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MUTAGEN AND ONCOGEN STUDY ON 1-METHYLAMINOANTHRAQUINONE

DAVID J. BRUSICK, Ph.D.

DALE W. MATHESON, Ph.D.

LITTON BIONETICS, INC.

5516 NICHOLSON LANE

KENSINGTON, MARYLAND 20795

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER



ANTHONY A. THOMAS, MD
Director
Toxic Hazards Division
Aerospace Medical Research Laboratory

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produced activity without activation. No significant in vivo responses were obtained. If the test compound produces only gene mutation and primary DNA damage in mammalian cells it might be expected to be negative in the dominant lethal studies.

PREFACE

This research was initiated by the Toxicology Branch, Toxic Hazards Division, Aerospace Medical Research Laboratory. Experiments were performed under Contract F33615-77-C-0518 by Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20795.

The experiments were conducted by David J. Brusick, Ph.D., and Dale W. Matheson, Ph.D., of Litton Bionetics, Inc., Kensington, Maryland 20795. Kenneth C. Back, Ph.D., was contract monitor for the Aerospace Medical Research Laboratory.

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GENERAL INTERPRETATION AND CONCLUSIONS

INTRODUCTION

The material evaluated in this study was subjected to a matrix of in vitro assays employing microbial cells, mammalian cells in culture and in vivo tests measuring potential germ cell effects in mice and rats.

This battery of tests is capable of detecting specific locus gene mutations, nonspecific DNA damage and chromosome aberration (as indicated by dominant lethality). The dosing regimens included acute and subchronic exposures and the in vivo nature of some of the tests permits parameters of pharmacodynamics to be considered.

The analysis of the data is made on a matrix consideration using the entire spectra of responses to formulate the evaluation. A single set of data might indicate activity but the significance of the results will be interpreted as part of the total matrix. If all other data are negative the impact of the positive response will be reduced.

Conversely, if all tests show positive effects, the application of this broad-based response to estimation of potential human risk may be made with greater confidence.

The interpretations of data outlined in this section are based primarily on criteria developed for each assay system. The criteria are described in the experimental sections of this report.

Genetic activity is a property of chemicals which in most cases also indicates carcinogenic activity. Genetic activity cannot be used as a definitive assessment of carcinogenic risk for mammals but can be used to identify chemicals with a high probability of having carcinogenic activity.

INTERPRETATION OF RESULTS

Microbial Assay

The results from the Ames Salmonella/microsome assay on 1-Methylaminoanthraquinone (MAA) were negative. All test data were within the control range for each strain.

Mouse Lymphoma Assay

MAA was mutagenic in this assay at concentrations of 200 µg/ml to 500 µg/ml. The test material was highly cytotoxic in this concentration range but sufficient data at survival values greater than 10% established mutagenic activity. The activity in activation (+S9) tests was clearly greater than nonactivation results.

Unscheduled DNA Synthesis Assay

MAA was active in this assay at concentrations of 50 µg/ml to 100 µg/ml. Similar to the results with the mouse lymphoma cells, the compound was quite cytotoxic and produced the strongest activity in activation tests. The results on page 21 show the narrow dose range for activity.

Dominant Lethal Assays

Mouse--Mouse test results for MAA were negative indicating no genetic activity by the test material at 0.2 g/kg, 0.67 g/kg and 2.0 g/kg dose levels.

Rat--The test results were considered negative even though there were significant data points at weeks 4 and 8. Although these data points cannot be disregarded entirely, they appear to be primarily the result of low negative control values rather than genetic activity.

CONCLUSIONS

MAA demonstrated significant genetic activity in two cell culture assays that measure gene mutation and DNA repair. Both assays responded to the chemical under activation test conditions and in one, MAA produced activity without activation. No significant in vivo responses were obtained. If MAA produces only gene mutation and primary DNA damage in mammalian cells, the compound might be expected to be negative in the dominant lethal studies.

PART I
MICROBIAL ASSAY

EVALUATION SUMMARY

The test material, 1-Methylaminoanthraquinone, did not demonstrate mutagenic activity in any of the assays conducted in this evaluation and was considered not mutagenic under these test conditions.

FINAL REPORT
MUTAGENICITY PLATE ASSAY

OBJECTIVE

The objective of this study was to evaluate the test compound, 1-methylamino-anthraquinone, for genetic activity in microbial assays with and without the addition of mammalian metabolic activation preparations.

MATERIALS

Test Compound

The test compound was received on August 30, 1977. The compound was a red powder.

Indicator Microorganisms

The indicator organisms used were:

- Salmonella typhimurium strains
TA-1535 TA-98
TA-1537 TA-100
TA-1538

- Saccharomyces cerevisiae strain
D4

Activation System*

The reaction mixture used for this test was composed of the following:

Component	Final concentration/ml
TPN	4 μ moles
Glucose-6-phosphate	5 μ moles
Sodium phosphate (dibasic)	100 μ moles
MgCl ₂	8 μ moles
KCl	33 μ moles
Homogenate fraction	0.1-0.15 ml 9,000 x g supernatant of rat liver

The 9,000 x g supernatant was prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 5 days prior to kill.

*Ames et al., Mutation Research, 31:347, 1975.

Positive Control Chemicals

The following table lists the chemicals used for positive controls in the nonactivation and activation assays.

Assay	Chemical ^a	Solvent	Probable mutagenic specificity
Nonactivation	Methylnitrosoguanidine (MNG)	Water or saline	BPS ^b
	2-Nitrofluorene (NF)	Dimethylsulfoxide ^c	FS ^b
	Quinacrine mustard (QM)	Water or saline	FS ^b
Activation	2-Anthramine (ANTH)	Dimethylsulfoxide ^c	BPS ^b
	2-Acetylaminofluorene (AAF)	Dimethylsulfoxide ^c	FS ^b
	8-Aminoquinoline (AMQ)	Dimethylsulfoxide ^c	FS ^b

^aConcentrations given in Results section.

^bBPS = base-pair substitution. FS = frameshift.

^cPreviously shown to be nonmutagenic.

Solvent

Either deionized water or dimethylsulfoxide (DMSO) was used to prepare stock solutions of solid materials. All dilutions of test materials were made in either deionized water or DMSO. The solvent employed and its concentration are recorded in the Results section.

EXPERIMENTAL DESIGN

Plate Test (Overlay Method*)

Approximately 10^8 cells from an overnight culture of each indicator strain were added to separate test tubes containing 2.0 ml of molten agar supplemented with biotin and a trace of histidine. For nonactivation tests, at least four 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, a minimum of four different concentrations of the test chemical was added to the appropriate tubes with cells. Just prior to

*Certain classes of chemicals known to be mutagens and carcinogens do not produce detectable responses using the standard Ames overlay 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.

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 hours at 37°C and scored for the number of colonies growing on each plate. The concentrations of all chemicals are given in the Results section. Positive and solvent controls using both directly active positive chemicals and those that require metabolic activation were run with each assay.

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 per plate for each indicator strain employed in the assay. The positive and the solvent controls are provided as reference points. Other relevant data are provided on the computer printout.

EXPLANATION OF EVALUATION PROCEDURES FOR PLATE ASSAYS

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 to 3 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 to 3 days.

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 plate. At high concentrations the surviving population is usually reduced by some fraction. Our protocol normally employs several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

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 selecting 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

SUMMARY OF PLATE TEST RESULTS

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: 1-METHYLAHINO ANTHRAQUINONE
 B. SOLVENT: DMSO
 C. TEST INITIATION DATE: NOV. 18, 1977
 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	D4*		
NONACTIVATION			1	2	1	2	1	2	1	2
SOLVENT CONTROL	---	---	33	20	28	29	42	117	68	
POSITIVE CONTROL**	---	---	>1000	633	>1000	>1000	>1000	>1000	766	
TEST COMPOUND										
0.100000 UG	---	---	14	16	18	46	114	49		
1.000000 UG	---	---	24	11	25	53	125	71		
10.000000 UG	---	---	22	26	19	49	116	62		
100.000000 UG	---	---	16	16	20	38	43	43		
500.000000 UG	---	---	17	20	19	93	51	50		
1000.000000 UG	---	---				44				

ACTIVATION

SOLVENT CONTROL	POSITIVE CONTROL***	TEST COMPOUND	RAT	RAT	LIVER	LIVER	25	288	16.	270	14	703	36	795	122	>1000	188	282
0.100000 UG	RAT	RAT	25	25	LIVER	LIVER	25	24	23	22	13	20	62	50	158	122	71	
1.000000 UG	RAT	RAT	30	30	LIVER	LIVER	30	30	20	20	19	19	48	48	136	136	111	
10.000000 UG	RAT	RAT	30	30	LIVER	LIVER	30	30	14	14	14	14	40	40	134	134	204	
500.000000 UG	RAT	RAT	29	29	LIVER	LIVER	29	29	19	19	12	12	41	41	104	104	101	

* TRY+ CONVERTANTS PER PLATE

** TA-1535 MNING 10 UG/PLATE
 TA-1537 QM 10 UG/PLATE
 TA-1538 NF 100 UG/PLATE
 TA-98 NF 100 UG/PLATE
 TA-100 MNING 10 UG/PLATE
 D4 MNING 10 UG/PLATE
 SOLVENT DMSO 50 UL/PLATE

*** TA-1535 ANTH 100 UG/PLATE
 TA-1537 AMQ 100 UG/PLATE
 TA-1538 AAF 100 UG/PLATE
 TA-98 AAF 100 UG/PLATE
 TA-100 ANTH 100 UG/PLATE
 D4 DMNA 100 MICROMOLES/PLATE
 SOLVENT DMSO 50 UL/PLATE

range selected. 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.

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.

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.

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.

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 two to three times the solvent control value for TA-98 and D4 is considered to be mutagenic. For these strains the dose response increase should start at approximately the solvent control value.

Pattern--Because TA-1535 and TA-100 were both derived from the same parental strain (G-46) and because TA-1538 and TA-98 were 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.

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.

Relationship Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/microsome 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 of 300 chemicals by McCann et al.* show an extremely good correlation between results of microbial mutagenesis tests and in vivo rodent carcinogenesis assays.

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

*McCann et al., Proc. Nat. Acad. Sci., USA, 72:5135-5139, 1975.

PART II
MOUSE LYMPHOMA ASSAY

EVALUATION SUMMARY

The test material, 1-Methylaminoanthraquinone, was mutagenic at the TK gene in L5178Y mouse lymphoma cells. The results from both nonactivation and activation tests were positive but greater activity was observed in the activation test.

FINAL REPORT

L5178Y MOUSE LYMPHOMA MUTAGENICITY ASSAY

OBJECTIVE

The objective of this study was to evaluate 1-Methylaminoanthraquinone for specific locus forward mutation induction in the L5178Y thymidine kinase (TK) mouse lymphoma cell assay.

MATERIALS

Test Compound

The test compound was received on August 30, 1977. The compound was a red powder.

Indicator Cells

The Fischer mouse lymphoma cell line used in this study was derived from L5178Y. The cells are heterozygous for a specific autosomal mutation at the TK locus and are bromodeoxyuridine (BUdR) sensitive. Scoring for mutation was based on selecting cells that have undergone forward mutation from a TK+/- to a TK-/- genotype by cloning them in soft agar with BUdR.

Media

The cells were maintained in Fischer's Medium for Leukemic Cells of Mice with 10% horse serum and sodium pyruvate. Cloning medium consisted of Fischer's Medium with 20% horse serum, sodium pyruvate and 0.37% agar. Selection medium was made from cloning medium by the addition of 5.0 mg of BUdR to 100 ml of cloning medium.

Control Compounds

Negative Control--The solvent in which the test compound was dissolved was used as a negative control and is designated as solvent control in the data table. The actual solvent is listed in the Results section.

Positive Controls--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 μ l/ml.

Dimethylnitrosamine (DMN), which requires metabolic biotransformation by microsomal enzymes, was used as a positive control substance for the activation studies at a final concentration of 0.5 μ l/ml.

EXPERIMENTAL DESIGN

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 4-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. Toxicity produced by chemical treatment was monitored during the experiment.

Assays

Nonactivation Assay--The procedure used is a modification of that reported by Clive and Spector.* Prior to each treatment, cells were cleansed of spontaneous TK-/- by growing them in a medium containing thymidine, hypoxanthine, methotrexate and glycine (THMG). This medium permits the survival of only those cells that produce the enzyme thymidine kinase and can therefore utilize the exogenous thymidine from the medium. The test compound was added to the cleansed cells in growth medium at the predetermined doses for 4 hours. The mutagenized cells were washed, fed and allowed to express in growth medium for 3 days. At the end of this expression period, TK-/- mutants were detected by cloning the cells in the selection medium for 10 days. Surviving cell populations were determined by plating diluted aliquots in nonselective growth medium.

Activation Assay--The activation assay differs from the nonactivation assay in the following manner only. Two milliliters of the reaction mixture were added to 10 ml of growth medium. The desired number of cleansed cells was added to this mixture and the flask was incubated on a rotary shaker for 4 hours. The incubation period was terminated by washing the cells twice with growth medium. The washed mutagenized cells were then allowed to express for 3 days and were cloned as indicated for the nonactivated cells.

Preparation of 9,000 x g Supernatant

Male random bred mice were killed by cranial blow, decapitated and bled. The liver was immediately dissected from the animal using aseptic technique and placed in ice-cold 0.25M sucrose buffered with Tris buffer at a pH of 7.4. When an adequate number of livers had been collected they were washed twice with fresh buffered sucrose and completely homogenized. The homogenate was centrifuged for 20 minutes at 9,000 x g in a refrigerated centrifuge. The supernatant from this centrifuged sample was retained and frozen at -80°C until used in the activation system. This microsome preparation was added to a "core" reaction mixture to form the activation system described below:

Component	Final concentration/ml
TPN (sodium salt)	6 µmoles
Isocitric acid	35 µmoles
Tris buffer, pH 7.4	28 µmoles
MgCl ₂	2 µmoles
Homogenate fraction	100 µliters

*Clive and Spector, Mutation Research, 31:17-29, 1975.

Screening

A mutation index was derived by dividing the number of clones formed in the BUdR-containing selection medium by the number found in the same medium without BUdR. The ratio was then compared to that obtained from other dose levels and from positive and negative controls. Colonies were counted on an electronic colony counter that resolves all colonies greater than 200 microns in diameter.

RESULTS

The data presented in the following table show the concentrations of the test compound employed, the number of mutant clones obtained, the surviving populations after the expression period and the calculated mutation frequencies.

CRITERIA USED IN THE EVALUATION

Several criteria have been established which, if met, provide a basis for declaring a material genetically active in the mouse lymphoma 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 evaluation since absolute criteria may not be applicable to all biological data.

A compound is considered mutagenic in the mouse lymphoma assay if:

- A dose response relationship is observed over three of the four dose levels employed.
- The minimum increase at the high level of the dose response curve is at least 2.5 times greater than the solvent control value.
- The solvent control data are within the normal range of the spontaneous background for the TK locus.

All evaluations of mutagenic activity are based upon the concurrent solvent control value run with the experiment in question. Positive control values are not used as reference points but are included to ensure 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.

SUMMARY OF MOUSE LYMPHOMA RESULTS

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: 1-Methylaminoanthraquinone

B. SOLVENT: DMSO

C. TEST DATE: 12/27/77

NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) OR MILLIGRAMS (ML) PER MILLILITER.

TEST	S-9	SOURCE TISSUE	DAILY COUNTS			TOTAL 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	---	---	16.5	17.0	8.3	100.0	32.0	255.0	100.0	100.0	12.5
NEGATIVE CONTROL	---	---	14.0	17.9	7.1	76.4	33.0	280.0	109.8	83.9	11.8
EMS .5UL/ML	---	---	11.2	13.6	6.0	39.3	361.0	122.0	47.8	18.8	295.9
TEST COMPOUND	---	---	10.2	11.0	7.6	36.6	65.0	239.0	93.7	34.3	27.2
0.10000 MG/ML	---	---	7.0	14.0	9.0	37.9	68.0	135.0	52.9	20.1	50.4
0.20000 MG/ML	---	---	7.2	9.4	5.2	15.1	59.0	182.0	71.4	10.8	32.4
0.25000 MG/ML	---	---	7.2	6.4	5.4	10.7	63.0	121.0	47.5	5.1	52.1
0.38000 MG/ML	---	---	4.2	4.6	5.0	4.1	35.0	72.0	28.2	1.2	48.6

ACTIVATION

SOLVENT CONTROL	MOUSE LIVER	12.0	14.6	7.9	100.0	30.0	297.5	100.0	100.0	10.1
NEGATIVE CONTROL	MOUSE LIVER	16.2	15.9	8.0	148.9	29.0	255.0	85.7	127.6	11.4
DMN .5UL/ML	MOUSE LIVER	8.8	11.0	4.4	30.8	172.0	70.0	23.5	7.2	245.7
TEST COMPOUND	MOUSE LIVER	8.4	12.8	4.6	35.7	119.0	173.0	58.2	20.8	68.8
0.05000 MG/ML	MOUSE LIVER	5.2	12.4	3.4	15.8	81.0	63.0	21.2	3.4	128.6
0.20000 MG/ML	MOUSE LIVER	6.6	7.4	4.0	14.1	52.0	47.0	15.8	2.2	110.6
0.25000 MG/ML	MOUSE LIVER	6.2	5.8	6.0	15.6	69.0	43.0	14.5	2.3	160.5
0.38000 MG/ML	MOUSE LIVER	5.0	7.8	4.4	12.4	41.0	64.0	21.5	2.7	64.1

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

** (MUTANT CLONES / VIABLE CLONES) X 10E-6

PART III

UNSCHEDULED DNA SYNTHESIS IN WI-38 CELLS

EVALUATION SUMMARY

The test material, 1-Methylaminoanthraquinone, was active in the unscheduled DNA synthesis (UDS) assay and increased the incorporation of ^3H -thymidine in WI-38 cells. The activity was only evident in the activation (+S9 mix) test. The test material was highly cytotoxic for all dose levels in the nonactivation test and for the two highest concentrations in the activation study.

FINAL REPORT

UNSCHEDULED DNA SYNTHESIS IN WI-38 CELLS

OBJECTIVE

The objective of this study was to evaluate the test chemical, 1-Methylamino-anthraquinone, for its ability to induce unscheduled DNA synthesis (UDS) in human diploid WI-38 cells blocked in G₁ phase.

MATERIALS

Test Compound

The test compound was received on August 30, 1977. The compound was a red powder.

Indicator Cells

Diploid WI-38 cells derived from human embryonic lung were used in this assay.

Media

Growth medium (GM) consisted of Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (PS).

Maintenance medium (SM) consisted of EMEM supplemented with 0.5% FCS and PS.

Hydroxyurea medium (HUM) consisted of SM plus hydroxyurea to a final concentration of 10⁻²M.

Control Compounds

Negative Control--The material used as the solvent for the test chemical was used as the negative control. The solvent is listed in the Results section. The volume of solvent in the negative control test will equal the total solvent added in the high dose for the test chemical.

Positive Controls--N-methylnitrosoguanidine (MNNG) at a concentration of 5 µg/ml was used as the positive control agent in nonactivation tests. The positive control agent in activation tests was 3,4-benzo(α)pyrene (BαP) at a concentration of 10 µg/ml.

EXPERIMENTAL DESIGN

Cell Preparation

Normal human diploid WI-38 cells were seeded at 2.5 x 10⁵ cells in a 60 mm tissue culture dish and grown to confluency in GM. Once reaching confluency the cells were switched to SM for 5 days. The contact inhibition imposed by confluency and the use of SM held the cells in a nonproliferating state.

Treatment

On the day of treatment, cells held in G₁ phase were placed in HUM. After 30 minutes this medium was replaced by 2 ml of HUM containing the control or test chemical and 1.0 μ Ci of ³H-TdR. Each treatment was at three concentrations. Exposure was terminated after 1.5 hours by washing the cells twice in cold balanced salt solution (BSS) containing an excess of cold thymidine. The test concentrations were selected from a large series of trial concentrations and covered toxic and nontoxic dose ranges.

DNA Extraction and Measurement of ³H-TdR Incorporation

Treated plates were frozen at -20°C until processed. After thawing, the cells on the 60-mm plate were covered with 2.5% sodium dodecyl sulfate (SDS) in 1 x (SSC) (0.15M NaCl - 0.015M Na citrate) and scraped from the dish with a rubber policeman. The cells were washed and precipitated from the SDS by three changes of 95% ethanol and centrifuged at 10,000 x g. Additional lipid components were removed by extraction in ethanol ether at 70°C. This pellet was washed in 70% ethanol, further incubated at 70°C in 0.3N NaOH and the DNA extracted in 50 μ l 1N perchloric acid (PCA) at 70°C. The DNA was separated into two 25 μ l aliquots. One of these was dissolved in 10 ml of hydromix scintillation cocktail (Yorktown Company) and counted in a Beckman liquid scintillation spectrometer. The second aliquot was added to 275 μ l of 1N PCA and read at 260 nm in a Gilford spectrophotometer. The values were corrected for light scatter and converted to micrograms of DNA. Following liquid scintillation counting the data were combined with the DNA extraction values and expressed as disintegrations per minute (DPM) per microgram of DNA (DPM/ μ g DNA).

Activation System

Because metabolic activation is essential for the expression of biological activity in some chemicals, a mouse liver activation system containing liver S9 was employed. The activation system consisted of the following:

Component	Final concentration/ml
TPN (sodium salt)	6 μ moles
Isocitric acid	35 μ moles
Tris buffer, pH 7.4	28 μ moles
MgCl ₂	2 μ moles
Homogenate fraction	100 μ liters

RESULTS

The results of the UDS assay in WI-38 cells are shown in the following tables.

SUMMARY OF UNSCHEDULED DNA SYNTHESIS IN WI-38 CELLS

Test Compound: 1-Methylaminoanthraquinone (MAA)
Solvent: DMSO

Assay No. 2298

Date of Test Initiation: March 16, 1978

Test	Compound concentration, $\mu\text{g}/\text{ml}$	DNA μg	DPM	DPM $\mu\text{g}/\text{DNA}$	Percent of control
Trial Nonactivation					
Solvent control	-	12.87	233.5	18.1	100
MNNG	5	13.86	543.5	39.2	217
Test compound, MAA	50	13.70	151.6	11.1	61
	100	12.54	99.7	7.9	44
	500	32.51	56.5	1.7	10
	1000	33.50	63.2	1.9	10
Activation					
Solvent control	-	11.72	54.2	4.6	100
Benzo(α)pyrene	10	12.21	110.6	9.1	197
Test compound, MAA	50	11.06	91.6	8.3	180
	100	11.88	153.2	12.9	280
	500	25.91	48.4	1.9	41
	1000	33.50	63.5	1.9	41

SUMMARY OF UNSCHEDULED DNA SYNTHESIS IN WI-38 CELLS

Test Compound: 1-Methylaminoanthraquinone (MAA)
Solvent: DMSO

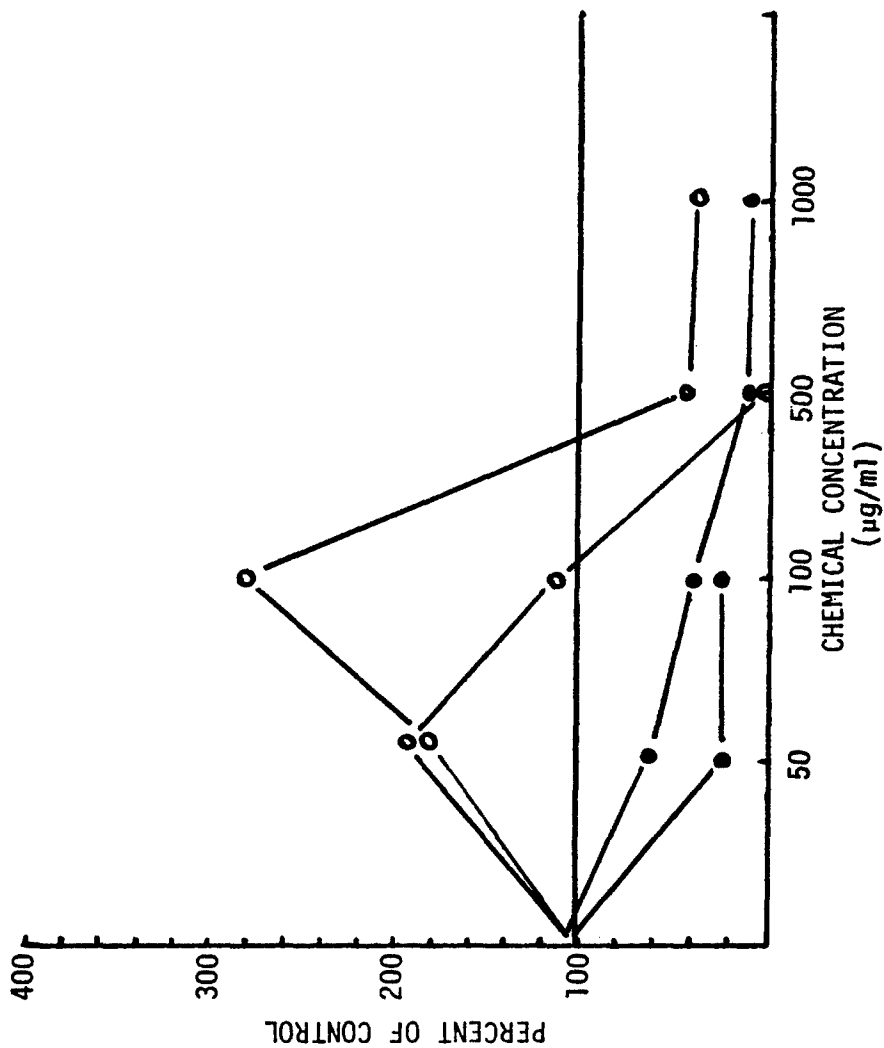
Assay No. 2298

Date of Test Initiation: February 2, 1978

Test	Compound concentration, $\mu\text{g/ml}$	DNA μg	DPM	DPM $\mu\text{g/DNA}$	Percent of control
Trial Nonactivation					
Solvent control	-	31.35	1826.5	58.3	100
MNNG	5	15.84	1955.5	123.5	212
Test compound, MAA	50	36.30	511.9	14.1	24
	100	29.70	481.3	16.2	28
	500	38.28	2.6	0.1	0.1
	1000	36.96	170.6	4.6	8
Activation					
Solvent control	-	32.34	1405.8	43.5	100
Benzo(α)pyrene	10	29.04	2562.6	88.2	203
Test compound, MAA	50	33.66	2882.3	85.6	197
	100	33.66	1602.9	47.6	109
	500	36.30	27.4	0.8	2.0
	1000	46.86	1.6	0.03	0.08

CRITERIA USED IN THE EVALUATION

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 an 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.



○ +S9 MIX
● -S9 MIX

Figure 1. INCORPORATION OF ³H-THYMIDINE IN WI-38 CELLS
TEST COMPOUND - 1-METHYLAMINOANTHRAQUINONE

A compound is considered active in the UDS assay if:

- A dose-response relationship is observed over two of the three dose levels employed.
- 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 at 5 µg/ml of MNNG was 206% of the control; for 10 µg/ml of benz(α)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 become more firmly established.

PART IV-A

MOUSE DOMINANT LETHAL ASSAY

EVALUATION SUMMARY

The test material, 1-Methylaminoanthraquinone, was not active in the mouse dominant lethal assay at 0.2 g/kg, 0.67 g/kg and 2.0 g/kg. The test data were all consistent with the range for the concurrent and historical negative control data for this mouse strain. The positive control was active at weeks 1 and 2 producing 39% and 64% dead implants, respectively. The only data from the test groups which appeared positive was the low dose at week 4. This appears to be an aberrant fluctuation since the intermediate and high dose levels at week 4 are back to the normal range.

FINAL REPORT

MUTAGENICITY EVALUATION OF MOUSE DOMINANT LETHAL ASSAY

OBJECTIVE

The objective of this study was to evaluate 1-Methylaminoanthraquinone for its ability to induce dominant lethality in mice.

MATERIALS

The test compound was received August 30, 1977. The compound was a red powder.

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 midpregnancy 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 was 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.

EXPERIMENTAL DESIGN

Ten random bred male mice from a closed colony were assigned to one of five groups. Three of these groups received different dose levels of the test compound, a fourth group received only the solvent and the fifth group received a known mutagen and served as the positive control group. The test compound and the solvent control were administered in the feed for 5 consecutive days. Triethylene melamine (TEM) was used as the positive control and was given as a single intraperitoneal injection 2 days before the animals were mated. Following treatment each male was rested for 2 days and then caged with two unexposed virgin females. At the end of 7 days these females were replaced with two new unexposed females. This weekly mating sequence was continued for 7 weeks. The mated females were transferred to a new cage and 14 days after the midweek of being caged with the male the females were killed with CO₂. At necropsy their uteri were examined for dead and living fetuses, resorption sites and total implantations.

Animals

Random bred male and female mice, strain CD-1, were purchased from The Charles River Breeding Laboratories (Portage, Michigan). Male and female mice were at least 8 weeks of age when purchased.

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 for 2 weeks prior to being used in the study to acclimate them to the new laboratory conditions. Purina Lab Chow was used as the basic diet and water was 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.

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 onto computer entry cards and analyzed for statistical significance as outlined in the Appendix.

Compound Administration

The dose levels used in this evaluation were determined by performing a preliminary range finding study. The vehicle for this test was corn oil and the route of administration was oral. Based on the low toxicity indicated by the range finding evaluation, dose levels employed were 2.0 g/kg, 0.67 g/kg and 0.2 g/kg. The negative control animals received 0.5 ml/mouse/day, this volume being equal to the largest volume of compound administered. Positive control animals received TEM at 0.5 mg/kg intraperitoneally acute in a vehicle of 0.1 ml of 0.85% saline.

Male numbers	Treatment	Dose, g/kg	Route	Total vol. admin., ml/mouse/day
31-40	MAA	0.2	PO	0.1
41-50	MAA	0.67	IP	0.2
51-60	MAA	2.0	PO	0.5
151-160	(NC) Corn oil	-	PO	0.5
171-180	(PC) TEM	0.5 mg	PO	0.1

IP = intraperitoneal

PO = per os

NC = negative control

PC = positive control

RESULTS

The results are presented in the following tables.

DOMINANT LETHALITY EVALUATION CRITERIA

Both pre- and postimplantation 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, preimplantation loss is not as rigorous an indication of dominant lethality as postimplantation loss. Corpora lutea cannot be reliably counted in mice and, therefore, preimplantation loss is not evaluated in studies using mice. Postimplantation losses are measured as early and late fetal deaths plus the number of resorption sites.

Dominant lethality is typically determined from: 1) A mutation index derived from the ratio of dead to total implants; or 2) the number of dead implants per pregnant female. In interpreting these values it must be remembered that the former measurement reflects both pre- and postimplantation 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 postimplantation 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 upon 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 upon the data gathered an estimate of dominant lethality and of risk to the gene pool should be obtainable.

FERTILITY INDEX

WEEK	COMPOUND: 1-METHYLAMINOANTHRAQUINONE		STUDY: SUBCHRONIC		SPECIES: MICE		LONG DOSE	ARITH DOSE
	HIST. NEG. CONT.	NEG. CONTROL	PUS. CONTROL	0.2000 GM/KG	0.6700 GM/KG	2.0000 GM/KG		
1	8/20 = 0.40	2/20 = 0.10	8/20 = 0.40	7/20 = 0.35	12/20 = 0.60			
2	13/20 = 0.65	2/20 = 0.10**	11/20 = 0.55	8/20 = 0.40	15/20 = 0.75			
3	10/20 = 0.50	9/20 = 0.45	10/20 = 0.50	10/20 = 0.50	13/20 = 0.65			
4	11/20 = 0.55	7/20 = 0.35	5/20 = 0.25	5/20 = 0.25	14/20 = 0.70			\$
5	10/20 = 0.50	10/20 = 0.50	6/20 = 0.30	12/20 = 0.60	11/20 = 0.55			
6	12/20 = 0.60	8/20 = 0.40	11/20 = 0.55	11/20 = 0.55	12/20 = 0.60			
7	10/20 = 0.50	10/20 = 0.50	9/20 = 0.45	14/20 = 0.70	10/20 = 0.50			

NOTE: THE SYMBOL * DENOTES ANY SIGNIFICANT DIFFERENCE AT THE DOSE LEVELS COMPARED WITH THE NEGATIVE CONTROL. THE TWO COLUMNS IDENTIFIED AS LGD DOSE AND ARITHMETIC DOSE ARE USED TO NOTE ANY SIGNIFICANT DEVIATION OF THE SLOPE OF THE REGRESSION LINE FROM ZERO. SIGNIFICANCE IN THE LGD 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.

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

WEEK	HIST. MEG. CONT.		NEG. CONTROL		POS. CONTROL		STUDY: SUBCHRONIC			SPECIES: MICE	
							0.2000 GM/KG	0.6700 GM/KG	2.0000 GM/KG	LOG DOSE	ARITH DOSE
1	90/ 8 = 11.25	18/ 2 = 9.00	75/ 8 = 9.38	69/ 7 = 9.86	132/ 12 = 11.00						
2	149/ 13 = 11.46	11/ 2 = 5.50*	118/ 11 = 10.73	85/ 8 = 10.63	161/ 15 = 10.73						
3	114/ 10 = 11.40	97/ 9 = 10.78	108/ 10 = 10.80	109/ 10 = 10.90	144/ 13 = 11.08						
4	119/ 11 = 10.82	74/ 7 = 10.57	54/ 5 = 10.80	57/ 5 = 11.40	138/ 14 = 9.86						
5	104/ 10 = 10.40	107/ 10 = 10.70	61/ 6 = 10.17	127/ 12 = 10.58	118/ 11 = 10.73						
6	138/ 12 = 11.50	69/ 8 = 8.63*	114/ 11 = 10.36	140/ 11 = 12.73	133/ 12 = 11.08						
7	114/ 10 = 11.40	105/ 10 = 10.50	97/ 9 = 10.78	143/ 14 = 10.21	113/ 10 = 11.30						

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.

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE

WEEK	NEG. CONTROL		POS. CONTROL		0.2000 GM/KG		0.6700 GM/KG		2.0000 GM/KG		LUG DOSE	AKITH DOSE
	HIST.	CONT.	HIST.	CONT.	HIST.	CONT.	HIST.	CONT.	HIST.	CONT.		
1	4/ 8 = 0.50	7/ 2 = 3.50**	17/ 8 = 2.13	8/ 7 = 1.14	6/ 12 = 0.50							
2	17/ 13 = 1.31	7/ 2 = 3.50	3/ 11 = 0.27	5/ 8 = 0.63	30/ 15 = 2.00							
3	9/ 10 = 0.90	23/ 9 = 2.56	6/ 10 = 0.60	17/ 10 = 1.70	20/ 13 = 1.54							
4	15/ 11 = 1.36	17/ 7 = 2.43	20/ 5 = 4.00	5/ 5 = 1.00	21/ 14 = 1.50							
5	5/ 10 = 0.50	29/ 10 = 2.90	8/ 6 = 1.33	8/ 12 = 0.67	18/ 11 = 1.64							
6	7/ 12 = 0.58	1/ 8 = 0.13	6/ 11 = 0.55	6/ 11 = 0.55	4/ 12 = 0.33							
7	6/ 10 = 0.60	8/ 10 = 0.80	4/ 9 = 0.44	9/ 14 = 0.64	5/ 10 = 0.50							

COMPOUND: 1-METHYLAMINOANTHRACINONE STUDY: SUBCHRONIC SPECIES: MICE

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.

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS

WEEK	HIST. NEG. CONT.		NEG. CONTROL		POS. CONTROL		STUDY: SUBCHRONIC		SPECIES: MICE		LOG DOSE	ARITH DOSE
	3/ 8 = 0.38	6/ 13 = 0.46	5/ 10 = 0.50	9/ 11 = 0.82	4/ 10 = 0.40	5/ 12 = 0.42	0.2000 GM/KG	0.6700 GM/KG	2.0000 GM/KG	5/ 12 = 0.42		
1			2/ 2 = 1.00	5/ 8 = 0.63	4/ 7 = 0.57	5/ 8 = 0.63						
2			2/ 2 = 1.00	2/ 11 = 0.18	5/ 8 = 0.63	8/ 15 = 0.53						
3			6/ 9 = 0.67	4/ 10 = 0.40	6/ 10 = 0.60	7/ 13 = 0.54						
4			4/ 7 = 0.57	3/ 5 = 0.60	2/ 5 = 0.40	8/ 14 = 0.57						
5			4/ 10 = 0.40	1/ 6 = 0.17	5/ 12 = 0.42	7/ 11 = 0.64						
6			5/ 12 = 0.42	4/ 11 = 0.36	5/ 11 = 0.45	4/ 12 = 0.33						
7			4/ 10 = 0.40	4/ 9 = 0.44	6/ 14 = 0.43	4/ 10 = 0.40						

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.

PROPORTION OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS

WEEK	COMPOUND: 1-METHYLAMINOANTHRAQUINONE		STUDY: SUBCHRONIC			SPECIES: MICE	
	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	0.2000 GM/KG	0.6700 GM/KG	2.0000 GM/KG	LOG DOSE
1	1/ 8 = 0.13	2/ 2 = 1.00	2/ 8 = 0.25	1/ 7 = 0.14	1/ 12 = 0.08		
2	3/ 13 = 0.23	2/ 2 = 1.00	1/ 11 = 0.09	0/ 8 = 0.0	4/ 15 = 0.27		
3	2/ 10 = 0.20	6/ 9 = 0.67	1/ 10 = 0.10	3/ 10 = 0.30	4/ 13 = 0.31		
4	3/ 11 = 0.27	3/ 7 = 0.43	2/ 5 = 0.40	2/ 5 = 0.40	5/ 14 = 0.36		
5	1/ 10 = 0.10	3/ 10 = 0.30	1/ 6 = 0.17	1/ 12 = 0.08	4/ 11 = 0.36		
6	2/ 12 = 0.17	0/ 8 = 0.0	2/ 11 = 0.18	1/ 11 = 0.09	0/ 12 = 0.0		
7	1/ 10 = 0.10	2/ 10 = 0.20	0/ 9 = 0.0	1/ 14 = 0.07	1/ 10 = 0.10		

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.

DEAD IMPLANTS / TOTAL IMPLANTS

WEEK	COMPOUND: 1-METHYLAMINOANTHRAQUINONE		STUDY: SUBCHRONIC		SPECIES: MICE		LOG DOSE	ARITH DOSE
	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	0.2000 GM/KG	0.6700 GM/KG	2.0000 GM/KG		
1	4/90 = 0.04	7/18 = 0.39**	17/75 = 0.23	8/69 = 0.12	6/132 = 0.05		\$	
2	17/149 = 0.11	7/11 = 0.64	3/118 = 0.03	5/85 = 0.06	30/161 = 0.19		\$	
3	9/114 = 0.08	23/97 = 0.24	6/108 = 0.06	17/109 = 0.16	20/144 = 0.14			
4	15/119 = 0.13	17/74 = 0.23	20/54 = 0.37	5/57 = 0.09	21/138 = 0.15		\$	
5	5/104 = 0.05	29/107 = 0.27	8/61 = 0.13	8/127 = 0.06	18/118 = 0.15		\$	
6	7/138 = 0.05	1/69 = 0.01	6/114 = 0.05	6/140 = 0.04	4/133 = 0.03			
7	6/114 = 0.05	8/105 = 0.08	4/97 = 0.04	9/143 = 0.06	5/113 = 0.04			

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.
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PART IV-B

RAT DOMINANT LETHAL ASSAY

EVALUATION SUMMARY

The test material, 1-Methylaminoanthraquinone (MAA), was evaluated for its ability to produce dominant lethality in rats at dose levels of 0.2 g/kg, 0.67 g/kg and 2.0 g/kg. The material was administered orally over 5 days. The results for MAA were considered negative. The data shown on page 46 indicate an increase in the dead implants/total implants over week 4. While the data appear significant at the two high dose levels, it was noted that the negative control was quite low and, therefore, most likely responsible for the significant effect. The same observation was made for the high dose of week 8.

FINAL REPORT

MUTAGENICITY EVALUATION OF RAT DOMINANT LETHAL ASSAY

OBJECTIVE

The objective of this study was to evaluate 1-Methylaminoanthraquinone for its ability to induce dominant lethality in rats.

MATERIALS

The test compound was received August 30, 1977. The compound was a red powder.

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 rats 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 rats 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 midpregnancy 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 was 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.

EXPERIMENTAL DESIGN

Ten random bred male rats from a closed colony were assigned to one of five groups. Three of these groups received different dose levels of the test compound, a fourth group received only the solvent and the fifth group received a known mutagen and served as the positive control group. The test compound and the solvent control were administered orally by gavage for 5 consecutive days. Triethylene melamine (TEM) was used as the positive control and was given as a single intraperitoneal injection 2 days before the animals were mated. Following treatment each male was rested for 2 days and then caged with two unexposed virgin females. At the end of 7 days these females were replaced with two new unexposed females. This weekly mating sequence was continued for 7 weeks. The mated females were transferred to a new cage and 14 days after the midweek of being caged with the male the females were killed with CO₂. At necropsy their uteri were examined for dead and living fetuses, resorption sites and total implantations.

Animals

Random bred male and female rats, strain CRL:COBS CD(SD)Br, were purchased from The Charles River Breeding Laboratories (Portage, Michigan). Male and female rats were at least 10 weeks of age when purchased.

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 for 2 weeks prior to being used in the study to acclimate them to the new laboratory conditions. Purina Rat Chow was used as the basic diet and water was 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.

Records

The number of corpora lutea, dead and living fetuses, resorption sites and total implantation sites were recorded on a standardized record form. Data were keypunched directly from these forms onto computer entry cards and analyzed for statistical significance as outlined in the Appendix.

Compound Administration

The dose levels used in this evaluation were determined by performing a preliminary range-finding study. The vehicle for this test was corn oil and the route of administration was oral. Based on the low toxicity indicated by the range finding evaluation, dose levels employed were 2.0 g/kg, 0.67 g/kg and 0.2 g/kg. The negative control animals received 1.0 ml/rat/day, this volume being equal to the largest volume of compound administered. Positive control animals received TEM at 0.5 mg/kg intraperitoneally acute in a vehicle of 0.1 ml/rat of 0.85% saline.

Male numbers	Treatment	Dose, g/kg	Route	Total vol. admin., ml/rat/day
1-10	MAA	0.2	PO	0.2
11-10	MAA	0.67	PO	0.4
21-30	MAA	2.0	PO	1.0
61-70	(NC) Corn oil	-	PO	1.0
71-80	(PC) TEM	0.5 mg/kg	IP	0.1

IP = intraperitoneal

PO = per os

NC = negative control

PC = positive control

RESULTS

The results are presented in the following tables.

DOMINANT LETHALITY EVALUATION CRITERIA

Both pre- and postimplantation 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, preimplantation loss is not as rigorous an indication of dominant lethality as postimplantation loss. Postimplantation losses are measured as early and late fetal deaths plus the number of resorption sites.

Dominant lethality is typically determined from: 1) A mutation index derived from the ratio of dead to total implants; or 2) the number of dead implants per pregnant female. In interpreting these values it must be remembered that the former measurement reflects both pre- and postimplantation 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 postimplantation 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 upon 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 upon the data gathered, an estimate of dominant lethality and of risk to the gene pool should be obtainable.

FERTILITY INDEX

WEEK	COMPOUND: 1-METHYLAMINOANTHRAQUINONE		STUDY: SUBCHRONIC		SPECIES: RATS	
	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	0.2000 G/M/KG	0.6700 G/KG	2.0000 G/M/KG
1	201/ 519 = 0.39	12/ 20 = 0.60	4/ 20 = 0.20*	9/ 20 = 0.45	12/ 20 = 0.60	11/ 20 = 0.55
2	230/ 520 = 0.44	13/ 20 = 0.65	7/ 20 = 0.35	10/ 20 = 0.50	12/ 20 = 0.60	13/ 20 = 0.65
3	260/ 520 = 0.50	11/ 20 = 0.55	5/ 20 = 0.25	8/ 20 = 0.40	11/ 20 = 0.55	11/ 20 = 0.55
4	308/ 520 = 0.59	9/ 20 = 0.45	2/ 20 = 0.10*	10/ 20 = 0.50	10/ 20 = 0.50	8/ 20 = 0.40
5	310/ 520 = 0.60	13/ 20 = 0.65	9/ 20 = 0.45	10/ 20 = 0.50	12/ 20 = 0.60	9/ 20 = 0.45
6	304/ 520 = 0.58	9/ 20 = 0.45	10/ 20 = 0.50	16/ 20 = 0.80*	12/ 20 = 0.60	10/ 20 = 0.50
7	306/ 480 = 0.64	12/ 20 = 0.60	9/ 20 = 0.45	9/ 20 = 0.45	8/ 20 = 0.40	12/ 20 = 0.60

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.

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

WEEK	COMPOUND: 1-METHYLAMINOANTHRAQUINONE		STUDY: SUBCHRONIC		SPECIES: RATS	
	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	0.2000 GM/KG	0.6700 G/KG	2.0000 GM/KG
1	2313/ 201 = 11.51	87/ 12 = 7.25	39/ 4 = 9.75	102/ 9 = 11.33*	116/ 12 = 9.67	110/ 11 = 10.00
2	2568/ 230 = 11.17	143/ 13 = 11.00	43/ 7 = 6.14*	112/ 10 = 11.20	150/ 12 = 12.50	116/ 13 = 8.92
3	3039/ 260 = 11.69	112/ 11 = 10.18	38/ 5 = 7.60	71/ 8 = 8.88	118/ 11 = 10.73	107/ 11 = 9.73
4	3943/ 308 = 12.80	89/ 9 = 9.89	19/ 2 = 9.50	89/ 10 = 8.90	50/ 10 = 5.00	91/ 8 = 11.38
5	3915/ 310 = 12.63	111/ 13 = 8.54	73/ 9 = 8.11	60/ 10 = 6.00	107/ 12 = 8.92	49/ 9 = 5.44
6	3571/ 304 = 11.75	65/ 9 = 7.22	74/ 10 = 7.40	129/ 16 = 8.06	120/ 12 = 10.00	48/ 10 = 4.80
7	3803/ 306 = 12.43	59/ 12 = 4.92	93/ 9 = 10.33*	96/ 9 = 10.67*	53/ 8 = 6.63	99/ 12 = 8.25

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AVERAGE CORPORA LUTEA PER PREGNANT FEMALE

WEEK	COMPOUND: 1-METHYLAMINOANTHRAQUINONE		STUDY: SUBCHRONIC		SPECIES: RATS		LOG DOSE	ARITH DOSE
	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	0.2000 GM/KG	0.6700 G/KG	2.0000 GM/KG		
1	3023/ 201 = 15.04	201/ 12 = 16.75	62/ 4 = 15.50	131/ 9 = 14.56	158/ 12 = 13.17**	153/ 11 = 13.91*		
2	3407/ 230 = 14.81	189/ 13 = 14.54	83/ 7 = 11.86	158/ 10 = 15.80	183/ 12 = 15.25	204/ 13 = 15.69		
3	3947/ 260 = 15.18	161/ 11 = 14.64	65/ 5 = 13.00	108/ 8 = 13.50	158/ 11 = 14.36	151/ 11 = 13.73		
4	4827/ 308 = 15.67	125/ 9 = 13.89	37/ 2 = 18.50	134/ 10 = 13.40	115/ 10 = 11.50	129/ 8 = 16.13		
5	5058/ 310 = 16.32	193/ 13 = 14.85	117/ 9 = 13.00	140/ 10 = 14.00	191/ 12 = 15.92	152/ 9 = 16.89		
6	4631/ 304 = 15.23	150/ 9 = 16.67	151/ 10 = 15.10	191/ 16 = 11.94**	176/ 12 = 14.67	125/ 10 = 12.50**		
7	4700/ 306 = 15.36	174/ 12 = 14.50	140/ 9 = 15.56	134/ 9 = 14.89	107/ 8 = 13.38	174/ 12 = 14.50		

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AVERAGE PREIMPLANTATION LOSSES PER PREGNANT FEMALE

COMPOUND: 1-METHYLANHINOANTHRAQUINONE STUDY: SUBCHRONIC SPECIES: RATS

WEEK	HIST.	NEG. CONT.	NEG. CONTROL	POS. CONTROL	0.2000 GM/KG	0.6700 G/KG	2.0000 GM/KG	LOG DOSE	ARITH DOSE
1	710/ 201 =	3.53	114/ 12 = 9.50	23/ 4 = 5.75	29/ 9 = 3.22**	42/ 12 = 3.50*	43/ 11 = 3.91*		
2	839/ 230 =	3.65	46/ 13 = 3.54	40/ 7 = 5.71	46/ 10 = 4.60	33/ 12 = 2.75	88/ 13 = 6.77		\$
3	908/ 260 =	3.49	49/ 11 = 4.45	27/ 5 = 5.40	37/ 8 = 4.63	40/ 11 = 3.64	44/ 11 = 4.00		
4	884/ 308 =	2.87	36/ 9 = 4.00	18/ 2 = 9.00	45/ 10 = 4.50	65/ 10 = 6.50	38/ 8 = 4.75		
5	1143/ 310 =	3.69	82/ 13 = 6.31	44/ 9 = 4.89	80/ 10 = 8.00	84/ 12 = 7.00	103/ 9 = 11.44		\$
6	1060/ 304 =	3.49	85/ 9 = 9.44	77/ 10 = 7.70	62/ 16 = 3.88	56/ 12 = 4.67	77/ 10 = 7.70		\$
7	897/ 306 =	2.93	115/ 12 = 9.58	47/ 9 = 5.22*	38/ 9 = 4.22*	54/ 8 = 6.75	75/ 12 = 6.25		

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

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE

WEEK	COMPOUND: 1-METHYLANTHRAQUINONE		STUDY: SUBCHRONIC		SPECIES: RATS		LOG DOSE	ARITH DOSE
	HIST. NEG. CONT.	NEG. CONTROL	POS. CONTROL	0.2000 GM/KG	0.6700 G/KG	2.0000 GM/KG		
1	258/ 201 = 1.28	37/ 12 = 3.08	21/ 4 = 5.25	33/ 9 = 3.67	38/ 12 = 3.17	66/ 11 = 6.00		
2	253/ 230 = 1.10	35/ 13 = 2.69	23/ 7 = 3.29	17/ 10 = 1.70	46/ 12 = 3.83	6/ 13 = 0.46		
3	354/ 260 = 1.36	65/ 11 = 5.91	14/ 5 = 2.80	51/ 8 = 6.38	87/ 11 = 7.91	38/ 11 = 3.45		
4	239/ 308 = 0.78	3/ 9 = 0.33	7/ 2 = 3.50**	19/ 10 = 1.90	17/ 10 = 1.70	66/ 8 = 8.25**	\$	\$\$
5	328/ 310 = 1.06	22/ 13 = 1.69	36/ 9 = 4.00	16/ 10 = 1.60	12/ 12 = 1.00	16/ 9 = 1.78		
6	366/ 304 = 1.20	7/ 9 = 0.78	20/ 10 = 2.00	16/ 16 = 1.00	10/ 12 = 0.83	10/ 10 = 1.00		
7	296/ 306 = 0.97	4/ 12 = 0.33	44/ 9 = 4.89*	1/ 9 = 0.11	3/ 8 = 0.38	32/ 12 = 2.67	\$	\$\$

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PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS

WEEK	COMPOUND: 1-METHYLAMINOANTHRACINONE		STUDY: SUBCHRONIC		SPECIES: RATS	
	HIST. MFG. CNT.	NEG. CONTROL	POS. CONTROL	0.2000 GM/KG	0.6700 G/KG	2.0000 GM/KG
1	97/ 201 = 0.48	5/ 12 = 0.42	3/ 4 = 0.75	7/ 9 = 0.78	8/ 12 = 0.67	8/ 11 = 0.73
2	115/ 230 = 0.50	8/ 13 = 0.62	6/ 7 = 0.86	5/ 10 = 0.50	8/ 12 = 0.67	6/ 13 = 0.46
3	129/ 260 = 0.50	8/ 11 = 0.73	4/ 5 = 0.80	6/ 8 = 0.75	10/ 11 = 0.91	7/ 11 = 0.64
4	142/ 308 = 0.46	2/ 9 = 0.22	2/ 2 = 1.00	4/ 10 = 0.40	5/ 10 = 0.50	7/ 8 = 0.88*
5	153/ 310 = 0.49	6/ 13 = 0.46	7/ 9 = 0.78	5/ 10 = 0.50	6/ 12 = 0.50	6/ 9 = 0.67
6	155/ 304 = 0.51	3/ 9 = 0.33	7/ 10 = 0.70	7/ 16 = 0.44	6/ 12 = 0.50	3/ 10 = 0.30
7	153/ 306 = 0.50	4/ 12 = 0.33	7/ 9 = 0.78	1/ 9 = 0.11	2/ 8 = 0.25	9/ 12 = 0.75

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

PROPORTION OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS

WEEK	COMPOUND: 1-METHYLAminoANTHRAQUINONE		STUDY: SUBCUTANEOUS			SPECIES: RATS			
	HIST.	NEG. CONT.	NEG. CONTROL	POS. CONTROL	0.2000 GM/KG	0.6700 G/KG	2.0000 GM/KG	LOG DOSE	ARITH DOSE
1	47/201 = 0.23	3/12 = 0.25	2/4 = 0.50	2/4 = 0.50	3/9 = 0.33	4/12 = 0.33	7/11 = 0.64		
2	45/230 = 0.20	4/13 = 0.31	5/7 = 0.71	2/10 = 0.20	2/10 = 0.20	7/12 = 0.58	0/13 = 0.0		
3	53/260 = 0.20	7/11 = 0.64	1/5 = 0.20	5/8 = 0.63	5/8 = 0.63	9/11 = 0.82	6/11 = 0.55		
4	43/308 = 0.14	1/9 = 0.11	2/2 = 1.00	2/10 = 0.20	2/10 = 0.20	3/10 = 0.30	7/8 = 0.88**	\$	\$\$
5	78/310 = 0.25	3/13 = 0.23	6/9 = 0.67	4/10 = 0.40	4/10 = 0.40	3/12 = 0.25	3/9 = 0.33		
6	72/304 = 0.24	2/9 = 0.22	3/10 = 0.30	4/16 = 0.25	4/16 = 0.25	3/12 = 0.25	1/10 = 0.10		
7	84/306 = 0.27	0/12 = 0.0	5/9 = 0.56*	0/9 = 0.0	0/9 = 0.0	1/8 = 0.13	5/12 = 0.42*	\$	\$\$

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DEAD IMPLANTS / TOTAL IMPLANTS

WEEK	HIST. NEG. CONT.		NEG. CONTROL		POS. CONTROL		STUDY: SUBCHRONIC		SPECIES: RATS		LOG DOSE	ARITH DOSE
	0.11	0.10	0.12	0.06	0.08	0.03	0.03	0.03	0.03	0.03		
1	258/2313	37/87	35/143	3/89	22/111	7/65	21/39	33/102	38/116	66/110	0.6700 G/KG	2.0000 GM/KG
2	253/2568	37/87	35/143	3/89	22/111	7/65	23/43	17/112	46/150	6/116	0.6700 G/KG	2.0000 GM/KG
3	354/3039	37/87	35/143	3/89	22/111	7/65	14/38	51/71	87/118	38/107	0.6700 G/KG	2.0000 GM/KG
4	239/3943	37/87	35/143	3/89	22/111	7/65	7/19	19/89	17/50	66/91	0.6700 G/KG	2.0000 GM/KG
5	328/3915	37/87	35/143	3/89	22/111	7/65	36/73	16/60	12/107	16/49	0.6700 G/KG	2.0000 GM/KG
6	366/3571	37/87	35/143	3/89	22/111	7/65	20/74	16/129	10/120	10/48	0.6700 G/KG	2.0000 GM/KG
7	296/3803	37/87	35/143	3/89	22/111	7/65	44/93	1/96	3/53	32/99	0.6700 G/KG	2.0000 GM/KG

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

STANDARD OPERATING PROCEDURES

To ensure an accurate and reliable mutagenicity testing program, LBI instituted the following procedures:

- The test compound was registered in a bound log book recording the date of receipt, complete client identification, physical description and LBI code number.
- Complete records of weights and dilutions associated with the testing of the submitted material were entered into a bound notebook.
- Raw data information was recorded on special printed forms that were dated and initialed by the individual performing the data collection at the time the observations were made. These forms were filed as permanent records.
- All animal tissue S-9 preparations used in the activation tests were taken from dated and pretested frozen lots, each identified by a unique number. The S-9 preparations were monitored for uniformity and the information was recorded.

APPENDIX A

Analysis of Data

1. Fertility Index

- a. The fertility index is defined as $F.I. = \# \text{ of pregnant females} / \# \text{ of mated females}$. 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.

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

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

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

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

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 .

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

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

$$t_i = \bar{u}_0 - \bar{u}_i / \left(\frac{s_0^2 + s_i^2}{n_0 + n_i - 2} \left(\frac{1}{n_0} + \frac{1}{n_i} \right) \right)^{\frac{1}{2}}$$

$$\text{d.f.} = n_0 + n_i - 2$$

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 = $\text{VARU} \left(\frac{1}{N} + \frac{\bar{x}^2}{SS_X} \right)$
TB	= $B/(\text{VARB})^{1/2}$ = 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 = $(\text{VARU.X})^{1/2}/\bar{U}$
VARU.X	= $\frac{1}{N-2} (SS_U - S_{XU}^2/SS_X)$
SDY	= standard deviation of U about the regression line = $(\text{VARU.X})^{1/2}$
SDS	= standard deviation of the slope = $(\text{VARB})^{1/2}$
SDA	= standard deviation of intercept = $(\text{VARA})^{1/2}$

Significant difference of the slope from zero is indicated at the 5 and 1% levels in Table 2. Table 2A shows detailed results of the regression analysis.

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

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

- 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

$$u_i = \# \text{ of corpora lutea for the } i\text{-th female}$$

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:

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

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

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

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

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

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

- 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

$$U_i = \# \text{ of preimplantation losses for the } i\text{-th female.}$$

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.