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Chemical Category	2,4,6-TRIBROMOPHENOL		

OFFICE OF TOXIC SUBSTANCES
CODING FORM FOR GLOBAL INDEXING

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Great Lakes Chemical Corporation

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ATTN: TSCA Section 8(e) Coordinator

RE: Follow-up Submission Regarding Document Control Number 8EHQ-99-14403
(When responding, please refer to JAB-99-073)

Dear TSCA 8(e) Coordinator:

Enclosed is a copy of two final reports. Both are entitled, "Mammalian Erythrocyte Micronucleus Test". One report is on 2,4,6-tribromophenol and the other is on 2,4,6-trichlorophenol. These final study reports are being submitted as a follow-up to a previous TSCA Section 8(e) substantial risk notification that was submitted by Great Lakes Chemical Corporation, West Lafayette, IN and the Griffin Corporation, Valdosta, GA and entered/filed under the EPA-TSCA Document Control Number 8EHQ-99-14403.

If you have any questions, please feel free to contact me at (765) 497-6223.



8EHQ-99-14403

Sincerely,

Handwritten signature: John A. Biesemeier

John A. Biesemeier
Manager, Corporate Toxicology

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cc: Dr. V. J. Piccirillo (NPC, Inc.)
Mr. A. Las (Griffin Corp.)

Certified Mail No.: Z 497 658 674
Internal ID No.: 99-01 (final report submitted)

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FINAL REPORT

Study Title

MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST

Test Article

2,4,6-Tribromophenol

Data Requirement

40 CFR Part 158
US-EPA-FIFRA, Section F, Guideline 84-2

Authors

Ramadevi Gudi, Ph.D.
Ljubica Krsmanovic, Ph.D.

Study Completion Date

May 20, 1999

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Study Number

AA11VU.123.BTL

Sponsor Project Number

GP99-019 (Griffin L.L.C.)

CONTAINS NO CBI

Sponsors

Great Lakes Chemical Corporation
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Highway 52 N.W.
West Lafayette, IN 47906

Griffin L.L.C.
P.O. Box 1847
2509 Rocky Ford Road
Valdosta, GA 31603-1847

CONFIDENTIALITY STATEMENT

STATEMENT OF COMPLIANCE

Study AA11VU.123.BTL was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

The stability of the test or control article under the test conditions was not determined by the testing facility.

Ramadevi Gudi
Ramadevi Gudi, Ph.D.
Study Director

5/20/99
Date

John A. Breseman
Sponsor

6/9/99
Date

Sponsor Submitter

Date

QUALITY ASSURANCE STATEMENT

Study Title: MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST
Study Number: AA11VU.123.BTL
Study Director: Ramadevi Gudi, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Regulations, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

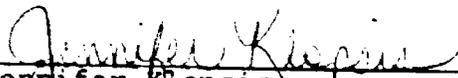
INSPECT ON 26 JAN 99, TO STUDY DIR 26 JAN 99, TO MGMT 26 JAN 99
PHASE: Protocol Review

INSPECT ON 03 MAR 99, TO STUDY DIR 03 MAR 99, TO MGMT 03 MAR 99
PHASE: Slide Preparation

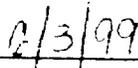
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PHASE: Draft Report

INSPECT ON 03 JUN 99, TO STUDY DIR 03 JUN 99, TO MGMT 03 JUN 99
PHASE: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.



Jennifer Klopsis
QUALITY ASSURANCE



DATE

MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST

FINAL REPORT

Sponsors: **Great Lakes Chemical Corporation**
P.O.Box 2200
Highway 52 N.W.
West Lafayette, IN 47906

Griffin L.L.C.
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2509 Rocky Ford Road
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Authorized Representative: **John A. Biesemeier (Great Lakes Chemical Corporation)**
W.A. Hawkins, Jr., Ph.D. (Griffin L.L.C.)

Performing Laboratory: **BioReliance**
9630 Medical Center Drive
Rockville, MD 20850

Test Article I.D.: **2,4,6-Tribromophenol**

Test Article Purity: **100% (Provided by Sponsor)**

Sponsor Project No.: **GP99-019 (Griffin L.L.C)**

BioReliance Study No.: **AA11VU.123.BTL**

Test Article Description: **White solid**

Storage Conditions: **Room temperature; in a cool, dry, well ventilated area away from incompatible materials and protected from exposure to light and moisture**

Test Article Receipt: **January 11, 1999**

Study Initiation: **January 25, 1999**

Study Director: Ramadevi Gudi 5/20/99
Ramadevi Gudi, Ph.D. Date

TABLE OF CONTENTS

	Page
Summary	6
Purpose	9
Characterization of Test and Control Articles	9
Materials and Methods	9
Results and Discussion	13
Conclusion	14
References	14
Data Tables	16
Appendix I: Mouse Micronucleus Test Historical Control Data	24
Appendix II: Study Protocol	26

SUMMARY

The test article, 2,4,6-Tribromophenol, was tested in the mouse micronucleus assay. The assay was performed in two phases. The first phase, designed to set dose levels for the definitive study, consisted of a pilot assay followed by a toxicity study. The second phase, the micronucleus study, evaluated the potential of the test article to increase the incidence of micronucleated polychromatic erythrocytes in bone marrow of male and female mice. In both phases of the study, test or control articles were administered in a constant volume of 20 mL/kg body weight by a single intraperitoneal injection.

Corn oil was determined to be the solvent of choice based on a solubility determination of the test article and compatibility of the vehicle with the test system animals. The test article was soluble in corn oil at 200 mg/mL, the maximum concentration tested. Dosing concentrations were delivered to the test system as yellow solutions.

In the pilot assay, male mice were dosed with 1, 10, 100, or 1000 mg test article/kg body weight and male and female mice were dosed with 2000 mg/kg. Mortality was observed in 2/2 male mice at 1000 mg/kg and in 5/5 male mice and 5/5 female mice at 2000 mg/kg. Clinical signs, observed immediately following dose administration, included: convulsions in male mice at 1000 mg/kg and in male and female mice at 2000 mg/kg.

In the toxicity assay, male and female mice were dosed with 200, 400, 600, or 800 mg test article/kg body weight. Mortality was observed in 5/5 male mice and 5/5 female mice at 600 and 800 mg/kg. Clinical signs, observed immediately following dose administration, included: convulsions in male and female mice at 400, 600 and 800 mg/kg. Lethargy was observed in male and female mice at 200 and 400 and 600 mg/kg, piloerection in male and female mice at 200 and 400 and crusty eyes in male and female mice at 400 and 600 mg/kg. Approximately two hours after dosing with 600 mg/kg, one male and one female mouse exhibited tremors. A reduction of approximately 10% and 15% in body weights in male and female groups treated with 400 mg/kg were observed three days after dose administration. Due to clinical signs and reductions in body weights, the maximum tolerated dose was estimated to be 300 mg/kg and was used as the high dose for the micronucleus test.

In the micronucleus assay, male and female mice were dosed with 75, 150 and 300 mg test article/kg body weight. No mortality was observed in any male or female mice in the micronucleus study. Clinical signs following dose administration included: lethargy, and piloerection in male and female mice at 150 and 300 mg/kg. All other animals treated with test or control articles appeared normal following dose administration. Bone marrow cells, collected 24 and 48 hours after treatment, were examined microscopically for micronucleated polychromatic erythrocytes. Statistically significant reductions in the number of polychromatic erythrocytes were observed in male mice treated with 150 and 300 mg/kg 24 hours after dose administration relative to the respective vehicle control ($p \leq 0.05$, Mann Whitney Test).

No significant increase in micronucleated polychromatic erythrocytes in test article-treated groups relative to the respective vehicle control groups was observed in male or female mice

at 24 or 48 hours after dose administration ($p > 0.05$, Kastenbaum-Bowman). The results of the assay indicate that under the conditions described in this report, 2,4,6-Tribromophenol did not induce a significant increase in micronucleated polychromatic erythrocytes in either male or female mice. The test article, 2,4,6-Tribromophenol was concluded to be negative in the mouse micronucleus assay.

PURPOSE

The purpose of this study was to evaluate the clastogenic potential of the test article as measured by its ability to induce micronucleated polychromatic erythrocytes in mouse bone marrow.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, 2,4,6-Tribromophenol, was received by BioReliance on January 11, 1999 and was assigned the code number AA11VU. The lot number of the test article was not provided. The test article was characterized by the Sponsor as a white to off-white powder which should be stored at room temperature in a cool, dry, well-ventilated area away from incompatible materials. Upon receipt, the test article was described as a white solid and was stored at room temperature, in a dry, well ventilated area away from incompatible materials and protected from exposure to light and moisture. The test article was reported by the Sponsor to be 100% pure.

The vehicle used to deliver 2,4,6-Tribromophenol to the test system was corn oil (CAS number 8001-30-1) obtained from Sigma Chemical Company.

Cyclophosphamide (CP, CAS number 6055-19-2), was obtained from Sigma Chemical Company and was dissolved in sterile distilled water at a concentration of 2.5 mg/mL for use as the positive control.

MATERIALS AND METHODS

Test System

ICR mice for pilot assay were obtained from Harlan Sprague Dawley, Inc., Frederick, MD and for toxicity and micronucleus assay from Harlan Sprague Dawley, Inc., Oregon, WI. At the initiation of the study, the mice were 6 to 8 weeks old. Animal body weights recorded at randomization were within the following ranges:

Pilot study: Males, 27.4 - 31.3 grams
Females, 25.3 - 27.7 grams

Toxicity study: Males, 30.6 - 34.3 grams
Females, 24.9 - 29.2 grams

Micronucleus assay: Males, 29.9 - 36.7 grams
Females, 24.7 - 30.0 grams

Animal Receipt and Quarantine

Mice were obtained from a source monitored for evidence of ectoparasites, endoparasites, pathogenic bacteria, mycoplasmas, and appropriate murine viruses and were quarantined for no less than 5 days after receipt. The mice were observed each working day for signs of illness, unusual food and water consumption, and other conditions of poor health. The animals were judged to be healthy prior to utilization in the assay.

Animal Care

The mice were housed in an AAALAC-accredited facility with a controlled environment of $74\pm 6^{\circ}\text{F}$, $50\pm 20\%$ relative humidity, and a 12 hour light/dark cycle. Mice of the same sex were housed up to five per cage in plastic autoclavable cages which were maintained on stainless steel racks equipped with automatic watering manifolds and which were covered with filter material. Heat-treated hardwood chips were used for bedding. Mice had free access to certified laboratory rodent chow which had been analyzed for environmental contaminants (Harlan TEKLAD certified Rodent 7012C) and to tap water (Washington Suburban Sanitary Commission, Potomac Plant). There were no contaminants in the feed which were considered to have influenced on the results of the study. The water used in the study met USEPA drinking water standards and is monitored at least annually for levels of organophosphorus pesticides, metals, and coliform and other contaminants.

Solubility Test

A solubility test was conducted to select the vehicle and to determine the vehicle that permit preparation of the highest soluble or workable concentration, up to 200 mg/mL. On the basis on information provided by the Sponsor, the test article was insoluble in water and thus a limited solubility test was conducted using corn oil.

Pilot Study

For the pilot study, mice were randomly assigned to one group of five males and five females and to four groups of two males each. Each mouse was given a sequential number and identified by ear tag. All mice were weighed immediately prior to dose administration and the dose volume based on individual body weights. Mice were observed after dose administration and daily thereafter for 3 days for clinical signs of chemical effect. Body weights were recorded prior to dose administration and 1 and 3 days after dose administration.

Toxicity Study

For the toxicity study, mice were randomly assigned to four groups of five males and five females each. Each mouse was given a sequential number and identified by ear tag. All mice in the experimental groups were weighed immediately prior to dose administration and the dose volume was based on individual body weights. Mice were observed after dose administration and daily thereafter for 3 days for clinical signs of chemical effect. Body

weights were recorded prior to dose administration and 1 and 3 days after dose administration.

Micronucleus Assay

The micronucleus assay was conducted using established and validated procedures (Heddle, 1973; Hayashi et al., 1994; Mavournin et al., 1990). The mice were assigned to seven experimental groups of five males and five females each according to a computer-generated program which is based on distribution according to body weight. An additional group of 5 males and 5 females was designated as replacement animals in the event of mortality prior to the scheduled sacrifice time and was dosed with the test article high dose level. Negative and positive control groups were shared between this and study AA11XA.123.BTL, both of which were performed for Great Lakes Chemical Corporation and Griffin L.L.C. Each mouse was given a sequential number and identified by ear tag. The study design was as follows:

	<u>Number of Mice Per Sex Dosed</u>	<u>Number of Mice Per Sex Used for Bone Marrow Collection After Dose Administration</u>	
		<u>24 hr</u>	<u>48 hr</u>
Vehicle Control Corn oil	10	5	5
Test Article			
Low test dose (75 mg/kg)	5	5	
Mid test dose (150 mg/kg)	5	5	
High test dose (300 mg/kg)	15	5	5
Positive Control CP, 50 mg/kg	5	5	

Dose Administration

The test article vehicle mixture, the vehicle alone, or the positive control was administered by a single intraperitoneal injection at a constant volume of 20 mL/kg body weight. Intraperitoneal injection was selected to maximize delivery of the test article to the test system. All mice in the experimental and control groups were weighed immediately prior to dose administration, and the dose volume was based on individual body weights. Mice were observed after dose administration for clinical signs of chemical effect.

Slide Preparation

At the scheduled sacrifice times, five mice per sex per treatment were sacrificed by CO₂ asphyxiation. Immediately following sacrifice, the femurs were exposed, cut just above the knee, and the bone marrow was aspirated into a syringe containing fetal bovine serum. The

bone marrow cells were transferred to a capped centrifuge tube containing approximately 1 mL fetal bovine serum. The bone marrow cells were pelleted by centrifugation at approximately 100 x g for five minutes and the supernatant was drawn off, leaving a small amount of serum with the remaining cell pellet. The cells were resuspended by aspiration with a capillary pipet and a small drop of bone marrow suspension was spread onto a clean glass slide. Two slides were prepared from each mouse. The slides were fixed in methanol, stained with May-Gruenwald-Giemsa and permanently mounted.

Scoring for Micronuclei

Slides were coded using a random number table by an individual not involved with the scoring process. Using medium magnification, an area of acceptable quality was selected such that the cells were well spread and stained. Using oil immersion, 2000 polychromatic erythrocytes were scored for the presence of micronuclei which are defined as round, darkly staining nuclear fragments, having a sharp contour with diameters usually from 1/20 to 1/5 of the erythrocyte. The number of micronucleated normochromatic erythrocytes in the field of 2000 polychromatic erythrocytes was enumerated. The proportion of polychromatic erythrocytes to total erythrocytes was also recorded per 1000 erythrocytes.

Evaluation of Test Results

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes was determined for each mouse and treatment group. Statistical significance was determined using the Kastenbaum-Bowman tables which are based on the binomial distribution (Kastenbaum and Bowman, 1970). All analyses were performed separately for each sex and sampling time.

In order to quantify the proliferation state of the bone marrow as an indicator of bone marrow toxicity, the proportion of polychromatic erythrocytes to total erythrocytes was determined for each animal and treatment group.

All conclusions were based on sound scientific judgement; however, as a guide to interpretation of the data, the test article was considered to induce a positive response if a dose-responsive increase in micronucleated polychromatic erythrocytes was observed and one or more doses were statistically elevated relative to the vehicle control ($p \leq 0.05$, Kastenbaum-Bowman Tables) at any sampling time. If a single treatment group was significantly elevated at one sacrifice time with no evidence of a dose-response, the assay was considered a suspect or unconfirmed positive and a repeat assay recommended. The test article was considered negative if no statistically significant increase in micronucleated polychromatic erythrocytes above the concurrent vehicle control was observed at any sampling time.

Criteria for a Valid Test

The mean incidence of micronucleated polychromatic erythrocytes must not exceed 5/1000 polychromatic erythrocytes (0.5%) in the vehicle control. The incidence of micronucleated

polychromatic erythrocytes in the positive control group must be significantly increased relative to the vehicle control group ($p \leq 0.05$, Kastenbaum-Bowman Tables).

Archives

All raw data, scored slides, and a copy of the final report will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance Regulatory Affairs/Quality Assurance Unit headquartered at: BioReliance, 14920 Broschart Rd., Rockville, MD. 20850.

RESULTS AND DISCUSSION

Solubility Test

Corn oil was determined to be the solvent of choice based on a solubility determination of the test article and compatibility of the vehicle with the test system. The test article was soluble in corn oil at 200 mg/mL, the maximum concentration tested. Dosing concentrations were delivered to the test system as yellow solutions.

Pilot Assay

For the pilot study, 2,4,6-Tribromophenol was administered by intraperitoneal injection to male mice at 1, 10, 100, or 1000 mg test article/kg body weight and to male and female mice at 2000 mg/kg which was administered in a total volume of 20 mL test article-vehicle mixture/kg body weight. Mortality, clinical signs and body weight data are reported in Tables 1 and 2. Mortality occurred after dose administration as follows: 5/5 males and 5/5 females at 2000 mg/kg and 2/2 males at 1000 mg/kg. Clinical signs, which were noted immediately after dose administration, included: convulsions in all male mice at 1000 mg/kg and in all male and female mice at 2000 mg/kg. All other animals appeared normal throughout the observation period. Due to excessive mortality at 2000 and 1000 mg/kg, a toxicity assay was performed.

Toxicity Assay

For the toxicity study, 2,4,6-Tribromophenol was administered by a single intraperitoneal injection to male and female mice at 200, 400, 600, or 800 mg test article/kg body weight which was administered in a total volume of 20 mL test article-vehicle mixture/kg body weight. Mortality, clinical signs and body weight data are reported in Tables 3 and 4. Mortality occurred within a day of dose administration in all male and female mice at 600 and 800 mg/kg. Clinical signs, which were noted immediately following dose administration, included: convulsions in all male and female mice at 400, 600 and 800 mg/kg. Lethargy was observed in male and female mice at 200, 400 and 600 mg/kg, piloerection in males and females at 200 and 400 mg/kg and crusty eyes in males and females at 400 and 600 mg/kg. Approximately two hours after dosing with 600 mg/kg, one male mouse and one female mouse exhibited tremors. A reduction of approximately 10% and 15% in body weights in male and female groups treated with 400 mg/kg were observed three days after dose administration. Due to clinical signs and a reduction in body weights, the maximum

tolerated dose was estimated to be 300 mg/kg and was used as the high dose for the micronucleus test.

Micronucleus Assay

For the micronucleus test, male and female mice were dosed with 2,4,6-Tribromophenol by a single intraperitoneal injection of 75, 150, or 300 mg test article/kg body weight which was administered in a total volume of 20 mL test article-vehicle mixture/kg body weight. Mortality and clinical signs are presented in Table 5. No mortality occurred at any dose level during the course of the micronucleus study. Clinical signs, which were noted on the days following dose administration, included: lethargy and piloerection in male and female mice at 150 and 300 mg/kg. All other mice treated with test and control articles appeared normal during the study.

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes scored and the proportion of polychromatic erythrocytes per total erythrocytes are summarized and presented for each treatment group by sacrifice time in Table 6. Individual animal data are presented in Tables 7 and 8. Statistically significant reductions in the number of polychromatic erythrocytes were observed in male mice treated with 150 and 300 mg/kg 24 hours after dose administration relative to the respective vehicle control. ($p \leq 0.05$, Mann Whitney Test).

The number of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes in test article-treated groups was not statistically increased relative to their respective vehicle control in either male or female mice, regardless of dose level or bone marrow collection time ($p > 0.05$, Kastenbaum-Bowman Tables). CP induced a significant increase in micronucleated polychromatic erythrocytes in both male and female mice ($p \leq 0.05$, Kastenbaum-Bowman Tables).

CONCLUSION

All criteria for a valid test were met. Under the conditions of the assay described in this report, 2,4,6-Tribromophenol did not induce a significant increase in the incidence of micronucleated polychromatic erythrocytes in bone marrow and was concluded to be negative in the micronucleus test using male and female mice.

REFERENCES

- Heddle, J.A. 1973. A rapid *in vivo* test for chromosomal damage. *Mutation Res.* 18:187-190.
- Hayashi, M., R.R. Tice, J.T. Macgregor, D. Anderson, D.H. Blakey, M. Dirsch-Volders, F.G. Oleson Jr., F. Pacchierotti, F. Romagna, H. Shimada, S. Sutou and B. Vannier. 1994. *In vivo* rodent erythrocyte micronucleus assay. *Mutation Res.* 312: 293-304.

Mavournin, K.H., D.H. Blakey, M.C. Cimino, M.F. Salamone and J.A. Heddle. 1990. The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutation Res.* 239:29-80.

Kastenbaum, M.A. and K.O. Bowman. 1970. Tables for determining the statistical significance of mutation frequencies. *Mutation Res.* 9:527-549.

Table 1
 Clinical Signs Following Dose Administration of 2,4,6-Tribromophenol
 Pilot Toxicity Study

Treatment	Clinical Observation	Number of Animals with Clinical Signs/Total Number of Animals Dosed		Number of Animals Died/Total Number of Animals Dosed	
		Males	Females	Males	Females
2,4,6-Tribromophenol, 1 mg/kg	Normal	2/2	N/A	0/2	N/A
2,4,6-Tribromophenol, 10 mg/kg	Normal	2/2	N/A	0/2	N/A
2,4,6-Tribromophenol, 100 mg/kg	Normal	2/2	N/A	0/2	N/A
2,4,6-Tribromophenol, 1000 mg/kg	Convulsions	2/2	N/A	2/2	N/A
2,4,6-Tribromophenol, 2000 mg/kg	Convulsions	5/5	5/5	5/5	5/5

Table 2
Pilot Toxicity Study Using 2,4,6-Tribromophenol In ICR Mice:
Body Weight and Mortality Data

Treatment	Sex	Group Mean Body Weights (gms)			% Change ¹		Mortality ²
		Pretreatment	Day 1	Day 3	Day 1	Day 3	
2,4,6-Tribromophenol, 1 mg/kg	M	30.2 ± 0.4	31.3 ± 1.0	31.9 ± 1.2	3.6%	5.6%	0 / 2
10 mg/kg	M	31.6 ± 0.6	30.6 ± 0.6	31.3 ± 1.1	-3.2%	-0.9%	0 / 2
100 mg/kg	M	30.4 ± 3.0	29.8 ± 3.0	31.0 ± 3.2	-2.0%	2.0%	0 / 2
1000 mg/kg	M	30.8 ± 1.4	ND	ND	ND	ND	2 / 2
2000 mg/kg	M	30.9 ± 1.0	ND	ND	ND	ND	5 / 5
	F	27.8 ± 1.1	ND	ND	ND	ND	5 / 5

¹% Change = $\frac{\text{Post-treatment weight} - \text{Pretreatment weight}}{\text{Pretreatment weight}} \times 100$

²Reported as number of animals dead 3 days after dose administration/total number tested.

ND = No data due to mortality.

Table 3

Clinical Signs Following Dose Administration of 2,4,6-Tribromophenol Toxicity Study

Treatment	Clinical Observation	Number of Animals with Clinical Signs/Total Number of Animals Dosed		Number of Animals Died/Total Number of Animals Dosed	
		Males	Females	Males	Females
2,4,6-Tribromophenol, 200 mg/kg	Lethargy Piloerection	5/5 5/5	3/5 3/5	0/5	0/5
2,4,6-Tribromophenol, 400 mg/kg	Convulsions Lethargy Piloerection Crusty eyes	5/5 5/5 5/5 5/5	5/5 5/5 5/5 5/5	0/5	0/5
2,4,6-Tribromophenol, 600 mg/kg	Convulsions Tremors Crusty eyes Lethargy	5/5 1/5 1/5 1/5	5/5 1/5 1/5 1/5	5/5	5/5
2,4,6-Tribromophenol, 800 mg/kg	Convulsions	5/5	5/5	5/5	5/5

Table 4
Toxicity Study Using 2,4,6-Tribromophenol In ICR Mice:
Body Weight and Mortality Data

Treatment	Sex	Group Mean Body Weights (gms)			% Change ¹		Mortality ²
		Pretreatment	Day 1	Day 3	Day 1	Day 3	
2,4,6- Tribromophenol, 200 mg/kg	M	33.7 ± 1.1	33.5 ± 1.1	34.1 ± 1.2	-0.6%	1.2%	0 / 5
	F	26.8 ± 1.2	26.9 ± 1.2	27.7 ± 1.9	0.4%	3.4%	0 / 5
400 mg/kg	M	34.2 ± 1.3	33.0 ± 1.0	30.8 ± 1.4	-3.5%	-9.9%	0 / 5
	F	27.0 ± 1.3	26.1 ± 1.7	22.7 ± 2.0	-3.3%	-15.9%	0 / 5
600 mg/kg	M	33.2 ± 1.5	³ ND	³ ND	³ ND	³ ND	5 / 5
	F	27.4 ± 1.6	³ ND	³ ND	³ ND	³ ND	5 / 5
800 mg/kg	M	33.1 ± 1.8	³ ND	³ ND	³ ND	³ ND	5 / 5
	F	27.0 ± 1.5	³ ND	³ ND	³ ND	³ ND	5 / 5

¹% Change = $\frac{(\text{Post-treatment weight} - \text{Pretreatment weight}) \times 100}{\text{Pretreatment weight}}$

²Reported as number of animals dead 3 days after dose administration/total number tested.

³No data due to mortality.

Table 5
 Clinical Signs Following Dose Administration of 2,4,6-Tribromophenol
 Micronucleus Assay

Treatment	Clinical Observation	Number of Animals with Clinical Signs/Total Number of Animals Dosed		Number of Animals Died/Total Number of Animals Dosed	
		Males	Females	Males	Females
Corn oil, 20 ml/kg	Normal	10/10	10/10	0/10	0/10
2,4,6-Tribromophenol, 75 mg/kg	Normal	5/5	5/5	0/5	0/5
2,4,6-Tribromophenol, 150 mg/kg	Lethargy Piloerection	4/5 3/5	2/5 2/5	0/5	0/5
2,4,6-Tribromophenol, 300 mg/kg	Lethargy Piloerection	15/15 15/15	15/15 15/15	0/15	0/15
CP, 50 mg/kg	Normal	5/5	5/5	0/5	0/5

Table 6
Summary of Bone Marrow Micronucleus Study Using 2,4,6-Tribromophenol

Treatment	Sex	Time (hr)	Number of Mice	PCE/Total Erythrocytes (Mean +/- sd)	Change From Control (%)	Micronucleated Polychromatic Erythrocytes Number per 1000 PCEs (Mean +/- sd)	Erythrocytes Number per PCEs Scored
Corn oil 20 mL/kg	M	24	5	0.54 ± 0.02	---	0.4 ± 0.42	4 / 10000
	F	24	5	0.57 ± 0.12	---	0.4 ± 0.55	
2,4,6-Tribromophenol 75 mg/kg	M	24	5	0.47 ± 0.06	-13	0.3 ± 0.27	3 / 10000
	F	24	5	0.47 ± 0.03	-18	0.2 ± 0.27	2 / 10000
150 mg/kg	M	24	5	0.42 ± 0.06	-22	0.2 ± 0.27	2 / 10000
	F	24	5	0.44 ± 0.03	-23	0.5 ± 0.35	5 / 10000
300 mg/kg	M	24	5	0.45 ± 0.04	-17	0.2 ± 0.27	2 / 10000
	F	24	5	0.47 ± 0.03	-18	0.3 ± 0.27	3 / 10000
CP, 50 mg/kg	M	24	5	0.33 ± 0.02	-39	25.5 ± 4.29	* 255 / 10000
	F	24	5	0.40 ± 0.05	-30	27.5 ± 4.91	* 275 / 10000
Corn oil 20 mL/kg	M	48	5	0.51 ± 0.02	---	0.2 ± 0.27	2 / 10000
	F	48	5	0.51 ± 0.05	---	0.3 ± 0.27	3 / 10000
2,4,6-Tribromophenol 300 mg/kg	M	48	5	0.48 ± 0.06	-6	0.6 ± 0.42	6 / 10000
	F	48	5	0.48 ± 0.04	-6	0.1 ± 0.22	1 / 10000

¹p<0.05 (Mann-Whitney test)
²p<0.05 (Kestevenbaum-Bowman Tables)

Table 7
 Induction of Micronucleated Polychromatic Erythrocytes in
 Bone Marrow Cells Collected 24 Hours After a Single Dose of
 2,4,6-Tribromophenol

Treatment	Sex	Animal Number	PCE/Total Erythrocytes	Micronucleated PCE (Number/PCE Scored)
Corn oil 20 mL/kg	M	1	0.52	1 / 2000
		2	0.55	0 / 2000
		3	0.52	0 / 2000
		4	0.57	2 / 2000
		5	0.53	1 / 2000
	F	6	0.62	0 / 2000
		7	0.59	0 / 2000
		8	0.40	2 / 2000
		9	0.72	0 / 2000
		10	0.52	2 / 2000
2,4,6-Tribromophenol 75 mg/kg	M	11	0.48	0 / 2000
		12	0.44	1 / 2000
		13	0.57	1 / 2000
		14	0.42	1 / 2000
		15	0.47	0 / 2000
	F	16	0.47	0 / 2000
		17	0.42	0 / 2000
		18	0.49	0 / 2000
		19	0.49	1 / 2000
		20	0.49	1 / 2000
150 mg/kg	M	21	0.43	0 / 2000
		22	0.43	0 / 2000
		23	0.31	1 / 2000
		24	0.44	1 / 2000
		25	0.48	0 / 2000
	F	26	0.42	1 / 2000
		27	0.40	1 / 2000
		28	0.43	2 / 2000
		29	0.49	1 / 2000
		30	0.45	0 / 2000
300 mg/kg	M	31	0.51	0 / 2000
		32	0.46	1 / 2000
		33	0.43	0 / 2000
		34	0.41	0 / 2000
		35	0.44	1 / 2000
	F	36	0.45	1 / 2000
		37	0.51	0 / 2000
		38	0.48	1 / 2000
		39	0.43	1 / 2000
		40	0.47	0 / 2000
CP, 50 mg/kg	M	71	0.35	53 / 2000
		72	0.35	57 / 2000
		73	0.33	53 / 2000
		74	0.33	56 / 2000
		75	0.32	36 / 2000
	F	76	0.39	64 / 2000
		77	0.44	58 / 2000
		78	0.32	41 / 2000
		79	0.39	63 / 2000
		80	0.46	49 / 2000

Table 8

Induction of Micronucleated Polychromatic Erythrocytes in Bone Marrow Cells Collected 48 Hours After a Single Dose of 2,4,6-Tribromophenol

Treatment	Sex	Animal Number	PCE/Total Erythrocytes	Micronucleated PCE (Number/PCE Scored)
Corn oil 20 mL/kg	M	81	0.48	0 / 2000
		82	0.52	1 / 2000
		83	0.51	1 / 2000
		84	0.54	0 / 2000
		85	0.49	0 / 2000
	F	86	0.56	1 / 2000
		87	0.44	0 / 2000
		88	0.54	1 / 2000
		89	0.47	1 / 2000
		90	0.51	0 / 2000
2,4,6-Tribromophenol 300 mg/kg	M	91	0.55	1 / 2000
		92	0.43	0 / 2000
		93	0.42	2 / 2000
		94	0.45	1 / 2000
		95	0.55	2 / 2000
	F	96	0.44	0 / 2000
		97	0.54	0 / 2000
		98	0.48	0 / 2000
		99	0.47	1 / 2000
		100	0.47	0 / 2000

APPENDIX I

Mouse Micronucleus Test Historical Control Data

**Mouse Micronucleus Test Historical Control Data
1995-1997**

Negative Control Animals¹

Parameter	Ratio of PCE/Total Erythrocytes		MPCE/1000 PCE Scored	
	Males	Females	Males	Females
Mean	0.54	0.55	0.79	0.82
Standard Deviation	0.08	0.08	0.90	0.94
Range	0.15 - 0.81	0.24 - 0.86	0 - 8	0 - 8

Positive Control Animals²

Parameter	Ratio of PCE/Total Erythrocytes		MPCE/1000 PCE Scored	
	Males	Females	Males	Females
Mean	0.46	0.48	31.92	28.37
Standard Deviation	0.12	0.10	17.18	14.37
Range	0.06 - 0.80	0.12 - 0.83	2 - 109	2 - 77

¹Negative controls include all vehicles and all routes of administration.

²Positive control is cyclophosphamide, 40 to 60 mg/kg, dosed by IV, IP or PO.

Bone marrow cells were collected at 24 hours after a single administration.

APPENDIX II
Study Protocol

Mammalian Erythrocyte Micronucleus Test

QA 12/19
APPROVED

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of the test articles as measured by their ability to induce micronucleated polychromatic erythrocytes in mouse bone marrow.

2.0 SPONSOR

2.1 Name: Great Lakes Chemical Corporation and Griffin L.L.C.

Address: Great Lakes Chemical Corporation
P.O. Box 2200
Highway 52 N.W.
West Lafayette, IN 47906

Griffin L.L.C.
PO Box 1847
2509 Rocky Ford Road
Valdosta, GA 31603-1847

2.3 Representatives: John A. Biesemeier (Great Lakes Chemical Corporation)
W. A. Hawkins, Jr., Ph.D. (Griffin L.L.C.)

2.4 Sponsor Project #: GP99-019 (Griffin L.L.C.)

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: 2,4,6-Tribromophenol

3.2 Controls: Vehicle: Test article vehicle
Positive: Cyclophosphamide (CP)

3.3 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at BioReliance will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test articles, and the stability and strength of the test articles in the solvent (or vehicle).



04

3.4 Test Article Retention Sample

The retention of a reserve sample of the test articles will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

- 4.1 Name: Toxicology Testing Facility
BioReliance
- 4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850
- 4.3 Study Director: Ramadevi Gudi, Ph.D.

5.0 TEST SCHEDULE

- 5.1 Proposed Experimental Initiation Date: 2/2/99
- 5.2 Proposed Experimental Completion Date: 3/26/99
- 5.3 Proposed Report Date: 4/2/99

6.0 TEST SYSTEM

Closed-colony, random-bred rodents are acceptable models for mutagenicity studies. ICR mice were selected because of the availability of historical control data.

- 6.1 Source: Harlan Sprague Dawley, Inc., Frederick, MD or
Charles River Breeding Laboratories, Kingston, NY or Raleigh, NC or
other approved alternates
- 6.2 Age at initiation of study: 6-8 weeks

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be conducted according to established procedures (Heddle, 1973; Mavoumin *et al.*, 1990; Hayashi *et al.*, 1994). Following the administration of three concentrations of test article as well as positive and negative (vehicle) controls to male and female mice, bone marrow cells will be collected at 24 and 48 hours and examined for the presence of micronucleated polychromatic erythrocytes. The clastogenic potential of the test article will be measured by its ability to increase micronucleated polychromatic erythrocytes in treated animals as compared to vehicle control animals.

The study design will be as follows:

	Number per Sex per to be Sacrificed After Dose Administration	
	24 Hr	48 Hr
Vehicle Control		
Vehicle alone	5	5
Test Article 1		
Low Dose	5	-
Mid Dose	5	-
High Dose	5	5
Test Article 2		
Low Dose	5	-
Mid Dose	5	-
High Dose	5	5
Positive Control		
Cyclophosphamide	5	-

*NA, corrected
consultation with
Sponsor 11/23/99*

7.1 Selection of Test Article Vehicle

Unless the Sponsor has indicated the test article vehicle, a solubility determination will be conducted to measure the maximum soluble concentration or workable suspension of test article in vehicle. Vehicles compatible with this test system, in order of preference, include, but are not limited to, distilled water or saline, 1% carboxymethylcellulose in water, and corn oil. The vehicle of choice will be that allowing preparation of dosing solutions required to achieve targeted doses.

7.2 Dose Selection

Selection of doses for the micronucleus assay will be based on the toxicity of the test articles but will not exceed 2000 mg/kg. In the absence of toxicity data, a pilot study will be performed with each test article at a dose of 2000 mg/kg using five male and five female mice. Three or more lower doses will be tested using two male mice each. If dose administration produces no treatment-related mortality, the high dose for the micronucleus test will be 2000 mg/kg. In the event of mortality in excess of 50% at 2000 mg/kg in the pilot study, an extensive toxicity study will be performed using at least four test article doses, each containing five male and five female mice. Mice will be observed after dose administration and each working day thereafter for 3 days for clinical signs of chemical effect. Body weights will be recorded prior to dose administration and at 1 and 3 days after dose administration.

Unless specified otherwise by the Sponsor or at the discretion of the Study Director, the high dose for the micronucleus test may be 50% to 80% of the LD₅₀ (the dose required to kill 50% of the animals within 3 days after administration) or the

required to kill 50% of the animals within 3 days after administration) or the maximum tolerated dose but will not exceed 2000 mg/kg. Two additional doses will be tested, one-half and one-fourth of the high dose.

7.3 Route and Frequency of Administration

Animals will be dosed by intraperitoneal (IP) injection. IP injection was selected to maximize delivery of the test article to the target system. IP injection is an acceptable method for administration of test article concentrations to laboratory animals. Animals will receive the test article as a single administration.

7.4 Controls

7.4.1 Vehicle control

The solvent vehicle for the test article will be used as the vehicle control.

7.4.2 Positive control

Cyclophosphamide monohydrate (CP, CAS number 6055-19-2) will be administered as the positive control at a dose of 30-60 mg/kg. CP will be administered by the same route as the test article.

Vehicle and positive controls will be shared between two test article groups

7.5 Animal Receipt and Quarantine

Virus antibody-free (VAF) mice will be quarantined for no less than 5 days prior to dose administration. The animals will be observed each working day for signs of illness, unusual food and water consumption, and other general conditions of poor health. All animals will be judged to be healthy prior to utilization in the study.

7.6 Animal Care

Animals will be housed in an AAALAC-accredited facility with a controlled environment of $50 \pm 20\%$ relative humidity and $74 \pm 6^\circ\text{F}$ with a 12 hour light/dark cycle. Mice of the same sex will be housed up to five per cage in plastic autoclavable cages. Heat-treated hardwood chips will be used for bedding. Animals will have free access to a certified laboratory rodent chow which has been analyzed for environmental contaminants and to tap water.

7.7 Randomization

The animals will be assigned to ^{eleven} ~~eleven~~ groups of five males and females each using a randomization procedure based on equalization of group mean body weights. At the time of randomization, the weight variation of animals will not exceed $\pm 20\%$ of the mean weight. Additional animals may be designated and dosed as replacement animals in the high dose group to be used in the event of mortality prior to the



07

scheduled sacrifice. This will be done at the discretion of the Study Director after evaluation of the toxicity data. Each animal will be given a sequential number and identified by ear tag.

7.8 Dose Preparation and Administration

The test article-vehicle mixture, the vehicle alone and the positive control (CP) will be given as a single administration. The rate of administration will be 10 ml/kg body weight unless larger volumes, up to 20 ml/kg, are required to deliver the targeted dose. All mice in the experimental groups will be weighed and the dose volume will be based on individual body weight.

7.9 Bone Marrow Collection

Twenty-four and 48 hours after dose administration, animals will be sacrificed by carbon dioxide asphyxiation. The positive control group will be sacrificed 24 hours after dose administration. Immediately following sacrifice, the femurs will be exposed, cut just above the knee and the bone marrow will be aspirated into a syringe containing fetal bovine serum. The bone marrow cells will be transferred to a capped centrifuge tube containing approximately 1 ml fetal bovine serum.

The bone marrow cells will be pelleted by centrifugation and the supernatant will be drawn off, leaving a small amount of fetal bovine serum with the remaining cell pellet. The cells will be resuspended by aspiration with a capillary pipette and a small drop of the bone marrow suspension will be spread onto a clean glass slide. Each slide will be identified by the experiment and animal number. At least two slides will be prepared from each animal, air dried, fixed by dipping in methanol, stained with May-Gruenwald-Giemsa and permanently mounted.

7.10 Scoring for Micronuclei

Slides will be coded using a random number table by an individual not involved with the scoring process. Using medium magnification, an area of acceptable quality will be selected such that the cells are well spread and stained. Using oil immersion, 2000 polychromatic erythrocytes will be scored per animal for the presence of micronuclei. The number of micronucleated normocytes in the field of 2000 polychromatic erythrocytes will also be enumerated. The proportion of polychromatic erythrocytes to total erythrocytes will also be recorded per 1000 erythrocytes. The proportion of polychromatic erythrocytes to total erythrocytes in test article-treated animals should not be less than 20% of the control value.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The mean incidence of micronucleated polychromatic erythrocytes must not exceed 5/1000 polychromatic erythrocytes (0.5%) in the negative (vehicle) control. The incidence of micronucleated polychromatic erythrocytes in the positive control group must be significantly increased relative to the negative control ($p \leq 0.05$, Kastenbaum-Bowman Tables).

9.0 EVALUATION OF TEST RESULTS

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes will be presented for each animal and treatment group. Statistical significance will be determined using the Kastenbaum-Bowman tables which are based on the binomial distribution.

In order to quantify the test article effect on erythropoiesis, as an indicator of bone marrow toxicity, the proportion of polychromatic erythrocytes to total erythrocytes will be presented for each animal and treatment group.

All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response if a dose-responsive increase in micronucleated polychromatic erythrocytes is observed and one or more doses are statistically elevated relative to the vehicle control ($p \leq 0.05$, Kastenbaum-Bowman Tables) at any sampling time. If a single treatment group is significantly elevated at one sacrifice time with no evidence of a dose-response, the assay will be considered a suspect or unconfirmed positive and a repeat experiment will be recommended. The test article will be judged negative if no statistically significant increase in micronucleated polychromatic erythrocytes above the concurrent vehicle control values are observed at any sampling time.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. The report will include:

- **Test article:** identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of the test article, if known.
- **Solvent/Vehicle:** justification for choice of vehicle; solubility and stability of the test article in the solvent/vehicle, if known.
- **Test animals:** species and strain of animals used; number, age and sex of animals; source, housing conditions, diet, etc.
- **Test conditions:** positive and negative (vehicle/solvent) control data; data from range-finding study, if conducted; rationale for dose level selection; details of test article preparation; details of the administration of test article; rationale for route of administration; methods for verifying that the test article reached the general circulation or target tissue, if applicable; details of food and water quality; description of treatment and sampling schedules; method of slide preparation; methods for measurement of toxicity; criteria for scoring micronucleated immature erythrocytes; number of cells analyzed per

animal: criteria for considering study as positive, negative or equivocal.

- Results: signs of toxicity; proportion of polychromatic erythrocytes among total erythrocytes; number of micronucleated polychromatic erythrocytes per animal; mean±standard deviation of micronucleated polychromatic erythrocytes per group; dose-response relationship, where possible; statistical analyses; concurrent negative control data; historical negative control data with ranges, means and standard deviations; concurrent positive control data.
- Discussion of results.
- Conclusion.

11.0 RECORDS AND ARCHIVES

All raw data, the protocol, and a copy of all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 474 (Genetic Toxicology: Mammalian Erythrocytes Micronucleus Test), July, 1997 and with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (1996 and 1997).

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? UNKNOWN
If so, which agency or agencies? EPA-FIFRA, EPA-TSCA

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Heddle, J.A. 1973. A rapid in vivo test for chromosomal damage. Mutation Res. 18:187-190.

Hayashi, M., R.R. Tice, J.T. MacGregor, D. Anderson, D.H. Blakey, M. Dirsch-Volders, F.G. Oleson Jr., F. Pacchierotti, F. Romagna, H. Shimada, S. Sutou and B. Vannier. 1994. *In vivo* rodent erythrocyte micronucleus assay. Mutation Res. 312: 293-304.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for

adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18,02. April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. Federal Register 62:16026-16030, November 21, 1997.

Kastenbaum, M.A. and K.O. Bowman. 1970. Tables for determining the statistical significance of mutation frequencies. Mutation Res. 9:527-549.

Mavourmin, K.H., D.H. Blakey, M.C. Cimino, M.F. Salamone and J.A. Heddle. 1990. The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res. 239:29-80.

OECD Guidelines for Testing of Chemicals. Guideline 474, Genetic Toxicology. Mammalian Erythrocyte Micronucleus Test. Organisation for Economic Co-operation and Development, Paris, France. July 1997.

14.0 APPROVAL

John A. Biesemeier 1/18/99
Sponsor Representative Date
Great Lakes Chemical Corporation

John A. Biesemeier
(Print or Type Name)

W. A. Hawkins, Jr. 1/18/99
Sponsor Representative Date
Griffin Corporation - L.L.C. 2/27/99
1/18/99

W. A. Hawkins, Jr., Ph.D.
(Print or Type Name)

Ramadevi Gudi 1/25/99
BioReliance Study Director Date

If submission to Japanese Regulatory Agency is indicated in section 12.0, BioReliance management will sign.

BioReliance Study Management Date



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FINAL REPORT

Study Title

MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST

Test Article

2,4,6-Trichlorophenol

Data Requirement

40 CFR Part 158
US-EPA-FIFRA, Section F, Guideline 84-2

Authors

Ramadevi Gudi, Ph.D.
Ljubica Krsmanovic, Ph.D.

Study Completion Date

May 20, 1999

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Study Number

AA11XA.123.BTL

Sponsor Project Number

GP99-019 (Griffin L.L.C.)

Sponsors

Great Lakes Chemical Corporation
P.O. Box 2200
Highway 52 N.W.
West Lafayette, IN 47906 and

Griffin L.L.C.
P.O. Box 1847
2509 Rocky Ford Road
Valdosta, GA 31603-1847

CONTAINS NO GL



CONFIDENTIALITY STATEMENT

CONTAINS NO CBI

STATEMENT OF COMPLIANCE

Study AA11XA.123.BTL was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

The stability of the test or control article under the test conditions was not determined by the testing facility.

Ramadevi Gudi
Ramadevi Gudi, Ph.D.
Study Director

5/20/99
Date

John A. Braseman
Sponsor

6/9/99
Date

Sponsor Submitter

Date

QUALITY ASSURANCE STATEMENT

Study Title: MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST
 Study Number: AA11XA.123.BTL
 Study Director: Ramadevi Gudi, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Regulations, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 29 JAN 99, TO STUDY DIR 29 JAN 99, TO MGMT 29 JAN 99
 PHASE: Protocol Review

INSPECT ON 03 MAR 99, TO STUDY DIR 03 MAR 99, TO MGMT 03 MAR 99
 PHASE: Bone Marrow Collection

INSPECT ON 24 MAR 99, TO STUDY DIR 25 MAR 99, TO MGMT 03 MAR 99
 PHASE: Draft Report

INSPECT ON 03 JUN 99, TO STUDY DIR 03 JUN 99, TO MGMT 03 JUN 99
 PHASE: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Jennifer Kloppis

 Jennifer Kloppis
 QUALITY ASSURANCE

6/3/99

 DATE

MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST**FINAL REPORT**

Sponsor: Great Lakes Chemical Corporation
P.O. Box 2200
Highway 52 N.W.
West Lafayette, IN 47906

Griffin L.L.C.
P.O.Box 1847
2509 Rocky Ford Road
Valdosta, GA 31603-1847

Authorized Representative: John A. Biesemeier (Great Lakes Chemical Corporation)
W.A. Hawkins, Jr., Ph.D. (Griffin L.L.C.)

Performing Laboratory: BioReliance
9630 Medical Center Drive
Rockville, MD 20850

Test Article I.D.: 2,4,6-Trichlorophenol

Test Article Lot No.: 12421LS

Test Article Purity: 98% (Provided by Sponsor)

Sponsor Project No.: GP99-019 (Griffin L.L.C.)

BioReliance Study No.: AA11XA.123.BTL

Test Article Description: off-white crystalline powder

Storage Conditions: Room temperature in a cool, dry, well ventilated area away from incompatible materials and protected from exposure to light and moisture

Test Article Receipt: January 13, 1999

Study Initiation: January 25, 1999

Study Director: Ramadevi Gudi 5/20/99
Ramadevi Gudi, Ph.D. Date

TABLE OF CONTENTS

	Page
Summary	7
Purpose	9
Characterization of Test and Control Articles	9
Materials and Methods	9
Results and Discussion	13
Conclusion	14
References	14
Data Tables	16
Appendix I: Mouse Micronucleus Test Historical Control Data	24
Appendix II: Study Protocol	26

SUMMARY

The test article, 2,4,6-Trichlorophenol, was tested in the mouse micronucleus assay. The assay was performed in two phases. The first phase, designed to set dose levels for the definitive study, consisted of a pilot assay followed by a toxicity study. The second phase, the micronucleus study, evaluated the potential of the test article to increase the incidence of micronucleated polychromatic erythrocytes in bone marrow of male and female mice. In both phases of the study, test and control articles were administered in a constant volume of 20 mL/kg body weight by a single intraperitoneal injection.

Corn oil was determined to be the solvent of choice based on a solubility determination of the test article and compatibility of the vehicle with the test system animals. The test article was soluble in corn oil at 200 mg/mL, the maximum concentration tested. Dosing concentrations were delivered to the test system as yellow solutions.

In the pilot assay, male mice were dosed with 1, 10, 100, or 1000 mg test article/kg body weight and male and female mice were dosed with 2000 mg/kg. Mortality was observed in 2/2 male mice at 1000 mg/kg and in 5/5 male mice and 5/5 female mice at 2000 mg/kg. Clinical signs, observed immediately following dose administration, included convulsions in all male mice at 1000 mg/kg and in all male and female mice at 2000 mg/kg.

In the toxicity assay, male and female mice were dosed with 200, 400, 600, or 800 mg test article/kg body weight. Mortality was observed in 4/5 male mice and 4/5 female mice at 400 mg/kg and in all male and female mice at 600 and 800 mg/kg. Clinical signs, observed immediately following dose administration, included convulsions in all male and female mice at 400, 600 and 800 mg/kg. Lethargy and piloerection were observed in male and female mice at 200 and 400 mg/kg, crusty eyes in one female mouse at 200 mg/kg and in one male and one female mouse at 400 mg/kg. Irregular breathing was observed in one male and one female mouse at 400 mg/kg. The high dose for the micronucleus test was set at 240 mg/kg which was estimated to be approximately 70% of the LD₅₀.

In the micronucleus assay, male and female mice were dosed with 60, 120 or 240 mg/kg body weight. No mortality was observed in any male or female mice in the micronucleus study. Clinical signs, observed immediately following dose administration, included tremors in all male and female mice at 240 mg/kg. Lethargy, piloerection and crusty eyes were observed in male and female mice at 240 mg/kg. Other animals treated with the test article or controls appeared normal following dose administration.

Bone marrow cells, collected 24 and 48 hours after treatment, were examined microscopically for micronucleated polychromatic erythrocytes. Statistically significant reduction in the number of polychromatic erythrocytes was observed only in the male 48 hour high dose test article treated group relative to the vehicle control ($p \leq 0.05$, Mann Whitney test).

No significant increase in micronucleated polychromatic erythrocytes in test article-treated groups relative to the respective vehicle control group was observed in male or female mice at 24 or 48 hours after dose administration ($p > 0.05$, Kastenbaum-Bowman). The results of

the assay indicate that under the conditions described in this report, 2,4,6-Trichlorophenol did not induce a significant increase in micronucleated polychromatic erythrocytes in either male or female mice. The test article, 2,4,6-Trichlorophenol was concluded to be negative in the mouse micronucleus assay.

PURPOSE

The purpose of this study was to evaluate the clastogenic potential of the test article as measured by its ability to induce micronucleated polychromatic erythrocytes in mouse bone marrow.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, 2,4,6-Trichlorophenol, was received by BioReliance on January 13, 1999 and was assigned the code number AA11XA. The lot number of the test article was 12421LS. The test article was characterized by the Sponsor as a white to off-white crystalline which should be stored at room temperature in a cool, dry place. An expiration date was not provided by the Sponsor. Upon receipt, the test article was described as a off-white crystalline powder and was stored at room temperature, protected from exposure to light and moisture. The test article was reported by the Sponsor to be 98% pure.

The vehicle used to deliver 2,4,6-Trichlorophenol to the test system was corn oil (CAS number 8001-30-1) obtained from Sigma Chemical Company.

Cyclophosphamide (CP, CAS number 6055-19-2), was obtained from Sigma Chemical Company and was dissolved in sterile distilled water at a concentration of 2.5 mg/mL for use as the positive control.

MATERIALS AND METHODS

Test System

ICR mice for pilot assay were obtained from Harlan Sprague Dawley, Inc., Frederick, MD and for toxicity study and micronucleus assay from Harlan Sparague Dawley, Inc., Oregon, WI. At the initiation of the study, the mice were 6 to 8 weeks old. Animal body weights recorded at randomization were within the following ranges:

Pilot study: Males, 28.3 - 31.3 grams
 Females, 25.4 - 27.6 grams

Toxicity study: Males, 29.2 - 34.5 grams
 Females, 24.6 - 28.8 grams

Micronucleus assay: Males, 29.9 - 36.7 grams
 Females, 24.7 - 30.0 grams

Animal Receipt and Quarantine

Mice were obtained from a source monitored for evidence of ectoparasites, endoparasites, pathogenic bacteria, mycoplasmas, and appropriate murine viruses and were quarantined for

no less than 5 days after receipt. The mice were observed each working day for signs of illness, unusual food and water consumption, and other conditions of poor health. The animals were judged to be healthy prior to utilization in the assay.

Animal Care

The mice were housed in an AAALAC-accredited facility with a controlled environment of $74\pm 6^{\circ}\text{F}$, $50\pm 20\%$ relative humidity, and a 12 hour light/dark cycle. Mice of the same sex were housed up to five per cage in plastic autoclavable cages which were maintained on stainless steel racks equipped with automatic watering manifolds and which were covered with filter material. Heat-treated hardwood chips were used for bedding. Mice had free access to certified laboratory rodent chow which had been analyzed for environmental contaminants (Harlan TEKLAD certified Rodent 7012C) and to tap water (Washington Suburban Sanitary Commission, Potomac Plant). There were no contaminants in the feed which were considered to have influenced the results of the study. The water used in the study met USEPA drinking water standards and is monitored at least annually for levels of organophosphorus pesticides, metals, and coliform and other contaminants.

Solubility Test

A solubility test was conducted to select the vehicle and to determine the vehicle that permit preparation of the highest soluble or workable concentration, up to 200 mg/mL. On the basis on information provided by the Sponsor, the test article was insoluble in water and thus a limited solubility test was conducted using corn oil.

Pilot Study

For the pilot study, mice were randomly assigned to one group of five males and five females and to four groups of two males each. Each mouse was given a sequential number and identified by ear tag. All mice were weighed immediately prior to dose administration and the dose volume based on individual body weights. Mice were observed after dose administration and daily thereafter for 3 days for clinical signs of chemical effect. Body weights were recorded prior to dose administration and 1 and 3 days after dose administration.

Toxicity Study

For the toxicity study, mice were randomly assigned to four groups of five males and five females each. Each mouse was given a sequential number and identified by ear tag. All mice in the experimental groups were weighed immediately prior to dose administration and the dose volume was based on individual body weights. Mice were observed after dose administration and daily thereafter for 3 days for clinical signs of chemical effect. Body weights were recorded prior to dose administration and 1 and 3 days after dose administration.

Micronucleus Assay

The micronucleus assay was conducted using established and validated procedures (Heddle, 1973; Hayashi et al., 1994; Mavournin et al., 1990). The mice were assigned to seven experimental groups of five males and five females each according to a computer-generated program which is based on distribution according to body weight. An additional group of 5 males and 5 females was designated as replacement animals in the event of mortality prior to the scheduled sacrifice time and was dosed with the test article high dose level. Negative and positive control groups were shared between this and study AA11VU.123.BTL, both of each were performed for Great Lakes Chemical Corporation and Griffin L.L.C. Each mouse was given a sequential number and identified by ear tag. The study design was as follows.

	Number of Mice Per Sex Dosed	Number of Mice Per Sex Used for Bone Marrow Collection After Dose Administration	
		24 hr	48 hr
Vehicle Control Corn oil	10	5	5
Test Article			
Low test dose (60 mg/kg)	5	5	
Mid test dose (120 mg/kg)	5	5	
High test dose (240 mg/kg)	15	5	5
Positive Control CP, 50 mg/kg	5	5	

Dose Administration

The test article-vehicle mixture, the vehicle alone, or the positive control was administered by a single intraperitoneal injection at a constant volume of 20 mL/kg body weight. Intraperitoneal injection was selected to maximize delivery of the test article to the test system. All mice in the experimental and control groups were weighed immediately prior to dose administration, and the dose volume was based on individual body weights. Mice were observed after dose administration for clinical signs of chemical effect.

Slide Preparation

At the scheduled sacrifice times, to five mice per sex per treatment were sacrificed by CO₂ asphyxiation. Immediately following sacrifice, the femurs were exposed, cut just above the knee, and the bone marrow was aspirated into a syringe containing fetal bovine serum. The bone marrow cells were transferred to a capped centrifuge tube containing approximately 1 mL fetal bovine serum. The bone marrow cells were pelleted by centrifugation at approximately 100 x g for five minutes and the supernatant was drawn off, leaving a small

amount of serum with the remaining cell pellet. The cells were resuspended by aspiration with a capillary pipet and a small drop of bone marrow suspension was spread onto a clean glass slide. Two slides were prepared from each mouse. The slides were fixed in methanol, stained with May-Gruenwald-Giemsa and permanently mounted.

Scoring for Micronuclei

Slides were coded using a random number table by an individual not involved with the scoring process. Using medium magnification, an area of acceptable quality was selected such that the cells were well spread and stained. Using oil immersion, 2000 polychromatic erythrocytes were scored for the presence of micronuclei which are defined as round, darkly staining nuclear fragments, having a sharp contour with diameters usually from 1/20 to 1/5 of the erythrocyte. The number of micronucleated normochromatic erythrocytes in the field of 2000 polychromatic erythrocytes was enumerated. The proportion of polychromatic erythrocytes to total erythrocytes was also recorded per 1000 erythrocytes.

Evaluation of Test Results

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes was determined for each mouse and treatment group. Statistical significance was determined using the Kastenbaum-Bowman tables which are based on the binomial distribution (Kastenbaum and Bowman, 1970). All analyses were performed separately for each sex and sampling time.

In order to quantify the proliferation state of the bone marrow as an indicator of bone marrow toxicity, the proportion of polychromatic erythrocytes to total erythrocytes was determined for each animal and treatment group.

All conclusions were based on sound scientific judgement; however, as a guide to interpretation of the data, the test article was considered to induce a positive response if a dose-responsive increase in micronucleated polychromatic erythrocytes was observed and one or more doses were statistically elevated relative to the vehicle control ($p \leq 0.05$, Kastenbaum-Bowman Tables) at any sampling time. If a single treatment group was significantly elevated at one sacrifice time with no evidence of a dose-response, the assay was considered a suspect or unconfirmed positive and a repeat assay recommended. The test article was considered negative if no statistically significant increase in micronucleated polychromatic erythrocytes above the concurrent vehicle control was observed at any sampling time.

Criteria for a Valid Test

The mean incidence of micronucleated polychromatic erythrocytes must not exceed 5/1000 polychromatic erythrocytes (0.5%) in the vehicle control. The incidence of micronucleated polychromatic erythrocytes in the positive control group must be significantly increased relative to the vehicle control group ($p \leq 0.05$, Kastenbaum-Bowman Tables).

Archives

All raw data, scored slides, and a copy of the final report will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance Regulatory Affairs/Quality Assurance Unit headquartered at: BioReliance, 14920 Broschart Rd., Rockville, MD. 20850.

RESULTS AND DISCUSSION**Solubility Test**

Corn oil was determined to be the solvent of choice based on a solubility determination of the test article and compatibility of the vehicle with the test system animals. The test article was soluble in corn oil at 200 mg/mL, the maximum concentration tested. Dosing concentrations were delivered to the test system as yellow solutions.

Pilot Assay

For the pilot study, 2,4,6-Trichlorophenol was administered by a single intraperitoneal injection to male mice at 1, 10, 100, or 1000 mg test article/kg body weight and to male and female mice at 2000 mg/kg which was administered in a total volume of 20 mL test article-vehicle mixture/kg body weight. Mortality, clinical signs and body weight data are reported in Tables 1 and 2. Mortality occurred within, approximately, three hours of dose administration as follows: 5/5 males and 5/5 females at 2000 mg/kg and 2/2 males at 1000 mg/kg. Clinical signs, which were noted immediately after dose administration, included: convulsions in all animals at 1000 and 2000 mg/kg. All other animals appeared normal throughout the observation period. Due to excessive mortality at 1000 and 2000 mg/kg, a toxicity assay was performed.

Toxicity Assay

For the toxicity study, 2,4,6-Trichlorophenol was administered by a single intraperitoneal injection to male and female mice at 200, 400, 600, or 800 mg test article/kg body weight which was administered in a total volume of 20 mL test article-vehicle mixture/kg body weight. Mortality, clinical signs and body weights are reported in Tables 3 and 4. Mortality occurred within three hours of dose administration as follows: 4/5 males and 4/5 females at 400 mg/kg and in 5/5 males and 5/5 females at 600 and 800 mg/kg. Clinical signs, which were noted immediately following dose administration, included: convulsions in all animals at 400, 600 and 800 mg/kg. Lethargy and piloerection were observed in all male and female mice at 200 mg/kg and in 1/5 male mice and 1/5 female mice at 400 mg/kg. Crusty eyes were observed in 1/5 female mice at 200 mg/kg and in 1/5 male mice and 1/5 female mice at 400 mg/kg. Irregular breathing was observed in 1/5 male mice and 1/5 female mice at 400 mg/kg. The LD₅₀ was calculated by probit analysis to be approximately 354.2 mg/kg for male and female mice. The high dose for the micronucleus test was set at 240 mg/kg for male and female mice which was estimated to be approximately 70% of the LD₅₀.

Micronucleus Assay

For the micronucleus test, male and female mice were dosed with 2,4,6-Trichlorophenol by a single intraperitoneal injection of 60, 120, or 240 mg test article/kg body weight which was administered in a total volume of 20 mL test article-vehicle mixture/kg body weight. Mortality and clinical signs are presented in Table 5. No mortality occurred at any dose level during the course of the micronucleus study. Clinical signs, which were noted immediately following dose administration, included tremors in all male and female mice at 240 mg/kg. Lethargy and piloerection were observed within four hours after dosing in male and female mice at 240 mg/kg and crusty eyes were observed in 5/15 male mice and 5/15 female mice at 240 mg/kg. All other mice treated with the test article and controls appeared normal during the course of the study.

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes scored and the proportion of polychromatic erythrocytes per total erythrocytes are summarized and presented for each treatment group by sacrifice time in Table 6. Individual animal data are presented in Tables 7 and 8. Statistically significant reduction in the number of polychromatic erythrocytes was observed in the male 48 hour high dose test article-treated group relative to the vehicle control ($p \leq 0.05$, Mann Whitney Test).

The number of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes in test article-treated groups was not statistically increased relative to their respective vehicle control in either male or female mice, regardless of dose level or bone marrow collection time ($p > 0.05$, Kastenbaum-Bowman Tables). CP induced a significant increase in micronucleated polychromatic erythrocytes in both male and female mice ($p \leq 0.05$, Kastenbaum-Bowman Tables).

CONCLUSION

All criteria for a valid test were met. Under the conditions of the assay described in this report, 2,4,6-Trichlorophenol did not induce a significant increase in the incidence of micronucleated polychromatic erythrocytes in bone marrow and was concluded to be negative in the micronucleus test using male and female mice.

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Table 1
 Clinical Signs Following Dose Administration of 2,4,6-Trichlorophenol
 Pilot Toxicity Study

Treatment	Clinical Observation	Number of Animals with Clinical Signs/Total Number of Animals Dosed		Number of Animals Died/Total Number of Animals Dosed	
		Males	Females	Males	Females
2,4,6-Trichlorophenol, 1 mg/kg	Normal	2/2	N/A	0/2	N/A
2,4,6-Trichlorophenol, 10 mg/kg	Normal	2/2	N/A	0/2	N/A
2,4,6-Trichlorophenol, 100 mg/kg	Normal	2/2	N/A	0/2	N/A
2,4,6-Trichlorophenol, 1000 mg/kg	Convulsions	2/2	N/A	2/2	N/A
2,4,6-Trichlorophenol, 2000 mg/kg	Convulsions	5/5	5/5	5/5	5/2

Table 2
Pilot Toxicity Study Using 2,4,5-Trichlorophenol In ICR Mice:
Body Weight and Mortality Data

Treatment	Sex	Group Mean Body Weights (gms)			% Change ¹		Mortality ²
		Pretreatment	Day 1	Day 3	Day 1	Day 3	
2,4,6-Trichlorophenol, 1 mg/kg	M	30.5 ± 1.5	29.8 ± 0.1	30.8 ± 0.1	-2.3%	1.0%	0 / 2
10 mg/kg	M	31.4 ± 0.8	30.6 ± 1.1	30.8 ± 0.2	-2.5%	-1.9%	0 / 2
100 mg/kg	M	31.5 ± 0.5	30.8 ± 0.4	31.4 ± 0.6	-2.2%	-0.3%	0 / 2
1000 mg/kg	M	30.8 ± 0.9	³ ND	³ ND	³ ND	³ ND	2 / 2
2000 mg/kg	M	30.7 ± 0.9	³ ND	³ ND	³ ND	³ ND	5 / 5
	F	26.8 ± 1.4	³ ND	³ ND	³ ND	³ ND	5 / 5

¹% Change = $\frac{(\text{Post-treatment weight} - \text{Pretreatment weight}) \times 100}{\text{Pretreatment weight}}$

²Reported as number of animals dead 3 days after dose administration/total number tested.

³No data due to mortality.

Table 3

Clinical Signs Following Dose Administration of 2,4,6-Trichlorophenol
Toxicity Study

Treatment	Clinical Observation	Number of Animals with Clinical Signs/Total Number of Animals Dosed		Number of Animals Died/Total Number of Animals Dosed	
		Males	Females	Males	Females
2,4,6-Trichlorophenol, 200 mg/kg	Lethargy Piloerection Crusty eyes	5/5 5/5	5/5 5/5 1/5	0/5	0/5
2,4,6-Trichlorophenol, 400 mg/kg	Convulsions Lethargy Piloerection Crusty eyes Irregular breathing	5/5 1/5 1/5 1/5 1/5	5/5 1/5 1/5 1/5 1/5	4/5	4/5
2,4,6-Trichlorophenol, 600 mg/kg	Convulsions	5/5	5/5	5/5	5/5
2,4,6-Trichlorophenol, 800 mg/kg	Convulsions	5/5	5/5	5/5	5/5

Table 4
Toxicity Study Using 2,4,6-Trichlorophenol In ICR Mice:
Body Weight and Mortality Data

Treatment	Sex	Group Mean Body Weights (gms)			% Change ¹		Mortality
		Pretreatment	Day 1	Day 3	Day 1	Day 3	
2,4,6-Trichlorophenol, 200 mg/kg	M	32.8 ± 2.3	32.5 ± 1.8	32.5 ± 2.5	-0.9%	-0.9%	0 / 5
	F	27.4 ± 0.8	27.2 ± 1.2	26.7 ± 0.6	-0.7%	-2.6%	0 / 5
400 mg/kg	M	33.0 ± 1.0	30.4 ND	27.4 ND	-7.9%	-17.0%	4 / 5
	F	27.3 ± 0.8	25.7 ND	23.5 ND	-5.9%	-13.9%	4 / 5
600 mg/kg	M	33.0 ± 1.8	ND	ND	ND	ND	5 / 5
	F	27.6 ± 1.6	ND	ND	ND	ND	5 / 5
800 mg/kg	M	33.5 ± 1.4	ND	ND	ND	ND	5 / 5
	F	27.9 ± 1.2	ND	ND	ND	ND	5 / 5

¹% Change = $\frac{(\text{Post-treatment weight} - \text{Pretreatment weight}) \times 100}{\text{Pretreatment weight}}$

²Reported as number of animals dead 3 days after dose administration/total number tested.

³Standard deviation not available due to single surviving animal.

⁴No data due to mortality.

Table 5
Clinical Signs Following Dose Administration of 2,4,6-Trichlorophenol
Micronucleus Assay

Treatment	Clinical Observation	Number of Animals with Clinical Signs/Total Number of Animals Dosed		Number of Animals Died/Total Number of Animals Dosed	
		Males	Females	Males	Females
Corn oil. 20 ml/kg	Normal	10/10	10/10	0/10	0/10
2,4,6-Trichlorophenol. 60 mg/kg	Normal	5/5	5/5	0/5	0/5
2,4,6-Trichlorophenol. 120 mg/kg	Normal	5/5	5/5	0/5	0/5
2,4,6-Trichlorophenol. 240 mg/kg	Lethargy Piloerection Tremor Crusty eyes	15/15 15/15 15/15 5/15	15/15 15/15 15/15 5/15	0/15	0/15
CP 50 mg/kg	Normal	5/5	5/5	0/5	0/5

Table 6
Summary of Bone Marrow Micronucleus Study Using 2,4,6-Trichlorophenol

Treatment	Sex	Time (hr)	Number of Mice	PCE/Total Erythrocytes (Mean +/- sd)	Change From Control (%)	Micronucleated Polychromatic Erythrocytes Number per 1000 PCEs (Mean +/- sd)	Erythrocytes Number per PCEs Scored
Corn oil 20 mL/kg	M	24	5	0.54 ± 0.02	---	0.4 ± 0.42	4 / 10000
	F	24	5	0.57 ± 0.12	---	0.4 ± 0.55	4 / 10000
2,4,6-Trichlorophenol 60 mg/kg	M	24	5	0.49 ± 0.06	-9	0.4 ± 0.42	4 / 10000
	F	24	5	0.46 ± 0.04	-19	0.4 ± 0.42	4 / 10000
120 mg/kg	M	24	5	0.46 ± 0.06	-15	0.3 ± 0.27	3 / 10000
	F	24	5	0.43 ± 0.02	-25	0.3 ± 0.27	3 / 10000
240 mg/kg	M	24	5	0.50 ± 0.06	-7	0.2 ± 0.27	2 / 10000
	F	24	5	0.46 ± 0.04	-19	0.3 ± 0.45	3 / 10000
CP, 50 mg/kg	M	24	5	0.33 ± 0.02	-39	25.5 ± 4.29	* 255 / 10000
	F	24	5	0.40 ± 0.05	-30	27.5 ± 4.91	* 275 / 10000
Corn oil 20 mL	M	48	5	0.51 ± 0.02	---	0.2 ± 0.27	2 / 10000
	F	48	5	0.51 ± 0.05	---	0.3 ± 0.27	3 / 10000
2,4,6-Trichlorophenol 240 mg/kg	M	48	5	0.44 ± 0.02	-14	0.4 ± 0.42	4 / 10000
	F	48	5	0.47 ± 0.03	-8	0.3 ± 0.27	3 / 10000

*, p<0.05 (Kestorbaum-Bowman Tables)

Table 7

Induction of Micronucleated Polychromatic Erythrocytes in
Bone Marrow Cells Collected 24 Hours After a Single Dose of
2,4,6-Trichlorophenol

Treatment	Sex	Animal Number	PCE/Total Erythrocytes	Micronucleated PCE (Number/PCE Scored)
Corn oil 20 mL/kg	M	1	0.52	1 / 2000
		2	0.55	0 / 2000
		3	0.52	0 / 2000
		4	0.57	2 / 2000
		5	0.53	1 / 2000
	F	6	0.62	0 / 2000
		7	0.59	0 / 2000
		8	0.40	2 / 2000
		9	0.72	0 / 2000
		10	0.52	2 / 2000
2,4,6-Trichlorophenol 60 mg/kg	M	41	0.46	0 / 2000
		42	0.59	0 / 2000
		43	0.48	2 / 2000
		44	0.44	1 / 2000
		45	0.47	1 / 2000
	F	46	0.44	0 / 2000
		47	0.47	1 / 2000
		48	0.45	0 / 2000
		49	0.52	2 / 2000
		50	0.40	1 / 2000
120 mg/kg	M	51	0.45	0 / 2000
		52	0.48	1 / 2000
		53	0.41	1 / 2000
		54	0.41	0 / 2000
		55	0.56	1 / 2000
	F	56	0.43	0 / 2000
		57	0.42	1 / 2000
		58	0.47	0 / 2000
		59	0.42	1 / 2000
		60	0.42	1 / 2000
240 mg/kg	M	61	0.45	1 / 2000
		62	0.56	0 / 2000
		63	0.56	1 / 2000
		64	0.50	0 / 2000
		65	0.44	0 / 2000
	F	66	0.52	0 / 2000
		67	0.48	0 / 2000
		68	0.44	1 / 2000
		69	0.42	2 / 2000
		70	0.46	0 / 2000
CP, 50 mg/kg	M	71	0.35	53 / 2000
		72	0.35	57 / 2000
		73	0.33	53 / 2000
		74	0.33	56 / 2000
		75	0.32	36 / 2000
	F	76	0.39	64 / 2000
		77	0.44	58 / 2000
		78	0.32	41 / 2000
		79	0.39	63 / 2000
		80	0.46	49 / 2000

Table 8

Induction of Micronucleated Polychromatic Erythrocytes in
Bone Marrow Cells Collected 48 Hours After a Single Dose of
2,4,6-Trichlorophenol

Treatment	Sex	Animal Number	PCE/Total Erythrocytes	Micronucleated PCE (Number/PCE Scored)
Corn oil 20 mL/kg	M	81	0.48	0 / 2000
		82	0.52	1 / 2000
		83	0.51	1 / 2000
		84	0.54	0 / 2000
		85	0.49	0 / 2000
	F	86	0.56	1 / 2000
		87	0.44	0 / 2000
		88	0.54	1 / 2000
		89	0.47	1 / 2000
		90	0.51	0 / 2000
2,4,6-Trichlorophenol 240 mg/kg	M	101	0.46	0 / 2000
		102	0.46	1 / 2000
		103	0.43	1 / 2000
		104	0.42	0 / 2000
		105	0.44	2 / 2000
	F	106	0.47	1 / 2000
		107	0.42	1 / 2000
		108	0.49	1 / 2000
		109	0.47	0 / 2000
		110	0.47	0 / 2000

APPENDIX I

Mouse Micronucleus Test Historical Control Data

**Mouse Micronucleus Test Historical Control Data
1995 - 1997**

Negative Control Animals¹

Parameter	Ratio of PCE/Total Erythrocytes		MPCE/1000 PCE Scored	
	Males	Females	Males	Females
Mean	0.54	0.55	0.79	0.82
Standard Deviation	0.08	0.08	0.90	0.94
Range	0.15 - 0.81	0.24 - 0.86	0 - 8	0 - 8

Positive Control Animals²

Parameter	Ratio of PCE/Total Erythrocytes		MPCE/1000 PCE Scored	
	Males	Females	Males	Females
Mean	0.46	0.48	31.92	28.37
Standard Deviation	0.12	0.10	17.18	14.37
Range	0.06 - 0.80	0.12 - 0.83	2 - 109	2 - 77

¹Negative controls include all vehicles and all routes of administration.
Bone marrow collected at 24 and 48 hours after dose administration.

²Positive control is cyclophosphamide, 40 to 60 mg/kg, dosed by IV, IP or PO.
Bone marrow cells were collected at 24 hours after dose administration.

APPENDIX II

Study Protocol

QA 3/15/99
APPROVED

PROTOCOL AMENDMENT 1

SPONSOR: Great Lakes Chemical Corporation and Griffin L.L.C.

TEST ARTICLE I.D.: 2,4,6-Trichlorophenol

BIORELIANCE STUDY NO: AA11XA.123.BTL

SPONSOR PROJECT NO.: GP99-019 (Griffin L.L.C.)

PROTOCOL TITLE: Mammalian Erythrocyte Micronucleus Test

1. LOCATION: Page 1, § 3.1 Test Article

AMENDMENT: Amend test article name to 2,4,6-Trichlorophenol

REASON FOR THE AMENDMENT: Error in protocol preparation.

APPROVALS:

Salvadevi Gudi
BIORELIANCE STUDY DIRECTOR

3/16/99
DATE

John A. Bismarck
SPONSOR REPRESENTATIVE

3/31/99
DATE



APPROVED

Mammalian Erythrocyte Micronucleus Test

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of the test articles as measured by their ability to induce micronucleated polychromatic erythrocytes in mouse bone marrow.

2.0 SPONSOR

2.1 Name: Great Lakes Chemical Corporation and Griffin L.L.C.

Address: Great Lakes Chemical Corporation
P.O. Box 2200
Highway 52 N.W.
West Lafayette, IN 47906

Griffin L.L.C.
PO Box 1847
2509 Rocky Ford Road
Valdosta, GA 31603-1847

2.3 Representatives: John A. Biesemeier (Great Lakes Chemical Corporation)
W. A. Hawkins, Jr., Ph.D. (Griffin L.L.C.)

2.4 Sponsor Project #: GP99-019 (Griffin L.L.C.)

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: 2,4,6-Trichlorophenol

3.2 Controls: Vehicle: Test article vehicle
Positive: Cyclophosphamide (CP)

3.3 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at BioReliance will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test articles, and the stability and strength of the test articles in the solvent (or vehicle).



3.4 Test Article Retention Sample

The retention of a reserve sample of the test articles will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Toxicology Testing Facility
BioReliance

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

4.3 Study Director: Ramadevi Gudi, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 2/2/99

5.2 Proposed Experimental Completion Date: 3/26/99

5.3 Proposed Report Date: 4/2/99

6.0 TEST SYSTEM

Closed-colony, random-bred rodents are acceptable models for mutagenicity studies. ICR mice were selected because of the availability of historical control data.

6.1 Source: Harlan Sprague Dawley, Inc., Frederick, MD or
Charles River Breeding Laboratories, Kingston, NY or Raleigh, NC or
other approved alternates

6.2 Age at initiation of study: 6-8 weeks

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be conducted according to established procedures (Heddle, 1973; Mavourin *et al.*, 1990; Hayashi *et al.*, 1994). Following the administration of three concentrations of test article as well as positive and negative (vehicle) controls to male and female mice, bone marrow cells will be collected at 24 and 48 hours and examined for the presence of micronucleated polychromatic erythrocytes. The clastogenic potential of the test article will be measured by its ability to increase micronucleated polychromatic erythrocytes in treated animals as compared to vehicle control animals.

The study design will be as follows:

	Number per Sex per to be Sacrificed After Dose Administration	
	24 Hr	48 Hr
Vehicle Control		
Vehicle alone	5	5
Test Article 1		
Low Dose	5	-
Mid Dose	5	-
High Dose	5	5
Test Article 2		
Low Dose	5	-
Mid Dose	5	-
High Dose	5	5
Positive Control		
Cyclophosphamide	5	-

NA, corrected... consultation with... done at 48 hr

7.1 Selection of Test Article Vehicle

Unless the Sponsor has indicated the test article vehicle, a solubility determination will be conducted to measure the maximum soluble concentration or workable suspension of test article in vehicle. Vehicles compatible with this test system, in order of preference, include, but are not limited to, distilled water or saline, 1% carboxymethylcellulose in water, and corn oil. The vehicle of choice will be that allowing preparation of dosing solutions required to achieve targeted doses.

7.2 Dose Selection

Selection of doses for the micronucleus assay will be based on the toxicity of the test articles but will not exceed 2000 mg/kg. In the absence of toxicity data, a pilot study will be performed with each test article at a dose of 2000 mg/kg using five male and five female mice. Three or more lower doses will be tested using two male mice each. If dose administration produces no treatment-related mortality, the high dose for the micronucleus test will be 2000 mg/kg. In the event of mortality in excess of 50% at 2000 mg/kg in the pilot study, an extensive toxicity study will be performed using at least four test article doses, each containing five male and five female mice. Mice will be observed after dose administration and each working day thereafter for 3 days for clinical signs of chemical effect. Body weights will be recorded prior to dose administration and at 1 and 3 days after dose administration.

Unless specified otherwise by the Sponsor or at the discretion of the Study Director the high dose for the micronucleus test may be 50% to 80% of the LD₅₀ (the dose

required to kill 50% of the animals within 3 days after administration of the maximum tolerated dose but will not exceed 2000 mg/kg. Two additional doses will be tested, one-half and one-fourth of the high dose.

7.3 Route and Frequency of Administration

Animals will be dosed by intraperitoneal (IP) injection. IP injection was selected to maximize delivery of the test article to the target system. IP injection is an acceptable method for administration of test article concentrations to laboratory animals. Animals will receive the test article as a single administration.

7.4 Controls

7.4.1 Vehicle control

The solvent vehicle for the test article will be used as the vehicle control.

7.4.2 Positive control

Cyclophosphamide monohydrate (CP, CAS number 6055-19-2) will be administered as the positive control at a dose of 30-60 mg/kg. CP will be administered by the same route as the test article.

Vehicle and positive controls will be shared between two test article groups

7.5 Animal Receipt and Quarantine

Virus antibody-free (VAF) mice will be quarantined for no less than 5 days prior to dose administration. The animals will be observed each working day for signs of illness, unusual food and water consumption, and other general conditions of poor health. All animals will be judged to be healthy prior to utilization in the study

7.6 Animal Care

Animals will be housed in an AAALAC-accredited facility with a controlled environment of 50 ± 20% relative humidity and 74 ± 6°F with a 12 hour light:dark cycle. Mice of the same sex will be housed up to five per cage in plastic autoclavable cages. Heat-treated hardwood chips will be used for bedding. Animals will have free access to a certified laboratory rodent chow which has been analyzed for environmental contaminants and to tap water.

7.7 Randomization

The animals will be assigned to eleven groups of five males and females each using a randomization procedure based on equalization of group mean body weights. At the time of randomization, the weight variation of animals will not exceed ±20% of the mean weight. Additional animals may be designated and dosed as replacement animals in the high dose group to be used in the event of mortality prior to the

consulted with Dr. Stenar on 1/25/99

scheduled sacrifice. This will be done at the discretion of the Study Director after evaluation of the toxicity data. Each animal will be given a sequential number and identified by ear tag.

7.8 Dose Preparation and Administration

The test article-vehicle mixture, the vehicle alone and the positive control (CP) will be given as a single administration. The rate of administration will be 10 ml/kg body weight unless larger volumes, up to 20 ml/kg, are required to deliver the targeted dose. All mice in the experimental groups will be weighed and the dose volume will be based on individual body weight.

7.9 Bone Marrow Collection

Twenty-four and 48 hours after dose administration, animals will be sacrificed by carbon dioxide asphyxiation. The positive control group will be sacrificed 24 hours after dose administration. Immediately following sacrifice, the femurs will be exposed, cut just above the knee and the bone marrow will be aspirated into a syringe containing fetal bovine serum. The bone marrow cells will be transferred to a capped centrifuge tube containing approximately 1 ml fetal bovine serum.

The bone marrow cells will be pelleted by centrifugation and the supernatant will be drawn off, leaving a small amount of fetal bovine serum with the remaining cell pellet. The cells will be resuspended by aspiration with a capillary pipette and a small drop of the bone marrow suspension will be spread onto a clean glass slide. Each slide will be identified by the experiment and animal number. At least two slides will be prepared from each animal, air dried, fixed by dipping in methanol, stained with May-Gruenwald-Giemsa and permanently mounted.

7.10 Scoring for Micronuclei

Slides will be coded using a random number table by an individual not involved with the scoring process. Using medium magnification, an area of acceptable quality will be selected such that the cells are well spread and stained. Using oil immersion, 2000 polychromatic erythrocytes will be scored per animal for the presence of micronuclei. The number of micronucleated normocytes in the field of 2000 polychromatic erythrocytes will also be enumerated. The proportion of polychromatic erythrocytes to total erythrocytes will also be recorded per 1000 erythrocytes. The proportion of polychromatic erythrocytes to total erythrocytes in test article-treated animals should not be less than 20% of the control value.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The mean incidence of micronucleated polychromatic erythrocytes must not exceed 5 micronucleated polychromatic erythrocytes (0.5%) in the negative (vehicle) control. The incidence of micronucleated polychromatic erythrocytes in the positive control group must be significantly increased relative to the negative control ($p \leq 0.05$, Kastenbaum-Bowman Tables).

9.0 EVALUATION OF TEST RESULTS

The incidence of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes will be presented for each animal and treatment group. Statistical significance will be determined using the Kastenbaum-Bowman tables which are based on the binomial distribution.

In order to quantify the test article effect on erythropoiesis, as an indicator of bone marrow toxicity, the proportion of polychromatic erythrocytes to total erythrocytes will be presented for each animal and treatment group.

All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response if a dose-responsive increase in micronucleated polychromatic erythrocytes is observed and one or more doses are statistically elevated relative to the vehicle control ($p \leq 0.05$, Kastenbaum-Bowman Tables) at any sampling time. If a single treatment group is significantly elevated at one sacrifice time with no evidence of a dose-response, the assay will be considered a suspect or unconfirmed positive and a repeat experiment will be recommended. The test article will be judged negative if no statistically significant increase in micronucleated polychromatic erythrocytes above the concurrent vehicle control values are observed at any sampling time.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. The report will include:

- Test article: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of the test article, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of the test article in the solvent/vehicle, if known.
- Test animals: species and strain of animals used; number, age and sex of animals, source, housing conditions, diet, etc.
- Test conditions: positive and negative (vehicle/solvent) control data; data from range-finding study, if conducted; rationale for dose level selection; details of test article preparation; details of the administration of test article; rationale for route of administration; methods for verifying that the test article reached the general circulation or target tissue, if applicable; details of food and water quality; description of treatment and sampling schedules; method of slide preparation; methods for measurement of toxicity; criteria for scoring micronucleated immature erythrocytes; number of cells analyzed per

animal: criteria for considering study as positive, negative or equivocal.

- Results: signs of toxicity; proportion of polychromatic erythrocytes among total erythrocytes; number of micronucleated polychromatic erythrocytes per animal; mean±standard deviation of micronucleated polychromatic erythrocytes per group; dose-response relationship, where possible; statistical analyses; concurrent negative control data; historical negative control data with ranges, means and standard deviations; concurrent positive control data.
- Discussion of results.
- Conclusion.

11.0 RECORDS AND ARCHIVES

All raw data, the protocol, and a copy of all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 474 (Genetic Toxicology: Mammalian Erythrocytes Micronucleus Test), July, 1997 and with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (1996 and 1997).

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? UNKNOWN
 If so, which agency or agencies? EPA-FIFRA, EPA-TSCA

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Heddle, J.A. 1973. A rapid in vivo test for chromosomal damage. Mutation Res. 18: 187-190.

Hayashi, M., R.R. Tice, J.T. MacGregor, D. Anderson, D.H. Blakey, M. Dirsch-Volders, F.G. Oteson Jr., F. Pacchierotti, F. Romagna, H. Shimada, S. Sutou and B. Vannier. 1994. In vivo rodent erythrocyte micronucleus assay. Mutation Res. 312: 293-304.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for

adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18202. April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. Federal Register 62:16026-16030. November 21, 1997.

Kastenbaum, M.A. and K.O. Bowman. 1970. Tables for determining the statistical significance of mutation frequencies. Mutation Res. 9:527-549.

Mavournin, K.H., D.H. Blakey, M.C. Cimino, M.F. Salamone and J.A. Heddle. 1990. The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res. 239:29-80.

OECD Guidelines for Testing of Chemicals. Guideline 474. Genetic Toxicology. Mammalian Erythrocyte Micronucleus Test. Organisation for Economic Co-operation and Development, Paris, France. July 1997.

14.0 APPROVAL

John A. Biesemeier 1/8/99
Sponsor Representative Date
Great Lakes Chemical Corporation

John A. Biesemeier
(Print or Type Name)

W. A. Hawkins, Jr., Ph.D. 1/18/99
Sponsor Representative Date
Griffin Corporation L.L.C. 2/18/99 1/18/99

W. A. Hawkins, Jr., Ph.D.
(Print or Type Name)

Ramadevi Gu di 1/25/99
BioReliance Study Director Date

If submission to Japanese Regulatory Agency is indicated in section 12.0, BioReliance management will sign.

BioReliance Study Management Date

CERTIFICATE OF AUTHENTICITY

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