 5 g/kg (or equivalent) is generally chosen.

4. METHODOLOGY

Table 3 shows the basic design of the test. Both acute (single dose) and subchronic (5 consecutive doses) sequences are provided. A total of 136 mice--104 in the acute study and 32 in the subchronic study--were used in the test as outlined in Table 3.

Three hours prior to kill, the animals were injected IP with 4.0 mg/kg colchicine. At times indicated in Table 3, mice were killed with CO_2 and the adhering soft tissue and epiphyses of one or both tibiae were removed. The marrow was aspirated from the bone and transferred to Hank's Balanced Salt Solution (HBSS). The marrow button was collected by centrifugation and then resuspended in 0.075M KCL. The centrifugation was repeated and the pellet resuspended in Carnoy's fixative. The fixative was changed after one-half hour and the cells left overnight at 4°C.

Slides were prepared by dropping the cells from the fixative onto a glass slide and the film air-dried. Spreads were stained with 10% Giemsa at pH 6.8.

Slides were coded and scored for chromosomal aberrations. Where possible, 50 spreads were read for each animal dosed.

Animals which died during dosing were not replaced unless the number of deaths at a single dose level was 4 or more. In that case, the entire dose level was repeated following consultation with the sponsor.

TABLE 3

MOUSE BONE MARROW CYTOGENETIC ANALYSIS
NUMBER OF ANIMALS DOSED

Treatment	ACUTE STUDY Number of Animals Killed at 6, 24, and 48 Hr After Dosing			SUBCHRONIC STUDIES 5 Exposures-24 Hr Apart Animals Killed 6 Hr After Last Exposure	Total Animals
High Level	6 Hr	24 Hr	48 Hr	6 Hr	
High Level	8	8	8	8	32
Intermediate Level	8	8	8	8	32
Low level	8	8	8	8	32
Positive Control	-	8	-	-	8
Negative Control	8	8	8	8	32

5. EVALUATION CRITERIA

A number of general guidelines has been established to serve as an aid in determining the meaning of bone marrow chromosomal aberrations.

A. General

Basically, we were trying to establish whether a substance or its metabolites could interact with chromosomes to produce gross lesions or changes in chromosome numbers, and whether these were of a type which could survive more than one mitotic cycle of the cell. The assay design was such that bone marrow samples were taken at 6, 24, and 48 hrs afer an acute administration of the compound. Since the cell transit time for bone marrow is 20-24 hrs, one can, based on the time of kill, obtain an indication of when in the cell cycle a compound may be active.

B. Aberrations/Records

All aberration figures detected by this assay resulted from breaks in the chromatin which either failed to repair or repaired in atypical combinations. We scored and recorded on standard forms gaps, breaks, fragments, and reunion figures which involved a single chromatid or both chromitids of a single chromosome. The number and type of aberration for each cell were recorded as was the number of chromosomes for every cell located and scored. Up to 50 cells were scored on each slide. Depending on the suitability of the material, it could have been necessary to prepare additional slides from the original fixed material. The location of cells bearing aberrations was identified by the use of coordinates on the mechanical stage.

C. Data Interpretation

Data were summarized in tabular form and evaluated. Gaps were not counted as significant aberrations unless they were present at exceptionally high frequency. Open breaks were considered as indicators of genetic damage as were configurations resulting from the repair of breaks. The latter include translocations. multiradials, rings, multicentrics, etc. Reunion figures such as these were weighted slightly higher than breaks since they usually result from more than one break and may lead to stable configurations.

The number of aberrations per cell is also considered to be significant; cells with more than one aberration were considered to indicate more genetic damage than those containing evidence of single events. Consistent variations from the euploid number were also considered in the evaluation of mutagenic potential.

5. EVALUATION CRITERIA (continued)

C. Data Interpretation

Comparison with a concurrent negative control which lacks aberrations can suggest statistical significance; therefore, treatment data may also be considered against historical control data. In either event the type of aberration, its frequency, and its correlation to dose trends within a given time period, were all considered in evaluating a compound as being mutagenically positive or negative.

#3737

MUTAGENICITY EVALUATION OF AMMONIUM PICRATE IN THE MOUSE DOMINANT LETHAL ASSAY

SEGMENT REPORT

SUBMITTED TO:
CODE 4444
U.S. NAVY
OFFICE OF NAVAL RESEARCH
ARLINGTON, VIRGINIA
22217

SUBMITTED BY:

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

LBI PROJECT NO. 21021

MAY 1979

Litton BIONETICS

I. SPONSOR: U.S. NAVY

II. MATERIAL

A. Identification: Ammonium Picrate

B. Date Received: November 7, 1978

C. Physical Description: Yellow Crystals

III. TYPE OF ASSAY: Mouse Dominant Lethal Assay

IV. STUDY DATES

A. Initiation Date: January 29, 1970

B. Completion Date: April 5, 1979

V. PROTOCOL NO.: 470

VI. The results are presented in Tables 2 - 7. Table 1 provides treatment information. The remaining Tables summarize the test results and statistical analyses.

VII. INTERPRETATION OF RESULTS AND CONCLUSIONS

Male mice were exposed to 2.23 mg/kg, 7.43 mg/kg and 22.3 mg/kg of Ammonium Picrate and mated to virgin females over the entire spermatogenic cycle. Administration of the test material was by oral gavage. Detailed dosing information is provided in Table 1.

The results of the dominant lethal scoring are given in Tables 2-7 and cover all significant parameters of mating, fertility and fetal wastage.

Fertility-The data were generally within the historical control range except for several values showing increased fertility. These data were not compound related and presumed to be normal variations.

Implants per pregnant female-The positive control values for week 2 were significantly reduced, otherwise all data were within the historical range.

Dead implants per pregnant female-The data for the test material were generally within the negative control range. Week 3 results appeared significantly low because of the unusually high negative control value. Compared to the historical control values, the test data were not unusual and the response was not considered indicative of dominant lethality.

VII INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

Females with 1, 2 or more dead implants-All data were within the historical control range. No compound related effects were observed.

Dead implants per total implants-TEM showed a positive effect in weeks 1 and 2. The test compound data were negative and all frequencies were within the negative control range.

Ammonium Picrate was not active in the dominant lethal assay conducted in mice.

Submitted by:

Study Director

_____ _____

Dan Stetka Date
Section Leader
Animal Genetics
and Cytogentics
Department of
Genetics and
Cell Biology

Reviewed by:

_____ _____

David J. Brusick, Ph.D. Date
Director
Department of Genetics
and Cell Biology

Table 1

Sponsor U.S. Navy **Study ID** Mouse Dominant Lethal **Initiation Date** January 29, 1979

Project No. 21021 **Strain/Species** CD-1 Mice **Termination Date** April 5, 1979

Compound Ammonium Picrate **Breeder** Charles River **Location** Kensington

Assay No. 3737 **Purchase Order No.** 87857 **Room Nos.** L, 5X

Treatment	Vehicle	Dosage	Route of Administration	Volume/ Animal [ml]	Number of Administrations	Animal Numbers
Ammonium Picrate	Deionized Water	2.23 mg/kg	PO	0.08	5	9833-9842
Ammonium Picrate	Deionized Water	7.43 mg/kg	PO	0.25	5	9843-9852
Ammonium Picrate	Deionized Water	22.3 mg/kg	PO	0.08	5	9853-9862
Deionized[a] Water	--	7.4 ml/kg	PO	0.25	5	9903-9912
TEM	0.85% Saline	0.35 mg/kg	IP	0.12	1	9913-9922

[a] Only five of the controls received deionized water; the other five were not treated with water.

Supervisory Personnel: Gary Roy

A Dosage levels based on client provided information.
B Dosage levels based on LD_{50} determination.
C Toxic signs noted:

TABLE 2

COMPOUND: AMMONIUM PICRATE FERTILITY INDEX 3737 STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	2.2300 MG/KG	7.4300 MG/KG	22.3000 MG/KG	LOG DOSE	ARITH DOSE
1	571/1020 = 0.56	7/ 20 = 0.35	10/ 20 = 0.50	4/ 16 = 0.25	9/ 18 = 0.50	9/ 16 = 0.56		
2	736/1060 = 0.69	13/ 20 = 0.65	14/ 20 = 0.70*	5/ 14 = 0.36	12/ 18 = 0.67	10/ 14 = 0.71		
3	702/1020 = 0.69	8/ 10 [a] 0.80	13/ 20 = 0.65	9/ 12 = 0.75	13/ 18 = 0.72	10/ 14 = 0.71		
4	620/1022 = 0.61	15/ 20 = 0.75	16/ 20 = 0.80**	7/ 12 = 0.58	15/ 18 = 0.81**	9/ 12 = 0.75		
5	644/1019 = 0.63	12/ 20 = 0.60	18/ 20 = 0.90**	3/ 11 = 0.27	15/ 18 = 0.83*	8/ 12 = 0.67		
6	678/1020 = 0.66	13/ 20 = 0.65	18/ 20 = 0.90**	7/ 12 = 0.58	10/ 18 = 0.56	9/ 12 = 0.75		
7	636/ 980 = 0.65	12/ 20 = 0.60	14/ 20 = 0.70*	6/ 12 = 0.50	10/ 18 = 0.56	7/ 12 = 0.58		

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

ONE * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

[a] Five negative control males were inadvertently not mated in week three.

TABLE 3

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

COMPOUND: AMMONIUM PICRATE 3737 STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	2.2300 MG/KG	7.4300 MG/KG	22.3000 MG/KG	LOG DOSE	ARITH DOSE
								$
1	6649/ 571 = 11.64	80/ 7 = 11.43	90/ 10 = 9.00	47/ 4 = 11.75	113/ 9 = 12.56	120/ 9 = 13.33		
2	8598/ 736 = 11.68	143/ 13 = 11.00	83/ 14 = 5.93**	59/ 5 = 11.80	146/ 12 = 12.17	125 / 10 = 12.5		
3	8394/ 702 = 11.94	100/ 8 = 12.50	154/ 13 = 11.85	119/ 9 = 13.22	171/ 13 = 13.15	118/ 10 = 11.80		
4	8141/ 690 = 11.80	163/ 15 = 10.87	174/ 16 = 10.88	79/ 7 = 11.29	173/ 16 = 10.81	93 / 9 = 10.3		
5	7591/ 644 = 11.77	134/ 12 = 11.17	199/ 18 = 11.06	38/ 3 = 12.67	188/ 15 = 12.53*	98/ 8 = 12.25		
6	8255/ 673 = 12.18	151/ 13 = 11.62	199/ 18 = 11.06	71/ 7 = 10.14	115/ 10 = 11.50	95/ 9 = 10.56		
7	7775/ 636 = 12.22	151/ 12 = 12.58	171/ 14 = 12.21	67/ 6 = 11.17*	125/ 10 = 12.50	83/ 7 = 11.86		

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SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

ONE * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 4

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE
COMPOUND: AMMONIUM PICRATE 3737 STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	2.2300 MG/KG	7.4300 MG/KG	22.3000 MG/KG	LOG DOSE	ARITH DOSE
1	393/ 571 = 0.67	6/ 7 = 0.80	54/ 10 = 5.40*	9/ 4 = 2.25	2/ 9 = 0.22	15/ 9 = 1.67		
2	621/ 736 = 0.84	11/ 13 = 0.85	58/ 14 = 4.14**	4/ 5 = 0.80	6/ 12 = 0.50	8/ 10 = 0.80		
3	537/ 702 = 0.76	10/ 8 = 2.38	27/ 13 = 2.08	4/ 9 = 0.44*	11/ 13 = 0.85*	11/ 10 = 1.10		
4	549/ 699 = 0.79	4/ 15 = 0.27	26/ 16 = 1.63	7/ 7 = 1.00	14/ 16 = 0.88	4/ 9 = 0.50		
5	404/ 666 = 0.61	8/ 12 = 0.67	13/ 18 = 0.72	1/ 3 = 0.33	18/ 15 = 1.20	6/ 8 = 0.75		
6	512/ 678 = 0.76	5/ 13 = 0.39	8/ 18 = 0.44	3/ 7 = 0.43	2/ 10 = 0.20	8/ 9 = 0.89		
7	383/ 636 = 0.60	7/ 12 = 0.58	5/ 14 = 0.36	6/ 6 = 1.00	12/ 10 = 1.20	7/ 7 = 1.00		

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

ONE * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 5

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS
COMPOUND: AMMONIUM PICRATE 3737 STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	2.2300 MG/KG	7.4300 MG/KG	22.3000 MG/KG	LOG DOSE	ARITH DOSE
1	251/ 571 = 0.44	3/ 7 = 0.43	10/ 10 = 1.00	4/ 4 = 1.00	2/ 9 = 0.22	5/ 9 = 0.56		
2	277/ 736 = 0.38	9/ 13 = 0.69	14/ 14 = 1.00	3/ 5 = 0.60	4/ 12 = 0.33	5/ 10 = 0.50		
3	326/ 702 = 0.46	7/ 9 = 0.89	10/ 13 = 0.77	4/ 9 = 0.44	7/ 13 = 0.54	6/ 10 = 0.60		
4	343/ 620 = 0.50	1/ 15 = 0.07	10/ 16 = 0.63	5/ 7 = 0.71	9/ 16 = 0.56	4/ 9 = 0.44		
5	254/ 644 = 0.39	5/ 12 = 0.42	7/ 18 = 0.39	1/ 3 = 0.33	9/ 15 = 0.60	5/ 8 = 0.63		
6	322/ 679 = 0.47	5/ 13 = 0.39	6/ 18 = 0.33	3/ 7 = 0.43	2/ 10 = 0.20	6/ 9 = 0.67		
7	269/ 636 = 0.42	5/ 12 = 0.42	5/ 14 = 0.36	5/ 6 = 0.83	5/ 10 = 0.50	5/ 7 = 0.71		

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

ONE * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 6

PROPORTION OF FEMALES WITH TWO OR MORE AD IMPLANTATIONS
COMPOUND: AMMONIUM PICRATE 3737 STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	2.2300 MG/KG	7.4300 MG/KG	22.3000 MG/KG	LOG DOSE	ARITH DOSE
1	98/ 571 = 0.17	2/ 7 = 0.29	8/ 10 = 0.8	2/ 4 = 0.50	0/ 9 = 0.0	4/ 9 = 0.44		
2	132/ 736 = 0.18	2/ 13 = 0.08	11/ 14 = 0.79	1/ 5 = 0.20	2/ 12 = 0.17	1/ 10 = 0.10		
3	113/ 702 = 0.16	5/ 8 = 0.63	9/ 13 = 0.69	0/ 9 = 0.0 *	3/ 13 = 0.23	2/ 10 = 0.20		
4	123/ 690 = 0.18	1/ 15 = 0.07	7/ 16 = 0.44	2/ 7 = 0.29	5/ 16 = 0.31	0/ 9 = 0.0		
5	91/ 644 = 0.14	3/ 12 = 0.25	3/ 18 = 0.17	0/ 3 = 0.0	4/ 15 = 0.27	1/ 8 = 0.13		
6	108/ 679 = 0.16	0/ 13 = 0.0	2/ 18 = 0.11	0/ 7 = 0.0	0/ 10 = 0.0	2/ 9 = 0.22		$
7	76/ 636 = 0.12	2/ 12 = 0/17	0/ 14 = 0.0	1/ 6 = 0.17	1/ 10 = 0.10	2/ 7 = 0.29		

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

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TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 7

DEAD IMPLANTS / TOTAL IMPLANTS

COMPOUND: AMMONIUM PICRATE 3737 STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	2.2300 MG/KG	7.4300 MG/KG	22.3000 MG/KG	LOG DOSE	ARITH DOSE
1	383/6648 = 0.06	6/ 80 = 0.08	54/ 90 = 0.60**	9/ 47 = 0.19	2/113 = 0.02	15/120 = 0.13		
2	621/8599 = 0.07	11/143 = 0.08	58/ 83 = 0.70**	4/ 59 = 0.07	6/146 = 0.04	8/125 = 0.06		
3	537/8384 = 0.06	19/190 = 0.10	27/154 = 0.18	4/119 = 0.03*	11/171 = 0.06	11/118 = 0.09		
4	548/8141 = 0.07	4/ 163 = 0.03	26/174 = 0.15	7/ 79 = 0.09	14/173 = 0.08	4/ 93 = 0.04		
5	404/7581 = 0.05	8/134 = 0.06	13/199 = 0.07	1/ 38 = 0.03	18/188 = 0.10	6/ 98 = 0.06		
6	512/8255 = 0.06	5/151 = 0.03	9/199 = 0.04	3/ 71 = 0.04	2/115 = 0.02	8/ 95 = 0.08		
7	383/7775 = 0.05	7/151 = 0.05	9/171 = 0.03	6/ 67 = 0.09	12/125 = 0.10	7/ 83 = 0.08		

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL.

THE TWO COLUMNS IDENTIFIED AS LOG DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO.

SIGNIFICANCE IN THE LOG DOSE COLUMN IS CALCULATED USING THE LEVELS OF THE TEST COMPOUND, WHEREAS THE SIGNIFICANCE IN THE ARITHMETIC DOSE COLUMN ALSO INCLUDES THE NEGATIVE CONTROL.

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TWO * OR $ INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for its ability to induce dominant lethality in mice.

2. OVERVIEW AND RATIONALE

The dominant lethal assay is designed to determine the ability of a compound to induce genetic damage in the germ cells of treated male mice leading to fetal wastage. Chromosome aberrations including breaks, rearrangements, and deletions are believed to produce the dominant lethality although ploidy changes and chromosome nondisjunction may also be detected in this assay. Male mice are exposed to several dose levels of the test compound for 5 days and then mated over the entire period of spermatogenesis to unexposed virgin females. At mid-pregnancy, the females are killed and scored for the number of living and dead implants as well as the level of fertility. These results are then compared to data from control animals and used to determine the degree of induced dominant lethality.

Evidence of dominant lethality emphasizes that the compound is able to reach the developing germ cells and induce genetic damage. It also suggests, but does not measure directly, that in addition to the detected gross chromosomal lesions, more subtle balanced lesions or specific locus gene mutations may be produced. These latter types have a good chance of being transmitted to the gene pool of future offspring.

3. EXPERIMENTAL DESIGN

Ten (10) random bred, male mice from a closed colony were assigned to 1 of 5 groups. Three of these groups received different dose levels of the test compound; a fourth group received only the solvent or vehicle; and the fifth group received a known mutagen and served as the positive control group. The test compound and control compounds were administered as specified in Table 1. Triethylenemelamine (TEM) was used as the positive control and was given as a single intraperitoneal injection. Following treatment, each male was rested for 2 days and then caged with 2 unexposed virgin females on the third day. At the end of 5 days, these females were removed. This weekly mating sequence was continued for 7 weeks. Each pair of mated females was transferred to a fresh cage, and approximately 14 days after the midweek of being caged with the male, the females were killed with CO_2. At necropsy, their uteri were examined for dead and living implants, and total implantations. Animals which died during dosing were not replaced unless there was 75% mortality at a single dose level. In that case the compound toxicity was reviewed, and the entire dose level was repeated.

3. EXPERIMENTAL DESIGN (Continued)

A. Animals

Random bred, adult male and female mice, strain CD-1 were purchased from the Charles River Breeding Laboratories, Inc. Male and female mice were at least 8 weeks of age when purchased.

B. Animal Husbandry

Males were housed individually and females housed in pairs (except during mating) in shoe box cages on AB-SORB-DRI bedding.

All animals were quarantined prior to being used in the study to acclimate them to the new laboratory conditions. Purina Lab Chow was used as the basic diet food, and water were offered ad libitum. Light was provided on a 12-hour light/dark cycle.

Personnel handling animals or working within the animal facility wore suitable protective laboratory garments, including face masks or respirators.

C. Dosage Determination

Dosage information was calculated on the basis of range finding studies using at least 5 groups of 6 mice each. LD50, LD5, and LD1 concentrations were computer generated based on the preliminary study. The high dose level was selected from these data. One-third and one-tenth of the high dose were used as the intermediate and low dose levels, respectively.

D. Records

The number of dead and living implants, and total implantation sites were recorded on a standardized record form. Data were keypunched directly from these forms to computer entry cards, and analyzed for statistical significance as outlined in Appendix A. Original copies of all data are stored in the Litton Bionetics, Inc. archival system.

4. EVALUATION CRITERIA

Both pre- and post-implantation losses contribute to dominant lethality. The former is reflected in the total number of implantation sites per pregnant female and strictly measured by the difference between the number of corpora lutea gravidus and the number of implantation sites. Toxic or physiological effects on sperm may also reduce the number of implantation sites. Therefore, unless subtle physiological effects on sperm can be discounted, pre-implantation loss is not as rigorous and indication of dominant lethality as post-implantation loss. Corpora lutea cannot be reliably counted in mice and, therefore, pre-implantation loss is not evaluated in studies using mice. Post-implantation losses are measured as early and late fetal deaths plus the number of resorption sites.

Dominant lethality is typically determined from: a) a mutation index derived from the ratio of dead to total implants; or b) the number of dead implants per pregnant female. In interpreting these values it must be remembered that the former measurement reflects both pre- and post-implantation losses and that the ratio is affected by changes in either the numerator or the denominator. For this reason the second parameter is perhaps a better indicator of post-implantation loss. This becomes especially so if one concurrently examines the number of living embryos per pregnant female. The two sets of data should be inversely related. In other words, if true dominant lethality is being observed, then a significant increase in the number of dead implants per pregnant female should be accompanied by a significant decrease in the number of living implants per pregnant female.

These ratios are compared with both concurrent and historical control data for significant statistical differences. Dose-related trends are also looked for, but may not always be found. For example, some compounds such as EMS tested in mice show a threshold value and then a very steep rise. Certain portions of the response might be missed, depending on the spacing of the dose levels used.

True, as opposed to spurious, dominant lethality also tends to cluster according to the stage of spermatogenesis affected and typically would not be expected to appear in widely spaced weeks or blocks of weeks.

All data which are indicated as being statistically significant must also be strongly evaluated for their biological significance. By bringing both statistical and biological selective pressures to bear on the data gathered, an estimate of dominant lethality and of risk to the gene pool should be obtainable.

REFERENCES

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Ray, V.A. and Hyneck, M.L. (1973). Some primary considerations in the interpretation of the dominant-lethal assay. Environmental Health Perspectives 6, 27.

Bateman, A.J. (1960). The induction of dominant lethal mutations in rats and mice with triethylenemelamine (TEM). Genet. Res. Comb. 1, 381.

APPENDIX A

STATISTICAL ANALYSIS

APPENDIX A

Analysis of Data

1. Fertility Index

a. The fertility index is defined as F.I. = # of pregnant females/ # of mated females. It is calculated for each week (in subacute study) or at the end of 8 weeks (in acute study) and for each dose level, negative control, and positive control.

b. A chi-square test is used to compare each treatment group and positive control to negative control.

$$\chi_i^2 = \frac{(N_0 + N_i)\left(n_0(N_i - n_i) - n_i(N_0 - n_0) - (N_0 + N_i)/2\right)^2}{(n_0 + n_i)(N_0 - n_0 + N_i - n_i)N_0N_i}$$

where

n_i = # impregnated in i-th test group

n_0 = # impregnated in negative control group

N_i = # of females mated in the i-th test group

N_0 = # of females mated in negative control group

A 2 x 2 table is formed as follows:

	control	test
# impreg	n_0	n_i
# not impreg	$N_0 - n_0$	$N_i - n_i$

Significance at the 5 and 1% levels is indicated with asterisks.

c. Armitage's trend for linear proportions is used to test whether the fertility index is linearly related to arithmetic or log dose.

The following table is set up:

	-control	dose 1	dose 2	dose 3	dose k	totals
# impreg	n_0	n_1	n_2	n_3	n_k	t
# not impreg	$N_0 - n_0$	$N_1 - n_1$	$N_2 - n_2$	$N_3 - n_3$	$N_k - n_k$	$T - t$
totals	N_0	N_1	N_2	N_3	N_k	T

and Armitage's chi-square is calculated:

$$\chi_A^2 = \chi_{(k-1)}^2 - \chi_1^2$$

where

$$\chi_1^2 = \frac{T(\sum_{i=0}^{k} n_i x_i - t\sum_{i=0}^{k} N_i x_i)^2}{t(T - t)(\sum_{i=0}^{k} N_i x_i^2 - (\sum_{i=0}^{k} N_i x_i)^2)}$$

$$\chi_{(k-1)}^2 = \frac{T^2(\sum_{i=0}^{k} n_i^2/N_i - t^2/T)}{t(T - t)}$$

and the x_i are the dose levels. This calculation is repeated with x replaced by $\log_{10} x$. The 5 and 1% significance levels are indicated by dollar signs.

2. Total Number of Implantations

a. The total number of implantations is evaluated by the Student's t-test to determine whether the average number of implantations per pregnant female for each treatment group and the positive control group differs significantly from the negative control group.

n_i = # of pregnant females at dose level i.
u_{ij} = # of implantations for pregnant female j in dose group i.

$$\bar{u}_i = 1/n_i \left(\sum_{j=1}^{n_i} u_{ij}\right)$$

$$S_i^2 = \sum_{j=1}^{n_i} (u_{ij} - \bar{u}_i)^2$$

$$t_i = \bar{u}_0 - \bar{u}_i \Big/ \left(\frac{S_0^2 + S_i^2}{n_0 + n_i - 2} \left(\frac{1}{n_0} + \frac{1}{n_i}\right)\right)^{\frac{1}{2}}$$

$$d.f. = n_0 + n_i - 2$$

Significance at the 5 and 1% levels is indicated by asterisks.

b. A regression fit of the average number of implantations, $\bar{u}_i$, is made for both the arithmetic and logarithmic dose (x_i and log x_i). The doses x_i are used as independent variables and the fit includes data from the three treatment groups and the control group.

N = total # of pregnant females in all groups.

x_i = dose/log (dose) for the i-th female.

U_i = # of implantations for the i-th female.

$$\bar{x} = \frac{1}{N}\sum_{i=1}^{N} x_i$$

$$SS_x = \sum_{i=1}^{N} (x_i - \bar{x})^2$$

$$\bar{U} = \frac{1}{N}\sum_{i=1}^{N} U_i$$

$$SS_u = \sum_{i=1}^{N} (U_i - \bar{U})^2$$

$$S_{xu} = \sum_{i=1}^{N} (x_i - \bar{x})(U_i - \bar{U})$$

B = estimate of slope of regression line = S_{xu}/SS_x

A = estimate of intercept of regression line = $\bar{U} - B\,\bar{x}$

VARU = variance of U about regression line

$$= \frac{SS_u - S_{xu}^2/SS_x}{N-2}$$

VARB = variance of B = $\frac{VARU}{SS_x}$

VARA = variance of A = $VARU\ (\frac{1}{N} + \frac{\bar{x}^2}{SS_x})$

TB = $B/(VARB)^{\frac{1}{2}}$ = t-statistic for testing the hypothesis that the regression slope is zero

DF = N-2 = # of degrees of freedom for T B

CVUX = coefficient of variation of U about x

= $(VARU.X)^{\frac{1}{2}}/\bar{U}$

VARU.X = $\frac{1}{N-2}\ (SS_U - S_{XU}^2/SS_X)$

SDY = standard deviation of U about the regression line

= $(VARU.X)^{\frac{1}{2}}$

SDS = standard deviation of the slope

= $(VARB)^{\frac{1}{2}}$

SDA = standard deviation of intercept

= $(VARA)^{\frac{1}{2}}$

Significant difference of the slope from zero is indicated at the 5 and 1% levels.

3. Total Number of Corpora Lutea
(For rats only)

a. The average number of corpora lutea per pregnant female is evaluated by t-test to determine whether each treatment group differed significantly from the control group. Use the equation described in Step 2 above with

u_{ij} = # of corpora lutea for pregnant female j in dose group i.

b. A regression fit of the average number of corpora lutea per pregnant female is made for both the arithmetic and logarithmic dose. Use the equations described in Step 2 above with

u_i = # of corpora lutea for the i-th female

4. Preimplantation Losses

(For rats only)

a. The number of preimplantation losses is the number of corpora lutea minus the number of implantations.

Y_{ij} = preimplantation losses for j-th female in i-th group

V_{ij} = # of corpora lutea for j-th female in the i-th group

b. The Freeman-Tukey transformation is applied to the Y_{ij} as follows:

$$f_{ij} = \sin^{-1}\sqrt{\frac{y_{ij}}{V_{ij}+1}} + \sin^{-1}\sqrt{\frac{y_{ij}+1}{V_{ij}+1}}$$

The t-test is then applied to the f's, comparing the test groups to the negative control. Let

$$\bar{f}_i = \frac{1}{n_i}\sum_{j=1}^{n_i} f_{ij}$$

$$s_i^2 = \sum_{j=1}^{n_i} (f_{ij} - \bar{f}_i)^2$$

where n_i = # of pregnant females at dose level i.

Then $t = (\bar{f}_0 - \bar{f}_i)/[\frac{s_0^2 + s_i^2}{n_0 + n_i - 2}(\frac{1}{n_0} + \frac{1}{n_i})]^{\frac{1}{2}}$

c. Regression analysis is used to determine whether the average number of preimplantation losses per female is related to the arithmetic or the log dose. The method is as used in Step 2 above substituting

U_i = # of preimplantation losses for the i-th female.

5. Dead Implantations

The dead implants were evaluated by the same statistical techniques that were used in evaluating the total number of implantations.

Substitute

u_{ij} = # of dead implants for j-th female in the i-th group in the equations in Step 2 above.

6. Proportion of Females with One or More Dead Implantations

The proportion of females with one or more dead implants is the number of females with dead implants/number of pregnant females. These proportions are analyzed by the same method used to analyze the fertility indices, i.e., by a chi-square test and Armitage's trend.

Substitute n_i = # of pregnant females with one or more dead implants at dose level i and

N_i = # of pregnant females at dose level i in Step 1 above.

Also a probit regression analysis is done using these proportions, p_i, to determine whether the probit of p_i is linearly related to the log or arithmetic dose. The Biomedical Computer Program BMD03S is used to compute A and B and the χ^2 statistic for the regression equations $y = A + B\,x$ and $y = A + B \log x$.

7. Proportion of Females with Two or More Dead Implantations

The proportion of females with two or more dead implantations is the number of females with two or more dead implants/number of pregnant females. The data are evaluated by the same method used for evaluating the proportion of females with one or more dead implants.

8. Dead Implants/Total Implants

Dead implants/total implants were computed for each female and transformed by way of the Freeman-Tukey arc-sine transformation prior to being evaluated by t-test to compare each treatment group and positive control to negative control.

Use y_{ij} = # dead implants for j-th female in i-th group

v_{ij} = # of total implants for j-th female in i-th group

in the equations in Step 4 above.

END

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