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Submitting Organization	EASTMAN KODAK CO		
Contractor			
Document Title	INITIAL SUBMISSION: LETTER FROM EASTMAN KODAK CO TO USEPA REGARDING HEALTH EFFECTS STUDIES WITH 3-AMINO-2,2,3-TRIMETHYL BUTYRIC ACID METHYL ESTER, W/ATTACHMENTS AND DATED 9/15/1999		
Chemical Category	3-AMINO-2,2,3-TRIMETHYLBUTYRIC ACID METHYL ESTER		

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September 15, 1999



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Document Control Office (7407)
Office of Pollution Prevention and Toxics
U. S. Environmental Protection Agency
401 M Street
Washington, DC 20460-001
ATTN: TSCA Section 8(e) Coordinator

MR 26501

Contain NO CBI

Dear Sir or Madam:

Subject: Report submitted in accordance with U. S. Environmental Protection Agency
Statement of Interpretation and Enforcement policy: Notification of Substantial
Risk-Section 8(e) - SCA.

The following information is submitted in accordance with the above statement. The submission pertains to 3-amino-2,2,3-trimethylbutyric acid, methyl ester [CAS # 090886-53-6], and is being submitted because of neurological and testicular effects observed in a four-week oral toxicity study in rats.

Male and female rats were treated daily with 500, 150, 50 or 0 mg/kg of the test substance in distilled water by oral gavage for 29 days. Mortality prior to scheduled necropsy was limited to one male and one female from the high dose group. Except for significantly higher scores for hair coat condition for 500 mg/kg female rats on Days 14, 21, and 28 and a significant decrease in hindlimb grip strength for 500 mg/kg male rats on Day 28, no other differences were observed in a functional observational battery (FOB). High-dose group male rats also had significantly lower mean total motor activity scores and lower total ambulation scores than controls.

At study termination, a statistically significant decrease in numbers of white blood cells and changes in the percentages of white blood cells was observed only in high-dose group animals. Clinical chemistry effects were limited to an increase in mean total protein, cholesterol, and triglyceride levels in high-dose group males and an increase in cholesterol and decrease in sodium values in high-dose group females.

No gross lesions were observed during necropsy that were attributed to exposure to the test substance. Histopathological effects included sciatic nerve fiber degeneration for the high-dose groups. Seminiferous tubule degeneration within the testis and degenerative cells within the epididymal tubules were seen only for 500 mg/kg male rats. No other treatment-related lesions were observed for any dose group tested. The no-observed-adverse-effect level (NOAEL) was determined to be 150 mg/kg.

R. Hays Bell, Ph.D., Director, Health, Safety, and Environment
Vice President, Eastman Kodak Company
Rochester, NY 14652-6256 • 716-722-5036 • FAX 716-722-0239



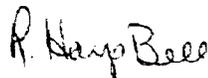
Document Control Office -- 2

In addition to the four-week oral toxicity study, we have also enclosed a copy of an acute oral toxicity study in the rat, an acute dermal toxicity study in the rat, an acute dermal irritation study in the rabbit, and two *in vitro* mutagenicity studies. The acute oral LD50 for this material was calculated to be 1414 mg/kg for male rats and 1231 mg/kg for female rats. The major treatment-related change observed at necropsy was gastric irritation. The acute dermal LD50 for this material was greater than 2000 mg/kg for male and female rats. Clinical signs and treatment-related changes provided evidence of dermal irritation and/or corrosion by the test material. Similar effects were observed in a four-hour dermal irritation study. The test material was negative in the *Salmonella - E. coli*/Mammalian-Microsome Reverse Mutation Assay and a Chromosomal Aberration Study in Chinese Hamster Ovary (CHO) Cells both in the presence and absence of metabolic activation.

The test material is a site-limited intermediate used in the manufacture of a chemical for photographic film and paper. Unreacted test material is not expected to be present in the final chemical or in the product. This material is currently purchased from an outside vendor. A copy of this submission letter will be forwarded to the vendor. We are not aware of any adverse health problems associated with the use of the test material. A copy of our revised material safety data sheet (MSDS) is also included with this submission packet.

Please contact me if additional information is required.

Sincerely,



R. Hays Bell
(716) 722-5036

RHB:JAF

A 05

MATERIAL SAFETY DATA SHEET

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FOR USE ONLY WITHIN EASTMAN KODAK COMPANY AND ITS SUBSIDIARIES.

PRIOR APPROVAL IS REQUIRED FOR DISTRIBUTION OF THIS MSDS TO ANYONE OTHER THAN A KODAK EMPLOYEE. PHONE: THE CHEMICAL AND REGULATORY INFORMATION SECTION (58-83743).

1. CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

Product Name: 3-Amino-2,2,3-trimethylbutyric acid, methyl ester

Product Identification Number(s): 812984

Manufacturer/Supplier: EASTMAN KODAK COMPANY, Rochester, New York 14650

For Emergency Health, Safety & Environmental Information, call (716) 722-5151

Synonym(s): KAN 812984, CIN 10082843

Molecular Formula: C8 H17 N O2

Molecular Weight: 159.23

2. COMPOSITION/INFORMATION ON INGREDIENTS

Weight % - Component - (CAS Registry No.)

100 3-amino-2,2,3-trimethylbutyric acid, methyl ester (090886-53-6)

3. HAZARDS IDENTIFICATION

COMBUSTIBLE LIQUID AND VAPOR
CAN DECOMPOSE AT ELEVATED TEMPERATURES
MAY CAUSE NERVOUS SYSTEM DAMAGE BASED ON ANIMAL DATA
MAY CAUSE TESTICULAR DAMAGE BASED ON ANIMAL DATA
HARMFUL IF INHALED, ABSORBED THROUGH SKIN, OR SWALLOWED
MIST OR VAPOR IRRITATING TO EYES AND RESPIRATORY TRACT
CAUSES SKIN AND EYE IRRITATION

Eastman Kodak Hazard Ratings: R-2, S-2, F-2, C-1T REPO

4. FIRST-AID MEASURES

Inhalation: If symptomatic, move to fresh air. Treat symptomatically. Get medical attention if symptoms persist.

Eyes: Immediately flush with plenty of water for at least 15 minutes. Get medical attention.

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Skin: Immediately flush with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Get medical attention. Wash contaminated clothing before reuse. Destroy or thoroughly clean contaminated shoes.

Ingestion: Do NOT induce vomiting. Give victim a glass of water. Never give anything by mouth to an unconscious person. Call a physician or poison control center immediately.

5. FIRE FIGHTING MEASURES

Extinguishing Media: Water spray, carbon dioxide (CO2), dry chemical, foam

Special Fire-Fighting Procedures: Wear self-contained breathing apparatus and protective clothing. Fire or excessive heat may produce hazardous decomposition products. Use water spray to keep fire-exposed containers cool.

Hazardous Combustion Products: Carbon dioxide, carbon monoxide, oxides of nitrogen

Unusual Fire and Explosion Hazards: Classified as combustible. Fire or high temperatures may cause decomposition.

6. ACCIDENTAL RELEASE MEASURES

Eliminate all ignition sources. Absorb spill with vermiculite or other inert material, then place in a container for chemical waste. Clean surface thoroughly to remove residual contamination.

7. HANDLING AND STORAGE

Personal Precautionary Measures: Avoid breathing mist or vapor. Avoid contact with eyes, skin, and clothing. Use with adequate ventilation. Wash thoroughly after handling.

Prevention of Fire and Explosion: Keep away from heat and flame. Keep from contact with oxidizing materials.

Storage: Keep container closed. Use with adequate ventilation.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Exposure Limits: Not established

Ventilation: Good general ventilation (typically 10 air changes per hour) should be used. Ventilation rates should be matched to conditions. Supplementary local exhaust ventilation, closed systems, or respiratory protection may be needed in

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special circumstances such as poorly ventilated spaces, evaporation from large surfaces, spraying, heating, etc.

Respiratory Protection: If engineering controls do not maintain airborne concentrations to an acceptable level, an approved respirator must be worn. Respirator type: Organic vapor. If respirators are used, a program should be instituted to assure compliance with OSHA Standard 29 CFR 1910.134.

Eye Protection: Wear safety glasses with side shields (or goggles).

Skin Protection: Wear impervious gloves and protective clothing appropriate for the risk of exposure.

Recommended Decontamination Facilities: Eye bath, washing facilities, safety shower

9. PHYSICAL AND CHEMICAL PROPERTIES

Physical Form: Liquid

Color: Colorless

Odor: Rancid

Specific Gravity (water = 1): Not available

Relative Density: 0.96528

Vapor Pressure at 25°C (77°F): 73.6 Pa

Vapor Density (Air = 1): Not available

Volatile Fraction by Weight: Not available

Boiling Point: 186.8°C (368.2°F)

Solubility in Water: 51.6 wt/wt%

pH: Not available

Flash Point (closed cup): 77°C (171°F)

Autoignition Temperature: 316°C (601°F)

10. STABILITY AND REACTIVITY

Stability: Stable. Safe handling temperatures are dependent on specific conditions of use and are typically substantially below the onset temperature. Consult your technical safety experts.

Exotherm onset temperature: 288°C by DSC

Incompatibility: Strong oxidizing agents

Hazardous Polymerization: Will not occur.

11. TOXICOLOGICAL INFORMATION

Effects of Exposure:

General: Based on animal data, may cause adverse effects on the following organs/systems: Nervous system, testes.

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Inhalation: Harmful if inhaled. Mist or vapor irritating.

Eyes: Causes irritation. Mist or vapor irritating.

Skin: Harmful if absorbed through skin. Causes irritation.

Ingestion: Harmful if swallowed. May cause irritation of the gastrointestinal tract.

Acute Toxicity Data:

Oral LD-50 (rat): 1920 mg/kg

Dermal LD-50: > 2000 mg/kg

Skin irritation: moderate

Skin sensitization: low potential (3/20)

Definitions for the following section(s): LOEL = lowest-observed-effect level, LOAEL = lowest-observed-adverse-effect, NOAEL = no observed-adverse-effect level, NOEL = no-observed-effect level.

Subchronic Toxicity Data:

Oral study (29 days, rat): NOAEL = 150 mg/kg (target organ effects: nervous system, testes)

Mutagenicity/Genotoxicity Data:

Salmonella-E. coli/Mammalian-Microsome Reverse Mutation Assay: negative (in presence and absence of activation)

Chromosomal aberration assay: negative (in presence and absence of activation)

12. ECOLOGICAL INFORMATION

Introduction: This environmental effects summary is written to assist in addressing emergencies created by an accidental spill which might occur during the shipment of this material, and, in general, it is not meant to address discharges to sanitary sewers or publically owned treatment works.

Summary: Data for chemically similar materials have been used to estimate the environmental impact of this material. However, this material, itself, has not been tested for environmental effects. It is expected to have the following properties: a moderate biochemical oxygen demand and may cause oxygen depletion in aqueous systems, a moderate potential to affect some aquatic organisms, a moderate potential to affect secondary waste treatment microbial metabolism, a moderate potential to affect algal growth, a moderate potential to biodegrade (moderate persistence) with unacclimated microorganisms from activated sludge, a low potential to bioconcentrate. After dilution with a large amount of water,

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followed by secondary waste treatment, this material is not expected to cause adverse environmental effects.

The following properties are ESTIMATED from the components of the preparations. The effects of are considered the most significant in this estimation:

Potential Toxicity

Fish LC50 mg/l:

Daphnid EC50 mg/l:

Algal IC50 mg/l:

Organics Readily Degradable (>70%): Yes (days)

Potential Bioaccumulation: Log Pow <1

COD (approximate g/l):

BOD5 (approximate g/l):

Potential Toxicity

Waste treatment microorganisms EC50 (mg/l):

13. DISPOSAL CONSIDERATIONS

Discharge, treatment, or disposal may be subject to national, state, or local laws. Incinerate. Since emptied containers retain product residue, follow label warnings even after container is emptied.

14. TRANSPORT INFORMATION

United Nations

UN Number: None, not regulated

USA Department of Transportation (DOT) Hazardous Materials Classification:

DOT (USA) Status: R

DOT Identification Number: NA 1993

DOT Proper Shipping Name: Combustible Liquid, n.o.s. (3-amino-2,2,3-trimethylbutyric acid, methyl ester)

DOT Hazard Class: Combustible liquid

DOT Packing Group: III

DOT Hazardous Substance(RQ): No

Marine Pollutant: No

R = Regulated, NR = Not Regulated, NA = Not Accepted, LQ = Limited Quantity, EX = Exempted Quantity, CAO = Cargo Aircraft Only

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For other transportation information, call the Kodak Worldwide Transportation Hazmat Hot Line: (716) 722-2400 between 8 a.m. and 5 p.m. (Eastern Standard Time), Monday through Friday.

15. REGULATORY INFORMATION

- Material(s) known to the State of California to cause cancer: None
 - Material(s) known to the State of California to cause adverse reproductive effects: None

 - Carcinogenicity Classification (components present at 0.1% or more):
 - International Agency for Research on Cancer (IARC): None
 - American Conference of Governmental Industrial Hygienists (ACGIH): None
 - National Toxicology Program (NTP): None
 - Occupational Safety and Health Administration (OSHA): None

 - Chemical(s) subject to the reporting requirements of Section 313 or Title III of the Superfund Amendments and Reauthorization Act (SARA) of 1986 and 40 CFR Part 372: None
-

16. OTHER INFORMATION

US/Canadian Label Statements:

WARNING!

MAY CAUSE NERVOUS SYSTEM DAMAGE BASED ON ANIMAL DATA
MAY CAUSE TESTICULAR DAMAGE BASED ON ANIMAL DATA
HARMFUL IF INHALED, ABSORBED THROUGH SKIN, OR SWALLOWED
MIST OR VAPOR IRRITATING TO EYES AND RESPIRATORY TRACT
CAUSES SKIN AND EYE IRRITATION
COMBUSTIBLE LIQUID AND VAPOR
CAN DECOMPOSE AT ELEVATED TEMPERATURES

Keep away from heat and flame.
Avoid breathing mist or vapor.
Avoid contact with eyes, skin, and clothing.
Keep container closed.
Use with adequate ventilation.
Wash thoroughly after handling.

FIRST AID: If swallowed, do NOT induce vomiting. Give victim a glass of water. Never give anything by mouth to an unconscious person. Call a physician or poison control center immediately. If inhaled, move to fresh air. Treat symptomatically. In case of contact, immediately flush eyes and skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Get medical attention. Wash contaminated clothing before reuse. Destroy or thoroughly clean contaminated shoes.

Keep out of reach of children.

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Do not handle or use until safety precautions in Material Safety Data Sheet (MSDS) have been read and understood.

Additional hazard precautions for containers greater than 1 gallon of liquid or 5 pounds of solid:

Since emptied containers retain product residue, follow label warnings even after container is emptied.

IN CASE OF FIRE: Use water spray, carbon dioxide (CO2), dry chemical, foam. Use water spray to keep fire-exposed containers cool.

IN CASE OF SPILL: Eliminate all ignition sources.

The information contained herein is furnished without warranty of any kind. Users should consider these data only as a supplement to other information gathered by them and must make independent determinations of suitability and completeness of information from all sources to assure proper use and disposal of these materials and the safety and health of employees and customers and the protection of the environment.

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FINAL REPORT

3-AMINO-2,2,5-TRIMETHYLBUTYRIC ACID METHYL ESTER

HAEL No.: 99-0005

KAN: 812984-5

CIN: 10082843

CAS: 90886-53-6

RAN: 322104P

SKIN SENSITIZATION STUDY (GPMT METHOD) IN THE GUINEA PIG

GUIDELINE

OECD: 406

EEC: Annex V., Test B.6

AUTHOR

Stephen D. Jessup, A.A.S.

TESTING FACILITY

Toxicological Sciences Laboratory
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-6272
USA

LABORATORY PROJECT ID

99-0005A3

STUDY SPONSOR

Eastman Kodak Company

STUDY COMPLETION DATE

August 10, 1999

QUALITY ASSURANCE INSPECTION STATEMENT
(21 CFR 58.35(B)(7), 40 CFR 792.35(B)(7), AND 40 CFR 160.35(B)(7))

STUDY: 99-0005-1 STUDY DIRECTOR: SHEPARD, K.P.
ACCESSION NUMBER: 812984

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08/11/99

STUDY TYPE: GUINEA PIG MAXIMIZATION TEST (GPMT)

M. [Signature]
(AUDITOR, QUALITY ASSURANCE UNIT)

8/11/99
DATE

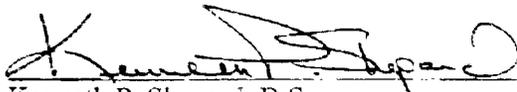
THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY ASSURANCE UNIT. WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES.

INSPECTION DATES	PHASE(S) INSPECTED	STATUS REPORT DATES
04/12/99	PROTOCOL APPENDIX/AMENDMENT SUBMISSION	
05/13/99	CLINICAL SIGNS AT 48 HRS. POST INJECTION	08/03/99
08/03/99	FINAL REPORT REVIEW	08/03/99

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted according to:

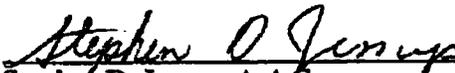
OECD Principles of Good Laboratory Practice (as revised in 1997)
[C(97)186/Final].



Kenneth P. Shepard, B.S.
Study Director

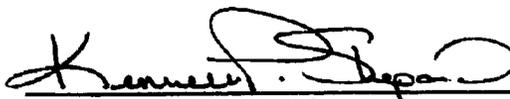
August 10, 1999
Month/Day/Year

SIGNATURE PAGE



Stephen D. Jessup, A.A.S.
Report Author

August 5, 1999
Month/Day/Year



Kenneth P. Shepard, B.S.
Study Director

August 10, 1999
Month/Day/Year



Douglas C. Topping, Ph.D.
Unit Director, Mammalian Toxicology

August 10, 1999
Month/Day/Year

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ABSTRACT**3-AMINO-2,2,3-TRIMETHYLBUTYRIC ACID METHYL ESTER****HAEL No.: 99-0005****KAN: 812984-5****CIN: 10082843****CAS: 90386-53-6****RAN: 322104P****SKIN SENSITIZATION STUDY (GPMT METHOD) IN THE GUINEA PIG**

A dermal sensitization study was conducted with this test substance using the Guinea Pig Maximization Test (GPMT). A primary irritation screen was conducted prior to the sensitization study to determine if the test substance was a dermal irritant. In addition, the screen was used to determine a concentration of the test substance in distilled water and in an emulsion of equal parts Freund's Complete Adjuvant (FCA) and distilled water that could be injected intradermally without eliciting a strong local or systemic toxic reaction. Based on results of the preliminary tests, a concentration of 1% test substance in distilled water and 1% test substance in the FCA emulsion were selected for the intradermal induction injections. The test substance was administered as a 25% concentration when used for both the topical induction and for the challenge exposure in the sensitization study.

For the 10 control animals, no dermal responses were noted at the 24- or 48- hour observation period after the challenge dose. For the 20 animals previously induced with the test substance, 15% (3 of 20) animals were graded as having a dermal response at 24- and/or 48- hours after the challenge exposure.

Based on these results, the test substance was not considered to be a dermal sensitizer in guinea pigs. The test substance requires no label for sensitization by skin contact, as defined in the 18th Adaptation of the EC Classification, Packaging, and Labelling of Dangerous Substances Directive.

STUDY AND TEST SUBSTANCE INFORMATION**Testing Facility**

Toxicological Sciences Laboratory
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-6272
USA

Project Participants

Study Director: Kenneth P. Shepard, B.S.
Principal Investigator: John W. Mosher, B.S.
Report Author: Stephen D. Jessup, A.A.S.

Sponsor

Eastman Kodak Company

Test Substance Characterization

Test Substance Name: 3-Amino-2,2,3-trimethylbutyric acid methyl ester
CAS No: 90886-53-6
HAEL No.: 99-0005
KAN: 812984-5
CIN: 10082843
SRID or Lot No.: BB8792-131J
Physical State and Appearance: Solid, Off-white chunks
Source of Test Substance: Eastman Kodak Company
Laboratory Project ID: 99-0005A3

Composition: Refer to composition information included in the notification when applicable.

Study Dates

Study Initiation Date: April 12, 1999
Experimental Start Date: April 12, 1999
Experimental Completion Date: June 4, 1999

PURPOSE

The purpose of the study was to determine whether the test substance has the ability to produce delayed contact hypersensitivity (skin sensitization).

MATERIALS AND METHODS

Test system

Male guinea pigs (Cri:(HA)BR) obtained from Charles River Kingston (Stone Ridge, NY) were used in the sensitization study. For the primary irritation phase, four guinea pigs were used to determine a concentration of the test substance that could be injected intradermally without eliciting a strong local or systemic toxic reaction. Six additional guinea pigs were used to determine the topical concentration of the test substance to be used in the induction and challenge phase of the sensitization study. The animals assigned to the primary irritation test were 9 to 10 weeks of age. For the induction and challenge phase of the sensitization study, 30 guinea pigs were randomly assigned to one of two groups (a control group of 10 animals or a test group of 20 animals). These guinea pigs were 6 to 7 weeks of age and weighed 391 to 512 grams at the start of the induction phase of the study. Guinea pigs were chosen for this study because they are the animal of choice for predictive sensitization studies and are recommended for use in the OECD Guideline.

Husbandry

Housing

Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The guinea pigs were singly housed in suspended, stainless-steel, wire mesh cages. Cages and racks were washed once a week. Absorbent paper used to collect excreta was changed at least three times a week.

Environmental Conditions

The study room was maintained at 19.7 to 24.7 °C and 37.0 to 68.5% relative humidity. A photoperiod of 12 hours light from approximately 6 a.m. to 6 p.m. was maintained.

Husbandry, continuedAcclimation Period

The animals were isolated upon arrival and allowed to acclimate for a period of 5 days. Animals were judged to be healthy prior to testing.

Feed

PMI Certified Guinea Pig Diet (5026) was available *ad libitum*. Feed containers were cleaned weekly and refilled at least once a week. No known contaminants which would interfere with the outcome of this study were present in the feed. Analyses of feed are maintained on file within the testing laboratory.

Water

Water was available *ad libitum* through an automatic watering system. The source of the water was the local public water system. There have been no contaminants identified in periodic water analyses that would be expected to interfere with the conduct of the study. Semiannual analyses of water are maintained on file within the testing laboratory.

Identification

Upon arrival, all guinea pigs were identified by uniquely-numbered metal ear tags. During randomization, study-specific animal numbers were assigned to each animal. Cage cards contained the study-specific animal number and the ear tag number.

Experimental DesignTest Procedures

This study was conducted according to the Organisation for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals Guideline: 406, Skin Sensitisation; and European Economic Community (EEC): Annex V., Test B.6, Skin Sensitization.

Experimental Design, continuedRandomization

A clinical examination was performed on each animal to ensure that only healthy animals were utilized. The procedure for including animals in the sensitization study was to randomly select and assign animals from the same shipment to each group (test and control). Randomization was done by a computer-generated list. After assignment of animals to individual groups, the body weights were determined to ensure that weights were no greater than 600 grams at the initiation of the induction phase.

Distribution of Animals

Animals assigned to the study were distributed as shown in Table 1.

TABLE 1
Animal Assignments

STUDY PHASE	GROUP	NUMBER OF ANIMALS	ANIMAL NUMBERS
Primary Irritation Screen	Intradermal Injections	4	101 - 104
	Topical Application	6	105 - 110
Sensitization: Study	Test	20	191 - 210
	Control	10	211 - 220

Primary Irritation Screen

The primary irritation screen determined the concentration of the test substance (up to 5%) in distilled water and in an emulsion of Freund's Complete Adjuvant (FCA) and distilled water (1:1) that could be injected intradermally without eliciting a strong local or systemic toxic reaction. In addition, the screen also determined the topical concentration of the test substance to be used in the sensitization study.

Intradermal Injections

Prior to intradermal injections, four emulsions were prepared by blending commercial FCA with an equal volume of distilled water. These emulsions were used to prepare concentrations of 0.1, 1.0, 3.0, and 5.0% of the test substance. Identical concentrations of the test substance were prepared in distilled water.

Experimental Design, continued

Primary Irritation Screen, continued

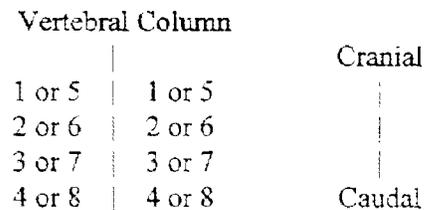
Intradermal Injections, continued

On the day of the intradermal injections, the hair was removed from the backs of four guinea pigs using an electric clipper. Two pairs of intradermal injections (0.1% and 1% or 3% and 5%) were made on each side of the vertebral column as described in Table 2. Approximately 48 hours after the injections, sites were evaluated for necrosis and ulceration. If these concentrations did not produce ulceration or strong necrosis, then the highest percentage was utilized for the intradermal induction phase of the sensitization study. Local necrosis without signs of systemic toxicity was considered indicative that the concentration was tolerated.

TABLE 2
Primary Irritation Screen-Intradermal Injections

ANIMAL NUMBER	INJECTION SITE *	TEST SUBSTANCE CONCENTRATION	VEHICLE
101 and	1	0.1%	FCA Emulsion
	2	0.1%	Distilled Water
102	3	1.0%	FCA Emulsion
	4	1.0%	Distilled Water
103 and	5	3.0%	FCA Emulsion
	6	3.0%	Distilled Water
104	7	5.0%	FCA Emulsion
	8	5.0%	Distilled Water

* Location of intradermal injections:



Experimental Design, continued**Primary Irritation Screen, continued****Topical Applications**

Hair was removed from the backs of six guinea pigs using an electric clipper. Two guinea pigs were placed in each group containing 12.5, 25, or 50% test substance. The test substance was applied to a patch approximately 2 cm square and placed on the clipped area of each guinea pig. The patch was secured using an elastic wrap. Approximately 24 hours after initiation of exposure, the wrap and patch were removed. Approximately 24 hours after patch removal, the application site was observed for signs of irritation. Topical application of the test substance is described in Table 3.

TABLE 3
Primary Irritation Screen-Topical Applications

ANIMAL NUMBERS	LOCATION OF APPLICATION SITE ON EACH ANIMAL	CONCENTRATION OF TEST SUBSTANCE
105 and 106	Dorsal Midline	12.5%
107 and 108	Dorsal Midline	25%
109 and 110	Dorsal Midline	50%

Sensitization Study**Induction Phase (Day 0)**

On the day of initiation of the study, the hair was removed from an area of approximately 4 x 6 cm from the shoulder region of each guinea pig. Prior to the injections, an emulsion was prepared by blending commercial FCA with an equal volume of distilled water. Three pairs of intradermal injections were made with a 1 mL syringe and an appropriate needle, each pair flanking the dorsal midline. For the test group, the three pairs of intradermal injections were as follows: (Site 1/Preparation 1) 0.1 mL of the FCA Emulsion, (Site 2/Preparation 2) 0.1 mL of the test substance in distilled water (concentration determined in primary irritation screen), and (Site 3/Preparation 3) 0.1 mL of the test substance in FCA Emulsion (concentration determined in primary irritation screen). For the control group, three pairs of injections were as follows: (Site 4/Preparation 4) 0.1 mL of the FCA Emulsion, (Site 5/Preparation 5) 0.1 mL of distilled water, and (Site 6/Preparation 6) 0.1 mL of distilled water in FCA Emulsion (1:1).

Experimental Design, continued

Sensitization Study, continued

Induction Phase (Day 0), continued

Location sites and preparations of the intradermal injections:

Test Group

Vertebral Column

Site/Prep.	Site/Prep.
1	1
2	2
3	3

Cranial

|

Caudal

Control Group

Vertebral Column

Site/Prep.	Site/Prep.
4	4
5	5
6	6

Cranial

|

Caudal

Local Irritation (Day 6)

With an electric clipper, the hair was removed on both control and test animals from the same area over the shoulder region that received the intradermal injections on Day 0. Since a 25% concentration of the test substance was not determined to be a skin irritant in the topical portion of the primary irritation screen, the application site of all animals (treated and control) was treated with 0.5 mL of 10% sodium lauryl sulfate in petrolatum to induce local irritation.

Induction Phase (Day 7)

For the twenty test animals, a 2 x 4 cm patch with 2.0 mL of a 25% concentration of the test substance, moistened with water (Preparation 7) was applied to each application site. The patches were secured in place with an elastic bandage. For the ten control animals, 2.0 mL of distilled water (Preparation 8) was applied to each animal using the same procedure used for test animals. The patches of both groups (test and control) were left in place for a period of approximately 48 hours (Day 7 to Day 9). Approximately 24 hours after patch removal on Day 10, the application sites were scored for local irritation using the 4-point scale of Magnusson and Kligman (1969).

Experimental Design, continuedSensitization Study, continued**Challenge Phase (Day 21)**

The hair was removed with an electric clipper from an area of approximately 3.75 x 3.75 cm on both flanks of all animals in the control and test groups. A patch (approximately 2.0 cm square) with 1.0 mL of a concentration of 25% test substance in distilled water (Preparation 9) was applied to the left flank of each animal in both groups. In addition, a second patch with 1.0 mL of distilled water (Preparation 10) was applied to the right flank of each animal in both groups. The torso of each animal was then wrapped with an elastic bandage to secure the patches. The patches were removed approximately 24 hours after initiation of exposure. Dermal reactions were scored at 24 and 48 hours after termination of the challenge exposure (Days 23 and 24) using the 4-point Magnusson and Kligman (1969) scale.

Grading the Dermal Reactions (Irritation and Sensitization Responses)

Dermal reactions were scored using the following 4-point scale developed by Magnusson and Kligman (1969):

0 - no reaction	2 - moderate and diffuse redness
1 - scattered and mild redness	3 - intense redness and swelling

Body Weight Determination

Body weights were collected on the day of the intradermal inductions (Day 0) and on the day of the last observations following the challenge dose.

Necropsy

Animals were not necropsied at the conclusion of the test.

Data Storage

The final report, data sheets, all nonperishable raw data, and an aliquot of the test substance have been stored in the testing facility archive managed under GLP-mandated conditions.

Data Analysis

The important statistic in the GPMT is the frequency of sensitization. A severity value of "1" is considered just as positive as a severity value of "3" (as long as the controls are zero). The test substance is considered to be a sensitizer if the frequency of the dermal reactions from the challenge exposure in the test group clearly outweigh those in the control group. The sensitization rate (% of positive responders) is based on the highest number of animals showing a positive response from either the 24-hour or 48-hour observation data.

Classification of the test substance is determined in accordance with the 18th Adaptation of the EC Classification, Packaging, and Labelling of Dangerous Substances Directive. For the GPMT method, a test substance that produces a dermal response in at least 30% of the animals is considered positive for skin sensitization.

Protocol and Standard Operating Procedure Deviations

There were no SOP or protocol deviations during the study.

RESULTS

Primary Irritation Screen

Intradermal Injections - The following table (Table 4) shows the results of the observations for strong local effects (necrosis and ulceration) for the two pairs of intradermal injections that were made on each side of the vertebral column of four guinea pigs. Signs of necrosis were noted for all sites that were injected with the 3% or 5% concentrations. Therefore, the 1% concentration (1.0% test substance in distilled water and 1.0% test substance in an FCA emulsion) was utilized for the intradermal injections in the induction phase (Day 0) of the sensitization study.

TABLE 4
Primary Irritation Screen (Intradermal Injections)

ANIMAL NUMBERS	DERMAL REACTIONS FROM INTRADERMAL INJECTIONS			
	PERCENT	VEHICLE	EFFECT (LEFT)*	EFFECT (RIGHT)*
101	0.1%	FCA Emulsion	0	0
	0.1%	Distilled Water	0	0
	1.0%	FCA Emulsion	0	0
	1.0%	Distilled Water	N	0
102	0.1%	FCA Emulsion	0	0
	0.1%	Distilled Water	0	0
	1.0%	FCA Emulsion	0	0
	1.0%	Distilled Water	N	0
103	3.0%	FCA Emulsion	N	N
	3.0%	Distilled Water	N	N
	5.0%	FCA Emulsion	N	N
	5.0%	Distilled Water	N	N
104	3.0%	FCA Emulsion	N	N
	3.0%	Distilled Water	N	N
	5.0%	FCA Emulsion	N	N
	5.0%	Distilled Water	N	N

* Dermal effects of the intradermal injections were graded as N = necrosis, U = ulceration, and 0 = no necrosis or ulceration.

Primary Irritation Screen, continued

Topical Applications - Two guinea pigs received concentrations of 50%, 25%, or 12.5% of the test substance in distilled water. Each animal assigned to the topical portion of the irritation screen received approximately 2 mLs of the respective concentration of the test substance for an exposure period of 24 hours.

Table 5 shows the scores for irritation following topical application of the test substance. Since signs of irritation were observed, the concentration used for the topical induction and challenge phases of the sensitization study was the highest concentration that did not elicit a local reaction.

TABLE 5
Primary Irritation Screen (Topical Applications)

ANIMAL NUMBERS	CONCENTRATION	DERMAL EFFECTS*
105	12.5% Test Substance	0
106	12.5% Test Substance	0
107	25.0% Test Substance	0
108	25.0% Test Substance	0
109	50.0% Test Substance	3
110	50.0% Test Substance	1

* The application sites were scored using the 4-point scale developed by Magnusson and Kligman (1969).

Sensitization Study

Intradermal Injections - On the day of initiation of the induction phase of the study, three pairs of intradermal injections were made for all control and treated animals as described in the experimental design under the heading Induction (Day 0). Table 6 shows the observations for local effects (necrosis or ulceration) that were recorded 48 hours after the intradermal injections for the treated and control animals.

Sensitization Study, continued

TABLE 6
Induction (Intradermal Injections)

TREATED GROUP													
Dermal Effects* At the Injection Sites (Left and Right of Midline)							Dermal Effects* At the Injection Sites (Left and Right of Midline)						
Animal Number	Left Sites			Right Sites			Animal Number	Left Sites			Right Sites		
	1	2	3	1	2	3		1	2	3	1	2	3
191	0	0	0	0	0	0	201	0	0	0	0	0	0
192	0	N	N	N	N	N	202	0	N	0	0	N	N
193	0	0	0	0	0	N	203	0	0	N	0	0	N
194	0	N	0	0	N	N	204	0	0	0	0	0	0
195	0	0	0	0	0	0	205	0	N	N	0	N	N
196	0	0	0	0	0	N	206	0	0	N	0	0	N
197	0	N	N	0	N	N	207	0	N	0	0	N	0
198	0	0	N	0	0	N	208	0	N	0	0	N	N
199	0	N	N	0	N	N	209	0	0	N	0	0	N
200	0	0	N	0	0	N	210	0	0	0	0	N	N
CONTROL GROUP													
Dermal Effects* At the Injection Sites (Left and Right of Midline)							Dermal Effects* At the Injection Sites (Left and Right of Midline)						
Animal Number	Left Sites			Right Sites			Animal Number	Left Sites			Right Sites		
	4	5	6	4	5	6		4	5	6	4	5	6
211	0	0	0	0	0	0	216	0	0	0	0	0	0
212	0	0	0	0	0	0	217	0	0	0	0	0	0
213	0	0	0	0	0	0	218	0	0	0	0	0	0
214	0	0	0	0	0	0	219	0	0	0	0	0	0
215	0	0	0	0	0	0	220	0	0	0	0	0	0

* Dermal effects were graded as N = local necrosis, U = ulceration, or 0 = no necrosis or ulceration).

Local Irritation - Since the concentration of test substance used in the induction phase was not a skin irritant in the primary irritation screen, the application site of each animal (treated and control) was treated on Day 6 with 0.5 mL of 10% sodium lauryl sulfate in petrolatum to induce local irritation. Table 7 shows the dermal irritation readings obtained 24 hours after application of the sodium lauryl sulfate and prior to the topical induction (Day 7) applications.

Sensitization Study, continued

TABLE 7
Irritation (Application of Sodium Lauryl Sulfate)

TREATED GROUP			
Animal Number	Dermal Effects*	Animal Number	Dermal Effects*
191	2	201	1
192	2	202	1
193	1	203	1
194	1	204	1
195	1	205	2
196	2	206	2
197	2	207	2
198	1	208	2
199	1	209	1
200	1	210	2
CONTROL GROUP			
Animal Number	Dermal Effects*	Animal Number	Dermal Effects*
211	2	216	2
212	1	217	2
213	2	218	2
214	1	219	1
215	1	220	2

- * The sites were scored using the following 4-point scale developed by Magnusson and Kligman (1969).

Sensitization Study, continued

Topical Applications - For the test animals, 2.0 mLs of a 25% concentration of the test substance was used for the topical induction phase (Day 7) of the sensitization study. For the control animals, 2.0 mL of distilled water was used. Table 8 shows the dermal reactions observed 24 hours after completion of the 48-hour topical inductions.

TABLE 8
Induction (Topical Application)

TREATED GROUP			
Animal Number	Dermal Effects*	Animal Number	Dermal Effects*
191	2	201	2
192	2	202	2
193	1	203	1
194	2	204	1
195	1	205	2
196	1	206	2
197	2	207	2
198	2	208	1
199	1	209	2
200	1	210	1
CONTROL GROUP			
Animal Number	Dermal Effects*	Animal Number	Dermal Effects*
211	1	216	0
212	1	217	1
213	1	218	1
214	0	219	1
215	0	220	1

* The sites were scored using the 4-point scale developed by Magnusson and Kligman (1969).

Challenge Application - For the test animals and control animals, a concentration of 25% of the test substance was applied to the left flank and a second patch with distilled water was applied to the right flank. Table 9 shows the individual responses following the challenge applications.

Sensitization Study, continued**TABLE 9**
Challenge

ANIMAL NUMBER (Treated Group)	SCORE*		ANIMAL NUMBER (Treated Group)	SCORE*	
	(Left Flank = Test Substance) (Right Flank = Vehicle)			(Left Flank = Test Substance) (Right Flank = Vehicle)	
	24 HOURS (Left, Right)	48 HOURS (Left, Right)		24 HOURS (Left, Right)	48 HOURS (Left, Right)
191	0,0	0,0	201	0,0	0,0
192	0,0	0,0	202	0,0	0,0
193	0,0	0,0	203	0,0	1,0
194	0,0	0,0	204	0,0	0,0
195	0,0	0,0	205	0,0	0,0
196	2,0	2,0	206	0,0	0,0
197	0,0	0,0	207	0,0	0,0
198	0,0	0,0	208	0,0	0,0
199	1,0	1,0	209	0,0	0,0
200	0,0	0,0	210	0,0	0,0
ANIMAL NUMBER (Control Group)	SCORE*		ANIMAL NUMBER (Control Group)	SCORE*	
	(Left Flank = Test Substance) (Right Flank = Vehicle)			(Left Flank = Test Substance) (Right Flank = Vehicle)	
	24 HOURS (Left, Right)	48 HOURS (Left, Right)		24 HOURS (Left, Right)	48 HOURS (Left, Right)
211	0,0	0,0	216	0,0	0,0
212	0,0	0,0	217	0,0	0,0
213	0,0	0,0	218	0,0	0,0
214	0,0	0,0	219	0,0	0,0
215	0,0	0,0	220	0,0	0,0

* Dermal reactions were scored at 24 and 48 hours after termination of the challenge exposure using the 4-point scale developed by Magnusson and Kligman (1969).

For three of the twenty test animals, minimal to moderate erythema was noted 24 or 48 hours after the challenge dose. No dermal responses were noted for the remaining 17 animals previously induced with the test substance or for the 10 control animals.

Weight Gain

All animals gained weight during the study. Table 10 lists the individual animal body weights.

TABLE 10
Individual Body Weights

ANIMAL NUMBER (Treated Group)	BODY WEIGHTS (grams)		ANIMAL NUMBER (Treated Group)	BODY WEIGHTS (grams)	
	START	END		START	END
191	497	627	201	447	567
192	444	567	202	428	461
193	512	715	203	466	518
194	467	606	204	452	637
195	505	683	205	449	565
196	391	492	206	471	571
197	440	567	207	456	593
198	416	536	208	413	536
199	428	528	209	470	560
200	506	587	210	448	533
ANIMAL NUMBER (Control Group)	BODY WEIGHTS (grams)		ANIMAL NUMBER (Control Group)	BODY WEIGHTS (grams)	
	START	END		START	END
211	504	703	216	428	514
212	451	608	217	419	533
213	400	570	218	445	575
214	451	501	219	426	545
215	451	491	220	452	605

DISCUSSION

A dermal sensitization study was initiated on April 12, 1999 with this test substance using the Guinea Pig Maximization Test (GPMT). A primary irritation screen was conducted prior to the sensitization study to determine if the test substance was a dermal irritant. In addition, the screen was used to determine concentrations of the test substance in distilled water or in an emulsion of equal parts Freund's Complete Adjuvant (FCA) and distilled water that could be injected via the intradermal route without eliciting a strong local or systemic toxic reaction. Based on results of the preliminary tests, a concentration of 1% test substance in distilled water and a concentration of 1% in the FCA emulsion were selected for the intradermal induction injections. A concentration of 25% test substance was used for both the topical inductions and the challenge exposure in the sensitization study.

For the 20 test animals, dermal responses were observed for three animals (15%) after the challenge dose. For the remaining 17 animals previously induced with the test substance and the 10 control animals, no dermal responses were noted.

A sensitization study conducted by the GPMT method with the known sensitizing agent, 2-mercaptobenzothiazole (CAS No. 149-30-4), was initiated (induction phase) in this laboratory on this strain of guinea pig on May 3, 1999 for method validation. In this instance, the known sensitizing agent produced a positive sensitization response.

CONCLUSION

For the GPMT method, a test substance which produced a dermal response in at least 30% of the test animals is considered positive for skin sensitization. In the current study, dermal responses were observed in only three of twenty animals, and therefore, the test substance was not considered to be a dermal sensitizer in guinea pigs. The test substance requires no label for sensitization by skin contact, as defined in the 18th Adaptation of the EC Classification, Packaging, and Labelling of Dangerous Substances Directive.

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HAEL #: 99-0005

KAN #: 812984-5

Final Report

**Mutagenicity Test on EK 99-0005, TMAE Measuring
Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells**

**PREPARED FOR:
Eastman Kodak Company**

**COVANCE STUDY NUMBER:
20309-0-437 OECD**



MUTAGENICITY TEST ON

EK 99-0005, TMAE

MEASURING CHROMOSOMAL ABERRATIONS IN
CHINESE HAMSTER OVARY (CHO) CELLS

FINAL REPORT

AUTHOR

Hemalatha Murli, Ph.D.

PERFORMING LABORATORY

Covance Laboratories Inc. (Covance)
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LABORATORY PROJECT IDENTIFICATION

Covance Study No.: 20309-0-437OECD

SUBMITTED TO

Eastman Kodak Company
Building 320, Kodak Park
Rochester, New York 14652-6272

STUDY COMPLETION DATE

May 17, 1999

QUALITY ASSURANCE STATEMENT

Project Title: Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells

Project No.: 20990

Assay No.: 20309

Protocol No.: 437OECD

Edition No.: 3

Quality Assurance inspections of the study and review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

<u>Inspection/Date</u>	<u>Findings Reported</u>	<u>Auditor</u>
Cell Seeding/03/10/1999	03/10/1999	J. Crouch
Draft Report Review/05/07/1999	05/07/1999	K. Groeninger
Final Report Review/05/17/1999	05/17/1999	K. Groeninger

K. Groeninger
Quality Assurance Unit

17 May 1999
Date Released

STUDY COMPLIANCE AND CERTIFICATION

The study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Organization for Economic Cooperation and Development Principles of Good Laboratory Practice C(97) 186/Final (effective 26 November 1997) with any applicable amendments. There were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results in this report are supported by an experimental data record and this record has been reviewed by the Study Director. All raw data, documentation, records, protocol and a copy of the final report generated as a result of this study will be archived in the storage facilities of Covance-Vienna for at least 1 year following submission of the final report to the Sponsor. After the 1-year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities of Covance-Vienna for an additional period of time, or sent to a storage facility designated by the Sponsor.

Submitted By:

Study Director:

Hemalatha Muri

Hemalatha Muri, Ph.D.

Mammalian Cytogenetics

Department of Genetic and Cellular Toxicology

5/17/99

Study Completion
Date

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ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of EK 99-0005, TMAE to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without metabolic activation.

The test substance was dissolved in sterile deionized water at a concentration of 160 mg/ml for the initial and confirmatory assays. The high dose tested, 1600 µg/ml, was achieved using a dosing volume of 1.0% (10.0 µl/ml) and the solvent control cultures were treated with 10.0 µl/ml of sterile deionized water. The high dose tested, 1600 µg/ml, is 10 mM of the test substance, the recommended high dose for this assay.

In the initial chromosomal aberrations assay, the treatment period was for 3.0 hours with and without metabolic activation and cultures were harvested 20.1 hours from the initiation of treatment. Concentrations of 10.9, 15.5, 22.2, 31.7, 45.3, 64.7, 92.4, 132, 188, 269, 384, 549, 784, 1120, and 1600 µg/ml were tested with and without metabolic activation. Cultures treated with concentrations of 549, 784, 1120, and 1600 µg/ml with and without metabolic activation were analyzed for chromosomal aberrations. No significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed in the cultures analyzed.

In a confirmatory chromosomal aberrations assay, the treatment period was for 17.8 hours without metabolic activation and 3.0 hours with metabolic activation, and cultures were harvested 20.0 hours from the initiation of treatment. Concentrations of 127, 253, 506, 675, 900, 1200, and 1600 µg/ml were tested without metabolic activation and 506, 675, 900, 1200, and 1600 µg/ml were tested with metabolic activation. Cultures treated with concentrations of 127, 253, 506, and 675 µg/ml without metabolic activation and 675, 900, 1200, and 1600 µg/ml with metabolic activation were analyzed for chromosomal aberrations. No significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed in the cultures analyzed.

EK 99-0005, TMAE was considered negative for inducing chromosome aberrations in CHO cells with and without metabolic activation.

Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells with EK 99-0005, TMAE

- 1.0 SPONSOR:** Eastman Kodak Company
- 2.0 MATERIAL (TEST SUBSTANCE)**
 - 2.1 Client's Identification:** 99-0005, TMAE, ACC No.: 812984-5
Internal Div. No.:10082843, Lot/Batch No.: BB8292-131J
 - 2.2 Date Received:** February 10, 1999
 - 2.3 Physical Description:** Transparent colorless liquid
 - 2.4 Storage Conditions:** Ambient
 - 2.5 Genetics Assay No.:** 20309
- 3.0 TYPE OF ASSAY:** Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells
- 4.0 PROTOCOL NO.:** 437OECD, Edition 3
- 5.0 STUDY DATES**
 - 5.1 Initiation Date:** February 11, 1999
 - 5.2 Experimental Start Date:** March 11, 1999
 - 5.3 Experimental Termination Date:** April 20, 1999
- 6.0 SUPERVISORY PERSONNEL**
 - 6.1 Study Director:** Hemalatha Murli, Ph.D.
 - 6.2 Laboratory Supervisor:** Emilie C. Beckelhimer, B.S.
- 7.0 OBJECTIVE**

The objective of this *in vitro* assay was to evaluate the ability of EK 99-0005, TMAE to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells, with and without metabolic activation. The assay design is based on OECD Guideline 473, updated and adopted July 21, 1997.

8.0 TEST SYSTEM RATIONALE

Aberrations are a consequence of failure or mistakes in repair processes such that breaks either do not rejoin or rejoin in abnormal configurations (Evans, 1962, 1976). Descriptions of the types of aberrations are provided in Section 16.0. Structural aberrations may be of two types, chromosome or chromatid:

- **Chromosome aberration:** structural chromosome damage expressed as breakage, or breakage followed by reunion, of both sister chromatids at an identical site.
- **Chromatid aberration:** structural chromosome damage expressed as breakage of single chromatids or breakage followed by reunion between chromatids. This is the most common type of structural aberration.

Most known chemical clastogens (chromosome-breaking agents) require a period of DNA synthesis to convert initial DNA damage into chromosome alterations visible at mitosis. At predetermined intervals after exposure to the test substance, the treated cells were treated with a metaphase-arresting substance, Colcemid[®], then harvested and stained, and metaphase cells were analyzed microscopically for the presence of chromosomal aberrations.

Many mutagenic chemicals do not act directly on DNA but do so after being converted to active intermediates by enzymes found in liver. CHO cells have little or no capacity to metabolize test substances, so an exogenous metabolic activation system (rat liver S9 homogenate) was included with a series of treatments to enhance the degree of conversion and the ability of the assay to detect clastogenic, metabolic intermediates.

Numerical aberrations (a change in the number of chromosomes from the modal number of 21 for the CHO cell line used in this assay) were not determined. However, the occurrence of polyploidy or endoreduplication, which were scored, might indicate that the test substance has the potential to induce numerical aberrations.

9.0 EXPERIMENTAL DESIGN

Replicate cultures were used at each dose level, and negative, solvent, and positive controls. The assays were conducted with approximately a 20-hour harvest time (approximately 1.5 normal cell cycle; Galloway *et al.*, 1994). Chromosomal aberrations were analyzed from the cultures treated at four concentrations, the negative and solvent control, and from one of the positive control concentrations. A summary of the treatment schedule for the chromosomal aberrations assays is given on the following page.

Summary of Chromosomal Aberrations Assay Treatment Schedule in Hours

Activation Condition	Test Substance	Wash	Colcemid®	Fixation
<u>Initial Trial</u>				
- S9	0	3.0	18.1	20.1
+ S9	0	3.0	18.1	20.1
<u>Confirmatory Trial</u>				
- S9	0	17.8	18.0	20.0
+ S9	0	3.0	18.1	20.0

10.0 MATERIALS AND METHODS**10.1 Test System**

The Chinese hamster ovary cells (CHO-WBL) used in this assay were from a permanent cell line and were originally obtained from the laboratory of Dr. S. Wolff, University of California, San Francisco. The cells have since been recloned to maintain karyotypic stability. This cell line has an average cycle time of 12 to 14 hours with a modal chromosome number of 21.

10.2 Cell Culture Medium

The CHO cells were grown in McCoy's 5a culture medium which was supplemented with 10% fetal bovine serum (FBS), L-glutamine (2mM), penicillin G (100 units/ml) and streptomycin (100 µg/ml). Cultures were incubated with loose caps in a humidified incubator at 37°C ± 2°C in an atmosphere of 5% ± 1.5% CO₂ in air.

10.3 Negative and Solvent Controls

In the nonactivation assays, negative controls were cultures which contain only cells and culture medium. Solvent controls were cultures containing the solvent for the test substance, sterile deionized water, at the highest concentration used in test cultures, 10.0 µl/ml. In the activation assays, the negative and solvent controls were the same as described in the nonactivation assays but with the S9 activation mix included.

10.4 Positive Control Agents

The positive control agents which were used in the assays were mitomycin C (MMC) for the nonactivation series and cyclophosphamide (CP) in the metabolic activation series. Mitomycin C (CAS# 50-07-7, Sigma, Lot # 116H2511) is a

clastogen that does not require metabolic activation. Cyclophosphamide (CAS # 6055-19-2, Sigma, Lot # 73H0846) does not act directly but must be converted to active intermediates by microsomal enzymes. In the chromosomal aberrations assays, two concentrations of MMC (0.75 and 1.5 $\mu\text{g/ml}$, initial assay; 0.10 and 0.15 $\mu\text{g/ml}$, confirmatory assay) and CP (5.0 and 10.0 $\mu\text{g/ml}$) were used to induce chromosomal aberrations in the CHO cells. One of the concentrations was analyzed in each of the aberration assays. Both MMC and CP were dissolved in water.

10.5 S9 Metabolic Activation System

The *in vitro* metabolic activation system (Maron and Ames, 1983) consisted of a rat liver post-mitochondrial fraction (S9) and an energy-producing system (NADP plus isocitric acid). Prior to collecting livers from male Sprague-Dawley rats, various P450 isoenzyme levels were increased by treatment of the rats with Aroclor™ 1254. Rats were treated once with 500 mg/kg of Aroclor™ 1254 and S9 prepared approximately 5 days later (Molecular Toxicology, Inc., Lot #897). The S9 fraction, prepared in sucrose or in potassium chloride, was retained frozen at $\leq -70^\circ\text{C}$ until use. Aliquots of S9 were thawed immediately before use and added to the other components to form the activation system described as follows:

<u>Component</u>	<u>Concentration in Cultures</u>
NADP (sodium salt)	1.5 mg/ml (1.8 mM)
Isocitric acid	2.7 mg/ml (10.5 mM)
Homogenate (S9 fraction)	15.0 $\mu\text{l/ml}$ * (1.5%)

* This concentration of rat S9, obtained from Molecular Toxicology Inc., Boone, NC, has consistently caused CP to be highly clastogenic for many different lots.

10.6 Aberrations Assay Without Metabolic Activation

Cultures were initiated by seeding 1.2×10^6 cells per 75 cm² flask into 10 ml of complete McCoy's 5a medium. One day after culture initiation, the cells were incubated at $\approx 37^\circ\text{C}$ with the test substance at predetermined concentrations for 3.0 hours for the initial assay or 17.8 hours in the confirmatory assay. The cultures were then washed with buffered saline. In the initial assay, the cells were refed with complete McCoy's 5a medium and incubated for the rest of the culture period up to the time of harvest with 0.1 $\mu\text{g/ml}$ Colcemid® present during the last 2.0 hours of incubation. In the confirmatory assay, cells were refed with complete McCoy's 5a medium with 0.1 $\mu\text{g/ml}$ Colcemid® and harvested 2.0 hours later.

10.7 Aberrations Assay With Metabolic Activation

Cultures were initiated by seeding 1.2×10^6 cells per 75 cm² flask into 10 ml of complete McCoy's 5a medium. One day after culture initiation, the cultures were incubated at $\approx 37^\circ\text{C}$ for 3.0 hours in the presence of the test substance and the S9 reaction mixture in McCoy's 5a medium without FBS. The cells were then washed twice with buffered saline and the cells were refed with complete McCoy's 5a medium. The cells were incubated for the rest of the culture period up to the time of harvest with 0.1 $\mu\text{g/ml}$ Colcemid[®] present during the last ≈ 2.0 hours of incubation. The cultures were then harvested.

10.8 Harvest Procedure

Prior to the harvest of the cultures, visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures were also evaluated for the presence of mitotic (large rounded cells) or dead cells floating in the medium. The cultures were then trypsinized to collect mitotic and interphase cells and were treated with 0.075 M KCl hypotonic solution. This treatment helps to swell the cells and thus disperse the chromosomes. The cultures were then fixed with an absolute methanol: glacial acetic acid (3:1, v:v) fixative before air-dried slides were prepared.

10.9 Slide Preparation and Staining

Slides were prepared by dropping the harvested cultures on clean slides. The slides were stained with 5% Giemsa solution for the analysis of mitotic index and chromosomal aberrations. All slides were then air-dried and mounted permanently.

10.10 Aberrations Analysis and Assay Evaluation

Cells were selected for good morphology and only cells with the number of centromeres equal to the modal number 21 ± 2 (range 19-23) were analyzed.

One hundred cells, if possible, from each replicate culture at four concentrations of the test substance and from the negative, solvent, and one dose of the positive control cultures were analyzed for the different types of chromosomal aberrations (Evans, 1962, 1976; See Section 16.0). At least 25 cells were analyzed from those cultures that had greater than 25% of cells with one or more aberrations. Mitotic index was evaluated from the negative control, vehicle control, and a range of concentrations by analyzing the number of mitotic cells in 1000 cells and the ratio expressed as a percentage of mitotic cells. Percent polyploidy and

endoreduplication were also analyzed by evaluating 100 metaphases, if available, and tabulated. For control of bias, all slides were coded prior to analysis. Cells with aberrations were recorded on the data sheets by the microscope stage location. Historical control data are presented in Table 9.

10.10.1 Data Presentation

Data were summarized in tables showing mitotic index, percent polyploidy, percent endoreduplication, the numbers of cells analyzed, types of aberrations found, frequencies of aberrations per cell, and percentages of cells bearing aberrations. Chromatid and isochromatid gaps were noted but were not added into the totals for aberration assessment since they are not considered to represent true breaks.

10.10.2 Assay Acceptance Criteria

An assay was considered acceptable for evaluation of test results only if all of the following criteria were satisfied. The metabolic activation and nonactivation sections of the aberrations assay were independent units and would be repeated independently, as needed, to satisfy the acceptance criteria.

Acceptable Controls

The negative (untreated) and the vehicle control cultures must contain less than approximately 5% cells with aberrations.

The positive control result must be significantly higher ($p < 0.01$) than the vehicle controls. If the positive control result in the test with S9 is adequate in an assay where activation and nonactivation assays are run concurrently, but the positive control in the nonactivation assay fails, the test need not be repeated because the S9 activation positive control demonstrates the sensitivity of the cells.

Acceptable High Dose

If the aberration results are negative and there is no significant reduction (approximately $\geq 50\%$) in mitotic index, the assay must include the highest applicable dose (a target dose of 10 mM or 5 mg/ml, whichever is lower) or a dose exceeding the solubility limit in culture medium. Testing was conducted at insoluble concentrations when a

well-dispersed suspension in culture medium was obtained that did not settle rapidly.

10.10.3 Acceptable Number of Doses

The assay must include at least three analyzable concentrations.

10.10.4 Assay Evaluation Criteria

The following factors are taken into account in evaluation of the test substance data:

- Percentage of cells with aberrations.
- Percentage of cells with more than one aberration.
- Evidence for increasing amounts of damage with increasing dose, i.e., a dose related increase in aberrations.

The experimental unit is the cell, and therefore the percentage of cells with structural aberrations was the basis for evaluation. Statistical analysis employed a Cochran-Armitage test for linear trend and Fisher's Exact Test (Thakur *et al.*, 1985) to compare the percentage of cells with aberrations (and, if applicable, the percentage of cells with more than one aberration) in treated cells to the results obtained for the vehicle controls

Statistical analysis was also performed for cells exhibiting polyploidy and/or endoreduplication in order to indicate significant ($p \leq 0.01$) increases in these events as indicators of possible induction of numerical aberrations; however, the test substance was evaluated only for structural aberrations and not for numerical aberrations by this protocol.

Evaluation of a Positive Response

A test substance was considered positive for inducing chromosomal aberrations if a significant increase (the difference was considered significant when $p \leq 0.01$) in the number of cells with chromosomal aberrations is observed at one or more concentrations. Statistical evaluation of the percentage of cells with more than one aberration provided an indication of the severity of the positive response observed. The linear trend test evaluated the dose responsiveness. If a significant increase was seen at one or more concentrations, a dose-response should be observed.

Evaluation of a Negative Response

A test substance was considered negative for inducing chromosomal aberrations if no significant increase was observed in the number of cells with chromosomal aberrations at any of the concentrations.

Equivocal Evaluation

Although most assays gave clearly positive or negative results, in rare cases the data set would preclude making a definitive judgment about the activity of the test substance. Results might remain equivocal or questionable regardless of the number of times the assay is repeated.

Although the evaluation criteria provided here normally was sufficient, the study director might use additional considerations to obtain a final evaluation of the test substance based upon the study director's scientific judgment.

11.0 RESULTS

11.1 Solubility and Dose Determination

The test substance was soluble in sterile deionized water and formed a transparent colorless solution at 160 mg/ml. This solution was dosed in the absence of cells using a 1% (10 µl/ml) dosing volume in McCoy's 5a culture medium. At a dosed concentration of 1600 µg/ml, the culture medium turned purple with no visible precipitate and the pH was 9.5. At 1600 µg/ml in HEPES buffered culture medium, the pH was 8.0 (the pH of the culture medium was 7.5). Based on the results of the solubility, a top stock solution of 160 mg/ml was selected with a dosing volume of 1% (10 µl/ml) to achieve top dose of 1600 µg/ml (10mM). Sterile deionized water (Covance, Lot # 31) was the vehicle of choice for this assay. All dosing was achieved with a 1.0% (10.0 µl/ml) dosing of the stock solutions prepared and the solvent control cultures were treated with 10.0 µl/ml of sterile deionized water. The culture medium was buffered with HEPES during the treatment period. The initial and confirmatory chromosomal aberrations assays were conducted with stock concentration of 160 mg/ml. Concentrations of 10.9, 15.5, 22.2, 31.7, 45.3, 64.7, 92.4, 132, 188, 269, 384, 549, 784, 1120, and 1600 µg/ml with and without metabolic activation were tested with a treatment period of 3.0 hours and a harvest of 20.1 hours from the initiation of treatment in the initial assay. The high dose tested, 1600 µg/ml, is 10 mM of the test substance, the recommended high dose for this assay. The stability of the test

substance under the preparation and dosing conditions used in this assay is the responsibility of the sponsor.

11.2 Chromosomal Aberrations Assay Without Metabolic Activation

Initial Assay

No visible signs of toxicity were observed in the cultures analyzed. Reductions of 6% and 21% were observed in the mitotic indices of the cultures treated with 269 and 384 $\mu\text{g/ml}$, respectively, as compared with the solvent control cultures (Table 1). Chromosomal aberrations were analyzed from the cultures treated with 549, 784, 1120, and 1600 $\mu\text{g/ml}$ (Table 2). No significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed in the cultures analyzed.

Based on the results from the initial assay, the confirmatory nonactivation aberrations assay was conducted testing concentrations of 127, 253, 506, 675, 900, 1200, and 1600 $\mu\text{g/ml}$. Treatment period was for 17.8 hours and cultures were harvested 20.0 hours from the initiation of treatment.

Confirmatory Assay

Unhealthy cell monolayers, floating dead cells, debris, and a severe reduction in the number of visible mitotic cells were observed in the cultures treated with 675, 900, 1200, and 1600 $\mu\text{g/ml}$. Slightly unhealthy cell monolayers and a slight reduction in the number of visible mitotic cells was observed in the cultures treated with 506 $\mu\text{g/ml}$. Reductions of 33%, 75%, 82%, 94%, and 95% were observed in the mitotic indices of the cultures treated with 506, 675, 900, 1200, and 1600 $\mu\text{g/ml}$, respectively, as compared with the solvent control cultures (Table 3). Chromosomal aberrations were analyzed from the cultures treated with 127, 253, 506, and 675 $\mu\text{g/ml}$ (Table 4). No significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed in the cultures analyzed.

The sensitivity of the cell cultures for induction of chromosomal aberrations was shown by the increased frequency of aberrations in the cells exposed to mitomycin C, the positive control agent. The test substance was considered negative for inducing chromosomal aberrations, polyploidy, and endoreduplication under nonactivation conditions.

11.3 Chromosomal Aberrations Assay With Metabolic Activation

Initial Trial

No visible signs of toxicity were observed in the cultures analyzed, except for a slight reduction of $\approx 15\%$ in the cell monolayer confluence in the cultures treated with 549 $\mu\text{g/ml}$. Reductions of 11%, 33%, 26%, 27%, 21%, and 22% were observed in the mitotic indices of the cultures treated with 269, 384, 549, 784, 1120, and 1600 $\mu\text{g/ml}$, respectively, as compared with the solvent control cultures (Table 5). Chromosomal aberrations were analyzed from the cultures treated with 549, 784, 1120, and 1600 $\mu\text{g/ml}$ (Table 6). No significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed in the cultures analyzed.

Based on the results from the initial assay, the confirmatory aberrations assay was conducted testing concentrations of 506, 675, 900, 1200, and 1600 $\mu\text{g/ml}$. Treatment period was for 3.0 hours and cultures were harvested 20.0 hours from the initiation of treatment.

Confirmatory Assay

No visible signs of toxicity were observed in the cultures analyzed. No reductions were observed in the mitotic index of the cultures analyzed, as compared with the solvent control cultures (Table 7). Chromosomal aberrations were analyzed from the cultures treated with 675, 900, 1200, and 1600 $\mu\text{g/ml}$ (Table 8). No significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed in the cultures analyzed.

The successful activation by the metabolic system was demonstrated by the increased incidence of cells with chromosomal aberrations in the cultures induced with cyclophosphamide, the positive control agent. The test substance was considered negative for inducing chromosomal aberrations or polyploidy, and weakly significant for endoreduplication under activation conditions.

12.0 CONCLUSION

The test substance was considered negative for inducing chromosome aberrations in CHO cells with and without metabolic activation.

13.0 REFERENCES

- Evans, H.J. (1962) Chromosomal aberrations produced by ionizing radiation, *International Review of Cytology*, **13**: 221-321.
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- Galloway, S.M., Aardema, M.J., Ishidate, M., Jr., Ivett, J.L., Kirkland, D.J., Morita, T., Mosesso, P., and Sofuni, T. (1994) Report from working group on in vitro tests for chromosomal aberrations, *Mutation Research*, **312 (3)**: 241-261.
- Maron, D.M., and Ames, B.N. (1983) Revised methods for the Salmonella mutagenicity test, *Mutation Research*, **113**: 173-215.
- Thakur, A.J., Berry, K.J., and Mielke, P.W., Jr. (1985) A FORTRAN program for testing trend and homogeneity in proportions, *Computer Programs in Biomedicine*, **19**: 229-233.

14.0 DEVIATION FROM THE SIGNED PROTOCOL

Inadvertently, the mitotic cells from one of the cultures treated with 15.5 µg/ml was added to the trypsinized monolayer of one of the solvent control cultures in the initial assay with metabolic activation. This had no impact on the study since mitotic index information was available from the other solvent control culture and the mitotic cells were available from both cultures for analysis; the cultures treated with 15.5 µg/ml were not needed for evaluation.

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KAN #: 812984-5

Final Report

Mutagenicity Test with EK 99-0005, TMAE
in the *Salmonella - Escherichia coli*/Mammalian-Microsome
Reverse Mutation Assay with a Confirmatory Assay

PREPARED FOR:
Eastman Kodak Company

COVANCE STUDY NUMBER:
20309-0-409R



MUTAGENICITY TEST WITH

EK 99-0005, TMAE

IN THE *SALMONELLA* - *ESCHERICHIA COLI*/MAMMALIAN-MICROSOME REVERSE
MUTATION ASSAY WITH A CONFIRMATORY ASSAY

FINAL REPORT

AUTHOR

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LABORATORY PROJECT ID

Covance Study No.: 20309-0-409R

SUBMITTED TO

Eastman Kodak Company
Bldg. 320, Kodak Park
Rochester, NY 14652-6272

STUDY COMPLETION DATE

April 13, 1999

QUALITY ASSURANCE STATEMENT

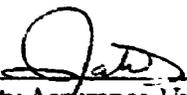
STUDY TITLE: *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay

ASSAY NO.: 20309-0-409R

PROTOCOL NO.: 409R, Edition 4, Modified for Eastman Kodak Company

Quality Assurance inspections of the study and review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

<u>Inspection/Date</u>	<u>Findings Reported</u>	<u>Auditor</u>
Weighing of Test Article - 02/18/99	02/18/99	K. Groeninger
Draft Report Review - 03/31/99	03/31/99	P. Cáceres
Final Report Review - 04/13/99	04/13/99	P. Cáceres


Quality Assurance Unit

4/13/99
Date Released

STUDY COMPLIANCE AND CERTIFICATION

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (21 CFR 58, 40 CFR 792, and 40 CFR 160) and Annex 2 of the OECD Guidelines for Testing of Chemicals (C(81)30 Final) as required by Council Directive 87/18/EEC of December 18, 1986. The study described herein also complies with the EEC Annex V Guideline number B.14, "Other Effects-Mutagenicity, *Salmonella Typhimurium*-Reverse Mutation Assay", and Guideline number B.13, "Other Effects-Mutagenicity, *Escherichia Coli*-Reverse Mutation Assay." There were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The stability of the test article under the conditions of administration was the responsibility of the Sponsor. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

Study Director:



Timothy E. Lawlor, M.A.
Bacterial Mutagenesis
Genetic and Cellular Toxicology

4.13.99

Study Completion Date

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SECTION I. SUMMARY

INTRODUCTION AND CONCLUSIONS

SUMMARY**A. Introduction**

At the request of Eastman Kodak Company, Covance Laboratories Inc. investigated the test article for mutagenic activity in the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay. This assay evaluated the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains and at the tryptophan locus in an *Escherichia coli* tester strain both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9).

The doses tested in the mutagenicity assay were selected based on the results of a dose ranging study using tester strains TA100 and WP2uvrA(pKM101) and ten doses of test article ranging from 5,000 to 6.67 µg per plate, one plate per dose, both in the presence and absence of S9 mix.

The tester strains used in the mutagenicity study were *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* tester strain WP2uvrA(pKM101). The assay was conducted with five dose levels of test article in both the presence and absence of S9 mix with concurrent vehicle and positive controls using three plates per dose. The doses tested ranged from 5,000 to 100 µg per plate in both the presence and absence of S9 mix. The results of the initial mutagenicity assay were confirmed in an independent experiment.

B. Conclusions

The results of the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay indicate that under the conditions of this study, Eastman Kodak Company's test article did not cause positive increases in the mean number of revertants per plate with any of the tester strains in either the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

SECTION II. STUDY INFORMATION

STUDY INFORMATION

- A. Sponsor: Eastman Kodak Company
- B. Test Article: EK 99-0005, TMAE (Lot number BB8292-131J)
Div. No.: 10082843
 - 1. Physical Description: transparent, colorless liquid
 - 2. Date Received: 02/10/99
- C. Type of Assay: *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay
 - 1. Protocol Number: Covance Protocol 409R, Edition 4,
Modified for Eastman Kodak Company
 - 2. Covance Study Number: 20309-0-409R
- D. Study Dates
 - 1. Study Initiation Date: 02/11/99
 - 2. Experimental Start Date: 02/15/99
 - 3. Experimental Termination Date: 03/02/99
- E. Study Supervisory Personnel
 - Study Director: Timothy E. Lawlor, M.A.
 - Laboratory Supervisor: Carlos E. Orantes, B.S.

SECTION III. MATERIALS AND METHODS

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames *et al.* (1975) and Green and Muriel (1976).

MATERIALS

A. Tester Strains

1. *Salmonella typhimurium*

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, and TA1537 as described by Ames *et al.* (1975). The specific genotypes of these strains are shown in Table I.

TABLE I. TESTER STRAIN GENOTYPES

Histidine Mutation			Additional Mutations		
<i>hisG46</i>	<i>hisC3076</i>	<i>hisD3052</i>	LPS	Repair	R Factor
TA1535	TA1537		<i>rfa</i>	<i>uvrB</i>	-
TA100		TA98	<i>rfa</i>	<i>uvrB</i>	+R

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The *rfa* wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the *uvrB* gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the *uvrB* deletion extends through the *bio* gene, all of the tester strains containing this deletion require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA1535 is reverted by base

substitution mutagens and TA100 is reverted by mutagens which cause both frameshift and base substitution mutations.

2. *Escherichia coli*

The tester strain used in the mutagenicity assay was the tryptophan auxotroph WP2*uvrA* as described by Green and Muriel (1976) and Brusick *et al.* (1980) containing the pKM101 plasmid.

In addition to a mutation in the tryptophan operon, the tester strain contains a *uvrA* DNA repair deficiency which enhances its sensitivity to some mutagenic compounds. This deficiency allows the strain to show enhanced mutability since the *uvrA* repair system would normally act to remove the damaged part of the DNA molecule and accurately repair it afterwards.

Tester strain WP2*uvrA*(pKM101) also contains the R-factor plasmid, pKM101, which further increases the sensitivity of this strain to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strain WP2*uvrA*(pKM101) is reverted from tryptophan dependence (auxotrophy) to tryptophan independence (prototrophy) by base substitution mutagens.

3. Source of Tester Strains

a. *Salmonella typhimurium*

The tester strains in use at Covance were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

b. *Escherichia coli*

The tester strain, WP2*uvrA*(pKM101), in use at Covance was received from The National Collection of Industrial Bacteria, Torrey Research Station, Scotland (United Kingdom).

4. Storage of the Tester Strains

a. Frozen Permanent Stocks

Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing small aliquots (0.5-1.5 ml) at $\leq -70^{\circ}\text{C}$.

b. Master Plates

Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with 1) for *Salmonella typhimurium*, an excess of histidine and biotin, and for tester strains TA98 and TA100, ampicillin (25 $\mu\text{g/ml}$), to ensure the stable maintenance of the pKM101 plasmid; and 2) for *Escherichia coli*, ampicillin (25 $\mu\text{g/ml}$) and an excess of tryptophan. Tester strain master plates were stored at $5 \pm 3^{\circ}\text{C}$.

5. Preparation of Overnight Cultures

a. Inoculation

Overnight cultures for use in all testing procedures were inoculated by transferring a colony from the appropriate master plate to a flask containing culture medium. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 ± 25 rpm; incubation, $37 \pm 2^{\circ}\text{C}$) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began.

b. Harvest

To ensure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring of culture turbidity. Cultures were harvested once a predetermined turbidity was reached as determined by a percent transmittance (%T) reading on a spectrophotometer. This target turbidity ensures that cultures have reached a density of at least 0.5×10^9 cells per ml and that the cultures have not overgrown. Overgrown (stationary) cultures may exhibit decreased sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached and were placed at $5 \pm 3^{\circ}\text{C}$.

6. Confirmation of Tester Strain Genotypes

Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

a. *Salmonella typhimurium*1) *rfa* Wall Mutation

The presence of the *rfa* wall mutation was confirmed by demonstration of the sensitivity of the culture to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

2) pKM101 Plasmid R-factor

The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

3) Characteristic Number of Spontaneous Revertants

The number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 µl aliquots of the culture along with the appropriate vehicle on selective media.

b. *Escherichia coli*

1) pKM101 Plasmid R-factor

The presence of the pKM101 plasmid was confirmed for tester strain WP2uvrA(pKM101) by demonstration of resistance to ampicillin. An aliquot of an overnight culture was overlaid onto a plate containing selective media and an antibiotic sensitivity disk containing 10 µg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone surrounding the disk.

2) Characteristic Number of Spontaneous Revertants

The number of spontaneous revertants per plate in the vehicle controls that is characteristic of the strain was demonstrated by plating 100 µl aliquots of the WP2uvrA(pKM101) culture along with the appropriate vehicle on selective media.

7. Tester Strain Media

a. Culturing Broth

The broth used to grow overnight cultures of the tester strains was Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

b. Agar Plates

Bottom agar (25 ml per 15 x 100 mm petri dish) was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 1.5% (w/v) agar and 0.2% (w/v) glucose.

c. Overlay Agar for Selection of Revertants

Overlay (top) agar was prepared with 0.7% agar (w/v) and 0.5% NaCl (w/v) and was supplemented with 10 ml of 1) 0.5 mM histidine/biotin solution per 100 ml agar for selection of histidine revertants, or 2) 0.5 mM tryptophan solution per 100 ml of agar for selection of tryptophan revertants. When S9 mix was required, 2.0 ml of the supplemented top agar was used in the overlay. However, when S9 mix was not required, water was added to the supplemented top agar (0.5 ml of water per 2 ml of supplemented top agar) and the resulting 2.5 ml of diluted supplemented top agar was used for the overlay. This dilution ensured that the final top agar and amino acid supplement concentrations remained the same both in the presence and absence of S9 mix.

B. Liver Microsomal Enzyme Reaction Mixture (S9 Mix)

1. S9 Homogenate

Liver microsomal enzymes (S9 homogenate) were purchased from Molecular Toxicology, Inc. (Batch 0904, 36.7 mg per ml). The homogenate was prepared from male Sprague-Dawley rats that had been injected (i.p.) with Aroclor™ 1254 (200 mg per ml in corn oil) at 500 mg/kg as described by Ames *et al.*, 1975.

2. S9 Mix

The S9 mix was prepared immediately prior to its use in any experimental procedure. The S9 mix contained the components indicated in Table II.

TABLE II. S9 MIX COMPONENTS

H ₂ O	0.70 ml
1M NaH ₂ PO ₄ /Na ₂ HPO ₄ , pH 7.4	0.10 ml
0.25M Glucose-6-phosphate	0.02 ml
0.10M NADP	0.04 ml
0.825M KCl/0.2M MgCl ₂	0.04 ml
S9 Homogenate	<u>0.10 ml</u>
	1.00 ml

C. Controls**1. Vehicle Controls**

Vehicle controls were plated for all tester strains both in the presence and absence of S9 mix. The vehicle control was plated, using a 50 µl aliquot of vehicle (equal to the maximum aliquot of test article dilution plated), along with a 100 µl aliquot of the appropriate tester strain and a 500 µl aliquot of S9 mix (when necessary), on selective agar.

2. Positive Controls

The combinations of positive controls, activation condition and tester strains plated concurrently with the assay are indicated in Table III.

TABLE III. POSITIVE CONTROLS

Tester Strain	S9 Mix	Positive Control	Conc. per plate
TA98	+	2-aminoanthracene	2.5 µg
TA98	-	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	-	sodium azide	2.0 µg
TA1535	+	2-aminoanthracene	2.5 µg
TA1535	-	sodium azide	2.0 µg
TA1537	+	2-aminoanthracene	2.5 µg
TA1537	-	ICR-191	2.0 µg
WP2uvrA(pKM101)	+	2-aminoanthracene	5.0 µg
WP2uvrA(pKM101)	-	4-nitroquinoline-N-oxide	2.0 µg

a. **Source and Grade of Positive Control Articles**

2-aminoanthracene (CAS# 613-13-8), Sigma Chemical Co., purity $\geq 97\%$; 2-nitrofluorene (CAS# 607-57-8), Aldrich Chemical Co., $\geq 98\%$; sodium azide (CAS# 26628-22-8), Sigma Chemical Co., purity $\geq 98\%$; ICR-191 (CAS# 1707-45-0), Sigma Chemical Co., purity $\geq 98\%$; 4-nitroquinoline-N-oxide (CAS# 56-57-5), Sigma Chemical Co., purity $\geq 99\%$.

3. **Sterility Controls**

a. **Test Article**

The most concentrated test article dilution was checked for sterility by plating a 50 μ l aliquot (the same volume used in the assay) on selective agar.

b. **S9 Mix**

The S9 mix was checked for sterility by plating 0.5 ml on selective agar.

METHODS

A. **Dose Ranging Study**

The growth inhibitory effect (cytotoxicity) of the test article to the test system was determined in order to allow the selection of appropriate doses to be tested in the mutagenicity assay.

1. **Design**

The dose ranging study was performed using tester strains TA100 and WP2uvrA(pKM101) both in the presence and absence of S9 mix. Ten doses of test article were tested at one plate per dose. The test article was checked for cytotoxicity up to a maximum concentration of 5 mg per plate if solubility/miscibility permitted.

a. **Rationale**

The cytotoxicity of the test article observed on tester strain TA100 is generally representative of that observed on the other tester strains and because of TA100's comparatively high number of spontaneous revertants per plate, gradations of cytotoxicity can be readily discerned from routine experimental variation. The *Escherichia coli* tester strain

WP2uvrA(pKM101) does not possess the *rfa* wall mutation that the *Salmonella typhimurium* strains have and thus, a different range of cytotoxicity may be observed. Also, the cytotoxicity induced by a test article in the presence of S9 mix may vary greatly from that observed in the absence of S9 mix. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the S9 mix.

2. Evaluation of the Dose Ranging Study

Cytotoxicity is detectable as a decrease in the number of revertant colonies per plate and/or by a thinning or disappearance of the bacterial background lawn.

3. Selection of the Maximum Dose for the Mutagenicity Assay

a. No Cytotoxicity Observed

Since no cytotoxicity was observed in the dose ranging study, the highest dose level of test article used in the mutagenicity assay was the same as that tested in the ranging study.

B. Mutagenicity Assay

1. Design

The assay was performed using tester strains TA98, TA100, TA1535, TA1537, and WP2uvrA(pKM101) both in the presence and absence of S9 mix. Five dose levels of test article were tested along with the appropriate vehicle and positive controls. The doses of test article were selected based on the results of the dose ranging study. The results of the initial mutagenicity assay were confirmed in an independent experiment.

2. Frequency and Route of Administration

The tester strains were exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. In the plate incorporation methodology, the test article, the tester strain and the S9 mix (where appropriate) were combined in molten agar which was overlaid onto a minimal agar plate. Following incubation at $37 \pm 2^\circ\text{C}$ for 48 ± 8 hours, revertant colonies were counted. All doses of the test article, the vehicle controls and the positive controls were plated in triplicate.

C. Plating Procedures

These procedures were used in both the dose ranging study and the mutagenicity assay.

Each plate was labeled with a code which identified the test article, test phase, tester strain, activation condition and dose. The S9 mix and dilutions of the test article were prepared immediately prior to their use.

When S9 mix was not required, 100 μ l of tester strain and 50 μ l of vehicle or test article dose were added to 2.5 ml of molten selective top agar (maintained at $45 \pm 2^\circ\text{C}$). When S9 mix was required, 500 μ l of S9 mix, 100 μ l of tester strain and 50 μ l of vehicle or test article dose were added to 2.0 ml of molten selective top agar. After the required components had been added, the mixture was vortexed and overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 48 ± 8 hours at $37 \pm 2^\circ\text{C}$. Positive control articles were plated using a 50 μ l plating aliquot.

D. Scoring the Plates

Plates which were not evaluated immediately following the incubation period were held at $5 \pm 3^\circ\text{C}$ until such time that colony counting and bacterial background lawn evaluation could take place.

1. Bacterial Background Lawn Evaluation

The condition of the bacterial background lawn was evaluated for evidence of cytotoxicity and test article precipitate. Evidence of cytotoxicity was scored relative to the vehicle control plate and was recorded along with the revertant counts for all plates at that dose on the data tables using the code system presented at the end of the Materials and Methods Section.

2. Counting Revertant Colonies

For the mutagenicity assay, the number of revertant colonies per plate for the vehicle controls and plates containing test article were counted manually except for the vehicle controls and plates containing test article with tester strain WP2uvrA(pKM101) in both the presence and absence of S9 mix in Experiment 20309-C1, which were counted by automated colony counter. The number of revertant colonies per plate for the positive controls were counted by automated colony counter with the exception of the positive controls for tester strain TA1537 in the absence of S9 mix in Experiment 20309-C1, which were counted manually.

E. Analysis of Data

For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The results of these calculations are presented in tabular form in the Data Tables Section of this report.

EVALUATION OF TEST RESULTS

Before assay data were evaluated, the criteria for a valid assay had to be met.

A. Criteria for a Valid Assay

The following criteria were used to determine a valid assay:

1. Tester Strain Integrity: *Salmonella typhimurium***a. *rfa* Wall Mutation**

To demonstrate the presence of the *rfa* wall mutation, tester strain cultures exhibited sensitivity to crystal violet.

b. pKM101 Plasmid

To demonstrate the presence of the R-factor plasmid, pKM101, cultures of tester strains TA98 and TA100 exhibited resistance to ampicillin.

c. Characteristic Number of Spontaneous Revertants

To demonstrate the requirement for histidine, the tester strain cultures exhibited a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable ranges for the vehicle controls were as follows:

TA98	8 - 60
TA100	60 - 240
TA1535	4 - 45
TA1537	2 - 25

2. Tester Strain Integrity: *Escherichia coli*

a. pKM101 Plasmid

To demonstrate the presence of the R-factor plasmid, pKM101, cultures of tester strain WP2uvrA(pKM101) exhibited resistance to ampicillin.

b. Characteristic Number of Spontaneous Revertants

To demonstrate the requirement for tryptophan, the tester strain culture exhibited a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable range for the WP2uvrA(pKM101) vehicle controls was 80 to 350 revertants per plate.

3. Tester Strain Culture Density

To demonstrate that appropriate numbers of bacteria are plated, the density of tester strain cultures were greater than or equal to 0.5×10^9 bacteria per ml and/or had reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 0.5×10^9 bacteria per ml.

4. Positive Control Values

a. Positive Control Values in the Absence of S9 Mix

To demonstrate that the tester strains were capable of identifying a mutagen, the mean value of a positive control for a respective tester strain exhibited at least a 3-fold increase over the mean value of the vehicle control for that strain.

b. Positive Control Values in the Presence of S9 Mix (S9 Mix Integrity)

To demonstrate that the S9 mix was capable of metabolizing a promutagen to its mutagenic form(s), the mean value of the positive control for a respective tester strain in the presence of the S9 mix exhibited at least a 3-fold increase over the mean value of the vehicle control for that strain.

An acceptable positive control in the presence of S9 mix for a specific strain was evaluated as having demonstrated both the integrity of the S9 mix and the ability of the tester strain to detect a mutagen.

5. Cytotoxicity

A minimum of three non-toxic doses were required to evaluate assay data.

B. Criteria for a Positive Response

Once the criteria for a valid assay had been met, responses observed in the assay were evaluated as follows:

1. Tester Strains TA98, TA100, and WP2uvrA(pKM101)

For a test article to be considered positive, it had to produce at least a 2-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate had to be accompanied by a dose response to increasing concentrations of the test article.

2. Tester Strains TA1535 and TA1537

For a test article to be considered positive, it had to produce at least a 3-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate had to be accompanied by a dose response to increasing concentrations of the test article.

RECORDS TO BE MAINTAINED

All raw data, documentation, records, the protocol, and the final report generated as a result of this study will be archived in the storage facilities of Covance-Vienna for at least one year following submission of the final report to the Sponsor. After the one year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities of Covance-Vienna for an additional period of time or sent to a storage facility designated by the Sponsor.

REFERENCES

- Ames, B.N., J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the *Salmonella*/Mammalian-Microsome Mutagenicity Test. *Mutation Research* 31:347-364 (1975).
- Brusick, D.J., V.F. Simmon, H.S. Rosenkranz, V.A. Ray, and R.S. Stafford. An evaluation of the *Escherichia coli* WP2 and WP2uvrA reverse mutation assay. *Mutation Research* 76:169-190 (1980).
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- Maron, D.M., and B. Ames. Revised methods for the *Salmonella* Mutagenicity Test. *Mutation Research* 113:173-215 (1983).
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BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

<u>CODE</u>	<u>DEFINITION</u>	<u>CHARACTERISTICS OF BACKGROUND LAWN</u>
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

sp	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
mp	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus requiring the plate to be hand counted.
hp	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4mp would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

SECTION IV. RESULTS AND CONCLUSIONS

RESULTS**A. Test Article Handling**

The test article was stored at room temperature. Water (Quality Biological Inc., Lot 707551) was used as the vehicle. At 100 mg per ml, which was the most concentrated stock dilution prepared for the mutagenicity assay, the test article was observed to form a transparent, colorless solution. The test article remained dissolved in all succeeding dilutions prepared for the mutagenicity assay.

B. Dose Rangefinding Study

Doses tested in the mutagenicity assay were selected based on the results of the dose rangefinding study conducted on the test article using tester strains TA100 and WP2uvrA(pKM101) in both the presence and absence of S9 mix (one plate per dose). Ten doses of test article, from 5,000 to 6.67 µg per plate, were tested and the results are presented in Tables 1 and 2. These data were generated in Experiment 20309-A1. In this experiment, no cytotoxicity was observed in either the presence or absence of S9 mix as evidenced by a normal background lawn and by no decrease in the number of revertants per plate. No test article precipitate was observed on the plates at any of the doses tested.

C. Mutagenicity Assay

The results of the dose rangefinding study were used to select the five doses tested in the initial mutagenicity assay. The doses tested were 5,000, 3,330, 1,000, 333, and 100 µg per plate in both the presence and absence of S9 mix.

The mutagenicity assay results for the test article are presented in Tables 3 through 6. These data were generated in Experiments 20309-B1 and 20309-C1. The data are presented as individual plate counts (Tables 3 and 5) and as mean revertants per plate ± standard deviation (Tables 4 and 6) for each treatment and control group.

In both the initial mutagenicity assay (Experiment 20309-B1, Tables 3 and 4), and the confirmatory assay (Experiment 20309-C1, Tables 5 and 6), all data generated were acceptable and no positive increases in the mean number of revertants per plate were observed with any of the tester strains in either the presence or absence of S9 mix.

All criteria for a valid study were met.

CONCLUSIONS

The results of the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay indicate that under the conditions of this study, Eastman Kodak Company's test article did not cause positive increases in the mean number of revertants per plate with any of the tester strains in either the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

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FINAL REPORT

3-AMINO-2,2,3-TRIMETHYLBUTYRIC ACID, METHYL ESTER

HAEL No.: 97-0132 KAN: 812984-5
CAS No.: 090886-53-6 CIN: 10082843
RAN: 317280Q

ACUTE DERMAL IRRITATION/CORROSION STUDY IN THE RABBIT

GUIDELINE

OECD: 404
EEC: Annex V., Test B.4

AUTHOR

Kenneth P. Shepard, B.S.

TESTING FACILITY

Toxicological Sciences Laboratory
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-6272
USA

LABORATORY PROJECT ID

97-0132A2

STUDY SPONSOR

Eastman Kodak Company

STUDY COMPLETION DATE

February 9, 1998

QUALITY ASSURANCE INSPECTION STATEMENT
(21 CFR 58.35(B) (7), 40 CFR 792.35(B) (7), AND 40 CFR 160.35(B) (7))

STUDY: 97-0132-1 STUDY DIRECTOR: SHEPARD, K.P.
ACCESSION NUMBER: 812984

PAGE 1
02/06/98

STUDY TYPE: ACUTE DERMAL IRRITATION TEST


(AUDITOR, QUALITY ASSURANCE UNIT)


DATE

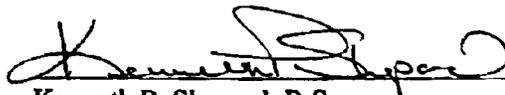
THIS STUDY WAS INSPECTED BY 1 OR MORE PERSONS OF THE QUALITY
ASSURANCE UNIT. WRITTEN STATUS REPORTS WERE SUBMITTED ON THE
FOLLOWING DATES.

INSPECTION DATES	PHASE(S) INSPECTED	STATUS REPORT DATES
-----	-----	-----
01/07/98	PROTOCOL APPENDIX/AMENDMENT SUBMISSION	
01/08/98	CLINICAL SIGNS AT 24 HRS.	02/06/98
02/05/98	FINAL REPORT REVIEW	02/05/98

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted according to:

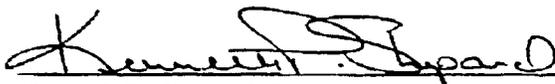
**Annex 2, Organisation for Economic Cooperation and Development, Guidelines
for Testing of Chemicals [C(81)30(Final)].**



Kenneth P. Shepard, B.S.
Study Director

February 9, 1998
Month/Day/Year

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Kenneth P. Shepard, B.S.
Study Director

February 9, 1998
Month/Day/Year



Douglas C. Topping, Ph.D.
Unit Director, Mammalian Toxicology

Feb 9, 1998
Month/Day/Year

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ABSTRACT**3-AMINO-2,2,3-TRIMETHYLBUTYRIC ACID, METHYL ESTER**

HAEL No.: 97-0132 KAN: 812984-5
CAS No.: 090886-53-6 CIN: 10082843
RAN: 317280Q

ACUTE DERMAL IRRITATION/CORROSION STUDY IN THE RABBIT

A dermal irritation/corrosion study was conducted by administering single topical doses of 0.5 milliliter of the test substance to three rabbits. The test substance was left in contact with the skin under an occlusive wrap for four hours. Skin lesions were graded according to OECD Guideline 404 (Annex V., Test B.4).

Signs of irritation included erythema (grade 2 or 3) and edema (grade 2) noted one hour after termination of exposure and erythema (grade 1) and edema (grade 1) noted at the 24-hour examinations. In addition, very slight to slight induration was noted at the three application sites from the 24-hour examinations to the Day 7 examinations. By the Day 14 examinations, slight desquamation was seen at all application sites, and at the Day 21 examinations, very slight desquamation or eschar formation was noted at the three application sites. The application site of each rabbit was also slightly discolored black from the initial one-hour examination to the 14-day examination. Reversibility of the irritant response was not seen by termination of the 21-day observation period.

Since inflammation of the skin on all three rabbits was still significant at the end of the observation period (21 days), the test substance is classified as "irritating to skin" as defined in the 18th Adaptation of the EC Classification, Packaging and Labelling of Dangerous Substances Directive.

STUDY AND TEST SUBSTANCE INFORMATION

Testing Facility

Toxicological Sciences Laboratory
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-6272
USA

Project Participants

Study Director:	Kenneth P. Shepard, B.S.
Principal Investigator:	John W. Mosher, B.S.
Report Author:	Kenneth P. Shepard, B.S.

Sponsor

Eastman Kodak Company

Test Substance Characterization

Test Substance Name:	3-Amino-2,2,3-trimethylbutyric acid, methyl ester
CAS No:	090886-53-6
HAEL No.:	97-0132
KAN:	812984-5
CIN:	10082843
SRID or Lot No.:	BB7527-184
Physical State and Appearance:	Liquid, Clear colorless
Source of Test Substance:	Eastman Kodak Company
Laboratory Project ID:	97-0132A2

Composition: Refer to composition information included in the notification when applicable.

Study Dates

Study Initiation Date:	January 7, 1998
Experimental Start Date:	January 7, 1998
Experimental Completion Date:	January 28, 1998

PURPOSE

The purpose of the study was to determine the potential of the test substance to cause primary irritation of mammalian skin.

MATERIALS AND METHODS

Test system

Three albino rabbits (Hra:(NZW)SPF) obtained from Covance Research Products Inc. (Denver, PA) were assigned to the study. The rabbits were young adults (at least three months old) and weighed at least 2000 grams at the start of the study. Rabbits were chosen for this study because they are a common representative species for dermal irritation studies. The rabbit is the preferred species recommended for use in the OECD Guideline.

Husbandry

Housing

Animals were housed in an American Association for Accreditation of Laboratory Animal Care-accredited vivarium in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The rabbits were singly housed in suspended, stainless-steel mesh cages. Cages and racks were washed once a week. Absorbent paper, used to collect excreta, was changed every other day.

Environmental Conditions

The study room was maintained at 18-21°C and 54-59% relative humidity. A photoperiod of 12 hours light from 6 a.m. to 6 p.m. was maintained.

Acclimation Period

The animals were isolated upon arrival and allowed to acclimate for a period of 5 days. Animals were judged to be healthy prior to testing.

Feed

Certified High Fiber Rabbit Diet (PMI® #5325) was available *ad libitum*. Feed containers were cleaned weekly and refilled at least once a week. No known contaminants which would interfere with the outcome of this study were present in the feed. Analyses of feed are maintained on file within the testing laboratory.

Husbandry, continued**Water**

Water was available *ad libitum* through an automatic watering system. The source of the water was the local public water system. There have been no contaminants identified in previous water analyses that would be expected to interfere with the conduct of the study. Semiannual analyses of water are maintained on file within the testing laboratory.

Identification

Upon arrival, all rabbits were identified by uniquely-numbered ear tags. Cage cards contained the study-specific animal number and the ear tag number.

Experimental Design**Test Procedures**

This study was conducted according to the Organisation for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals: Guideline 404, Acute Dermal Irritation/Corrosion; and European Economic Community (EEC): Annex V., Test B.4, Acute Toxicity (Skin Irritation).

Identification Numbers of Animals Used

Animal numbers 379, 380, and 381 were used in this study.

Preparation of Test Substance

The test substance, a liquid, was administered as received.

Test Substance Exposure

The hair was removed from an area of the dorsal skin with an electric clipper. A single dose of 0.5 milliliter of the test substance was applied topically to each animal using a fiber pad. An occlusive wrap was used to hold the pad with the test substance in place for a period of four hours. At the end of the exposure period, the application site was rinsed with acetone and running water.

Experimental Design, continued**Control Substance**

No control substance was used. Adjacent areas of untreated skin of each animal served as control sites for the test areas.

Vehicle

No vehicle was used.

Clinical Observations

The site of application was examined at 1, 24, 48, and 72 hours, and 7, 14, and 21 days after removal of the occlusive patch. Observations included estimation of erythema, edema, necrosis, eschar formation, scarring, erosion, and staining caused by the test substance as well as general systemic effects.

Body Weight

Body weights were measured on the day of initiation of the study.

Necropsy

No necropsies were conducted at the conclusion of the observation period.

Data Storage

The final report, data sheets, all nonperishable raw data, and an aliquot of the test substance have been stored in the testing facility archive managed under GLP-mandated conditions.

Statistical Procedures

Evaluation of data was not done statistically, but rather by the following method. The application site of each animal was graded as described in OECD Guideline 404 (Annex V., Test B.4) (Grading of Skin Reaction).

Protocol and Standard Operating Procedure Deviations

There were no SOP or protocol deviations during the study.

RESULTS**Observations for Erythema and Edema**

The application site of each animal was examined for signs of erythema and edema and the responses scored at 1, 24, 48, and 72 hours, and 7, 14, and 21 days after termination of exposure to the test substance. Additional observations of induration, desquamation, and eschar formation are footnoted.

ANIMAL NUMBER	RESPONSE AT APPLICATION SITE (Erythema, Edema) ¹						
	1 Hour	24 Hours	48 Hours	72 Hours	7 Days	14 Days	21 Days
379	2,2	1,1 ²	0,0 ²	0,0 ²	0,0 ²	0,0 ³	4 ⁴ ,0
380	2,2	1,1 ²	0,0 ²	0,0 ²	0,0 ²	0,0 ³	4 ⁴ ,0
381	3,2	1,1 ²	0,0 ⁵	0,0 ⁵	0,0 ⁵	0,0 ³	0,0 ⁶

¹ Graded as described in OECD Guideline 404 (Annex V., Test B.4) (Grading of Skin Reaction)

² Very slight induration noted at the application site.

³ Slight desquamation noted at the application site.

⁴ Erythema graded as 4 due to eschar formation (approximately 2x2 cm).

⁵ Slight induration noted at the application site.

⁶ Very slight desquamation noted at the application site.

Description of Observations Other Irritation

The application site for each rabbit was slightly discolored black from the initial one-hour examinations to the 14-day examinations.

Toxic Effects

No toxic effects were noted during the study.

Body Weights

At initiation of the study, Rabbit Numbers 379, 380, and 381 weighed 2685, 2639, and 2620 grams, respectively.

DISCUSSION

Prior to initiation of the dermal irritation study, the pH of the test substance was measured in an attempt to avoid testing substances which might produce significant irritation to the skin of rabbits. The pH value of the test substance was 10.2 which did not classify the substance as a strong acid ($\text{pH} \leq 2.0$) or as a strong alkali ($\text{pH} \geq 11.0$). Therefore, a dermal irritation study was conducted in which single doses of 0.5 milliliter of the test substance was applied to the skin of three rabbits.

Signs of irritation included erythema (grade 2 or 3) and edema (grade 2) noted one hour after termination of exposure and erythema (grade 1) and edema (grade 1) noted at the 24-hour examinations. In addition, very slight to slight induration was noted at the three application sites from the 24-hour examinations to the Day 7 examinations. By the Day 14 examinations, slight desquamation was seen at all application sites, and at the Day 21 examinations, very slight desquamation or eschar formation was noted at the three application sites. The application site of each rabbit was also slightly discolored black from the initial one-hour examination to the 14-day examination. Reversibility of the irritant response was not seen by termination of the 21-day observation period

CONCLUSION

Since inflammation of the skin on all three rabbits was still significant at the end of the observation period (21 days), the test substance is classified as "irritating to skin" as defined in the 18th Adaptation of the EC Classification, Packaging and Labelling of Dangerous Substances Directive.

REFERENCES

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FINAL REPORT

3-AMINO-2,2,3-TRIMETHYLBUTYRIC ACID, METHYL ESTER

HAEL No.: 97-0132 KAN: 812984-5
CAS No.: 090886-53-6 CIN: 10082843
RAN: 317406G

ACUTE ORAL TOXICITY STUDY IN THE RAT

GUIDELINE

OECD: 401
EEC: Annex V., Test B.1

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LABORATORY PROJECT ID

97-0132A0

STUDY SPONSOR

Eastman Kodak Company

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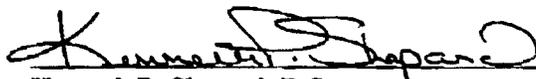
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

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**Annex 2, Organisation for Economic Cooperation and Development, Guidelines
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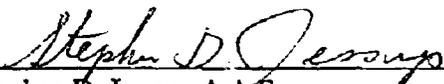


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March 23, 1998

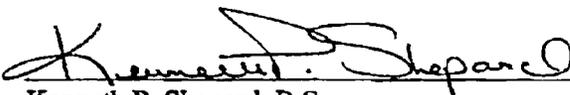
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March 19, 1998
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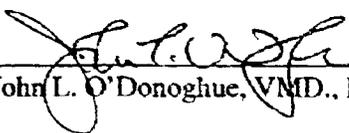
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March 19, 1998
Month/Day/Year



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3/21/98
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ABSTRACT**3-AMINO-2,2,3-TRIMETHYLBUTYRIC ACID, METHYL ESTER**

HAEL No.: 97-0132 KAN: 812984-5
CAS No.: 090886-53-6 CIN: 10082843
RAN: 317406G

ACUTE ORAL TOXICITY STUDY IN THE RAT

An acute oral toxicity study was conducted with three groups of five male and female rats administered 2000, 1000, or 500 mg/kg of the test substance by gavage. The test substance, a liquid, was administered as received. All animals at the 2000 mg/kg dose level died within 24 hours of dosing. Additional mortality was limited to a single female at the 1000 mg/kg dose level which was found dead at the 48-hour examinations. The acute oral LD₅₀ for this test substance was calculated to be 1414 mg/kg for male rats and 1231 mg/kg for female rats.

Abnormal clinical signs observed on the day of dosing and prior to death for the 2000 mg/kg animals were limited to convulsions and tremors. For the single female which died after administration of 1000 mg/kg of the test substance, no abnormal signs were seen on the day of dosing. On the day following dosing, a reduced amount of feces, staining (urine) of the inguinal hair, and staining (porphyrin) of the hair around the nose area were noted for this female. For the remaining animals at the 1000 mg/kg dose level, abnormal clinical signs included tremors (only on the day of dosing), a reduced amount of feces, staining (urine) of the inguinal hair, and dry staining (porphyrin) of the hair of the face. Gasping and reduced activity were noted for a single female at the 1000 mg/kg dose level on Day 2 of the study, but by the following day, these two signs were no longer evident. For the 500 mg/kg dose group, all males appeared clinically normal throughout the 14-day observation period. Abnormal signs noted for females at the 500 mg/kg dose level were limited to tremors (for a single female on the day of dosing), a reduced amount of feces, and staining (urine) of the inguinal hair. All surviving animals gained weight during both weeks of the study.

The cause of death for the rats which died after exposure to the test substance was not determined. The major treatment-related change observed at necropsy indicated that the test substance was a gastric irritant as evidenced by hemorrhage, edema, and necrosis of the glandular gastric mucosa.

Based on the oral LD₅₀ calculated by combining the male and female mortality data (1320 mg/kg), the test substance was classified as slightly toxic in rats according to the criteria set forth by Hodge and Sterner (1949) and harmful if swallowed as defined in the 18th Adaptation of the EC Classification, Packaging and Labelling of Dangerous Substances Directive.

STUDY AND TEST SUBSTANCE INFORMATION

Testing Facility

Toxicological Sciences Laboratory
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-6272
USA

Project Participants

Study Director:	Kenneth P. Shepard, B.S.
Principal Investigator:	John W. Mosher, B.S.
Report Authors:	Kenneth P. Shepard, B.S. Stephen D. Jessup, A.A.S.

Sponsor

Eastman Kodak Company

Test Substance Characterization

Test Substance Name:	3-Amino-2,2,3-trimethylbutyric acid, methyl ester
CAS No:	090886-53-6
HAEL No.:	97-0132
KAN:	812984-5
CIN:	10082843
SRID or Lot No.:	BB7527-184
Physical State and Appearance:	Liquid, Clear colorless
Source of Test Substance:	Eastman Kodak Company
Laboratory Project ID:	97-0132A0

Composition: Refer to composition information included in the notification when applicable.

Study Dates

Study Initiation Date:	January 12, 1998
Experimental Start Date:	January 12, 1998
Experimental Completion Date:	February 11, 1998

PURPOSE

The purpose of the study was to determine the estimated oral LD₅₀ of the test substance in male and female rats and the clinical signs of toxicity associated with a single oral dose.

MATERIALS AND METHODS

Test system

Male and female Sprague-Dawley® rats (SAS:VAF⁶(SD) obtained from SASCO, Inc., (Kingston, NY) were randomly assigned to each dose group. At initiation of the study, male rats were 8-10 weeks of age and weighed 212 to 271 grams and female rats were 9-11 weeks of age and weighed 157 to 182 grams. Rats were chosen for this study because they are a common representative species for toxicity studies. The rat is one of two primary rodent species recommended for use in the OECD Guideline.

Husbandry

Housing

Animals were housed in an American Association for Accreditation of Laboratory Animal Care-accredited vivarium in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The rats were singly housed in suspended, stainless-steel, wire mesh cages. Cages and racks were washed once a week. Absorbent paper, used to collect excreta, was changed at least three times a week.

Environmental Conditions

The study room was maintained at 20-22 °C and 50-51% relative humidity. A photoperiod of 12 hours light from 6 a.m. to 6 p.m. was maintained.

Acclimation Period

The animals were isolated upon arrival and allowed to acclimate for a period of 5 days. Animals were judged to be healthy prior to testing.

Husbandry, continued**Feed**

Certified Rodent Diet (PMI® #5002, pelleted) was available *ad libitum*. Feed containers were cleaned weekly and refilled at least once a week. No known contaminants which would interfere with the outcome of this study were present in the feed. Analyses of feed are maintained on file within the testing laboratory.

Water

Water was available *ad libitum* through an automatic watering system. The source of the water was the local public water system. There have been no contaminants identified in periodic water analyses that would be expected to interfere with the conduct of the study. Semiannual analyses of water are maintained on file within the testing laboratory.

Identification

Upon arrival, all rats were identified by uniquely-numbered metal ear tags. During randomization, study-specific animal numbers were assigned to each animal. Cage cards contained the study-specific animal number and the ear tag number.

Experimental Design**Test Procedures**

This study was conducted according to the Organisation for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals Guideline: 401, Acute Oral Toxicity; and European Economic Community (EEC): Annex V., Test B.1, Acute Toxicity (Oral).

Randomization

The procedure for including animals in the study was to randomly select and assign animals from the same shipment to the study. Randomization was done by computer-generated lists. After assignment of animals to the study, the body weights were determined to ensure that individual body weights did not exceed 20% of the mean weight for each sex.

Experimental Design, continued**Determination of Dose Levels**

Initially, a limit dose of 2000 mg/kg of the test substance was administered to the animals. Due to the mortality rate at this dose level, additional dose levels of 1000 and 500 mg/kg of the test substance were selected for the oral toxicity study.

Preparation of Test Substance in the Vehicle

The test substance, a liquid, was administered as received.

Test Substance Exposure

A single dose of the test substance was administered by gavage to animals that had been fasted overnight.

Distribution of Animals**TABLE 1**

Dose Level	Number Of Animals	Animal Numbers	
		Males	Females
Range-Finding Test			
1000 mg/kg	1 Male & 1 Female	53	56
500 mg/kg	1 Male & 1 Female	52	55
250 mg/kg	1 Male & 1 Female	51	54
Oral Toxicity Study			
2000 mg/kg	5 Males & 5 Females	1 - 5	6 - 10
1000 mg/kg	5 Males & 5 Females	96 - 100	106 - 110
500 mg/kg	5 Males & 5 Females	91 - 95	101 - 105

Body Weights

Body weights were collected on Days 0 (prior to treatment), 7, and 14.

Experimental Design, continued**Clinical Observations**

Animals were observed three times on the day of dosing (Day 0), and once each day thereafter for the duration of the experiment. Observations included, but were not limited to, examination of the hair, skin, eyes, mucous membranes, motor activity, feces, urine, respiratory system, circulatory system, autonomic nervous system, central nervous system, and behavior patterns.

Necropsy

Any animal that died during the study was necropsied on the day of death. All surviving animals were euthanatized and necropsied at the completion of the 14-day observation period.

Data Storage

The final report, data sheets, all nonperishable raw data, and an aliquot of the test substance have been stored in the testing facility archive managed under GLP-mandated conditions.

Statistical Procedures

No statistical procedures were required during the study. No dose/mortality curve was constructed since graphs become statistically useful only with the use of large numbers of animals and dose groups.

The LD₅₀ values and 95% confidence intervals were determined separately for male and female rats and, if different, for male and female rats combined according to the method of Weil (Weil, C.S., 1952).

Protocol and Standard Operating Procedure Deviations

There were no SOP or protocol deviations during the study.

RESULTS**RANGE-FINDING TEST**

Due to the mortality rate at a dose level of 2000 mg/kg in the oral toxicity study, a range-finding test was conducted using one animal of each sex administered a single oral dose of 250, 500, or 1000 mg/kg. Mortality in the range-finding test was limited to the female rat at the 1000 mg/kg dose level. Based on the range-finding test, additional dose levels of 500 and 1000 mg/kg were selected for the oral toxicity study.

ORAL TOXICITY STUDYMortality

Mortality was 100% for both sexes at a dose level of 2000 mg/kg, 0% for male rats and 20% for female rats at a dose level of 1000 mg/kg, and 0% for male and female rats at a dose level of 500 mg/kg. The dose level, the number of animals dosed, the number of deaths, and the day of death are listed in Table 2.

TABLE 2
Mortality Table

Dose (mg/kg)	Number Of Rats Exposed (Male, Female)	Number Of Deaths (Male, Female)	Time Of Death
2000	5,5	5,5	Days 0-1
1000	5,5	0,1	Day 2
500	5,5	0,0	Not Applicable

LD₅₀ for male rats: 1414 mg/kg (95% C.I. = 1072 - 1866 mg/kg)¹
 LD₅₀ for female rats: 1231 mg/kg (95% C.I. = 933 - 1625 mg/kg)¹
 LD₅₀ for the combined sexes: 1320 mg/kg (95% C.I. = 1149 - 1516 mg/kg)¹

¹ Calculated according to the method of Weil (Weil, C.S., 1952).

Clinical Signs

The time of each observation during the 14-day period and the number of animals involved at each dose level are listed in Table 3.

TABLE 3
Table Of Clinical Observations

Dose (mg/kg)	Time	Clinical Signs	Number of Animals Affected
2000	Day 0 (Immediate)	Appeared Clinically Normal	5/5 Males, 5/5 Females
2000	Day 0 (1-Hour)	Died Appeared Clinically Normal Convulsions (Prior to Death) Tremors (Prior to Death)	1/5 Males, 2/5 Females 2/4 Males, 1/3 Females 1/4 Males 1/4 Males, 2/3 Females
2000	Day 0 (4-Hours)	Died Tremors (Prior to Death)	3/4 Males, 3/3 Females 1/1 Males
2000	Day 1	Died	1/1 Males

1000	Day 0 (Immediate)	Appeared Clinically Normal	5/5 Males, 5/5 Females
1000	Day 0 (1 Hour)	Appeared Clinically Normal Tremors	4/5 Males, 4/5 Females 1/5 Males, 1/5 Females
1000	Day 0 (4-Hours)	Appeared Clinically Normal Tremors	1/5 Males, 4/5 Females 4/5 Males, 1/5 Females
1000	Day 1	Reduced Amount of Feces Staining (Urine) of Inguinal Hair Dry Stain (Porphyrin) of Hair of Face	5/5 Males, 5/5 Females 3/5 Males, 3/5 Females 3/5 Males, 1/5 Females

Table continued on next page.

TABLE 3, continued

Table Of Clinical Observations

1000	Day 2	Died Reduced Amount of Feces Staining (Urine) of Inguinal Hair Dry Stain (Porphyrin) of Hair of Face Reduced Activity and Gasping	1/5 Females 5/5 Males, 4/4 Females 3/5 Males, 1/4 Females 1/4 Females 1/4 Females
1000	Day 3	Appeared Clinically Normal Reduced Feces Amount	4/5 Males, 2/4 Females 1/5 Males, 2/4 Females
1000	Days 4-14	Appeared Clinically Normal	5/5 Males, 4/4 Females

500	Day 0 (Immediate)	Appeared Clinically Normal	5/5 Males, 5/5 Females
500	Day 0 (1 Hour)	Appeared Clinically Normal Tremors	5/5 Males, 4/5 Females 1/5 Females
500	Day 0 (4-Hours)	Appeared Clinically Normal	5/5 Males, 5/5 Females
500	Day 1	Appeared Clinically Normal Reduced Amount of Feces Staining (Urine) of Inguinal Hair	5/5 Males 5/5 Females 2/5 Females
500	Day 2	Appeared Clinically Normal Reduced Amount of Feces Staining (Urine) of Inguinal Hair	5/5 Males, 3/5 Females 2/5 Females 2/5 Females
500	Day 3	Appeared Clinically Normal Reduced Amount of Feces Staining (Urine) of Inguinal Hair	5/5 Males, 3/5 Females 1/5 Females 2/5 Females
500	Day 4	Appeared Clinically Normal	5/5 Males, 5/5 Females
500	Days 5-8	Appeared Clinically Normal Staining (Urine) of Inguinal Hair	5/5 Males, 4/5 Females 1/5 Females
500	Days 9-14	Appeared Clinically Normal	5/5 Males, 5/5 Females

Weight Gain

All animals which survived to termination of the 14-day observation period gained weight during both weeks of the study.

Individual Body Weights

The individual body weights are listed in Table 4.

TABLE 4
Table Of Individual Body Weights (grams)

Dose (mg/kg)	Animal Number	Day 0	Day 7	Day 14
MALE RATS				
2000	1	266	Died Day 1	*
2000	2	268	Died Day 0	*
2000	3	252	Died Day 0	*
2000	4	248	Died Day 0	*
2000	5	231	Died Day 0	*
1000	96	271	313	344
1000	97	260	294	332
1000	98	250	300	330
1000	99	265	298	326
1000	100	212	220	249
500	91	215	255	268
500	92	253	298	327
500	93	243	282	300
500	94	244	289	311
500	95	268	328	358

* A terminal body weight was not recorded for any animal which died within 24 hours of dosing.

Table continued on next page.

TABLE 4, continued
Table Of Individual Body Weights (grams)

Dose (mg/kg)	Animal Number	Day 0	Day 7	Day 14 (Terminal)
FEMALE RATS				
2000	6	176	Died Day 0	*
2000	7	172	Died Day 0	*
2000	8	161	Died Day 0	*
2000	9	167	Died Day 0	*
2000	10	172	Died Day 0	*
1000	106	174	Died Day 2	(150)
1000	107	169	214	228
1000	108	177	192	214
1000	109	174	202	207
1000	110	181	212	231
500	101	164	186	206
500	102	180	200	207
500	103	182	204	213
500	104	157	175	189
500	105	168	190	204

* A terminal body weight was not recorded for any animal which died within 24 hours of dosing.

Necropsy Findings

For animals at the 2000 mg/kg dose level, all of which died within 24 hours after administration of the test substance, changes seen at necropsy included incomplete collapse of the lungs; red discoloration of the lungs; hemorrhage and edema of the glandular gastric mucosa; and red fluid present in the stomach, duodenum, jejunum, and ileum. Moderate autolysis was noted for the male rat which was found dead on the morning following dosing.

For the single female in the 1000 mg/kg dose group that died on Day 2 of the study, similar gastrointestinal changes were noted. These changes included necrosis and hemorrhage of the glandular gastric mucosa; red fluid present in the stomach, duodenum, and jejunum; and distension of the stomach, jejunum, ileum, and cecum due to gas. Additional changes seen for this female included a dark liver, a wet urine stain on the hair of the inguinal area, and a dried porphyrin stain on the hair around the nose.

For all remaining animals at the 1000 mg/kg dose level and all animals at the 500 mg/kg dose level which survived to completion of the 14-day observation period, changes noted at necropsy were limited to hydrometra of the uterus for a single female at the 1000 mg/kg dose and for three females at the 500 mg/kg dose level.

No tissue was collected for microscopic examination. A detailed record of the incidence and severity of all gross abnormalities is presented in computer-generated tables which are included in the Appendix.

DISCUSSION

Due to 100% mortality at a dose level of 2000 mg/kg in the oral toxicity study, a range-finding test was conducted using one animal of each sex administered a single oral dose of 250, 500, or 1000 mg/kg. Mortality in the range-finding test was limited to the female rat at the 1000 mg/kg dose level. Based on the range-finding test, additional dose levels of 500 and 1000 mg/kg were selected for the oral toxicity study. At the 1000 and 500 mg/kg dose levels, mortality was 0% for male rats and 20% for female rats at the 1000 mg/kg dose level and 0% for male and female rats at a dose level of 500 mg/kg. The acute oral LD₅₀ for this test substance was calculated to be 1414 mg/kg for male rats and 1231 mg/kg for female rats.

Abnormal clinical signs observed on the day of dosing and prior to death for the 2000 mg/kg animals were limited to convulsions and tremors. For the single female which died after administration of 1000 mg/kg of the test substance, abnormal signs included a reduced amount of feces, staining (urine) of the inguinal hair, and dry staining (porphyrin) of the hair of the face on the day following dosing. For the remaining animals at the 1000 mg/kg dose level, abnormal clinical signs included tremors only on the day of dosing, a reduced amount of feces, staining (urine) of the inguinal hair, and dry staining (porphyrin) of the hair of the face. Gasping and reduced activity were noted for a single female at the 1000 mg/kg dose level on Day 2, but by the following day, these two signs were no longer evident. For the 500 mg/kg dose group, all males appeared clinically normal throughout the study. Abnormal signs noted for females at the 500 mg/kg dose level were limited to tremors (for a single female on the day of dosing), a reduced amount of feces, and staining (urine) of the inguinal hair. All surviving animals at the 1000 mg/kg dose level and all animals at the 500 mg/kg dose level gained weight during both weeks of the study.

The cause of death for rats which died after exposure to the test substance was not determined. For these animals, treatment-related changes observed at necropsy suggest that the test substance was a gastric irritant as evidenced by hemorrhage, edema, and necrosis of the glandular gastric mucosa and the presence of red fluid in the stomach, duodenum, jejunum, and ileum.

The incomplete collapse of the lungs which was seen only in rats that had died on the day of dosing was considered an agonal effect occurring shortly prior to death. Red discoloration of the lungs, probably hemorrhage, was also observed only in rats which died on the day of dosing. Hemorrhage in the lungs of rats is often an agonal phenomenon related to the mode of death (Innes et. al., 1967).

Moderate autolysis, a dark liver, and distension of the gastrointestinal tract with gas were noted for the male rat that died after the last examination on the day of dosing but prior to examinations on the following morning. These changes were not considered significant because they are typically seen in animals where there is a delay between death and necropsy.

A dried porphyrin stain was present on hair around the nose of the single female rat assigned to the 1000 mg/kg dose level which died on Day 2. Stress was considered the most probable cause for the porphyrin formation. Porphyrin discharge is also occasionally observed in normal, untreated control rats. Porphyrin staining was not considered treatment-related. A wet urine stain was also seen on the hair of the inguinal area for this female. This change was probably treatment-related since it was seen in all dose groups during clinical observations.

Hydrometra was an incidental finding. Hydrometra is the dilation of the uterus with an accumulation of ovulatory intraluminal fluid during the estrus cycle.

In the absence of significant gross organ lesions, other than the obvious signs of gastric irritation, no tissue was collected for microscopic examination.

CONCLUSION

The test substance was a gastric irritant as evidenced by hemorrhage, edema, and necrosis in the glandular gastric mucosa of rats that died from the 1000 and 2000 mg/kg dose groups. The acute oral LD₅₀ for this test substance was calculated to be 1414 mg/kg for male rats and 1231 for female rats. Based on the oral LD₅₀ calculated by combining the male and female mortality data (1320 mg/kg), the test substance was classified as slightly toxic in rats according to the criteria set forth by Hodge and Sterner (1949) and harmful if swallowed as defined in the 18th Adaptation of the EC Classification, Packaging and Labelling of Dangerous Substances Directive.

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FINAL REPORT

3-AMINO-2,2,3-TRIMETHYLBUTYRIC ACID METHYL ESTER

HAEL No.: 99-0005

KAN: 812984-5

CIN: 10082843

CAS: 90886-53-6

RAN: 322258P

ACUTE DERMAL TOXICITY STUDY IN THE RAT

GUIDELINE

OECD: 402

EEC: Annex V., Test B.3

AUTHOR

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TESTING FACILITY

Toxicological Sciences Laboratory
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-6272
USA

LABORATORY PROJECT ID

99-0005A1

STUDY SPONSOR

Eastman Kodak Company

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August 24, 1999

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STUDY: 99-0005-1 STUDY DIRECTOR: SHEPARD, K.P.
ACCESSION NUMBER: 812984

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(AUDITOR, QUALITY ASSURANCE UNIT)

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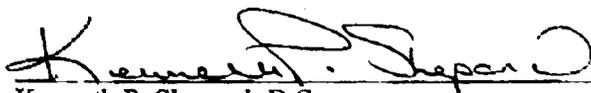
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02/25/99	SCALE/BALANCE CALIBRATION CLINICAL SIGNS AT 1 WEEK TEST SYSTEMS WEIGHTS	08/18/99
08/18/99	FINAL REPORT REVIEW	08/18/99

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted according to:

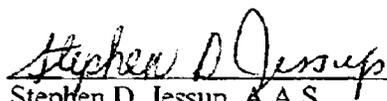
OECD Principles of Good Laboratory Practice (as revised in 1997)
[C(97)186/Final].



Kenneth P. Shepard, B.S.
Study Director

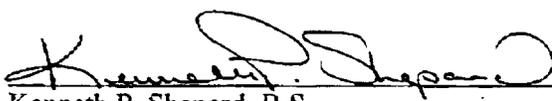
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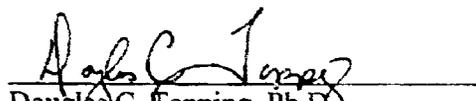
Stephen D. Jessup, A.A.S.
Report Author

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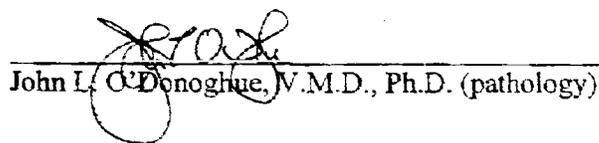
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Unit Director, Mammalian Toxicology

Aug 23, 1999
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ABSTRACT**3-AMINO-2,2,3-TRIMETHYLBUTYRIC ACID METHYL ESTER**

HAEL No.: 99-0005

KAN: 812984-5

CIN: 10082843

CAS: 90886-53-6

RAN: 322258P

ACUTE DERMAL TOXICITY STUDY IN THE RAT

An acute dermal toxicity study was conducted in male and female rats administered a single limit dose of 2000 mg/kg of the test substance topically. The test substance, a liquid, was administered as received. Mortality was limited to one female rat on Day 2 and a second female rat on Day 3. The acute dermal LD₅₀ for this test substance was greater than 2000 mg/kg for male and female rats.

Signs seen at the application site of all animals provided evidence of dermal irritation and/or corrosion by the test substance after a 24-hour topical exposure. These signs included erythema, desquamation, induration, necrosis, and scar formation. Tremors, a lack of feces, dehydration, and hypothermia were seen for one or both of the two female rats that died after topical exposure to the test substance. Other abnormal clinical signs were limited to a reduced amount of feces for a single female on Day 2 of the study. All animals that survived to termination of the observation period gained weight during both weeks of the study.

Treatment-related changes observed at necropsy were limited to female rats. These included desquamation, induration, scar formation, and a thickened white area on the muscle of the back below the application site. Black foci were also noted in the glandular stomach of a single female rat which died.

The test substance was classified as, at most, slightly toxic by the dermal route according to the criteria set forth by Hodge and Sterner (1949) and requires no toxicity classification as defined in the 18th Adaptation of the EC Classification, Packaging and Labelling of Dangerous Substances Directive.

STUDY AND TEST SUBSTANCE INFORMATION**Testing Facility**

Toxicological Sciences Laboratory
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-6272
USA

Project Participants

Study Director:	Kenneth P. Shepard, B.S.
Principal Investigator:	John W. Mosher, B.S.
Report Author:	Stephen D. Jessup, A.A.S.

Sponsor

Eastman Kodak Company

Test Substance Characterization

Test Substance Name:	3-Amino-2,2,3-trimethylbutyric acid methyl ester
CAS No:	90886-53-6
HAEL No.:	99-0005
KAN:	812984-5
CIN:	10082843
SRID or Lot No.:	BB8292-131J
Physical State and Appearance:	Liquid, Clear colorless
Source of Test Substance:	Eastman Kodak Company
Laboratory Project ID:	99-0005A1

Composition: Refer to composition information included in the notification when applicable.

Study Dates

Study Initiation Date:	February 18, 1999
Experimental Start Date:	February 18, 1999
Experimental Completion Date:	March 4, 1999

PURPOSE

The purpose of the study was to determine the estimated dermal LD₅₀ of the test substance in male and female rats and the clinical signs of toxicity associated with a single topical dose.

MATERIALS AND METHODS

Test System

Five male Sprague-Dawley rats (CD(SD)IGS BR) obtained from Charles River Laboratories, Stone Ridge (Kingston), NY and five female Sprague-Dawley rats (CD(SD)IGS BR) obtained from Charles River Laboratories Montreal, Canada were randomly assigned to each dose group. The male rats were 9 to 10 weeks of age and weighed 279 to 292 grams at the start of the study. The female rats were 11 to 12 weeks of age and weighed 221 to 248 grams at the start of the study. Rats were chosen for this study because they are a common representative species for toxicity studies. The rat is one of three species recommended for use in the OECD Guideline.

Husbandry

Housing

Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The rats were singly housed in suspended, stainless-steel, wire mesh cages. Cages and racks were washed once a week. Absorbent paper, used to collect excreta, was changed at least three times a week.

Environmental Conditions

The study room was maintained at 19.4-22.8°C and 38-56% relative humidity. A photoperiod of 12 hours light from 6 a.m. to 6 p.m. was maintained.

Acclimation Period

The animals were isolated upon arrival and allowed to acclimate for a period of 5 days. Animals were judged to be healthy prior to testing.

Husbandry, continuedFeed

Certified Rodent Diet (Purina Rodent Chow #5002, pellets) was available *ad libitum*. Feed containers were cleaned and refilled at least once a week. No known contaminants which would interfere with the outcome of this study were present in the feed. Analyses of feed are maintained on file within the testing laboratory.

Water

Water was available *ad libitum* through an automatic watering system. The source of the water was the local public water system. There have been no contaminants identified in periodic water analyses that would be expected to interfere with the conduct of the study. Semiannual analyses of water are maintained on file within the testing laboratory.

Identification

Upon arrival, all rats were identified by uniquely-numbered metal ear tags. During randomization, study-specific animal numbers were assigned to each animal. Cage cards contained the study-specific animal number and the ear tag number.

Experimental DesignTest Procedures

This study was conducted according to the Organisation for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals Guideline: 402, Acute Dermal Toxicity; and European Economic Community (EEC): Annex V., Test B.3, Acute Toxicity (Dermal).

Randomization

Animals were randomly selected and assigned to the study. Randomization was done by computer-generated lists. After assignment of animals to the study, the body weights were determined to ensure that variation in individual body weights did not exceed 20% of the mean weight for each sex.

Determination of Dose Levels

A limit dose of 2000 mg of the test substance/kg body weight was selected as the dose level for the dermal toxicity study.

Experimental Design, continuedPreparation of Test Substance

The test substance, a liquid, was administered as received.

Test Substance Exposure

The hair was removed from an area of the dorsal skin with an electric clipper. A single dose of the test substance was placed in contact with the skin using a fiber pad and an occlusive wrap to hold the test substance in place for 24 hours. At the end of the exposure period, any residual test substance was removed with running water.

Distribution of Animals

TABLE 1

Dose Level	Number Of Animals	Animal Numbers	
		Males	Females
2000 mg/kg	5 Males & 5 Females	811 - 815	816 - 820

Body Weights

Body weights were measured on Days 0 (prior to treatment), 7, and 14.

Clinical Observations

Animals were observed at least once during the exposure period, and once each day thereafter for the duration of the experiment. Observations included, but were not limited to, examination of the hair, skin, eyes, mucous membranes, motor activity, feces, urine, respiratory system, circulatory system, autonomic nervous system, central nervous system, and behavior patterns.

Necropsy

Any animal that died during the study was necropsied as soon as possible. All surviving animals were euthanatized and necropsied at the completion of the 14-day observation period.

Data Storage

The final report, data sheets, all nonperishable raw data, and an aliquot of the test substance have been stored in the testing facility archive managed under GLP-mandated conditions.

Data Analysis

No statistical procedures were required during the study. No dose/mortality curve was constructed since graphs become statistically useful only with the use of large numbers of animals and dose groups.

Protocol and Standard Operating Procedure Deviations

There were no SOP or protocol deviations during the study.

RESULTS

Mortality

The dose level, the number of animals administered the test substance, the number of deaths, and the day of death are listed in Table 2.

TABLE 2
Mortality Table

Dose (mg/kg)	Number Of Rats Exposed (Male, Female)	Number Of Deaths (Male, Female)	Time Of Death
2000	5,5	0,2	Day 2 and Day 3

LD₅₀ for male rats: > 2000 mg/kg (95% C.I. = No Range Calculable)
LD₅₀ for female rats: > 2000 mg/kg (95% C.I. = No Range Calculable)

Clinical Signs

Signs seen at the application site of all animals provided evidence of dermal irritation and/or corrosion by the test substance. These signs included erythema, desquamation, induration, necrosis, and scar formation. Tremors, a lack of feces, dehydration, and hypothermia were seen for one or both of the two female rats that died after topical exposure to the test substance. Other abnormal clinical signs were limited to a reduced amount of feces for a single female on Day 2 of the study. The time of each observation and the number of animals involved at each dose level are listed in Table 3.

TABLE 3
Table Of Clinical Observations

Dose (mg/kg)	Time	Clinical Signs	Number Of Animals Affected	
2000	1 Hour	Appeared Clinically Normal	5/5 Males	5/5 Females
2000	Day 1	Appeared Clinically Normal	5/5 Males	
		Necrosis (Application Site)		4/5 Females
		Tremors		2/5 Females
		Lack of Feces		2/5 Females
		Induration (Application Site)		1/5 Females

Table continued on the next page.

TABLE 3, continued
Table Of Clinical Observations

Dose (mg/kg)	Time	Clinical Signs	Number Of Animals Affected	
2000	Day 2	Found Dead Erythema (Application Site) Necrosis (Application Site) Tremors Lack of Feces Induration (Application Site) Reduced Feces Amount Dehydration Hypothermia	5/5 Males	1/5 Females 3/4 Females 1/4 Females 1/4 Females 3/4 Females 1/4 Females 1/4 Females 1/4 Females
2000	Day 3	Found Dead Erythema (Application Site) Necrosis (Application Site) Induration (Application Site)	5/5 Males	1/4 Females 1/3 Females 2/3 Females 3/3 Females
2000	Day 4	Erythema (Application Site) Desquamation (Application Site) Induration (Application Site)	5/5 Males 5/5 Males	3/3 Females
2000	Day 5-Day 6	Desquamation (Application Site) Induration (Application Site)	5/5 Males	3/3 Females
2000	Day 7-Day 10	Desquamation (Application Site) Induration (Application Site)	5/5 Males	1/3 Females 3/3 Females
2000	Day 11	Appeared Clinically Normal Desquamation (Application Site) Induration (Application Site)	2/5 Males 3/5 Males	3/3 Females 3/3 Females
2000	Day 12	Appeared Clinically Normal Desquamation (Application Site) Induration (Application Site) Scar (Application Site)	4/5 Males 1/5 Males	3/3 Females 2/3 Females 1/3 Females
2000	Day 13-Day 14	Appeared Clinically Normal Desquamation (Application Site) Induration (Application Site) Scar (Application Site)	5/5 Males	3/3 Females 2/3 Females 1/3 Females

Body Weights

All animals that survived to termination of the study gained weight during both weeks. The individual body weights are listed in Table 4.

TABLE 4
Table Of Individual Body Weights (grams)

Dose (mg/kg)	Animal Number	Day 0	Day 7	Day 14 or (Terminal)
MALE RATS				
2000	811	291	351	392
2000	812	287	352	395
2000	813	292	359	401
2000	814	283	343	375
2000	815	279	331	366
FEMALE RATS				
2000	816	238	248	254
2000	817	248	262	271
2000	818	234	273	284
2000	819	247	Died on Day 3	(225)
2000	820	221	Died on Day 2	(199)

Necropsy Findings

Abnormal findings noted at necropsy were limited to female rats. These included desquamation, induration, scar formation, and a thickened white area on the muscle of the back below the application site. Black foci were also noted in the glandular stomach of one female rat. A record of the incidence and severity of all gross abnormalities is presented in computer-generated tables which are included in the Appendix.

DISCUSSION

An acute dermal toxicity study was conducted in male and female rats administered a single limit dose of 2000 mg/kg of the test substance topically. The test substance, a liquid, was administered as received.

Mortality was limited to one female rat on Day 2 and a second female rat on Day 3. Signs seen at the application site of all animals provided evidence of dermal irritation and/or corrosion by the test substance after a 24-hour topical exposure. These signs included erythema, desquamation, induration, necrosis, and scar formation. Tremors, a lack of feces, dehydration, and hypothermia were seen for one or both of the two female rats that died after topical exposure to the test substance. Other abnormal clinical signs were limited to a reduced amount of feces for a single female on Day 2 of the study. All animals that survived to termination of the study gained weight during both weeks of the study.

Treatment-related changes observed at necropsy were limited to female rats. These included Desquamation, induration, scar formation, and a thickened white area on the muscle of the back below the application site. Black foci were also noted in the glandular stomach of a single female rat which died.

No treatment related changes were observed for male rats. The acute dermal LD₅₀ for this test substance was greater than 2000 mg/kg for male and female rats.

CONCLUSION

Based on the dermal LD₅₀ for male and female rats, the test substance was classified as, at most, slightly toxic by the dermal route in rats according to the criteria set forth by Hodge and Sterner (1949) and requires no toxicity classification as defined in the 18th Adaptation of the EC Classification, Packaging and Labelling of Dangerous Substances Directive.

REFERENCES

- Hodge, H.C. and Sterner, J.H. (1949). Tabulation of toxicity classes. *Am. Indust. Hyg. Quart.*, 10, 93-96.
- National Research Council (1996). *Guide for the Care and Use of Laboratory Animals*. National Academy Press. Washington, D.C.

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FINAL REPORT

3-AMINO - 2,2,3-TRIMETHYLBUTYRIC ACID METHYL ESTER

HAEL No.: 99-0005

KAN : 812984-5

CAS No.: 90886-53-6

CIN: 10082843

RAN: 321704N

A FOUR-WEEK ORAL TOXICITY STUDY IN THE RAT

GUIDELINE

OECD: TG-407

EBC: Annex V., Test B.7

AUTHOR

Stephen D. Jessup, A.A.S.

TESTING FACILITY

Toxicological Sciences Laboratory
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-6272
USA

LABORATORY PROJECT ID

990005G1

STUDY SPONSOR

Eastman Kodak Company

STUDY COMPLETION DATE

September 13, 1999

QUALITY ASSURANCE INSPECTION STATEMENT
(21 CFR 58.35(B)(7), 40 CFR 792.35(B)(7), AND 40 CFR 160.35(B)(7))

STUDY: 99-0005-1 STUDY DIRECTOR: DAVID, R.M.
ACCESSION NUMBER: 812984

PAGE 1
09/07/99

STUDY TYPE: BASIC REPEATED GAVAGE (28-DAY)

M. L. James
(AUDITOR, QUALITY ASSURANCE UNIT)

9/7/99
DATE

THIS STUDY WAS INSPECTED BY 1 OF MORE PERSONS OF THE QUALITY ASSURANCE UNIT. WRITTEN STATUS REPORTS WERE SUBMITTED ON THE FOLLOWING DATES.

INSPECTION DATES	PHASE(S) INSPECTED	STATUS REPORT DATES
04/01/99	PROTOCOL SUBMISSION	04/01/99
04/27/99	TEST SYSTEM IDENTIFICATION FUNCTIONAL OBSERVATIONAL BATTERY TESTING CONTROL SUBSTANCE DOSING OF TEST SYSTEM TEST SUBSTANCE CARRIER MIXTURE DOSING OF TEST SYSTEMS DAY 21 FOB	04/27/99
05/05/99	TEST SYSTEM BLEEDING NECROPSY SPECIMEN COLLECTION SPECIMEN/SAMPLE WEIGHT HEMATOLOGY	05/05/99
05/17/99	RECORDS REVIEW FUNCTIONAL OBSERVATIONAL BATTERY TESTING DAYS -1, 8, 14, 21, 28	05/17/99
05/25/99	RECORDS REVIEW FUNCTIONAL OBSERVATIONAL BATTERY TESTING SUMMARY	05/25/99
06/28/99	RECORDS REVIEW HEMATOLOGY CLINICAL CHEMISTRY CELL MORPHOLOGY AND ORGAN WEIGHTS	06/28/99
07/28/99	RECORDS REVIEW GROSS PATHOLOGY HISTOPATHOLOGY PATHOLOGY REPORT	07/28/99
08/04/99	FINAL REPORT REVIEW	08/05/99
08/05/99	FINAL REPORT REVIEW	08/05/99

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted according to:

United States Environmental Protection Agency, Toxic Substances Control Act,
Good Laboratory Practice Standards, 40 CFR Part 792;

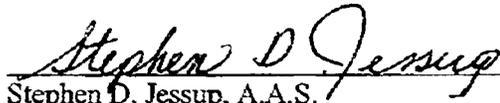
OECD Principles of Good Laboratory Practice (as revised in 1997)
[C(97)186/Final].



Raymond M. David, Ph.D.
Study Director

9/13/99
Month/Day/Year

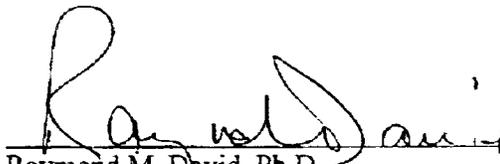
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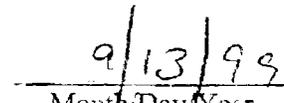
Stephen D. Jessup, A.A.S.
Report Author



Month/Day/Year



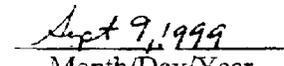
Raymond M. David, Ph.D.
Study Director



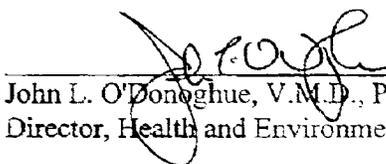
Month/Day/Year



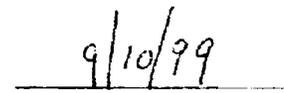
Douglas C. Topping, Ph.D.
Unit Director, Mammalian Toxicology



Month/Day/Year



John L. O'Donoghue, V.M.D., Ph.D.
Director, Health and Environment Laboratories



Month/Day/Year

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ABSTRACT**3-AMINO - 2,2,3-TRIMETHYLBUTYRIC ACID METHYL ESTER**

HAEL No.: 99-0005

KAN : 812984-5

CAS No.: 90886-53-6

CIN: 10082843

RAN: 321704N

A FOUR-WEEK ORAL TOXICITY STUDY IN RATS

Male and female Sprague-Dawley rats were treated daily with 500, 150, 50 or 0 mg/kg of the test substance in distilled water by oral gavage for 29 days. Animals were observed daily for clinical signs of toxicity. Body weights and feed consumption were measured weekly. Motor activity was measured at study termination. A functional observational battery (FOB) was performed prior to treatment and weekly thereafter. Mortality prior to scheduled necropsy was limited to one male and one female rat from the 500 mg/kg group. An abnormal clinical sign which may be attributed to test substance exposure was excessive salivation. Significantly higher scores ($p \leq 0.05$) were noted for the condition of haircoat for the 500 mg/kg female rats on Days 14, 21, and 28. Hindlimb grip strength for the 500 mg/kg male rats was significantly less than for the male control rats on Day 28. No other differences in the FOB were observed. Significantly lower mean body weights were noted for the 500 mg/kg male rats on Days 14, 21, and 28. Lower mean feed consumption was also noted on Day 3 for this group. There were no other significant differences in mean body weight or mean feed consumption among any of the groups. Male rats in the 500 mg/kg group had significantly lower mean total motor activity scores and lower total ambulation scores than the control male rats. There were no other significant differences in either mean total motor activity counts or mean total ambulations for male or female rats of any group.

At study termination, animals were anesthetized with carbon dioxide and blood was obtained from the posterior vena cava for clinical chemistry and hematology analyses. Fasted body weights and selected organ weights were measured at necropsy. Selected tissues were collected from all animals. A number of statistically significant differences were noted in hematology. These differences include reduced numbers of white blood cells and changes in the percentages of white blood cell types (neutrophils, lymphocytes, monocytes, myelocytes, metamyelocytes, and blast cells) for the 500 mg/kg male and female rats. A significantly lower ($p \leq 0.05$) mean lymphocyte percentage and a significantly higher blast cell percentage were noted for the 150 mg/kg female rats. However, the percentage of lymphocytes for the 150 mg/kg group was within normal limits for this strain of rat and this laboratory. The increase in the percentage of blast cells, while suggestive of enhanced bone marrow activity, was not accompanied by histopathologic changes in the bone marrow or adverse changes in other hematologic parameters. Therefore, the effects observed for the 150 mg/kg group were not considered to be adverse. Changes in red blood cell morphology did not follow a pattern indicative of any specific clinical condition. The mean total protein, cholesterol, and triglyceride values for the 500 mg/kg male group were significantly ($p \leq 0.05$) higher than those of the control group. Significantly higher

cholesterol and lower sodium values were also seen for the 500 mg/kg female rats. There were no other toxicologically significant differences in clinical chemistry. The mean terminal body weight for the male 500 mg/kg group was significantly lower than for the control group, and lower for the female 500 mg/kg group but not statistically different.

Absolute weights for the thymus, spleen, testes, and epididymides of the 500 mg/kg male rats were significantly ($p \leq 0.05$) lower when compared to the male control group. For female rats, absolute thymus weights for 500 mg/kg rats were also significantly lower when compared to the control group. Mean relative (to body weight) liver, kidney, and adrenal gland weights for the 500 mg/kg male and female dose groups were significantly ($p \leq 0.05$) higher than the respective control groups. The 500 mg/kg male rats also had significantly higher mean relative heart and brain weights than did the male control animals. The 150 mg/kg male rats mean relative (to body weight) liver weights were significantly ($p \leq 0.05$) higher and the 50 mg/kg male rats mean relative liver weights were significantly lower than the control group. Mean relative (to body weight) thymus weights for 500 mg/kg male and female rats were significantly ($p \leq 0.05$) lower than their control group. The 500 mg/kg rats also had a significantly ($p \leq 0.05$) lower mean relative (to body weight) epididymal weight than the control group. Organ weight changes in the liver, kidney, adrenal gland, and heart appear to be adaptive in that no microscopic lesions were observed in those organs from the high-dose group.

No gross lesions were observed during necropsy that were attributed to exposure to the test substance. Microscopic findings include sciatic nerve fiber degeneration for the 500 mg/kg groups. Seminiferous tubule degeneration within the testis and degenerative cells within the epididymal tubules were seen for 500 mg/kg male rats. Similar effects were not observed at the mid- and low-dose levels. No other treatment-related lesions were identified for any dose group.

Based on the histopathologic findings in the sciatic nerve and testes, the decrease in grip strength, and increases in serum cholesterol and triglycerides for the 500 mg/kg animals, and the lack of effect at other dose levels, the no-observed-adverse-effect level (NOAEL) was determined to be 150 mg/kg.

STUDY AND TEST SUBSTANCE INFORMATION**Testing Facility**

Toxicological Sciences Laboratory
Health and Environment Laboratories
Eastman Kodak Company
Rochester, New York 14652-6272
USA

Project Participants

Study Director:	Raymond M. David, Ph.D.
Study Technician:	Stephen D. Jessup, A.A.S.
FOB Observer:	Lisa G. Bernard, M.S.
Hematologist/Clinical Chemist:	Robert. E. Emmons, B.S.
Histopathologist:	Robert H. Garman, D.V.M., DACVP, Consultants in Veterinary Pathology
Analytic Chemist:	Christine M. Hoffman, M.S.
Statistician:	Mary L. Ritter, M.S.
Report Author:	Stephen D. Jessup, A.A.S.

Sponsor

Eastman Kodak Company

Test Substance Characterization

Test Substance Name:	3-Amino - 2,2,3-Trimethylbutyric Acid Methyl Ester
HAEL No.:	99-0005
CAS:	90886-53-6
CIN:	10082843
KAN:	812984-5
SRID No.:	BB8292-131-J
Physical State and Appearance:	clear liquid
Source of Test Substance:	Eastman Kodak Company
Laboratory Project ID:	990005G1

Study Dates

Study Initiation Date:	March 29, 1999
Experimental Start Date:	April 6, 1999
Experimental Completion Date:	August 31, 1999

Purity, Structure Confirmation, and Stability Determination

The purity of the test substance was determined by gas chromatography with flame ionization detection (GC/FID). At the beginning of the study, the purity of the test substance was determined to be 99.3%, with the remainder being 0.7% of an unknown material. At study termination, the purity of the test substance was determined to be 98.7%. Based on these data, the test substance was considered to be stable during the test period. The structure of the test substance was confirmed using a Fourier transform infrared (FTIR) spectrum. The spectrum of the test substance was determined to be consistent with the proposed structure by assigning the major functional groups. The analytical reports are provided in Appendix B beginning on page 2.

PURPOSE

The purpose of this study was to evaluate the sub-acute effects of the test substance when given to rats orally for four weeks.

MATERIALS AND METHODS

Test system

Five male and five female Sprague-Dawley rats (CD(SD)IGS BR) obtained from Charles River Laboratories, Stone Ridge (Kingston), NY were randomly assigned to each exposure group. The male and female rats were 41 or 50 days of age and weighed 238.2 ± 10.6 or 180.1 ± 9.2 grams (mean \pm SD), respectively, at the start of the study. Rats were chosen for this study because they are a common representative species for toxicity studies. The rat is one of the two primary rodent species recommended for use in USEPA and OECD test guidelines.

Husbandry

Housing

Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The rats were singly housed in suspended, stainless-steel mesh cages. No other study was housed in the same room as this study. Cages and racks were washed once a week. Absorbent paper, used to collect excreta, was changed daily.

Husbandry, continued

Environmental Conditions

The study room was maintained at 20.3 - 24.1°C and 44.8 - 63.0% relative humidity. A photoperiod of approximately 12 hours light from 6 a.m. to 6 p.m. was maintained.

Acclimation Period

The animals were isolated upon arrival and allowed to acclimate for a period of at least five days prior to assignment to this study. Animals were judged to be healthy prior to testing.

Feed

Certified Rodent Diet (Purina Rodent Chow #5002, meal; PMI Feed, Inc. Richmond, IN) was available *ad libitum*. Feed containers were cleaned and refilled at least once a week. No known contaminants which would interfere with the outcome of this study were present in the feed. Analyses of feed are maintained on file within the testing laboratory.

Water

Water was available *ad libitum* through an automatic watering system. The source of the water was the local public water system. There have been no contaminants identified in periodic water analyses that would be expected to interfere with the conduct of the study. Semiannual analyses of water are maintained on file within the testing laboratory.

Identification

Upon arrival, all rats were identified by uniquely-numbered metal ear tags. During randomization, study-specific animal numbers were assigned to each animal. Cage cards, color-coded for each group, contained the study-specific animal number and the ear tag number.

Experimental Design

Randomization

The test animals were culled from the stock population based on body weight and were randomly assigned to groups using computer-generated lists. Variation among the body weights of individual animals did not vary more than 20% from the mean for each sex. Following randomization, the body weights of all groups were compared by analysis of variance to insure that there were no statistically significant differences prior to initiation of exposure.

Experimental Design, continued**Test Procedures**

This study was conducted according to the Organisation for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals: Guideline TG-407, Repeated Dose 28-day Oral Toxicity Study in Rodents (Adopted July 27, 1995) and European Economic Community (EEC): Annex V., Test B.7, Repeated Dose (28 days) Toxicity (Oral), EC 22nd Adaptation, May 1996.

Determination of Dose Levels

A probe study was conducted using 9 female rats (3 per dose level). Animals were dosed with 750, 500 or 250 mg/kg of the test substance in distilled water for four days and observed daily for signs of toxicity. Body weights were measured on Days 0, 2 and 4. Feed consumption was measured on Days 2 and 4. Based on the results of this probe study, the following dose levels were selected for the four-week study: 500, 150, 50, and 0 mg/kg body weight.

Test Substance Exposure

Rats were treated by oral gavage for 29 days with daily doses of 500, 150, 50, or 0 mg/kg of the test substance in distilled water. Dosing solutions were prepared so that animals from each dose group received equal volumes on a per kilogram body weight basis. Control animals received a volume of distilled water equal to dose volumes administered to the test groups. Dose levels were selected based on the results of a 5-day probe study.

Preparation of Test Substance in Vehicle Mixtures

The test substance was mixed with distilled water to yield a high dose concentration of 100 mg/mL. Dilutions of this mixture were made to yield concentrations of 30 mg/mL and 10mg/mL, respectively, for the mid and low-dose groups. Test substance in vehicle mixtures were prepared every four days based on the stability of the mixtures.

Characterization of Test Substance in Vehicle

Stability of the test substance in distilled water was determined by repeated analysis of 150, 30, and 10 mg/mL mixtures of the test substance. Initially, the 150 mg/mL mixture was analyzed on Days 4, 8, and 14. The Day 4 mean concentration of the 150 mg/mL mixture was 100% of the Day 0 mean. However, the measured concentrations of the 150 mg/kg mixtures on Days 8 and 14 were less than 90% of the Day 0 mean concentration. Based on this lack of stability, analysis of 30 and 10 mg/mL mixtures were performed on Days 0, 1, 2, and 4. The Day 4 concentrations of the 30 and

Experimental Design, continued**Characterization of Test Substance in Vehicle, continued**

10 mg/mL mixtures were 95.1% and 93.2% of the Day 0 mean, respectively. Therefore, this indicated the test substance was stable for at least four days in distilled water. Mixtures were analyzed using gas chromatography with flame ionization detection (GC/FID). Homogeneity of the test substance in distilled water was evaluated by measuring the concentration of the test substance at three levels (top, middle, and bottom) of 100, 30, and 10 mg/mL mixtures of the test substance. The analytical concentrations of the test substance in the top, middle, and bottom layers for the 100 mg/mL mixture varied between 102.4 and 115.4 mg/mL, with a standard deviation of 3.5. For the 30 mg/mL mixture, the analytical concentrations varied between 29.49 and 34.07 mg/mL with a standard deviation of 1.15. For the 10 mg/mL mixture, the analytical concentrations varied between 9.42 and 11.19 mg/mL, with a standard deviation of 0.43. Based on these results, the preparations were considered homogeneous. The homogeneity of the test substance mixtures was maintained by using a stirring bar to continuously mix the test solutions while removing aliquots for dosing purposes. The mean concentrations of the test substance were 104, 32, and 10 mg/mL compared to the target concentrations of 100, 30, and 10 mg/mL, respectively. The analytical report for stability, homogeneity, and concentration verification can be found in Appendix B beginning on page 58.

Disposition of Groups

Animals were distributed into groups as follows:

Group	Dose Level	Number of Animals	Animal Numbers	
			Males	Females
1	Control / 0 mg/kg	5 Males & 5 Females	401 - 405	421 - 425
2	Low / 50 mg/kg	5 Males & 5 Females	406 - 410	426 - 430
3	Mid / 150 mg/kg	5 Males & 5 Females	411 - 415	431 - 435
4	High / 500 mg/kg	5 Males & 5 Females	416 - 420	436 - 440

Animals were treated for 29 consecutive days. All surviving animals were euthanized and necropsied on the day following the last treatment.

Daily Clinical Observations

Clinical (hands-on) examinations were conducted every morning, except for days on which the functional observational battery was conducted. Except for holidays, animals were observed for moribundity/mortality each weekday afternoon. Clinical observations included, but were not limited to, examination of the hair, skin, eyes, mucous membranes, motor activity, feces, urine, respiratory system, circulatory system, autonomic nervous system, central nervous system, and behavior patterns.

Experimental Design, continued**Weekly Functional Observational Battery (FOB)**

Detailed functional observation of animals was conducted prior to study start, and prior to daily treatment on Days 7, 14, 21, and 28. If possible, these observations were made at approximately the same time each day. The FOB observations followed an acclimation to the home cage of at least 30 minutes after motor activity measurements.

The animals were observed for:

- Severity and degree of lacrimation, salivation, or discharges
- Piloerection and hair coat
- Pupillary size
- Exophthalmus
- Mucous Membranes/Skin Color
- Unusual respiration
- Feces (amount and consistency)
- Urine (amount and color)
- Description of body position, coordination of movement, and gait abnormalities
- Description, incidence, and severity of convulsions and tremors
- Stereotypy and bizarre behavior

Additionally, prior to study start and on Day 28, the animals were observed for:

- Sensory function (vision and audition)
- Proprioceptive reflex
- Forelimb and hindlimb grip strength

The observations were rated with a score of 1 indicating typical behavior and scores of 2-4 indicating levels of behavior different from the typical pattern. Descriptive categories of abnormal behavior were also noted.

Two people, one functioning as an observer and the other as a recorder, performed the FOB examinations. The observer was blind to treatment status. The same observer was used for each replicate throughout the study. Each animal was evaluated in random order using the ear tag as identification. A copy of the FOB scoring reference sheet is provided in Appendix A.

Historical positive control data demonstrating the sensitivity of the FOB procedure are available.

Experimental Design, continued

Body Weight and Feed Consumption

Body weights were measured on Days 0, 3, 7, 14, 21, and 28. Feed consumption was determined on Days 3, 7, 14, 21, and 28. Animals were fasted the day prior to necropsy. Terminal body weights were measured after exsanguination, but prior to necropsy.

Motor Activity Determination

Motor activity was determined on Day 28. Motor activity was monitored sequentially in two replicates because of the number of motor activity measurement systems available. All exposure groups were evaluated concurrently.

Motor activity was measured in 10 minute intervals for a total of 60 minutes using an automated cage rack photobeam activity system (San Diego Instruments, San Diego, CA). To reduce variability during data collection, the animals were randomly placed into the motor activity units within 5 minutes of each other. Motor activity was measured in an isolated room within the vivarium. No entry to the room was allowed during the measurement period.

Motor activity data were collected from the automated cage rack photobeam activity system (PAS) using a Compaq 386SX computer. The motor activity system is composed of individual animal enclosures equipped with three pairs of infrared beams and sensors per frame. Each frame is placed over a clear plastic animal cage; as the animal moves about the cage, the infrared beams are broken. The number of breaks of each beam in the frame is transmitted to a digital input/output interface and is then stored in files on the hard disc of the Compaq computer. The system distinguishes and records two types of horizontal movement: 1) simple motor activity (single beam break) and 2) ambulation (multiple beam breaks over the 60 minute time period). During this study, motor activity (single beam breaks) was compiled every ten minutes for one hour. The total number of ambulations and total motor activity were calculated for the entire one-hour period.

Experimental Design, continued

Blood Collection and Euthanasia

Animals were fasted overnight beginning in the afternoon of the last day of treatment. The following day, animals were anesthetized with carbon dioxide and blood was collected from the posterior vena cava. The blood was placed into vacutainer tubes and allowed to clot for serum analyses. Other tubes containing an anticoagulant were used for analyses of whole blood samples. Blood smears were also prepared for blood cell counts. Following blood collection, the animals were exsanguinated. Animals were bled and humanely killed in a random order based on a computer-generated list.

Hematology and Clinical Chemistry Examinations

Clinical pathology assays were conducted using the following instruments: Roche Analytical Instruments Cobas Fara II serum chemistry analyzer (Roche Diagnostic Systems, Nutley, NJ), Technicon H-1 System hematology analyzer (Bayer Corporation, Diagnostics Division, Tarrytown, NY), Helena Laboratories Titan Gel Electrophoresis System [Helena Laboratories, Beaumont, TX (A/G ratio and albumin)], BBL Fibrosystems analyzer [BBL division of Becton, Dickinson and Company, Cockeysville, MD (prothrombin times)], and Corning Flame 480 Photometer [Corning Medical and Scientific, Corning Glass Works, Medfield, MA (sodium and potassium)].

Hematology tests included: hemoglobin concentration, hematocrit, red blood cell count, white blood cell count, red blood cell indices, prothrombin time, and platelet count. Slides containing blood smears were examined for cellular morphology and differential white blood cell count. Clinical chemistry tests included: alanine aminotransferase, sorbitol dehydrogenase, creatinine, urea nitrogen, glucose, total bilirubin, total protein, albumin, albumin/globulin ratio, total cholesterol, triglycerides, calcium, phosphorus, sodium, and potassium.

Necropsy

Following exsanguination, the animals were weighed and necropsied. The following tissues were fixed in 10% neutral buffered formalin: trachea, lungs, heart, stomach, duodenum, jejunum, ileum, cecum, colon, liver, salivary glands, kidneys, urinary bladder, adrenal glands, thyroid glands, thymus, spleen, sternum (with bone marrow), mesenteric lymph nodes, cervical lymph nodes, brain (including sections of medulla/pons, cerebellar cortex, and cerebral cortex), sciatic nerve, cervical spinal cord, testes, epididymides, male accessory sex glands, ovaries, vagina, uterus, Fallopian tubes, and gross lesions.

Experimental Design, continued**Organ Weights**

The liver, kidneys, adrenal glands, spleen, thymus, heart, brain, testes, and epididymides were weighed. Paired organs were weighed together.

Histopathology

For the control and high-dose groups, all tissues were embedded in paraffin and sectioned at 4 μm except the brain which was sectioned at 5 μm . The sternum was decalcified prior to being embedded and sectioned. The lungs were sectioned along a plane to allow visual examination of the major bronchi and bronchioles. The resulting tissue sections were stained with hematoxylin and eosin (H&E) stains and examined for histopathology. The liver, thymus, and sciatic nerve from the male and female low- and mid-dose animals were examined microscopically. In addition, the kidneys, testes, epididymides, seminal vesicles, coagulating gland, and prostate from the low- and mid-dose male animals were also examined microscopically. Microscopic examinations were performed on bones of the skull for one female and two male low-dose rats, and cervical lymph nodes from a single low-dose male rat. The lungs of one male and one female mid-dose animals were also examined.

Data Storage

The final report, tissues, paraffin blocks, slides, data sheets, all nonperishable raw data, and an aliquot of the test substance have been stored in the testing facility archive managed under GLP-mandated conditions.

Calculations and Statistical Procedures

Mean values were calculated for body weight, feed consumption, total motor activity, total ambulations, grip strength, FOB behavior scores, serum chemistries, hematology values, organ weights, and organ-to-body weight ratios. Homogeneity of variance was evaluated using Bartlett's test ($p \leq 0.01$) for the following data: body weight, feed consumption, FOB behavior scores, motor activity scores, clinical pathology results, and organ weight. Body weight, feed consumption, clinical pathology data, and organ weight data were evaluated using a one-way analysis of variance (ANOVA) ($p \leq 0.05$) and Duncan's multiple range test ($p \leq 0.05$). All other continuous data (grip strength, total motor activity, and total ambulations) and FOB behavior scores were evaluated using a repeated-measures/multivariate analysis of variance ($p \leq 0.05$). If significant time components were detected, then one-way analyses of each test day were conducted. Post-hoc multiple comparison procedures such as Dunnett's t-test using appropriate error terms were employed to identify the statistically significant means. Tests for linear trend over time in each group were conducted using linear regression ($p \leq 0.05$). Categorical FOB data were analyzed using a two-way or multiway frequency table analysis. If significant dose-behavior interactions were detected, then a Fisher's Exact test or Likelihood Ratio Chi-Square comparison was used to compare treated and control incidence at each weekly observation period.

Protocol and Standard Operating Procedure Deviations

There were no SOP or protocol deviations during the study.

RESULTS

Probe Study

Groups of three female rats were treated for four days with 750, 500 or 250 mg/kg of the test substance in distilled water. Mortality was limited to two rats in the 750 mg/kg dose group which died on Day 0 or Day 1 of the study. For the 750 mg/kg rat which died on Day 1, clinical signs of toxicity observed prior to death included a reduced amount of feces (minor), dry urine staining (moderate), and perioral red discoloration (minor). For the surviving 750 mg/kg animal, reduced amounts of feces (minor) were observed on Days 2 and 4. Other abnormal clinical signs were limited to a reduced amount of feces (minimal) for one 250 mg/kg rat seen on Day 3.

Mean feed consumptions for the surviving 750 mg/kg rat and one of three 500 mg/kg rats were reduced throughout the probe study. These two animals lost a minimal amount of weight (11.8g and 6.9g respectively) between Days 0 and 4.

All other rats survived to study termination and gained weight. No significant differences in feed consumption were noted in any of these animals.

Based on the signs of toxicity observed in the probe study, a dose of 500 mg/kg was selected as the highest dose level for the four-week study. Dose levels of 150 mg/kg and 50 mg/kg were used to provide evidence of dose-response relationships.

Mortality

One 500 mg/kg female rat (#440) and one 500 mg/kg male rat (#420) died on Day 4 and Day 28 respectively. No other mortality occurred during the study.

Daily Clinical Observations

Clinical observations noted during the study are summarized on pages 27 - 30 (males) and 31 - 34 (females). Individual animal data are presented in Appendix A. Abnormal clinical signs which may be attributed to test substance exposure include sialorrhea (minimal) and salivation (minor). These clinical signs were seen on either Day 11 or Days 16 and 17 for 500 mg/kg female rats #436 and #438, respectively. In addition, poor general body condition with increased activity was observed for three 500 mg/kg female rats (#436, #437, #439) for one to four days between Day 16 and Day 26. Wet or dry urine staining of the inguinal hair (minimal to moderate) was also observed for these three 500 mg/kg female rats intermittently between Days 16 and 29.

Daily Clinical Observations, continued

For a single 500 mg/kg female rat (#440) clinical signs observed prior to induced death (on Day 4) included rales, labored breathing, dehydration, decreased and softened feces, perioral red discoloration, wet urine staining of the inguinal hair, pallor of the body as a whole, abdomen distension and alopecia on the skin of the arm. This was most likely the result of aspiration of a small amount of the test substance.

Rales, decreased feces, dried porphyrin discharge around the eyes and peri-oral red discoloration were all noted for one 500 mg/kg male rat (#420) on Day 12. By Day 13, rales were still audible. This rat appeared normal on Day 14 - Day 27. On Day 28 however, it was found dead prior to the scheduled FOB evaluation.

Other clinical abnormalities that were not considered to be related to test substance exposure included wet and/or dry porphyrin nasal discharges, alopecia (skin of the arm, abdomen and thigh) and crust/scale on the skin of the neck. Crust/scale on the neck is most likely attributed to the method of test substance administration and is not considered related to exposure to the test substance. For a single 500 mg/kg female rat (#438), the skin of the foot and toe was discolored red for eight days of the study, most likely the result of injury. A single control male rat was observed to have decreased feces volumes for two days and to be dehydrated for one of these two days. Upon examination of this rat's home cage, an inoperable water nipple was found, and the problem was corrected.

No other clinical abnormalities were observed throughout the 29-day observation period.

Weekly Functional Observational Battery (FOB)

Mean scores and incidences from the weekly functional observational battery are summarized on pages 35 - 49 (males) and pages 50 - 64 (females). Individual animal data are presented in Appendix A.

Significantly higher scores ($p \leq 0.05$) were noted for haircoat condition for the 500 mg/kg female rats on Days 14, 21, and 28. For 50 mg/kg male rats, mean forelimb grip strength values were higher ($p \leq 0.05$) than the control animals on Day -1. Hindlimb grip strength for 500 mg/kg male rats was significantly lower than for the male control rats on Day 28. No other differences in FOB parameters were observed.

Body Weight and Feed Consumption

Mean body weights are presented in graph and tabular forms on pages 69 - 70 (males) and 71 - 72 (females). Individual animal data are presented in Appendix A. Mean feed consumption data are presented in graph and tabular forms on pages 65 - 66 (males) and 67 - 68 (females). Individual animal data are presented in Appendix A. Significantly lower mean body weights were noted for the 500 mg/kg male rats on Days 14, 21, and 28. Lower mean feed consumptions were also noted on Day 3 for this group. There were no other significant differences in mean body weight or mean feed consumption among any of the groups.

Motor Activity Determination

Mean motor activity values are presented in graph and tabular forms on pages 74 - 76. Individual motor activity data are presented in the Appendix A. Male rats in the 500 mg/kg group had significantly lower mean total ambulation and lower total motor activity scores than the control male rats. There were no other significant differences in either mean total motor activity counts or mean total ambulations for male or female rats of any group.

Hematology and Clinical Chemistry

Mean hematology and clinical chemistry values and analyses of blood cell morphology are presented in summary tables on pages 76 - 80. Individual animal data are presented in Appendix A.

Hematology findings included significantly ($p \leq 0.05$) higher polymorphonuclear leukocyte, banded leukocyte, monocyte, and blast cell counts, and significantly lower lymphocyte counts for 500 mg/kg male and female rats. Additionally, female rats in the 500 mg/kg group had significantly ($p \leq 0.05$) lower white cell counts and significantly higher mean myelocyte and myelocyte values. Female rats in the 150 mg/kg group also had significantly ($p \leq 0.05$) lower lymphocyte counts and significantly higher blast cell counts when compared to the female control rats. However, the percentage of lymphocytes for the 150 mg/kg group was within normal limits for this strain of rat and this laboratory. There were no other significant differences in hematology parameters between treated and control male or female rats.

For red blood cell morphology, minimal to minor poikilocytosis was seen in the 500 mg/kg (2/4), 150 mg/kg (2/5), 50 mg/kg (1/5), and 0 mg/kg (2/5) male groups and in the 500 mg/kg (4/4), 150 mg/kg (4/5), 50 mg/kg (2/5), and 0 mg/kg (2/5) female groups. Minimal anisocytosis was noted in three 500 mg/kg male rats, two 150 mg/kg female rats, and four 500 mg/kg female rats. Minimal microcytosis was noted in one 500 mg/kg female rat. Minimal macrocytosis was noted in 3/4 500 mg/kg male and 4/4 500 mg/kg female rats. Minimal spherocytosis was seen in one 150 mg/kg female rat. Minimal Howell-Jolly bodies were seen in one 0 mg/kg male, one 500 mg/kg male, and one 150 mg/kg female. These changes in red blood cell morphology did not follow a pattern indicative of any specific clinical condition. There were no other differences in red blood cell morphology between treated and control groups of male or female rats. The mean total protein, cholesterol, and triglyceride values for the 500 mg/kg male group were significantly

($p \leq 0.05$) higher than those of the control group. For female rats, significantly ($p \leq 0.05$) lower mean creatinine values were noted for the 50 mg/kg and 500 mg/kg rats. Significantly higher cholesterol and lower sodium values were also seen for the 500 mg/kg female rats. There were no other significant differences in mean clinical chemistry parameters between treated and control groups for male or female animals.

Organ Weights

The mean terminal body weights, absolute organ weights, and relative (to body weight) organ weights are presented in the summary tables on pages 81 (male) and 82 (female). Individual animal data are presented in Appendix A.

The mean terminal body weight for the male 500 mg/kg group was significantly lower than for the control group. Mean terminal body weight for the female 500 mg/kg group was lower than controls, but the difference was not statistically significant. Absolute weights for the thymus, spleen, testis, and epididymides of the 500 mg/kg male rats were significantly ($p \leq 0.05$) lower when compared with the male control group. For female rats, absolute thymus weights for 500 mg/kg rats were also significantly lower when compared with the control group. Mean relative (to body weight) liver, kidney, and adrenal gland weights for the 500 mg/kg male and female dose groups were significantly ($p \leq 0.05$) higher than the respective control groups. The 500 mg/kg male rats also had significantly higher mean relative heart and brain weights than did the male control animals. The 150 mg/kg male rats' mean relative (to body weight) liver weights were significantly ($p \leq 0.05$) higher and the 50 mg/kg male rats mean relative liver weights were significantly lower than the control group. Mean relative (to body weight) thymus weights for 500 mg/kg male and female rats were significantly ($p \leq 0.05$) lower than their control group. Mean relative epididymides weight from the 500 mg/kg rats was also significantly ($p \leq 0.05$) lower than the control group. All other mean relative (to body weight) organ weights for male and female rats were comparable to those of the respective control groups.

Gross Pathology

Gross lesions observed at necropsy included minor to moderate thymus hemorrhage for single male and female rats from each of the 50 mg/kg and 150 mg/kg dose groups. Minimal cervical lymph node hemorrhage was seen for one 50 mg/kg male. Moderate red focal discoloration of the lungs was observed for one 150 mg/kg male rat and one 150 mg/kg female rat. Minimal focal red discoloration of the esophagus was seen for a single control female. Minimal tan discoloration on the tip of the median lobe of the liver was seen for one 150 mg/kg female rat. For one 500 mg/kg male rat, a small and soft testis (minor) was noted. For another 500 mg/kg male rat, the gastrointestinal tract from the stomach to the rectum was distended with gas and empty of contents. For this animal, an accessory median lobe of the liver was noted. Minor hematomas on the bones of the skull were observed for two male rats and a one female rat from the 50 mg/kg dose group. Minor inguinal hair urine staining was seen for two 500 mg/kg female rats, and minimal porphyrin discharge of the nose was seen for one 150 mg/kg male rat. A single 500 mg/kg female was also observed to have a decreased amount of adipose tissue. No other gross lesions were observed at necropsy.

Histopathology

Microscopic examinations were performed on the protocol-specified tissues from rats assigned to the 500 mg/kg and control groups. Tissues with gross lesions and target organs from other dose groups were also examined microscopically. For details of the microscopic examination of the tissues, see the Pathology Report beginning on page 83. Microscopic findings which may be related to test substance exposure were limited to the tissues of the 500 mg/kg male and female rats. For these animals, varying degrees of sciatic nerve fiber degeneration were seen. Seminiferous tubule degeneration within the testis and degenerative cells within the epididymal tubules were also seen for 500 mg/kg rats. Microscopic findings in the single high-dose male rat which died prior to termination of the study included centrilobular necrosis within the liver, tubular necrosis in the kidney, and necrosis of the epithelia of the secretory ducts of the submandibular salivary glands. No histopathologic lesions were noted for the single 500 mg/kg female that was euthanatized prior to the scheduled necropsy. Other microscopic lesions were found in the liver, thymus, prostate, and kidneys; however, these lesions were not considered treatment related due to their limited incidence or occurrence among all study groups at similar rates.

DISCUSSION

Treatment of male and female rats with oral doses of the test substance resulted in overt signs of toxicity. Mortality, poor body condition, poor haircoat, and decreased body weight were observed in animals treated with 500 mg/kg. In addition, effects were observed in the sciatic nerve and testes of animals treated with 500 mg/kg, and serum cholesterol and triglycerides were significantly elevated.

Decreased hindlimb grip strength was observed in male rats from the 500 mg/kg group. These animals also had a decrease in mean total ambulations, and a lower mean total motor activity score. Whether the decrease in grip strength is associated with the decrease in motor activity is not clear. However, it seems likely that the decrease in grip strength is associated with the sciatic nerve fiber degeneration observed for animals in this dose group. No histopathologic lesions were reported for the brain or spinal cord. Treatment related peripheral nerve lesions were not observed at the 150 mg/kg and 50 mg/kg dose levels.

Decreased leukocyte counts and alterations in the percentages of leukocytes in the differential count have been observed in animals treated by injection with *E. coli* endotoxin (Schalm's *Veterinary Hematology*, 1986) and chemotherapeutic agents (Zimmerman *et al.*, 1991). The profile of changes in relative leukocyte counts for the 500 mg/kg group appears to mimic a degenerative response although there was no evidence of degenerative changes in the microscopic evaluation of the bone marrow. The percentage of lymphocytes for the 150 mg/kg group was within normal limits for this strain of rat and this laboratory. The increase in the percentage of blast cells, while suggestive of enhanced bone marrow activity, was not accompanied by histopathologic changes in the bone marrow or adverse changes in other hematologic parameters. Therefore, the effects observed for the 150 mg/kg group were not considered to be adverse. The increase in cholesterol and triglycerides observed for the 500 mg/kg group were considered toxicologically significant. Mean cholesterol and triglyceride levels for the 500 mg/kg groups were nearly double the values for the control groups.

Effects were also observed for male reproductive organs which included decreased testes weight and seminiferous tubule degeneration in animals treated with 500 mg/kg. No effects were observed for female reproductive organs. Other organ weight changes (liver, kidney, adrenal gland, and heart) appear to be adaptive in that no microscopic lesions were observed in those organs from the high-dose group.

CONCLUSION

Based on the histopathologic findings in the sciatic nerve and testes, the decrease in grip strength, and the increase in cholesterol and triglycerides for the 500 mg/kg group, and the lack of effect at other dose levels, the no-observed-adverse-effect level (NOAEL) was determined to be 150 mg/kg.

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