

FYI-0197-1281



FYI-97-001281

CHEMICAL MANUFACTURERS ASSOCIATION

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November 26, 1996



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Dr. Lynn Goldman
Assistant Administrator
Office of Prevention, Pesticides and Toxic Substances TS-7101
Environmental Protection Agency
401 M Street, SW, Room 637, East Tower
Washington, DC 20460

84970000004

Contains No CBI

Dear Dr. Goldman:

The Chemical Manufacturers Association makes available to the public and appropriate government agencies final reports of environmental, health and safety research that it manages. In keeping with this policy, the following recently completed reports are enclosed:

- ✓ HEXABROMOCYCLODODECANE (HBCD): Chromosome Aberrations in Human Peripheral Blood Lymphocytes; 3194-55-6
- ✓ HEXABROMOCYCLODODECANE (HBCD): Maximization Test in Guinea Pigs;
- ✓ PENTABROMODIPHENYL OXIDE (PBDPO): Maximization Test in Guinea Pigs;
32534-81-9
- ✓ OCTABROMODIPHENYL OXIDE (OBDPO): Maximization Test in Guinea Pigs;
32536-52-0
- ✓ HEXABROMOCYCLODODECANE (HBCD): Closed Bottle Test; and,
OCTABROMODIPHENYL OXIDE (OBDPO): Closed Bottle Test.

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EPA/PTAC
NOV 29 11:57

These reports do not include confidential information.

If you have any questions, please call Has Shah of my staff at 703-741-5637.

Sincerely,

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Enclosure



FINAL REPORT

Study Title

**CHROMOSOME ABERRATIONS IN
HUMAN PERIPHERAL BLOOD LYMPHOCYTES**

Test Article

Hexabromocyclododecane

Authors

Ramadevi Gudi, Ph.D.
Elizabeth H. Schadly, B.S.

Study Completion Date

November 12, 1996

Performing Laboratory

Microbiological Associates, Inc.
9630 Medical Center Drive
Rockville, Maryland 20850

Laboratory Study Number

G96A061.342

Sponsor

Chemical Manufacturers Association
1300 Wilson Boulevard
Arlington VA 22209

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 **MICROBIOLOGICAL
ASSOCIATES, INC.**

STATEMENT OF COMPLIANCE

Study G96AO61.342 was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US 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.

The stability of the test or control article under the test conditions was 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.

Ramadevi Gudi
Ramadevi Gudi, Ph.D.
Study Director

11/12/96
Date

QUALITY ASSURANCE STATEMENT

Study Title: CHROMOSOME ABERRATIONS IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES
Study Number: G96AO61.342
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 Compliance Programme, 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 07 JUN 96, TO STUDY DIR 07 JUN 96, TO MGMT 07 JUN 96
PHASE: Protocol Review

INSPECT ON 13 JUN 96, TO STUDY DIR 13 JUN 96, TO MGMT 24 JUN 96
PHASE: Colcemid treatment of the test system

INSPECT ON 12 SEP 96-13 EP 96, TO STUDY DIR 13 SEP 96, TO MGMT 17 SEP 96
PHASE: Draft Report

INSPECT ON 13 NOV 96, TO STUDY DIR 13 NOV 96, TO MGMT 15 NOV 96
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.



Diane B. Madsen
QUALITY ASSURANCE

11-15-96

DATE

**CHROMOSOME ABERRATIONS IN
HUMAN PERIPHERAL BLOOD LYMPHOCYTES**

FINAL REPORT

**Sponsor: Chemical Manufacturers Association
1300 Wilson Boulevard
Arlington, VA 22209**

Authorized Representative: Dr. Hasmukh C. Shah

**Performing Laboratory: Microbiological Associates, Inc. (MA)
9630 Medical Center Drive
Rockville, Maryland 20850**

Test Article I.D.: Hexabromocyclododecane

MA Study No.: G96AO61.342

Test Article Description: white powder

Storage Conditions: room temperature, protected from light and moisture

Test Article Receipt: May 21, 1996

Study Initiation: June 5, 1996

Laboratory Supervisor: Elizabeth H. Schadly, B.S.

Study Director:

Ramadevi Gudi
Ramadevi Gudi, Ph.D.

11/12/96
Date

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SUMMARY

The test article, Hexabromocyclododecane, was tested in the *in vitro* mammalian cytogenetic test using human peripheral blood lymphocytes (HPBL) both in the absence and presence of metabolic activation. The assay was performed in two phases. The first phase, the initial chromosomal aberration assay, was conducted to establish the dose range for testing and to evaluate the clastogenic potential of the test article. The second phase, the independent repeat chromosome aberration assay, was performed to confirm the test system response to the test article seen in the initial assay.

Dimethylsulfoxide (DMSO) was the solvent of choice based on information provided by the Sponsor, the solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at approximately 500 mg/ml, the maximum concentration tested.

In the initial chromosome aberration assay, the maximum dose tested was 2500 µg/ml. This dose was achieved using a stock concentration of 250 mg/ml, and a 100 µl dosing aliquot added to 10 ml fresh complete medium or S9 reaction mix. Dose levels greater than 2500 µg/ml were insoluble in treatment medium and not tested in the assay. Visible precipitate was observed in treatment medium at dose levels 750 and 2500 µg/ml and was soluble but cloudy (no visible precipitate) at dose levels 75 and 250 µg/ml. The test article was soluble in treatment medium at all other dose levels tested. In the non-activated portion of the initial assay HPBL cells were exposed to the test article continuously for 20 hours; in the S9-activated portion of the initial chromosome aberration assay, HPBL cells were exposed to the test article for 4 hours. Metaphase cells were collected for microscopic evaluation at 20 hours after the initiation of treatment. Dose levels of 2500 µg/ml in the non-activated study and 750 and 2500 µg/ml in the S9-activated study were not analyzed for chromosome aberrations due to complete mitotic inhibition. Toxicity (mitotic inhibition) of approximately 56% was observed at the highest dose level (750 µg/ml) evaluated for chromosome aberrations, in the non-activated study. In the S9-activated study, 13% toxicity was observed at the highest dose level (250 µg/ml) evaluated for chromosome aberrations. No statistically significant increases in chromosome aberrations were observed in either the non-activated or S9-activated test systems relative to the solvent control group regardless of dose level ($p > 0.05$, Fisher's exact test).

Based on the results of the initial assay, an independent repeat chromosome aberration assay was conducted in the absence and presence of an Aroclor-induced S9 metabolic activation system at dose levels of 10, 19, 38, 75, 150, 300, and 600 µg/ml. The test article was soluble in treatment medium at dose level 75 µg/ml and was workable in treatment medium at dose levels 150 µg/ml and higher. The test article was soluble in treatment medium at all other concentrations tested. In the independent repeat assay, HPBL cells were exposed to the test article continuously for 20 or 44 hours in the non-activated test system and for 4 hours in the S9-activated test system. Metaphase cells were collected for microscopic evaluation in both the non-activated and S9-activated studies at 20 and 44 hours after the initiation of treatment. Toxicity, measured by mitotic inhibition, was

approximately 55% and 94% at the 20 and 44 hour harvests, respectively, at the highest dose levels (600 and 300 $\mu\text{g/ml}$) evaluated for chromosome aberrations in the non-activated studies. In the S9-activated studies, toxicity was approximately 71% and 69% at the 20 and 44 hour harvests, respectively, at the highest dose levels (300 and 600 $\mu\text{g/ml}$) evaluated for chromosome aberrations. Dose level 600 $\mu\text{g/ml}$ in the non-activated 44 hour harvest and in the S9-activated 20 hour harvest was not analyzed for chromosome aberrations due to an insufficient number of scorable metaphase cells. No statistically significant increases in structural chromosome aberrations were observed in either the non-activated or S9-activated studies, regardless of dose level or harvest time ($p > 0.05$, Fisher's exact test). No statistically significant increases in numerical chromosome aberrations were observed in either the non-activated or S9-activated studies at the 44 hour harvest time, regardless of dose level ($p > 0.05$, Fisher's exact test).

Based on the findings of this study, Hexabromocyclododecane was concluded to be negative for the induction of structural and numerical chromosome aberrations in human peripheral blood lymphocytes.

PURPOSE

The purpose of this study was to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in human peripheral lymphocytes.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Hexabromocyclododecane, was received by Microbiological Associates, Inc. on May 21, 1996 and was assigned the code number 96AO61. The test article was characterized by the Sponsor as an off-white powder with a characteristic odor. No expiration date was provided. Upon receipt, the test article was described as a white powder and was stored at room temperature, protected from exposure to light and moisture. The solvent used to deliver Hexabromocyclododecane to the test system was DMSO (CAS No.: 67-68-5), supplied by the Fisher Scientific Company.

Mitomycin C (MMC; CAS No.: 50-07-7), was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 12.5 and 25 µg/ml for use as the positive control in the non-activated test system. Cyclophosphamide (CP; CAS No.: 6055-19-2), was obtained from Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 2.5 and 5 mg/ml for use as the positive control in the S9-activated test system. For each positive control one dose with sufficient scorable metaphase cells was selected for analysis. The solvent for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. RPMI-1640 complete medium (containing 15% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin and 100 µg streptomycin/ml) supplemented with 1% PHA, or S9 reaction mixture was used in the untreated control.

MATERIALS AND METHODS

Test System

Peripheral blood lymphocytes were obtained from a healthy 40 year old adult female with no recent history of either radiotherapy, viral infection or the administration of drugs. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston, et al., 1981).

Activation System

Aroclor 1254-induced rat liver S-9 was used as the metabolic activation system. The S-9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S-9 was batch prepared and stored at ≤-70°C until used. Each bulk preparation of S-9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethyl-benz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S-9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 μ l S-9 per milliliter medium (RPMI 1640 serum-free medium supplemented with 100 units penicillin and 100 μ g streptomycin/ml, and 2 mM L-glutamine).

Solubility Test

A solubility test was conducted using dimethylsulfoxide (DMSO) which was selected by the Sponsor as the solvent of choice. The test was performed to determine the level of solubility of the test article in DMSO, which permitted preparation of the highest soluble or workable stock concentration, up to 500 mg/ml.

Chromosome Aberration Assays (Initial and Independent Repeat)

The initial chromosome aberration assay was performed using standard procedures (Evans, 1976), by exposing duplicate cultures of HPBL to nine concentrations of the test article as well as positive, solvent and untreated controls. In the initial assay, the dividing cells were harvested at approximately 20 hours from the initiation of treatment.

The independent repeat chromosome aberration assay was performed by exposing duplicate cultures of HPBL to at least four concentrations of the test article, as well as positive, solvent and untreated controls. The concentrations tested were selected based on the findings of the initial assay. For the independent repeat assay, the dividing cells were harvested at two time points, 20 and 44 hours after the initiation of treatment.

For the chromosome aberration assays, 0.6 ml heparinized blood was inoculated into centrifuge tubes containing 9.4 ml complete medium supplemented with 1% PHA. The tubes were incubated at $37\pm 1^\circ\text{C}$ in a humidified atmosphere of $5\pm 1\%$ CO_2 in air for approximately 44-48 hours. Treatment was carried out by refeeding with approximately 10 ml fresh complete medium or S9 reaction mixture to which was added 100 μ l of dosing solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium or S9 reaction mixture was also included.

In the non-activated study, the cells were exposed for 20 or 44 hours at $37\pm 1^\circ\text{C}$ in a humidified atmosphere of $5\pm 1\%$ CO_2 in air. In the S9-activated studies, the cells were exposed for 4 hours at $37\pm 1^\circ\text{C}$ in a humidified atmosphere of $5\pm 1\%$ CO_2 in air. After the exposure period, the treatment medium was removed, the cells washed with calcium and magnesium free-phosphate buffered saline (CMF-PBS), refeed with complete medium containing 1% PHA and returned to the incubator for an additional 16 hours for the first harvests or 40 hours for the delayed harvest. For all treatment groups, two hours prior to the scheduled cell harvests at 20 or 44 hours after treatment initiation, Colcemid® was added to the cultures at a final concentration of 0.1 μ g/ml.

Collection of Metaphase Cells

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the activated and non-activated studies by centrifugation. The cells were collected by centrifugation at approximately 1200 rpm for about 5 minutes. The cell pellet was resuspended in 5 ml 0.075 M KCl and incubated at $37\pm 1^\circ\text{C}$ for 20 minutes. At the end of the KCl treatment and immediately prior to centrifuging, the cells were gently mixed and approximately 0.5 ml of fixative (methanol:glacial acetic acid, 3:1 v/v) was added to each tube. The cells were collected by centrifugation, the supernatant aspirated, and the cells were fixed with two washes with approximately 3-5 ml fixative and stored in fixative overnight or longer at approximately $2-6^\circ\text{C}$.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 1200 rpm for 5 minutes, the supernatant fluid was aspirated, and the cells were resuspended in 1 ml cold fresh fixative. The cells were collected by centrifugation and the supernatant aspirated, leaving 0.1 to 0.3 ml fixative above the cell pellet. An aliquot of cell suspension was dropped onto a glass slide and allowed to air dry overnight. Slides were identified by the study number, dose level, activation condition, harvest time, replicate tube designation, and date prepared. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 46 centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate treatment condition) were examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (>10 aberrations) also were recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted. In the delayed harvests, the percent polyploid cells was recorded per 100 metaphase cells.

Controls

Mitomycin C was used as the positive control in the non-activated study at final concentrations of 0.13 and 0.25 µg/ml. Cyclophosphamide was used as the positive control in the S-9 activated study at final concentrations of 25 and 50 µg/ml. For both positive controls one dose with sufficient scorable metaphase cells was selected for analysis. The solvent vehicle for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Growth medium or S9 reaction mixture was used in the untreated control.

Evaluation of Test Results

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and are presented for both the initial and the independent repeat chromosome aberration assays. The number and types of aberrations, the percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined, the percentage of numerically damaged cells in the total population of cells examined, and the frequency of structural aberrations per cell (mean aberrations per cell) was reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage test was used to determine dose-responsiveness.

All conclusions were based on sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentages of cells with aberrations are increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A reproducible and significant increase at a single dose level will be considered positive. The test article was concluded to be negative if no statistically significant increase was observed relative to the solvent control.

Criteria for Determination of a Valid Test

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

Record and Specimen Archives

Upon completion of the final report, all raw data, reports, and stained and cover slides are maintained in the archives of Microbiological Associates, Inc. located in Rockville, Maryland.

RESULTS AND DISCUSSION

Solubility Test

DMSO was selected by the Sponsor as the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at approximately 500 µg/ml, the maximum concentration tested.

Initial Assay

In the initial chromosome aberration assay HPBL were exposed to nine concentrations of test article ranging from 0.25 µg/ml to 2500 µg/ml. Test article concentrations greater than 2500 µg/ml were insoluble in treatment medium and not tested in the assay. The test article was soluble but cloudy in treatment medium at dose levels 75 and 250 µg/ml and workable in treatment medium at dose levels 750 and 2500 µg/ml. The test article was soluble in treatment medium at all other dose levels tested. The osmolality and pH of the highest concentration tested, 2500 µg/ml, were 419 mmol/kg and approximately 8, respectively. The osmolality of the solvent, DMSO, was 447 mmol/kg. Metaphase cells were collected for microscopic evaluation at 20 hours after the initiation of treatment.

The activity of Hexabromocyclododecane in the induction of chromosome aberrations in HPBL was tested following a 20 hour exposure period in the absence of an exogenous source of metabolic activation. The findings of the cytogenetic analysis are presented by treatment culture in Table 1 and summarized by group in Table 3. At the highest test concentration evaluated (750 µg/ml) microscopically for chromosome aberrations, the mitotic index was reduced 56% relative to the solvent control. Dose level 2500 µg/ml was not selected for analysis due to an insufficient number of scorable cells. Dose levels 0.25, 0.75, 2.5 and 7.5 µg/ml were tested but not required for analysis. The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control at any dose level ($p > 0.05$, Fisher's exact test). The percentage of aberrant cells in the MMC group was 8.5% ($p \leq 0.01$, Fisher's exact test).

The activity of Hexabromocyclododecane in the induction of chromosome aberrations in HPBL cells was tested using a 4 hour exposure and a 16 hour recovery period in the presence of an exogenous source of metabolic activation. The findings of the cytogenetic analysis are presented by treatment flask in Table 2 and summarized by group in Table 3. At the highest test concentration evaluated (250 µg/ml) for chromosome aberrations, the mitotic index was reduced 13% relative to the solvent control. Dose levels 750 and 2500 µg/ml were not selected for analysis due to an insufficient number of scorable cells. Dose

levels 0.25, 0.75, and 2.5 µg/ml were tested but not required for analysis. The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control at any dose level ($p > 0.05$, Fisher's exact test). The percentage of aberrant cells in the CP group was 6.5% ($p \leq 0.01$, Fisher's exact test).

Independent Repeat Assay

Based on the results of the initial assay, dose levels of 10, 19, 38, 75, 150, 300 and 600 µg/ml were selected for further study in both the non-activated and S9-activated portions of the independent repeat assay with 20 and 44 hour cell harvest times. The test article was soluble but cloudy in treatment medium at dose level 75 µg/ml, and was workable in treatment medium at dose levels 150 µg/ml and higher. The test article was soluble in treatment medium at all other concentrations tested. The osmolality of the highest concentration (600 µg/ml) tested was 381 mmol/kg. The osmolality of the solvent, DMSO, was 393 mmol/kg. The pH of the highest concentrations tested was approximately 8. At the time of test article administration, due to technical oversight, the cultures were treated approximately 43 minutes to 1 hour 13 minutes beyond the 44-48 hour time limit; since the result of the study was negative, the Study Director determined this deviation to have had no significant effect upon the test system.

The findings of the cytogenetic analysis for groups tested in the absence of an exogenous source of metabolic activation are presented by treatment culture in Tables 4 and 5 and summarized by group in Table 8. At the highest test concentrations evaluated for chromosome aberrations, 500 and 300 µg/ml, the mitotic indices were reduced 55% and 94% relative to the solvent control at the 20 hour and 44 hour harvests, respectively. Dose level 600 µg/ml in the 44 hour harvest was not analyzed due to complete mitotic inhibition. Dose levels 10, 19, and 38 µg/ml in the 20 hour harvest were tested but not required for analysis. In the 44 hour harvest, dose levels 10 and 19 µg/ml were not required for analysis. The percentage of cells with structural aberrations in the test article-treated group was not significantly increased above that of the solvent control at any dose level or harvest time ($p > 0.05$, Fisher's exact test). The percentage of cells with numerical aberrations in the test article-treated groups was not significantly increased above that of the solvent control at the 44 hour harvest time, regardless of dose level ($p > 0.05$, Fisher's exact test). The percentages of aberrant cells in the MMC groups were 7.5% and 35% ($p \leq 0.01$, Fisher's exact test) for the 20 and 44 hour harvest times, respectively.

The findings of the cytogenetic analysis for groups tested in the presence of an exogenous source of metabolic activation are presented by treatment flask in Tables 6 and 7 and summarized by group in Table 8. At the highest test concentrations evaluated for chromosome aberrations, 500 and 600 µg/ml, in the 20 and 44 hour harvests, respectively, the mitotic indices were reduced 71% and 69% relative to the solvent control. Dose level 600 µg/ml in the 20 hour harvest was not analyzed due to complete mitotic inhibition. Dose levels 10 and 19 µg/ml in the 20 hour harvest and 10, 19, and 38 µg/ml in the 44 hour harvest were tested but not required for analysis. The percentage of cells with structural aberrations in the test article-treated groups was not statistically increased above that of the solvent control at any dose level or harvest time ($p > 0.05$, Fisher's exact test). The percentage of cells with numerical aberrations in the test article-treated groups was

not statistically increased above that of the solvent control at the 44 hour harvest time, regardless of dose level ($p > 0.05$, Fisher's exact test). The percentages of aberrant cells in the CP groups were 6% and 8.5% ($p \leq 0.01$, Fisher's exact test) for the 20 and 44 hour harvests, respectively.

CONCLUSION

The positive and negative controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, Hexabromocyclododecane was concluded to be negative in the non-activated and S9-activated test systems in the *in vitro* mammalian cytogenetics test using human peripheral blood lymphocytes.

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TABLE 1
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED
WITH Hexabromocyclododecane IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INITIAL ASSAY: 20 HOUR HARVEST

Treatment ¹	Culture	Mitotic Index ² (%)	Cells Scored	Aberrant Cells ³ (%)	Total Number of Structural Aberrations						Severely Damaged Cells ⁵	Average Aberrations Per Cell ⁶
					Chromatid-type			Chromosome-type				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	1.4	100	0	0	0	0	0	0	0	0	0.000
	B	1.6	100	1	1	1	0	0	0	0	0	0.010
DMSO	A	1.4	100	0	0	0	0	0	0	0	0	0.000
	B	2.2	100	0	0	0	0	0	0	0	0	0.000
Hexabromocyclododecane												
0.25 µg/ml ⁷												
0.75 µg/ml ⁷												
2.5 µg/ml ⁷												
7.5 µg/ml ⁷												
25 µg/ml	A	1.8	100	0	0	0	0	0	0	0	0	0.000
	B	1.2	100	1	0	0	0	0	1	0	0	0.010
75 µg/ml	A	1.6	100	0	0	0	0	0	0	0	0	0.000
	B	1.0	100	0	0	0	0	0	0	0	0	0.000
250 µg/ml	A	1.2	100	0	0	0	0	0	0	0	0	0.000
	B	0.8	100	0	1	0	0	0	0	0	0	0.000
750 µg/ml	A	1.0	9	0	0	0	0	0	0	0	0	0.000
	B	0.6	16	0	0	0	0	0	0	0	0	0.000
2500 µg/ml ⁷												
MMC, 0.25 µg/ml	A	1.8	100	6	2	7	1	0	0	0	0	0.080
	B	1.4	100	11	1	8	6	1	0	0	0	0.150

¹HPBL were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.

²Mitotic index = number mitotic figures x 100/500 cells counted.

³Excluding cells with only gaps.

⁴Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

⁵Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

⁶Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

⁷Severely damaged cells and pulverizations were counted as 10 aberrations.

⁸Lower dose levels were also tested as a guard against excessive toxicity at higher dose levels but were not required for analysis. Dose level 2500 µg/ml was not evaluated due to complete mitotic inhibition.

TABLE 2
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED
WITH Hexabromocyclododecane IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INITIAL ASSAY: 20 HOUR HARVEST

Treatment ¹	Culture	Mitotic Index (%)	Cells Scored	Aberrant Cells (%)	Total Number of Structural Aberrations						Severely Damaged Cells ⁷	Average Aberrations Per Cell ²
					Chromatid-type ³			Chromosome-type ⁴				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	1.4	100	0	0	0	0	0	0	0	0	0.000
	B	1.6	100	0	0	0	0	0	0	0	0	0.000
DMSO	A	1.4	100	0	1	0	0	0	0	0	0	0.000
	B	1.6	100	0	1	0	0	0	0	0	0	0.000
Hexabromocyclododecane												
0.25 µg/ml ⁵												
0.75 µg/ml ⁵												
2.5 µg/ml ⁵												
7.5 µg/ml	A	2.2	100	0	2	0	0	0	0	0	0	0.000
	B	1.8	100	0	0	0	0	0	0	0	0	0.000
25 µg/ml	A	1.4	100	0	0	0	0	0	0	0	0	0.000
	B	1.8	100	0	0	0	0	0	0	0	0	0.000
75 µg/ml	A	1.4	100	2	0	0	0	1	0	1	0	0.020
	B	2.0	100	1	1	1	0	0	0	0	0	0.010
250 µg/ml	A	1.0	100	0	0	0	0	0	0	0	0	0.000
	B	1.6	100	0	0	0	0	0	0	0	0	0.000
750 µg/ml ⁵												
2500 µg/ml ⁵												
CP, ⁶ 25 µg/ml	A	0.4	100	7	2	10	1	0	0	0	0	0.110
	B	0.6	100	6	1	9	1	0	0	1	0	0.110

¹HPBL were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.

²Mitotic index = number mitotic figures x 100/500 cells counted.

³Excluding cells with only gaps.

⁴Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

⁵Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

⁶Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

⁷Severely damaged cells and pulverizations were counted as 10 aberrations.

⁸Lower dose levels were tested as a guard against excessive toxicity at higher dose levels but

not required for analysis. Dose levels 750 and 2500 µg/ml were not analyzed due to complete mitotic inhibition.

TABLE 3
SUMMARY
INITIAL ASSAY

Treatment	S9 Activation	Treatment/ Harvest Time	Mitotic Index	% Change ¹ from Control MI	Cells Scored	Aberrations Per Cell ² (Mean ± SD)	Cells With Aberrations ³ (%)
Untreated	-	20/20	1.5	-	200	0.005 ± 0.071	0.5
DMSO	-	20/20	1.8	-	200	0.000 ± 0.000	0.0
Hexabromocyclododecane							
25 µg/ml	-	20/20	1.5	17	200	0.005 ± 0.071	0.5
75 µg/ml	-	20/20	1.3	28	200	0.000 ± 0.000	0.0
250 µg/ml	-	20/20	1.0	44	200	0.000 ± 0.000	0.0
750 µg/ml	-	20/20	0.8	56	25	0.000 ± 0.000	0.0
MMC, 0.25 µg/ml	-	20/20	1.6	-7	200	0.115 ± 0.439	8.5**
Untreated	+	4/20	1.5	-	200	0.000 ± 0.000	0.0
DMSO	+	4/20	1.5	-	200	0.000 ± 0.000	0.0
Hexabromocyclododecane							
7.5 µg/ml	+	4/20	2.0	-33	200	0.000 ± 0.000	0.0
25 µg/ml	+	4/20	1.6	-7	200	0.000 ± 0.000	0.0
75 µg/ml	+	4/20	1.7	-13	200	0.015 ± 0.122	1.5
250 µg/ml	+	4/20	1.3	13	200	0.000 ± 0.000	0.0
CP, 25 µg/ml	+	4/20	0.5	67	200	0.110 ± 0.446	6.5**

¹% reduction in mitotic index relative to negative (solvent or untreated) control. Test article concentrations are compared to the solvent control and the positive control is compared to the untreated control.

²Severely damaged cells were counted as 10 aberrations.

³**, p<0.01; Fisher's exact test.

TABLE 4
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED
WITH Hexabromocyclododecane IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 20 HOUR HARVEST

Treatment ¹	Culture	Mitotic Index (%)	Cells Scored	Aberrant Cells (%)	Total Number of Structural Aberrations						Severely Damaged Cells ⁵	Average Aberrations Per Cell ⁶
					Chromatid-type			Chromosome-type				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	1.4	100	0	0	0	0	0	0	0	0	0.000
	B	1.4	100	0	0	0	0	0	0	0	0	0.000
DMSO	A	1.4	100	0	0	0	0	0	0	0	0	0.000
	B	0.8	100	0	0	0	0	0	0	0	0	0.000
Hexabromocyclododecane												
10 µg/ml ⁷												
19 µg/ml ⁷												
38 µg/ml ⁷												
75 µg/ml	A	1.0	100	0	0	0	0	0	0	0	0	0.000
	B	0.8	100	0	0	0	0	0	0	0	0	0.000
150 µg/ml	A	1.6	100	0	0	0	0	0	0	0	0	0.000
	B	1.6	100	0	0	0	0	0	0	0	0	0.000
300 µg/ml	A	1.4	100	1	0	1	0	0	0	0	0	0.000
	B	0.2	100	0	0	0	0	0	0	0	0	0.000
600 µg/ml	A	0.4	100	0	0	0	0	0	0	0	0	0.000
	B	0.6	100	0	0	0	0	0	0	0	0	0.000
MMC 0.13 µg/ml	A	1.8	100	8	0	4	3	0	0	1	0	0.080
	B	0.4	100	7	0	5	2	0	0	0	0	0.070

¹HPBL were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.

²Mitotic index = number mitotic figures x 100/500 cells counted.

³Excluding cells with only gaps.

⁴Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

⁵Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

⁶Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations. Severely damaged cells and pulverizations were counted as 10 aberrations.

⁷Lower dose levels were tested as a guard against excessive toxicity at higher dose levels but were not required for analysis.

TABLE 5
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED
WITH Hexabromocyclododecane IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 44 HOUR HARVEST

Treatment ¹	Flask	Mitotic Index ²	Cells Scored	Cells with Aberrations ³		Number of Structural Aberrations						Severely Damaged Cells ⁵	Average Structural Aberrations Per Cell ⁷
				(%)		Chromatid-type			Chromosome-type				
				Numerical	Structural	Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	4.6	100	0	2	1	1	0	1	0	0	0	0.020
	B	5.8	100	0	2	0	2	0	0	0	0	0	0.020
DMSO	A	5.2	100	0	0	0	0	0	0	0	0	0	0.000
	B	4.0	100	0	0	0	0	0	0	0	0	0	0.000
Hexabromocyclododecane													
10 µg/ml ⁸													
19 µg/ml ⁸													
38 µg/ml	A	4.4	100	0	0	0	0	0	0	0	0	0	0.000
	B	5.6	100	0	1	0	1	0	0	0	0	0	0.010
75 µg/ml	A	5.6	100	0	0	0	0	0	0	0	0	0	0.000
	B	4.4	100	0	1	0	1	0	0	0	0	0	0.010
150 µg/ml	A	2.8	100	2	0	1	0	0	0	0	0	0	0.000
	B	3.6	100	1	1	0	1	0	0	0	0	0	0.010
300 µg/ml	A	0.2	29	2 ⁹	0	0	0	0	0	0	0	0	0.000
	B	0.4	59	0 ¹⁰	2	0	0	0	0	0	0	1	0.169
600 µg/ml ⁸													
MMC 0.15 µg/ml	A	2.4	100	1	37	1	23	27	0	0	0	0	0.500
	B	3.8	100	2	33	3	26	22	0	0	1	0	0.490

¹HPBL were treated for 44 hours at 37±1°C in the absence of an exogenous source of metabolic activation.

²Mitotic index = number mitotic figures x 100/500 cells counted.

³Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.

⁴Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

⁵Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

⁶Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

⁷Severely damaged cells and pulverizations were counted as 10 aberrations.

⁸Lower dose levels were tested as a guard against excessive toxicity at higher dose levels but were not required for analysis. Dose level 600 µg/ml was not evaluated due to complete mitotic inhibition.

⁹Percentage based on a total of 56 cells scored for numerical aberrations.

¹⁰Percentage based on a total of 75 cells scored for numerical aberrations.

TABLE 6
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED
WITH Hexabromocyclododecane IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 20 HOUR HARVEST

Treatment	Culture	Mitotic Index (%)	Cells Scored	Aberrant Cells (%)	Total Number of Structural Aberrations						Severely Damaged Cells	Average Aberrations Per Cell
					Chromatid-type			Chromosome-type				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	1.2	100	0	0	0	0	0	0	0	0	0.000
	B	1.8	100	0	0	0	0	0	0	0	0	0.000
DMSO	A	1.6	100	0	0	0	0	0	0	0	0	0.000
	B	1.2	100	0	0	0	0	0	0	0	0	0.000
Hexabromocyclododecane												
10 µg/ml ¹												
19 µg/ml ²												
38 µg/ml	A	1.4	100	0	0	0	0	0	0	0	0	0.000
	B	1.2	100	0	0	0	0	0	0	0	0	0.000
75 µg/ml	A	1.0	100	0	0	0	0	0	0	0	0	0.000
	B	1.6	100	0	0	0	0	0	0	0	0	0.000
150 µg/ml	A	1.8	100	0	0	0	0	0	0	0	0	0.000
	B	1.8	100	0	0	0	0	0	0	0	0	0.000
300 µg/ml	A	0.6	100	0	0	0	0	0	0	0	0	0.000
	B	0.2	100	0	0	0	0	0	0	0	0	0.000
600 µg/ml ³												
CP 25 µg/ml	A	0.4	100	6	0	5	2	0	0	0	0	0.070
	B	0.6	100	6	0	3	3	0	0	0	0	0.060

¹HPBL were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.

²Mitotic index = number mitotic figures x 100/500 cells counted.

³Excluding cells with only gaps.

⁴Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.

⁵Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

⁶Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

⁷Severely damaged cells and pulverizations were counted as 10 aberrations.

⁸Lower dose levels were tested as a guard against excessive toxicity at higher dose levels but were not required for analysis. Dose level 600 µg/ml was not analyzed due to an insufficient number of scorable cells.

TABLE 7
CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED
WITH Hexabromocyclododecane IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

INDEPENDENT REPEAT ASSAY: 44 HOUR HARVEST

Treatment ^{1,2}	Flask	Mitotic Index ²	Cells Scored	Cells with Aberrations ³ (%)		Number of Structural Aberrations						Severely Damaged Cells ⁵	Average Structural Aberrations Per Cell ⁶
				Numerical	Structural	Chromatid-type		Chromosome-type					
						Gaps	Breaks	Exch	Breaks	Dic	Ring		
Untreated cells	A	7.6	100	0	0	0	0	0	0	0	0	0	0.000
	B	4.2	100	0	0	0	0	0	0	0	0	0	0.000
DMSO	A	6.4	100	0	0	0	0	0	0	0	0	0	0.000
	B	8.0	100	0	1	0	0	0	1	0	0	0	0.010
Hexabromocyclododecane													
10 µg/ml ⁷													
75 µg/ml	A	3.8	100	0	0	0	0	0	0	0	0	0	0.000
	B	5.8	100	0	0	0	0	0	0	0	0	0	0.000
150 µg/ml	A	4.0	100	3	1	0	1	0	0	0	0	0	0.010
	B	2.6	100	0	1	0	0	0	1	0	0	0	0.010
300 µg/ml	A	3.6	100	1	2	0	1	0	1	0	0	0	0.020
	B	3.0	100	1	1	0	1	0	0	0	0	0	0.010
600 µg/ml	A	1.2	100	1	4	1	5	0	0	0	0	0	0.050
	B	3.2	100	1	2	0	2	0	1	0	0	0	0.030
CP 25 µg/ml	A	5.2	100	0	9	1	10	1	4	0	0	0	0.150
	B	4.0	100	0	8	0	10	1	1	0	0	0	0.120

¹HPBL were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.

²Mitotic index = number mitotic figures x 100/500 cells counted.

³Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.

⁴Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.

⁵Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

⁶Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

⁷Severely damaged cells and pulverizations were counted as 10 aberrations.

⁸Lower dose levels were tested as a guard against excessive toxicity at higher dose levels but were not required for analysis.

TABLE 8
SUMMARY
INDEPENDENT REPEAT ASSAY

Treatment	SV Activation	Treatment/ Harvest Time (Hrs.)	Mitotic Index (Mean)	% Change from Control MI	Total Cells Scored	Aberrations Per Cell ^a (Mean ± SD)	Cells With Aberrations (%)	
							Numerical	Structural
Untreated	-	20/20	1.4	-	200	0.000 ± 0.000		0.0
DMSO	-	20/20	1.1	-	200	0.000 ± 0.000		0.0
Hexabromocyclododecane								
75 µg/ml	-	20/20	0.9	18	200	0.000 ± 0.000		0.0
150 µg/ml	-	20/20	1.6	-45	200	0.000 ± 0.000		0.0
300 µg/ml	-	20/20	0.8	27	200	0.005 ± 0.071		0.5
600 µg/ml	-	20/20	0.5	55	200	0.000 ± 0.000		0.0
MMC 0.13 µg/ml	-	20/20	1.1	21	200	0.075 ± 0.264		7.5**
Untreated	-	44/44	5.2	-	200	0.020 ± 0.140	0.0	2.0
DMSO	-	44/44	4.6	-	200	0.000 ± 0.000	0.0	0.0
Hexabromocyclododecane								
38 µg/ml	-	44/44	5.0	-9	200	0.005 ± 0.071	0.0	0.5
75 µg/ml	-	44/44	5.0	-9	200	0.005 ± 0.071	0.0	0.5
150 µg/ml	-	44/44	3.2	30	200	0.005 ± 0.071	1.5	0.5
300 µg/ml	-	44/44	0.3	93	88	0.114 ± 1.066	0.8	1.1
MMC 0.13 µg/ml	-	44/44	3.1	38	200	0.495 ± 0.802	1.5	35.0**
Untreated	+	4/20	1.5	-	200	0.000 ± 0.000		0.0
DMSO	+	4/20	1.4	-	200	0.000 ± 0.000		0.0
Hexabromocyclododecane								
38 µg/ml	+	4/20	1.3	7	200	0.000 ± 0.000		0.0
75 µg/ml	+	4/20	1.3	7	200	0.000 ± 0.000		0.0
150 µg/ml	+	4/20	1.8	-22	200	0.000 ± 0.000		0.0
300 µg/ml	+	4/20	0.4	71	200	0.000 ± 0.000		0.0
CP 25 µg/ml	+	4/20	0.5	67	200	0.065 ± 0.267		6.0**
Untreated	+	4/44	5.9	-	200	0.000 ± 0.000	0.0	0.0
DMSO	+	4/44	7.2	-	200	0.005 ± 0.071	0.0	0.5
Hexabromocyclododecane								
75 µg/ml	+	4/44	4.8	33	200	0.000 ± 0.000	0.0	0.0
150 µg/ml	+	4/44	3.3	54	200	0.010 ± 0.100	1.5	1.0
300 µg/ml	+	4/44	3.3	54	200	0.015 ± 0.122	1.0	1.5
600 µg/ml	+	4/44	2.2	69	200	0.040 ± 0.242	1.0	3.0
CP 25 µg/ml	+	4/44	4.6	22	200	0.135 ± 0.508	0.0	8.5**

^a% reduction in mitotic index relative to negative (solvent or untreated) control. Test article concentrations are compared to the solvent control and the positive control is compared to the untreated control.

^bSeverely damaged cells were counted as 10 aberrations.

**_p < 0.01; Fisher's exact test.

Data not collected for 20 hour harvest time.

Percentage based on a total of 131 cells scored for numerical aberrations.

APPENDIX I

Historical Control Data

IN VITRO MAMMALIAN CYTOGENETIC TEST USING
HUMAN PERIPHERAL LYMPHOCYTES

HISTORICAL CONTROL VALUES
1993-1995

NON-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control ¹	Positive Control ²
Mean	0.2%	0.3%	19.9%
Standard Deviation	0.5%	0.6%	13.9%
Range	0.0% to 2.5%	0.0% to 4.5%	6.5% to 87.5%

S-9 ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control ¹	Positive Control ³
Mean	0.3%	0.3%	15.0%
Standard Deviation	0.5%	0.5%	5.8%
Range	0.0% to 2.0%	0.0% to 3.0%	7.0% to 34.0%

¹Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, lactic acid buffer, placebo, 0.105N HCl, AD284 diluent, and 45% hydroxypropyl- β -cyclodextrin

²Positive control for non-activated studies, mitomycin C, (MMC)

³Positive control for S-9 activated studies, cyclophosphamide, (CP)

APPENDIX II

Study Protocol

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QA
APPROVED

Received by RA/OA 6-7-96

MA Study Number: G96A061.342

**Chromosome Aberrations in Human Peripheral Blood
Lymphocytes**

1.0 PURPOSE

The purpose of this study is to test the mutagenic potential of a test article based upon its ability to induce chromosome aberrations in human peripheral blood lymphocytes (HPBL).

2.0 SPONSOR

- 2.1 Name: **Chemical Manufacturers Association**
- 2.2 Address: 1300 Wilson Boulevard
Arlington, VA 22209
- 2.3 Representative: Dr. Hasmukh C. Shah

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

- 3.1 Test Article: **Hexabromocyclododecane**
- 3.2 Controls: Untreated: Untreated cells
Solvent: Test Article Solvent
Positive: Mitomycin C (MMC), Cyclophosphamide (CP)

3.3 Determination of Purity

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article.

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Microbiological Associates, Inc.

4.0 TESTING FACILITY AND KEY PERSONNEL

- 4.1 Name: Genetic Toxicology Division
Microbiological Associates, Inc.
- 4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850
- 4.3 Study Director: Ramadevi Gudi, Ph.D.

Protocol No. SPGT342

01/03/96

1 of 8

 **MICROBIOLOGICAL
ASSOCIATES, INC.**

MA Study No. G96A061.342

27

4.4 Associate Study Director: Donald Putman, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 6/11/96

5.2 Proposed Experimental Completion Date: 8/30/96

5.3 Proposed Report Date: 9/13/96

6.0 TEST SYSTEM

Peripheral blood lymphocytes will be obtained from healthy adults without six months exposure to either radiotherapy, viral infections or the administration of drugs. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston et al., 1981).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The initial assay will be performed by exposing lymphocytes to a minimum of nine concentrations of the test article as well as positive, untreated and solvent controls in duplicate cultures (Evans and O'Riordan, 1975). In the non-activated test system, exposure will be continuous up to the time of cell collection; in the S9-activated system, exposure will be for 4 hours. The dividing cells will be harvested at a single time point, approximately 20 hours from the initiation of treatment. The highest scorable dose level with at least 50% mitotic inhibition and three lower doses will be evaluated microscopically for structural chromosome aberrations. In the independent repeat assay, a minimum of four concentrations will be selected based on the findings of the initial assay. The exposure conditions will remain the same but two harvests will be carried out, approximately 20 hours after the initiation of treatment and 24 hours later (44 hours after initiation of treatment). The clastogenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group. The test article will also be assessed for its ability to induce numerical chromosome aberrations.

7.1 Solubility Determination

Unless the Sponsor has indicated the test article solvent, a solubility determination will be conducted to determine solvent and the maximum soluble concentration up to a maximum of 500 mg/ml. Vehicles compatible with this test system, in order of preference, include but are not limited to distilled water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5), and acetone (CAS 67-64-1). The vehicle will be the solvent, selected in order of preference, that permits preparation of the highest stock concentration, up to 500 mg/ml.

7.2 Dose Levels

For the initial cytogenetics assay, HPBL cells will be exposed to at least nine concentrations of test article at half-log dilutions. The highest concentration tested will be 5 mg/ml for freely soluble test articles, or the maximum concentration resulting in a workable suspension for poorly soluble test articles. The pH will be measured at the highest test article treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest treatment condition will also be measured. If excessive precipitation of the test article-solvent solution occurs upon addition to treatment medium, or if the osmolality of the treatment medium is excessive, the Sponsor will be consulted.

For the independent repeat assay, HPBL cells will be exposed to at least four concentrations of test article at no greater than half-fold dilutions, the highest concentration being that which is estimated to cause at least 50% mitotic inhibition (regardless of solubility) relative to the solvent control. In the absence of at least 50% mitotic inhibition the concentration resulting in minimum precipitation in test medium will be the highest concentration tested. Minimum precipitation will be determined by direct visual inspection. If excessive precipitation of the test article-solvent solution occurs upon addition to treatment medium, or if the osmolality of the test article-solvent solution in treatment medium exceeds the osmolality of the treatment medium-solvent solution by approximately 100 mOs/kg or greater, the Sponsor will be consulted. The pH and osmolality will be monitored as in the initial assay.

7.3 Frequency and Route of Administration

Target cells will be treated for approximately 20 or 44 hours in the non-activated system, depending upon harvest time, and for 4 hours in the S9-activated system. Treatment will be achieved by incorporation of the test article-solvent mixture into the medium. This technique has demonstrated to be an effective method of detection of chemical clastogens in this test system (Evans, 1976).

7.4 Metabolic Activation System

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S9 will be thawed and mixed with cofactors to contain 2 mM magnesium chloride ($MgCl_2$), 6 mM potassium chloride (KCl), 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 μ l S9 per ml serum-free medium.

7.5 Controls

7.5.1 Untreated Controls

Untreated cells will be used as the untreated control.

7.5.2 Solvent Control

The solvent vehicle for the test article will be used as the solvent control. For solvents other than water or medium, the final concentration in treatment medium will not exceed 1%.

7.5.3 Positive Controls

Mitomycin C (MMC) will be used at a concentration within 0.10-0.25 µg/ml as the positive control in the non-activated study. Cyclophosphamide (CP) will be used at a concentration within 25-100 µg/ml as the positive control in the S9-activated study.

7.6 Preparation of Target Cells

Peripheral blood cells will be cultured in complete medium (RPMI-1640 containing 15% fetal bovine serum, 2mM L-glutamine, 100 units penicillin and 100 ug streptomycin/ml) containing 1% phytohemagglutinin (PHA) by adding 0.6 ml heparinized blood to a centrifuge tube containing 9.4 ml complete medium with 1% PHA. The tubes will be incubated upright at 37±1°C in a humidified atmosphere of 5±1% CO₂ in air for 44-48 hours.

7.7 Identification of Test System

Using a permanent marking pen, the culture flasks/test tubes, etc. used in the test system will be identified by the study number, the treatment condition and test phase.

7.8 Treatment of Target Cells

All treatments will be carried out in duplicate. About 44-48 hours after culture initiation, tubes will be re-fed (centrifugation at approximately 1200 rpm for approximately 5 minutes) with 10 ml of complete medium for the non-activated exposure or 10 ml of S9 reaction mixture for the activated exposure to which will be added 100 µl of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if water or medium is used as the solvent. An untreated control consisting of cells in complete medium or S9 reaction mixture will also be included.

In the non-activated study, the cells will be exposed for 20 hours (harvest 1) or for 44 hours (harvest 2) at 37±1°C in a humidified atmosphere of 5±1% CO₂ in air. Exposure will continue until collection of metaphase cells.

In the S9-activated study, the cells will be exposed for 4 hours at $37\pm 1^\circ\text{C}$ in a humidified atmosphere of $5\pm 1\%$ CO_2 in air. After the exposure period, the cells will be centrifuged at approximately 1200 rpm for approximately 5 minutes, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium containing 1% PHA, and returned to the incubator for an additional 16 hours (harvest 1) or 40 hours (harvest 2).

7.9 Collection of Metaphase Cells

Cells will be collected approximately 20 hours and 44 hours after initiation of treatment. These times are selected to assure analysis of first division metaphase cells after initiation of test article treatment as well as to allow for analysis of delayed cells. Two hours prior to harvest, Colcemid® will be added to the cultures at a final concentration of 0.1 $\mu\text{g}/\text{ml}$. The cells will be collected by centrifugation (approximately 1200 rpm for 5 minutes), treated with 0.075M KCl, washed with two charges of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately $2-6^\circ\text{C}$. To prepare slides, the cells will be collected by centrifugation, resuspended in fresh fixative. One to two drops of fixed cells will be dropped onto a wet slide and air-dried. The slide will be identified by the MA study number, treatment condition and date. At least two slides will be prepared from each treatment tube. The slides will be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

Prior to analysis, slides from the initial and independent repeat studies will be scanned for scorable metaphases. In each study, whenever possible, the high dose will be selected to yield approximately 50% or greater mitotic inhibition. Slides from the highest scorable dose level and three lower dose levels will be selected for analysis.

Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 46 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentric and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥ 10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations will be recorded using a calibrated microscope stage. In the delayed harvest, the

percent polyploid cells will be evaluated per 100 metaphase cells. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Untreated and Solvent Controls

The frequency of cells with structural and numerical chromosome aberrations in the untreated or solvent control must be no greater than 6%.

8.2 Positive Controls

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water is used.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and will be presented for both the initial and the independent repeat chromosome aberration assays. The number and types of aberrations, the percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined, the percentage of numerically damaged cells in the total population of cells examined, and the frequency of structural aberrations per cell (mean aberrations per cell) will be reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells will be performed using the Fisher's exact test. Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage trend test will be used to measure dose-responsiveness.

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 when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A reproducible and significant increase at a single dose level will be considered positive. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

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.

Protocol No. SPGT342

01/03/96

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 MICROBIOLOGICAL
ASSOCIATES, INC.

MA Study No. G96A061.342

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Results presented will include, but not be limited to:

- cells used
- test conditions: composition of medium, CO₂ concentration, incubation time, concentration of test article and rationale for selection of concentration, duration of treatment, duration of treatment with and concentration of Colcemid®, type of metabolic activation system used, positive, solvent and untreated controls
- number of cell cultures
- number of metaphases analyzed (data given separately for each culture)
- mitotic index
- criteria for scoring aberrations
- type and number of aberrations, given separately for each treated and control culture
- historical control data

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Quality Assurance Unit of Microbiological Associates, Inc., Rockville, MD, in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with EU Legislation L 383 a/148, B.10 Mutagenicity (*In Vitro* Mammalian Cytogenetic Test), December, 1992; OECD Guideline 473 (Genetic Toxicology: *In Vitro* Mammalian Cytogenetic Test), May, 1983; and EPA Health Effects Testing Guidelines, Subpart 798.5375 (*In Vitro* Mammalian Cytogenetics), Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for EPA TSCA Regulations, 40 CFR 792 and OECD ISBN 922-84-12367-9.

Will this study be submitted to a regulatory agency? Yes

If so, to which agency or agencies? EU and 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

Evans, H.J. and M.L. O'Riordan. 1975. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. *Mutation Res.* 31:135-148.

Evans, H.J. 1976. Cytological methods for detecting chemical mutagens. In: *Chemical Mutagens, Principles and Methods for their Detection*, vol. 4. A. Hollaender (ed). Plenum Press, New York, NY.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff, J.S. Wassom. 1981. Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the Gene-Tox Program. *Mutation Res.* 87:143-188.

Scott, D., N.D. Danford, B.J. Dean and D.J. Kirkland. 1990. Metaphase Chromosome Aberration Assays In Vitro. In: *Basic Mutagenicity Tests: UKEMS Recommended Procedures*. D.J Kirkland (ed). Cambridge University Press, New York, NY.

14.0 APPROVAL

<u>Hasmukh Shah</u>	<u>5-29-96</u>
SPONSOR REPRESENTATIVE	DATE
<u>Hasmukh Shah</u>	
(Print or Type Name)	
<u>Ramona Tudi</u>	<u>6/5/96</u>
MA STUDY DIRECTOR	DATE

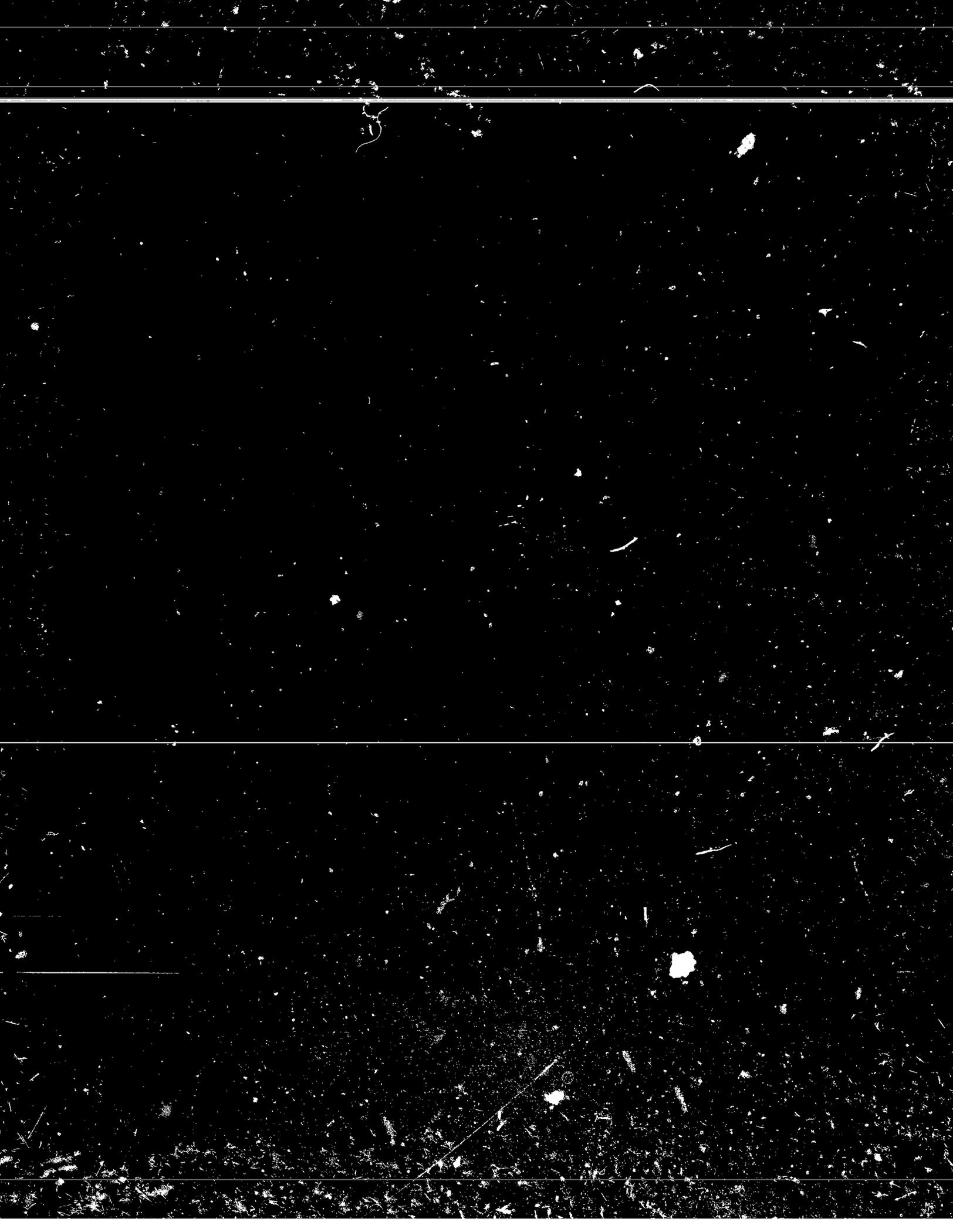
Protocol No. SPGT342 01/03/96

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 MICROBIOLOGICAL
ASSOCIATES, INC.

MA Study No. G96A061.342

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FYI - 0197 - 1281

MAXIMIZATION TEST IN GUINEA PIGS

Test Article

Hexabromocyclododecane

Author

Martin L. Wenk, Ph.D., D.A.B.T.

Study Completion Date

November 8, 1996

Performing Laboratory

Microbiological Associates, Inc. (MA)
9630 Medical Center Drive
Rockville, MD 20850

Laboratory Project Identification

M96AO61.1X64

Sponsor

Chemical Manufacturers Association
1300 Wilson Boulevard
Arlington, VA 22209

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study No. M96AO61.1X64 - Maximization Test in Guinea Pigs, was conducted in compliance with the Good Laboratory Practice Standards as published by the Organization for Economic Co-Operation and Development (OECD), OCDE/GD (92)32; and the United States Environmental Protection Agency (EPA) in 40 CFR Part 160 and 40 CFR 792 in all material aspects with the following exceptions:

The identity, strength, purity, and composition or other characteristics to define the test, control or reference substances have not been determined by the testing facility [(Section 105 (a)).

The stability of the test, control or reference substances under the test conditions has not been determined by the testing facility and is not included in the final report [(Sections 105 (a) and (b) and 185 (a) (5)).

Analyses to determine the uniformity, solubility or stability of the test, control or reference mixtures were not performed by the testing facility [(Section 113 (a)).

Signatures:

Martin L. Wenk 11/8/96
Martin L. Wenk, Ph.D., D.A.B.T. Date
Study Director

Sponsor: Hamukh Shah 11/19/96
Date

Submitter: Hamukh Shah 11/19/96
Date

QUALITY ASSURANCE STATEMENT

Study Title: MAXIMIZATION TEST IN GUINEA PIGS
Study Number: M96AO61.1X64
Study Director: Martin L. Wenk, Ph.D., D.A.B.T.

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 Compliance Programme, 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 09 MAY 96, TO STUDY DIR 09 MAY 96, TO MGMT 09 MAY 96
PHASE: Protocol Review

INSPECT ON 06 JUN 96, TO STUDY DIR 06 JUN 96, TO MGMT 10 JUN 96
PHASE: Randomization, allocation to housing and/or animal
identification - Range finder

INSPECT ON 17 JUL 96, TO STUDY DIR 17 JUL 96, TO MGMT 09 AUG 96
PHASE: Raw Data Audit

INSPECT ON 09 AUG 96, TO STUDY DIR 09 AUG 96, TO MGMT 14 AUG 96
PHASE: Draft Report

INSPECT ON 08 NOV 96, TO STUDY DIR 08 NOV 96, TO MGMT 08 NOV 96
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.

Juan M. McGowan
Juan M. McGowan
QUALITY ASSURANCE

Nov. 8, 1996
DATE

STUDY INFORMATION PAGE

Test article Identity: Hexabromocyclododecane

Vehicle Identities: Corn Oil and Petroleum Jelly

Study Initiation Date: May 31, 1996

Initiation of Dosing (Range-finding Test): June 13, 1996

Completion of In-Life Phase (Main Study): July 17, 1996

MA Study Number: M96AO61.1X64

Sponsor: Chemical Manufacturers Association
1300 Wilson Boulevard
Arlington, VA 22209

Sponsor Representative: Dr. Hasmukh C. Shah

Testing Facility: Microbiological Associates, Inc.
9630 Medical Center Drive
Rockville, Maryland 20850

Technical Staff: Cynthia Horner, B.S., LATG, RILAM - Toxicology Testing Facility
Manager
Geof Hunt, LAT - Laboratory Supervisor
Thomas J. Downs, B.S. - Lead Technician
Harry Paulin, M.S. - Analytical Chemist
Anton Allen, D.V.M., Ph.D. - Director of Veterinary Medicine

Study Director: Martin L. Wenk
Martin L. Wenk, Ph.D., D.A.B.T.

10/8/96
Date

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I. SUMMARY

The test article, hexabromocyclododecane, was administered in two stages (induction and challenge doses) to male guinea pigs. The induction doses were administered to the interscapular region as intradermal injections (phase I) and topical applications (phase II). The challenge dose was administered as topical applications to the flanks of each animal. Phase I of the induction doses consisted of three pairs of intradermal injections of 1) Freund's adjuvant and corn oil (50:50), 2) the test article at a concentration of 5.0% in corn oil, or 3) the test article at a concentration of 5.0% in the 50:50 corn oil/Freund's adjuvant solution which were administered to each treated animal (Group 2). The control animals (Group 1) received the same regimen but the test article was omitted. After a period of seven days, the treated animals (Group 2) received topical applications of neat test article (moistened in corn oil) on the previously treated interscapular sites.

Following a two week rest period, the challenge doses were administered. Each animal received a topical application of neat test article (moistened with corn oil) on the left flank and an empty Hilltop® Chamber on the right flank. Subsequent examination of the test sites indicated the test article produced no erythema or edema in any animal at 24, 48, 72, 96, or 120 hours after the challenge dose. Based on the percentage of animals responding (0%), the test article, Hexabromocyclododecane was considered a non-sensitizer.

II. INTRODUCTION

The purpose of this study was to assess whether the test article caused delayed contact hypersensitivity in guinea pigs as measured by the Magnusson and Kligman method (Magnusson and Kligman, 1969) and Kligman and Basketter's paper "A critical commentary and updating of the guinea pig maximization test. Kligman A. and Basketter, D Contact Dermatitis, 1995, 32, 129-134.". The experimental design complied with the EPA Health Effects Test Guidelines 40 CFR 798.4100 and the Organization for Economic Co-Operation and Development (OECD) Guidelines 406 for Testing of Chemicals, 1992.

III. MATERIALS AND METHODS

A. Test Article

Hexabromocyclododecane, a white powder, was received on May 21, 1996, assigned MA chemical number 96AO61, and stored at room temperature. The Sponsor was responsible for the determination and documentation of the identification, composition and potency of the test article.

NJ) to absorb liquids. The animals were transferred to clean cages weekly, and the racks were changed at least once every other week. The cage size (22 in. x 12.5 in. x 8 in.) conformed to the dimensions recommended in The Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication No. 86-23. All animals were housed in the same room from receipt until study completion. During the course of the study, the environmental conditions in the animal room were generally maintained with a temperature of 69-75°F and relative humidity of 30-70% on a 12 hour light/12 hour dark illumination cycle.

3. Husbandry

The animals were provided *ad libitum* access to drinking water (Washington Suburban Sanitary Commission Potomac Plant, Potomac, MD) via automatic watering devices and to Harlan TEKLAD Certified Guinea Pig #7006C feed (Harlan TEKLAD, Madison, WI) from arrival to termination. Annual water analyses and individual feed-lot analyses failed to reveal any contaminants which would have an effect on the results of the study. The results of these analyses are on file at MA. The animals were quarantined for approximately 1 week prior to test article administration, during which time they were observed daily for signs of illness or death. All animals assigned to the study appeared normal. No disease related observations were made during quarantine and all the animals were approved for use by the Clinical Veterinarian prior to being placed on test.

D. Test Procedure

1. Range-Finding Test

a. Randomization

Four animals to be used in the range-finding test were randomized 7 days prior to dosing. The animals were chosen from the stock population using a computer-generated random number table and assigned to one of two test groups as indicated in the table presented below. Each animal was given a unique identification number which was displayed on an ear tag and on a cage card which was placed on the front of each cage. The cage card also listed the study number, test article identification, dose level and displayed color coded indicators of those parameters. Raw data records and specimens were also identified by the unique animal identification number. A summary of the design of the Range-finding test is presented on the next page:

Final Report
MA Study No. M96AO61.1X64

	Route of Administration	Dose	No. of Animals
Neat ^A , 25%, 12.5%, 6.25% (in petroleum jelly)	Topical Application	Hilltop® Chamber with test material	2
5%, 2.5%, 1% and 0.5% (in corn oil)	Intradermal Injection	0.1 ml	2
TOTAL			4

^A The neat test article was moistened with corn oil for the topical application.

b. Treatment

Approximately twenty-four hours prior to dosing, the fur of the interscapular area of the two animals to be treated by intradermal injection was clipped to expose an area roughly 4 by 6 cm. For the animals to be treated topically, an area large enough to accept two Hilltop® Chambers was shaved on each flank prior to application. Dosing for the Range-finding test was performed on June 13, 1996.

(1) Intradermal Injection

Two animals each received four intradermal injections of the test article at concentrations of 0.5%, 1.0%, 2.5% and 5.0%, at separate sites on the shaved interscapular region. Each injection was administered just inside the periphery of the shaved area at a volume of 0.1 ml.

(2) Topical Applications

The two animals which were treated topically on the shaved flanks with four concentrations (neat-moistened with corn oil, 25%, 12.5%, and 6.25%) of the test article using 2 Hilltop® Chambers per flank. Each Hilltop® Chamber was loaded with 0.5 gms of one of the four concentrations. The Hilltop® Chambers were placed on separate locations and secured in place with 2" hypoallergenic cloth tape and adhesive tape. The Hilltop® Chambers were left in place for 48 hours.

c. Types and Frequency of Observations and Measurements

The animals which were administered intradermal injections of the test article were evaluated for necrosis, ulcerations, erythema, and edema 48 hours after dosing. The animals which were

administered topical applications of the test article were evaluated for dermal lesions (erythema and edema) at the time of unwrapping according to Draize (see section III.D.2.c).

d. Dose Selection for the Main Study

The highest concentration which caused mild to moderate irritation (for each route of administration) was selected to be used for the corresponding route of administration in the induction phase of the main study. The highest concentration of the topical application route of administration that did not cause erythema was selected to be used in the challenge phase of the main study. No ulcerations or necrosis was noted at any intradermal injection site during the Range-finding test. None of the concentration levels administered by intradermal injection produced mild to moderate irritation, but the 5.0% level exhibited very slight edema. Therefore, the 5.0% level was used for the intradermal injections (phase I) of the induction dose of the main study. No concentration of the topical application produced irritation. The test article was administered neat in the topical application (phase II) of the induction dose of the main study and in the challenge dose of the main study.

e. Sacrifice

After final evaluation of the treatment sites, the animals were sacrificed by lethal injection of Beuthanasia®-D Special euthanasia solution. No necropsies were performed.

2. Main Study

a. Randomization

The animals used in the main study were randomized two days prior to dosing. The weight ranges of guinea pigs (252.7 - 351.6 grams) did not exceed $\pm 20\%$ of the mean weight. Using a stratified body weight program (Innovative Programming Associates, Inc., Princeton, NJ), each animal was randomly assigned to a test group and given an identification number which was displayed on a cage card on the front of each cage and on an ear tag. The cage card also listed the study number, test article identification, dose level and displayed color coded indicators of those parameters. Raw data records and specimens were labelled with the animal identification number. A summary of the experimental design is presented on the following page:

Final Report
 MA Study No. M96AO61.1X64

Group	Treatment Regimen	Dose	No. of Animals
1	Induction Dose: Intradermal Injections: One pair each of Freund's Adjuvant, Vehicle and Vehicle plus Freund's Adjuvant. Topical Application: No treatment because neat material was applied to test group.	0.1 ml	10
	Challenge Dose: Topical Application: Separate and single application of neat test article and an empty Hilltop® Chamber.	0.5 g	
2	Induction Dose: Intradermal Injections: One pair each of Freund's Adjuvant, Test Material and Test Material plus Freund's Adjuvant. Topical Application: Single application of neat test article.	0.1 ml 0.5 g	20
	Challenge Dose: Topical Application: Separate and single application of the neat test article and an empty Hilltop® Chamber.	0.5 g	

b

Treatment

(i) Induction Doses

Phase 1 (intradermal injections) induction dosing was performed on June 19, 1996. Approximately twenty-four hours prior to dosing, the fur of the interscapular region of each animal was clipped to expose an area roughly 4 by 6 cm.

(a) Intradermal injections

During phase 1, three pairs of intradermal injections were given to each animal at injection sites 1, 2 and 3 as shown in Diagram 1. Each injection (0.1 ml) was administered just inside the periphery of the shaved area.

The first pair of injections (site 1) consisted of a 50:50 solution of corn oil and Freund's complete adjuvant. The second pair of injections (site 2) consisted of corn oil (Group 1) or the test article at a concentration of 5.0% in corn oil (Group 2). The third pair of injections (site 3) consisted of corn oil (Group 1) or the test article (Group 2) at a concentration level of 5.0% in Freund's adjuvant.

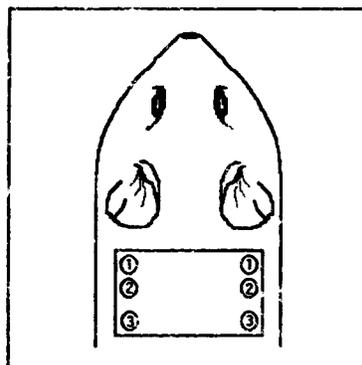


Diagram 1
Intradermal Injection Sites

On Day 7, the interscapular area which received the intradermal injections was shaved again (approximately 4 x 6 cm) with an electric clipper. Since the test article did not cause irritation when applied topically in the Range-finding test, the test area was treated with 0.5 gms of 10% sodium lauryl sulphate in petroleum jelly, in order to create a local irritation.

(b) **Topical Application**

On Day 8, a topical application (phase II) of the neat test material (moistened with corn oil) was applied to the shaved interscapular area using a Hilltop® Chamber which was loaded with 0.5 gms of the test article and placed in the interscapular area of each Group 2 animal. Since the test article was administered neat, the Group 1 animals were not treated during Phase II. The chambers were secured in place with 2" hypoallergenic cloth tape and adhesive tape. The chambers were left in place for 48 hours.

(2) Challenge Dose

After the completion of the induction phase, all animals were given a two week rest period, after which, a challenge dose (24-hour exposure) was given to both groups. Approximately twenty-four hours prior to the challenge dose, the fur of both flanks was clipped to expose an area approximately 5 by 5 cm. For each animal, one empty Hilltop® chamber was applied to the right flank and a second chamber was loaded with 0.5 gms of neat test article (moistened with corn oil) and applied to the left flank. The chambers were secured as before and removed 24 hours later.

Approximately 21 hours after removing the chambers the challenge area was cleaned using a gauze moistened with the vehicle. The animals were scored according to Draize (see section III.D.2.c).

c. Types and Frequency of Observations and Measurements

All animals were observed daily for mortality/morbidity. Body weights for all surviving animals were recorded on Test Days 1 (prior to dosing) and following the last day of observation (Day 29).

Dermal lesions were scored approximately 24, 48, 72, 96 and 120 hours after the challenge dose.

o Erythema and eschar formation:

No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Total possible erythema score	4

○ Edema formation:	
No edema	0
Very slight edema (barely perceptible)	1
Slightly edema (edges of area well defined by definite raising)	2
Moderate edema (raised approx. 1.0 mm)	3
Severe edema (raised more than 1.0 mm extending beyond the area of exposure)	4
Total possible erythema score	4

Total possible primary irritation	8
-----------------------------------	---

d. Terminal Sacrifice

After the last examination, the animals were sacrificed by lethal injection of Beuthanasia®-D Special euthanasia solution. No necropsies were performed.

E. Data Analyses

1. Calculations

a. Body Weights

Means and standard deviations were calculated for the body weights of each group of the Main Study on Days 1 and 29. An Analysis of Variance Test (ANOVA) was performed on body weights comparing the treated animals (Group 2) to the control animals (Group 1).

b. Grading of Test Material

The grading of the test material was based on the number of guinea pigs showing a response (defined as a score of 1 or greater) at 48 hours post-challenge dose.

Percentage of Animals Responding (Based on 20 animals)	No. of Animals Responding	Grade	Classification
5	1	I	Weak
10 - 30	2 - 6	II	Mild
35 - 65	7 - 13	III	Moderate
70 - 80	14 - 16	IV	Strong
85 - 100	17 - 20	V	Extreme

F. Archives

Upon completion of the final report, all raw data and reports will be maintained by the Quality Assurance Unit of Microbiological Associates, Incorporated, Rockville, Maryland in accordance with the relevant Good Laboratory Practices regulations. Unused dosage preparations and all residual test article were discarded following administration to the test system. The retention of any required reserve sample of test article will be the responsibility of the Sponsor.

Written permission from the Sponsor will be obtained to discard or transfer original records during the required retention period. If a transfer of original records occurs, true copies (i.e. photocopies, microfilm, or other accurate reproductions of the original records) will be retained for at least the minimum retention period specified by the relevant regulations in accordance with MA Standard Operating Procedures (SOP) No. OPQP2601.

G. Deviations from the Protocol

For the topical application of the challenge phase, cloth bandages were used rather than Coban elastic bandages.

The temperature and/or humidity were sporadically out of the protocol-prescribed range. The duration of these excursions was from 2 to 6 hours. The temperature exceeded the range by 1°F and the humidity exceeded the range by 4 percentage points.

The animals were transferred to clean cages twice a week rather than three times as prescribed by the protocol.

These deviations from the protocol did not have any negative effects on the results of the study.

IV. RESULTS

A. Dermal Scores (Range-Finding Test)

The individual scores for ulceration, necrosis, erythema and edema for the Range-Finding Test were reviewed by the Study Director. The animals which received the intradermal injections exhibited no ulcerations or necrosis. Very slight edema was noted in 2 of 4 test sites (2.5 and 5.0%) in both animals. No irritation was noted at any test site of the two animals which received topical applications of the test article. Therefore, the 5.0% dose level was selected for use in the intradermal injections of induction phase of the main study and neat test article was used for the topical application of the induction and challenge phases.

B. Dermal Scores (Main Study)

Individual scores for erythema and edema for the Main Study animals are presented in Table 1 (scores at 24, 48, 72, 96, and 120 hours post challenge phase dose application).

Examination of the flank test sites at 24, 48, 72, 96, and 120 hours post challenge dose failed to reveal any irritation at any test site in any animal from either group.

C. Body Weights

Individual and group mean body weights are presented in Table 2. Mean body weights are represented graphically in Figure 1.

Statistical analysis failed to show any significant ($p \leq 0.05$) difference in the Day 29 body weights of the treated animals when compared to the control animals.

V. DISCUSSION

Due to the lack of a response to the test article after the challenge dose, hexabromocyclododecane is considered a non-sensitizer when administered to guinea pigs via intradermal injections and topical applications and requires no grading characterization (Table 3).

VI. REFERENCES

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Magnusson, B., Kligman, A.M. *The Identification of Contact Allergens by Animal Assay. The Guinea Pig Maximization Test*, The Journal of Investigative Dermatology. 52: 268-275, 1969.

Kligman, A.M., Basketter, D.A. *A Critical Commentary and Updating of the Guinea Pig Maximization Test*, Contact Dermatitis. 32: 129-134, 1995.

TABLES AND FIGURES

MAXIMIZATION TEST IN GUINEA PIGS

TABLE 1
 DERMAL SCORES (CHALLENGE DOSE)
 CONTROL SITE

Treatment Group	Animal Number	24 Hours ¹		48 Hours		72 Hours		96 Hours		120 Hours	
		Ery.	Edema	Ery.	Edema	Ery.	Edema	Ery.	Edema	Ery.	Edema
1 (Challenge Only)	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0
2 (Treated)	11	0	0	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0	0	0
	13	0	0	0	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0
	16	0	0	0	0	0	0	0	0	0	0
	17	0	0	0	0	0	0	0	0	0	0
	18	0	0	0	0	0	0	0	0	0	0
	19	0	0	0	0	0	0	0	0	0	0
	20	0	0	0	0	0	0	0	0	0	0
	21	0	0	0	0	0	0	0	0	0	0
	22	0	0	0	0	0	0	0	0	0	0
	23	0	0	0	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0	0	0	0
	25	0	0	0	0	0	0	0	0	0	0
	26	0	0	0	0	0	0	0	0	0	0
	27	0	0	0	0	0	0	0	0	0	0
	28	0	0	0	0	0	0	0	0	0	0
	29	0	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0	0

Ery. = Erythema

¹Hours after the challenge dose application.

MAXIMIZATION TEST IN GUINEA PIGS

TABLE 1 (cont.)
 DERMAL SCORES (CHALLENGE DOSE)
 TEST ARTICLE SITE

Treatment Group	Animal Number	24 Hours ¹		48 Hours		72 Hours		96 Hours		120 Hours	
		Ery.	Edema	Ery.	Edema	Ery.	Edema	Ery.	Edema	Ery.	Edema
1 (Challenge Only)	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0
2 (Treated)	11	0	0	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0	0	0
	13	0	0	0	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0
	16	0	0	0	0	0	0	0	0	0	0
	17	0	0	0	0	0	0	0	0	0	0
	18	0	0	0	0	0	0	0	0	0	0
	19	0	0	0	0	0	0	0	0	0	0
	20	0	0	0	0	0	0	0	0	0	0
	21	0	0	0	0	0	0	0	0	0	0
	22	0	0	0	0	0	0	0	0	0	0
	23	0	0	0	0	0	0	0	0	0	0
	24	0	0	0	0	0	0	0	0	0	0
	25	0	0	0	0	0	0	0	0	0	0
	26	0	0	0	0	0	0	0	0	0	0
	27	0	0	0	0	0	0	0	0	0	0
	28	0	0	0	0	0	0	0	0	0	0
	29	0	0	0	0	0	0	0	0	0	0
	30	0	0	0	0	0	0	0	0	0	0

Ery. = Erythema

¹Hours after the challenge dose application.

MAXIMIZATION TEST IN GUINEA PIGS

TABLE 2
 BODY WEIGHTS (Grams)

<u>Group No.</u>	<u>Animal No.</u>	<u>Day 1</u>	<u>Day 29</u>
1	1	302.2	537.6
	2	333.8	530.7
	3	343.7	556.9
	4	334.7	553.1
	5	348.0	581.0
	6	316.1	555.1
	7	327.2	580.3
	8	316.7	612.4
	9	355.3	555.6
	10	328.2	519.8
		Mean	330.6
	S.D.	16.1	27.2
	(N)	(10)	(10)
2	11	351.9	535.8
	12	328.7	534.0
	13	314.4	527.9
	14	333.7	538.4
	15	323.3	574.5
	16	317.1	553.9
	17	332.4	570.7
	18	346.1	511.9
	19	348.0	593.2
	20	337.2	616.4
	21	369.9	626.0
	22	316.2	473.8
	23	322.5	458.1
	24	340.6	531.5
	25	320.6	517.1
	26	317.3	556.2
	27	338.1	566.0
	28	353.4	510.3
	29	353.9	587.3
30	306.4	500.3	
	Mean	333.6	546.7
	S.D.	16.6	46.7
	(N)	(20)	(20)

MAXIMIZATION TEST IN GUINEA PIGS

TABLE 3

TEST MATERIAL GRADE

Percentage of Animals Responding	No. of Animals Responding (Based on a Group of 20)	Grade	Classification
0%	0	NA*	Non-irritant

NOTE: The grading of the test material is based on the number of guinea pigs showing a response (defined as a score of 1 or greater) 48 hours post-chamber removal.

* No animals responded to the test article as defined above. A minimum of one animal responding is necessary for a grade of I and a classification of "weak".

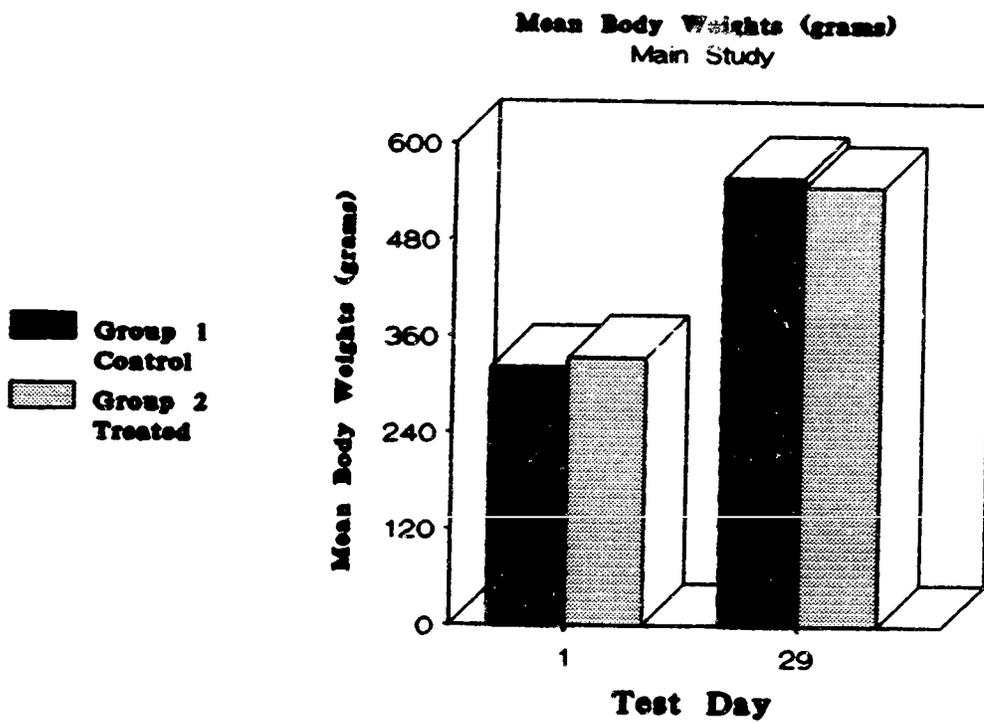


FIGURE 1

VIII. APPENDIX

**APPENDIX A
PROTOCOL AND AMENDMENTS**

QA
APPROVED

PROTOCOL AMENDMENT
#1

DATE: July 31, 1996
SPONSOR: Chemical Manufacturers Association
STUDY NO.: M96AO61.1X64
STUDY TITLE: Maximization Test in Guinea Pigs

Revisions:

1. Page 2, Section 4.3.1, "Storage Conditions to Maintain Stability"; Add data for sections 4.3.1.1 to 4.3.1.4. Room temperature, ambient, ambient and none, respectively.

Reason: Omitted from protocol.

2. Page 3, Section 6.5, "Source of Animals"; Revise to Harlan-Sprague Dawley, Madison, WI.

Reason: Error.

Walter J. Wend
Study Director

7/31/96
Date

Harmukh Shah
Sponsor's Approval

8/5/96
Date

 MICROBIOLOGICAL
ASSOCIATES, INC.

 MICROBIOLOGICAL
ASSOCIATES, INC.

MA Study Number: M96AO61.1X64

4.0 TEST ARTICLE:

4.1 Name or Code No.: Hexabromocyclododecane

4.2 MA Test Article Number: 96AO61

4.3 Stability and Handling of Test Article:

4.3.1 Storage Conditions to Maintain Stability:

4.3.1.1 Temperature: Room temperature

4.3.1.2 Humidity: Ambient

4.3.1.3 Light: Ambient

4.3.1.4 Special Requirements: None

4.3.2 Special Handling Procedures: *N/A M2017/5/19/96*

4.4 Vehicle: For injection, sterile physiological saline (0.9% w/v) will be used for aqueous-soluble test articles. Aqueous-insoluble materials will be given as a solution in light mineral oil, corn oil, polyethylene glycol 400, DMSO or propylene glycol (in that order of preference). If solutions are not possible, 2% carboxymethylcellulose, corn oil, or mineral oil will be used to form an injectable suspension for a 23 gauge needle. For topical applications of solids, the test article will be applied as a neat paste formed by wetting with the appropriate vehicle or a w/w concentration in petroleum jelly. Acetone but not DMSO may be added as a solvent used for topical applications. If a liquid, the test article will be applied neat or solubilized or suspended with the appropriate vehicle (solution first choice) as determined for injections.

4.5 Test Article Analysis: The Sponsor will be responsible for the determination and documentation of the composition and stability of the test article.

Protocol No. 13.1X64 04/09/96

2 of 14

THIS PAGE REVISED	Study M96AO61.1X64
<i>Martin L. Wenk</i>	July 31, 1996
Martin L. Wenk, Ph.D., D.A.S.T.	Date
Study Director	

 MICROBIOLOGICAL
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MA Study Number: M96AO61.1X64

4.6 Test Article Retention Sample:

The retention of any required reserve sample of the test article will be the responsibility of MA.

5.0 TESTING SCHEDULE:

- 5.1 Proposed Initiation of Dosing: June 4, 1996
- 5.2 Proposed Completion of Observations: July 12, 1996
- 5.3 Proposed Submission Date of Draft Report to Sponsor: August 12, 1996
- 5.4 Proposed Submission Date of Final Report: Two weeks following receipt of Sponsor comments.

5.0 TEST SYSTEM:

- 6.1 Species: Guinea Pigs
- 6.2 Strain: Hartley (DHA)
- 6.3 Number per Sex: Thirty-four Males
- 6.4 Body Weight: The test animals will weigh at least 300 g at the start of the study.
- 6.5 Source of Animals: Harlan-Srage Dawley, Madison, WI.
- 6.6 Justification for Selection of Test System: Guinea pigs are recommended for sensitization testing in the EPA Health Effects Test Guidelines and the OECD Guidelines.
- 6.7 Procedure for Unique Identification of Test System: Upon arrival, each animal will be given a study-unique pretest number. During the test animal selection process, each test animal will be assigned a test animal number unique to it within

Protocol No. 18.1X64 04/09/96 3 of 14

THIS PAGE REVISED	Study M96AO61.1X64
<i>Martin L. Wenk</i>	July 31, 1996
Martin L. Wenk, Ph.D., D.A.B.T.	Date
Study Director	

 MICROBIOLOGICAL
ASSOCIATES, INC.

 MICROBIOLOGICAL
ASSOCIATES, INC.

MA Study Number: M96AO61.1X64

MAXIMIZATION TEST IN GUINEA PIGS

1.0 PURPOSE OF THE STUDY:

The purpose of this study is to assess whether a test article causes delayed contact hypersensitivity in guinea pigs as measured by the Magnusson and Kligman method (Magnusson and Kligman, 1969) and Kligman and Basketter's recent paper "A critical commentary and updating of the guinea pig maximization test. Kligman A. and Basketter, D. Contact Dermatitis, 1995, 32, 129-134." The experimental design complies with the EPA Health Effects Test Guidelines 40 CFR 798.4100 and the Organization for Economic Co-Operation and Development (OECD) Guidelines 406 for Testing of Chemicals, 1992.

2.0 SPONSOR:

- 2.1 Name: Chemical Manufacturers Association
- 2.2 Address: 1300 Wilson Boulevard
Arlington, VA 22209
- 2.3 Representative: Dr. Hasmukh C. Shah

3.0 TESTING FACILITY:

- 3.1 Name: Microbiological Associates Inc. (MA)
- 3.2 Address: 9630 Medical Center Drive
Rockville, Maryland 20850
- 3.3 Study Director: Martin L. Wenk, Ph.D. D.A.B.T.
- 3.4 Animal Facility Manager: Cynthia Horner, B.S., LATG, RILAM
- 3.5 Laboratory Supervisor: Geof Hunt, LAT
- 3.6 Lead Technician: Thomas J. Downs, B.S.

Protocol No. 18.1X64 04/09/96 1 of 14

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MA Study Number: M96AO61.1X64

4.0 TEST ARTICLE:

4.1 Name or Code No.: Hexabromocyclododecane

4.2 MA Test Article Number: 96AO61

4.3 Stability and Handling of Test Article:

4.3.1 Storage Conditions to Maintain Stability:

4.3.1.1 Temperature:

4.3.1.2 Humidity:

4.3.1.3 Light:

4.3.1.4 Special Requirements:

4.3.2 Special Handling Procedures:

4.4 Vehicle: For injection, sterile physiological saline (0.9% w/v) will be used for aqueous-soluble test articles. Aqueous-insoluble materials will be given as a solution in light mineral oil, corn oil, polyethylene glycol 400, DMSO or propylene glycol (in that order of preference). If solutions are not possible, 2% carboxymethylcellulose, corn oil, or mineral oil will be used to form an injectable suspension for a 23 gauge needle. For topical applications of solids, the test article will be applied as a neat paste formed by wetting with the appropriate vehicle or a w/w concentration in petroleum jelly. Acetone but not DMSO may be added as a solvent used for topical applications. If a liquid, the test article will be applied neat or solubilized or suspended with the appropriate vehicle (solution first choice) as determined for injections.

4.5 Test Article Analysis: The Sponsor will be responsible for the determination and documentation of the composition and stability of the test article.

MA Study Number: M96AO61.1X64

4.6 Test Article Retention Sample:

The retention of any required reserve sample of the test article will be the responsibility of MA.

5.0 TESTING SCHEDULE:

- 5.1 Proposed Initiation of Dosing: June 4, 1996
- 5.2 Proposed Completion of Observations: July 12, 1996
- 5.3 Proposed Submission Date of Draft Report to Sponsor: August 12, 1996
- 5.4 Proposed Submission Date of Final Report: Two weeks following receipt of Sponsor comments.

6.0 TEST SYSTEM:

- 6.1 Species: Guinea Pigs
- 6.2 Strain: Hartley (DHA)
- 6.3 Number per Sex: Thirty-four Males
- 6.4 Body Weight: The test animals will weigh at least 300 g at the start of the study.
- 6.5 Source of Animals: Hazleton Research Products, Denver, PA.
- 6.6 Justification for Selection of Test System: Guinea pigs are recommended for sensitization testing in the EPA Health Effects Test Guidelines and the OECD Guidelines.
- 6.7 Procedure for Unique Identification of Test System: Upon arrival, each animal will be given a study-unique pretest number. During the test animal selection process, each test animal will be assigned a test animal number unique to it within

MA Study Number: M96AO61.1X64

the population making up the study. This number will appear as an ear tag and will also appear on a cage card visible on the front of each cage. The cage card will additionally contain the study number, test article, dose level and study type. Raw data records and specimens will also be identified by the unique test animal number.

- 6.8 Housing: Animals will be housed in polycarbonate cages with Sani-Chip heat-treated hardwood bedding (P.J. Murphy Forest Products Corporation, Montville, NY). Animals will be housed in an environmentally controlled room at a temperature of 69-75°F and at a relative humidity of 30-70%, with a 12 hr light/12 hr dark cycle. Animals will be housed two per cage during quarantine and individually following test animal selection. The cage size will conform to the upper weight range as recommended in the Guide for the Care and Use of Laboratory Animals, DHEW (NIH) No. 86-23. All animals will be transferred to clean cages with fresh bedding three times a week.
- 6.9 Acclimation Period: All animals will be acclimated for at least one week. During this time, animals will be observed daily for signs of illness or death and all unusual observations will be reported to the Study Director or Clinical Veterinarian. Animals will be examined and approved for use by the Clinical Veterinarian (RAQA #11). Any sickly animal will be eliminated prior to the test animal selection process.
- 6.10 Food: Harlan TEKLAD Guinea Pig 7006C (Harlan TEKLAD, Madison, WI) will be provided ad libitum from arrival until termination. The lot number or milling date will be recorded in the raw data. There are no contaminants in the feed which are expected to influence the study.
- 6.11 Water: Tap water from an automatic watering system will be provided ad libitum from arrival until termination. The water source is Washington Suburban Sanitary Commission, Potomac Plant; no additional treatment. The water meets USEPA drinking water standards and is monitored at least annually for levels of organophosphorus pesticides, metals, and coliform bacteria and other contaminants.
- 6.12 Animal Welfare Compliance Statements: Daily observations and the ability to euthanize animals for humane reasons will minimize unavoidable discomfort and

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pain to study animals. The Clinical Veterinarian will be consulted if animal health or welfare issues arise during the course of the study.

7.0 EXPERIMENTAL DESIGN:

- 7.1 Procedure Used to Assign Animals to Treatment Groups: Following the quarantine period, a sufficient number of animals for the range-finder will be chosen from the stock using a computer generated random number table for the Range-Finding Test(s). Following the Range-Finding Test(s), thirty animals from the remaining stock will be randomized into two experimental groups on the basis of body weight using a computer-generated randomization program.
- 7.2 Range-Finding Test(s): Four animals will be used for the initial range-finding test (2 animals/route of administration):

Dose Level(s)	Route of Administration	Dose	No. of Animals
If a liquid: Neat, 75%, 40%, 10% If a solid: Neat as a paste, 25%, 12.5% and 6.25% in petroleum jelly	Topical Application	load patch or Hilltop Chamber with test material ¹	2
As a solution (preferably) or an injectable suspension: 5%, 2.5%, 1% and 0.5%	Intradermal Injections	0.1 ml	2

¹ Exact amount to be documented in the raw data.

- 7.2.1 Test Procedure: An approximate 4 by 6 cm area of the interscapular region of each of two animals will be clipped free of fur approximately 24 hours prior to the intradermal injections. An area large enough to accept two 2 x 4 cm patches will be shaved from each flank of two other animals prior to the topical application.

- 7.2.1.1 Intradermal Injections: Twenty-four hours later two animals will receive 4 intradermal injections of four different concentrations, each one at a separate interscapular site. Each injection will be 0.1 ml and will be given just inside the periphery of the shaved area. The location and

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concentration of the injection will be documented in the raw data for each animal.

Necrosis and ulcerations will be evaluated 48 hours after injection. The highest concentration that causes mild to moderate skin irritation will be used for the intracutaneous induction phase of the Main Study. However, if the material produces necrosis or ulcerations at all doses tested, then additional Range-Finding Tests will be conducted until a suitable dose level is determined. If additional Range-Finding Tests are necessary, they will be performed at an additional cost and with the Sponsor's approval.

7.2.1.2

Topical Applications: The other two animals will receive 4 topical applications of the four different concentrations using two 2 x 4 cm pieces of filter paper or two suitable size Hilltop® Chambers on each flank of each animal. If a patch is used, it will be loaded with the test material as described in Section 7.2. A plastic cover (e.g. dam with occluded surface) will be placed over the patch to form an occlusive barrier. If a Hilltop® Chamber is used, it will be loaded with the test material as described in Section 7.2. The patch or Hilltop® Chamber will be secured in place with a Coban elastic bandage and adhesive tape. This will be left in place for 48 hours.

Animals will be scored at the time of unwrapping according to the Section 7.4.3. The highest concentration that causes mild to moderate irritation will be used for the induction phase of the Main Study. The highest concentration causing no erythema will be used for the challenge phase of the Main Study. However, if the material produces levels of irritation greater than necessary to select induction and challenge doses then additional Range-Finding Tests will be conducted until suitable dose levels are determined. If additional Range-Finding Tests are necessary, they will be

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performed at an additional cost and with the Sponsor's approval.

7.3 Main Study: Thirty animals will be used for the main study:

Group	Treatment Regimen	Dose	No. of Animals
1 (Challenge Only)	Induction Dose: Intradermal Injections: One pair each of Freund's Adjuvant, Vehicle and Vehicle plus Freund's Adjuvant).	0.1 ml	10
	Topical Application: Single application of the vehicle if vehicle employed OR no treatment if heat material applied.	load patch	
	Challenge Dose: Topical Application: Separate and single application of test article and vehicle (if used).	load patch	
2 (Treated)	Induction Dose: Intradermal Injections: One pair each of Freund's Adjuvant, Test Material and Test Material plus Freund's Adjuvant).	0.1 ml	20
	Topical Application: Single application of test article.	load patch	
	Challenge Dose: Topical Application: Separate and single application of the test article and vehicle (if used).	load patch	

7.3.1 Test Procedure: An approximate 4 by 6 cm area of the interscapular region of each animal will be clipped free of fur approximately 24 hours prior to each induction dose. An approximate 5 by 5 cm area of each flank of each animal will be clipped free of fur approximately 24 hours prior to the challenge dose.

7.3.1.1 Induction Doses: Induction doses shall be in two phases: intradermal injections and a topical application. During phase 1, three pairs of intradermal injections will be given to each animal at the injection sites (1, 2 and 3) as shown in Figure 1.

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Cranial
Clipped interscapular region

1	1
2	2
3	3

Caudal

Figure 1 - Location of intradermal injection sites. For the **Challenge Only Group** (Group 1), the first pair of injections (1) will be adjuvant alone. The second pair of injections (2) will be the vehicle, selected in the range-finder for intradermal injections, alone. The third pair (3) of injections will be the chosen vehicle in Freund's complete adjuvant. Similar injections will be given to each **Treated Group** (Group 2) with the exception the test material in the selected vehicle and at the concentration determined in the range-finder will be substituted for the vehicle.

Freund's complete adjuvant will be prepared for Groups 1 and 2 as an emulsified 50:50 mixture with the vehicle selected for the intradermal injections of the test article. It will then be injected alone or in combination with the test article at the concentration determined by the range-finder or the test article vehicle.

Each injection will be 0.1 ml and will be given just inside

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the periphery of the shaved interscapular area that will bear the 2 x 4 cm patch during the topical phase of induction (see below).

On Day 7, the interscapular area which received the intradermal injections will be shaved again (approximately 4 x 6 cm) with an electric clipper. If the test article is not a skin irritant, the test area will be treated with 0.5 ml of 10% sodium lauryl sulphate in vaseline, in order to create a local irritation.

On Day 8, a topical application (phase 2) of the test material or vehicle (if appropriate) will be applied to the shaved interscapular area, used for the intradermal injections, using a 2 x 4 cm piece of filter paper or suitable size Hilltop® Chambers. The concentration of the topically applied test material will be at doses set by the Range-Finding Test (mild to moderate irritation).

If a patch is used, it will be loaded with the test material. A plastic cover (e.g. dam with occluded surface) will be placed over the patch to form an occlusive barrier. If a Hilltop® Chamber is used, it will be loaded with the test material. The patch or Hilltop® Chamber will be secured in place with a Coban elastic bandage and adhesive tape. The exact amount of test material applied will be recorded in the study data. This will be left in place for 48 hours.

7.3.1.2

Challenge Dose: After the completion of the induction phase, all animals will be given a two week rest period. Following the rest period, a final challenge dose (24-hour exposure) will be given to both groups.

An approximate 5 by 5 cm area of each flank of each animal will be clipped free of fur approximately 24 hours prior to the challenge dose.

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A topical application of the test material or vehicle (if appropriate) will be applied to the shaved flank using a 2 x 2 cm piece of filter paper or a Hilltop® Chamber. The concentration of the topically applied test material will be at doses set by the Range-Finding Test (non-irritating). If a patch is used, it will be loaded (e.g. saturated) with the test material or vehicle (if appropriate) and the exact amount will be recorded in the study data. A plastic cover (e.g. dam with occluded surface) will be placed over the patch to form an occlusive barrier. If a Hilltop® Chamber is used, it will be loaded (e.g. saturated) with the test material or vehicle (if appropriate). The patch or Hilltop® Chamber will be secured in place with a Coban elastic bandage and adhesive tape. Both the Challenge Only Group (Group 1) and the Treated Group (Group 2) will be treated as follows:

- Single topical dose of the test material given on the left flank.
- Single topical dose of the vehicle (if appropriate) given on the right flank.

The patch or Hilltop Chamber® will be removed 24 hours later. Approximately 21 hours after removing the patch the challenge area is cleaned and shaved if necessary. The animals will be scored as described in Sections 7.4.2 and 7.4.3. At the time dermal lesions are scored after the challenge exposure, consideration shall be given to the need to re-challenge all animals. A re-challenge may be needed in order to distinguish a hyperirritability syndrome from true sensitization (Kligman and Basketter, 1995). This can be especially important in differentiating the etiology of mild skin responses. The need for a re-challenge shall be performed after a three (3) week rest period and at an additional cost to the sponsor. The re-challenge shall be

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carried out as described in Section 7.3.1.2 and the type and frequency of observations and measurements shall be the same as that described in Section 7.4 for the initial challenge.

7.4 Type of Frequency of Observations and Measurements:

7.4.1 Body Weights: Body weights will be recorded on Test Day 1 and on the last day of observation for all animals.

7.4.2 Evaluation of Dermal Irritation: Dermal lesions will be scored approximately 24, 48, 72, 96 and 120 hours after the challenge dose application (at unwrapping, 24, 48, 72 and 96 hours after unwrapping).

7.4.3 Scale for Scoring Dermal Lesions: Draize, J.H., Woodward, G., Calvery, H.O. *Methods for the Study of Irritation and Toxicity Substances Applied Topically to the Skin and Mucous Membranes*, Journal of Pharmacology and Experimental Therapeutics, 83:377-390, 1944.

o	Erythema and eschar formation:	
	No erythema	0
	Very slight erythema (barely perceptible)	1
	Well defined erythema	2
	Moderate to severe erythema	3
	Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
	Total possible erythema score	4
<hr/>		
o	Edema formation:	
	No edema	0
	Very slight edema (barely perceptible)	1
	Slightly edema (edges of area well defined by definite raising)	2
	Moderate edema (raised approx. 1.0 mm)	3
	Severe edema (raised more than 1.0 mm extending beyond the area of exposure)	4
	Total possible erythema score	4
	Total possible primary irritation	8

MA Study Number: M96AO61.1X64

- 7.4.4 Mortality/Unusual Reactions: Animals will be observed once daily during the course of the study. Any unusual reactions will be recorded if observed.
- 7.4.5 Method of Euthanasia: After the last examination in both the Range-finding Test and Main Study, the animals will be euthanized by lethal injection of Beuthanasia®-D Special euthanasia solution. No necropsies will be performed.

8.0 RECORDS AND SAMPLE ARCHIVES:

- 8.1 Records: Upon completion of the final report, all raw data and reports will be maintained by the Quality Assurance Unit of Microbiological Associates, Rockville, MD in accordance with the relevant Good Laboratory Practice Regulations.
- 8.2 Test Article: Test article will be stored under appropriate conditions with access restricted to authorized personnel. Unless arrangements are made to the contrary, unused dosage preparations will be disposed of following administration to the test system and all residual test article will be returned to the Sponsor following finalization of the report.

9.0 DATA AND REPORTING:

- 9.1 Grading of Test Material: The grading of the test material is based on the number of guinea pigs showing a response (defined as a score of 1 or greater) at 48 hour post-challenge dose.

MA Study Number: M96AO61.1X64

Percentage of Animals Responding	No. of Animals (Based on a Group of 20)	Grade	Classification
0 - 8	1	I	Weak
9 - 28	2 - 6	II	Mild
29 - 64	7 - 13	III	Moderate
65 - 80	14 - 16	IV	Strong
81 - 100	17 - 20	V	Extreme

- 9.2 Treatment of Results: Data will be summarized in tabular form, showing for each individual animal the skin reaction results of the challenge exposure at 24, 48, 72, and 96 hours after exposure.
- 9.3 Study Report Requirements: Results presented in the final report will include, but not be limited to, the following:
- o Summary
 - o Materials and method
 - o Individual irritation scores
 - o Results of the Range-Finding Test
 - o Initial and final individual body weights of the animals with a mean and standard deviation.
 - o Interpretation of the test is based on the Grading and Classification as defined by the Magnusson and Kligman method and as discussed by Kligman and Basketter, 1995.

MA Study Number: M96AO61.1X64

9.4 Interpretation of the Results: The test results will provide an estimate of the overall sensitization potential of the test material, i.e. essentially a non-sensitizer, a weak sensitizer, a moderate sensitizer, or a potent sensitizer.

10.0 STANDARD OPERATING PROCEDURES AND PROTOCOL ALTERATION:

All procedures not specified in this protocol will be performed in accordance with the Microbiological Associates Inc. Standard Operating Procedures Manual and in compliance with the appropriate EPA Good Laboratory Practice Standards. This study will be conducted in compliance with the appropriate EPA or OECD guidelines.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for EPA TSCA Regulations, 40 CFR 792 and OECD ISBN 922-84-12367-9.

Alterations of the study protocol may be made after the study has been initiated. In the event that the sponsor verbally authorizes a change in protocol, such change will be honored by Microbiological Associates Inc. A protocol amendment will then be issued by Microbiological Associates Inc. which will include the change and the justification, to be dated and signed by both study director and sponsor.

Will this study be submitted to an Agency? YES

Please list the agency(ies). EU, EPA

Please state the appropriate guidelines to be followed. EU, EPA, OECD

11.0 SIGNATURES:

Harmukh Shah 5-27-96
Sponsor Approval Date

Martha L. Wood 5/31/96
Study Director Date

Protocol No. 18.1X64 04/09/96 14 of 14

 MICROBIOLOGICAL ASSOCIATES, INC.

 MICROBIOLOGICAL ASSOCIATES, INC.

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HEXABROMOCYCLODODECANE (HBCD): CLOSED BOTTLE TEST

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 439E-102

AUTHORS:
Edward C. Schaefer
Doug Haberlein

STUDY INITIATION DATE: June 11, 1996

STUDY COMPLETION DATE: November 11, 1996

Submitted to

Chemical Manufactures Association's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209



WILDLIFE INTERNATIONAL LTD.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600



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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Chemical Manufacturer's Association Brominated Flame Retardant Industry Panel

TITLE: Hexabromocyclododecane (HBCD): Closed Bottle Test

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 439E-102

STUDY COMPLETION: November 11, 1996

This study was conducted to conform with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Part 792, 17 August 1989; and OECD, ISBN 92-84-12367-9 Paris 1982, with the following exceptions:

The reference substance, obtained from J.T. Baker (Phillipsburg, NJ), was not characterized in compliance with Good Laboratory Practice Standards.

Characterization and stability of the test substance was not conducted in compliance with Good Laboratory Practice Standards.

Verification of the test concentrations, stability, and homogeneity of the test and reference substances in the test medium were not determined.

STUDY DIRECTOR:



Edward C. Schaefer
Study Director

11/11/96

DATE

SPONSOR APPROVAL:

Sponsor

DATE

Applicant/Submitter

DATE

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**QUALITY ASSURANCE
HEXABROMOCYCLODODECANE (HBCD): CLOSED BOTTLE TEST
PROJECT NO.: 439E-102**

This study was examined for conformance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Part 792, 17 August 1989; and OECD, ISBN 92-84-12367-9, Paris 1982. The dates of all audits and inspections and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT:
Test substance preparation	June 17, 1996	June 18, 1996	June 19, 1996
Test initiation	June 18, 1996	June 25, 1996	June 27, 1996
DO measurements	July 9, 1996	July 9, 1996	July 10, 1996
Data and Draft Report	August 6 and 7, 1996	August 7, 1996	August 8, 1996
Final Report	November 11, 1996	November 11, 1996	November 11, 1996



Lisa T. Drottar
Quality Assurance Representative

11-11-96

DATE

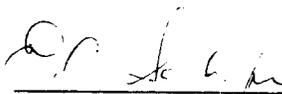
REPORT APPROVAL

SPONSOR: Chemical Manufacturer's Association Brominated Flame Retardant Industry Panel

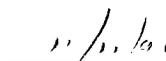
TITLE: Hexabromocyclododecane (HBCD): Closed Bottle Test

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439E-102

STUDY DIRECTOR:

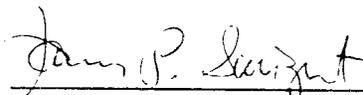


Edward C. Schaefer
Study Director



DATE

MANAGEMENT:



James P. Swigert, Ph.D.
Manager, Analytical Chemistry



DATE

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SUMMARY

SPONSOR:	Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel
CONTACT:	Hasmukh Shah, Ph.D.
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International Ltd. Easton, MD 21601

WILDLIFE INTERNATIONAL LTD.	
PROJECT NUMBER:	439E-102
STUDY:	Hexabromocyclododecane (HBCD): Closed Bottle Test
TEST CONCENTRATION:	7.7 mg/L
TEST DATES:	Experimental Start - June 18, 1996 Experimental Termination - July 16, 1996
LENGTH OF EXPERIMENTAL PHASE:	28 Days

TEST SUBSTANCE:	PERCENT BIODEGRADATION:
Hexabromocyclododecane (HBCD)	0.0

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INTRODUCTION

Tests of ready biodegradability, by definition, provide limited opportunity for acclimation and biodegradation to occur. A positive result in a test of ready biodegradability is an indication that the test substance will undergo rapid and ultimate biodegradation in the environment. A negative result in a test of ready biodegradability does not necessarily mean that the test substance will not be biodegraded under relevant environmental conditions but that additional testing may be needed.

OBJECTIVE

The objective of the study was to measure dissolved oxygen uptake over a 28 day period and express it as a percentage of the theoretical oxygen demand (ThOD) or chemical oxygen demand (COD).

EXPERIMENTAL DESIGN

The test contained an inoculum control group, a reference group, and a treatment group. The blank control, reference, and treatment groups contained ten replicate test chambers. The inoculum control was used to measure the dissolved oxygen consumption of the inoculum and was not dosed with a carbon source. The reference chambers were dosed with sodium benzoate, a substance known to be biodegradable, at a concentration of 2 mg/L. The treatment group test chambers were used to evaluate the test substance at 7.7 mg/L. Measurements of oxygen consumption were performed on two test chambers from the control, reference and treatment groups on days 0, 7, 14, 21, and 28.

MATERIALS AND METHODS

This study was conducted according to the procedures outlined in the protocol, Hexabromocyclododecane (HBCD): Closed Bottle Test, (Appendix I). The protocol was based on the procedures specified in the OECD Guideline for Testing of Chemicals, Guideline 301D (1); Council of the European Communities, Guideline C.4-E, *Closed Bottle* (2); and TSCA Title 40 of the Federal Code of Regulations, Part 796, Section 3200: *Ready Biodegradability: Closed Bottle Test* (3).

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Test Substance

The test substance used in this study was a composite of the following three samples of hexabromocyclododecane (HBCD):

Manufacturer: Bromide Compounds
Sample ID: Hexabromocyclododecane FR106
Date: 14-12-95
Ref. No.: 4117
Batch No.: 950303
Expiration Date: Not Given
Date Received: February 5, 1996
Wildlife ID: 3551

Manufacturer: Great Lakes Chemical
Sample ID: Hexabromocyclododecane
Lot. No.: 6352976-1
Expiration Date: Not Given
Date Received: October 26, 1995
Wildlife ID: 3462

Manufacturer: Albemarle
Sample ID: SAYTEX HBCD-LM 6.6# Flame Retardant (Powder)
Lot. No.: 33449-15x
Expiration Date: December 16, 1996
Date Received: December 20, 1995
Wildlife ID: 3519

The composite HBCD sample was prepared on February 19, 1996 and was assigned Wildlife International Ltd. identification number 3577. The composite was prepared by combining equal parts of the three manufacturers' products and mixing for approximately two hours. Subsamples of the

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composite sample were collected from the left and right sides of the top, middle, and bottom of the container. The subsamples were shipped to Albemarle Corporation for analysis to determine the homogeneity of the mixture. An additional sample of the composite was collected from the mixture and shipped to Albemarle Corporation for characterization of the test substance.

The theoretical oxygen demand (ThOD) value used to calculate the percent degradation of the test substance was 0.75 mg O₂/mg.

Stock Solution Preparation

A suspension of the test substance was prepared at a nominal concentration of 100 mg/L in Nanopure water. In addition, a stock solution of the reference substance, sodium benzoate, was prepared at a nominal concentration of 100 mg/L in Nanopure water.

Test Medium

The test medium was a modified biochemical oxygen demand (BOD) test dilution water and was prepared using Nanopure water as described in Protocol Appendix I. The mineral medium was aerated for approximately 20 minutes and then allowed to stand overnight at test temperature. Prior to use, the dilution water was aerated for approximately 10 minutes to achieve a dissolved oxygen (DO) concentration of ≥ 8.5 mg O₂/L.

Test Apparatus and Conditions

The test chambers were 300 mL BOD bottles. The test was conducted at 20±3°C. Test chambers were identified by project number, test substance ID, and test concentration, and bottle number.

Test Inoculum

Secondary clarifier supernatant was collected from Prospect Bay Wastewater Treatment Facility, Grasonville, Maryland on June 18, 1996. The supernatant was filtered through glass wool and then aerated until used. A standard plate count was performed on the inoculum. Plates were incubated at 20 ± 3°C for approximately 48 hours.

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Preparation of Test Chambers

Aerated mineral medium was added to the BOD bottles so that they are about one-half full. Adequate volumes of test substance and reference substance stock solution necessary to achieve a concentrations of 7.7 and 2 mg/L, respectively, were added to the appropriate bottles. Approximately 0.5 mL of inoculum was added to all bottles. Sufficient mineral medium was then added to each bottle so that all bottles were completely full. The bottles were stoppered and capped.

Sample Analysis

The dissolved oxygen concentration was determined using a Yellow Springs Instruments Dissolved Oxygen Meter. Dissolved oxygen measurements were performed on two test chambers from the control, reference and treatment groups on days 0, 7, 14, 21, and 28. Day zero samples were analyzed immediately after all bottles had been prepared.

Calculations

The average oxygen uptake exhibited by the control, reference, and treatment groups was calculated for each sampling interval. The biochemical oxygen demand (BOD) was calculated for each sampling interval using the following equation:

$$BOD = \frac{mgO_2/L \text{ uptake test substance} - mgO_2/L \text{ uptake blank}}{mg \text{ test substance}/L \text{ in vessel}}$$

The percent degradation was calculated using the following equation:

$$\% \text{ degradation} = \frac{BOD (mg O_2/mg \text{ test substance})}{ThOD (mg O_2/mg \text{ test substance})} \times 100$$

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RESULTS AND DISCUSSION

Observations and Measurements

The temperature range recorded during the test was 18 to 20°C and was within the protocol specified range throughout the test. The result of the standard plate count performed on the inoculum was 3.7×10^4 CFU/mL.

The average oxygen uptake exhibited by the control, reference, and treatment groups at each sampling interval is presented in Table 1. The oxygen depletion of the inoculum control was ≤ 1.5 mg O₂/L. The calculated biochemical oxygen demand (BOD) and percent degradation results are also presented in Table 1. Degradation of the test substance was not observed over the 28-day test period.

The viability of the inoculum and validity of the test was supported by the results of the reference substance, sodium benzoate, degrading approximately 94%. An average percent biodegradation of greater than 60% was achieved by day 7, thereby fulfilling the criteria for a valid test (1).

CONCLUSION

Degradation of the test substance, hexabromocyclododecane (HBCD) at 7.7 mg/L was not observed over the 28-day test period.

REFERENCES

- 1 **Organisation for Economic Cooperation and Development.** 1992. *Ready Biodegradability: Closed Bottle Test.* OECD Guideline 301D.
- 2 **Council of the European Communities.** Directive 67/548/EEC. Annex V. Guideline C.4-E, *Closed Bottle.*
3. **TSCA Title 40 of the Federal Code of Regulations.** 1994. Part 796 Section 3200: *Ready Biodegradability: Closed Bottle Test.*

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TABLE 1

Average Oxygen Uptake, Biochemical Oxygen Demand (BOD) and Percent Degradation Results

Test Substance	Conc. (mg/L)	Day	DO (mg/L)	DO Uptake	Average Uptake	BOD (mg/hr)	Percent Degradation
Control	NA	0	9.0	NA	NA	NA	NA
Control	NA	0	9.0	NA			
Control	NA	7	8.9	0.1	0.1	NA	NA
Control	NA	7	8.9	0.1			
Control	NA	14	8.2	0.8	0.8	NA	NA
Control	NA	14	8.2	0.8			
Control	NA	21	8.4	0.6	0.6	NA	NA
Control	NA	21	8.4	0.6			
Control	NA	28	8.5	0.5	0.5	NA	NA
Control	NA	28	8.6	0.4			
Benzoate	2	0	9.0	NA	NA	NA	NA
Benzoate	2	0	9.0	NA			
Benzoate	2	7	6.3	2.7	2.8	1.4	82
Benzoate	2	7	6.1	2.9			
Benzoate	2	14	5.6	3.4	3.4	1.3	76
Benzoate	2	14	5.7	3.3			
Benzoate	2	21	5.6	3.4	3.5	1.5	88
Benzoate	2	21	5.5	3.5			
Benzoate	2	28	5.2	3.8	3.7	1.6	94
Benzoate	2	28	5.4	3.6			
HBCD	7.7	0	9.0	NA	NA	NA	NA
HBCD	7.7	0	9.0	NA			
HBCD	7.7	7	8.8	0.2	0.2	0.0	0
HBCD	7.7	7	8.8	0.2			
HBCD	7.7	14	8.2	0.2	0.8	0.0	0
HBCD	7.7	14	8.2	0.8			
HBCD	7.7	21	8.4	0.6	0.6	0.0	0
HBCD	7.7	21	8.4	0.6			
HBCD	7.7	28	8.5	0.5	0.6	0.0	0
HBCD	7.7	28	8.4	0.6			

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APPENDIX I

Study Protocol and Protocol Deviation

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APPENDIX I

PROTOCOL

HEXABROMOCYCLODODECANE (HBCD): CLOSED BOTTLE TEST

Organisation for Economic Cooperation and Development
OECD Guideline 301D

and

Council of the European Communities Directive 67/548/EEC
Annex V, Guideline C.4-E

and

TSCA Title 40 of the Federal Code of Regulations
Part 796, Section 3200

Submitted To

Chemical Manufacturers Association's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209



WILDLIFE INTERNATIONAL LTD.



8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

April 17, 1996

PROTOCOL NO.: 439/041796/CBTa/SUB439

APPENDIX I

WILDLIFE INTERNATIONAL LTD.

HEXABROMOCYCLODODECANE (HBCD): CLOSED BOTTLE TEST

SPONSOR: Chemical Manufacturers Association's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

SPONSOR'S REPRESENTATIVE: Dr. Hasmukh Shah

TESTING FACILITY: Wildlife International Ltd.
8598 Commerce Drive
Easton, Maryland 21601

STUDY DIRECTOR: Edward C. Schaefer

LABORATORY MANAGEMENT: James P. Swigert Ph.D.
Manager of Aquatic Toxicology

FOR LABORATORY USE ONLY

Proposed Dates:	
Experimental Start Date: <u>6-18-96</u>	Experimental Termination Date: <u>7-16-96</u>
Project No.: <u>489E-102</u>	Study Room: <u>BIODEGRADATION</u>
Test Concentrations: <u>7.5 mg/L</u>	Int/Date: <u>AMS 6-11-96</u>
Test Substance No.: <u>3577</u>	Receipt Date: <u>2-19-96</u>

PROTOCOL APPROVAL

E.C. Schaefer
STUDY DIRECTOR

6-11-96
DATE

James P. Swigert
LABORATORY MANAGEMENT

6-11-96
DATE

Hasmukh Shah
SPONSOR'S REPRESENTATIVE

May 28, 1996
DATE

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APPENDIX I

WILDLIFE INTERNATIONAL LTD.

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INTRODUCTION

Tests of ready biodegradability, by definition, provide limited opportunity for acclimation and prolonged biodegradation to occur. A positive result in a test of ready biodegradability is an indication that the test substance will undergo rapid and ultimate biodegradation in the environment. A negative result in a test of ready biodegradability does not necessarily mean that the test substance will not be biodegraded under relevant environmental conditions but that additional testing may be needed.

OBJECTIVE

The objective of the study will be to measure dissolved oxygen uptake over a 28 day period and express it as a percentage of the theoretical oxygen demand (ThOD) or chemical oxygen demand (COD).

EXPERIMENTAL DESIGN

The test will contain an inoculum control group, a reference group, and a treatment group. The blank control, reference, and treatment groups will contain ten replicate test chambers. The inoculum control is used to measure the dissolved oxygen consumption of the inoculum and will not be dosed with a carbon source. The reference chambers will be dosed with sodium benzoate, a substance known to be biodegradable, at a concentration of 2 mg/L. The treatment group test chambers within the treatment group will be used to evaluate the test substance at 7.5 mg/L. Measurements of oxygen consumption will be performed on two test chambers from the control, reference and treatment groups on days 0, 7, 14, 21, and 28.

MATERIALS AND METHODS

Test methods are based on the procedures specified in the OECD Guideline for Testing of Chemicals, Guideline 301D (1); Council of the European Communities, Guideline C.4-E, *Closed Bottle* (2); and TSCA Title 40 of the Federal Code of Regulations, Part 796, Section 3200: *Ready Biodegradability: Closed Bottle Test* (3).

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APPENDIX I

WILDLIFE INTERNATIONAL LTD.

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Test Substance

The test substance will be a composite sample of hexabromocyclododecane (HBCD) produced by three manufacturers (Albemarle Corporation, Akerbrom LTD, and Great Lakes Chemical Corporation). The composite HBCD sample will be composed of equal parts of each of the three manufacturers' product. Prior to use in the study, equal weights of test substance from each manufacturer will be placed in an appropriate plastic container and mixed on a laboratory shaker for a minimum of two hours to form a composite HBCD sample for use as the test substance. Subsamples of the composite sample will be collected from the left and right sides of the top, middle and bottom of the container. The subsamples will be analyzed by Albemarle Corporation to determine the homogeneity of the mixture. An additional sample of the composite will be collected indiscriminately from the mixture and analyzed by Albemarle Corporation to characterize the test substance. If additional mixing or other procedures are necessary based on the results of the analysis, details of the procedure will be amended to the protocol. The mean HBCD percent of the homogeneity samples will be used for the study.

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice Standards (GLP), 40 CFR Part 792.31. The Sponsor is responsible for providing Wildlife International Ltd. written verification that the HBCD composite sample has been characterized according to GLP's prior to its use in the study. If written verification of GLP test substance characterization is not provided to Wildlife International Ltd., it will be noted in the compliance statement of the final report. The attached form IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR (Appendix I) will be used to provide information necessary for GLP compliance.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Stock Solution Preparation

A suspension of the test substance will be prepared at a nominal concentration of 100 mg/L in high quality water (e.g. Nanopure). In addition, a stock solution of the reference substance,

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APPENDIX I

WILDLIFE INTERNATIONAL LTD.

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sodium benzoate, will be prepared at a nominal concentration of 100 mg/L in high quality water. The stock solutions may be stored in a refrigerator for a maximum of three days.

Test Medium

The test medium is a modified biochemical oxygen demand (BOD) test dilution water and will be prepared using high quality water as described in Appendix I. The mineral medium will be aerated for approximately 20 minutes and then allowed to stand overnight at test temperature. The dissolved oxygen (DO) concentration of the mineral medium will be measured. The mineral medium will be reaerated if the measured DO is less than 8.5 mgO₂/L.

Test Apparatus and Conditions

The test chambers will be 300 mL BOD bottles. The test will be conducted at 20±3°C. Test chambers will be identified by project number, test substance ID, and test concentration, and bottle number.

Test Inoculum

Secondary clarifier supernatant will be collected from Prospect Bay Wastewater Treatment Facility, Grasonville, Maryland. The supernatant will be filtered through glass wool and then aerated until used. A standard plate count will be performed on the inoculum. Plates will be incubated at 20 ± 3°C for approximately 48 hours.

Preparation of Test Chambers

Aerated mineral medium will added to the BOD bottles so that they are about one-half full. Adequate volumes of test and reference substance stock solutions necessary to achieve a concentration of 7.5 and 2 mg/L, respectively, will be added to the appropriate bottles. Approximately 0.5 mL of inoculum will be added to all bottles. Sufficient mineral medium will then be added to each bottle so that all bottles are completely full. The bottles will be stoppered and capped.

Sample Analysis

The dissolved oxygen concentration will be determined using a Yellow Springs Instruments Dissolved Oxygen Meter or equivalent. Dissolved oxygen measurements will be performed on two

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WILDLIFE INTERNATIONAL LTD.

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test chambers from the control, reference and treatment groups on days 0, 7, 14, 21, and 28. Day zero samples will be analyzed immediately after all bottles have been prepared.

Calculations

The average oxygen uptake exhibited by the control, reference, and treatment groups will be calculated at each sampling interval. The biochemical oxygen demand (BOD) will be calculated after for each sampling interval using the following equation:

$$BOD = \frac{mgO_2/L \text{ uptake test substance} - mgO_2/L \text{ uptake blank}}{mg \text{ test substance}/L \text{ in vessel}}$$

The percent degradation will be calculated using the following equation:

$$\% \text{ degradation} = \frac{EOD (mg O_2/mg \text{ test substance})}{ThOD (mg O_2/mg \text{ test substance})} \times 100$$

Results

No bias is expected in this study and statistical methods will not be used in the analysis of the data. Interpretation of the results will be based on the following:

1. Oxygen depletion in the inoculum blank is $\leq 1.5 \text{ mgO}_2/\text{L}$.
2. The reference substance must achieve 60% degradation by day 14.

RECORDS TO BE MAINTAINED

Records to be maintained will include, but not limited to, the following:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance as provided by Sponsor.
3. Study initiation and termination dates.
4. Experimental initiation and termination dates.
5. Stock solution concentration calculations and solution preparation.
6. Inoculum source and pretreatment data.

PROTOCOL NO.: 439/041796/CBTa/SUB439

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APPENDIX I

WILDLIFE INTERNATIONAL LTD.

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7. Results of DO analysis.
8. Temperature range recorded during test period.
9. Copy of final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International Ltd. The report is to include, but is not limited to, the following, when applicable:

1. Name and address of facility performing the study.
2. Dates on which the study was initiated and completed.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.
5. Identification and characterization of the test substance as provided by Sponsor including name, CAS number, percent active, percent carbon, theoretical ThOD, and other characteristics, if provided by the Sponsor.
6. A description of the transformations and calculations performed on the data.
7. A description of the methods used and reference to any standard method employed.
8. A description of the test system.
9. A description of the preparation of the test solutions, the testing concentrations, the route of administration, and the duration of the test.
10. A description of all circumstances that may have affected the quality or integrity of the data.
11. The name of the study director, the names of other scientists or professionals, and the names of all supervisory personnel, involved in the study.
12. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
13. The location where the raw data and final report are to be stored.
14. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and findings reported to the Study Director and Management.

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CHANGES TO THE FINAL REPORT

If it is necessary to make corrections or additions to the final report after it has been accepted, such changes shall be made in the form of an amendment issued by the Study Director. The amendment shall clearly identify the part of the study that is being amended and the reasons for the alteration. Amendments shall be signed and dated by the Study Director and Laboratory QA.

CHANGING OF PROTOCOL

Changes in the protocol will be made in the form of a written amendment that is signed by the Study Director, Laboratory Management, and the Sponsor. The amendment will become part of the original protocol.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 792) and OECD (ISBN 92-84-12367-9). Each study conducted by Wildlife International Ltd. is routinely examined by the Wildlife International Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site or at an alternative location to be specified in the final report.

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REFERENCES

- 1 **Organisation for Economic Cooperation and Development.** 1992. *Ready Biodegradability: Closed Bottle Test.* OECD Guideline 301D.
- 2 **Council of the European Communities.** Directive 67/548/EEC. Annex V. Guideline C.4-E, *Closed Bottle.*
- 3 **TSCA Title 40 of the Federal Code of Regulations.** 1994. Part 796, Section 3200: *Ready Biodegradability: Closed Bottle Test.*

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APPENDIX I. Test Medium

The following stock solutions used to prepare the test medium may be purchased or prepared as described below:

- (a) Potassium dihydrogen orthophosphate, KH_2PO_4 8.50 g
Dipotassium hydrogen orthophosphate, K_2HPO_4 21.75 g
Disodium hydrogen orthophosphate dihydrate,
 $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 33.40 g
Ammonium chloride, NH_4Cl 0.50 g

Dissolve in high quality water and make up to 1 liter.

- (b) Calcium chloride solution, CaCl_2 27.50 g

Dissolve in high quality water and make up to 1 liter.

- (c) Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 22.50 g

Dissolve in high quality water and make up to 1 liter.

- (d) Ferric chloride, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.25 g

The test medium will contain the following standard reagent solutions per liter of high quality water (e.g. Nanopure):

- 1 mL of phosphate buffer solution, APHA, pH 7.2
1 mL of calcium chloride solution, APHA, 2.75%
1 mL of magnesium sulfate solution, APHA, 2.25%
1 mL of ferric chloride solution, APHA, 0.025%

The constituents of the test medium are not known to contain any contaminants that are reasonably expected to be present and are known to be capable of interfering with the study.

APPENDIX I

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APPENDIX II
IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

I. Test Substance Identity (name to be used in the report): _____
Test Substance Sample Code or Batch Number: _____
Test Substance Purity (% Active Ingredient): _____ Expiration Date: _____
Solubility: Water: _____ Theoretical Oxygen Demand: _____

II. Test Substance Characterization
Have the identity, strength, purity and composition or other characteristics which appropriately define the test substance and reference standard been determined prior to its use in this study in accordance with GLP Standards? Yes ___ No ___

III. Test Substance Storage Conditions
Please indicate the recommended storage conditions at Wildlife International Ltd.

Has the stability of the test substance under these storage conditions been determined in accordance with GLP Standards? Yes ___ No ___
Other pertinent stability information: _____

IV. Test Concentrations: Adjust test concentration to 100% a.i. based upon the purity (%) given above.
 Do not adjust test concentration to 100% a.i. Test the material AS IS.

V. Toxicity Information:
Mammalian: Rat LD50 ___ Mouse LD50 ___
Aquatic: Invertebrate Toxicity (EC/LC50) Fish Toxicity (LC50)

Other Toxicity Information (including findings of chronic and subchronic tests):

VI. Classification of the Compound:
____ Insecticide _____ Herbicide _____ Fungicide
____ Microbial Agent _____ Economic Poison
Other: _____

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APPENDIX I

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PROJECT NO.: 439E-102
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DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Hexabromocyclododecane (HBCD): Clos'd Bottle Test

PROTOCOL NO.: 439/041796/CBTa/SUB439

DEVIATION NO.: 1

SPONSOR: Chemical Manufacturers Association

PROJECT NO.: 439E-102

DATE OF DEFACTO DEVIATION: June 18, 1996

DEVIATION: Page 3, Experimental Design; and Page 5, Preparation of Test Chambers:

The test substance concentration in the test chambers was approximately 7.7 mg/L.

REASON:

Rounding of the dosing volume of test substance stock solution increased the testing concentration.

IMPACT:

This deviation had no impact on the integrity of the study.


STUDY DIRECTOR

8-8-96
DATE


LABORATORY MANAGEMENT

8/8/96
DATE

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APPENDIX II

Personnel Involved in the Study

The following key Wildlife International Ltd. personnel were involved in the conduct or management of this study:

- (1) James P. Swigert, Manager, Aquatic Toxicology
- (2) Edward C. Schaefer, Study Director, Biodegradation
- (3) Doug Haberlein, Biologist, Biodegradation
- (4) Joel I. Stenzel, Senior Chemist, Analytical Chemistry

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